Oxidative Medicine and Cellular Longevity

Targeting Molecular Mediators of Oxidative Stress for Neurolongevity and Neuroprotection

Lead Guest Editor: Gaurav Kumar
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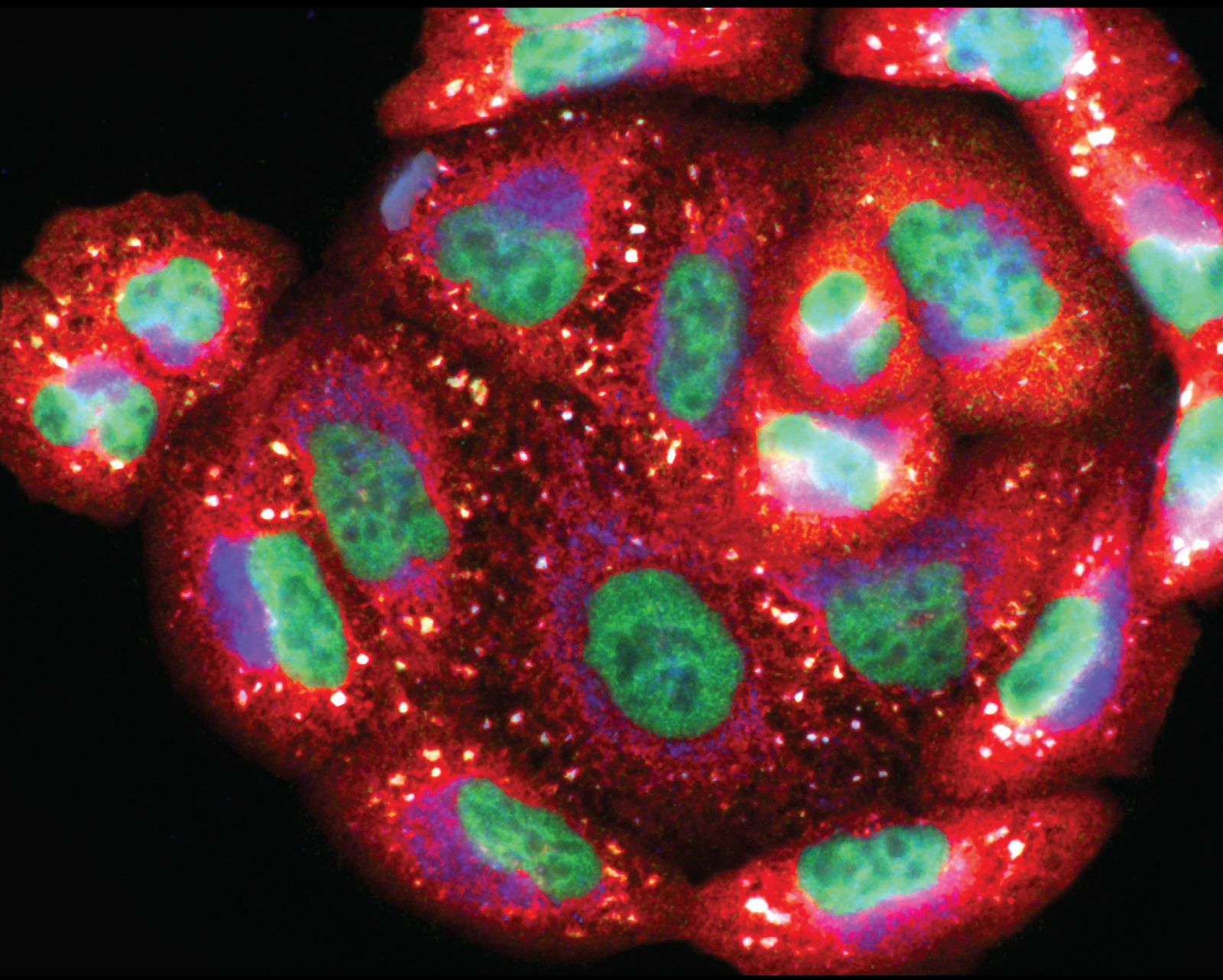

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Research Article

A Multifunctional (-)-Meptazinol-Serotonin Hybrid Ameliorates Oxidative Stress-Associated Apoptotic Neuronal Death and Memory Deficits via Activating the Nrf2/Antioxidant Enzyme Pathway

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The pathogenesis of Alzheimer's disease (AD) involves multiple pathophysiological processes. Oxidative stress is a major cause of AD-associated neuronal injury. The current research was designed to examine whether a novel (-)-meptazinol-serotonin hybrid (Mep-S) with potent antioxidant activity and additional inhibitory properties for acetylcholinesterase (AChE) activity could attenuate oxidative neuronal damage and cognitive deficits. In human SH-SY5Y cells, Mep-S suppressed H_2O_2-induced apoptosis by restoring mitochondrial membrane potential and inhibiting caspase-3 activation. Meanwhile, it attenuated oxidative stress elicited by H_2O_2 through lessening generation of reactive oxygen species as well as enhancing production of glutathione (GSH) and activity of superoxide dismutase (SOD). Mechanistically, Mep-S promoted nuclear translocation of a transcription factor nuclear factor E2-related factor-2 (Nrf2) in H_2O_2-challenged cells. This effect was accompanied by reduction in Kelch-like ECH-associated protein-1 (Keap1) levels as well as augmentation of Akt phosphorylation and expression of heme oxygenase-1 (HO-1) and NAD(P)H quinine oxidoreductase-1 (NQO-1). Molecular docking analysis revealed that Mep-S may disrupt the protein-protein interactions between Keap1 and Nrf2. In an *in vivo* mouse model, Mep-S attenuated scopolamine-caused cognitive deficits with inhibition of apoptotic neuronal death and brain AChE activity. Furthermore, the scopolamine-induced impairment of total antioxidant capacity and reduction in SOD1, SOD2, and γ-glutamate-cysteine ligase expression in the brain were counteracted by Mep-S, accompanied by decreased Keap1 levels, increased Akt catalytic subunit and Nrf2 phosphorylation, and decreased Nrf2, HO-1, and NQO-1 expression. Collectively, our results suggest that Mep-S ameliorates apoptotic neuronal death and memory dysfunction associated with oxidative stress by regulating the Nrf2/antioxidant enzyme pathway through inactivating Keap1 and phosphorylating Nrf2 via Akt activation. Therefore, Mep-S may be a potential lead for multitarget neuroprotective agents to treat AD-like symptoms.

1. Introduction

Alzheimer's disease (AD), predominantly affecting the elderly people and causing progressive cognitive decline, involves a multifactorial pathogenesis. The primary neuropathological hallmarks in AD brains include the presence of extracellular amyloid-β (Aβ) deposition as senile plaques, intracellular accumulation of hyperphosphorylated tau as

neurofibrillary tangles, and loss of cholinergic neurons [1]. Currently, no disease-modifying treatment has been established. To date, FDA-approved drugs for AD therapy include inhibitors of acetylcholinesterase (AChE) or N-methyl-D-aspartate receptors [2–4]. Although benefiting for cognitive symptoms, they fail to prevent pathological processes of the disease [1, 5]. Notably, the Aβ-directed antibody aducanumab received accelerated approval from the FDA in 2021; however, its efficacy for AD is controversial [6]. Since the increasing AD population may pose a significant burden on family and public health, it is urgent to develop innovative disease-modifying medications for this disease.

AD pathogenesis involves multiple pathological processes that are closely linked and interact [1]. Substantial evidence shows that oxidative stress, caused by reactive oxygen species (ROS) overproduction, is an early and sustained event in AD brain [7–9]. In addition to directly causing neuronal damage (particularly in cognition-related areas, e.g., the hippocampus), oxidative stress may aggravate Aβ and tau pathology [9, 10]. Therefore, oxidative neuronal damage is considered a major factor associated with both the onset and progression of AD pathogenesis. Evidence shows that agents with antioxidant activity could benefit for AD therapy [11–13].

The multifactorial nature of AD warrants multitarget intervention for the disease. To this end, the exploration of multi-target-directed ligands (MTDLs) has become a hotspot in the development of new therapies for AD [14–17]. Considering the critical roles of oxidative stress in AD pathogenesis, MTDLs with antioxidant potency may be favorable for AD therapy. In fact, the development of anti-AD MTDLs with antioxidant activity has recently attracted much attention [15, 18]. In a previous research, we reported a number of (-)-meptazinol-melatonin/serotonin hybrids with multiple pharmacological properties [19]. The results of *in vitro* testing demonstrate that a novel (-)-meptazinol-serotonin hybrid, Mep-S (Figure 1(a)), in addition to inhibiting AChE activity, exhibits high antioxidant potency (7.2-fold of Trolox). Additionally, it displays a favorable effect on neuronal cell viability and shows sufficient blood-brain barrier penetrability. Thus, this compound could serve as a potential MTDL lead for AD therapy.

In this work, we examined whether Mep-S could ameliorate oxidative neuronal injury by *in vitro* and *in vivo* testing. The results showed that Mep-S protects against H_2O_2-induced neuronal apoptosis and ameliorates scopolamine-caused cognitive deficits. Further mechanistic studies revealed that the nuclear factor E2-related factor-2 (Nrf2)/antioxidant enzyme pathway was associated with the neuroprotective effect of Mep-S.

2. Materials and Methods

2.1. Reagents. Mep-S was synthesized in our laboratory as previously described [19]. Scopolamine, H_2O_2, and N-acetylcysteine (NAC) were the products of Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM)/F-12 (1 : 1), phosphate-buffered saline (PBS), and a mixture of penicillin and streptomycin were the products of Gibco (Grand Island, NY, USA). The rabbit-derived antibodies against NAD(P)H quinine oxidoreductase-1 (NQO-1; cat #ab80588), heme oxygenase-1 (HO-1), Kelch-like ECH-associated protein-1 (Keap1), Nrf2, phospho-Nrf2 (S40), and B-cell lymphoma 2 (Bcl-2) were the products of Abcam (Cambridge, MA, USA). The rabbit-derived antibodies against caspase-3 (D3R6Y), phospho-Akt (Ser473), Akt, and Bax were the products of Cell Signaling Technology (CST; Danvers, MA, USA). Mep-S was dissolved in dimethyl sulfoxide (DMSO; Sigma), and NAC was dissolved in normal saline to obtain stock solutions (20 mg/ml), which were diluted with saline (for *in vivo* experiments) or culture medium (for *in vitro* tests). The Mep-S working solutions contain less than 2.5% of DMSO.

2.2. Animals. ICR mice (male, weighing 25–30 g; Shanghai Laboratory Animal Center, Chinese Academy of Science, Shanghai, China) were kept under a light/dark cycle of 12 h/12 h in a room with constant temperature, with free access to water and food. The experimental procedures were performed following principles of the Declaration of Helsinki. The Ethics Committee of Shanghai Jiao Tong University School of Medicine approved them.

2.3. Cell Culture. SH-SY5Y human neuroblastoma cells were cultured in a DMEM/F-12 mixture containing fetal bovine serum (FBS, 10%; Sigma), L-glutamine (2 mM; Gibco), penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C. They were used for experiments after growing to the required confluence, with culture medium changed to serum-free medium prior to addition of drugs.

2.4. Detection of Cell Viability. Cell viability was assessed with a Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan). After being seeded in 96-well plates at 2×10^4 cells per well and growing to the required confluence, SH-SY5Y cells received 24 h exposure to H_2O_2 with or without preincubation with Mep-S or NAC (serving as a positive control of antioxidants) for 1 h. Thereafter, the cells were incubated with CCK-8 (20 μl, 5 mg/ml) at 37°C for 2 h. Cell viability, which was evaluated based on the absorbance measured with a test wavelength (570 nm) and a reference wavelength (655 nm), was normalized to the percentage of control group.

2.5. Detection of Lactate Dehydrogenase (LDH) Release. The cell-released LDH content was detected using an LDH cytotoxicity assay kit (Beyotime Institute of Biotechnology, Shanghai, China) following instructions of the manufacturer. In brief, the cells were subjected to 24 h stimulation with H_2O_2 with or without preincubation with Mep-S or NAC. Then, the culture medium (120 μl) was aspirated to mix with LDH test working solution (60 μl). The absorbance was detected at a wavelength of 490 nm after 30 min incubation at room temperature.

2.6. Annexin V/PI Staining. Neuronal apoptosis was measured by flow cytometry using a commercial FITC Annexin V Apoptosis Detection Kit (BD Pharmingen, Franklin Lakes, NJ, USA). In brief, the cells, which were seeded in 6-well plates at 5×10^5 cells per well, were stimulated with H_2O_2 for 24 h with or without preincubation with Mep-S or

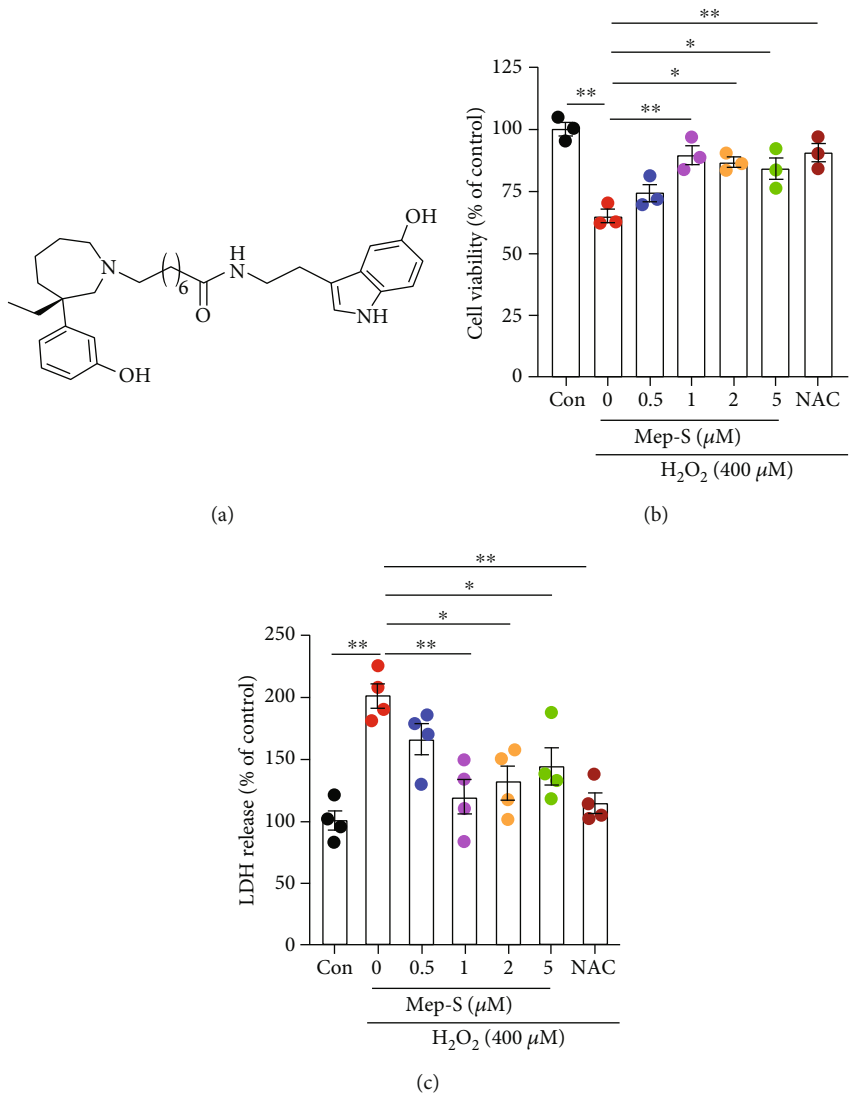


FIGURE 1: Mep-S protects against H_2O_2-induced neurotoxicity in SH-SY5Y cells. (a) Chemical structure of Mep-S. Mep-S protected against H_2O_2-induced cell death (b) and LDH release (c) in SH-SY5Y cells. The cells were pretreated with Mep-S (0.5-5 μM) or N-acetyl-L-cysteine (NAC, 1 mM) for 1 h prior to stimulation with H_2O_2 (400 μM) for 24 h. Data are presented as the mean ± SEM of 3-4 independent experiments. $^*P < 0.05$ and $^{**}P < 0.01$.

NAC. Afterwards, they were harvested with trypsin-ethylenediaminetetraacetic acid (EDTA, 0.25%; Gibco) and centrifugated at 1500 rpm for 5 min at 4°C. Thereafter, cell pellets were resuspended in FITC Annexin V- and propidium iodide-containing binding buffer, followed by 15 min incubation at room temperature and subsequent detection on a flow cytometer (Thermo Fisher Scientific, Rockford, IL, USA).

2.7. Mitochondrial Membrane Potential (MMP) Detection. MMP was evaluated with JC-1 fluorescence dye. It exhibits potential-dependent accumulation in mitochondria, where it forms J-aggregates and exhibits red fluorescence. Decreased MMP favors JC-1 monomers that yield green fluorescence. Briefly, the cells were challenged with H_2O_2 for 24 h with or without preincubation with Mep-S or NAC. Afterwards, they were centrifuged for 5 min at 1500 rpm at 4°C after 20 min incubation with JC-1 dye (2 μM; Beyotime) at 37°C. The cell pellets were resuspended in JC-1 staining buffer and detected on a flow cytometer.

2.8. Measurement of Intracellular ROS Accumulation. The intracellular ROS was detected with 2′7′-dichlorofluorescein-diacetate (DCFH-DA; Sigma), a fluorescent dye. After deacetylation, it reacts with intracellular radicals (predominantly generated by hydrogen peroxide) and is transformed into DCF, an intracellularly retained fluorescent product. The cells were challenged with H_2O_2 for 2 h with or without preincubation with Mep-S or NAC. Afterwards, they were probed with DCFH-DA (10 μM; Sigma) at 37°C for 30 min. The fluorescence intensity (Ex 488 nm/Em 530 nm) was examined under a confocal microscope (Leica TCS SP2 AOBS; Leica, Wetzlar, Germany).

2.9. Detection of Glutathione (GSH) Content and Superoxide Dismutase (SOD) Activity. A GSH assay kit (Abbkine Scientific Co., Ltd, Wuhan, China) and SOD assay kit (Beyotime) were applied for detection of GSH content and SOD activity, respectively. The cells received 24 h H_2O_2 stimulation with or without preincubation with Mep-S or NAC. Afterwards, the supernatants of cell homogenates were collected and used for assay of GSH content and SOD activity.

2.10. Molecular Docking. Molecular docking simulation was conducted to explore the potential impact of Mep-S on Keap1-Nrf2 protein-protein interactions (PPI). The X-ray crystal structure of mouse Keap1 Kelch domain (at 1.21 Å resolution), which serves as the recognition module for Nrf2 [20, 21], was acquired from the Protein Data Bank (code: 6ZF4). Water molecules and heteroatoms in protein structures were discarded before addition of hydrogen atoms. The 3D coordinates of Mep-S (serving as the ligand) were obtained using CORINA Version 3.0 (Molecular Networks GmbH, Erlangen, Germany). After acquirement of the final ligand conformations by energy minimization with Tripos force field, molecular docking simulation was conducted with AutoDock Vina 1.1.2 to obtain an ensemble of docked conformations. The best conformation was chosen according to the lowest docked energy.

2.11. Design of Animal Experiments. Mice were randomized into 6 groups ($n = 10$). The control animals were given intraperitoneal (i.p.) injection of saline per day while the scopolamine-treated animals were daily challenged with scopolamine (2 mg/kg) 15 min before the behavioral test. The scopolamine+Mep-S-treated groups received a daily injection of Mep-S at 0.5, 1, 2, and 5 mg/kg, respectively, 15 min prior to the delivery of scopolamine. On days 1-6, the Morris water maze test was performed for behavioral assessment. In a separate experiment, 4 groups of mice received a daily injection of saline and Mep-S (at 0.5, 2, and 5 mg/kg), respectively, prior to the injection of scopolamine for 6 days. The control group received daily saline injection. The mice were transcardially perfused with saline after euthanasia 24 h after the last drug administration. After removal of the brain, one hemisphere was immediately postfixed for 2 h in 10% formalin solution and then subjected to paraffin embedding and sectioning for histological analyses. The other hemisphere was immediately stored at -80°C for subsequent quantitative real-time polymerase chain reaction (qPCR), western blot, and biochemical analyses.

2.12. Morris Water Maze Test. The Morris water maze test was performed as previously described [22]. Briefly, mice were placed in the experimental room to adapt to the environment 24 h before the testing. The testing was conducted in a black circular tank with a diameter of 140 cm, which contained water (22 ± 1.0°C) to a 30 cm depth. During the 5-day acquisition testing, each mouse received 4 trials daily. The interval between trials was 30 s. The test animals that were able to find the submerged platform (9 cm diameter, located on the center of a fixed quadrant) within 60 s were allowed to stay for 30 s on it. Animals failing to find the platform within 60 s were guided to stay for 30 s on it. During the probe test on day 6, retention of spatial memory was assessed, with the platform being removed. The test animals were allowed to swim for 60 s. Swimming traces were analyzed to evaluate spatial learning capacity.

2.13. Western Blot. SH-SY5Y cells as well as mouse hippocampi and cerebral cortex were lysed in radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitor cocktail (1%) and phenylmethanesulfonyl fluoride (PMSF, 1%) protease inhibitor, followed by 10 min centrifugation at 15,000 rpm at 4°C. For cellular nuclear and cytoplasmic fractions, a commercial CelLytic NuCLEAR Extraction Kit (Sigma) was used to separate cellular cytoplasmic and nuclear proteins. Protein samples were separated on 10% sodium dodecyl sulfate-polyacrylamide gel. Thereafter, they were transferred to a polyvinylidene fluoride (PVDF) membrane (Merck Millipore, Tullagreen, Ireland), followed by blockade with 5% nonfat milk. Afterwards, the membrane was subjected to overnight incubation at 4°C with primary antibodies (1 : 1000) against caspase-3, HO-1, NQO-1, Keap1, phospho-Akt, Akt, Nrf2, phospho-Nrf2, Bcl-2, Bax, β-actin (CST), glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Abcam), or H3 (Abcam), followed by 1 h incubation at room temperature with a diluted (1 : 10000) horseradish peroxidase-conjugated antibody. After visualization with an enhanced chemiluminescence (Pierce, Rockford, IL, USA), the bands were analyzed and quantified.

2.14. qPCR. After extraction with a commercial RNA Extraction Kit (Takara, Otsu, Shiga, Japan), 1 μg of RNA extracted from mouse brain tissues was reverse-transcribed to cDNA. qPCR was conducted on LightCycler 480 Instrument II (Roche, Indianapolis, IN, USA) with a Takara SYBR Premix Ex Taq Kit. The following are primer sequences used: SOD1, 5′-GTGATTGGGATTGCGCAGTA-3′ (forward) and 5′-TGGTTTGAGGGTAGCAGATGAGT-3′ (reverse); SOD2, 5′-TTAACGCGCAGATCATGCA-3′ (forward) and 5′-GGTGGCGTTGAGATTGTTCA-3′ (reverse); γ-glutamate-cysteine ligase catalytic subunit (GCLC), 5′-AGCACAGGG TGACAGAAGAG-3′ (forward) and 5′-GAGGGACTCTG GTCTTTGTG-3′ (reverse); and GAPDH, 5′-AACGAC CCCTTCATTGAC-3′ (forward) and 5′-TCCACGACATA CTCAGCAC-3′ (reverse). Target gene expression was evaluated with the $2^{-\Delta\Delta ct}$ method.

2.15. Hematoxylin and Eosin (H&E) Staining. Serial coronal sections of mouse brains were deparaffinized and rehydrated. After hematoxylin and eosin staining, the histopathological properties were evaluated under a Leica DFC 320 digital camera.

2.16. Immunofluorescence Staining. Serial coronal sections of mouse brains were incubated at 4°C overnight with an antibody specific for Nrf2 (1 : 100), followed by 1 h incubation at room temperature with a Alexa Fluor 488-conjugated antibody (Invitrogen, Carlsbad, CA, USA). The nucleus was stained with 4′,6-diamidino-2-phenylindole (DAPI). The fluorescent intensity was evaluated under a Leica confocal microscope.

2.17. Detection of AChE Activity. Mouse brain samples were subjected to homogenate and 10 min centrifugation (2500 rpm) at 4°C. After addition of PBS (0.05 M, pH 7.2) containing 0.25 mM 5,5′-dithiobis-(2-nitrobenzoic) acid (DTNB; Sigma), the supernatants were added with 1 mM acetylthiocholine (Sigma). The absorbance change at the wavelength of 412 nm was detected using a Varioskan Flash multimode reader (Thermo Fisher). AChE activity was presented as units/mg protein.

2.18. Detection of Total Antioxidant Capacity. Mouse brain samples were homogenized and centrifuged at 12,000 rpm for 5 min at 4°C. Total antioxidant capacity was detected with a commercial kit (Beyotime) following the instructions of the manufacturer.

2.19. Statistical Analysis. Experimental data were presented as the mean ± standard error of the mean (SEM) and statistically analyzed with analysis of variance (ANOVA; one-way or two-way) and Bonferroni's posttest. A P value of less than 0.05 was regarded as statistically significant.

3. Results

3.1. Protection of Mep-S against H_2O_2-Evoked Cytotoxicity. We previously found potent antioxidant activity of Mep-S (7.2-fold of Trolox) in a cell-free system [19]. In this work, we further examined the antioxidant effect of this compound in human SH-SY5Y neuronal cells stimulated with H_2O_2. We first detected the effect of Mep-S on viability of neuronal cells. The results showed that Mep-S did not influence the survival of SH-SY5Y cells at concentrations of 1-20 μM (one-way ANOVA, $F(5, 18) = 0.25$, $P = 0.93$; Supplementary material, Figure S1A). We then treated the cells with H_2O_2 and found that H_2O_2 induced dose-dependent cell death at concentrations of 50-800 μM (one-way ANOVA, $F(5, 12) = 59.81$, $P < 0.0001$; Supplementary material, Figure S1B). A dose of 400 μM H_2O_2 was adopted in the subsequent *in vitro* testing.

To detect whether Mep-S could inhibit H_2O_2-induced neurotoxicity, the cells received 1 h pretreatment with Mep-S (0.5-5 μM) before H_2O_2 stimulation. As demonstrated in Figure 1(b), cell viability was obviously different between groups (one-way ANOVA, $F(6, 14) = 11.65$, $P < 0.0001$). The viability of cells incubated with H_2O_2 (65.20 ± 2.64%) was higher than control group ($P < 0.01$). The survival rate was markedly increased to 89.79 ± 3.90%, 86.91 ± 1.98%, and 84.20 ± 4.49% in cells pretreated with Mep-S at 1, 2, and 5 μM, respectively ($P < 0.05$ or $P < 0.01$ vs. H_2O_2-treated cells). Similarly, survival of cells receiving NAC (1 mM; as a positive control) pretreatment (90.72 ± 3.65%) was overtly elevated compared with H_2O_2-treated cells ($P < 0.01$). In addition, LDH release was obviously different between groups (one-way ANOVA, $F(6, 21) = 8.46$, $P < 0.0001$; Figure 1(c)). Mep-S at doses of 1, 2, and 5 μM significantly suppressed H_2O_2-evoked LDH release ($P < 0.01$ at 1 μM and $P < 0.05$ at 2 or 5 μM vs. H_2O_2-treated group). LDH release from cells pretreated with NAC was also significantly lower than that from H_2O_2-challenged cells ($P < 0.01$).

3.2. Protection of Mep-S against H_2O_2-Evoked Apoptotic Neuronal Death. We then examined neuronal apoptosis to further explore the protective effect of Mep-S (at 1 μM, the concentration at which Mep-S showed the most obvious effect) on H_2O_2-challenged SH-SY5Y cells. Based on the results of Annexin V/PI staining in SH-SY5Y cells (one-way ANOVA, $F(3, 8) = 10.17$, $P = 0.0042$; Figure 2(a)), the apoptotic rate of cells challenged with H_2O_2 increased by 1.47-fold relative to control group ($P < 0.01$). Pretreatment with Mep-S or NAC markedly decreased the cellular apoptotic rate compared with H_2O_2-treated group ($P < 0.05$).

Dissipation of MMP is closely associated with the initiation of the intrinsic pathway of apoptosis and, thus, is regarded as a marker of apoptotic cells [23]. As shown in Figure 2(b) (one-way ANOVA, $F(3, 8) = 19.40$, $P = 0.0005$), the emission of JC-1 in H_2O_2-treated SH-SY5Y cells was shifted from red to green (indicating a loss of MMP and depolarization of the mitochondrial membrane), with an increased intensity of JC-1 green fluorescence compared to H_2O_2-treated cells ($P < 0.01$). Mep-S or NAC pretreatment markedly decreased the intensity of JC-1 green fluorescence ($P < 0.01$ vs. H_2O_2-challenged group). Activation of caspase-3 is a downstream event of MMP collapse [23]. As shown in Figure 2(c) (one-way ANOVA, $F(3, 12) = 6.75$, $P = 0.0064$), H_2O_2 markedly elevated cleaved caspase-3 level to 177.3 ± 21.5% relative to control values ($P < 0.01$). Mep-S or NAC pretreatment significantly repressed the upregulation of cleaved caspase-3 levels compared to H_2O_2-challenged group ($P < 0.05$).

3.3. Mep-S Inhibits H_2O_2-Induced ROS Production along with Decrease in GSH Content and SOD Activity. It was shown based on one-way ANOVA that intracellular ROS level is obviously different between groups ($F(5, 12) = 4.89$, $P = 0.011$; Figure 3(a)). Stimulation with H_2O_2 increased ROS levels within SH-SY5Y cells, with significantly higher DCHF-DA fluorescence intensity compared to control cells ($P < 0.01$). Pretreatment with Mep-S (1 and 2 μM) or NAC markedly inhibited intracellular ROS accumulation ($P < 0.05$ or $P < 0.01$ vs. H_2O_2-treated cells) (Figure 3(a)). In addition, one-way ANOVA showed significantly different GSH content ($F(5, 18) = 5.06$, $P = 0.0045$; Figure 3(b)) and SOD activity ($F(5, 12) = 4.61$, $P = 0.014$; Figure 3(c)) among groups. The H_2O_2-challenged cells showed significantly decreased GSH content and SOD activity compared to control group ($P < 0.01$). Mep-S (1 and 2 μM) or NAC pretreatment significantly increased GSH content and SOD activity compared to H_2O_2-treated group ($P < 0.05$).

3.4. Mep-S Activates the Nrf2/Antioxidant Enzyme Signaling Axis in H_2O_2-Challenged Cells. Evidence shows that Keap1/Nrf2 axis is crucial for protection of neurons against oxidative injury by inducing antioxidant enzyme (e.g., HO-1 and NQO-1) expression to suppress the intracellular ROS level [24]. We therefore explored the effect of Mep-S on this pathway. As shown in Figure 4(a), HO-1 and NQO-1 levels in H_2O_2-challenged SH-SY5Y cells overtly declined ($P < 0.01$ vs. control group). Concurrently, Keap1 (a negative Nrf2 modulator) and cytoplasmic Nrf2 levels increased, while

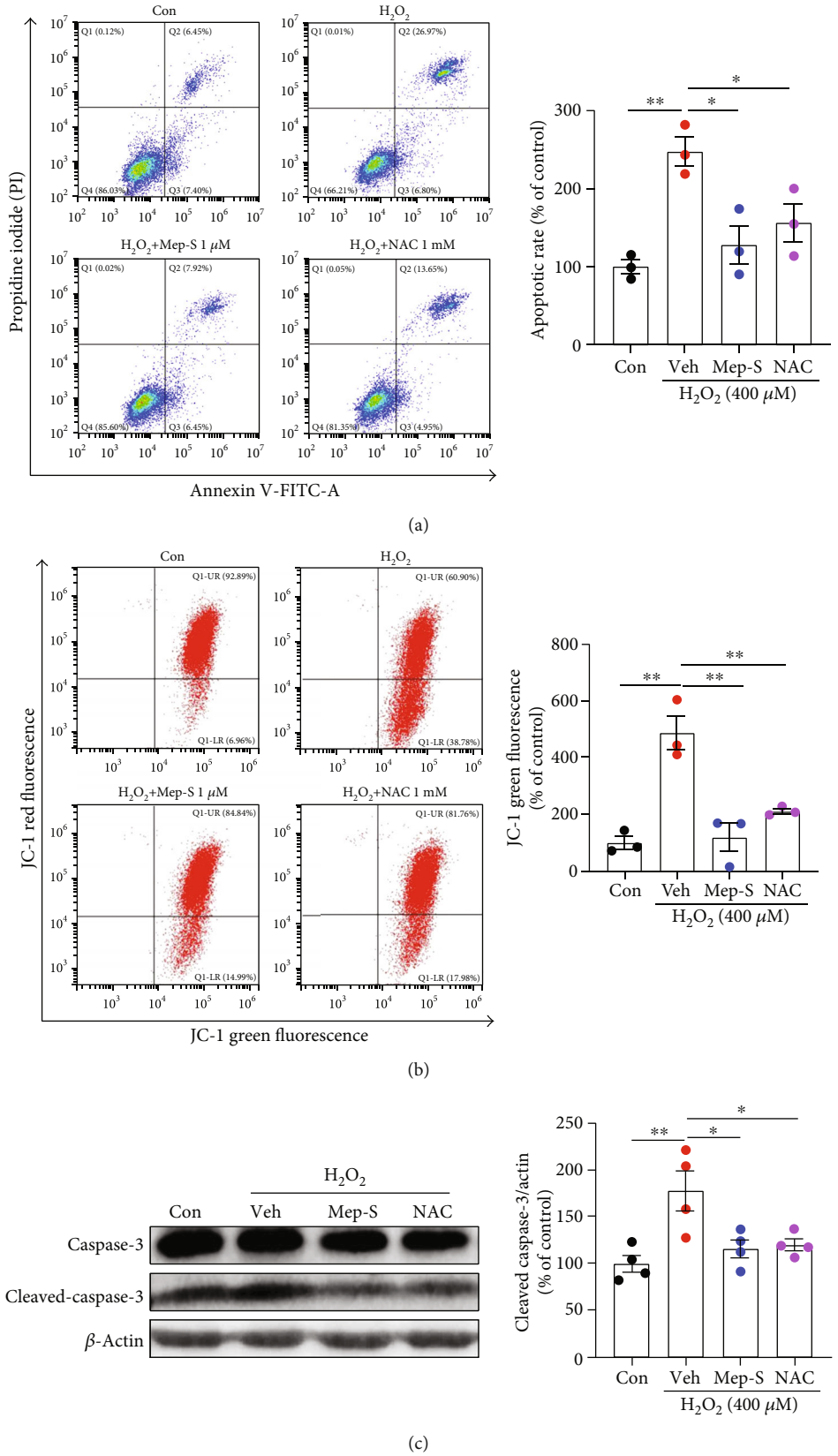
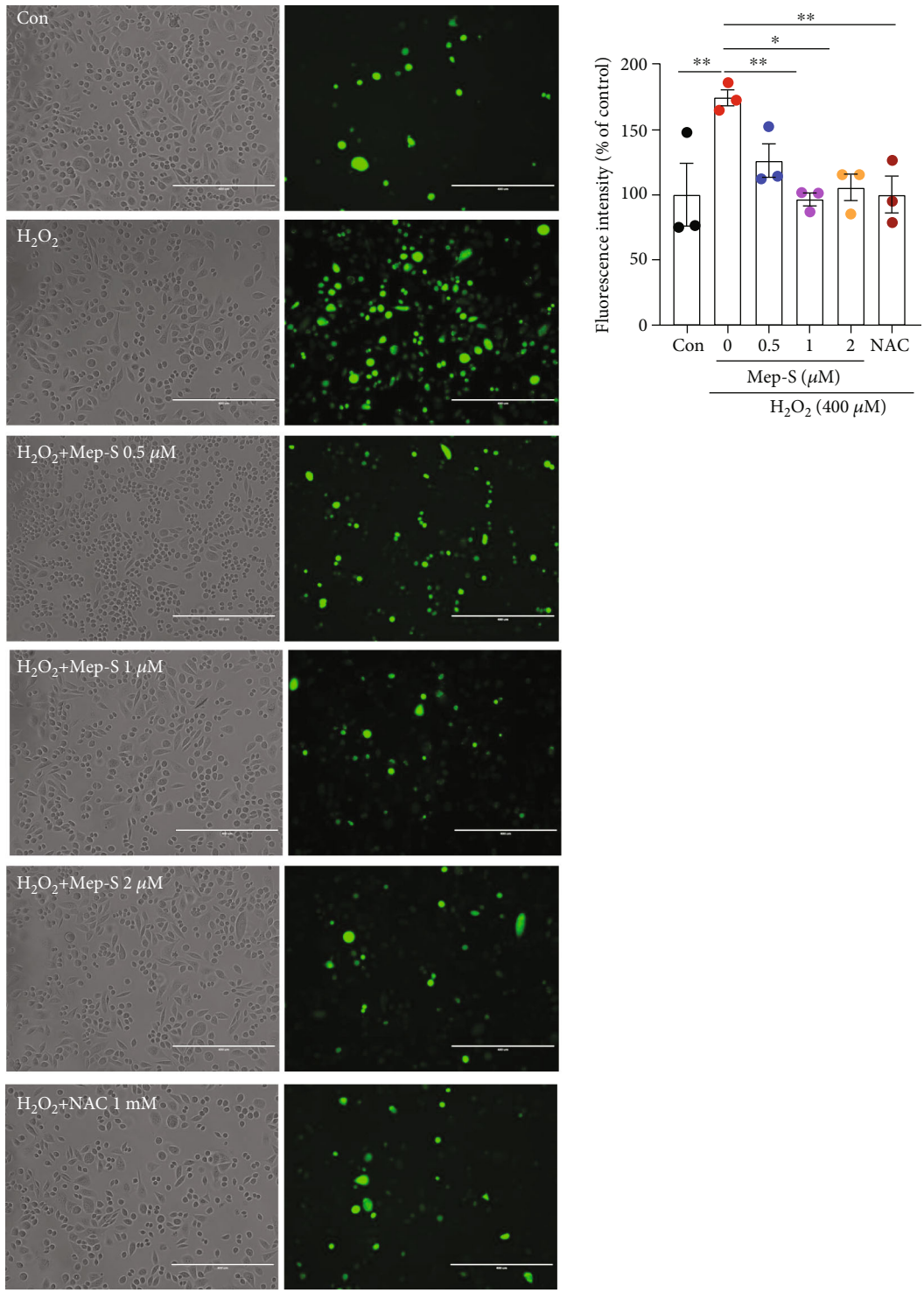


FIGURE 2: Mep-S protects against H_2O_2-induced neuronal apoptosis in SH-SY5Y cells. Mep-S inhibited H_2O_2-induced apoptosis (a) and loss of mitochondrial membrane potential (b) in SH-SY5Y cells. (c) Mep-S inhibited H_2O_2-induced upregulation of cleaved caspase-3 levels. The cells were pretreated with Mep-S (1 μM) or NAC (1 mM) for 1 h and then exposed to H_2O_2 (400 μM) for another 24 h. Data are presented as the mean ± SEM of 2-4 independent experiments. $^*P < 0.05$ and $^{**}P < 0.01$.



(a)

Figure 3: Continued.

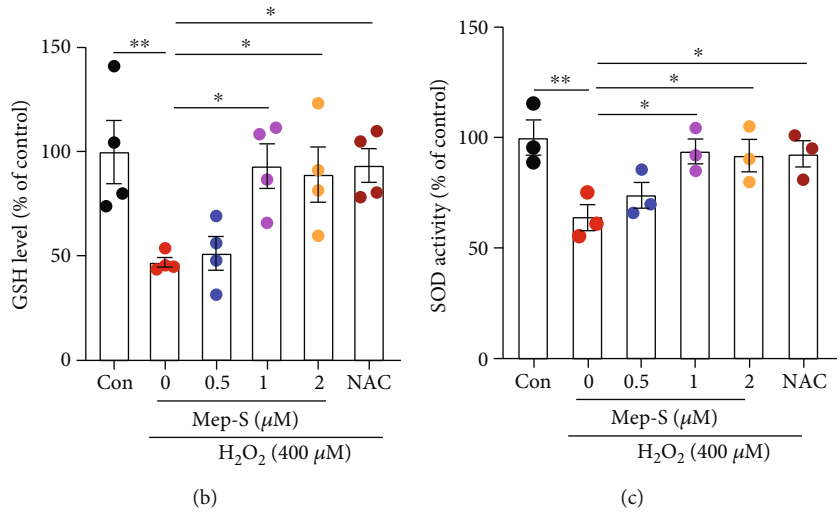


Figure 3: Mep-S inhibits H_2O_2-induced ROS generation and decreases GSH content and SOD activity in SH-SY5Y cells. (a) Mep-S inhibited H_2O_2-induced intracellular ROS accumulation, as demonstrated by decreased DCFH-DA fluorescence intensity. Scale bar = 400 μm. Mep-S inhibited the H_2O_2-induced decrease in GSH content (b) and SOD activity (c). The cells were pretreated with Mep-S (0.5-2 μM) or NAC (1 mM) for 1 h prior to stimulation with H_2O_2 (400 μM) for 2 h (a) or 24 h (b, c). Data are presented as the mean ± SEM of 2-4 independent experiments. $^*P < 0.05$ and $^{**}P < 0.01$.

nuclear Nrf2 level decreased after H_2O_2 stimulation compared with control group ($P < 0.05$ or $P < 0.01$). Mep-S or NAC pretreatment markedly inhibited the H_2O_2-induced reduction in HO-1, NQO-1, and nuclear Nrf2 levels as well as elevation of Keap1 and cytoplasmic Nrf2 levels compared to H_2O_2-treated group ($P < 0.05$). Phosphatidylinositol-3-kinase (PI3K)/Akt pathway is an important upstream regulator of Nrf2 signaling that promotes Nrf2 phosphorylation and nuclear translocation [25–27]. We found that Akt phosphorylation in H_2O_2-stimulated cells was overtly lower ($P < 0.01$ vs. control group). Mep-S or NAC pretreatment significantly suppressed this effect compared with H_2O_2-challenged group ($P < 0.05$).

It has been suggested that small-molecule regulators can enhance Nrf2 signaling through directly binding with Keap1 or Nrf2 to impair Keap1-Nrf2 PPI [28, 29]. We next performed molecular docking analysis to investigate whether Mep-S could affect PPI between Keap1 and Nrf2. As demonstrated in Figure 4(b), Mep-S inserted into the central pocket containing the Nrf2 binding domain and established multiple interactions with several key residues of Kelch domain in mouse Keap1. Its benzene ring established a π–π stacking interaction with Tyr334. The phenolic hydroxyl established a favorable hydrogen bond with Ser602. Additionally, the nitrogen in the indole ring established two hydrogen bonds with Val418 and Val465. Meanwhile, the hydroxyl group at the 5 position interacted with Val606 and Gly367 via three hydrogen bonds. These results indicate that Mep-S may bind to the Kelch domain of Keap1 to disrupt the Keap1-Nrf2 PPI.

3.5. Mep-S Attenuates Scopolamine-Induced Cognitive Deficits in Mice. Based on the *in vitro* neuroprotective effect of Mep-S, we subsequently examined whether Mep-S could enhance learning ability in scopolamine-challenged mice. The Morris water maze task was conducted to assess whether Mep-S could improve the hippocampus-dependent learning capacity. Two-way ANOVA showed overt effects of Mep-S treatment ($F(5, 270) = 12.50$) and training days ($F(4, 270) = 39.61$) on escape latency (both $P < 0.0001$). As demonstrated in Figures 5(a) and 5(b), control mice displayed gradually reduced escape latency from 64.40 s to 18.48 s over the 5 consecutive training days. Mice in the scopolamine-treated group showed longer latency (from 66.77 s to 52.10 s) over the training days ($P < 0.01$ vs. control group on days 3-5), suggesting that scopolamine induced an impairment of spatial memory. The latency on days 4 and 5 in mice pretreated with Mep-S (1, 2, or 5 mg/kg) was obviously shorter compared with scopolamine-challenged animals. The average swimming speeds of mice during the 5-day training sessions were similar among groups (two-way ANOVA, $F(5, 270) = 0.15$, $P = 0.98$ for Mep-S treatment and $F(4, 270) = 0.10$, $P = 0.98$ for days; Figure 5(c)). As shown in Figures 5(d) and 5(e), during the probe test on the 6th day, scopolamine-treated mice displayed markedly decreased proportions of swimming distance and time in target quadrant ($P < 0.01$ vs. control animals). In mice receiving Mep-S (2 or 5 mg/kg) pretreatment, the proportions of swimming distance and time in target quadrant were significantly higher ($P < 0.05$ vs. scopolamine-challenged mice). The average swimming speeds of mice on day 6 showed no significant difference between groups (Figure 5(f)). Mice did not show thigmotaxis during the Morris water maze test (data not shown).

3.6. Mep-S Attenuates Neuronal Apoptosis and Inhibits AChE Activity in Scopolamine-Challenged Mice. We subsequently detected whether Mep-S could attenuate scopolamine-induced neuronal death in brain. H&E staining

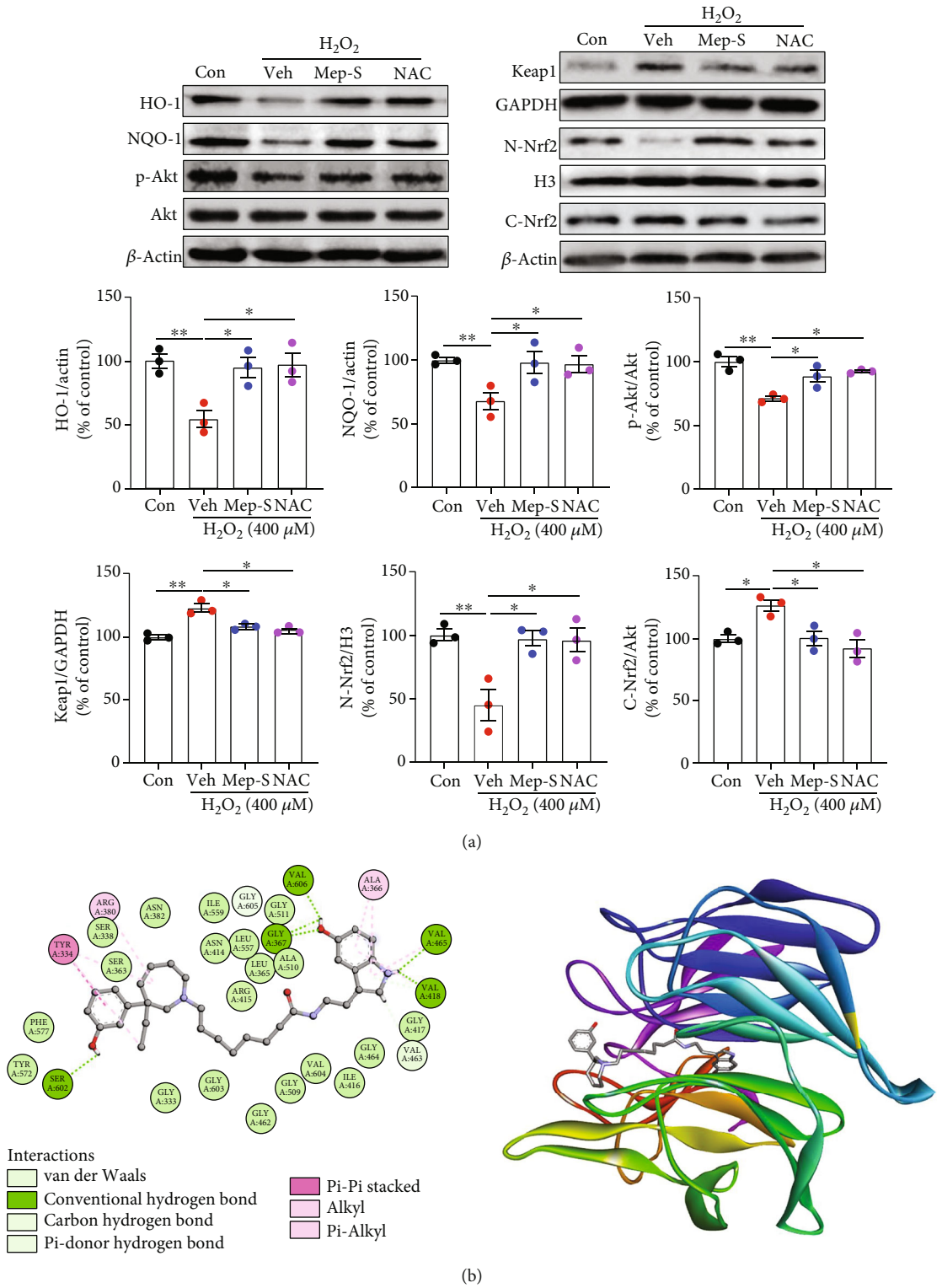


Figure 4: Effect of Mep-S on the Nrf2/antioxidant enzyme pathway in H_2O_2-stimulated SH-SY5Y cells. (a) Protein levels of HO-1, NQO-1, Keap1, Akt, phosphorylated Akt, and nuclear and cytoplasmic Nrf2 in SH-SY5Y cells detected by western blot analysis. The cells were pretreated with Mep-S (1 μM) or NAC (1 mM) for 1 h prior to stimulation with H_2O_2 (400 μM) for 24 h. Data are presented as the mean ± SEM of 3 independent experiments. One-way ANOVA: $F(3, 8) = 8.34$, $P = 0.0076$ (HO-1); $F(3, 8) = 5.35$, $P = 0.026$ (NQO-1); $F(3, 8) = 14.07$, $P = 0.0015$(p-Akt); $F(3, 8) = 21.24$, $P = 0.0004$ (Keap1); $F(3, 8) = 9.83$, $P = 0.0046$ (N-Nrf2); $F(3, 8) = 8.35$, $P = 0.0076$ (C-Nrf2). $^*P < 0.05$ and $^{**}P < 0.01$. (b) Interactions (left) and docking representation (right) of Mep-S with the Kelch domain of mouse Keap1 (PDB code: 6ZF4). Mep-S is shown as a gray stick model, and Keap1 is shown as a cartoon. In the 2D diagram (the left panel), key residues of Keap1 are represented as circular shapes and colored according to the interaction types.

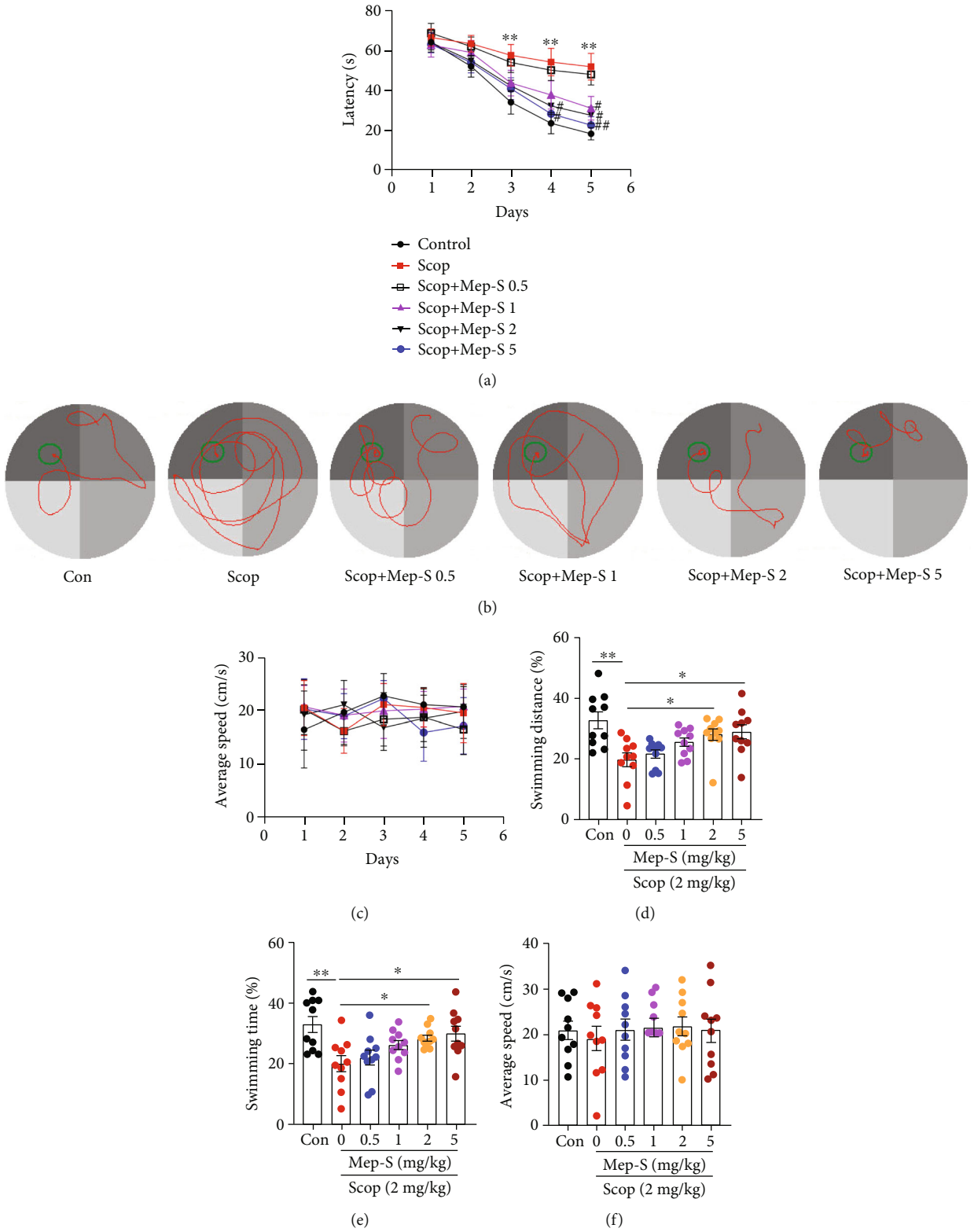


Figure 5: Mep-S improves scopolamine-induced learning and memory dysfunction in mice. The escape latency to the platform (a), swimming tracks (b), and average swimming speeds (c) of mice during the 5-day training sessions. $^{**}P < 0.01$ vs. control group; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs. scopolamine-treated group. The proportions of swimming distance (d) and time (e) in the target quadrant and average swimming speeds (f) of mice during the probe trial on day 6. One-way ANOVA: $F(5, 54) = 5.30$, $P = 0.0005$ (distance); $F(5, 54) = 4.89$, $P = 0.009$ (time); $F(5, 54) = 0.17$, $P = 0.97$ (speeds). Data are expressed as the mean ± SEM ($n = 10$). $^{*}P < 0.05$ and $^{**}P < 0.01$.

revealed markedly lower amount of surviving neuronal cells in CA1 and CA3 regions of hippocampus in mice receiving scopolamine challenge ($P < 0.01$ vs. control mice). Pretreatment with Mep-S at 2 and 5 mg/kg markedly inhibited the scopolamine-induced decrease in surviving neuronal number in mouse hippocampus (Figure 6(a)). We then examined the expression of some antiapoptotic and apoptotic markers. It was found that scopolamine reduced expression of the antiapoptotic protein Bcl-2 and enhanced expression of the apoptotic protein Bax, leading to decreased Bcl-2 to Bax ratios in mouse hippocampus and cortex ($P < 0.01$ vs. the control animals). Mep-S pretreatment resulted in enhanced Bcl-2 levels and reduced Bax levels, leading to increased Bcl-2 to Bax ratios in the hippocampus (2 mg/kg: by 0.81-fold, $P < 0.05$; 5 mg/kg: by 1.53-fold, $P < 0.01$) and cortex (2 mg/kg: by 0.76-fold, $P < 0.05$; 5 mg/kg: by 1.16-fold, $P < 0.01$) of mice relative to those in mice receiving scopolamine alone (Figure 6(b)). These results indicate that Mep-S attenuated scopolamine-induced neuronal apoptosis *in vivo*.

We also measured AChE activity in mouse brains. Brain AChE activity showed obviously between-group difference (one-way ANOVA, $F(4, 15) = 13.00$, $P < 0.0001$; Supplementary material, Figure S2). AChE activity in brain tissues markedly elevated in mice challenged with scopolamine ($P < 0.01$ vs. control group). Brain AChE activity in mice pretreated with Mep-S was markedly lower compared to that of mice receiving scopolamine alone ($P < 0.05$).

3.7. Antioxidant Effect of Mep-S via Activating the Nrf2/Antioxidant Enzyme Pathway in Scopolamine-Challenged Mice. We next detected the effect of Mep-S on brain oxidative status in scopolamine-treated mice. As shown in Figure 7, the total antioxidant capacity along with mRNA levels of SOD1, SOD2, and GCLC in brain tissues markedly declined in scopolamine-treated mice compared with the control mice. Mep-S markedly inhibited the scopolamine-induced effects ($P < 0.05$). Moreover, levels of HO-1, NQO-1, and phosphorylated Akt and Nrf2 in the hippocampal and cortex regions were lower in scopolamine-challenged mice than in control mice ($P < 0.05$). Mep-S at 5 mg/kg resulted in elevated levels of these proteins in mouse hippocampal and cortex regions ($P < 0.05$ vs. mice receiving scopolamine alone). Mep-S at 2 mg/kg led to elevated levels of HO-1 and Nrf2 in the hippocampus and cortex ($P < 0.05$ vs. mice receiving scopolamine alone). Scopolamine did not obviously affect Keap1 and Nrf2 levels. However, mice receiving Mep-S (2 and 5 mg/kg) treatment showed decreased Keap1 levels and increased Nrf2 expression in mouse hippocampal and cortex regions relative to mice receiving scopolamine alone (Figure 8). The results of immunofluorescence staining showed that scopolamine decreased Nrf2 immunoreactivity in mouse hippocampal region, while Mep-S (5 mg/kg) overtly counteracted this effect (Supplementary material, Figure S3).

4. Discussion

AD is a neurodegenerative disease associated with multiple etiological factors. As oxidative stress is a key mechanism for AD-associated neuronal injury [7], pharmacological interventions targeting oxidative neurotoxicity may be an important strategy for AD treatment. Here, we demonstrate that Mep-S, a novel multifunctional (-)-meptazinol-serotonin hybrid with the potential for Keap1-Nrf2 PPI inhibition, attenuates apoptotic neuronal death and memory dysfunction associated with oxidative stress. These effects may be exerted through activating Nrf2/antioxidant enzyme axis. Therefore, Mep-S may be a potential MTDL lead with antioxidant activity for the intervention of AD-like symptoms.

The brain, with high oxygen consumption, is especially at risk of oxidative stress [30]. ROS (including superoxide, hydroxyl radicals, H_2O_2, and singlet oxygen) are regarded as key factors for oxidative pathology [26, 31, 32]. In *in vitro* experiments, we examined the effect of Mep-S in H_2O_2-stimulated neuronal cells. The results showed that Mep-S inhibited H_2O_2-induced cytotoxicity in SH-SY5Y cells, as demonstrated with increased cell viability and decreased LDH release. Furthermore, Mep-S suppressed H_2O_2-induced neuronal apoptosis (detected by Annexin V/PI staining) by restoring MMP and inhibiting caspase-3 cleavage. These findings indicate a neuroprotective effect of Mep-S *in vitro*. We subsequently detected whether Mep-S attenuates oxidative stress trigger by H_2O_2. We found that Mep-S suppressed H_2O_2-induced intracellular ROS accumulation (detected by the fluorescence probe DCFH-DA) in neuronal cells. Moreover, the reduction in GSH content and SOD activity caused by H_2O_2 was markedly inhibited by Mep-S. These results suggest that Mep-S repressed H_2O_2-elicited oxidative neuronal damage, and the antioxidant effect may contribute to the neuroprotective effect of Mep-S.

The Nrf2/antioxidant enzyme pathway is essential for cells to prevent oxidative injury [27, 33]. Physiologically, intracellular Nrf2 level is controlled by the cytosolic repressor Keap1, which can promote ubiquitination and the following proteasomal degradation of Nrf2 via PPI between Keap1 and Nrf2. During the oxidative stress-triggered adaptive responses, Keap1 is inactivated due to a conformational change, facilitating Nrf2 dissociation from the Keap1-Nrf2 complex and nuclear translocation to bind to the antioxidant response element (ARE) and transcriptionally induce expression of antioxidant enzymes [34, 35]. However, overproduction of ROS may cause deregulation of redox balance and, thus, hinder nuclear translocation of Nrf2, resulting in impaired antioxidant defense responses [15, 36, 37]. Accordingly, enhancement of Keap1/Nrf2 signaling is reported to protect against AD-associated neurodegeneration and memory dysfunction by restoring redox homeostasis, and thus, this pathway may be an attractive drug target for AD treatment [24, 38, 39]. In addition, activation of this pathway contributes to the neuroprotective effect of bioactive agents in animal models of other neurological disorders, such as Parkinson's disease and traumatic brain injury [40, 41]. To get a deeper understanding of the mechanisms for neuroprotection of Mep-S, we explored whether Mep-S influences this pathway. We found that in H_2O_2-challenged SH-SY5Y cells, Mep-S promoted expression of HO-1 and NQO-1. Meanwhile, Mep-S reduced the protein level of Keap1 while

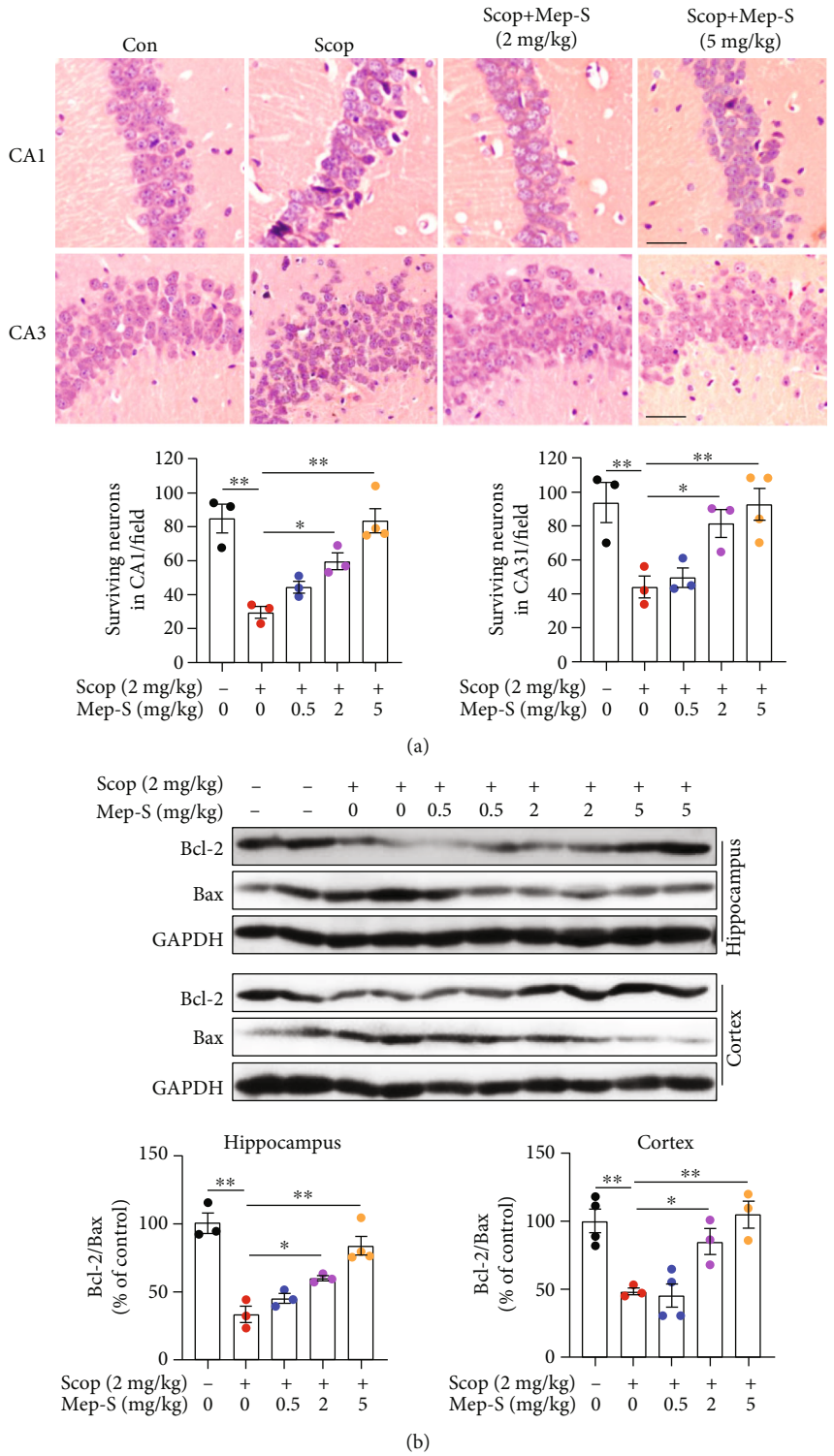


Figure 6: Mep-S attenuates neuronal apoptosis in the brains of scopolamine-treated mice. (a) Representative images of hematoxylin and eosin staining (upper panel) and quantitative analysis of the number of surviving neurons (lower panel) in the hippocampal CA1 and CA3 regions. Scale bar, 50 μm. One-way ANOVA: $F(4, 11) = 16.15$, $P = 0.0001$ (CA1); $F(4, 11) = 7.17$, $P = 0.0043$ (CA3). (b) Protein expression of Bcl-2 and Bax in the hippocampus and cerebral cortex of mice was determined by western blot analysis. One-way ANOVA: $F(4, 11) = 21.66$, $P < 0.0001$ (hippocampus); $F(4, 12) = 11.08$, $P = 0.0005$ (cortex). Data are presented as the mean ± SEM ($n = 3 - 4$ per group). $^{*}P < 0.05$ and $^{**}P < 0.01$.

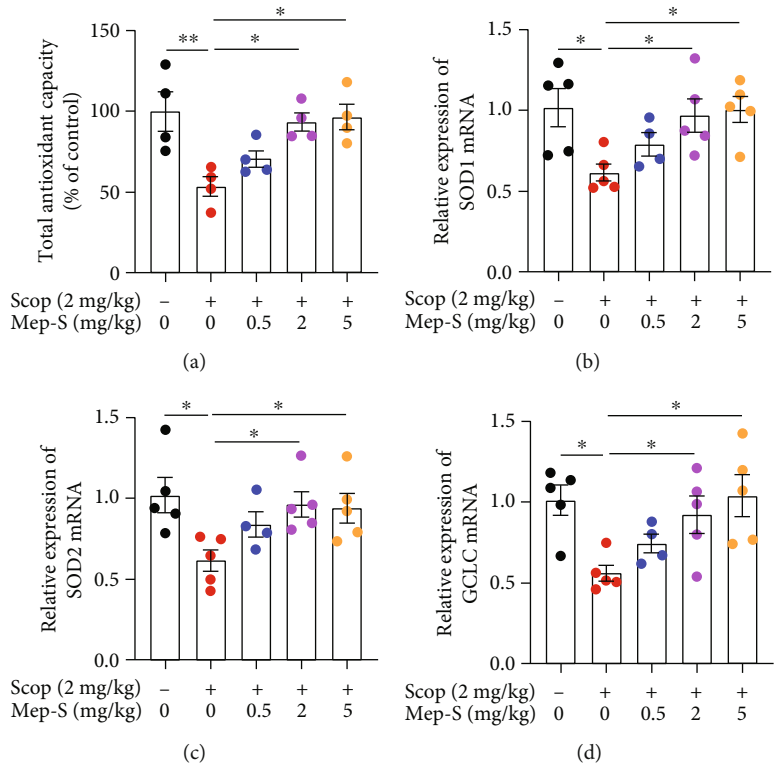


FIGURE 7: Effect of Mep-S on oxidative status in the brains of scopolamine-treated mice. (a) The total antioxidant capacity in brain tissues of mice. One-way ANOVA: $F(4, 15) = 6.49$, $P = 0.0031$.The mRNA levels of SOD1 (b), SOD2 (c), and GCLC (d) in the brain tissues of mice. One-way ANOVA: $F(4, 19) = 3.76$, $P = 0.020$ (SOD1); $F(4, 19) = 3.46$, $P = 0.028$ (SOD2); $F(4, 19) = 4.38$, $P = 0.011$ (GCLC). Data are presented as the mean ± SEM ($n = 4 - 5$ per group). $^{*}P < 0.05$ and $^{**}P < 0.01$.

promoted nuclear translocation of Nrf2. Therefore, Mep-S could attenuate oxidative neurotoxicity via enhancing Keap1/Nrf2/antioxidant enzyme signaling. It was also observed that Mep-S promoted Akt phosphorylation in H_2O_2-challenged SH-SY5Y cells. Since PI3K/Akt signaling can facilitate Nrf2 phosphorylation, thereby promoting its dissociation from Keap1 [25–27, 35], our results indicate that Akt activation may also be involved in Nrf2 activation induced by Mep-S. The *in vitro* and *in vivo* studies have reported that Keap1-Nrf2 PPI inhibitors protect neuronal functioning in AD [38, 39]. We next performed molecular docking simulations to explore whether Mep-S could directly disrupt the Keap1-Nrf2 PPI. It was shown that Mep-S can form a π–π stacking interaction with Tyr334 and form hydrogen bonds with Ser602, Val418, Val465, Val606, and Gly367 of the Keap1 Kelch domain at the Keap1-Nrf2 interface. These results indicate that Mep-S directly binds with Keap1 to interfere with Keap1-Nrf2 PPI, leading to Nrf2 activation. Taken together, the above results suggest that Mep-S ameliorates oxidative neuronal damage via enhancing Nrf2/antioxidant enzyme signaling, in which Mep-S may promote the separation of Nrf2 from Keap1 (by inactivating Keap1) and phosphorylation of Nrf2 (by activating PI3K/Akt axis).

Scopolamine can easily enter into the brain to block muscarinic acetylcholine receptors. It has been applied to elicit memory dysfunction in AD-associated animal experiments. It induces cholinergic dysfunction and increases Aβ and tau deposition in the central nervous system [42–44]. It also causes mitochondrial dysfunction and neuroinflammation [44]. Moreover, it causes oxidative stress and promotes neuronal apoptosis [15, 44, 45]. Therefore, scopolamine has been applied in animal models to elicit AD-like symptoms involving both cholinergic dysfunction and oxidative stress [15, 18]. We first performed behavioral experiments using the Morris water maze test to detect the effect of Mep-S in scopolamine-challenged mice. It was found that scopolamine-treated mice displayed cognitive dysfunction with impaired acquisition and retention of spatial memory, while Mep-S protected against scopolamine-induced cognitive deficits. Further experiments revealed that Mep-S improved the survival of hippocampal neurons and increased Bcl-2 to Bax ratios (an antiapoptotic index) in the hippocampal and cortex regions of scopolamine-challenged mice, indicating that the effect of Mep-S on scopolamine-induced AD-like symptoms may be associated with its protection against neuronal apoptosis.

We next examined whether the antioxidant activity of Mep-S could have a role in its *in vivo* effect. The total antioxidant capacity as well as expression of SOD1, SOD2, and GCLC (the rate-limiting enzyme for GSH synthesis [46]) in mouse brains decreased after scopolamine exposure, while Mep-S suppressed the effect of scopolamine. These results indicate that Mep-S could provide protection against scopolamine-induced oxidative neurotoxicity. To investigate the underlying mechanisms, the effect of Mep-S on the Nrf2/

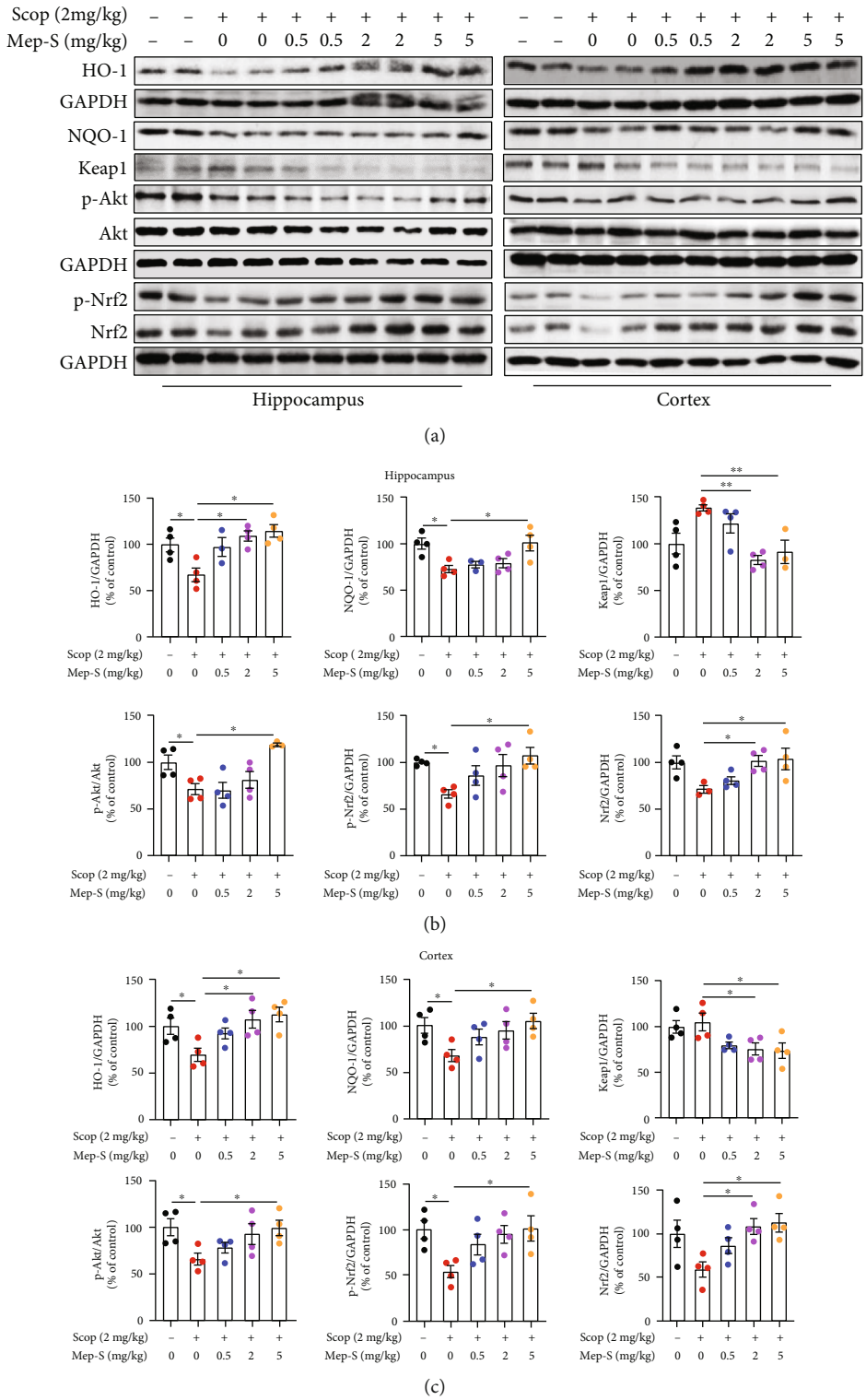


Figure 8: Mep-S activates the Nrf2/antioxidant enzyme pathway in the brains of scopolamine-treated mice. (a) Protein levels of HO-1, NQO-1, Keap1, and phosphorylated Akt and Nrf2 in the hippocampus and cerebral cortex of mice detected by western blot analysis. Densitometric analysis of protein expression in the Nrf2/antioxidant enzyme pathway in the hippocampus (b) and cortex (c) of mice in the control, scopolamine-treated, and scopolamine+Mep-S-treated groups. One-way ANOVA for hippocampus: $F(4, 14) = 6.58$, $P = 0.0034$ (HO-1); $F(4, 14) = 5.51$, $P = 0.071$ (NQO-1); $F(4, 14) = 6.97$, $P = 0.0026$ (Keap1); $F(4, 14) = 7.16$, $P = 0.0023$ (p-Akt); $F(4, 15) = 3.52$, $P = 0.0032$ (p-Nrf2); $F(4, 14) = 3.67$, $P = 0.030$ (Nrf2). One-way ANOVA for cortex: $F(4, 15) = 4.59$, $P = 0.013$ (HO-1); $F(4, 15) = 3.10$, $P = 0.048$ (NQO-1); $F(4, 15) = 4.08$, $P = 0.020$ (Keap1); $F(4, 15) = 3.18$, $P = 0.044$ (p-Akt); $F(4, 15) = 3.38$, $P = 0.037$ (p-Nrf2); $F(4, 15) = 3.87$, $P = 0.024$ (Nrf2). Data are presented as the mean ± SEM ($n = 3 - 4$ per group). $^{*}P < 0.05$ and $^{**}P < 0.01$.

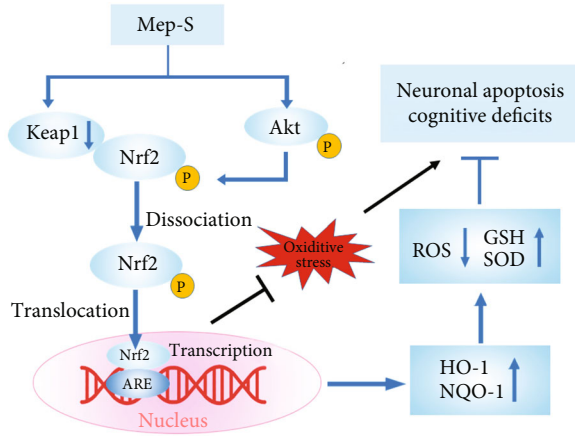


FIGURE 9: Proposed schematic summary of the protective effect of Mep-S against oxidative stress-mediated neuronal apoptosis and cognitive deficits. Mep-S promotes the nuclear translocation of Nrf2 to transcriptionally induce the expression of antioxidant enzymes such as HO-1 and NQO-1 by promoting the dissociation of Nrf2 from Keap1 by inactivating Keap1 and the phosphorylation of Nrf2 by activating Akt. The activation of the Nrf2/antioxidant enzyme pathway causes decreased ROS levels, increased GSH levels and SOD activity, and enhanced oxidant resistance, which may contribute to the protective effect of Mep-s against oxidative stress-mediated neuronal apoptosis and cognitive deficits.

antioxidant enzyme pathway was tested. As expected, Mep-S increased HO-1 and NQO-1 expression along with the levels of Nrf2 and phosphorylated Akt and Nrf2, accompanied with reduced Keap1 levels in the hippocampal and cortex regions of scopolamine-challenged mice. Collectively, the above findings indicate that the nootropic effect of Mep-S in the scopolamine-elicited cognitive dysfunction model may involve neuroprotection against oxidative stress. We also observed that Mep-S suppressed scopolamine-promoted AChE activity in mouse brains. Notably, evidence suggests an association between brain AChE activity and oxidative stress [17, 47]. Therefore, the inhibition of AChE activity by Mep-S could also contribute to neuroprotective and nootropic effects by attenuating oxidative neurotoxicity in the brains of scopolamine-treated mice, in addition to improving neurotransmission by elevating acetylcholine levels in the synaptic cleft.

Our research has some limitations. We only used male mice to explore the protection of Mep-S. Notably, both sexes are appropriate in AD research, and female animals may be more prominent since AD shows a higher incidence in women [48, 49]. Evidence shows that male and female animals exhibit similar variability of proxies for physiological and neurological outputs across multiple timescales [50]. In contrast, recent studies demonstrate that cognitive impairment of AD animals were detected in males but not in females [51, 52]. Therefore, sex differences should also be considered in the exploration of neuroprotective mechanisms. Since our results cannot reflect drug effect in females, future research is needed to investigate the potential of Mep-S for AD therapy in female AD models. In addition, although scopolamine have been used to induce memory dysfunction in animal models [15, 18], the pathological changes and cognitive impairment caused by scopolamine may not precisely mimic AD pathology and thus are not specific for AD. In other words, it only induces AD-like pathology and symptoms. Researchers are using transgenic AD animal models to evaluate the therapeutic effect of MTDLs [53]. Therefore, future studies using transgenic AD animal models are warranted.

Collectively, this work demonstrates that Mep-S, a potential Keap1-Nrf2 PPI blocker, attenuates apoptotic neuronal death and scopolamine-elicited memory dysfunction associated with oxidative stress. These effects could be exerted via enhancement of Nrf2/antioxidant enzyme signaling by promoting separation of Nrf2 from Keap1 by inactivating Keap1 and phosphorylation of Nrf2 by activating Akt (Figure 9). Additionally, it inhibits brain AChE activity to improve neurotransmission impairment and oxidative neuronal injury in scopolamine-treated mice. Therefore, Mep-S may be a potential lead for multitarget neuroprotective agents to treat AD-like symptoms.

Data Availability

Data in support of the observations of this work were included in the article and the supplementary files.

Ethical Approval

This study was performed following the principles of the Declaration of Helsinki and approved by the Ethics Committee of Shanghai Jiao Tong University School of Medicine (protocol code A-2019-039).

Disclosure

Faxue Zhao current address is Department of Cardiology, Pan-Vascular Research Institute, Shanghai Tenth People's Hospital, Tongji University School of Medicine, 301 Middle Yanchang Road, Shanghai 200072.

Conflicts of Interest

The authors declare no conflicts of interest.

Authors' Contributions

J.L., Y.Q., and H.C. were responsible for the conceptualization. J.L., Y.Q., and W.Z. were responsible for the methodology. F.Z., L.Z., Y.Z., X.T., Y.Y., and W.N. were responsible for the investigation. F.Z., L.Z., Y.Z., Y.Y., and J.L. were responsible for the data analysis. F.Z., L.Z., J.L., Y.Q., and W.Z. were responsible for writing the manuscript. J.L., Y.Q., and H.C. were responsible for the supervision. Faxue Zhao and Lin Zhao contributed equally to this work.

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Supplementary Materials

Supplementary Figure 1: The viability of SH-SY5Y cells treated with the indicated concentrations of Mep-S (A) or H_2O_2 (B) for 24 h. Data are presented as the mean ± SEM of 3-4 independent experiments. $^*P < 0.05$ and $^{**}P < 0.01$ compared with the control group. Supplementary Figure 2: Measurement of acetylcholinesterase (AChE) activity in mouse brains. After 6-day scopolamine treatment, AChE activity was determined in homogenates of brain tissues of mice in each group. Data are presented as the mean ± SEM ($n = 3 - 5$ per group). $^*P < 0.05$ and $^{**}P < 0.01$. Supplementary Figure 3: Representative photographs of immunofluorescence staining of Nrf2 (green) and DAPI (blue) in mouse hippocampus of the control, scopolamine-treated, and scopolamine+Mep-S-treated groups. *(Supplementary Materials)*

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Review Article

The Innate and Adaptive Immune Cells in Alzheimer's and Parkinson's Diseases

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Alzheimer's disease (AD) and Parkinson's disease (PD) are the most common neurodegenerative disorders of the central nervous system (CNS). Increasing evidence supports the view that dysfunction of innate immune cells initiated by accumulated and misfolded proteins plays essential roles in the pathogenesis and progression of these diseases. The TLR family was found to be involved in the regulation of microglial function in the pathogenesis and progression of AD or PD, making it as double-edged sword in these diseases. Altered function of peripheral innate immune cells was found in AD and PD and thus contributed to the development and progression of AD and PD. Alteration of different subsets of T cells was found in the peripheral blood and CNS in AD and PD. The CNS-infiltrating T cells can exert both neuroprotective and neurotoxic effects in the pathogenesis and progression. Here, we review recent evidences for the roles of innate and adaptive immune cells in the pathogenesis and progression of AD and PD.

1. Introduction

Alzheimer's disease (AD) and Parkinson's disease (PD) are the most common age-related neurodegenerative diseases in the world [1, 2]. AD is clinically characterized by progressive cognitive impairment, irreversible loss of memory, language disorders, and impairment of visuospatial skills. The hallmarks of AD pathology include extracellular aggregation of β-amyloid (Aβ) plaques and intracellular formation of neurofibrillary tangles (NFTs, also called tau aggregates), which inevitably lead to death of neurons and loss of synaptic transmission in hippocampal and cortical regions responsible for memory and learning [3–6]. Unfortunately, most cases of AD develop over tens of years without obvious symptoms of clinical dementia. Clinically apparent AD begins with mild cognitive impairment (MCI), which, after an average of 10 years, gradually progresses to moderate and severe AD. On the other hand, PD is characterized by progressive degeneration and loss of dopaminergic (DA) neurons in the substantia nigra and loss of nerve terminals in the striatum, which results in rigidity, bradykinesia, resting tremor, and postural instability [7–10]. The loss of DA neurons in PD is coupled with intracellular filamentous deposits called Lewy bodies, which consist of aggregates of

parkin, α-synuclein (α-Syn), phosphorylated neurofilament, and components of the ubiquitin-proteasome pathway [11, 12]. However, there are conflicting views about whether or not the large aggregates (amyloid plaques, NFTs, Lewy bodies) are responsible for neuronal death in AD and PD [12–15]. Although distinct from each other in clinical manifestations and pathological mechanisms, PD and AD are considered "protein misfolding" diseases due to deposition of improperly folded, modified protein aggregates in specific anatomical areas. Furthermore, the neurodegenerative processes in these two diseases are generally accompanied by neuroinflammation [13–16], which may play complicated roles in these diseases: both beneficial and detrimental, both cause and effect.

Neuroinflammation is a physiological response to protect CNS from exogenous and endogenous insults, but uncontrolled and prolonged inflammatory responses are detrimental to the CNS [16]. Traditionally, resident immune cells of the CNS (i.e., microglia) were thought to play a major role in this neuroinflammation [17]. Recently, it has also been demonstrated that both innate and adaptive immune cells from the periphery can penetrate through the blood-brain barrier (BBB) into the CNS and thus involved in the neuroinflammation [18, 19]. In the acute phase of neuroinflammation, these cells can exert protection by surveillance functions and maintaining homeostasis. Conversely, during chronic phase such as AD or PD, the cells may exert detrimental effect on neurons. However, how the innate and adaptive immune systems influence the onset and development of AD and PD remains elusive. Here, we review recent literatures related to the role of innate and adaptive immune cells in PD and AD.

2. Microglia in AD and PD

Innate immune cells include neutrophils, dendritic cells (DCs), monocytes/macrophages, natural killer (NK) cells, and other cells that are capable of rapidly generating inflammatory responses directed towards extra- and intracellular pathogens. So far as CNS is concerned, it should be extended some immune cells circulating in the cerebral spinal fluid and found in the brain (e.g., resident T cells, Astrocytes), as well as microglia, which were investigated most in CNS.

Microglia are resident immune cells which have been demonstrated to play essential roles in CNS tissue maintenance, injury response, and pathogen defense as well as recruitment of peripheral immune cells [20]. The conventional classification of microglia defined M1 phenotype as pro-inflammatory and neurotoxic, and M2 phenotype as anti-inflammatory and neuroprotective [21, 22]. Recently, accumulating evidence challenged this simplified classification and suggested microglia showed a range of intermediate states expressing mixed phenotypes in neurodegenerative diseases.

Recent evidence suggested that activated microglia can form a protective barrier around amyloid deposits making it less toxic, which can not only prevent new Aβ accumulation but also reduce axonal dystrophy in the nearby neuropil [23]. It was demonstrated that in the early stage of AD, activated microglia can serve as neuroprotective by releasing several proteases for clearance of Aβ plaque [24]. Moreover, the M2 phenotype was found to help in phagocytosis of Aβ plaques and creates a physical barrier to prevent further spreading of plaque [23]. Actually, acute microglia activation appeared to be neuroprotective, but the sustained chronic microglial activation seemed to be detrimental. Accordingly, such unbridled microglial malfunction may lead to overproduction of pro-inflammatory cytokines which will result in a shift towards M1 polarization contributing to the neurotoxic effect and synaptic loss resulting from the Aβ accumulation [25]. In addition, activated microglia can release some neurotoxic inflammatory cytokines such as TNF-α and INF-γ. However, their downstream signaling involves activation of MAPK and NF-κB which usually exerts neuroprotective effect [26]. With respect to PD, α-Syn was found to promote microglial phagocytic activity which was associated to the DA neurons loss in vivo [27, 28]. In PD patients, a negative correlation was observed between the microglial phagocytosis marker CD68 and the disease duration [29]. Interestingly, CD68 was overexpressed at early stage implying an increase in phagocytosis when cells start to die and tissue clearance is needed. Moreover, CD68 positive cells were found increased over the course of PD in substantia nigra (SN) which showed a more amoeboid cell shape suggesting that these cells were also in a pro-inflammatory state rather than just clearing dead cells [30]. Thus, whether a beneficial or harmful role that microglia plays in neurodegenerative diseases depends on their functional status (Figure 1).

Numerous receptors have been demonstrated to be involved in microglial stimulation and subsequent secretion of inflammatory cytokines. In the context of AD and PD, some members of the Toll-like receptor (TLR) family, which can recognize protein aggregates in these diseases, are involved in the disease progression [31]. As far as AD is concerned, TLRs have been demonstrated to contribute to the pathology by upregulation of mRNA levels in mice [32, 33] and different mechanisms activated by Aβ in microglial cells. For example, TLR2 and TLR4 expressed on microglia have been shown to play significant roles in the clearance of protein deposits in neurodegenerative disorders [32]. It has also been shown that plaque-associated microglia can upregulate mRNA expression of TLR4 and TLR2 in a mouse model of cerebral amyloidosis [34]. TLR4 has been verified to have beneficial roles in AD pathophysiology through Aβ phagocytosis in APP/PS1 mice [35]. Moreover, TLR4 stimulation can attenuate tauopathy in human tau transgenic mice [36] and TLR2 blockade can reduce gliosis and Aβ burden with associated improvement in learning in APP/PS1 mice [37]. In a recent study, TLR5 has been validated upregulated in the frontal cortex of moderate AD cases. GAS6, one ligand of TAM (Tyro3, Axl, Mer) family which play crucial roles in limiting inflammatory responses upon TLR stimulation, has also been demonstrated to exert negative impact on AD progression. In this study, the author found that upregulation of GSA6 induced by co-stimulation with Aβ and flagellin in THP-1 cells could be prevented by neutralization of TLR5 [38]. This study demonstrated presence of an immunosuppressive response in moderate AD cases, arguably mediated

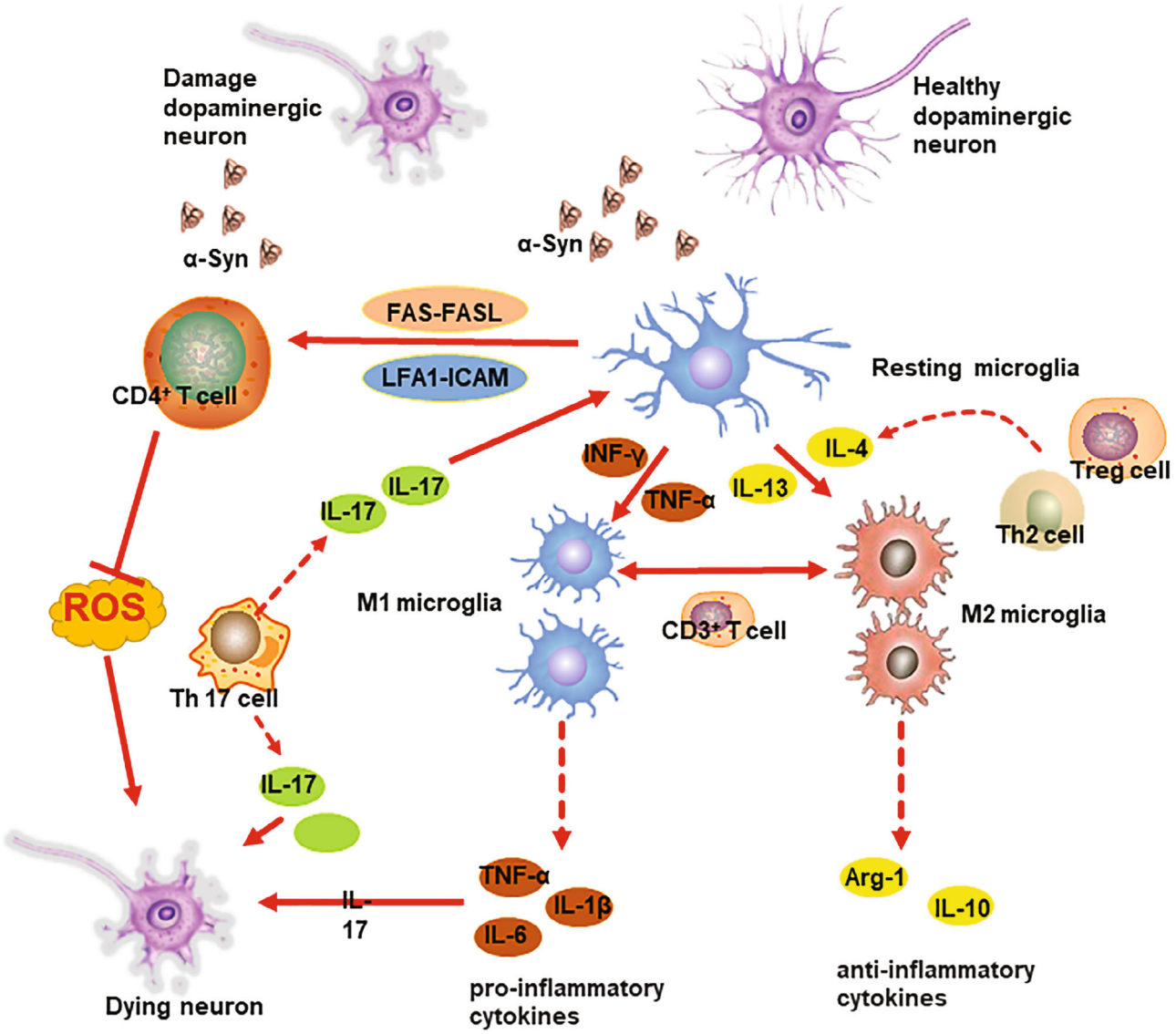

Figure 1: Potential mechanisms of T cell contributions to PD pathogenesis. T cells may directly contribute to PD pathogenesis through interaction of LFA1 with ICAM or by Fas-FasL signaling. T cells can also indirectly influence PD pathogenesis by mediating microglial transformation from a M2 phenotype (anti-inflammatory) to M1 phenotype (pro-inflammatory). Moreover, α-Syn deposits may be presented to activate T cells, thus initiating an autoimmune inflammatory response, which in turn exacerbates PD pathology by disturbing the balance of effector Th subsets. The production of IL-4 and IL-13, inducing alternative activation of microglia—known as the M2 state—can exert a protective effect against neuronal damage.

through the TAM system, and the potential implication of TLR5 signaling, upon prolonged immune stimulation in the presence of Aβ. In addition, this evidence also suggested that "TLRs" are engaged in disease progression inhibition.

It has been suggested that TLR4 also plays beneficial roles in PD by inducing α-Syn clearance from microglia, both in PD patients and mouse models [30, 39–41]. In addition, TLR2 has also been shown to be upregulated in microglia in both PD patients and transgenic Thy1.2-α-Syn mice [42–44]. Recently, several studies suggested that α-Syn secreted by neurons could stimulate TLR2 and induce subsequent inflammatory responses in microglia, thus contributing to neurodegeneration [44, 45].

As mentioned above, AD and PD are regarded as "protein misfolding" diseases due to characteristic aggregation of improperly folded modified proteins. The extracellular aggregation of Aβ is believed to be a crucial pathogenic mechanism in AD, resulting from an imbalance in the production versus clearance of Aβ, in which microglia may play an important role [46, 47]. Excessive production of amyloidogenic Aβ has been regarded as a major cause of early-onset AD (EOAD) [46, 47].

Genome-wide association studies (GWAS) have identified over 20 single-nucleotide polymorphisms (SNPs) that are strongly related to AD risk in late-onset AD (LOAD), also called sporadic AD, which constitutes the majority of

cases [48, 49]. Intriguingly, most of the identified risk genes for AD are expressed preferentially or selectively in microglia compared to other types of cells in the brain [50, 51]. In addition to these common variants, it has also been demonstrated that microglia can abnormally express some rare genetic variants related to AD [52, 53]. For example, a missense mutation (R47H) in triggering receptor expressed in myeloid cells 2 (TREM2), a cell surface protein highly and selectively expressed on microglia, was found to increase the risk of AD approximately three-fold [54, 55]. Traditionally, stimulation of microglial TREM2 through interaction with the activating adaptor protein DAP12 has been thought to initiate signal transduction pathways associated with enhanced proliferation, chemotaxis, and phagocytosis [56, 57]. TREM2 binds to apolipoprotein (APOE), encoded by the APOE gene, which is known to contribute to a significant fraction of the heritable risk for late-onset AD [57]. In addition, overexpression of wild-type TREM2 was sufficient to enhance uptake of low-density lipoprotein (LDL), Clusterin (CLU) (identified by unbiased protein microarray screen) in heterologous cells, whereas TREM2 disease variants were impaired in this activity. TREM2 knockout microglia showed reduced internalization of LDL and CLU. Aβ binds to lipoproteins and this complex is efficiently taken up by microglia in a TREM2-dependent fashion [58].

Consistent with these observations, researchers have found that TREM2-deficient microglia show diminished Aβ internalization in vivo and decreased uptake of Aβ-lipoprotein complexes in vitro [55, 59]. However, the impact of TREM2 deficiency has been unclear because studies have shown both increased and reduced Aβ deposition in TREM2-deficient AD mouse models [44, 56, 60]. A recent study using the APP/PS1-21 mouse model showed that TREM2 deficiency could ameliorate Aβ aggregation early in the disease but was associated with exacerbation in the late stage of disease [61]. In addition, it has been shown that microglia could form a protective barrier around Aβ aggregates and force amyloid fibrils into a tightly packed and potentially less toxic form, thereby reducing the accumulation of new Aβ deposits onto existing plaques and ameliorating axonal injury in nearby neurons [23]. Overall, studies related to TREM2 have so far suggested several mechanisms by which microglia may protect against Aβ aggregation and development of AD: phagocytosis of insoluble fibrillar Aβ aggregates, clearance of soluble Aβ species, and sequestration and compaction of Aβ plaques.

In addition to TLRs and TREM2, genome-wide association study (GWAS) has identified cluster of differentiation 33 (CD33) as another immune receptor conferring risk for AD. CD33 shows elevated expression in AD and functions as a modifier of microglial stimulation, thereby inhibiting Aβ clearance [62]. It has also been shown that the CD33 risk allele is associated with elevated expression of TREM2 on mononuclear phagocytes [63]. There is also strong evidence that elevated expression of CD33 is related to greater disease burden in PD [64].

Recently, it has been shown that the stimulation of microglia by α-Syn can in turn promote α-Syn phagocytosis [65]. Moreover, α-Syn phagocytosis can also be facilitated by leucine-rich repeat kinase 2 (LRRK2), a regulator of microglial response [66]. LRRK2 is one of the most commonly mutated genes in both familial and sporadic PD and can influence microglial internalization and degradation of α-Syn, thus exacerbating microglial pathology mediated by α-Syn [66].

Another crucial signaling pathway involved in microglial stimulation in PD is the nuclear factor-kappa B (NF-κB) pathway [67], which can promote microglial secretion of pro-inflammatory cytokines such as TNF-α and interleukin-1β (IL-1β) [68]. Cytokines released by stimulated microglia can attract peripheral immune cells to the brain.

Some studies have demonstrated that overexpression of α-Syn can induce microglial expression of major histocompatibility complex-II (MHC-II), which plays an essential role in both innate and adaptive immune responses in PD [69]. In addition, microglia can also express multiple receptors for neurotransmitters, including glutamate, acetylcholine, gamma-aminobutyric acid, norepinephrine, and cannabinoid receptors, all of which can exert neurotoxic or neuroprotective effects [21]. For example, cannabinoid receptor CB2 was found to be overexpressed in animal models of PD, conferring a neuroprotective effect [70, 71]. Moreover, it has also been demonstrated that cannabinoid agonists can exert protective effects against nigrostriatal neuronal loss in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of PD [72]. Another study found that senile plaques in AD patients express cannabinoid 1 (CB1) receptor together with markers of microglial activation, and that CB1-positive neurons, present in high numbers in control cases, are greatly reduced in areas of microglial activation. Cannabinoid agonists can inhibit Aβ toxicity in vivo as well as Aβ-induced microglial activation in vitro [73].

Together, both TLR family and CD33 are involved in the regulation of microglial function during the pathogenesis and progression of AD and PD. TREM2 and LRRK2, respectively, impact the microglial function in AD and PD. Moreover, microglia can also express multiple receptors for neurotransmitters to exert neurotoxic or neuroprotective effects. All these findings suggest that microglia play a role as double-edged sword in both the pathogenesis and progression of AD and PD. Targeting the regulation of microglial functions may be a promising therapeutic strategy.

3. Peripheral Innate Immune Cells in AD and PD

AD and PD have been historically viewed as diseases restricted to the brain even though neuroinflammation was regarded as a crucial component of these neurodegenerative disorders. Recently, the observation that the integrity of the BBB is compromised in neurodegenerative diseases provided a clue to explain the migration of peripheral immune cells into the brain [74]. Importantly, these immune cells may not only infiltrate into the CNS but may also return to the peripheral circulation, thus establishing a cycle between the periphery and the brain. It has already been established that a continuous cross-talk between the brain and the peripheral immune system exists [75]. This relationship suggested a

further hypothesis that neurodegenerative disorders such as AD and PD are systemic diseases in which the immune system exerts an influence on pathogenesis and progression.

3.1. NK Cells. As described above, the innate immune cell population includes NK cells, neutrophils, dendritic cells (DCs), monocytes/macrophages, and other immune cells. NK cells are potent cytotoxic effectors against pathogen-infected cells and tumor cells [75–77]. NK cells play a crucial role in bridging the innate and adaptive immune systems by secreting multiple cytokines and interacting with other immune cells. It was shown that NK cell populations did not differ among AD patients and age-matched healthy elderly controls [77]. However, there is evidence of increased NK cell activity in patients with amnestic mild cognitive impairment (aMCI), an antecedent to AD [76]. For example, elevated levels of TNFα, Interferonγ (IFNγ), and granzyme B coming from NK cells were observed in aMCI, compared with those of confirmed mild AD (mAD) patients. Cluster of differentiation 95 (CD95), a prototypical death receptor belonging to the TNF receptor superfamily that regulates tissue homeostasis through induction of apoptosis, was also observed to be increased in mAD and amnestic mild cognitive impairment (aMCI). Chemokine ligand 19- (CCL19-) dependent chemotaxis was reported to be decreased in aMCI and mAD patients, while Chemokine C Receptor 7 (CCR7) expression was increased in aMCI [76, 77]. We suggest that this state of activation reflects an innate immune response against an undefined challenge and may contribute to neuroinflammation. According to this assumption, rather than serving a protective role, NK cells may contribute to pro-inflammatory conditions in the setting of AD.

In other words, NK cells in peripheral blood of PD patients were found increased compared to those of non-PD patients [78]. Moreover, NK cells were also found in the PD mouse brain and substantia nigra of postmortem PD patients [79]. Recently, NK cells were demonstrated to efficiently internalize and degrade α-Syn aggregates via the endosomal/lysosomal pathway. Human NK cells were found capable to scavenge various forms of α-Syn species. Within NK cells, α-Syn aggregates are degraded and cytoplasmic α-Syn is colocalized with endosomal and lysosomal protein markers, implicating NK cells in the clearance of extracellular α-Syn [80]. It was also worth noting that NK cell cytotoxicity was attenuated and IFN-γ secretion was decreased along with the presence of α-Syn aggregates [80]. In addition, NK cells can interact with microglia to trigger NK cell-mediated cytotoxicity towards hyperactive microglia which is induced by sustained α-Syn burden [81]. Recently, an analysis about peripheral immune cells in PD observed an elevation in less cytotoxic NK cells and a decline in highly cytotoxic NK cells along with disease progression. Accordingly, this implies that NK cells may initially play a protective role in PD by the clearance of α-Syn pathology, which may further be lost with disease progression and decreasing NK cell activity [82].

3.2. Monocytes/Macrophages. In the blood, three subsets of monocytes have been identified based on the surface markers CD14 and CD16: the classical monocytes ($CD14^{++}/CD16^{-}$), the intermediate monocytes ($CD14^{++}/CD16^{+}$), and the non-classical monocytes ($CD14^{+}/CD16^{++}$) [83]. In neurodegenerative diseases such as AD and PD, the CNS is damaged which usually leads to an increase in the BBB permeability and thus favoring the peripheral monocytes infiltration. It has been demonstrated that Aβ can induce chemokine release such as monocyte chemoattractant proteins (MCPs) which can attract monocytes. After that, monocytes start to produce pro-inflammatory cytokines like IL-6 and TNF-a and improve their phagocytic capacity of toxic elements, including Aβ [84]. A recent study showed that Aβ-stimulated cell cultures from AD patients contained elevated numbers of inflammatory monocytes/macrophages. These cells could express TLR2, TLR4, IL-6, and CCR2, which could in turn promote the migration of monocytes/macrophages through the blood-brain barrier (BBB) into the brain [82, 83] (Figure 2).

The three subsets of monocytes may play different roles in AD. Non-classical monocytes ($CD14^{+}/CD16^{++}$) are observed decreased in AD patients compared to aMCI patients or healthy people suggesting a protective role of this subset in this disease [85]. Similarly, a progressive reduction of classical monocytes was observed in AD patients, also suggesting a more prominent role in this disease [86]. Moreover, a dysregulation of the monocyte subset distribution was also found in this study. However, it remains to be clarified whether this dysregulating distribution resulted from a shift of the monocyte phenotype or a progressive death of classical monocytes.

With respect to PD, monocytes were supposed to be a contributing role to DA neuronal loss [87]. Several studies have demonstrated an enrichment of classical monocytes in peripheral blood of PD patients, especially in those with a high risk of developing early dementia (HR-PD), of which monocytes express higher levels of TREM2 [88, 89]. Moreover, classical monocytes that express CCR2 were found to be enriched in peripheral blood of PD patients along with a strong reduction of CCR2-positive cells [90]. In addition, it was observed a relationship between CCR2+ monocytes and disease duration, suggesting a crucial role of the activation of CCL2-CCR2 axis in PD [91]. Considering CCR2 is important for peripheral monocytes to recruit to inflamed tissue, that the deletion of CCR2 failed in protecting DA neuronal loss in MPTP mouse model may suggest a potential neurotoxic role of monocytes [92]. Another remarkable phenomenon was that P11 protein, which is involved in depression, was found to be expressed almost 10-fold higher in monocytes than in the other leukocytes in PD patients who was experiencing depression many years after disease diagnosis. However, in the PD patients without depression, the expression of P11 protein in monocytes was not observed at the same high levels [93]. Accordingly, the P11 protein could be a potential biomarker for assessing the severity of PD, especially in those patients with depression.

3.3. Polymorphonuclear Neutrophils. The roles of polymorphonuclear neutrophils (PMNs) in the pathogenesis of AD have also been recently investigated. Neutrophils may exert effects during the initiation phases of AD pathogenesis,

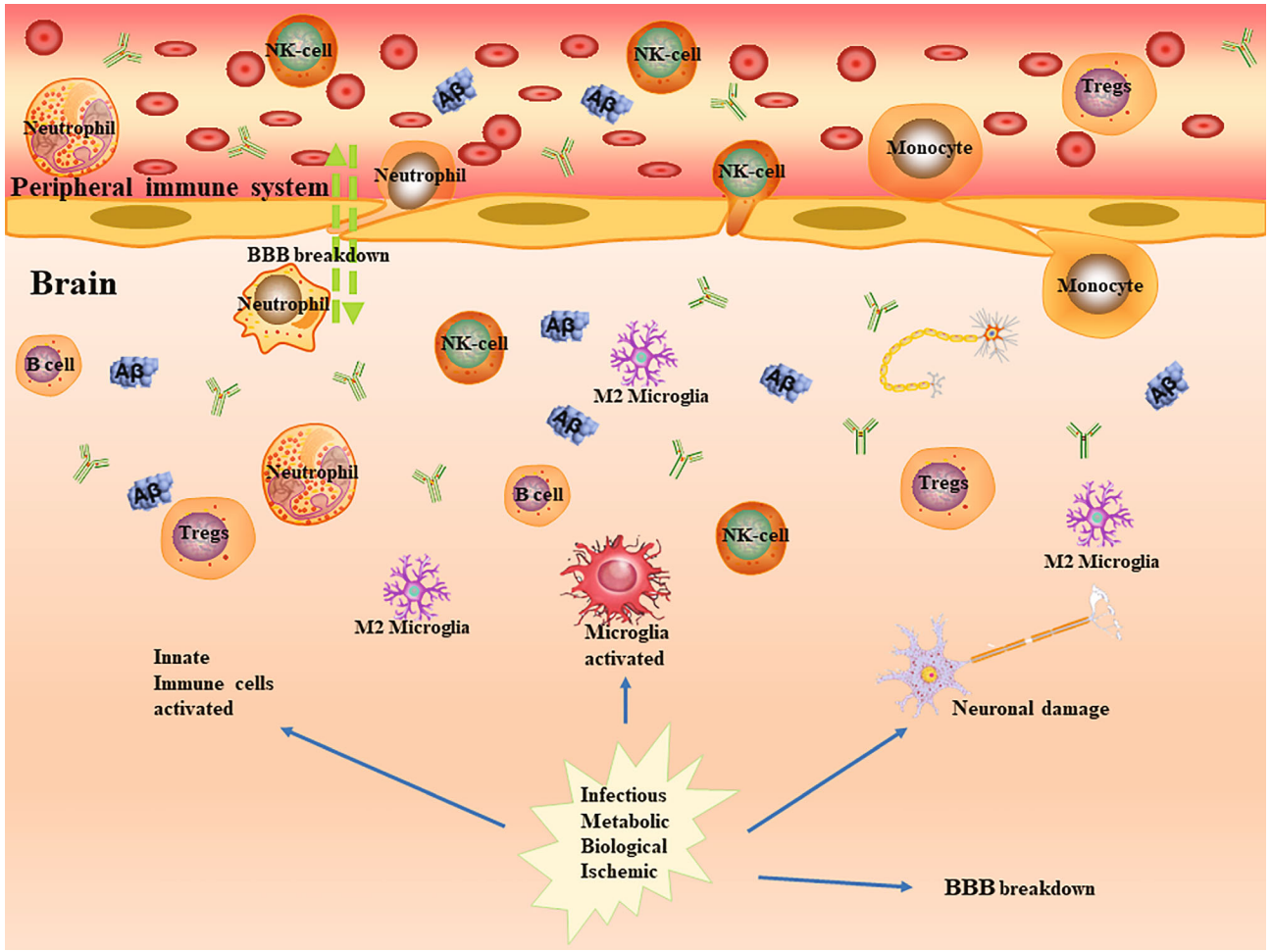

Figure 2: Peripheral innate immune cells and cerebral innate immune cells involved in the pathogenesis of AD. Insults begin in the brain along with impaired BBB function, promoting release of inflammatory mediators to the periphery. Peripheral innate immune cells become stimulated and infiltrate into the brain. These cells can either help to resolve or perpetuate inflammation. They can return to the periphery where they may amplify the inflammatory process.

although their role is controversial. Some studies reported reduced production of superoxide anion and diminished phagocytic capacity in AD, while others showed an increase [94]. Another study observed altered PMN function between healthy elderly individuals, aMCI subjects, and mAD patients [85]. For instance, CD177 was found to be overexpressed in mAD patients, while CD14 and CD16 expressions were reduced in PMN of mAD [77]. These data suggest altered PMN function in aMCI and mAD, which may be attributable to the variety of pathological stimuli associated with AD development.

With respect to PD, the number of neutrophils was found elevated in the peripheral blood of PD patients which seemed to occur years before diagnosis [95]. Several studies also found increased neutrophils in the peripheral blood during prodromal and clinical PD which is correlated with different symptomatic presentations [96, 97]. However, it remains to be clarified the role of the neutrophils in PD.

In summary, resident microglia have multiple roles in the CNS, including protecting the brain from various insults such as infection and injury and regulating inflammatory responses. Peripheral innate immune cells including NK cells and monocytes/macrophages have been demonstrated to interact with the brain. Considering the view that microglia are responsible for the maintenance of homeostasis and protection against pathogens, it is reasonable to hypothesize that sustained neuroinflammation plays a role in the pathogenesis of neurodegenerative diseases. This sustained neuroinflammation may result from the failure of microglia to either recruit innate immune cells to promote and sustain their activities or to terminate their activation after pathogens are eliminated.

4. Adaptive Immune Cells in AD and PD

Adaptive immunity includes humoral immunity dependent on the specific recognition of antigens by B cells and cellular immunity dependent on specific recognition of T cell receptors. Based on specific types of activities, T cells can be further categorized into CD8+ cytotoxic T (Tc) cells, which function to eliminate infected somatic cells, and CD4+ T helper (Th) cells that help regulate other immune cells.

Moreover, Th cells can be further subdivided into different effector T helper (Th) subgroups: immunosuppressive regulatory T cells (Tregs), pro-inflammatory Th9 cells, pro-inflammatory Th17 (secreting IL-17) and Th1 (secreting IFNγ) cells, and anti-inflammatory Th2 cells (secreting IL-4).

Increasing evidences suggest that T cells play crucial roles in the pathogenesis and progression of AD and PD. Studies of lymphocyte populations in blood from AD patients showed an elevated frequency of CD4+ T helper cells and reduced frequency of CD8+ cytotoxic T cells [98, 99]. However, a reduced frequency of CD4+ T helper cells and elevated frequency of CD8+ were detected in blood from patients with PD [98]. Another study reported that Tregs expressing fork head box P3+ (FoxP3+) and CD4+ were also increased in AD patients [100]. Moreover, Th9 cells and Th17 cells have also been found to be increased in AD patients [101]. Hypothesizing that Th17 cells and Tregs represent pro- and anti-inflammatory populations, respectively, these findings demonstrate that adaptive immune cell populations are altered in AD patients.

Analysis of peripheral blood from patients with PD showed distinct alterations in frequencies of lymphocyte subpopulations, which also suggested stimulation of adaptive immunity in PD pathogenesis. Some researchers studied the phenotype of circulating lymphocytes in 30 untreated and 34 levodopa-treated patients. They found that Th cells were decreased and activated, and CD4+CD25+ lymphocytes were increased which were independent of levodopa treatment [102]. Other researchers have found that CD4+ Th cells were decreased in PD patients' peripheral blood, of which CD4+CD29+ cells and CD4+CD45RO+ memory T cells were more decreased. Moreover, the reduction of CD4+CD29+ cells was correlated with clinical stage of PD [103]. Another study reported a lower ratio of IL-4/IFNγ-secreting Th cells in 33 PD patients, implying a shift towards a Th1-type immune response [104]. Moreover, elevated frequencies of IL-17-secreting cells were found in 29 PD patients and 18 patients with early PD, indicating a potential role for Th17 cells in the development and progression of PD [78, 105]. Tregs have also been demonstrated to be involved in the progression of PD. Some authors defined CD4+CD25+ cells as Tregs and found that these cells were decreased in PD patients [102, 104]. However, when FoxP3 and CD127 expression were taken into account, the frequency of Tregs defined as CD4+FoxP3+ or CD4+CD25+CD127- showed no differences between PD patients and healthy controls [106, 107]. Intriguingly, although not reduced in quantity, Tregs from PD patients in the aforementioned study showed impaired capacity to suppress proliferation of effector T cells [107]. Numerous factors may influence findings related to investigation of blood cell populations in PD patients, including patient age, medication use, and genetic variants [108–113].

Alterations of T cells in the peripheral blood of PD patients suggested a significant role in PD pathology, which gave rise to investigations on T cell infiltration into the CNS in human postmortem brain tissues and animal models of PD. Using the MPTP model of PD, Sommer et al. recently demonstrated that Th cells could cause neuronal death in the SN through factor-associated suicide/factor-associated suicide ligand (Fas/FasL) signaling [114]. Moreover, in the Thy1-WTS transgenic mouse model, CNS-infiltrating CD3+ T cells were demonstrated to contribute to PD pathogenesis by mediating microglial transformation from the anti-inflammatory M2 phenotype to the pro-inflammatory M1 phenotype, which were able to reduce microglial clearance of α-Syn deposits and thus promote α-Syn pathology [115]. Furthermore, this research team found that Th17 cells isolated from PD patients could aggravate neuronal cell death in co-cultures of iPSC-derived midbrain DA neurons [116]. Using the MPTP mouse model, it was recently demonstrated that Th17 cells could directly contact neurons through adhesion molecules and thus induce neurotoxicity [117]. So far as CD8+ T cells were concerned, the role remains unclear. It was found that CD8+ T cells infiltrated into the brain in response to increased α-Syn levels, which was early in the disease course prior to significant DA loss [118, 119]. This suggested that CD8+ T cells may contribute little to neuronal cell loss, but may contribute neuroinflammation in response to pathogenic changes of α-Syn aggregation.

Apart from neurotoxicity, T cells may also exert neuroprotective effects in neurodegenerative disease. Tregs have been demonstrated in vitro to suppress release of reactive oxygen species (ROS) from microglia and thus prevent ROS-induced neuronal damage [120]. This could explain, to some extent, how adoptively transferred activated Tregs could confer protection against neurotoxicity in another study using the MPTP mouse model [121]. Furthermore, a recent phase I clinical trial in a small cohort of PD patients has started to investigate sargramostim, a human recombinant granulocyte-macrophage colony stimulating factor that can stimulate Tregs-mediated suppression [122]. Excitingly, this trial has shown that sargramostim treatment for PD patients could boost the frequency of Tregs among total CD4+ T cells and enhance their suppressive capacity. More importantly, patients showed improvement on the unified PD rating scale III and magnetoencephalography-recorded cortical motor activities [122]. From another perspective, these observations also support the hypothesis that PD pathogenesis may result at least in part from an imbalance between anti- and pro-inflammatory effector T cell subsets.

The effects of CNS-infiltrating T cells in AD appear to be complex. It has been demonstrated that T cells may be neuroprotective through release of neurotrophic factors, stimulation of microglial phagocytic activity, and assistance in reducing Aβ deposition [123]. However, some Aβ-reactive T cells may also exacerbate AD progression through secretion of pro-inflammatory cytokines, thus leading to chronic inflammation [123]. Moreover, in the APP/PS1 mouse model of AD, Th1-released IFNγ was shown to impair cognitive function by promoting microglial stimulation and increasing Aβ aggregation. Treatment with an anti-IFNγ antibody could alleviate disease progression in APP/PS1 mice, which supported the view that Th1 cells may exert a neurotoxic effect in AD pathology [124]. In addition, Aβ-specific Th2 cells can prevent the production of cytokines by glial cells, and Aβ-specific T1 cells possess the properties of inducing the production of pro-inflammatory cytokines

by microglial cells [123]. According to these evidences, we speculate that different stages of AD progression have distinct profiles of T cell subpopulations and that the immune cells may play contradictory roles at different stages of AD.

These evidences suggest that alteration of T cells in the peripheral blood in AD and PD plays a significant role in pathology. And CNS-infiltrating T cells in AD and PD can exert both neuroprotective and neurotoxic effects.

5. Conclusion

Taken together, numerous sources of evidences demonstrated the contributions of innate and adaptive immune cells to the pathology of neurodegeneration in AD and PD. Numerous evidences suggested that TLR family, TREM2, CD33, and LRRK2 were involved in the regulation of microglial function in the pathogenesis and progression of AD or PD, making it as double-edged sword in these diseases. Altered function of peripheral innate immune cells has also been demonstrated in AD and PD which can interact with the brain, and modulating the functions of these cells may become a beneficial approach to modify the progression of neurodegeneration. Alteration of T cells in the peripheral blood has been found in AD and PD, and CNS-infiltrating T cells can exert both neuroprotective and neurotoxic effect in the pathogenesis and progression. In addition, an imbalance of Tregs and pro-inflammatory T (Th17 or Th1) cells, which can impair neuroprotective effects and induce neuronal damage, is associated with neurodegeneration. Thus, developing compounds that target peripheral innate immune cells or promote expansion of neuroprotective Tregs and anti-inflammatory T cells may also be promising approaches for the treatment of AD and PD, as examples of neurodegenerative diseases with underlying neuroinflammation.

Conflicts of Interest

There are no conflicts of interest to disclose relevant to this paper.

Authors' Contributions

Zhang HB and Huang BY wrote the initial draft. Figures were prepared by Ye YZ. Gu LJ prepared the final version. Xiong XX and Zhang SZ recommended a structure for the review and substantially advanced the draft. Boyuan Huang and Zhenxin Yan contributed equally to this work.

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Review Article

Neuroprotective Treatments for Digestive Forms of Chagas Disease in Experimental Models: A Systematic Review

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Chagas disease is an anthropozoonosis caused by the protozoan *Trypanosoma cruzi* and is characterized as a neglected disease. It is currently endemic in 21 countries on the Latin American continent, including Bolivia, Argentina, and Paraguay. Unfortunately, there are no optimally effective treatments that can reduce the damage caused in the digestive form of the disease, such as the neuronal destruction of the myenteric plexus of both the esophagus and the colon. Therefore, the objective of this systematic review was to report the possible pharmacological neuroprotective agents that were tested in murine models of the digestive form of Chagas disease. Inclusion criteria are *in vivo* experimental studies that used different murine models for digestive forms of Chagas disease related to pharmacological interventions with neuroprotective potential, without year and language restriction. On the other hand, the exclusion criteria were studies that did not approach murine models with the digestive form of the disease or did not use neuroprotective treatments, among others. The search in the PubMed, Web of Science, Embase, and LILACS databases was performed on September 4, 2021. In addition, a manual search was performed using the references of the included articles. The risk of bias assessment of the studies was performed based on the SYRCLE tool guidelines, and the data from the selected articles are presented in this review as a narrative description and in tables. Eight articles were included, 4 of which addressed treatment with acetylsalicylic acid, 3 with cyclophosphamide, and 1 with Lycopodium clavatum 13c. In view of the results of the studies, most of them show neuroprotective activity of the treatments, with the potential to reduce the number of damaged neurons, as well as positive changes in the structure of these cells. However, more studies are needed to understand the mechanisms triggered by each drug, as well as their safety and immunogenicity. Systematic review registration is as follows: PROSPERO database (CRD42022289746).

1. Introduction

Chagas disease (CD), caused by the flagellate protozoan *Trypanosoma cruzi*, represents a neglected disease that affects 8 to 11 million people worldwide. In general, the disease can be divided into two phases: acute with nonspecific symptoms in most cases and chronic, which can be symptomatic or asymptomatic [1]. In the symptomatic chronic phase, the disease is related to the development of cardiac and/or digestive tract changes (megaesophagus and/or megacolon). Digestive forms comprise up to 10-21% of symptomatic CD cases, with megaesophagus having the highest incidence,

followed by megacolon [2]. Unfortunately, there is a lack of effective treatments at this stage [3].

Although the pathogenesis of the digestive forms is not so clear, it is suggested that the inflammation induced by the infection is one of the essential points for the progression of the disease, mainly because it affects neurons of the myenteric plexus in both the esophagus and the colon [4]. Studies report that *T. cruzi* infection in experimental models induces neuronal destruction, starting in the acute phase. Thus, it is suggested that immune system components such as macrophages, NK cells, eosinophils [5], nitric oxide (NO) and IFN-γ play a role in neuronal destruction [6–9]. In addition, homeostasis-related components of the enteric nervous system have also been reported to be altered after infection.

While neuronal destruction occurs, processes such as neuronal hypertrophy or atrophy increase in the wall of the esophagus and colon and in the muscular layers of these organs which have also been reported. With the passage of time, all these processes culminate in the alteration of the functioning of the organ, loss of peristalsis, and, consequently, the stoppage of the passage of food or fecal bolus.

Isosorbide and nifedipine are drugs tested in clinical trials and used for the treatment of megaesophagus in humans, with the aim of improving the passage of food through the organ [10–14]. Although the use of isosorbide has shown lower rates of esophageal retention and severity of dysphagia, few studies have actually evaluated the impact of these treatments [15]. In addition, side effects such as headache are common during treatment with isosorbide, which decreases patients' adherence to therapy [16]. Pneumatic dilatation or surgery is also indicated depending on the stage of the megaesophagus. For chagasic megacolon, changes in life habits, such as diets rich in fiber and high-water intake, are indicated. Pharmacologically, laxatives are also used. Surgical interventions are only used in severe cases, such as severe refractory constipation and other complications [2]. Thus, most treatments for CD help with the symptoms of digestive forms and can be invasive.

Due to this problem, there is a need to develop new alternatives for the treatment of digestive forms that aim to destroy the parasite and reduce the inflammatory response and consequently neuronal protection. Therefore, the purpose of this systematic review is to report the possible pharmacological neuroprotective agents that were tested in experimental animal models for CD, in its digestive form.

2. Methods

2.1. Protocol and Record of the Systematic Review. The present systematic review was conducted in accordance with the methodological guidelines proposed by the Key Items for Reporting Systematic Reviews and Meta-analyses (PRISMA) [17]. The protocol of this review was registered in the database called PROSPERO (International Prospective Register of Systematic Reviews), with registration number CRD42022289746.

2.2. Eligibility Criteria. The development of the systematic review in question was based on a guiding question: "Are there pharmacological interventions that prevent neuronal loss in the myenteric plexus in digestive forms of CD in experimental murine models?". Thus, for the assembly of the search strategy and the establishment of eligibility criteria, the acronym model PICOT (population, intervention, comparator, outcome, and types of studies) was used:

P: murine models of digestive Chagas disease
I: pharmacological treatment
C: no treatment (control group)
O: neuroprotection
T: *in vivo* studies

Therefore, only experimental *in vivo* studies were included, which used different experimental models for digestive forms of CD related to pharmacological interventions with neuroprotective potential. For the exclusion criteria, the following points were followed:

(1) Not murine models of digestive forms of Chagas disease

(2) Not pharmacological treatment with neuroprotective potential in acute or/and chronic phases of Chagas disease

(3) Studies that focus on treatment and do not assess neuronal counts

(4) Studies that do not use pharmacological treatment and evaluate neuronal counts (example: effect of physical activity and neuronal protection)

(5) Studies that do not compare infected and treated animals with infected and untreated animals

(6) Letter to the editor, editorial, conference documents, commentary, news, descriptive and systematic reviews, and book chapters

(7) Any measurement that does not show a biological effect

2.3. Sources of Information and Search. According to the indications of the Peer Review of Electronic Search Strategies (PRESS) [18], the search strategy was developed and submitted for evaluation by a subject specialist. For setting up the strategy (presented in supplementary materials S1), the PubMed database was considered as the standard, and year of publication and language were not considered as exclusion factors.

To carry out the bibliographic search, four research bases were used: PubMed, Web of Science, Embase, and LILACS. The details of the searches in each database are exposed in supplementary materials. In addition to these bases, manual searches focused on the reference list of the included articles were performed. After the search was completed, duplicate articles were tracked and removed using the EndNote X9® program.

2.4. Selection of Studies and Data Extraction. The first step of article selection was performed by two evaluators (J.R.C.N and R.O.G) independently and blindly. The titles and abstracts of all articles obtained through the search were evaluated for inclusion or exclusion. To assist in this step, the Rayyan–Intelligent Systematic Review program was used. After analyzing

the articles, possible disagreements between the reviewers were agreed upon in a discussion between them.

In the second step, the articles selected in the first step were transferred to an Excel table with the following information: authors and year, article title, inclusion or exclusion, final status, and justification for exclusion. Then, only articles focusing on the use of some intervention with neuroprotective potential in murine models for digestive forms of CD were included. All those who did not meet the inclusion criteria were excluded. The reviewers performed this step blindly and independently (J.R.C.N and R.O.G). In addition, possible disagreements between the reviewers were agreed upon in discussion between them.

Relevant data were extracted from all included studies by two independent evaluators (J.R.C.N and R.O.G). Thus, the information collected in the *in vivo* studies was as follows: intervention used, experimental model, groups evaluated and number of animals used, strain used, route of infection/inoculum used, evaluation phases, form of induction of the chronic phase, treatment regimen, concentration of the intervention used/treatment route, mortality rate, organ and region evaluated, methodology used for neuronal analysis/analyzed region, number of fields and neurons analyzed, number of neurons per group, area of the neuronal body per group (μm^2 or cm^2), neuronal cytoplasm area per group (μm^2), neuronal nucleus area per group (μm^2), other observed biological phenomena, and reference.

The WebPlotDigitizer tool was used to obtain apparently hidden data in the article. Through this tool, it was possible to extract values present only in graphs.

2.5. Risk of Bias in In Vivo Studies. To assess the risk of bias, the Systematic Review Center for Laboratory Animal Experimentation (SYRCLE) tool was used [19]. This step was also performed by two reviewers independently (J.R.C.N and R.O.G). Possible disagreements between the reviewers were agreed upon in discussion between them. The tool consists of six categories: selection bias, performance bias, detection bias, attrition bias, reporting bias, and other sources of bias. Each category had some questions, which are exposed in supplementary materials, totaling 10 to help reviewers classify each article included. For each question, it was necessary to answer "yes," "no," or "uncertain," with each of these judgments corresponding to a color: red, green, or yellow, respectively. The 10 questions used for the in vivo risk of bias assessment are listed in Supplemental Materials S2.

2.6. Synthesis Methods. The main findings of the studies were presented through a narrative description, and, whenever possible, a comparison between them was performed. In addition, data from the articles (in topic 2.4) were tabulated (Table 1). Statistical analyses such as meta-analysis, heterogeneity, and sensitivity analyses were not applied.

3. Results

3.1. Search for PRISMA Studies and Flowchart. The search in the databases for studies that evaluate different neuroprotective interventions for digestive forms of CD in experimental models resulted in 419 articles. In addition to this amount, 2 articles were obtained from a manual search through the reference list of articles, totaling 421. In the duplicate article tracking step, 142 articles were excluded, with a total of 279 for analysis. Then, analysis by title and abstract was performed, totaling 9 potential articles included and 270 excluded. Of those included in the previous step, the full articles were read and only 8 studies were included, with the exclusion of 1. The exclusion of the article was based on the nonuse of an experimental model for Chagas disease. Thus, 8 articles were considered eligible and followed with the qualitative analysis (Figure 1). The list of screened articles and the final status of each are listed in the Supplemental Materials S3.

3.2. Study Characteristics. The first article focusing on neuroprotective interventions for the digestive forms of CD in experimental models was published in 2006. As of that year, 2017 was the year with the highest number of publications (3), representing 37.5% of articles. The last article published on the topic was in 2019.

To conduct the experiments, only two strains were used to infect the animals: strain Y (5-62.5) and Morc-1 (3-37.5%) (Figure 2(a)). Finally, the colon (4) and esophagus (4) were evaluated in the same number of articles (Figure 2(b)).

Three different interventions were used in the included articles: acetylsalicylic acid (ASA) (4), cyclophosphamide (3), and Lycopodium clavatum 13c (LC) (1). Thus, ASA is the most focused intervention in the studies, with 50%, followed by cyclophosphamide, with 37.5% and LC with 12.5% (Figure 2(c)).

Furthermore, to assess the impact of each intervention, three experimental models were approached: *Mus musculus* (Swiss mice) (4), *Calomys callosus* (3), and *Rattus norvegicus* (Wistar lineage) (1). Swiss mice represented the most used model in 50% of the articles, followed by *Calomys callosus* (37.5%) and *Rattus norvegicus* (Wistar lineage) (12.5%) (Figure 2(d)).

3.3. Risk of Bias Assessment of In Vivo Studies. For the assessment of risk of bias, all 8 articles included were analyzed. As shown in Figure 3, most articles did not clearly address the selection bias criteria (items 1, 2, and 3), detection (items 6 and 7), and others (item 10). Within these items, the lack of exposure and reporting of allocation criteria, baseline characteristics such as initial animal weight, allocation concealment, random housing, blinding of caregivers and outcome assessors, and randomization of animals were unclear. Finally, 75% of the selected works did not declare information regarding a possible conflict of interest.

3.4. Effects of Interventions on Experimental Models. The effects of the interventions were subdivided according to the type of intervention in each study, they are arranged below, and the main information is summarized in Table 1.

3.4.1. Lycopodium Clavatum 13c (LC). Among the eight articles, LC was evaluated in only one study [20].

Table 1: Summary of the main data regarding interventions with neuroprotective potential in experimental models of the digestive form of Chagas disease.

Intervention	Experimental model	Evaluated groups and number of animals used	Strain	Route of infection/ inoculum used	Phases	Chronic phase induction method	Treatment schedule	Intervention concentration of/treatment route	Mortality rate	Organ and region evaluated	Methodology used for neuronal analysis/ analyzed region	Number of fields and neurons analyzed	Number of neurons per group	Neuronal body area per group (μm^2 or cm^{2*})	Area of neuronal cytoplasm per group (μm^2)	Neuronal nucleus area per group (μm^2)	Reference
Cyclophosphamide (CY)	*Calomys callosus*	Not infected (NI) (5) Infected without treatment (IC) (5) Infected treated with cy (IC-cy) (5)	MORC-1	Intraperitoneal 1×10^5	Chronic	Natural time of infection	3 consecutive days	0.2 mL of a 0.4 mg/mL solution of the drug in water/ orally	Not informed	Distal esophagus	Cresyl violet staining myenteric plexus	Performed in the total area between the inner and outer muscles of the esophagus	NI: 59 ± 30.125 IC: 34 ± 19.937 IC-cy: 39 ± 9.910	NI: 28.66 ± 9.08 μm^2 IC: 29.29 ± 8.56 μm^2 IC-cy: 35.12 ± 11.54 μm^2	Unvalued	Unvalued	[22]
Lycopodium clavatum 13c (Ly)	*Rattus norvegicus*, Wistar lineage	IC ($n = 21$) Infected treated with Ly (IC-Ly) ($n = 21$)	Y	Intraperitoneal 5×10^6	125 days (acute phase) 322 days (chronic phase)	Natural time of infection	2 days before infection and on days 2, 5, and 8 postinfection	10 μL/mL water *ad libitum*	Not informed	Intestine proximal (PC) and distal (DC) colon	GIEMSA staining myenteric plexus	120 fields for neuron quantification 300 neurons for cell body, cytoplasm, and nucleus measurement	IC PC 125 days: 96.6 ± 18.5 IC PC 322 days: 103.7 ± 27.9 IC-Ly PC 125 days: 141.9 ± 57.0 IC-Ly PC 322 days: 107.8 ± 16.1 IC DC 125 days: 116.7 ± 13.3 IC DC 322 days: 52.9 ± 8.1 IC-Ly DC 125 days: 114.4 ± 13.0 IC-Ly DC 322 days: 93.2 ± 9.0	IC PC 125 days: 184.4 ± 135.5 IC PC 322 days: 344.4 ± 163.0 IC-Ly PC 125 days: 339.2 ± 133.0 IC-Ly PC 322 days: 403.4 ± 206.5 IC DC 125 days: 131.0 ± 108.8 IC DC 322 days: 361.9 ± 184.7 IC-Ly DC 125 days: 201.5 ± 103.9 IC-Ly DC 322 days: 358.3 ± 238.3	IC PC 125 days: 122.8 ± 102.1 IC PC 322 days: 241.0 ± 135.7 IC-Ly PC 125 days: 229.1 ± 113.0 IC-Ly PC 322 days: 291.7 ± 176.2 IC DC 125 days: 88.8 ± 79.2 IC DC 322 days: 260.2 ± 157.6 IC-Ly DC 125 days: 139.1 ± 86.4 IC-Ly DC 322 days: 256.8 ± 205.5	IC PC 125 days: 61.5 ± 42.6 IC PC 322 days: 103.4 ± 43.0 IC-Ly PC 125 days: 110.1 ± 37.6 IC-Ly PC 322 days: 111.6 ± 42.7 IC DC 125 days: 42.2 ± 35.2 IC DC 322 days: 101.6 ± 41.0 IC-Ly DC 125 days: 62.4 ± 31.2 IC-Ly DC 322 days: 101.6 ± 42.3	[20]
Acetylsalicylic acid (ASA)	Swiss mice (*Mus musculus*)	NI ($n = 5$) NI treated with ASA (NIASA) ($n = 10$) IC ($n = 10$) IC treated with ASA (ICASA) ($n = 10$)	Y	Intraperitoneal 1.300	81 days (chronic phase)	Six doses of benznidazole (Lafepe, PE, Brazil; 100 mg/kg) were administered by oral gavage 11, 13, 15, 25, 29, and 48 days after infection	Intraperitoneal consecutive days (55-63 days postinfection) and on days 65, 67, 69, 71, 73, and 75 postinfection	100 μL ASA of stock solution (50 mg/kg) via not informed	Not informed	Intestine distal colon	GIEMSA staining myenteric plexus	120 neuron quantification fields 300 neurons for cell body, cytoplasm, and nucleus measurement	NI: 5819.20 ± 754.80 NI-ASA: 5415.75 ± 259.34 IC: 4769.00 ± 65.00 IC-ASA: 3987.25 ± 529.41	NI: 132.6 (96.3; 182.7) NI-ASA: 209.7 (163.7; 311.3) IC: 114.6 (82.0; 156.5) IC-ASA: 159.8 (119.0; 227.2)	NI: 75.4 (50.3; 111.6) NI-ASA: 132.0 (91.3; 227.7) IC: 65.0 (43.2; 97.8) IC-ASA: 98.2 (65.5; 153.2)	NI: 54.5 (40.3; 68.6) NI-ASA: 68.6 (52.3; 87.4) IC: 46.8 (35.4; 61.0) IC-ASA: 57.3 (43.2; 77.6)	[27]
Cyclophosphamide (cy)	*Calomys callosus*	IC ($n = 5$) Infected treated with cy (IC-cy) ($n = 5$)	MORC-1	Intraperitoneal 100.000	10 days (acute phase) 450 days (chronic phase)	Natural time of infection	Acute phase: cyclophosphamide in water from day of infection to 21 postinfection Chronic phase: 0.4 mg/mL cyclophosphamide in water 10 days before euthanasia (440 days)	Intraperitoneal acute phase: 0.2 mg/mL of Genuxal Chronic phase: 0.4 mg/mL of Genuxal	Not informed	Distal annular esophagus segments	Cresyl violet staining myenteric plexus	Total neurons count in total area between inner and outer muscle layer	IC 10 days: 23 IC 450 days: 12.6 IC-cy 10 days: 18 IC-cy 450 days: 22	IC 10 days: 64.17 ± 15.27 IC 450 days: 29.75 ± 10.89 IC-cy 10 days: 62.61 ± 18.87 IC-cy 450 days: 22.94 ± 6.60	Unvalued	Unvalued	[23]
Acetylsalicylic acid (ASA)	Swiss mice (*Mus musculus*)	NI ($n = 10$) NI treated with ASA (NIASA) ($n = 10$) IC ($n = 10$) IC treated with ASA (ICASA) ($n = 10$)	Y	Intraperitoneal 1.300	75 days (chronic phase)	Six doses of benznidazole (Lafepe, PE, Brazil; 100 mg/kg) were administered by oral gavage 11, 13, 15, 25, 29, and 48 days after infection	Treatment performed intraperitoneally daily from the 5th to the 11th day after infection	20 mg/kg	Not informed	Esophagus distal part	Nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-dp) staining myenteric plexus	100 neuron quantification fields 100 neurons for cell body, cytoplasm, and nucleus measurement	NI: 287.30 ± 7.54 NI-ASA: 304.40 ± 6.52 IC: 339.60 ± 8.24 IC-ASA: 342.10 ± 7.62	NI: 245.60 ± 4.82 NI-ASA: 221.80 ± 4.25 IC: 206.50 ± 4.22 IC-ASA: 248.40 ± 4.35	NI: 177.00 ± 4.18 NI-ASA: 153.30 ± 3.55 IC: 143.10 ± 3.80 IC-ASA: 174.10 ± 3.83	NI: 68.62 ± 1.07 NI-ASA: 68.51 ± 1.18 IC: 63.36 ± 1.12 IC-ASA: 74.29 ± 1.11	[24]

Table 1: Continued.

Intervention	Experimental model	Evaluated groups and number of animals used	Strain	Route of infection/ inoculum used	Phases	Chronic phase induction method	Treatment schedule	Intervention concentration of/treatment route	Mortality rate	Organ and region evaluated	Methodology used for neuronal analysis/ analyzed region	Number of fields and neurons analyzed	Number of neurons per group	Neuronal body area per group (μm^2 or cm^{2*})	Area of neuronal cytoplasm per group (μm^2)	Neuronal nucleus area per group (μm^2)	Reference
Acetylsalicylic acid (ASA)	Swiss mice (*Mus musculus*)	NI ($n = 5$) NI treated with ASA (NIASA) ($n = 5$) IC ($n = 5$) IC treated with ASA (ICASA) ($n = 5$)	Y	Intraperitoneal 1.300	75 days (chronic phase)	Six doses of benznidazole (Lafepe, PE, Brazil; 100 mg/kg) were administered by oral gavage 11, 13, 15, 25, 29, and 48 days after infection	Intraperitoneal consecutive days (55-63 days postinfection) and on days 65, 67, 69, 71, 73, and 75 postinfection	50 mg/kg	Not informed	Esophagus distal part	Nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-dp) staining myenteric plexus	100 neuron quantification fields 100 neurons for cell body, cytoplasm, and nucleus measurement	NI: 1.48 ± 0.27 NI-ASA: 1.41 ± 0.31 IC: 1.61 ± 0.06 IC-ASA: 1.61 ± 0.23	NI: 244.80 ± 4.56 NI-ASA: 249.90 ± 5.33 IC: 219.60 ± 3.95 IC-ASA: 191.60 ± 3.37	NI: 172.10 ± 3.94 NI-ASA: 178.90 ± 4.67 IC: 151.20 ± 3.31 IC-ASA: 131.50 ± 2.77	NI: 69.62 ± 1.14 NI-ASA: 68.98 ± 1.56 IC: 65.90 ± 1.15 IC-ASA: 57.15 ± 1.11	[25]
Ácido acetilsalicílico (ASA)	Swiss mice (*Mus musculus*)	NI IC NI treated with ASA in the acute phase (NIaASA) IC treated with ASA in the acute phase (ICaASA) NI treated with ASA in the chronic phase (NIcASA) IC treated with ASA in the chronic phase	Y	Intraperitoneal 1.300	75 days (chronic phase)	Six doses of benznidazole (Lafepe, PE, Brazil; 100 mg/kg) were administered by oral gavage 11, 13, 15, 25, 29, and 48 days after infection	Acute phase: treatment performed intraperitoneally daily from the 5th to the 11th day after infection Chronic phase: treatment performed intraperitoneally daily from the 55th to the 63rd day after infection. Then alternate treatment from day 65 to day 75	Acute phase: 20 mg/kg Chronic phase: 50 mg/kg	No deaths reported in any group	Intestine colon	Immunofluorescence for nNOS, VIP, SP, and myosin-V/ myenteric plexus	35 fields for quantification of neurons for each marker 300 neurons for area measurement for each marker	*Myosin-V: NI: 83552.63 ± 0.06 IC: 28289.47 ± 4276.32 NIa-ASA: 72039.47 ± 3618.42 ICa-ASA: 56578.95 ± 3289.47 NIc-ASA: 70394.74 ± 2631.58 ICc-ASA: 43092.11 ± 4605.26 *nNOS: NI: 12613.42 ± 447.28 IC: 6261.98 ± 492.01 NIa-ASA: 10153.35 ± 984.03 ICa-ASA: 9661.34 ± 357.83 NIc-ASA: 11316.29 ± 849.84 ICc-ASA: 9214.06 ± 357.82 VIP: NI: 1791.24 + 34.26 IC: 1097.64 + 50.01 NIa-ASA: 1757.57 + 47.79 ICa-ASA: 1548.82 + 82.05 NIc-ASA: 1198.65 + 150.34 ICc-ASA: 1703.70 + 41.26	Myosin-V: NI: 193.01 ± 7.73 IC: 250.37 ± 9.92 NIa-AAS: 142.28 ± 9.93 ICa-ASA: 201.84 ± 11.03 NIc-ASA: 178.68 ± 8.82 ICc-ASA: 210.66 ± 8.83 nNOS: NI: 146.52 ± 12.45 IC: 183.15 ± 3.66 NIa-ASA: 142.86 ± 11.44 ICa-ASA: 157.51 ± 9.52 NIc-ASA: 159.71 ± 9.52 ICc-ASA: 171.43 ± 8.79 VIP: NI: 462.04 + 19.71 IC: 554.01 + 24.09 NIa-ASA: 295.62 + 15.32 ICa-ASA: 424.81 + 24.09 NIc-ASA: 321.89 + 17.52 ICc-ASA: 486.13 + 19.7	Unvalued	Unvalued	[26]
Cyclophosphamide (cy)	*Calomys callosus*	IC ($n = 5$) Infected treated with cy (IC-cy) ($n = 5$)	MORC-1	Intraperitoneal 100.000	10 days (acute phase) 450 days (chronic phase)	Natural time of infection	Acute phase: cyclophosphamide in water from day of infection to 21 postinfection Chronic phase: 0.4 mg/mL cyclophosphamide in water 10 days before euthanasia (440 days)	Intraperitoneal acute phase: 0.2 mg/mL of Genuxal Chronic phase: 0.4 mg/mL of Genuxal	Not informed	Colon	Coloração por Cresyl violet staining Myenteric plexus	Contagem total neurons in the total area between the inner and outer muscle layer	IC 10 days: 20.3 IC 450 days: 5.2 IC-cy 10 days: 14 IC-cy 450 days: 13.5	IC 10 days: 125.59 ± 36.29 IC 450 days: 117.±47.56 IC-cy 10 days: 108.56 ± 26.49 IC-cy 450 days: 228.88 ± 111.10	Unvalued	Unvalued	[21]

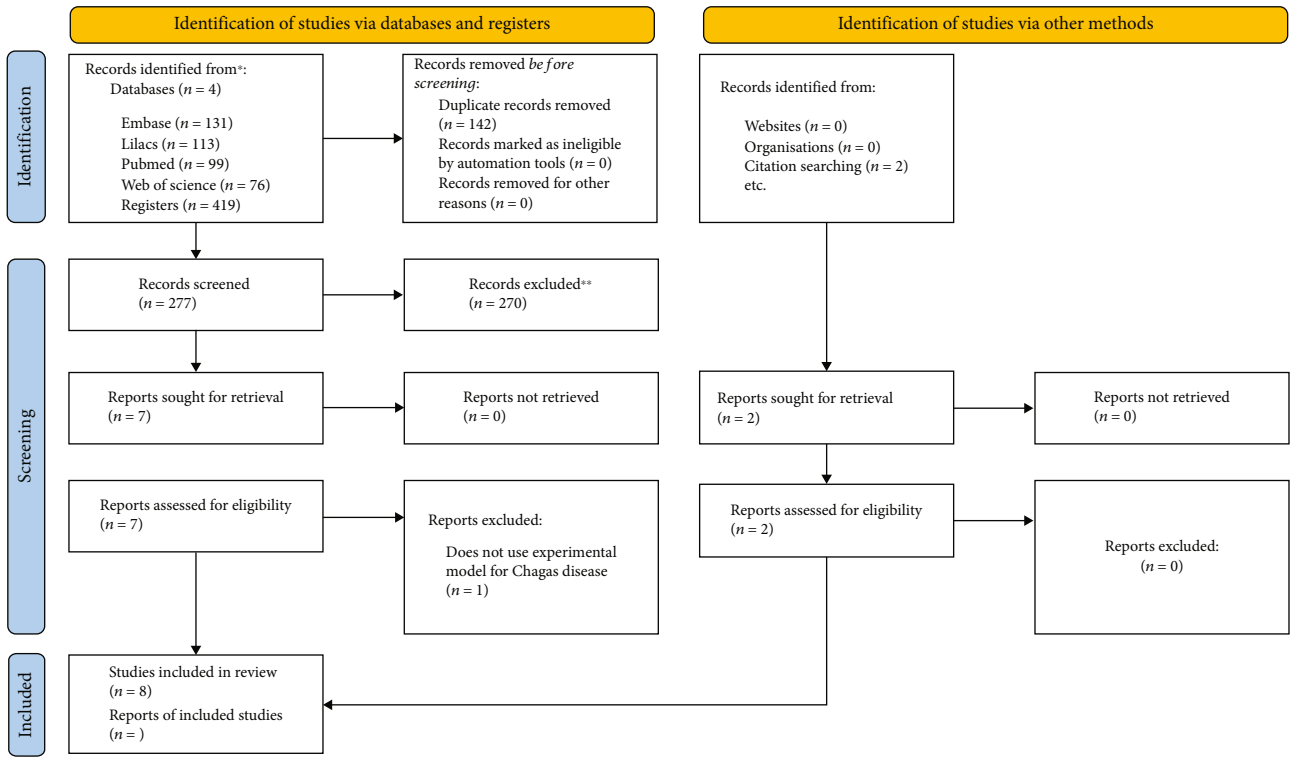

Figure 1: PRISMA flow chart of the study selection and inclusion process. PRISMA: Preferred Reporting Items for Systematic Reviews and Meta-analyses.

To analyze the impact of LC, *Rattus norvegicus* of the Wistar strain were infected with the Y strain of *T. cruzi*. The treatment induced a predominant proinflammatory profile at the beginning of infection, at 10-day postinfection, with an increase in IFN-γ and at 24 days with an increase in serum IL-12. Interestingly, at 24 days, anti-inflammatory cytokines (IL-10 and IL-4) were also found to be increased in the treated group compared to the untreated group, which demonstrates a balance between proinflammatory and anti-inflammatory/regulatory responses. Regarding the number of neurons in the distal colon at 322-day postinfection, the authors observed that the treatment induced neuronal protection when compared to the untreated group. Furthermore, the use of LC induced maintenance of the number of these cells, while in the untreated group, there was a reduction along the 125×322-day postinfection. LC treatment also induced hypertrophy in neurons present in the distal and proximal colon 125-day postinfection, but after 322 days of infection, this effect was observed only in the distal colon. Thus, an increase in the body area, cytoplasm, and nucleus of neurons was reported when compared to the untreated group [20].

Although the authors demonstrate that LC induces neuronal protection and even suggest that this protection is mediated by the establishment of a treatment-mediated immune balance profile, there is a lack of an uninfected control to further refine the comparisons between the experimental groups [20].

3.4.2. Cyclophosphamide. Among the eight articles, cyclophosphamide was evaluated in three studies, one focused on the impact of treatment on the colon [21] and the other two on the esophagus [22, 23].

All studies showed that cyclophosphamide treatment induces an increase in parasitaemia when compared to the infected and untreated group, especially after 10 days of infection. Furthermore, the use of cyclophosphamide reduced the production of NO in exudate from peritoneal macrophages from young (10 days of infection) and old mice (450 days of infection). The intervention also acted on the proliferation of splenocytes, reducing the proliferative capacity of these cells when exposed to polyclonal stimuli. Regarding neuronal count, the treatment resulted in protection of these cells both in the esophagus [22, 23] and in the colon [21] in both phases evaluated (10 days of infection and 450 days of infection). Regarding morphometric analyses of esophageal neurons (diameter, perimeter, area, and volume), treatment with cyclophosphamide did not induce any changes when the treated and infected groups were compared with the untreated infected group [22, 23]. However, older animals showed lower values in all parameters analyzed when compared to younger ones [23]. For colon neurons [21], the treatment did not induce morphometric changes at 10 days of infection. Only at 450 days of infection, it was observed that the use of cyclophosphamide increased the perimeter, area, and volume of neurons when compared to the respective untreated group.

3.4.3. Acetylsalicylic Acid (ASA). Among the eight articles, ASA was evaluated in four studies, two focused on the esophagus [24, 25], and two on the colon [26, 27].

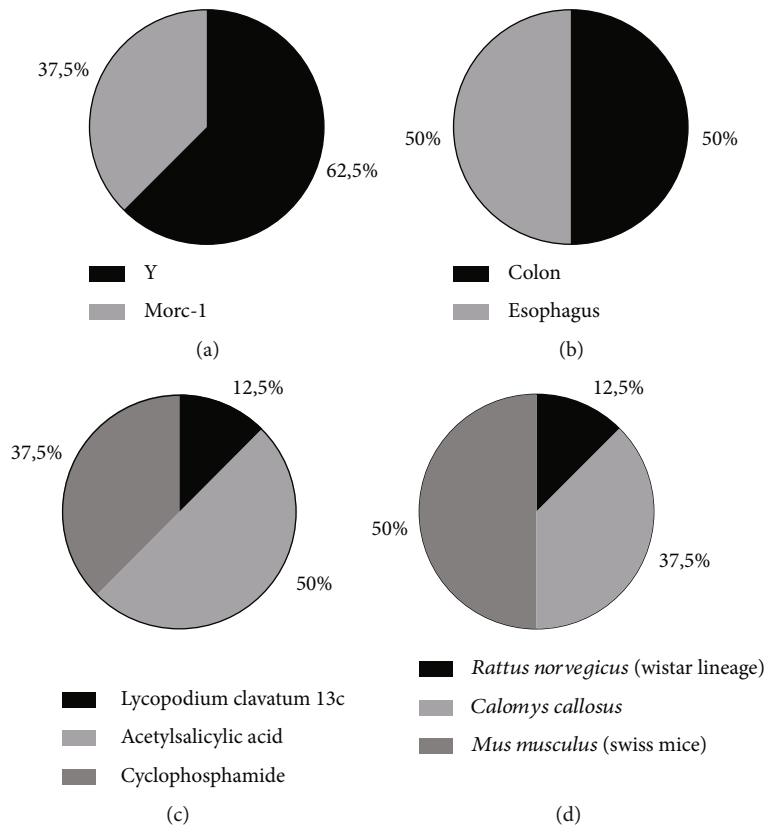

Figure 2: General characteristics of studies included in the systematic review ($n = 8$). List of *Trypanosoma cruzi* strains used (a), as well as organs used to study the digestive form of Chagas disease (b). In addition, there is also a list of pharmacological interventions (c) and the experimental models used (d).

		SYRCLE's risk of bias									
Authors	Year	Selection			Performance		Detection		Attrition	Relatórios	Reports
		1	2	3	4	5	6	7	8	9	10
Massocatto et al	2015										
Caetano et al	2007										
Caetano et al	2008										
Oda et al	2017										
Souza et al	2019										
Massocatto et al	2017										
Brustolin Aleixo et al	2017										
Caetano et al	2006										

Figure 3: Risk of bias assessment of *in vivo* studies. Prepared based on the SYRCLE tool [19]. Green (low risk of bias), red (high risk of bias), and yellow (uncertain risk of bias).

The first study that used ASA for Chagas disease aimed to assess the impact of this intervention on esophageal nitrergic neurons in an experimental model of chronic phase [24]. For this, Swiss mice were infected with the Y strain. When evaluating the total parasitaemia, it was reported that treated mice showed an increase of 13.52% in this parameter. Although infection increases the neuronal nitrergic population and this increase is maintained in the infected and treated groups, the use of ASA did not result in neuronal protection or destruction. However, the intervention was shown to prevent infection-induced atrophy in 20.33% of neurons by increasing the nuclear (17.28%) and cytoplasmic (21.68%) area of the cells. Regarding the esophageal structure in general, the infection, regardless of whether treated or not, induced a reduction in the diameter of the organ, without significantly affecting the thickness of the wall and the muscular layer. From these results, the authors suggested that, in fact, ASA represents an interesting intervention to prevent atrophy of esophageal nitrergic neurons [24].

To continue evaluating the potential of ASA in experimental Chagas disease (chronic phase), Massocatto et al. [25] observed that increasing the concentration from 20 mg/kg [24] to 50 mg/kg [25] induced neuronal protection to esophageal nitrergic neurons of the myenteric plexus.

However, the increase in concentration was also accompanied by neuronal atrophy, with a reduction in body areas (12.75%), nucleus (13.28%), and cytoplasm (13.03%). Interestingly, it was reported that the treatment partially prevented esophageal hypertrophy caused by the infection, by reducing the thickness of the tunica muscularis (4.33%) and of the circular muscle (11.80%) and allowing an increase of only 6.46% of increase of total organ thickness (compared to 20.37% of the untreated infected group). In addition to these parameters evaluated, the authors demonstrated that ASA improves the passage time of food through the animals' gastrointestinal tract. Thus, it is argued that ASA represents an alternative treatment for inducing nitrergic neuronal protection and reducing esophageal hypertrophy [25].

To assess the impact of ASA on the total population of colon myenteric plexus neurons, Swiss mice were infected with the Y strain of *T. cruzi* [27]. The treatment, as previously reported [24, 25], did not affect the evolution of the infection in relation to parasitemia [27]. Regarding the total number of neurons, ASA was not able to induce protection of these cells in the myenteric plexus in the distal colon, demonstrating a count similar to the reduction observed in the infected and untreated groups. In addition, the intervention induced neuronal hypertrophy with an increase in the cytoplasmic area (51.0%), nuclear (22.4%), and neuron body (39.4%) [27].

In a deeper analysis in relation to different neuronal subpopulations in the colon of Swiss mice infected with the Y strain of *T. cruzi*, Oda et al. [26] demonstrated the impact of using ASA in the acute and chronic phases. Treatment in the acute phase was able to reduce total parasitaemia, as well as the peak on different days, while treatment in the chronic phase did not change the course of infection. In addition, it was reported that the intervention was not able to change NO levels in the intestines of the animals, but rather to reduce the amount of inflammatory infiltrate in the organ, both with treatment in the acute and chronic phases. Regarding neurons, the authors demonstrated that the infection induces intense neuronal destruction, resulting in the total reduction of neurons in the myenteric plexus (60.7%), nitrergic (49%), vipergic (38%), and cholinergic (67%) subpopulations. Treatment with ASA, regardless of disease stage, reduced the destruction of all neuronal subpopulations. There was also a slight reduction in the number of these cells in uninfected treated animals. However, this reduction did not impact the animals' gastrointestinal transit. As long as the transit of the gastrointestinal tract was affected by the infection, the intervention was able to normalize the flow, regardless of the phase of exposure to ASA. The treatment was able to reduce infection-induced hypertrophy in all neuronal subpopulations only when the intervention was performed in the acute phase. In the chronic phase, it was not able to control the hypertrophy of nitrergic neurons.

Furthermore, Oda et al. [26] also evaluated the profile of neuropeptides, substance P (SP), and intestinal vasoactive peptide (VIP), involved in the pathophysiology of Chagas disease. It was shown that ASA treatment reversed the P/VIP substance profile found in *T. cruzi* infection, in which the presence of SP-containing varicosities was greater than those containing VIP. Thus, the use of ASA normalized SP levels and increased VIP levels.

4. Discussion

The present study summarizes potential preclinical pharmacological treatments for the digestive forms of CD. Thus, it was demonstrated that only 3 interventions have evidence applied to these conditions and with beneficial effects: LC, cyclophosphamide, and ASA (Figure 4).

Thus, LC is a plant of the Lycopodiaceae family commonly associated with anti-inflammatory [28], antimicrobial, and antioxidant phenomena [29, 30]. These functions may be associated with the diverse composition of secondary metabolites found in their spores [31], such as serratan triterpenoids [32]. Besides the impact on CD, the use of LC has also been evaluated in other conditions, such as in experimental *Toxoplasma gondii* infection [33, 34], *in vitro* cytotoxic effect on colon cancer cells [35, 36], and in a pilot study in humans with irritable bowel syndrome [37].

In fact, in the work of Brustolin Aleixo et al. [20], it was observed that the use of this plant, formulated in a dynamized way, resulted in immunoregulation and, consequently, neuronal protection in mice infected with *T. cruzi*. Although only one article has evaluated the neuroprotective potential, other studies have shown that the use of CL induces protection against *T. cruzi* infection, mainly by improving clinical signs and increasing the survival of infected animals [38, 39]. The authors suggest that these findings are due to the ability of LC to induce immune homeostasis on behalf of the host. For this, it was reported that the highly diluted intervention is able to favor the Th1 profile at the beginning of the infection, at 8 [38, 39] and 10 days [20], which controls the infection by the parasite. Later (24 days), as demonstrated by Brustolin Aleixo et al. [20], there is an inversion of cytokines, favoring the control of the proinflammatory profile by anti-inflammatory (IL-4) and regulatory (IL-10) cytokines, which reduces tissue damage and is associated with the neuronal protection described. In addition to immunoregulatory activity, it was observed that LC was also able to reduce parasitaemia and amastigote nests in the heart and intestine of mice infected with *T. cruzi* in the acute phase [40]. Thus, this intervention stimulates several mechanisms of action that help the host to control the infection and consequently reduce tissue damage.

Cyclophosphamide, in turn, was used in three articles included in this systematic review [21–23]. The authors demonstrate that this intervention was able to induce neuronal protection in the colon and esophagus, as well as reduce the proinflammatory response via NO and the proliferative capacity of splenocytes [21–23]. This drug is widely known for its immunosuppressive function and marked cytotoxic effect, especially on lymphocytes [41]. This action is related to the low cellular expression of aldehyde dehydrogenase by lymphocytes, an enzyme that participates in the detoxification process of the active form of cyclophosphamide [41, 42]. In this way, these cells become more susceptible and die faster. Although no intestinal assessment related to

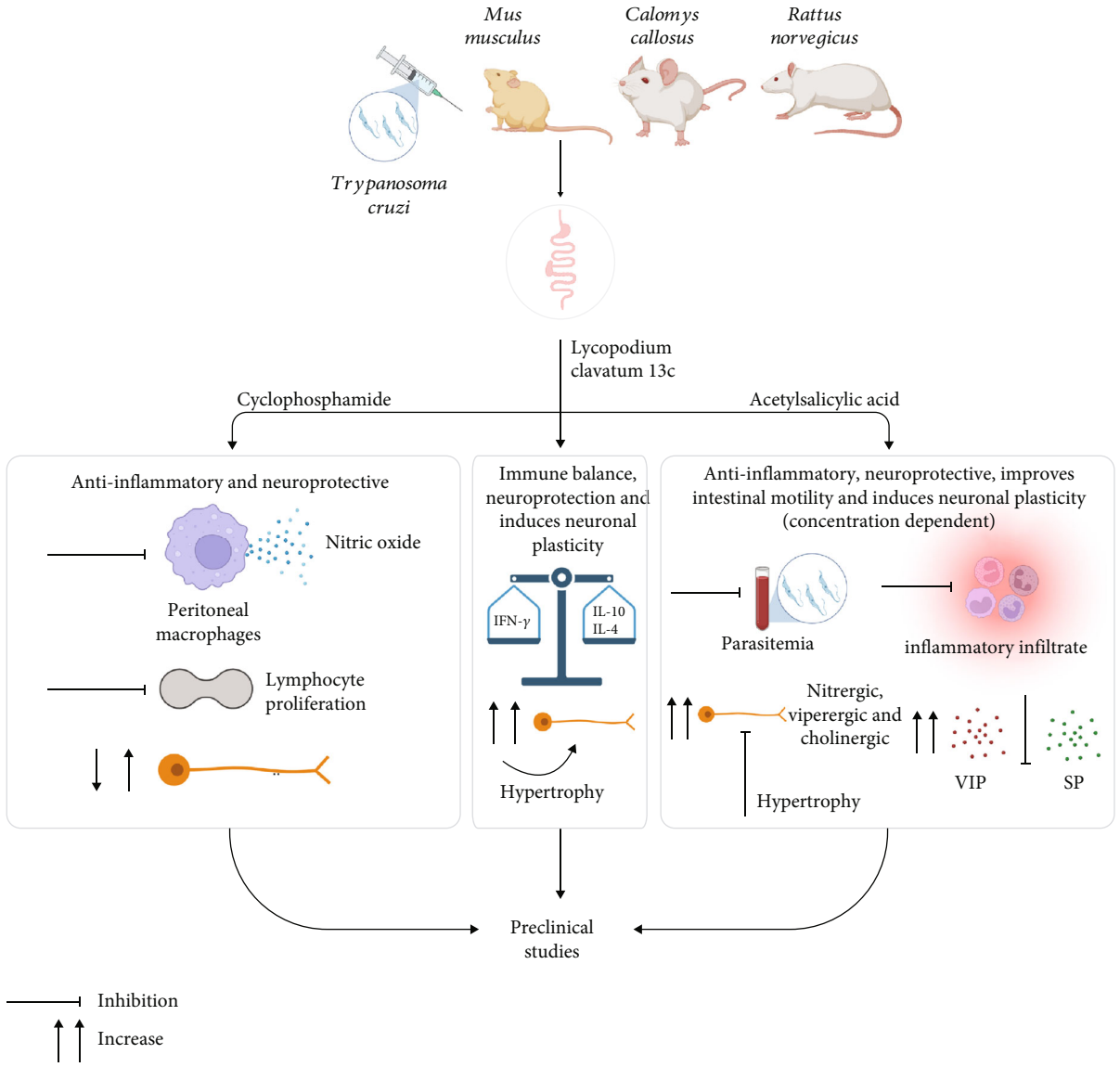

Figure 4: Beneficial effects of pharmacological interventions in experimental models of the digestive form of Chagas (made in ©BioRender: https://biorender.com).

cytokine profile, inflammatory cells, and the presence of the parasite was addressed in the studies included in this work, other studies have analyzed the effect of cyclophosphamide on the heart of animals infected with *T. cruzi* [43]. In both mice [44–46] and dogs [47], the intervention increased the myocarditis process established by the infection. However, for rats, the opposite was observed, with cyclophosphamide preventing acute myocarditis and sympathetic denervation, indicating that the inflammatory process may be one of the pathways of neuronal death [48]. The differences found in the studies may be due to the difference in the therapeutic regimen (concentration and treatment time), the experimental models used, the parasite strains, and the inoculum. Besides CD, cyclophosphamide is one of the most successful antineoplastics known today [49]. Its potential has also been described in kidney diseases [50], autoimmune rheumatic diseases [51], and dermatological diseases [52].

ASA is one of the most used drugs in the world, related to different potentials, such as antiplatelet effect, cancer prevention and treatment, prevention of preeclampsia, therapeutic potential for diabetes, and mental and neurobiological diseases [53]. It is widely known as an anti-inflammatory, mainly because it inhibits the NF-κB pathway [54]. In addition, it has also been described as an inhibitor of COX1 and COX2 [55] and peripheral production of cytokines such as IL-6 and TNF-α [56], all proinflammatory markers. This set of anti-inflammatory actions results in the neuroprotective potential of ASA observed in experimental models of the digestive forms of CD. This drug may be related to the reduction of the inflammatory process in general in affected organs (esophagus or colon) and in a systemic way in animals, which consequently results in neuronal protection against *T. cruzi* infection [25, 26]. In fact, it has already been demonstrated that the presence of inflammatory cells such as NK cells and

cytotoxic T lymphocytes, presence of the parasite (kDNA) [5, 57], and increased production of TLR8, IFN-β [58], TNF-α, and IFN-γ [6] by peripheral mononuclear cells are components present in individuals with digestive forms of CD and, consequently, are part of neuronal death mechanisms. This same pattern, with a proinflammatory profile and neuronal death, is also observed in experimental models, whether in the acute or chronic phases, especially in the colon [8, 9, 59–61]. Thus, it is suggested that ASA has an immunomodulatory action by favoring the inhibition of the proinflammatory and neurotoxic profile induced by the infection. However, studies focused on the immune response after ASA intervention in *T. cruzi* infection are necessary, as none of the articles included in this work focused on this point.

Interestingly, only one of the articles included in the study aimed to use ASA during the chronic phase [26]. When using the treatment in this phase, the authors observed results similar to those found when the intervention was used in the acute phase in relation to neuroprotection. Evaluating treatments in the chronic phase are extremely important, since approximately 2-27% of individuals diagnosed in the chronic phase develop digestive forms [62]. Thus, it is very important to use a drug that can reduce neuronal destruction so that it does not progress and/or can stabilize progression when the individual already has megacolon or megaesophagus. However, the evidence found in the literature focused on this aspect is rare.

In addition, it is believed that the differences found in the results of articles using ASA may be due to methodological differences for staining and subsequent neuronal counts (GIEMSA, NADPH-dp, or immunofluorescence), as well as drug concentration (20 or 50 mg/kg), route of administration (oral or intraperitoneal), therapeutic regimen, and the organ analyzed (esophagus or colon).

Besides these factors, the use of different experimental models also impacts the results obtained. Three species of animals were used in the studies included in this work: *Mus musculus* (Swiss mice), *Calomys callosus*, and *Rattus norvegicus* (Wistar lineage). The easy and practical handling, low cost, and need for low concentration of interventions in the new drug discovery phase are advantages that increase the incidence of using these experimental models in studies focused on CD [63]. With pathogenesis similar to that of CD in humans (immunological, pathological, and physiological), it is essential to consider that models such as mice and rats may not accurately reflect the progression and manifestations of CD, with dependence on the strain used in infection, concentration, route, and form of the protozoan used in inoculum and the genetic background of the experimental model [63–65]. As an example, depending on the strain, inoculum, and experimental model used, infection in the acute experimental phase can result in up to 100% mortality rate, while for humans, the rate is 5% [63, 66]. On the other hand, cardiac changes close to human chagasic heart disease are extensively reported in *T. cruzi*-infected mice and rats (cardiac fibrosis, electrocardiogram changes, inflammation, etc.) [67–74] as well as digestive changes (delayed intestinal transit time, intestinal dilatation, neuronal loss, etc.) [24–27, 75–78]. Although factors of host-parasite dynamics are related to CD progression, the studies included in this work demonstrate that the three interventions (LC, cyclophosphamide, and ASA) have neuroprotective potential, regardless of whether the model used was mouse or rat.

Neuron morphometry was also another point that showed different results between studies. When the interventions were used, three phenomena were observed: (1) induction of neuronal hypertrophy or (2) maintenance of neuronal proportions or (3) protection against the hypertrophy of these cells. These phenomena, although different, were associated with the same factors: neuroprotection and/or compensation of neuronal reduction/death caused by the infection through cellular adaptation and neuronal plasticity, in order to maintain peristalsis.

Several studies show that *T. cruzi* infection causes changes in the profile of different neuropeptides essential for the functioning of the enteric nervous system-gastrointestinal system axis, such as SP, VIP, glial fibrillary acidic protein (GFAP), morphogenetic protein type 2 (BMP2), NOS, S100, nerve growth factor (NGF), growth-associated protein 43 (GAP-43), and glial-derived neurotrophic factor (GDNF), among others [4, 8, 79–82]. These components influence and are influenced by the cellular microenvironment. The inflammatory process, for example, induced by the protozoan can alter the balance of all these systems: immune, nervous, and endocrine. Thus, developing interventions capable of inducing the balance of these systems is extremely important.

In addition, most of the included studies did not clearly report all items evaluated using the SYRCLE tool, thus making a complete analysis of methodological quality impossible. To circumvent this limitation at the level of studies, it is interesting that authors of future studies seek to describe the study methodology in more detail, to ensure better reproducibility and reliability of studies. Furthermore, it is noteworthy that due to the heterogeneity of the included studies, the meta-analysis was not tested, which is a limitation at the level of this systematic review. On the other hand, a comprehensive search, including a Latin American database, was carried out to find all articles that fit the guiding theme.

5. Conclusion

This systematic review addressed studies that tested possible pharmacological and neuroprotective interventions for cases of the digestive form caused by *T. cruzi* infection in murine models. Thus, three different types of therapeutic agents have been described, so far, in the literature, being acetylsalicylic acid, cyclophosphamide, and Lycopodium clavatum 13c, which showed different modes of action. Lycopodium clavatum 13c suggests an immunomodulatory activity, resulting in neuronal protection in the distal and proximal colon. Similarly, cyclophosphamide showed a neuroprotective effect in the colon, with improvement in the morphological parameters of neurons. Although it also protected esophageal neurons, these did not undergo morphological

changes. On the other hand, the results of studies that evaluated acetylsalicylic acid were contradictory, as it may act as neuroprotective or neurodestructive agents. It is worth noting that acetylsalicylic acid was able to normalize the transit of the gastrointestinal tract, as well as reduce the inflammatory infiltrate in the colon in both the acute and chronic phases. Furthermore, it was able to act on the structure of the esophagus, preventing its hypertrophy. In general, it is clear that the action of treatments is dependent on different factors, including drug concentration, stage of the disease evaluated, and the region evaluated (colon or esophagus). Thus, these parameters need to be considered in future articles and comparative studies are valid to better define the magnitude of each factor in the face of interventions. Approaches to understand how these treatments influence the behavior of immune cells in the neuronal environment are also needed. Thus, it is evident that the guiding theme of this systematic review is recent and deserves more attention, since neuroprotective interventions are crucial to reduce the digestive impact caused by Chagas disease to patients.

Data Availability

All the data used to support the findings of this study are included within the article and references.

Disclosure

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Supplementary Materials

S1: database used and search strategy used in each one. S2: screened articles and final status for inclusion in the systematic review. S3: SYRCLE tool risk of bias criteria adapted and met. *(Supplementary Materials)*

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Review Article

Insights into the Promising Prospect of G Protein and GPCR-Mediated Signaling in Neuropathophysiology and Its Therapeutic Regulation

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G protein-coupled receptors (GPCRs) are intricately involved in the conversion of extracellular feedback to intracellular responses. These specialized receptors possess a crucial role in neurological and psychiatric disorders. Most nonsensory GPCRs are active in almost 90% of complex brain functions. At the time of receptor phosphorylation, a GPCR pathway is essentially activated through a G protein signaling mechanism via a G protein-coupled receptor kinase (GRK). Dopamine, an important neurotransmitter, is primarily involved in the pathophysiology of several CNS disorders; for instance, bipolar disorder, schizophrenia, Parkinson's disease, and ADHD. Since dopamine, acetylcholine, and glutamate are potent neuropharmacological targets, dopamine itself has potential therapeutic effects in several CNS disorders. GPCRs essentially regulate brain functions by modulating downstream signaling pathways. GPR6, GPR52, and GPR8 are termed orphan GPCRs because they colocalize with dopamine D1 and D2 receptors in neurons of the basal ganglia, either alone or with both receptors. Among the orphan GPCRs, the GPR52 is recognized for being an effective psychiatric receptor. Various antipsychotics like aripiprazole and quetiapine mainly target GPCRs to exert their actions. One of the most important parts of signal transduction is the regulation of G protein signaling (RGS). These substances inhibit the activation of the G protein that initiates GPCR signaling. Developing a combination of RGS inhibitors with GPCR agonists may prove to have promising therapeutic potential. Indeed, several recent studies have suggested that GPCRs represent potentially valuable therapeutic targets for various psychiatric disorders. Molecular biology and genetically modified animal model studies recommend that these enriched GPCRs may also act as potential therapeutic psychoreceptors. Neurotransmitter and neuropeptide GPCR malfunction in the frontal cortex and limbic-related regions, including the hippocampus, hypothalamus, and brainstem, is likely responsible for the complex clinical picture that includes cognitive, perceptual, emotional, and motor symptoms. G protein and GPCR-mediated signaling play a critical role in developing new treatment options for mental health issues, and this study is aimed at offering a thorough picture of that involvement. For patients who are resistant to current therapies, the development of new drugs that target GPCR signaling cascades remains an interesting possibility. These discoveries might serve as a fresh foundation for the creation of creative methods for pharmacologically useful modulation of GPCR function.

1. Introduction

Several genetic alterations and mutations have been recognized as hazardous factors in the pathogenesis of mental illnesses, like schizophrenia, bipolar disorder, and depression. However, although there have been substantial advancements in strategies to aid develop potent drugs as therapeutics for psychiatric disorders, currently, there is a gap in bridging treatment modalities with disease progression. This provides a continuing impetus to develop effective treatment options for such health conditions [1]. In the face of multiple complex genetic and environmental risk factor challenges faced when designing a targeted novel treatment for such pathological conditions, multiacting receptor-targeted antipsychotics (MARTA) have been considered as antipsychiatric drugs [2, 3, 4]. Two kinds of receptors that have different synaptic transmission modalities are how neurotransmitters carry out their tasks. Fast synaptic transmission is induced by ligand-gated ion channels, which are made up of ionotropic receptors. Contrarily, metabotropic receptors are made up of GPCRs that bind to neurotransmitters and activate intracellular signaling pathways to reduce synaptic transmission and induce the gene expression required for antipsychotic effects [5, 6]. Notably, it is recognized that the majority of neuropharmacological medications control GPCR activity in the central nervous system (CNS) [7, 8, 9].

G protein-coupled receptors are 7-transmembrane domain proteins that form a multiprotein complex with members of an intracellular family of heterotrimeric G proteins comprised of a Gα subunit and a $\beta\gamma$ dimer (Figure 1). There are several members of the Gα family that couple to different cohorts of effectors in the cell. GPCRs are the largest transmembrane proteins in the human genome. Half of such GPCRs possess sensory function, whereas the remaining half possess nonsensory function [10]. The nonsensory GPCRs regulate communications between various cells following activation via ligands like amino acids, peptides, amides, and lipid-derived products. Indeed, a vast majority of the currently available drugs work through GPCRs. In addition, GPCRs regulate a wide range of physiological functions, including chemosensory identification, endocrine regulation, and behavioral events [11].

Almost 90% of the nonsensory GPCRs exert their roles in both normal and complex brain functions and in regulating CNS physiology [12]. In this light, GPCRs have formed a primary target for antipsychotics like aripiprazole, olanzapine, and quetiapine. Some 367 receptors have been reported to contribute towards the nonsensory GPCRs [13]. Additionally, classical GPCRs, namely, dopamine, acetylcholine, and glutamate, have long been neuropharmacological targets in neurological/neuropsychiatric drug development. As a consequence, such protein binding sites can be considered to mediate potential therapeutic effects in CNS disorders [14], and supporting this, a substantial proportion of current medications that are related to neuropharmacology act via GPCR-pathways within the central nervous system [15]. GPCR pathways are activated when they receive signals from the G protein. Receptor phosphorylation through G protein-coupled receptor kinases (GRKs) provides the main impetus for this type of signaling pathway [16, 17]. Antipsychotic drugs, mediated through dopamine receptors, are widely employed in CNS disorders, and the dopaminergic system is additionally important and provides a drug target in bipolar disorder, schizophrenia, Parkinson's disease (PD), and ADHD [18, 19, 20]. Dopamine essentially acts on GPCRs with the potential ability to activate the G protein through either the Gi, Gs, or Gq pathways [21]. Mitochondrial complexes [22] and altered gene expressions [23] have been reported in such signaling cascades. Oxidative stress is a common feature too, due to the oxidative metabolism of dopamine [24], which may additionally induce glutathione [25].

Furthermore, glutathione's precursor N-acetyl-cysteine is a potent antioxidant and has, likewise, been extensively investigated in psychiatric disorders [26–28]. Mental health diseases have yet to benefit from the present molecular medicine revolution, which has had an immediate influence on other areas of medicine. Research into the genesis and pathophysiology of complicated mental diseases is hampered by more than simply the CNS's intrinsic complexity. These include an absence of a clearly defined pathology, limited access to tissues, and the humbling realization that behavior is more complicated than the sum of its parts [29]. Appropriate diagnosis is one of the most challenging issues in the development of a novel treatment approach when it comes to psychiatric disorders with limited understanding of their mechanism impacting cognition, behavior, and emotion. In this regard, an altered psychological function is strongly linked to psychiatric conditions. Consequently, GPCRs have been widely reported to possess huge potential with respect to potentially developing novel drugs or treatment approaches for psychiatric disorders [8, 30].

Therefore, this review is additionally aimed at investigating the pharmacokinetics, pharmacodynamics, and the significance GPCRs for developing novel treatment strategies for psychiatric disorders. The involvement of G protein-coupled receptors (GPCRs) in the development and treatment of serious mental diseases has nevertheless made significant progress in our knowledge. In this position essay, we analyze and integrate the knowledge that is presently available and discusses how it might be used to generate better drugs strategically. Due to space constraints, this review's analysis has been limited to the most important mental illnesses and the most important GPCR families that have been implicated. Wherever possible, we have used reviews in place of source materials.

2. GPCRs in Central Nervous System

Humans and mice each contain 367 nonodorant GPCR superfamily members, with 343 common receptors, based on thorough analysis of public databases for mouse and human genome sequences, according to the study. There are 83 mouse and 26 human GPCRs that have not yet been discovered. Unexpected levels of orthology are found when GPCRs from the two species are directly compared. The survival of these molecules over time disproves functional redundancy among closely comparable receptors [31]. Nonodorant GPCRs are highly expressed in the central nervous

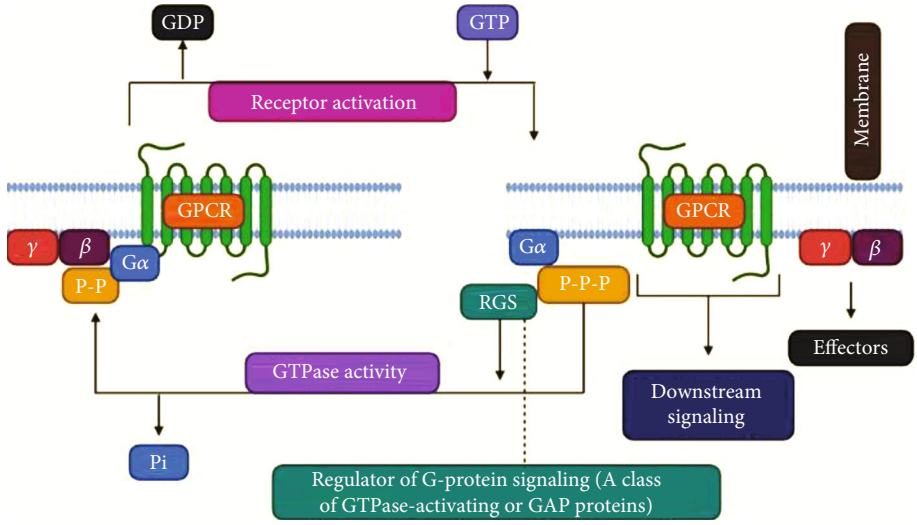

Figure 1: Heterotrimeric G protein signaling with RGS regulation. GPCR activation, either due to agonist binding or constitutive activity, causes downstream signaling through both the α and $\beta\gamma$ subunits. Various antidepressants modulate this process directly (e.g., buspirone) or indirectly (e.g., SSRIs). RGS proteins interact with active Gα and accelerate its GTPase activity, facilitating a return to the GDP-bound inactive state. Preclinical models suggest that direct manipulation of the RGS or G proteins can affect antidepressant response.

system, notably in the brain, with orphan receptors accounting for one-fourth of those found [32]. Nonodorant GPCRs make up around 80% of the 353 nonodorant GPCRs that are expressed in the mouse CNS. It has been shown by Komatsu that the CNS expresses considerable and selective levels of mRNA for six clusters of GPCRs, which collectively account for approximately 40% of the 322 nonodorant GPCRs discovered in mice. These six clusters are rich in neuropharmacological targets like dopamine, serotonin, acetylcholine, and glutamate which all have receptors for these chemicals in these clusters. The nonodorant GPCRs that are peculiar to the brain have a lot of potential as CNS medication targets, according to these findings [33].

3. GPCRs and Mood Disorders

3.1. Noradrenergic Receptors and Mood Disturbances with G Protein-Coupled Receptors. The distinctive primary synaptic components responsible for influencing emotional problems include the noradrenergic framework. After investigating the structure of peripheral blood cells, several critical assessments can be made with regard to the role of noradrenergic receptors in emotional disorders.

3.1.1. Studies on Peripheral Blood Cells

(1) Alpha2-Adrenergic Receptors. Several studies have reported alterations in blood platelets and alpha2-adrenergic receptor (alpha2-AR) mechanisms in subjects with mood disorders. In addition, it has also been observed that there were minimal changes in the receptor numbers in studies conducted with yohimbine-alkaloid radio ligands; one of the first radioligands developed to allow selective labeling of alpha2-ARs. Studies conducted with partial or complete agonists demonstrated wide variations in their activities, especially in weaker patients [34]. However, these findings highlighted several uncertainties, as the ligands involved here were bound to noradrenergic imidazoline receptors that were present on platelets [35, 36, 37]. Considering these findings, the sensitivity of alpha2 receptors seems inconclusive. This subject has also revealed the intricacies of assessments involving the bipolar issue. In addition, it was observed that the thickness of the alpha2-AR was enhanced in the platelets patients' who had bipolar disorder [38]

(2) Beta2-Adrenergic Receptors. Studies on the quantities of beta2-adrenergic receptors in peripheral cells, however, have shown inconsistent outcomes, with one finding being that depressed individuals' leukocytes have lower levels of beta-AR-stimulated AC activity [39, 40, 41, 42]. It has been postulated that this could be due to dysfunction of the receptor/Gs/AC complex. To date, it remains unclear which abnormalities in peripheral cells are identified with the pathophysiology of mood disorders and are associated with nonspecific stress or homeostatic mechanisms through both alpha2 and beta2 observations [43–45].

3.1.2. Impacts of Antidepressants and Mood Stabilizers

(1) Alpha-Adrenergic Receptors. Antidepressants have been demonstrated to lower alpha2-ARs in animal tissues, whereas alpha1-ARs have been shown to be increased [46]. Interestingly, prolonged lithium treatment has been found to reduce alpha2-AR-mediated behavioral consequences [47].

(2) Beta-Adrenergic Receptors. The majority of studies have discovered a predictable pattern of beta-AR downregulation in response to long-term antidepressant or electroconvulsive therapy treatment (ECT) [48, 49, 50, 51]. In the brain, long-term use of desipramine has been linked to an uncoupling of beta-AR and Gs [52, 51]. Receptor downregulation and receptor-G protein uncoupling both have been reported

responsible for reduced downstream of cAMP signaling [53]. Moreover, desipramine prevents the dissolution of the beta-AR high-affinity complex, which prevents adenylate cyclase from being activated downstream [54]. Lithium has been the subject of critical inquiries focusing on the results of evacuation stabilizers. There have been several studies assessing the effect of lithium on the actual adrenergic receptor in rodent cerebrums. However, an obstacle to beta-AR was that cAMP [55] was destroyed. Moreover, lithium does not avoid stimulant-instigated beta-AR downregulation [56].

3.1.3. Receptor Polymorphisms. Whereas there have been no reported associations between alpha2-AR polymorphisms and suicide or depression [57, 58], one study identified a nonsignificant link between a beta1-AR polymorphism, G1165C, and an increased antidepressant efficacy rate in depressed patients [59]. In recent research, the outcomes of yohimbine infusion on people with an alpha2C-adrenoreceptor subtype an-frame deletion (alpha2CDel322-325) [60] was studied. There was more whole-body noradrenaline spillover in homozygotes for the alpha2CDel322-325 polymorphism while they were at rest than in heterozygous individuals. In homozygotes, the effects of yohimbine were more pronounced and persisted longer than in the other groups. Because catecholaminergic responses have a similar impact on mood disorders, this receptor polymorphism is being studied more thoroughly in this area.

3.2. Serotonergic Receptors Coupled with G Protein and Mood Disturbances. The efficacy of serotonergic-mediated medications in the management of depression has been a driving force behind the idea that serotonin plays a crucial role in sadness and mood disorders. In this regard, the "serotonin hypothesis" of clinical depression is now over 50 years old and, in a nutshell, proposes that reduced serotonin-mediated signaling has a pivotal role in the pathophysiology that culminates in depression. This view is based on the adverse depressive actions of amine depleting drugs exemplified by reserpine, the antidepressant properties of tricyclic antidepressant and monoamine oxidase inhibitor drugs that augment serotonin and other monoamines within the synapse, and extensive studies showing aberrant serotonergic signaling in patients with sadness and mood disorders. Parallel information for the bipolar disorder is less extensive but is growing. Further evidence that serotonin plays a role in such dispositions comes from the discovery of receptor polymorphisms linked to illness susceptibility and treatment response in specific regions of the brain.

3.2.1. Impacts of Antidepressants and Mood Stabilizers

(1) Receptors of 5HT1A. During long-term antidepressant therapy, serotonin transmission increases in the hippocampus, leading to desensitization of dorsal raphe somatodendritic 5HT1A autoreceptors and support for 5HT1A receptor function in mood disorders [61]. In patients with bipolar disorder, 5HT1A receptor impairments can be mitigated by a stable lithium treatment [62]. A consistent energizer therapy has also been established to enhance the uncoupling of the 5HT1A receptor-G protein [63].

(2) Receptors of 5HT2. Antidepressants have been shown to decrease the cortex-authoritative 5HT2A receptor functions [64]. The evidence for the part of the 5HT2A receptor in the disposal stabilizer operation instrument is significantly less clear. Blended results have been provided by exploring the 5HT2A receptors after a consistent lithium association. Lithium induces a typical activity within the cutoff retention of the 5HT2 receptor with the most grounded hippocampal confirmation [62] at the same time when platelets were affected in bipolar sufferers, thereby, limiting the upward thrust of lithium inside the 5HT2A receptors [65].

3.2.2. Polymorphisms of Receptors

(1) Receptors for 5HT1A and 5HT1B. Terock et al. and Han et al. [65, 66] have reported that the C-1019G 5HT1A polymorphism is related to a significant self-destruction of the 5HT1A agonist stimulant response to flibanserin. A relationship between the stimulant response and both this polymorphism [67, 68] and Gly272Asp [68] has been revealed in a number of studies. Albert et al. [69] found that the hereditary impact of the 5HT1A receptor-encoding quality of HTR1A and the serotonin carrier encoding quality of SLC6A4 impacts the clinical results of patients treated with citalopram. It has been reported that the G861C locus of the 5HTR1B quality is implicated with distress, yet not with bipolar disorder [70]; albeit, no such association was found in other investigations [66].

3.3. Dopaminergic Receptors with G Protein-Coupled and Mood Disorders. Dopamine has long been a focus of schizophrenia-related research, but its role in mood disorders has gotten far less attention. Despite this, mounting data suggests that dopamine plays an important role in a wide range of illnesses. Goal-directed behavior is influenced by the dopamine systems in the midbrain and mesolimbic systems, which are linked to motor activity, motivation, and reward pathways respectively. All of these functions can be substantially damaged in states of depression and mania. Pharmacological therapies for mood disorders have been demonstrated to ameliorate mood symptoms, showing that G protein-coupled dopamine receptors play an important role in the development of mood disorders.

3.3.1. Polymorphisms of Receptors

(1) Receptor D1. Whereas various investigations have failed to see any bipolar changes in the D1 receptor gene [71, 72], a connection between polymorphism of the D1 receptor A48G and bipolar disease has been observed [73, 74].

(2) Receptor D2. Different research has sought to find a link between polymorphism of the D2 receptor and bipolar illness; however, such a link has not been discovered. Nevertheless, two evaluations have reported cautious, but optimistic outcomes. Overs et al. [75] investigated and reported a critical connection between the D2 receptor and bipolar

disorder in an European multicenter examination. Han chinese patients with a bipolar exacerbation was not underlined when attempted in Caucasians. Kulmala et al. [76] saw a relationship with a D2 receptor polymorphism recommending a potential race-express threat issue.

(3) Receptor D3. A relationship between D3 receptor polymorphism and unipolar despondency has been observed in one study [75]. Thus far, examinations have failed to show any evidence for the D3 receptor locus association in bipolar issues.

(4) Receptor D4. The possible role of the D4 receptor in misery has been investigated in few studies. A meta-assessment of these reported by Lopez Leon and colleagues found a liberal relationship between the existence of the D4 receptor 48 base pair found to be involved through polymorphism and unipolar weakness at any time, but not in a bipolar problem.

3.4. Cholinergic Receptors with G Protein-Coupled and Mood Disturbance. There has long been speculation about the role of the cholinergic framework in bipolar disorder. This was based on experiments showing that cholinergic agonists and antagonists have significant mood and behavioral effects. Most of the examinations detailing openness to cholinergic receptors in disposition issues have been exceptionally backhanded. For instance, REM occurs during particular patterns of sleep, yet its initiation might be set off by cholinergic actions as observed in healthy volunteers. The induction of REM sleep in persons with mood disorders who are not taking medication is more rapid with the administration of arecoline (a cholinergic agonist) (primarily focused on bipolar disorder) [62]. Several antidepressants possess anticholinergic effects, including tricyclic and serotonin reuptake inhibitors, and hence, their useful biological actions can sometimes be adversely affected by sedation, psychomotor and memory impairment, a dry mouth, and blurred vision. Several studies have provided contradicting reports on the impact of lithium on function of the muscarinic receptor. It has been reported [77] that lithium obstructs the super sensitivity of the muscarinic receptor despite influencing the quantity of receptor within restricted areas, suggesting that it works at a postreceptor site [78, 79].

3.5. GABAergic Receptors and Mood Disturbances That Are G Protein Coupled. G protein-coupled GABA-B receptors have been demonstrated to be beneficial in the treatment of mood disorders in a modest number of trials. GABA-B agonist baclofen has been demonstrated to cause depression in a tiny percentage of individuals; therefore, discontinuing the medication can help alleviate depression [80]. Lithium, valproic acid, and carbamazepine, among other mood stabilizers, increase GABA-B receptors in the hippocampus when taken on a long-term basis [81, 82].

3.6. Glutamatergic Receptors Coupled with G Protein and Mood Disorders. Most neurotransmitter systems are thought to have a threshold for excitation that is regulated by the glutamatergic system, which is the principal excitatory neurotransmitter of the CNS. Recently, research has focused on the possible significance of mood disorders in a possible link between the two. According to current investigations, the glutamatergic system appears to be important in the development and treatment of mental health issues [83–85]. Currently, investigations are jointly evaluating both NMDA [85] and AMPA [65] ionotropic receptors in the treatment of mood disorders. These will not be detailed as our focus is GPCRs; however, there are recent reviews that include a comprehensive discussion of the glutamatergic system's function in mood disorders [83–85].

3.7. Receptors of G Protein-Coupled Neuropeptides and Mood Disorders. As a neurotransmitter, neuropeptides are a group of short-chain amino acids. One of the initial interests in biological psychiatry was the role of modified endocrine function in mood disorders [86].

3.7.1. Receptors of Corticotrophin-Releasing Factor (CRF). Because of its growing importance in neurological illnesses and the numerous unanswered questions it has raised in people and animal models, CRF has become one of the most intensively studied neuropeptides [62, 87, 88]. Anxiolytic and overactivity effects have been demonstrated in preclinical studies employing direct models for antagonists of CRF receptors, in particular for subtype CRF receptor-1 [89]. These findings led to the open trial of R121919, a CRF receptor-1 antagonist, in 24 individuals with depression [90]. Initial results have been promising, and further clinical trial results are awaited. At least two genetic linkage and association studies, however, have found no evidence linking CRF polymorphisms to bipolar disorder [91–93].

4. Schizophrenia and GPCRs

4.1. Dopaminergic Receptors Coupled to G Protein and Schizophrenia. Despite the fact that the underlying pathogenesis of schizophrenia is clearly highly complex, the dopamine hypothesis and its various revisions provide a valuable and scientifically based explanation for the disease's psychotic symptoms (particularly hallucinations and delusions). Dopamine dysregulation in emotional processing areas like the amygdala and hyperactivity of dopamine transmission in the mesolimbic areas are hypothesized to be associated with schizophrenia, as is the hypoactivity of dopamine transmission in the prefrontal cortex.

4.1.1. Impacts of Antipsychotic Medications. To begin with, the idea of overactive dopamine was put up due to research showing that conventional antipsychotics have a high D2 affinity and that receptor affinity has a direct correlation to the clinical response [94]. Antipsychotic effectiveness appears to be best around 65% to 70% D2 receptor occupancy, while higher than 80% occupancy greatly raises the incidence of extrapyramidal symptoms (EPS), a well-known adverse effect. PET and SPECT investigations have supported this connection. However, several studies have shown that D2 receptor binding alone is not adequate to explain schizophrenia's physiology. In the first place, despite 70 percent or more receptor occupancy, standard antipsychotics are not helpful for all

people with schizophrenia [3]. Second, traditional antipsychotics are only successful in treating about 70% of positive symptoms [4] and have been reported ineffective in treating negative and cognitive symptoms [95].

4.1.2. Receptor Polymorphisms. In the light of the pivotal role of D2 receptors in the pharmacological action of antipsychotics, numerous investigators have looked into the role of D2 receptor polymorphisms in schizophrenia. Several studies, that have included different groups of independent investigators undertaking metaexaminations, have reported a positive relationship between polymorphisms and schizophrenia related to the D2 receptor Ser311Cys [96–99]. Nonetheless, other evaluations have found no connections [99]. A relationship between Ser311Cys and adverse effects was found in one of the investigations [100]. Furthermore, a few further studies have revealed that schizophrenia is linked to a second polymorphism in the D2 receptor, which highlight a specific region, -141C Ins/Del [101, 102]. Various assessments have additionally described assorted other polymorphisms of the D2 receptor, for instance, C957T [103], His313 [104], and Taq1A locus [105]. Several studies have linked the -141C Ins/Del allele to the time or degree of response to antipsychotic drugs, with the -141C Ins allele being associated with a better response; however, other studies have contradicted these findings [60, 104, 106, 107]. The association between treatment effects and the Taq1A locus [108], Ser311Cys polymorphism [68], and His452Tyr polymorphism [107] has been evaluated in a number of studies. The D3 receptor polymorphism, Ser9Gly, is the most frequently analyzed in relation to the D3 receptor, regardless of a lack of schizophrenic symptoms associated with it [109, 110]. The connection between this polymorphism and the response to antipsychotics has been reported in a few studies [111, 112]. There is some distinction from these examinations concerning which subset of indications principally is connected with this polymorphism [113]. The Gly9 allele is reported to be associated with the response to atypical antipsychotics [104]. G-205A [114], G-7685C [111], and G-712C [114] are other D3 receptor variations that have demonstrated a potential relationship with schizophrenia that some consider to be relatively nominal. Indeed, studies are yet to demonstrate a truly extensive association with schizophrenia [115, 116]. Other studies still have evaluated the relationship between polymorphism and the response to the atypical antipsychotic clozapine [24] which was observed, but not in all studies [117, 118].

4.2. Serotonergic Receptors Coupled to G Protein and Schizophrenia. Although there is contradictory evidence that serotonin receptors are changed in schizophrenia, pharmacological results of the serotonergic action of propsychotic drugs of abuse and atypical antipsychotic medications show that serotonin plays a crucial role in such illnesses. The strongest evidence for serotonin's significance in schizophrenia has come from pharmacological studies. D-Lysergic acid diethylamide (LSD) can elicit psychotic symptoms in healthy people because of its structural similarity to serotonin, prompting further inquiry into this neurotransmitter system in psychosis. LSD affects the raphe nucleus' serotonin system via 5HT1A receptors, and hallucinations are almost certainly induced by the agonist activity of the 5HT2A receptor [119]. The last two decades of research described at least fifteen 5-HT receptor subtypes based on the specific biochemical signaling pathways, as presented in Table 1.

4.3. Glutamatergic Receptors Coupled to G Protein and Schizophrenia. A great deal of evidence suggests that the glutamatergic system is involved in schizophrenia. The majority of the existing evidence, as with mood disorders, is focused on the role of ionotropic glutamatergic receptors. There are several reviews that cover this topic [121, 122]. Support of a glutamate role in schizophrenia is centered largely on pharmacological investigations. Studies have shown that phencyclidine (PCP) and amphetamine-instigated schizophrenia-like social symptoms are potentiated by group-I enemies of metabotropic glutamate receptors (mGluR1 and mGluR5) [123]. Get-together, group-I agonists limit sensorimotor gating impairments mediated by amphetamine and PCP-induced dopamine discharge in rodents in the prefrontal cortex [124]. Agonists of the get-together II receptor (mGluR2/3) block direct incitement achieved by PCP and working memory impairment [125, 126]. In sensorimotor gating, which aggravated schizophrenia, knockout mice mGluR1 and mGluR5 exhibit exacerbation [123].

5. GPCRS in Depression

5.1. Depression and Antidepressant Activity G Protein Subunits. The majority of the current antidepressant medications interact with G protein-coupled receptors (GPCRs). For example, 5-HT1A receptor acting partial agonists buspirone or aripiprazole mediates their effects through a variety of monoaminergic GPCRs, thereby affecting endogenous synapse levels. They produce serotonin reuptake inhibitors (SSRIs) like fluoxetine and monoamine oxidase inhibitors (MAOIs) like selegiline, as well as serotonin reuptake inhibitors (SSRIs). Members of an intracellular family of heterotrimeric G proteins, consisting of a $G\alpha$ subunit and a $\beta\gamma$ dimer, form a multiprotein complex with seven transmembrane domains with G protein-linked receptors [54].

5.1.1. Levels of G Protein Expression. Antidepressant drug therapy does not appear to alter G protein expression levels in the central nervous system (CNS) consistently in preclinical trials. Chronic treatment with the tricyclic antidepressant imipramine results in relatively stable mRNA expression of Gαs, Gαo, and Gαi in the rat hippocampus, according to one study [127]. However, the measurement of G protein mRNA levels does not necessarily provide insight into its protein expression [128]. As a result, the amount of G protein present is not always accurately reflected in these observations. In studies focused on protein level measurements, those of Gαs, Gαo, and Gαi in the rat cerebral cortex were unaffected by chronic treatment with amitriptyline, a dual serotonin-norepinephrine reuptake

Table 1: The family of 5-HT receptors [120].

Receptor	Selective agonists	Potential	Type	G protein effector	Mechanism of action	CNS distribution
5-HT1 (5-HT1A, 1B, 1D–F)	1A=8-OH-DPAT, 1B=sumatriptan, 1C=sumatriptan, 1F=LY 334370	Inhibitory	Gi/G0-protein coupled	Gi/o	Decrease in intracellular cAMP levels and inhibition of AC	Cerebral and frontal cortex, hippocampus
5-HT2 (5-HT2A–C)	DOI[d], BW 723C86, Ro 600175	Excitatory	Gq11-protein coupled	Gq/11	Activation of PLC, enhancing in IP3 and DAG intracellular concentration, and rise in intracellular calcium	Nucleus accumbens, basal ganglia, cerebellum
5-HT3 (5-HT3A, 3B)	DOI[d], BW 723C86, Ro 600175	Excitatory	Ligand-gated Na+/K+ channel	—	Depolarization of cell plasma membrane	Hippocampus, amygdala, nucleus accumbens
5-HT4 (5-HT4A–H)	DOI[d], BW 723C86, Ro 600175	Excitatory	Gs-protein coupled	Gs	Increased intracellular cAMP concentration and activation of AC	Hippocampal membranes
5-HT5 (5-HT5A)	—	Inhibitory	Gi/G0-protein coupled	Gi/o	Decrease in intracellular cAMP levels and inhibition of AC	Olfactory bulb, neocortex, hippocampus

inhibitor (SNRI), or by desipramine, tranylcypromine, or electroconvulsive shock [54, 129].

5.1.2. Impact on Gαs Localization and Signaling. Notwithstanding the absence of any immediate consequence on G protein verbalization levels, treatment with solutions of energizers (checking for amitriptyline, desipramine, and iprindole) brings cAMP focuses up in the brain of rodents, not in the liver or kidney, in a GAPS-subordinate manner [130–132]. Notwithstanding energizer arrangement items, constant electroconvulsive treatment reinforces the coupling among GHAs and adenyl cyclase. Also, extended cAMP-subordinate kinase movement (for example, protein kinase A) was seen in the rodent cerebrum after predictable upper treatment, reliable with this all-encompassing creation of adenylyl cyclase [133]. When desmethylimipramine was led constructively, but not brutally, these modifications in the cerebral cortex were not observed in the hippocampus, striatum, or cerebellum [134].

6. G Protein Signaling Regulators as a Potential Drug Target for the Central Nervous System (CNS)

G protein-coupled receptors (GPCRs) are colossal focuses for medication exposure. In the transduction of GPCR signals, the G protein signaling- (RGS-) protein family controller has a primary breaking point. GPCRs are common pharmacological targets. RGS proteins quicken the deactivation of G proteins. Likewise, they produce communication signals to diminish GPCR flagging. The blend of GPCR agonists with inhibitors of RGS could potentiate reactions and could essentially expand the geographic particularity of the agonist. Because of their diversity and highly localized and dynamically regulated distributions in the brain, RGS proteins are intriguing targets for the pharmacotherapy of central nervous system illnesses [135].

6.1. The Dual Role of RGS Proteins as Either Inhibitors Or Effectors in GPCR Signaling. (a) RGS proteins such as Gα GTPase accelerating proteins (GAPs): the binding of agonists to G protein-coupled receptors (GPCRs) induces the receptor guanine nucleotide-trade (GEF) action, leading the loading of GTP by the Gα subunit, conformational changes in the Gα switch areas (I, II, and III), making the dynamic $*$ α-accreditation, separation of the Gα–G$\beta\gamma$ complex, and resultant effector interactions. RGS proteins decrease GPCR motioning by inducing the speed of GTP hydrolysis by the G protein α-subunit, which prompts Gα–G$\beta\gamma$ reassociation. Blocking the binding of the RGS-box to Gα•GTP, for the current condition, would induce a delayed lifetime of the Gα subunit in the GTP-bound state, improving the receptor-engaged reaction through elevated levels of free Gα•GTP and G$\beta\gamma$ subunits. (b) RGS proteins as Gα effectors: RGS proteins, in a similar way, have a positive occupation in GPCR motioning as a result of p115-RhoGEF and the related proteins PDZ-RhoGEF and LARG. On binding, G13α, the GEF action of p115-RhoGEF broadened, affecting RhoA incitation, accomplishes the downstream impacts of cytoskeletal changes and transcriptional control. P115-RhoGEF additionally deactivates G13α by procedures for RGS-box GAP improvement, at any rate not before the signal is transmitted forwards. For the current situation, inhibiting the RGS-box–Gα interactions by small particle mediation should decrease G13α-subordinate RhoA beginning. LARG stands for leukemia-associated RhoGEF;

Pi stands for phosphate group; RGS stands for the regulator of G protein signaling [135].

6.2. Role of Endogenous RGS Protein. The dormant synaptic potential formed when heterotrimeric G proteins change molecule channels are signaled by metabotropic G protein-coupled receptors. Numerous neurons create excitatory postsynaptic possibilities interceded by G proteins of the Gαq/11 family, thus enacting phospholipase C-β. GTPase-actuating proteins (GAPs) are believed to be needed to quicken GTP hydrolysis and quickly turn off G proteins. However, the contribution of GAPs in neuronal Gαq/11 flagging has not been analyzed. It has been shown that controllers of G protein flagging (RGS) proteins provide a vital GAP movement at neuronal Gαq/11 subunits. Obstruction of neighbourhood 2-pore region potassium coordinates in cerebellar granule neurons was reconstituted by conveying fanciful Gα subunits that are activated by Gαi/o-coupled receptors, in this way bypassing endogenous Gαq/11 subunits [136, 137] .

6.3. Specificity of RGS Protein. RGS proteins are common negative G protein signaling regulators that work by speeding up the rate of GTP hydrolysis on G protein α subunits [138]. The large number of signaling pathways involving RGS and G proteins raises the question of how specificity in mutual recognition is achieved. In examinations using scrubbed proteins or proteins conveyed in cell culture [135, 139, 140], progression towards this way is made by portraying the instances of unique RGS-G protein associations. To fully comprehend the specificity of RGS protein function within a given G protein cascade, however, this knowledge is insufficient on its own. RGS proteins may also be needed to distinguish between the free activated G protein α subunits and their complexes with effectors or other regulatory proteins in order to make sure that the timing of the full signaling event delivered by a certain route is physiologically acceptable [141].

6.4. RGS Proteins as Drug Targets. Numerous neurological diseases are linked to RGS proteins. The development of RGS-insensitive Gα subunits and RGS-insensitive knock-in mice has greatly contributed to our knowledge of the role RGS proteins play in these disorders [142]. These mutant Gα subunits cause an uncoupled RGS-Gα state, which is critical to understanding the implications of disrupting this interaction on the body's physiological functions. RGS proteins play a critical role in signal transduction, as demonstrated by subsequent RGS deletion or knockdown studies that replicate an environment in which GPCR signaling is not restrained by RGS proteins [143–145].

6.5. RGS Inhibitors as Clinical Therapeutic Agents. RGS proteins, which offer an alternate approach of controlling the activity of G protein-coupled receptors, the target of many medications, have recently come to light as possible therapeutic targets. RGS protein inhibitors must be able to cross the blood-brain barrier, be permeable to cells, and disrupt protein-protein interactions (RGS-Gα), depending on the therapeutic target [135].

7. GPCRS in CNS

Extensive examinations of the genome sequence databases of humans and mice have revealed that the nonodorant GPCR superfamily consists of 392 receptors in mice and 367 receptors in humans, with 343 receptors being mutually expressed between these two species. Within the CNS, nonodorant GPCRs are abundantly expressed, with 6 clusters being noted [17, 146].

7.1. Medium-Sized Spiny Neurons (MSNs) Control Psychiatric Symptoms in the Striatum. The striatum is the basal ganglia's main input structure, and dopamine primarily regulates how information is processed there [147]. The nigra pars compacta (SNc) and the ventral tegmental area (VTA), containing nuclei associated with reward processing, reinforcement learning, and motor control, both innervate the striatum, with dysfunction in the VTA, in particular, being associated with schizophrenia. Within the striatum, MSNs, which represent a specialized type of GABAergic inhibitory cells, represent some 95% of neurons [148, 149]. MSNs have striatonigral and striatopallidal pathways and, based on their projection, can be classified into two types of neuronal populations. The striatonigral (direct) pathways project onto the medial globus pallidus (MGP) and the SNr (substantia nigra pars reticulate) and express dopamine D1 receptors and neuropeptide substance P. Adenosine A2A receptors, neuropeptide encephalin, and dopamine D2 receptors can be found in the striatopallidal (indirect) pathways, which project onto the lateral globus pallidus (LGP) [150, 151]. In the light of the known involvement of MSNs in the development of schizophrenia and PD [147], their differential functions require elucidation.

7.2. Antipsychotics Exerts Therapeutic Action through Dopamine D2 and D1 Receptors. Dopamine D2 and Gs-coupled dopamine D1 receptors are both strongly expressed in the striatum, which is where the majority of routinely prescribed antipsychotics are taken [147]. Schizophrenia has complex inheritance patterns and includes cognitive deficits and positive and negative symptoms [152]. These schizophrenic symptoms indicate a hyperactive dopaminergic transmission (mesolimbic pathway) as well as a lessened dopamine release in the prefrontal cortex [153]. Aripiprazole is a partial dopamine D2 receptor agonist and is classified as an atypical antipsychotic. In a situation of excessive dopaminergic neurotransmission, it also functions as a D2 receptor antagonist [154]. In schizophrenia, D2 signaling is considerably hyperactive [155], and in the prefrontal cortex, the D1 receptor is decreased [156].

7.3. Striatal-Enriched GPCRs Are a Potential Drug Target for Psychiatric Disorders. The striatum is an important part of the brain that might be studied as a possible treatment target for mental disorders. GPR88, GPR52, and GPR6, as well as dopamine D1 and D2 receptors and adenosine A2a receptors, are the most highly expressed GPCRs in the mouse striatum, according to transcriptional analyses [17]. GPR88, GPR52, and GPR6 are orphan GPCRs, and their transcriptional expression patterns are almost indistinguishable from

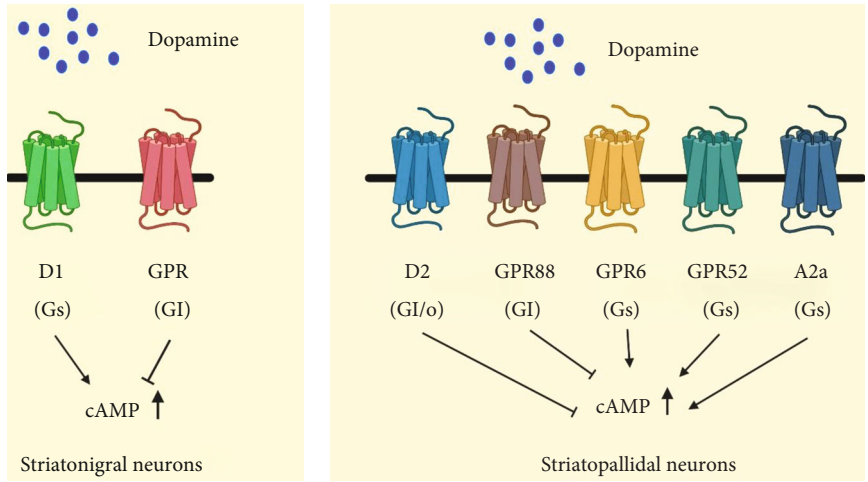

Figure 2: GPCRs expressed in the striatum in medium-sized sharp neurons (MSNs). MSNs are isolated into two primary categories: striatonigral (a) and striatopallidal (b) neurons.

D2 and D1 receptors in the cerebrum. In both striatonigral, GPR88 is communicated, and striatopallidal neurons communicate D2 and D1 receptors, individually, as per in situ hybridization (ISH) investigation (basal ganglia), while GPR6 and GPR52 are communicated in the striatopallidal neurons (Figure 2). Within the taking after segment, these three vagrant GPCRs, as well as the A2a receptor, are briefly clarified.

7.3.1. Adenosine A2a Receptor. Adenosine regulates a wide range of brain activities by acting on adenosine A1 and A2a receptors. The A2a receptor is reported to have a major impact on a variety of neuropsychiatric functions, fundamentally through glutamatergic and dopaminergic neurotransmission, which may have potential value in a number of neurological disorders. Niemann-Pick disease, autism-spectrum disorders, and schizophrenia have been linked to A2A receptor agonists, whereas A2A receptor antagonists have been linked to Alzheimer's disease, attention deficit hyperactivity disorder, fragile X syndrome, depression, and anxiety [9, 157]. The Gs-coupled A2a receptor is preferentially expressed in the striatopallidal MSNs (Figure 2) and is reported to play a key role in motor function regulation as its ligand can trigger characteristic motor effects [158]. In this light, GABAergic communication from striatopallidal MSNs guided by A2a receptor antagonists may provide a PD treatment strategy [159, 160]. As the A2a receptor can regulate dopaminergic neurotransmission, this may, hence, have psychopharmacological implications. A key component that is considered to allow A2a inhibitors to intervene in motor activity is by modulating GABA discharge.

Some researchers believe that future treatments for mental illness will be based on an inhibitory interaction between D2 and A2a receptors. Antipsychotic effects of psychostimulants can be altered by A2a agonists in animal models [161]. Pharmacological and hereditary investigations show that the movement of the A2a receptor impacts patients' schizophrenia-like behavior. Caffeine, as a nonselective A1 and A2a receptor antagonist, has demonstrated both positive and negative effects in schizophrenia, depending on when it is administered, for how long and when its actions are evaluated [162]. Most schizophrenia symptoms primarily derive from perturbed dopaminergic and glutamatergic neurotransmission; however, alterations in the adenosinergic system also have been reported. As the adenosinergic system is associated with motivational and cognitive processes, the impact of adenosine on dopaminergic- and glutamatergic-mediated neurotransmission likely depends on the baseline status of these processes when the adenosinergic system is being manipulated. As noted, adenosine A1 and A2A receptor agonists reverse both NMDA receptor hypo- and dopaminergic hyperactivity. Whereas caffeine may worsen the positive symptoms of schizophrenia subjects (such as delusions and hallucination), a number of studies (but not all) have reported that it positively impacts learning and memory tasks [162]. However, cognitive performance can be affected by multiple potentially competing factors including attention and motivation as well as stress, and differential effects on these can well account for overall improvements in learning and memory under some conditions and worsening under others. Cellular investigations have demonstrated that adenosine A_{2A} receptor activation decreases the affinity of dopamine D_2 receptor agonist binding sites on striatal neurons, without altering the affinity of dopamine D_2 receptor antagonist binding or the expression of D_2 receptors. Extensive studies reviewed by Ferré and colleagues support the concept that GPCR oligomerization can readily occur and that GPCR homodimers represent both functional and structural building blocks whereby heteromers, comprising of two different homodimers, can be formed that are each able to signal via their chosen G protein. This appears to be particularly likely in relation to A_{2A} receptor-D_2 receptor heteromers and potentially accounts for their known allosteric mechanisms and multiple unique pharmacological and biochemical properties. On chromosome 22q12–13, a single nucleotide polymorphism (SNP) on the

A2a receptor provides a candidate schizophrenia susceptibility gene [163], which, again, ties the adenosine A2a receptor to various mental disorders that include schizophrenia, as well as depression and anxiety. As indicated by a variety of studies, the adenosine A2a receptor "fine tunes" glutamatergic and dopaminergic network balance [164]. In this regard and as noted, the opposing interaction of D2 and A2a receptors in the striatum, in relation to dopaminergic action, provides potential antipsychotic activities in schizophrenia, as an antagonist of the dopamine receptor [161]. In relation to glutamatergic action and as reviewed by Komatsu [165], both A2a and A1 receptor agonists have been reported to reduce the electroencephalogram (EEG) and behavioral effects of NMDA receptor inhibitors [166]. It has been shown that NMDA receptor activity may be influenced by both A1 and A2A receptor function in schizophrenia models of NMDA receptor hypofunction. While the NMDA antagonist effects in animal models of schizophrenia are reversed by the blockage of A2a receptors and the genetic deletion of the receptor, this suggests that correcting the imbalance in adenosine A2a receptors may rectify the hypofunction of NMDA antagonists [167].

7.3.2. GPR88. Human gene association studies show a rising correlation between GPR88 function and mental health issues, including schizophrenia and bipolar disorder as well as neurodevelopmental and neurodegenerative disease [168–170]. Following exposure to a variety of psychoactive substances, including antidepressants, mood stabilizers, and drugs of abuse, mRNA levels of Gpr88 have been observed to vary in animal models. Hence, in this light, GPR88 may provide a promising target in the development of new treatment strategies for neurological disorders. By deleting the Gpr88 gene in mice, striatal-dependent physiology, neural networks, and behaviors are affected, resulting in hyperactivity, stereotypies, and deficits in learning and motor coordination, as well as changes in reward-driven behaviors. The expression of GPR88 beyond the basal ganglia and its knockout suggest that GPR88 has potential influence on a broad range of behaviors and brain physiology; however, how this is specifically achieved from a molecular basis remains to be elucidated as, currently, selective pharmacological probes to manipulate GPR88 remain relatively few. Available structural studies suggest that GPR88 is an atypical GPCR. Although considered as part of the class A (rhodopsin-like) family of GPCRs, which is the largest group and accounts for approx. 80% of GPCRs (including hormones, neurotransmitters, and light receptors), GPR88 minimally shares amino acid sequence and functional similarities to other members of this class. So far as we know, the structure of the GPR88 transmembrane binding pocket is more in line with class C GPCRs (which include the metabotropic glutamate family, GABA receptors, calcium-sensing receptors, and taste receptors). Although significant efforts have been made to deorphanize GPR88 (i.e., discover ligands that are highly selective), several hurdles persist, and hence, the signaling pathway(s) and receptor capabilities of GPR88 remain mostly unknown. Studies including the electrophysiological evaluation of MSNs taken from knockout mice lowered tonic GABAergic inhibition and responses to synaptically generated GABA, whereas glutamatergic excitatory synaptic transmission was increased. This shows that GPR88 may play a role in glutamatergic transmission, together with the phosphorylation of the AMPA-type glutamate receptor GluR1 in GPR88 knockout mice. According to the findings, GPR88 appears to have a role in the response of GPR88 knockout mice to agonists of the muscarinic and opioid delta and mu receptors. GPR88 has been discovered as a genetic risk factor for bipolar illness and schizophrenia by genetic association analysis. GRP88 dimerization with nonorphan GPCRs is supported by recent findings [171–174] and demonstrates an ability of GPR88 to dampen the signaling of many GPCR receptors in close proximity as well as impede β-arrestin recruitment. As ligands for GPR88 are recently being described, including 2-PCCA, RTI-13951–33, and phenylglycinol derivatives, our understanding of the signaling pathways associated with GPR88 and its physiological roles will likely become clearer in the near future.

7.3.3. GPR6. GPR6 is mostly expressed in the basal ganglia's striatopallidal neurons (Figure 2) [17]. GPR6 is an orphan receptor that has a ubiquitous function and generates an increase in intracellular cAMP levels when it is linked to a stimulatory Gs-protein. GPR6 was initially described as a lysophospholipid sphingosine 1-phosphate (S1P) receptor; however, this view has not been confirmed by others [175]. The corticostriatal circuitry and the dopaminergic system are considered engaged with human instrumental learning [176]. In rodent essential cerebellar granule neurons, overexpression of GPR6 augments neurite outgrowth [177]. Nishi and Shuto examined the neurochemical and behavioral phenotypes of GPR6 knockout mice to aid define the receptor's function. GPR6 deletion-mice demonstrated a decrease in striatal cAMP and an elevation in dopamine, as well as increased movement actions. A reduction in dyskinesia in a PD mouse model after therapy with apomorphine and quinpirole was also noted, implying that GPR6 inhibition may provide benefit PD. In GPR6 knockout animals, the phosphorylation of dopamine and the cAMP-regulated phosphoprotein of 32 kDa (DARPP-32) at Thr34 increased significantly, while the production of DARPP-32 in the striatum did not. The cAMP/PKA pathway phosphorylates DARPP-32 at Thr34, which is essential for the signal transduction of dopamine D2 and D1 receptors [178]. The D2 receptor antagonist haloperidol has been shown to increase DARPP-32 phosphorylation at Thr34 in striatopallidal neurons [178–180]. Increasing evidence supports the concept that GPR6 could potentially be targeted as a treatment strategy for schizophrenia.

7.3.4. GPR52. Despite the fact that the antipsychotic medicine reserpine is reported to be a surrogate ligand for actuating intracellular cAMP accumulating and receptor internalization, GPR52 is an orphan GPCR, indicating that it may be a Gs-coupled receptor [165]. Over 90% of the amino acid corrosive grouping property of GPR52 is conserved in vertebrates, and it is broadly distributed in the brain, notably the striatum, with no apparent alterations between species. Despite the fact that

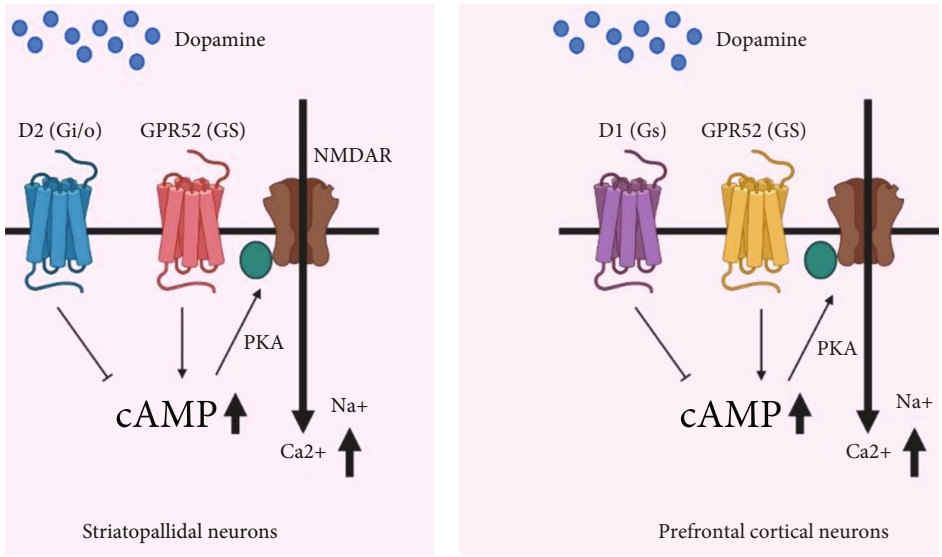

Figure 3: A potential sign transduction pathway for GPR52.

GPR52 is expressed in the basal ganglia, it also communicates with a high number of D1-expressing neurons in the prefrontal cortex. This is an unusual expression profile (Figures 2 and 3). As a result of this, GPR52 activation may promote good effects of schizophrenia by antagonizing Gi/o-coupled D2 receptor action in striatopallidal MSNs, while also reducing schizophrenic adverse effects and cognitive disability by upgrading NMDA receptor movement in prefrontal cortical neurons via protein kinase A (PKA), as can be seen in D1 receptor-NMDA signal transduction [181, 182].

An extensive study of GPR52-expressing neurons' axonal extensions shows that the limbic neural circuit, which is critical for memory and spatial memory, is communicated by GPR52. Dopaminergic neurons in the midbrain get a negative compensatory signal from GPR52-expressing neurons in the habenular nucleus [183]. Prefrontal cortex GPR52-expressing neurons are almost entirely glutamatergic, while only around 10% are GABAergic. The GFP-fused human GPR52 is useful in vitro in hGPR52-GFP transgenic (Tg) mice, and hGPR52-GFP and D2 receptor proteins are clearly separated across striatal regions. The GPR52-GFP is located largely in the LGP, while the bulk of D2 receptor proteins are detected in the striatum. LGP's axon terminals contain GPR52, whereas the dendritic spines of striatal neurons are home to D2 [17]. Up until now, GPR52 transgenic (hGPR52) and GPR52 knockout (KO) mice seemed to be created, as well as examined. The methamphetamine-(MAP-) interceded hyperlocomotion of hGPR52 Tg mice is less articulated than non-Tg mice, despite how hGPR52 Tg mice have ordinary locomotor action under typical conditions, showing that overexpression of GPR52 can neutralize hyperdopaminergic transmission prompted by MAP. GPR52 KO mice spend more time in the center zone of the open field test, indicating that they have anxiolytic-like behavior. NMDA receptor antagonist MK-801 makes GPR52 KO mice more susceptible to the startle reaction of the induced hindrance test (PPI). The startle reflex's habituation and PPI are slowed in schizophrenia patients. However, both conventional and atypical antipsychotics enhance PPI deficiencies and reduce startle responsiveness [184]. These data demonstrate that GPR52 appears to affect not just dopamine transmission but also NMDA signaling [185]. An intense, orally accessible GPR52 agonist with excellent pharmacokinetic properties was recently developed. After the oral administration of 3 mg/kg of methamphetamine, this agonist generously diminishes methamphetamine-instigated hyperactivity in mice and has reported extrapyramidal indications (EPS), proposing that GPR52 enactment checks effective dopaminergic transmission in the limbic framework. The disclosure of GPR52's atomic component could prepare for a new examination for the dopamine and NMDA frameworks, just as the advancement of new antipsychotic drugs. Some GPCRs in the specific types of psychiatric disorders with the function, polymorphisms, and drugs targeting receptors are shown in Table 2.

8. Treatment of Psychiatric Disorders

8.1. Modes of Action of Antipsychotics in Psychiatric Disorders. Catecholamine neurotransmitters, such as dopamine, are found in the brain [186]. Parkinson's disease, schizophrenia, obsessive-compulsive disorder, bipolar disorder, and hyperactivity-hyperactivity disorder are only a few of the CNS illnesses where dopamine plays a major role [187, 188]. D1 and D2 dopamine receptors are two subclasses of G protein-coupled receptors (GPCRs), which are responsible for the action of dopamine. When it comes to receptor subclasses, D2 has D2R, whereas D1 has the D1 receptor (D1R), while receptor subclasses D5 and D2 and subclasses D4 and D3 each have their specific receptor (D5R) [189]. The basal ganglia receive their information mostly from the striatum, which has the greatest number of D1R and D2R neurons [190]. In schizophrenia, the SNc and the VTA both have a substantial impact on striatum

Table 2: Some GPCRs in the specific types of psychiatric disorders with the function, polymorphisms, and drugs targeting receptors.

Some GPCRs in psychiatric disorders	Normal function	Polymorphism/change in expression	Drugs targeting receptors
5HT1A receptors	Evidence that continuous antidepressant treatment results in desensitization of somatodendritic 5HT1A auto receptors in the dorsal raphe, and subsequent increase serotonergic transmission in the hippocampus supports the hypothesis that 5HT1A receptors play a role in mood disorders.	Five-HT1A receptor data from numerous research suggest that the 5HT1A receptor has a role in depression and treatment response. Lemonde and colleagues establish a link between the C-1019G 5HT1A promoter polymorphism and serious depression, suicide, and the effectiveness of the antidepressant flibanserin, a 5HT1A agonist.	Chronic lithium therapy in bipolar individuals normalizes abnormalities in 5HT1A receptor binding.
5HT2A receptors	It has been demonstrated that antidepressants decrease the cortex's 5HT2A receptor binding.	Five-HT2A receptor A-1438G, a promoter polymorphism in the 5HTR2A gene, has been linked to serious depression. There have been numerous attempts to find a link between 5HT2A receptor polymorphisms and bipolar disorder, but these studies have not consistently shown any.	Studies examining the binding of the 5HT2A receptor after chronic lithium medication have yielded conflicting findings. Although studies in platelets have shown that lithium-induced increases in 5HT2A receptor binding capacity in bipolar individuals, the majority of research imply that lithium generates a decrease in 5HT2 receptor binding, with the strongest evidence in the hippocampus.

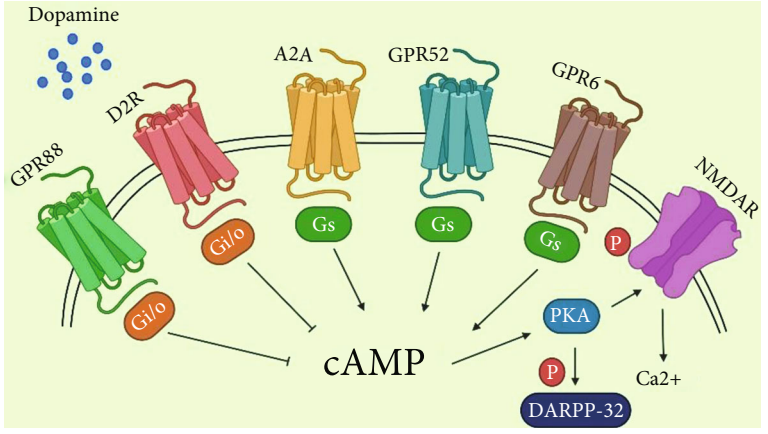

Figure 4: Model of GPCR-mediated NMDA receptor pathways in striatopallidal MSNs by phosphorylating it with cAMP.

innervation; it is considered that the VTA is hyperactive in positive symptoms [191]. The striatum includes 5% of interneurons including large aspiny cholinergic neurons and 95% gamma-amino butyric acid (GABAergic) medium-sized spiny neurons (MSNs) [151], composed of direct (striatonigral) and indirect (striatopallidal) pathways [192]. The striatonigral neurons transmit onto the MGP (medial globus pallidus) and SNr (substantia nigra pars reticulate) and prompt D1R and P, neuropeptide substances. The striatopallidal neurons project toward the globus pallidus (LPG) and contact the SNr/MGP. Moreover, it can express D2R, A2a adenosine receptor, neuropeptide encephalin, and two orphans GPCR, GPR6 and GPR52. The direct and indirect pathways regulate motor behavior by smoothing locomotion and revoking movements [153, 193, 194].

Reactivation of D2R stimulates Gi/o-facilitated signalling in striatopallidal neurons, which inhibits the PKA (protein kinase A)/dopamine and cAMP-regulated phosphoprotein of 32 (DARPP-32) kDa channel, which can help with many dopamine-related behaviors [152]. Some other examples that can also regulate this pathway are Gs-coupled GPR52, GPR6, Gi-coupled GPR88, and adenosine A2a (Figure 4). These GPRs Gs-coupled are helpful to enrich NMDA (N-methyl-D-aspartate) receptor activity via cAMP/PKA. Dopamine shows a remarkably attenuated response to amphetamine in mice lacking βarr2. A protein-protein interface occurs in D2R, and D1SC1 is found as an inducer of hyperactive activities in mice that needs to signal βarr2 to promote phosphorylation of AKT and consequent activation of GSK3β [153]. The mood stabilizer lithium hinders the triggering of GSK3β and collaboration in βarr2 and AKT [21]. The initial mode of action of antipsychotics in basal ganglia is antagonism of D2Rs and the first-line treatment for bipolar disorder and schizophrenia. As a result, a

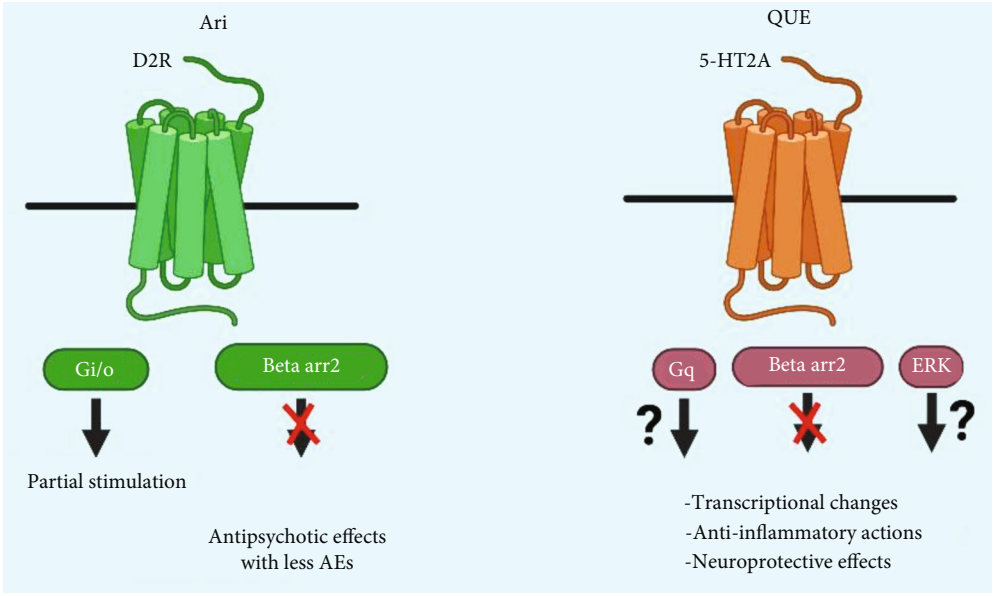

Figure 5: Proposed antipsychotic actions to confer bias of GPCR signalling.

genome-wide association study (GWAS) discovered the gene D2R within a schizophrenia-associated region to be effective for etiology and therapy [195], although antipsychotic medicines are not medically practicable for reducing cortical-related symptoms [196]. The classification of antipsychotics is typical and atypical. Haloperidol is a typical antipsychotic (first-generation antipsychotics) which principally has an inhibitory activity for D2Rs. Quetiapine, risperidone, and some others are examples of atypical antipsychotics (second-generation antipsychotics). They have an antagonistic activity for D2Rs, 5-HT2A (serotonin 2A receptor), and other several GPCRs, and they have fewer side effects than typical antipsychotics (Figure 5).

Different investigations have argued that a hyperdopaminergic state is the only reason for the development of schizophrenia. Additional evidence shows that the hyperdopaminergic state in the basal ganglia and the hyperdopaminergic state in the frontal cortex are also responsible (according to the dopamine hypothesis). Based on the reorganized dopamine hypothesis of psychosis [122], antipsychotics that block D2Rs were thought to simply reverse striatal hyperdopaminergic activity. So, the antipsychotic drug should simultaneously activate and inhibit dopamine signalling according to the brain region [197]. Antipsychotic medication might benefit from the discovery that D2Rs are capable of signaling not just through standard G protein pathways but also through noncanonical pathways that induce the creation of a signaling complex involving protein phosphatase2 (PP2A), GSK3β, 5AKT, and βarr2. Haloperidol initiates AKT phosphorylation in the brains of mice that could recompense for the defective activities of βarr2-GSK3β pathways in schizophrenia. D2R-arr2 recruitment is blocked by all clinically effective antipsychotic medications. Antipsychotics which mainly target the pathway of D2R-βarr2 might have more therapeutically favourable effects but have fewer side effect like EPS [21, 178]. BRD5814, an arr2-biased D2R antagonist, improves dopamine-induced hyperlocomotion in mice while reducing motoric side effects [198]. Hence, the mechanisms of quetiapine and aripiprazole which are frequently used as antipsychotic drugs are discussed in the following sections.

8.1.1. Aripiprazole. Aripiprazole is an antipsychotic medication that was first approved for the treatment of a neuropsychiatric disorder called schizophrenia, which affects more than 1% population of the world. At D2R-Gi/o pathways, aripiprazole works as a partial agonist beneath high dopaminergic tone. They act like a biased D2R-Gi/o inhibitor [199]. Aripiprazole's agonist activity can completely block the D2R-arr2 translocation, which is triggered by dopamine. Aripiprazole also has antagonistic effects on other antipsychotics, such as hyperprolactinemia, metabolic disorder, extrapyramidal symptoms (EPS), weight gain, and sedation [20]. Aripiprazole does not affect the progression of cognitive impairment in schizophrenia patients. Parvalbumin, a calcium-binding protein, and mRNA transcription of the GABA-producing enzyme glutamic acid decarboxylase 67 (GAD67) are both downregulated in an autopsy of a patient with schizophrenia [200]. D2Rs are mediated in GABAergic FSIs in the prefrontal cortex (PFC), which control the activity possibilities of glutamatergic pyramidal neurons. Aripiprazole is only a partial agonist of D2R-Barr2 in the presence of GPCR kinase 2 (GRK2) in the cortical FSIs, although it has a limited effect on action potential firing [201]. Therefore, aripiprazole completely blocks the D2R-βarr2 pathway whereas stabilizing the D2R-Gi/o channel in the hyperdopaminergic striatum of the syndrome.

8.1.2. Quetiapine. Quetiapine, a typical antipsychotic agent, has been approved for diseases like schizophrenia and bipolar disorder [64]. Metabolic nor-quetiapine may mediate the antidepressant action of quetiapine, at least in part, via noradrenaline transporter inhibition [202]. Patients with bipolar illness have been found to benefit from

immediate-release (IR) and extended-release tablets (XR) of quetiapine, according to several studies [203]. Further investigation uncovers that a patient with schizophrenia has abnormal cytokine expression. Quetiapine shows unique anti-inflammatory and neuroprotective properties [107]. Quetiapine reduces the activation of astrocytes and microglia, as well as the synthesis and release of two cytokines, TNF-alpha and MCP-1, in mice (MCP-1). These results recommend that quetiapine may block injury by releasing cytokines and inhibiting glial cells' neuroinflammatory response. Quetiapine also shows activity by neuroprotective effect by increasing the deliverance of brain-derived neuroprotective factor (BDNF) in contradiction of amyloid toxicity from cultured astrocytes [204]. Quetiapine shows unique regional and temporal transportation of epidermal growth factor receptor (EGFR) channel in mice, exhibiting EGFR-dependent striatal ERK1 activation and cortical ERK1 phosphorylation via aripiprazole is EGFR autonomous. The molecular actions of quetiapine are composite and could include influenced signaling [205].

9. Drug Discovery Areas for Psychiatric Disorders

Drug discovery for psychiatric disorders is slow and tedious [206]. There are several pathophysiological processes associated with genetic and environmental factors that may obstruct the drug development of such diseases [206, 207]. One of the major areas is target validation which contributes to the development of new drugs. Human brains are capable of storing a large amount of data, which can render our brains vulnerable in response to a stressful experience. For instance, PTSD is a psychiatric disease where patients can memorize horrible experiences like rape from their entire life. Trauma coupled with remembering past events activates neural plasticity in this situation [208]. Dysregulation of neuronal plasticity and brain atrophy is responsible for stress-related psychiatric disorders. Therefore, it has been suggested that the drug which possesses inhibitory effects on neuronal plasticity will be helpful in the management of this disorder [206]. Stress and depression are caused by glutamate hyperactivation, which is controlled by presynaptic proteins such as CaM kinase II. As a result, it may provide an opportunity to develop novel antidepressant medications [209]. Neuroimaging can provide an exact diagnosis of disease as well as the location of brain dysfunction. Developing new drugs will be feasible when the diagnosis is specific [206]. Additionally, imaging-based diagnosis has been an aid to the clinical management of psychiatric disorders.

10. Conclusions and Future Perspectives

Neurotransmitter imbalance leads to brain dysfunction in people with psychiatric illnesses. The GPCR may have a role in these conditions, as well as in psychiatric difficulties. As a result, the creation of new treatment options and pharmacological tactics is made easier with an in-depth knowledge of G protein-coupled receptor activities and signaling pathways. Several studies suggest that this dysfunction of ROS, glutathione, and oxidative stress is responsible for the alteration of brain function. The fact is that innovative drugs that simply imitate "traditional" treatments by altering neurotransmitter levels directly or indirectly may be of limited help to many patients with refractory illnesses. Altering synaptic activity will alter the system's postsynaptic "throughput," because such tactics presuppose that target receptors and downstream signal mediators are functionally intact. The direct targeting of postreceptor sites may be the sole strategy to enhance therapy for patients who are resistant to traditional medications, given the likely existence of GPCR abnormalities (and perhaps their signal transduction pathways). Research into the generation and deactivation of second messengers may lead to novel pharmacological medicines. Developing novel treatments that target second messenger systems may be possible since they are exceedingly varied at the molecular and cellular level, are connected to receptors in several ways, and are expressed in a wide variety of cell types and tissues. This, on the other hand, is more difficult than the development of receptor-targeted medications. These sites on the substrate serve as built-in targets for relative specificity of action since signal transduction pathways show varied properties based on their active state. Very recent achievements in new medicines for the long-term management of these serious mental illnesses are hugely encouraging. When it comes to the next phase of neuropsychopharmacology, it will be critical to apply the information obtained from advances in neuroscience and pharmacology in clinical settings. Moreover, a number of compounds and strategies have been discussed in this review for managing mental illness. Further investigations are required to develop standard treatment strategies with effective neuropotential effects. The present treatment approaches should be observed carefully to ensure their efficacy, or it should go for further development. However, more in-depth studies are required to identify more effective treatment approaches that aid in the improvement of current treatment methods and strategies. GPCR expression data from mice and humans can be used to model GPCR function in mental illnesses in a manner that is appropriate for animals. This data may also be used to find new therapeutic targets and anticipate on-target side effects.

Data Availability

All data are available within the text.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Review Article

PARP-1 Is a Potential Marker of Retinal Photooxidation and a Key Signal Regulator in Retinal Light Injury

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Advancements in technology have resulted in increasing concerns over the safety of eye exposure to light illumination, since prolonged exposure to intensive visible light, especially to short-wavelength light in the visible spectrum, can cause photochemical damage to the retina through a photooxidation-triggered cascade reaction. Poly(ADP-ribose) polymerase-1 (PARP-1) is the ribozyme responsible for repairing DNA damage. When damage to DNA occurs, including nicks and breaks, PARP-1 is rapidly activated, synthesizing a large amount of PAR and recruiting other nuclear factors to repair the damaged DNA. However, retinal photochemical damage may lead to the overactivation of PARP-1, triggering PARP-dependent cell death, including parthanatos, necroptosis, and autophagy. In this review, we retrieved targeted articles with the keywords such as "PARP-1," "photoreceptor," "retinal light damage," and "photooxidation" from databases and summarized the molecular mechanisms involved in retinal photooxidation, PARP activation, and DNA repair to clarify the key regulatory role of PARP-1 in retinal light injury and to determine whether PARP-1 may be a potential marker in response to retinal photooxidation. The highly sensitive detection of PARP-1 activity may facilitate early evaluation of the effects of light on the retina, which will provide an evidentiary basis for the future assessment of the safety of light illumination from optoelectronic products and medical devices.

1. Introduction

The retina is responsible for sensing light signals from the outside world and converting these light signals into bioelectrical signals to form vision [1]. The frequency and duration of human eye exposure to excessive, strong, artificial lighting has gradually increased, including prolonged exposure to mobile phone screens, televisions, computers, excessive indoor illumination, and ophthalmic examinations with intensive lighting [2]. Although certain compensatory mechanisms exist in the retina to autorepair such damage, prolonged exposure to intensive light can cause acute or chronic retinal injury [3]. Therefore, how to effectively and accurately evaluate the safety of light illumination to the eye, especially to the retina, is still a necessary and urgent scientific issue.

Light-induced damage to the retina can be classified as photothermal damage and photochemical damage [4]. High-power light irradiation is able to cause a rapid increase in the temperature of local retinal tissue and can lead to irreversible retinal photothermal damage due to the protein denaturation and the inactivation of enzymes if the temperature rises more than 10°C above the basal level [5–8]. However, the light intensity that causes retinal photochemical damage is near the level of light illumination used in daily life; thus, retinal damage caused by photochemical reactions has become a growing concern [9]. Photooxidation is the initial molecular step that triggers the retinal photochemical

damage via the oxidative cascade reactions and may even further activate the death signals in retinal cells [10]. *In vivo* studies have shown that retinal photochemical damage predominantly occurs in the outer layers of the retina, including the photoreceptor and retinal pigment epithelial (RPE) layers [11–13], as a large number of photosensitive substances, such as rhodopsin, melanin, and all-*trans*-retinal, are present in the cells located in these regions of the eye [14]. Energy from only an individual photon can be transferred to these photoactive groups, resulting in the modification of molecular structures and photooxidation [15].

Currently, the techniques most widely used to evaluate retinal light damage in vitro and in vivo assess three factors. First, retinal structural changes are determined using retinal slice hematoxylin and eosin (HE) staining, transparent electron microscope observation of ultrastructure, in vivo imaging optical coherence tomograms (SD-OCT), and autofluorescence (AF). Second, retinal functional changes are assessed via electroretinography, multifocal electroretinogram, visual field, or microvisual field. Third, the protein and mRNA levels of specific molecular markers retinal damage are measured. Light-induced changes in molecular levels may precede structural and functional changes, which may be an early indicator of the hazards of light illumination and retinal light damage and may be used a marker for early detection and assessment of these hazards [10]. Studies have shown that some markers, such as heme oxygenase 1 (HO-1) [16], 8-oxoG (related to oxidative stresse damage), caspase 3 [17], caspase 8 [18] (caspase-dependent apoptotic markers), as well as in situ terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) [19], and rhodopsin levels, may indicate the severity of retinal light damage; however, whether these molecular markers are suitable for accurate and rapid assessment of retinal light damage requires further investigation.

Poly(ADP-ribose) (PAR) polymerase-1 (PARP-1) is a ribozyme that is involved in repairing DNA damage [20]. Studies have shown that PARP-1 is rapidly activated when DNA is damaged, and activated PARP-1 can synthesize a large amount of PAR to facilitate the recruitment of nuclear factors for DNA repair [21]. We and other teams have shown that excessive light radiation can lead to the significant upregulation of PARP-1 *in vivo* and *in vitro*, indicating that light radiation may cause nuclear DNA damage in retinal cells [22–24]. However, the overactivation of PARP-1 may lead to the exhaustion of cellular energy or trigger PARP-1-dependent death [25, 26]. Multiple lines of evidence support the notion that the inhibition of PARP-1 activity or knockdown of PARP-1 may play a key protective role against light damage in photoreceptor cells [27, 28]. Therefore, in this review, we summarize the molecular mechanisms of retinal photooxidation, PARP activation, and DNA repair and illustrate the connections among them. We determine the possibility of using PARP-1 as a standard marker for response to retinal light damage and elucidate the key regulatory role of PARP-1 in light-induced retinal injury. Highly sensitive and accurate detection of PARP-1 activity may facilitate the rapid assessment of the effects of light on the retina and evaluation of the safety of light illumination from various optoelectronic products and medical devices.

2. Methods

The MEDLINE, Scopus, and Wiley online databases were searched using multiple combinations of the keywords "PARP-1," "photoreceptor," "retinal light damage," "photooxidation," "parthanatos," "necroptosis," "autophagy," and "mTOR." Articles published between January 1, 1990 and January 1, 2022 were retrieved; articles without an available English translation were excluded.

3. ADP Ribosylation and PARP-1

ADP ribosylation is a reversible posttranslational modification (PTM) that covalently links one or more ADP ribose unit(s) to a target protein using β-nicotinamide adenine dinucleotide (β-NAD^+) as a donor [29]. This modification is mediated by PARP family proteins, and the target protein can be modified by a single ADP ribose unit or by a polymer chain composed of multiple ADP ribose units [30]. The modification is classified as mono ADP ribosylation or poly ADP ribosylation, according to the amount of ADP ribose added to the target protein [31]. *In vitro* studies have shown that the modification of poly ADP ribosylation can contain up to 200 ADP ribosomes, including linear chains and branched chains [32]. ADP ribosylation can cause functional changes in the modified protein or can serve as a scaffold molecule to recruit other proteins to perform their functions after the protein is modified [33, 34]. Poly ADP ribosylation can regulate a variety of cellular processes, including cell division, apoptosis, chromatin structure regulation, transcription, and protein degradation [35].

The PARP family of proteins is responsible for catalyzing the ADP ribosylation modification [36]. The family consists of 17 members, and the catalytic domains of all members contain a classical conservative sequence H-Y-E, in which histidine and tyrosine are necessary for binding NAD^+, while glutamic acid is related to catalytic activity [37]. According to the catalytic activity, the members can be classified into mono (ADP ribosyl) transferases (MARTS), poly(ADP ribosyl) transferases (PARTS), and inactive enzymes. MARTS include PARP-3, PARP-4, PARP-6, PARP-10, PARP-14, PARP-15, and PARP-16; PARTS include PARP-1, PARP-2, PARP-5a, and PARP-5b; and PARP-9 and PARP-13 do not have catalytic activity [38].

PARP-1 was the first to be discovered, and it is also the most well-studied member of the PARP family. PARP-1 can uniquely catalyze ADPr residues to form long- branched chains of poly-ADPr polymers (PARylation) [20]. The human PARP-1 gene consists of 23 exons and is located on chromosome 1q42.12. The full-length PARP-1 protein is composed of 1,014 aa and has a molecular weight of approximately 116 kDa [39]. The structure of PARP-1 is composed of three functional domains: (1) an N-terminal DNA binding domain, containing three zinc finger motifs (Zn1-3) and a nuclear localization sequence (NLS), which can identify DNA double-stranded break and single-stranded breaks; (2) a central BRCA1 C-terminal (BRCT), which is an automodification domain that mediates

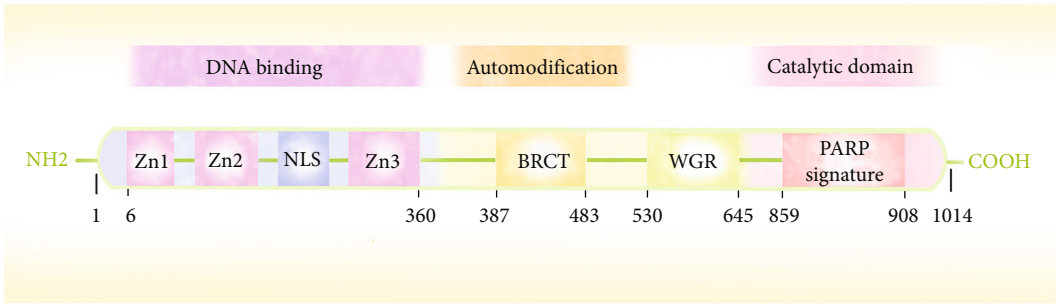

Figure 1: Three main domains of PARP-1 protein: (1) an N-terminal DNA binding domain, containing three zinc finger motifs (Zn1-3) and a nuclear localization sequence (NLS) that can identify DNA double- and single-stranded breaks; (2) a central BRCA1 C-terminal (BRCT), which is mainly for auto-modification; and (3) a C-terminal catalytic domain containing a tryptophan-glycine-arginine–rich domain (WGR) and PARP featured motifs, which is the binding site of the nicotinamide adenine dinucleotide (NAD) required for PAR synthesis.

protein-protein interactions; and (3) a C-terminal catalytic domain containing a tryptophan-glycine-arginine–rich domain (WGR) and PARP featured motifs, which is the NAD+ binding site required for PAR synthesis [40] (Figure 1). As a ribozyme, PARP-1 not only participates in DNA damage repair but is also involved in various biological functions, such as DNA replication, transcription regulation, cell cycle modulation, inflammation, differentiation, aging, and RNA processing [20].

4. Photooxidation and PARP-1 Activation

Prolonged exposure to intense visible light, especially exposure to the short-wavelength visible light with high energy, such as blue, violet, and green light, is prone to induce retinal photochemical damage [9]. The occurrence and severity of retinal photochemical damage is positively correlated with light energy intensity and exposure duration in a dose-dependent manner. Photooxidation is the initial step that triggers retinal photochemical damage [10]. As a crucial part of the visual system, the retina contains a large number of photosensitive groups for receiving light signals [41]. Cones contain three types of opsins that are sensitive to blue light (absorption peak at 430 nm), green light (absorption peak at 540 nm), and red light (absorption peak at 570 nm). All-*trans*-retinal in the outer segment of photoreceptor cells participates in the visual cycle with an absorption peak at 382 nm. In addition, A2E in RPE cells is one of the components of lipofuscin and may function as a potent photosensitizer with absorption peaks at 336 and 430–439 nm. Melanin is also present in RPE cells with an absorption peak at 335 nm [42]. The high energy carried by the photons of short-wavelength light in the visible light spectrum can trigger the orbital transition of electrons or break chemical bonds, resulting in modifications of molecular structures, once absorbed by the photosensitive groups of the retina [43]. The photon energy transferred to these photosensitive molecules causes the electron orbital transition of oxygen to generate singlet oxygen ($1O^2$), which can react with other molecules to break their chemical bonds and further generate superoxide radicals ($O^{2\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radicals (·OH), and other reactive oxygen species (ROS) [44]. This process is called photooxidation [45].

Excessive light irradiation can induce the production of a large amount of ROS in the retina. However, the imbalance between excessive accumulation of ROS and the ability of antioxidant defense systems to combat it may result in oxidative-stress damage, which can transduce the oxidative damage to cellular macromolecules, such as proteins, lipids, and DNA [46]. Notably, nitric oxide can penetrate the nuclear membrane and cause oxidative damage to DNA [47]. The bases, nucleotides, and single and double strands of nuclear DNA are all targets of ROS. Oxidative damage to DNA includes the modification of bases and the breaking of chemical bonds. Excessive ROS can even lead to structural modification of the four DNA bases (adenine, cytosine, guanine, and thymine). As these structures are modified, normal base pairing is disrupted. Oxidative damage to bases can cause base misincorporation, mismatches, and substitutions, ultimately leading to genetic mutations [48]. Guanine is the most easily oxidized DNA base because of its low oxidation potential, and the most common oxidized form is 8-oxo-2′-deoxyguanosine (8-oxoG) [49]. The major oxidized forms of the other three DNA bases are 8-oxo-2′-deoxyadenosine (8-oxoA, the oxidation product of adenine), thymidinediol (the oxidation product of thymine), and 5-hydroxy-2′-deoxycytidine (the oxidation product of cytosine) [50]. Oxidative damage can also break the hydrogen bonds between nucleotides, resulting in DNA single- or double-strand breaks or DNA gaps [51]. Free radicals then bind a hydrogen atom from the pentose of DNA to form a free radical with an unpaired electron at the C4 position, which in turn causes a break in the DNA chain at the β-position [52]. O^2 can also decompose nucleotides, especially guanylate [53]. After oxidative damage, DNA may undergo fragmentation, mutation, and changes in thermal stability, which markedly affects gene transcription and translation [54].

As a ribozyme, PARP-1 is responsible for repairing damaged DNA. The DNA damage such as DNA alkylation, strand gaps, and breaks can rapidly lead to the activation of PARP-1 [55, 56]. The catalytic activity of PARP-1 depends on its interaction with damaged DNA. As PARP-1 binds to DNA strand breaks, the PARP-1 activity increases and the resulting PAR synthesis is more than 500-fold higher than at basal levels [57]. The automodification of PARP-1 in response to DNA damage is crucial for rapid

DNA repair and recruitment of nuclear factors to DNA injury sites [58–61]. In light-induced retinal damage, many studies have shown that PARP-1 responds rapidly and the expression level of PARP-1 increases significantly. Lara et al. found that exposure to white light (2200 lux, 24 hours) significantly increased the number of TUNEL-positive cells in the outer nuclear layer of the rat retina, accompanied by a significant increase in PARP-1 expression, while treatment with the antioxidant EGCG significantly mitigated this retinal light damage [62]. Moreover, our team showed that exposure to visible light markedly induced oxidative-stress damage in retinal ganglion cell 5 (RGC-5) cells *in vitro*, accompanied by a significant increase in the expression of PARP-1, and the PARP-1 inhibitor NU-1025 significantly protected RGC-5 cells from light damage [23]. Additionally, Liu et al. confirmed that visible light irradiation significantly induced excessive production of intracellular ROS, decreased the ratio of reduced/oxidized glutathione (GSH/GSSG), and overexpressed PARP-1 in cultured 661W cells *in vitro* [27]. Lv et al. demonstrated that exposure to light for 12 hours resulted in significant structural damage of the inner nuclear layer (INL) and ganglion cell layer (GCL) in mouse retinas and that this light exposure caused a significant upregulation of PARP-1 in a time-dependent manner [24]. Thus, these studies indicate that photooxidation may result in the damage of nuclear DNA in retinal cells, while the ribozyme PARP-1 may be rapidly activated by damaged DNA and participates in the repair of the DNA damage (Figure 2).

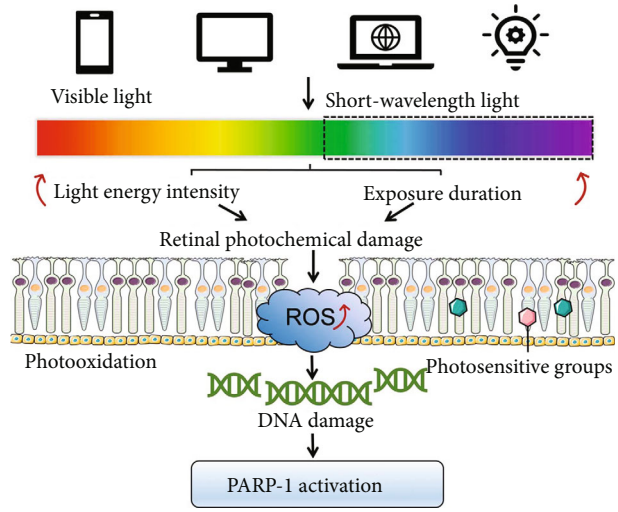

Figure 2: Correlation between PARP-1 activation and photooxidation. Prolonged exposure to intense visible light can induce retinal photochemical damage in a dose- and time-dependent manner. Photooxidation results in DNA damage in retinal cells; PARP-1 is then rapidly activated by the damaged DNA and participates in DNA repair. ROS: reactive oxygen species.

5. Role of PARP-1 in DNA Repair

Often, the damaged DNA sites are on only one strand, which are referred to as single-strand breaks (SSBs). There are many types of SSBs including breaks of the DNA backbone with intact base pairs, abasic sites caused by base (pyrimidine/purine) deletion, and nucleotide deletion. Since repair of SSBs can be guided by genetic information from the complementary strand, SSBs are usually easily mended [63]. However, a damaged site at the same position on both strands of DNA at the same time results in a double-strand break (DSB). DSBs are a more severe type of DNA damage and require the activation of special signaling pathways, such as the homologous recombination (HR) or nonhomologous end joining (NHEJ) [64].

PARP-1 can rapidly identify DNA damage through zinc finger structures [65, 66]. Two of the single zinc fingers (Zn1 and Zn2) of human PARP-1 can form complexes with nucleotide bases exposed from on DNA double-strand gaps through a loop structure connecting with two β-strands [66]. In addition, zinc fingers can also identify the continuity of nucleotide bases and phosphate backbones through a "backbone grasping" mechanism [67]. Thus, PARP-1 is highly sensitive to DNA damage. Once identified DNA free ends caused by single- or double-stranded DNA gaps are sensed, PARP-1 can rapidly bind SSBs and DSBs with its N-terminal DNA binding domain. The binding of DNA gaps triggers a conformational transformation exposing the enzymatic site of PARP-1, resulting in PARP-1 activation and PARylation [56] (Figure 3). This PARylation response

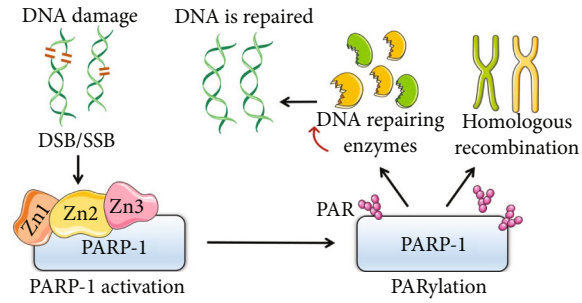

Figure 3: Role of PARP-1 in DNA repair response. PARP-1 can rapidly bind single-strand breaks (SSBs) and double-strand breaks (DSBs) with its zinc finger structures and trigger PARP-1 activation and PARylation, resulting in homologous recombination and the recruitment of DNA-repairing enzymes to repair the damaged DNA.

is very rapid; upon activation of PARP-1, intranuclear PAR levels can rise as much as 500-fold above baseline, consuming up to 90% of intracellular NAD^+ [57]. Long or branched PARs on PARP-1 and other protein substrates serve as scaffolds for the recruitment of DNA repairing enzymes, facilitating the localization of repairing factors to DNA damaged sites [68]. For instance, X-ray repair cross-complementary protein 1 (XRCC1) is recruited by PAR chains and is a key scaffolding protein for the assembly and activation of DNA base excision repair machinery [69]. In the case of DSBs, the chromatin surrounding a break is rapidly and transiently PARylated to recruit the nucleosome remodeling and deacetylase (NuRD) complex, which can further result in ATP-dependent chromatin remodeling, histone deacetylation, and the recruitment of DNA repairing factors [70]. PAR at DSB lesions can also rapidly recruit meiotic recombination 11 (MRE11) to detect DSBs via HR or

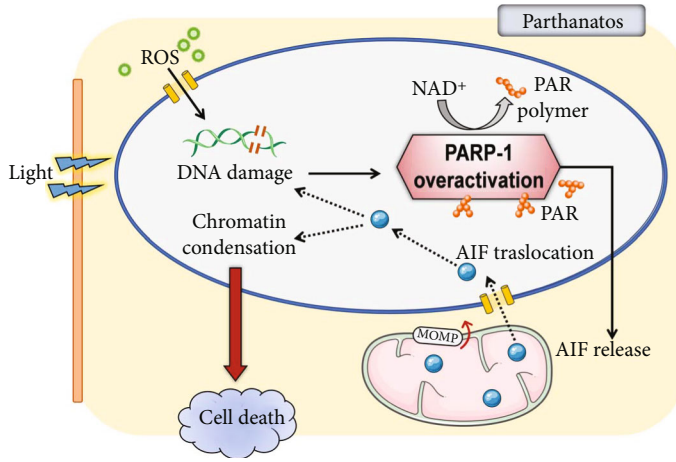

Figure 4: Parthanatos induced by light injury. Prolonged light exposure results in extensive DNA damage in the nucleus. PARP-1 is then overactivated leading to synthesis of a large amount of PAR and increase in mitochondrial outer membrane permeabilization (MOMP), triggering the release of apoptosis-inducing factor (AIF) from mitochondria, causing chromatin condensation/more DNA fragmentation and eventual cell death.

NHEJ, causing the halting of the cell cycle and the activation of downstream repair factors [70]. PARylation of chromatin-associated PARP-1 with negative charges causes chromatin decondensation and changes chromatin into an open conformation that facilitates DNA repair [71]. Furthermore, in alternative NHEJ pathways, PARylation caused by PARP-1 interacting with DSB ends leads to the recruitment of repairing proteins, DNA ligase III/XRCC1, and polynucleotide kinase phosphatase (PNKP) [72, 73]. PARylation modification at DNA damage sites and protein targets is highly dynamic, since PARylation can be rapidly degraded with half-lives ranging from 40 seconds to 6 minutes [74]. The degrading enzymes of PAR include poly(ADP-ribose) glycohydrolase (PARG), Nudix hydrolase 9 (NUDT9) and NUDT16, terminal ADP-ribose protein glycohydrolase 1 (TARG1), MacroD1, and MacroD2, which all contain the domain recognizing PAR and ADP-ribose [75–77]. PARG, as the major de-PARylation enzyme that localizes to the nucleus, can hydrolyze ribose-ribose bonds between ADP-ribose units [75], while NUDT9 and NUDT16 may hydrolyze phosphodiester bonds between ADP-ribose moieties and proteins [76, 77]. Removal of a terminal ADP-ribose is regarded as a rate-limiting step in PAR degradation, and TARG1, MacroD1, and MacroD2 may hydrolyze PARylation by cleaving a glutamate-banded ADP-ribose [75]. Accumulating evidence supports the important role of de-PARylation in DNA repair, however, the detailed mechanism of PARylation needs to be further elucidated.

When the retina suffers from photochemical damage, photooxidation is triggered and causes excessive accumulation of ROS within the retina, resulting in DNA damage of the outer layers of retinal cells. PARP-1 is then activated rapidly and promotes the repair of damaged DNA, to a certain extent, through PARylation. However, if the DNA damage is severe, PARP-1 will be overactivated, leading to cellular energy depletion and ultimately triggering PARP-1-dependent death.

6. PARP-1 Involvement in Signaling Pathways and PARP-Dependent Cell Death

6.1. Parthanatos. Parthanatos is a caspase-independent cell death characterized by the activation of PARP-1 and the nuclear translocation of apoptosis-inducing factor (AIF) [78]. Increasing evidence suggests that the parthanatos plays a crucial role in the progression of various neurodegenerative diseases [79, 80]. When death stimuli result in extensive DNA damage in the nucleus, PARP-1 is overactivated and synthesizes a large amount of PAR, which consumes massive intracellular NAD^+ and ATP, eventually leading to energy exhaustion and cell death [81]. In addition, the intracellular accumulation of PAR may increase mitochondrial outer membrane permeabilization (MOMP), triggering the release of AIF from mitochondria [82]. AIF is a flavoprotein synthesized in the cytosol with a full-length precursor of 67 kDa (pre-AIF) [78]. The 67 kDa AIF is guided by its mitochondrial localization sequence (MLS) and translocates into the mitochondrial intermembrane space, where it is cleaved into the mature form 62 kDa AIF and participates in mitochondrial energy synthesis. Under death stimuli, 62 kDa AIF is hydrolyzed into the 57 kDa soluble form, tAIF, and released from the mitochondria into the cytosol. In the cytosol, tAIF can further translocate into the nucleus and interact with histone H2AX and endonucleases/DNases, causing chromatin condensation, large-scale DNA fragmentation, and cell death [83, 84] (Figure 4). Our recent study showed that exposure to visible light significantly induced the upregulated expression of PARP-1 in photoreceptor cells (661 W) *in vitro*, accompanied by the nuclear translocation of AIF, and knock down of PARP-1 with lentivirus-mediated shRNA significantly blocked nuclear translocation of AIF, thus protecting photoreceptor cells from light damage [22]. Lv et al. found that exposure to light for 12 h resulted in significant structural damage of the inner nuclear layer (INL) and ganglion cell layer (GCL) of the mouse retina and that

light irradiation significantly increased the level of PARP-1 in a time-dependent manner. In addition, they found that light exposure also caused the activation of the PARP-1/AIF signaling pathway in RGC-5 cells (retinal precursor neurons) cultured *in vitro* and that the PARP inhibitor NU1025 significantly attenuated the light-induced death of RGC-5 cells [24].

6.2. Necroptosis. Necroptosis, a form of programmed cell death, is initiated by tumor necrosis factor-α (TNF-α) signaling and mediated by receptor-interacting protein kinase 1 (RIPK1) and RIPK3 [85], though it is caspase-independent [86] and can be blocked by necrostatin-1 (Nec-1) [87]. Studies have shown that PARP-1 is involved in TNF-α-induced necroptosis. PARP-1 can regulate necroptosis directly by interacting with RIP kinases or indirectly by generating PAR, which in turn can target necroptosic effectors [88]. The activity of PARP-1 is also influenced by upstream RIP kinases, and its activity is significantly increased during TNF-α-induced necroptosis, while inhibition of PARP-1 blocks necroptosis, suggesting that PARP-1 plays an active role in necroptosis [89]. Xu et al. showed that the activation of PARP-1 was involved in glutamate-induced necroptosis in HT-22 cells, and necrostatin-1, an inhibitor of necroptosis, could reduce the activity of PARP-1 [90]. Hitomi et al. demonstrated that PARP-2 played a key role in necroptosis in L929 cells induced by TNF-α and a caspase inhibitor (zVAD-fmk), while knockout of PARP-2 significantly inhibited necroptosis [91]. However, the *in vitro* evidence from Sosna et al. suggested that TNF-α-induced necroptosis and PARP-1 signaling represent two distinct and independent programmed necroptosis pathways [92]. Although studies have shown that PARP-1 is closely related to the necroptosis pathway, the exact molecular mechanism of this interaction remains to be elucidated. In addition, there is a lack of *in vivo* experimental evidence determining whether PARP-1 is directly or indirectly involved in necroptosis regulation, particularly clarifying the specific crosstalk between PARP-1 and TNF-α or RIP.

6.3. Autophagy. Autophagy is a programmed self-degradation process used to maintain cellular energy homeostasis. By degrading damaged or dysfunctional organelles, cells may recycle amino acids, lipids, and other molecules via autophagy [93]. The activation of autophagy is closely related to intracellular energy status and is regulated by the mammalian target of rapamycin (mTOR)/AMP-activated protein kinase (AMPK) signaling pathway [94]. For example, mTOR may negatively regulate the activation of autophagy. Mammalian target of rapamycin (mTOR) remains active under conditions where there is sufficient nutrient supply to keep autophagy "off," while mTOR is significantly inhibited as cells are starved, which in turn results in the activation of autophagy to promote energy production [95]. AMPK is able to sense the changes of cellular ATP/AMP and regulate autophagy activation through its downstream signal, mTOR [96]. The massive synthesis of PAR by the overactivation of PARP-1 leads to NAD^+ and ATP depletion as well as significant increases in intracellular AMP, which remarkably influence the activation of autophagy through the interaction between the AMPK/mTOR signaling pathways [97] (Figure 5). Chen et al. showed that PARP-1 promotes autophagy in CNE-2 cells after ionizing radiation by activating AMPK and inhibiting mTOR [98]. Huang et al. showed that, in addition to causing ATP consumption, the overactivation of PARP-1 also promotes autophagy through the liver kinase B1 (LKB1)-AMPK-mTOR pathway, thereby enhancing cell survival in oxidative stress-induced DNA damage [99]. Excessive light exposure can not only cause an increase in the level of PARP-1 but also in the activation of mTOR in photoreceptors [22, 100]. Pan et al. demonstrated that PARP-1 knockdown reduced the phosphorylation level of mTOR in photoreceptors *in vitro*, while knockdown of mTOR also resulted in a significant decrease in the levels of PARP-1 and PAR and that sirtuin 1 (SIRT1) may be the signal hub between PARP-1 and the mTOR signaling pathway [22]. However, as the underlying modulator of the interaction between mTOR and PARP-1, the role of SIRT-1 in regulating photodamage-induced autophagy still needs to be further elucidated.

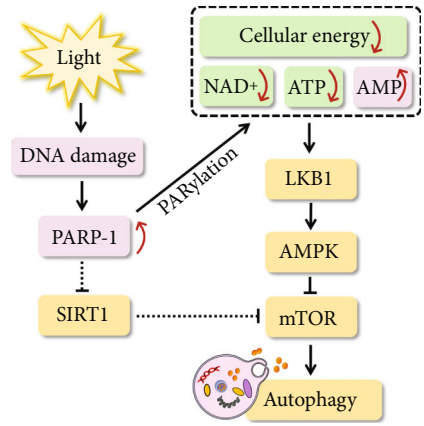

Figure 5: PARP-1-dependent autophagy. The massive synthesis of PAR (PARylation) by the overactivation of PARP-1 leads to NAD^+ and ATP depletion and significant increases in intracellular AMP, which remarkably activates autophagy through the interaction between the AMP-activated protein kinase (AMPK) and mammalian target of rapamycin (mTOR) signaling pathways. In addition, sirtuin 1 (SIRT1) may be the signal hub between PARP-1 and the mTOR signaling pathway.

7. Conclusions and Perspectives

Prolonged exposure to intense visible light, especially short-wavelength light such as blue light, may cause retinal photochemical damage, which is predominantly caused by intracellular cascade reactions triggered by photooxidation [9]. Intracellular oxidative stress damage can cause DNA breaks in the nucleus, in turn triggering the activation of the ribozyme, PARP-1, for DNA repair [99]. Therefore, determination of PARP-1 levels may indirectly reflect retinal light damage. Since PARP-1 is highly sensitive to DNA damage, it can be rapidly activated and is significantly upregulated once DNA damage occurs [101], ultimately triggering

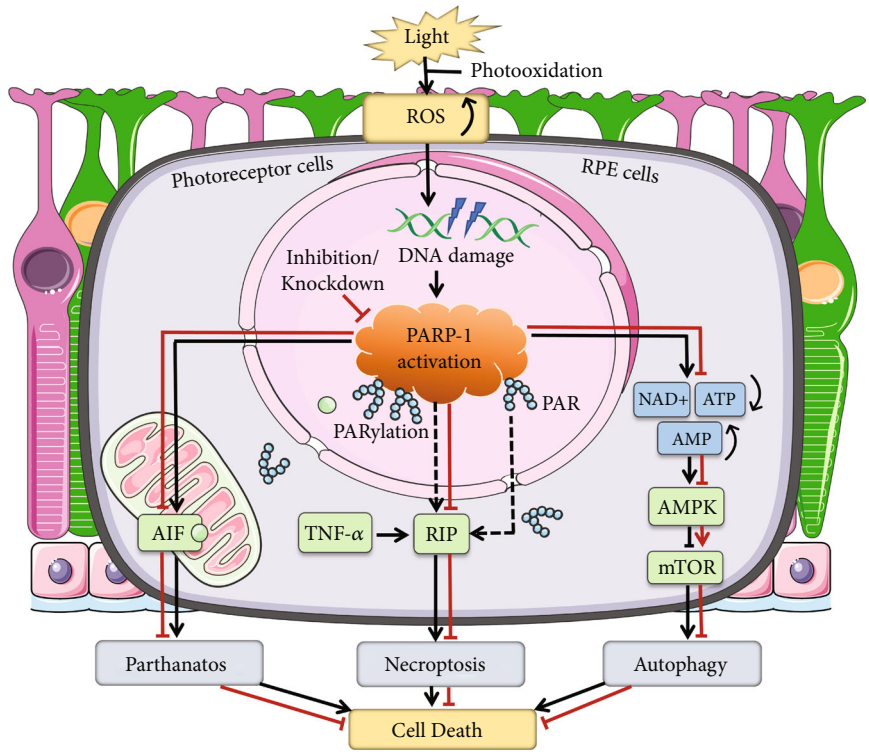

Figure 6: Schematic diagram illustrating that PARP-1 may be a key signal regulator in retinal light injury. Prolonged exposure to intense visible light may cause retinal photochemical damage that is triggered by photooxidation. Oxidative stress damage can cause nuclear DNA breaks and lead to rapid and significant activation of PARP-1 to repair damaged DNA. Additionally, inhibition or knockdown of PARP-1 can play a crucial protective role against light damage in the outer layers of retinal cells.

PARP-1-dependent cell death (Figure 6). PARP-1 has better stability and sensitivity compared with other oxidative stress markers for evaluating retinal light damage, such as HO-1 or 8-oxoG. Experimental evidence for assessing the safety of light illumination on the retina may be provided by determining the level of PARP-1 after light irradiation, using *in vitro* and *in vivo* experimental models and may also be indicative of light illumination safety in daily work.

The detection of PARP-1 activity is crucial as PARP-1 may serve as a potential biomarker. Frequently used techniques for detecting PARP-1 include enzyme-linked immunosorbent assay (ELISA), biotin labeling, immunoblotting, fluorescence, and colorimetry [102–106]. Recently, novel methods have been developed for more efficient detection of PARP-1 activity. Liu et al. indicated a potential tool for PARP-1 activity detection based on the large impact of PARP-1 on the diffusion flux of ferricyanide in anodic aluminum oxide (AAO) nanochannels [107]. Zhou et al. proposed a method to linearly detect PARP-1 activity based on host–guest recognition using a renewable electrochemical (EC) sensor modified with mono-(6-mercapto-6-deoxy)-beta-cyclodextrin on the electrode surface to avoid unspecific adsorption and improve detection accuracy [108]. Liu et al. developed an ultrasensitive EC detection for PARP-1 activity on basis of the electrostatic interaction of PAR and polyaniline [109]. Wang et al. designed a label-free photoelectrochemical (PEC) biosensor, also based on the electrostatic interaction of PAR and another chemical compound, poly [9,9-bis(6′-N, N, N-trimethylammonium) hexyl] fluorenylene phenylene for detection of PARP-1 activity [110]. In addition, Xu et al. further developed a dual-mode (both EC and PEC), label-free strategy for the detection of PARP-1 activity through gold nanocluster (AuNCs). The AuNCs adsorbed by PAR produced both strong fluorescence and chemiluminescence by catalyzing the luminol-H_2O_2 system, which provided higher sensitivity and stability, a wider linear range, and better biocompatibility [111]. In conclusion, these highly sensitive methods for detecting PARP-1 activity may facilitate rapid assessment of retinal light damage, providing an evidentiary basis for future evaluation of the safety of light illumination produced by optoelectronic products and medical devices.

Conflicts of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Authors' Contributions

Xun Li and Guang-Yu Li prepared the first draft of the manuscript. All authors edited the review article. Guang-Yu Li approved the submission of the manuscript. All authors contributed to the writing and editing and agreed to the submission of the manuscript.

Acknowledgments

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Research Article

Bu Shen Yi Sui Capsule Promotes Myelin Repair by Modulating the Transformation of A1/A2 Reactive Astrocytes *In Vivo* and *In Vitro*

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Background/Aims. Multiple sclerosis (MS) is an autoimmune disorder that affects the central nervous system (CNS) primarily hallmarked by neuroinflammation and demyelination. The activation of astrocytes exerts double-edged sword effects, which perform an integral function in demyelination and remyelination. In this research, we examined the therapeutic effects of the Bu Shen Yi Sui capsule (BSYS), a traditional Chinese medicine prescription, in a cuprizone- (CPZ-) triggered demyelination model of MS (CPZ mice). This research intended to evaluate if BSYS might promote remyelination by shifting A1 astrocytes to A2 astrocytes. *Methods*. The effects of BSYS on astrocyte polarization and the potential mechanisms were explored *in vitro* and *in vivo* utilizing real-time quantitative reverse transcription PCR, immunofluorescence, and Western blotting. Histopathology, expression of inflammatory cytokines (IL-10, IL-1β, and IL-6), growth factors (TGF-β, BDNF), and motor coordination were assessed to verify the effects of BSYS (3.02 g/kg/d) on CPZ mice. In vitro, A1 astrocytes were induced by TNF-α (30 ng/mL), IL-1α (3 ng/mL), and C1q (400 ng/mL), following which the effect of BSYS-containing serum (concentration of 15%) on the transformation of A1/A2 reactive astrocytes was also evaluated. *Results and Conclusions*. BSYS treatment improved motor function in CPZ mice as assessed by rotarod tests. Intragastric administration of BSYS considerably lowered the proportion of A1 astrocytes, but the number of A2 astrocytes, MOG+, PLP+, CNPase+, and MBP+ cells was upregulated. Meanwhile, dysregulation of glutathione peroxidase, malondialdehyde, and superoxide dismutase was reversed in CPZ mice after treatment with BSYS. In addition, the lesion area and expression of proinflammatory cytokines were decreased and neuronal protection factors and anti-inflammatory cytokines were increased. *In vitro*, BSYS-containing serum suppressed the A1 astrocytic markers' expression and elevated the expression levels of A2 markers in primary astrocytes triggered by C1q, TNF-α, and IL-1α. Importantly, the miR-155/SOCS1 signaling pathway was involved in the modulation of the A1/A2 phenotype shift. Overall, this study demonstrated that BSYS has neuroprotective effects in myelin repair by modulating astrocyte polarization via the miR-155/SOCS1 pathway.

1. Introduction

Multiple sclerosis (MS) is a heterogeneous neurodegenerative condition [1] that leads to nontraumatic neurological disability as a result of demyelination and axonal injury of the central nervous system (CNS) [2], for which there is still a lack of effective therapies. Approximately 2.8 million people are estimated to have MS globally [3], which imposes a heavy burden on individuals and their families. Accumulating evidence shows that autoimmune-mediated neuroinflammation is

associated with inefficient remyelination [4], leading to the deteriorated repair of demyelinating lesions, which might influence the poor prognosis of MS [5].

The activation of astrocytes and microglia were upregulated dramatically within the demyelination foci, implying that neuroinflammation initiated by innate immune resident cells of the CNS may result in axonal injury and oligodendrocyte loss [6]. Astrocytes, which are the most predominant glial cells found in the brain, perform crucial functions in homeostasis and response to CNS injury [7]. Astrocyte polarization has been widely studied in recent years, and the astrocytic phenotype has been well elucidated. Astrocytes play a harmful or favorable role depending on their polarization phenotypes. According to the findings of a series of research reports, reactive astrocytes may be classified into two main categories: the A1 phenotype has neurotoxic characteristics, whereas the A2 astrocytes are considered to be neurotrophic and can provide neuroprotection [8]. In MS, astrocytes transform to the A1 phenotype [9], secreting several neurotoxic cytokines, such as tumor necrosis factor-alpha (TNF-α), interleukin-1β (IL-1β), and IL-6, leading to the formation of a proinflammatory milieu in the CNS and the subsequent myelin injury as well as impaired remyelination process [10]. Therefore, the development of new drugs that skew A2 polarization to increase the production of anti-inflammatory cytokines and neuroprotective mediators and thus enhance myelin repair may provide an effective therapeutic strategy for MS [11].

MicroRNAs (miRs) are small noncoding RNAs, containing approximately 21 nucleotides, that can downregulate messenger RNA (mRNA) expression by targeting the three prime untranslated regions (3′UTR) [12]. In recent years, several research reports have indicated that specific miRs play an instrumental function in the process of demyelination and remyelination [13], which are critical in MS pathologies [14]. Particularly, upregulation of miR-155 was shown to inhibit the repair of myelin by promoting a proinflammatory microenvironment in the CNS [15, 16]. Moreover, miR-155 was confirmed to drive the polarization of astrocytes towards the neurotoxic phenotype (A1) via regulating the suppressor of the cytokine signaling 1 (SOCS1) pathway [17]. Several human neurodegenerative conditions, including MS, have been linked to upregulated expression levels of A1 astrocytes. The A1 phenotype induces oligodendrocytes death and inhibits oligodendrocyte precursor cells (OPCs) differentiation and maturation, thereby exacerbating demyelination and preventing myelin repair [9]. In MS, oligodendrocytes are the target of inflammatory and immune attacks and their progressive death leads to demyelinated lesions and remyelination failure [18]. The A2-like type enhances the maturity of OPCs and protects against white matter injury progression. Consequently, because miR-155 regulates the transition of A1/A2 reactive astrocytes, we hypothesized that reducing its expression level might be a viable treatment strategy for myelin repair in MS.

Growing evidence has illustrated that traditional therapies are successful in the treatment of neurodegenerative diseases (ND) of the CNS. Traditional therapies in China and India provide a holistic treatment for ND. The herbs and formulations of Ayurveda, traditional Chinese medicine (TCM), are rich in antioxidants, immunoregulatory, neuroprotective, and anti-inflammatory compounds, which have been shown to regulate neuroendocrine-immune functions, restore brain function, and improve quality of life [19]. The works of Sharma et al. have well summarized the therapeutic roles of Ayurveda, TCM, and other alternative therapies in epilepsy, depression, Parkinson's disease (PD), and Alzheimer's disease (AD). It lays a solid theoretical foundation for the development of novel drugs in ND and paves the pathways for further study on traditional herbal medicine as well as for its standardization in the future [20]. In recent years, Chinese herbal medicine has made significant contributions to the prevention and treatment of MS [21]. It has been effective in modulating immune response [22], reducing neuroinflammation, alleviating nerve injury [23], and strengthening myelin repair [24]. For more than a decade, the Bu Shen Yi Sui capsule (BSYS), a traditional Chinese medicine, has been utilized in clinical trials to treat MS. This formulation consists of several Chinese medicines including *Rehmanniae Radix* (Shengdihuang), *Rehmanniae Radix Praeparata* (Shudihuang), *Polygoni Multiflori Radix Praeparata* (Heshouwu), *Forsythiae Fructus* (Lianqiao), *Gastrodiae Rhiazoma* (Tianma), *Scorpio* (Quanxie), *Hirudo* (Shuizhi), *Fritillariae Thunbergii Bulbus* (Zhebeimu), *Rhei Radix et Rhizoma* (Dahuang), and *Leonuri Herba* (Yimucao). Our earlier research illustrated that the therapeutic impacts of BSYS in experimental autoimmune encephalomyelitis (EAE) mice were linked to the promotion of microglia polarization towards M2, which could be associated with alterations in miR-155 and miR-124 *in vivo* [25]. Nevertheless, it remains unclear whether BSYS can directly modulate astrocyte polarization in a cuprizone- (CPZ-) triggered demyelination model of MS (CPZ mice). Given BSYS's antineuroinflammatory effects, it would be quite interesting to examine whether it could be utilized to enhance myelin repair by altering astrocyte polarization, which would be a significant advancement. The focus of this research was to investigate the proremyelinating impacts of BSYS on CPZ mice, as well as the possible processes of astrocytic polarization modulation by BSYS.

2. Materials and Methods

2.1. Animals and Drugs. All animal experimentations were done in compliance with the standards stipulated in the guidelines of the International Council for Laboratory Animal Science, and the approval was granted by the Capital Medical University's Animal Experiments and Experimental Animal Welfare Committee (permit number: AEEI-2020-181). Female C57BL/6 J mice between the ages of 6 and 8 weeks and weighed between 15 and 18 g were procured from Huafukang Biotechnology Co., Ltd. (Beijing, China). We housed the mice in a specific pathogen-free (SPF) laboratory at the Capital Medical University's Experimental Animal Center (SYXK (Beijing) 2018-0003). The animals were given water and food *ad libitum* under constant humidity (40-50%) and temperature ($22 \pm 3^{\circ}$C), as well as a 12-hour

darkness/light cycle. Regular rodent chow that contained 0.2% CPZ (Sigma-Aldrich, United States) was provided by Keao Xieli Feed Co., Ltd. (Beijing, China). Asia-East Biopharmaceutical Co., Ltd. (Beijing, China) synthesized and supplied the BSYS utilized in this experiment. The production process of BSYS was similar to that in our previous reports [26]. BSYS was composed of *Rehmanniae Radix Praeparata*, *Rehmanniae Radix*, *Polygoni Multiflori Radix Praeparata*, *Rhei Radix et Rhizoma*, *Fritillariae Thunbergii Bulbus*, *Hirudo, Scorpio*, *Gastrodiae Rhiazoma, Forsythiae Fructus*, and *Leonuri Herba*. These herbs were combined in the following ratios: 10 : 10 : 10 : 2 : 6 : 3 : 2 : 3 : 6 : 10. All herbs, except *Fritillariae Thunbergii Bulbus*, were immersed for half an hour in water that had been distilled and then subjected to heating for two hours until boiling. After the inspissation of the filtered solution was carried out at a lowered pressure and a temperature of 70°C, the dry powdered sample was mixed evenly with the flour that was made from *Fritillariae Thunbergii Bulbus*. The powder mixture that was produced was then put into capsules. The chemical components of BSYS were identified and quantified by UPLC-QTOF-MS/MS and UPLC-LTQ-Orbitrap-MSn, which have been published elsewhere [27].

2.2. Establishment of Demyelination Model and Drug Treatment. The experimental mice were categorized at random into three groups: the normal control group (CON), CPZ model group (CPZ), and BSYS treatment group (CPZ +BSYS). In a paper published by our team in 2018, Zhao et al. tested the drug-only group by gavage of CON mice with BSYS 3.02 g/kg/d. They found no significant difference between mice in the CON group and mice in the CON +BSYS group [26]. Therefore, the BSYS drug-only control group was not retained in this experiment. Dietary supplements of CPZ were not supplied to the animals in the CON group. The CPZ-mediated demyelination model was established as previously described; to trigger demyelination, the mice received a diet that contains 0.2 percent (0.2% *w*/*w*) cuprizone for six weeks. Afterward, they were switched to a regular diet for another two weeks. During the recovery period, the mice participants in the BSYS treatment group were subjected to BSYS by gavage at a dose of 3.02 g/kg one time per day for two weeks. In addition, the therapeutic equivalent daily dosage of BSYS administered to the mice was set to be 3.02 g/kg, which was the optimum dose for treating the MS mouse model in our previous study [25, 28], whereas mice in the CPZ group were administered an equivalent volume of vehicle (distilled water) (Figure 1).

2.3. Body Weight Measurement and the Rotarod Test. After receiving CPZ-containing chow, each mouse's weight was recorded two times a week starting on the initial day of treatment. Subsequently, the mice were trained on the rotating rod for 180 s per day for three days before the behavioral assessment. A rotarod test was administered twice weekly to the mice once they had become acclimated to the apparatus. For 180 seconds, the rotation speed was steadily raised from 5 rpm to 40 rpm. The test was completed when the mouse either dropped off or was able to hold onto the rod

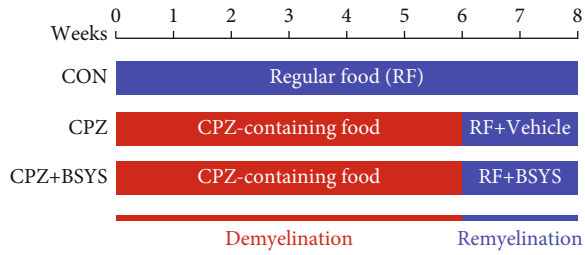


Figure 1: Schematic timeline of the experimental procedures. Blue represents regular food (RF), whereas red represents a diet containing 0.2% CPZ (CPZ-containing food). In the CPZ mouse model, the first 6 weeks are the demyelination phase and the last 2 weeks are the remyelination phase.

for 2 or even more spins [29]. To get an accurate value of the mice's motor function, we recorded the amount of time they spent on the rod.

2.4. Luxol Fast Blue (LFB) Staining. The pentobarbital sodium (60 mg/kg) administered intraperitoneally was utilized to anesthetize the mice before perfusing them transcardially with phosphate-buffered saline (PBS), followed by the administration of PBS comprising 4 percent ice-cold paraformaldehyde (PFA). A 12-hour fixation in 4% PFA solution at 4°C and subsequent dehydration and embedding in paraffin were performed after dissection of the brain tissues. Brain paraffin coronal sections (5 μm thick) were prepared, including the corpus callosum (CC). The extent of remyelination was assessed using LFB staining, and the sections were viewed and digitally photographed using a microscope. Three microscopic fields were randomly sampled from CC, caudoputamen (CPu), and anterior commissure (AC). The LFB results were quantified as the relative expression level of integrated optical density (IOD).

2.5. Preparation of BSYS-Containing Serum (BSYS-Serum). BSYS-serum and blank serum were prepared as previously described [30]. In brief, a dose of BSYS equal to 11.7 g/kg of body weight was administered intragastrically to Sprague-Dawley rats two times daily for one week. On the 7th day of the experiment, blood samples were extracted from the rats that had been sacrificed 2 hours after they had been fed by gavage. The serum was then isolated by centrifuging these samples. To produce blank serum, the equivalent amount of distilled water was administered to the control rats. The main chemical constituents in BSYS-serum analyzed using UPLC-MS/MS have been reported in our previous studies [31].

2.6. Cell Culture and Treatment. Primary astrocytes (ProCell, Wuhan, China) were extracted from C57BL/6 mice aged between 1 and 3 days old. The astrocytes were grown in Dulbecco's modified essential medium (DMEM) (Analysis Quiz, Beijing, China) containing 10% fetal bovine serum (FBS; Corning, NewYork, USA) and 1% penicillin-streptomycin (P/S; KeyGen, Nanjing, China) and preserved in 5% CO_2 at 37°C in a humid incubator. Subsequent tests were carried out using astrocytes of the third generation,

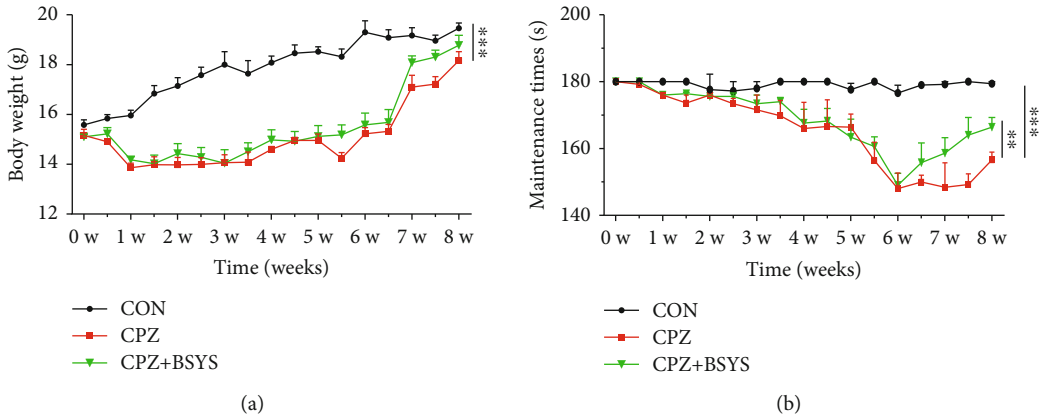


FIGURE 2: BSYS alleviates CPZ-induced motor function defect. (a) Variations in the mice's body weight per group ($n = 5$). (b) Variations in mouse motor performance as measured by the rotarod test ($n = 5$). The data are presented as mean ± SEM, in comparison to the CPZ group, $^{**}P < 0.01$, $^{***}P < 0.001$.

which were seeded in six-well plates. Afterward, miR-155 mimics (Lenti-miR-155) and miR-Negative Controls (Lenti-miR-NC) were transfected into astrocytes using a lentivirus system (Hanheng, Shanghai, China) after reaching 30-50% confluence. Once 24 hours had elapsed, the cells were treated with TNF-α (30 ng/mL, Novoprotein, Shanghai, China), IL-1α (3 ng/mL, Novoprotein, Shanghai, China), and C1q (400 ng/mL, MyBioSource, San Diego, USA), named Astrocyte Stimulation Cocktail (ASC) for 24 h to induce A1 phenotype [9]. Then, BSYS-serum (5%, 10%, 15%, 20%, and 25%) was added to astrocytes and the medium was removed 24 hours later and replaced with a full culture medium. In the next step, the culture medium was obtained after 24 hours and utilized as an astrocyte-conditioned medium (ACM).

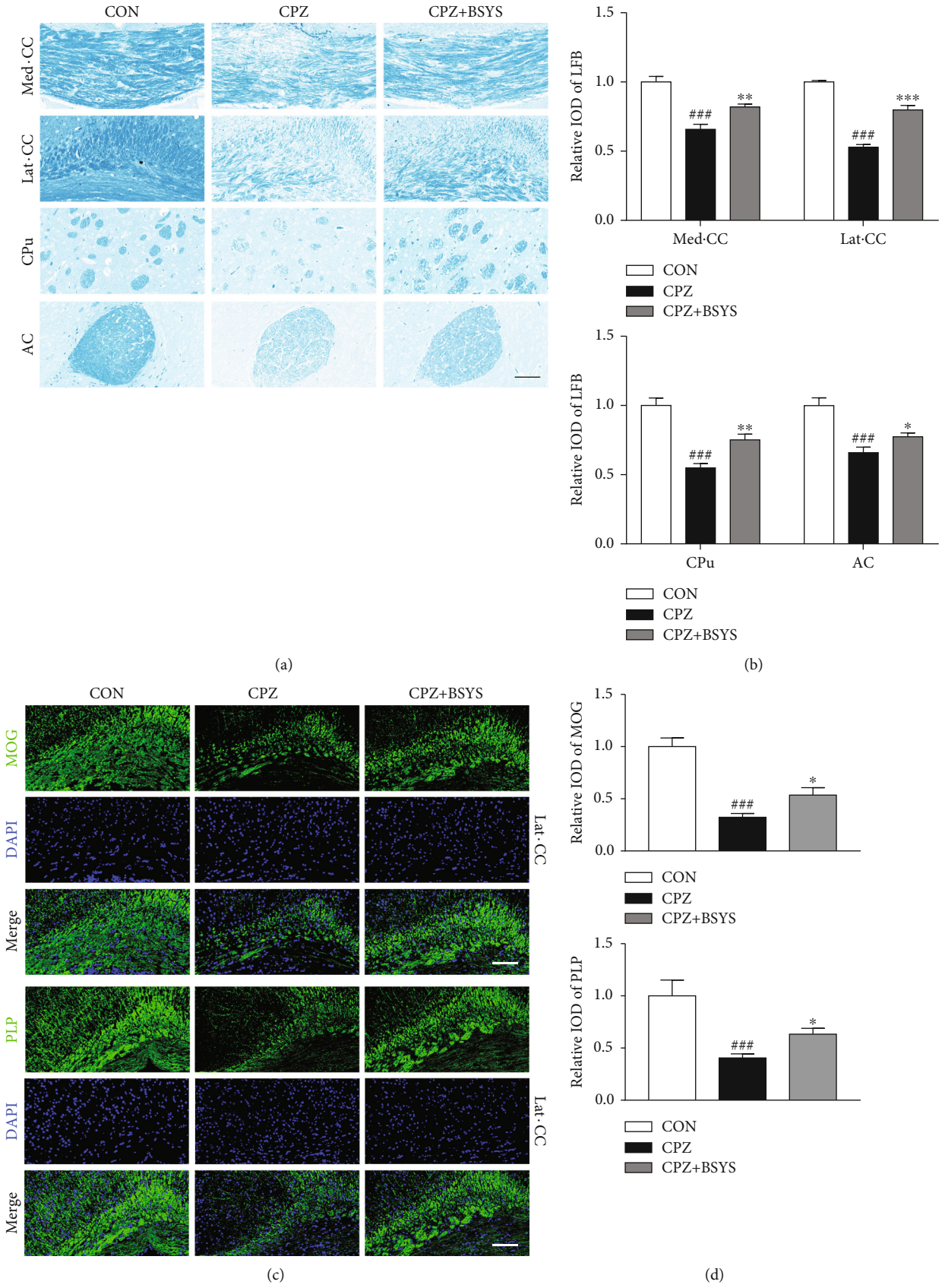
BeNa Culture Collection (BNCC, Beijing, China) supplied the OLN-93, an immature oligodendroglial cell line. Afterward, the OLN-93 cells were cultured at 37°C in a 5% CO_2 humidified atmosphere on 6-well plates in DMEM that contained 10% FBS and 1% P/S. Next, the OLN-93 medium was replaced using DMEM containing 0.5% FBS (low serum differentiation medium (LSM)). The previously collected ACM and LSM were added to a 6-well plate (2.4 mL in total) in the ratio of 1 : 5 (the concentrations of ACM and LSM were 16.7% and 83.3%, respectively) and incubated with OLN-93 for 3 days, to assess the differentiation and the maturation of oligodendrocytes mediated by astrocytes.

2.7. Cell Counting Kit-8 (CCK-8) Assay. The CCK-8 test was utilized to ascertain the viability of the cells. Astrocytes (0.8×10^4) were plated in 96-well plates for 24 hours, stimulated by ASC, and subjected to 24 hours treatment with BSYS-serum (5, 10, 15, 20, and 25%). The viability of the cells was evaluated utilizing the CCK-8 kit in a manner consistent with the manufacturer's guidelines. A microplate reader (Molecular Devices, Sunnyvale, USA) was utilized to detect the absorbance at 450 nm in the experiments. The control group's absorbance was interpreted as 100% cell viability.

2.8. Immunohistochemical (IHC) and Immunocytofluorescence Staining. The mice's brain tissue segments were deparaffinized and boiled with citrate buffer (pH 6.0) before being blocked in 10% normal goat serum (ZSGB-Bio, Beijing, China) in PBS for 1 hour at 37°C and labeling with rabbit anti-MOG antibody (1 : 500, ab233549, Abcam, Cambridge, UK), rabbit anti-PLP antibody (1 : 400, HA500202, Huabio, Hangzhou, China), rabbit anti-C3 antibody (1 : 200, ab97462, Abcam, Cambridge, UK), rabbit anti-S100A10 antibody (1 : 200, YT4198, Immunoway, Newark, USA), and mouse anti-GFAP antibody (1 : 400, YM3059, Immunoway, Newark, USA) at 4°C throughout the night. Once the sections had been rinsed in PBS, they were incubated at 37°C for 1 hour with Alexa 488-conjugated (1 : 400) and Alexa 594-conjugated (1 : 200) secondary antibodies (ZSGB-Bio, Beijing, China). Antifluorescence quenching (Solarbio, Beijing, China) and 4′,6-diamidino-2-phenylindole (DAPI) mounting medium were employed to label the nuclei.

To perform *in vitro* trials, astrocytes and OLN-93 cells were administrated according to the experimental design. After incubation, cells were fixed for 30 minutes PFA at 4% before being subjected to 5 min of permeabilization using 0.1% Triton X-100 (Beyotime, Shanghai, China) and blocking for 1 h using 5% bovine serum albumin blocking buffer. The next step involved incubating the cells with rabbit anti-C3 antibody (1 : 100, ab97462, Abcam, Cambridge, UK), rabbit anti-S100A10 antibody (1 : 100, PA5-82082, Invitrogen, Carlsbad, USA), mouse anti-GFAP antibody (1 : 200, YM3059, Immunoway, Newark, USA), and anti-PLP antibody (1 : 200, HA500202, Huabio, Hangzhou, China). Once the cells had been rinsed in PBS, they were subjected to 1 hour of incubation at 37°C with CoraLite488-conjugated (1 : 200) and CoraLite594-conjugated-conjugated (1 : 200) secondary antibodies (Proteintech, Wuhan, China) and then counterstained with DAPI for nuclei identification. All pictures were captured with the aid of a fluorescence microscope and analyses were conducted with ImageJ software (National Institutes of Health, Bethesda, USA).

2.9. Real-Time Quantitative Reverse Transcription PCR (qRT-PCR). Total RNA from the CC of the brain tissue and astrocytes was isolated using TRIzol reagent (Thermo



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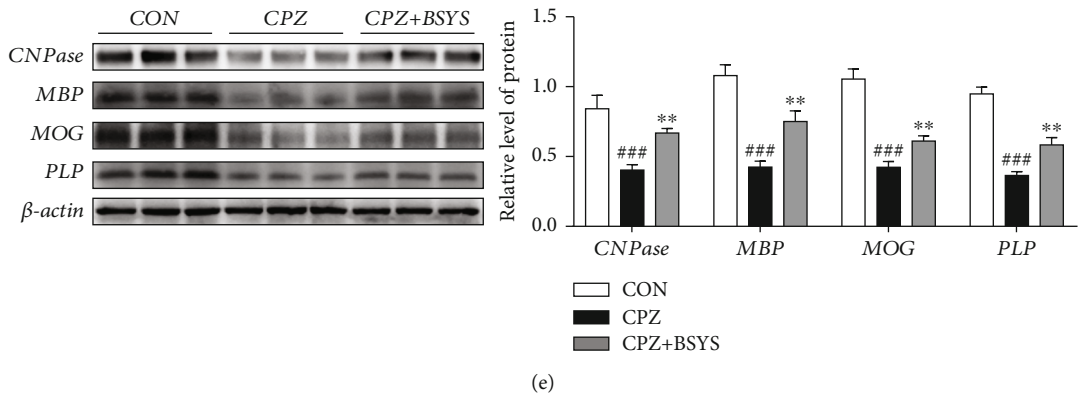


Figure 3: BSYS enhances myelin repair in CPZ-induced demyelination. (a) Changes in demyelination of the Med-CC, Lat-CC, AC, and CPu of mice per group were visualized by LFB staining. Scale bars: 100 μm. (b) Quantification of LFB-stained segments histologically within each group. (c) The MOG and PLP expressions in Lat-CC were detected by immunofluorescence staining. Scale bars: 100 μm. (d) Relative IOD expression of MOG and PLP in Lat-CC. (e) The relative protein expression levels of MOG, MBP, CNPase, and PLP in CC were shown by representative blots and statistical graphs (the protein expressions were standardized vs. β-actin). Data are reported as means ± SD ($n = 3$ /each group); as opposed to the CON group, $^{###}P < 0.001$; as opposed to the CPZ group, $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

Fisher, Waltham, USA) as per the instructions provided by the manufacturer and the concentration measured. qRT-PCR was conducted utilizing the One-Step qRT-PCR kit (Toyobo, Osaka, Japan) with a CFX Connect real-time PCR detection system (Bio-Rad, Hercules, USA) based on the SYBR Green technique. Supplementary Table 1 contains a collection of the primers that were utilized.

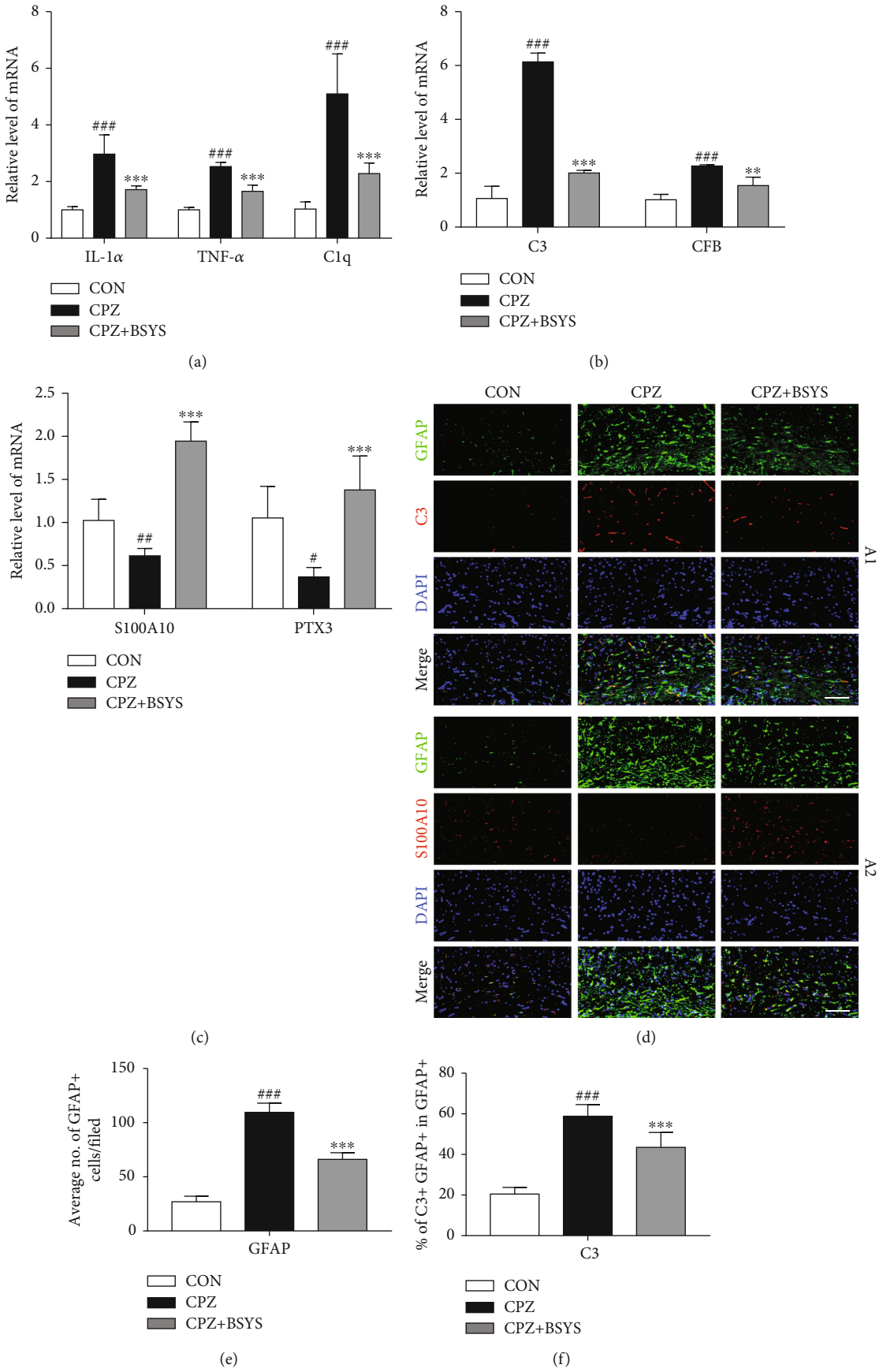
2.10. Measurement of the Level of GSH-Px, SOD, and MDA. CC homogenate and OLN-93 cell lysates were collected for the determination of malondialdehyde (MDA), superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px). The relative levels of GSH-Px, SOD, and MDA were detected utilizing their corresponding commercial kits (Beyotime, Shanghai, China) as per the guidelines stipulated by the manufacturer.

2.11. Western Blot Analysis. From both the CC and cell homogenates, the total protein was isolated. The protein concentration of the cell/tissue lysates was ascertained utilizing the bicinchoninic acid (BCA) protein assay kit (Applygen, Beijing, China). Extracted total proteins were denatured and subjected to electrophoresis separation in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Total proteins were loaded onto polyvinylidene fluoride (PVDF) membranes (Millipore, Darmstadt, Germany) following the manufacturer's specifications, blocked using StartingBlock blocking buffer (ThermoFisher, Waltham, USA), and subjected to incubation overnight at 4°C in universal antibody diluent (New Cell & Molecular Biotech, Suzhou, China) with primary antibodies against myelin oligodendrocyte glycoprotein (MOG, 1 : 2000, ab233549, Abcam, Cambridge, UK), CNPase (1 : 3000, ab6319, Abcam, Cambridge, UK), myelin basic protein (MBP, 1 : 2500, ab216668, Abcam, Cambridge, UK), proteolipid protein (PLP, 1 : 4000, HA500202, Huabio, Hangzhou, China), C3 (1 : 1000, ab97462, Abcam, Cambridge, UK), S100A10 (1 : 1000, YT4198, Immunoway, Newark, USA), glial fibrillary acidic protein (GFAP, 1 : 3000, YM3059, Immunoway, Newark, USA), IL-1β (1 : 1000, 26048-1-AP, Proteintech, Wuhan, China), IL-6 (1 : 1000, # 12912, Cell Signaling Technology, Danvers, USA), IL-10 (1 : 1000, 60269-1-Ig, Proteintech, Wuhan, China), tissue growth factor-β (TGF-β, 1 : 1000, 19999-1-AP, Proteintech, Wuhan, China), brain-derived neurotrophic factor (BDNF, 1 : 1000, ab108319, Abcam, Cambridge, UK), and β-actin (1 : 10000, GTX109639, Genetex, Irvine, USA). The blots were treated for 1 h at an ambient temperature with a horseradish peroxidase-conjugated antibody (Proteintech, Wuhan, China). Immunoblotting bands were identified utilizing enhanced chemiluminescence (ECL, Millipore, Darmstadt, Germany) and visualized on the Fusion FX imaging system (Vilber Lourmat, Torcy, France). When necessary, the membranes were stripped by Western blot stripping buffer (Takara, Kusatsu, Japan) and reprobed with corresponding antibodies. Relative band intensity was quantified by ImageJ software.

2.12. Statistical Analysis. GraphPad Prism 7 (GraphPad Software, San Diego, USA) was utilized to undertake all analyses of statistical data. The rotarod data and body weight changes were evaluated utilizing two-way repeated-measures ANOVA with post hoc Turkey tests. Other data from three or more groups were examined with one-way ANOVA accompanied by Turkey's post hoc test. Statistical significance was deemed to have been attained at P values of <0.05.

3. Results

3.1. BSYS Alleviates CPZ-Induced Motor Function Defect. The mice's body weight fluctuations, as well as their locomotor coordination, were measured to examine the therapeutic impacts of BSYS on CPZ mice. CPZ-containing diet significantly decreased the body weight of mice. However, two weeks of BSYS treatment ameliorated CPZ-caused body weight loss, but without statistical significance



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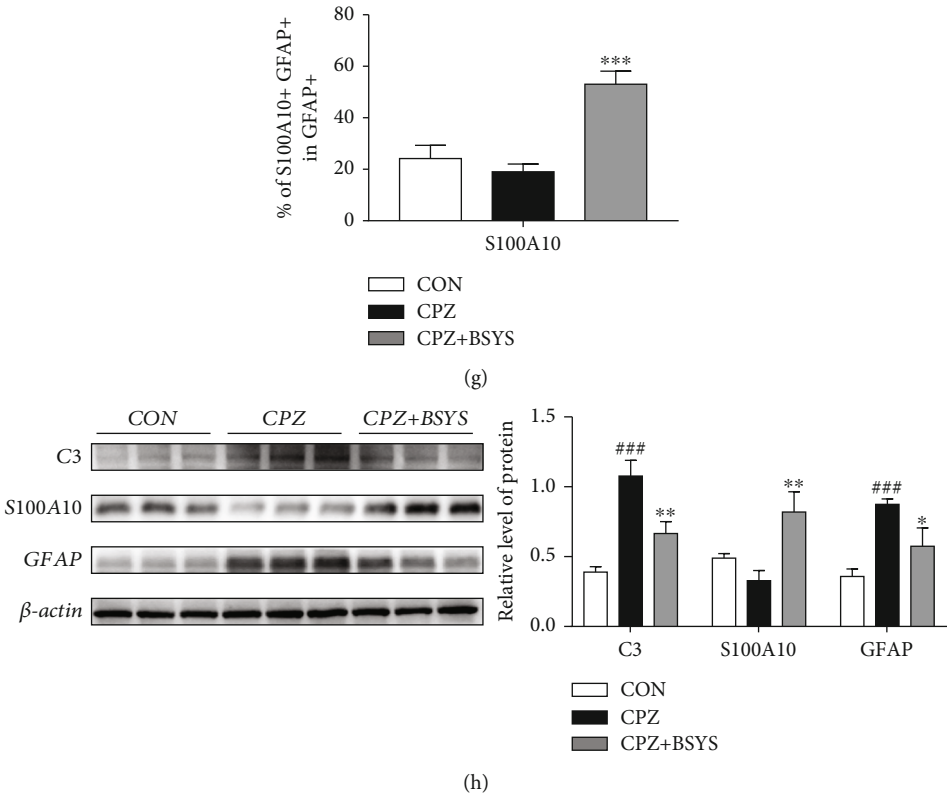
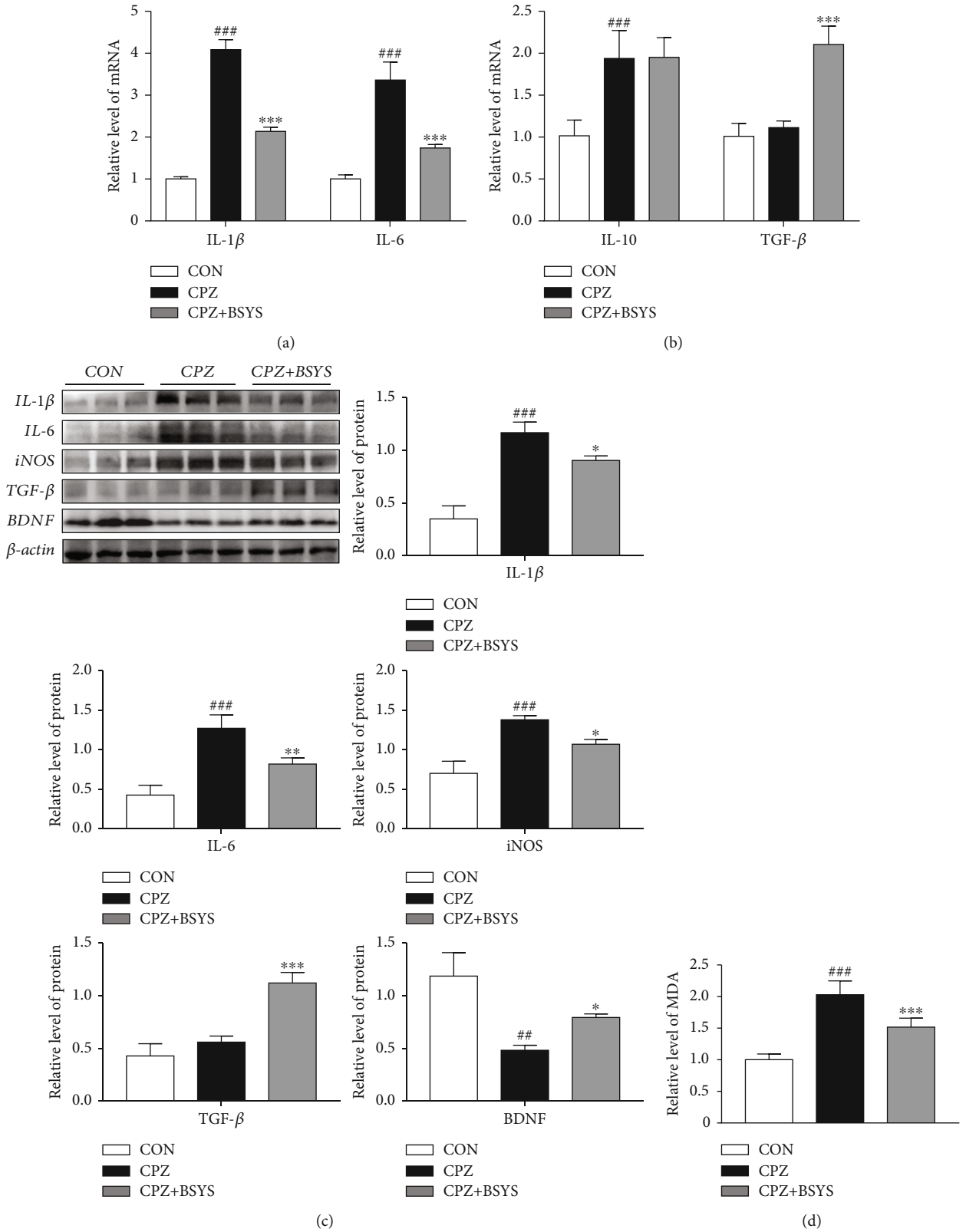


Figure 4: BSYS modulates the phenotype of reactive astrocytes in vivo. (a) Changes in relative expressions of IL-1α, TNF-α, and C1q in mice per group were determined by qRT-PCR ($n = 5$). (b, c) A1 phenotype markers (CFB, C3) and A2 phenotype markers (PTX3, S100A10) in CC were detected by qRT-PCR ($n = 5$). (d) Immunofluorescence of mice's CC per group utilizing antibodies specific for GFAP (green), C3 (red), and S100A10 (red) ($n = 3$). Scale bars: 100 μm. (e) Measurement of GFAP+ cells density in CC. (f, g) Measurement of the proportion of C3+/GFAP+ and S100A10+/GFAP+ cells in CC. (h) Illustrative Western blotting images and quantitative data of C3, S100A10, and GFAP in CC. Data are displayed as means ± SD; versus the CON group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$; in contrast with the CPZ group, $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

(Figure 2(a)). CPZ-exposed mice suffered significant motor dysfunction, as shown by a decline in the locomotion time in the rotarod test. Mice subjects that were given CPZ demonstrated decreased latency to fall off a rotarod in contrast with the control group. Additionally, mice that were treated with BSYS had much longer on-rod time in contrast with CPZ mice who were just given a vehicle treatment (Figure 2(b)).

3.2. BSYS Enhances Myelin Repair after CPZ-Mediated Demyelination. It is reported that CPZ intake in mice can induce extensive demyelination in many regions of the brain white matter. To evaluate the degree of myelin repair in demyelinated lesions, histological changes in white matter areas, including the lateral corpus callosum (Lat-CC) and medial corpus callosum (Med-CC) [32], anterior commissure (AC), and caudoputamen (CPu) [33] were detected by LFB staining. Compared with the CON group, relative LFB-IOD of Med-CC, Lat-CC, AC, and CPu declined sharply in CPZ mice. Conversely, relative IOD was increased in BSYS-treated mice (Figures 3(a) and 3(b)). Comparable findings were recorded by immunofluorescence staining of MOG and PLP (Figures 3(c) and 3(d)). Compared with vehicle-treated mice, BSYS-treated mice exhibited a stronger recovery from demyelination and a remarkably larger remyelination area 2 weeks after treatment. Moreover, the effects of BSYS in promoting myelin repair were assessed by Western blotting, and the findings illustrated that BSYS treatment dramatically elevated the protein expression levels of CNPase, MBP, MOG, and PLP in the CC comparison with CPZ mice (Figure 3(e)).

3.3. BSYS Modulates the Phenotype of Reactive Astrocytes In Vivo. Previous studies demonstrated that a substantial number of A1 astrocytes were activated in CPZ-induced demyelination lesions [34], and the A1 phenotype was mainly triggered by C1q, TNF-α, and IL-1α derived from microglia. Thus, the mRNA expression of C1q, TNF-α, and IL-1α in the brain was quantified by qRT-PCR. The IL-1α, TNF-α, and C1q mRNA levels in the CC were remarkably upregulated in CPZ mice. However, the mRNA levels were downregulated after BSYS treatment (Figure 4(a)). The gene expression of A1 markers (C3 and CFB) and A2 markers (PTX3 and S100A10) were analyzed to examine the involvement of BSYS in astrocyte polarization. Results illustrated that CPZ markedly enhanced the C3 and CFB expression at week 8 in contrast with the control group. Nevertheless, the administration of BSYS substantially lowered the mRNA



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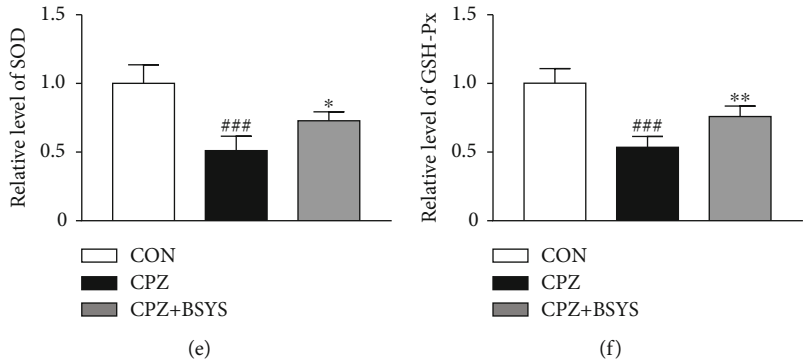


Figure 5: The influence of BSYS on CNS cytokine production and oxidative stress in CPZ mice. (a, b) The levels of anti-inflammatory markers (TGF-β, IL-10) and proinflammatory markers (IL-1β, IL-6) in CC were identified by qRT-PCR ($n = 5$). (c) Illustrative Western blotting images and quantitative data of proinflammatory cytokines (IL-1β, iNOS, and IL-6) and neuroprotective factors (TGF-β, BDNF) in CC ($n = 3$). (d) MDA content, (e) SOD levels, and (f) GSH-Px activities of the CC were measured by a corresponding commercial kit ($n = 5$). Data are reported as means ± SD; in contrast with the CON group, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$; versus the CPZ group, $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

levels of A1 markers. When comparing the BSYS-treated group to the CPZ group, levels of A2 astrocyte markers were shown to be elevated in the former (Figures 4(b) and 4(c)).

To confirm whether the results of protein levels were consistent with mRNA levels, immunofluorescence and Western blotting were performed to further identify the astrocytic phenotypic marker. Double-staining was used on brain samples for GFAP+ (astrocytic marker)/C3+ (A1 marker) or GFAP+/S100A10+ (A2 marker). CPZ significantly increased the proportion of A1 astrocytes. However, BSYS administration decreased the numbers of activated astrocytes (GFAP+ cells) and A1 astrocytes (GFAP+/C3+ cells) and raised the ratio of A2 astrocytes in the BSYS groups in contrast with the CPZ group (Figures 4(d)–4(g)). Western blot analysis illustrated that GFAP and C3 were highly expressed, while S100A10 was lowly expressed in the CC of CPZ mice. However, the BSYS administration significantly reversed the trend (Figure 4(h)).

A1 and A2 astrocytes have been confirmed to generate pro- and anti-inflammatory factors, respectively. To further confirm the efficacy of BSYS on the regulation of astrocyte polarization, inflammatory cytokines and neuroprotective factors in CC were detected. qRT-PCR and Western blotting findings illustrated that CPZ markedly increased the expressions of inflammation-associated markers IL-1β, IL-6, and IL-10 in contrast with the CON group. BSYS treatment considerably reduced the levels of proinflammatory cytokines IL-6 and IL-1β in contrast with the vehicle-treated group. Furthermore, BSYS treatment increased the expressions of TGF-β (Figures 5(a) and 5(b)). In addition, Western blotting analysis illustrated that BSYS administration enhanced the expression of BDNF and inhibited oxidative stress factors (inducible nitric oxide synthase (iNOS)) compared to the CPZ group (Figure 5(c)).

Previous studies suggested that CPZ intoxication induces astrocyte activation, while neurotoxic reactive astrocytes increase oxidative stress, which inhibits remyelination [35, 36]. Thus, the impacts of BSYS on the expression of MDA, SOD, and GSH-Px in the CC of CPZ mice were investigated. SOD and GSH-Px levels were dramatically lowered, and the MDA level was markedly elevated in the CPZ group as opposed to the CON group. Interestingly, BSYS administration significantly reduced MDA levels compared with CPZ mice (Figure 5(d)). Treatment with BSYS dramatically enhanced the SOD and GSH-Px expressions in CPZ mice. (Figures 5(e) and 5(f)).

3.4. Impacts of BSYS on the miR-155 Signaling Pathway in Demyelinated Mice Brains. The microarray data set with the accession number GSE100662 was extracted from the Gene Expression Omnibus (GEO) database to analyze the differences in miRs in CC between CPZ mice and normal mice. The parameters used for screening included $|\log_2 \text{fold change(FC)}| > 1.0$ and $P < 0.05$. Utilizing a volcano plot, we identified an aggregate of 14 miRs with differential expression (Figure 6(a)). Under the $|FC| > 2.0$ and $P < 0.01$ parameters, 8 miRNAs with differential expressions are presented in the heat map. CPZ considerably elevated the expression levels of multiple miRs in the CC of mice, especially miR-155 (Figure 6(b)). miR-155 has been proved to inhibit the repair of myelin by promoting a proinflammatory microenvironment in the CNS. Moreover, miR-155 was confirmed to drive the polarization of astrocytes towards a neurotoxic phenotype (A1) via regulating the SOCS1 pathway. Combined with our previous studies, we speculate that BSYS promotes myelin repair by modulating astrocyte polarization via inhibiting miR-155 in a CPZ mouse model.

Next, the effect of BSYS on the miR-155 signaling pathway in CPZ mice was evaluated. qRT-PCR illustrated that the miR-155 expression level in the CC was remarkably elevated in CPZ mice in contrast with normal mice. However, treatment with BSYS significantly decreased relative miR-155 expression in CPZ mice (Figure 6(c)). SOCS1 is a gene that is targeted by miR-155. Several research reports have illustrated that alterations in SOCS1 can modulate the neuroimmune response of astrocytes and might have therapeutic value in MS. Therefore, qRT-PCR and Western blotting were utilized to examine the SOCS1 expression patterns.

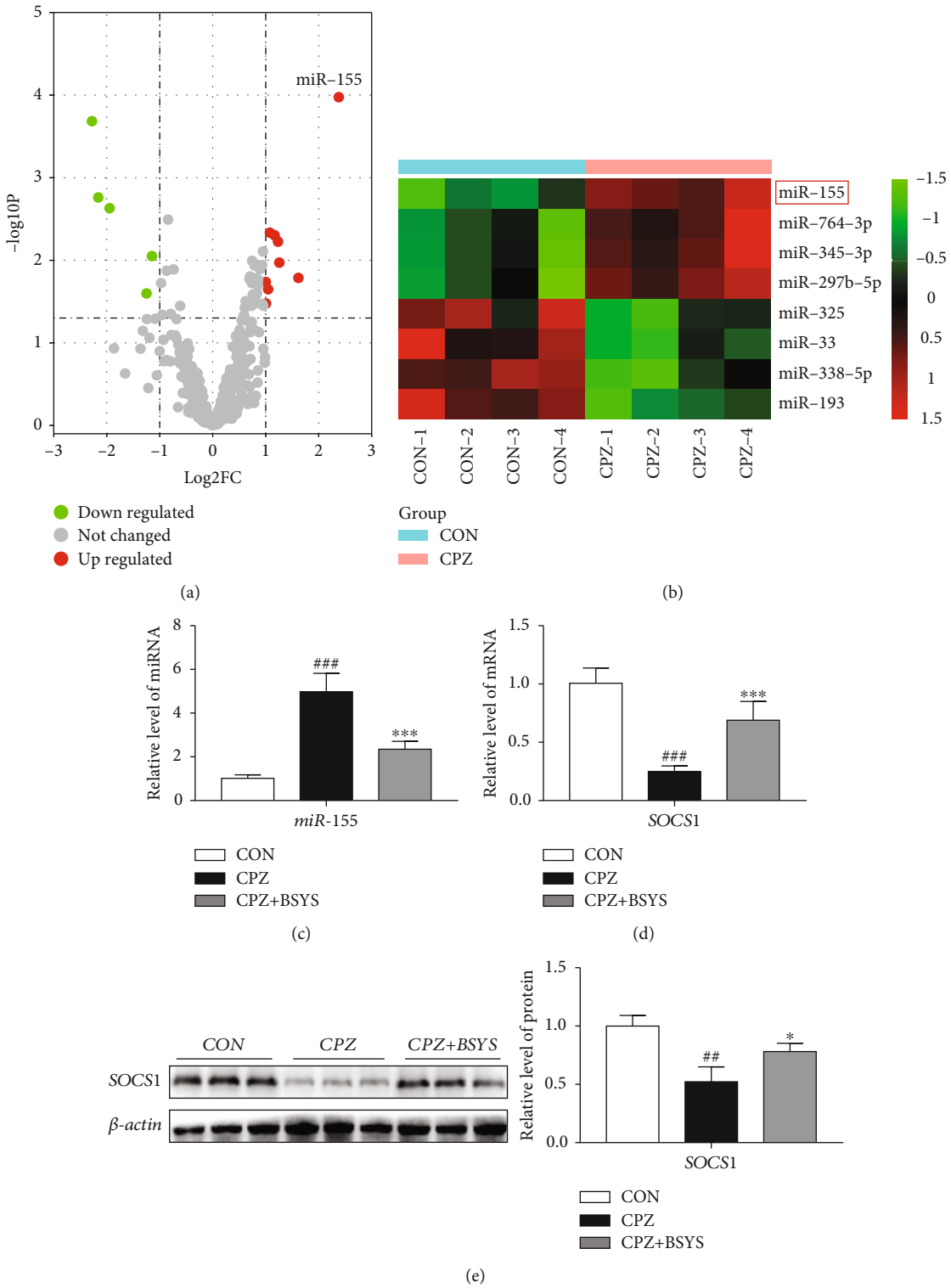


FIGURE 6: BSYS regulates the miR-155 signaling pathway in the brains of demyelinated mice. (a) Volcano plot and (b) heat map showing the differential miRs between CPZ mice and normal mice. (c, d) The levels of miR-155 and SOCS1 expressions in mice's CC per group as identified by qRT-PCR ($n = 5$). (e) Illustrative blots and statistical graphs of relative protein expression of SOCS1 in CC ($n = 3$). Data are displayed as means ± SD; in contrast with the CON group, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$; versus the CPZ group, $^{*}P < 0.05$, $^{***}P < 0.001$.

When CPZ mice were compared with CON mice, the level of SOCS1 expression was found to be lower in the former. However, treatment with BSYS enhanced SOCS1 expression in CPZ mice (Figures 6(d) and 6(e)).

3.5. Screening the Optimal Concentration of BSYS-Serum for Astrocytes. To corroborate the findings of the *in vivo* investigations, BSYS-serum was administrated to primary astrocytes cultured *in vitro*. CCK-8 assay was conducted to

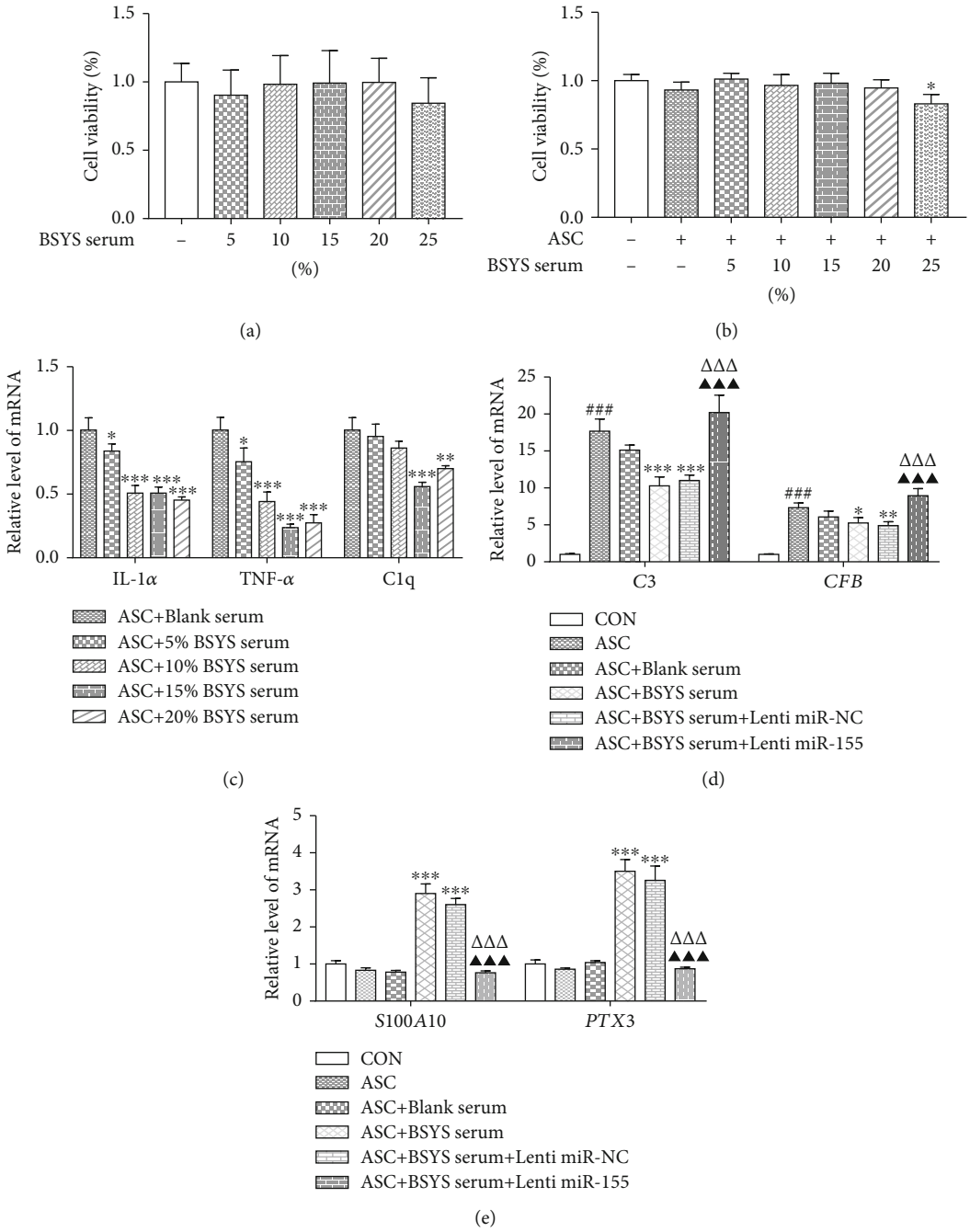
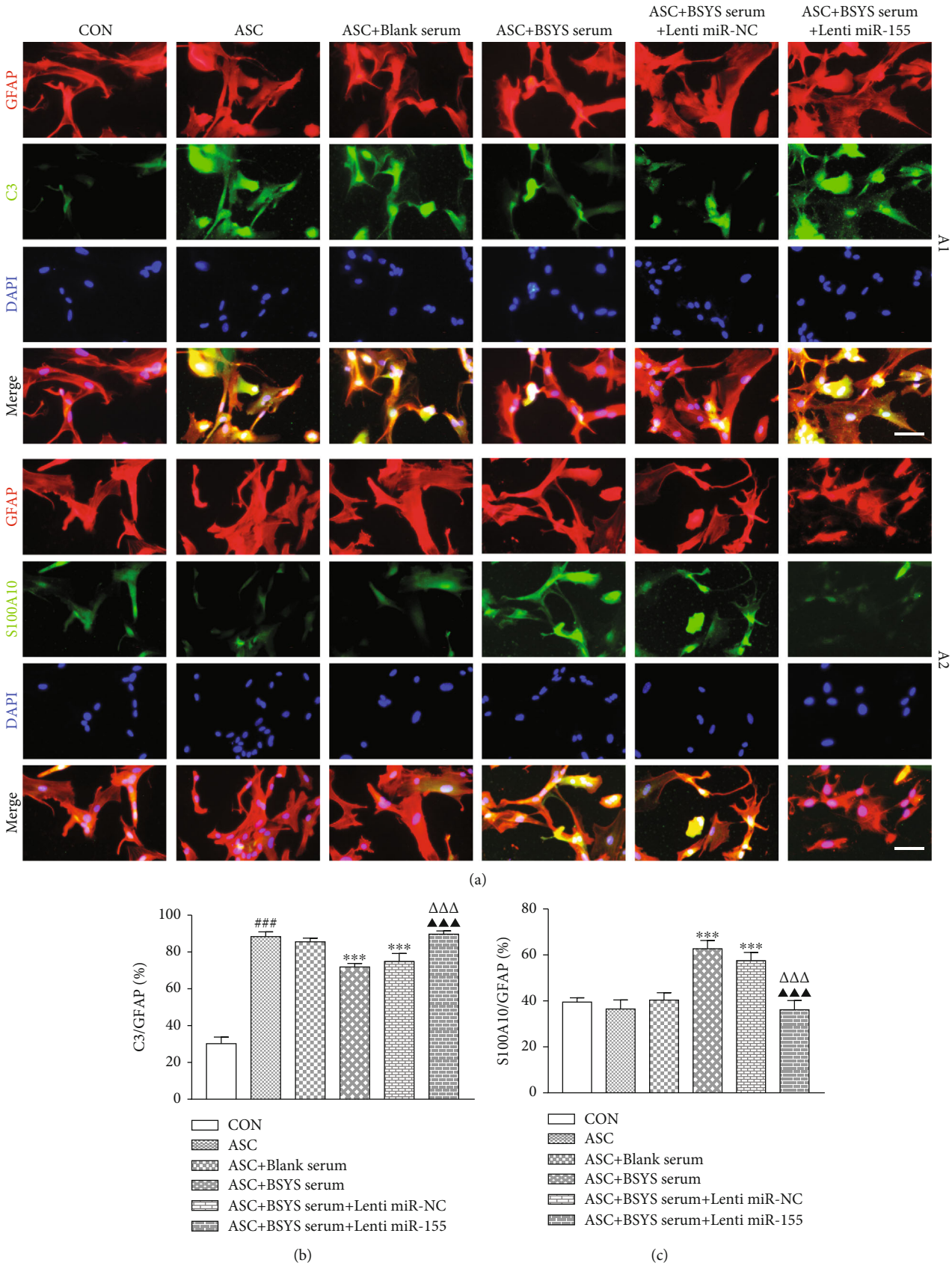


Figure 7: Effect of BSYS-serum on primary astrocytes. (a, b) With or without the stimulation of ASC, astrocytes were treated with 5%-25% BSYS-serum for 24 hours, and the CCK-8 test was utilized to ascertain the viability of the cells. (c) Changes in relative expression levels of C1q, TNF-α, and IL-1α of astrocytes in each group were evaluated by qRT-PCR. (d, e) A1 phenotype markers (CFB, C3) and A2 phenotype markers (PTX3 and S100A10) in astrocytes were evaluated by qRT-PCR. Data are reported as means ± SD; in contrast to the CON group, $^{\#\#\#}P < 0.001$; in contrast to the ASC group, $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$; in contrast with the ASC+BSYS group, $^{\triangle\triangle\triangle}P < 0.001$; in contrast with the ASC+BSYS+miR-NC group, $^{\blacktriangle\blacktriangle\blacktriangle}P < 0.001$.

ascertain if the concentrations of BSYS-serum used in this experiment influenced the cell viability of astrocytes. CCK-8 assay illustrated that treating astrocytes with 5-25% of BSYS-serum for 24 h had no cytotoxic effect (Figure 7(a)). ASC was used to stimulate astrocytes to investigate the underlying process of A1/A2 astrocyte transition, which is mediated by BSYS. Then, the cell viability (Figure 7(b)) and expression profiles of C1q, TNF-α, and IL-1α in astrocytes were assessed to determine the optimum concentration of BSYS-serum. C1q, TNF-α, and IL-1α levels were elevated substantially in ASC-induced astrocytes, and BSYS-serum considerably lowered the expression of these proinflammatory indicators in a dosage-dependent way (Figure 7(c)). The effects of 15% and 20% concentrations were the most



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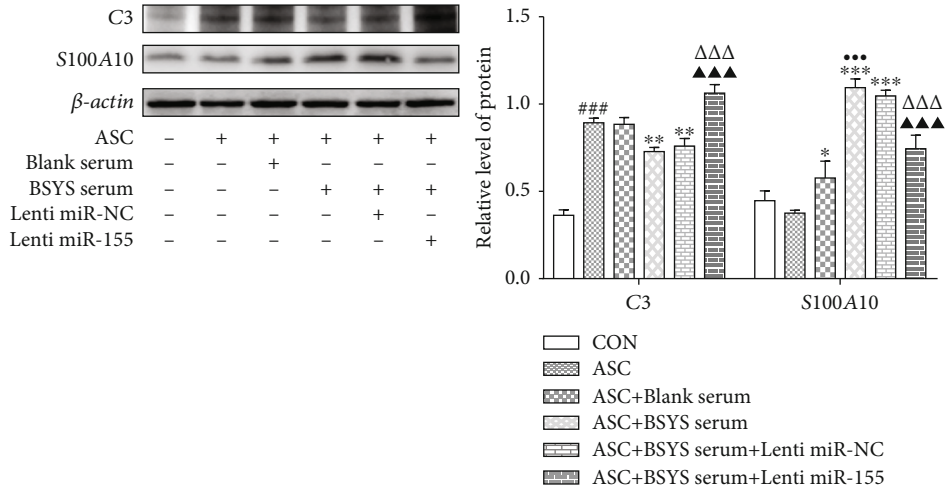


(d)

Figure 8: BSYS modulates the A1/A2 Astrocytes transformation by suppressing the miR-155 levels in vitro. (a) Immunocytofluorescence staining of astrocytes per group utilizing antibodies specific for GFAP (red), C3 (green), and S100A10 (green). Scale bars: 50 μm. (b, c) Measurement of the proportion of C3+/GFAP+ and S100A10+/GFAP+ cells in astrocytes. (d) Illustrative Western blotting images and quantitative data of C3 and S100A10 in astrocytes. Data are reported as means ± SD; as opposed to the CON group, $^{\#\#\#}P < 0.001$; versus the ASC group, $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$; contrasted with the ASC+blank serum group, $^{\bullet\bullet\bullet}P < 0.001$; vs. the ASC+BSYS group, $^{\triangle\triangle\triangle}P < 0.001$; vs. the ASC+BSYS+miR-NC group, $^{\blacktriangle\blacktriangle\blacktriangle}P < 0.001$.

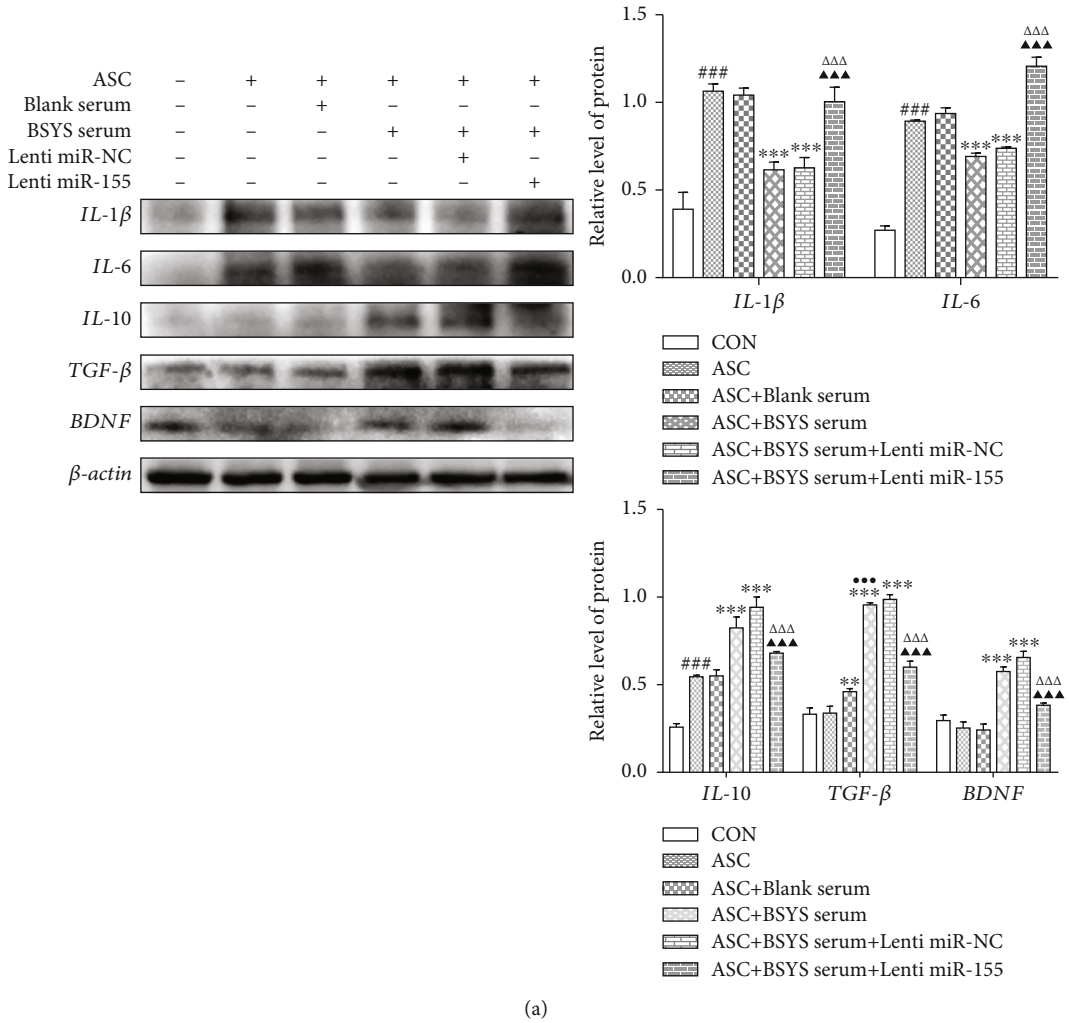
significant, but no meaningful difference was discovered between the two concentrations. Therefore, BSYS-serum with a concentration of 15% was selected for subsequent analyses.

3.6. BSYS Modulates the Transition of A1/A2 Astrocytes through the Suppression of the miR-155 Expression In Vitro. To ascertain if BSYS influenced the level of miR-155, which is required to modulate astrocyte polarization *in vitro*, astrocytes were firstly transfected with miR-155 mimics before ASC stimulation and then treated with BSYS-serum. As displayed in Figure 7(d), the level of the A1 phenotype marker (C3 and CFB) expression was considerably upregulated at 24 h after ASC treatment. This trend was suppressed by BSYS-serum. Nevertheless, the inhibition properties of BSYS-serum on the A1 markers were markedly attenuated after miR-155 mimic administration. Meanwhile, the effect of BSYS-serum promoting the A2 markers (S100A10 and PTX3) was also weakened by transfection with the miR-155 mimics (Figure 7(e)). Immunocytofluorescence staining showed that BSYS-serum lowered the expression level of C3 and elevated the S100A10 expression level in GFAP+ cells in contrast with ASC and blank serum-treated groups, while the effects were considerably diminished in the miR-155 overexpression group (Figures 8(a)–8(c)). Western blot results are consistent with those of immunofluorescence staining (Figure 8(d)).

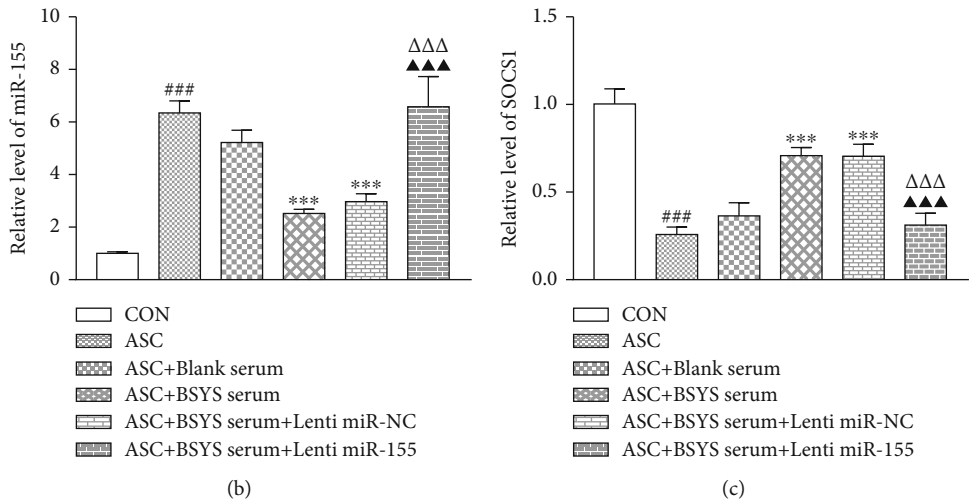
As shown in Figure 9(a), levels of neurotoxic markers, such as IL-6 and IL-1β, were substantially increased in astrocytes after ASC stimulation but were reversed to the baseline after treatment with BSYS-serum. Furthermore, the low expression of neuroprotective factors in the ASC group, including IL-10, TGF-β, and BDNF, were upregulated after BSYS-serum treatment. Similarly, these therapeutic effects were offset by the combination of miR-155-mimic transfection. In addition, miR-155 and its downstream target SOCS1 were detected in astrocytes. It was found that as opposed to the ASC group and the blank-serum treatment group, miR-155 was remarkably inhibited and SOCS1 was upregulated in the BSYS-serum treatment group. However, miR-155 mimic transfection decreased the effects (Figures 9(b)–9(d)). These data suggested that BSYS modulates astrocyte polarization by suppressing the level of miR-155 *in vitro*.

3.7. BSYS Stimulates the Oligodendrocytes Maturation In Vitro by Alleviating the Neurotoxic Impacts of A1 Astrocytes. An earlier study illustrated that A1 astrocytes contribute to oligodendrocytes' death and inhibit OPC differentiation [37] by producing neurotoxic mediators [38]. Neurotoxic reactive astrocytes have been proven to cause oxidative stress, which disrupts oligodendrocyte maturation [39, 40]. Therefore, oxidant biomarkers were measured to evaluate the oxidative stress level in oligodendrocytes [41, 42]. An immature oligodendroglial cell line (OLN-93) was incubated with ACM, and GSH-Px, SOD, and MDA levels were evaluated. Compared with culture medium from A1 astrocytes (A1CM), ACM from BSYS-serum treatment considerably lowered MDA expression level and elevated GSH-Px and SOD levels in OLN-93 cells. Conversely, these therapeutic effects were dramatically attenuated by miR-155 overexpression (Figures 10(a)–10(c)).

Furthermore, to assess the impacts of BSYS on the maturation of oligodendrocytes mediated by A1 astrocytes, PLP expression level in OLN93 cells was detected by immunocytofluorescence. After OLN-93 cells were added to A1CM, low expression of PLP was observed; this phenomenon was counteracted in the BSYS-serum treatment group. However, the effect on promoting oligodendrocyte maturation of



(a)



(b) (c)

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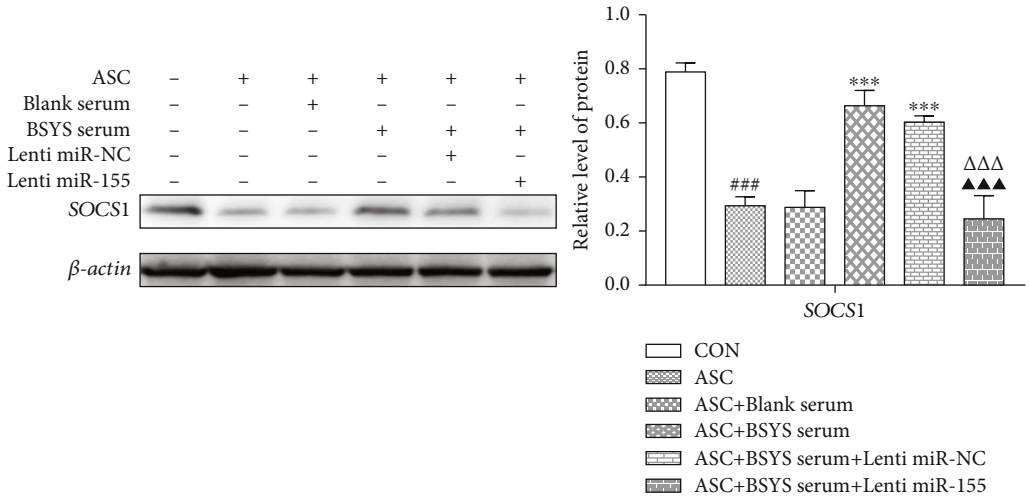


(d)

Figure 9: BSYS modulates cytokine levels by regulating miR-155 signaling pathways in astrocytes. (a) Illustrative Western blotting images and quantitative data of inflammatory indicators (IL-6, IL-1β) and neuroprotective factors (IL-10, TGF-β, and BDNF) in astrocytes. (b, c) The level of miR-155 and SOCS1 expression in astrocytes per group were identified by qRT-PCR. (d) The relative protein expression level of SOCS1 in astrocytes is shown using illustrative blots and statistical graphs. Data are presented as means ± SD; contrasted with the CON group, $^{\#\#\#}P < 0.001$; contrasted to the ASC group, $^{**}P < 0.01$, $^{***}P < 0.001$; contrasted to the ASC+blank serum group, $^{\bullet\bullet\bullet}P < 0.001$; contrasted with the ASC+BSYS group, $^{\Delta\Delta}P < 0.01$, $^{\Delta\Delta\Delta}P < 0.001$; contrasted with the ASC+BSYS+miR-NC group, $^{\blacktriangle\blacktriangle\blacktriangle}P < 0.001$.

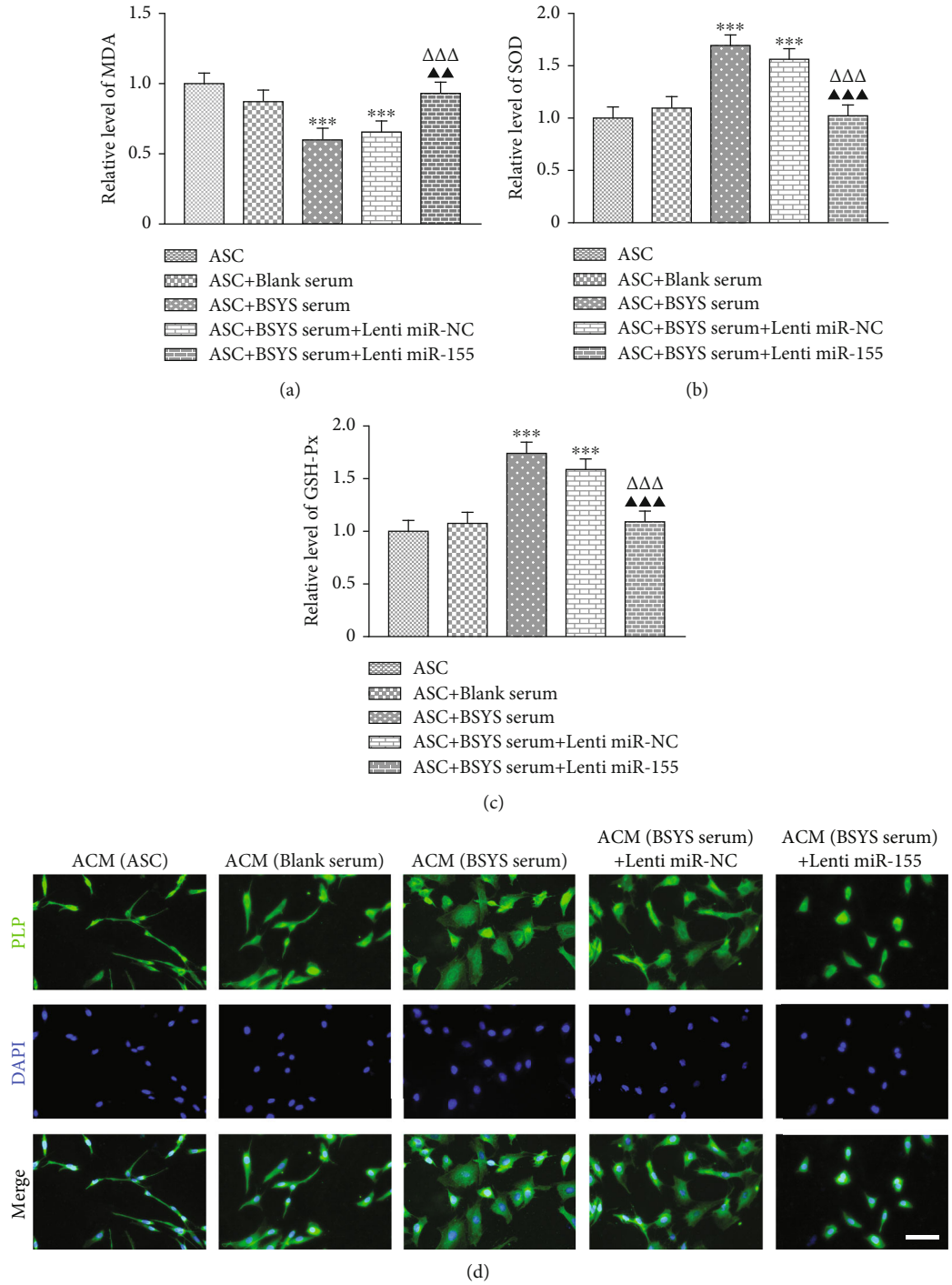
ACM in the BSYS-serum-treated group was attenuated by miR-155 transfection (Figure 10(d)). The results of oligodendrocyte maturation markers, including CNPase, MOG, MBP, and PLP in Western blot analysis were consistent with PLP immunofluorescence (Figure 10(e)). It was inferred that BSYS promotes the maturation of oligodendrocytes by alleviating neurotoxic effects of A1 astrocytes via inhibition of miR-155 in vitro.

4. Discussion

Multiple sclerosis (MS) is a chronic, disabling neurological condition hallmarked by neurodegeneration and demyelination [43]. In China, the incidence of MS in children is 0.055/100,000 and that in adults is 0.288/100,000 [44]. Numerous studies have been conducted to develop drugs to suppress neuroinflammation and promote myelin repair in patients with MS. Several Chinese herbal medicines have been investigated for their potential to treat MS given their multichannel and multitarget therapeutic effects. BSYS capsule, a traditional Chinese medicine prescription was reported to exert good clinical effects on MS [45]. Preliminary studies from our laboratory showed that BSYS alleviated myelin and axon damage via modulating Th17/Treg [46] and microglia M1/M2 ratio [25] in EAE mice. Additionally, our previous studies also illustrated that treatment with BSYS-serum promoted axonal regeneration *in vitro* [31, 47]. Based on these findings, we examined the mechanisms of the treatment effects of BSYS using the CPZ-mediated demyelination model from the perspectives of antineuroinflammation and remyelination.

Cuprizone is a chelating agent for copper ions which impairs the respiratory chain of oligodendrocytes, leading to oxidative stress and degeneration effects [48]. Continued dietary consumption of CPZ triggers reversible demyelination in distinct brain regions by inducing oligodendrocyte apoptosis [49] and activation of astrocytes and microglia [50]. The CPZ model has been used to explore the mechanisms leading to the destruction and restoration of myelin sheaths, which independently of invading peripheral immune cells [51]. In this research, mice were supplied with a normal diet comprising 0.2% CPZ, which contributed to a remarkable decrease in body weight and impaired motor function. Rotarod behavioral tests showed that BSYS administration ameliorated motor deficits in the CPZ-induced demyelination in mice. CPZ ingestion induced severe demyelination; however, BSYS administration resulted in dense LFB-positive staining in myelin tissues from different brain regions as determined by the LFB staining assay. Moreover, mature oligodendrocytes express myelin proteins such as the MBP and PLP (which are present in compact myelin), CNPase, and MOG, (which are expressed in noncompact myelin) [52, 53]. During the process of remyelination, these proteins agglomerate to neuronal axons and enwrap axons to form myelin sheaths. Consistent with the results of LFB, the expression levels of CNPase, MOG, MBP, and PLP were dramatically elevated in corpus callosum after BSYS treatment, suggesting that BSYS administration during the remyelination phase promoted myelin repair in the CPZ mouse model.

It has been shown that glial cell-mediated neuroinflammation exacerbates oligodendrocyte injury and remyelination failure in the CPZ model [54]. Therefore, modification of the glial cell-mediated neuroinflammatory microenvironment can ameliorate myelin injury and promote repair processes. Myelin fragments stimulate microglia activation in MS [55], which triggers the generation of many cytokines. In particular, TNF-α, C1q, and IL-1α derived from activated



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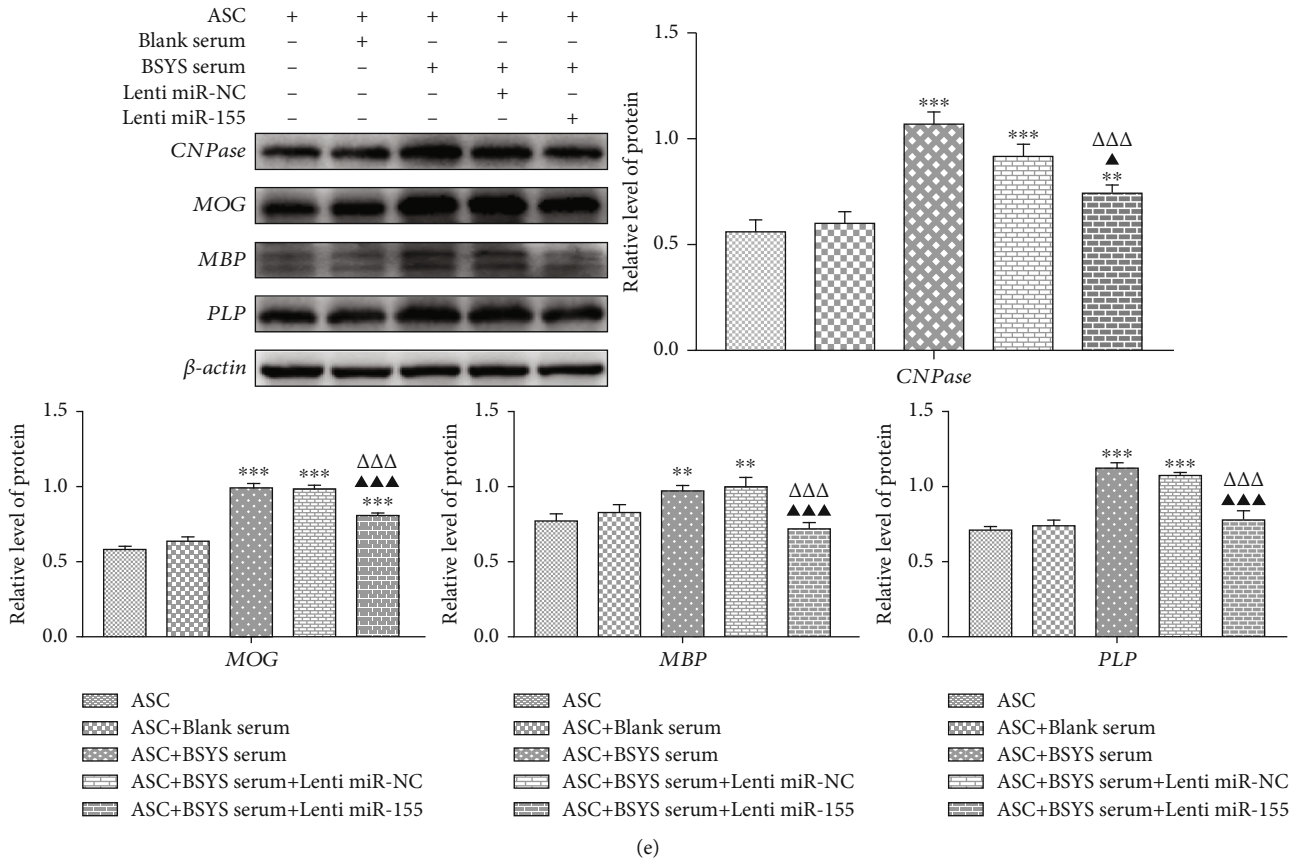

(e)

Figure 10: BSYS promotes oligodendrocyte maturation in vitro by ameliorating the neurotoxic impacts of A1 astrocytes. (a) MDA content, (b) SOD level, and (c) GSH-Px activity of OLN-93 cells were determined utilizing the respective commercial kit. (d) The expression of PLP in OLN-93 cells was determined by immunocytofluorescence staining. Scale bars: 50 μm. (e) The relative protein expression level of MOG, PLP, MBP, and CNPase in astrocytes is shown using illustrative blots and statistical graphs. Data are reported as means ± SD; contrasted to the ASC group, $^{**}P < 0.01$, $^{***}P < 0.001$; contrasted with the ASC+BSYS group, $^{\triangle\triangle\triangle}P < 0.001$; contrasted to the ASC+BSYS+miR-NC group, $^{\blacktriangle}P < 0.05$, $^{\blacktriangle\blacktriangle}P < 0.01$, and $^{\blacktriangle\blacktriangle\blacktriangle}P < 0.001$.

microglia promote inflammation triggered by neurotoxicity astrocytes, referred to as the A1 phenotype [9]. Consistent with the pathological changes of the demyelinated lesions in MS patients, many astrocytes were strongly activated in the demyelinating area of the CPZ mouse model [56]. Several studies have suggested that astrocyte activation leads to a more severe inflammatory response than microglia activation [57]. Evidence from prior studies indicates that the complement component 3 (C3), which is an A1 astrocytes marker, is highly expressed in the demyelinated region. A1 astrocytes inhibit OPC proliferation and differentiation, kill newly generated oligodendrocytes, and promote axon degeneration in demyelinating plaques. On the contrary, the A2 phenotype confers neuroprotective effects, thereby increasing the number of mature oligodendrocytes and inhibiting white matter injury [58]. It has been discovered that A2 astrocytes specifically express the proteins S100A10, PTX3, and TGM1 [59]. As a corollary, regulation of the conversion of reactive astrocytes from A1 to A2 will inhibit neuroinflammation and promote myelin repair, thus accelerating MS recovery. In light of these observations, we investigated whether astrocyte polarization mediated the effects of BSYS on the CPZ mouse model. Results showed that a substantial number of A1 astrocytes were found in the lesions caused by demyelination. Interestingly, BSYS treatment significantly prevented A1 formation and increased A2 astrocytic polarization in the demyelination area.

Astrocytes regulate CNS immune responses and neuroinflammation during MS [60]. A1 astrocytes are known to be critical sources of proinflammatory and oxidative stress markers, including iNOS, IL-6, TNF-α, and IL-1β, and can cause neurotoxicity [61]. The demyelination and remyelination processes in MS lesions are influenced by various proinflammatory chemokines and cytokines. Astrocyte-derived IL-1β and TNF-α have been reported to strongly inhibit OPC differentiation and oligodendrocyte survival [62, 63]. Therefore, reducing the production of proinflammatory factors by restraining A1 polarization may promote oligodendrocyte growth and myelin repair. In this study, *in vitro* experiments revealed that A1 astrocytes increased C3 and CFB mRNA levels and also the staining intensity and protein expression of C3. However, treatment with BSYS drug-containing serum after ASC stimulation decreased the A1 phenotype and lowered the IL-6 and IL-1β levels. These results were in agreement with those obtained from *in vivo*

studies. Interestingly, the expression levels of A2 astrocyte markers, S100A10 and PTX3, were increased after BSYS administration *in vivo* and *in vitro*. BSYS treatment increased the secretion of neuroprotective and anti-inflammatory markers, including BDNF, TGF-β, and IL-10, from astrocytes to enhance the capacity of oligodendrocytes to differentiate and mature, thus facilitating the remyelination process.

Moreover, neurotoxic reactive astrocytes have been demonstrated to cause oxidative stress, which hinders the differentiation and maturation of oligodendrocytes. The prooxidant biomarker, MDA, serves as the end product of lipid peroxidation. The SOD and GSH-Px can remove free radicals and are regarded as crucial antioxidative enzymes. A1 astrocytes culture supernatants cocultured with OLN-93 cells resulted in the abnormal expression of MDA, SOD, and GSH-Px. However, this phenomenon was reversed by BSYS-serum treatment. Moreover, the culture supernatants from astrocytes treated with BSYS-serum significantly increased the expression levels of oligodendrocyte maturation-related proteins, including CNPase, MOG, MBP, and PLP. These findings suggest that BSYS exerts pro-remyelination effects by improving oligodendrocyte differentiation and maturation, a process mediated by astrocyte polarization. However, the molecular mechanism by which BSYS induces astrocyte polarization remains uncertain.

Although the mechanism underlying A1/A2 phenotype transformation by astrocytes is not well understood, several studies have shown that some miRs, especially miR-155, modulate astrocytic polarization. It has been demonstrated that miR-155 overexpression in activated astrocytes drives astrocytic polarization towards the A1 proinflammatory phenotype. By contrast, miR-155 downregulation in astrocytes decreases the generation of proinflammatory factors and astrocyte-mediated neurotoxicity via enhancing the expression of suppressor of cytokine signaling 1 (SOCS1) [17]. SOCS1 is an intracellular, cytokine-inducible protein that restrains cytokine signaling in astrocytes and other inflammatory immune cells in the CNS, thereby alleviating neuroinflammation [64]. It has been demonstrated to play therapeutic roles against MS [65]. In previous studies, the expression of miR-155 was considerably upregulated in reactive astrocytes [66] and white matter lesions of MS tissue samples [67, 68]. Similarly, a substantial elevation of the miR-155 expression level has been reported in brain samples of the CPZ-mediated demyelination mouse model. These findings illustrate that the miR-155/SOCS1 axis participates in myelin damage and astrocyte polarization. Hence, the miR-155 pathway is a viable treatment target for MS. Indeed, we previously found that BSYS regulated miR-155 and miR-124 to facilitate microglial M2 polarization and reduce demyelination in EAE mice. However, the mechanisms through which BSYS regulates astrocyte polarization-mediated remyelination are unclear. In this study, the regulatory function of BSYS on A1/A2 phenotype transformation and promoting effect on oligodendrocyte maturation was significantly attenuated following miR-155 overexpression in astrocytes *in vitro*. In the future, we will combine gene knockout and knockin techniques to further clarify the effect of BSYS on the link between miR-155 and astrocytic polarization in a mouse model of MS.

5. Conclusions

Collectively, the results reported here demonstrate that BSYS ameliorates neuroinflammatory and promotes myelin repair by modulating astrocytic polarization and secretion of pro-/anti-inflammatory factors. Mechanistically, we show that BSYS regulates A1/A2 polarization by inhibiting miR-155 leading to the upregulation of SOCS1 signaling pathways. Therefore, BSYS might present a promising therapeutic agent for the management of MS as well as other CNS demyelinating disorders.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors warrant that they do not have any conflicting interests to disclose.

Authors' Contributions

Zheng Zha drafted this manuscript and conceived the study. Zheng Zha, Yi-Jiang Liu, Si-Si Liu, Nan Zhang, Jun-Ling Li, Fang Qi, and Liang-Yun Jin performed LFB, IHC, IF, Western blot, and qRT-PCR. Zheng Zha and Tao Yang performed the statistical analysis. Bing Xue, Hui Zhao, Yong-Ping Fan, and Lei Wang designed and coordinated the study. The final version of the manuscript was reviewed and approved by all the authors.

Acknowledgments

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Supplementary Materials

Supplementary Table 1: primer sequences. *(Supplementary Materials)*

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Research Article

Neuroprotective Effect and Possible Mechanisms of Ginsenoside-Rd for Cerebral Ischemia/Reperfusion Damage in Experimental Animal: A Meta-Analysis and Systematic Review

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Ischemic stroke, the most common type of stroke, can lead to a long-term disability with the limitation of effective therapeutic approaches. Ginsenoside-Rd (G-Rd) has been found as a neuroprotective agent. In order to investigate and discuss the neuroprotective function and underlying mechanism of G-Rd in experimental animal models following cerebral ischemic/reperfusion (I/R) injury, PubMed, Embase, SinoMed, and China National Knowledge Infrastructure were searched from their inception dates to May 2022, with no language restriction. Studies that G-Rd was used to treat cerebral I/R damage *in vivo* were selected. A total of 18 articles were included in this paper, and it was showed that after cerebral I/R damage, G-Rd administration could significantly attenuate infarct volume (19 studies, SMD = −1.75 [−2.21 to − 1.30], $P < 0.00001$). Subgroup analysis concluded that G-Rd at the moderate doses of >10- <50 mg/kg reduced the infarct volume to the greatest extent, and increasing the dose beyond 50 mg/kg did not produce better results. The neuroprotective effect of G-Rd was not affected by other factors, such as the animal species, the order of administration, and the ischemia time. In comparison with the control group, G-Rd administration could improve neurological recovery (lower score means better recovery: 14 studies, SMD = −1.50 [−2.00 to − 1.00], $P < 0.00001$; higher score means better recovery: 8 studies, SMD = 1.57 [0.93 to 2.21], $P < 0.00001$). In addition, this review suggested that G-Rd *in vivo* can antagonize the reduced oxidative stress, regulate Ca^{2+}, and inhibit inflammatory, resistance to apoptosis, and antipyroptosis on cerebral I/R damage. Collectively, G-Rd is a promising natural neuroprotective agent on cerebral I/R injury with unique advantages and a clear mechanism of action. More clinical randomized, blind-controlled trials are also needed to confirm the neuroprotective effect of G-Rd on cerebral I/R injury.

1. Introduction

Ischemic stroke is the leading cause of hospitalization for cerebrovascular disease, accounting for 85% of all stroke incidences [1]. It is associated with a variety of complications, including insomnia, depression, or poststroke dementia, which can lead to adverse outcomes [2–4]. As a leading cause of high morbidity, mortality, and disability rate, stroke imposes a severe financial and psychological burden on patients and families worldwide [5]. Making an urgent therapy is the most important and effective hotspot. Yet now, to achieve neuroprotection, majority of the therapeutic approaches for acute ischemic stroke are to recanalized the occluded arteries [5]. Currently, thrombolysis with recombined tissue plasminogen

activator and thrombectomy are effective treatments [6, 7]. However, limited by a narrow therapeutic window and the risk of hemorrhagic complications, these methods only can be used in the minority [8, 9]. Just as importantly, cerebral reperfusion caused secondary damage to the brain can also lead to severe adverse reaction [10].

When acute ischemic stroke happened, blood flow sharply decreased which gives rise to a rapid increasing in the production of reactive oxygen species (ROS) [11, 12]. Early restoration of blood supply can salvage ischemic and hypoxic tissue, but reperfusion itself also can cause cerebral ischemia/reperfusion (I/R) damage [13, 14]. Most importantly, ROS are produced and bursts after reperfusion [15]. Excess ROS is the main cause of oxidative stress and one of the major hazards leading to the direct neuron damage [11, 16–18]. Oxidative stress triggered nuclear factor kappa-light-chain-enhancer of the activated B cell (NF-κb) signaling pathway, leading to nucleotide-binding oligomerization domain-like receptor 3 (NLRP3) activation, those which contribute to the blood-brain barrier (BBB) damage, inflammatory environment formation, neuron apoptosis, and pyroptosis [17, 19–22]. Meanwhile, the occurrence of oxidative stress further attacks carbohydrates, lipids, proteins, nucleic acid, and release of Ca^{2+} from intracellular stores [23]. In addition, this microenvironment in turn further exacerbates oxidative stress [24, 25]. Thus, establishing a balance of ROS generation and consumption to attenuate I/R injury may be an effective therapeutic strategy.

After thousands of years of practice, traditional herbal extracts and its effective components are used worldwide as drug to prevent and treat ischemic stroke which has been collected both *in vivo*/*vitro* and in clinical application. Ginsenoside-Rd (G-Rd) (the chemical structure is shown in Figure 1), as a main bioactive saponin, belongs to the protopanaxadiol group [26]. G-Rd is an important metabolite in the transformation pathway of protopanaxadiol-type ginsenosides in human intestine [27]. To date, G-Rd can be obtained from structurally similar ginsenoside-Rb1 and ginsenoside-Rc by microbial-based biotransformation and enzymatic transformation [28, 29]. G-Rd has outstanding advantages in multisite and multitarget global regulations. Extensive studies showed G-Rd with multiplied pharmacological properties possesses a broad spectrum of therapeutic effects on the central nervous system [30, 31]. G-Rd directly makes contribution to the nuclear factor erythroid-2-related factor 2 (Nrf2) antioxidant pathway to promote the ability of eliminating ROS to inhibit lipid peroxidation [22, 32, 33]. Besides, G-Rd plays an anti-inflammatory role in Alzheimer's disease [34]. Furthermore, G-Rd with highly lipophilic ability can spread through biological membranes and BBB easily [31, 35]. Currently, preclinical studies have confirmed the effectiveness of G-Rd in the treatment of cerebral I/R, and then, G-Rd treatment of cerebral I/R has entered the second phase of clinical trials [31, 36, 37]. Its neuroprotective function has attracted an increasing attention. In order to review the beneficial effects of G-Rd on I/R damaged animal models and summarize the underlying molecular mechanisms, a comprehensive systematic review was performed.

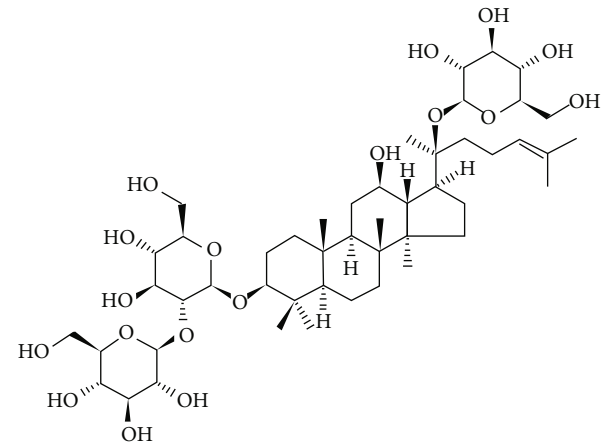

Figure 1: Chemical structures of G-Rd. G-Rd: ginsenoside-Rd.

2. Methods and Materials

The Preferred Reporting Items for Systematic Reviews and Meta-Analyses (the PRISMA) statement was selected to report for this systematic evaluation and meta-analysis [38].

2.1. Search Strategy. The following electronic databases were retrieved from their inception to May 2022 to identify relevant *in vivo* studies without language limitations: PubMed, Embase, China National Knowledge Infrastructure, and SinoMed. The lists of references included in this study are screened to identify if there are any other relevant studies. MeSH terms such as "brain anoxia ischemia", "brain hypoxia ischemia", "brain ischaemia", "brain ischaemics", "brain ischemia", "brain ischemias", "cerebral anoxia ischemia", "cerebral ischaemia", "cerebral ischaemia hypoxia", "cerebral ischemia", "cerebral ischemias", "ischemic stroke", "ischemized reperfusate", "ischemized reperfusion injury", "reperfusion injury", "stroke", "ginsenoside", "ginsenosides", and "ginsenoside Rd" were used. These search terms were translated into Chinese to be searched in Chinese databases. Supplementary material contains the PubMed database search strategy.

2.2. Inclusion Criteria. All controlled studies evaluating the neuroprotection effect and discussing the possible mechanisms of G-Rd on brain I/R damage in animal models were included. Specify the following inclusion criteria in advance to prevent bias (1) based on an animal experiment, no restriction on animal species, gender, age, weight, and sample size; (2) involve a focal cerebral I/R damage model, caused by transient or permanent middle cerebral artery occlusion (MCAO); (3) the experimental group was treated with G-Rd monotherapy in no restriction on dosage, mode, and time of initial treatment; (4) the control group was administered by saline, vehicle, or positive control drug or no treatment; (5) have one of the following outcomes available: infarct volume, neurological function score (NFS), and biochemical examinations.

2.3. Exclusion Criteria. The following exclusion criteria were also prespecified: (1) reviews, comments, case reports, editorials, clinical articles, and *in vitro* studies; (2) nonfocal brain

I/R model, adopting global models (e.g., bilateral common carotid occlusion), traumatic models, or only hypoxic ischemic models; (3) the experimental group was intervened of G-Rd combined with some other drugs; (4) absence of control group; (5) outcome measures are not included in the literature; and (6) duplicated publications.

2.4. Selection and Data Extraction. Two reviewers (ZAF and ZK) screened the abstracts and full texts of the included literature and excerpted the following details independently: (1) the first author's name, the publication year, methods of establishing ischemia model, and ischemia duration; (2) the characteristics of animals, such as the number, the species, the gender, and the weight of animals; (3) treatment information including dosage, timing, and the route of G-Rd delivery; and (4) outcome assessment. Disregarding the outcomes presented at different time points of each animal in the experiment, only extracted the data at the last time point. If the research results were incomplete or only showed in the form of graphs, the authors were e-mailed for these data, and if a response was not received, the data was got from the graphs using Engauge Digitizer 11.1 commercial software. If several independent experiments are carried out in a paper, it was broken down into several complete sections. These two reviewers extracted relevant data from the papers independently to avoid errors. Take the average data of two when the error was within the acceptable range (error $\leq$ 1%average data). If not, the third one (YM) shall reextract the data and then use the average of two data which were more closely related.

2.5. Quality Assessment. Study quality was evaluated by the Collaborative Approach to Meta-Analysis and Review of Animal Data in Experimental Studies (CAMARADES), a ten-item modified scale [39]. Two investigators (ZYY and LZY) independently evaluated the methodological quality of the included literature according to the following list: (1) published in a peer-reviewed journal; (2) temperature control statement; (3) randomization to treat or control group; (4) allocation concealment; (5) outcome assessed blinding; (6) no obvious intrinsic neuroprotective effect of anesthetic; (7) appropriate animal model such as aged, diabetic, or hypertensive; (8) sample size estimation; (9) compliance with animal welfare regulations; and (10) declared any potential conflict of interest. A ten-item (1 point for each item) was included in the modified scale, and an aggregate quality score was obtained for each study. If the weighted kappa (K_w) value was >0.75, the quality assessment was accepted, consulting with the corresponding author to solve any disagreements.

2.6. Statistical Analysis. Different scales were used in a different study to assess the same outcome index, so the standardized mean difference (SMD) with 95% CI was used in this analysis. The I^2 test was used to assess the statistical heterogeneity of the included studies. $I^2 > 50\%$ means significant heterogeneity exists, and then, the random-effect model test was conducted. Or then, the fixed-effect model test was selected. The factors modifying on the infarct volume were explored through the source of heterogeneity of subgroup analysis. $P \leq 0.05$ was considered statistically significant. Review Manager version 5.4 software was used for data analyses.

2.7. Publishing Bias. Publishing bias means that the published research literature does not systematically or comprehensively represent the completed research in the field. Therefore, in order to further test for the publishing bias, funnel plot and Egger's test were used in this meta-analysis stage.

3. Results

3.1. Selection of Studies. The process of screening is summarized in Figure 2. In total, 374 unique references were identified by searching electronic databases and removing the duplicates. 351 papers were excluded after going through the titles and abstracts. For the reason: the studies of solely *in vitro*, 4 articles were deleted from the remaining articles by reading the full text [40–43]. Wan et al. used a model of bilateral common carotid occlusion which was a nonfocal brain I/R model so that it was excluded [44]. Eventually, 18 articles were obtained and assessed these for eligibility [22, 33, 35, 45–59].

3.2. The Characteristics of the Included Studies. All of these studies were conducted in China and reported in English except for three studies which were published in Chinese. The animal species included Wistar rats [48], C57BL/6 mice [22, 58], and Sprague-Dawley rats [33, 35, 45–47, 49–53, 57, 59]. In order to keep the baseline status of experimental animals consistent and to be studied independently, Ye et al.'s study was split into five complete parts: dose-response study, therapeutic window study, permanent ischemia study, older study, and female study [45]. Ye et al. were separated into three parts, including dose-response study, therapeutic window study, and sustained neuroprotection study [58], while in Ye et al.'s article, the authors introduced the protection conferred by G-Rd in two parts, including its sustained effects [35]. All the animals included were male except in the Ye et al.'s female study. Most of the studies were transient MCAO models, with cerebral artery occlusion varied from 1 to 2 hours. While Ye et al. reported a study in permanent MCAO. All studied animals conditioned with G-Rd by intraperitoneal injection. The dosing of G-Rd treatment varied substantially, five of the included studies performed a dose gradient study of G-Rd [33, 45, 47, 55, 58]. And single-dose administration was conducted in the remaining. Moreover, the duration of G-Rd intervention ranged from 3 days before ischemia to 1 day after the ischemia stroke happened [48, 55]. For comparison, in some studies, G-Rd also was tested and compared/combined with edaravone and PBN, LY294002, and MG132 [45, 50, 55] (Table 1).

3.3. Risk of Bias within Studies. The quality score of the included studies ranged from 3 to 7 out of 10 points (Table 2). Consensus was built on 100% with $K_w = 0.89$. Of whom, six studies received more than 5 points. All the included studies were published in peer review, 16 studies illustrate the control of temperature. Randomization and

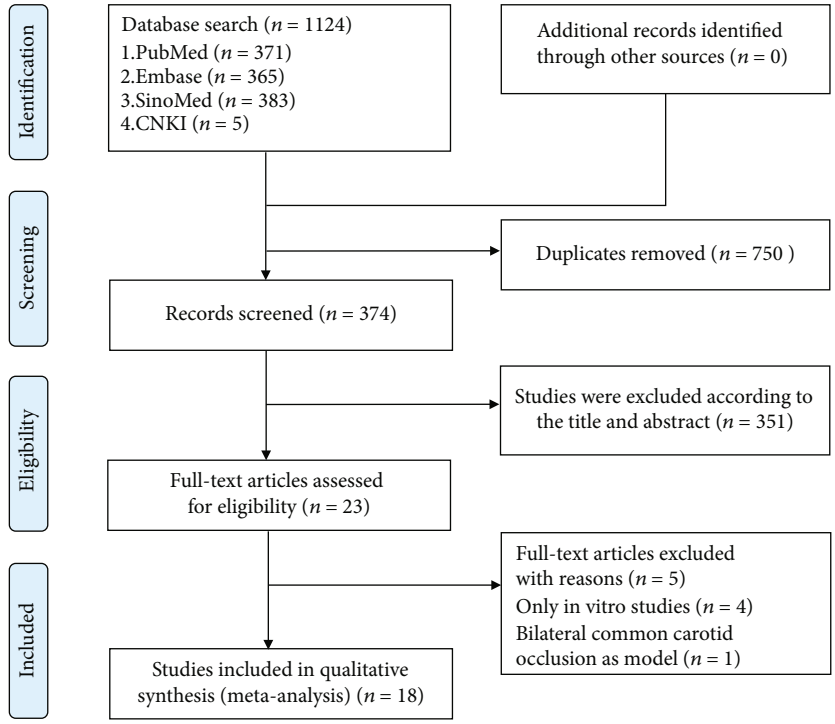

Figure 2: Summary of the literature identification and selection process.

blinded assessment were reported in 11 and 8 studies, respectively, but none of them described the sample-size calculation and allocation concealment. 13 studies reported without significant neuroprotective activity from anesthetics, while others did not describe which anesthetic agent they were used. 8 studies declared without potential conflicts of interest. Moreover, only 3 studies stated they were in compliance with animal welfare laws. In terms of compliance with appropriate model animals, 2 studies pointed out they researched on older or/and female animals. As proposed by Bederson et al. [60], 5 studies pointed out a completed stroke model was identified with forelimb flexion, whereas Du's study described with score of 2-3 according to Longa scale is considered as a successful stroke model.

3.4. The Effective of G-Rd for Cerebral Infarct Volume in Cerebral I/R Injury and Meta-Analysis. There were 12 studies with 18 comparisons included in this study. Based on the TTC staining, G-Rd was found to have significant effects on diminishing the infarct size in the comparison with the control group which received normal saline or with no treatment. After excluding one study, which calculated the actual infarct volume only through deducting the area of brain edema [56], while others used the adjusted infarct volume and use the percentage of the contralateral structure to expression (19 studies, SMD = −1.75 [−2.21 to − 1.30], $P < 0.00001$) (Figure 3).

Subgroup analysis showed that the pooled estimates of infarct size improvement did not depend on the species, ischemic time, timing regimen, and so on, but was associated with the dose (Table 3). Subgroup analysis was conducted to identify G-Rd lower the cerebral infarct volume on experimental cerebral I/R. The results illustrated a dose-response relationship in a dose no more than 50 mg/kg, in the studies using doses of >10-<50 mg/kg (4 studies, SMD = −5.08 [−7.58 to − 2.58], $P < 0.0001$) is more preferable than less than 10 mg/kg (6 studies, SMD = −1.48 [−2.52 to − 0.45], $P = 0.005$), 10 mg/kg (6 studies, SMD = −2.25 [−3.00 to − 1.50], $P < 0.00001$), or 50 mg/kg (11 studies, SMD = −1.53 [−1.86 to − 1.21], $P < 0.00001$). But when the dose was greater than 50 mg/kg, the protection of G-Rd on reducing the cerebral infarct volume was ineffective (3 studies, SMD = −1.18 [−3.10 to 0.73], $P = 0.22$).

3.5. The Effective of G-Rd for Cerebral NFS in Cerebral I/R Damage and Meta-Analysis. The reduction of infarct volume was associated with notable behavioral improvement. The NFS was still significantly improved with G-Rd treatment in the focal I/R injury setting. Twelve studies assessed neurological scores using different scoring systems. The 3-18 grading scale, which developed by Garcia et al. [61], was used in four studies [35, 46, 47, 58]. The 0-12 grading scale [35, 58], Zea-Longa score [22, 55, 57], modified neurological severity score [33, 45], Bederson's score [56], and other neurological scores [59] together to assess motor and sensory recovery after I/R injury. All of the included studies pointed the protective effect of G-Rd in improvement of the neurological deficits. In the scale category with a higher score indicating a better functional recovery (8 studies, SMD = 1.57 [0.93 to 2.21], $P < 0.00001$) (Figure 4). And in another scale category with a lower score indicating a better functional recovery into (14 studies, SMD = −1.50 [−2.00 to − 1.00], $P < 0.00001$) (Figure 5).

Table 1: Basic characteristics of the included studies.

Study	Animals	Model	No. of animals	Groups	Treatment	Assessment
Yao et al. [22]	Male C57BL/6 (22-25 g)	MCAO 1 h	8/8/8/8/8	1. Sham 2. MCAO+1,3-propanediol 3. MCAO+Rd (10 mg/kg) 4. MCAO+Rd (20 mg/kg) 5. MCAO+Rd (40 mg/kg)	0.5 h pre- and 2 post-MCAO, *i.p.*	IV, NF, edema, no. of neurons, caspase 1-TUNEL$^+$, NLRP3, ASC, caspase 1, GSDMD-FL, GSDMD-N, IL-18, IL-1β, TXNIP, ROS, HO-1, NOQ1, Trx1, Keap1, FoxO1, nuclear Nrf2, TXNIP-NLRP3
Ye et al. [33]	Male SD (270-320 g)	MCAO 2 h	161 (total)	1. Sham 2. MCAO+VEC 3. MCAO+Rd (0.1 mg/kg) 4. MCAO+Rd (1 mg/kg) 5. MCAO+Rd (10 mg/kg) 6. MCAO+Rd (50 mg/kg) 7. MCAO+Rd (200 mg/kg)	0.5 h pre-MCAO, *i.p.*	IV, NF, 2,3-DHBA, 2,5-DHBA, 8-OHdG$^+$, 4-HNE, MDA, AGEs, carbonyls, GPX, CAT, SOD1/2, GR, IBal$^+$, GSH/GSSG, iNOS, COX-2
Ye et al. [35]	Male SD (270-320 g)	**A/B.** MCAO 2 h	**A/B.** 12/12 **C.** 6/6/6/6	**A/B. neuroprotection and sustained neuroprotection study** 1. MCAO+VEC 2. MCAO+Rd (50 mg/kg)	**A**. 0.5 h pre-MCA **B**. 0.5 h pre-MCAO then continued until POD 7, *i.p.*	IV, NF, complex I~IV activity, MMP, ROS, aconitase activity, glucose, lactate, LPR, TUNEL$^+$, AIF, pro-/cleaved caspase-3, pyruvate, Cyto C
Ye et al. [45]	Male, female, and old male SD	**A/B/D.** MCAO 2 h **C.** MCAO 24 h	/	**A. Dose-response study** 1. MCAO+VEC 2. MCAO+PBN 3. MCAO+Edaravone 4. MCAO+Rd (1 mg/kg) 5. MCAO+Rd (10 mg/kg) 6. MCAO+Rd (50 mg/kg) **B/C. therapeutic window and permanent MCAO study** 1. MCAO+VEC 2. MCAO+Rd (50 mg/kg) (at different times) **D. Female and aged rat study** 1. MCAO+VEC 2. MCAO+Rd (50 mg/kg)	**A.** 0.5 h pre-MCA **B/C.** 0, 2, 4, 8 h post- MCAO **D.** Post-MCAO, *i.p.*	IV, NF
Yuan et al. [46]	Male SD (280-320 g)	MCAO 2 h	10/10/10/10/10/10	1. Sham 2. MCAO 3. MCAO+Rd (30 mg/kg) 4.MCAO+Rd+Pur 5.MCAO+Rd+TMP 6.MCAO+Rd+Pur+TMP	1 h pre-MCAO, *i.p.*	IV, NF

Table 1: Continued.

Study	Animals	Model	No. of animals	Groups	Treatment	Assessment
Lu et al. [47]	Male SD (280-320 g)	MCAO 2 h	10/10/10/10/10/10/10	1. MCAO 2. MCAO+VEC 3. MCAO+Rd (5 mg/kg) 4. MCAO+Rd (10 mg/kg) 5. MCAO+Rd (20 mg/kg) 6. MCAO+Rd (40 mg/kg) 7. MCAO+Rd (80 mg/kg)	1 h pre-MCAO, *i.p.*	IV, NF
Du et al. [48]	Male Wistar (250-300 g)	MCAO 1 h	9/27/27/27	1. Control 2. Sham 3. MCAO 4. MCAO+Rd (2 mg/kg)	3 d pre-MCAO, *i.p.*	IV, TUNEL, NR2B, EndoG
Zhang et al. [49]	Male SD (250-300 g)	MCAO 2 h	12/12/12/12	1. Sham 2. MCAO+VEC 3. Sham+Rd (10 mg/kg) 4. MCAO+Rd (10 mg/kg)	15-minute pre-MCAO, *i.p.*	TRPM7, ASIC1a, ASIC2a, NR1, NR2A, NR2B
Zhang et al. [50]	Male SD (270-320 g)	MCAO 2 h	15/15/15/6	1. Sham 2. MCAO 3. MCAO+Rd (30 mg/kg) 4. MCAO+LY294002+Rd (30 mg/kg)	1 h pre-MCAO and 10 mg/kg/d until sacrificed, *i.p.*	IV, NF, no. of neurons, S199/202, PHF-1, tau-5, p-GSK-3b, p-Akt
Zhang et al. [51]	Male SD (270-320 g)	MCAO 1.5 h	16/15/17	1. Sham 2. MCAO+SA 3. MCAO+Rd (30 mg/kg)	1 h pre-MCAO, *i.p.*	GR, GLT-1
Zhang [52]	Male SD (270-320 g)	MCAO 2 h	8/8/8/8	1. Sham 2. MCAO 3. MCAO+Rd (30 mg/kg) 4. MCAO+MG132	1 h pre-MCAO, *i.p.*	Cerebral edema, MMP-9, 20S proteasome activities, P65, NF-κB, IκBα, BBB function
Zhang et al. [53]	Male SD (250-300 g)	MCAO 2 h	/	1. Sham 2. MCAO+VEC 3. Sham+Rd (10 mg/kg) 4. MCAO+Rd (10 mg/kg)	4 h post-MCAO, *i.p.*	IBa1+, IL-1β, IL-6, IL-18, TNF-α, IFN-γ, p-IκBα, IκBα, nuclear and cytosolic p65
Hu et al. [54]	Male SD (280-300 g)	MCAO 2 h	10/30/10/30	1. Sham+SA 2. MCAO+SA 3. Sham+Rd (10 mg/kg) 4. MCAO+Rd (10 mg/kg)	0.5 h pre-MCAO, *i.p.*	PAR, PARP-1, nuclear p65, nuclear and mitochondria AIF
Liu et al. [55]	Male SD (220-240 g)	MCAO 1.5 h	19/19/9/9/19/10	1. Sham 2. MCAO 3. MCAO+Rd (1 mg/kg) 4. MCAO+Rd (2.5 mg/kg) 5. MCAO+Rd (5 mg/kg) 6. MCAO+LY294002+Rd (5 mg/kg)	POD1 to POD3, *i.p.*	IV, NF, p-Akt/Akt, GFAP, DCX^+

Table 1: Continued.

Study	Animals	Model	No. of animals	Groups	Treatment	Assessment
Xie et al. [56]	Male SD (270-320 g)	MCAO 2 h	21/42/21/42	1. Sham+VEC 2. MCAO+VEC 3. Sham+Rd (50 mg/kg) 4. MCAO+Rd (50 mg/kg)	0.5 h pre-MCAO, *i.p.*	IV, NF, NR2B, p-Ser-1303, p-Tyr-1472, p-Ser-1480
Yang et al. [57]	Male SD (270-320 g)	MCAO 2 h	24/24/24/24	1. Sham 2. MCAO 3. MCAO+Rd (30 mg/kg)	1 h pre-MCAO and 10 mg/kg/d until sacrifice, *i.p.*	NF, NEIL1, NEIL2, NEIL3, cleaved caspase-3, TUNEL$^+$, mtDNA, nDNA, survival rate
Ye et al. [58]	Male C57BL/6 (16-18-month)	MCAO 1 h	/	**A. Dose-response study:** 1. MCAO+VEC 2. MCAO+Rd (0.1 mg/kg) 3. MCAO+Rd (1 mg/kg) 4. MCAO+Rd (10 mg/kg) 5. MCAO+Rd (50 mg/kg) 6. MCAO+Rd (200 mg/kg) **B. Therapeutic window study** 1. MCAO+VEC 2. MCAO+Rd (50 mg/kg) (at different times) **C. Sustained neuroprotection study** 1.MCAO+VEC 2. MCAO+Rd (50 mg/kg)	**A.** 0.5 h pre-MCA **B.** 0, 2, 4, 6, 8 h post-MCAO **C.** 0.5 h pre-MCAO then continued until POD 7, *i.p.*	IV, NF, body weight, MDA, 8-OHdG$^+$, carbonyl levels, MMP, ROS, SOD1/2, CAT, GPX, GST, GSH/GSSG aconitase activity, complex I-IV activity
Zhang et al. [59]	Male SD (280-300 g)	MCAO 2 h	20/20/20/20	1. Sham 2. MCAO 3. Sham+Rd (10 mg/kg) 4. MCAO+Rd (10 mg/kg)	Immediately post-MCAO, *i.p.*	IV, NF, p-ser-1303, p-tyr1472, p-ser1480, p-DAPK

MCAO: middle cerebral artery occlusion; Rd: ginsenoside Rd; i.p.: intraperitoneally; IV: infarct volume; NF: neurological functions; TUNEL: terminal deoxynucleotidyl transferase-mediated dUTP biotin nick end labeling; NLRP3: nucleotide-binding oligomerization domain- (NOD-) like receptor 3; ASC: apoptosis-associated speck-like protein containing a CARD; GSDMD: gasdermin D; IL-18: interleukin 18; IL-1β: interleukin 1β; TXNIP: thioredoxin-interacting protein; ROS: reactive oxygen species; HO-1: heme oxygenase-1; NOQ1: reduced coenzyme/quinone oxidoreductase 1; Trx1: thioredoxin; Keap1: Kelch-like ECH-associated protein 1; FoxO1: forkhead box transcription factor O1; Nrf2: nuclear factor erythroid-2-related factor 2; SD: Sprague-Dawley; VEC: vehicle; DHBA: dihydroxybenzoic acids; 8-OHdG: 8-hydroxy-deoxyguanosine; 4-HNE: 4-hydroxynonenal; MDA: malondialdehyde; AGEs: advanced glycosylation end products; GPX: glutathione peroxidase; CAT: catalase; SOD: superoxide dismutase; GR: glutathione reductase; GSH: glutathione; GSSG: glutathione disulfide; iNOS: inducible nitric oxide synthase; COX-2: cyclooxygenase-2; MMP: mitochondrial membrane potential; LPR: lactate/pyruvate ratio; AIF: apoptosis-inducing factor; Cyto C: cytochrome c; PBN: N-tert-butyl-alpha-phenylnitrone; Pur: puerarin; TMP: tetramethylpyrazine; NR2B: N-methyl-D-aspartate receptor 2B; Endo G: endonuclease G; TRPM 7: transient receptor potential melastatin-7; ASIC: acid sensing ion channels; NR1: N-methyl-D-aspartate receptor 1; NR2A: N-methyl-D-aspartate receptor 2A; PHF-1: pairedhelicalfilaments-1; GSK-3b: glycogen synthase kinase-3b; Akt: proteinserine-threonine kinase; SA: saline; GLT-1: glial glutamate transporter-1; MMP-9: neuroinflammation-mediated matrix metalloproteases-9; IκBα: nuclear factor of kappa light polypeptide gene enhancer in B cell inhibitor: alpha; NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells; BBB: the blood-brain barrier; IL-6: interleukin 6; TNF-α: tumor necrosis factor alpha; IFN-γ: interferon gamma; PAR: poly(ADP-ribose); PARP-1: poly (ADP-ribose) polymerase-1; GFAP: glial fibrillary acidic protein; NEIL: endonuclease VIII-like; GST: glutathione S-transferase; DAPK: death-associated protein kinase.

Table 2: Risk of bias of the included studies according to CAMARADES checklist.

Study	1	2	3	4	5	6	7	8	9	10	Score
Yao et al. [22]	√	√	√	/	√	√	/	/	√	√	7
Ye et al. [33]	√	√	/	/	√	√	/	/	/	/	4
Ye et al. [35]	√	√	/	/	√	√	/	/	/	/	4
Ye et al. [45]	√	√	√	/	√	√	√	/	/	√	7
Yuan et al. [46]	√	√	√	/	√	√	/	/	/	/	5
Lu et al. [47]	√	√	√	/	√	√	/	/	/	/	5
Du et al. [48]	√	√	√	/	/	√	/	/	/	/	4
Zhang et al. [49]	√	√	√	/	/	/	/	/	/	√	4
Zhang et al. [50]	√	√	/	/	/	√	/	/	/	√	4
Zhang et al. [51]	√	√	/	/	/	√	/	/	/	√	4
Zhang et al. [52]	√	/	/	/	/	√	/	/	/	√	3
Zhang et al. [53]	√	√	√	/	/	√	/	/	√	√	6
Hu et al. [54]	√	√	√	/	/	/	/	/	/	/	3
Liu et al. [55]	√	/	√	/	√	/	/	/	√	/	4
Xie et al. [56]	√	√	√	/	/	/	/	/	/	/	3
Yang et al. [57]	√	√	√	/	/	√	/	/	/	√	5
Ye et al. [58]	√	√	/	/	√	/	√	/	/	/	4
Zhang et al. [59]	√	√	/	/	/	√	/	/	/	/	3

(1) Publication in a peer-reviewed journal; (2) statement of control of temperature; (3) randomization to treatment or control; (4) allocation concealment; (5) blinded assessment of outcome; (6) no obvious intrinsic neuroprotective effect of anesthetic; (7) appropriate animal model such as aged, diabetic, or hypertensive; (8) sample size estimation; (9) compliance with animal welfare regulations; (10) declared any potential conflict of interest.

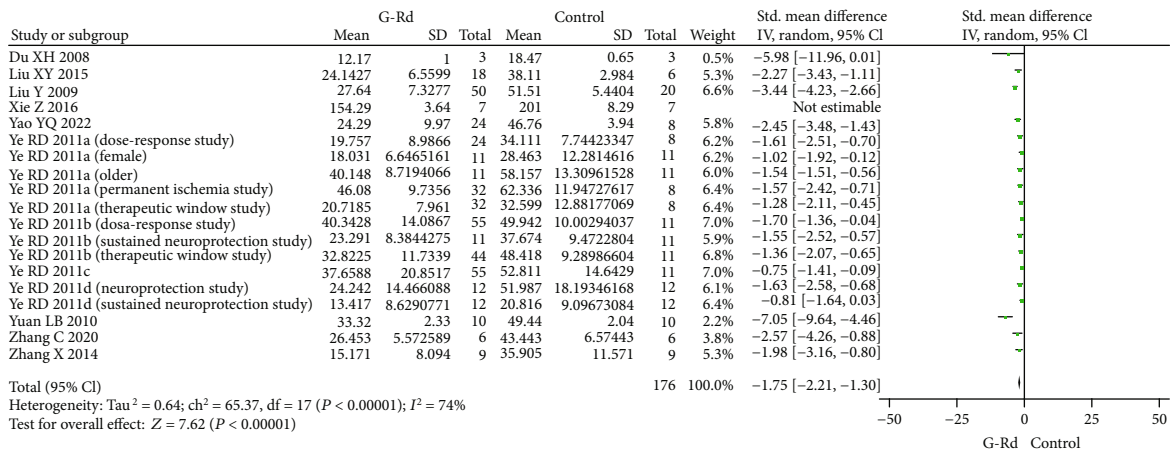

Figure 3: The pooled estimate of G-Rd on decrementing cerebral infarct volume after cerebral I/R damage. G-Rd: ginsenoside-Rd; I/R: ischemia/reperfusion.

3.6. Publishing Bias Test. The funnel plot test was used to check the meta-analysis publication bias, there was asymmetric for the effect of G-Rd on infarct volume (Figure 6(a)) and the funnel plot of the NFS (the lower score means better recovery) was essentially symmetrical (Figure 6(c)). Then, Egger's tests were conducted, the P values for the Egger's intercept suggested a moderate likelihood of publication bias for the effect of G-Rd on infarct volume analysis ($P = 0.046 < 0.05$) (Figure 6(b)), while the NFS (the lower score means better recovery) is with a low risk of publication bias for all analysis ($P = 0.279 > 0.05$) (Figure 6(d)).

4. Discussion

4.1. Summary of the Main Results. Systematic review and meta-analysis have already demonstrated the preclinical evidence that G-Rg1 and G-Rb1 have potential neuroprotective role in substantially reduced infarct volume and improved NFS in animal models of cerebral I/R injury [62, 63]. Up to now, this is the first meta-analysis to evaluate the promising therapeutic effect of G-Rd in focal brain I/R animal models. The qualities of the included studies were generally moderate. Evidence has showed that G-Rd treatment before

Table 3: Subgroup analysis of decrement in infarct volume with G-Rd.

Pooled estimates	No. of studies	Std. MD (95% CI)	*P* value	Subgroup (*P* value)
Species				
SD rats	13	-1.84 [-2.41, -1.28]	<0.00001	<0.00001
Wistar rats	1	-5.98 [-11.96, 0.01]	=0.05	
C57BL/6 mice	4	-1.44 [-2.12, -0.75]	<0.0001	
Dosage				
<10 mg/kg	6	-1.48 [-2.52, -0.45]	=0.005	
10 mg/kg	6	-2.25 [-3.00, -1.50]	<0.00001	<0.00001
>10-<50 mg/kg	4	-5.08 [-7.58, -2.58]	<0.0001	
50 mg/kg	11	-1.53 [-1.86, -1.21]	<0.00001	
>50 mg/kg	3	-1.18 [-3.10, 0.73]	=0.22	
Administration time				
Before I/R	7	-2.28 [-3.43, -1.13]	=0.0001	<0.00001
After I/R	7	-1.49 [-1.84, -1,14]	<0.00001	
Before and after I/R	4	-1.64 [-2.38, -0.91]	<0.0001	
Occlusion time				
60 min	5	-1.51 [-2.24, -0.78]	<0.0001	
90 min	1	-2.27 [-3.43, -1.11]	=0.0001	<0.00001
120 min	11	-1.77 [-2.35, -1.18]	<0.00001	
Permanent	1	-1.57 [-2.42, -0.71]	=0.0003	
Model animal				
Normal male	13	-1.77 [-2.04, -1.50]	<0.00001	<0.00001
Female	1	-1.02 [-1.92, -0.12]	=0.03	
Older male	4	-1.18 [-1.58, -0.79]	<0.00001	
Risk of bias				
<5	7	-1.32 [-1.87, -0.77]	<0.00001	<0.00001
≥5	4	-3.12 [-4.99, -1.24]	=0.001	

G-Rd: ginsenoside-Rd; SD: Sprague-Dawley; I/R: ischemia/reperfusion; MD: mean difference; CI: confidence interval.

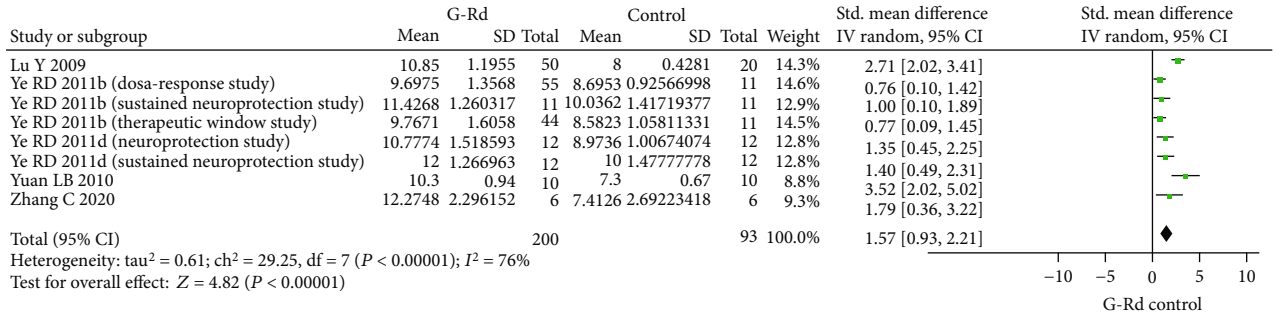

Figure 4: The pooled estimate of G-Rd in the improvement of neurological function score (higher score means better recovery). G-Rd: ginsenoside-Rd.

and/or after I/R stroke can reduce infarct volume, enhance neurological function. Subgroup analysis showed that G-Rd in the range of >10-<50 mg/kg dose substantially lower the infarct size, while beyond this range the effect was abrogated. Maybe G-Rd at 50 mg/kg dose has reached the upper limit of the blood concentration, and too high a dose may produce drug toxicity. Among the G-Rd administration dose-response studies, two studies revealed that in their test paradigms treat with 40 mg/kg performed better [22, 47]. Lu Y et al. explained the protective effect decrease may be related to the increased dosages lead to the animals cannot tolerate the drug toxicity [47]. Some studies found that the reduction in infarct volume was greatest in 50 mg/kg, but their experimental was limited by a wide range of doses, they did not design a dose study between 10 and 50 mg/kg, and the result comes from the same teamwork [33, 45, 58]. Although >10-<50 mg/kg showed the best effect on reduce infarct volume, we also found that most of the studies involving dose-response relationship were not set the dose between >10-<50 mg/kg, which may affect the reliability of our

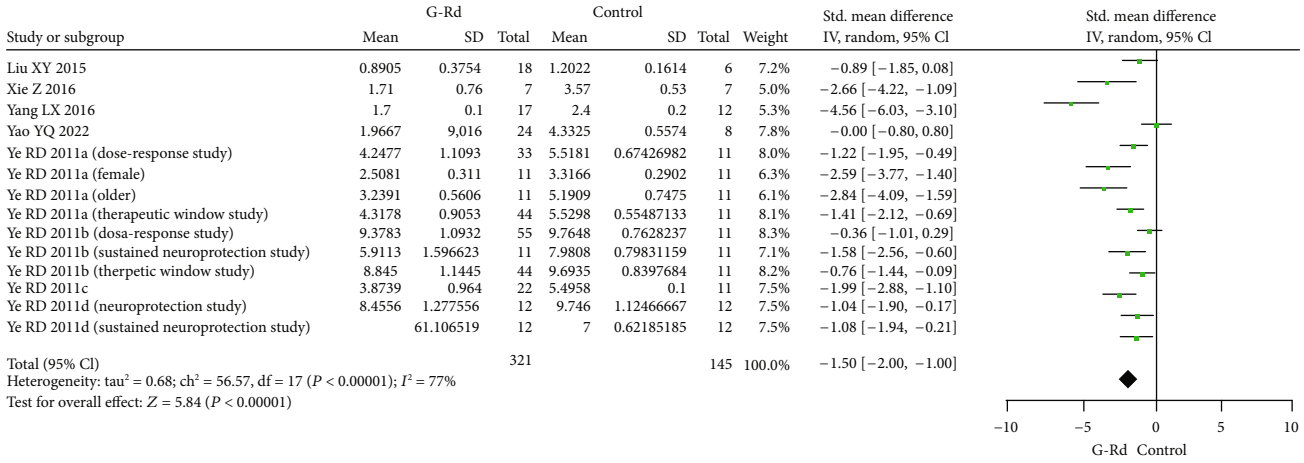

Figure 5: The pooled estimate of G-Rd in the improvement of neurological function score (lower score means better recovery). G-Rd: ginsenoside-Rd.

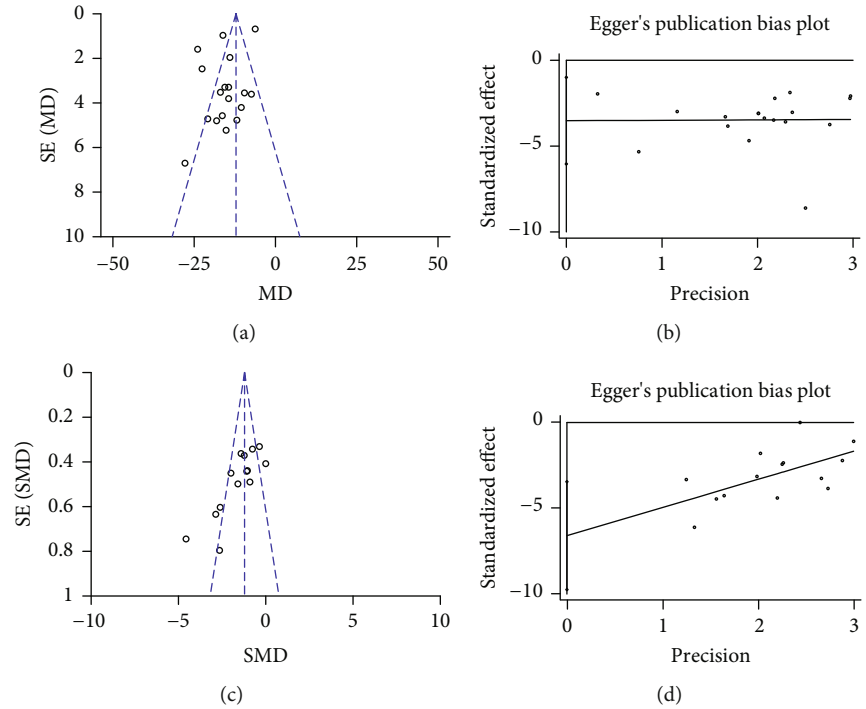

Figure 6: Bias assessment plot for the effect of G-Rd on infarct volume by funnel blot (a) and Egger's test (b); neurological function score (lower score means better recovery) by funnel blot (c) and Egger's test (d). G-Rd: ginsenoside-Rd.

conclusion. Refer to the subgroup analysis, further research about dose-response of G-Rd is needed at the concentrations ranging from 10 to 50 mg/kg to determine the optimal dose in the management of brain I/R damage.

4.2. Neuroprotective Strategies of G-Rd in Experimental I/R Injury Animal Model. G-Rd is the main bioactive saponins in Panax notoginseng and ginseng extracts. G-Rd contributes to neuroprotective with extensive biological activity. The possible mechanisms of G-Rd on focal cerebral ischemia animal models were discussed in this study (Table 4).

4.3. G-Rd Ameliorates ROS Production to Antioxidation on Cerebral I/R Damage to Impeded Injury. Oxidative stress plays a crucial role in cerebral I/R damage [16]. ROS generation was start during ischemia and burst after reperfusion. Importantly, the chemical structure of G-Rd (with sugar moiety located at the 20th position of the triterpene dammarane) determines its direct antioxidant properties [64]. G-Rd directly impeded the inactivation of cerebral I/R-induced Nrf2/heme oxygenase-1(HO-1)/NAD(P)H: quinine oxidoreductase 1(NQO-1) antioxidant pathway inactivation [22]. The activation of the signaling pathway can induce

Table 4: The neuroprotective mechanism of G-Rd *in vivo* in the treatment of cerebral I/R injury.

Study	Proposed mechanism	Outcome
Yao et al. [22]	Anti-inflammation and antioxidation	NLRP3, ASC, caspase 1, GSDMD-N, IL-18, IL-1β, TXNIP, ROS, Keap1, FoxO1, TXNIP-NLRP3↓; HO-1, NQO1, Trx1, nuclear Nrf2↑
Ye et al. [33]	Antioxidation and anti-inflammation	2,3-DHBA, 2,5-DHBA, 8-OHdG$^+$, 4-HNE, MDA, AGEs, carbonyls, extracellular glutamate, SOD1, GPX, IBal$^+$, iNOS, and COX-2↓; CAT, SOD2, GR, GSH/GSSG↑
Ye et al. [35]	Mitochondrial protection and antiapoptosis	Complex I-IV activity, ROS, lactate, LPR, cleaved caspase-3, Cyto C, AIF↓; MMP, aconitase activity, glucose, pyruvate↑
Du et al. [48]	Regulating Ca^{2+} and antiapoptosis	NR2B, EndoG↓
Zhang et al. [49]	Regulating Ca^{2+}	TRPM7, ASIC1a↓; ASIC2a↑
Zhang et al. [50]	/	S199/202, PHF-1, and tau-5↓; p-GSK-3b and p-Akt↑
Zhang et al. [51]	Regulating Ca^{2+}	Extracellular glutamate↓; GR, GLT-1↑
Zhang et al. [52]	Anti-inflammation	20S proteasome activities, nuclear P65, NF-κbp65; MMP-9↓; IκBα and BBB function↑
Zhang et al. [53]	Anti-inflammation	IL-1β, IL-6, IL-18, TNF-α, IFN-γ, p-IκBα, and nuclear NF-κb p65↓; IκBα and cytosolic p65↑
Hu et al. [54]	Anti-inflammation and antiapoptosis	PAR, nuclear p65, nuclear AIF↓; AIF in mitochondria↑
Liu et al. [55]	Antiapoptosis	p-Akt/Akt, GFAP$^+$, and DCX$^+$↑
Xie et al. [56]	Antiapoptosis	NR2B, p-Ser-1303, p-Tyr-1472, and p-Ser-1480↓
Yang et al. [57]	Antiapoptosis and antioxidation	Cleaved caspase-3, mtDNA, and nDNA↓; NEIL1, NEIL2, and NEIL3↑
Ye et al. [58]	Antioxidation and mitochondrial protection	8-OHdG$^+$, MDA, carbonyl levels, ROS, SOD1↓; MMP, SOD2, CAT, GPX, GST, and GSH/GSSG Aconitase activity, complex I-IV activity↑
Zhang et al. [59]	Regulating Ca^{2+}	p-ser-1303, p-tyr1472, p-ser1480, p-DAPK↓

G-Rd: ginsenoside-Rd; I/R: ischemia/reperfusion; NR2B: N-methyl-D-aspartate receptor 2B; Endo G: endonuclease G; 8-OHdG: 8-hydroxy-deoxyguanosine; MDA: malondialdehyde; ROS: reactive oxygen species; SOD: superoxide dismutase; MMP: mitochondrial membrane potential; CAT: catalase; GPX: glutathione peroxidase; GST: glutathione S-transferase; GSH: glutathione; GSSG: glutathione disulfide; DHBA: dihydroxybenzoic acids; 4-HNE: 4-hydroxynonenal; AGEs: advanced glycosylation end products; iNOS: inducible nitric oxide synthase; COX-2: cyclooxygenase-2; GR: glutathione reductase; LPR: lactate/pyruvate ratio; Cyto C: cytochrome c; AIF: apoptosis-inducing factor; TRPM 7: transient receptor potential melastatin-7; ASIC: acid sensing ion channels; PAR: poly(ADP-ribose); GLT-1: glial glutamate transporter-1; PHF-1: pairedhelicalfilaments-1; GSK-3b: glycogen synthase kinase-3b; Akt: proteinserine-threonine kinase; GFAP: glial fibrillary acidic protein; NEIL: endonuclease VIII-like; IL-1β: interleukin 1β; IL-6: interleukin 6; IL-18: interleukin 18; TNF-α: tumor necrosis factor alpha; IFN-γ: interferon gamma; NF-κb: nuclear factor kappa-light-chain-enhancer of activated B cells; IκBα: nuclear factor of kappa light polypeptide gene enhancer in B cell inhibitor: alpha; DAPK: death-associated protein kinase; MMP-9: neuroinflammation-mediated matrix metalloproteases-9; BBB: the blood-brain barrier; NLRP3: nucleotide-binding oligomerization domain (NOD)-like receptor 3; ASC: apoptosis-associated speck-like protein containing a CARD; GSDMD: gasdermin D; TXNIP: thioredoxin-interacting protein; Keap1: Kelch-like ECH-associated protein 1; FoxO1: forkhead box transcription factor O1; HO-1: heme oxygenase-1; NQO1: NAD(P)H: quinine oxidoreductase 1; Trx1: thioredoxin; Nrf2: nuclear factor erythroid-2-related factor 2.

protective genes through transcription to eliminate the production of reactive oxygen species, thereby resisting the oxidative stress injury caused by ischemia/reperfusion [65]. G-Rd inhibited the reduction of Nrf2, HO-1, and NQO-1, which further increased the superoxide dismutase (SOD) activity, and improve the level of glutathione peroxidase (GSH-Px) and catalase (CAT). In the ischemic penumbra, G-Rd dampened the accumulation of malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) to inhibit lipid peroxidation, G-Rd suppressed the expression of advanced glycosylation end products (AGEs) to alleviate protein denaturation, as well as it reduced 8-hydroxy-deoxyguanosine (8-OHdG$^+$) to improve nucleic acid and DNA damage against neuron injury [33, 58]. Overwhelming evidence implied that G-Rd acted as an antioxidant also through protecting mitochondrial metabolism. G-Rd significantly attenuated the loss of aconitase to antioxidative stress damage [33, 35]. It might diminish the mitochondrial dysfunction significantly associated with mitochondrial membrane potential (MMP) hyperpolarization as well as the elevation of mitochondrial electron transport complexes activities to ameliorate ROS production [35, 58]. In addition, G-Rd lowers the accumulation of lactate and increase pyruvate, respectively, hence improving energy status (Figure 7(a)) [35].

4.4. G-Rd Regulating Ca^{2+} to Impeded Excitatory Toxicity after Cerebral I/R Damage. Many studies have shown that excitatory toxicity is the trigger of all downstream events. Glutamate-induced excitotoxicity is an important factor [66]. In the early stage of cerebral ischemic, glutamate is markedly elevated in the extracellular space [51]. Excessive glutamate leads the N-methyl-d-aspartate (NMDA) receptor overactivation, which results in cytosolic Ca^{2+} overload and triggers a cascade of molecular events. To modulate glutamate-/NMDA-induced Ca^{2+} influx, G-Rd significantly upregulates glial glutamate transporter glutamate transporter-1 (GLT-1) via the phosphatidylinositol 3-kinase (PI3K)/extracellular regulated protein kinases 1/2 (ERK1/2) pathways to promote glutamate clearance [51]. Meanwhile, G-Rd inhibited the phosphorylation of NMDA receptor 2B (NR2b) induced by cerebral ischemia to interfere the NMDA receptor function, whose overactivation-induced Ca^{2+} overload to causes nervous excitatory [48, 56, 59]. Zhang et al. investigated G-Rd attenuated death-associated protein kinase (DAPK1) by reducing calcineurin (CaN) activity-mediated NR2b phosphorylation [59]. G-Rd antagonizes the accumulated Ca^{2+} also via regulating the nonglutamate dependent calcium-permeable cation channels. Such as acid sensing ion channels (ASIC) and transient receptor potential (TRP). G-Rd enhanced ASIC2a, inhibited ASIC1a expression, and downregulated transient receptor potential melastatin-7 (TRPM7) to mediated neuroprotection (Figure 7(b)) [5, 49].

4.5. G-Rd Makes Effect on Anti-Inflammation to Impeded Injury after Cerebral I/R Damage. Sequential inflammatory response plays a critical role in the pathophysiology of acute cerebral ischemic. G-Rd has also been noted to mitigate the acute stage of cerebral ischemia inflammatory response. G-Rd decreased Iba-1-positive microglia contribute to neuron death via secreting the proinflammatory cytokines including interleukin 1β (IL-1β), interleukin 6 (IL-6), and tumor necrosis factor alpha (TNF-α) [33, 53], and lowered inducible nitric oxide synthase (iNOS) released from activated astrocyte to stop triggering a stronger inflammatory cascade [33]. The neuroprotection of G-Rd was related to the suppression of cyclooxygenase-2 (COX-2) enzyme concentrations targeted in the inhibition of arachidonic acid release and metabolism [33]. NF-κb participates in these anti-inflammation progress, G-Rd pretreatment restored nuclear factor of kappa light polypeptide gene enhancer in B cell inhibitor alph (IκBα) expression in the cytoplasm, reduced the phosphorylation of IκBα, and blocked p65 nuclear translation by promoting the formation of IκBα-p65 complex [52–54]. In addition, G-Rd makes effect on maintain the blood-brain barrier integrity, not only to prevent peripheral leukocyte infiltration but also reduce brain edema in aggravating neurological deficit, and the machine is considered to be mediated by suppressing the NF-κb/neuroinflammation-mediated matrix metalloproteases-9 (MMP-9) pathway (Figure 7(c)) [52].

4.6. G-Rd Plays a Role in Antiapoptosis on Cerebral I/R Damage to Impeded Injury. Ischemia induced the elevation of intracellular ROS and Ca^{2+} levels leading the MMP to open, which enhanced the mitochondria permeability and many mitochondrial proapoptotic factors release are the important event leading to neuronal apoptosis [67]. The TUNEL-positive cell in the G-Rd treatment group was lowered significantly [35, 48, 57]. G-Rd counteracts apoptosis was related to the inhibition of apoptosis-inducing factor (AIF) and cytochrome c [35, 54]. The expression of cleaved caspase-3 significantly depressed in the G-Rd-treated rats compared with the control group [35, 57]. G-Rd management inhibits AIF release from mitochondria initiating the caspase-independent apoptotic cascade through the adenosine 5′-monophosphate-activated protein kinase/poly ADP-ribose polymerase-1 (AMPK/PARP-1) single pathway [35, 54]. G-Rd promoted the numbers of GFAP$^+$ and DCX$^+$ cells increased after focal I/R through the PI3K/Akt [55]. On the other hand, G-Rd serves as a promising drug to treat poststroke dementia by preventing the phosphorylation level of tau in the PI3K/AKT pathway (Figure 7(d)) [50].

4.7. G-Rd Anti-NLRP3-Mediated Pyroptosis on Cerebral I/R Damaged Tissues. Brain I/R damage involves a range of complex pathological mechanisms, and ROS trigger the NLRP3-mediated pyroptosis which has been implicated in cerebral I/R damage [22, 68]. The effective of antipyroptosis on G-Rd management has been investigated [22]. G-Rd promotes NLRP3 inflammasome inactivation by reducing ROS level to impeded procaspase 1 autocleaved into active caspase 1, which not only converts the precursors of IL-18 and IL-1β into their mature forms but also promotes the gasdermin D (GSDMD) maturation then causes the neuron pyroptosis [69]. G-Rd upregulated miR-139-5p to inhibit forkhead box transcription factor O1 (FOXO1), which regulates Kelch-like ECH-associated protein 1 (Keap1) transcriptional activity and subsequently triggers the Nrf2 antioxidant pathway. It is essential for the reduction of excessive ROS and inhibits the ROS/

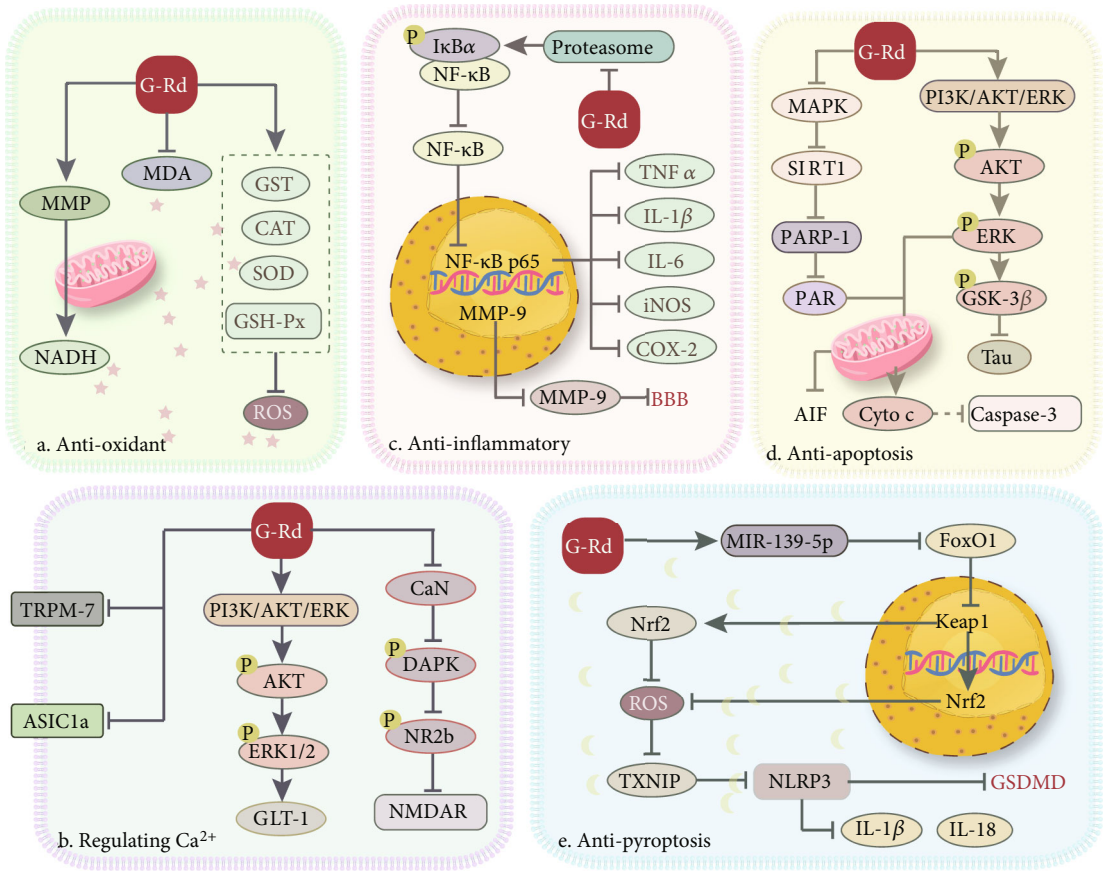

Figure 7: The neuroprotective mechanisms of G-Rd for I/R damage in experimental animal. MMP: mitochondrial membrane potential; NADH: nicotinamide adenine dinucleotide; MDA: malondialdehyde; GST: glutathione S-transferase; CAT: catalase; SOD: superoxide dismutase; GSH-Px: glutathione peroxidase; ROS: reactive oxygen species; IκBα: nuclear factor of kappa light polypeptide gene enhancer in B cell inhibitor, alpha; NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells; MMP-9: neuroinflammation-mediated matrix metalloproteases-9; TNF-α: tumor necrosis factor alpha; IL-1β: interleukin 1β; IL-6: interleukin 6; iNOS: inducible nitric oxide synthase; COX-2: cyclooxygenase-2; BBB: the blood-brain barrier; MAPK: mitogen-activated protein kinase; SIRT1: sirtuin1; PARP-1: poly (ADP-ribose) polymerase-1; PAR: poly(ADP-ribose); AIF: apoptosis-inducing factor; PI3K: phosphatidylinositol 3-kinase; Akt: proteinserine-threonine kinase; ERK: extracellular regulated protein kinases; GSK-3b: glycogen synthase kinase-3b; Cyto C: cytochrome c; GLT-1: glial glutamate transporter-1; CaN: calcineurin; DAPK: death-associated protein kinase; NR2b: N-methyl-D-aspartate receptor 2B; NMDAR: N-methyl-d-aspartate receptor; TRPM 7: transient receptor potential melastatin-7; ASIC1a: acid sensing ion channels 1a; FoxO1: forkhead box transcription factor O1; Keap1: Kelch-like ECH-associated protein 1; Nrf2: nuclear factor erythroid-2-related factor 2; TXNIP: thioredoxin-interacting protein; NLRP3: nucleotide-binding oligomerization domain- (NOD-) like receptor 3; GSDMD: gasdermin D; G-Rd: ginsenoside-Rd; I/R: ischemia/reperfusion.

thioredoxin-interacting protein (TXNIP)/NLRP3 inflammasome axis-mediated pyroptotic in ischemic cortical tissues (Figure 7(e)).

Several limitations should be considered, and first of all, due to time constraints, the protocol of this study was not registered in any registration platform, which was important to restrict the likelihood of biased post hoc decisions in review methods. Secondly, despite the quality of the included studies was acceptable, there are still some shortcomings. For example, no studies included here have reported sample size calculation and allocation concealment. More than half of the studies did not report blind evaluation of results. Only a half study was clearly proposed how to define a completed stroke model according to neurological function score. Then ischemic stroke affects elder patients preferentially accompanied by multiple risk factors, such as diabetes, hypertension, hyperlipidemia, obesity [70, 71], and certain female-specific risk factors may explain their higher risk of stroke [72]. G-Rd was well tolerated and with no dose-related adverse event patterns were assessed in healthy volunteers [73]. Preliminary, multicenter randomized, double-blind, placebo-controlled, phase II clinical trials also have showed that G-Rd had significantly effective on reduced neurological deficits and improved the scores of National Institutes of Health Stroke Scale (NIHSS) [36, 37, 53]. Further investigations are needed to be included in clinical trials to validate the results.

5. Conclusion

Pooled data analysis from this study approved that treatment with G-Rd prior- and/or post-I/R reduced infarct

volume and enhanced neurological functions in brain I/R injury animal model. The recommended dosage is >10-<50 mg/kg. Based on the literature data, G-Rd can reduce oxidative stress, antagonize the accumulated Ca^{2+}, and inhibit inflammatory resistance to apoptosis and antipyroptosis on cerebral ischemia. In the future, the protective effects of G-Rd are needed to be confirmed in clinical trial.

Data Availability

All data included in this study are available upon request by contact with the corresponding author.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Authors' Contributions

All authors have seen the manuscript and approved to submit to your journal. Ai-fang Zhou and Ke Zhu conceived the study, and Ai-fang Zhou and Ke Zhu participated in the collection of cases. Xue-jun Cui and Bing Shu checked as external advisers. Ai-fang Zhou and Ke Zhu completed the data extraction and the analysis of data with Min Yao. Pei-min Pu and Zhuo-yao Li undertook the assessment of the methodological quality, and Ai-fang Zhou, Ke Zhu, and Ya-yun Zhang wrote the initial draft. Yong-jun Wang, Min Yao, and Xue-jun Cui critically revised the manuscript. Ai-fang Zhou and Ke Zhu contributed equally to this work, both of them were the first authors.

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Supplementary Materials

The search strategy of PubMed. *(Supplementary Materials)*

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Review Article

Exploring the Paradox of COVID-19 in Neurological Complications with Emphasis on Parkinson's and Alzheimer's Disease

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Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a human coronavirus (HCoV) that has created a pandemic situation worldwide as COVID-19. This virus can invade human cells via angiotensin-converting enzyme 2 (ACE2) receptor-based mechanisms, affecting the human respiratory tract. However, several reports of neurological symptoms suggest a neuroinvasive development of coronavirus. SARS-CoV-2 can damage the brain via several routes, along with direct neural cell infection with the coronavirus. The chronic inflammatory reactions surge the brain with proinflammatory elements, damaging the neural cells, causing brain ischemia associated with other health issues. SARS-CoV-2 exhibited neuropsychiatric and neurological manifestations, including cognitive impairment, depression, dizziness, delirium, and disturbed sleep. These symptoms show nervous tissue damage that enhances the occurrence of neurodegenerative disorders and aids dementia. SARS-CoV-2 has been seen in brain necropsy and isolated from the cerebrospinal fluid of COVID-19 patients. The associated inflammatory reaction in some COVID-19 patients has increased proinflammatory cytokines, which have been investigated as a prognostic factor. Therefore, the immunogenic changes observed in Parkinson's and Alzheimer's patients include their pathogenetic role. Inflammatory events have been an important pathophysiological feature of neurodegenerative diseases (NDs) such as Parkinson's and Alzheimer's. The neuroinflammation observed in AD has exacerbated the Aβ burden and tau hyperphosphorylation. The resident microglia and other immune cells are responsible for the enhanced burden of Aβ and subsequently mediate tau phosphorylation and ultimately disease progression. Similarly, neuroinflammation also plays a key role in the progression of PD. Several studies have demonstrated an interplay between neuroinflammation and pathogenic mechanisms of PD. The dynamic proinflammation stage guides the accumulation of α-synuclein and neurodegenerative progression. Besides, few viruses may have a role as stimulators and generate a cross-autoimmune response for α-synuclein. Hence, neurological complications in patients suffering from COVID-19 cannot be ruled out. In this review article, our primary focus is on discussing the neuroinvasive effect of the SARS-CoV-2 virus, its impact on the blood-brain barrier, and ultimately its impact on the people affected with neurodegenerative disorders such as Parkinson's and Alzheimer's.

1. Introduction

The SARS-CoV-2, coronavirus disease-2019 (COVID-19), pandemic has made the daily lives of people all across the world challenging with a significant family burden. Millions of people have lost their lives, and the number is increasing day by day [1]. Both economy and health have been seriously affected as a result of this pandemic [2, 3]. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the strain of coronavirus that has caused this COVID-19 pandemic [4–6]. This pandemic's global lockdown has resulted in severe psychological and mental stress among children and adolescents [7, 8]. Mental and psychological health is very important to maintain homeostasis in the central nervous system (CNS) [9]. This pandemic has led to a disturbance in the homeostasis of the CNS by affecting the cytokine concentrations and may be responsible for the initiation of many neurodegenerative diseases [10, 11]. The blood-brain barrier (BBB) is mainly responsible for preventing harmful substances from entering the CNS [12–14]. COVID-19 also causes harmful changes in the permeability of the BBB [15]. The spike in SARS-CoV-2 is mainly responsible for the alteration in the permeability of the human BBB, as suggested by 2D static and 3D microfluidic *in vitro* models [16]. Consequently, altered permeability and integrity cause the BBB to be more prone to developing different neurological and neuroinflammatory diseases in the CNS [13, 14]. Parkinson's disease (PD) and Alzheimer's disease (AD) are the two most common neurodegenerative diseases found all over the world that share almost similar mechanisms of progression [17, 18].

The research studies focusing on delineating the relationship between coronavirus disease and neurodegenerative disorders have provided conflicting results. Thus, a clear causal link could not be established. However, several mechanisms have been proposed by which COVID-19 might contribute to the development of PD and AD, such as the cytokine storm associated with severe COVID-19 which might trigger neuroinflammation and eventually lead to neurodegeneration. Several studies have found significantly high levels of inflammatory cytokines such as TNF-α and IL-6 in COVID-19 patients [19]. Similarly, earlier studies on Parkinson's disease have correlated high plasma IL-6 concentration with an increased risk of PD development [20]. Therefore, an increased possibility of neuroinflammation due to cytokine storm associated with COVID-19 cannot be ruled out.

The meta-analysis of the Parkinson's disease gut microbiome suggested alterations linked to intestinal inflammation [21]. As revealed by various studies, the development of PD is also linked to the gut microbiome and its dysbiosis [21, 22]. In a study, SARS-CoV-2 imbalanced the gut microbiome (dysbiosis) and intestinal inflammation indicated by elevated fecal calprotectin in COVID. Similarly, cognitive decline was observed during acute covid infection, but cognitive impairment was also observed post COVID [23, 24].

Medicinal plants like *Mucuna pruriens*, *Withania somnifera*, and *Tinospora cordifolia* and their active components like ursolic acid and chlorogenic acid, which are very beneficial for PD and AD, also show significant therapeutic activity in the management of COVID-19 patients. Recent data suggest that these plants and their bioactive components play a very crucial role in the clinical and preclinical studies for the management of COVID-19 patients [25–31]. Researchers are also working on the derivatives of the medicinal plants and their bioactive components to investigate their possible interaction among COVID-19 patients and in animal models. In this review, we present the viewpoint on the effect of COVID-19 on permeability of the blood-brain barrier. Then, we discussed the effect of this pandemic on PD and AD neurotropism, neuropathology, and neuroinflammation in a sequential manner. We have also discussed the infection and mortality rate among AD- and PD-related dementia.

2. Effect of COVID-19 on the Permeability of the Blood-Brain Barrier

The microvascular endothelial cells can form a blood-brain barrier and cover the central nervous system from heterogenic microorganisms and toxins present in the blood [32]. These cells express tight-junction proteins that prevent adjacent cell movement [33]. However, several identified viruses that can disrupt the blood-brain barrier are the Zika virus, West Nile virus, and the arbovirus [34, 35]. Several experimental procedures conducted using *in vivo* and *in vitro* BBB models have determined that these viruses can replicate in the brain's microvascular endothelial cells and stimulate tight-junction protein degradation and downregulation leading to BBB disruption [34, 36]. Likewise, Bleau et al. demonstrated the penetration ability of the coronaviruses in the CNS via the extreme hepatotropic mouse hepatitis type 3 virus and a weakly typed A59 hepatotropic hepatitis mouse. The infected mice type 3 have brain invasion related to increased BBB permeability.

The impact was linked with an induced level of zona occludens protein 1 expression, occluding, and VE-cadherin [37]. Considering the molecular relatedness of coronaviruses in their replication mode to infect the brain's microvascular endothelial cells, it was determined that other kinds of coronaviruses can also use similar activity pathways to do the same [38]. Significantly, CoV's existence has been recognized in the microvascular endothelial cells (brain) of the frontal lobe tissues, obtained from the postmortem examination of COVID-19 patients [39]. Also, a viral particle and CoV genomic content have been identified in the brain's neuronal cytoplasm, particularly in the region of the cortex and hypothalamus [40]. Evidence suggests that SARS-CoV-2 can cross the BBB accompanied by basement membrane disruption [41], and in another study, the S1 subunit of the spike protein of severe acute respiratory syndrome coronavirus 2 was found to cross the blood-brain barrier by adsorptive transcytosis and angiotensin-converting enzyme 2 was involved in its brain and lung uptake [42]. Hence, the infection via various respiratory viruses and SARS-CoV-2 may disturb the BBB integrity via distinct mechanisms. The viral particles can directly cause cellular stress associated with cytotoxic effects, which may lead to the disruption of the infected cell; for example, coronaviruses can stimulate cell apoptosis [43]. However, the activation of endothelial cells of the inflammatory reactions can increase protease expression, such as matrix metalloproteinase, promoting tight-junction protein degradation [44]. This evidence suggests that

inflammatory responses probably play a significant role in stimulating BBB disruption.

The coronaviruses can be a source of BBB damage via the activation of inflammatory reactions linked with the dysregulation of these mechanisms [45]. Likewise, the stimulation of microvascular endothelial cells is associated with changes in the permeability of the BBB. For example, in the physiological state, the immune cell displacement into the CNS is precisely managed through processes conducted at the BBB stage [46]. Interestingly, the circulation of immune cells to the CNS is deficient and restricted to the particular immune subsets (innate and adaptive immune cell subsets), such as antigen-presenting cells, like dendritic cells, macrophages, and lymphocytes, which can manage the immune control in the CNS [47]. At the time of viral infection, the level of immune cell migration is increased. Therefore, it can be concluded that the enhanced CNS inflammatory reaction and systemic inflammation resulting from a viral infection may trigger the integrity of BBB and further progress into neurodegenerative disorders. These mechanisms may be supported via a histopathologic examination of the brain tissue in people with a SARS-CoV condition, where $CD3^+$ T lymphocyte and $CD68^+$ monocyte/macrophage pathological infiltration was found in the mesenchymal cells of the human brain [48].

Moreover, the process of infiltration is associated with the interaction of $\beta 1$ and $\beta 2$ integrins, which have an expression on leukocytes and their ligands, such as vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecules (ICAM-1, ICAM-2) [49]. The ICAM and VCAM-1 are present on the microendothelial cell surfaces that stimulate the extravasation over the BBB; inflammatory responses have been cited. The activation and infection of microvascular endothelial cells via the typical neurotrophic viruses may increase the endothelial adhesion molecules [36]. This mechanism promotes viral infected immune cell trafficking to the CNS via a "Trojan horse" [50]. However, at the time of viral replication in the host cells, the impairment may be created due to SARS-CoV-2 (cytoplasmic virus), which stimulates the discharge of damage-associated molecular patterns (DAMPs) [51]. Damage-associated molecular patterns (DAMPs) are endogenous compounds released through impaired cells that interact with a pattern-recognition receptor (PRR), which stimulates the endothelial cells, neighboring epithelial cells like macrophages, and a high inflammatory condition. Once the viral-host cell interaction has been accomplished, the viral protein and genomes can also be identified through PPRs that induce an immune cell response [52].

Distinct PPRs can identify SARS-CoV-2, such as Toll-like receptors (TLRs), expressed in various cell lines along with macrophages, endothelial cells, and dendritic cells. The TLRs (TLR3, TLR7, TLR8, and TLR9) stimulate different activation pathways that provide the proinflammatory cytokines and other antiviral drug molecules in controlling the infection. Therefore, this reaction can be exacerbated and dysregulate cytokine production [53]. The other PPRs and NOD-like receptors (NLR) may induce the inflammasome complex and stimulate the activation state in a few cells: the epithelial, macrophage, and microvascular endothelial cells may lead to the overproduction of interleukin (IL)-1β and IL-18 [54]. However, these processes need to be examined in detail for novel coronaviruses. Evidence suggests that viral RNA can stimulate the typical melanoma differentiation-associated gene 5 (antiviral state) and retinoic acid-inducible gene 1, into which interferons (IFN) release IFN types I and III. Interferons are important molecules that help prevent viral infection by stopping viral replication [55].

COVID-19 patients having high IFN levels, mainly IFN I, have been recognized; these molecules avoid replicating viruses from the adjacent cells. They impact the viral infection, that is, the activation of interferon-stimulated gene expression, cytokine production, and induction of immunoreactive cells (monocyte, macrophages, and neutrophils) [56]. Likewise, the other coronaviruses may have a similar pattern of responses. Moreover, COVID-19 patients have an enhanced level of chemokines and cytokines, such as interleukin-1 receptor antagonist (IL-1RA), IL-2, IL-6, IL-7, IL-8, IL-9, IL-10, IFN-γ, TNF-α, and the granulocyte-macrophage colony-stimulating factor [57]. Interestingly, an increased level of IL-6 is associated with a poor prognosis for SARS-CoV-2 [58]. The overproduction and imbalanced amount of the existing molecules may be defined as a cytokine storm, significantly creating BBB disruption. The cytokine storm stimulates the induction of macrophages, neutrophils, monocytes, and platelets; few of these molecules may be associated with coagulation and complement systems and involved in pathogenic inflammatory responses. Few chemokines might attract some innate immune response cells: natural killer cells, monocytes, T cells, and dendritic cells. It activates the production of other kinds of cytokines, such as granulocyte colony-stimulating factor, monocyte chemotactic protein-1, macrophage inflammatory protein 1-α, monocyte chemotactic protein-1, and IL-10, which help in the recruitment of monocytes and lymphocytes and initialize the humoral cell response [59]. These processes can contribute to the severity of the neurological manifestation of COVID-19 infection in the BBB. SARS-CoV-2-infected patients with overinflammatory alteration may lead to excessive thrombin production that prevents fibrinolysis and induces complement pathways. This reaction leads to microthrombin deposition, thrombin inflammatory reaction, and microvascular dysfunction, related to the impaired BBB [60]. The undetermined processes that can aid the damage in the BBB are adaptive immune reactions. The antibody (Ab) generation across COVID-19 can react with a few brain microvascular endothelial cells and be disrupted via activation of the complement system (C3 and C4 proteins). Besides, the AB-based enrichment phenomenon may enhance the contribution and infection to the damage. This mechanism has been extensively used in Zika and Dengue viral infections, where the antibodies produced in the primary exposure cross-react with the second exposure and increase the rate of disease rather than neutralizing it [61].

2.1. COVID-19 and Parkinson's Disease. The current pandemic COVID-19 caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has resulted in a severe health crisis worldwide and led to a global virtual deadlock [62]. Even though significant preventive steps have been taken as a complete lockdown of economic and social life, social distancing has become a severe health problem for people with Parkinson's disease (PD) [63, 64]. Increased evidence determines

the oxidative stress involvement that develops NF-κB in COVID-19 [65].

The effect of SARS-CoV-2 infection on PD patients has been extensively discussed by several researchers worldwide. The potential impact of SARS-CoV-2 illness on PD patients is still unknown; thus, there is a shortage of scientific studies. Nevertheless, the pandemic situation has resulted in an emphasis on psychological factors. Considering the standard features, which show an essential role in PD, oxidative stress may worsen PD progression in COVID-19 patients and vice versa. Therefore, the pathophysiology of PD may put the individuals at a high risk of severe stress and lead to one of the several unseen miseries of the COVID-19 epidemic. The enhanced stress during a pandemic may have various adverse short- and long-term effects on PD individuals [66].

On the one hand, enhanced psychological stress can disrupt several motor functions, such as dyskinesias and tremors, if it decreases the potency of the dopaminergic drugs [67, 68]. On the other hand, enhanced stress may also reveal the potency of the hypokinetic rigid syndrome, probably through a decreased remunerative system [69]. It could lead to an improved PD diagnosis level throughout the epidemic as various animal trials reveal that prolonged exposure to severe stress may lead to loss of dopaminergic cells in response to a toxin [70–73]. Besides, this pandemic has resulted in reduced physical activities due to complete lockdown and social distancing; as a result, people have not been able to do physical work [74]. Current studies provide evidence that physical exercises may reduce the clinical features of PD progression [75, 76]. A more rigorous workout was linked with a better outcome than a moderately intense workout [77]. The fundamental mechanisms are still unknown, but it is beneficial to note that reduced physical activity during the COVID-19 epidemic may lead to worse motor symptoms in PD individuals. It has also contributed to enhanced psychological stress, another exasperating indication of PD [78]. Likewise, it also leads to severe non-motor issues such as constipation or insomnia, because of reduced physical activeness [79]. Simultaneous evidence suggests that the COVID-19 pandemic will severely impact individuals having neurodegenerative disorders as they have enhanced exposure to negative consequences of decreased physical activity and increased stress. Moreover, both melancholies can aggravate their motor as well as non-motor manifestations. These pandemic situations work as stressors aligned in time for such individuals [65, 80, 81]. It provides novel events for health professionals to analyze how the current pandemic directed PD progression in the present longitudinal discipline by taking biological biomarkers' assets. It also allows healthcare professionals to test which factors can protect the patients from the adverse impact of this catastrophe, by adding PD resilience. In essence, the current detrimental situations will also bring long-term positive consequences for several individuals with PD globally.

The SARS-CoV-2 infection causes significant concern for people who have already suffered from PD. Chaudhry et al. revealed the evidence of the impact of COVID-19 on PD patients [65]. They discovered that both COVID-19 infection and 6-hydroxydopamine (6-OHDA)-induced toxicity prompted caspase-2, 3, and 8 stimulation through the NF-κB pathway culminating in the death of dopamine-containing neurons (dDCNs) [65]. Their findings prove that SARS-CoV-2 and PD might have common inflammatory pathways beneath the oxidative stress, as they have the essential indulgence of NF-κB. Although it has a clear correlation with oxidative stress biomarkers and the chronicity of viral infections, such as hepatitis C, the SARS-CoV's clinical data is limited [82]. Therefore, in the presymptomatic situation, several facts suggest that surplus reactive oxygen species (ROS) and impoverished antioxidant systems play a significant role in the pathologic process of COVID-19 infection and lung infection progression [83].

The animal trial of the COVID-19 disease has shown an increased ROS level and disturbed antioxidant defensive mechanisms during the SARS-CoV-2 infection [38]. The healthcare professionals suggested that the onset of chronic lung injury in a COVID-19 condition may depend on the stimulating oxidative-stress machinery associated with innate immunity, which may induce transcription factor NF-κB, resulting in aggravated proinflammatory host behavior [84, 85].

The coronavirus has been shown to spur caspase-based apoptosis required for the viral replication process [86]. According to various literature surveys, the PI3K/Akt signaling pathway induced via a collection of viruses helps in establishing a chronic infection, by delaying apoptosis in virus-infected cells, thus helping to complete the virus life cycle [87]. Accordingly, PI3K/Akt signaling may be a potential target against this COVID-19 pandemic and should be seriously considered. In addition, the role of oxidative stress, JNK, Akt, p38, and NF-κB signal pathways has also been proven in influenza A virus (IAV)-induced acute lung injury; thus, these can also act as possible biomarkers in COVID-19-induced lung injuries [88]. The oxidative stress, such as IAV-mediated TLR4 and NF-κB signaling pathways, may trigger SARS-CoV, increasing the host inflammatory reaction and leading to acute lung injury. Several studies have delineated the role of oxidative stress-mediated TLR4-TRIF-TRAF6 signaling pathways as an inflammatory response pathologic pathway that induces the implacability of chronic lung injury. The oxidative phospholipid is generated through the overproduction of lung macrophage-stimulated cytokines and leads to lung injury by TLR4-TRIF [89].

These oxidative phospholipids have also been recognized in animal and human lungs infected with the COVID-19 virus [90]. As is evident from these studies, a research focus should be kept on oxidative stress, as it might be the connecting link between the COVID-19 pandemic and neurodegenerative disorders. Similarly, in another study, upregulation of mitochondrial genes was also determined. These genes responded to oxidative stress, in the isolated peripheral blood mononuclear cells of recovered SARS-CoV patients [91]. Some of the genes included, such as *FOS*, *FTH1*, and *PRDX1*, have precise oxidative stress and provide remarkable ascent. The results suggest evidence of an association between inflammation, oxidative stress, and the pathogenic process of the COVID-19 infection. The virus infection has always been treated as a predisposing factor for developing PD and long-term neuron loss.

Several studies have shown the impact of dysfunctional mitochondria, mitochondrial gene upregulation, and the genes

that respond to oxidative stress (an essential feature of neurodegenerative disorders) in cells recovered from COVID-19 patients. A COVID-19 study revealed the production of proinflammatory cytokines (CXCL-8, IL-6, CCL20, CCL3, CCL4, and IL-12) by dysfunctional mitochondria [41, 45, 92]. Among these proinflammatory cytokines, chemo-attractants, such as CXCL-8, promote neutrophil infiltration, thus contributing to ROS generation and protease activation that further contribute to damaged mitochondria [93].

Similarly, the consideration has kept on COVID-19 infection due to induction of a marked fundamental proinflammatory reaction. An eventual case-control analysis revealed that males with a high plasma IL-6 concentration have an increased risk of developing PD [94]. It is already known that SARS-CoV-2 infection occurs through TMPRSS2 and ACE2 [95]. Likewise, when compared with untreated rats, the TMPRSS2 expression was upregulated in rats treated with 6-hydroxydopamine, which is extensively used as a tool to model PD [96]. This examining procedure demonstrates that genes may codify, as these proteins are differentially regulated and crucial in disease development. Interestingly, in the past, coronaviruses have been associated with PD individuals. Significantly, intravenous antibodies for coronavirus types, MHV-A59, and MHV-JHM are increased in PD individuals, compared to people with other neurological disorders [97].

However, the influential role of viral infection in PD patients is still not known. Hence, a long investigating period of post-COVID-19 patients is required, but some characteristics of the chronic phase of this disease are very distinct. For example, a prior detection of gastrointestinal problems and anosmia and a high predominance of reduced recognition are identified in more chronic phases [98]. It is believed that the neurotropic pattern of the COVID virus is related to its activity, which produces respiratory symptoms, with approximately 89% of the people in intensive care units not being able to breathe voluntarily. Apparently, this is caused by a central dysfunction [99, 100]. However, gastrointestinal problems and hyposmia are the common non-motor symptoms in PD individuals during the prodromal stage; this is when neurodegeneration has been initiated [101]. Based on Braak's hypothesis, these indications suggest that it is the beginning phase of PD progression, which entails α-synuclein deposition in the anterior olfactory nucleus region and dorsal motor region. Most coronaviruses share similar viral anatomy, infection mechanism, and pathogenic processes. The viral penetration in the host cells is induced through dipeptidyl peptidase 4 (DPP4) and angiotensin-converting enzyme 2 (ACE2) [100]. Furthermore, human SARS-CoV-2 infection can cause chronic neurological difficulty with refractory status epilepticus, seizures, encephalomyelitis, encephalitis, leukoencephalopathy, cerebellitis, Guillain-Barré syndrome, and severe neuromyopathy. Evidence suggests that neurotropism and neuroinvasion are common manifestations of coronaviruses [102].

Like other coronaviruses, SARS-CoV-2 can infect cells via associating its spike proteins and the ACE2 receptor. For interacting like this, the spike protein must be cleaved through the transmembrane serine protease enzyme. The cells expressing both TMPRSS2 and ACE2 receptors are more affected by the SARS-CoV-2 infection [103]. Chen et al. revealed the expression of ACE2 by examining the information obtained from the brain transcriptome database. These receptors are overexpressed in SN and brain ventricles, scattered in excitatory and preventive neurons, and present in oligodendrocytes and astrocytes [104]. Besides, there is no strong evidence for the co-expressive mechanism of ACE2/TMPRSS2 in the brain region. Brann et al. revealed that the non-neurological sensory olfactory epithelium cells such as Bowman's gland cells, horizontal basal cells, and sustentacular cells express both TMPRSS2 and ACE2 receptors [105].

Hence, these cells may be the primary ones to be affected by SARS-CoV-2 infection. However, these non-neuronal cells can support the developed olfactory sensory neurons of the sensory epithelium. Supportive cells can be influenced by HCoV, sequentially transmitting the virus to the olfactory sensory neurons via axonal transport after those invading neurons in the olfactory bulb and then spreading to the CNS, where they cause inflammation [106]. The process of viral invasion by the olfactory bulb to the brain has already been reported to show a role in neurodegenerative disorder. It may trigger pathological aggregated protein transmission in a prion-like manner [107]. In the case of a retrospective study of COVID-19-infected individuals hospitalized in Wuhan, China, the researchers have found that out of 214 patients, 78 may have neurologic symptoms, defective memory, and cerebrovascular disorder occurrence in the more acute cases [98]. Dysgeusia and anosmia are also commonly reported in COVID-19 patients [108]. These investigations support the speculation that SARS-CoVs like coronaviruses can infect human brain cells.

2.2. Trigger for Future Neurodegeneration. The substantia nigra and cortex are the brain regions having a high risk of HCoV penetration through an ACE2 receptor. Often, this is the exact correlation in a neurodegenerative disorder and must not be taken as only a coincidence [39]. Lippi et al. have hypothesized the significant role of coronaviruses in the future development of neurological disorders, particularly in PD [109]. The author has hypothesized a new model for the neurodegenerative disease, as coronaviruses can promote α-synuclein accumulation, which is a significant element of Lewy bodies in the human brain, as has been suggested (Figure 1). The author focused on cellular pathways infected through the SARS-CoV-2 virus (proteostasis) to explain the phenomenon [109]. It is precise in providing efficient equilibrium and activates stress-induced mechanisms, which appear to be the same targets in neurodegeneration. Evidence has suggested that the infection of H1N1 of dopaminergic cells may result in the formation of α-synuclein aggregates and not of any other distinct protein recommended to focus on this mechanism positively. Therefore, *in vitro* models that determine the triggering changes in proteostasis may lead to the persistence of toxic impenetrable proteins [110]. These findings demonstrate that coronavirus infection may alter the PD neurodegeneration through progressive aging in brain cells or tissues (Figure 1).

2.3. The Impact of Coronavirus. SARS-CoV-2 has been recognized in brain autopsies and extracted from the cerebrospinal fluid of COVID-19-infected patients [111–113]. Currently,

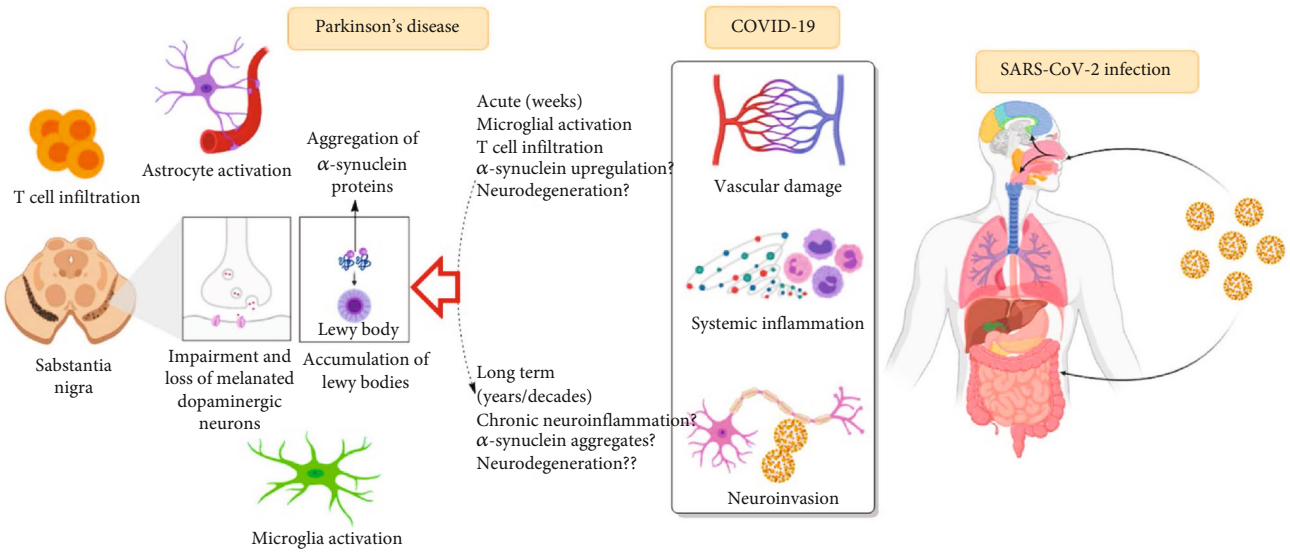

Figure 1: Schematic illustration of how COVID-19 infection impacts the pathology of Parkinson's disease. The COVID-19 infection might affect the brain in the following ways: through vascular damage, through systemic inflammation, and via direct neuroinvasion. These changes may lead to development of acute Parkinsonism due to microglial activation, T cell infiltration, and resulting in neurodegeneration due to α-synuclein upregulation. In addition to the development of acute Parkinsonism, COVID-19 may also elevate the PD risk in the long term due to sustained chronic inflammation and aggregation of α-synuclein.

there are at least two well-developed facts about the association between neurodegenerative disorder and COVID-19, particularly for PD. On the one hand, there is a presence of antibodies against HCoV in the cerebrospinal fluid of patients who have already suffered from PD [97]. On the other hand, the second evidence suggests the penetration of the virus in the brain via the nasal cavity, which causes anosmia and hyposmia [114]. It has been reported that hyposmia is a common premotor characteristic of PD and the olfactory bulb is a chosen target for the deposition of α-synuclein pathogenicity [115]. The role of neurotropic virus pathogenicity in a neurodegenerative disorder can selectively target the basal ganglionic cells. Furthermore, few researchers also demonstrate that the longevity of the viruses in the nervous system infected cells for a longer time.

2.4. Modification of the Neurological Disease and Care Strategies. Donadio and coworkers state that the primary community depends on case-control analysis that describes the impact of symptomatic SARS-CoV infection on the motor or non-motor manifestation. In their group, 141 people who already had PD, in Lombardy, where there were 12 SARS-CoV-2-infected cases, had a mean age and course of disease duration similar to the controls [116]. The PD individuals may experience substantial damage to both motor and non-motor manifestations (mainly fatigue and urinary disorder) during the period of mild-to-tolerable COVID-19 infection, explicitly of aging and disease duration (Figure 1). Commonly, fatigue is reported as a dominant non-motor symptom during the COVID-19 infection in PD cases [114].

Comparatively, cognitive features were imperceptibly indulged; hence, none of them experienced autonomic damage. Medical disturbance can be observed in PD patients. It can be explained via infection-based mechanisms and damaged pharmacokinetics of dopaminergic treatment, requiring therapy adjustments in one-third of the cases [116]. The SARS-CoV-2 infection in PD patients cannot be limited to motor symptoms due to the incidental impact of the more prolonged exposure under lockdown, causing self-isolation, increased stress, and anxiety that should be considered. A current Iranian cross-sectional, case-control study evaluated the anxiety level among PD patients as compared to caretakers and familiar individuals. The occurrence of anxiety has been investigated in PD patient groups, who followed through with their caregivers [117]. Evidence suggests that the researchers have also demonstrated a strong association between severe anxiety in PD individuals and fear of having COVID-19 infection [117]. However, the other significant consequences of COVID-19 conditions are concerns about the considerable reduction in health activity.

The impact of reduced physical activity must not be neglected, as it may attenuate the progression of the medical symptoms in PD individuals. This evidence has been investigated by Shalash et al., who have determined the effects of COVID-19 on mental health, physical activeness, and quality of life in PD individuals. PD individuals harm their health care, cognitive ability, and interest in social life compared to PD controls [118]. Besides, Prasad et al. have demonstrated the implications and perceptions of SARS-CoV-2 infection in around 100 Indian PD individuals and their caretakers, which proved to have damaged the mental ability, physical wellness, and quality of life of these individuals [119]. This analysis may also shed light on the significance of managing these conditions preceding PD patients' care, specifically via taking telemedicine. It is not surprising to see PD individuals having impaired mental health during the lockdown. The immediate changes commonly need a flexible adjustment to new assets; these situations are strongly associated with the

regular dopaminergic activity. Likewise, PD patients have experienced cognitive inability resulting in nigrostriatal dopaminergic depletion, which makes up the pathophysiologic substrate of PD [120]. To complicate this precarious situation of COVID-19 coexistence, there is less access to routine visits to hospitals to preserve PD patients from infection. Therefore, in the new era, taking care of patients has significantly changed, the most challenging concept for clinicians, as it has reinvented the work processes such as telemedicine, including digital visits, simple telephone consultations, and mail or text messages. As we advance in this direction, Goetz et al. accessed the validity and reliability of virtual-based MDS Unified Parkinson's Disease Rating Scale examination for comparing to official work. The E-Rehabilitation scheme can include virtual improvement programs as alternative strategies to deliver rehabilitation at a considerable level, which must be appreciated [121]. Although the telemedicine strategies are not well established universally, superior to the quality of regular in-person visits, evidence suggests that it associates with comparable results and can offer an excellent service and efficiency to PD patients. In short, the COVID-19 infection can complicate the PD clinical course, resulting from the severity of motor and non-motor manifestations, increasing stress and anxiety, and affecting the quality of life and mental wellness. In the era of COVID-19, telemedicine has played a significant role. Finally, various longitudinal and cross-sectional studies are required to find a causal association between clinical and severity of the SARS-CoV-2 infection, sequential inflammatory reactions having cytokine levels, and via identification in the CSF of PD individuals.

2.5. COVID-19 and Alzheimer's Disease. In our senior community, dementia is associated with a pandemic situation. However, the administration in the COVID-19 pandemic situation may also bring us more concerns [122]. The first concern is combining two potent risk factors, aging and dementia, for fatalities in COVID-19-infected patients. Likewise, the second concern is about the effect of associated coronavirus outbreak and dementia and the consequences of social distancing on the mental health of these weak patients, which needed a better prognosis [123]. Table 1 shows the global burden and mortality of Alzheimer's disease and other dementias, population, age, structure, and COVID-19. Results are classified according to (A) global burden of disease super-region classification and (B) World Bank income level-based classification. Similarly, Figure 2 exhibits a scatter plot between Alzheimer's disease and other dementia disability-adjusted life year (DALY) rates. It shows the total number of COVID-19 cases per million and the total number of COVID-19 deaths per million.

It is well understood that aged people are at high risk for fatalities after COVID-19 infection [124]. Very little data is available on older individuals infected with COVID-19, and limited reports have concentrated on above 80 individuals [125]. All of this has been demonstrated among older individuals having no dementia. Covino et al. provided results among dementia individuals having COVID-19 infection. Their results from this retrospect, single-centered, and observational examination in a Central Italy referral center for COVID-19 determine that the death risk does not depend on age [126].

In contrast, acute dementia itself might be a prominent risk factor among these individuals. Based on this evidence, Bianchetti et al. have determined the predominance, clinical representation, and results of individuals with dementia among those admitted in the hospitals with a COVID-19 condition [127]. Data obtained from 627 patients hospitalized in the Acute Hospital in Brescia province, Northern Italy, were examined retrospectively. Compared to people without dementia, patients with dementia have shown a high mortality rate of up to 40% [127]. Combining this investigation on dementia, mainly with the late onset of disease, may significantly risk fatality in corona patients. The neurological disease modification and care scheme are more vulnerable in AD patients due to neurological damage and a high neuropsychiatric symptomatology rate. This has been specifically proven at the stage of the compassionate outbreak that is SARS-CoV-2 infectious condition. Around 80% of AD individuals may show at least one or two neuropsychiatric manifestations throughout the infection. These factors are flexible and appear more progressive in AD, even if there may be early symptoms of prodromal stages. The neuropsychiatric manifestations of the AD medical spectrum include anxiety, depression, agitation, and emerging apathy, being exposed as frequent damages [128]. However, it is also considered being the effect of factors such as the enhanced rate of AD progression, altering meditational responses and reducing the patients' life quality. During the COVID-19 outbreak, for the first time, Boutoleau-Bretonniere et al. provided evidence of the impact of detention on the neuropsychiatric manifestation among AD individuals. The results suggest that only 30% of AD patients displayed neuropsychiatric alterations during the period of confinement. The confining period is significantly associated with their symptom severity and their caretaker's afflictions. The restrictions can worsen neuropsychiatric symptoms in AD patients, whereas no such manifestations were stimulated in people having more conserved cognition [129].

Other authors determined the severity of neuropsychiatric manifestations of agitation and impaired motor function, as the most affected indication among AD and MCI, during the complete lockdown during this COVID pandemic [130, 131]. The classic symptoms of AD patients with COVID-19 infection such as dyspnea, fever, and cough were less usual, while they specifically experienced drowsiness and diarrhea. Finally, that delirium can cause hypoxia, which is a prominent feature of COVID-19 infection and it can complicate the representation of dementia, although it is needed for dementia support and care [127]. However, these investigations can confirm the medical spectrum's anticipated risk, mainly the neuropsychiatric manifestations during the epidemic in AD individuals.

3. Trigger for Future Neurodegeneration

Despite having a long-term association between HCoV and its impact on the brain, it is not well understood; its efficient role in future neurodegeneration can be significant in AD research. Chronic results after the infection of COVID-19, often linked

Table 1: The regional and national data regarding COVID-19 confirmed cases, deaths, and the burden of Alzheimer's disease and other dementias (data accessed from World Bank [143], Ritchie et al. [144], and IHME [145]).

(a) Global burden of disease study super regions

	COVID-19		Alzheimer's disease and other dementias		Population age structure		
Super regions	Cases per million (95% CI)	Deaths per million (95% CI)	DALY per 100,000	Death per 100,000	Age, median (IQR)	Population aged ≥65 years (%)	Population aged ≥70 years (%)
Central Europe, Eastern Europe, and Central Asia region	16866.33 (16613.94–17118.72)	287.84 (254.59–321.09)	501.02 (230.07–1078.94)	31.87 (7.76–85.40)	40.75 (36.65–43.15)	13.49	8.81
High-income region	24477.67 (24174.80–24780.54)	562.46 (515.99–608.93)	800.59 (372.40–1656.48)	59.25 (15.49–147.57)	41.60 (37.9–43.3)	18.31	12.24
Latin America and Caribbean region	18662.06 (18396.82–18927.31)	670.30 (619.57–721.03)	328.89 (140.60–717.62)	22.15 (5.59–56.73)	29.35 (26.9–32.85)	7.61	4.62
North Africa and Middle East region	7188.25 (7022.67–7353.82)	186.36 (159.60–213.11)	198.46 (87.40–439.09)	11.58 (2.83–30.52)	30.7 (23.2–32.4)	5.38	3.21
South Asia region	5810.05 (5661.09–5959.01)	84.72 (66.68–102.76)	145.00 (58.78–344.85)	8.76 (2.09–24.21)	26.25 (23.5–28.2)	5.65	3.27
Southeast Asia, East Asia, and Oceania region	570.42 (523.62–617.21)	14.82 (7.27–22.36)	359.62 (157.95–795.89)	19.86 (4.76–51.92)	29.3 (25.2–34.1)	9.21	5.19
Sub-Saharan Africa	1250.44 (1181.18–1319.71)	28.46 (18.00–38.92)	92.11 (37.71–210.29)	5.67 (1.41–15.43)	19.25 (17.9–21.75)	3.11	1.78

(b) World Bank classifications

	COVID-19		Alzheimer's disease and other dementias		Population age structure		
Income levels	Cases per million (95% CI)	Deaths per million (95% CI)	DALY per 100,000	Death per 100,000	Age, median (IQR)	Population aged ≥65 years (%)	Population aged ≥70 years (%)
High income	23552.65 (23255.42–23849.89)	512.73 (468.36–557.10)	771.05 (358.61–1596.43)	56.63 (14.78–141.36)	41.1 (36.2–43.2)	17.73	11.79
Upper middle income	6578.98 (6420.53–6737.44)	203.22 (175.29–231.16)	391.57 (173.18–854.67)	22.83 (5.63–59.56)	31.25 (28.8–38.35)	9.77	5.68
Lower middle income	4472.26 (4341.47–4603.04)	72.83 (56.11–89.56)	155.83 (64.15–361.11)	9.35 (2.28–25.53)	25.05 (20.35–28.4)	5.38	3.16
Low income	498.46 (454.71–542.21)	12.46 (5.53–19.36)	98.95 (41.17–227.55)	5.91 (1.47–16.09)	18.75 (17.5–19.4)	3.1	1.8

Global burden and mortality of Alzheimer's disease and other dementias, population age structure, and COVID-19. Results classified according to the (a) global burden of disease super-region classification and (b) World Bank income level based classification.

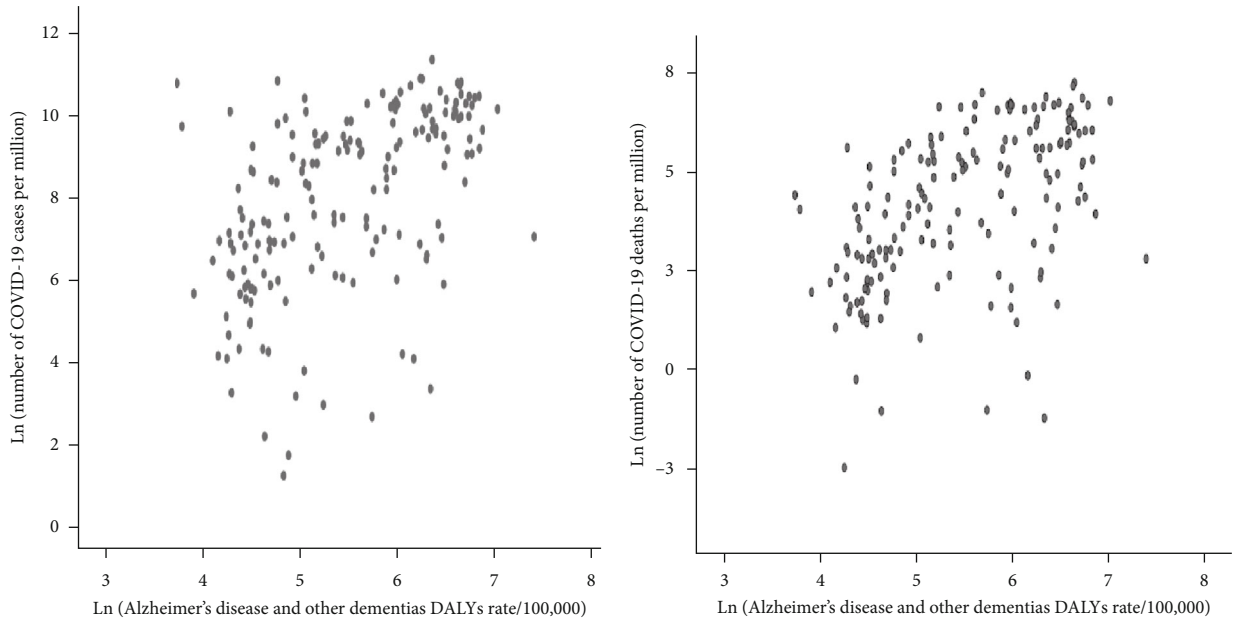

Figure 2: Scatter plot between Alzheimer's disease and other dementia disability-adjusted life year (DALY) rates. (a) The total number of COVID-19 cases per million. (b) The total number of COVID-19 deaths per million.

with the cytokine storm of distinct inflammatory responses, trigger enhanced proinflammatory cytokines (IL-1 and IL-6) [132]. In the case of AD patients, amyloid stimulates type I interferon (IFN) response; hence, it can create a perfect storm [133]. IFNs have an important role in AD pathology, thereby indicating nucleic acid containing amyloid fibrils stimulating the expression of genes responsible for IFN production. Microglia get activated by IFN, which gets associated with the nucleic acid-containing amyloid plaques, thus stimulating a proinflammatory response. IFN further activates complement cascade and leads to synapse degeneration (Figure 3).

It explains one of the questions that arise from presymptomatic individuals with undetected AD, who may see the stimulation of symptoms due to systemic inflammatory responses resulting from viral infection. Besides, several researchers have deliberated that infected people may have a high risk of impaired cognition after recovering from primary SARS-CoV-2 illness. The condition may directly negatively impact immune responses, and it also accelerates the preexisting cognitive deficiency and de novo stimulation of a neurodegenerative disorder. Based on the suggested evidence, it is possible to hypothesize that they may be at a high risk of generating neurodegenerative symptoms, which are unmasked via silent COVID-19 infection in the brain.

3.1. Management of Clinical Trial for the Alzheimer's and Parkinson's Patients Affected by COVID-19. The clinical features of COVID-infected patients are under great study. Globally, there have been 550 million confirmed cases of COVID-19, including 6.3 million deaths, reported as per WHO [134]. As of 11 July 2022, 12,130,881,147 vaccine doses have been administered. Therefore, it is crucial to scrutinize the risk condition of COVID-19-infected patients and their deaths. The lead factors affecting the enhanced transmission rate and medical severity remain unclear. The patients are suffering from other medical conditions such as cardiovascular disease, diabetes, and respiratory pathologies, and the elders especially are highly prone to the virus.

It is reported that age is a crucial risk factor for the SARS-CoVs-2 mortality rate. Age is the predominant feature of neurodegenerative diseases among older people. Thus, it is significant to analyze Alzheimer's disease and Parkinson's disease in SARS-CoV-2 infection [135]. SARS-CoV-2 is hazardous to older individuals with illness and neurodegenerative diseases like Parkinson's disease (PD) and Alzheimer's disease (AD). Currently, there is no proven evidence regarding the enhanced risk of COVID-19 for PD. Besides, severe PD symptoms associated with anxiety are often seen during the pandemic. PD patients, especially those who receive advanced therapy (brain stimulation or levodopa immersion), having a high fatality rate (40%–50%) [99]. In the case of patients with neurodegenerative disease, they are severely hampered by SARS-CoV-2 infection and require admission to the hospital (ICU) [136]. As previously mentioned, PD patients may have respiratory complications due to respiratory muscle bradykinin rigidity and dystonia, which can make life more challenging. Ingestion may also negatively impact these individuals, where saliva may pool in their mouth and cause breathing problems, which can complicate the management of COVID-19 patients. Even though no proven evidence supports the association of NDs with COVID-19 patients, it is examined worldwide as a future retrospective analysis concerning the clinical care strategy for ND patients admitted in the ICU. There are no current clinical guidelines. Therefore, an attempt should be made to ensure PD and AD patients continue to receive anti-PD and anti-AD therapy. For pneumonia patients on supporting previous

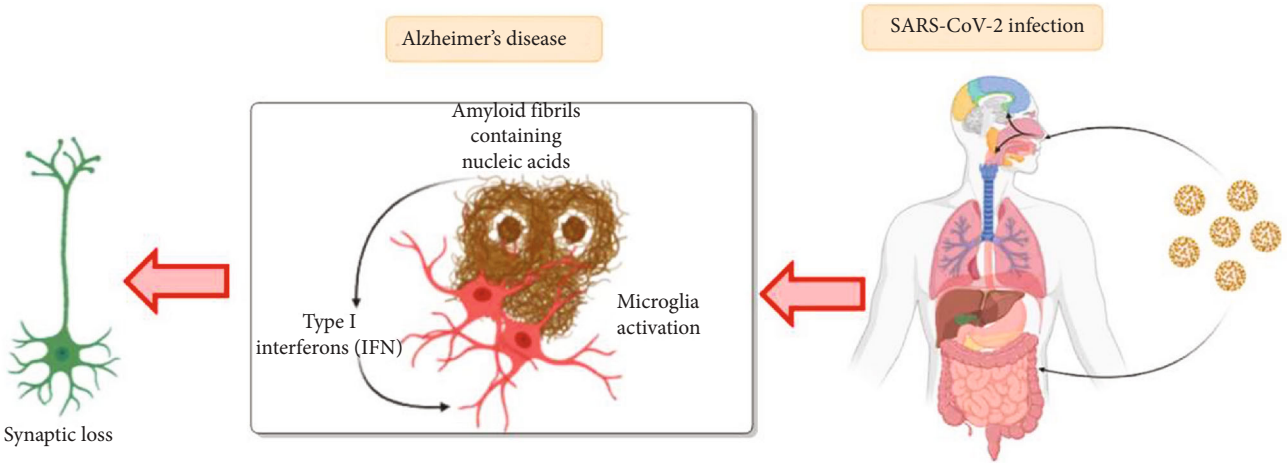

Figure 3: The potential interactions between Alzheimer's disease and SARS-CoV-2 infection. Synaptic loss takes place by inflammation mediated by type I interferon (IFN) after viral infection and in response to nucleic acid containing amyloid fibrils. The progression of IFN response occurs due to entrapment of viral particles by amyloid fibrils.

PD treatment medicine, an equivalent levodopa dose is significant for avoiding muscle bradykinin rigidity and impaired breathing due to dopaminergic withdrawal. The apomorphine pump therapy and levodopa/carbidopa intestinal gel therapy on patients should be continued if it has already been implemented.

In some cases, PD medications should be adapted in hospitals, like severe kinetic individuals having dysplasia symptoms, where oral drug administration is no longer viable [136]. The easiest and cost-effective method to administer levodopa solution to patients is via a nasogastric tube. Initially, the apomorphic pump is not advisable to be used on patients in the ICU; however, they can be examined only if akinesia constitutes chronic effects. Likewise, another medicine that is trending is transdermal rotigotine, considerably less effective than apomorphine/levodopa. Until now, there is no strategic guideline to dictate an alternative therapy for ND patients in hospitals; it may be regulated using the clinical teams' experiences on a case-by-case basis [136].

3.2. Impact of Prolonged Lockdown due to COVID-19 in PD and AD Patients. The SARS-CoV-2 infection can drastically change the usual routine of PD patients through a lockdown and social distancing worldwide. Thus, many AD and PD patients experience a negative impact on their mental health due to prolonged lockdown (Figure 4). There are several reports which describe that the symptoms of AD and PD patients became worse and they have developed depressive and anxious symptoms, affecting their quality of life, compared to matched controls, during the epidemic. However, a detailed explanation is that the PD and AD pathophysiology naturally enhances the risk of severe depressive disorder, as decreasing dopamine levels reduce the stress-coping mechanisms. Currently, the data suggest that older individuals with concurrent medical problems (such as diabetes and hypertension) and patients having dementia are associated with an enhanced risk of COVID-19 and a high mortality rate. Thus, health-related interventions such as lockdown and social distancing have severely affected AD and PD patients' well-being (Figure 4). Social distancing is crucial for preventing COVID-19 infection and also in protecting at-risk populations such as older individuals with high mortality risk from SARS-CoV-2 condition [129]. Likewise, in India, elder individuals account for up to 104 million of the total population. The number of individuals living with Alzheimer's or dementia in India has been evaluated as 5.29 million (prediction for 2020) by the Alzheimer's and Related Disorders Society of India [137]. The effect of the epidemic on the symptoms of older individuals and patients with dementia is of grave concern to the healthcare professionals and dementia support organizations in the country. It is problematic, as stress has short- and long-term negative impacts on AD and PD patients. It has shown that cognitive stress can impair motor symptoms such as dyskinesia, tremor, and gait. Due to stress, the impact of the dopaminergic medications may also reduce, like the effect of levodopa on PD tremors [138].

It is significant to consider that physical workouts can impair PD progression and related stress. Moreover, social distancing during an epidemic significantly decreases mobility and physical activity, leading to a sedentary lifestyle. Accordingly, regular home-based exercise such as online classes for AD and PD patients is substantial for maintaining their overall health quality during COVID-19 [139].

3.3. Role of Aging-Related Neuroinflammation in Parkinson's and Alzheimer's Disease due to COVID-19 Pandemic. In human and animal models, SARS-CoV-2 can enter the brain along the brainstem precisely via the olfactory nerves and without any initial respiratory involvement [140]. The COVID-19 infection develops when the viral glycoprotein spikes get associated with ACE2 receptors. The ACE2 receptors are ubiquitously present in the human brain, not only in the medulla's cardiorespiratory centers but also in the striatum (dopamine neurons) [141]. However, two well-integrated

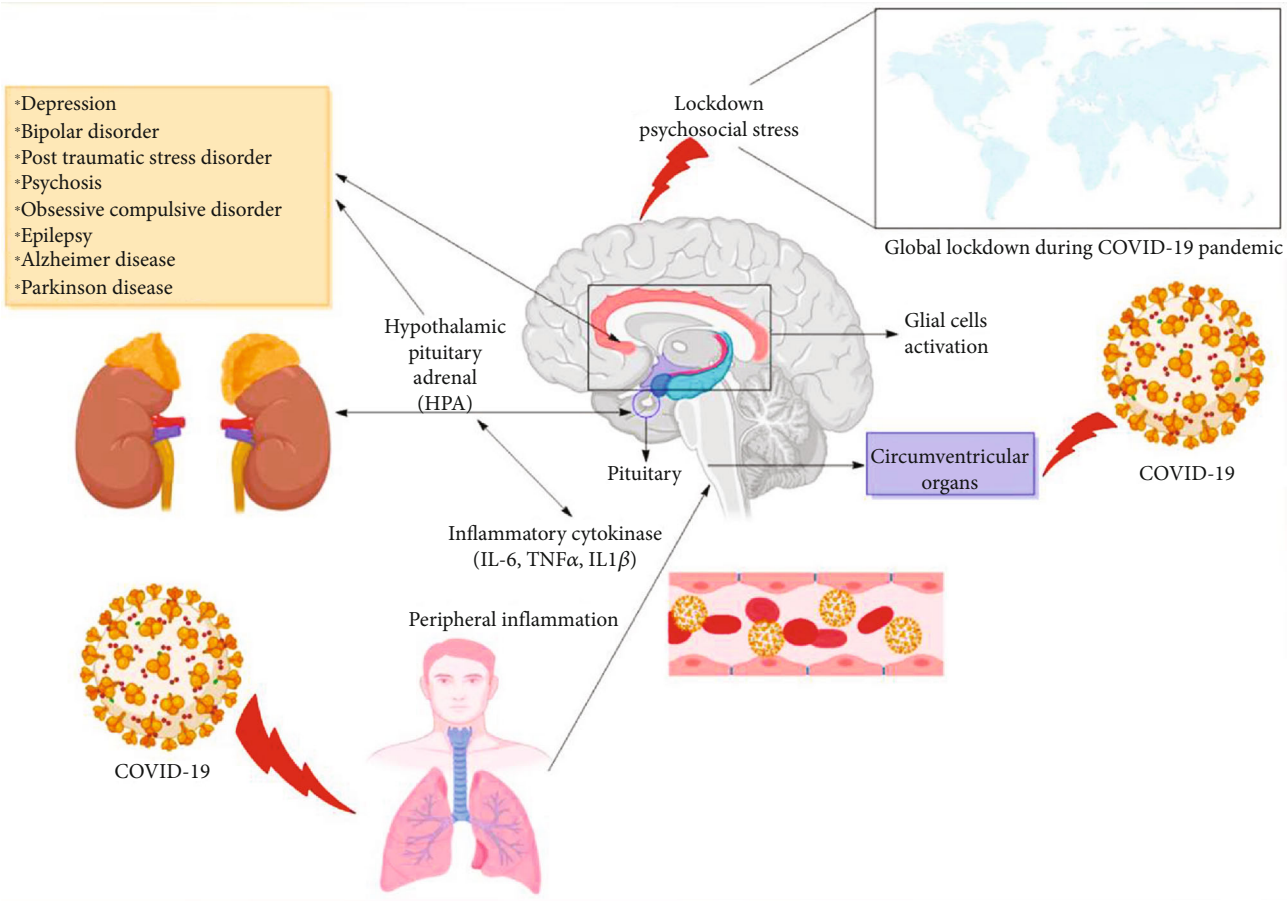

Figure 4: Schematic illustration of how global lockdown and SARS-CoV-2 lead to neuropsychiatric complications such as depression, anxiety, psychosis, and eventually leading towards neurodegenerative disorders. SARS-CoV-2 enters the body and causes systemic and tissue inflammation which compromises the blood-brain barrier (BBB). The combination of both psychosocial stress (caused by global lockdown) and disruption in the integrity of blood-brain barrier (BBB) floods the brain with an increased production and secretion of proinflammatory cytokines resulting in neuroinflammation.

pieces of evidence associate SARS-CoV-2 infection with movement disorder, particularly for PD. The first evidence, from over two decades ago, explains that antibodies exist against coronavirus in the CSF of PD patients [97]. Likewise, the second piece of evidence demonstrates the entrance of coronavirus into the brain via the nasal cavity, causing anosmia or hyposmia. It has been reported that hyposmia is a well-known premotor feature of PD. The olfactory bulb is the optimized target for alpha-synuclein pathologic deposition, which may be more than just fate [142].

3.4. The Relation between SARS-CoV-2 and Glial Cells in Parkinson's and Alzheimer's Disease. The glial cells (astrocyte and microglia) play a vital role in maintaining brain homeostasis and CNS response during neuropsychiatric and neurodegenerative disease conditions. These conditions often indicate a common neuroinflammatory need identified by the activated glial cells, releasing anti-inflammatory and proinflammatory cytokines, free radicals, chemokines, and neurotrophic factors. Evidence suggests that due to a large number of functions performed by the glial cells in the neuroinflammatory reactions, it is expected that the astrocytes and microglia have a significant effect on brain functioning during a viral infection, such as in COVID-19 patients. The extensive participation of glial cells in brain homeostasis management and viral infection expects that dysregulated glial cell function may explicitly and implicitly affect COVID-19 development. Aging is a crucial factor affecting the mortality rate in COVID-19 patients, even though the relation between SARS-CoV-2 and aging is still unclear. The age-based remodeling process is observed in astrocytes and microglia, which may ultimately cause damaged functional characteristics and contribute to the development of NDs (Figures 1 and 4) [142].

Similarly, the glial cells are present in various functional/morphological abnormalities in the older brain, such as increased ROS, decreased phagocytic activity, and mortality, proinflammatory cytokine production, and enhanced DNA mitochondrial impairment. These changes may result in the loss of the normal glial cell neuroprotective behavior and induce neuroprotective and neurodegenerative disorder; therefore, it is not seen in older COVID-19 patients. It is well known that impaired proinflammatory cytokine release may cause enhanced systemic symptoms and neurological damage in COVID-19-infected patients; however, whether glial cell activation is beneficial or harmful to the brain of COVID-19 patients is still a matter of examination.

4. Conclusion

The novel coronavirus infection has disrupted the world and its healthcare system unprecedentedly. This pandemic has also scared the viability and integrity of the present and near-future AD and PD research. However, local consequences will evolve and become distinct based on certain particular factors such as incidence, the linked death cases, the resource availability, and social changes that may control the COVID-19 pandemic. These local changes associated with AD and PD heterogeneity make simple generic suggestion inefficient. Therefore, the awareness about their probable consequences and alleviation approach adopted by the individual may prevent the harmful impact on patients with AD, PD, and their caretakers.

Moreover, further research is needed to demonstrate the detailed HCoV infection mechanism that leads to neuronal impairment, particularly if HCoV can truly exploit the associated pathways of CNS invasion. In that case, it may be quite interesting to analyze where the specific mechanisms combine with the specific neurological disease symptoms; this is yet to be resolved.

Data Availability

No data were used to support this study.

Conflicts of Interest

There is no conflict of interest in this manuscript.

Authors' Contributions

Sachchida Nand Rai and Neeraj Tiwari contributed equally to this work.

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Review Article

Flavonoids as Promising Neuroprotectants and Their Therapeutic Potential against Alzheimer's Disease

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Alzheimer's disease (AD) is one of the serious and progressive neurodegenerative disorders in the elderly worldwide. Various genetic, environmental, and lifestyle factors are associated with its pathogenesis that affect neuronal cells to degenerate over the period of time. AD is characterized by cognitive dysfunctions, behavioural disability, and psychological impairments due to the accumulation of amyloid beta (Aβ) peptides and neurofibrillary tangles (NFT). Several research reports have shown that flavonoids are the polyphenolic compounds that significantly improve cognitive functions and inhibit or delay the amyloid beta aggregation or NFT formation in AD. Current research has uncovered that dietary use of flavonoid-rich food sources essentially increases intellectual abilities and postpones or hinders the senescence cycle and related neurodegenerative problems including AD. During AD pathogenesis, multiple signalling pathways are involved and to target a single pathway may relieve the symptoms but not provides the permanent cure. Flavonoids communicate with different signalling pathways and adjust their activities, accordingly prompting valuable neuroprotective impacts. Flavonoids likewise hamper the movement of obsessive indications of neurodegenerative disorders by hindering neuronal apoptosis incited by neurotoxic substances. In this short review, we briefly discussed about the classification of flavonoids and their neuroprotective properties that could be used as a potential source for the treatment of AD. In this review, we also highlight the structural features of flavonoids, their beneficial roles in human health, and significance in plants as well as their microbial production.

1. Introduction

AD is one of the most prevalent neurodegenerative disorders that affects millions of people all over the world [1]. The social and economic burden of AD is high and the number of cases is rising dramatically and may reach to 88 million by 2050 [2]. AD is attributed by several pathological manifestations including profound oxidative stress, synaptic connection loss, cumulative emergence of intracellular tau pathology, accumulation of extracellular amyloid beta (Aβ) plaques, and other aspects [3]. During the onset and progression of AD, various neurological dysfunctions take place that ultimately leads to loss of memory and cognitive deficits that cause interference in daily life of the individuals [4]. Plenty of significant resources have been exhausted to date in its treatment, but the overall results are still disappointing and challenging. AD occures due to disturbance of multiple pathway, therefore, the multi-target approche thruogh hole plant extract will be more promising among the possible therapies. Ongoing analyses have demonstrated that consumption of flavonoid-rich diet can actually improve mental health and cognitive ability in people [5–8]. Furthermore, several flavonoids have been accounted to limit the progression of AD, and this has been originating from their capacity to improve cognitive function in various preclinical models [8]. The flavonoid-rich foods like cocoa, citrus, green tea, and berry can be ascribed to the connections of flavonoids and their metabolites with various subcellular targets [9, 10]. Consequently, flavonoids thus exert their neuroprotective effects by maintaining the neuronal quality and prevent the development of AD, involved in cognitive improvement. Overall, this review summarizes the classification, physiochemical properties, hallmarks of AD, and pharmacological effects of flavonoids against the pathogenesis of AD. This review also includes the molecular pathways targeted by flavonoids to prevent the progression of AD and provide a deep understanding in research of flavonoids.

1.1. Flavonoid Overview. A broad and varied range of organic chemicals are produced by plants, the vast majority of which do not appear to be directly involved in growth and development. Historically, these compounds have been referred to as secondary metabolites specially flavonoids. During the last few years, a myriad of studies has ignited the attention of polyphenolic compounds in the treatment of AD because of their possible therapeutic applications [11]. Flavonoids belong to one of the diverse groups of plant polyphenols, and more than 10,000 flavonoids have been extracted to date from natural resources including wines, vegetables, restorative plants, and organic products [12]. Flavonoids have emerged as a promising leading molecule either alone or in association with other compounds for showing the effective plan and improvement as anti-AD drugs [13].

1.2. Flavonoids and Neuroprotection. Their incredible diversity, circulation, availability, and simple detachment have made them a class of potent candidates for AD therapy. Various in vitro as well as in vivo studies have been conducted defining the antioxidant and neuroprotective property of flavonoids. In addition multiple preclinical and clinical studies also reported the role of flavonoids in the prevention and treatment of cognitive dysfunction, learning, and memory deficits [8]. Acute or chronic administration of flavonoids crosses through the blood-brain barrier suggesting that these compounds can feasibly have a direct effect on the brain and hence could be used as a prophylactic agent [14]. Furthermore, natural and synthetic flavonoids have gained substantial attention not only because of their antioxidative, anti-inflammatory, and antiamyloidogenic properties [15] but also because of multiple range of pharmacological effects that ameliorate learning and memory in the AD patients [16]. Moreover, most of the flavonoids are accounted for limiting the movement of AD pathologies, suppress the psychological shortfalls, and viably improve psychological capacities in humans [17]. Besides that, a few specialists are engaged in the various periods of clinical preliminaries, but none of the antiamyloid medication is presently clinically accessible. Because of the harmfulness related to the utilization of accessible medications and their restricted restorative viability, the quest for new anti-AD drugs is as yet in progress [18].

2. Classification of Flavonoids

More than 10000 different flavonoids have been identified to date which have enormous therapeutic potential. Despite having great diversity, the classification of flavonoids has restricted into six groups based on their molecular structure [17]. These groups include flavonols (rutin and quercetin), flavanols (catechin, epicatechin, and epigallocatechin), isoflavones (genistein, daidzein, glycetin, and formanantine), anthocyanidins (cyanidin, malvidine, and delphinidine), flavanones (hesperetin and naringenin), and flavones (apigenin and luteolin) [19]. Among the above mentioned six classes, the flavones are the most common and abundant within the families and genera of the higher plants [20]. Besides that, flavonoids are separated from one another based on contrasts in the condition of oxidation/reduction and aglycon ring design. Moreover, based on the extent of hydroxylation of aglycon, positions of the hydroxyl groups, saturation of pyran ring, and differences in the derivatization of the hydroxyl groups are major differentiating features among the various classes of flavonoids [21].

3. Distribution of Flavonoids in Nature

Flavonoids are the essential components of the plant cells hence, ubiquitously distributed in green plants [22] (Table 1). Additionally, they are part of the human diet due to their abundance in vegetables, fruits, seeds, and beverages such as coffee, tea, and red wine, as well as in medicinal herbs [23]. Being ubiquitous in nature, these secondary metabolites have emerged as an indispensable tool in a variety of nutraceutical, pharmaceutical, medicinal, and cosmetic applications and impose benevolent effects on human health [24]. Different dietary source contains different type of flavonoids with a range of conc. Like *Capparis*

Table 1: Classification of flavonoids and their dietary sources.

S. no.	Class	Flavonoids	Dietary sources	References
1.	Flavanones	Naringin Naringenin Hesperetin Eriodictyol	Cabbage, banana, kiwi, garlic, olives, onion, sprout vegetables, lemon	[25]
2.	Flavanols	Epigallocatechin gallate Epigallocatechin Epicatechin Catechin	Green tea, red wine, grapes, cocoa beans, apricots, berries, apple	[25]
3.	Flavones	Luteolin Diosmin Apigenin Wogonin	Kiwi, green tea, oregano, spinach, lettuce, broccoli, watermelon, peas, chamomile flower, orange, grapes, pumpkin, chickpea, brown rice, rosemary	[26]
4.	Flavonols	Quercetin Morin Galangin Kaempferol	Green pea, grape seeds, apple, citrus fruits, soyabean, onions, cucumber strawberries, tomatoes	[26]
5.	Anthocyanins	Malvidin Cyanidin Hirsutidin Pelargonidin	Berries (red, purple and blue), red grapes, fruits (pomegranate, red apple, apricot), vegetables (black beans, red cabbage, purple carrot, eggplant, coloured potatoes, red onions, radish), coloured cereals	[27, 28]
6.	Isoflavones	Genistein Glycitein Equol Daidzein	Soybeans, soy foods, legumes, parsley, tofu, fava beans, red clover	[27]

spinosa contain kaempferol (59 to 247 mg/100 gm) and quercetin (45-519 mg/100 gm), fresh rosemary contains high amount of naringenin (24.86 mg/ml). Petroselinum crispum commonly know as parsley is native species to European region that contains high amount of apigenin (1774-13506?mg/100 gm) and isorhamnetin (331?mg/100 gm). Very few human studies reported regarding concertation of flavonoid intake during the regular diet or from dietary components, because its concertation varies from one region to another in the same product. Future research needed to focus on dietary concentration of flavonoids and their absorption in human body.

4. Hallmarks of Alzheimer's Disease

The neuropathological hallmarks of AD include positive lesions such as amyloid plaques, neurofibrillary tangles, and cerebral amyloid angiopathy and negative lesions such as neuronal and synaptic loss [29].

4.1. Amyloid Plaques. Amyloid plaques also named senile plagues mainly composed of Aβ are one of the major hallmarks, and its accumulation leads to pathogenesis of AD [30]. Amyloid precursor protein (APP) is the precursor molecule in the generation of 37 to 49 amino acid residue peptide called Aβ with the help of β- and γ-secretase enzymes in neurons (Figure 1) [31]. The sequential cleavage leads to the deposition of two forms of Aβ with 42 or 40 amino acids (Aβ-40 and Aβ-42) and out of which Aβ-42 is more abundant and pathogenic as compared to Aβ-40 due to its insoluble nature [29]. In case of neurological disorders, the imbalance between Aβ formation and its clearance gets disrupted that ultimately leads to the formation of senile plaques [32]. An *in vivo* study reported that in the case of healthy people, the production rate of Aβ is lower than its clearance rate [33]; however, in the advancement of AD, the proportion of production and clearance gets disrupted in the brain.

4.2. Neurofibrillary Tangles. NFTs are considered to be another major pathological hallmark of AD. The ultraimages of AD brain revealed that NFTs are composed of fibrils of ~10 nm in diameter that form pairs with a helical conformation to give 65 nm paired helical filaments [34]. NFTs are found in both degenerated and dying neurons and composed of hyperphosphorylated tau protein (Figure 1). Tau is a microtubule-associated protein and its phosphorylated form is found in the somatodendritic region of neurons, and any abnormalities in its production lead to the production of NFTs and neuronal death [35]. The accumulation of

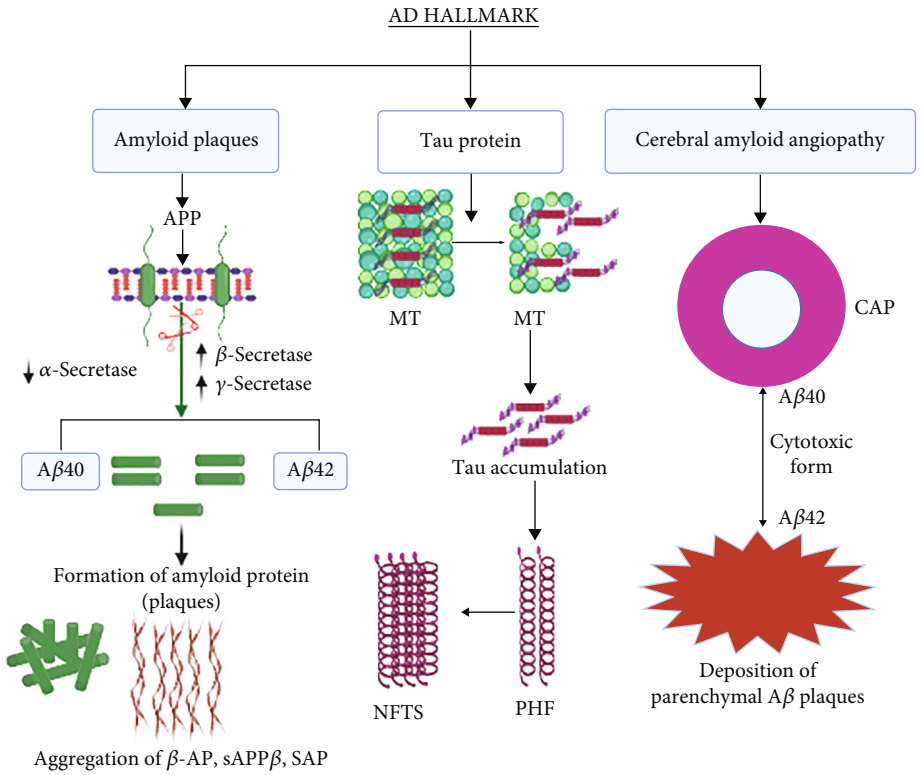

Figure 1: This figure depicts the overview of pathological hallmark of AD through amyloid plaque formation, NFT formation through Tau protein, and deposition of parenchymal Aβ plaques. APP: amyloid precursor protein; MT: microtubules; AD: Alzheimer's disease; NFTs: neurofibrillary tangles; PHF: paired helical filament.

NFTs is associated with neuronal dysfunction; that ultimately leads to dementia in AD patients.

4.3. Cerebral Amyloid Angiopathy (CAA). CAA has been recognized as one of the morphologic hallmarks of AD, but its presence is also reported in the neurologically healthy brains of elderly patients (Figure 1) [36]. CAA results from deposition of β-amyloid in cerebral cortex region and the ultraimages of AD brain revealed that CAA is a major cause of pathologies in AD, including loss of smooth muscle cells, haemorrhage, capillary occlusion, and dementia in the elderly [37, 38]. We recently provided the first evidence that in a mouse model of CAA, oral administration of taxifolin, a natural bioactive flavonoid, prevented cognitive impairment through pleiotropic beneficial effects [39]. However, how CAA contributes to vascular dysfunction in AD is unclear, and effective treatments for CAA have not been established.

5. Biochemical Properties of Flavonoids

Flavonoids are of huge interest in numerous nutraceutical, pharmacological, medical, and cosmetic applications; they are a crucial ingredient. This is explained by their ability to control important cellular enzyme functions as well as their antioxidative [40], anti-inflammatory [41, 42], antimutagenic [43], and anticarcinogenic capabilities [44–46]. According to numerous researches, flavonoids possess biological properties such as antiallergenic, antiviral, anti-inflammatory, and vasodilation effects. These flavonoids can be found in a variety of foods, including cereals, tea, red wine, and fruits and vegetables [47]. The antioxidant activity of flavonoids, however, has drawn the greatest attention because of their capacity to both prevent the creation of free radicals and scavenge existing ones. Cellular damage brought on by oxidative stress has been linked to a number of diseases including diabetes, cancer, cardiovascular disease (CVD), neurological disorders, and ageing. Numerous biological molecules are harmed by oxidative stress, and the easy target is proteins and DNA which involved in every biological processes. Numerous researches have examined the ability of flavonoids to function as antioxidants in vitro, and significant structure-activity connections of the antioxidant activity have been identified [25] In the past years, researcher provided the molecular mechanisms of flavonoids to suppress the activities of specific P450 isozymes, in order to prevent carcinogens [48]. Recent research demonstrated that the flavonoids are also work as antiviral (e.g., quercetin, naringin, hesperetin, and catechin) and antibacterial (apigenin, along with quercetin) against the different kinds of infections [49, 50]. The effect of flavonoids, especially quercetin, is reported in the modulation of various inflammatory processes and immunological functions [51]. Tannins, stilbenes, curcuminoids, coumarins, lignans, quinones, and other flavonoids have chemopreventive properties as well

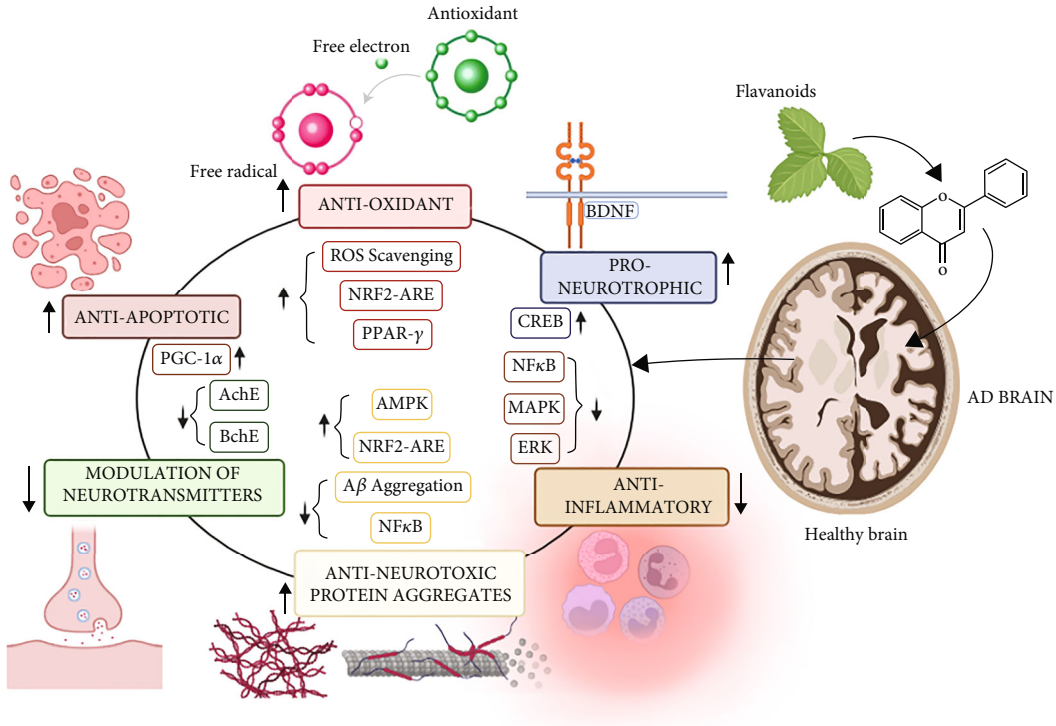

Figure 2: Role of flavonoids is regulation of different pathways. This figure shows flavonoids regulating various pathways including anti-inflammatory and antiapoptotic and play role as strong antioxidative agent to combat oxidative stress in Alzheimer's disease.

as contribute to the induction of apoptosis by arresting the cell cycle, regulating carcinogen metabolism, and controlling ontogenesis expression, according to research by Ren et al. [48, 52]. They also mentioned that the flavonoids' potential mechanisms of action in the prevention of cancer include antioxidant activity and scavenging free radicals, modulation of carcinogen metabolism, control of oncogene and tumor-suppressor gene expression in cell proliferation and differentiation, induction of cell cycle arrest and apoptosis, and modulation of enzyme activities in detoxification. There is a lot of knowledge about flavonoids and how they protect the central nervous system, especially when it comes to neurodegenerative diseases brought on by the interaction of oxidative stress, inflammation, and transition metal accumulation. Like flavonols, flavonoids are linked to a decrease in the prevalence of dementia [53–55]. Citrus flavanones such hesperidin, hesperetin, and naringenin, according to Hwang et al. [56] and Wróbel-Biedrawa et al. and Silva dos Santos et al. [57, 58], may be able to cross the blood-brain barrier and be an effective treatment for neurodegenerative illnesses. It has also been claimed that of flavonoids have antiaging and antidiabetic properties [59, 60].

6. The Interaction of Flavonoids with Multiple Signalling Pathways in Alzheimer's Disease

Various studies have revealed that flavonoids and their metabolites can exert useful actions on neurological processes through interactions with protein kinase and lipid kinase-signalling cascades [61]. These neuronal signalling pathways include nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway (Figure 2) [62] as well as the protein kinase C (PKC), tyrosine kinase [63], phosphoinositide-3-kinase (PI3K)/Akt [64], and mitogen-activated kinase (MAPK) signalling pathways [16]. MAPKs are found to regulate a variety of cellular processes by converting extracellular signals into intracellular responses [65]. Flavonoids and their metabolites, on the other hand, can selectively interact with MAPK signalling pathways by interacting with MAPK kinases such as MAP kinase 1 (MEK1), MEK2, and membrane receptors (Figure 2) [66]. These kinases appear to promote the effect of flavonoids on the extracellular signal-regulated kinase (ERK) pathway [67]. Flavonoids have a structural similarity to a number of pharmacological ERK signalling pathway modulators [68]. Activation of the cAMP response element-binding protein (CREB) has also been observed as a result of ERK activation, which can result in upregulation of neuroprotective pathways as well as changes in memory and synaptic plasticity [69]. The nuclear factors erythroid 2-related factor 2 (Nrf2) and hypoxia-inducible factor-1, which act as modulators of PPAR-γ and activate the PGC-1α pathway, can be stabilised by flavonoids [70]. Flavonoids modify these molecular pathways, slowing the development of Alzheimer's disease by reducing oxidative stress, improving mitochondrial dysfunction, lowering insulin resistance, and improving memory impairment [71]. In addition, flavonoids also regulate PI3K/Akt signalling cascade through direct interactions with their adenosine triphosphate (ATP) binding site. In a study, it has been reported that the flavanone hesperetin has been found to cause activation of the Akt/protein kinase B (PKB) signal transduction cascade to provide prosurvival

Table 2: Role of flavonoids in Alzheimer's disease.

S. no.	Flavonoids	Study model	Reported dose	Route of administration	Natural source	Outcome of the studies	Reference
1.	Epigallocatechin-3-gallate	APPswe/PS1dE9 mice	40 mg/kg	Oral	Green tea	Decreases Aβ plaques, neuroinflammation, increases memory and learning	[81]
2.	Cocoa flavonols	Human	550 mg, 994 mg	Oral	Dark chocolate	Improve cognitive function	[82]
3.	Apigenin, luteolin, kaempferol, quercetin	Human	2 gm to 20 gm	Oral	Parsley	Bioavailability, antioxidative, and biomarker	[83] [84]
4.	Hesperidin, neohesperidin, naringenin, quercetin, rutin, etc.	Human	3-5 citrus per week	Oral	Citrus	Lower the risk of dementia	[85]
5	Flavanone	Human	Daily for 8 weeks	Oral	Orange	Cognitive benefits	[86]
6.	Combination of quercetin and dasatinib	Clinical trial in AD patients	—	Oral	Apple, honey, onion, citrus fruits	—	NCT04063124, NCT04785300, NCT04685590
7.	Epigallocatechin gallate	Clinical trial on AD patients	—	Oral	Tea, fruits, nuts		NCT03978052

attributes in cortical neurons [72], while the flavonol quercetin was found to modulate the prosurvival Akt/PKB and ERK1/2 signalling cascade by hampering the activity of PI3K (Figure 2) [73]. Furthermore, it appears that cellular responses differ depending on the degree of interaction with downstream kinases or receptors, implying the possibility of structure-dependent signalling pathways.

Autophagy is a key process that is involved in synaptic plasticity and clearance of aggregate prone proteins in neuronal cells and aberrant autophagy plays a crucial role in neurodegenerative disorders [74]. Currently, flavonoids are being executed as a potential therapeutic tool for autophagy signalling involved in neurodegenerative disorders [75]. The group of few researchers reported that high dose of genistein (150 mg/kg/day) triggered the autophagy induction and promoted the degradation of Aβ and hyperphosphorylated tau in a streptozotocin-induced rat model of the sporadic AD [76]. In another study, it was found that resveratrol, a flavonoid, activated the first step of autophagy through AMPK/mTORC1 pathway to mitigate cognitive impairment in AD mice. Quercetin, the most potent flavonoid, not only cleared the Aβ aggregates but also delayed paralysis via activation of macroautophagy and limit brain damage *in vitro* and *in vivo* [77]. An *in vitro* study in SH-SY5Y neuronal cells revealed that flavonoid, wogonin, not only reduced the secretion of Aβ in the primary cortical astrocytes but also promoted its clearance through mTOR/autophagy signalling pathway [78]. Silibinin, another flavonoid, has also alleviated neuronal damage by inhibiting autophagy in the hippocampus region of the brain [79]. In addition, the combination of quercetin with silibinin and wogonin have been successfully used in preclinical and clinical studies to clear out the amyloid substance via induction of autophagy by ULK1/mTOR pathway [80]. Some studies reported on dietary intake of flavonoid and their benefits in AD patients (Figure 2) (Table 2).

7. Role of Flavonoids in Neuroprotection

Recently, flavonoid derivatives are being used as a neuroprotective agent in several neurodegenerative disorders including AD. The various aspects of flavonoids as a neuroprotectants are as follows.

7.1. Flavonoids as Neuroprotective Agents. Flavonoids exert a multiplicity of neuroprotective effects against neurodegenerative diseases to suppress neuroinflammation and to improve cognitive function (Figure 3) [87–89]. Recently, one study from Gu et al. showed antiamyloid property of the flavonoid prepared from the caper leaf and fruit [90]. Another study from the Szwajgier conferred the neuroprotective effect of a flavonoid (Icariin) derived from the Chinese herb *Epimedium brevicornum*. Szwajgier showed the ability of icariin to decrease both amyloids-β (Aβ) and APP levels and increase neurogenesis in the brain of Tg2576 mice significantly [91]. Another flavonoid baicalein (5,6,7-trihydroxy-2-phenyl-chromen-4-one) extracted from *Scutellaria baicalensis*, has strong neuroprotective potential and significantly rescues synaptic plasticity and memory deficits in AD mouse model [92]. In addition, baicalein is able to prevent the damage in the hippocampal long-term potentiation (LTP) induced by Aβ and improve cognitive deficit associated with AD. People have also studied the anticholinesterase activity of few flavonoids [93], and as we know, there is increased activity of cholinesterase in case of AD so flavonoid having anticholinesterase activity will be useful to protect the neuronal damage in the hippocampus area of the AD patient.

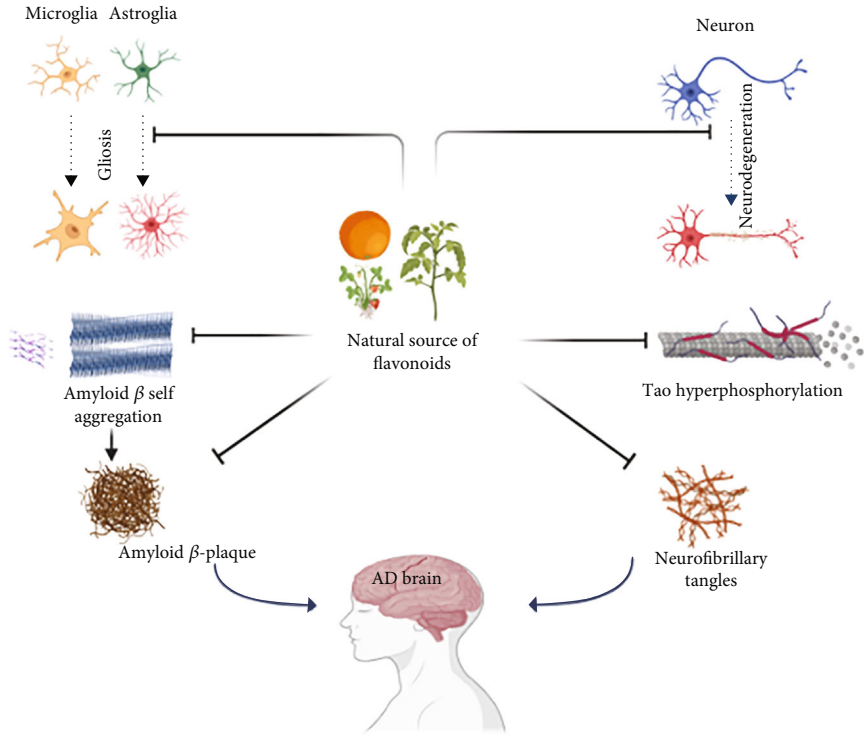

Figure 3: The neuroprotective role of flavonoids in inhibiting different signalling pathways responsible for Alzheimer's disease.

7.2. Flavonoids as Cholinesterase Inhibitors. Acetylcholine (ACh) is the most versatile neurotransmitter that plays its role in transmission of impulse across different neurotransmitters. Cholinesterase enzymes including acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) are completely engaged in the degradation of ACh responsible for the neurotransmission among synapses [94, 95]. A variety of studies revealed the low levels of ACh in AD brains and cholinesterase inhibitors not only increase ACh levels but also conduct the transmission of impulse at synaptic junctions [96]. Uriarte-Pueyo and Calvo summarized and represented 128 flavonoids exhibiting AChE-inhibiting activity making flavonoids anti-Alzheimer agents [97]. A myriad of flavonoid has been employed as inhibitors of AChE, but among them, the quercetin was found to be potent inhibitor of AChE [98]. The information in regard to the presently accessible medications demonstrates that utilizing this methodology is the most successful remedy in AD indicative treatment, along these lines smoothing out the inevitable clinical endorsement of four medications [99]. Attributable to the undesirable impacts and restricted adequacy of the presently accessible medications, there is a critical need to grow more protected and compelling medications [100–102]. In view of their intensity as AChE inhibitors, they were viewed as promising remedial specialists in the improvement of new enemy of Alzheimer drugs [103, 104].

7.3. Flavonoids as Cognition Enhancers. Flavonoids have been shown to reverse cognitive impairments and inhibit the progression of Alzheimer's disease, implying that they may have therapeutic potential [105]. Flavonoid-rich diets including grape juice, cocoa, and blueberry are used as memory enhancers [82, 106, 107]. In a randomized controlled clinical study, daily ingestion of anthocyanin-rich cherry juice improved fluency, short-term memory, and long-term memory in aged people (70+) with dementia [108]. Administration of quercetin along with daily usage of flavonoid-rich fruits increases cognition, memory retention, and recovery of short- and long-term memory loss [109].

Various studies have looked at flavonoids' antiamyloidogenic effects as a potential natural treatment for Alzheimer's disease. Moreover, flavonoids' effectiveness in learning and memory has been documented in a number of studies [110]. However, there are fewer studies on cognition and memory in animal models fed with fruits with high flavonoid-rich diet sources [111], e.g., anthocyanin-rich mulberry extracts corrected the cognitive impairment in mice with accelerated senescence and AD-like neurodegeneration [112]. A metanalysis revealed that dietary flavonoids improved the cognition function and showed its positive effect against AD pathogenesis [7]. Moreover, several recent studies reported on the beneficial effects of anthocyanin-enriched extracts or anthocyanin-enriched wheat grain diet on cognitive function in mouse AD models [113–115]. In a transgenic AD mouse model, a citrus flavonoid called nobiletin was found to reduce the strain of Aβ and plaques in the hippocampus, thus enhancing memory deficits caused by Aβ [116]. In both human Swedish mutant APP transgene-bearing neuron-like cells and primary neurons, the citrus

flavonoid luteolin was found to minimise APP processing by amyloidogenic γ-secretase activity and decrease the generation of Aβ. Furthermore, the accumulation of Aβ in the brains of AD mouse models was prevented by administering curcumin or polyphenol-rich grape seed extracts for nine months [117]. However, further research is required to determine which flavonoid structures have potent beneficial properties as well as their underlying mechanisms of action. Three structural features of natural products have been suggested in a recent study to clarify their inhibitory function against Aβ-42 aggregation [118]. The first feature is carboxy acid derivatives of anthraquinonoids or triterpenoids that can form a salt bridge with basic amino acid residues like Lys28 and Lys16 in Aβ-42 trimers or dimers [119]. The second property is that noncatechol-type flavonoids have molecular planarity due to $\alpha\beta$-unsaturated carbonyl groups that can interact with the intermolecular β-sheet region in Aβ-42 aggregates, especially aromatic rings like Phe20 and Phe19 (Figure 3) [120]. Catechol-type flavonoids can form Michael adducts with the side chains of Lys28 and Lys16 in monomeric Aβ-42 through flavonoid autoxidation, which is the third characteristic [121].

7.4. Flavonoids as Free Radical Scavenger. Flavonoids possess several biochemical properties, but the most important biological role of flavonoids has been reported to have antioxidant and hydrogen-donating capacity [122]. The functional groups associated with the structure are prominently responsible for the antioxidant property of flavonoids. Oxidative stress is generated because of the imbalance between reactive oxygen species (ROS) and antioxidants and one of the important events in any neurodegenerative disorders. Apart from neurodegenerative disorders, oxidative stress has been linked to various other diseases including ischemic injury, cancer, atherosclerosis and inflammation. Oxidative stress and metal toxicity played a crucial role in the pathogenesis of AD [55]. Flavonoids play a crucial role in preventing the adverse effect of ROS by protecting the neurons against oxidative stress and hence suppress neuronal damage and improve cognitive function [20] [123]. Besides flavonoids, its derivatives have been reported to have potential antioxidant and neuroprotective activity *in vitro* and *in vivo* [89, 124–127]. Various studies have suggested that flavonoids exert its action especially in neuroprotection against AD by affecting gene expression and interacting with mitochondria and the intracellular signalling modulation of cascades that control neuronal differentiation, survival, and death [73]. Moreover, being a potent scavenger molecule, flavones exert an important indirect antioxidant activity contributing to the homeostasis of metal chelation, enzymatic activity modulation, and stabilization of membranes through antilipid peroxidation [122, 128]. Apart from the antioxidant activity of the flavonoids, these polyphenolic compounds also play very important role in human health and exhibit several other significant properties including antimicrobial, antiviral, and anticancer [129]. Antioxidant mechanism of the flavonoids reduces the free radical load and improves the health status through activation of antioxidant enzyme system that scavenges the reactive oxygen species (ROS) [130].

8. Demerits of Flavonoids

The wide accessibility of flavonoids and their new expansion in utilization by people has brought up significant issues with respect to the expected harmfulness of these dietary parts [131]. Albeit most of characteristic items are all around endured; nonetheless, flavonoids and related phytochemicals have been appeared to incite neurobehavioural and endocrine-disturbing impacts [132]. It has been stated that the quercetin possesses very low toxicity for humans and doses of approximately 1 mg/adult/per day have been recommended [133]. It has been reported that long-term consumption of quercetin over several years resulted in the formation of tumors in mice [134]. Afterwards, several studies were conducted but no study had reported the carcinogenicity and other ill effects of flavonoids [135]. The collaborations of flavonoids with CYP3A4, the prevalent human hepatic and intestinal CYP liable for processing half of restorative specialists, are exceptionally compelling. The concurrent organization of flavonoids and clinically utilized medications may cause drug-flavonoid interactions by balancing the pharmacokinetics of specific medications [133]. Dosage also seems to be an essential issue. For example, high doses of resveratrol caused various side effects including nausea, diarrhea, and abdominal discomfort [136]. Moreover, some flavonoids such as resveratrol or epigallocatechin-3-gallate are regarded as pan-assay-interference (PAINS) compounds [137]. Their effect on an organism might be nonspecific, including cell membrane perturbations, rather than specific protein binding [138], especially while supraphysiological doses are applied. Very few studies reported on side effect of flavonoids. Early studies reported that flavonoids (quercetin) act as mutagenic and cause toxicity for gene expression [139, 140]. Hodnick et al. observed that myricetin and quercetin caused mitochondrial enzyme inhibition, damage endogenous antioxidant, and accelerate oxidative stress [141]. Some flavonoids also inhibit topoisomerase II, although this help to recover the cancer patients, but beside this, it also increases the risk of leukemia. Further studies needed to further validate this result. Flavonoids also found to interfere with thyroid hormone production in infant with autoimmune disease. But no studies reported regarding flavonoids and thyroid production in healthy population. Tons of data present regarding role of flavonoids in treatment of various disease but still very few clinical studies carried out. Beside this, the rate of translation of preclinical to clinical studies is very less.

9. Future Prospective

During the last decade, flavonoids received much attention and a variety of beneficial effects have been elucidated against a myriad of neurodegenerative disorders. Further research is needed to discover new flavonoids from nature's bounty so that this may replace using synthetic drugs which are harmful to the body. In this context, there is a need of studies and development programmers related to in vivo studies so one can provide a hopeful and safe picture for the destiny. Presently, the consumption of fruit, greens,

and drinks containing flavonoids is recommended, although it is too early to make hints on every day flavonoid intakes. Further studies are needed especially well-designed clinical trials to endorse the clinical effectiveness of flavonoids in neurodegeneration so that it might be used as a better prophylactic agent to improve the human health including AD patients. Another issue that needs to be addressed in the flavonoid research is to identify the amount of flavonoids absorbed, either they work in synergistic way or individual in improvement of human health.

10. Conclusion

This review will provide a brief insight about the pathology of AD and potential of natural flavonoids and their derivatives as active agents against neurological diseases. This should encourage research community in a new direction to exploit such information for the rational design of flavonoid based pharmaceutical drugs for AD. Flavonoid is very vast and present in almost natural sources with range of concentration. Various preclinical/clinical studies reported that flavonoids are effective against various diseases including AD. Further research needed especially clinical studies to standardise the dose, side effect, and bioavailability of flavonoids in AD patients.

Conflicts of Interest

The authors declare no conflict of interest.

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Review Article

Perspectives on the Molecular Mediators of Oxidative Stress and Antioxidant Strategies in the Context of Neuroprotection and Neurolongevity: An Extensive Review

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Molecules with at least one unpaired electron in their outermost shell are known as free radicals. Free radical molecules are produced either within our bodies or by external sources such as ozone, cigarette smoking, X-rays, industrial chemicals, and air pollution. Disruption of normal cellular homeostasis by redox signaling may result in cardiovascular, neurodegenerative diseases and cancer. Although ROS (reactive oxygen species) are formed in the GI tract, little is known about how they contribute to pathophysiology and disease etiology. When reactive oxygen species and antioxidants are in imbalance in our bodies, they can cause cell structure damage, neurodegenerative diseases, diabetes, hypercholesterolemia, atherosclerosis, cancer, cardiovascular diseases, metabolic disorders, and other obesity-related disorders, as well as protein misfolding, mitochondrial dysfunction, glial cell activation, and subsequent cellular apoptosis. Neuron cells are gradually destroyed in neurodegenerative diseases. The production of inappropriately aggregated proteins is strongly linked to oxidative stress. This review's goal is to provide as much information as possible about the numerous neurodegenerative illnesses linked to oxidative stress. The possibilities of multimodal and neuroprotective therapy in human illness, using already accessible medications and demonstrating neuroprotective promise in animal models, are highlighted. Neuroprotection and neurolongevity may improve from the use of bioactive substances from medicinal herbs like *Allium stadium*, *Celastrus paniculatus*, and *Centella asiatica*. Many neuroprotective drugs' possible role has been addressed. Preventing neuroinflammation has been demonstrated in several animal models.

1. Introduction

A disruption in the balance of reactive oxygen species such as superoxide, hydroxy radicals, and nitric oxide radicals, as well as antioxidants such as vitamins A, C, and E, selenium, and carotenoids, which can cause tissue damage, is known as oxidative stress. In biological systems, oxidative stress is spread as metabolic by-products [1, 2]. It can come from both endogenous (in mitochondria during oxidative phosphorylation, during inflammation, the respiratory burst, endoplasmic reticulum, phagocytic cells, and peroxisomes for example) and exogenous (pollution, alcohol,

tobacco smoke, heavy metals, transition metals, industrial solvents, pesticides, certain drugs like paracetamol, and UV radiation, halothane, and exogenous sources) [3]. When ROS and RNS are produced in our bodies at moderate or low levels, they help the heart pump more blood in stressful situations and also perform a variety of physiological functions such as immune function and a variety of cellular signaling pathways in the redox regulation pathway; nitric oxide (NO°) helps to regulate blood pressure during mitogenic responses, and phagocytic cells use them to kill bacteria during bacterial infections [4, 5]. Exogenously or endogenously produced highly reactive oxidizing chemicals are always a threat to living organisms. They can take electrons from oxidizing compounds with ease. This results in cell structural damage, diabetes, hypercholesterolemia, atherosclerosis, cancer, cardiovascular diseases, metabolic disorders, and other obesity-related problems [6–9]. When a person is under oxidative stress, free radicals assault his neural cells, putting them at risk of degeneration. The cytotoxic effect of ROS can cause protein misfolding, mitochondrial malfunction, glial cell activation, and subsequent cellular apoptosis [10].

There are two terms in "neurodegeneration": "neuro" represents for nerve cells, and "degeneration" means for deterioration. The loss of neuron function is a characteristic feature of neurodegenerative disorders [11–13]. Degenerative nerve illnesses can affect a person's mobility, vision, memory, IQ, speech, respiration, heart function, and much more. The gradual loss of the structure or functionality of neurons, or neurodegeneration, is the root cause of neurodegenerative diseases. From the molecular to the systemic levels of neural circuitry, neurodegeneration may be seen in the brain. Alzheimer's disease and associated memory disorders ataxia, multiple system atrophy, Huntington's disease, motor neuron disease, Parkinson's disease, progressive supranuclear palsy, and amyotrophic lateral sclerosis are all examples of neurodegenerative disorders [14]. There is no proven treatment for Alzheimer's disease (AD) [15]. Phytochemicals have been shown to be beneficial in the treatment of neurodegenerative illnesses including Alzheimer's and Parkinson's disease (PD). Traditional herbs and phytochemicals may delay its development and decrease its course and enable healing by addressing several pathogenic reasons. They control mitochondrial stress, apoptosis, free radical scavenging, and neurotrophic factors. The most prevalent neurodegenerative disorders include amyloidoses, tauopathies, alpha-synucleinopathies, and TDP-43 proteinopathies [16]. Many of these disorders are inherited, but they can also be caused by toxins, chemicals, or viruses. When neurodegenerative disorders are inherited in an autosomal dominant pattern, they have a 50% chance of recurrence [17]. Vitamin C (ascorbic acid) is an important antioxidant molecule in the brain because it aids in the preservation of the integrity and function of numerous brain processes [18]. However, an abnormally high vitamin C concentration can cause neurological problems. Many investigations have shown that preterm babies' neurological damage is caused by impairment of vitamin C transfer [19, 20]. Mortality from presenile dementia (PSD), Alzheimer's disease (AD), Parkinson's disease (PD), and motor neuron disease (MND) was examined for 27 states in national occupation mortality surveillance (NOMS) systems from 1982 to 1991, totaling 130,420 deaths, according to the CDC (Centers for Disease Control and Prevention) [21].

Oxidative stress is caused by an imbalance between the amount of reactive oxygen species (ROS) in the body and the ability of a biological system to quickly get rid of the reactive intermediates or fix the damage they cause. When the normal redox state of a cell is upset, free radicals and peroxides are made that damage proteins, lipids, and DNA [22]. Damage to DNA's bases and breaks in its strands are caused by oxidative stress, which comes from oxidative metabolism. The development of chronic fatigue syndrome (ME/CFS), cancer, Parkinson's disease, Lafora disease, Alzheimer's disease, atherosclerosis, heart failure, myocardial infarction, fragile X syndrome, sickle-cell disease [23], vitiligo, lichen planus, and ADHD [24] are all believed to be influenced by oxidative stress in humans. However, ROS can also be helpful because they are employed by the immune system to attack and kill pathogens.

Around 5 million Americans endure Alzheimer's disease, 1 million from Parkinson's disease, 400000 from multiple sclerosis (MS), 30,000 from amyotrophic lateral sclerosis (ALS), and 3000 from Huntington's disease in contemporary times (HD) [25]. Neuron cells have been found to be protected by a variety of phytochemicals. The purpose of this research covers oxidative stress and its link to neurodegenerative diseases, and thus a number of neuroprotective drugs along with several phytochemicals that have recently been shown to have neuroprotective effects in a lot of species.

2. Neurological Abnormalities and Oxidative Stress

Free radicals are reactive chemicals that are spontaneously created in the human body. They have either beneficial or detrimental effects on the immune system. An antioxidant system is required to reduce these adverse effects on an organism. When there is an imbalance between the production of reactive oxygen species and the antioxidant defense, a situation known as oxidative stress may be described. It is more difficult to define an antioxidant. Halliwell proposed a popular definition: Low concentrations of an antioxidant inhibit or significantly reduce the oxidation of an oxygen-soluble compound. Antioxidants are used to protect off oxidative damage. It is essential to mention that reducing agents are not the same as antioxidants, because they use different chemical words to explain the same activity. A lowering agent could even be used. If it converts transition metal ions to free radicals or reduces oxygen to free radicals, it is a prooxidant. Peroxides react more rapidly with lower oxidation states. Several biological lowering agents Janus-faced agents: Depending on the amounts of O_2, they can be antioxidants or prooxidants [26].

Oxidants are reactive molecules that are formed both within the body and in the environment. These molecules have the potential to react with other biological components

found in the body, including protein, DNA, and lipids. Several biological processes, such as aging, cancer, atherosclerosis, and dementia, are associated with tissue damage induced by oxidative stress and mediated by excessive free radicals. The genesis of oxidative stress is an imbalance between the creation of reactive oxygen/nitrogen species and the antioxidant capabilities of cells and organs. Reactive oxygen species (ROS) consist of superoxide anion (O2-), hydroxyl radicals (.OH), and hydrogen peroxide (H2O2), while antioxidants consist of numerous vitamins and endogenous enzymes, such as superoxide dismutase (SOD), catalase, and glutathione peroxidase [27]. Endogenous or exogenous reactive oxygen species can exist mitochondrial electron transport chain that is the predominant endogenous ROS emitter. The reduction of O_2 to H_2O_2 occurs in four phases each of which produces ROS [28].

(1) Superoxide radical $O_2 + e\ O_2^{\circ}$

(2) Hydroperoxyl radical $O_2^{\circ} + H_2O\ H_2O^{\circ}$

(3) Hydrogen peroxide ($H_2O^{\circ} + e + HH_2O_2$)

(4) Hydroxyl radical $H_2O_2 + H + OH^{\circ}$

Mitochondrial-targeted neuroprotective therapeutics mitochondria are a significant source of reactive oxygen species (ROS) in the central nervous system. They have redox carriers that may transport single electrons to oxygen, resulting in the production of ROS superoxide (O_2). The tricarboxylic acid cycle (complexes I, II, and III) and electron transport chain (complexes I, II, and III) enzymes, as well as monoamine oxidases, are among the mitochondrial redox carriers that generate superoxide. Other production of reactive oxygen species enzymes can also be found in mitochondria. Superoxide is depleted and converted into hydrogen peroxide during a dismutation reaction by superoxide dismutase (SOD) (H_2O_2). To remove H_2O_2 from mitochondria, SOD enzymes cooperate with catalases and glutathione peroxidases. Nevertheless, O_2 and H_2O_2 can generate hydroxyl radicals and peroxynitrites when those who react with other molecules in the cell, such as redox-active metals (Fenton reaction involving iron) and nitric oxide. These chemical processes occur a precise balance of Ros generation and eradication under standard circumstances. This balance is thrown off by aging or Alzheimer's disease increasing reactive oxygen species (ROS) and oxidative damage. Elevated numbers of mutations in mitochondrial DNA, as well as increased quantities of 8-hydroxy-2-deoxyguanosine, a hallmark of oxidative DNA damage, has been identified in AD [29, 30]. Mitochondrial dysfunction and apoptosis can result from either of these deletions or point mutations, which can be exacerbated by oxidative stress [31]. Several mitochondrial essential enzymes involved in ROS detoxification are also impacted in addition to DNA damage. The ketoglutarate dehydrogenase complex (KGDHC) [32], pyruvate dehydrogenase complex (PDHC), and cytochrome oxidase (COX) [33] are all significantly reduced in adult AD brains. In animal models, mitochondria have also been connected to the etiology of Alzheimer's disease.

3. Neuroregeneration in Neurodegenerative Disorders

The term "neuroregeneration" refers to generating new neurons, glial cells, axons, synapses, or myelin to heal or regenerate damaged neural tissue [26]. The term "neurodegeneration" is nonspecific. Degeneration can range from severe neuronal death and brain atrophy, as in late-stage AD, to degeneration in tiny neuronal structures, such as dendrites, spines, and axons, without neuronal death [34]. The central nervous system is mostly incapable of self-healing and regeneration, but the peripheral nervous system can. For all time, there has been no cure for CNS damage. There were no effective CNS regeneration therapy regimen available, and repeated efforts at neural re-growth failed owing to a lack of information regarding CNS regeneration. The discovery that mature neurons in the CNS may recover after injury has just put an end to this therapeutic nihilism [35]. Unlike PNS injuries, CNS injuries have poor prognoses due to their inability to repair neurons. This disorder may be caused by the human CNS's more complicated neural networks than other species [36]. By adding neurons to already complex brain networks, one runs the danger of producing system confusion, which is analogous to accidentally short-circuiting electrical equipment, which in turn raises the chance of having seizures. With illness or injury, these restrictions become obstacles to rehabilitation (Figure 1) [31, 32].

The capacity for axonal regeneration is mostly determined by inhibitory components of the environment that are extrinsic as well as the inherent regenerative potential of neurons. In wounded adult CNS neurons, the intrinsic neural pathways launching a growth program are similarly quite restricted [32, 33]. Axons quickly regenerate after damage to a peripheral nerve (PNS). Wallerian degeneration occurs in the axon's distal part, which is not attached to the cell body. The axon is disintegrated and fragmented as a consequence of this dynamic process. Glial cells, mostly macrophages, clear debris from the brain. It is therefore possible to rejuvenate and regenerate their targets so that function may be restored [37].

4. Neuroplasticity in Neurodegeneration Disorders

The ability of the brain to change through time is referred to as neuroplasticity. Adaptive behaviors, learning, and memory are at the top of the neuroplasticity hierarchy. Neuroplasticity couples functional changes with structural changes [38]. During ontogeny, phylogenesis, physiological learning, and brain damage, nervous system plasticity improves neuronal networks [36]. The molecular and cellular levels are both involved in the process of neuroplasticity, which manifests itself as short-term (STP), long-term (LTP), and long-term potentiation depression (LTD) [39]. Neuroplasticity is classified into two categories such as functional plasticity and structural plasticity. Learning and memory are the two fundamental mechanisms that underlie functional neuroplasticity. This sort of neural and synaptic plasticity is based on particular types of synaptic plasticity that

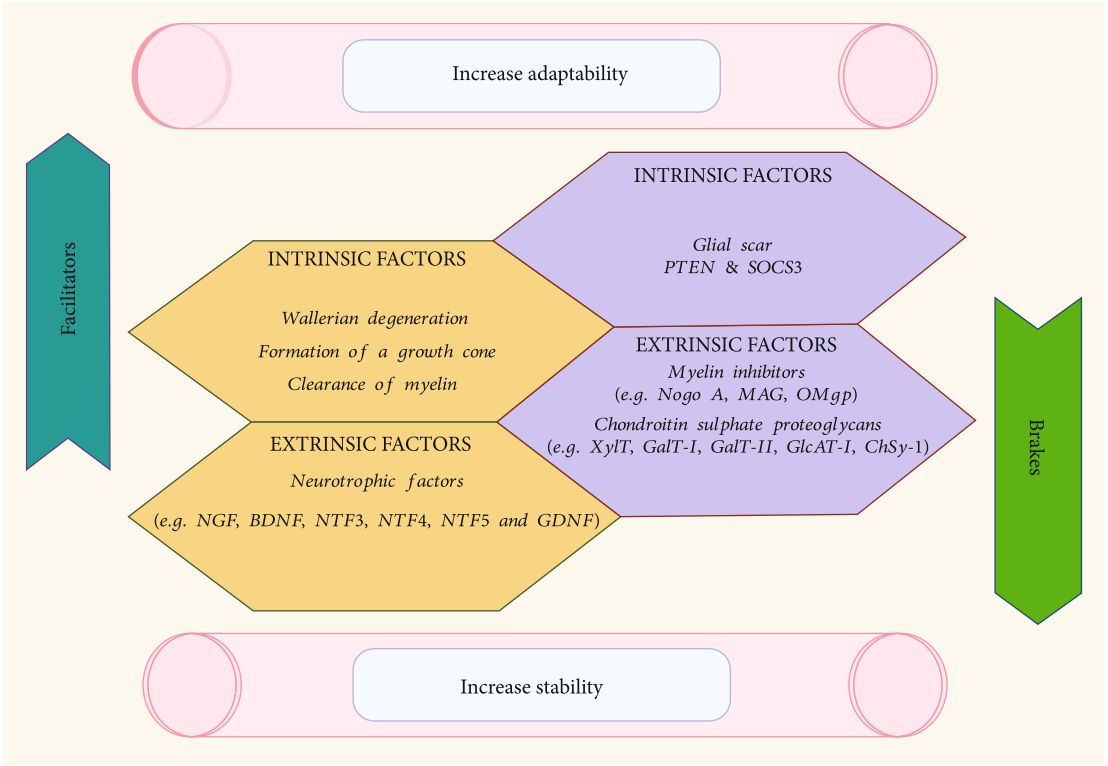

Figure 1: Central and peripheral nervous system neuroregeneration is influenced by extrinsic and intrinsic factors. For instance, the suppressor of cytokine signaling; PTEN (phosphatase and tensin homolog); NGF (nerve growth factor); SOCS3; MAG (myelin glycoprotein); keratin sulfate proteoglycans; myelin-associated glycoprotein; chondroitin sulfate proteoglycans; and oligodendrocyte [36].

cause persistent changes in synaptic efficacy. The synaptic connections between neurons change permanently throughout learning and memory as a result of structural alterations or intracellular metabolic activities [40]. The brain's capacity to modify neural connections is called structural plasticity. Neuroplasticity produces and integrates new neurons into the CNS during life. Researchers employ cross-sectional imaging (MRI, CT) to analyze structural brain changes [41]. Neuroplasticity investigates the influence of internal or external stimuli on brain remodeling. Structural neuroplasticity includes changes in grey matter percentage or synaptic strength. Current neuroscience research focuses on structural neuroplasticity [42]. The neuroplasticity hypothesis explains why individuals who have had a brain injury or have had a CVA recover abnormally [43]. Brain alterations are commonly seen as a sign of progress, although this is not necessarily true. There are several ways in which the brain's structure and function may be influenced or changed. A common illustration of how the brain's adaptability may be a problem is when drug abuse, sickness, or trauma leads to undesirable alterations (Involving damage to the brain or stressful events that lead to PTSD). Lead poisoning may impair your brain's capacity to adapt. Several medical problems may potentially restrict or inhibit brain plasticity [44]. As a result, children may be affected by a variety of pediatric neurological diseases, such as epilepsy and cerebral palsy [45]. Neuroplasticity may be guided to restore function and cure undesired symptoms in clinical settings by using a variety of therapeutic approaches [46]. Constraint-induced movement therapy (CIMT) is a type of physical therapy that has been studied a lot. Patients who have had a stroke may benefit from this technique because it forces the afflicted limb to perform repeated tasks and develop new behaviors. Contralateral premotor and secondary somatosensory cortex activity in the brains of patients who undergo this treatment has been shown to rise in correlation with better function [47]. Researchers have spent a lot of time and money studying how environmental influences might influence neuroplasticity. Studies have demonstrated that music therapy may have a favorable impact on neuroplasticity. Cognition and other executive functions have been proven to benefit from it. Researchers are looking at a variety of dietary supplements to see whether or not they might assist promote neuroplasticity [48].

5. Cellular and Molecular Immune Mediators of Neuroprotection

A neuroprotection system or approach aims to prevent the nervous system from being damaged or injured, especially in those who have been injured or diagnosed with a neurological condition [49]. The purpose of neuroprotection is to protect the central nervous system against early degeneration and other factors that may lead to the loss of nerve cells, as well as to limit the amount of nerve death [50]. It would seem that the central nervous system and the immune system (CNS) have developed a diverse set of mechanisms throughout the course of evolution in order to fight

infections and react to stress. This is because the CNS immune response has a large number of failsafe mechanisms that facilitate a well-regulated response to injury and the commencement of healing and repair [51]. Neurotrophins are important regulators of neural growth function, [52] survival, and the ability to change (plasticity) [53]. Nerve growth factor, often known as NGF, is a neurotrophic factor that, in mammals like humans, helps to foster the development of sympathetic nerve cells and peripheral sensory, and ensures that they are able to survive. It was identified in 1950 [54]. NGF functions by activating two transmembrane receptors. Two examples are the trkA and p75 receptors, both members of the tropomyosin receptor kinase (trk) family [55].

Exosomes are important mediators of neurodegenerative disorders, carrying beta amyloid and prions from their source cells to other cells [56]. Exosomes may regulate neuroinflammation, enhance neurogenesis and neurogenic physiological location, and cure neurological disorders [57]. It has been shown that the CREB (cAMP-responsive element-binding protein) pathway is involved in the control of neuronal function. This is achieved by its involvement in two major cascades of gene expression. The first one explains that CREB is an important component of the molecular switch that controls more long-term forms of brain plasticity and learning. The second one connects CREB to the maintenance and protection of neuronal survival [58]. CREB is involved in a wide number of cellular functions, some of the most significant of which include cell differentiation, proliferation, metabolism, and survival [59].

6. Neuroprotective Agents and their Functions

Trying to protect the nervous system from neuronal loss and neurodegeneration can be achieved by using capable of differentiating to suppress pathophysiological pathways that can ultimately lead to damage to the nervous system (Table 1) [60]. Immunosuppressive calcineurin inhibitors are responsible such as NOS inhibitor, ca2+ channel blocker, cationic arginine-rich peptides, benzoic acid-derived nitrones, edaravone, AMPA antagonist cyclosporine A, and sulfur-containing secondary metabolites [61–63] . Neuronal cell death prevention was shown to be beneficial for several drugs in animal models, but human therapeutic studies have yet to confirm these finding Here, we will take a look at several promising neuroprotective drugs such as magnesium sulfate, statins, melatonin, erythropoietin, free radical scavengers, immunosuppressant drugs, N-acetyl-L-cysteine (NAC), β-blockers, COX-2 selective inhibitors, and curcumin that might be useful for patients in the intensive care unit [64].

6.1. Glutamate Blockers. The amino acid glutamate is the most prevalent in the brain. Glutamate has an increasing contribution on nerve cells even though glutamate receptors are present on some of these cells. It can cause cells to die. Glutamate activates several metabotropic receptors as well as three important ionotropic receptors: To avoid excitotoxicity, kainite amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), and N-methyl-D-aspartate (NMDA), glutamate blockers reduce NMDA and AMPA [65].

Glutamate transporters are a type of neurotransmitter transporter that removes extracellular glutamate to prevent excitotoxicity in neurons when glutamate tries to enter the synaptic cleft, the excitatory amino acid transporter (EAAT) family involves removing it, and when it wants to enter the cell cytoplasm, it required to transport it into synaptic vesicles. When EAAT 2 (human glutamate transporter 2) is not operating, it can cause traumatic brain damage, stroke, amyotrophic lateral sclerosis, and Alzheimer's disease. Some of the most widely accepted stroke professionals in rodents while also nonhuman primates and humans were using the glutamate blockers polyarginine R18 and NA-1 (TAT-NR2B9C). It lowers mitochondrial oxidative stress in neurons [66].

6.2. Statins. Statins are the first-choice therapeutic medicines for the prevention of cardiovascular disease (CVD) and atherosclerotic diseases due to high levels of cholesterol in the blood [84]. A growing body of research indicates that statins have other pleiotropic effects in addition to their vascular effects, such as stability of atherosclerotic plaques and reduced carotid intimal medial thickness, which are unrelated to their cholesterol-lowering impact [85]. These activities include decreasing the thickness of the carotid intimal medial layer. On the other hand, it is common knowledge that both theoretical and empirical research have shown that inflammation plays a crucial part in the mediation of every stage of atherosclerotic illnesses. In addition to having antioxidant, anti-inflammatory, and anti-platelet actions, statins also have a role in the protection of endothelial cells by acting on the enzyme that produces nitric oxide [86]. These effects of statins might have significant therapeutic significance in the treatment of a wide variety of neurological illnesses. There is an expanding body of research that points to a connection between neurodegenerative disorders and vascular risk factors including atherosclerosis; nevertheless, this connection is still considered to be speculative. In this study, we highlight and discuss the current state of knowledge regarding the effects of statins in stroke, Alzheimer's disease, Parkinson's disease, multiple sclerosis, and primary brain tumors. In addition, we report the potential adverse effects of statins as well as the restrictions placed on the use of these drugs [87].

Research on the effectiveness of statins as treatments for Alzheimer's disease and stroke is advancing at a breakneck pace. There is a wealth of evidence to support the use of these medicines in the pretreatment of ischemic stroke as well as in patients who have a history of cerebrovascular illness [88]. In the acute period of stroke, the use of statins for their brain-protective impact is being studied, along with many other possible therapies.

6.3. Withania somnifera (Ashwagandha). One of the most expensive herbal medicines used in Indian traditional medicine (Ayurveda) is Ashwagandha, which is derived from the roots of the *Withania somnifera* Dunal plant and is used as a Rasayana medication to promote long life, young energy,

Table 1: Neuroprotective agent in intensive care for hemorrhagic stroke, acute ischemic stroke, and brain trauma management.

Neuroprotective agents	Class	Therapeutic applications	Recommended dosage	Witness the people	Result	References
Polyarginine R18 NA-1 (TAT-NR2B9c)	Glutamate blockers	Ischemic stroke	1000 nmol/kg	Rat	Maintained functional outcomes as well as reduced infarct volume.	[67–71]
Magnesium sulfate	Glutamate blockers/ NMDA channels blocker	Hemorrhagic and ischemic stroke, traumatic brain injuries	Up to 65 mmol/day	Patients were indeed human beings.	One explanation Mgso4 had such a successful aspect was the fact that it reduced late-onset ischemia of the central nervous system (DCI)	[60, 72–78]
Rosuvastatin and simvastatin atorvastatin, mevastatin	Statins	Ischemic stroke	Up to 20 mg/kg/ day	Mice and humans are the subjects of this investigation	a decrease in mortality, a decrease in the size of something like the infarct, and an increase in cerebral blood flow	[61–63, 65]
Melatonin	Hormone	Hemorrhagic and ischemic stroke, traumatic brain injuries	Up to 200 mg/kg/ day	Mice and rats from New Zealand's white rabbit electorate	Reduce oxidative damage by attempting to prevent endothelial cell vasospasm and apoptosis	[66, 79]
Erythropoietin	Growth factor for determining the ability	Stroke, both hemorrhagic and ischemic, and traumatic injury toward the brain	Up to 5000 units/ kg	Rabbit, rat, mice	a smaller infarct, less vasospasm, and an effective therapy in terms of function	[80, 81]
Cyclosporin A (CsA) and FK506 (tacrolimus)	Immunosuppressant	Strokes, brain trauma, ischemic stroke	As much as 10 milligrams per kilogram for CsA FK506 has a full dose of 6 milligrams per kilogram	Rat	Improved functional recovery, significantly reduced volume of infarct tissue	[82, 83]

and strong mental faculties [89]. Clinical studies have shown that Ashwagandha may cure a variety of conditions, including overall sluggishness, consumption, nervous weariness, sleeplessness, memory loss, and more [90]. By virtue of its historical applications, Ashwagandha could be able to treat neurodegenerative disorders. In fact, this herbal medication has been shown to have a variety of pharmacological actions, including those that are anti-inflammatory, antitumor, antioxidant, immunomodulatory, and antineuropsychiatric illness effects [91]. It is anticipated that therapeutic applications of Ashwagandha and its ingredients may result in improvements in neurodegenerative illnesses due to the plant's actions against these conditions [92]. Some organizations have in the past stated that Ashwagandha and its components are safe to use. In rats, giving them 100 milligrams per kilogram per day of an Ashwagandha water extract along with their drinking water for a period of eight months did not produce any harmful effects. The results of repeated oral administration of a methanol extract of Ashwagandha containing 80% (2000 mg/kg/day for 28 days) revealed no signs of toxicity [93]. However, acute toxicity was caused in mice when the alcoholic extract from the defatted seeds of Ashwagandha was given to them by oral administration; the LD50 value was 1750 41 mg/kg. After intraperitoneal injection of an ethanol extract of Ashwagandha, the LD50 in mice was determined to be 1259 mg/kg [94].

7. Neuroprotective Roles of Phytochemicals

Phytochemicals, from the Greek "Phyto" meaning "plant," are chemical substances produced by plants. That phytochemical is used by plants to defend themselves against microbes [95]. Plants employ phytochemicals to protect themselves not only against microbes, but also from environmental threats like pollution, stress, and UV exposure [66]. It is responsible for the colors, perfume, and flavor of plants.

Antifungal, chemopreventive, anti-inflammatory hepatoprotective, hypolipidemic, neuroprotective, hypolipidemic, and hypotensive properties are all antifungal, antiallergenic, anti-inflammatory, antiallergenic, antispasmodic, hypolipidemic, and hypotensive properties of phytochemicals [96].

More than 4500 phytochemicals have been identified, although only 350 have been thoroughly investigated [41]. Protective properties, as well as physical and chemical features, are used to classify phytochemicals. More than 120 traditional remedies have been used in Asian countries to treat central nervous system disorders [97].

Over antiquity, medicinal plants have provided an assortment of bioactive components that support in the health and quite well of humans [98]. When used as a neuroprotective agent, they also have few adverse effects [41]. Plants produce bioactive chemicals as secondary metabolites. On humans and animals, it has pharmacological or toxicological effects. Bioactive chemicals have health-promoting effects on the body. They are being researched for cancer, heart disease, and other disorders prevention. Lycopene, lignan, tennis, indoles, terpenoids, glycosides, alkaloids, flavonoids, phenolic compounds, and other bioactive chemicals are examples. Plants and some foods, such like fruits, vegetables, nuts, and whole grains, contain modest levels of this substance. Carotenoids, choline, flavonoids, carnitine, coenzyme Q, dithiolthiones, phytosterols, phytosterols, glucosinolates, certain vitamins and minerals, polyphenols, tocotrienols, organosulfur compounds including isothiocyanates, lycopene, lignan, tennis, indoles, terpenoids, glycosides, and alkaloids are also included.

Medicinal plants are high in phytochemicals and antioxidants, which may assist to control the disease by reducing the progression of symptoms and problems while having few or no adverse effects [99]. About 80% of people in poor nations rely on primary healthcare for men and cattle [47]. Some medicinal plants and their bioactive compounds for neuroprotection are depicted in Table 2 [100].

7.1. Bacopa monnieri. Phytochemical *Bacopa monnieri*, rather than Brahmi, has neuroprotective properties [101]. It has been widely utilized in neuro medicine to treat a variety of ailments, including anxiety, depression, and memory loss. It possesses antioxidant, adaptogenic, and memory-enhancing properties. Bacoside A is a Bacoside chemical molecule with neuropharmacological effects. Bascopaside III, bacopaside X, bascoside A3, and bacopasaponin make up this chemical molecule. As a result, passive diffusion over the blood-brain barrier is accomplished by the non-polar glycosidic structure of A.

Several studies have shown that backside A, a bioactive component, protects the brain from oxidative damage and age-related cognitive decline through a variety of methods [102]. As illustrated by a high-resolution liquid chromatography (HPLC) study, it reduces Abeta aggregation and fibril formation that could interact directly or indirectly with neurotransmitter systems to improve memory and learning potential.

7.2. Allium Stadium. Allium stadium is renowned as "garlic" by the general public. You can get it in a range of methods, and it is a well-known and valuable spice [103]. Antioxidant, hypotensive, antimicrobial, antifungal, antitumorigenic, immunomodulatory, anti-inflammatory, hepatoprotective, anthelmintic, anticoagulant, and fibrinolytic characteristics are all present in it [104–107]. Garlic is high in potassium, phosphorus, zinc, sulfur (at least 30 sulfur-containing compounds including alliin, allyl propyl disulfide, vitamin A, C, and B complex, allicin dats, s-allyl cysteine, vinyldithiins, same), and myrosinase and low in sodium, ajoene, and various types of enzymes such as alliance and peroxidase. Garlic consumption has numerous health benefits due to its therapeutic properties against cancer, diabetes, hyperlipidemia, lowering blood pressure, bone and skin diseases, Parkinson disease, atherosclerosis, type 2 diabetes, and aged garlic extract, and its components exert neuroprotective effects in Alzheimer's disease, cardiovascular disease, Huntington disease, and cerebral ischemia models [108]. Garlic extract has been shown to preserve dopaminergic neurons in Parkinson's disease research. Oxidative stress, inflammation, and

Table 2: Neuropsychopharmacological consequences of medicinal plants.

Bioactive compounds	Medicinal plants	Therapeutic applications	Impact/action	References
Carvone, allyl tetrasulfide diallyl disulfide, and diallyl trisulfide	*Allium sativum*	Neuroprotection	Simple recollection, gliosis, and oxidative stress are all directly affected	[110]
Bacoside	*Bacopa monnieri*	Protective lead for Alzheimer's chronic conditions	Defend the brain from oxidative damage and the decreased cognitive function that comes with increased age	[111]
Asiatic acid, M-adeacamic acid, and brahmaside as well as flavonoids madecassoside and madesiatic acid	*Centella asiatica*	Antimicrobial, anti-inflammatory, anticancer, neuroprotective, cytotoxic	Preventing the emergence of amyloid plaque in Alzheimer's disease, as well as reducing dopamine neurotoxicity in Parkinson's disease, is the highest priorities of enzyme inhibition	[112]
Monoterpenes (linalool, alpha-terpinyl acetate, andnerol acetate) sesquiterpene esters (Suchar malhangunoil, Balkan gun in, valerenal, global) Vioridiflorol, cubenol, agarouran derivatives, diterpenoids such as lupeol, pristine in, pristine in, zyeylosteral, alkaloids such as celapenin, celapenigin, panculatine, celestine maymyrone, tatty acids, steroids such as serpentine, flavonoids, benzoic acid, and vitamin C	*Celastrus paniculatus*	An expected performance for neuroprotection in the management of neurodegenerative diseases the same as Alzheimer's and other neuronal disorders	Inhibits the levels of noradrenaline, dopamine, and 5-hydroxy tryptamine	[113]
Sesquiterpene alkaloid	*Huperzia Serrata*		Neuroprotective against a beta-amyloid peptide fragment, potent AChE inhibitor	[114]
6-gingerol	*Zingiber Officinale*	Treatment of Alzheimer's disease		[115]
Clerodane diterpenes	*Croton yanhuii*	Treatment of Alzheimer's disease		[116]
Xylocarpin B, Xylocarpin G	*Xylocarpus granatum*	Neuroprotective property		[117]
Resveratrol	*Vitis vinifera*	Neuritogenesis, neuroinflammation, neuroprotection property		[118]

mitochondrial malfunction have also been prevented by the meal replacement, along with cell death. Adult male Wistar rats were given AGE (aged garlic extract) in doses of 125, 250, and 500 mg/kg, respectively, based on their body weight. It was repeated every day for 56 days. They were subsequently given a bilateral injection of 1 l of aggregated A (-amyloid) into the lateral ventricles. An NUR test seven days apparently showed that AGE dosages of 250 mg and 500 mg/kg BW significantly improved short-term recognition memory in cognitively impaired rats and also reduced the inflammatory response by minimizing microglia activation (-amyloid) in the cerebral hemispheres. In cognitively challenged rats, AGE dosages of 250 mg and 500 mg/kg BW drastically enhanced short-term recognition memory and also reduced the inflammation response by lowering microglia activation seven days later [109].

8. Potential Role of Antioxidant Vitamins and Synthetic Compounds for Neuroprotection

The breakdown of the equilibrium between pro-oxidant and antioxidant owing to an excessive buildup of reactive oxygen species is known as oxidative stress (ROS). The central nervous system is especially vulnerable to ROS due to its high energy demand and metabolic rate, as well as an insufficient antioxidant defense mechanism and reduced ability for cellular regeneration [119]. An excessive amount of ROS may result in significant pathological damage. These pathological damages include inflammation, cell cycle regulation, stressor responses, enzyme and receptor activation, phagocytosis, more signal transduction, and gene expression. ROS, when present at quantities that are not excessive, are essential for the proper functioning of a

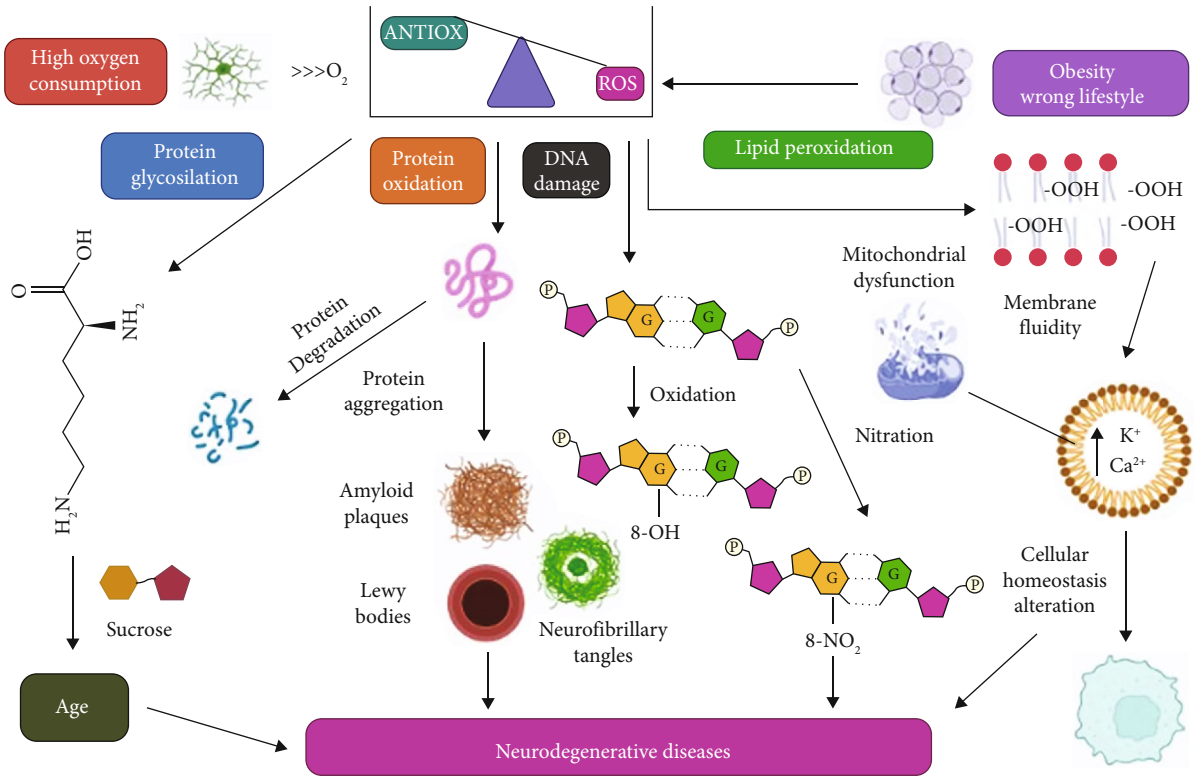

Figure 2: Oxidative stress hypothesis as well as its effects on a cellular level. What happens to free radicals when they are formed in cells? Because of the brain's high oxygen demand, ROS (reactive oxygen species) are constantly being produced (ROS). Since their high reaction rate increases oxidative stress and thus the formation of AGE and/or protein function loss, they also cause (i) protein oxidation and glycosylation, which leads to protein degradation; (ii) cell peroxidation, which reduces membrane fluidity and increases cellular permeability, which alters homeostasis in cells, and neurodegenerative diseases may be caused by any of these; and (iii) reasons DNA damage through guanine nucleotide oxidation or reduction [127].

number of physiological processes, such as signal transduction and gene expression [120]. Specifically, ROS oxidize polyunsaturated fatty acids, which are key biological targets. Nucleic acids are another potential biological target of free radicals. Ascorbate, commonly known as AA (ascorbic acid), is a potent antioxidant that is water-soluble and serves as a cofactor for a variety of enzymes. It is one of the most prevalent antioxidants [121, 122]. It is able to prevent the production of reactive oxygen species (ROS), directly remove ROS and RNS from the environment, and restore the functionality of other scavengers that have been damaged by oxidation. The AA concentration in striatal extracellular fluid was lowered in a transgenic HD mouse model; therefore, high dosages of ascorbate were used to restore the mice's normal behavior [123]. Tocopherols and tocotrienols are the two main classes of lipid-soluble antioxidants that make up vitamin E [124]. Among them, alpha-tocopherol is the type of vitamin E that exhibits the highest level of biological activity [125]. It is an antioxidant that can break chains while maintaining a low molar ratio in comparison to unsaturated phospholipids. Vitamin E protects cellular membranes from oxygen free radicals produced by polyunsaturated fatty acids and scavenges superoxide and hydroxyl radicals. Vitamin E recycling by vitamin C, ubiquinols, and thiols restores its antioxidant activity (Figure 2) [126].

8.1. Cellular and Molecular Immune Mediators of Neuroprotection. A neuroprotection system or technique aims to prevent the nervous system from being damaged or injured, especially in people who have been injured or diagnosed with a degenerative disease [128]. Neuroprotection aims to reduce nerve death following a CNS injury and to protect the CNS from early degeneration and other causes of nerve cell loss [50]. In order to beat infections and cope with stress, the immune system and the CNS appear to have evolved a wide and varied set of mechanisms. This is because the CNS immune response has a large number of failsafe mechanisms that facilitate a well-regulated response to injury and the commencement of healing and repair [51]. Neurotrophins are important regulators of neural growth function, survival, and the ability to change (plasticity) [122, 129]. Nerve growth factor (NGF) is a neurotrophic factor that promotes the development and survival of peripheral sensory and sympathetic nerve cells in mammals, including humans. It was identified in 1950 [54]. NGF functions by activating two transmembrane receptors. Another one is the p75 receptor, which actually applies to the tropomyosin receptor kinase (trk) family [130]. Exosomes are important mediators of neurodegenerative disorders, carrying beta amyloid and prions from their source cells to other cells [56]. Exosomes may regulate neuroinflammation, enhance neurogenesis and neurogenic

physiological location, and cure neurological disorders [131]. Gene expression is regulated by the cAMP-responsive element-binding protein (CREB) pathway, which participates in two key gene expression cascades. The first one describes CREB as an essential part of the molecular switch that regulates more permanent kinds of neural plasticity and learning. The second one connects CREB to the maintenance and protection of neuronal survival [58]. CREB plays an important role in a variety of cell processes, metabolism, including proliferation, differentiation, and survival [59].

9. Turmeric and Choline in Diet Can Increase the Neuroplasticity

9.1. Turmeric. In Southeast Asia, turmeric is widely used as a spice. It contains numerous health benefits, particularly in the cases of Alzheimer's disease and Parkinson's disease. Turmeric contains polyphenolic curcuminoids, diferuloylmethane, bisdemethoxycurcumin, dimethoxy curcumin, and cyclocurcumin, among other beneficial compounds. Curcumin contains numerous important physiological effects, anti-rheumatoid, including anti-inflammatory, antispasmodic, anti-allergy, and anticancer characteristics [132–134]. Curcumin activated extracellular signals, which regulated neuronal plasticity and stress response transmission [59].

9.2. Choline. Among the foods that are high in cholesterol per gram are eggs, beef liver, chicken liver, and meats. Choline has a variety of functions in the human body, ranging from cell structure to neurotransmitter production. Its deficiency, on the other hand, has an impact on a variety of ailments, including liver disease, atherosclerosis, and neurological disorders. Neuroplasticity in the adult central nervous system can be altered by replenishing with B vitamins like folic acid and riboflavin as well as choline [135, 136]. On days 0 and 14, Balb/c mice were sensitized with 100 g ovalbumin and then challenged with aerosolized ovalbumin on days 25–27. On days 14–27, mice were given 1 mg kg–1 choline either oral gavage or intranasal method. In addition, mice were given 100 mg kg–1 of lipoic acid as a conventional antioxidant. In bronchoalveolar lavage (BAL) fluid, total cell counts, eosinophils, and eosinophil peroxidase (EPO) activity were measured. In BAL fluid, levels of reactive oxygen species (ROS), lipid peroxidation, and isoprostanes were assessed. The levels of IL-13 and tumor necrosis factor-alpha (TNF-) in BAL fluid and spleen cell culture supernatant were also evaluated. After the final ovalbumin challenge, the expression of the nuclear factor B (NFB) p65 protein was examined in lungs' nuclear and cytosolic extracts. Treatment with choline and lipoic acid significantly reduced eosinophilic infiltration and EPO activity in BAL fluid compared to mice that were exposed to ovalbumin. Reducing the levels of ROS and isoprostanes in BAL fluid was achieved through the use of choline and lipoic acid therapy [137, 138].

10. Novel Therapeutic Agent

10.1. Phytocannabin. There has recently been a surge in interest in researching cannabis-based products for medical purposes [139]. However, research in the field of neurology is still lacking, and further randomized double-blind placebo studies are needed [140]. People who have seizures linked to certain epileptic syndromes and MS patients who experience spasms have been shown to benefit from Class I evidence of the goods being tested for suitability keeping in mind that not all cannabis-based products are created equal is essential in the process of labeling [141]. In the absence of FDA oversight, products sold and marketed for human consumption are neither regulated nor subjected to rigorous testing. Furthermore, there is still debate on brain inhibition. Other potential symptom exacerbating characteristics of various marijuana compounds Marijuana's chemical constituents interact with other drugs. There is still more to learn [139]. Obtaining information regarding the use of these products is common, not by medical providers, but by other patients, advertisements, or media sources [142].

MS symptoms can be relieved with a combination of 9-THC and CBD, which led to the development of the first legal PCB medication, Sativex. As a neuroprotective agent, CBD's ability to influence immune cell activity in the central nervous system (CNS) and limit oxidative stress is very promising. In particular, CBD's ability to modulate CNS immune cell activity and limit oxidative stress is very promising. However, it is important to note that previous antioxidant and anti-inflammatory-based treatments for neurodegenerative diseases have had minimal clinical efficacy in many cases [143]. There is currently very little human data on PCB effects in neurodegenerative illnesses aside from the favorable evidence on the benefits of SCEs on MS symptoms gathered over the last decade. For patients with Alzheimer's disease, Parkinson's disease, and other neurodegenerative diseases, clinical trials examining the effects of PCBs on both disease progression and symptom control are needed. It does seem that PCB-based therapies, regardless of the target condition, are well tolerated, which is a positive sign for future trials. Antioxidant properties of other PCBs have been overlooked, but they will be better suited to specific illness. 9-THCV, for example, negatively regulates both neuronal cell death and probably results immunological response in models of Parkinson's disease, while reducing signs of bradykinesia.

10.2. Rosmarinus officinalis. Neuroprotective properties have been found in Rosmarinus officinalis, and it is a surprising discovery. Alzheimer's disease and dementia are examples of such disorders. Rosemary is a woman who proved inhibitory activity using the two enzymes such as butyryl cholinesterase (BChE) and acetylcholinesterase (AChE). Acetylcholine-cholinesterase is responsible for its breakdown. These are also responsible for the essential oils of the plant [144]. Enhancing Rosemary has been shown to reduce total choline levels in the brain. Memory loss, anxiety, and depression are all symptoms of Alzheimer's disease [135, 136]. Two more studies show that the ability to protect the brain is beneficial. R. officinalis is a medicinal plant. The first is polyphenols, which are found in rosemary. Stress proteins, which play a role in disease, were found to be inhibited by the extract the neurodegenerative process [145]. Rosemary has also been shown to increase the production of

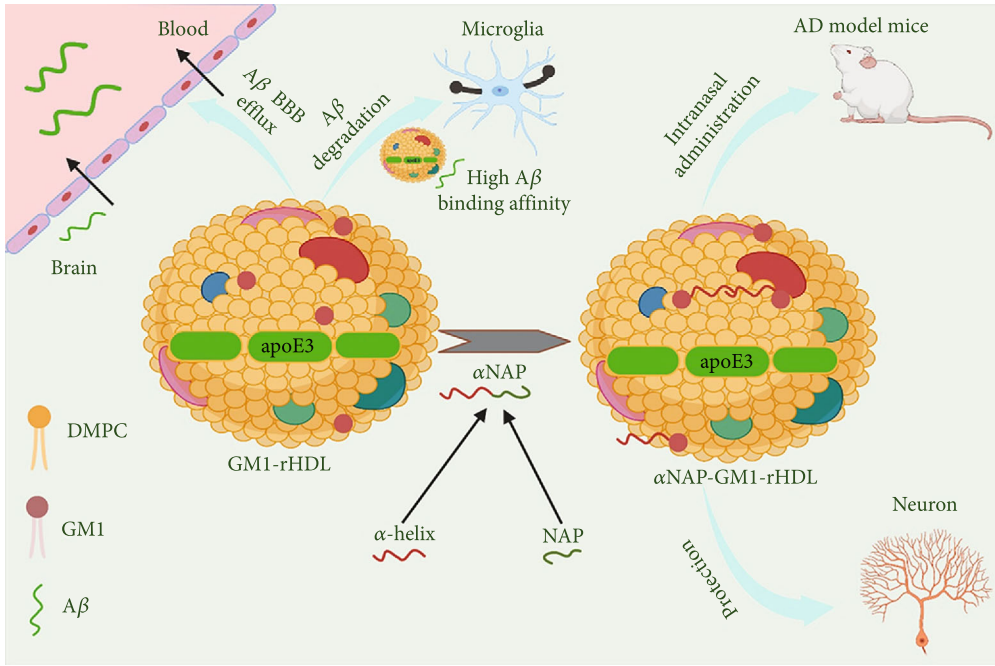

Figure 3: Monosialotetrahexosyl ganglioside-incorporated reconstituted high-density lipoprotein (GM1-rHDL) possesses antibody-like high binding affinity to Aβ, facilitates Aβ degradation by microglia and Aβ efflux across the blood-brain barrier (BBB), and simultaneously allows the efficient loading of neuroprotective agents, serving as a nanoparticulate drug delivery system for the combination therapy of AD [155].

nerve growth factor (NGF), a protein that is essential for nerve growth and maintenance. Alzheimer's disease may benefit from increased NGF levels. So, this study has showed that Rosemary has a lot of roles to expand as a plant's neuroprotective substance.

10.3. Nano Therapy. Alzheimer's disease treatment and prevention have used nanotherapeutic methods (Figure 3). Three barriers protect the brain and spinal cord: the blood-brain barrier (BBB), the blood-cerebrospinal fluid barrier (BCSFB), and the ependymal barrier (CNS). As the cerebrospinal fluid (CSF) flows through into the choroid plexus of the ventricles, the BCSFB acts as a barrier between the fluid and brain tissue. Communication between the central nervous and blood is managed by the biological membrane (BBB) [146]. Aside from protecting the CNS from harmful chemicals and promoting hemostasis, the BBB also blocks the delivery of medications to the CNS. The BBB [147] allows only extremely lipid-soluble molecules with a molecular weight (MW) of less than 400 Da to pass through. The BBB is changed in AD, according to various findings.

BBB disruption could be both a cause and a symptom of Alzheimer's disease. Three types of BBB damage have been identified as contributing to the start of AD: leaking of unwanted substances from the bloodstream into the CNS, transport system malfunction, and changes in protein expression in endothelial cells [146]. Medication delivery may be managed to improve by trying to disrupt the BBB, which increases BBB permeability, reduces efflux transporter expression, and reduces CSF reabsorption. Due to a decreased BBB in some cases of Alzheimer's disease, medication distribution to the CNS is diminished, which is extremely damaging to drug delivery [146].

Because such nanoparticles have a high surface to volume ratio and can be synthesized and characterized with desired ligands, nanotherapeutic methods that would be used in noninvasive frameworks have shown promise in overcoming the BBB impediment. Multiple nanotherapeutic methods, such as targeting A, cholinesterase downregulation, and dissolving fibrinogen clots, outperformed conventional therapy significantly [148]. Genetically restricted or regulated expression of the A peptide, suppression of the fibrillation process, and removing accumulated A amyloids from the brain have all been utilized to target A plaques. The amyloid precursor protein (APP) is cleaved at the N-terminus by BACE1's protease activity, resulting in the A-amyloid peptide. The BACE1-siRNA was efficiently scattered using synthesis of nanomethods and was using naturally inert exosomes to limit its activity. To reduce immunogenicity and maximize the efficacy of silencing, the targeted exosome nanocarriers were obtained from C57BL/6 mice's bone marrow cells. Another way to counter A plaques is to slow or stop the nucleation-dependent process that produces fibrils and the resulting plaques. DSPC-Chol liposome NPs functionalized with an A monoclonal antibody were checked on postmortem AD brains (A-MAb) [149] that backed up the effectiveness of the proposed technique. The removal of brain plaques caused by decrepitude is the third procedure. Gold (Au) NPs and reconstituted high-density lipoprotein NPS expanded BBB permeability and, as a result, targeted efficacy in resolving and destroying aggregates. It was highly suggested by innumerable

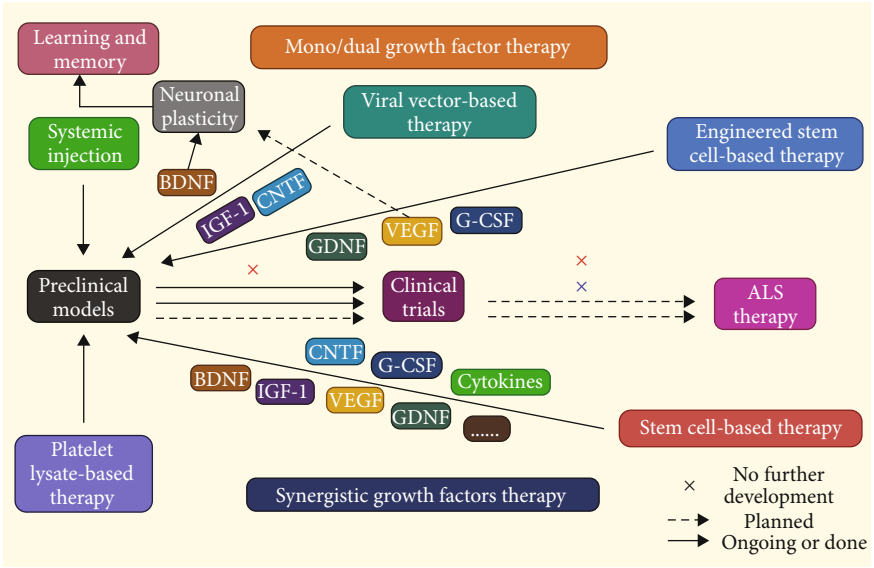

Figure 4: Individual neurotrophic growth factor therapy for synergistic effects. (i) Synergistic growth factor therapy. (ii) Mono/dual growth factor therapy. (iii) ALS therapy [177].

epidemiological studies, which was eventually approved by the FDA, following the failures of many clinical trials connected to the A cascade concept. Using modified poly (n-butyl cyanoacrylate) NPs with polysorbate 80 and additionally chitosan NPs, nanotechnology-based treatments deliver the cholinesterase inhibitor rivastigmine to the brains of Wistar rats via intravenous and intranasal injection, respectively [150, 151]. Cerebrovascular risk factors have been linked to the severity of Alzheimer's disease and cognitive impairment in some epidemiological studies. When cerebrovascular dysfunction occurs alongside Alzheimer's disease, the pathological symptoms are increased [152]. Fibrinogen can enter AD brains through disrupted BBBs, resulting in abnormal clots, especially when A is present. Based on this information, EMT zeolite NPs were developed to inhibit A interaction with fibrinogen and the rate of A fibrillation. Like uncovered EMT zeolite NPs, their fibrinogen binding strength was inhibited after they were covered by the corona layer [153]. Safety and toxicological methods have limited translational pathways even though clinical research based on nanotechnology is still active, as safety concerns about nanoparticle-mediated adverse effects have become more significant when medication delivery to the central nervous [154]. It is becoming easier for pharmaceutical companies to conduct preclinical evaluations and high-throughput screenings of small compounds because of the development of brain-mimetic 3D-culture models.

11. Neuroprotection: Challenges and Opportunities

Demonstrating the effectiveness of any neuroprotective therapy in people is the main challenge in neuroprotection. Classical pharmacology, complicated approaches such as deep brain stimulation, and traditional pharmaceutical equipment are all being explored as part of neuroprotective therapy (ENT) [156]. Recent developments in both experimental technique and the design of clinical trials have prompted cautious hope. Such concerns as therapeutic window, dose-response profile, and CNS penetration have been more consistently addressed in experimental pharmacological investigations [157]. There are numerous methods and assays that may be used to determine the status of the OxS biomarkers in a range of biological samples, which is a major advantage but also a downside. Each method has its own set of pros and limitations, as well as practicality and economic considerations [158]. The interpretation of data may also be complicated by a number of pre- and post-analytical difficulties, which require further standardization such as preanalytical issues, analytical issues and, postanalytical issues [159]. However, in diseases such as Parkinson's (PD) and Alzheimer's (AD), neuroprotective interventions are required to reduce neuronal death, whereas in conditions such as ALS (Figure 4), autism spectrum disorders (ASD), spinal cord injuries, and other such conditions, restoration or both protection and restoration are required to restore neurons [156]. A lethal form of persistent neurodegeneration, ALS (amyotrophic lateral sclerosis) is characterized by the presence of proteinaceous, ubiquitinated cytoplasmic inclusions in affected motor neurons as well as in cells surrounding these neurons [160]. Apoptosis may have a role in neurodegeneration, according to some research, but others disagree. This controversy must be settled since it is therapeutically significant. Clarifying MN death pathways gives reasonable targets for future ALS treatments. A primary focus of study needs to be on finding models of motor neuron degeneration that are the most accurate representations of motor neuron death in clinical ALS (outstanding issues). Identification of MN cell death pathways and evaluation of medications and biological substances for neuroprotective properties are both made possible via the use of animal and cell model system research [161]. Traumatic brain injury (TBI) is the leading

cause of mortality and disability in those under 45 worldwide. The knowledge of pathophysiological events has been expanded as a result of numerous experimental and clinical studies of biomechanical injury and tissue damage. This information has the potential to serve as the foundation for the development of novel treatment techniques as well as the enhancement of treatment strategies that are already in use [162]. Dementia treatment differs based on the kind of dementia detected in people who have had traumatic brain injuries. When treating Alzheimer's disease or any form of dementia, patients with and without a previous history of traumatic brain injury should adhere to the same treatment protocols [163]. In the case of mild traumatic brain injury (TBI), neuropsychological tests are often used in combination with imaging to assess brain function. Memory, focus, information processing, executive functioning, and response speed are some of the cognitive abilities that may be assessed with these tests [164].

Neuroprotection is a treatment method that modifies the consequences of the ischemia cascade or facilitates reperfusion in order to protect neurons from suffering irreparable damage. This is done in the hopes of preventing neuronal death [165]. Despite the fact that various medicines have shown neuroprotective effects in preclinical studies, the translation of those findings to clinical trials has failed to show any significant impact. The NDDs may be distinguished from one another in large part by the anatomical locations that exhibit neuronal dysfunction, biochemical and structural changes in protein indicators, and neuronal cell diseases including the deposition of protein(s), as well as alterations in genetics and epigenetics [166]. Since it is undeniable that OxS plays a role in the etiology of many chronic degenerative illnesses as well as the aging process, several investigations into the potential advantages of antioxidant treatment have been made. Exogenous supplementation is justified in order to preserve the general public's wellness and health by avoiding the onset of illness, its progression, and its repercussions. Genome instability is mostly caused by endogenous stress. It is true that the main force for genomic evolution, genetic diversity, is necessary for physiological activities. The goal of the most recent diagnostic studies is to create readily identifiable biomarkers from saliva or blood to discriminate between the many types of neurological diseases.

11.1. Neuro-Imaging in the Identification of Severe Disorders Caused by Neurodegeneration. The unique protein that aggregates characterizes each neurodegenerative disease type. Extensive research has recently been conducted on disease-modifying medicines for neurodegenerative diseases (e.g., Alzheimer's disease and tauopathies), with the hope of developing them in the near future. Urgently needed for the correct diagnosis of neurodegenerative diseases as well as the facilitation of the creation of disease-modifying therapies are disease-specific biomarkers that are both straightforward and applicable [167]. Characteristic imaging findings in neurodegenerative diseases are described in several diagnostic criteria. Furthermore, only a small number of current diagnostic criteria for neurodegenerative disorders have identified neuroimaging methods as biomarkers that might gauge the pathological changes taking place in neurodegenerative disease patients' brains. The importance of neuroimaging methods as biomarkers are discussed here for various neurodegenerative disorders such as magnetic resonance imaging, single photon emission computed tomography (SPeCT) and positron emission tomography (PeT), dopamine transporter imaging, 123iodine-metaiodobenzylguanidine, myocardial scintigraphy, Aβ imaging, and Tau imaging. Functional neuroimaging methods are used to diagnose the most common CNS illnesses (Parkinson's disease (PD), Alzheimer's disease (AD), Huntington's disease (HD), amyotrophic lateral sclerosis (ALS), and Multiple sclerosis (MA)) [168].

11.2. Anatomical Identifications of Neuronal Losses in relation to Clinical Symptoms. In order to get a proper grasp of the early symptoms, identification of the anatomical locations is required. For instance, the regions of the brain known as the entorhinal cortex, neocortex, hippocampus, and limbic system are responsible for symptoms such as cognitive decline, dementia, and other alterations in high-order brain functions, whereas thalamus, the basal ganglia, motor cortical, and brain stem areas are primarily responsible for disturbances in body movements. The majority of the time and combinations of these different kinds of symptoms are found throughout the evolution of illnesses that follow region-specific neurodegenerations.

The computational models that are going to be given here perform certain alterations on three-dimensional (3D) ensembles of neurons that represent healthy persons. The data from digital photographs of brain tissue were utilized to construct a reasonable three-dimensional arrangement of neurons with statistical features that were compatible with experimental data in order to establish these first ensembles [169]. This computational technology was developed in the past. To be more specific, the prior technique measured the microcolumnarity of a particular tissue slice in order to generate, with the help of a density map computation, the 3D representation of neurons that had the same statistical features as those seen in the experimental tissue [170]. After they have been created, these preliminary ensembles will be used as the foundation for stochastic simulations that will investigate different models of deletion and displacement. These simulations will begin after the preliminary ensembles have been formed. The models that are going to be described here consider groups of neurons that are arranged in a Cartesian coordinate system. This is done in order to facilitate the computations being performed with more ease [171]. We find that this limitation has no discernible impact on the scope of either our work or our conclusions when we contrast the results from our models with data obtained from straight sections of tissue that were locally fitted to this coordinate system. This method is widely used and has been implemented by others in order to keep from becoming confused by the impacts of curved areas. However, due to the nature of the methodology that has been presented in this article, it is possible to make straightforward generalizations to other coordinate systems. These other coordinate systems might be able to better describe curved regions of the brain, such as those that can be found in the lip or fundus of any sulcus [172].

11.3. Brain Mapping: A Diagnostic Tool for Neurodegenerative Diseases. The scientific community has been captivated for a very long time by the prospect of producing maps that may localize cognitive processes and alterations caused by illness to specific areas of the brain. Brodmann's cytoarchitectonic map, which was created in the early twentieth century and separated 52 cortical subregions based on their distinctions in thickness, lamination, neuronal type, and staining characteristics, is perhaps the most lauded brain mapping feat to date. Since the development of noninvasive neuroimaging techniques, brain maps have progressed to the point that they are now digital atlases that are highly complex, multidimensional, and multimodal. These atlases cover the whole of a human's life and depict the course of a number of disorders [173]. Since the middle of the 1990s, many potent brain mapping methods have been developed. A lot of people rely on computational anatomy, a mathematical approach to modeling the brain in which brain surfaces and subvolumes are viewed as complex geometrical patterns and are modeled as 3D continuous mesh models or deformable shapes that can be averaged and combined across subjects, and on which statistics can be defined [174]. Thus, the anatomical pictures are changeable templates that may be elastically or fluidly reshaped into a comparable shape, most often onto the average of the research group, an atlas average, or the brain shape of another individual [175]. Some of these methods employ surface markers as constraints to explicitly represent the anatomy of the brain (e.g., sulci). Without losing the underlying subtleties in the measure of interest, these strategies easily enable the correct alignment of surface-specific geometrical patterns (such as gyri) and assist in accurately colocalizing identical cortical and subcortical areas (e.g., cortical thickness, functional activation, or gray matter density). The ability to describe cortical and subcortical illness patterns and identify subtle disease-associated alterations is enhanced by the ensuing anatomical coregistration [176].

12. Conclusion and Future Perspective

Many neurodegenerative diseases, such as Alzheimer's, Parkinson's, and amyotrophic lateral sclerosis, are caused by free radical damage to human nerve cells. This gang of neurodegenerative diseases is to start blaming for the obliteration of DNA, lipids, and proteins. Phytochemicals, magnesium, choline, and turmeric in our diet all play a role in neuroplasticity by acting as neuroprotective agents. This review illustrates the different neuroprotective agents and their functions.

Neuroprotective agents have been shown to be beneficial in a variety of models, including rats, mice, and drosophila, as well as humans. Various neurodegenerative infections can be treated with bioactive molecules found in medicinal plants because medicinal plants are shown to have neuroprotective properties. Alzheimer's disease people can benefit from AGE, a supplement food that improves cognitive function, as a supplement.

Dopaminergic neurons in Parkinson's disease are protected by Bacoside A and Donezil, and allium stadium has been shown to protect cells from oxidative stress while also inhibiting A aggregation and fibril formation. Clinical trials have shown neuroprotective effects, but this is not sufficient. A large sample size is needed for the clinical trial. This review could be helpful for future research priorities on various phytochemicals that can be used as a medicine because they had been shown already a wide range of health benefits such as inhibiting of Aβ aggregation and formation of fibrils, decreasing the amount of Aβ fibris, and protecting the dopaminergic neurons in Parkinson's disease and also are capable of protecting cells from oxidative stress. Despite numerous clinical studies demonstrating neuroprotective financial advantages, more research needs to be done. Large numbers of people should be involved in the research. There are numerous phytochemicals that can be used in medicine, and a review of this material could help guide future research because a wide range of health benefits had already been illustrated.

Data Availability

All data are available within the text.

Conflicts of Interest

All authors declare that they have no conflicts of interest.

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Review Article

Polyphenols Targeting Oxidative Stress in Spinal Cord Injury: Current Status and Future Vision

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A spinal cord injury (SCI) occurs when the spinal cord is deteriorated or traumatized, leading to motor and sensory functions lost even totally or partially. An imbalance within the generation of reactive oxygen species and antioxidant defense levels results in oxidative stress (OS) and neuroinflammation. After SCI, OS and occurring pathways of inflammations are significant strenuous drivers of cross-linked dysregulated pathways. It emphasizes the significance of multitarget therapy in combating SCI consequences. Polyphenols, which are secondary metabolites originating from plants, have the promise to be used as alternative therapeutic agents to treat SCI. Secondary metabolites have activity on neuroinflammatory, neuronal OS, and extrinsic axonal dysregulated pathways during the early stages of SCI. Experimental and clinical investigations have noted the possible importance of phenolic compounds as important phytochemicals in moderating upstream dysregulated OS/inflammatory signaling mediators and axonal regeneration's extrinsic pathways after the SCI probable significance of phenolic compounds as important phytochemicals in mediating upstream dysregulated OS/inflammatory signaling mediators. Furthermore, combining polyphenols could be a way to lessen the effects of SCI.

1. Introduction

Neurodegenerative disorders (NDDs) progressively affect millions worldwide as significant causes of disability and death, despite progress in considering various dysregulated routes in the pathophysiology of NDDs. The main pathophysiological processes of NDDs are still unknown [1–4]. Spinal cord injury (SCI) is an NDD that causes sensory-

motor impairment and significantly lowers the standard of living. SCI is becoming more common among people aged 14.6 to 67.6 years old, and men are four times more likely than women [5, 6]. SCI has primary and secondary phases from a pathophysiological standpoint. The secondary step comprises inherent oxidative stress (OS), autophagic, apoptotic, and inflammatory routes. Direct injuries occur after spinal mechanical trauma [7].

In contrast, extrinsic routes have an essential role in SCI, such as glial scar development and destruction [8]. Extrinsic pathways are coupled with intrinsic processes such as OS, neuroinflammation, and neuroapoptosis (e.g., axonal signaling). Thus, the preceding pathogenic pathways negatively affect neurodegeneration and neurodegenerative mechanisms, eventually leading to apoptosis. Antioxidant defenses modulate neuroinflammatory and neuroapoptosis responses, which influence microglia, astrocytes, and related mediators and have a considerable position in the initiation and development of SCI [9, 10].

It is crucial to highlight that developing new plant medications has a compelling track record in producing unconventional therapeutics. Incidentally, the plant kingdom has demonstrated encouraging outcomes in that against SCI. Polyphenols/phenolic combinations are obtainable phytochemicals and can act as multiple targeted drugs with excellent selectivity and minimal toxicity among natural substances, because of their broad biological activity and therapeutic properties are now used in contemporary medications to construct and acquire novel treatments. In many NDDs, these substances have been regarded as reliable nutritional mediators with potent repressive impacts on OS and inflammation [11]. Emerging research has recently focused on utilizing organic neuroprotective polyphenols with putative antioxidant properties to treat SCI and NDDs [12]. This review discussed about the oxidative-mediated polyphenols' role in controlling and managing SCI.

2. Methodology

PubMed, Scopus, and Web of Science were all used to conduct this literature review. The terms polyphenols, SCI, oxidative stress, reactive oxygen species, preclinical studies, and clinical studies were utilized. We selected and analyzed English-published research papers, narrative review articles, and primary research articles until June 2022. An algorithm used the flowchart imposed in Figure 1 (according to Page et al.'s guidelines [13]) and contained all of the steps/selection constraints for the required literature.

3. Spinal Cord Injury Pathophysiology

SCI is categorized into primary, secondary, and chronic [14, 15]. The first stage is the physical forces related to the original traumatic event, often the essential factors of injury severity, causing the first stage. Compression, shearing, laceration, and severe stretch/distraction are examples of these forces [16]. Following the original injury, a series of subsequent occurrences occur. The damage worsens in the second stage, and neurological impairments and consequences worsen [17, 18]. After the first injury, secondary SCI is a gradual and progressive injury (Figure 2).

Furthermore, a chronic stage could affect the orthograde and retrograde routes and brian-specific regions; moreover, according to the time scale, chronic stages can start from days to years following the damage [19, 20]. Several vascular alterations are detected during the secondary cascade [21]—neutrophils and macrophages and role in releasing superoxide anion and hydrogen peroxide to sanitize the wounded area. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is a significant superoxide anion originator of superoxide anion that plays a role in activating the hematogenous phagocytic cells [22]. Moreover, the phagocytic inflammatory cells work as reactive oxygen species (ROS) producers. At the same time, the free radicals respond to polyunsaturated fatty acids, which lead to a phospholipid structural design disruption of cellular and subcellular organelle membranes. Furthermore, aldehyde molecules produced by lipid peroxidation prevent metabolic enzymes, such as Na^+/K^+-ATPase, from working precisely [23].

SCI causes an increase in cytokines containing tumor necrosis factor-alpha (TNF-α), interleukin-1 (IL-1), and interleukin-6 (IL-6), as well as overexpression of nuclear factor kappa B (NF-κB), activator protein 1 (AP-1), c-Jun N-terminal Kinase (JNK), and other inflammatory and apoptotic factors like p38, mitogen-activated protein kinase (MAPK), and prostaglandin E2 (PGE2) [24]. The generation of excitation amino acids involving glutamate from damaged cells increases the discharge of excitation amino acids after SCI [25, 26].

Additionally, the glial scar formation, microglia/macrophages, reactive astrocytes, and extracellular matrix molecules—particularly chondroitin sulfate proteoglycans—at the chronic phase play a vital part in preventing axon growth by acting as a protective border [27–29]. Therefore, developing reliable methods and treatments for SCI patients becomes imperative. Reduced ROS levels are an essential approach for SCI management, which can be accomplished by employing antioxidants or drugs that standardize or modulate ROS signaling routes [30, 31].

4. Spinal Cord Injury and Oxidative Stress

Reactive nitrogen species (RNS) and ROS are frequently formed endogenously. However, an increased ROS construction may outpace the antioxidant defense capability, leading to OS and oxidative destruction (Figure 3) [32–35]. Superoxide is created by the NADPH oxidase (NOX), mitochondrial electron transport chain, and xanthine oxidase (XO), which response to nitric oxide (NO) manufactured by the nitric oxide synthase (NOS) to generate the peroxynitrite (ONOO) [36, 37].

Superoxide dismutase (SOD) is an enzyme that transforms oxygen (O_2) into hydrogen peroxide (H_2O_2). There are two similar forms of SOD: (1) copper (Cu)/zinc(Zn)-SOD and (2) manganese(Mn)-SOD. Zn plays a considerable part in the antioxidant defense scheme. According to the databases, the Zn condition and time-dependent modifications following SCI are still unknown [38–42]. The analysis

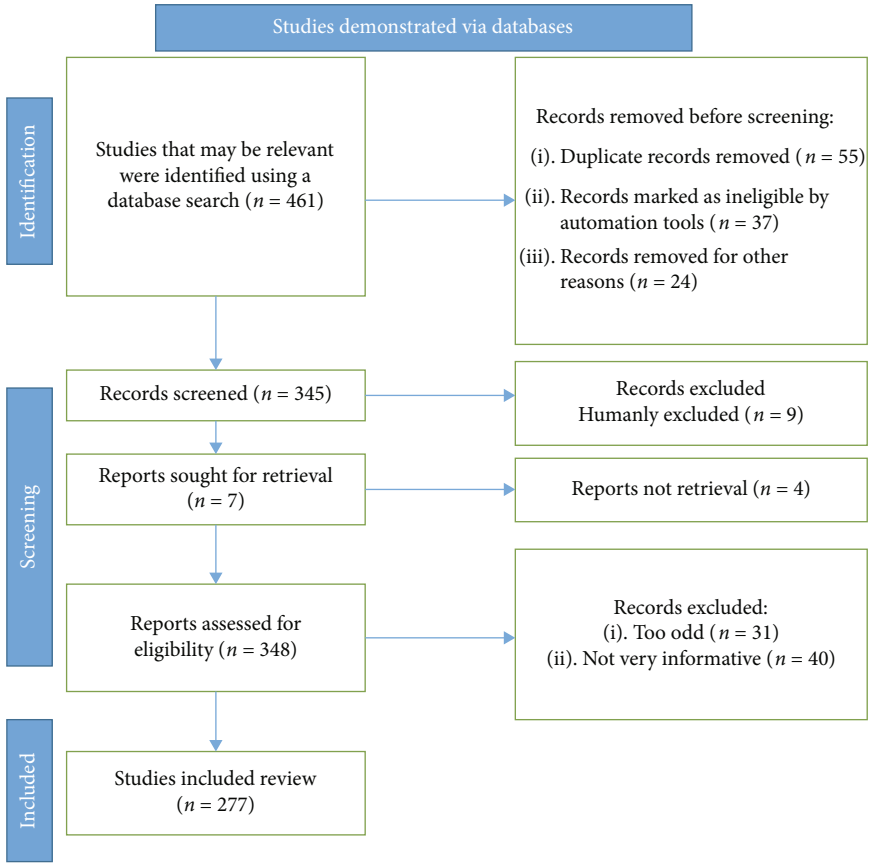

Figure 1: The stages of picking data for inclusion in the existing research are illustrated in a flow chart; n = number of literature reports.

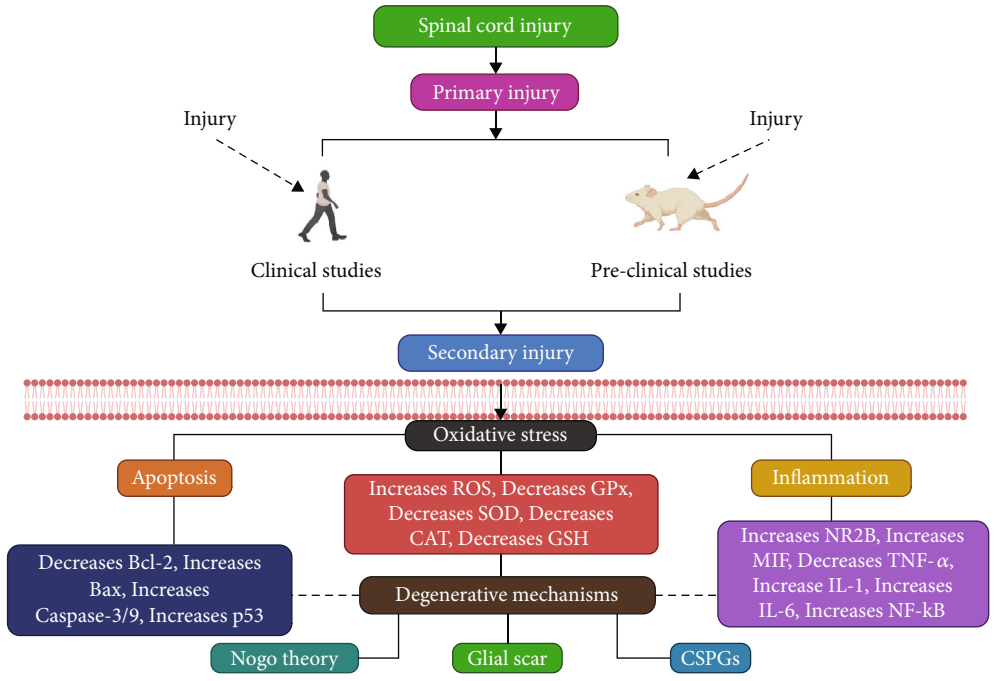

Figure 2: Pathophysiology of SCI: spinal cord injury. ROS: reactive oxygen species; GPx: glutathione peroxidase; SOD: superoxide dismutase; CAT: catalase; GSH: glutathione; MIF: macrophage migration inhibitory factor; TNF-α: tumor necrosis factor-alpha; and NF-κB: nuclear factor kappa B.

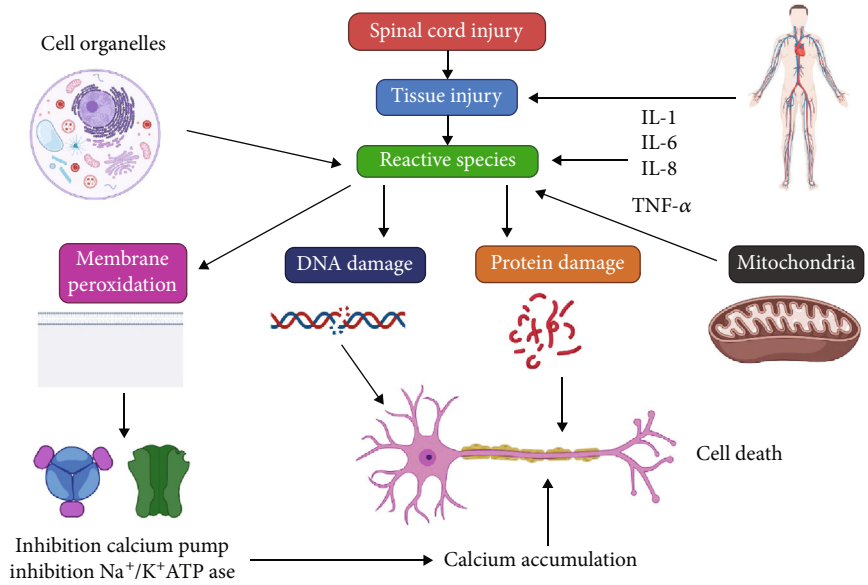

Figure 3: SCI can be facilitated by oxidative stress. TNF-α: tumor necrosis factor-alpha.

of Zn dynamics in 38 cervically damaged SCI patients yielded a prediction prototype for continuing functional prediction [41]. Heller and colleagues [42] looked at the vigorous variations in serum Zn intensity in short periods throughout the preliminary 72 hours after injury to see a link between early changes in total Zn serum levels and NDDs and patient outcomes. They discovered that patients with the cognitive disease have higher median Zn concentrations in the initial 9 hours after injury than patients with vertebral fractures who do not have neurological dysfunction. They established that the result is associated with early Zn level dynamics and could be an investigative tool for these patients. Alterations in serum Zn levels allow early assessing the risk of neurological damage [42].

In this context, it was discovered that Zn therapy aided motor control restoration in the 28 days that followed SCI and reduced ROS and increased antioxidant potential [43]. The Fenton reaction allows H_2O_2 to produce the highly reactive hydroxyl radical (HO•), that considers the leading cause of lipid peroxidation in the presence of iron. Catalase (CAT) and glutathione peroxidase (GPx) convert H_2O_2 to water and oxygen [44]. SOD, CAT, GPX, and glutathione reductase are the primary endogenous antioxidant enzymes [34].

The enzyme GPX is selenium (Se) dependent. By neutralizing reactive oxygen species (ROS) via GPX and reversible oxidation to glutathione disulfide, GSH acts as an antioxidant (GSSG). Glutathione reductase transforms into GSH. Meanwhile, XO produces superoxide but catalyzes the conversion of xanthine to UA, a compound that may scavenge superoxide. HO is the primary antioxidant in biological fluids. In rats, Se nanoparticles were shown to treat OS-induced SCI [45]. According to Seelig et al., Cu and Se concentrations upon intake and Se and ceruloplasmin levels after one day were indications of likely SCI clearance [46]. Within the secondary injury stage, magnesium (Mg) is assumed to play an important role. A better probability of neurological recovery has been associated with reduced Mg serum concentrations during the first seven days [47]. Mg acts by blocking ROS generation and lipid peroxidation precisely [48].

Acrolein, a reactive aldehyde generated endogenously by lipid peroxidation and involved in SCI, is more responsive than the other HNEs and causes glutathione deprivation [49]. To investigate the antioxidant potential of SCI patients, Bastani et al. examined a vast scope of antioxidant and OS markers. When evaluating persons with SCI to controls, they observed that urine F2-IsoP and specific enzymes (NOX and XO) in vastus lateralis biopsies enhanced while SOD decreased [50, 51].

5. Polyphenols in Spinal Cord Injury

To reduce OS after SCI, many natural polyphenolic combinations have been used [52]. These compounds impede the restoration of molecules following free radical damage and control various dysregulated pathways/mediators, such as blocking production. OH. Such polyphenols have formerly been prospective neuroprotective therapeutics in other OS-related NDDs (Figure 4) [53–55].

5.1. Epigallocatechin Gallate. The primary compound of tea catechins is epigallocatechin gallate (EGCG) (Figure 5), often called epicatechin. This composition is related to the biological functions of green tea extracts [56]. EGCG's anti-apoptotic, anti-inflammatory, and antioxidant actions have been demonstrated to prevent against NDDs [57], brain injury [58], SCI [59], and peripheral nerve damage [60] in many experiments conducted. The hydroxyl groups in the catechins ring B and D cause them to interact with free radicals [61]. For 24 hours, various doses of green tea polyphenols (Table 1) (50–200 μg/mL) prevented spinal neurons from oxidative damage caused by H_2O_2 [62].

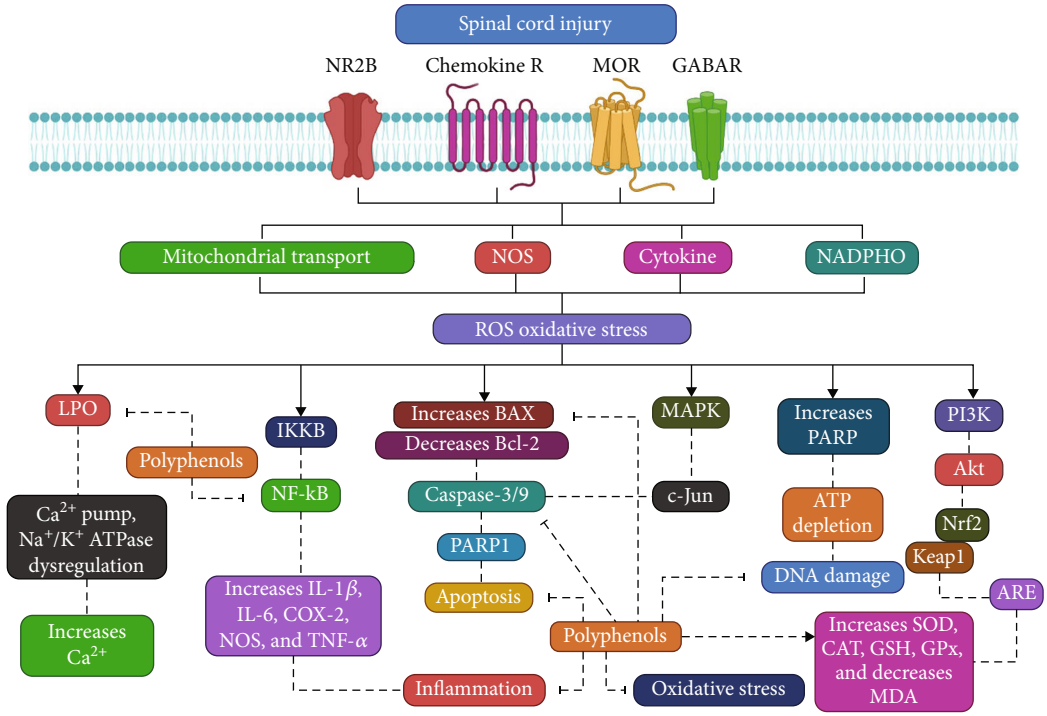

Figure 4: Action mechanism illustration of polyphenols blocking spinal cord injury. LPO: lactoperoxidase; TNF-α: tumor necrosis factor-alpha; NF-κB: nuclear factor kaa-B; GPx: glutathione peroxidase; SOD: superoxide dismutase; CAT: catalase; GSH: glutathione; COX-2: cyclooxygenase-2; MDA: malondialdehyde; Nrf2: nuclear factor erythroid 2–related factor 2; PARP1: poly-ADP ribose polymerase 1 (PARP-1).

In vitro experiments revealed that PC12 cells to 0-2000 mol/L of EGCG hindered ROS generation [63]. Dosages of EGCG (10, 25, or 50 mg/kg, i.p.) drastically diminished NADPH/neuronal nitric oxide synthase (nNOS) representation following nerve damage in mice [53] and inhibited neurodegeneration by activating the cyclic adenosine monophosphate (cAMP) for 18 days with 25-75 mg/kg dosage scale of myeloperoxidase (MPO) function, inducible TNF-α, interleukin 1 beta (IL-1β), poly-ADP ribose polymerase (PARP), nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2) representation were all reduced in the rat spinal cord after a 50 mg/kg dose of EGCG [60, 64].

Khalatbary et al. also swiftly exhibited a 50 mg/kg i.p. injection of EGCG and 1 hour after SCI lowered malonaldehyde (MDA) [65]. In a rat spinal cord organic culture, EGCG at a five-molar level for 48 hours suppressed OS and preserved motor neurons, according to *in vitro* experiments [66]. Thermal hyperalgesia was minimized in mice after administering 30 mg/kg of EGCG for a week following SCI, inhibiting the expression of RhoA and TNF-α [67].

5.2. Resveratrol. Resveratrol (3,4′,5-trihydroxystilbene) (Figure 5) is a natural phytoalexin identified in *Veratrum grandiflorum*, grape, and peanut that protects counter to stress damage fungal growth [68, 69]. Resveratrol is a potent antioxidant because it scavenges free radicals, protects against ROS-stimulated DNA damage [70], and reduces the generation of H_2O_2. Resveratrol significantly suppressed oxidized glutathione reductase [63], GSH function, TNF-α, and IL-1β production [64]. Additionally, resveratrol promoted autophagy by stimulating the nuclear factor erythroid 2–related factor 2 (Nrf2) gene and prevented programmed cell death by increased expression of the sirtuin 1 (SIRT1) gene [71, 72].

According to some studies, resveratrol is a SIRT1 activator that may prevent OS, inflammation, and apoptotic neurons, according to some studies [73]. The SIRT1/Akt1 pathway was developed by resveratrol, resulting in cell survival [74]. Suppressing the TLR-4/MyD88/NF-κB enhanced mitochondrial function/biogenesis [75].

By surpassing the NF-κB signaling pathway, the resveratrol might reduce the SCI health consequence severity [76]. Resveratrol (Table 1) (100 mg/kg, i.p.) induced the activity of p-AMPK, Bcl-2, and SIRT1, while lowering the transcription of p62, caspase-3, caspase-9, and Bax, which following SCI. Resveratrol was also reported to protect neurons by downregulating via the SIRT1/AMPK signaling pathway [77, 78].

Apoptosis-related genes were revealed to be helpful in the SCI rat model by Liu et al. [79]. Resveratrol exhibited anti-apoptotic impacts after SCI, according to Zhang et al., by reducing associated p53, caspase-3, and cytochrome C [80]. Additionally, resveratrol suppressed neuroinflammation following SCI by triggering autophagy by the AMPK/mTOR pathway [81]. Resveratrol significantly benefited neuronal autophagic flux to minimize programmed cell death and stimulate operational repair in rats to post to SCI [82].

A further study demonstrated that resveratrol (200 mg/kg) diminished programmed cell death, OS, and inflammation [30]. In mice, a particular quantity of resveratrol improved autophagic proteins while reducing apoptotic ones [83]. Senturk et al. reported that resveratrol (Table 1) (10 mg/kg)

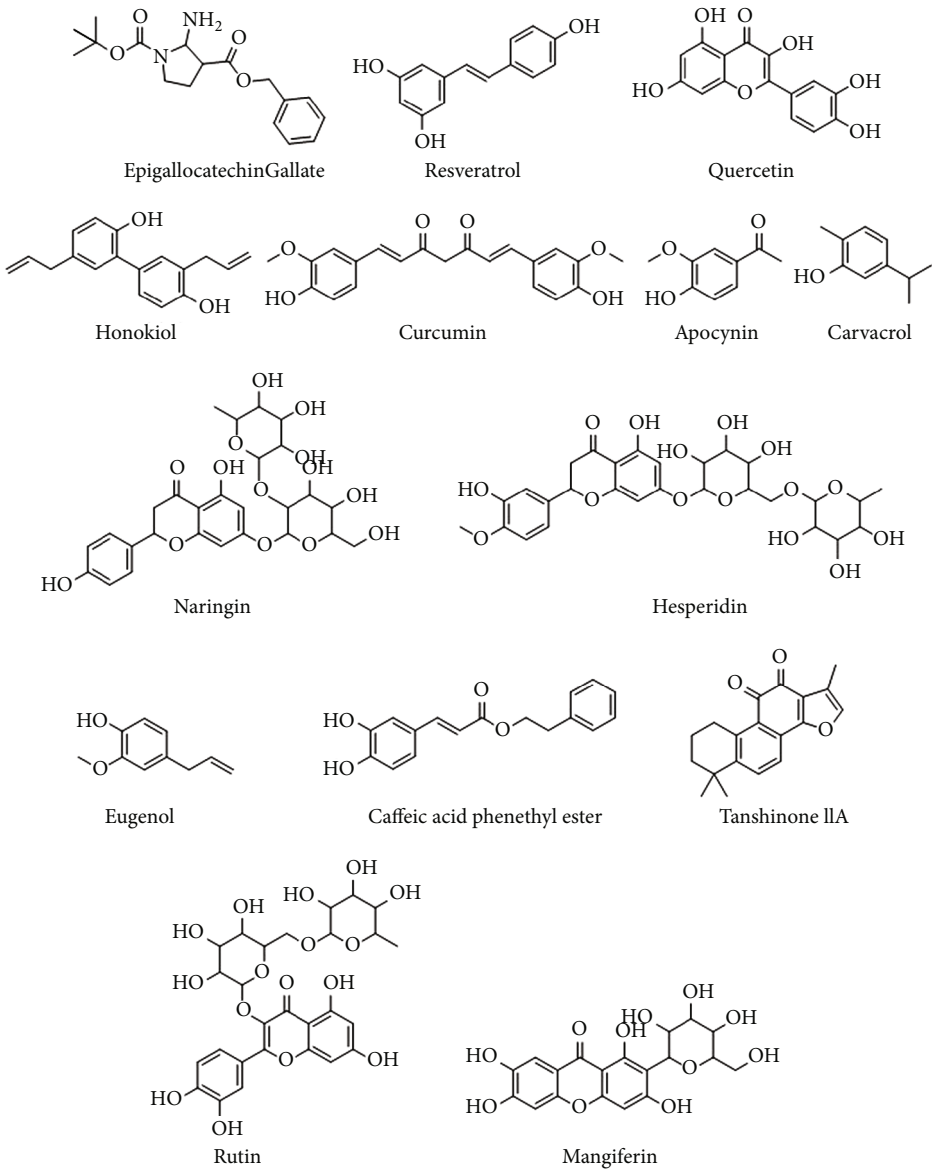

Figure 5: Chemical configurations of some efficient chemical complexes as opposed to spinal cord injury.

exhibited anti-inflammatory characteristics after SCI [84]. Polydatin (20, 40 mg/kg), a glucoside of resveratrol [85], via the Nrf2/heme oxygenase-1 (HO-1) pathway, suppressed OS and protected apoptosis post-SCI [86].

5.3. Quercetin. Flavonoids such as Quercetin (Figure 5) are observed in several fruits, vegetables, and grains. It exhibits anti-inflammatory, anti-carcinogenic, antioxidant, and antiviral activities, among other pharmacological attributes. Quercetin has also been demonstrated to enhance neuronal dysregulation and mental/physical malfunction by inhibiting lipid peroxidation and capillary penetrability and encouraging mitochondrial biogenesis [87–90]. Quercetin's phenolic hydroxyl groups can effectively scavenge. OH, superoxide anions, and LPO [91]. Quercetin can also connect to conversion metals and inhibit oxidation and decrease, forming metal chelates that can be used to neutralize transition metals, notably copper and iron [92]. Quercetin's neuroprotective properties have been widely exhibited in several in vivo studies. After brain damage considerably reduced GSH levels and MPO function [93]. In traumatic brain damage [94], quercetin boosted the activities of SOD, GPx, and AT, lowered the increased MMP-9 level [95], and regulated the tropomyosin receptor kinase B (TrkB) and brain-derived neurotrophic factor (BDNF) [96].

Quercetin (Table 1) (30 mg/kg) also reduced OS, spinal cytokine secretion, and glial cell facilitation of GFAP [97]. Additional studies revealed that a ten-day i.p. quercetin management at a 20 mg/kg/day dosage scale could mitigate monosodium Glu-induced neurotoxicity by lowering p38MAPK, decreasing OS, and boosting GFAP transcription [98]. According to Azevedo et al. [89], quercetin (25, 50, and 100 mg/kg) mitigated OS-induced degeneration by lowering LPO, which was in agreement with Liu et al. [99, 100].

Table 1: Various preclinical investigations have investigated the effect of polyphenols in combating OS and in the after of SCI.

Polyphenol	Dose/concentration	Study model	Pharmacological mode of actions	References
Epigallocatechin gallate	50 mg/kg (i.p), instantly and one h after SCI	Female SD rats	Diminished Bax and MDA; improved Bcl-2	[65]
	30 mg/kg (i.p.); 7 days after SCI	Female BALB/c mice	Decreased TNF-α and RhoA	[67]
	10, 20 mg/kg (i.t)	Female SD rats	Decreased Bax; increased Bcl-2 and BDNF	[156]
	25 mg/kg (p.o), 1 and 6 h later to SCI	Male adult CD1 mice	Decreased Bax, TNF-α, MPO, MDA, NF-κB, iNOS, PARP; increased Bcl-2	[157]
Resveratrol	1 and 10 mg/kg (p.o); 30 min earlier to SCI	Wistar male rats	Decreased NO and MDA	[159]
	400 mg/kg (p.o.); 10 days after SCI	SD male rats	Decreased MDA and IL-6	
	50, 100 mg/kg (i.p.)	SD male and female rats	Decreased MDA; improved Na^+, K^+-ATPase activities	[160]
	200 mg/kg (i.p.); until three days after SCI	SD rats	Decreased MDA, MPO, IL-1β, IL-10, and TNF-α; increased SOD	[79]
	50, 100, 200 mg/kg (i.v.); until seven days after SCI	Female mice	Decreased p38MAPK; NF-κB	[158]
	100 mg/kg (i.p.)	Long Evans female rats	Decreased MDA, NO, and TBARS	[161]
	200 mg/kg (i.p.); directly after SCI	Wistar male rats	Enhanced SOD, GPx, and CAT	[162]
	100 mg/kg (i.p), directly after SCI	Male SD rats	Diminished TNF-α, IL-1β, IL-10, and mTOR; enhanced AMPK, LC3, and Beclin-1	[81]
	200 mg/kg (i.p), Immediately after SCI	Male C57BL/6 mice	Decreased Bax; increased Bcl-2, LC3, and Beclin-1	[83]
Quercetin	10,100mg/kg (i.p), first 3 days after SCI	Wistar male rats	Decreased MDA and NO	[103]
	100 mg/kg (i.p.) for three days following SCI	Male SD rats	Decreased ROS, IL-1β, IL-18, and TNF-α	[108]
	20 mg/kg (i.p.), twice per day for seven days following SCI	Wistar albino rats	Decreased MDA, IL-6, TNF-α, and caspase-3	[105]
	Up until ten days following SCI, 7.5 mg/kg (i.p.), two times per day	Female SD rats	Decreased TNF-α, iNOS, NF-κB, and IL 12; enhanced IL-4 IL-10, and TGF-β	[107]
Honokiol	20 mg/kg (i.p.)	Female SD rats	Decreased MPO, iNOS, COX-2, IL-1β, IL-6, and TNF-α	[126]
	50, 100 mg/kg (i.p.), until three days following SCI	Female SD mice	Decreased MDA, ROS, and TNF-α	[163]
Curcumin	100 mg/kg (i.p), 15 min following SCI	Male SD rats,	Decreased IL6, IL1β, TNF-α, NF-κB, and TLR4	[164]
	200 mg/kg (i.p), 1week before SCI	Male Wistar albino rats	Degraded caspase-3, IL1β, TNF-α, MDA, SOD, and GSH	[145]
	60 mg/kg (i.t), directly after SCI, until three weeks, once weekly	Wistar rats	Decreased IL4, IL1β, IL12, and TNF-α,	[143]
	200 mg/kg (i.m), until eight weeks after SCI	Male SD rats	Decreased caspase-3, Bax, and Bcl-2	[140]
	60 mg/kg (i.m), 30 min after SCI, until three weeks	Male SD rats	Decreased mTOR, p62, and Akt	[165]

Table 1: Continued.

Polyphenol	Dose/concentration	Study model	Pharmacological mode of actions	References
Naringin	50, 100 mg/kg (p.o.), three days before SCI until seven days after SCI	Male SD rats	Diminished TNF-α, IL8, IL-1β, and IL-6	[166]
	20 mg/kg (i.p.), directly and one h after SCI	Female SD rats	Reduced MDA and Bax; enhanced Bcl-2 and GSH	[167]
	50, 100 mg/kg (i.p), 1week before SCI	Female SD rats	Decreased TNF-α, IL-1β, IL-6, NF-κB, MPO, MDA, and SOD; increased GSH, and CAT	[168]
	20, 40 mg/kg (p.o), until six weeks after SCI	Female SD rats	Decreased caspase-3 and Bax; increased Bcl-2 and BDNF	[151]
Apocynin	0.1 mg/kg (i.t)	Male SD rats,	Decreased ROS	[169]
	100 mg/kg (i.p)	Male SD rats	Decreased Caspase-1, ROS, NF-κB, JNK, and p38	[170]
	5 mg/kg (i.p), 1 and 6 h after SCI	Male CD1 mice	Decreased NADPH oxidase, JNK, p38, FasL, MPO, and Bcl-2	[171]
	5 mg/kg (i.p), 1 and 6 h after SCI until 1week	C57BL/6 female mice	Decreased ROS	[172]
Carvacrol	25,75 and 150 mg/kg (i.p)	Male SD rats	Diminished TNF-α, IL-1β, MPO, and NF-κB	[173]
Hesperidin	100 mg/kg; 7 days before SCI until seven days after SCI	Female SD rats	Decreased IL-1β, NF-κB, and PARP; increased SOD, HO-1, and p-p38	[174]
Rutin	30 mg/kg (i.p.)	Rats	Diminished MDA; IL-6; TNF-α; and NF-κB; increased SOD; GSH; CAT	[175]
	30 mg/kg (i.p.), until 3 days	Male SD rats	Decreased TNF-α; MDA; ROS; TGF-β1; and Smad2	[176]
Mangiferin	20, 40 mg/kg (i.p.), until 30 days after SCI	Male SD rats	Decreased MDA, NF-κB; increased SOD, GPx, and CAT	[177]
	10, 25, 50 mg/kg (i.p.)	SD rats	Decreased MDA, NF-κB, TNF-α, and caspase-9; increased CAT, SOD, and GSH	[178]
	0.2 mg/kg (i.p.), 1 h after SCI	Male SD rats	Decreased iNOS, p38MAPK, MDA, and SOD	[92]
	0.25 μmol/kg (i.p.), 1 h after SCI	Wistar male rats	Decreased MPO	[102]
Caffeic acid phenethyl ester	10 μL; 1 μg/kg (i.t.), 1 h after SCI	Wistar female mice	Decreased MDA, SOD, and TOA; increased TAC	[179]
	10 μg/kg (i.p.), 30 min after SCI	Wistar female rats	Increased IL-1β, and TNF-α	[180]
Tanshinone IIA	50 mg/kg (i.p) 1h before SCI (20 mg/kg) until 7 days after SCI	Male SD rats	Decreased TNF-α, NF-κB, MAPK, and JNK	[181]
Eugenol	25, 50 mg/kg (p.o), until seven weeks after SCI	Female SD rats	Decreased, NF-κB, and iNOS; increased SOD, and CAT	[182]

Following SCI, a 7-day i.p. processing of 20 mg/kg quercetin inhibits the p38MAPK/iNOS signaling pathway and synchronizes secondary OS by blocking the BDNF and JAK2/STAT3 signaling pathways [101]. Quercetin administration at a frequency of 0.25 mol/kg diminished MPO expression, according to Schültke et al. [102]. In addition, a particular dose of quercetin provided during three days of SCI enhanced overall antioxidant levels while lowering NO and MDA levels [103]. Quercetin raised overall antioxidant potential and paraoxonase function in rats following SCI [104].

A further research paper discovered that delivering 20 mg/kg of quercetin could safeguard against SCI-stimulated OS by behaving as an antioxidant and anti-inflammatory [105]. Wang et al. observed that quercetin (50 mol/kg) attenuated proinflammatory cytokines while elevating anti-inflammatory cytokines relevant to oxidative mechanisms. The treatment significantly attenuated the cystic cavity size while enhancing macrophage polarization, neuronal function, and axonal survival [106]. Based on in vivo and in vitro investigations, quercetin (7.5 mg/kg) suppressed oligodendrocyte necroptosis after SCI by modulating the STAT1 and NF-κB pathways [107]. Jiang et al. discovered that 100 mg/kg of quercetin lowered ROS construction, IL-1, TNF-α, and IL-18 in female rats following SCI [108]. Therefore, quercetin appears to be a favorable treatment for reducing OS after neurodegeneration and SCI.

5.4. Honokiol. *Magnolia grandiflora* has a pleiotropic lignan called honokiol (Figure 5) [109]. Antioxidant [110], anti-

inflammatory [111], analgesic [112], depressive [113], antitumorigenic [114], and neuroprotective [115] actions are among its therapeutic benefits. Honokiol has been shown to reduce OS factors in tissue diversity, involving the heart [116], liver [117], kidney [118], and brain [119]. Honokiol reduced ROS generation in microglial cells via the ERK/NADPH oxidase pathway [120]. To exhibit neuroprotective effects, it also triggered Nrf2 [121], suppressed xanthine oxidase (XO), and regulated the PI3K/Akt pathway [122]. Furthermore, honokiol protected mitochondrial respiratory chain enzymes by targeting PKC, MAPKs, and NF-κB [123–125]. 20 mg/kg of honokiol decreased the generation of proinflammatory cytokines and prevented neutrophil permeation and microglial stimulation in a rat version of SCI, all of which are linked to oxidative factors [126]. In ischemic brains, 10 g/kg of honokiol reduced neutrophil infiltration and ROS production while maintaining Na^+/K^+-ATPase function and mitochondrial biogenesis against OS [113]. Honokiol also conserved mitochondrial respiratory chain enzyme [125]. In a rat model of SCI, 20 mg/kg of honokiol lowered the manufacture of proinflammatory cytokines, blocked neutrophil penetration, and prevented microglial activation, all associated with oxidative factors [126]. 10 g/kg of honokiol (Table 1) reduced neutrophil infiltration and ROS generation in ischemic brains while maintaining Na^+/K^+-ATPase activity and mitochondrial biogenesis [113].

5.5. Curcumin. Curcumin (Figure 5) is an organic polyphenol substance isolated from the *Curcuma longa* rhizome [127, 128]. In many studies, curcumin has antioxidant, anti-inflammatory, and anticancer estates, which have antioxidant, anti-inflammatory, and anticancer properties. Curcumin exerts anti-inflammatory actions via upregulating the PPAR- linked with the NF-κB pathway [129, 130]. Curcumin inhibited the stimulation of NF-κB, lowered the production of COX-2, IL-1, IL-6, IL-8, and TNF-α [131], and boosted the SOD activity [132]. Curcumin's anti-inflammatory impact after SCI has been linked to suppression of NF-κB, IL-1β, IL-6, and TNF-α activity, as well as an enhancement in Nrf2 [133] and stimulation of the TLR4/NF-κB signaling route [134].

Curcumin generated antioxidative preservation via Nrf2 routes and a reduction in ROS as a consequence of NF-κB stimulation [135]. In treating SCI, curcumin also affects the mTOR signaling pathway [136]. Curcumin, a more potent antioxidant that targets antioxidant enzymes such as GPx and SOD than vitamin E, has been reduced by methoxy and phenolic groups [137]. Curcumin elevated the CDGSH iron sulfur domain 2 (CISD2) as a durability gene due to its activities in Ca^{2+} metabolism after SCI. CISD2 improved BCL-2/Beclin-1 binding. It is guarded against programmed cell death and mitochondrial dysfunction. At the ER stress, CISD2 reduced a rise in excitotoxic Ca^{2+} [138].

Curcumin reduced neuron death and inhibited neuronal death following SCI, according to Lin et al. [139]. In the long-term treatment of SCI, curcumin outperformed methylprednisolone by lowering Bax and caspase-3 while increasing Bcl-2 [140]. Following curcumin therapy in humans or mice, tetrahydrocurcumin is among the most common curcumin metabolites isolated from the liver cytoplasm and small intestine [141]. In SCI patients, tetrahydrocurcumin (80 mg/kg/day) has been reported to lower OS and death [142]. Curcumin decreased inflammatory cytokines with pro-apoptotic effects in rats after SCI [143].

Curcumin entirely inhibited TGF-β following SCI. They also discovered that curcumin inhibits NF-κB, a protein implicated in the apoptotic and inflammatory mechanisms [144]. Curcumin's anti-apoptotic action was also exhibited in the spinal cord damage rat model, later being given intravenously. Curcumin was also found to decline caspase-3 [145], enhance Bcl-2 [146], and have anti-inflammatory antioxidant estates [147]. In a rabbit model of SCI, curcumin was discovered to block apoptotic (caspase-3) [147].

5.6. Naringin. Naringin (Figure 5) is considered a flavanone glycoside attained from citrus fruits. Naringinase hydrolyzes it to yield naringenin, which can effortlessly intersect the blood-brain barrier [148]. The inflammatory and OS reactions in adults' brains were controlled by naringin therapy. Naringin also has neuroprotective estates by stimulating neurotrophic factors and constraining apoptosis [149, 150]. Naringin can be an apoptotic inhibitor because the inflammatory factors and apoptotic mediators are linked. Following SCI, naringin (Table 1) (20, 40 mg/kg, p.o.) raised BDNF and vascular endothelial growth factor (VEGF) levels while inhibiting brain apoptosis [151]. BDNF reduced apoptosis and MAPK pathways via interacting with TrkB [152, 153], although the β-catenin/GSK-3β signaling route has been found to promote remyelination following SCI [154]. Naringenin, a naringin aglycone analog, has shown promising neuroprotective benefits and may be used in SCI in the future. Naringenin diminished the expression of IL-6, TNF-α, and CXCL10 mRNA in the spinal cord, which is an essential factor in apoptosis [155].

5.7. Apocynin. Apocynin (Figure 5), also known as acetovanillone, is an organic polyphenolic substance extracted from the rhizomes of *Apocynum androsaemifolium* [183]. Apocynin is a nicotinamide adenine dinucleotide phosphate (NADPH) oxidase inhibitor that suppresses p47phox's serine phosphorylation and prevents it from binding to gp91phox, delaying NADPH oxidase activity [184]. H_2O_2 and myeloperoxidase (MPO) stimulate apocynin, resulting in the formation of an apocynin radical. NADPH oxidase is inhibited by thiol-oxidizing compounds [185], a significant source of ROS in the cell [186]. This method has significantly altered redox-sensitive signaling pathways in neuroinflammation in different NDDs, particularly SCI. Sun and colleagues have found that apocynin (50 mg/kg) (Table 1) reduced SCI-induced neurodegenerative in rats by diminishing inflammatory cytokine production, improving glutathione (GSH)/SOD activity, and decreasing MPO and malondialdehyde levels (MDA). Apocynin (5 mg/kg) inhibited apoptosis after SCI by lowering FasL stimulation and phospho-JNK, P38, inflammatory cytokines (IL-1, TNF-α), and NF-κB representation levels [171]. Corresponding to research by Liu et al., apocynin can aid histology

results and forelimb motor control restoration following SCI. Furthermore, Zhang and coworkers demonstrated the prospective neuroprotective estates of apocynin by decreasing neuroinflammation in spinal cord injured rats by suppressing the growth of NADPH oxidase-mediated ROS [172]. In an SCI chronic animal experiment, ROS and lipid peroxidation were similarly reduced by apocynin, implying an indirect control of apoptosis [169].

5.8. Carvacrol. Carvacrol (Figure 5) is a monoterpenoid phenolic product of cymene and has been demonstrated to have anxiolytic [187], depressive [188], antibacterial, antioxidant [189], anticancer, antimutagenic [190], anti-inflammatory [191], and antihepatotoxic properties [192]. Carvacrol strengthened the regulations of Nrf2 and ERK1 in PC12 cells that had been suppressed by cadmium [193]. Cells following exposure to iron ions and in cells exposed to H_2O_2 exhibited anti-carcinogenic characteristics via HO-1 [194, 195]. The Fenton reaction combines an excess of iron ions with oxygen, causing oxidative damage such as mitochondrial dysfunction and LPO [19]. Carvacrol also has anti-inflammatory and pro-inflammatory cytokine modulating properties [196]. After administering (25, 75, and 150 mg/kg), it inhibited OS factors like MDA, GSH, and NO [173]. However, more investigations are required to identify the neuroprotective properties of carvacrol following SCI via oxidative mechanisms.

5.9. Hesperidin. Hesperidin (Figure 5) is an anti-inflammatory, antioxidant, anticancer, and anti-apoptotic flavanoglycone obtained from citrus fruits [197, 198]. Hesperidin regulated Nrf2/ARE/HO 1 and TGF1/Smad3 signaling, which decreased OS and inflammation [199]. Hesperidin modulation of the ERK/MAPK pathway is implicated in the production of HO-1 and Nrf2 in an *in vitro* investigation based on OS [200]. *In vitro*, hesperidin triggered Nrf2/ARE/HO-1 and upregulated the Keap1-Nrf2/HO-1 pathway, enhancing the action of antioxidant enzymes in kidney tissue [201]. As a result of stimulating the Nrf2/HO-1/ARE and PPAR mechanisms, it reduced OS and inflammation [201, 202].

5.10. Rutin. The flavonol glycoside rutin, commonly identified as vitamin P, is derived from buckwheat [203]. Rutin (Figure 5) has a number of pharmacological properties, such as cytoprotection, antioxidant [204], anticancer [205], vasoprotection [206], neuroprotective effects [207], and anti-inflammation [163]. Rutin lowered OS by increasing CAT function, decreasing LPO and protein carbonyl content, and modulating the MAPK [208] and iNOS/ Nrf2 signaling pathways. In ischemic neuronal apoptosis, rutin suppressed LPO and p53 expression, enhanced antioxidant defense enzymes, and lowered ROS generation [209]. In mice, it alleviated diabetic neuropathy by lowering OS via HO-1 and Nrf2 [210]. Rutin boosted the transcription of BDNF, CREB, and ERK1 genes in the hippocampus at 100 mg/kg [211] and shielded PC12 cells against sodium nitroprusside stimulation by regulating the PI3K/Akt/mTOR and ERK1/2 pathways [212]. Oral medication with 10 mg/kg rutin for three weeks reduced OS [213].

A further study noticed that three-day rutin (Table 1) (50 and 100 mg/kg) substantially reduced ROS, MDA, IL-1, IL-18, and TNF-α [163]. Rutin protected cells from OS and apoptosis caused by H2O2 in vitro studies by directing the Bax/Bcl-2 ratio and the NF-κB/p65 signaling route, managing ROS, reducing LPO, and maintaining the intracellular antioxidant enzyme activities [214]. Rutin also safeguarded neurons from oxidative DNA damage and degeneration resulting from a lack of food [215]. Furthermore, 30 mg/kg rutin in the SCI animal paired with mild hypothermia for three days after SCI decreased inflammatory factors by blocking the TGF-β/Smad route [215].

5.11. Mangiferin. Mangiferin (Figure 5) is a bioactive xanthonoid extracted from various mango components. It is a potent antioxidant [216] with a variety of health benefits, notably immunomodulatory [217], antiviral [218], anti-inflammatory [219], antidiabetic [220], anticancer [221], and analgesic [222] activities. Mangiferin inhibits LPO and DNA damage by neutralizing free radicals and generating mangiferin-iron complexes [216, 223]. In an *in vivo* study, mice were recovered from cadmium chloride contamination by administering 50 mol/L of mangiferin for 4 hours, which reduced LPO rates and increased GSH, CAT, GST, and SOD activity [224]. Mangiferin increased Nrf2 levels, altered NQO1 expression, and increased ROS levels *in vitro* research [225]. Interestingly, 20 and 100 mg/kg of mangiferin triggered the Nrf2/HO-1 pathway in a dose-dependent approach in a brain injury model [177]. Mangiferin (Table 1) (20 and 100 mg/kg) for 30 days after SCI significantly decreased MDA at the same time as significantly boosted SOD, CAT, and GPx [178]. Mangiferin's neuroprotective properties in concentrations of 10, 25, and 50 mg/kg 30 days following SCI were connected with diminished spinal cord edema, reduction of OS, and inflammatory condition [226].

5.12. Caffeic Acid Phenethyl Ester. Honeybee propolis contains phenethyl caffeate [227]. Because of the associated hydroxyl groups in the catechol ring, it has antioxidant [228], anti-inflammatory [229], antibacterial [230], anticancer, and cytotoxic effects [231]. The phenethyl ester of caffeic acid inhibits NF-κB [232] and protein tyrosine kinase [233]. Hypoxic-ischemic brain injury models inhibit lipoxygenase activity [234] and limit calcium-induced cytochrome c release [235]. Following ischemia-reperfusion injury, caffeic acid phenethyl ester suppressed superoxide anion generation and XO [236] and decreased MPO and Na^+/K^+ ATPase capacities [237]. Caffeine's phenethyl ester increased HO-1 synthesis by activating Nrf2 and the extracellular signal-regulated kinases (ERK) signaling route [238]. It binds to Keap1, allowing Nrf2 to better connect to ARE [239]. MDA, LPO, and total oxidant action were reduced after SCI with an intrathecal infusion of 1 g/kg caffeic acid phenethyl ester. After SCI, it boosted antioxidative mediators [240], even as it decreased IL-6 levels in tissue and serum [241]. In a similar vein, Ak et al. found that caffeic acid phenethyl ester (10 g/kg) infusions lowered TNF-α and IL-1β levels after SCI [179].

After SCI, 10 mol/kg of this phytochemical enhanced motor function and decreased lesion size by lowering IL-1β, NOS, and COX-2 expression [180]. Caffeic acid phenethyl ester, 10 mol/kg (Table 1), was given before surgery to minimize ischemic damage in the spinal cord and to enhance microcirculation by blocking endothelial cell lysis by activated leukocyte proteases [242]. It also inhibited ROS and iNOS catalytic performance at a 50 mol/mL dosage, which had neuroinflammatory effects [243].

5.13. Tanshinone IIA. Tanshinone IIA (Figure 5) is extracted from the roots of *Salvia miltiorrhiza*. Tanshinone IIA has been found to have anti-apoptotic and anti-inflammatory properties in investigations [244]. Tanshinone IIA's antioxidant development is associated with efficient communication among DNA and lipid peroxidation product avoidance, DNA conservation by inhibiting NADPH oxidase, lipid peroxidation, and lipid-free radical clearance [245, 246]. Tanshinone IIA also inhibited the onset of neuroinflammation in neurodegenerative pathologies by preventing the production [247]. MAPKs are also critical signaling mediators that control cell development and death [248]. Tanshinone IIA (20, 50 mg/kg) (Table 1) has been demonstrated to suppress inflammation and apoptosis during SCI by decreasing NF-κB, MAPK, IL-1β, TNF-α, IL-6, iNOS, and caspase-3 boosting Bcl-2 [181]. Other investigations [249, 250] determined the spinal levels of inflammatory factors after tanshinone IIA treatment. These inflammatory factors also interact with apoptotic factors, as aforementioned. Tanshinone IIA has been shown to have the ability to improve neuronal autophagic factors and pathways (PI3K/Akt/mTOR) [251].

5.14. Eugenol. Eugenol (Figure 5), often known as clove oil (4-allyl-2-methoxy phenol), is an organic chemical derived from the *Syzygium aromaticum* (clove) plant [252]. Eugenol has antitumor [253], anti-microbial [254], anti-inflammatory [255], and antioxidant properties. It has been proven that proinflammatory cytokines, inflammation enzymes, and antioxidative enzymes reduce inflammation [256]. Eugenol has been shown to have therapeutic efficacy by lowering TRPV1 and sodium channels [257], connecting with Ca^{2+} channels [258], and boosting autophagy via the AMPK/mTOR pathway [259]. Eugenol lowered OS, inflammatory markers, and caspase-3 [182]. In neuroprotective effects, Eugenol increased Bcl-2 but decreased Bax [238] and TNF-α [239]. It has also been demonstrated to stimulate neuronal autophagy by the Akt/AMPK route [259].

6. Clinical Studies

Polyphenols are potential secondary metabolites with a comprehensive scale of favorable health outcomes. The US Food and Drug Administration (FDA) has acknowledged curcuminoids as relatively reliable and highly allowed effective forms in clinical studies, even at concentrations of up to 12,000 mg/day [260]. In controlled clinical research, curcumin's impacts on inflammatory and stress markers in 100 osteoarthritis patients of both genders have been investigated [261]. In a prospective randomized open-end blinded examination (PROBE) of 80 individuals with knee osteoarthritis, researchers discovered that consuming 30 mg of curcumin three times a day (p.o.) for four weeks decreased COX-2 concentrations [262]. Another RDBPC analysis [263] shows the anti-inflammatory efficacy of oral curcumin (400mg/3 times a day, p.o.) in type 2 diabetic cases and a substantial decrease in MDA, IL-6, and TNF-α levels.

In one hundred individuals with SCI, curcumin was significantly connected to decrease osteoporosis development and bone metabolism markers after six months [264]. According to randomized, parallel-group outcomes controlled clinical research on 20 participants, the InflanNox tablet (curcumin 1200 mg/day) has additional anti-inflammatory and antioxidant characteristics, lowers IL-1β, and improves depression and anxiety in SCI patients [265]. In 50 individuals with multiple sclerosis, administration of nanocurcumin (80 mg/day) was linked to a considerable increase in TGF-β and IL-10 expression [266]. Nanocurcumin was governed in a randomized of 40 diabetes people. In this investigation, nanocurcumin was discovered to be an antioxidant that may minimize OS and free radicals [267].

Polyphenol supplements (200mL/day) reportedly regulated plasma homocysteine concentrations in 48 Alzheimer's patients in an eight-month multiple center RDBC experiment [268]. In a multicenter, double-blind clinical investigation, thirty-four diabetic patients with neuropathy (aged 21 to 72) were given a topical preparation including quercetin to reduce OS [269]. Verlaet et al. showed antioxidant properties in a randomized controlled experiment examining the treatment properties of the herbal, polyphenol-rich extract [270]. Furthermore, another study found that meals high in polyphenols could increase cognitive reserve [271]. Another polyphenol-rich extract has shown promising antioxidative consequences in healthful people and those suffering from NDDs [272–274].

7. Conclusion and Future Perspectives

The complicated pathophysiological mechanisms in SCI seem to be orchestrated by OS to influence other interrelated pathways, such as neuroinflammation. Thus, an interaction between OS and neuroinflammatory/apoptotic pathways is complex.

In this line, Nrf2/Keap1/ARE, SOD, CAT, GSH, MDA, HO-1, and XO have significantly reduced the associated pathways/mediators contributing to neuroprotection in NDDs and SCI. Because of the polyphenol's shortcomings, researchers must apply novel drug delivery strategies in clinical studies, such as nanoformulations. Nanoformulations of polyphenols are proposed to overcome such restrictions due to the management indicated above and the advantageous effect of nanoparticles in boosting spinal cord medication distribution. It will enable the chemical's favorable impacts on SCI and other NDDs. To address SCI difficulties, metal nanoparticles (iron oxide, gold, silver, and so on), liposomes, and inorganics have all been utilized to create nanoparticles [275].

Equivalent recommendations will aid in raising understanding of the complexities of dysregulated signal transduction

pathways after the SCI and the significance of discovering new and more effective multitarget alternative natural intermediaries with more excellent safety and efficacy among the scientific community. The exact molecular pathogenesis and signaling pathways associated with NDDs and the secondary phase of SCI must be revealed in further research studies. The mediators represent promising options to prevent associated pathogenicity in an oxidative way. Polyphenols are suggested to be the primary focus in this line of work as alternatives to interventions with fewer complications and greater efficacy.

Polyphenols/phenolic compounds are secondary metabolites with a broad scale of biological activity and health improvements exploited in modern medication to generate novel drugs [276]. Clinical studies are currently evaluating the therapeutic effect of polyphenols in the treatment of NDDs; however, clinical research to investigate the promise of polyphenols in treating following SCI consequences is lacking [277]. Therefore, well-designed clinical trials will aid in revealing polyphenols' therapeutic promise in addressing sensory-motor dysfunction after SCI and pave the way to address any recommendations for the future of their administration. The role of OS in modifying the inflammatory and apoptotic pathways in NDDs, with a particular focus on SCI, was investigated in this work. As potential multitarget neuroprotective treatments, we also emphasized the need to synthesize polyphenols and phenolic compounds that proinflammatory cytokines, extrinsic axonal related pathways, and other pathways involved with OS. Co-administering polyphenols/phenolic chemicals may also help treat SCI side effects. These research projects will explore potential pharmacological targets for avoiding, controlling, and treating NDDs and SCI.

Data Availability

All data used to establish the conclusions of this study are integrated into the article.

Conflicts of Interest

The authors proclaim that they have no conflicts of interest.

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Review Article

Emerging Role of Neuron-Glia in Neurological Disorders: At a Glance

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Based on the diverse physiological influence, the impact of glial cells has become much more evident on neurological illnesses, resulting in the origins of many diseases appearing to be more convoluted than previously happened. Since neurological disorders are often random and unknown, hence the construction of animal models is difficult to build, representing a small fraction of people with a gene mutation. As a result, an immediate necessity is grown to work within in vitro techniques for examining these illnesses. As the scientific community recognizes cell-autonomous contributions to a variety of central nervous system illnesses, therapeutic techniques involving stem cells for treating neurological diseases are gaining traction. The use of stem cells derived from a variety of sources is increasingly being used to replace both neuronal and glial tissue. The brain's energy demands necessitate the reliance of neurons on glial cells in order for it to function properly. Furthermore, glial cells have diverse functions in terms of regulating their own metabolic activities, as well as collaborating with neurons via secreted signaling or guidance molecules, forming a complex network of neuron-glial connections in health and sickness. Emerging data reveals that metabolic changes in glial cells can cause morphological and functional changes in conjunction with neuronal dysfunction under disease situations, highlighting the importance of neuron-glia interactions in the pathophysiology of neurological illnesses. In this context, it is required to improve our understanding of disease mechanisms and create potential novel therapeutics. According to research, synaptic malfunction is one of the features of various mental diseases, and glial cells are acting as key ingredients not only in synapse formation, growth, and plasticity but also in neuroinflammation and synaptic homeostasis which creates critical physiological capacity in the focused sensory system. The goal of this review article is to elaborate state-of-the-art information on a few glial cell types situated in the central nervous system (CNS) and highlight their role in the onset and progression of neurological disorders.

1. Introduction

Kettenmann and Verkhratsky used the term "glia" from the Greek word "stick" to describe the filling of gaps between neurons in the focal sensory system in 1856. Despite the relentlessness of neuron-focused exploration for a long period of time, Kettenmann and Verkhratsky successfully noted the significance of glial cells in understanding the role of the central nervous system in his 1858 address: "Up to this point, courteous fellows, in having thought about the sense organs, I have discussed the deeply anxious pieces of it. But, if we need to examine the sensory system in its

standard-setting in the body, we should initially comprehend the substance between the legitimate neural components, hold them together, and give the whole its shape somewhat" [1]. The intricacy of the collaboration among neurons and glial cells is simply starting to be seen today. Improved coculture strategies have helped in the exploration of a few issues more noteworthy profundity. In the CNS, three glial cells (astrocytes, oligodendrocytes, and microglia) are generally perceived, each with individual capacities [2]. All four cell types have been linked to the development of essentially all known CNS pathologic conditions, including neurodegenerative diseases like Alzheimer's disease (AD), Huntington's disease (HD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), spinal muscular atrophy (SMA), multiple sclerosis (MS), and various pathologies like Rett syndrome [3, 4]. Accordingly, powerful model frameworks for unwinding the exceptional job of every particular glial cell type in an infection state, just as contemplating their dynamic interaction, might be incredibly valuable in recognizing novel therapeutics [5]. The first few years of life are crucial for neurodevelopment. Synapse formation and pruning, as well as the consolidation of neural circuitry, are all part of maturation [6]. The creation and pruning of synapses have traditionally been researched in conjunction with the formation and removal of synapses. Synapses were considered to operate as a messenger among two adjusting neurons, and pruning synapses might cause synapse patterns that influenced neuronal communication [7, 8]. Previously, the role of glial cells was unknown, but recent research has revealed few information such as the generation of gliotransmitters and cytokines, which allow them to interact with neurons during brain development. Moreover, neuron-glia interactions are being studied more and more in terms of synaptic alterations made by glial cells at various stages of development, like synaptic patterning as a consequence of pruning [9]. Glial cells secrete gliotransmitters such as glutamate, gamma-aminobutyric acid (GABA), and cytokines, which have direct and indirect effects on neurons [10]. These drugs have been found to affect tripartite synapses. Tonic gliotransmitter release, which is usually visible as extracellular glutamate and GABA, is also another key part of neuron-glia interactions. Tonic inhibition has been discovered to be particularly useful in the research of neurodegenerative diseases including AD and PD [11]. The chronic stimulation of nerve cells, on the other hand, has only been studied sporadically in neurodevelopmental diseases [12, 13]. For proper brain function during development and adulthood, the connections between neurons and glia are essential. According to recent studies, glia play a crucial role in bidirectional communication with neurons, adaptation to various diseases, modulation of neuronal activity, and phenotypic changes in response to neuronal injury [14, 15]. Neurons are strongly connected with glial cells such as astrocytes, oligodendrocytes, and microglia throughout the brain tissue, and their dynamic interactions are critical for appropriate brain function [5, 16]. Every type of neurological disease is thought to include a glial component, which could be the primary or secondary cause [17]. As a result, glia's protective and homeostatic abilities define their central position in neuropathology. The mechanisms governing glial cells' varied reactive states are yet unknown; however, they can be linked to changes in their metabolic profiles. In the context of these phenotypic changes in neurological illnesses, the unique metabolic changes combined with mitochondrial modifications in activated glial cells are receiving attention [5, 13, 18]. Changes in glial cells' metabolic profiles have been found to disrupt neuron-glial and interglial interactions, increasing the ongoing reaction to the initial cause. In numerous neurological illnesses, abnormal neuron-glial interactions have been identified in several studies. The rising body of evidence demonstrates the close relationship between glia and neuronal cells, as well as their impact on neurons. Several studies have found that metabolic changes in glial cells modify neuron-glial connections, hence amplifying the pathophysiology of many neurological illnesses [19–23]. In preliminary research on neurodegenerative, ischemic brain injury, and demyelinating illnesses, glial metabolism was found to have a lower oxidative burden, lower generation of proinflammatory cytokines, and less neuronal damage. As a result, future research into the underlying processes that regulate metabolic changes in reactive glial cells will lead the way for the creation of new therapeutic approaches [24–26]. In this review work, we looked into neuron-glia interactions in the setting of a number of neurodevelopmental disorders, including autism spectrum disorder (ASD), attention deficit hyperactivity disorder (ADHD), and epilepsy (Figure 1). Besides from that, many neurotransmitters and their actions have been discussed, as well as the significance of glial cells in neurological disorders.

2. Neurotransmitters

2.1. Endocannabinoids. Endocannabinoids (ECBs) are retrograde-acting molecules that are delivered from neurons in light of depolarization-initiated Ca^{2+} inundation. Depolarization-induced suppression of inhibition (DSI)/excitation flagging gave the evident primary proof to retrograde ECB flagging depolarization-induced suppression of excitation (DSE). Later examination uncovered that the ECB framework is involved in both excitatory and inhibitory neural connections as well as present moment and long-term depression (LTD). From that point forward, the ECB framework has become the mind's most investigated retrograde flagging framework. Much of the time, ECB-interceded retrograde flagging starts with the union of 2-arachidonoylglycerol (2-AG), which is set off by raised intracellular Ca^{2+} fixation and initiated Gq/11-coupled receptors. 2-AG is in this way delivered into the extracellular space, where it goes until it comes to the presynaptic terminal, where it ties to the cannabinoid receptor type 1 (CB1R). Initiated CB1R hinders synapse discharge as follows: first, by obstructing voltage-gated Ca^{2+} channels, which limit presynaptic Ca^{2+} deluge, and second, by restraining adenylyl cyclase (AC) and the cAMP/PKA pathway, which is associated with LTD [27, 28].

2.2. Glutamate. Glutamate is the essential excitatory synapse in the focal sensory system, which deals with astrocytes in an

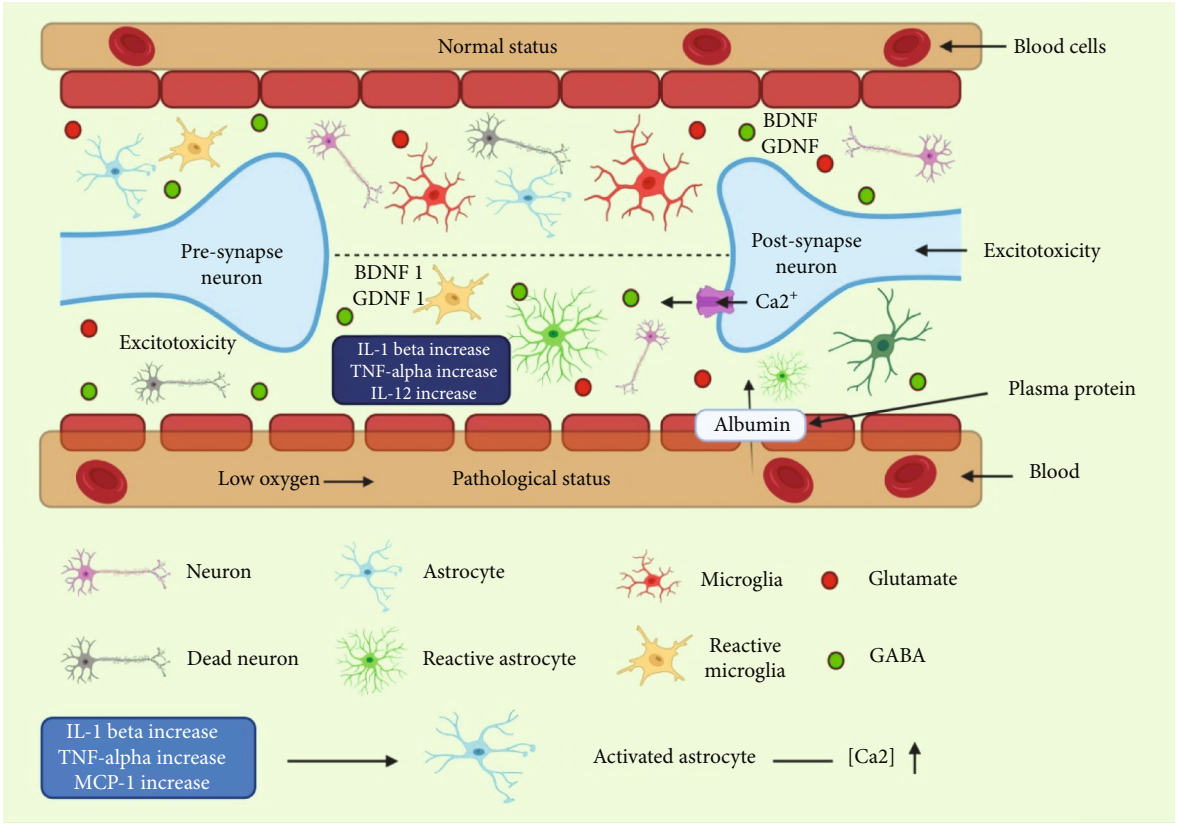

Figure 1: Changes that are caused by interactions between neurons and glia during neurodevelopment. Increased levels of the inflammatory cytokines IL-12, IL-1, and TNF-α are associated with neurodevelopmental disorders. The resulting astrocyte and microglia cell proliferation leads to their enlargement, which causes more of the molecules to be released into the extracellular space, leading to neuronal death. Albumin levels increase in patients with epilepsy due to increased permeability of the blood-brain barrier (BBB) and activation of astrocytes. In epilepsy, released cytokines have been demonstrated to promote effective neurogenesis and the synthesis of neurotrophins such as BDNF, NGF, and GDNF (NGF, BDNF, and GDNF).

assortment of cerebrum regions. Despite the fact that a few studies have shown that astrocytes have ionotropic glutamate receptors, it is commonly assumed that glutamatergic communication in such glial cells occurs mostly through metabotropic glutamate receptors (mGluR). Based on their organization homology, G-protein coupling, and ligand selectivity, mGluRs are categorized into three classes. Group I contains mGluRs 1 and 5, group II contains mGluRs 2 and 3, and group III contains mGluRs 4, 6, 7, and 8. The measures of mGlu1, mGlu3, and mGlu5 receptors in astrocytes fluctuate contingent upon the formative stage and cerebrum area. According to a large research collection, mGluR5 is the most important glutamate receptor transmitted in astrocytes in culture and in situ. Ca^{2+} waves in astrocytes are triggered by glutamatergic neuronal afferent fibers in hippocampal slices, which are blocked by mGluR5 antagonists. mGluR5s are additionally significant in astrocytic reactions to glutamatergic neurotransmission in other brain regions like the core accumbens and the thalamus. Moreover, astrocyte Ca^{2+} reactions are defenseless to mGluR5 opponents when tangible incitement is acted in vivo. The statement of mGlu5 in astrocytes is high during pregnancy and reduces during advancement, when mGluR3 is upregulated, recommending that mGluR5 may just play an unobtrusive capacity in grown-up stages [29, 30].

2.3. Acetylcholine. Brown was the first to identify acetylcholine (ACh) as a neurotransmitter based on its effects on the heart [31]. ACh's many roles in synaptic communication have been discovered [32]. Cholinergic circuit disruption is assumed to be at least partly responsible for the cognitive impairments seen in neurodegenerative illnesses. Cholinergic circuit disruption has been connected to both normal and abnormal cognitive performance [33, 34]. While direct cholinergic activation of pre- and postsynaptic neuronal receptors is thought to be the method by which cholinergic signaling modulates cognitive functions, the involvement of ACh in astrocytes has been overlooked. Muscarinic and nicotinic receptors have been found in astrocytes [35–38]. In hippocampal slices, cholinergic agonists or synaptically produced ACh causes astrocyte Ca^{2+} increases. Such effects are mediated via muscarinic receptors, according to pharmacological techniques [39–41].

2.4. Norepinephrine. The neural connection norepinephrine is essentially conveyed by the locus coeruleus (LC) and has

a vast extent of effects across the frontal cortex. Norepinephrine, as various catecholamines, is transmitted through axonal varicosities in neurons in this way acts through "volume transmission." Astrocytes have adrenergic receptors a1, a2, and ß1 [42, 43] and can respond to norepinephrine made by neurons [44, 45]. *In vivo*, LC neuron impelling causes short statures in cortical astrocytic Ca^{2+}, controlled by phentolamine, an ambiguous adrenergic receptor blocker [44]. Plus, advancement authorizes astrocyte networks across many psyche areas, curbed by adrenoceptor adversaries [46, 47].

2.5. Dopamine. Although the transport of dopamine in cultured astrocytes has been studied in great depth, the existence of down-to-earth dopamine receptors in situ has stayed a question mark until continuous assessments revealed astrocyte response to dopamine in a variety of brain locations. Dopamine D2R order of astrocytes has been demonstrated to suppress a crystallin-mediated neuroinflammation *in vivo* [48] and lower intracellular Ca^{2+} levels in hippocampal [49] and ventral midbrain astrocytes. In contrast, exogenous dopamine inception of D1R lifts intracellular Ca^{2+} in hippocampal astrocytes [49]. Moreover, we have displayed the presence of D1Rs in astrocytes of the center accumbens using electron microscopy. That inception of these receptors *in vivo* and situ by synaptically conveyed dopamine causes intracellular Ca^{2+} ascends through a GPCR hailing course, including IP3R2 and intracellular Ca^{2+} arrangement [50].

2.6. GABAA Receptors. GABAARs are integral membrane ion channels with five sections that govern the most common type of fast regulatory neurotransmission in the brain [51, 52]. They are sensitive to Cl and HCO3 anions. There should be at least 19 different GABAAR subunits genes, including 6α (α1-α6), 3β (β1-β3), 3γ (γ1-γ3), 3ρ (ρ1-ρ3), and also 1 gene for each of the subunits [53]. As a consequence of this diversity, various homomeric or heteromeric subunit mixtures exist, each with its location in the CNS and functional and pharmacology features [54]. A few elements impact the subunit profile that creates GABAARs, for example, cerebrum area, cell type, formative stage, and physiological or neurotic conditions [55–57]. As of late, 11 GABAAR subtypes with various subunit arrangements have likewise been found, most of which are heteromeric receptors shaped by αxβxγx or αxβxδ, while some are homomeric receptors comprising of subunits [58, 59].

2.7. GABAB Receptors. GABABRs are slow and long-acting G-protein-coupled receptors (GPCRs) that are occupied with GABA-intervened restraining transmission. It was first distinguished pharmacologically as bicuculline-harsh metabotropic receptors that were animated by the GABA simple baclofen [60]. GABABRs are heterodimers made out of GABAB1 and GABAB2 receptor subunits that act together to initiate signals [61, 62]. GABAB1 has ligand binding sites, while GABAB2 has allosteric modulator binding sites [63, 64]. It is needed to get the heterodimer to the cell membrane, where the receptors can activate [65, 66]. It has an interaction with the Gi/o protein. Voltage-gated Ca^{2+}channels (VGCC), inwardly rectifying potassium channels (Kir), and adenylyl cyclase are some of the effector components involved with GABABR flagging pathways in neurons [67]. However, depending on the cell type and location studied, the specific coupling of GABABRs to the molecular effector can vary [68].

2.8. Serotonin. Serotonergic neurotransmission is suspected to be involved in several mental diseases [69]. Even though the evidence appears to be conflicting, the role of serotonin in learning and memory has attracted interest [70]. Furthermore, experimental evidence suggests that stimulating serotonergic neurotransmission reduces behavioral performance, while inhibiting it, improves it. 5-HT 3 antagonists [71], which were shown to improve rodent and primate performance in a variety of cognitive tests [72], have yielded promising results. As a result, it is not unexpected that many substances have been created to treat AD (for example, ICS 205930, Ondansetron, and Zacopride; see [73]). A putative neurochemical mechanism of action has also been proposed by many studies. 5-HT 3 receptors appear to regulate cortical ACh release and may work through another 5-HT receptor subtype [71]. The 5-HT 3 antagonists' apparent cognition-enhancing benefits are hypothesized to be amplified by their effects on ACh generation in the brain. Because the entorhinal cortex has a higher density of 5-HT1A receptors [74], and this receptor subtype is involved in learning and memory, it has been hypothesized that it could be a target for cognitive-enhancing drugs. Several research looked into the involvement of the 5-HT1A receptor subtype in learning and memory; however, most found no evidence of improved learning or memory after using 5-HTIA agonists [75–77]. In actuality, there is no influence or impairment in learning performance. Only one study [78, 79] discovered that ipsapirone, a partial agonist of the 5-HTjA receptor, improved performance in a conditional delayed discriminating task. It will be fascinating to see if a 5-HT1A antagonist will improve both memory and learning.

2.9. Excitatory Amino Acids. Glutamate, the most prevalent endogenous excitatory amino acid in the brain, has attracted a lot of attention because of its possible role in neurological and psychiatric disorders [80]. The importance of NMDA and AMPA receptors in long-term potentiation indicates a link between excitatory amino acids and learning and memory activities. The physiological correlate of memory formation has been considered to be long-term potentiation [81]. Although there is evidence that blocking the NMDA receptor can affect both long-term potentiation and memory, increased glutamatergic signaling may have detrimental repercussions for behavior since excessive levels of glutamate are neurotoxic [78]. As a consequence, excitatory amino acid receptor agonists' memory-enhancing effects may be limited. However, a newly developed pharmaceutical (i.e., 1-(1,3-benzodioxol-5-ylcarbonyl)piperidine), which was expected to boost AMPA receptor activity, was found to improve cognition in different learning and memory models [79].

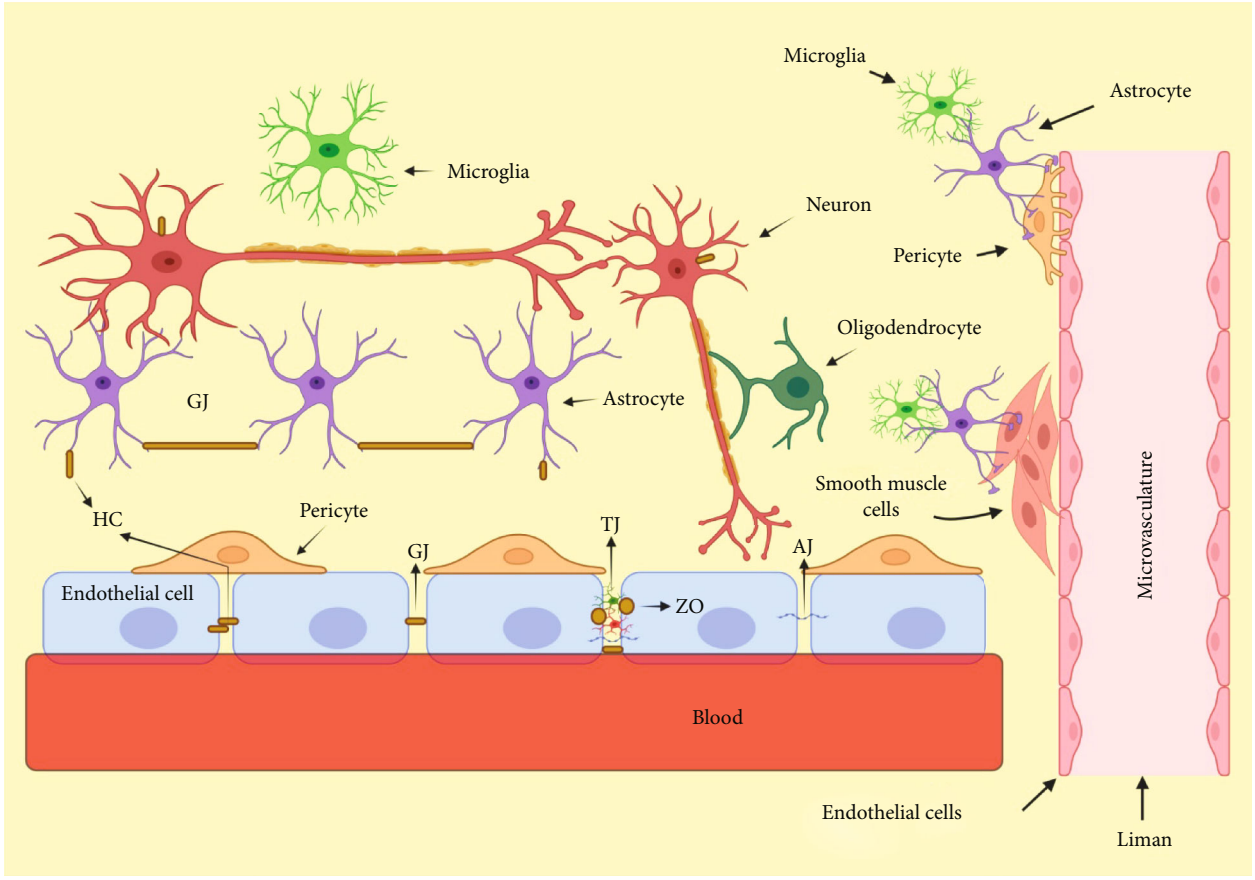

Figure 2: Connexin-based channels in the blood-brain barrier. Capillary endothelial cells (ECs), pericytes, neurons, glial cells, and microvasculature involvement make up a functional unit.

Finally, there seems to be a glutamatergic shortage in AD [82].

3. BBB Structure

An essential constituent structure in the blood-brain barrier (BBB) is glial cells. Pericytes and endothelial cells (ECs) collaborate to form a continuous, membrane network around blood vessels that allows for molecular signaling (Figure 2). The barriers have strong selectivity for necessary nutrients, which prevents hazardous materials from entering the brain and keeps brain homeostasis stable. BBB thus serves a vital role in keeping the unique neuronal function in the systemic circulation safe from biochemical attack.

4. Glia's Role in a Healthy CNS

In the CNS, three kinds of glial cells (astrocytes, oligodendrocytes, and microglia) are traditionally differentiated, each with specific roles. Due to their diverse functions, polydendrocytes or NG2(+) oligodendrocyte precursor cells (OPCs) could be respected as a fourth glial cell type [80]. Practically completely known CNS pathologic conditions, including neurodegenerative problems like AD, PD, ALS, HD, MS, and SCI, are influenced by each of the four cell types [83–91]. Therefore, reasonable model frameworks for explaining the unmistakable jobs of each glial cell type in a sickness state and looking at their dynamic interchange could be gigantically significant in the advancement of new therapies. In the CNS, astrocytes are the most common cell type. As the nervous system becomes more complicated, their ratio and amount to neurons increases, demonstrating their importance in the development and maintenance of this complex system [92, 93]. The astrocyte population is exceptionally diverse in shape and gene expression, which aligns with the numerous roles of this cell type [94–96]. The fundamental job of astrocytes in the CNS is to maintain and provide homeostasis. Ion, neurotransmitter and neurohormone trafficking, metabolic support for storing and dispersing energy substrates like lactate, cellular homeostasis (neurogenesis), and organ homeostasis for constructing and maintaining the blood-brain barrier (BBB) are all examples of this [91]. Additionally, astrocytes integrate and coordinate synaptic and nonsynaptic impulses, as well as impact neighboring cell activity in a flexible manner [97, 98]. Initially, astrocytes were thought to overlap, but new data reveals that they are structured systematically, with individual cells covering separate territories and interacting with both the microvasculature and neurons. They create a tripartite synapse with neuronal transmission, and activation is modulated by pre- and postsynaptic neurons. A single astrocyte may touch hundreds of synapses simultaneously due to

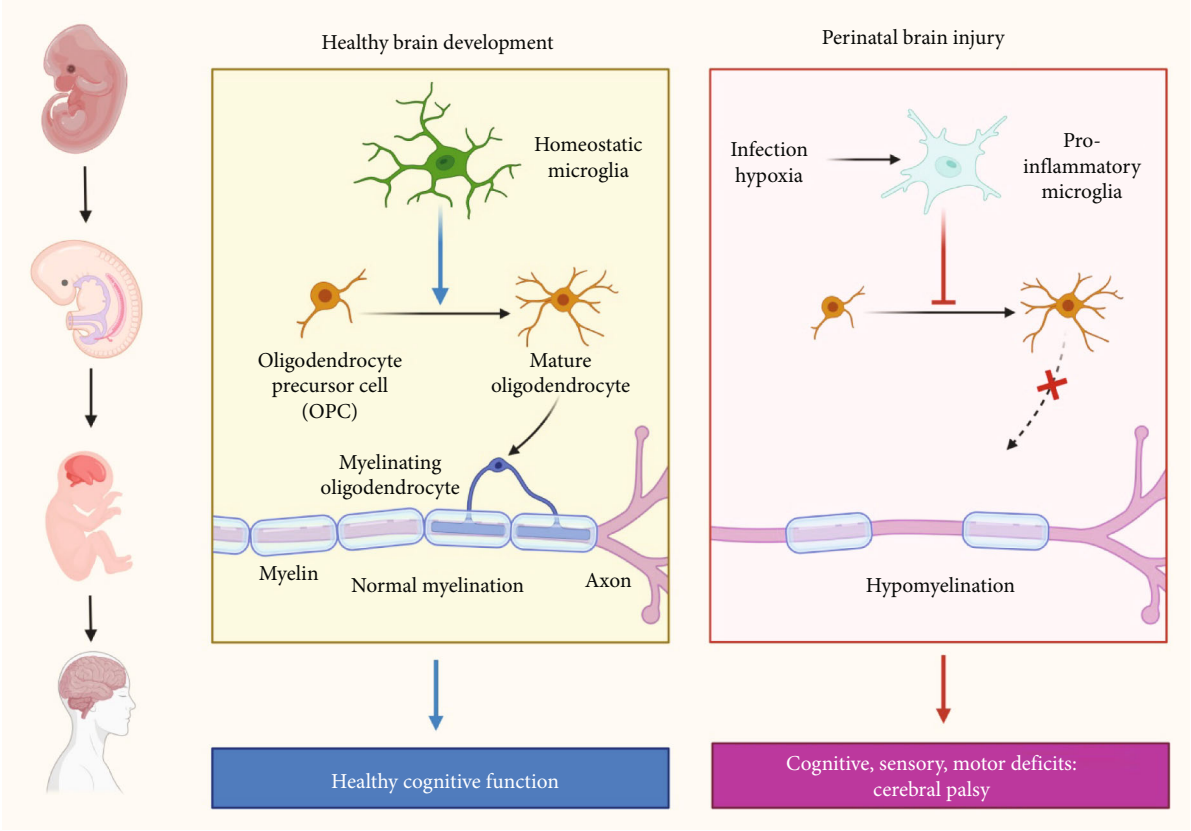

Figure 3: Differential roles of microglia in the developing brain. By phagocytosing dead or dying cells, microglia can control the amount of neurons in the growing brain and give neural progenitor cells trophic support for growth and maturation.

its numerous processes and branches [99]. Furthermore, astrocytes are linked by gap junctions that connect neurons to form a complex network that sends messages via Ca^{2+} waves at a much slower rate than neuronal communication [100].

4.1. Microglia. Microglia are CNS tissue-explicit macrophages with an extended life expectancy that make around 15–20 percent of the synapses. They come from the yolk sac's mesodermal hematopoietic undifferentiated cells, in contrast to the ectodermal produced neurons, astrocytes, and oligodendrocytes. Microglia antecedents (myeloid forebear cells) arrive at the CNS during early-stage improvement before the BBB is made [101]. As the name says, microglia are a lot more modest than astrocytes. They exist in an amoeboid transitory state when they enter the CNS or are set off and a ramified "resting" shape with a minuscule soma and broad praiseworthy cycles when they are not enacted. They are equally scattered all through the grown-up CNS, with every cell having its particular area (like astrocytes). Because of their immobility and lack of activation markers, "resting" microglia were thought to be dormant until recent research revealed that their fine ramified processes are constantly monitoring the environment [102]. Microglia (Figure 3) go about as immunological assessors in the sound CNS and are fundamentally liable for eliminating waste. To consistently pass on their amazing well-being to the microglia, neurotransmitters and neurotrophins are delivered by neurons and astrocytes [92, 93, 103–105]. Microglia, similar to neurons, have an assortment of synapse receptors that distinguish neuronal action and direct microglia movement, provocative reactions, cytokine delivery, neuroprotection, and neurotoxicity [106, 107]. Microglia are insusceptible cells with chemokine, cytokine, and supplement factor receptors that produce modulatory substances like cytokines and responsive oxygen species (ROS). Antigens are conveyed to attacking T lymphocytes through the significant histocompatibility complex (MHC) class II complex. After detecting a physical issue or obsessive affront, microglial cells quickly change into an amoeboid shape and move towards the site of the sore [108–110]. Microglia cells relocate towards injured or dead neurons because of glutamate-initiated Ca^{2+} waves, as per new exploration [111].

4.2. Oligodendrocytes. Myelination of neuronal axons is carried out by oligodendrocytes in the CNS, which is required for rapid electrical signal transmission. OPCs arise in many brain areas throughout development and travel great distances to reach their eventual destination. OPCs go through complicated proliferation and differentiation processes throughout this process. Myelination begins immediately after birth, once the OPCs have finished their migration to their site of activity. In humans, the majority of myelination

occurs within the first year of life; it continues in some areas of the CNS until young adulthood [112] and throughout adulthood [113]. The development of cognitive ability in specific regions appears to be linked to myelination in those areas [97, 98, 112]. When immature oligodendrocytes come into touch with target axons, they undergo a complicated process of differentiation that entails wrapping their plasma membranes around the neurons [108, 114]. The cytoplasm is drained as the membrane layers thicken, and the residual sheets contain up to 160 compact membrane layers of myelin lipids and proteins [109, 115]. The myelination process and the development of OPCs into adult oligodendrocytes are carefully controlled. Due to a lacking of model systems, the signaling pathways and substances involved are currently unknown [116, 117]. During their development from OPCs, oligodendrocytes appear to only myelinate for a brief period [118]. While electrical driving forces in neurons are essential for myelination to start, astrocytes assume a part in the wrapping's effectiveness and speed. Oligodendrocytes have a layer that can uphold multiple times the heaviness of their cell body. An oligodendrocyte can create up to 5000 m^2 of new layer each day during top myelination, which is a huge metabolic exertion that requires a ton of oxygen and adenosine triphosphate (ATP), just as a ton of endoplasmic reticulum limit [106, 115, 116]. Despite the way that these phones live for quite a while in a solid neurological framework, with a turnover pace of just a single cell in 300 every year, they are helpless against injury and stressors such aggravation and oxygen hardship [107]. The leftover NG2(+) OPCs scattered all through the grown-up CNS can supplant lost oligodendrocytes (for additional data on NG2(+) cells, see the part underneath). Oligodendrocytes give trophic variables to neurons and control axon width and particle direct dissemination as well as giving protection [117]. Hole intersections (like those found between astrocytes) interface oligodendrocytes with astrocytes, taking into consideration the dispersion of particles and little atoms, metabolic trade, spatial buffering, and electrical coupling [119]. To get signals, for example, certain MHC subtypes, supplement factors, cytokines, chemokines, glutamate receptors, and cost-like receptors, oligodendrocytes produce and express invulnerable administrative synthetic compounds and receptors [110]. This shows that oligodendrocytes play a part in irritation and are firmly connected to microglia.

5. Extracellular Vesicles as Neuron-Glia Communication Mediators

Glia cells assume a basic part in the creation, upkeep, and capacity of the focal sensory system, all of which requires critical cell-cell associations among glia and neurons. Direct cell-cell association or the paracrine activity of delivered atoms can help intercellular correspondence. A novel contact method based on cell-to-cell exchange of extracellular vesicles (EVs) has emerged in recent years. Numerous cell types discharge EVs into the climate, which can move an assortment of biomolecules between cells over brief distances or longer distances. EVs are discharged by both glia and neurons, and new examination shows that EV intercellular correspondence in the CNS has an assortment of practical implications [91, 116, 119, 120]. Size, payload, layer piece, and beginning of extracellular vesicles, for example, shedding microvesicles (MVs), exosomes, and apoptotic bodies, shift. Apoptotic bodies are delivered during apoptosis, while sound cells make various types of vesicles. EVs can be detected in practically all bodily fluids, and distinguishing between them has proven difficult due to several classification criteria overlapping [121]. In contrast to MVs, which are generated directly from the plasma membrane and range in size from 50 to 100 nm, exosomes are formed through the endosomal system (up to 1000 nm in diameter). Exosomes are intraluminal vesicles of multivesicular bodies (MVBs); hence, the ESCRT (endosomal sorting complex required for transport) process is necessary to sort them at the endosomal limiting membrane [80, 110] or aided by ceramide and tetraspanins, two sphingolipids [89, 122]. Exosomes are framed when MVBs combine with the plasma film and Rab GTPases; for example, this cycle is controlled by Rab27 in epithelial cells and Rab35 in oligodendrocytes [102, 108]. Tetraspanins, integrins, heat shock proteins, biogenesis-related proteins (such as Tsg101 and Alix), and components specific to different cell types can all be present in exosomes. However, other intracellular structures such as the mitochondria and endoplasmic reticulum are not included [92, 123]. The composition and biogenesis of MVs are less well understood. Exosome-forming components may, interestingly, be necessary for MV production by the molecular machinery [97, 113].

6. Neuronal Doctrine Challenged: Glial Cell Shape Brain

Only a million years ago, the quick growth of thinking, and thus of humanity, remained the major puzzle in our understanding of ourselves. The sudden advent of intelligence, and thus an only around a million years ago, human beings appeared. The critical question for our self-understanding has not been solved yet. In the same way, we have no idea how the human mind is more developed than animals. And actual variation lies between animal and human being. Since the turn of the twentieth century, this neuronal doctrine has governed current neuroscience [124, 125]; the neuron is a fundamental data processing unit made up of neurons in the brain that transfer messages unidirectionally from receiving dendrites to the integrating cell. The axon's terminal branches connect the body to the axon's terminal branches. The substrate of our intellect is widely thought to be a neuronal network connected by synaptic connections. The diameter and length of neuron cells increase according to the size of the brain (mammal). Surprisingly, the structure and physiology of neurons in humans and animals are essentially comparable, as is the quantity of neurons in humans and animals. The number of synapses in rodents and human brains is approximately 1100–1300 million per mm that is more or less stable [126]. Human protoplasmic astroglial cells, the most common glia form in grey matter, have the following linear dimensions. They are about 2.75 times more significant, and their density is about 27 times

higher than in a mouse brain for the same cells. Moreover, the protoplasmic astrocytes of humans have roughly 40 major processes, with far more sophisticated branching than mouse astrocytes (they have only 3–4 main functions) [127]. In contrast to all of these quantitative alterations, the CNS of Homo sapiens and other primates acquired distinct forms of astroglia, such as interlaminar astrocytes and astrocytes. Polarized astrocytes [127, 128] are not found in the brains of different species. Glial cells play a significant role in brain development. Astroglia are neuronal-glial-vascular units that compose the CNS's well-being and provide all lines of protection.

7. Glial Networks of Neurons (Integral Gear of Brain Activity)

In the brain, information processing is usually drawn from neural activity, with neurons and their dynamic signaling pathway responsible for data transit and processing [129]. Despite this, great progress has been made in understanding the molecular and physiological characteristics of astrocytes, a kind of glial cell revealed to play a role in neurotransmission and neuronal function [130, 131]. Moreover, the active astrocyte participation in synaptic transmission, usually as neuroactive name particles released by astrocytes, describes productive signal transduction among both astrocytes and neurons [132]. There is a broad consensus that astrocytes show a critical function in the case of maintaining homeostasis of surrounding synapses, including crucial involvement in energy metabolite supply [133, 134] and clearance of extracellular potassium [135–137]. Furthermore, astrocytes that surround synapses control the extracellular space volume, as well as the amounts and flow of neuroactive chemicals outside the cell [138]. Aside from their homeostatic functions, astrocytes dynamically connect with neurons and synapses. To monitor neuronal and synaptic action, ion channels, neurotransmitter transporters, and receptors are engaged. These are activated. Molecular interactions may result in complex Ca^{2+} signals being sent to astrocytes. Astrocytes, for addition, can modify surrounding pre- and postsynaptic neuronal elements, generating functional but also morphological changes; gliotransmitters such as glutamate, ATP, and D-serine are consumed or released in the brain [139]. Synapses are the connections between neurons. Whether astrocytes are active constituents, however, remains to be shown in how neural networks work and whether or not they play a role in dynamic functions in information processing in the brain (Figure 4) [140–142].

8. Glial Cells' Importance in Neurological Disorders

Glial cells had originally thought to offer primarily structural and trophic aid for neurons, gluing them together (glia seems to be the Greek word for "sticky," a phrase for glue) and providing them with essential nutrients for life. Only neurons were given the responsibility of conveying and data processing. As a result of this idea, drama has changed [143].

8.1. Acute Insults to the CNS

8.1.1. Toxins. Astrocytes are predominantly targeted by heavy metals, which cause substantial brain damage and cognitive deficits. Because heavy metals (such as manganese, Pb, Al, and Hg) are predominantly segregated into astrocytes by diverse mechanisms, this is the case. Plasmalemmal carriers are a type of transporter found in plasma cells. Heavy metals, on average, reduce astroglial transcription of carriers of glutamate, resulting in a reduction in glutamate discharge and neuroinflammation [144–147]. Minamata illness is the name given to methylmercury poisoning, which was initially identified in the Japanese city of Minamata [148]. Ocular defects, peripheral deficits, central defect, deafness, weakness, and convulsion are signs of Minamata disease. Methylmercury is mainly found in astrocytes where glutamate and cystine uptake is inhibited [149].

8.1.2. Neurotrauma. The traumatic brain and neurological disorders are categorized per their origin (penetrating wounds or concussions; it is referred to medically as cervical cord neurapraxia when it occurs in the cervical spinal cord), and severity like it can be more harmful or less. It can influence life risk, physical disabilities, mild impairment, healing location, and anatomical location [150]. A severe event to the central nervous system, by its character, has complicated pathogenesis connected mostly with direct damage to neural cells and the whole system, including loss of the brain stem capillaries and the BBB. Neurotrauma primarily induces an astrogliosis response, reaching highly reliant on the pathogenic environment [151–155]. But in the aftermath, a neurotrauma astroglial scar border develops, identifying and segregating all sites of the focused lesion from the brain, which is sound. Prevention during astrogliosis, resulting in a distorted astroglial defect, worsens cellular injury and the neurologic deficiency [156–161].

8.1.3. Stroke. Astrocytes assist neurons in the case of ischemic penumbra via several homeostatic mechanisms. Importantly, the astrocyte regulates the balance of glutamate in the ischemic area of the brain. They also provide metabolism resources like lactate to neurons. In the situation of ischemia, the neuroprotective effect is increased by lactate [162]. Glutamate inflammation, which invariably occurs after a heart attack, is nearly entirely the responsibility of astroglial units. The infarct size is increased when the astroglial glutamate channel GLT-1 is found [163].

8.2. Epilepsy. A gradual depolarization in neurons is known as paroxysmal depolarization shift, and it is the cellular substrate of epilepsy and coincides with all cells inside an epileptic focus. Ionotropic glutamate receptors were engaged to release glutamate continuously in the multiple surrounding neurons in epileptic focus, which are responsible for such a depolarization. Epilepsy is linked to a large amount of reactive astrogliosis and the formation of a glial scar. Reactive astrogliosis germinates itself in the initial phases of these diseases, even before the clinical presentation of seizures. In epileptic tissue, the reactive astrocytes lose their domain structure. Such trait has been detected in human

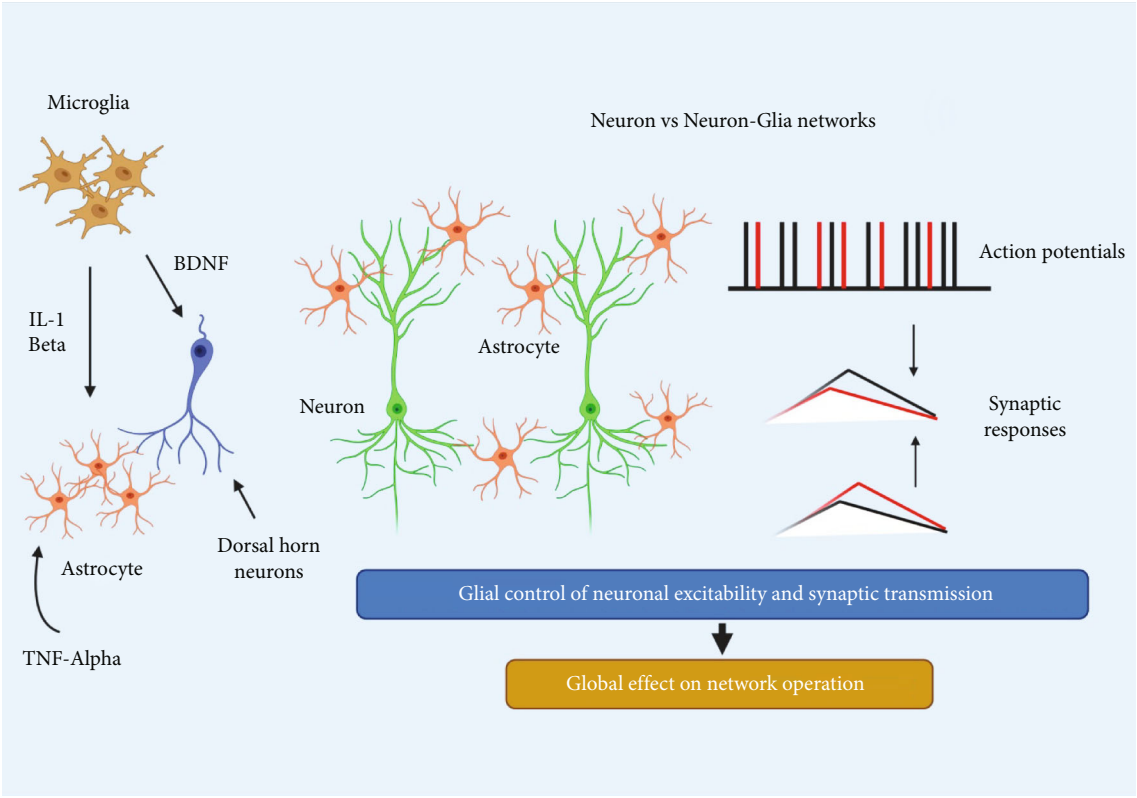

Figure 4: A representation of the neuron-glia network. The (red) astrocytes interact with the neurons (gray) and influence cellular excitability (top-left panel) and synaptic responses (bottom-right panel), affecting neural network function. On the right, the black and red markers show neuron-glia activity.

postmortem samples and animal models [164]. Ionotropic and metabotropic glutamate receptors are overexpressed in astrocytes from epileptic tissues, while inwardly rectifying K^+ channels and aquaporins are underexpressed. Additionally, epilepsy decreases glutamine synthetase and astroglial plasma membrane glutamate transporter expression and function, resulting in abnormal glutamate and GABA homeostasis [137, 165–169]. Epilepsy has been linked to changes in astrocytic intracellular and intercellular Ca^{2+} dynamics [170].

8.3. Alexander Disease. Alexander disease (AxD) is a neurodegenerative disease that is rare, chronic, and generally fatal. It is named after William Stewart Alexander, a neuropathologist who first identified it. In pathophysiological terms, AxD is a leukodystrophy with a distinct phenotype and a primary hereditary astrogliopathology [171]. A significant gain of function variation in the GFAP gene causes AxD. This causes astrological pathology which causes significant harm to the increasing white matter. AxD is characterized by the development of protein aggregates known as Rosenthal fibers around astroglial nuclei and endfeet [171]. Megalencephaly, seizures, spasticity, speech issues, and swallowing are just a few examples of the severe mental and physical difficulties that are present in type I AxD. Type II AxD has a later onset and slightly different and less severe clinical manifestations, such as ataxia, visual and motor difficulties, autonomic dysregulation, sleep disturbances, hyperreflexia, and problems speaking and swallowing [172].

8.4. Neurodegenerative Disorders. As recently said, neurodegenerative infections, for example, AD, PD, and HD, are brought about by an assortment of pathologies with an assortment of hidden causes. Therefore, we have a restricted handle of the beginning phases of numerous illnesses, making it hard to recognize causes from results and hindering a total cognizance of the essential components [173]. One of the most important variables in increasing therapy outcomes is early intervention. New reprogramming and culturing approaches are fascinating tools for understanding disease development early on. Even though diverse subtypes of neurons are destroyed, they all develop distinct characteristics as the disease progresses. Because aging is a significant risk factor for many diseases, cellular care may have a significant impact on disease progression. Protein aggregation, protein trafficking, and energy metabolism disturbance, as well as oxidative stress and the production of free radicals, are all common occurrences [165, 174]. Glial cells of the CNS, which are in charge of metabolic, cellular, and transfer signals, play a big role in these systems. Receptive gliosis, which is characterized as glial cell initiation and multiplication because of injury, happens in every single neurodegenerative ailment [175]. All glial cell types are implicated in neurodegenerative diseases, and a discussion of them would be

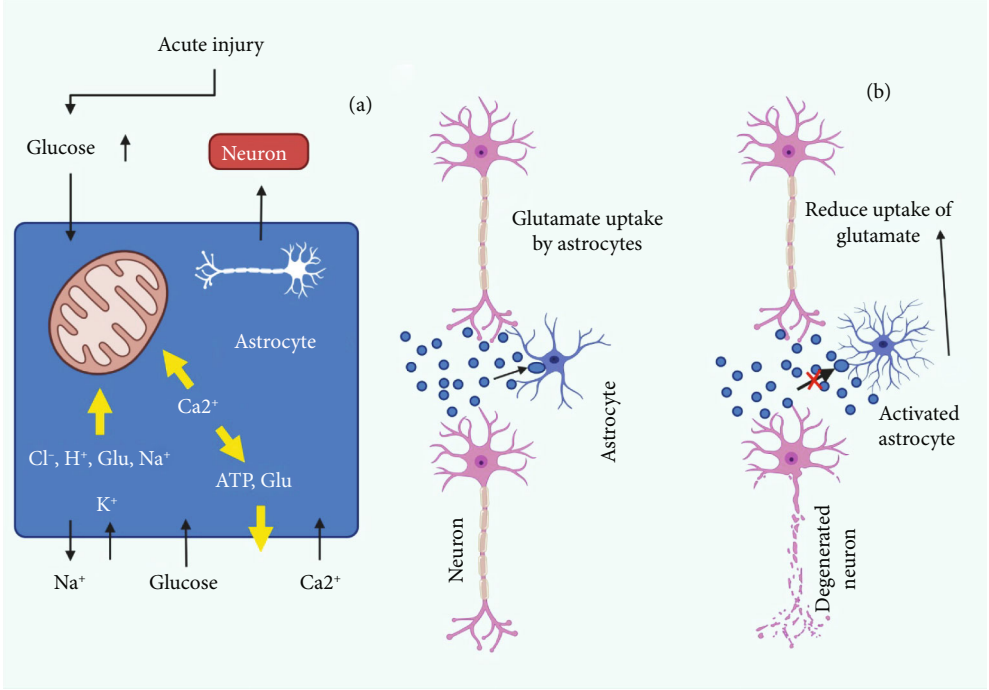

Figure 5: Reduced glutamate uptake by activated astrocytes.

beyond the scope of this article [176–178]. It has been discovered that in ALS, engine neurons, then again, control the beginning of the illness, while astrocytes and microglia are dominatingly engaged with infection movement, suggesting that altering the reactions of these phone types could bring about significant advantages for patients [179–181].

8.5. The Interaction of Neurons and Glia in Alzheimer's Disease. The majority of neurodegenerative diseases are classified as "proteinopathies," or toxic protein clumps [182–184]. Protein aggregation most commonly happens in synapses, resulting in synaptic dysfunction [185]. Aside from proteinopathies and neuron degeneration, new research suggests that glial cells are active actors in neurodegenerative illnesses. Microglia and astrocytes are part of the network that helps the brain connect by deleting superfluous synapses [186]. Glial-based synapse elimination is reduced with age and disease. Increased complement cascade activation generates extra chemical disposition in the synapse, which disrupts the clearance process, especially in neurodegenerative conditions. Several animal models of dementia and human investigations have shown that elevated levels of C3 and C1q (components of the complement cascade) contribute to synapse loss. In AD, glutamate excitotoxicity leads to synaptic weakening due to glutaminergic signaling abnormalities [4, 187, 188]. In amyloidopathy AD models with activated microglia-mediated synaptic engulfment, C1q knockout aged mice lacking C1q-protected synaptophysin loss in the hippocampus were studied [189]. The buildup of A peptide at synaptic locations in the AD brain occurs long before the extracellular plaque aggregation that causes synaptic structural abnormalities [185, 190]. Astrocytes can change their shape and function in response to a diseased situation, which is known as astrogliosis [191]. According to De Strooper and Karran, astrogliosis is characterized by hypertrophy, multiplication, and overexpression of the glial fibrillary acidic protein (GFAP) [192]. Under pathological situations, astrocytes fail to perform their natural functions, such as maintaining K^+ and glutamate balance, resulting in neuron depolarization (i.e., elevated Ca^{2+}) [191]. Glutamate uptake by astrocytes is required for neuronal cell protection; abnormal glutamate uptake causes neuronal cell injury [193]. Several investigations have suggested that amyloid protein buildup triggers astrogliosis; nevertheless, activated astrocytes produce an A42-immunopositive substance that changes in concentration across the cerebral cortex in AD brains depending on the severity of the disease. Although the precise method by which A42 material collects inside active astrocytes is unknown, it is thought to be owing to either phagocytosis or endocytosis [194–199] (Figures 5 and 6).

Reactive astrogliosis can produce inflammatory molecular mediators that cause persistent inflammation, which is related to the development of AD [200]. Inflammatory cytokines released by microglia and astrocytes stimulate the release of secretase-1, a critical enzyme in the synthesis of amyloid protein; they also affect phagocytic activity, which is responsible for amyloid protein breakdown and clearance. Aside from the modification in the synthesis of neurotrophic mediators, another function of cytokines is the suppression of LTP, a crucial chemical for memory in the hippocampus; both processes will result in cognitive symptoms of AD [201]. The buildup of A causes upregulation of NF-κB, which drives astrocytes to produce C3. Dendritic shape and network dysfunction are altered when C3 binds to the c3aR receptor (Figure 6) [192].

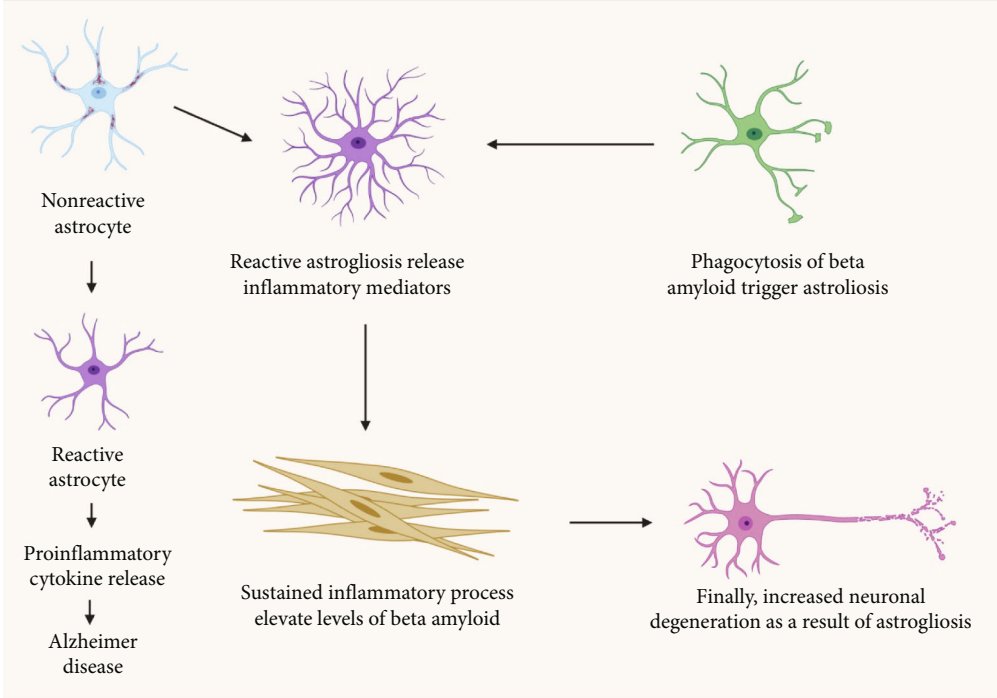

Figure 6: Representation of astrogliosis causing for neurodegeneration.

8.6. Multiple Sclerosis, Inflammation, and Injury. Multiple sclerosis (MS) is a CNS inflammatory disease with an unknown etiology that may include metabolic, genetic, and immunological factors [202]. This is one of the most prevalent CNS inflammatory disorders, and it is thought to be caused by an autoimmune reaction directed targeting myelin [203]. An abatement in oligodendrocyte number is found in a relationship with the collection of provocative cells and responsive glial cells. In creature models of MS, microglial initiation is seen before infection starts, and it is expected to assume a part in tweaking the provocative reaction [204]. Astrocytes have a significant influence on the provocative cycles in the CNS by enacting microglia, drawing leukocytes from the outskirts, altering BBB porousness, and emitting chemokines. Astrocytes assume a part in MS improvement and other CNS fiery cycles. Oligodendrocytes are powerless against irritation-initiated injury brought about by supportive incendiary synthetic compounds and nitric oxide (NO) foster anomalies and pass on during these cycles. NG2(+) OPCs might make up for the deficiency of oligodendrocytes and remyelinate axons in the beginning phases of MS, as indicated by mice models [205]. Be that as it may, NG2(+) cells are very powerless against aggravation instigated harm, and their numbers decay extensively as MS advances [206].

9. Neuro-Glial Coagulonome in Diseases of Peripheral Nervous System

In the PNS, Schwann cells discharge an enormous number of proteins that are all in all known as the secretome. Neurotrophic factors and extracellular matrix proteins are among the proteins produced in response to various brain impulses [207].

9.1. Peripheral Nerve Injury. Many studies show that coagulation factors, particularly the thrombin pathway, have a role in Schwann cell-mediated reanimation and axonal activity. The thrombin inhibitor protease nexin 1 (PN1) is secreted in the media of Schwann cells, which was discovered in the early 1990s [208]. In the peripheral nerve crush model, thrombin and prothrombin levels are elevated. Following the injury, this improvement is associated with PN1. The Schwann cells were shown to be the producer of this PN1 [209]. The participation of coagulation is indicated by a substantial elevation of factor V levels in Schwann cells following damage [210].

The activation of protease-activated receptor 1 (PAR1) could have beneficial or harmful consequences on nerve regeneration transmission. As previously stated, increasing the thrombin levels in node of Ranvier (NOR) results in a conduction block [211]. In reaction to a sciatic nerve crush injury, thrombin levels increase [212, 213]. High amounts of this substance have been related to a negative impact on nerve function. Small quantities of thrombin produce APC (activated protein C), which can stimulate PAR1 when linked in its receptor, EPCR (endothelial protein C receptor) [214]. This stimulation has a therapeutic effect on the brain [215, 216]. PAR1 stimulation boosts cultured Schwann cells' neuroprotective and neurotrophic abilities [217]. Surprisingly, the EPCR pathway activates PAR1, which reduces thrombin in vascular permeability while boosting the protective characteristics of BBB endothelial cells. Activation of sphingosine-phosphate mediates these effects [218]. The action of sphingosine-phosphate channel stimulator fingolimod on Schwann cells also supports positive PAR1 channel activation. Fingolimod, a drug used to treat MS, encourages the synthesis of proteins that stimulate neurite growth in

Schwann cells, resulting in a "regenerative phenotype" [219]. Thrombin levels rose in the first hour after damage and then returned to normal state later a week. After four days of injury, the levels of EPCR were high, notably distal for injury. EPCR has also been identified in the NOR's Schwann microvilli. The PAR1 activity for the therapeutic impact is supported by the discovery of greater EPCR levels distal to the damaged location, where Schwann cells develop quickly [212]. It has also been investigated how active thrombin is produced at wounded nerve locations. TF (tissue factor) and Xa are two crucial participants in the thrombin production system in the blood coagulation pathway. Factor X is split by TF/factor VIIa complex to produce the factor Xa, which stimulates prothrombin to active thrombin. Data indicating that medication of apixaban which is a specific factor of Xa inhibitor progress the function of motor following damage adds to the clinical concern [220].

9.2. Guillain-Barre Syndrome. Inflammatory neuropathies are a set of disorders in which axons, myelin, or both are damaged. Because an inflammatory response influences the NG-coagulonome and can alter Schwann-axon function, it is critical to assess its position in diseases like these. The acute inflammatory neuropathies are referred to as GBS, but inflammation of polyneuropathy is common [221]. GBS patient needs mechanical ventilation in 25% of cases, and also, patient cannot be able to walk in 20% of cases after six months, and death occurs in 3-7 percent of cases. The typical mouse model for GBS is the autoimmune animal model, which shows nerve conduction velocity reduction and NOR structure degeneration. These functional and structural changes are associated with increases in thrombin phases in the sciatic nerve and a decrease in nodal PAR1 stages. The animal flow velocity and NOR structure are balanced when given thrombin inhibitors [222].

9.3. Chronic Inflammatory Demyelinating Polyradiculoneuropathy. CIDP (chronic inflammatory demyelinating polyradiculoneuropathy) is a peripheral nervous system (PNS) demyelinating illness that affects gradual loss of motor and sensory abilities [223]. With the exception that it is chronic and has relapsed, CIDP has a clinical course similar to GBS. The onset is gradual, and it disproportionately affects people of a given age range [224, 225]. The resistant framework assaults and obliterates the myelin sheath of the PNS, causing demyelination and axonal degeneration in fragments [226]. Histological outcomes from the CIDP show a meager myelin sheath with more limited internodes, commonly referred to as onion bulbs. The slow nerve conduction rate, which indicates conduction block, indicates demyelination [226]. Autoimmunity to neurofascin-155 (NF155) and contactin-1 (CNTN1) has recently been discovered in a large number of patients [227, 228]. NF155 is a glial paranode-expressed adhesion molecule that binds to CNTN1, a key axonal adhesion molecule [229]. CIDP symptoms appear gradually but steadily, with neurological impairments peaking after 8 weeks of disease initiation [223]. There are a number of signs and symptoms, such as tingling and numbness in the extremities, symmetrical sluggishness and paresthesia in the arms and legs, fatigue, ataxia, and limb incoordination [226]. Therapy with oral glucocorticoids usually results in a positive outcome. Plasmapheresis and intravenous immunoglobulin (IVIG) are also successful treatments [194, 223, 230–236].

9.4. Neuropathy Caused by Anti-Myelin-Associated Glycoprotein. IgM monoclonal gammopathy against MAG in fringe nerves causes myelin-associated glycoprotein (MAG) neuropathy [237]. MAG is a sort I transmembrane glycoprotein present in the periaxonal SC and oligodendroglial layers of myelin sheaths, where it keeps in touch and axonal capacity [238]. At the point when MAG is lost, the integrity of the myelin sheath and axonal capacity are impaired. MAG has a carb epitope with other glycoconjugates that go about as significant antigenic destinations for IgM paraproteins [237]. Chickens are administered with serum that contains IgM antagonistic to MAG paraproteins, which results in segmental demyelination and conduction obstruction [239]. Increasing mild to severe distal muscular weakness and growing sensory ataxia and frequent tremors are all symptoms of the condition. The clinical course is usually uneventful, with only minor functional decline over time. Supportive therapy, such as exercise and balance training, is employed because anti-MAG neuropathy symptoms are typically mild and initially do not interfere with the patient's daily activities. Medication should be used to address sensorimotor weakness. Only in extreme cases are steroids, intravenous immunoglobulin, and plasmapheresis used. Rituximab, a monoclonal antibody that targets the CD20 antigen on the cell surface, is efficacious [240].

9.5. Nerve Pathology. Demyelination caused by macrophages is the first lesion, followed by Schwann cell growth and remyelination. Furthermore, there is a varying degree of axonal degeneration, which can become severe with time but is rarely the main feature. The motor axons' ventral roots and terminal portions are the first to be impacted. Many nerve trunks have demyelination that runs the length of them. Dispersed lesions with various degrees of demyelination and remyelination, as well as axonal degeneration, result from the pattern of evolution and distribution of lesions across the peripheral nervous system [241].

9.6. Vasculitic Neuropathy. PNS vasculitis can occur as a component of a larger systemic vasculitis or as a separate illness (nonsystemic vasculitic neuropathy) [242–245]. Primary systemic vasculitis [246] includes Takayasu syndrome, thromboangiitis obliterans, Kawasaki disease, Churg-Strauss syndrome, Wegener granulomatosis, cryoglobulinemic vasculitis, Behçet's disease, giant cell arteritis, classical panarteritis nodosa, microscopic polyangiitis, and Henoch-Schönlein. Vasculitis of the peripheral nervous system can take the form of a single mononeuropathy, overlapping mononeuropathies, or symmetric polyneuropathies that are far apart. Peripheral neuropathy is an important clinical feature of vasculitis, and it is often the first symptom [247–251]. The expression "mononeuritis multiplex" has been authored to depict the most common and trademark

kind of vasculitic neuropathy. It alludes to the imbalanced successive contribution of explicit nerves or trunks from distal to proximal [252, 253]. Neuropathy is habitually unexpected, with torment in the influenced nerve's field, showing that both engine and tactile modalities are involved. Fleeting and Takayasu arteritis are the two sorts of monster cell arteritis, albeit just transient arteritis causes fringe neuropathy. Patients might foster a few mononeuropathies, radiculopathies, plexopathies, or a wide tangible fringe neuropathy [254].

10. In Central Nervous System Diseases, the Neuro-Glia Coagulonome

A growing body of literature indicates that thrombin and its related route play a significant function in CNS physiology. Some thrombin's features are moderated by glia cells inside the CNS, just because they are in the PNS. The thrombin pathway has an effect on a variety of cellular processes, which can be beneficial or detrimental depending on dosage, receptor activation technique, and downstream indicating. Extrinsic sources of thrombin include inflammation events and blood-brain barrier disruption, while intrinsic sources include glial cells. The impact of thrombin on brain activity is profound, regardless of source. As previously stated, the PAR1 pathway is involved in myelin modulation and is required for nerve function [255, 256]. In PAR1 mutant mice, the direct placement of myelin proteins throughout the spinal cord was shown to be dysregulated. An *in vitro* study found that an oligodendroglia cell lacking PAR1 had higher levels of proteolipid protein and simple myelin protein, confirming the impact on myelin regulation [255]. The stimulation of PAR1 by thrombin causes a rise in cytosol calcium and mitogen-triggered protein kinases in cultivated microglia [257]. The generation of tumor necrosis factor and nitric oxide is responsible for this [258]. Thrombin activates PAR1 in astrocytes, causing structural and physiological alterations resulting in extended retract and astrogliosis [259]. As previously stated, the NG-coagulonome affects neuronal electrical activities and nerve function in physiology, implying that it may play a role in CNS disorders. Minimal thrombin values were discovered to have protective effects inside the CNS in the last decades, giving rise to thrombin preconditioning. Before ischemia, a trim level of thrombin administered directly to a rat caudate nucleus decreased ischemia injury and brain edema [260]. The dual impact of thrombin presents where the good and detrimental impacts meet at what concentration [261].

10.1. Epilepsy. The widespread incidence of brain damage and seizures has prompted scientists to wonder about the mechanism that links the two. Injecting thrombin directly into the brains of rats triggered motor seizures, according to a major study [262]. This was the first of many investigations exploring the complex relationship between thrombin, trauma, and epileptic activity. In the context of brain traumas and overly high levels of protein, the dangers of thrombin in the CNS have been investigated. The PAR1 pathway is implicated in epileptic activity in mice with either too much or too little PN1, indicating that the PAR1 pathway is involved [263]. Thrombin impacts neuronal plasticity in a dose-dependent manner, similar to how it affects the PNS. Low amounts activate the aPC, but high levels cause a gradual, LTP that is NMDA-dependent [264]. PAR1 is involved in both impacts. PAR1 and aPC promote LTP in hippocampal slices in the presence of a brief tetanic stimulation via a mechanism involving sphingosine-phosphate receptor 1 [265]. Thrombin changes the electrophysiological of mouse hippocampus brain slices via activating PAR1. It induces a decrease in the epileptic threshold in CA3 neurons as well as an increase in the responsiveness of CA1 neurons to afferent stimuli (which is inhibited by NMDA receptor antagonist) [266]. On the one hand, these two well-known thrombin adverse effects are associated with memory and learning impairments and, on the other, hyperexcitability and increased seizure susceptibility. Thrombin generates more spontaneous activity potentials in CA3 neurons, which is associated with higher PAR1 expression in CA3 neurons, according to whole-cell patch monitoring of pyramidal neurons in the hippocampus [267]. Seizures are triggered by a positive feedback loop of depolarization in a PAR1-dependent manner, a rupture of the blood-brain barrier, and the entry of more thrombin, according to this discovery [268]. Epileptic seizures, status epilepticus, and brain damage can all be caused by paraoxon and other organophosphates. The brains of paraoxon-treated mice contain high levels of thrombin, PAR1, and pERK, as well as enhanced electrical impulses in the CA1 and CA3 neurons of the hippocampal. A PAR1 antagonist is used to minimize excessive electrical activity [269–275]. These findings suggest that thrombin is implicated in an epileptic activity that is not triggered by trauma, raising new issues concerning the origins of thrombin in the CNS [276]. Epilepsy models with observed neuron-glia interactions are as follows [13]:

Genetic: AP- (activator protein-) 1 KO, SCN1A KO, and DBA/2 KO; Lafora disease: Malin KO, Epm2a, and Epm2b

Pharmacological: pentylenetetrazol (PTZ), pilocarpine (PA), and kainic acid (KA)

Environmental: electrical stimulation and brain injury

∗To study epilepsy, pharmacological models are commonly and widely used.

10.2. Traumatic Neuronal Damage (TBI). The term "traumatic brain injury" (TBI) covers a wide range of conditions in which the brain has been subjected to external force. Although it can cause headaches and short- and long-term cognitive impairment, mild traumatic brain injury (mTBI), which is defined as a Glasgow Coma Scale of 13 to 15 following a head injury, is relatively harmless. TBI damages the blood-brain barrier either microscopically or macroscopically; it may allow plasma proteins like thrombin to touch brain tissue, as well as the synthesis and release of endogenous brain proteases. TBI animal models come in a range of shapes and sizes, each indicating a different sort of injury mechanism. Among these are fluid percussion injuries, free weight drop devices, and piercing mechanisms. After spinal cord damage, better motor outcomes, less astrogliosis, and lower levels of proinflammatory cytokines like interleukin-

1 and interleukin-6 are all observed in PAR1 deletion rats. *In vitro*, thrombin stimulates the production of inflammatory cytokines by astrocytes, while astrocytes exposed to interleukin-6 upregulate PAR1 and thrombin, creating a feedback loop [277, 278]. Thrombin was thought to play a function in the development of hyperalgesia in rats after spinal cord injury. Fibrin levels rise one day after nerve root compression as an indirect indicator of thrombin activity. To prevent hyperalgesia, the mice were administered hirudin, a thrombin inhibitor, or PAR1 antagonists. Furthermore, even when there was no prior trauma, intrathecal thrombin injection generated hyperalgesia, which could be avoided by inhibiting spinal PAR1 before thrombin injection [279]. These two spinal cord investigations show the importance of the thrombin-PAR1 pathway in a variety of inflammatory processes and their clinical consequences following CNS damage, albeit they do not explain thrombin's origin. Amnesia following a mild traumatic brain injury (mTBI) is a common event that has been explored in an animal model induced by free weight loss, which is equivalent to the definition of mild TBI. According to behavioral and memory tests, brain thrombin levels rise after mTBI, and the animals become amnestic. Amnesia is caused in the same way by intraventricular injections of thrombin or a PAR1 agonist. PAR1 antagonists prevent this amnestic effect, indicating that the PAR1 pathway is implicated in the formation of amnesia after mTBI [279].

The stabilization of thrombin activity levels in the hippocampus marks the end of trauma-induced amnesia, according to a second study employing the same animal model. In the context of brain trauma, this shows a link between cognitive abnormalities and brain thrombin levels [280]. It is tempting to believe that the presence of thrombin in the CNS after an injury is due to plasma leakage caused by a blood-brain barrier rupture. Researchers looked at the kinetics of thrombin activity after damage to figure out where it comes from. In the mTBI model, two thrombin peaks were measured. The elevation was measured immediately after the insult and then adjusted an hour later. A breach of the blood-brain barrier is most likely to blame for this increase. The second peak occurred 72 hours later, followed by an increase in PAR1 and, more intriguingly, an increase in the thrombin inhibitor PN1. This late rise is very certainly due to inflammation mediated by astrocytes [281–284]. Injecting thrombin into the brain ventricle enhances seizure susceptibility 72 hours after the insult, according to the same study, when compared to animals who did not receive thrombin but did not suffer mTBI. After mTBI, an increase in PAR1 was assumed to be the reason for sensitivity [276].

10.3. Ischemic Injury. Thrombosis, embolism, or systemic hypoperfusion can all induce ischemic stroke. When global ischemia was induced in rats [285], prothrombin mRNA levels increased, while PN1 and PAR1 levels remained unchanged. This calls into question the role of thrombin in ischemic injury. When a brief carotid artery blockage causes *in vivo* ischemia, it increases prothrombin and factor X mRNA levels in the ischemic core as well as thrombin activity across the ischemic hemisphere, including the peri-infract areas [286]. Synaptic responses in hippocampal slices exposed to thrombin concentrations equivalent to those found in the ischemic hemisphere are changed [286]. A considerable increase in brain thrombin level over time was seen (up to 24 hours after ischemia) in an irreversible ischemic animal model, followed by a decline in PAR1 activity in the ischemic core [287]. A factor Xa inhibitor (apixaban) taken systemically reduces brain thrombin levels and diminishes infract size shortly after ischemia induction [288]. PAR1 knockout mice have less brain edema and neuronal damage, as well as fewer behavioral problems than wild-type mice [289]. In mouse mind cuts, *in vitro* ischemia brought about by intense oxygen and glucose hardship increments hippocampal thrombin presence and action while diminishing prothrombin mRNA. During oxygen and glucose hardship, *in vitro* accounts from CA1 neurons in the hippocampus demonstrated the development of ischemia LTP, which communicates the useful harm brought about by thrombin increment. Ischemic LTP is intervened by thrombin through PAR1 and is repressed when either thrombin or PAR1 is restrained. The NG-coagulonome may be a therapeutic target for ischemic stroke, according to these studies [290].

10.4. Neoplasms. In the United States, primary brain neoplasms occur at a rate of 29.9 per 100,000 people [291]. Glioblastoma (GBM) is a type of glial tumor that accounts for 15% of all primary CNS cancers. It is cancer that is quite aggressive, with a 10- to 12-month median overall survival rate [292, 293]. Current therapies for tumors in conveniently accessible locations include complete excision [294]. Adjuvant treatment with radiation and the alkylating medication temozolomide [295] is essential due to the tumor's infiltrative nature. Despite this combined strategy, these drugs seldom extend median survival time by more than a few months and are severely damaging to patients. GBM processes necessitate more precise treatments. Thrombin has been associated with the formation of GBM [296–298], and PAR1 expression in glioma cells has been identified. In culture, glioma cells synthesize and secrete active thrombin, which promotes proliferation and is inhibited by dabigatran, a thrombin-specific inhibitor [299]. In PAR1 knockout mice, GBM edema volume and glioma development indicators (vascular endothelial growth factor and hypoxia-inducible factor 1) are reduced [297]. The PAR1 gene (F2R) is also in the top 2% of overexpressed genes in GBM patients, according to differential analysis of its expression in human GBM patients [300]. In human GBM patients [301], increased PAR1 levels exhibit a positive correlation with TF expression and a negative correlation with tumor suppressor factors, implying that this pathway is involved in GBM pathogenesis. In a rat GBM model, thrombin activity is increased in vivo and is linked to brain edema volume. The rise in PAR1 is only seen in the tumor and does not affect the surrounding tissues [302]. SIXAC, a PAR1 proteolytic activation inhibitor that is selective and irreversible, inhibits glioma cell proliferation, invasive, and colony formation in vitro. SIXAC was demonstrated to minimize cerebral edema and extend survival in a rat GBM model when

administered directly to the tumor bed [300]. For this incurable condition, the NG-coagulonome has been proposed as a therapeutic target.

11. ASD and ADHD

There is presently no effective treatment for ASD as it is a neurological illness that affects 1% of the population. In ASD, there is a large gender gap, with boys having a rate that is 4.3 times higher than girls [303]. For the vast majority of persons who are impacted by ASD, the cause is unclear. Social and communication difficulties, repetitive behaviors, and excessive interests are all characteristics of ASD (Table 1). Autism is linked to both inherited and environmental factors, according to a previous study. One of the environmental elements connected to maternal immunological activation is linked to ASD. Maternal viral infection, exposure to toxins, and obesity have all been linked to inflammatory and immunological system failure, which may increase the likelihood of behavioral issues in offspring [304–306]. Mothers of children with ASD, for example, have an increased chance of allergies and autoimmune illnesses than mothers of children who are developing typically [307]. IFN-α, IL-α4, and IL-α5 levels were shown to be greater in the midgestation serum of women expecting a child with ASD. Neuron-glia interactions are also executed in ADHD models (Table 2).

12. Metabolic Interaction between Neuron and Glia: The Significance of Glia-Secreted Metabolites

Glial cells provide the majority of the energy that neurons require. For brain function, the bioenergetic connection between neurons and glial cells is essential. The metabolic coupling of neurons and glia is a multistep process involving a variety of enzymes that convert biomolecules, transporters that transfer substances between the two cells, and cell surface receptors. To carry out their jobs and preserve mitochondrial integrity and membrane potential, neurons require a lot of ATP. Endothelial cells, neurons, and glia make up the neurovascular unit, which controls the delivery of nutrients and energy supplies to neurons. Endothelial cells have receptors for a wide range of metabolic substrates, including glucose transporters 1 (GLUT1), monocarboxylate transporters for ketone bodies (MCT1 and 2), and fatty acid transporters (CD36,) [318, 319]. Endothelial cells have transporters that operate as a gatekeeper for nutrients entering the brain in a concentration-dependent manner. Intercellular contact between astrocytes and endothelial cells is essential for nutrient delivery to the brain. It was recently discovered that NO produced by endothelial cells boosts astrocyte glycolytic activity [320]. Endothelial cells that come into close contact with astrocyte end foot and therefore become active components of the neurovascular unit prevent neurons from oxidative destruction, create gliotransmitters, and give energy substrates for neurons. The astrocytic-neuron lactate shuttle (ANLS), which focuses on astrocytic responses to neuronal metabolic supports, illustrates the role of neuron-glial connections in preserving brain homeostasis [321]. In addition to astrocytes, recent studies have revealed the importance of oligodendrocytes in the metabolic maintenance of neurons, particularly the axonal regions of neurons. Both astrocytes and oligodendrocytes exhibit neuronal activity, which is detected by the extracellular glutamate that neurons release. Through GLUT1, glutamate binding to the appropriate receptors on both cell types facilitates glucose absorption. Inside the cells, glucose is transformed into either lactate by glycolysis or pyruvate for oxidative phosphorylation by the mitochondria. Glycogen, which is intracellular glucose that has been stored, can also be used by astrocytes to generate energy. Lactate is produced by oligodendrocytes and astrocytes and can either be actively transported to neurons by MCT transporters or converted to pyruvate for fatty acid or ATP generation. In order to prevent neuronal excitotoxicity, astrocytes also play a crucial role in glutamate synaptic clearance. Inside astrocytes, glutamine synthase transforms glutamate into glutamine, which is subsequently transferred to neurons to produce glutamate. Glutamate, glutamine, and tricarboxylic acid (TCA) cycle metabolism are closely related in neurons, astrocytes, and oligodendrocytes [321, 322]. Krebs cycle and citric acid cycle are other names for the TCA cycle. It is the most crucial metabolic pathway for the body's energy source. TCA is the most significant central metabolic route, connecting nearly all of the individual metabolic pathways. Since neurons can generate ATP from a variety of substrates depending on different circumstances, such as fasting or hyperactivity, glial cell metabolites are essential for maintaining neuronal energy requirements. Among the energy substrates, lactate is currently acknowledged as the predominant source of ATP synthesis during hyperactivity [323]. Since MCT2 reduction in the rat hippocampus led to memory impairment, lactate—which is mostly generated by astrocytes and oligodendrocytes—is known to have a function in memory formation. Injections of glucose did not improve the memory deficits, showing the relevance of glial-derived lactate in memory processing [324]. During hyperactivity, astrocytes use a variety of strategies to regulate neuronal metabolism, in addition to supplying lactate. Reactive oxygen species, which are generated during times of information processing, cause the phospholipids that make up cell membranes' fatty acid content to peroxide. Peroxidized fatty acids generated during vigorous neuronal activity may contribute to neurodegeneration because neurons' mitochondrial capability to utilise fatty acids for ATP production is constrained and they are unable to make lipid droplets. Recent research has demonstrated that astrocytes absorb peroxided fatty acids via lipoprotein particles and produce ATP by oxidizing fatty acids to protect neurons during times of hyperactivity. Increased reactive oxygen species (ROS) formation is countered by higher detoxifying gene expression. Additionally, enhanced inhibitory interneuron activity, which regulates excitotoxicity, is upregulated in response to glutamate released from overactive neurons by astrocytic ATP [325]. During neuronal excitation, astrocytes regulate cholesterol and fatty acid production to maintain synapse integrity and transmission. In

Table 1: Neuron-glia interactions are executed in ASD models.

Serial no.	ASD	Model of ASD	References
1.	Genetic	PTEN mutant, MeCP2 mutant, TSC1 HT, BTBR, Scn1a HT, Shank2 KO, Shank3 KO, NLGN3R451C KI, TSC1 HT, NLXN1 KO, BTBR, MeCP2 mutant, and Scn1a HT	[308, 309].
2.	Pharmacological	VPA (valproic acid)	[310]
3.	Environmental	MIA (maternal immune activation), methyl mercury, and polyinosinic: polycytidylic acid (poly I: C)	[307, 311–314].

Table 2: Neuron-glia interactions are executed in ADHD models.

Serial no.	ADHD	Model of ADHD	References
1.	Genetic	nAChR (nicotinic acetylcholine receptor) β2-KO, DAT (dopamine transporter) mutant, NK1R-KO, SNAP25 mutant, Cdk5 KO, Git1 KO, and DAT (dopamine transporter) mutant	[315]
2.	Pharmacological	Ethanol, methyl azoxy methanol	[316, 317].
3.	Environmental	Neonatal X-rays, hypoxia, heavy metal exposure (cadmium, lead), and oncogenic environmental exposure (polychlorinated biphenyl (PCB)) are all factors that might cause cancer in children	[315]

hippocampal astrocytes, sterol regulatory element binding proteins (SREBPs) are highly expressed, govern cholesterol production in astrocytes. SREBP cleavage-activating protein (SCAP) deletion in astrocytes reduced cholesterol and phospholipid secretion. In SCAP mutant mice, immature synapses increased while presynaptic proteins decreased, inadequate short- and long-term hippocampal synaptic plasticity as a result [326]. These results demonstrate that astrocytic control of neuronal activity is mostly mediated by cholesterol and fatty acid metabolism [24].

13. Concluding Remarks and Future Directions

The release of trophic factors and immunomodulatory substances by cellular replacement therapy is a defining moment in neuroscience, with a promising future in replacing lost cells and fostering a neuronal survival environment. Progress has been achieved in producing human iPSC lines from a variety of CNS illnesses since the discovery of iPSCs. In a recent study, iPSC-derived NPCs from a PD patient were transplanted into a monkey model, bringing iPSC research to the preclinical stage. Krencik and coworkers revealed a groundbreaking approach for generating astrocytes from iPSCs that showed functional features of glutamate absorption, synaptogenesis, and calcium wave propagation, as well as astrocyte lineage markers. This approach can now be used to generate glial cells from sick iPSC lines, laying the groundwork for future glial therapies. Other neurological disorders, such as depression, stroke, ischemia, spinal cord injury, autism, schizophrenia, and others, are being studied for glial involvement. Glial cells are a potentially attractive therapeutic target for cell replacement therapies due to ongoing efforts to recognize glial contributions to illnesses and efforts to replace them. Much about the communication between neurons and glia is still uncertain. For example, a wide controversy rages about how many of the many chemicals released by glia in response to physiological stimuli come from the release of adenosine triphosphate (ATP); other chemical messengers include glutamate and glutamine. Glial Ca^{2+} responses are generated by a diverse set of chemical messengers via a variety of pathways and hence could be linked to neuron-glial and glial-glial communication. Because Ca^{2+} is ubiquitous as a second messenger, it is even more difficult to distinguish direct from indirect effects. We know far too little about the vast range of glia variety. It is hard to discover the processes of interaction and communication between neurons and glial cells at this early period of research because of the numerous distinctions that are expected to exist between these cells. A prominent example of this phenomenon is the similarities and contrasts between cell culture and in situ neuron-glial communication. Information currently is not sufficient to reflect the full complexity of the events. Future studies on glial variety and cell-cell communication processes will lead to an improved understanding of the roles played by nonneuronal cells in neural processing. The neural connections glial cells make with neurons in several neurodevelopmental diseases were highlighted in our review. Autism, ADHD, and epilepsy are each characterized by some mechanisms that are present in ASD, ADHD, and epilepsy, such as neuroinflammation, imbalance of excitation and inhibition, and neurotransmitters. Complementary approaches using patients and animal models indicate that there is an increase in cytokines in the brain in neurodevelopmental disorders. Neurotransmitter alteration can potentially result in a neurotransmitter imbalance. An imbalance in neurotransmitter concentrations could be caused by changes in receptor and transporter expression levels, changes in released gliotransmitters, or a malfunction in uptake.

Abbreviations

CNS: Central nervous system
AD: Alzheimer's disease

HD: Huntington's disease
PD: Parkinson's disease
MS: Multiple sclerosis
ALS: Amyotrophic lateral sclerosis
SMA: Spinal muscular atrophy
BBB: Blood-brain barrier
NGF: Nerve growth factor
ECSs: Endocannabinoids
DSE: Depolarization-induced suppression of excitation
LTD: Long-term depression
CB1R: Cannabinoid receptor type 1
AC: Adenylyl cyclase
mGluR: Metabotropic glutamate receptors
ACh: Acetylcholine
LC: Locus coeruleus
GPCRs: G-protein-coupled receptors
AMPA: α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
NMDA: N-Methyl-D-aspartate
OPCs: Oligodendrocyte antecedent cells
SCI: Spinal cord injury
ATP: Adenosine triphosphate
AxD: Alexander disease
GFAP: Glial fibrillary acidic protein
PNS: Peripheral nervous system
PN1: Protease nexin 1
NOR: Node of Ranvier
EPCR: Endothelial protein C receptor
CIDP: Chronic inflammatory demyelinating polyradiculoneuropathy
MAG: Myelin-associated glycoprotein
TBI: Traumatic brain injury
NLGN: Neuroligin
NLXN: Neurexin
TSC: Tuberous sclerosis
MeCP2: Methyl CpG binding protein 2
Scn1: Sodium channel protein type 1
PTEN: Phosphatase and tensin homolog
NK1: Neurokinin
SNAP: Synaptosome-associated protein
ASD: Autism spectrum disorder
ADHD: Attention-deficit/hyperactivity disorder
GJ: Gap junctions
HC: Hemichannels
AJ: Adherens junctions
TJ: Tight junctions
BDNF: Brain-derived neurotrophic factor
GDNF: Glial cell-derived neurotrophic factor
BDNF 1: Brain-derived neurotrophic factor 1
GDNF 1: Glial cell-derived neurotrophic factor 1
IL1: Interleukin-1
IL12: Interleukin-12
TNFα: Tumor necrosis factor α
MCP-1: Monocyte chemotactic protein-1
GABA: Gamma-aminobutyric acid.

Data Availability

All data are available in the text.

Conflicts of Interest

The authors declare no conflict of interest.

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Review Article

Tacrine Derivatives in Neurological Disorders: Focus on Molecular Mechanisms and Neurotherapeutic Potential

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Tacrine is a drug used in the treatment of Alzheimer's disease as a cognitive enhancer and inhibitor of the enzyme acetylcholinesterase (AChE). However, its clinical application has been restricted due to its poor therapeutic efficacy and high prevalence of detrimental effects. An attempt was made to understand the molecular mechanisms that underlie tacrine and its analogues influence over neurotherapeutic activity by focusing on modulation of neurogenesis, neuroinflammation, endoplasmic reticulum stress, apoptosis, and regulatory role in gene and protein expression, energy metabolism, Ca^{2+} homeostasis modulation, and osmotic regulation. Regardless of this, analogues of tacrine are considered as a model inhibitor of cholinesterase in the therapy of Alzheimer's disease. The variety both in structural make-up and biological functions of these substances is the main appeal for researchers' interest in them. A new paradigm for treating neurological diseases is presented in this review, which includes treatment strategies for Alzheimer's disease, as well as other neurological disorders like Parkinson's disease and the synthesis and biological properties of newly identified versatile tacrine analogues and hybrids. We have also shown that these analogues may have therapeutic promise in the treatment of neurological diseases in a variety of experimental systems.

1. Introduction

Neurological disorders are very complex and multifaceted, necessitating multitarget medications that can cause multiple subpathologies at once. These illnesses are characterized by progressive failure of certain neurotransmitter system and the improper functioning of neural networks in the brain [1, 2]. There have been a growing number of studies undertaken to discover changes linked with neurological disorders, including in neurotransmitters, transporters, receptors, and metabolizing enzymes. Through nicotinic and muscarinic cholinergic receptors on monoaminergic

neurons, the cholinergic system can influence monoaminergic systems [3]. As a result, manipulating the cholinergic system can affect the signaling of other monoaminergic systems, and age-related changes in neurotransmitter systems can affect the response to such treatment techniques [4]. The use of small compounds to treat neurological disorders like Alzheimer's disease (AD) by modulating cholinergic and glutamatergic neurotransmissions has been suggested. A powerful acetylcholinesterase inhibitor (AChE), tacrine, was the first medicine approved by the US Food and Drug Administration for the treatment of AD [5]. It is a low-affinity N-methyl-D-aspartate receptor (NMDAR) antagonist. In addition to being an AChE inhibitor, tacrine has been revealed to have a variety of cholinergic actions, including boosting the synthesis and release of acetylcholine (ACh) and modulating the muscarinic and nicotinic receptors. It has also been shown that tacrine interacts with monoaminergic systems. A number of investigations have indicated that the memory-enhancing qualities of tacrine are more nuanced than previously thought. It has been shown to target a variety of pathways, including the thenitrinergic, gamma-aminobutyric acid (GABA)ergic, glutamatergic, and AChE pathways [6–10].

Although tacrine was the first drug approved for the treatment of AD, it was banned in 2013 by US Food and Drug Administration (FDA) due to its toxic effects on liver. After the prohibition, most of the research focused on finding safer analogues and of tacrine and its complex with other hepatoprotective agents to treat AD in a multitarget-directed ligand approach. Tacrine has effect against neurological disorders in a molecular basis [11].

This article explains the therapeutic effect of tacrine, its analogues and derivatives discovered in several preclinical studies in a variety of neurological disorders, especially Alzheimer's disease particularly from a pharmaceutical standpoint. Furthermore, this article includes an overview of tacrine and examines the molecular mechanistic role of tacrine effects against the neurological disorders.

2. Overview of Tacrine

Tacrine's pharmacological actions were initially characterized by Shaw and Bently in 1946 [7, 12]. It was first used in clinical practice to treat anesthetic-induced delirium and to enhance the muscle-relaxing effects of succinylcholine [13]. It was first thought that tacrine would help counteract the respiratory depression caused by morphine; however, it was later revealed that it also acted as an inhibitor of both AChE and butyrlcholine esterase (BChE) [9, 14]. In 1993, tacrine became the first medicine to be licensed for the treatment of Alzheimer's disease; however, its hepatotoxicity limited its usage [1]. Additional effects of tacrine have been discovered throughout time, including reduction of monoamine oxidase activity, inhibition of 5-hydroxytryptamine receptor and dopamine neuronal uptake, blockage of certain potassium ion channels, and interaction with muscarinic ACh receptors [15, 16].

Tacrine (9-amino-1,2,3,4-tetrahydroacridine, or THA) is an acridine containing three rings, but only a little substitution of an amino group in the fifth position is a white crystalline powder that dissolves in water, Tacrine's pKa value is 9.85, and it has a planar chemical structure [7]. Though it is totally ionized at pH 7, it is likely that the positive charge is spread out between the mesomeric structures [16]. Tacrine is quickly absorbed and has a bioavailability between 10% and 30%. Absorption can be reduced by as much as 40% when taken with food. Plasma proteins account for approximately 55% of the binding of tacrine. Its clinical half-life is approximately 3-6 hours after a single oral dose. Oral tacrine has a therapeutic dosage range of ten-fold, necessitating individualized dosing. The distribution volume appears to be 182 liters. During the last elimination phase, the plasma half-life is 2.5 hours on an average [7]. It has been reported incorrectly that 1-OH-tacrine, a pharmacologically active form of tacrine, is formed during metabolism. In reality, tacrine is broken down into up to seven distinct compounds during digestion, which include dextro-chiral (stereospecific) version of 1-OH-tacrine [14, 17].

Multiple modes of action exist for tacrine. Tacrine's primary mechanism of action in neurological disorders is as an acetylcholinesterase inhibitor that acts noncompetitively but selectively on the central nervous system (CNS). Moreover, CNS abnormalities extend beyond the cholinergic system and include serotonin neurotransmitter deficits, noradrenaline neurotransmitter deficits, and reduced vascular perfusion [18, 19]. Tacrine has some BChE selectivity over AChE. There are several other consequences on cholinergic system apart from enzyme inhibition: boost in production and secretion of ACh, activation of M1 subtype muscarinic receptors, suppression of M2 subtype muscarinic receptors and a raise in brain nicotinic receptors, and their potentiation at low and inhibition at high concentrations as well as a general potentiation of neuromuscular communication [20, 21]. Additionally, tacrine affects a wide range of biological targets, including neuromuscular junctions, potassium channels, sodium channels, and phosphorylation. Tacrine has been demonstrated to limit the absorption and increase 5-HT, noradrenaline, and GABA release; it also has an inhibitory action both monoamine oxidase (MAO)-A and MAO-B monoamine oxidase subtypes, and it suppresses brain histamine N-methyltransferase. As an example of this, tacrine has been demonstrated to reduce potassium-induced release of excitatory amino acids and block neuronal calcium channels in large dosages [21, 22].

In addition, tacrine was found to have a positive effect on ketamine side effects. It is mostly used to initiate and maintain anesthesia with the help of ketamine. Pseudo-hallucinations, delirium, and hypnagogic states were all common side effects of this medication's treatment. Postoperatively, tacrine has been shown to reverse psychotic episodes. It was discovered that tacrine's effects on the CNS were due to its interaction with adrenergic and cholinergic receptors [21, 23].

Pure anticholinesterase inhibitors like physostigmine and donepezil do not share two other peculiar properties of tacrine. An important benefit of tacrine in people with neurological disorders is that it greatly enhances cerebral blood flow. Decreased cerebral blood flow is linked to many neurological illnesses. Second, tacrine prevents the release of

β-precursor protein from the intestinal tract. There are numerous metabolites that can be formed by metabolization of tacrine by cytochrome P450 IA2 system. In the early stages of tacrine administration, the hepatocellular response that is typical of many pharmacological drugs can occur. It is not known if tacrine and/or its metabolites are excreted in the bile or if they are transported via the enterohepatic system. The clearance of tacrine, however, does not appear to be affected by renal impairment [9, 15, 21].

3. Tacrine Derivatives

Tacrine (9-amino-1,2,3,4-tetrahydroacridine) acts by three times more effectively inhibiting butyrylcholinesterase (BChE) than acetylcholinesterase (AChE). When the carbocyclic ring was modified from a cyclopentyl (compound (1)) to a cycloheptyl ring (compound (2)), the efficiency against BChE activity enhanced approximately 4-fold, while the efficacy against AChE activity increased 2-fold. On the other hand, the anticholinesterase activity of the cyclooctyl derivative (compound (3)) was 100 times lower than that of tacrine, presumably owing to the carbocyclic ring's increased flexibility and bulk. The introduction of a methylene bridge in tacrine's cyclohexyl ring (compound (4)) leads to a reduction in anticholinesterase efficacy (IC_{50} against AChE: 0.24 μM) that further significantly improved while the substituent bulk on the bridge is increased by three methyl groups compound (5) (IC_{50} against AChE:110.0 μM). The inclusion of a fourth benzene ring led to a change in compounds with high AChE selectivity over BChE; just like in comparison to tacrine, compound (6) was extremely selective for AChE rather than BChE, with an IC_{50} of 0.35 μM and 3.1 μM against AChE and BChE, respectively. Against BChE (IC_{50}: 6.7 μM), tetracyclic compound (7) was 50 times more powerful than AChE ($IC_{50:}$ 391.1 μM) [24].

In addition to being more effective AChE inhibitors over tacrine, certain halogenated analogues have a greater affinity for AChE over BChE. Additional study has attempted at what emerges when a halogen atom is incorporated into the benzene ring of parent cyclopentyl analogue compound (1). The insertion of Cl at position 6, compound (10), elevated the strength by six fold; nevertheless, the 6F compound was equiactive with the parent cyclopentyl analogue. In aspects of selectivity against AChE being preferred over BChE (the B/A ratio), the 5Cl, 6Cl, 8Cl, 7F, and 8F derivatives, with B/A ratios of 4.1, 13, 3.8, 6.2, and 19, respectively, were the most selective compounds [25].

León et al. assessed the biological activity of new tacrine analogues possessing a heterocyclic ring moiety at position C-4, such as 2-thienyl, 3- or 4-pyridyl, and 2-(N-acetyl)-pyrrolyl groups. Among the pyrano[3,2-*e*]pyridines and pyrano[2,3-*b*]quinolines groups, the pyrrolyl possessing molecule compound (8) is by far the most effective, with an IC_{50} against AChE: 1.7 μM, approximately 10-fold decline in levels of activity in comparison to tacrine. Compound (9) was the most powerful of the [1,8]naphthyridine derivatives, with an IC_{50} against AChE: 1.4 μM [26].

Friedländer-type reactions of 2-aminopyridine-3-carbonitriles with 1-benzyl-4-piperidone or cyclohexanone have yielded new tacrine analogues. The biological analysis demonstrated that, in nanomolar range, certain of these compounds were effective AChE inhibitors, as well as selective inhibitors of BChE. Compound (11) is the shortest N2-alkyl substituted compound, and it performs better than the molecules with longer alkyl chain. This inhibitor has an identical AChE inhibition profile (IC_{50}:14 nM) as tacrine, even though it is a weaker BChE inhibitor (IC_{50}: 5.2 μM). The insertion of an electron-withdrawing substituent like Cl at C-2 contributed in a substantial reduction in anticholinesterase activity. Furthermore, to enhance AChE selectivity, an N-benzyl was substituted for the methylene group at C-7, as demonstrated in two instances: (i) from compound (13) to compound (15) and (ii) from compound (14) to compound (16). Meanwhile, inhibitor A was 5.8 times more effective against EeAChE (AChE from *Electrophorus electricus*) in relative to compound B; both of these compounds possess a methoxy group at C-2. Nonetheless inhibitor C was 14 times less powerful than inhibitor D against EeAChE, and both compounds included a chlorine atom at C-2 [27].

Tacrine is covalently bonded to some other pharmacologically active compounds, including such as a M1 agonist xanomeline and (Z)-3-(4-chlorophenyl)-N′-((4-chlorophenyl)sulfonyl)-N-methyl-4-phenyl-4,5-dihydro-1*H*-pyrazole-1-carboximidamide known as an antagonist of CB1 receptor, which enables to consolidate tacrine's profound AChE inhibition with other pharmacological features [28]. The AChE inhibition of compound (17) is 1,000-fold broader than that of tacrine [29]. In respect to compound (17), compound (18) has a lower toxicity, yet will inhibit AChE and BChE in the same frequency as tacrine [28, 30].

Tang et al. created a variety of tacrine-oxoisoaporphine hybrids in their pursuit for more effective tacrine derivatives. In most instances, these compounds display significant AChE inhibitory potential, IC_{50} values being in nanomolar range, with the most impactful AChE inhibitor reported being compound (20) (IC_{50}: 3.4 nM) [31].

Mao et al. discussed the design, synthesis, and assessment of a variety of o-amino benzylamine and o-hydroxyl tacrine hybrids, as well as o-hydroxyl benzylamine-(7-chlorotacrine) hybrids as multifunctional anti-AD drugs. Over these variants, compound (21) inhibits AChE (IC_{50}: 0.55 nM) more effectively than tacrine [32].

7-MEOTA (9-amino-7-methoxy-1,2,3,4-tetrahydroacridine), a new AChE inhibitor, was shown to be exempt of several toxicities particularly in comparison to tacrine (Figures 1 and 2). Korabecny et al. generated a number of 7-MEOTA analogues by alkylating the aromatic amino moiety of this new AChE inhibitor, with intriguing findings. C5–C9 chains outperformed their parent molecule 7-MEOTA and also in specific instance of inhibition of hAChE (human recombinant AChE), compound with a hexyl chain being the most effective (IC_{50}: 0.10 μM) [33].

4. Molecular Basis of Tacrine Derivatives' Action against Neurological Disorders

4.1. Modulation of Neurogenesis. Animal and human brains undergo a process known as neurogenesis, in which new

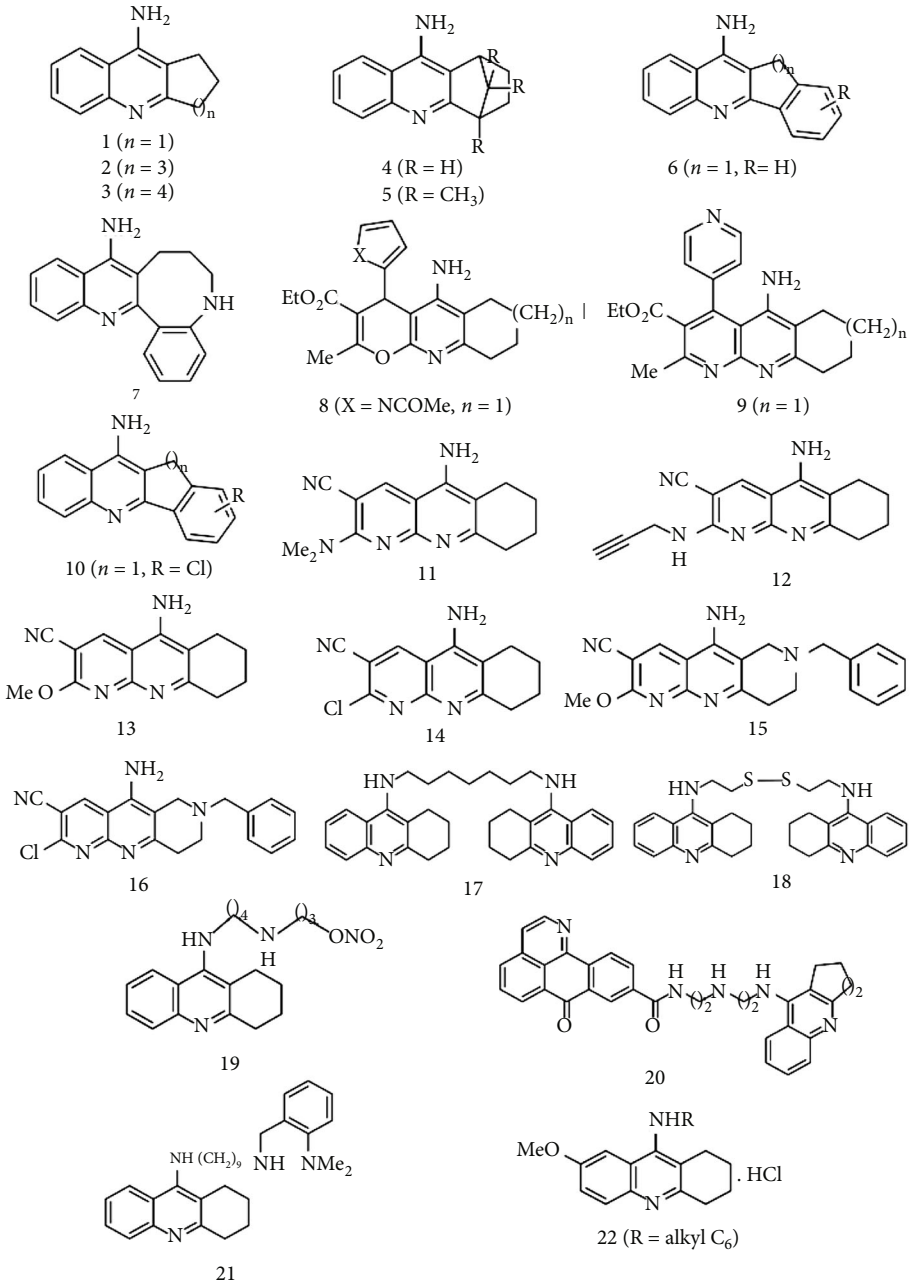

Figure 1: Illustration of compounds of tacrine analogues. The compound number denoted by (1) 9-Amino-2,3-dihydro-1H-cyclopenta[1,2-b]quinoline. (2) 11-Amino-2,3,4,5-tetrahydro-1H-cyclohepta[1,2-b]quinoline. (3) 12-Amino-1,2,3,4,5,6-hexahydrocycloocta[1,2-b] quinoline. (4) 9-Amino-1,4-methano-1,2,3,4-tetrahydroacridine. (5) 9-Amino-1,4-methano-1,2,3,4-tetrahydro-4,11,11-trimethylacridine. (6) 6-Amino-4,5-benzo-5H-cyclopenta[1,2-b]quinoline. (7) 9-Amino-5,6,7,8-tetrahydroquinolino[3,2-e]-1-benzazocine. (8) Ethyl 4-(1-acetyl-1H-pyrrol-2-yl)-5-amino-6,7,8,9- tetrahydro-2-methyl-4H-pyran[2,3-b]quinoline-3-carboxylate. (9) Ethyl 5-amino-6,7,8,9-tetrahydro-2-methyl-4-(4- pyridyl)-benzo[b][1–8]naphthyridine-3-carboxylate. (10) 9-amino-6-chloro-2,3-dihydro-[1H] cyclopenta [1,2-b]-quinoline. (11) 5-Amino-2-(dimethylamino)-6,7,8,9-tetrahydrobenzo[1,8-b]-naphthyridine-3-carbonitrile. (12) 5-Amino-2-(prop-2-yn-1-ylamino)-6,7,8,9-tetrahydrobenzo[1,8-b]-naphthyridine-3-carbonitrile. (13) 5-Amino-2-(methyloxy)-6,7,8,9-tetrahydrobenzo[1,8-b]-naphthyridine-3-carbonitrile. (14) 5-Amino-2-chloro-6,7,8,9-tetrahydrobenzo[1,8-b]- naphthyridine-3-carbonitrile. (15) 5-Amino-7-benzyl-2-methoxy-6,7,8,9-tetrahydropyrido[2,3-b][1,6]naphthyridine-3-carbonitrile. (16) 5-Amino-7-benzyl-2-chloro-6,7,8,9- tetrahydropyrido[2,3-b][1,6]naphthyridin-3-carbonitrile. (17) Bis(7)tacrine dimer. (18) Cystamine-tacrine dimer. (19) Nontoxic tacrine-organic nitrates, compound E (as the name wasn't found in the paper). (20) N-(7-Oxo-7H-dibenzo[de,h]quinolin-9-yl)-3-((2-((1,2,3,4-tetrahydroacridin-9-yl)amino)ethyl)amino)propanamide. (21) N1-(2-(Dimethylamino)benzyl)-N9-(1,2,3,4-tetrahydroacridin-9-yl)nonane-1,9-diamine. (22) N-alkyl-7-methoxytacrine.

nerve cells are continuously created throughout the life span of the organism. They are capable of differentiating into functional cells of the central nervous system and integrating into existing neural circuits in the brain after they have been born [6, 34]. Therapy for Alzheimer's disease may be possible if neuronal regeneration (neurogenesis) is targeted in the

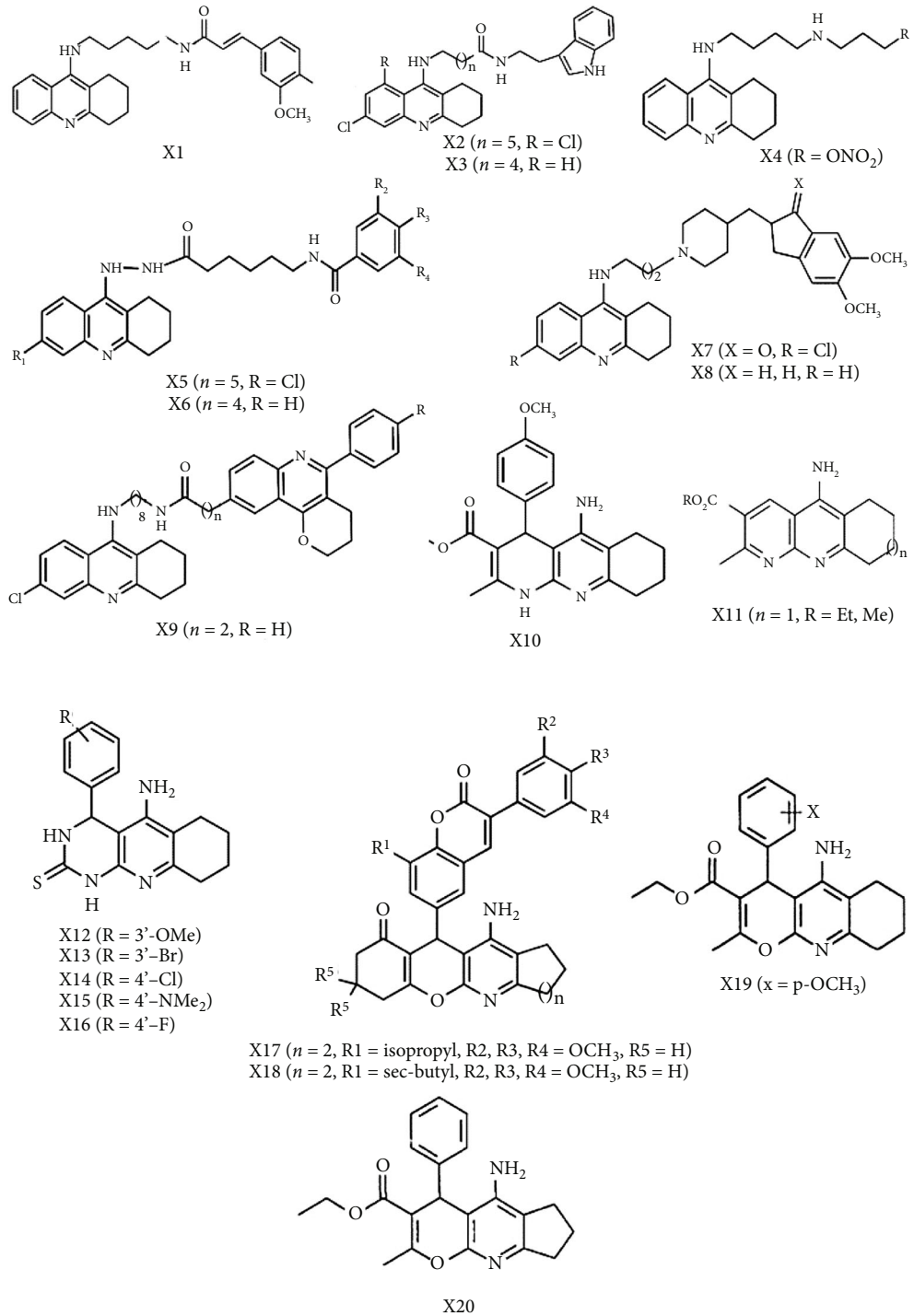

Figure 2: Illustration of compounds for neuropharmacological potential of tacrine hybrids.

hippocampus [35]. Subventricular zone (SVZ) and dentate gyrus (DG) of the hippocampus are two regions of the mammalian brain where neurogenesis continues into adulthood. When NPCs are multiplied in many locations, new neurons and glial cells can be generated in the brain [8, 36, 37]. With the help of the bromodeoxyurine (BrdU)-labeling paradigm, Jin et al. determined the effects of tacrine on neurogenesis in adult mice. According to the findings, it increases neurogenesis in the DG and SVZ by 26–45%, although it has no effect on BrdU labeling in the DG [35]. In order to study the effects of compound (17), an acetylcholinesterase (AChE) inhibitor, on cognitive performance, neuronal apoptosis, and neurogenesis in the hippocampal region of adult male Sprague-Dawley rats, was permanently ligated of the bilateral common carotid arteries [38]. The neuroprotective effects of tacrine and its analogues have been proven in several investigations, including those by Li et al. and Luo et al. Tacrine and its analogues antagonize glutamate excitotoxicity

and reduce neuronal nitric oxide synthase activity [39, 40]. Farkas et al. discovered that administering compound (17) to a chronic cerebral ischemia rat corrected the impairments in spatial learning and memory that had previously been seen. In the hippocampus, the neuroprotective impact of compound (17) may be exerted through the promotion of neurogenesis in the DG and the inhibition of apoptosis in the CA1 area, respectively. They observed that this impact could potentially be due to activation of cholinergic receptors that are expressed on neural progenitors and that these receptors in turn may promote neurogenic factors [41].

4.2. Modulation of Neuroinflammation. The recognition of the role played by inflammation in the progression of a wide range of diseases has prompted innovative efforts to discover therapeutic approaches to reduce inflammation. Cholinergic deficiency and neuroinflammation are critical factors in both acute and long-term CNS disorders [2, 21, 42]. Glial cell activation (astrocytes and microglia) is a frequent feature of many neurodegenerative illnesses, and it is largely responsible for this process. Glial cells activated during brain inflammation may produce proinflammatory mediators, which have been linked to the neuropathology that produces cognitive impairments in neurological diseases [43, 44]. In neurodegenerative illnesses, the balance between proinflammatory and anti-inflammatory cytokines also has an impact on the course of the disease [15, 45]. According to Zhang et al., hippocampal injection of a tacrine analogue known as 4-(bis(pyridin-2-ylmethyl)amino)-N-(3- ((1,2,3,4-tetrahydroacridin-9-yl) amino)propyl)butanamide could effectively attenuate Aβ1-42 oligomers-induced cognitive dysfunction by activating the CREB/BDNF signaling pathway, decreasing tau phosphorylation [46]. According to the findings of a study conducted by Tyagi et al., tacrine may have an inhibitory effect on lipopolysaccharide (LPS-)-induced neuroinflammation in adult Swiss albino mice, as evidenced by a decrease in the high levels of IL-2 in the brain [42]. Being AChE inhibitors, tacrine and its analogues work by increasing the availability of acetylcholine in central cholinergic synapses and are hence considered as the most promising medications now available for the treatment of neurological diseases like AD [47, 48]. As per Jeřábek et al., tacrine-resveratrol-fused hybrids have the potential to be exploited as multitargeted ligands against neurological diseases especially AD due to their fascinating anti-inflammatory and immunomodulatory capabilities in neuronal and glial AD cell models [49]. Moreover, in a cell-based experiment conducted by Alfadly et al., the antineuroinflammatory and neuroprotective characteristics of tacrine were demonstrated by a reduction in the expression of IL-1β and TNF-α, as well as the formation of ROS, in LPS-challenged PC12 cells following treatment with the chosen inhibitors [50]. Tacrine, as discovered by Xu and Xu, alleviated thermal hyperalgesia and mechanical allodynia following sciatic nerve chronic construction injury while also restoring functional morphological damage in rats. Besides that, tacrine inhibited the growth and activation of glia and decreased IL-1, IL-6, and TNF-α level in the blood. Tacrine also had an anti-inflammatory effect on the JAK2/STAT3 signaling pathway, which is involved in neuroinflammatory processes. These findings suggest that tacrine may be a good option for use as an analgesic drug in the treatment of neuropathic pain [51].

4.3. Modulation of Endoplasmic Reticulum Stress. Inhibition of DNA synthesis by tacrine results in functional and structural alterations in the liver's endoplasmic reticulum, ribosome, and mitochondria. As an acetylcholinesterase inhibitor (AChEI), tacrine has the potential to interfere with the normal trafficking of AChE in the endoplasmic reticulum (ER) [48, 52]. Tacrine exposure in neuronal cells with AChE (e.g., neurons) resulted in accumulation of misfolded AChE. Due to ER stress and the unfolded protein response's downstream signaling cascade, this misfolded enzyme is incapable of moving to its target, resulting in neuronal death via apoptosis [7, 53]. ER-mitochondrial collaboration enhanced mitochondrial membrane potential loss when the stress level was too high. After a while, the tacrine-exposed cells were unable to maintain homeostasis any longer and eventually died. Tacrine promoted ER stress and apoptosis, which were inversely related to the concentration of AChE [9]. By causing ER stress in neurons, other AChEIs (rivastigmine and bis(3)-cognitin) could create the same problem as tacrine. Tacrine and other AChEIs interfere with AChEe's normal transport across the ER. Once neurons and other cells have been damaged, they die. AChEIs for the treatment of neurological disorders like AD may benefit from these findings, which can be used to guide the development of new drugs [10, 39, 54, 55]. Using cultured neuronal cells, Liu et al. found that tacrine disrupts oligomeric acetylcholinesterase's ability to properly assemble, leading to endoplasmic reticulum–stressed death [54].

4.4. Modulation of Apoptosis. Apoptosis is a process in which cells actively participate in their own death. The regulation of apoptotic cell death can be regulated by a number of genes like bcl-2 family [56, 57]. Although the exact origin and mechanism of neuron death in neurological disorders like AD are still unknown, research suggests that oxidative stress may play a role. During neurodegeneration, reactive oxygen species (ROS) have been proposed to play a significant role in oxidative damage, which can be created by the cell lysis, by an excessive amount of free transition metals, or by an oxidative burst [7, 17]. Alzheimer's disease patients' brains appear to degenerate by an apoptotic process, including the presence of damaged DNA, nuclear apoptotic bodies, and other apoptosis-related markers in postmortem tissue samples. As a result of these findings, it is possible that apoptosis can be prevented or delayed by using ROS-inhibiting therapies, which may be a viable treatment option for the disease [58, 59].

Wang et al. reported that tacrine protects against H_2O_2-induced apoptosis, presumably by preventing the production of proapoptotic genes such as p53 and bax in the cells [58]. A tacrine analogue called as tacrine(2)–ferulic acid was found to inhibit 6-hydroxydopamine-induced apoptosis in rat pheochromocytoma (PC12) cells by activating the phosphoinositide 3-kinase (PI3-K)/Akt signaling pathway, according to the findings of Zhang et al. [57]. Gao et al. (2013 and 2014) reported that tacrine causes apoptosis, thereby imposing cytotoxicity in HepG2 (American Type

Culture Collection HB-8065) cells via a lysosome- and mitochondria-dependent mechanism and increased intracellular ROS production [17, 60]. Apoptosis has been linked to oxidative stress, and prior research has shown that tacrine, huperzine A, and donepezil protect against exogenous β-amyloid-induced damage and ameliorate redox disequilibrium in aged rats [56, 57, 61, 62]. Tacrine may be a helpful neuroprotective medication that lowers oxidative stress-induced cell death, which may complement its AChE inhibitory capabilities for the treatment of neurological illnesses. One of the hallmarks of apoptosis is oxidative stress, which has been linked to a wide range of cell death mechanisms and which in turn is triggered by ROS. YCG063 was tested as a ROS inhibitor by Gao et al., to see if it affected tacrine-induced apoptosis. The results show that YCG063 greatly suppresses tacrine-induced apoptosis, implying that tacrine-induced apoptosis is directly linked to ROS generation [17]. Apoptosis is triggered by tacrine, and ROS is a key player in this process. To put it another way, oxidative stress can lead to mitochondrial permeability transition (MPT) if ROS levels are greater than the cell's capacity to deal with them, which in turn increases ROS levels and the stress [17, 60, 63].

AChE activation by tacrine and its analogues might also possibly play a crucial role in apoptosis, as suggested by various research studies [7, 26, 48, 64, 65]. Apoptosis was increased in retinal cells from chick embryos transfected with AChE sense vectors, and highly purified AChE proteins have been demonstrated to be harmful to cells via the apoptotic mechanism. These findings suggest that AChE is involved not only in mammalian organ development but also in regulating how well it can respond to external stimuli [57, 58]. These noncholinergic functions of AChE may be crucial in neurological disorders, and tacrine may be able to influence them positively [4, 7].

4.5. Role in Energy Metabolism. Alterations in cellular energy metabolism and mitochondrial dysfunction are two further aspects that contribute to the pathophysiology of neurological disorders [5, 66]. Multitarget-directed ligands combine cholinesterase inhibition with antioxidant capabilities to favorably alter neuronal energy metabolism and mitochondrial function to reduce illness symptoms [67–69]. The effect of ChE inhibitors (tacrine and 7-methoxytacrine) on the activity of mitochondrial complex I in the brain of a pig was investigated by Korábečný et al. and Hroudová et al., in mitochondria from the brain of the animal [70, 71]. Tacrine dramatically reduced the activity of mitochondrial complex I, which may result in tacrine-induced deleterious consequences associated with imbalances of electron transport chain [7, 72]. A disruption in the activity of complex I, which is critical in the regulation of oxidative phosphorylation, could result in impairment of cellular energy metabolism and, as a result, abnormalities in neuronal activity. As a result of inhibition of oxidative phosphorylation, glycolytic energy generation can become more prominent in tissues with high-energy requirements, such as the brain, which is especially true for the brain [71, 73]. Berson et al. reported that the weak base tacrine has a protonophoric action in mitochondria due to its high proton concentration. When the cell's energy expenditure increases without a concurrent increase in ATP production, the result is cell malfunction at low dosages and cell death at high levels, according to the literature. These mitochondrial effects are observable in cells from either rats or humans who have been treated to tacrine at clinically relevant concentrations or dosages [74].

4.6. Regulatory Role in Gene and Protein Expression. Tacrine regulates Kv2.1 channel gene expression and cell proliferation, according to Hu et al. [34]. Transcription factors have been shown to influence the expression of proapoptotic Bcl-2 and antiapoptotic Bax genes, respectively, in response to the p53 tumor suppressor gene [75]. In both developed and undifferentiated human neuroblastoma cell lines, Lahiri et al. found that tacrine altered the secretion of the β-amyloid precursor protein. Fu et al. found that compound (17) has been shown to be effective in protecting primary cultured astrocytes, pheochromocytoma cells, and neurons from hydrogen peroxide-induced apoptosis as well as against amyloid beta protein and glutamate-induced apoptosis. Tacrine derivatives accelerate human glioma SF295 cell death and change glioblastoma-related proteins such as p53, β-catenin, MAP2c, Iba-1, Olig-2, HLA-DR, and IDH1-proteins related to disease development [76]. ChAT and M3 and M5 muscarinic receptor subtypes downregulation in chronic hypoperfusion-induced cognitive deficits can be alleviated by tacrine therapy, according to the findings of Zhao et al. [77]. By protecting neurons against amyloid β protein-induced cell death and apoptosis, tacrine may serve as more than just an AChEI. It may also stimulate antiapoptotic gene expression by lowering oxygen-free radicals during neuronal injury [78]. Alfirevic et al. reported that tacrine-induced transaminitis was linked to genetic variations in ABCB4, which encodes the MDR3 phosphatidylcholine transporter. Tacrine does not appear to be a substrate or inhibitor of MDR3 [79].

4.7. Role in Ca^{2+} Homeostasis Modulation. Cell death can be controlled by calcium, which is a second messenger in the body. It has even been proposed that the final common mechanism for all types of cell death is Ca^{2+} overload [80]. The imbalance of Ca^{2+} in the body is now recognized as a common factor in the pathophysiology of Alzheimer's disease [81]. Ca2+ has recently been linked to the toxicity and buildup of amyloid protein, which, in turn, shows collaboration with tau protein [82]. Since nimodipine, a well-known calcium channel blocker, and tacrine, a well-studied calcium channel blocker, have been combined structurally, Marco-Contelles et al. have produced tacripyrines, a new therapeutic candidate (lead drug candidate RL2/101) [83]. It has been demonstrated that RL2/101 interacts with the peripheral anionic site of AChE as well as the L-type of Ca^{2+} channels concomitantly. Several studies have shown that tacrine affects the homeostasis of Ca^{2+}. Tacrine has been shown to inhibit high-threshold calcium currents by Kelly et al. [82] and Lermontova et al. [84]; however, their investigation required significantly larger concentrations of tacrine, and they were unable to identify the channels involved. Tacrine

had no effect on calcium levels in synaptosomes, according to Gibson et al. [85] and Marco-Contelles et al. [83]. Doležal et al. [86] showed that tacrine attenuates the influx of calcium by blocking L-type and N-type calcium channels in cholinergic SN56 neuronal cell lines. This inhibitory action is not a consequence of the AChE activity of tacrine. An important consideration in determining tacrine's therapeutic potential is whether or not low micromolar concentrations of the drug interfere with calcium-dependent processes. Tacrine-dihydropyridine hybrids have a great neuroprotective profile and a modest blocking action on L-type voltage-dependent calcium channels due to the moderation of $[Ca^{2+}]$ elevation triggered by K^+ depolarization. Chioua et al. found that blocking the entry of Ca^{2+} ions through L-type voltage-gated calcium channels by tacrine is a valuable strategy to prevent neuronal damage in neurological disorders like AD [73]. Tacrine treatment in male Crl:CD mice significantly increased the mitochondria's vulnerability to calcium-induced mitochondrial permeability transition, according to Mansouri et al. [59].

4.8. Osmotic Regulation. Tacrine is structurally similar to 9-aminoacridine, which is an open Na^+ channel blocker, as well as 4-aminopyridine, which is a K^+ channel blocker. Several investigations have demonstrated that tacrine has a direct effect on Na^+, K^+, and Ca^{2+} channels [7]. Tacrine reduces Na+ currents at a rate that is much larger than K+ currents, according to Adem [87], who performed voltage clamp tests on myelinated axons. It was recently reported that THA-induced action potential lengthening is caused by a modified Na+ current inactivation and delayed K+ current activation, which was proven using a similar preparation. Neuronal firing patterns and direct transmitter release are theorized to be affected by an action potential prolongation in some systems [9]. They further suggest that tacrine preferentially inhibits Na+ channels in the open state, whereas tacrine largely inhibits K+ channels the closed state, further supporting the previous findings. After an initial period of inhibition, ACh is administered to pyramidal neurons in the cerebral cortex, resulting in a progressive increase in activity followed by another period of inhibition. Muscarinic receptors, which may be selectively inhibited by pirenzepine, appear to be responsible for the delayed excitatory response, and these responses are mediated through voltage-dependent K+ conductance, which decreases with voltage [22, 44, 70]. Tacrine (and ACh in the synapse) act on M1 muscarinic receptors, which are blocked by pirenzepine, preventing the release of ACh from the slices, as previously explained. Pirenzepine blocks the release of ACh from the slices. A reduction in voltage-dependent K+ conductances may occur as a result of the impact of tacrine (and ACh) on muscarinic M1 receptors, which would lead to an increase in the release of Ach [3, 21]. However, a direct action of tacrine on K+ channels may boost ACh release, which cannot be totally ruled out. Thus, tacrine's direct and/or indirect effects on the different ion channels may contribute to its therapeutic success in the treatment of Alzheimer's disease [88, 89]. A low osmotic pressure makes tacrine unsuitable for use as an osmotic dose form in the gastro-intestinal tract [7, 87].

5. Therapeutic Potential of Tacrine Derivatives against Neurological Disorders

By blocking the enzyme acetylcholinesterase, tacrine prevents acetylcholine from being degraded, therefore augmenting acetylcholine levels. The therapeutic utilization of tacrine for AD therapy has been halted due to its toxicity to the liver, incurred by an elevation in liver transaminase and a decline in liver albumin. Meanwhile, medications that regulate a single target may not be therapeutically successful in multifactorial disorders like Alzheimer's. As a corollary, a number of medicines acting on various targets particular to multifactorial diseases have been derived. Multitargeted-directed ligands (MTDLs) are the term for those kinds of novel medications.

5.1. Alzheimer's Disease. There are millions of older people throughout the world who are suffering with Alzheimer's disease (AD), a debilitating and incurable neurological illness. The treatment options available currently only achieve some short-term relief from the cognitive symptoms. The major mechanisms of this disease include accumulation of amyloid beta (Aβ) proteins forming neurofibrillary tangles, loss of cholinergic activity, elevation of oxidative stress, and disruption of homeostasis of transition metal ions [90]. Tacrine was the first approved drug against Alzheimer's disease which was approved by the FDA in 1993. It worked as an inhibitor of cholinesterase [91]. But due to its liver toxicity, it was discontinued in the USA in 2013. Since then, tacrine has been used as a lead compound and combined with numerous ligands to create multitarget-directed ligands (MTDLs) or drugs to combat against the multifactorial aspects of AD.

Homodimer of tacrine known as compound (17) is a potential lead compound of novel MTDLs. The heptamethylene linker of compound (17) was replaced with the structure of cystamine, forming compound (18) (Figure 3). This dimer exhibited a lower toxicity and greater ability to act as cholinesterase inhibitor, against both AchE (acetylcholinesterase) and BchE (butyrylcholinesterase). It is also able to inhibit beta-amyloid aggregation and displays a neuroprotective action against oxidative injury induced by H_2O_2 on SH-SY5Y cell line. According to research, the chemical (18) exerts its neuroprotective effects through activating kinase 1 and 2 (ERK1/2) and the Akt/protein kinase B (PKB) pathways [92].

By synthesizing new multifunctional tacrine-trolox hybrids, which have both ChE inhibitory and potent antioxidant activities akin to the mother molecule, trolox, Xie, Lan et al. were able to create compounds with both of these features. Compound 6d was shown to be the most effective inhibitor of AChE in male Sprague-Dawley (SD) rats (IC50 value of 9.8 nM for eeAChE and 23.5 nM for hAChE). A powerful inhibitor of BuChE was also found (IC50 value of 22.2 nM for eqBuChE and 20.5 nM for hBuChE). Studies in molecular modeling and kinetics reveal that 6d is a mixed-nature inhibitor that binds to both the CAS and PAS regions of AChE concurrently. The hepatotoxicity of 6d was shown to be substantially lower than that of tacrine in in vivo tests.

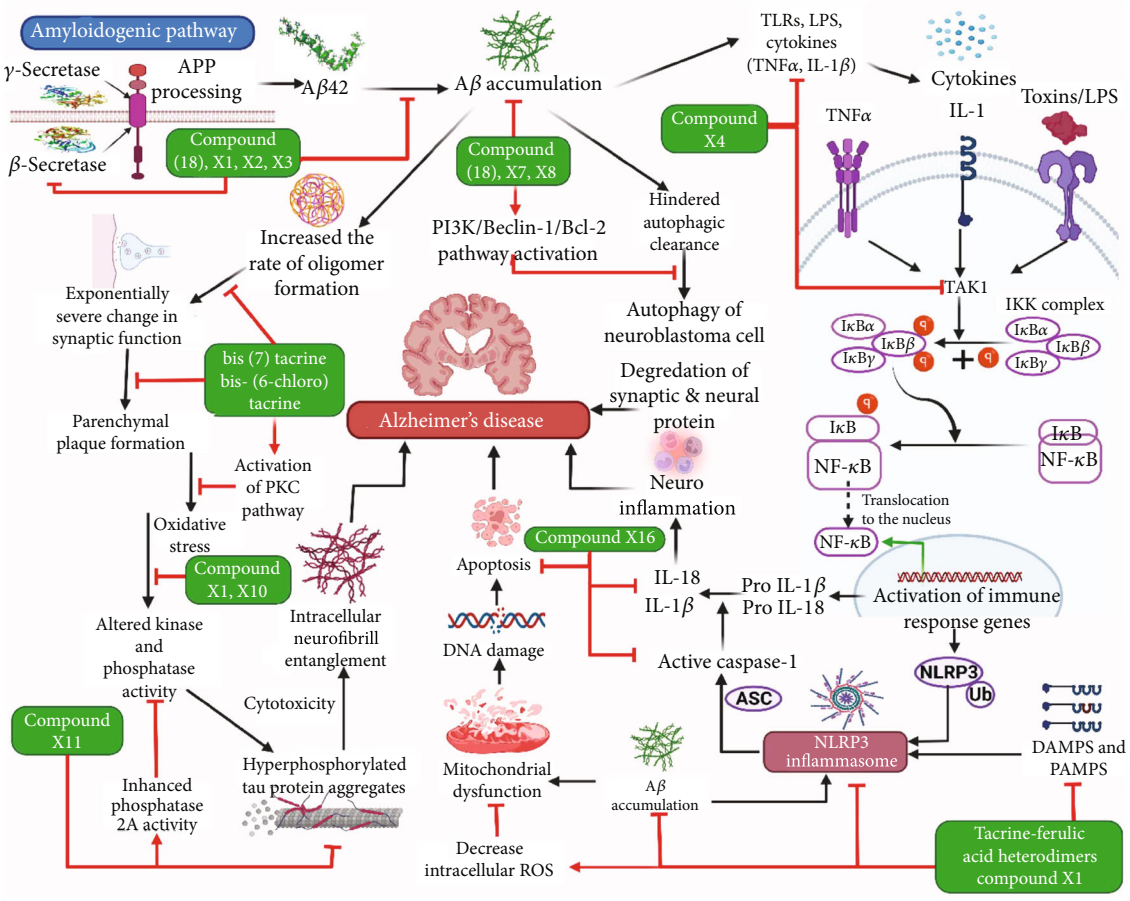

Figure 3: Illustration representing the site of action of different tacrine derivatives in Alzheimer's disease.

A neuroprotective impact as well as excellent BBB permeability was also demonstrated. Overall, 6d can be considered a multifunctional drug for the treatment of Alzheimer's disease (AD) [93].

Xie, Wang, et al. designed, synthesized, and tested another class of new multitarget compounds against Alzheimer's disease: tacrine-coumarin hybrids. Most of the substances studied have robust AChE and BuChE inhibitory action, as well as clearly selective MAO-B inhibitory activity. Inhibition of AChE and BuChE by 14c (IC50 values for eeAChE and 16.11 nM and 33.63 nM, respectively) and BuChE was observed in the produced compounds (IC50 values of 80.72 nM for eqBuChE and 112.72 nM for hBuChE). As a competitive inhibitor of MAO-B, 14c is an effective anti-AD multitargeted drug with increased CNS penetration and reduced cell toxicity [94].

In this study, Zha et al. developed and synthesized twenty-six new tacrine–benzofuran hybrids and investigated their antiefficacy Alzheimer's in vitro on recombinant human AChE (hAChE) and BChE (hBChE) from human serum. Inhibiting human acetylcholinesterase at a subnanomolar level (IC50 = 0.86 nM) and suppressing both hBACE-1 activity (IC50 = 1.35 M) and aggregation of -amyloid (IC50 = 0.86 nM) (hAChE- and self-induced, 61.3 percent and 58.4 percent, respectively). Tests on scopolamine-treated ICR mice found that 2e significantly improved their performance and had no hepatotoxic effects [95].

Experiments using in-silico docking modeling chose a linker that best suited the bimodal drug's interaction with the active site of acetylcholinesterase enzyme (AChE). It was shown that compounds 9d and 9l had the highest results for AChE inhibition and for preventing superoxide formation as well as A-induced cellular toxicity, respectively, when compared to all other compounds tested in this study [96].

Bis(7)tacrine, which has a heptamethylene connection between two tacrine units, seems to be the most intriguing tacrine homodimer. It has a greater IC_{50} value of 1.5 nM for AChE inhibition as well as a superior pharmacological characteristic than tacrine. The potential of bis(7)tacrine to operate on several AD targets was linked to its neuroprotective action.

Designing novel anti-AD-modifying drugs entails inhibiting BACE-1, an enzyme concerned for β-amyloid peptide (Aβ) formation. Apparently, bis(7)tacrine has been reported to suppress BACE-1 with an IC_{50} of 7.5 μM. In Neuro2a APPswe cells, bis(7)tacrine reduced the quantities of both intracellular and secreted Aβ without affecting APP expression (Figure 3). BiS(7)tacrine, like other multifunctional tacrine compounds, reduced AChE-induced Aβ aggregation. The multifunctional nature of bis(7)tacrine for AD therapy was further emphasized by the fact that it blocked both KV1.2-encoded potassium channels and native delayed rectifier potassium channels like tacrine. Furthermore, bis(7)tacrine is an

antagonist of the γ- aminobutyric acid type A ($GABA_A$) receptor, having an IC_{50} of 6.28 μM. This observation leads to the inference that AChEIs with considerable $GABA_A$ receptor antagonism would be more effective for treating AD than simple AChE inhibition alone [7].

Heptylene-linked bis-(6-chloro) tacrine, another homodimeric congener, has a substantially higher potency, with an IC_{50} of 0.07 nM. Heptylene-linked bis-(6-chloro)tacrine was shown to be 3000 times more effective than tacrine and interestingly 3 times more potent than bis(7)tacrine in inhibiting rat AChE. The inclusion of a halogen at the 6-position of such homodimeric tacrines resulted in a remarkable enhancement in AChE inhibitory efficacy with AChE/BChE selectivity [97].

Tacrine-ferulic acid heterodimers are generated by connecting ferulic acid and tacrine with a polymethylene diamine-type spacer. The antioxidant characteristics of ferulic acid, along with its neuroprotective efficacy against $A\beta_{42}$ toxicity in both in vitro and in vivo experiments, prompted the selection of this compound. It was found that compound X1 was simultaneously noncompetitive and reversible AChEI and implies that the enzyme's PAS may interact with it. In an oxygen radical absorbance capacity (ORAC) analysis, compound X1 demonstrated a high ability to lessen the quantity of reactive oxygen species (ROS), like one might predict given the inclusion of the ferulic moiety [98–101].

Hybrids of tacrine and melatonin were also developed as AD therapy drugs with several functions. Melatonin was considered owing to its potential to neutralize a wide range of ROS in the body. It moreover promotes the activity of endogenous antioxidant enzymes, protects against Aβ, and suppresses neurofilament hyperphosphorylation. The most powerful AChEIs in this series, with IC_{50} values in the subnanomolar range and antioxidant activity in an ORAC experiment, were X2 and X3. Comparing to propidium, compound X3 suppressed the self-promoted aggregation of the Aβ (63% at 10 M). It has a 16.2% neuroprotection against $A\beta_{25\text{-}35}$ at 1 μM as well as cell viability of 100% at 1 μM and 80% at 10 μM. Furthermore, in neuroblastoma cells, X3 reduced rotenone-induced oxidative damage by 30% at 3 μM. Rotenone acts as an apoptosis inducer and suppresses mitochondrial complex I specifically [102, 103].

NO enhances blood flow and minimizes inflammatory responses, while both of which may be pharmacologically advantageous for Alzheimer's patients. On this premise, tacrine was coupled to a NO donor unit using a suitable alkylenediamine spacer to generate novel tacrine heterodimers. In vitro study using PGF2-precontracted porcine pulmonary arteries demonstrates NO donor unit's vasorelaxing properties. X4 was the most powerful of the heterodimers studied, with an EC50 of 8.80 μM, whereas isosorbide dinitrate as a positive control had an EC50 of 0.42 μM and tacrine as a negative control had an EC50 of 97.3 μM. Unlike tacrine, X4 seemed to have no consequence of hepatotoxicity, since albumin, lactate dehydrogenase (LDH), and aspartate aminotransferase (ASAT) levels were unaffected by this heterodimer. Interestingly, in behavioral tests, X4 showed a significantly greater capacity to manage scopolamine-induced memory impairment compared to tacrine [104].

In order to target both the AChE and the muscarinic M_2 receptor allosteric site, tacrine-gallamine heterodimers were generated. This is because tacrine additionally functions as an allosteric modulator for muscarinic receptors, while gallamine blocks the AChE PAS and acts as selective allosteric modulator for muscarinic M_2 receptor subtypes. X5 was the most powerful AChEI of the heterodimers studied in this series, with an IC_{50} of 5.44 nM. Tacrine-gallamine heterodimers slowed [^{3}H]N-methyl scopolamine dissociation, particularly X6 with an EC50 value of 0.9 nM outperforms tacrine and gallamine by 4800 and 100 times, respectively. The ability of X6 to bind concurrently with the core and peripheral portions of the muscarinic M_2 receptor allosteric site might explain this augmentation in efficiency [105, 106].

Tacrine-donepezil heterodimers were generated by substituting donepezil's benzyl moiety with tacrine connected with a polymethylene spacer. With an IC_{50} value of 0.27 nM, X7 was found to be the most powerful AChEI. The AChE-induced Aβ aggregation was suppressed by X7 and X8, by 46.1% and 65.9% at 100 μM, hence demonstrating their interaction with the AChE PAS and their possible involvement in modulating AD pathology [107].

Among tacrine-propidium heterodimers, one of the balanced and intriguing compounds is X9. It decreased AChE-induced Ab aggregation by 45.7% at 100 μM and modestly inhibited $A\beta_{1\text{-}42}$ aggregation, according to molecular modeling studies. Furthermore, this molecule blocked BACE-1 and was demonstrated to pass the blood-brain barrier, allowing it to approach its therapeutic targets in the CNS [7].

Tacripyrines, a new class of hybrid compounds, were developed by combining the calcium antagonist 1,4-dihydropyridine (DHP) skeleton with the tacrine in order to suppress AChE while also suspending L-type Ca^{2+} channels. Cell death happens as a consequence of excess Ca^{2+} influx via L-type Ca^{2+} channels. In Alzheimer's disease, Ca^{2+} dysfunction promotes tau hyperphosphorylation and Aβ production [7]. Compound X10 was found to be the most promising because of its ability to inhibit hAChE with an IC_{50} of 105 nM, as well as 34.9% moderate inhibition of self-aggregation of Aβ and 30.7% inhibition of AChE-induced Aβ aggregation [108]. Furthermore, it is enriched with the capability to block Ca^{2+} (albeit lower than nimodipine) and to protect the brain from oxidative stress. It also inhibits AChE-induced Ab aggregation and may penetrate the BBB, as shown by an in vitro BBB permeability experiment [7]. Furthermore, X10 was revealed to be as effective as 3-{4-[(benzylmethylamino)methyl]phenyl}-6,7-dimethoxy-2*H*-2-chromenone (AP2238) and even more powerful than donepezil [108].

The PyridoTacrine family, which possesses the 1,8-naphthyridine motif, exhibits increased ChE inhibition and reduced hepatotoxicity while losing VGCC blocking efficacy. X11 is an MTDL for Alzheimer's disease since it offers pharmacological activity against 2 different therapeutic targets related to neurodegeneration: ChE and PP2A. In animal models of AD and stroke, this compound demonstrated its therapeutic potential. In fact, when compared to BuChE, tacrine derivative X11 is a more powerful and selective AChEI. By activating PP2A, it may be shielding against glutamate-induced excitotoxicity. PP2A inhibition causes

tau hyperphosphorylation, which culminates in the generation of neurofibrillary tangles inside neurons, one of the most prominent indications of Alzheimer's disease. PyrTac X11 lowers scopolamine-induced memory impairment, which might be encouraging outcomes for therapeutic application [2].

A tacripyrimidine series was generated by merging tacrine and 3,4-dihydropyrimidin-2(1H)-thiones. Concerning the biological profile of tacripyrimidines, compound X12 appeared as the well-balanced cholinesterase inhibitor and calcium channel blocker, with an IC_{50} value of 3.05 μM against hAChE and 3.19 μM against hBChE. This compound had modest calcium channel blocking activity of 30.4% at 1 μM; further, it did not cause any toxicity to HepG2 cells. X13 inhibits hAChE 10 times more effectively than tacrine, the strongest AChEI in the tacripyrimidine family. On the basis of hBuChE inhibition, X14 was the most effective with an IC50 of 0.372 μM. In the test of $Ca2^{+}$ channel blocking activity in SH-SY5Y neuroblastoma cells, X15 is the most strong, inhibiting 59.01%, followed by X16, which inhibited 66.79%, and the reference compound, nimodipine, which inhibited 49.62% [73].

Vilazodone and tacrine were combined to create a novel class of hybrid molecules for the treatment of depression associated with Alzheimer's disease. Additionally, vilazodone is an inhibitor of 5-HT reuptake and a 5-HT1A receptor partial agonist. Serotonin deficiency has been linked to a number of mental diseases, including depression. Compound IC50 values for acetylcholinesterase inhibition (3.319 M), 5-HT reuptake inhibition (76.3 nM), and 5-HT1A agonist (EC50 = 107 nM) were all within a reasonable range for 3-(4-(4-(4-(2,3-dihydro-1H-cyclopenta[b]quinolin-9-yl)piperazin-1-yl)butyl). It also significantly reduced hERG activity, exhibited tolerable hepatotoxicity, and could pass the blood-brain barrier in vivo. Cycle length has an effect on activity, which can be seen when the cycle size reduces: cycloheptane ring >cyclohexane ring >cyclopentane ring >no ring. The 5-HT1A antagonist's action increases as the cycle length lowers. Adding the naphthenic ring to 5-HT reuptake inhibitory activity resulted in a significant increase in potency. 3 (Butyl)-1H indole-5 carbonitrile-HCl was found to considerably boost cognitive function and reduce depressive features in mice [109].

Among all benzochromenoquinolinones, 14-amino-13-(3-nitrophenyl)-3,4-dihydro-1H-benzo[6,7]chromeno[2,3-b]quinoline-7,12(2H,13H)-dione had the most suppressive activities on the cholinesterase enzyme. This chemical inhibited AChE with an IC_{50} of 0.86 μM and BChE with IC_{50} 6.03 μM. Additionally, the IC_{50} value of this compound for BACE1 inhibition was found to be 19.60 μM. It is capable of binding to both the PAS and CAS sites found on AChE and BChE. Considering they possess the suitable biological attributes and have a minimal hepatotoxicity, benzochromenoquinolinones have evolved as a flexible anti-AD drug [110].

A chloro substitution of tacrine and several of its analogues has been effective in managing the ChEI action in the hunt for powerful AChEI with minimal hepatotoxicity for the cure of Alzheimer's disease. Adding chlorine atom to tacrine's phenyl ring generated chlorinated tetrahydroacridines (THA) with considerable AChE inhibition. Furthermore, 3-cyano-2-substituted tetrahydroquinolines bearing a phenyl ring at the 4-position are also notable chlorinated analogues. When the 2-position of the pyridine ring had a chlorine atom added to it, this indicated the compound to be more efficacious than tacrine in inhibiting AChEI while also being less harmful to the liver. The chlorinated 4-phenyltetrahydroquinolines have improved AChE inhibitory potential and minimal hepatotoxicity compared with the unchlorinated analogues. In addition to this, compound 4-(pchlorophenyl)pyrazolo[3,4-b]tetrahydroquinoline showed encouraging activity, and its formation requires joining the pyridine ring with the pyrazolo nucleus. In addition, this compound was depicted to being completely risk-free [111].

Within the central nervous systems of mammals, glutamate is one of the most prominent excitatory neurotransmitters. Glutamate excitotoxicity, which is accountable for neurodegeneration in ischemic and traumatic brain damage, is triggered by an overstimulation of postsynaptic glutamate receptors, specifically NMDA subtype. Evidence shows that mitochondrial dysfunction is a key and initial mechanism in glutamate excitotoxicity. In terms of reducing glutamate-induced excitotoxicity, posttreatment with compound (17) is much more effective than posttreatment with NG-monomethyl-L-arginine. Compound (17) posttreatment may reduce glutamate excitotoxicity by blocking signaling pathways of mitochondrion apoptosis, while preserving proper mitochondrial performance. Besides these qualities, compound (17) suppresses glutamate-induced oxidative stress and NOS activity. Thus, this tacrine homodimer might pave the way for a new approach for curing neurodegenerative disorders [112].

In terms of hindering DMPP-mediated Ca^{2+} uptake, the novel tacrine derivatives were quite far highly effective and powerful. Compounds X19 and ethyl 5-amino-6,7,8,9-tetrahydro-4-(m-methoxyphenyl)-2-methylpyrano[2,3-b] quinoline-3-carboxylate were the most efficient blockers, inhibiting 90% and 88.5% of DMPP-stimulated Ca^{2+} entry, respectively. On the basis of animal studies, compound X20 has the most effective Ca^{2+}-blocking efficacy (IC_{50} 0.3 μM) in comparison to the commonly used L-type Ca^{2+} channel blocker, diltiazem (IC_{50} 0.03 μM) [113].

Besides, tacrine forms hybrid molecules other many other compounds, such as 7-hydroxycoumarin, ferulic acid, acridine, cinnamic acid, phenolic acid, hydroxyphenyl benzimidazole, tryptophan, and indoles, which possess potential therapeutic activity against Alzheimer's disease (Table 1).

5.2. Parkinson's Disease. Parkinson's disease is caused by a decrease in the number of dopaminergic neurons in the substantia nigra portion of the brain. The presence and buildup of alpha-synuclein protein are the key pathological characteristic of Parkinson's disease. Due to the excessive buildup, levels of dopamine begin to decline over time, leading to a range of motor and nonmotor disorder. It can be treated but not cured completely. For the purpose of treating Parkinson's disease, a number of multifunctional targeted 3-arylcoumarin-tetracyclic tacrine derivatives were developed. Compounds X17 and X18 were shown to reduce

Table 1: Experimental findings on the use of tacrine derivatives in neurological disorders.

Types of compound	Type of study	Study models	Dose/ concentration	Assay type	Findings/activity	References
Tacrine-benzoate (phenyl acetates or cinnamates) hybrid	*In-vitro*	AChE, from electric eel, BuChE, from equine serum	5.63 nM	Ellman method	Inhibit AChE with highest selectivity ratio against BuChE	[114]
7-MEOTA-donepezil-like hybrids	*In-vivo*	Male Wistar rats	25.6, 12.3, 5.7, 5.2 mg/kg	Water maze test, passive avoidance test	Significant effect of the swim order indicating maintenance of learning ability	[115]
Tacrine-ferulic acid hybrids	*In-vitro*	AChE BuChE	61.7 ± 5.2 106.9 ± 13.1	Spectrophotometric method	Good inhibitory activity to both AChE and BuChE, better selectivity for AChE compared with tacrine	[100]
	In-vitro	Aβ (1–42)	20 μM	Thioflavin T-based fluorometric assay	Similar inhibitory activity as curcumin and ferulic acid	[100]
Ferulic acid-tacrine-melatonin hybrids (FATMHs)	*In-vitro*	SH-SY5Y cells	1 μM and 3 μM	Neuroprotection analyses	Significant neuroprotection was observed against all toxic insults assayed	[116]
Tacrine-trolox, tryptoline hybrids	*In-vitro*	TcAChE from electric eel, eqBuChE (from equine serum)	49.31 nM 17.37 nM	Ellman's assay	Hybrids with longer linker chain lengths show increased AChE inhibitory activities compared to the shorter ones	[117]
Tacrine-cinnamic acid hybrids	*In vivo*	Adult ICR mice	15 mg/kg	Morris water Maze test	Considerably ameliorated the cognitive impairment of the treated mice And was much better than tacrine	[118]
				ALT & AST level test	Did not show any hepatotoxicity at all the time points	[118]
	In vitroIn vivo	AChE, BuChE, Aβ (1–42) ICR mice	10.2 nM 6.3 nM 30 mg/kg	Ellman's Assay Thioflavin T-based fluorometric assay Morris water Maze test	Cholinesterase inhibitory activities, amelioration of scopolamine-induced cognition impairment, preliminary safety in hepatotoxicity evaluation	[119]
(Benz)imidazopyridino tacrines	*In-vitro*	EeAChE eqBuChE	0.50 ± 0.03 μM	Ellman protocol	Nonhepatotoxic shows moderate and selective EeAChE inhibition	[120]
Tacrine-O-protected phenolics heterodimers	*In-vitro*	AChE, from electric eel, BuChE, from equine serum	3.5 μM	Ellman method	Safe, nonhepatotoxic, potent, and selective inhibitor of hBuChE	[121]
Tacrine-resveratrol-fused hybrids	*In-vitro*	AChE, from electric eel, BuChE, from equine serum	8.8 μM	Ellman method	AChE inhibition, Aβ self-aggregation modulation, anti-inflammatory, and immunomodulatory properties, high-predicted blood-brain barrier permeability, and low cytotoxicity	[49]

Table 1: Continued.

Types of compound	Type of study	Study models	Dose/ concentration	Assay type	Findings/activity	References
Tacrine-ferulic acid hybrids	*In-vitro*	Aβ (1–42)	20 μM	Thioflavin T-based fluorometric assay	Inhibited amyloid β-protein self-aggregation by 65.49%	[122]
	In-vitro	AChE, from electric eel, BuChE, from equine serum	37.02 nM	Ellman method	Potent inhibitor against AChE and strong inhibitor against BuChE	[122]
	In vivo	Adult ICR mice	30 mg/kg	Morris water maze test, serum ALT, AST test	Ameliorated the cognition impairment and showed preliminary safety in hepatotoxicity evaluation	[122]
Tacrine-acridine hybrids	*In-vitro*	AChE, from electric eel, BuChE, from equine serum	7.6 pM 1.7 pM	Ellman method	More active inhibitor than tacrine	[123]
	In-vitro	Aβ (1–42)	50 μM	Thioflavin T (ThT) fluorescence assay	54.74% inhibition of Aβ aggregation	[123]
Tacrine-deferiprone hybrids	*In-vitro*	AChE, from electric eel	0.64 μM	Ellman method	Control of cholinergic dysfunction, amyloid peptide aggregation, oxidative stress, and metal modulation	[124]
Tacrine, phenolic acid, and ligustrazine hybrids	*In-vitro*	AChE, from electric eel	3.9 nM	Ellman method	Potent inhibition activity towards cholinesterases (ChEs)	[125]
	In-vitro	—	85.8 ± 3.5 μM	DPPH assay	Very potent peroxyl radical scavenging capacity	[125]
Cystamine-tacrine dimer	*In-vitro*	SH-SY5Y cell line	0.005-0.5 μM	MTT assay Enzymatic assay Fluorometric assay	AChE and BChE inhibitor; activates kinase 1 and 2 (ERK1/2) and Akt/(PKB) pathways	[92]
Tacrine-trolox hybrid	*In-vivo*	Male Sprague-Dawley (SD) rats	6 mmol/100 g b. wt	(AST) and (ALT) activity	Introduction of trolox could reduce the hepatotoxicity of tacrine Inhibitor against AChE and BuChE	[93]
	In-vitro	Electric eel, Ellman's reagent, DTNB	9.8-23.5 nM 20.5-22.2 nM	Ellman's assay	More potent inhibitory activity for BuChE than for AChE	[93]
		PC12 cells	3.125 μM and 6.25 μM	MTT assay	Significantly inhibit cell death	[93]
Tacrine-propargylamine derivatives	*In-vitro*	Human neuroblastoma cell line, SH-SY5Y	10, 50, and 100 μM	MTT assay	Nearly no effect on the viability of SH-SY5Y cells, lower cytotoxicity than tacrine	[126]

Table 1: Continued.

Types of compound	Type of study	Study models	Dose/ concentration	Assay type	Findings/activity	References
Tacrine-coumarin hybrids	*In-vitro*	hMAO-A, hMAO-B	0.24 μM	Fluorimetric method	Selective MAO-B inhibitor	[94]
	In-vitro	eeAChE, hBuChE	16.11 ± 0.09 nM 112.72 ± 0.93nM	Ellman's method	Potent inhibitory action for AChE and BuChE	[94]
	In-silico	Recombinant hAChE	—	—	Simultaneously bind to PAS and CAS and the mid-gorge site of AChE	[94]
	In-vitro	hAChE, hBuChE	38 nM 63 nM	Ellman's method	Potent and selective inhibitory activities towards both hAChE and hBuChE	[127]
	In-vitro	A-β1-40 peptide	1 μM	Thioflavin T assay	Inhibit A-β40 amyloid self-assembly	[127]
	In-silico	hAChE(1ACJ), hBuChE (4 BDS), β-secretase (BACE1)	—	AutoDock 4.2 and Vina	Fingerprints studies showed 34 ligands to be effective in their docking binding energies and high binding natures	[128]
Tacrine-benzofuran hybrids	*In-vitro*	Recombinant hAChE and hBChE	0.86 nM 1.35 μM	Ellman's assay	Selectively inhibited hAChE, suppressed both hBACE-1 activity and β-amyloid aggregation	[95]
	In-vivo	ICR mice	20 μmol/kg	Morris water maze test	Considerably ameliorated the cognition impairment of the treated mice	[95]
Tacrine/cysteine-conjugated compounds	*In-vitro*	Amyloid-β peptide	70 μM	Fluorescence assay	Decreased Aβ_{42} (40 μM) self-aggregation	[96]
	In-vitro	Human neuroblastoma SH-SY5Y cells	2.5 μM	MTT assay	Cell viability is not significantly affected after a 24-h treatment	[96]
		TcAChE	0.30 μM	Ellman0s assay	High inhibitory activity in submicromolar range	[96]
	In-silico	—	—	Molecular docking	Target both the CAS and PAS of AChE	[96]
Tacrine-phenylbenzothiazole hybrids	*In-vitro*	AChE from electric eel Aβ (1–42)	0.15 μM 40 μM	Modified method of Ellman's assay Thioflavin T (ThT) assay	Excellent AChE inhibitory activity and moderate inhibition values for amyloid-β (Aβ) self-aggregation (27–44.6%)	[129]
Tacrine-1,2,4-thiadiazole derivatives conjugates	*In-vitro*	Human erythrocytes AChE equine serum BChE, porcine liver CES	17.1 μM 44.8 μM	Ellman method	Effectively inhibited cholinesterases with a predominant effect on (BChE), could block AChE-induced β-amyloid aggregation	[130]

Table 1: Continued.

Types of compound	Type of study	Study models	Dose/ concentration	Assay type	Findings/activity	References
Tacrine-hydroxamate derivatives	*In-vitro*	AChE BChE HDAC	0.12 nM 361.52 nM 0.23 nM	Ellman method Fluorescence assay	Potent and selective inhibition on ache, potent inhibition on HDAC, recognitive impairments inhibitory activity on Aβ1-42 self-aggregation as well as disaggregation activity on preformed Aβ fibrils	[47]
Tacrine-pyrimidone hybrids	*In-vitro*	Murine AChE Recombinant human GSK-3β	51.1 nM 89.3 nM	Ellman's method with modification	Possessed excellent dual AChE/GSK-3 inhibition both in terms of potency and equilibrium	[48]
	In-vivo	Female ICR mice	15 mg/kg	Morris water maze (MWM) tests	Displayed significant amelioration on cognitive deficits in scopolamine-induced amnesia mice	[48]
Tacrine(10)-hupyridone dimer	*In-vivo*	Wild-type (WT) mice	0.36 or 0.72 μmol/kg	Morris water maze (MWM) tests	Long-term treatment of the compound could attenuate precognitive impairments in APP/PS1 transgenic mice	[131]
Tacrine and salicylamide conjugates	*In vitro*	AChE BChE	0.22 μM 0.01 μM	Propidium iodide fluorescence	Exhibited high dual anticholinesterase activity with selectivity towards BChE	[132]

synuclein protein aggregation as well as having antioxidant properties. These two compounds were found to be fascinating. In the case of Parkinson's disease, they also elevated levels of dopamine, which are essential and advantageous for relaying nerve impulses [133].

Tacrine is used to induce tremulous jaw movements, a characteristic feature of Parkinson's disease in rat models to test the effect of anti-Parkinsonian agents [134–138]. Parkinson's disease cannot be treated with tacrine alone. 6-Hydroxydopamine (6-OHDA) is a neurotoxin used as a tool to induce Parkinson's disease in PC12 cells and rat models. 6-OHDA-induced Parkinson's disease in PC-12 cells can be lessened by the dimer of tacrine (2)-ferulic acid, which has been proven to reduce the symptoms of the condition. TnFA, $n = 2 - 7$, acetylcholinesterase inhibitory tacrine–-ferulic acid dimers connected by an alkylenediamine side chain, were produced to test their effect on 6-OHDA-affected PC12 cells, according to a research conducted in 2011. At high concentrations and over a long period of time, 6-OHDA caused cell death in PC12 cells. T2FA (20 M) provided neuroprotection against 6-OHDA-induced cell death (69.35 3.69 percent survival, compared with control, p 0.01), whereas TnFA ($n = 3 - 7$) did not provide any protection. The authors also mentioned that ferulic acid or tacrine alone could not prevent 6-OHDA-induced cell death in the test subject. So, the protective effect is not caused by any monomer but the T2FA dimer (Figure 4). In addition, 20 M T2FA lowered the frequency of cells with apoptotic bodies and nuclear condensation, decreased intracellular ROS, blocked GSK3b activation, and reversed the inhibition of the PI3-K/Akt pathway produced by 6-OHDA. Researchers found that T2FA, an AChE inhibitor, reduced 6-OHDA-induced apoptosis in PC12 cells and so might be used to change the etiology of Parkinson's disease (PD) [57].

5.3. Ischemia. Tacrine has no therapeutic activity against cerebral ischemia. But a dimer of tacrine, compound (17) and has shown notable activities against cerebral ischemia according to a few studies. Comparable to memantine, a well-known NMDA receptor antagonist used to treat Alzheimer's disease, compound (17) has promising properties on blocking the NMDA receptor. For this reason, the ability of compound (17) to impede focal cerebral ischemic injury in rats with middle cerebral artery occlusion was examined. The results showed that compound (17) (0.1-0.2 mg/kg) could notably decrease the neurological problems and improve the neurological score and reduced infarction and brain edema after 2-h occlusion/2411 reperfusion. The neuroprotective activity of compound (17) was almost 260 times higher than that of memantine. So compound (17) could be useful as a neuroprotective agent for treating stroke as it can reduce neurological consequences of ischemic injury by NMDA receptor inhibition [139].

In another experiment, the influence of compound (17) on apoptosis caused by ischemia in astrocytes of mice cerebral cortex was examined. The astrocyte cell cultures were incubated in ischemic condition for 6 hours, and lactate dehydrogenase (LDH) release assay showed significant reduction in

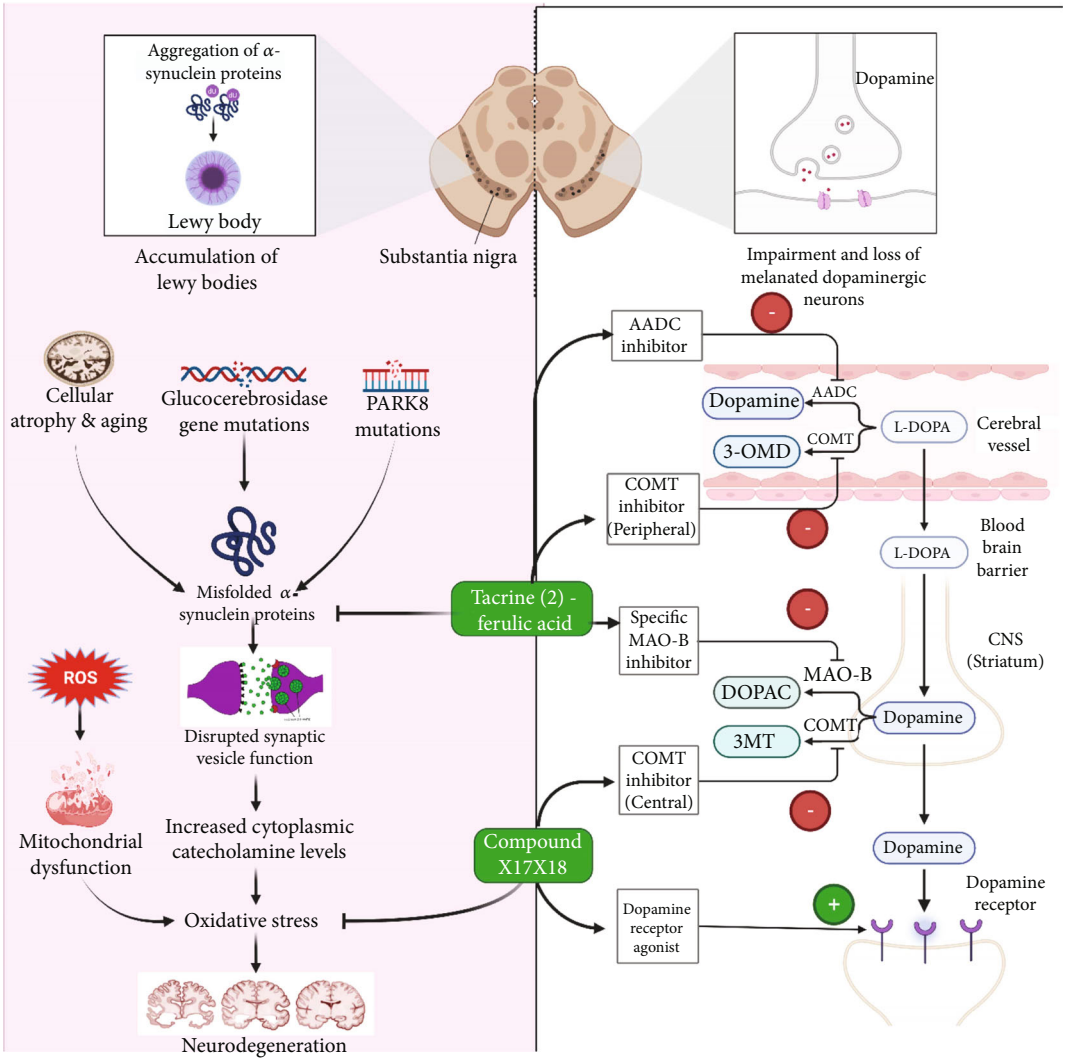

Figure 4: Illustration representing the site of action of different tacrine derivatives in Parkinson's disease.

viable cells' percentage. Also, bisbenzimide staining determined that the anaerobic chamber caused apoptosis of 65% of the cells. During the ischemic incubation, the treatment with 1-10 nM concentration of compound (17) prevented this apoptosis of cells caused by oxygen deficiency, which was confirmed by both biochemical and morphological tests. So this drug can be beneficial against vascular dementia, which is caused by ischemia-induced death of the astrocytes [140].

Sprague-Dawley rats were used in another investigation to test the in vivo effects of compound (17) against localized cerebral ischemic injury. There was ischemia in the brain for 2 hours and 24 hours, and compound (17) was administered intraperitoneally after 15 minutes of this time period. Depending on the dose, the neurological abnormalities were improved, and both edema and cerebral infarct volume were decreased. It was shown is TUNEL staining assay that compound (17) reduced apoptosis of the neurons of penumbral region. The therapeutic window of compound (17) lasted until 6 hours which was wider than a similar NMDA receptor blocker memantine. But it did not change the blood flow of cerebral regions or any physiological variables in any stage of the experiment. So it can be concluded that the dose- and time-dependent effects of compound (17) can give protection against acute focal cerebral ischemic injury, probably through its antiapoptotic activity during numerous stages of the ischemic cascade [141].

Shu et al. stated that compound (17) at the dose of 0.2 mg/kg shortened the escape latency of adult male Sprague-Dawley rats whose spatial learning ability was impaired by chronic cerebral ischemia. Compound (17) lowered the rate of neuronal apoptosis in CA1 region of the hippocampus and enhanced neurogenesis in that region, compared to the saline-treated rats, as shown by immunohistochemical assay. Thus, it was suggested that compound (17) inhibits apoptosis and promotes neurogenesis in 2VO rats to exert its neuroprotective effects [38].

The neuroprotective impact of compound (17) on ischemia retinal injury was substantial. Compound (17) further prevented the reduction of amplitude of a- and b-wave in electroretinography, caused by ischemia. Ischemic damage led to a rise in the protein quantities of p53, which is a tumor suppressor gene well-known for its ability to trigger

apoptosis. In such scenario, compound (17) therapy lowered the protein's expression. The treatment with compound (17) offered protection from damages of acute focal cerebral ischemia. Furthermore, glutamate excitotoxicity in the extracellular space of the retina is hypothesized to be one of the processes underlying neuronal cell death in glaucoma. Compound (17) outperformed memantine in glutamate-induced neurotoxicity in rats by specifically inhibiting the NMDA-activated current. In addition, compound (17) has the ability to inhibit the formation of NO which is induced by glutamate. As a corollary, compound (17) may hold promise as a neuroprotectant therapeutic agent for the treatment of glaucoma [142].

6. Concluding Remarks

This study provided a concise summary of the existing understanding about tacrine analogues that have genuinely valuable therapeutic effects. This article reviews the literature in the fields of medicinal chemistry, pharmacology, and biology pertaining to the topic. The authors described NO-donor-tacrine hybrids that displayed hepatoprotective features, as well as fused with natural components that protect cells from oxidative damage, such as melatonin, hydroxyquinoline, or thioflavine. Additionally, addresses a broad range of hybrid compounds that were developed by researching into the pharmacophores of currently marketed drugs. Other examples of hybrid compounds that were developed by studying the pharmacophore of commercial medications have been described. Structured hybrid inhibitors of cholinesterases have effectiveness in inhibiting cholinesterases activity, as well as antioxidant capacity and the ability to prevent aggregation Aβ (β-amyloid). As a result, these compounds have the potential to operate as neuroprotective medications while also displaying increased biological activity. The currently targetable pathways have been outlined in detail in order to facilitate the development of neurotherapeutic drugs. The article provides a concise update as well as their viewpoint on actual and possibly druggable pharmacological targets for the illness of the central nervous system.

This review highlighted a number of innovative multitarget-directed tacrine analogues that have been developed to date and are categorized based on their biological target and chemical composition. Novel therapeutic prototype candidates have been uncovered, and several are already undergoing preclinical testing. It is the starting point for a radical shift in how we approach medication development. System biology may help medical scientists uncover novel chemical entities capable of comprehending and efficiently modifying this severe neurodegenerative condition, despite the fact that none of these MTDL medication candidates has reached the clinical stage.

Data Availability

All data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

The Mechanism of TNF-α-Mediated Accumulation of Phosphorylated Tau Protein and Its Modulation by Propofol in Primary Mouse Hippocampal Neurons: Role of Mitophagy, NLRP3, and p62/Keap1/Nrf2 Pathway

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Background. Neuroinflammation-induced phosphorylated Tau (p-Tau) deposition in central nervous system contributes to neurodegenerative disorders. Propofol possesses neuroprotective properties. We investigated its impacts on tumor necrosis factor-α (TNF-α)-mediated p-Tau deposition in neurons. *Methods*. Mouse hippocampal neurons were exposed to propofol followed by TNF-α. Cell viability, p-Tau, mitophagy, reactive oxygen species (ROS), NOD-like receptor protein 3 (NLRP3), antioxidant enzymes, and p62/Keap1/Nrf2 pathway were investigated. *Results*. TNF-α promoted p-Tau accumulation in a concentration- and time-dependent manner. TNF-α (20 ng/mL, 4 h) inhibited mitophagy while increased ROS accumulation and NLRP3 activation. It also induced glycogen synthase kinase-3β (GSK3β) while inhibited protein phosphatase 2A (PP2A) phosphorylation. All these effects were attenuated by 25 μM propofol. In addition, TNF-α-induced p-Tau accumulation was attenuated by ROS scavenger, NLRP3 inhibitor, GSK3β inhibitor, or PP2A activator. Besides, compared with control neurons, 100 μM propofol decreased p-Tau accumulation. It also decreased ROS and NLRP3 activation, modulated GSK3β/PP2A phosphorylation, leaving mitophagy unchanged. Further, 100 μM propofol induced p62 expression, reduced Keap1 expression, triggered the nuclear translocation of Nrf2, and upregulated superoxide dismutase (SOD) and heme oxygenase-1 (HO-1) expression, which was abolished by p62 knockdown, Keap1 overexpression, or Nrf2 inhibitor. Consistently, the inhibitory effect of 100 μM propofol on ROS and p-Tau accumulation was mitigated by p62 knockdown, Keap1 overexpression, or Nrf2 inhibitor. *Conclusions*. In hippocampal neurons, TNF-α inhibited mitophagy, caused oxidative stress and NLRP3 activation, leading to GSK3β/PP2A-dependent Tau phosphorylation. Propofol may reduce p-Tau accumulation by reversing mitophagy and oxidative stress-related events. Besides, propofol may reduce p-Tau accumulation by modulating SOD and HO-1 expression through p62/Keap1/Nrf2 pathway.

1. Introduction

Neurodegenerative disorders, such as Parkinson's disease (PD), Alzheimer's disease (AD), and perioperative neurocognitive disorder (PND), are diseases in which the structure and function of neurons are impaired, leading to dysfunction of central nervous system (CNS). While the causes associated with neuronal impairment remain poorly understood, increasing evidence proved systemic inflammatory response especially neuroinflammation is crucial in the progression of neurodegenerative diseases [1, 2]. The core of neuroinflammation is likely the same in aging, metabolic diseases such as hypertension and diabetes, or cerebral insults such as stroke and injury [3], and inflammatory mediator tumor necrosis factor-α (TNF-α) was proved to serve as a key player and biomarker of neuroinflammation [4]. Speaking of molecular mechanisms, plenty of evidence suggested that the aggregation of β-amyloid, α-synuclein, and Tau protein plays a crucial role in neurodegenerative cascades [5–7]. However, tauopathy especially hyperphosphorylation of Tau, rather than Tau itself, was believed to lead to dementia and neurodegenerative diseases [8]. A recent clinical study also revealed a robust relationship between phosphorylated Tau protein (p-Tau) in the brain and the extent of neurodegeneration in AD patients [9]. Nevertheless, whether neuroinflammation caused p-Tau accumulation was still unknown, and if so, how neuroinflammation triggered p-Tau accumulation was also rarely studied. Although posttranslational Tau protein modifications may be mediated by many factors, mitochondrial autophagy (also known as mitophagy), reactive oxygen species (ROS), the activation of the NOD-like receptor protein 3 (NLRP3) inflammasome, and the regulation of kinases and phosphatases have attracted attention due to their upstream and downstream effects on tauopathy [10–12].

Propofol is widely used as an intravenous anesthetic/sedative agent in clinical practice. Apart from hypnotic advantages, it possesses anti-inflammation [13] and antioxidation effects [14] as well as neuro-protective properties [15]. On cellular and molecular levels, propofol has been shown to attenuate TNF-α-induced neuronal dysfunction [16, 17]. It has also been reported to diminish mitochondrial dysfunction and ROS production in isolated rat hippocampal neurons [18, 19]. In addition, animal studies revealed that propofol exerted cognitive protection by regulating the expression and posttranslational modification, mostly phosphorylation of Tau in rat models [20, 21]. Nevertheless, deeper investigation concerning how propofol modulates Tau protein phosphorylation needs to be carried out.

In the present *in vitro* study, we examined whether and how TNF-α caused p-Tau accumulation in hippocampal neurons. More importantly, we investigated the protective effects and mechanisms of propofol in neurons. The findings of this study may provide potential therapeutic targets for the prevention and treatment of p-Tau accumulation and resultant neuron injury as well as neurodegenerative disorders.

2. Materials and Methods

2.1. Experimental Design. To examine the effects of TNF-α on p-Tau accumulation, neurons were exposed to different concentrations of TNF-α (10, 20, 40, 80, and 160 ng/mL) for different durations (1, 2, 4, and 8 h). Neuron viability and the amount of Tau as well as p-Tau were examined, and the optimal TNF-α treatment condition was determined. To investigate the protective effects of propofol, neurons were incubated with different concentrations (1, 5, 10, 25, 50, and 100 μM) of propofol for 1 h followed by TNF-α treatment. We intended to examine the effect of propofol on TNF-α-induced p-Tau accumulation, and further investigated the mechanisms including mitophagy, ROS, NLRP3 inflammasome, glycogen synthase kinase-3β (GSK3β), cAMP-dependent protein kinase (PKA), protein phosphatase 2A (PP2A), antioxidant enzyme superoxide dismutase (SOD), heme oxygenase-1 (HO-1), NADPH quinine oxidoreductase 1 (NQO1), and p62/Keap1/Nrf2 pathway. To confirm their roles, inhibitors and activators as well as overexpression/knockdown technique were applied.

2.2. Cell Culture. Cryopreserved primary mouse hippocampal neurons were commercially obtained from Gibco-Life Technologies (Carlsbad, CA, USA) and cultured in B-27 Plus Neuronal Culture System (Gibco-Life Technologies, Carlsbad, CA, USA). After thawed and seeded in tissue culture flasks containing 5 mL media supplemented with neuronal growth supplement, 1% penicillin/streptomycin and 5% fetal bovine serum, neurons were kept in a humidified incubator filled with 5% CO_2 and 95% air at 37°C. The culture media were replaced every other day, until neurons reached about 70% confluency and were ready for experiments without subculturing.

2.3. TNF-α Treatment and Propofol Pretreatment Protocol. Recombinant mouse TNF-α was obtained from Sigma-Aldrich (St. Louis, MO, USA) and was reconstituted with sterile water to a stock concentration of 0.1 mg/mL. To investigate the effect of TNF-α on p-Tau accumulation, neurons were exposed to different concentrations of TNF-α (10, 20, 40, 80, and 160 ng/mL) for different durations (1, 2, 4, and 8 h). By measuring the expression and phosphorylation of Tau protein, we aimed to determine the optimal condition, under which TNF-α exerted significant effect on the accumulation of p-Tau.

To investigate the protective effects of propofol against TNF-α in hippocampal neurons, we incubated neurons with different concentrations (1, 5, 10, 25, 50, and 100 μM) of propofol (Sigma-Aldrich, St Louis, MO, USA) or its solvent 0.1% dimethyl sulfoxide (DMSO, Sigma-Aldrich, St Louis, MO, USA) for 1 h followed by TNF-α treatment (with the presence of propofol or DMSO). By observing the expression and phosphorylation of Tau protein, we intended to identify the optimal concentration, at which propofol exerted protective effects against p-Tau accumulation.

2.4. Cell Viability Assay. 3-4,5-dimethylthiazol-2,5-diphenyltetrazolium bromide (MTT) assay was used to assess the viability of neurons. Briefly, neurons were seeded in 6-well

culture plates and exposed to respective treatment. After removing culture media, neurons were rinsed with phosphate-buffered saline (PBS). MTT was dissolved in serum-free medium at a final concentration of 0.5 mg/mL and each well was loaded with 150 μL MTT. After incubating at 37°C for 30 min, 150 μL dimethyl formamide was added, and the incubation continued for 4 h, during which formazan crystals formed. Then, 150 μL DMSO was added to dissolve formazan crystals. A microplate reader (Bio-Rad, Hercules, CA, USA) was used to determine the absorbance values at 570 nm, and optical density served as unit. Cell viability was expressed as the percentage of absorbance of treated neurons compared with that of untreated control neurons.

2.5. Mitochondrial Membrane Potential (MMP) Determination. MMP was determined through fluorescent dye rhodamine-123 (Rh123), which is a lipophilic cationic fluorescent probe for mitochondria, and the fluorescence was examined through flow cytometry with the use of fluorescence-activated cell sorter (FACS). Briefly, Rh123 (Beyotime Institute of Biotechnology, Shanghai, China) was dissolved in DMSO to make a 5 mM stock solution. After treatment, neurons were washed with PBS and incubated with 5 μM Rh123 at 37°C in a dark chamber for 30 min. Then, neurons were washed with PBS to remove excess dye, and BD FACSPresto™ System (BD Biosciences, San Jose, CA, USA) was applied to detect the fluorescent signal at an excitation wavelength of 490 nm and an emission wavelength of 585 nm. Data were expressed as mean ± standard deviation of fluorescent intensity of Rh123 staining.

2.6. Mitophagy Assessment. The extent of mitophagy was evaluated by using Mitophagy Detection Kit (Beyotime Institute of Biotechnology, Shanghai, China) according to the manufacturer's instructions. In brief, after treatment, neurons were washed with Hanks' HEPES solution, incubated with 0.1 μM mitophagy dye working solution at 37°C for 30 min, and incubated with 1 μM lysosome dye working solution at 37°C for 30 min. After washing with Hanks' HEPES solution to remove excessive dye, the fluorescence was detected at an excitation wavelength of 550 nm and an emission wavelength of 610 nm. Data were expressed as mean ± standard deviation of fluorescent intensity.

2.7. Intracellular ROS Measurement. Intracellular ROS was monitored using a ROS-sensitive fluorogenic dye. The method is based on fluorescent 2',7'-dichlorofluorescein (DCF), which is oxidatively converted from non-fluorescent 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA, Beyotime Institute of Biotechnology, Shanghai, China). In brief, neurons were seeded in 6-well culture plates and exposed to respective treatment. Thereafter, neurons were incubated with 10 μM DCFH-DA for 30 min at 37°C. Then, the reaction mixture was aspirated and replaced with 200 μL PBS in each well. The plates were placed on a shaker for 10 min at room temperature in the dark, and subject to fluorescence microplate reader with an excitation wavelength of 485 nm and an emission wavelength of 535 nm. The data were recorded as folds of increased fluorescence intensity in treated neurons compared with that of untreated neurons.

2.8. Mitochondrial ROS Assessment. ROS generation within mitochondrial compartment was assessed in live cells using MitoSOX Red, a fluorogenic dye that is taken up by mitochondria where it is readily oxidized by superoxide anion and serves as mitochondrial ROS indicator. Briefly, neurons were seeded in 6-well culture plates, and MitoSOX Red (Beyotime Institute of Biotechnology, Shanghai, China) was dissolved in DMSO to form 5 mM stock solution. After treatment, neurons were loaded with 1 μM MitoSOX Red for 10 min at 37°C in the dark. Then, neurons were washed with PBS, and fluorescence intensity was determined with fluorescence microplate reader at 510 nm excitation and 580 nm emission, respectively. Data were recorded as folds of increased fluorescence intensity in treated neurons compared with that of untreated neurons.

2.9. Enzyme-Linked Immunosorbent Assay (ELISA). The production of interleukin-1β (IL-lβ) and interleukin-18 (IL-18) was evaluated by SimpleStep ELISA kit (Beyotime Institute of Biotechnology, Shanghai, China) according to the manufacturer's instructions. Briefly, after treatment, the culture media were harvested. In addition, neurons were scrapped off, suspended in PBS, and subject to ultrasonic homogenizer. Then, the homogenates and culture media were centrifuged at 2000 revolutions per minute (rpm) for 10 min at 4°C, and the supernatant was collected and transferred to 24-well plates precoated with antibody against IL-lβ or IL-18. After incubating at 4°C for 30 min, the capture and detector antibody cocktail were added, and incubation lasted for 30 min at 4°C. Then, the supernatant was removed, and the wells were washed with PBS. Subsequently, the detection reagent was added, and the absorbance at 450 nm was measured with a microplate reader (Bio-Rad, Hercules, CA, USA). A standard curve was plotted using standard IL-1β or IL-18 supplied by the kit, and data were expressed as pg/mL.

2.10. Preparation of Whole Cell Extracts. After treatment, culture media were removed, and neurons were washed with PBS and then scraped off the culture flasks. After centrifugation for 5 min at 1000 rpm at 4°C, neuron pellets were suspended in radioimmunoprecipitation assay (RIPA) lysis solution (Santa Cruz Biotechnology, Santa Cruz, CA, USA) containing 1% protease inhibitor and 0.1% phosphatase inhibitor, and placed on ice for 10 min with intermittent homogenization by vortexing. Then, the whole cell proteins were obtained by centrifuging for 10 min at 5000 rpm, and the protein concentration was determined by BCA protein assay kit (Beyotime Institute of Biotechnology, Shanghai, China).

2.11. Preparation of Nuclear Extracts. Nuclear extracts were prepared using nuclear extract kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer's protocol. After treatment, culture media were thoroughly removed. Then, neurons were washed with PBS, scraped off, transferred to prechilled tubes, pelleted by centrifugation at 1000 rpm for 5 min at 4°C, suspended in hypotonic buffer, and incubated

on ice for 15 min. After adding detergent and intermittent vortexing for 10 sec, the suspensions were centrifuged at 14,000 rpm for 1 min at 4°C. Then, the pellets were suspended in complete lysis buffer, vortexed for 10 sec, and incubated for 30 min at 4°C. The suspensions were centrifuged at 14,000 rpm for 10 min at 4°C, and the supernatant was collected. Protein concentration was quantified by BCA protein assay kit (Beyotime Institute of Biotechnology, Shanghai, China), and the purity of nuclear fractions was verified by the absence of cytosolic marker α-tubulin.

2.12. Preparation of Cytosolic Extract. Cytosolic fractions were separated using ProteoExtract subcellular proteome extraction kit (Calbiochem, La Jolla, CA) according to the manufacturer's protocol. Briefly, after treatment, neurons were harvested and washed twice with wash buffer and were suspended in 50 μL extraction buffer I containing protease inhibitor cocktail and lysed by gently rocking for 5 min. Cell debris and heavy membrane organelles were pelleted by centrifugation at 10,000 rpm for 10 min. The supernatant containing cytosolic fraction was collected, and protein concentrations were quantified by BCA protein assay kit (Beyotime Institute of Biotechnology, Shanghai, China). The purity of cytosolic fractions was verified by the absence of nuclear marker Histone H3.

2.13. Protein Analysis by Western Blot Analysis. Equal amounts of protein (40 μg per lane) were heated to 95°C for 5 min followed by storing on ice for 5 min, separated with 8% or 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and electrophoretically transferred to polyvinylidinene fluoride membranes (Millipore, Bedford, MA, USA) for 90 min at a constant current of 200 mA. After sealing the membranes with 5% skimmed milk at room temperature for 2 h, 1 : 500~1000 dilution of specific primary antibodies (Cell Signaling Technology, Beverly, MA, USA) against NLRP3, cleaved-caspase-1, pro-caspase-1, GSK3β, phosphorylated GSK3β, PKA, phosphorylated PKA, PP2A, phosphorylated PP2A, p62, Keap1, Nrf2, SOD, HO-1, NQO1, Tau, p-Tau, α-tubulin, Histone H3, or GAPDH were incubated with the membranes for overnight at 4°C. Subsequently, the membranes were washed with TBST (Tris-buffered saline containing 0.1% Tween 20) and incubated with 1 : 5000 dilution of HRP-conjugated species-specific secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at room temperature for 2 h. The immunoreactive bands were detected with Amersham ECL plus Western blotting detection reagent (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and images were scanned and recorded with Odyssey System (LI-COR Biosciences, Lincoln, NE, USA). The gray values of protein bands were analyzed with Image J v1.8.0 software. The values of GAPDH served as internal control for whole cell or cytosolic proteins, and values of Histone H3 served as an internal control for nuclear proteins. During the examination of nuclear proteins, α-tubulin was used to rule out contamination of cytosolic component. The relative expression of target protein was calculated according to the equation: gray value of target protein band/gray value of control protein band.

2.14. Transient Transfection. Small interfering RNA (siRNA) and plasmid were transiently transfected with lipofectamine RNAiMAX transfection reagent (Thermo Fisher Scientific, Waltham, USA). siRNA against mouse p62 (5′-CGAGGA ATTGACAATGGCCAT-3′) and scramble control siRNA (5′-UUCUCCGAACGUGUCACGUTT-3′) were purchased from Cell Signaling Technology (Beverly, MA, USA), and Keap1 overexpression plasmid (5′-AGTGGCGAATGATC ACAGCAAT-3′) and random control plasmid (5′-ACGU GACACGUUCGGAGAATT-3′) were designed and constructed by GenePharma (Shanghai, China). Briefly, on reaching 50% confluency, 10 μL lipofectamine and 5 μg siRNA/plasmid were mixed for 20 min, followed by incubation with neurons for 6 h at 37°C. Thereafter, neurons were washed with PBS and cultured in culture media for 48 h and exposed to respective treatment. The transfection efficiency was examined via Western blot analysis 48 h after transfection.

2.15. Statistical Analysis. Data were presented as mean values with standard deviations. All experiments were performed with 5 independent repeats carried out in different cultures. Group differences were assessed with paired two-tailed Student's *t*-test or one-way ANOVA, followed by post hoc Tukey testing. All analyses were performed using SPSS version 13.0, and $p \leq 0.05$ was considered 95% confidence limits as a significant difference.

3. Results

3.1. TNF-α Induced p-Tau Accumulation in Hippocampal Neurons in a Concentration- and Time-Related Manner. To mimic *in vivo* neuroinflammation, we treated primary mouse hippocampal neurons with different concentrations of inflammation mediator TNF-α (10, 20, 40, 80, and 160 ng/mL) for 4 h. By Western blot analysis, we found that TNF-α (10~80 ng/mL) had minor effect on Tau protein expression, which was reduced by 160 ng/mL TNF-α (Figure 1(a)). In addition, we found that 20~80 ng/mL TNF-α induced the amount of p-Tau, which was also inhibited by 160 ng/mL TNF-α (Figure 1(a)). Next, we treated neurons with 20 ng/mL TNF-α for different durations (1, 2, 4, and 8 h), and showed that although TNF-α (1~8 h) had no effect on Tau protein expression, 4- and 8-h treatment induced its phosphorylation status (Figure 1(b)). We also examined the effect of TNF-α on neuron viability and showed that 10, 20, 40, and 80 ng/mL TNF-α treatment for 4 h had minor effect on neuron viability, which was greatly reduced by 160 ng/mL TNF-α (Figure 1(c)). In addition, we showed that treatment of neurons with 20 ng/mL TNF-α for different durations (1, 2, 4, and 8 h) did not affect cell viability (Figure 1(c)). We postulated the reduced expression and phosphorylation of Tau after 160 ng/mL TNF-α exposure was due to suppress cell viability. Thereafter, 20 ng/mL TNF-α incubation for 4 h was considered an optimal stimulus to induce p-Tau without affecting neuron viability and was applied in the following experiments.

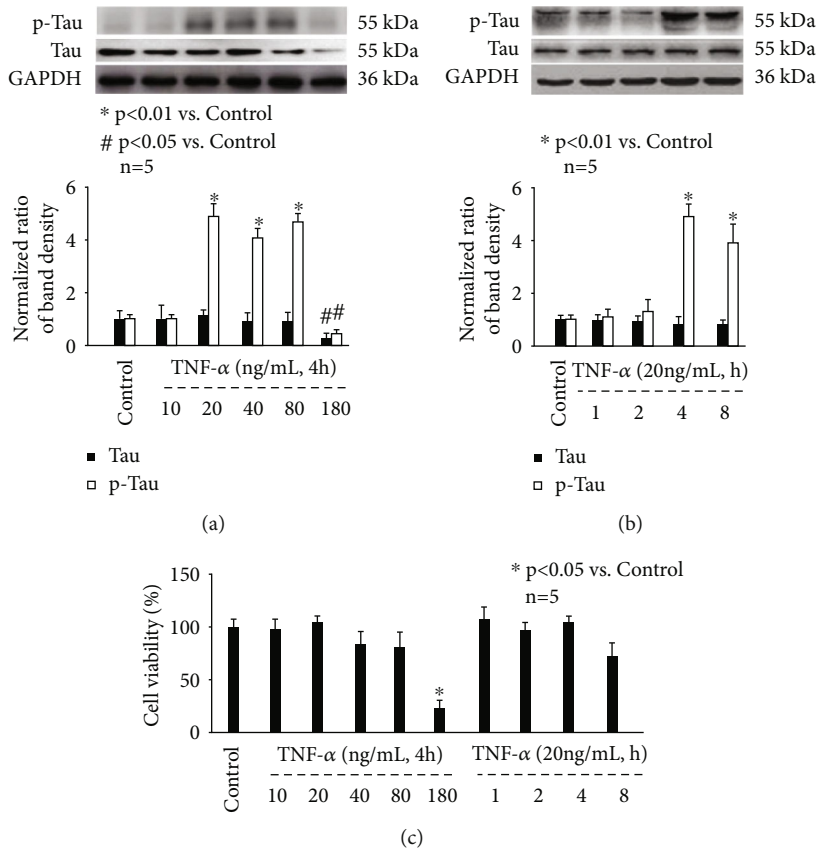


Figure 1: The effect of TNF-α on p-Tau accumulation and cell viability in mouse hippocampal neurons. (a) TNF-α treatment for 4 h induced p-Tau in a concentration-dependent manner. The upper panel was a representative experiment, and the lower panel was the summary of densitometric data from 5 separate experiments. GAPDH served as loading control. Data were expressed as normalized ratio of protein band density of Tau or p-Tau against GAPDH and were presented as mean ± standard deviation. (b) 20 ng/mL TNF-α induced p-Tau in a time-dependent manner. (c) The effect of TNF-α treatment on cell viability. Data were expressed as the percentage of absorbance of treated neurons compared with control neurons.

3.2. Propofol Pretreatment Concentration Dependently Prevented TNF-α-Induced p-Tau Accumulation in Hippocampal Neurons. To mimic clinical administration of propofol, and to exam the effect of propofol on p-Tau accumulation, we incubated hippocampal neurons with different concentrations (1, 5, 10, 25, 50, and 100 μM) of propofol or 0.1% DMSO for 1 h, followed by TNF-α treatment (20 ng/mL, 4 h). Please note that this concentration range covers plasma concentrations of propofol during general anesthesia and sedation in clinical practice. As shown in Figure 2(a), we indicate that both propofol and DMSO had no effect on Tau protein expression. However, 25, 50, and 100 μM propofol attenuated TNF-α-induced p-Tau accumulation, which was not affected by DMSO. In addition, we observed the effect of propofol or DMSO alone in neurons and revealed they had no effect on basal status of Tau protein expression (Figure 2(b)). Interestingly, we detected 100 μM propofol reduced p-Tau accumulation, while DMSO had no such effect (Figure 2(b)). We also reported that neither propofol nor DMSO affected neuron viability (Figure 2(c)). Accordingly, we believed that the effect of propofol was independent of its solvent DMSO and thereafter further investigated detailed mechanisms for 25 and 100 μM propofol-regulated p-Tau accumulation in hippocampal neurons.

3.3. The Effect of TNF-α and Propofol on Mitophagy, ROS, NLRP3 Inflammasome, and GSK3β/PP2A in Hippocampal Neurons. Recently, a large body of evidence implied the correlation between NLRP3 inflammasome activation and Tau phosphorylation, which relies on the balance between kinases (GSK3β and PKA) and phosphatase (PP2A) activity [22–24]. As such, we examined the effect of TNF-α and propofol on the activation of NLRP3 inflammasome, GSK3β, PKA, and PP2A. In this *in vitro* study, NLRP3 inflammasome activation was evaluated by measuring NLRP3 expression, cleavage of pro-caspase-1, and release of matured IL-lβ and IL-18. As shown in Figure 3, we demonstrate that TNF-α (20 ng/mL, 4 h) increased NLRP3 expression (Figure 3(a)), induced the cleavage of pro-caspase-1 (Figure 3(b)), and increased matured IL-lβ and IL-18 release (Figure 3(c)), which were all attenuated by 25 μM propofol pretreatment (Figure 3). Besides, we identified that TNF-α (20 ng/mL, 4 h) increased the phosphorylation of GSK3β (Figure 4(a)) and PKA (Figure 4(b)), while reduced PP2A phosphorylation (Figure 4(c)). Although 25 μM propofol did not affect

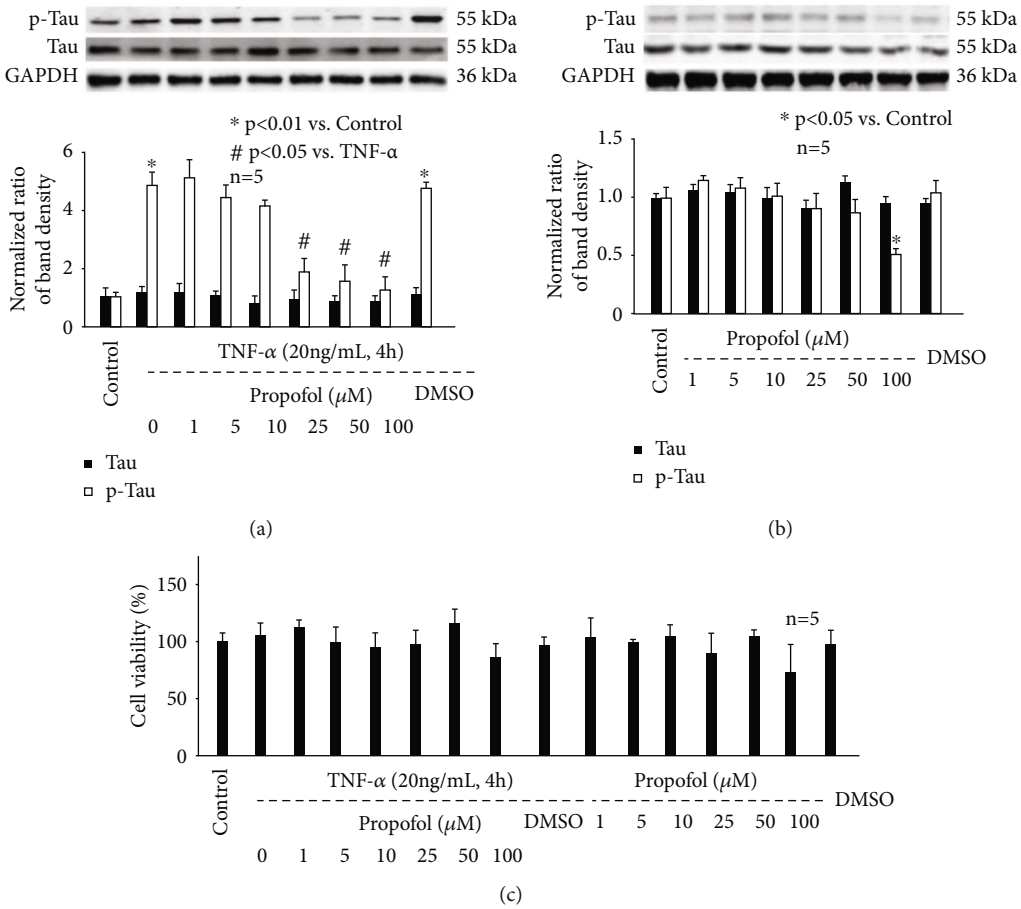


Figure 2: The effect of propofol on p-Tau accumulation and cell viability in mouse hippocampal neurons. (a) Propofol reduced p-Tau in hippocampal neurons exposed to TNF-α (20 ng/mL, 4 h). The upper panel was a representative experiment, and the lower panel was the summary of densitometric data from 5 separate experiments. GAPDH served as loading control. Data were expressed as normalized ratio of protein band density of Tau or p-Tau against GAPDH and were presented as mean ± standard deviation. (b) The effect of propofol on p-Tau in untreated hippocampal neurons. (c) The effect of propofol on neuron viability. Data were expressed as the percentage of absorbance of treated neurons compared with untreated control neurons.

TNF-α-modulated PKA phosphorylation (Figure 4(b)), it ameliorated GSK3β phosphorylation and increased PP2A phosphorylation (Figures 4(a) and 4(c)). In addition, compared with control neurons, 25 μM propofol alone did not affect NLRP3 expression, pro-caspase-1 cleavage, or matured IL-lβ/IL-18 release, while 100 μM propofol marked reduced NLRP3 expression and pro-caspase-1 cleavage (Figures 3(a) and 3(b)). In consistence, 25 μM propofol alone had no significant effect on the phosphorylation of GSK3β, PKA, or PP2A; however, 100 μM propofol decreased GSK3β phosphorylation and increased PP2A phosphorylation (Figure 4).

Furthermore, it has been suggested that abnormal mitophagy and resultant ROS dysregulation serve as an important mediator for NLRP3 inflammasome activation [22–24]. So, we examined the effect of TNF-α and propofol on mitophagy and ROS balance. As shown in Figure 5, we show that TNF-α (20 ng/mL, 4 h) inhibited the extent of mitophagy (Figure 5(a)), disrupted MMP values (Figure 5(b)), and induced intracellular ROS (Figure 5(c)) as well as mitochondrial ROS (Figure 5(d)). And all these effects were ameliorated by 25 μM propofol pretreatment (Figure 5). Please note that 25 μM propofol alone did not affect mitophagy or ROS. While interestingly, we discovered that compared with control neurons, 100 μM propofol had no effect on mitophagy (Figures 5(a) and 5(b)), but it reduced intracellular ROS (Figure 5(c)) and mitochondrial ROS (Figure 5(d)) to a lower extent than the basic levels.

To confirm the role of ROS, NLRP3 inflammasome, GSK3β, and PP2A in modulating p-Tau accumulation, we pretreated neurons with specific inhibitors or activators. As shown in Figure 6(a), TNF-α-induced p-Tau accumulation is attenuated by 40 μM ebselen (a ROS scavenger), 1 μM YQ128 (a NLRP3 inhibitor), 10 μM SB216763 (a GSK3β inhibitor), and 1 μM PP2A activator. In addition, we revealed that 40 μM ebselen inhibited TNF-α-induced activation of NLRP3 inflammasome (Figure 3). It also ameliorated TNF-α-mediated phosphorylation of GSK3β (Figure 4(a)) and PP2A (Figure 4(c)). Consistently, 1 μM YQ128 reduced GSK3β phosphorylation (Figure 4(a)), while induced PP2A phosphorylation (Figure 4(c)). Summarized from above data,

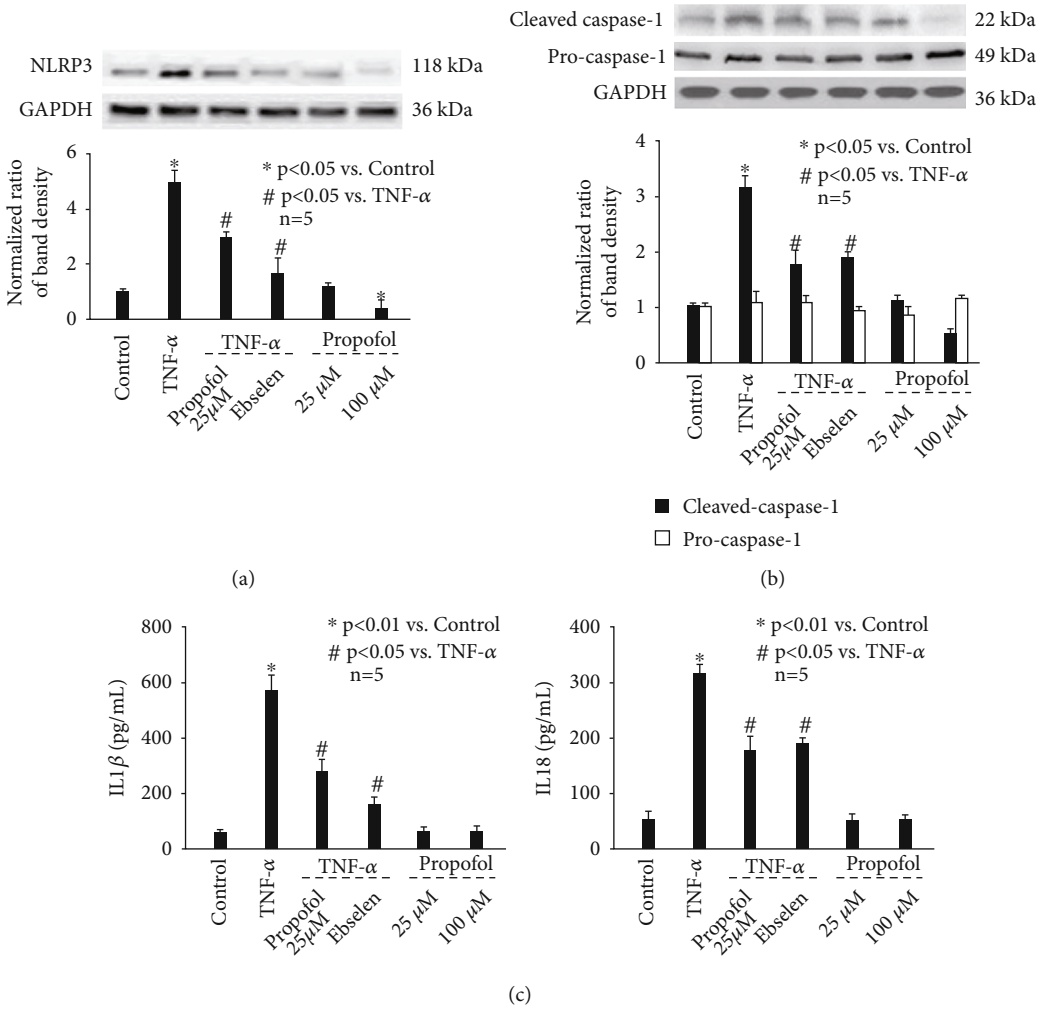


Figure 3: The effect of TNF-α and propofol on NLRP3 inflammasome activation. (a) The effect of TNF-α and propofol on NLRP3 protein expression. The upper panel was a representative experiment, and the lower panel was the summary of densitometric data from 5 separate experiments. GAPDH served as loading control. Data were expressed as normalized ratio of protein band density of NLRP3 against GAPDH and were presented as mean ± standard deviation. (b) The effect of TNF-α and propofol on pro-caspase-1 cleavage. (c) The effect of TNF-α and propofol on the release of matured IL-lβ (left) and IL-18 (right). Data were expressed as mean ± standard deviation, and pg/mL served as unit.

we believed ROS functioned upstream of NLRP3 inflammasome, which modulates GSK3β and PP2A phosphorylation, leading to p-Tau accumulation.

3.4. The Effect and Mechanism of Propofol on Antioxidant Enzyme Expression. As shown in Figure 2(b), we report that compared with untreated neurons, 100 μM propofol reduced basic levels of p-Tau. In addition, 100 μM propofol reduced ROS (Figures 5(c) and 5(d)) without affecting the extent of mitophagy (Figures 5(a) and 5(b)). The underlying mechanism of how ROS was reduced was of great interest. It is known that cellular ROS homeostasis is modulated by their synthesis and their scavenging through the antioxidant machinery with SOD, HO-1, and NQO1 acting as major antioxidants in CNS [25]. We found that 25 μM propofol alone had no effect on the expression of SOD and HO-1, which was markedly induced by 100 μM propofol (Figure 7(a) left and 7a middle). In contrast, 25 μM and 100 μM propofol had no effect on NQO1 expression (Figure 7(a) right).

Previous findings implied p62/Keap1/Nrf2 pathway as a key mechanism for SOD and HO-1 expression [26, 27]. Our data demonstrated that 25 μM propofol did not affect the expression of p62 (Figure 7(b) left) or Keap1 (Figure 7(b) middle), and did not affect the nuclear translocation of Nrf2 (Figure 7(b) right). However, 100 μM propofol induced p62 (Figure 7(b) left), reduced Keap1 expression (Figure 7(b) middle), and triggered the nuclear translocation of Nrf2 (Figure 7(b) right). More importantly, we found that 100 μM propofol-induced SOD and HO-1 expression was mitigated by blocking p62 expression through p62 siRNA, by enhancing Keap1 expression through Keap1 overexpression, and by 10 μM ML385 (Nrf2 inhibitor) treatment (Figure 7(c)), suggesting the critical role of p62, Keap1 and Nrf2. Consistently, 100 μM propofol-modulated ROS and p-Tau accumulation was abolished by p62 knockdown,

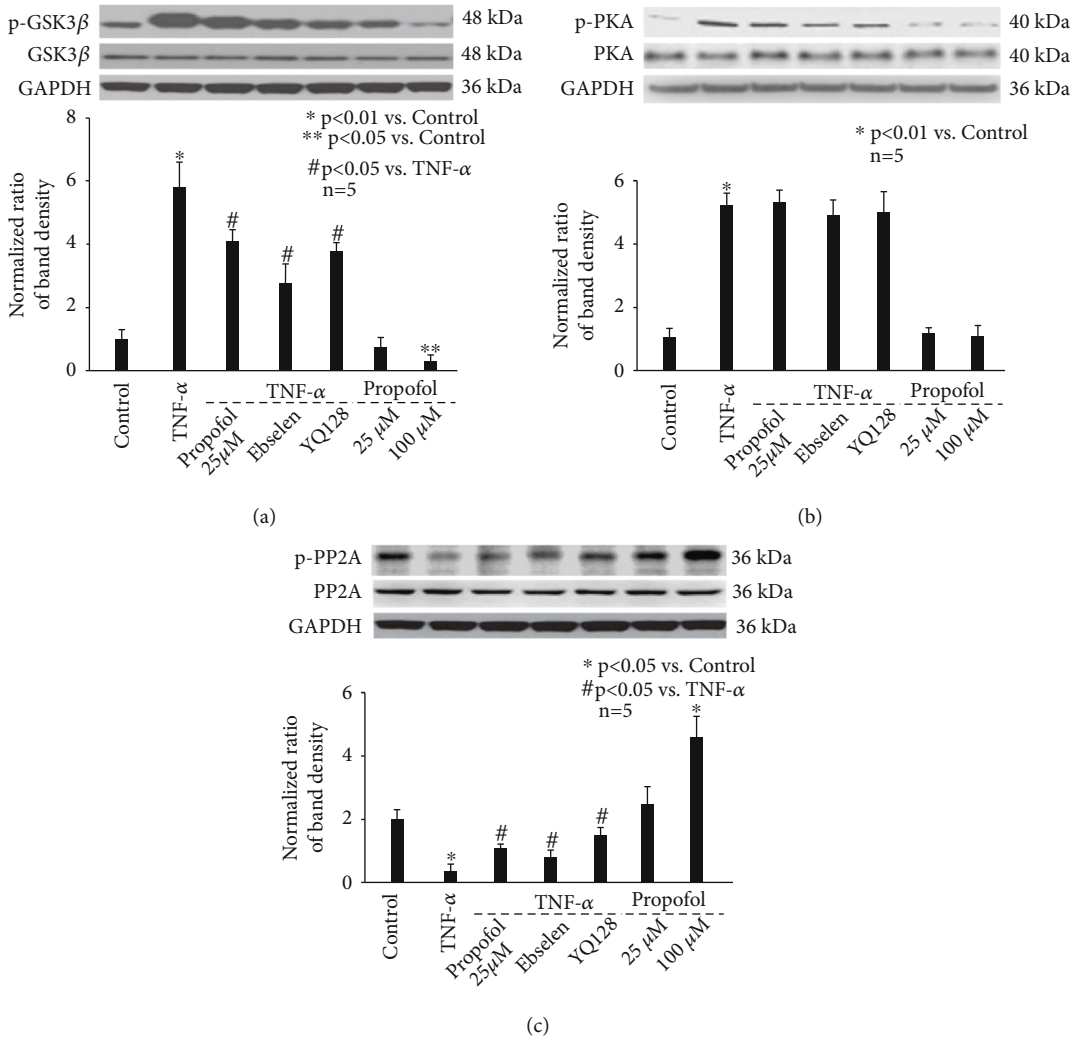


Figure 4: The effect of TNF-α and propofol on kinase/phosphatase phosphorylation. (a) The effect of TNF-α and propofol on the expression and phosphorylation of GSK3β. The upper panel was a representative experiment and the lower panel was the summary of densitometric data from 5 separate experiments. GAPDH served as loading control. Data were expressed as normalized ratio of protein band density of phosphorylated GSK3β against GSK3β, which was normalized with GAPDH, and were presented as mean ± standard deviation. (b) The effect of TNF-α and propofol on the expression and phosphorylation of PKA. (c) The effect of TNF-α and propofol on the expression and phosphorylation of PP2A.

Keap1 overexpression, and ML385 treatment (Figures 5(c) and 5(d) and 6(b)). The efficiency of p62 knockdown and Keap1 overexpression was demonstrated by immunostaining (Figure 7(d)).

4. Discussion

In the present study, we investigated the effect and mechanism of inflammation mediator TNF-α and anesthetic agent propofol on p-Tau accumulation in mouse hippocampal neurons. Our data implied that TNF-α may induce p-Tau accumulation via inhibiting mitophagy, inducing ROS, which modulated NLRP3 and GSK3β/PP2A activity. More meaningfully, we proved that propofol may inhibit p-Tau accumulation through modulating mitophagy, ROS, and related events, and through enhancing SOD and HO-1 expression via p62/Keap1/Nrf2 signal pathway.

Tau is a microtubule-associated protein that is predominantly expressed in the brain. In healthy neurons, Tau is almost exclusively located in the axon and is closely associated with the proper functioning of the cytoskeletal network in terms of microtubule assembly. Under normal conditions, Tau contributes to maintain neuronal functions such as transport of mRNA and proteins along the axons, microtubule stabilization, actin reorganization, and synaptic activity as well as neurite extension. In contrast, Tau pathology may cause neurofibrillary tangles and neuronal dysfunction, which are closely correlated with neurodegenerative disorders [5, 28]. Besides protein expression level, posttranslational modifications of Tau, such as phosphorylation, nitration, ubiquitination, truncation, glycosylation, and isomerization, are proved to influence its function [28]. During the past decades, increasing evidence indicated that abnormally phosphorylated Tau plays a critical role during

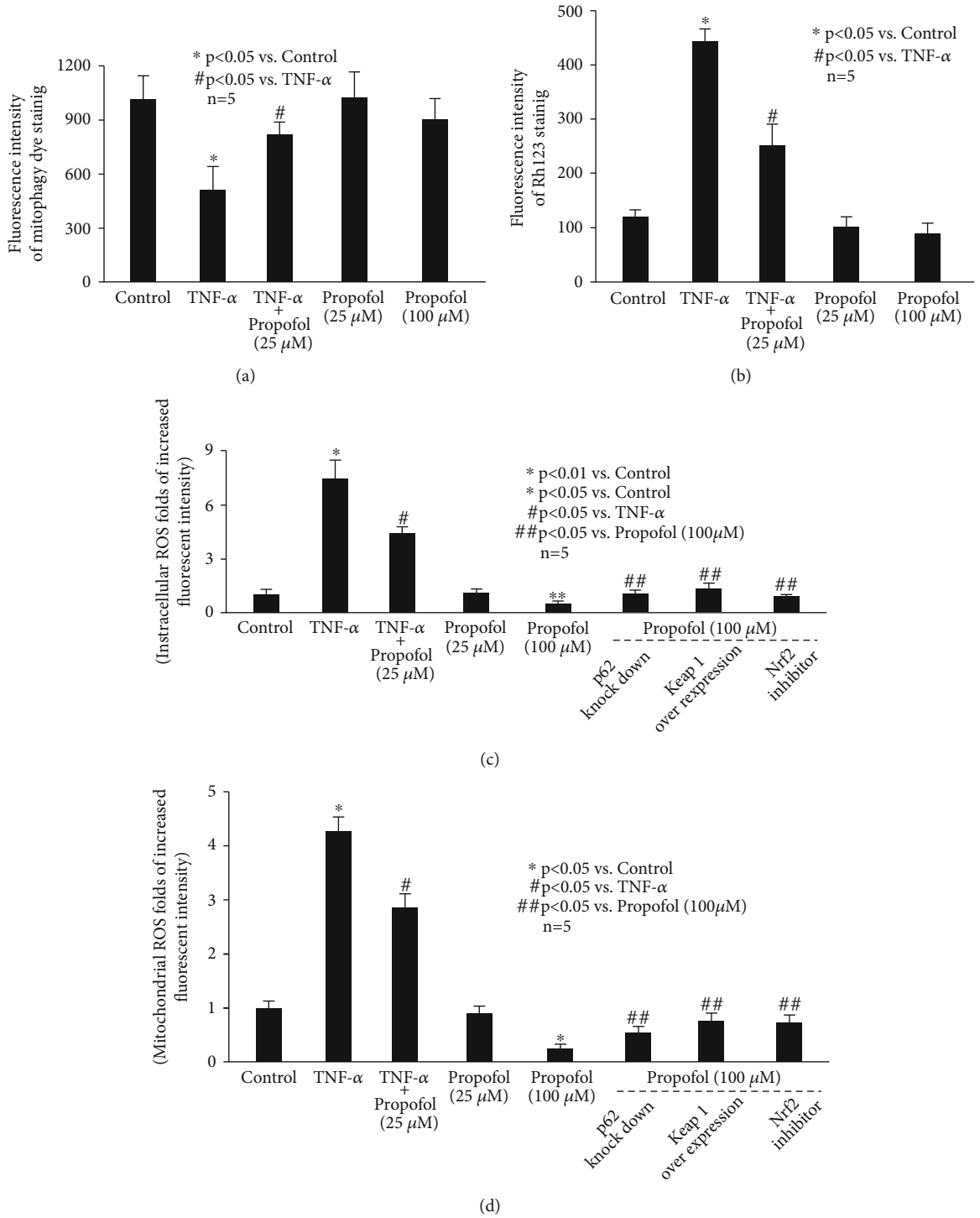


Figure 5: The effect of TNF-α and propofol on mitophagy and ROS. (a) The effect of TNF-α and propofol on the extent of mitophagy. Data were expressed as mean ± standard deviation of fluorescence intensity of mitophagy dye staining. (b) The effect of TNF-α and propofol on MMP values. Data were expressed as mean ± standard deviation of fluorescence intensity of Rh123 staining. (c) The effect of TNF-α and propofol on intracellular ROS. p62 siRNA, Keap1 overexpression plasmid, and Nrf2 inhibitor were used to modulate p62/Keap1/Nrf2 pathway. The data were recorded as folds of increased fluorescence intensity in treated neurons compared with that of untreated neurons. (d) The effect of TNF-α and propofol on mitochondrial ROS. Data were recorded as folds of increased fluorescence intensity in treated neurons compared with that of untreated neurons.

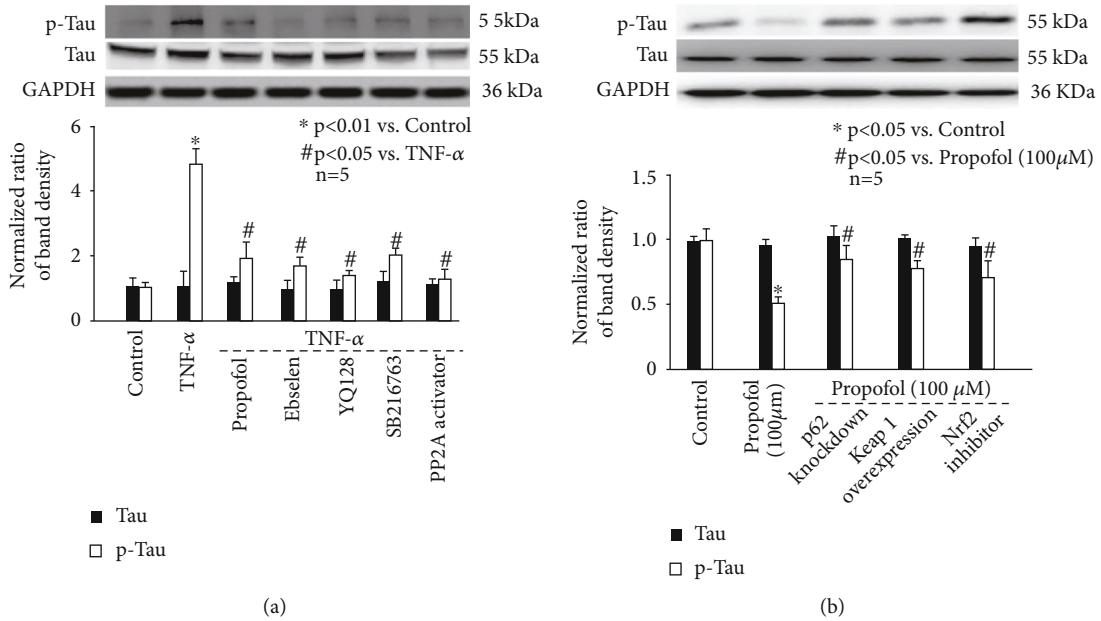


Figure 6: The effect of specific signal modulators on p-Tau accumulation in hippocampal neurons. The upper panel was a representative experiment and the lower panel was the summary of densitometric data from 5 separate experiments. GAPDH served as loading control. Data were expressed as normalized ratio of protein band density of Tau or p-Tau against GAPDH and were presented as mean ± standard deviation. (a) The effect of TNF-α, propofol, ROS scavenger, NLRP3 inhibitor, GSK3β inhibitor, and PP2A activator. (b) The effect of propofol and p62/Keap1/Nrf2 pathway regulators: p62 siRNA, Keap1 overexpression plasmid, and Nrf2 inhibitor.

the development of AD in animal studies and clinical trials [9, 21, 28]. Although systematic inflammation and neuroinflammation are widely accepted as a central process to the pathogenesis of neurodegenerative disorders [1–3], detailed mechanism is far from clear. Here, in the present, *in vitro* study, we treated mouse hippocampal neurons with inflammation mediator TNF-α to mimic *in vivo* neuroinflammation status and proved that TNF-α may induce the accumulation of p-Tau in neurons (Figure 1(a)). Our data at least provide a potential linkage between inflammation and neuron dysfunction, and this needs to be further studied in animal models. In addition, we reported that TNF-α-induced accumulation of p-Tau was attenuated by propofol pretreatment (Figure 2(a)), and one of the astonishing findings of our study is that propofol, within clinically achieved concentrations, may reduce the basic level of p-Tau (Figure 2(b)). Although whether propofol exerts beneficial or detrimental effects to CNS in the clinical practice is debatable [29–31], a large body of *in vitro* evidence from us and other researchers proved the anti-inflammation and neuro-protective property of propofol [16–19]. The current findings implied a novel research field to the neuroprotective effect of propofol and more importantly provided a novel target for the protection of neurons and neurodegenerative disorders against inflammation.

The phosphorylation status of Tau relies on the balance between kinases (including but not limited to glycogen synthase kinase, cyclin-dependent kinase, mitogen-activated protein kinase, and microtubule affinity regulating kinase) that phosphorylate it and one major phosphatase (PP2A) that dephosphorylates it [32]. It is noted that different kinases are responsible for the hyperphosphorylation of Tau protein in response to various stimuli. For example, ischemia/reperfusion injury induced Tau phosphorylation through cyclin-dependent kinase 5 (CDK5) [33]; sleep disturbances-associated Tau phosphorylation was mediated via p38 mitogen-activated protein kinase (p38MAPK) [34]; virus infection enhanced Tau phosphorylation by double-stranded RNA-dependent protein kinase [35]; sepsis triggered Tau phosphorylation through GSK-3β [36]; and metal dysregulation activated rapamycin/ribosomal S6 protein kinase and thus caused Tau phosphorylation [37]. In a previous study, streptozotocin was intracranially injected into the rats to induce neuroinflammation, and it was revealed that plasma TNF-α release was increased and p-Tau was induced by GSK-3β in the hippocampus area [38]. Consistently, a recent animal study carried out in mice demonstrated chronic systemic exposure to lipopolysaccharide caused neuroinflammation by promoting TNF-α release and triggered Tau hyperphosphorylation through activating GSK-3β [39]. In addition to GSK-3β, the role of protein kinase A (PKA) in hippocampus Tau phosphorylation and neuroinflammation-related cognitive deficits has been confirmed in animal AD models [40, 41]. Since we focused on inflammation-induced Tau phosphorylation in the current study, we only examined GSK-3β and PKA and proved that both were activated by TNF-α (Figure 4). However, our data implied that propofol only modulated GSK-3β activation (Figure 4). In addition, we examined phosphatase PP2A and showed that TNF-α inhibited PP2A activity, which was induced by propofol (Figure 4). In consistence, we showed that TNF-α-induced p-Tau accumulation was attenuated by propofol, GSK-3β inhibitor, and PP2A activator (Figure 6). Taken together, we inferred that the effect of

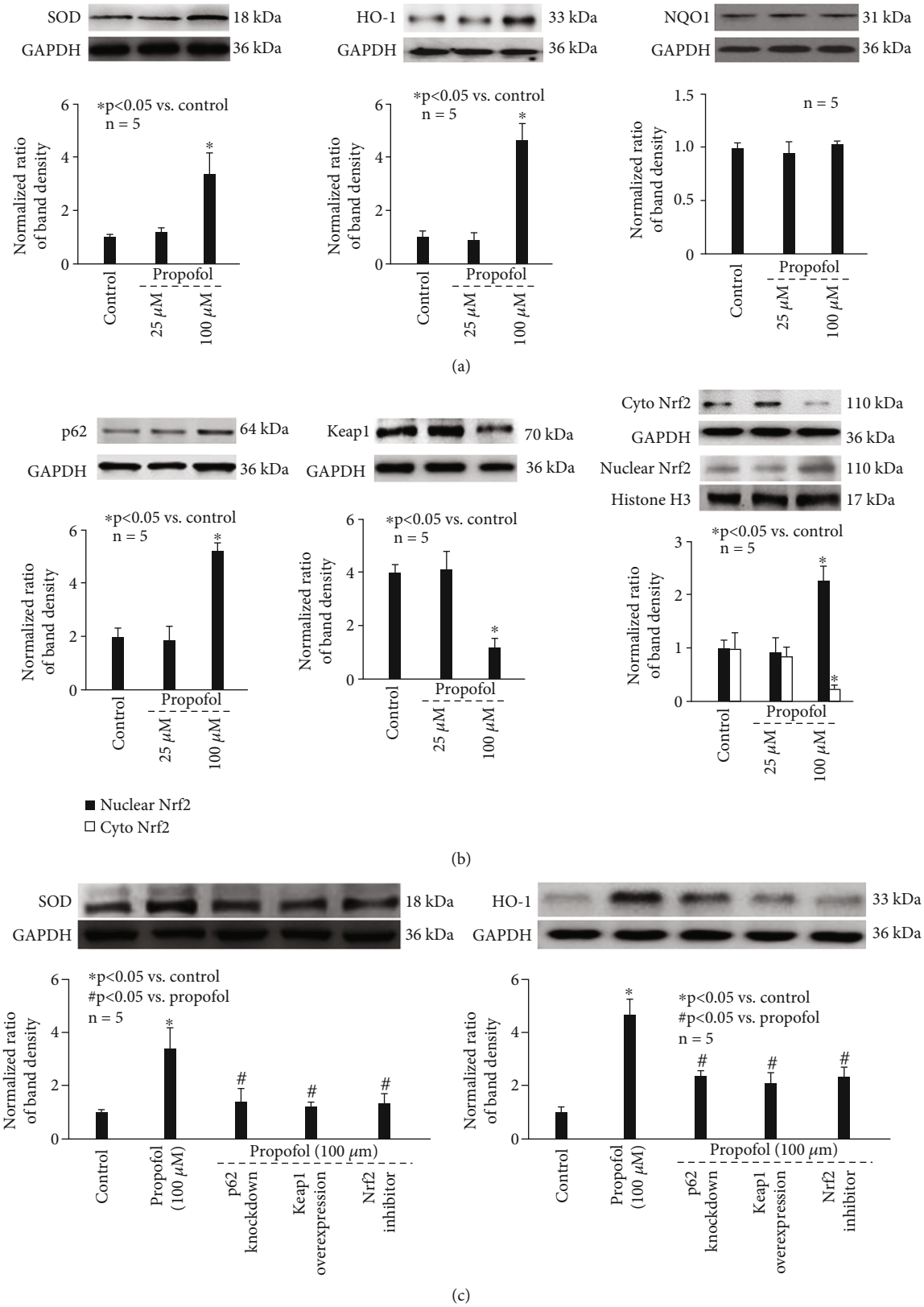
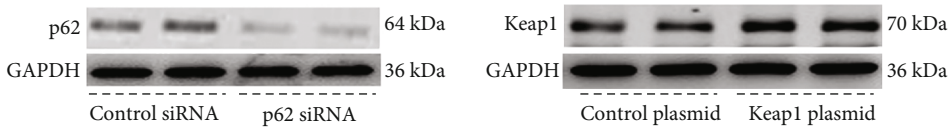


Figure 7: Continued.



(d)

Figure 7: The effect and mechanism of propofol on antioxidant enzyme expression. (a) Left: SOD; middle: HO-1; and right: NQO1. The upper panel was a representative experiment, and the lower panel was the summary of densitometric data from 5 separate experiments. GAPDH served as loading control. Data were expressed as normalized ratio of protein band density of SOD, HO-1, or NQO1 against GAPDH and were presented as mean ± standard deviation. (b) Left: p62; middle: Keap1; and right: Nrf2. The upper panel was a representative experiment, and the lower panel was the summary of densitometric data from 5 separate experiments. GAPDH served as loading control for p62, Keap1, and cytosolic Nrf2. Histone H3 served as loading control for nuclear Nrf2. Data were expressed as normalized ratio of protein band density of p62, Keap1, or Nrf2 against loading control and were presented as mean ± standard deviation. (c) The effect of p62/Keap1/Nrf2 pathway regulators on antioxidant enzyme expression. Left: SOD; middle: HO-1; and right: NQO1. The upper panel was a representative experiment, and the lower panel was the summary of densitometric data from 5 separate experiments. GAPDH served as loading control. (d) The siRNA and plasmid transfection efficiency. Left: a representative immunostaining of duplicate p62 siRNA and duplicate scramble siRNA transfection. Right: a representative immunostaining of duplicate Keap1 plasmid and duplicate control plasmid transfection.

propofol on p-Tau accumulation was mediated through modulating kinase (GSK-3β) and phosphatase (PP2A) simultaneously.

Inflammasome is a type of cytosolic multiprotein complex and plays a crucial role in innate immunity. Among reported inflammasomes, NLRP3 inflammasome is the most studied. More and more experimental evidence showed that the activation of NLRP3 inflammasome is closely related to neurodegenerative diseases [22]. Recently, the role of NLRP3 inflammasome in tauopathy-induced neurodegeneration attracts extensive attention [24]. It was published that tauopathy (Tau hyperphosphorylation) and neurodegeneration (hippocampal atrophy) were decreased in the NLRP3-deficient mice compared with wild type mice, implying its critical role [42]. However, how NLRP3 inflammasome modulates Tau phosphorylation is far from clear. It was shown that GSK-3β activity was reduced, while PP2A activity was increased in NLRP3 knockout mice [24]. This study also revealed that the kinase activity of CDK5 and p38MAPK remained unchanged in NLRP3 knockout mice [24]. Consistently, it was recognized that GSK-3β and PP2A were subject to NLRP3 inflammasome activation and were responsible for regulating Tau protein phosphorylation in neuronal cells [43]. Combined with our findings that propofol inhibited TNF-α-modulated NLRP3 inflammasome activation and GSK-3β/PP2A activity (Figure 4) as well as Tau phosphorylation (Figure 6), we believed that the beneficial effects of propofol on p-Tau accumulation were via inhibiting NLRP3 inflammasome-mediated GSK-3β/PP2A activity.

NLRP3 inflammasome activation generally requires two steps: priming and protein complex assembly. Priming is triggered by pattern recognition receptor signals, leading to transcriptional activation of NLRP3 inflammasome components. Protein complex assembly is correlated with NLRP3 inflammasome activation, leading to inflammatory response through caspase-1 activation and inflammatory cytokine IL-1β maturation and secretion. Although a variety of external or host-derived stimuli, such as mitochondrial dysfunction, ion flux, and lysosomal damage are involved in the activation of NLRP3 inflammasome [43], recent studies focused on mitochondrial autophagy and subsequent oxidative stress [44–46]. It was reported that in intracerebral hemorrhage brain injury model, ROS was elevated, and NLRP3 inflammasome pathway was activated [47]. It also reported that ROS scavenger may inhibit NLRP3 inflammatory response and alleviate brain injury [47]. Another study proved during ischemia/reperfusion injury, mitochondria malfunction caused ROS accumulation and stimulated NLRP3 inflammasome activation [48]. In addition, it was shown that increased ROS, which is duo to impaired mitophagy, contributed to NLRP3 inflammasome signaling activation in neurodegenerative diseases [49, 50]. Here in this study, our data also suggested a correlation between mitophagy, ROS, and NLRP3 inflammasome activation after TNF-α treatment (Figures 3 and 5). Based on the findings that propofol and ROS scavenger could reduce intracellular ROS and NLRP3 inflammasome activation (Figures 3 and 5), we concluded that ROS was indispensable for NLRP3 inflammasome activation.

In general, we deduced from this *in vitro* study that TNF-α impaired neuron mitophagy, caused excessive oxidative stress, which activated NLRP3 inflammasome, resulting in dysregulation of GSK-3β/PP2A activity and advanced p-Tau accumulation. Further, we believed that propofol may decrease p-Tau accumulation via enhancing mitophagy, reducing oxidative stress and subsequent events. Nevertheless, we discovered an interesting phenomenon that relative high concentration of propofol (100 μM) reduced basic level of p-Tau (Figure 2(a)). It also modulated GSK-3β/PP2A phosphorylation (Figure 4), NLRP3 inflammasome activity (Figure 3), and ROS (Figures 5(c) and 5(d)). However, 100 μM propofol had no significant effect on mitophagy (Figures 5(a) and 5(b)). The potential mechanisms for reduced ROS in this scenario deserve investigations.

It is recognized that the cellular ROS homeostasis is modulated by their synthesis, mainly through NADPH oxidase complex, and their scavenging through the antioxidant machinery with glutathione and ascorbate acting as major

antioxidants. Among multiple antioxidant enzymes, SOD, HO-1, and NQO1 were extensively studied in hippocampal neurons and neurological disorders [25, 51–54]. SOD is a group of metal-containing enzymes that catalyze the dismutation of superoxide radicals to molecular oxygen and hydrogen peroxide, providing cellular defense against reactive oxygen species. It was reported that sinomenine may improve hippocampal and cognitive dysfunction through modulating SOD activity and ROS, while it was also suggested that catalase, glutathione reductase, glutathione peroxidase, and myeloperoxidase were not involved [55]. HO-1 is a cytoprotective enzyme that catalyzes the degradation of heme to carbon monoxide, iron, and biliverdin, and its induction has been regarded as an adaptive cellular response against inflammatory response and oxidative injury. It was reported that increased expression of HO-1 was correlated with less intracellular ROS and improved neuronal and neurological function in mice [56]. NQO1 is a cytosolic enzyme which catalyzes the reduction of quinones and a wide variety of other compounds. It is often upregulated in response to cellular stress, and it has a role in minimizing free radical load within cells. Previous study showed that increased NQO-1 activation was correlated with less ROS and improved cell viability in hippocampal neuronal cells [57]. Animal study also proved that upregulated NQO-1 expression was correlated with reduced oxidative stress and improved neurological status in rats following traumatic brain injury [58]. Here in the present study, we demonstrated that SOD and HO-1 expression were induced by 100 μM propofol; however, we did not detect modulation of NQO-1 (Figure 7).

Speaking of the molecular mechanisms for antioxidant enzyme activation in CNS, plenty of data pointed to p62/Keap1/Nrf2 pathway [51, 56, 58]. It is believed that p62 aggregation leads to Keap1 degradation by autophagosomes. Under normal conditions, Keap1 functions as an adapter protein of the Cul3-ubiquitin E3 ligase complex responsible for degrading Nrf2. Accordingly, increased p62 leaves Nrf2 free to transfer to the nucleus and binds to antioxidant-responsive elements in the promoter of antioxidant enzymes. We believed that p62/Keap1/Nrf2 pathway was responsible for propofol-modulated SOD/HO-1 expression and p-Tau accumulation, since p62 knockdown/Keap1 overexpression/Nrf2 inhibitor almost completely blocked the beneficial effects of propofol (Figure 7(c) and 6(b)).

We realized there are several issues that are unsolved in this study and deserve further investigations. Firstly, it is known that neuronal TNF-α receptor (TNFR), such as TNFR-I and TNFR-II perform fundamentally different roles in CNS pathology [59], while we did not examine which receptor is responsible for TNF-α-induced p-Tau accumulation in hippocampal neurons. Also, we did not investigate whether propofol affects the expression and activation of specific TNFR. The answer to these questions may reveal a novel therapeutic target for tauopathy. Secondly, in this study, in order to investigate the protective effects of propofol, its exposure was 1 h ahead of TNF-α. While we did not know whether propofol could reverse the accumulation of p-Tau when the neurons have already been exposed to TNF-α for a while, this needs to be revealed, and the results may provide solid evidence for the beneficial property of propofol in those patients who have been suffered from neuroinflammation.

5. Conclusions

In conclusion, we testified that TNF-α may induce p-Tau accumulation via inhibiting mitophagy, inducing ROS, which modulated NLRP3 and GSK3β/PP2A activity. We also proposed that propofol may inhibit p-Tau accumulation through modulating mitophagy, ROS, and resultant events and through enhancing SOD and HO-1 expression via p62/Keap1/Nrf2 pathway.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

Authors' Contributions

Lin Zhang performed experiments and wrote the manuscript; Hong Song performed experiments and wrote the manuscript; Jie Ding performed experiments and wrote the manuscript; and Dong-jie Wang performed cell culture and statistical analysis. Shi-peng Zhu performed experiments. Chi Liu designed and supervised the research; Xian Jin designed and supervised the research; Jia-wei Chen designed research and revised as well as approved the manuscript. Lin Zhang, Hong Song, and Jie Ding are equal contributors as the first author; Chi Liu, Xian Jin, and Jia-wei Chen are equal contributors as the corresponding author.

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Research Article

Chronic Cerebral Hypoperfusion Aggravates Parkinson's Disease Dementia-Like Symptoms and Pathology in 6-OHDA-Lesioned Rat through Interfering with Sphingolipid Metabolism

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Chronic cerebral hypoperfusion (CCH) is a cardinal risk factor for Parkinson's disease dementia (PDD), but this potential causality lacks mechanistic evidence. We selected bilateral common carotid artery occlusion (BCCAO) to simulate chronic cerebral hypoperfusion in the rat model of PD induced by typical neurotoxin 6-hydroxy dopamine (6-OHDA). Four weeks after unilateral injection of 6-OHDA into the medial forebrain bundle, rats underwent BCCAO. Male Sprague-Dawley rats were divided into five groups of ten, including sham, PD+BCCAO 2 weeks, PD+BCCAO 1 week, PD, and BCCAO 2 weeks. Then, open field test (OFT) and *Morris* water maze test (MWM) were used to assess the PDD-like symptoms in rats. Also, the pathological manifestations and mechanisms of BCCAO impairing cognitive functions have been explored via hematoxylin-eosin staining, Nissl staining, immunohistochemistry, immunofluorescence, RNA sequencing analysis, lipidomics, and quantitative real-time polymerase chain reaction. In this study, we found that CCH could aggravate PDD-like cognitive symptoms (i.e., learning memory and spatial cognition) and PDD-like pathology (higher expression of α-Syn and Aβ in prefrontal cortex and striatum). Moreover, a potential relationship between differentially expressed mRNAs and lipid metabolism was revealed by RNA sequencing analysis. Lipidomics showed that CCH could affect the intensity of 5 lipids, including sphingomyelin (SM 9:0;2O/26:2; SM 8:1;2O/25:0; and SM 8:0;2O/28:4), cardiolipin, lysophosphatidylcholine, cholesteryl ester, and triacylglycerol. Interestingly, the KEGG pathway analysis of both RNA sequencing analysis and lipidomics suggested that CCH leaded to learning impairment by affecting sphingolipid metabolism. Finally, we found that CCH disrupts the sphingolipid metabolism by affecting the mRNA expression of SMPD1 and SMS2, leading to the accumulation of sphingomyelin in the prefrontal cortex. In summary, CCH, an independent exacerbating reason for impairment in learning and memory within the pathopoiesis of PD, aggravates Parkinson's disease dementia-like symptoms and pathology in 6-OHDA-lesioned rat through interfering with sphingolipid metabolism.

1. Introduction

Cognitive impairment is one of the most prominent and disabling nonmotor symptoms of Parkinson's disease (PD) [1]. At present, in PD patients with a course of 10 years or more, the cumulative prevalence of Parkinson's disease dementia (PDD) is 75-90% [2]. With increasing life expectancy of PD patients, PDD is set to become even more prevalent in the future. It would seriously affect the patient's quality of life. The etiology of PDD involves genetics, neurophysiology,

neuropathology, neuroimaging, environment, vascular factors, and other aspects of research [3]. On the one hand, a growing number of studies showed that synergistic effects of alpha-synuclein (α-Syn) and beta-amyloid (Aβ) are closely associated with PDD severity [4, 5]. α-Syn inclusion formation could impair cognitive behaviors associated with functions of prefrontal cortex [6]. On the other hand, brain magnetic resonance imaging (MRI) studies showed white matter hyperintensities and a postural drop in blood pressure among PD patients, which revealed recurrent episodic hypotension results in cerebral hypoperfusion (CH), in turn causing anoxic damage to vulnerable areas of the brain and impaired cognitive function [7, 8]. Several studies found that chronic cerebral hypoperfusion (CCH) independently exacerbated cognitive impairment in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine- (MPTP-) lesioned mice via impairing microvascular in the hippocampus and white matter [9, 10]. Despite the efforts to improve the knowledge pertaining the pathological bases of PDD, the contribution of CCH is still unclear. Therefore, in-depth study of the mechanism of CCH in the occurrence and development of PDD will help to improve the pathological understanding of PDD and promote the development of new treatment methods for PDD.

Bilateral common carotid artery occlusion (BCCAO) is a common method to induce CCH in rat [11, 12]. Therefore, we selected BCCAO to simulate chronic cerebral hypoperfusion in the rat model of PD induced by typical neurotoxin 6-hydroxy dopamine (6-OHDA) [3, 13, 14]. Then, we explored the autonomic motor and cognitive functions of those rats through open field test (OFT) and *Morris* water maze test (MWM). Furthermore, the pathological manifestations and mechanisms of BCCAO impairing cognitive functions have been explored via immunohistochemistry (IHC), immunofluorescence (IF), ribonucleic acid (RNA) sequencing analysis, lipidomics, and quantitative real-time polymerase chain reaction (qRT-PCR). Our results would provide new ideas for the study of mechanism of CCH enhancing PDD-like symptoms and pathology.

2. Materials and Methods

2.1. Animal. Male Sprague-Dawley rats (200-220 g) were purchased from Experimental Animal Center of Guangzhou University of Chinese Medicine (Guangzhou, China) and maintained in cages under standard conditions with a 12 h light/dark cycle with free access to food and water. All experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals and were approved by the Ethics Committee for Animal Research of Guangzhou University of Chinese Medicine (No. 20210318006). For all experiments, rats were acclimated to their surroundings for 3 days in the animal room prior to surgery, and they had free access to food and water. Then, rats were divided into five groups of ten. Body weight was measured once a week. The intervention methods of rats in each group are shown in Figure 1.

2.2. Unilateral Injection of 6-Hydroxy Dopamine into the Medial Forebrain Bundle (MFB). The rat model of PD was established by unilateral injection of 6-hydroxy dopamine (6-OHDA) into MFB as previously described with minor modifications [15]. Rats were anaesthetized with isoflurane and treated with carprofen as an analgesic. Also, rats are prone to death due to hypothermia during anesthesia. Thus, surgery was carried on a heating pad to prevent hypothermia. Rat skulls were exposed and then drilling a hole in the right side of the MFB (2.2 mm posterior to the anterior fonticulus, 1.5 mm on the right side of the center line, and 8.0 mm subdural). Subsequently, 4 μL of 6-OHDA (MedChemExpress, USA) solution (5 μg/μL; dissolved in saline containing 2 mg/mL ascorbic acid) was injected into the right MFB at a rate of 1 μL/min. Rats injected with the same volume of saline containing 2 mg/mL ascorbic acid were used as the sham group. Apomorphine (Wako, Japan) solution (0.05 mg/kg, dissolved in saline containing 0.02% ascorbic acid) was subcutaneously used to induce the spontaneous rotational movement in rat 4 weeks after model establishment and before sacrificing. The number of rotations contralateral to the lesion during 30 min after apomorphine injection was evaluated [16].

2.3. Bilateral Common Carotid Artery Occlusion Surgery. Rats were anaesthetized with isoflurane and treated with carprofen as an analgesic. Also, surgery was carried on a heating pad to prevent hypothermia. BCCAO was performed as described in the literature [17]. A midline ventral cervical incision was made to expose both common carotid arteries. The carotid arteries were isolated from the carotid sheath and vagus nerve, and carotid arteries were ligated with a 5/0 silk suture. Rats in the sham group were operated in the same surgical procedure except that the arteries were not ligated.

2.4. Behavioral Analysis. The autonomic motor of rats was assessed by OFT, and spatial memory capability of rats was assessed by MWM test as previously reported [18, 19]. The rats were transferred to the behavioral test room (soundproof dark room) before behavioral test. OFT was performed from 19:00 to 24:00. The open field is a black box ($100 \times 100 \times 40$ cm), and for data analysis, the total area was equally divided into 25 equal squares virtually. Each rat was gently placed in the center of the field and observed for 5 min. Between trials, the floor of the box was wiped with 50% ethanol to remove the scent marks. The total distance (mm) and average velocity (mm/s) were recorded and analyzed. MWM was performed in the experimental apparatus consisted of a circular tank (150 cm in diameter and 50 cm in depth) divided into four quadrants and filled with water dyed with black food colorant ($24 \pm 1^{\circ}$C). A circular platform (12 cm in diameter and 30 cm in depth) was submerged about 1 cm below the water surface in the southwest quadrant. Space navigation test was conducted during days 1-5 with 4 trials per day, and one trial each day was from each of the four positions selected randomly. The interval between trials was 10 min. The rats were given 60 s to locate the hidden platform and allowed to rest on the platform for 10 s. The space exploration test was allowed to swim freely for 60 s. Escape latency (s) in the space navigation test, number of crossing platform, the time during platform quadrant (s), and average speed (mm/s) in space

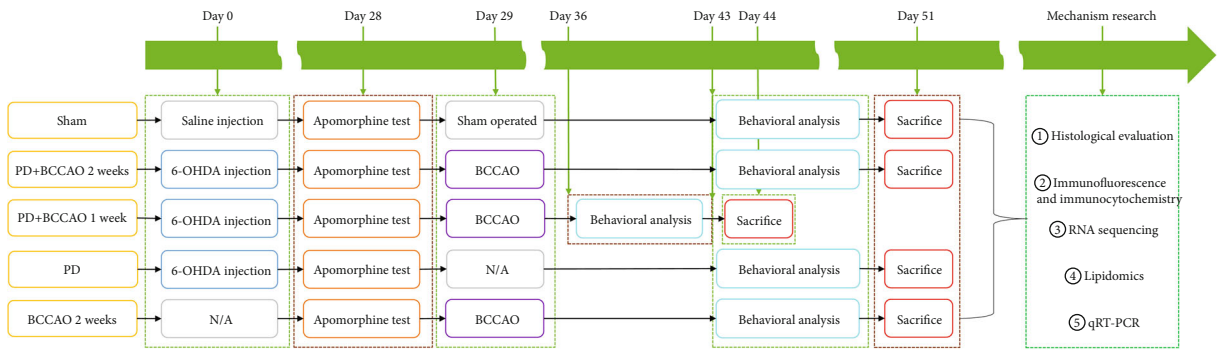


Figure 1: The timeline of the experiment. Abbreviations: PD: Parkinson's disease; BCCAO: bilateral common carotid artery occlusion; 6-OHDA: 6-hydroxy dopamine; N/A: no operation; RNA: ribonucleic acid; qRT-PCR: quantitative real-time polymerase chain reaction.

exploration test were recorded and analyzed. All behavioral results were analyzed by video-tracking system (Flydy Co., Ltd., Guangzhou, China).

2.5. Histological Evaluation. The animal used for histological evaluation were perfused, firstly with 0.9% saline and then with cold 10% paraformaldehyde. The brains were removed, washed, and protected in the 10% paraformaldehyde. Formalin-fixed brains were dehydrated in a series of graded ethanol and xylene baths and embedded in paraffin. Brains were cut into 6 μM thick coronal sections using a RM2255 microtome (Leica, Switzerland), mounted on glass slides. Distribution of neural cells in the rat prefrontal cortex and striatum was assessed by microscopy following standard hematoxylin-eosin (HE) staining (Servicebio, China). Nissl bodies in rat brain tissues were detected by a previously described Nissl staining method (Solarbio, China) [18].

2.6. Immunofluorescence. After dewaxing and rehydrating and antigen retrieval, the paraffin sections were blocked endogenous peroxidase activity by citric buffer (Pondus Hydrogenii (PH) 6.0). Brain sections were blocked with normal goat serum for 1 h at room temperature. The primary antibody was diluted in phosphate buffer saline (PBS) (PH 7.4) in a certain proportion (tyrosine hydroxylase (TH), Proteintech, 1 : 200) and is added to the sections, and the sections are placed flat in a wet box and incubated overnight at 4°C. Following three washes with PBS, rat brain sections were incubated in the dark with green fluorophore-conjugated secondary antibodies for 1.5 h at room temperature. After counterstaining with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI), sections were mounted and observed by fluorescence microscopy.

2.7. Immunocytochemistry. The steps for dewaxing and rehydrating, antigen retrieval, blocking endogenous peroxidase activity, and serum sealing are similar to immunofluorescence. The primary antibody was diluted in PBS (PH 7.4) in a certain proportion (α-Syn, Abcam, 1 : 16000; Aβ, Abcam, 1 : 200) and is added to the sections, and the sections are placed flat in a wet box and incubated overnight at 4°C. After the sections are slightly shaken and dried, the tissues are covered with secondary antibody (horseradish peroxidase labeled) from the corresponding species of primary antibody and incubated at room temperature for 50 minutes. 3,3-N-diaminobenzidine tertrahydrochloride (DAB) color developing solution newly prepared is added in the circle after the sections are slightly dried. After counterstaining nucleus, the brain sections were dehydrated and mounted. Then, sections were observed by microscopy.

2.8. RNA Sequencing and Bioinformatics. Differential expressions of messenger RNA (mRNA) in prefrontal cortex of the sham group and PD+BCCAO 2 weeks were characterized by RNA sequencing (RNA-Seq). Total RNA was prepared from prefrontal cortex using TRIzol reagent (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. The concentrations of RNA were assessed by NanoDrop 2000 spectrophotometer (Thermo Scientific, USA), and the quality of RNA was analyzed calculating RNA integrity number with Agilent Bioanalyzer 2100 (Agilent Technologies). Transcriptome sequencing was performed on RNA samples that passed the quality control indicators (2100 RNA integrity number (RIN) $\geq$ 7.0 and 28S/18S > 1.0). Subsequently, the RNA-Seq libraries were constructed and sequenced on the DNBSEQ platform (Beijing Genomics Institution, China). In order to display the number of genes in different expression intervals of each sample more intuitively, we carried out statistics on the number of genes in three cases of expression amount (expression $\leq$ 1, expression = 1 − 10, and expression $\geq$ 10). Following adapter trimming and other processing, we used HISAT (http://ccb.jhu.edu/software/hisat/index.shtml) and Bowtie2 (http://bowtie-bio.sourceforge.net/bowtie2/index.shtml) to align clean reads to the reference genome sequence (NCBI, GCF_000001895.5_Rnor_6.0) to get the alignment results. Differential expressions of mRNAs between the two groups were defined as the combination of a log2 Ratio (PD +BCCAO 2 weeks/PD) value of $\geq$1 and a P value $\leq$ 0.05 based on reads per kilobase million using DESeq2 software. Hierarchical clustering and scatter plots of differentially expressed mRNAs were constructed using R language. Enrichment of Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways for

differentiation expressed mRNAs was performed using the Database for Annotation, Visualization, and Integrated Discovery system (DAVID, https://david.ncifcrf.gov/). The GO and KEGG pathways with P values $(P) < 0.05$ were considered significant.

2.9. Sample Preparation and Ultraperformance Liquid Chromatography-High-Definition Mass Spectrometry (UPLC-HDMS) Analysis. The collected samples in the prefrontal cortex were thawed on ice, and metabolites were extracted with 50% methanol buffer. Pooled quality control (QC) samples were also prepared by combining 10 μL of each extraction mixture. All samples were acquired by the LC-MS system following machine orders. Firstly, all chromatographic separations were performed using a Thermo Scientific UltiMate 3000 HPLC (Thermo Scientific, USA). An ACQUITY UPLC BEH C18 column (100 mm $*$ 2.1 mm , 1.8 μm, Waters, UK) was used for the reversed phase separation. The column oven was maintained at 35°C. The flow rate was 0.4 mL/min, and the mobile phase consisted of solvent A (water, 0.1% formic acid) and solvent B (acetonitrile, 0.1% formic acid). Gradient elution conditions were set as follows: 0~0.5 min, 5% B; 0.5~7 min, 5% to 100% B; 7~8 min, 100% B; 8~8.1 min, 100% to 5% B; and 8.1~10 min, 5%B. The injection volume for each sample was 4 μL. A high-resolution tandem mass spectrometer Q-Exactive (Thermo Scientific, USA) was used to detect metabolites eluted from the column. The Q-Exactive was operated in both positive and negative ion modes. Precursor spectra (70–1050 m/z) were collected at 70,000 resolution to hit an AGC target of 3e6. The maximum inject time was set to 100 ms. A top 3 configuration to acquire data was set in DDA mode. Fragment spectra were collected at 17,500 resolution to hit an AGC target of 1e5 with a maximum inject time of 80 ms. In order to evaluate the stability of the LC-MS during the whole acquisition, a quality control sample (pool of all samples) was acquired after every 10 samples. MetaX software was used to quantify and screen the differential metabolites. Those features that were detected in less than 50% of QC samples or 80% of biological samples were removed, the remaining peaks with missing values were imputed with the k-nearest neighbor algorithm to further improve the data quality. PCA was performed for outlier detection and batch effect evaluation using the preprocessed dataset. Quality control-based robust LOESS signal correction was fitted to the QC data with respect to the order of injection to minimize signal intensity drift over time. In addition, the relative standard deviations of the metabolic features were calculated across all QC samples, and those >30% were then removed.

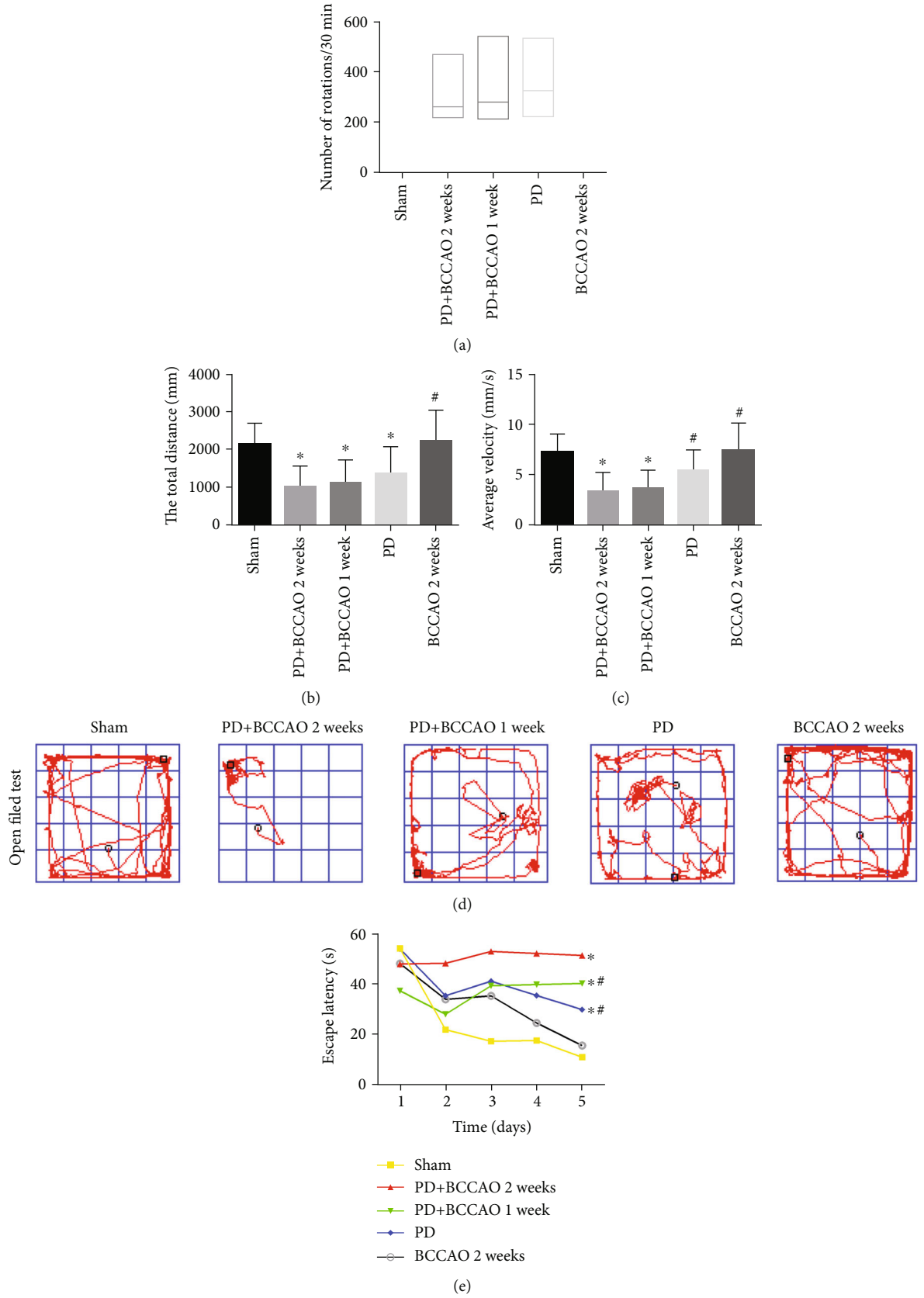
2.10. Pattern Recognition Analysis and Data Processing. The raw data of mass spectrometry was converted into readable data mzXML using MSConvert software. Peak extraction was performed using XCMS software, and peak extraction quality control was performed. The extracted substances were annotated by summation ions using CAMERA and then identified by MetaX software. The MS primary information was used for identification, and the MS secondary information was matched to an in-house standard database, respectively. Metabolite annotation was performed on candidate identification substances using databases such as HMDB (https://hmdb.ca/) and KEGG, to explain the physicochemical properties and biological functions of metabolites. Student's t-tests were conducted to detect differences in metabolite concentrations between 2 groups. The P value was adjusted for multiple tests using an false discovery rate (Benjamini–Hochberg). Supervised PLS-DA was conducted through MetaX to discriminate the different variables between groups. The variable important in the projection (VIP) value was calculated. Differential metabolic ions between the two groups were defined as the combination of a ratio ≥ 1.5 or $\leq 1/1.5$, a P value ≤ 0.05, and VIP ≥ 1. Enrichment of KEGG pathways for differentiation lipids was performed using the DAVID.

Table 1: A list of primers used for qRT-PCR assay.

Primer ID	Primer sequences (5′-3′)
β-Actin-F	AAGATCCTGACCGAGCGTGG
β-Actin-R	CACAGGATTCCATACCCAGGAAG
SMPD1-F	ATGAGGAAACTCTGAGCCGC
SMPD1-R	GGTAAACTCGGTAGCCAGGA
SMPD2-F	CCTACGTGACCCATCTGCAC
SMPD2-R	TCCTCGGTCTCAACAAAGGC
SMS2-F	CAGTGTGCTCCAAAGCTCAA
SMS2-R	TCGACCGTGTAGTGTTCGTG

2.11. Quantitative Real-Time Polymerase Chain Reaction. Total RNA samples were extracted from prefrontal cortex using TRIzol according the manufacturer's instructions. Concentrations and quality of total RNA samples were measured using a NanoDrop 2000 spectrophotometer. Complementary deoxyribonucleic acid (cDNA) was synthesized from 500 ng of RNA from each group by reverse transcription using a RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA). In CFX connect Real-Time System (Bio-Rad, USA), a GoTaq qPCR Master Mix kit (Promega, USA) was used for quantitate expression levels by quantitative PCR. Relative expressions of mRNAs were calculated using the standard $2^{-\Delta\Delta Ct}$ method for at least three biological replicates [18]. Also, β-actin levels are used for calibration. Primer sequences are listed in Table 1.

2.12. Statistical Analysis. Statistical tests were carried out using SPSS 26.0 software. Mauchly's test of sphericity was used to analyze repeated measurement data. Data considered to conform to a normal distribution (P value of the Shapiro-Wilk test > 0.05) were presented as the mean ± standard deviation and analyzed by one-way analysis of variance (ANOVA). Data that did not conform to a normal distribution were presented as median (mix-max) and analyzed by nonparametric tests. $P < 0.05$ was considered significant. GraphPad Prism 6.0 software was used to draw images.



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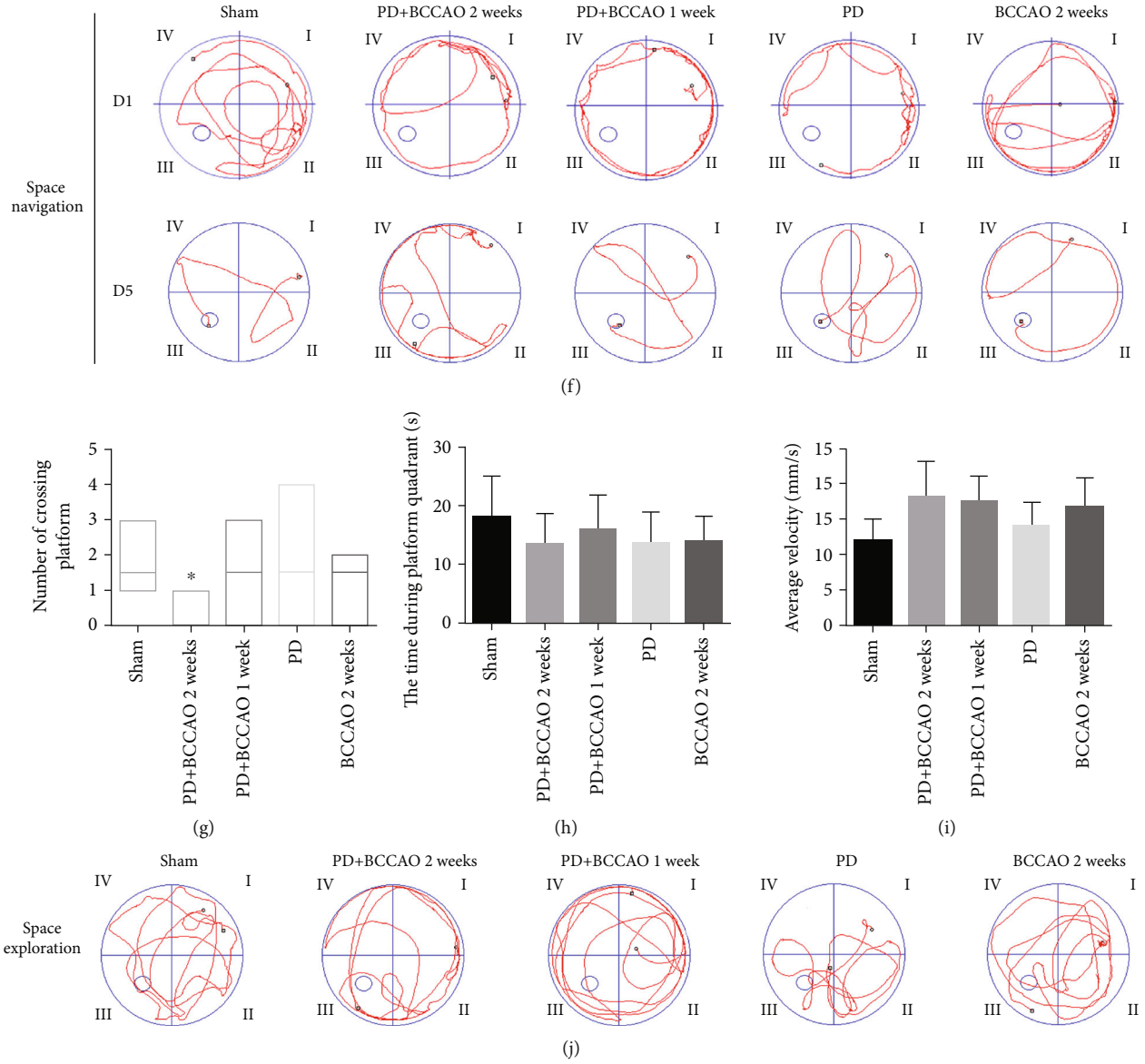


FIGURE 2: Behavioral performance in apomorphine-induced rotation tests, OFT, and MWM ($n = 10$). (a) The number of rotations of each group in apomorphine-induced rotation tests. (b) The total distance of each group in OFT test. (c) Average velocity of each group in OFT test. (d) Representative movement trajectories of rats in the OFT. (e) Escape latency of each group in space navigation of MWM. (f) Representative movement trajectories of rats on the first and fifth days of space navigation. (g) Number of crossing platform of each group in space exploration of MWM. (h) The time during platform quadrant of each group in space exploration of MWM. (i) Average speed of each group in space exploration of MWM. (j) Representative movement trajectories of rats in space exploration of MWM. $^{*}P < 0.05$ compared with the sham group and $^{\#}P < 0.05$ compared to the BCCAO 2 weeks group.

3. Results

3.1. CCH Aggravated PDD-Like Symptoms in 6-OHDA-Lesioned Rat. To validate the effect of CCH on the behavior of 6-OHDA-lesioned rats, we assessed the motor and cognitive functions of the rats in each group by apomorphine-induced rotation, OFT, and MWM (Figure 2). Firstly, apomorphine-induced rotation tests were used to characterize the dopamine depletion levels after unilateral 6-OHDA infusion in rats. The 6-OHDA-lesioned rats with contralateral rotations were divided into three groups: PD+BCCAO 2 weeks group, PD+BCCAO 1 week group, and PD group for the next step of the experiment (supplementary Figure 1A). Then, compared with PD group, we found that CCH could not aggravate the number of rotations induced by apomorphine in PD+BCCAO 2 weeks group and PD +BCCAO 1 week group ($P > 0.05$, Figure 2(a)). Interestingly, we found that there was no significant difference in the body weight of the rats in each group during the experimental period (supplementary Figure 1B).

In addition, compared with the sham group, OFT results demonstrated a significant decrease in total distance and

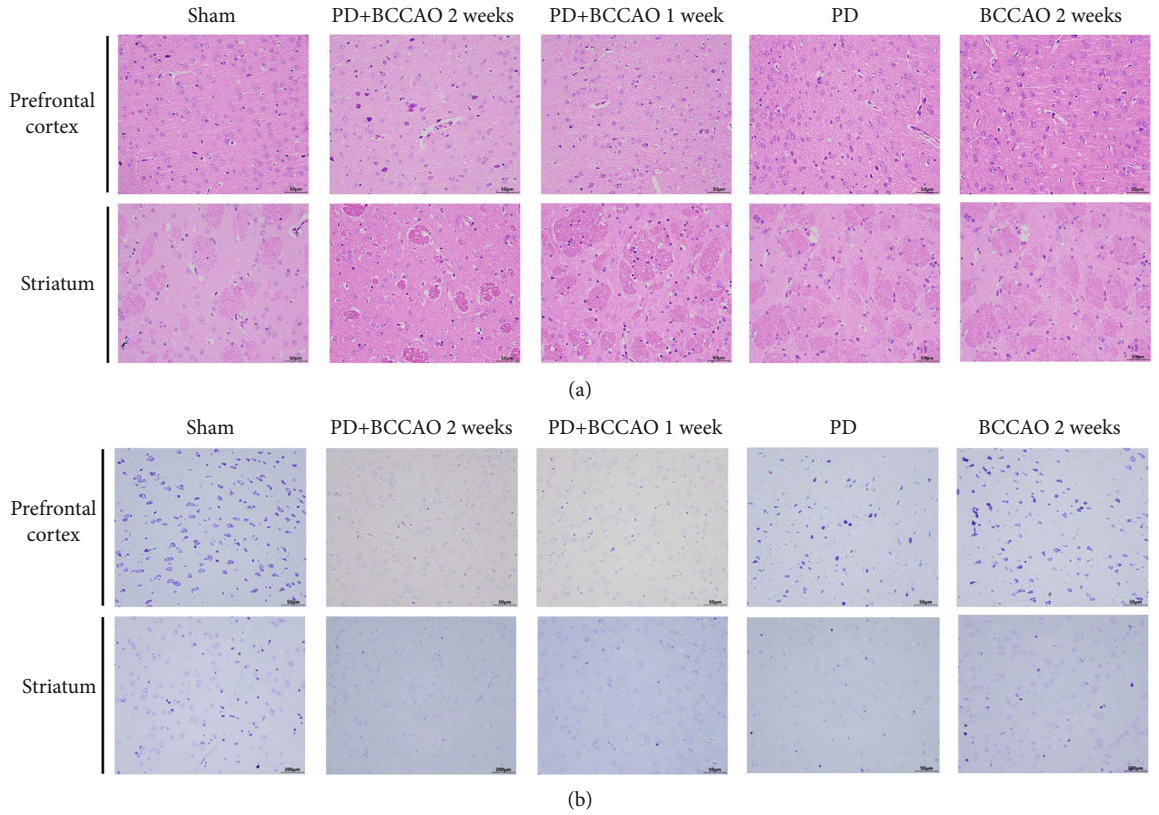


Figure 3: Hematoxylin and eosin (H&E) staining and Nissl staining sections from prefrontal cortex and striatum ($n = 3$). (a) Representative images of HE staining in prefrontal cortex and striatum of each group. In PD+BCCAO 2 weeks group, the interstitial space of the striatum is relatively loose, while the boundary of the striatal interstitial space is unclear, and many vacuoles can be seen throughout the striatum. The prefrontal cortex in PD+BCCAO 2 weeks group showed that a large number of neurons in the prefrontal cortex were shrunken, the staining of the cells was deepened, the boundary between the nucleus and cytoplasm was unclear, and the cells were loosely arranged. (b) Representative images of Nissl staining in prefrontal cortex and striatum of each group. Dark blue represents Nissl bodies. Nissl bodies are large and numerous, indicating that nerve cells have a strong function of synthesizing proteins; on the contrary, when nerve cells are damaged, the number of Nissl bodies will decrease or even disappear. The numbers of Nissl-stained neurons in prefrontal cortex and striatum were significantly decreased in the PD+BCCAO 2 weeks group. Scale bars represent 50 μm.

average velocity traveled by rats in the PD group, PD +BCCAO 2 weeks group, and PD+BCCAO 1 week group ($P < 0.05$, Figures 2(b)–2(d)). Interestingly, compared with the PD group, CCH caused the animal to exhibit a slower average velocity ($P < 0.05$, Figure 2(c)). The above results indicated that CCH could aggravate PDD-like motor symptom, especially voluntary motor velocity.

Cognitive functions, including learning-memory and spatial cognition were evaluated using the MWM [20]. In the space navigation tests, PD+BCCAO 2 weeks group showed a significantly increased in the time of escape latency, compared to the sham group, PD group, and PD+BCCAO 1 week group ($P < 0.05$, Figures 2(e) and 2(f)). Also, compared to the sham group, PD+BCCAO 2 weeks group showed a significantly decreased in the number of crossing platform in the space exploration test ($P < 0.05$, Figures 2(g) and 2(j)). But the time during platform quadrants and average speed in the space exploration test, there was no significant difference in the results of each group ($P > 0.05$, Figures 2(h)–2(j)). It manifested that the number of crossing platform in the space exploration test was not affected by the swimming speed of the animal in the water. In addition, the time of escape latency and the number of crossing platform reflect learning memory and spatial cognition, respectively. Thus, these results indicated that CCH could aggravate PDD-like cognitive symptoms, including learning memory and spatial cognition, especially learning memory.

3.2. CCH Damaged the Structure and Neuronal Cells in Prefrontal Cortex and Striatum of 6-OHDA-Lesioned Rat. Previous literature reported that learning memory is related to prefrontal cortex and spatial cognition is related to striatum [21–24]. To explore the effect of CCH on brain tissue, we further analyzed the morphological structures of prefrontal cortex and striatum. In PD+BCCAO 2 weeks group, the interstitial space of the striatum is relatively loose, while the boundary of the striatal interstitial space is unclear, and many vacuoles can be seen throughout the striatum (Figure 3(a)). Also, compared with the others groups, the prefrontal cortex in PD+BCCAO 2 weeks group showed that a large number of neurons in the prefrontal cortex were shrunken, the staining of the cells was deepened, the

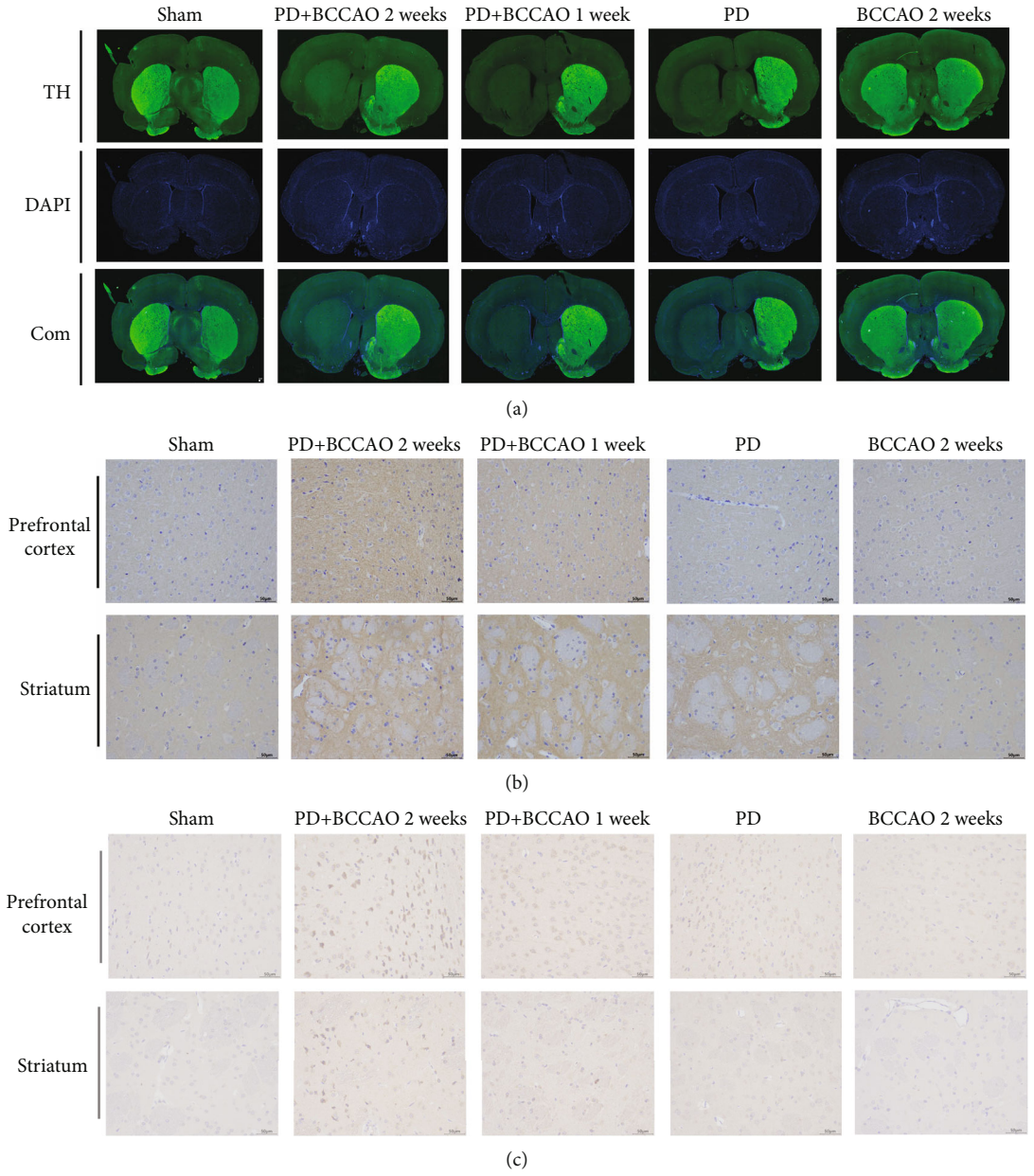
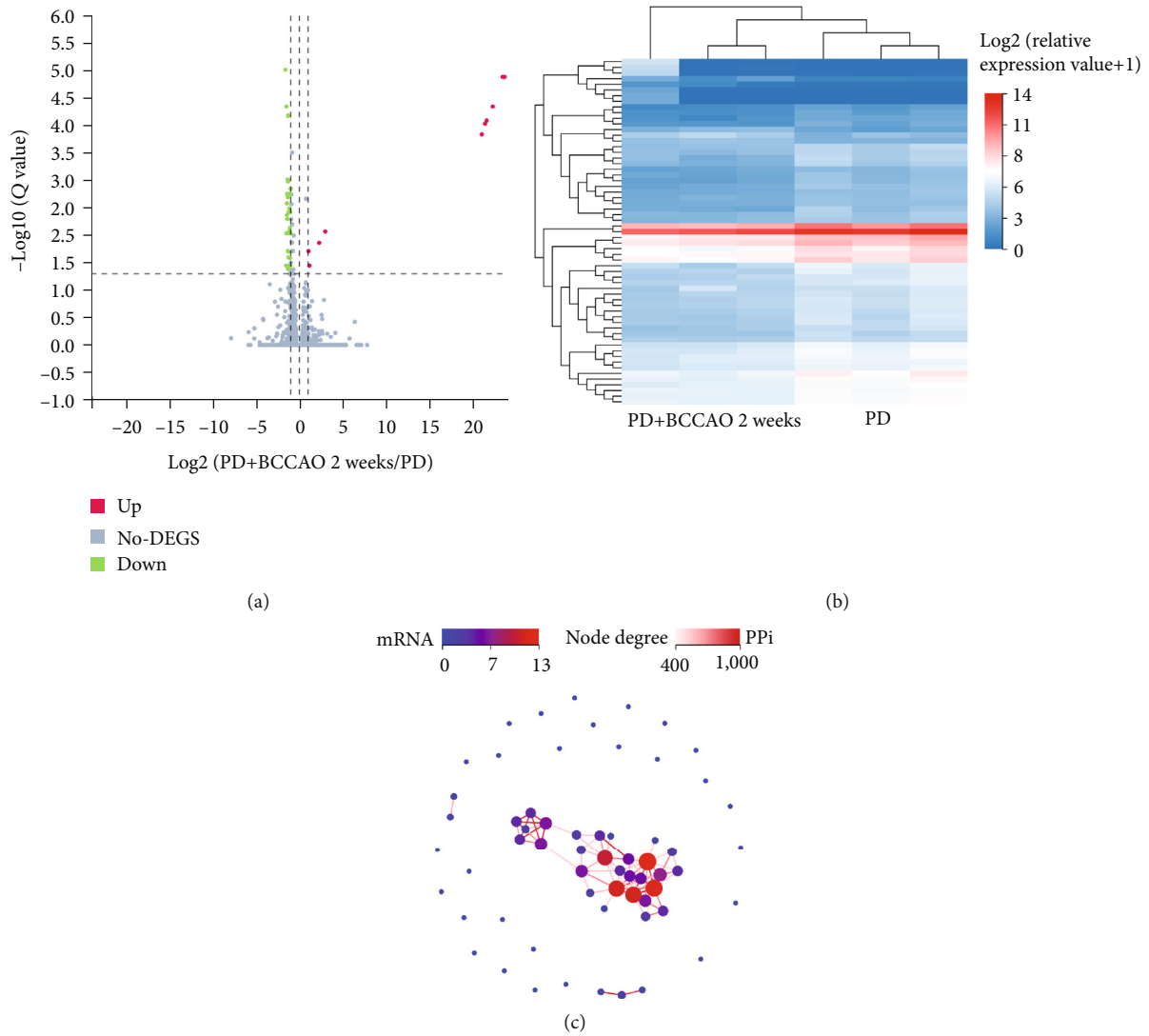


Figure 4: CCH aggravated PDD-like pathology in prefrontal cortex and striatum of 6-OHDA-lesioned rat ($n = 3$). (a) Representative images of TH expression in prefrontal cortex and striatum of each group. TH (green) and DAPI nuclear stain (blue). CCH could not aggravate the loss of TH-positive cells in striatum, compared to the PD group. (b) Representative images of α-Syn expression in prefrontal cortex and striatum of each group. α-Syn (brown) and cell nucleus (blue). CCH combined with 6-OHDA treatment could increase the expression of α-Syn in prefrontal cortex. (c) Representative images of Aβ expression in prefrontal cortex and striatum of each group. Aβ (brown) and cell nucleus (blue). The expression of Aβ in striatum and prefrontal cortex of PD+BCCAO 2 weeks was higher than that of other groups. Scale bars represent 50 μm.

boundary between the nucleus and cytoplasm was unclear, and the cells were loosely arranged (Figure 3(a)). In addition, numbers of Nissl-stained neurons in prefrontal cortex and striatum were significantly decreased in the PD+BCCAO 2 weeks group compared with the other groups (Figure 3(b)).

3.3. CCH Aggravated PDD-Like Pathology in Prefrontal Cortex and Striatum of 6-OHDA-Lesioned Rat. TH is a critical marker of dopaminergic neurons. Therefore, we explored the effect of CCH on dopaminergic neurons of striatum. As shown in Figure 4(a), compared with the sham group, 6-OHDA treatment led to decrease of TH-positive cells in the 6-OHDA-lesioned striatum. However, CCH could not aggravate the loss of TH-positive cells in striatum, compared to PD group.

In addition, α-Syn and Aβ are the major pathological markers of PDD. We found that 6-OHDA treatment would increase the expression of α-Syn in striatum, compared with



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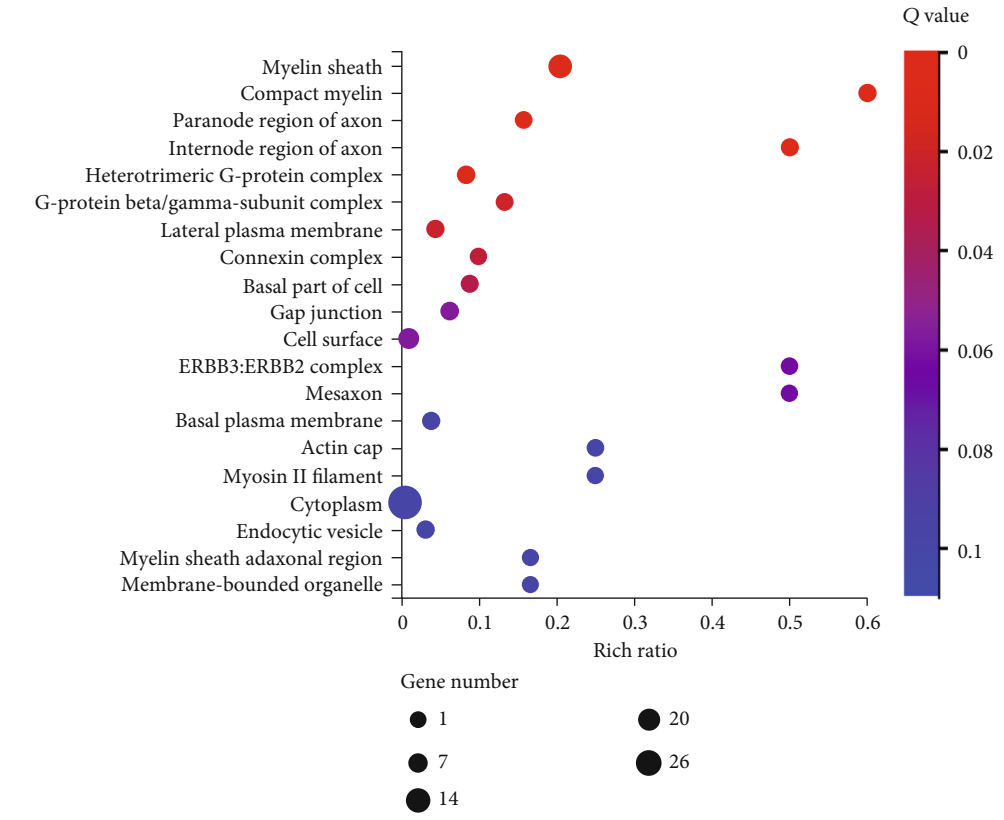
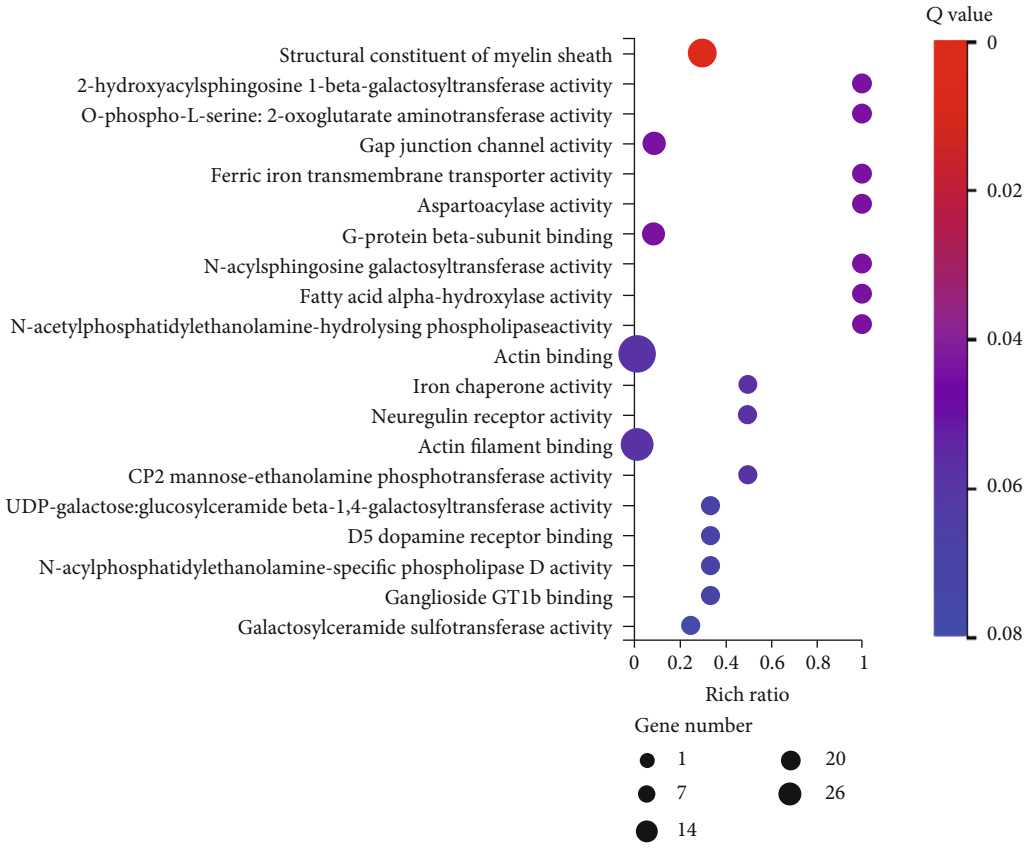


(d)



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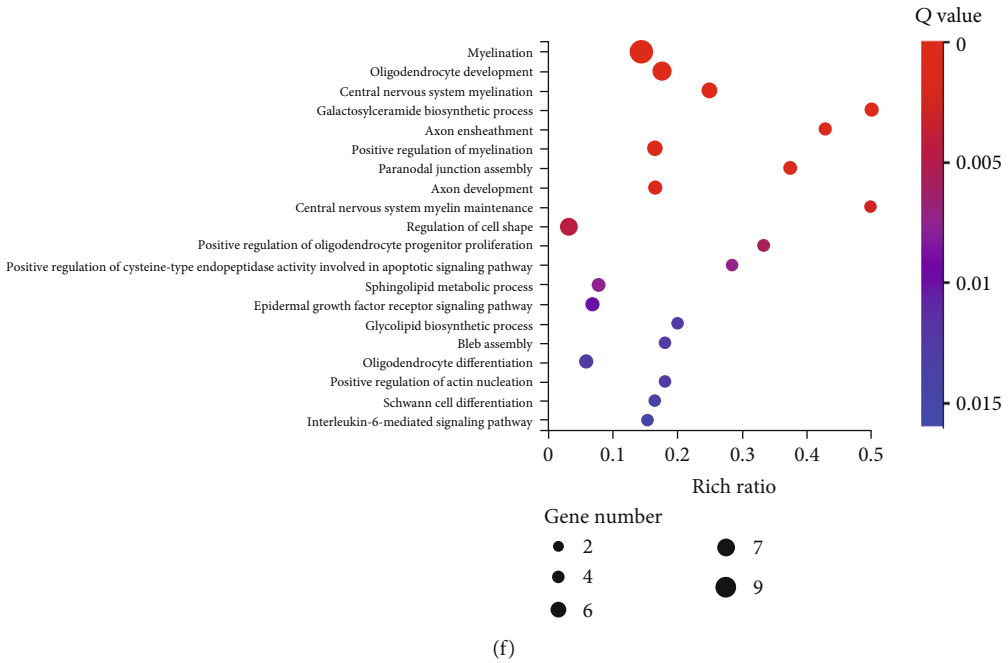

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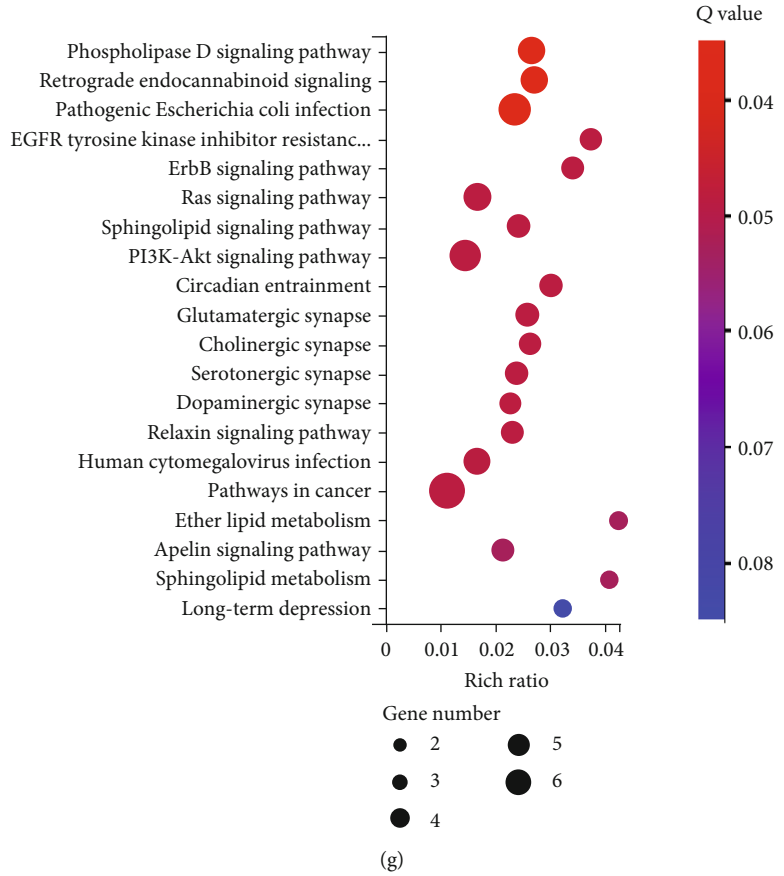
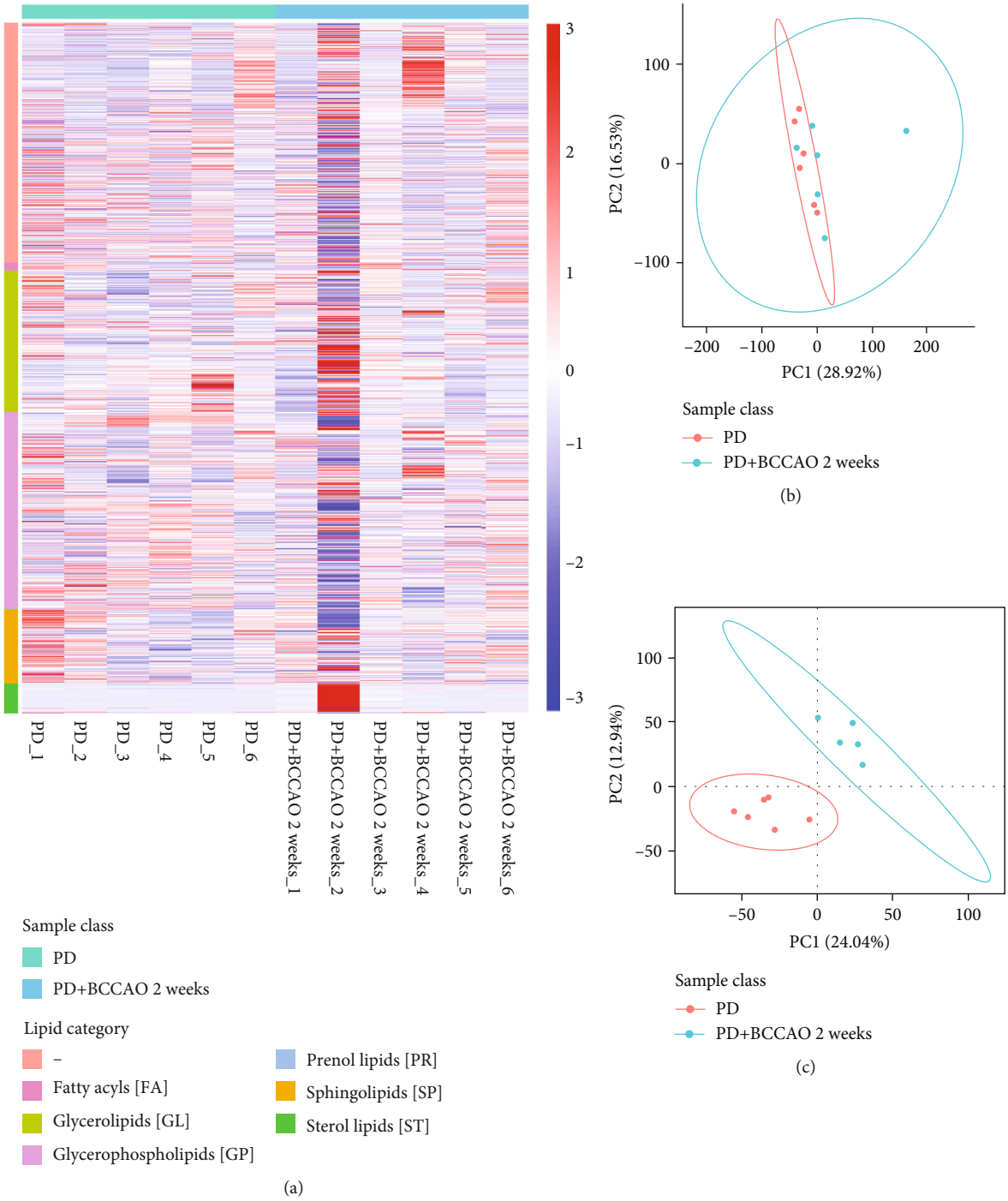


(g)

Figure 5: CCH modulated mRNA expression in prefrontal cortex of 6-OHDA-lesioned rat ($n = 3$). (a) Volcano map of differentially expressed mRNAs characterized by RNA sequencing. Compared with the PD group, green represents decrease in the PD+BCCAO 2 weeks group, and red represents increase. (b) Correlation heatmap of differentially expressed mRNAs. The color represents the expression level; the higher the expression level, the redder the color, otherwise the bluer. (c) PPI of differentially expressed mRNAs. (d) Enrichment of GO biological process of differentially expressed mRNAs. (e) Enrichment of GO cellular component of differentially expressed mRNAs. (f) Enrichment of GO molecular function of differentially expressed mRNAs. (g) Enrichment of KEGG pathways of differentially expressed mRNAs.



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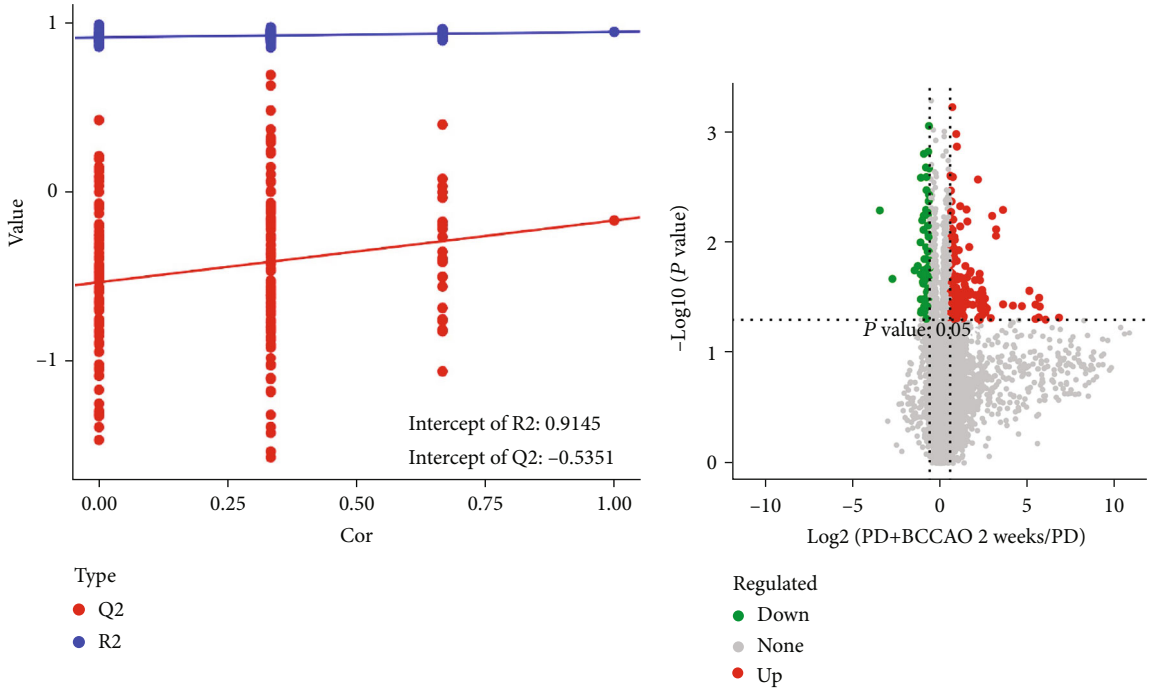

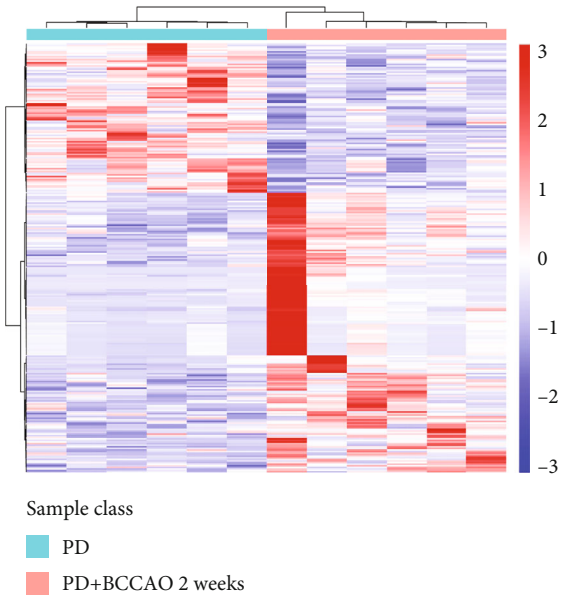

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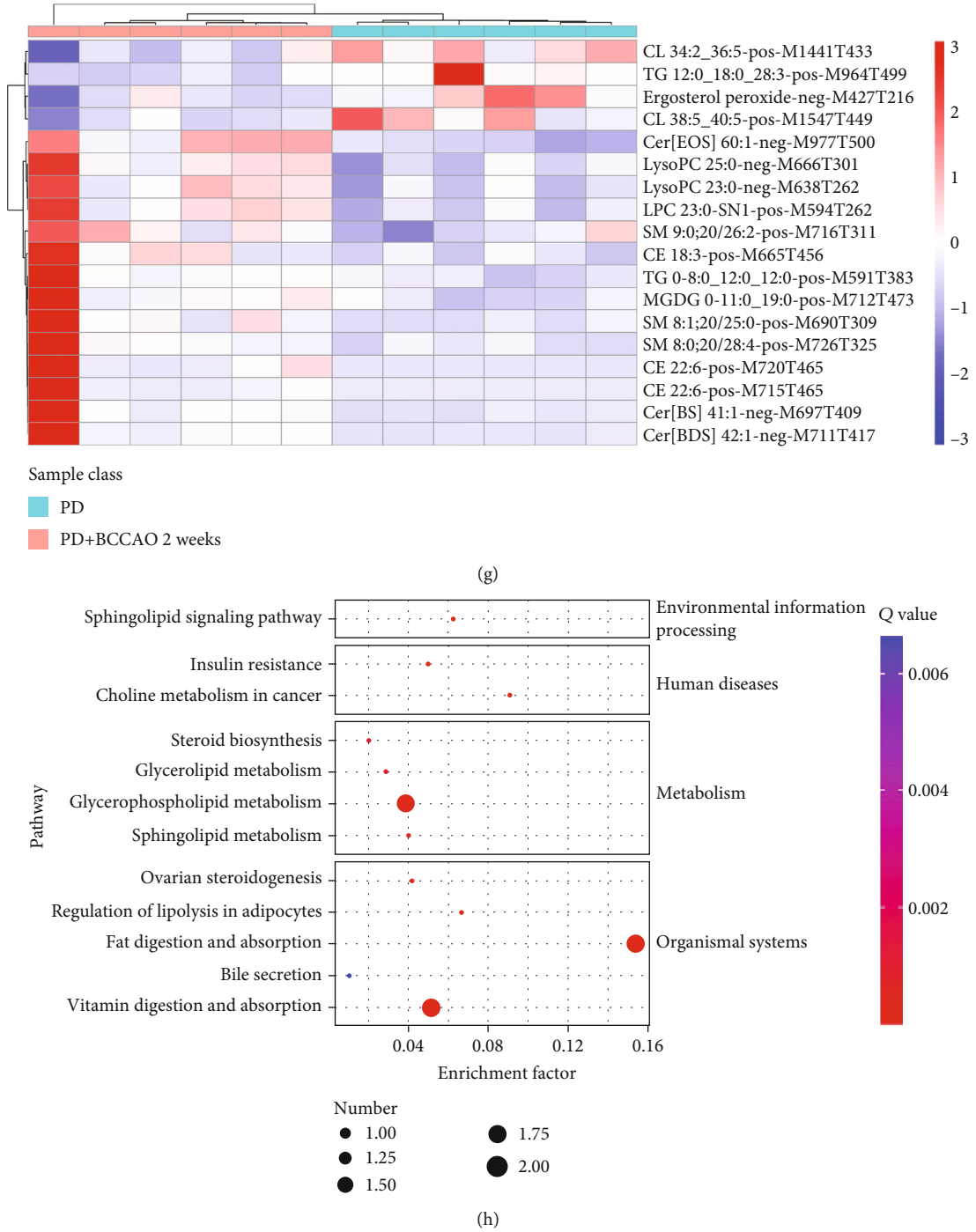


Figure 6: CCH aggravates PDD-like symptoms and pathology in 6-OHDA-lesioned rat through interfering with sphingolipid metabolism ($n = 6$). (a) Metabolic ions identified in positive and negative ion mode into six major categories of lipids. (b) PCA analysis of differentially metabolic ions. The horizontal axis is the first principal component, and the vertical axis is the second principal component. (c) PLS-DA analysis of differentially metabolic ions. (d) Response ranking test plot for the PLS-DA analytical model. (e) Volcano map of differentially metabolic ions. (f) Correlation heatmap of differentially metabolic ions. (g) Correlation heatmap of differentially secondary metabolites. (h) Enrichment of KEGG pathways of differentially metabolic ions.

the sham group and BCCAO 2 weeks group (Figure 4(b)). Also, compared with the sham group, PD group, and BCCAO 2 weeks group, CCH combined with 6-OHDA treatment could increase the expression of α-Syn in prefrontal cortex (Figure 4(b)). Moreover, the expression of Aβ in striatum and prefrontal cortex of PD+BCCAO 2 weeks was higher than that of other groups (Figure 4(c)). The above results indicated that CCH combined with 6-OHDA treatment aggravated PDD-like pathology in prefrontal cortex and striatum, especially prefrontal cortex. Simultaneously, the pathology of PD +BCCAO 2 weeks group was more obvious than PD+BCCAO 1 week. Therefore, to further explore the mechanism by which

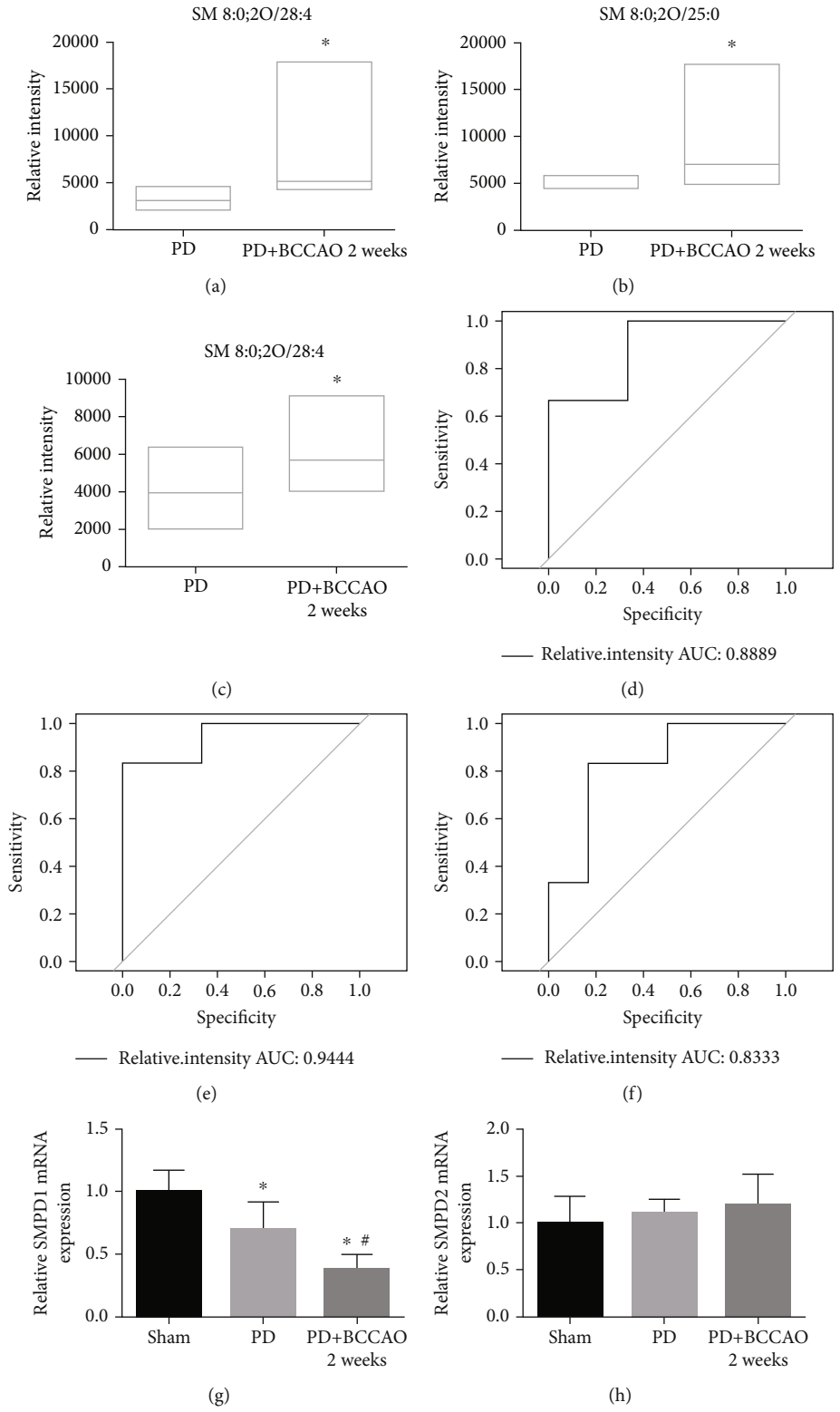
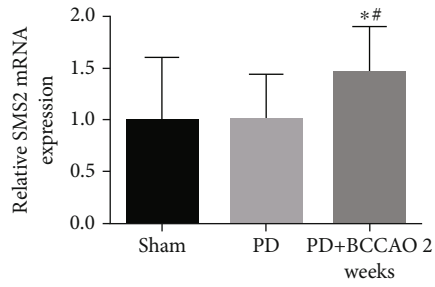


Figure 7: Continued.



(i)

Figure 7: CCH disrupts the sphingolipid metabolism by affecting the mRNA expression of SMPD1 and SMS2. (a–c) Relative intensity analysis of metabolic ions. pos-M716T311 (SM 9:0;2O/26:2), pos-M690T309 (SM 8:1;2O/25:0), and pos-M726T325 (SM 8:0;2O/28:4) are higher in the PD+BCCAO 2 weeks group compared to the PD group. (d) Receiver operating characteristic curve analysis showed the AUC of pos-M726T325 (SM 8:0;2O/28:4) was 0.8889. (e) Receiver operating characteristic curve analysis showed the AUC of pos-M690T309 (SM 8:1;2O/25:0) was 0.9444. (e) Receiver operating characteristic curve analysis showed the AUC of pos-M716T311 (SM 9:0;2O/26:2) was 0.8333. (g–i) Relative mRNA expression of each group. The relative expression of SMPD1 was significantly decreased in the prefrontal cortex of PD+BCCAO 2 weeks group, but SMS2 was significantly elevated, while SMPD2 did not change. $^{*}P < 0.05$ compared with the sham group and $^{\#}P < 0.05$ compared to BCCAO 2 weeks group.

CCH causes PDD-like pathology, we selected prefrontal cortex form PD group and PD+BCCAO 2 weeks group for RNA-Seq.

3.4. CCH Modulated mRNA Expression in Prefrontal Cortex of 6-OHDA-Lesioned Rat. Overall, 61 differentially expressed mRNAs, including 11 upregulated and 50 downregulated mRNAs, were identified in the PD+BCCAO 2 weeks group compared with the PD group (Figures 5(a)–5(c)). Also, protein-protein interaction network (PPI) showed that Mag, Mbp, Plp1, Mobp, and Fa2h have more associations with other mRNAs (Figure 5(c)). Enrichment of GO cellular component showed that the differentially expressed mRNAs involve in myelin sheath, compact myelin, and paranode region of axon (Figure 5(d)). Enrichment of GO molecular function showed that the differential mRNAs possess molecular functions including structural constituent of myelin sheath, fatty acid alpha-hydroxylase activity, and N-acetylphosphatidylethanolamine-hydrolysing phospholipase activity (Figure 5(e)). Moreover, differentially expressed mRNAs are involved in biological processes, including myelination, galactosylceramide biosynthetic process and sphingolipid metabolic process (Figure 5(f)). Then, KEGG analysis demonstrated that differentially expressed mRNAs related to CCH were significantly enriched in multiple signaling pathways, including sphingolipid signaling pathway, sphingolipid metabolism, phospholipase D signaling pathway, and ether lipid metabolism (Figure 5(g)). It is clear that there is a potential relationship between differentially expressed mRNAs and lipid metabolism. In order to elucidate the metabolism of which lipids are specifically affected by CCH, we performed lipidomics on prefrontal cortex.

3.5. CCH Aggravates PDD-Like Symptoms and Pathology in 6-OHDA-Lesioned Rat through Interfering with Sphingolipid Metabolism. The QC information of samples was shown in supplemental Figure 2. As shown in Figure 6(a), we have classified the 7069 metabolic ions identified by negative ion mode and 13970 metabolic ions identified in positive ion mode into six major categories of lipids including fatty acyls (FA), glycerolipids (GL), glycerophospholipids (GP), prenol lipids (PR), sphingolipids (SP), and sterol lipids (ST). Then, differential lipids in the prefrontal cortex of the PD group and the PD+BCCAO 2 weeks group were screened using multivariate statistical analysis methods principal component analysis (PCA) (PC1 = 28.92%, PC2 = 16.53%, PC3 = 11.39 %) and partial least squares method-discriminant analysis (PLS-DA) ($R2 = 0.946889$, $Q2 = -0.1709$, P value-$R2 = 0.195$, P value-$Q2 = 0.245$, intercept-$R2 = 0.91$, intercept-$R2 = -0.54$) (Figures 6(b)–6(d)). The results suggested that the main metabolic ions of this study can be analyzed by the above two models.

Differential metabolic ions between the two groups were defined as the combination of a ratio ≥ 1.5 or $\leq 1/1.5$, a $P \leq 0.05$, and VIP ≥ 1. Overall, 273 differential metabolic ions, including 178 upregulated and 95 downregulated metabolic ions, were identified in the PD+BCCAO 2 weeks group compared with the PD group (Figures 6(e) and 6(f)). Among, there are 18 metabolic ions for which secondary metabolites can be identified, including pos-M716T311 (SM 9:0;2O/26:2), pos-M690T309 (SM 8:1;2O/25:0), pos-M726T325 (SM 8:0;2O/28:4), neg-M711T417 (Cer[BDS 42:1]), neg-M697T409 (Cer[BS]), neg-M977T500 (Cer[EOS]), etc. (Figure 6(g)). Finally, compared with the PD group, the content of 5 kinds of lipids, including sphingomyelin, cardiolipin, lysophosphatidylcholine, cholesteryl ester, and triacylglycerol, significantly changed in the PD+BCCAO 2 weeks group. Interestingly, enrichment of KEGG signaling pathway of 5 kinds of lipids also suggested that CCH mainly affected the sphingolipid signaling pathway and sphingolipid metabolism (Figure 6(h)).

Since the lipid involved in the sphingolipid metabolism is sphingomyelin, we further showed the relative intensity of metabolic ions, including pos-M716T311, pos-M690T309, pos-M726T325 (Figures 7(a)–7(c)). To further identify biomarkers, the screened differential metabolic ions were analyzed using partial least squares-based ROC curves. We found that the area under the curve (AUC) of pos-M716T311, pos-M690T309, and pos-M726T325 is more than 0.8. Also, sensitivity and specificity of above metabolic ions are

more than 80% (Figures 7(d)–7(f)). These results suggested that sphingomyelin may serve as a potential biomarker for a PDD-like model induced by CCH combined with 6-OHDA treatment.

In order to explore the abnormal increase in the level of Sphingomyelin, we detected the mRNA associated with the synthesis (SMPD1 and SMPD2) and decomposition (SMS2) of sphingomyelin. Compared with the sham group and PD group, we found the relative expression of SMPD1 was significantly decreased in the prefrontal cortex of PD+BCCAO 2 weeks group, but SMS2 was significantly elevated, while SMPD2 did not change (Figures 7(g)–7(i)). These results suggested that CCH disrupts the sphingolipid metabolism by affecting the mRNA expression of SMPD1 and SMS2, leading to the accumulation of sphingomyelin in the prefrontal cortex.

4. Discussion

According to the diagnosis and treatment guidelines, the two important symptoms of PDD are motor deficits (i.e., bradykinesia, postural instability, asymmetric resting, and rigidity) and cognitive deficits in memory, attention, executive function, and visuoconstructive ability [25]. Meanwhile, CCH is a cardinal risk factor for PDD, especially for cognitive impairment [7, 26–29]. To elucidate the mechanism of CCH exacerbating cognitive impairment in PD, we used BCCAO to simulate CCH in rats with reference to previous literature [30, 31]. Importantly, our results also certified that CCH could aggravate PDD-like symptoms in 6-OHDA-lesioned rats including bradykinesia (Figures 2(b)–2(d)) and memory impairment (Figures 2(e)–2(j)). Usually, WMW is mainly used to assess learning memory impairment and spatial orientation memory impairment in animals. As shown in Figure 2(f), during the learning process of spatial navigation, the PD +BCCAO 2 weeks group still showed marginal movement after 4 days of learning. Also, the PD+BCCAO 2 weeks group showed marginal movement and spatial memory impairment in the recall of spatial exploration (Figure 2(j)). These results suggested that CCH could seriously affect the learning ability of animals, resulting in animals unable to remember the location of the platform.

Then, we found that decreased neuronal cells (Figure 3) and increased expression of α-Syn and Aβ (Figures 4(b) and 4(c)) in the prefrontal cortex and striatum. The most persuasive evidence to date suggests that α-Syn pathology and Aβ pathology, which are widely distributed in brain of PDD patients, plays a paramount role in PDD [32–34]. Therefore, CCH can aggravate not only PDD-like symptoms but also PDD-like pathology in 6-OHDA-lesioned rat. Also, the complete circle of Willis in the rat affords incessant (but reduced) blood flow from the vertebral arteries after the onset of occlusion. The combination of BCCAO and 6-OHDA-lesioned can better simulate the disease state of PDD.

Prefrontal cortex is closely related to higher cognitive functions such as attention, memory, and learning. Also, ipsilateral nigrostriatal dopaminergic function was related to prefrontal cortex perfusion [35]. Coincidently, the PD+BCCAO 2 weeks group performed serious learning impairment in MWM, which is related to prefrontal cortex (Figure 2). Meantime, PDD-like pathology could be found in prefrontal cortex (Figure 4). Thus, we speculated that prefrontal cortex is an important lesion location in relation to cognitive impairment. So, we further explore the mechanism of effect of CHH on prefrontal cortex by RNA-Seq and lipidomics.

According to the analysis of RNA-Seq and lipidomics, we found that CCH mainly disrupts the sphingolipid metabolism by affecting the mRNA expression of SMPD1 and SMS2, leading to the accumulation of sphingomyelin in the prefrontal cortex (Figures 5–7). Sphingolipids are a group of structurally diverse lipids which play an integral role in body functions, including participation in cell proliferation, death, migration, membrane domains and signaling, and central nervous system development [36]. Sphingolipids mainly include sphingosine, sphingomyelin, sphingolipid intermediates, ceramides, and sphingosine-1-phosphate. Sphingomyelin is a complex sphingolipid, consisting of phosphorylcholine and ceramide, in a cylindrical structure, which is responsible for myelination, impulse transmission, synaptic plasticity, localization of neurotransmitter receptors, and blood circulation. Integrity of the brain barrier is critical [37, 38]. Elevated levels of SM (C18:1) and SM (C20:0) in the anterior cingulate cortex of PD patients, which is similar to our findings [39, 40].

The catabolism and synthesis of sphingomyelin are mediated by sphingomyelinase (SMases, encoded by SMPD1 and SMPD2) and sphingomyelin synthase 2 (SMS2, encoded by SMS2), respectively. Thus, high-level of sphingomyelin might be related to SMPD1, SMPD2, and SMS2. Our results showed that mRNAs of low-expressed SMPD1 and high-expressed SMS2, leading to accumulation of sphingomyelin in the prefrontal cortex (Figure 7). On the one hand, abnormally elevated sphingomyelin promotes the accumulation of α-Syn in the plasma membrane region, which disrupts plasma membrane organization and leads to the degradation of synaptic function [41, 42]. On the other hand, sphingomyelin could promote the expression of α-Syn and hinder the decomposition of α-Syn [43]. Mutation of the gene SMPD1 encoding lysosomal acid SMase can lead to abnormal lysosomal function and affect the degradation of α-Syn in lysosomes [44, 45]. Therefore, we speculated that CCH can aggravate the aggregation and expression of α-Syn, which is related to cognitive functions, by promoting the accumulation of sphingomyelin in the prefrontal cortex.

5. Conclusion

In present study, the effects and mechanisms of CCH on PDD-like symptoms and pathology were evaluated in the classical PD rat model of 6-OHDA-lesions. In summary, we found that CCH is an independent exacerbating reason for impairment in learning and memory within the pathopoiesis of PD. Also, CCH disrupts the sphingolipid metabolism by affecting the mRNA expression of SMPD1 and SMS2, leading to the accumulation of sphingomyelin in the prefrontal cortex. Elevated levels of sphingomyelin could aggravate the aggregation and expression of α-Syn, which is related to cognitive functions. Thus, maintenance of adequate cerebral perfusion and stable sphingolipid metabolism

may be critical to prevent further cognitive impairment in PD patients.

Data Availability

The datasets generated and/or analysed during the current study are not publicly available because the data still need further analysis, but are available from the corresponding author on reasonable request.

Ethical Approval

All studies involving animals were approved by the Ethics Committee for Animal Research of Guangzhou University of Chinese Medicine (No. 20210318006). All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This article does not contain any studies with human participants performed by any of the authors.

Conflicts of Interest

The authors declare that they have no competing interests.

Authors' Contributions

MZ and DC designed the research. YF, ML, YW, and PW performed the study. YF and LZ analyzed the data. YF and CW wrote the manuscript. CW and ZH revised the manuscript. QW and JH provided technical support. All authors contributed to the article and approved the submitted version.

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Supplementary Materials

Supplemental Figure 1: (A) the number of rotations of each group in apomorphine-induced rotation tests. (B) Weight monitoring during the experiment. Supplemental Figure 2: the QC information of samples. (A) Negative ion mode TIC overlay of QC samples. (B) Metabolic ions distribution in negative ion mode. (C) Positive ion mode TIC overlay of QC samples. (D) Metabolic ions distribution in positive ion mode. (E) PCA analysis of all identified metabolic ions. (F) Correlation analysis of all identified metabolic ions. *(Supplementary Materials)*

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Review Article

A Review on Recent Advances of Cerebral Palsy

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This narrative review summarizes the latest advances in cerebral palsy and identifies where more research is required. Several studies on cerebral palsy were analyzed to generate a general idea of the prevalence of, risk factors associated with, and classification of cerebral palsy (CP). Different classification systems used for the classification of CP on a functional basis were also analyzed. Diagnosis systems used along with the prevention techniques were discussed. State-of-the-art treatment strategies for CP were also analyzed. Statistical distribution was performed based on the selected studies. Prevalence was found to be 2-3/1000 lives; the factors that can be correlated are gestational age and birth weight. The risk factors identified were preconception, prenatal, perinatal, and postnatal categories. According to the evidence, CP is classified into spastic (80%), dyskinetic (15%), and ataxic (5%) forms. Diagnosis approaches were based on clinical investigation and neurological examinations that include magnetic resonance imaging (MRI), biomarkers, and cranial ultrasound. The treatment procedures found were medical and surgical interventions, physiotherapy, occupational therapy, umbilical milking, nanomedicine, and stem cell therapy. Technological advancements in CP were also discussed. CP is the most common neuromotor disability with a prevalence of 2-3/1000 lives. The highest contributing risk factor is prematurity and being underweight. Several preventions and diagnostic techniques like MRI and ultrasound were being used. Treatment like cord blood treatment nanomedicine and stem cell therapy needs to be investigated further in the future to apply in clinical practice. Future studies are indicated in the context of technological advancements among cerebral palsy children.

1. Introduction

Cerebral palsy is the most common disability of childhood that affects motor function as a result of injury to the developing brain [1]. It is also known as Little's disease as the term was first described by William John Little in the year 1843 in which he mentioned spasticity occurs due to damage to the brain during infancy, preterm birth, or birth asphyxia. This was followed by extensive contributions of Osler, Sach, and Peterson, Sigmund Frued, Mac Keith and Polani, and many others until 2006 when an expert executive panel defined CP as a group of permanent disorders of the development of movement and posture, causing activity limitation, which is attributed to nonprogressive disturbances occurring in the developing fetal or infant brain. CP symptoms are heterogeneous, a child having limited brain injury may find difficulty in just one component of the musculoskeletal system, and another child with a broad range of symptoms may suffer from activities that hamper the activities of daily living of the child along with other life-threatening comorbidities; however, its symptom may improve in due course of time owing to the maturity of the nervous system with age. Damage to the developing brain before, during, or just after delivery affects both neurological and musculoskeletal systems of the body producing symptoms such as abnormal contraction of muscles, postural changes, and movement and activity limitation which are accompanied by sensory disturbances along with perceptual disorders, cognitive issues, inability to communicate, behavioral issues, epilepsy, and secondary musculoskeletal problems. Previously, it was thought that lack of oxygen at birth is responsible for cerebral palsy; however, with emergent

research, it is evident that along with this, there are many other causes and risk factors responsible for the development of cerebral palsy. It is now believed that CP results from a series of events that combines to cause injury to the brain during the developmental period [2, 3].

The epidemiology of CP has changed over time. It occurs in 2-3 in every 1000 live births; however, it is relatively stable over decades [1]. The prevalence of CP was found to be in increasing trend in studies done before 1990 as there was better survival of preterm infants due to advances in medical technology; however, the prevalence decreased subsequently as there was an improvement in prenatal care too after that. The prevalence has remained the same from 1990 to 2003 and was found to be between 2.2 and 2.3; however, it has decreased now [2, 4]. According to another study, the prevalence of CP has decreased from 2.1 to 1.4 in Australian children since 1995; apart from these, various studies show that due to the financial burden of the developing countries, children cannot get the best service for prevention and management of CP which has led to its increased severity; these population trends indicate that changes in preventive and management studies are successful; however, more research is required in these areas. Increasing evidence of various low-cost novel treatment techniques that can be made easily available to the population promises to deliver better outcomes [5, 6]. The incidence of CP is found to be stable in worldwide epidemiological studies, but the management of premature birth complications is still a contributory factor in increasing the incidence of this disease [7]. In the last decade, various prevention and management strategies have been identified in the literature that helped in decreasing the occurrence of this disease. Prescribing magnesium sulfate, progesterone, and corticosteroids to pregnant women for their neuroprotective nature and application of therapeutic hypothermia are some of the evident methods of preventing prematurity which is a major causative factor of CP [5, 6]. This review is aimed at summarizing the strategies that will improve the status of children with CP. The review will begin by compiling research done on the risk factors and etiologies of CP in the last 5 years including efforts to standardize diagnostic criteria along with classification and clinical features of CP. The prevention and management strategies will then be focused on in detail.

2. Methodology

The study is aimed at throwing light on recent developments in cerebral palsy and showing new paths for future research in this field. For this purpose, various types of published articles including original research, review articles, and systematic reviews that we consider relevant to our study were selected. A search in Medline via PubMed, Google Scholar, and manually extracted relevant publications by cross-referencing was done to find publications made in the English language. Search terms included cerebral palsy or early brain lesion or perinatal stroke along with management and prevention techniques. We focused on publications of the last five years, i.e., 2017 to 2021, to provide an updated overview.

3. Results

3.1. Risk Factors for and Etiology of Cerebral Palsy. It is now well known that the prime risk factors for CP are delivery before 37 weeks and birth weight of less than 2.5 kg; however, there are some other problems evident in the literature which are found to be some of the prominent reasons for brain damage, some of which includes malformation of the brain in the developmental period, genetic causes, in utero mother and fetus infections, and various other issues [8]. Factors that may put the developing brain prone to injury were divided into risk factors that develop during preconception, during pregnancy, and after birth [2]. A study confirmed that the health of the mother before conception is one of the reasons that affect the central nervous system of the fetus later during the gestational period which might lead to CP. According to this study, preconception is defined as the health conditions of the mother before conception, prenatal is defined as the period of gestation, perinatal is during delivery, and postnatal is after delivery [2]. A study in 2021 also found that the risk factors for cerebral palsy were 21%, 30.5%, 17.1%, and 31.4% when grouped under prenatal, perinatal, postnatal, and unidentified categories [9]. Preconception risk factors include the mother's systemic illness, substance abuse, maternal undernutrition, swallowing harmful substances, fertility issues, and previous spontaneous termination of pregnancy [7]. Factors that may lead to brain damage during gestation include maternal abnormalities of the central nervous system, gestational diabetes, excessive bleeding per vagina, and preeclampsia. Multiple gestations, cotwin death, genetic contributions, and encephalopathy of prematurity are also strong risk factors for CP [7]. Risk factors during delivery are premature birth, C-section, vacuum-assisted delivery, forceps delivery, delivery after the due date, labor induction, prolonged labor, asphyxia, and meconium aspiration syndrome [10]. Various other risk factors before, during, and after delivery that may lead to brain damage are summed up in Table 1 and Figure 1.

Multiple etiologies are responsible for various developmental defects in the fetal brain that results in brain injury which affects the physical functioning of the body [11]. Nearly 75% of CP occurs due to prenatal etiologies whereas 92% of causes are perinatal [2, 12]. It is now well known that CP results from various reasons during pregnancy or during delivery, but in various studies, it is found that it occurs due to brain injury in the postneonatal period also [8, 12]. Postnatal CP is defined as any trauma or disease in the brain after a neonatal period and before 5 years of age [13]. Immediately after delivery, CP may occur in 10-18% of cases due to conditions like hypoglycemia, jaundice, and infections [2, 12, 14, 15]. Though preterm is considered an important benchmark in the etiology of CP, term babies are also high in percentage; this might serve as an indication of a genetic basis associated with CP [2]. For term-birth children, pieces of evidence also suggest that sudden genetic mutations in genes may also be responsible for the development of CP without any other probable causes [16]. Placenta abruption, prolapsed cord, birth asphyxia, congenital anomalies, and maternal conditions during labor like high fever are

Table 1: Risk factors for cerebral palsy [2, 7, 10].

Preconception	Before birth	During birth	After birth
Systemic illness of the mother	Premature birth	Premature birth	Hypoxic ischemic encephalopathy
Use of drugs and stimulants	Low birth weight	C-section	Infection
Immune system disorders preceding pregnancy	CNS malformation	Vacuum-assisted delivery	Hyperbilirubinemia
Spontaneous abortions	Maternal DM	Delivery after the due date	Cerebrovascular accidents
Socioeconomic factors	Prolonged rupture of membrane	Prolonged labor	Intracranial hemorrhage
Poisoning	Maternal hemorrhage	Asphyxia	CNS infection
Infections	Multiple gestations	Meconium aspiration	Respiratory distress syndrome
Impaired fertility	Cotwin death	Breech vaginal delivery	Artificial respiratory support
Treatment of fertility	Genetic factors	A high fever during delivery	Hypoglycemia neonatal convulsions
	Encephalopathy of prematurity		Traumatic brain injury
	Congenital malformation		Near drowning
	Hypoxic ischemic encephalopathy		Meningitis
	In utero stroke		Sepsis
Genetic factor	In vitro fertilization	Perinatal stroke	
	Kernicterus		
	Maternal disorder of clotting		Neonatal encephalopathy
	Meconium aspiration		
	Fetal growth restriction		
	Preeclampsia		

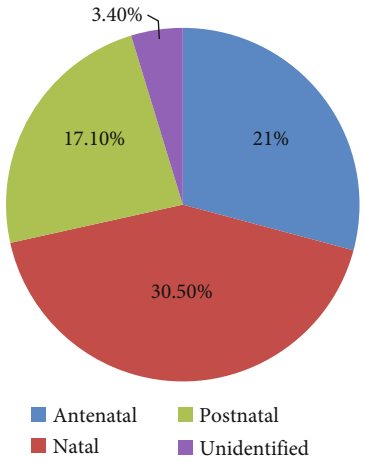

Figure 1: Risk factors for cerebral palsy [2, 7, 10].

common causative agents that may lead to brain damage in the fetus. Congenital etiologies such as failure of closure of the neural tube, schizencephaly, chromosomal defects, and microcephaly are also some of the causes [8]. In children born in less than 32 weeks, white matter injury in 84.6% of children was evident which was found to increase with decreasing gestational age [13, 16]. Grey matter injury was seen in moderately preterm infants. Bilateral CP was found to be in a higher percentage than unilateral CP with a decrease in gestational age [15]. A study in 2019 among 2-15-year-old children in Nigeria revealed that most cases were due to birth asphyxia, hyperbilirubinemia, and rubella [2]. An SCPE collaborative study in 2021 referred to the common causes of various types of CP as PVL, congenital infections, asphyxia, hyperbilirubinemia, genetic, neonatal stroke, etc. [15]. Various other causes of CP are listed in Table 2 and Figure 2. Apart from this, events that lead to CP are demonstrated in Figure 3.

3.2. Classification of Cerebral Palsy. As injury to the developing brain occurs due to numerous causes and manifests in different clinical presentations and severity, it has been described under various headings based on the type of movement disorder, area of involvement, and level of damage. According to the type of movement disorder, CP is classified as spastic, dyskinetic, and ataxic. Based on the area of presentation, it can be classified into involvement in one side or both sides of the body, i.e., quadriplegic, hemiplegic, diplegic, and monoplegic, diplegic being most common followed by hemiplegic (20–30%) and quadriplegic (10–15%) (Figure 4). In quadriplegic CP, all four limbs are affected. In this condition, the hands are more affected than the legs, and this occurs due to acute hypoxic asphyxia during the perinatal period, excessive cystic degeneration of the brain, and developmental abnormalities such as polymicrogyria and schizencephaly. The condition presents with

Table 2: Etiologies of cerebral palsy [2, 8, 11–13, 15].

Prenatal	Perinatal	Postnatal
Infection and fever during pregnancy	Obstructed labor	Hypoglycemia
Metabolic disorders	Cord prolapses	Jaundice
Intrauterine infection	Antepartum hemorrhage	Neonatal meningitis
Chorioamnionitis	Metabolic acidosis	Septicemia
Maternal ingestion of toxins	Use of assisted reproductive technology	Malaria
Preeclampsia		Malaria with seizures
Maternal trauma in pregnancy		Malaria with coma
Exposure to methylmercury		
Genetic syndromes		Meningitis
Multiple pregnancies		
IUGR		Tuberculosis
Fetal growth restriction	Intrapartum hypoxia	Sickle cell disease
Placenta abruption		HIV
Failure of closure of the neural tube		PVL
Schizencephaly		Congenital infections
Chromosomal defects		Asphyxia
Microcephaly		Hyperbilirubinemia
Rubella		Genetic causes
		Neonatal stroke

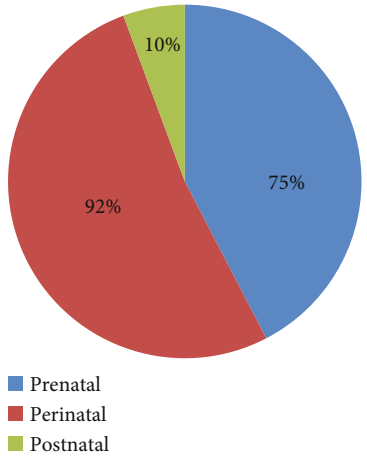

Figure 2: Causes of cerebral palsy [2, 8, 11–13, 15].

limited voluntary movements of all the extremities, pseudobulbar signs, accidental food entry in the airways, difficulty in swallowing, optic atrophy, seizures, and severe intellectual abnormality. In hemiplegic CP, hand functions are mostly affected. Dorsiflexion and aversion of the foot are severely impaired in the lower limb. Increased spasticity in flexor muscles, sensory abnormalities, seizures, and visual problems are common findings. In diplegic, CP cystic periventricular leukomalacia is the most common neurological feature seen in premature infants. In the case of hemiplegic CP, only one side of the body is affected with a high tone in flexor muscles and sensory loss. Apart from this, hand function is severely impaired when compared to legs. In the foot, dorsiflexion and eversion are affected. Both matured and premature-born children are at risk of hemiplegic CP [14].

Popularly, CP was classified according to the Ingram and Hagbergs classification; however, surveillance of cerebral palsy in Europe (SCPE) has simplified the classification of CP as spastic, ataxic, and dyskinetic (Table 3) [2]. Ingram classified CP based on location and severity of neurological symptoms. He classified cerebral palsy into diplegic, hemiplegic, tetraplegic, ataxic, dyskinetic, and mixed [21]. Hagberg however classified CP into spasticity, dyskinetic syndromes, and ataxia. The spastic syndrome occurs due to damage to the brain and tracks controlling movement. It can be divided into monoparesis, hemiparesis, triparesis, tetraparesis, and spastic diplegia. Dyskinetic symptoms are seen due to injury to the subcortical structure, and ataxic symptoms are seen due to cerebellar injuries dividing CP into spastic, affecting one or both sides of the body, dyskinetic involving involuntary movements with altered tone or choreoathetosis movements, and ataxic. Around 80% of CP cases are found to be spastic [22]. Spastic CP in this context is characterized by increased muscle tone and increased reflexes. It has been subcategorized into unilateral or bilateral along with the area of involvement. SCPE refers to dyskinetic CP accounting for 10 to 20% of CP cases and presents as having involuntary, uncontrolled, repetitive, and sometimes stereotypical movements with a fluctuating muscle tone. A faulty posture with enhanced muscle tone is defined as dystonic; a quick, uncontrolled, and twisting movement with hypotonia is called choreoathetosis. On the other hand, ataxic CP consists of 5-10% of CP cases and presents with loss of coordination with hypotonia. In some children, damage may occur in different parts of the developing brain which causes them to develop symptoms of having a combination of two or more types of cerebral palsy. This type is called mixed CP which accounts for 15.4 percent

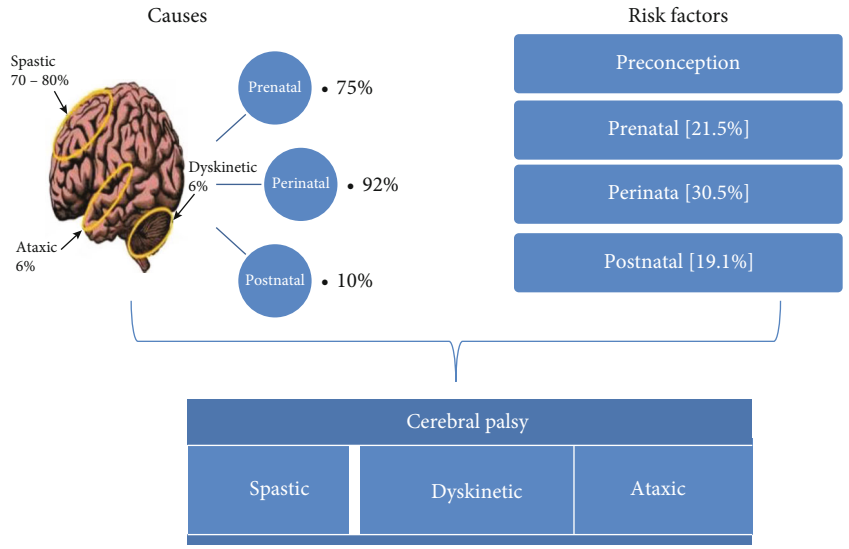

Figure 3: Events leading to cerebral palsy [2, 8, 11–13, 15].

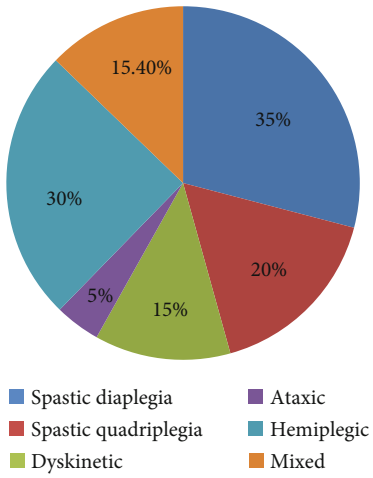

Figure 4: Different types of cerebral palsy [2, 14].

of all cases. The most common presenting symptoms of mixed type are a combination of spastic and athetoid features [13].

Evaluating the severity of motor disorders is important for predicting the functioning of the affected limbs and the outcome of the treatments. For this purpose, four systems are used for functional classification of CP which include GMFCS, MACS, CFCS, and EDACS [2, 11, 12, 23]. GMFCS developed by Palisano et al. in 1997 is used worldwide for the functional classification of CP (Table 4) [2, 11, 12, 23]. It is easy to use and describes gross motor function. It has levels that describe voluntary movement and the use of aid for movement. It was first designed to measure gross movements in children of 2–12 years of age, but in 2007, it was revised and ages 12–18 were included. According to the new revised version of GMFCS, a child is considered to be in GMFCS level 1 if the individual can walk without any aids. However, there are considerations and limitations according to the age of the child. In level 2, the child can do all these activities, but limitations are present in the form of speed, balance, and endurance. The child finds difficulty walking long distances and requires a handheld or wheeled device for long distances. Gross motor skills are minimal. Level 3 children walk with handheld mobility devices in indoor settings, need supervision during stair climbing, and require wheeled devices for long distances. In level 4, the child lacks self-mobility. The child can sit with support, but transportation requires a manual or powered wheelchair. In level 5, children are dependent on all settings and have limitations to maintain antigravity posture. They strictly require wheelchair transportation. Another classification system, namely, the manual ability classification system (MACS), is a five-level scale used for 4–18 years old which was developed by Eliasson et al. in 2006, to evaluate the functions of the upper limb. In level I, the child can handle objects with ease, there are some limitations with accuracy, but that does not hamper activities of daily living. In level II, the child's activities are slower and of reduced quality. A different way to perform the activity can be used by the child, but it does not affect the daily activities performed by the child. The child in MACS III has reduced speed while performing hand activities and often with limited success. Some activities need help, but others can be done without any help. A child in MACS IV performs various simple activities with lots of effort. They require constant help and adapted types of equipment for performing simple activities. Individuals in MACS V are dependent [24].

A classification system, namely, the communication function classification system (CFCS), is also a five-level scale that is used to evaluate everyday communication. At an individual level, level I can communicate at a comfortable pace. The person can send and receive information from different people in different individuals. In level II, the pace of communication is slow; however, they can communicate properly. In level III, communication is effective only with a familiar partner. In level IV, the person is not always

Table 3: SCPE classification of cerebral palsy [2, 13].

Type of CP	Description
Spastic	Presents with hypertonicity and hyperreflexia
	May be unilateral or bilateral
Dyskinetic	Presents with involuntary, uncontrolled, repetitive, and sometimes stereotype movements with altered muscle tone
	Abnormal posture with hypertonicity is termed dystonic
	A quick, uncontrolled, and twisting movement with hypotonia is called choreoathetosis
Ataxic	In coordination with a decreased muscle tone

Table 4: Functional classification of children with cerebral palsy [2, 11, 12, 23].

Classification type	Description
GMFCS	Evaluates the gross motor function of the individual with CP
MACS	Evaluates functions of upper limb
CFCS	Evaluates everyday communication
EDACS	Evaluates the ability to eat for children with CP after 3 years

consistent in communication with known people, whereas in level V, the individual cannot communicate affectively and consistently with unknown people. The eating and drinking ability classification system is again a five-level classification system used to assess how efficiently a CP child eats and drinks. It is used for more than 3-year-old children. It has extra three levels that help to find out how much help is required while performing these activities. In EDACS level I, an individual can eat and drink safely without any help, but hard food can cause difficulty swallowing. In level II, the individual can eat and drink safely but has very less speed. The child may present with a cough when food is given at an increased speed. In level III, the child cannot eat hard food; he/she needs very soft and mashed food. However, an individual in EDACS IV or V cannot swallow food and drink safely. Tube feeding is required to provide nutrition [24].

3.3. Clinical Presentations of Cerebral Palsy. The presenting signs and symptoms of CP are diverse and mainly consist of motor disorders, sensory deficits, and associated comorbidities which occur due to a static lesion to the developing brain. These signs and symptoms change as the child ages and new features are added to the list. Thus, with advanced age, there is a worsening of the neuromuscular system and functional capability of the child even though the damage in the brain is static [25]. Injury to the fetal brain can be generally diagnosed by its presenting features; however, often, some of these symptoms resolve after 2 years in many infants owing to the maturation of the CNS [12]. A recent study revealed that the most common symptoms seen were using one hand before 2 years of age, inability to reach normal milestones within the appropriate time, and presence of primitive reflexes after a definite period (Table 5) [22]. Some comorbidities are also associated with cerebral palsy which are summarized in Table 6 [11, 17–20] and demonstrated in Figure 5 [11, 17–20]. Hypertonicity of the muscles owing to brain injury is the most common symptom seen in CP patients along with other motor issues such as impaired balance, coordination, hand function, etc. [10]. A recent study found that this may be due to three causes, i.e., more muscle fibers are required to perform a certain task than healthy individuals, excessive level of collagen deposition in myofibers decreases the efficiency of the muscles by making them stiff, and a disturbance in the neuromuscular junction causes a problem in muscle contraction. The study also revealed that collagen accumulation occurs due to damage to CNS in the developing brain, and this causes issues in the motor abilities of the child as mentioned above. However, there is scarce evidence on the prevention and treatment of this finding [25].

Spastic diplegia is the most common type that accounts for 35% of cases and occurs due to damage to the immature oligodendroglia in the second trimester. In 3- to 6-month-old babies, some of the features seen are decreased neck control, stiffness, floppiness, arching of the back, lower extremity stiffness, and leg crossing while raising from the bed, and in babies older than 6 months of age, there is no rolling. Incoordination of the upper extremities is also evident. Babies older than 10 months of age depict abnormal crawling. Periventricular leukomalacia is the most common neurological finding seen in such cases. Another type of spastic CP apart from diplegic is spastic quadriplegia which accounts for 20% of CP children; the most common reason is premature birth. The child has severe motor and sensory problems, cognitive deficit, seizures, vision problems, and other associated problems which make the child completely dependent. Term infants who are at risk of in utero or perinatal stroke suffer from spastic hemiplegia. They have good cognition and can maintain independent mobility. 15% of CP results from extrapyramidal lesions in term babies. They consist of involuntary movements termed choreoathetosis, dystonic, or dyskinetic clinical features. Hemiplegic CP cases are mostly term babies having causes like brain injury due to lack of oxygen, kernicterus, and neurometabolic or genetic disorders [2]. A high mortality rate is seen in CP children due to respiratory problems [26].

Table 5: Early signs of cerebral palsy [22].

Early signs of CP
Early hand dominance
Delayed motor milestones
Persistent primitive reflexes
Scissored legs below 6 months
Floppiness
Stiffness

Table 6: Comorbidities associated with cerebral palsy [11, 17–20].

Comorbidities present in CP children	
Pain	75%
Intellectual disability	50%
Gait disorders	33%
Hip displacement	33%
Speech problems	25%
Epilepsy	25%
Incontinence	85%
Behavior disorders	25%
Sleep disorders	40%
Hearing impairment	9%
Vision impairment	10%
Cognitive impairment	77%
Thyroid dysfunction	3%
G.I. disturbances	2%

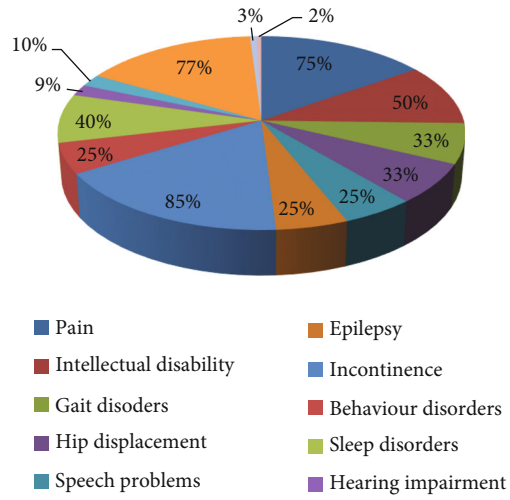

Figure 5: Comorbidities associated with cerebral palsy [11, 17–20].

3.4. Diagnosis. Early diagnosis is necessary as it helps to provide early intervention during the earliest period of development. It is a special service to prevent developmental delay which optimizes the impact of the interventions on the developing brain's neuroplasticity [4, 27]. Diagnosis of cerebral palsy is based on the combined use of clinical presentations along with physical assessments and neuroimaging, which can provide various implications for this disease. Assessment of maternal history including the child's performance of motor functions brings out important points of diagnosis. Owing to the complexity of the condition, psychological tests, vision evaluation, audiometric tests, and electroencephalography are carried out [2]. Close monitoring of early signs in the form of neurobehavioral signs, presence of developmental reflexes that did not disappear with time, abnormal tone and posture, and delayed milestones along with associated comorbidities is essential to screen risk infants. The history of early diagnosis started in the 1800s when William Little urged that the earliest diagnosis will lead to early intervention. It is very important to find out the cause of CP and give the required treatment so that the disease process can be minimized along with increased neuroplasticity and functional outcome. In the 1970s, the idea of risk factors, retaining of abnormal primitive reflexes, and the cranial US in neonatal intensive care units were introduced which helped to identify children who were at risk for CP. In high-income countries, diagnosis of CP was previously done after 1 year, but now, it can be done before 6 months [4]. In 2017, a systematic review was published which inferred that certain tools can be used to diagnose high-risk infants for the development of CP as early as 6 months. A list of such tools is given in Table 7. Prechtl's Qualitative Assessment of General Movements and the Hammersmith Infant Neurological Examination can also be used as predictive tools along with clinical examination in infants below 5 months of age. After 5 months, magnetic resonance imaging, the Hammersmith Infant Neurological Examination, and the Developmental Assessment of Young Children are used to predict CP in extremely low birth weight infants [4]. A combination of two MRI biomarkers fractional anisotropy of superior thalamic radiations and radial diffusivity of the corticospinal tract was used to evaluate the brain's sensory and motor tracts, respectively [5]. In 2011 a study named "cerebral palsy—don't delay" summed up the call for early detection and accurate prediction of CP in the earliest months of life by referring to general movement assessment (GMA), first introduced in 1990 which is an assessment of the spontaneous movement of an infant along with another standardized neurological examination called the Hammersmith Infant Neurological Examination (HINE) [4]. In 2019, a paper was published on the topic of international expert recommendations of clinical features to prompt referral for diagnostic assessment of cerebral palsy where a survey was conducted among 51 international experts in Asia, USA, Australia, Canada, and Europe to find out agreement upon early motor signs and diagnosis of CP and their referral to other health professionals under a project called PROMPT (primary care referral of motor impaired children). The international experts provided a strong agreement on six clinical features and two warning signs along with five referral recommendations based on which a child should be immediately referred for diagnosis to other health care professionals or specialized health services [28]. The American Academy of Neurology recommends a stepwise protocol to help diagnose a cerebral palsy child. The first step is the

Table 7: Tools for early diagnosis of CP [4, 13].

Below 5 months	Above 5 months
GMA	Magnetic resonance imaging
MRI	
HINE	The Hammersmith Infant Neurological Examination
Prechtl's Qualitative Assessment of General Movements	The Developmental Assessment of Young Children
The Hammersmith Infant Neurological Examination	

recognition of the disease by clinical history taking and physical examinations followed by screening for associated comorbidities. This is followed by studying perinatal histories such as fetal anatomy surveys and newborn transcranial ultrasounds. If no abnormalities are detected, MRI is recommended for finding out intracranial abnormalities. Further, if the test is nondiagnostic, then screening for inborn errors of metabolism or genetic abnormality is followed [13]. The entire process is described in Figure 6 [13]. Based on some studies done on dead CP children, Little found that there is some venous and capillary congestion in the brain and spinal cord which led him to refer to this disease as a cerebrospinal disorder. However, William Osler was the person who gave the name cerebral palsy to this condition. Though modern definitions of CP are refined to the context of the cerebral cortex, a critical evaluation study on the concept of CP urges that more studies should be done on the original concept of CP as a "cerebrospinal" disorder, both in clinical work and in animal models [29].

3.5. Prevention. Based on the time of insult to the brain, CP can be divided into individuals whose brain injury occurred during the gestational period, during delivery, and postdelivery. Thus, prevention strategies can depend on the prevention of factors that will decrease the risk of CP in the antenatal, perinatal, and postnatal periods [30]. Prevention strategies include prevention of risk factors, treatments that affect the disease process, and treatment of neonates who are exposed to risk. Various techniques in the literature are present for the prevention of brain injury during the gestational period and delivery. Administration of magnesium sulfate is an important preventive measure for high-risk mothers [28, 31]. There is moderate quality evidence of an increased rate of CP in mothers who used prophylactic antibiotics during pregnancy. Latest reports show that prenatal and perinatal causes of CP have decreased. This has occurred due to various strategies that are used for the early treatment of neonates [30]. The worldwide incidence of birth before 37 weeks is 12% and is one of the main causes of death and illness in neonates. Various studies infer that prophylactic use of progesterone decreases early birth in women with previous birth complications. Universal cervix screening is recommended by midtrimester transvaginal ultrasonography. Management of IUGR, administration of magnesium support, and corticosteroids for fetal lung maturity are equally important strategies [7, 32]. Decreasing the rate of early birth and low weight in neonates is the most significant consideration in reducing the overall incidence of CP. Therapeutic cooling or hypothermia is helpful in cases of brain injury due to a lack of oxygen. It decreases the risk of CP in term and late preterm infants in such cases. It is started within 6 hours after birth which helps to decrease the temperature by 2°C for 48 hours [28, 32, 33]. Prevention of preeclampsia is done by screening and administration of acetylsalicylic acid along with aspirin which should be started before 16 weeks of gestation, with a daily dose higher than 100 mg in high-risk patients. This issue, however, requires further prospective research. Antenatal steroid therapy is evident in newborns in preventing perinatal death of newborns and preventing the risk of disability and development of sepsis in the initial days of birth. Delayed cord clamping is another intervention used in preterm babies that lowers the risk of bleeding, necrotizing enterocolitis, and anemia that requires blood transfusion and late onset of sepsis which has an impact on the neurological development of the baby. Preventive techniques during pregnancy also include corticosteroids for the mother for accelerating lung maturation in the case of early-birth infants. The literature also has evidence of caffeine for apnea of prematurity. Apart from preterm infants, those who are born at the expected time and suffer from a lack of oxygen during delivery have also benefitted from therapeutic hypothermia. Factors that can prevent postnatal CP that are evident from the literature include finding hidden cases of group B streptococcus, administration of antibiotics and vaccines during and after delivery, safe car seating, safety measures in swimming pools, and preventing shaking of the baby [30]. As CP is seen to occur mostly due to prenatal causes (45%), preventing strategies during this period can decrease the overall incidence of CP. To implement this, a recent study in 2018 advised certain interventions to reduce preterm birth: midwife-led continuity models of care, finding and treating urinary tract infections, augmenting zinc supplementation among pregnant women, and cervical cerclage for high-risk mothers [30, 34]. A schematic diagram for the prevention of cerebral palsy is given in Figure 7.

3.6. Management. Cerebral palsy management is aimed at improving functional ability and independency and managing secondary complications. Physical and occupational therapies, mechanical aids, orthopedic surgery to address patients' motor problems, and optimal medical and surgical treatment of medical comorbidities are the main management strategies [35]. An increase in neonatal care and decreased prevalence showed a promising impact on early diagnosis [4]. Early intervention programs are the most

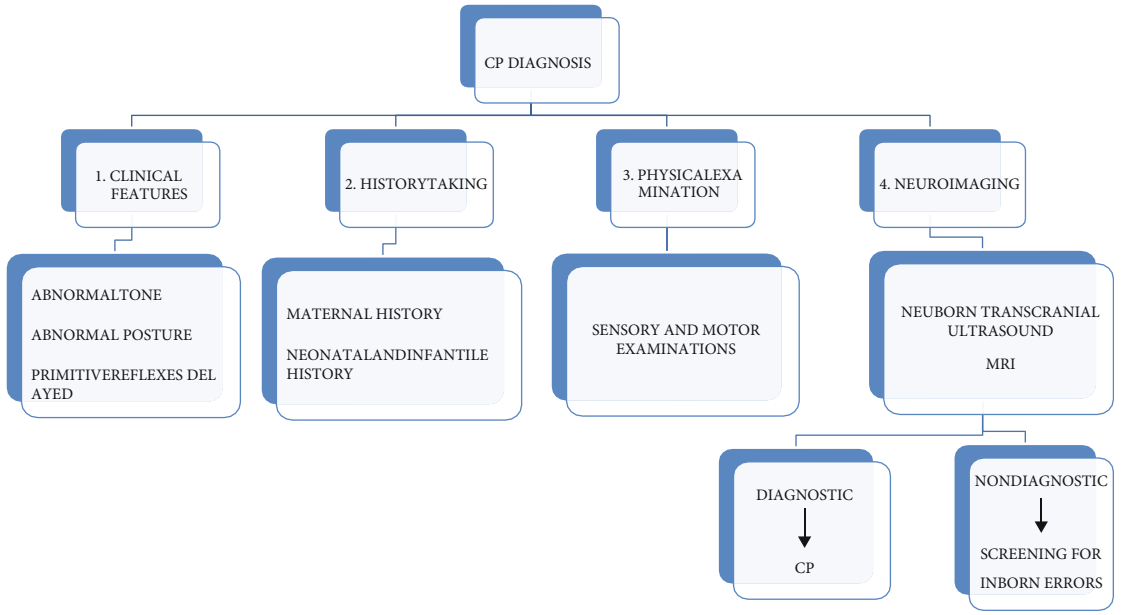

Figure 6: Diagnostic criteria for detecting cerebral palsy [2, 13].

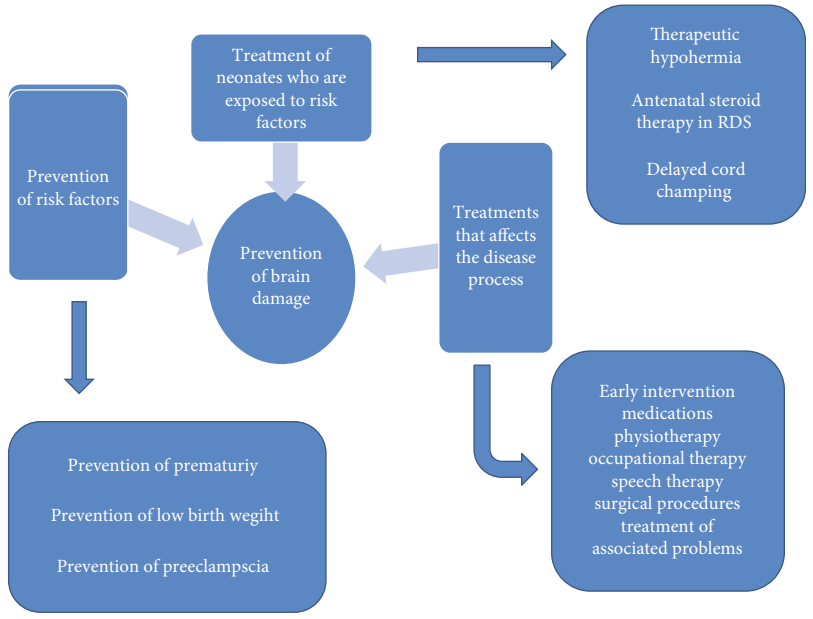

Figure 7: Prevention and management of cerebral palsy [28, 30, 31].

essential component of the management of CP as it addresses the disease process at the earliest and helps in early neuroplasticity of the brain [36]. Two trials, namely, GAME (goals, activity, and motor enrichment) and REACH (rehabilitation early for congenital hemiplegia), are under investigation in Australia to establish evidence for early intervention in children with CP [37]. Addressing functions like physical issues, cognition, communication, eating and drinking, vision, and sleep helps in improving the overall health of the child, and cooperation of the family and environment modification are the major factors for improvement [38]. Management of CP children requires a team approach which includes a list of multidisciplinary team members such as an audiologist, medical social worker, nurse, nutritionist, occupational therapist, pediatric gastroenterologist, pediatric neurologist, pediatric orthopedic, surgeon, pediatric pulmonologist, pediatric surgeon, pediatrician, physiatrist, physiotherapist, psychologist, speech-language therapist, and special educator [10]. Many recent advances in the management of CP have come up including intrathecal baclofen, selective dorsal rhizotomy, and sensory integration [14]. Various medical managements are effective

in the treatment of associated problems in CP children such as multilevel surgery for epilepsy, benzhexol hydrochloride for saliva control, and laxatives for constipation [27]. NSAIDs reduce pain; gabapentin is effective in dystonic CP [39]. Certain treatment exposures such as cooling, umbilical cord blood treatment, glial cell transplantation, nanomedicine, and stem cell therapy are under investigation and extremely reviewed [14]. Therapies such as physical, occupational, speech, and behavioral therapies help in enhancing patient and caregiver interactions while providing family support [27]. Various novel techniques such as telemedicine with outreach programs of physiotherapy services are found to be beneficial [40].

3.6.1. Spasticity Management. The most common movement disorders seen in cerebral palsy are spastic muscles and dystonia with difficulties in coordination, strength, and selective motor control. Spasticity is the major challenge in the management of CP children. It causes spasticity-induced bone and joint deformity, pain, and functional loss [10]. Commonly used medicines found in the literature to relieve spasticity are baclofen, diazepam, clonazepam, dantrolene, and tizanidine. Baclofen and diazepam help in relaxing the muscles but have many side effects [27]. First-line treatment for spasticity is physiotherapy, occupational therapy and botulinum toxin injections, selective dorsal rhizotomy, and intrathecal baclofen [8]. A selective dorsal rhizotomy is a surgical procedure that is effective in CP children that improve their walking ability and range of movement. It rectifies the spasticity that impairs gait by improving the ankle joint junctions [41]. Administration of intrathecal baclofen is done via an implantable pump. It is reserved for GMFCS levels IV and VI as it is used in extreme spasticity cases. However, it is expensive, and relief is of short duration. Intramuscular onabotulinum toxin type (Botox) weakens the skeletal muscles by impairing the release of neurotransmitters at the NMJ. This slows down the contraction of skeletal muscle. The injection is first given between 18 and 24 months of age [8, 10]. Surgical management including lengthening of the soft tissues such as adductors and hamstrings, multilevel surgery of the ankle and foot, nerve blocking, tendon transfers, and joint stabilization are some of the surgical techniques used in CP age appropriately [27].

3.6.2. Management of Balance and Movement Disorder. Balance and movement disorders are crucial management issues in CP children as they are necessary for maximum activities of daily living. The traditional management approach in CP includes physiotherapy, occupational therapy, hyperbaric oxygen therapy, sensory integration, NDT, hippotherapy, CIMT, BWSTT, acupuncture, and the Vojta method [8, 42]. Two treatment techniques called whole-body vibration along with core stability exercises are found to be effective in managing balance issues, the former being more effective [43]. Another therapy having prospects of improving balance is virtual reality. Giving it for 20 minutes, twice a week for 6 weeks gives very good results in balance improvement [44]. Nintendo Wii therapy is another balance-improving treatment that can be considered an effective treatment for improving functional and dynamic balance. It can be combined with physiotherapy techniques for 30 minutes for a minimum of 3 weeks for effective results [44]. Management of movement disorders however for CP patients includes trihexyphenidyl, tetrabenazine, baclofen, levodopa, benzodiazepines, and deep brain stimulation [45].

3.6.3. Management of Hand Dysfunction. Injury in the brain may cause disturbances in hand functioning which may be unilateral or bilateral. In the former case, motor control and function on one side of the body are affected. In this type of CP, children experience difficulties using their hands on the affected side. Constraint-induced movement therapy (CIMT) is a technique that is being used to improve the function of the affected hand. It is based on the principles that not using the good hand and intensive use of the affected hand improves hand function by neuroplasticity of the brain. A study reveals that the therapeutic effect of CIMT is independent of age. No differences were found between boys and girls for this therapy. CIMT is found to be effective in the literature to improve hand function; however, its effect on muscle tone and protective extension is yet to be investigated. Hand-arm intensive bimanual therapy is a similar technique that improves hand function; however, both hands need to be used in this technique. In a study of children with hemiplegic cerebral palsy, it is found that both of these strategies are promising techniques to improve hand function; however, the latter is more tolerable in children than CIMT. CIMT has also shown improvement in somatosensory functioning and neural processing in such children [46–49].

3.6.4. Management of Hip and Ankle Deformities. 36% of CP children suffer from hip disorders which lead to problems such as dislocation, subluxation, and other related problems which can be managed surgically. Hip surveillance programs are recommended to screen cases of hip deformities [40]. In younger children who cannot walk due to hip disorders, reconstructive procedures are useful as they provide long-term results; in cases of degeneration of the hip, reconstruction surgeries such as osteotomy or arthroplasty are done [41]. In ankle equines, the deformity is seen in cerebral palsy children. Orthotic devices can help improve the ankle range which is beneficial in improving the gait of the child. Specific types of AFOs improve joint function and gait parameters. AFOs reduce energy expenditure in children with spastic CP. The HKAFO is very helpful in improving gait parameters and is evident in energy conservation in hemiplegic CP children. Further studies are required for better evidence regarding this [50–52].

3.7. Management of Associated Problems of CP

3.7.1. Epilepsy. Epilepsy is a common comorbidity seen in CP children. Children who are grouped under the quadriplegic category with microcephaly often present with seizures. Various systemic and postzygotic genetic mutations are thought to be responsible for neonatal epilepsies. Levetiracetam, valproic acid, topiramate, phenobarbital, levetiracetam phenobarbital, vigabatrin, lamotrigine, clonazepam, clobazam, and gabapentin are choice of drugs for epilepsy in CP

children. Polytherapy and monotherapy are compared using the first-line and second-line antiepileptic drugs; it is found that polyepileptic is more effective though full control of the seizures is not always achieved [53–55].

3.7.2. Osteoporosis. Osteoporosis causes weak bones. It causes the bone to break easily with very little stress or a light impact fall. It is often seen in CP patients due to lack of nutrition, decreased weight bearing, and use of certain medicines that causes weakening of the bones. Older patients are advised for screening bone mass before treatment using the fracture risk assessment tool or the Q fracture tool and dual-energy X-ray absorptiometry. Calcium, vitamin D supplements, and bisphosphonates are useful in managing osteoporosis [10, 46]. Weight-bearing exercises are found to improve bone mineral density in cerebral palsy children and hence need to be implemented to improve bone conditions [55].

3.7.3. Behavior Issues. Various behavior disorders are prevalent in CP children. They suffer from conditions such as attention-deficit/hyperactivity disorder, conduct disorders, anxiety, and depression. Cognitive behavior therapy and mental health screening help patients identify and manage behavior issues [10]. "Cool Kids" is a CBT program that is used to manage behavior problems like anxiety and ADHD children. It has been a promising anxiety management program that has been accepted internationally with several clinical trials that have proven to be effective. Experienced psychologists are required to provide the treatment that helps the child and parent develop skills and strategies to manage anxiety and associated impairments [56].

3.7.4. Dysphasia. Swallowing disorders are common in CP children as they occur due to neurological involvement. Its treatment consists of oral care, careful feeding techniques, food modifications, and stimulation of the oral musculature. Drooling occurs in CP children due to weakness of facial muscles and neck muscles. It can be managed with neck posture control, mouth closing, tongue control, behavioral therapies, intraoral appliances, and certain medications like anticholinergic drugs beneficial for this condition. Surgical management includes removal of the salivary glands and duct ligation [57].

3.7.5. Respiratory Problems. Respiratory problems are often seen in CP children which are the main cause of death in adults struggling with this disease. Due to factors such as weakness of the muscles, bad posture, and bad postural control, there is the accidental entry of food particles into the respiratory system which sometimes leads to bacterial growth causing respiratory failures. Management of such conditions includes lifestyle modifications such as postural modifications, food modifications, and weight loss. Improvement of motor functions and respiratory hygiene include improving lung functions, improving lung expansion and aerobic fitness along with airway clearance, and producing effective cough [47]. Oromotor techniques such as sensory awareness training, neck control exercises, general postural management, certain medications, and surgical interventions such as duct transposition and duct ligation are important strategies for managing feeding and swallowing problems [58].

3.7.6. Vision Problems. Abnormal brain development or damage to the brain results in cerebral visual impairment (CVI) previously known as cortical blindness which presents with visual deficits and perceptual deficits. The CVI inventory and assessment are used to diagnose the functional deficits that occur due to cerebral visual impairment [59]. Treatment of hypoxic-ischemic encephalopathy can prevent incidences of CVI, and other treatment approaches like visual stimulation techniques and stem cell therapy need to be assessed further [60]. Vision impairment may be an important aftereffect of brain damage and especially in those born preterm. Some probable aspects that can help these children are family counseling and involving the family in the rehabilitation process and various welfare services from the government like education allowances, special books, scholarship, readers, permission to use assistive devices, large print question paper, scribe for writing examination, extra time in the examination, and substituting visual questions [61].

3.7.7. Sleep Disorders. Sleep disorders are very common in CP children which produces a huge psychological burden on their families. Sleep disorders also cause decreased function. Apart from this, sleep disorders lead to behavioral changes which cause functional problems in the body structure and affect the quality of life of the patient and family. A systematic review was conducted in 2021 to gather information on sleep disorders of CP children under 2 years which found polysomnography as a good assessment technique for CP children. Treatment includes cannabis, surgical interventions, and stimulation of the sensory system [62, 63].

3.8. Various Approaches Used in the Management of Cerebral Palsy

3.8.1. Physiotherapy. Physiotherapy has provided great achievement in the field of cerebral palsy. It helps in improving the muscle structure and function and joint range of motion and reduces contractures; some techniques used to achieve this are muscle stretching, joint range of motion exercises, low resistance repetitive exercises, progressive resistance training, functional strength training, balance training, plyometrics, and selective muscle activation by techniques such as constraint-induced movement therapy. A study was done on the effects of neurodevelopmental therapy in CP children which revealed improved function in various activities of children after the application of the intervention technique. NDT also reduced spasticity and improved overall function in CP children; however, there was not much improvement in walking, running, and jumping [8, 10, 17, 21, 64]. Another emergent therapy called hippotherapy has improved neck control and posture control in sitting along with the upper extremity and trunk. There is an overall posture improvement due to stimulation of balance reactions which has a positive effect on balance and spasticity. 30–45 min sessions, twice weekly for 8–12 weeks, produce a positive effect on gross motor function in

children with CP [18]. Deep brain stimulation in the case of dyskinetic CP and electrical stimulation via tens and NMES in spastic CP are two techniques to improve the strength and function of muscles [8]. Serial casting is a technique used to stretch tight muscles to improve the range of motion by application of a cast to the affected part [10]. The robot-assisted gait training regimen is effective in improving gross motor function in children whose both sides are affected. There was a positive effect on all the measures of gross motor function after this intervention. It also improved the locomotor ability in ambulatory children [19]. Functional gait training or practice walking on a treadmill with limited body weight support helps in standing erect with a decreased load on the lower extremity joint. This facilitates gait training with good posture and control and is most effective in GMFCS grades IV and V. It can be done with or without a treadmill. Virtual reality and biofeedback can be incorporated; it produces a positive effect [19, 20]. Biofeedback is a common strategy used in rehabilitation that can be used to represent any biological parameters and their changes. The changes can be detected in a variety of ways such as visual, audio, and haptic responses. It is effective in improving motor function by identifying effective motor performance and motor learning [65].

3.8.2. Speech Therapy. There are multiple levels of speech impairment in cerebral palsy children which includes problems such as drooling, swallowing, and feeding having a high rate of 44.0%, 50.4%, and 53.5%, respectively, and as much as half of the children with vertebral palsy are affected with speech problems. Due to the abnormal tone of CP children and impaired musculoskeletal control, speech production and swallowing are difficult in these children. Speech therapy for such conditions helps improve oromotor skills, disarticulation problems, and communication skills [57].

3.8.3. Stem Cell Therapy. Stem cells derived from five areas are present in the literature; they are human umbilical cord blood which is the most common followed by the bone marrow, fetal brain, adipose, and peripheral blood. Autologous stem cells are preferred for children with CP as it shows low immunogenicity. Brain tissue has the maximum amount of neural stem cells, but they have many fewer clinical trials. Peripheral blood cells are highly used stem cells; however, adipose tissue effectiveness is under investigation. Greater improvement was seen in younger children between 10 months and 10 years old. The most efficient route for the administration of stem cells found in the literature is lumbar puncture and intravenous lines; however, it is seen that the prognosis is different in different cases of CP about the administration of stem cells. Apart from this, the effectiveness of the therapy also depends upon the appropriate dose of stem cells. Side effects found in studies with stem cells were fever, nausea, vomiting, and pain at the site of injection, particularly as the lumbar puncture is the preferred method. Hypotonicity is another side effect observed in stem cell therapy using bone marrow. Fine motor function improvement is less compared to gross motor; however, fewer studies have been done in this context [63].

3.9. Technological Advances in Cerebral Palsy

3.9.1. Robot-Assisted Devices. Robotics is a novel technique that works on a computerized control system and helps in motor learning and cortical reorganization to improve function in the upper and lower limbs. As functional movements are more fruitful than normal movement patterns, it is found that gait rehabilitation has a more positive impact on lower limb function. With advanced technology, robot-assisted gait training has taken over traditional gait rehabilitation. RAGT is beneficial as it works with increased duration, repetition, constant speed, and pattern. Lower limb robotic exoskeletons are found to be evident in improving the quality of life in CP children. The most evident robotic systems in the literature are Lokomat, Innowalk, Robogait, and Waltbox-K, but due to lack of literature, their efficacy is still a question. It is found that ankle foot orthosis is most beneficial for lower limbs in CP children, and an electronic variance of such device is scarce. There are many clinical trials, but review studies are lacking in the literature. Extensive studies are required for upper limb robotic assistance. Apart from these, social robots are also another milestone in artificial intelligence that has to improve communication and participation among CP children along with motivation in rehabilitation [66, 67]. Some of such major technological advances are described in Table 8.

3.9.2. Virtual Reality. Virtual reality is a recent development in the field of neurorehabilitation that induces imaginations as real as reality, and patients are allowed to perform functional activities in such environments. It was developed in the 1960s and is used as a diagnostic tool in certain psychiatric cases. Many clinical trials are being conducted on VR among stroke patients, COPD patients, and most recently in obstetric and gynaec cases. Though VR is used as a pain management strategy in CP patients, functional outcome studies are scarce in this population [68].

3.9.3. Augmentative and Alternative Communication Devices. The communication problem is found to be present in 25% of cases of cerebral palsy children, and most of this population has some or other oromotor problems. Augmentative and alternative communication devices are used to improve the communication abilities of speech-impaired children. It helps develop a communication pattern among the CP child and various other members of the community. There are certain manual boards used in this strategy that can be used in the form of figures, number symbols, etc. Other AAC devices used with speech and language problems are some form of technological device that helps to expose the thinking of the child. All studies with AAC shows a good result, but very less studies are done on CP children [69].

3.9.4. Mobile Applications for Cerebral Palsy Children. The use of mobile applications has drastically changed the scenario of the health care delivery system. These apps are reliable and valid and have become very common and handy that are beneficial in transferring information, forming analysis, and monitoring and treatment. 23 mobile applications

Table 8: A statistical analysis of technological advancement in cerebral palsy management from 2016 to 2022.

Author (year)	Title	Summary
A Shierk et al. (2016)	Review of therapeutic interventions for the upper limb classified by manual ability in children with cerebral palsy	There was some form of improvement in the hands using various intervention techniques but only in MAC levels II and III. Extensive studies are required in levels IV and V [70].
Atefehaboutorabi et al. (2017)	Efficacy of ankle foot orthoses types on walking in children with cerebral palsy: a systematic review	Specific types of orthosis improve ankle and knee range of motion, walking speed, and stride length in CP children. The reduced energy expenditure was found to be effective in improving stride length, speed of walking, single limb support, and gait symmetry; it also helped in decreasing energy expenditure of hemiplegic CP as compared with the barefoot condition. Further studies are required for better evidence regarding this [71].
Anna Alves Pinto (2016)	The case for musical instrument training in cerebral palsy for neurorehabilitation	The study inferred that playing musical instruments may help produce plastic changes in the brain for developing skills of CP children [72].
Adam T C Booth et al. (2018)	The efficacy of functional gait training in children and young adults with cerebral palsy: a systematic review and meta-analysis	Functional gait using a treadmill with little body weight support helps present an upright posture and improves gait. The authors suggested that virtual reality and biofeedback improve function [73].
Ali Reza Jamali (2018)	The effects of constraint-induced movement therapy on functions of cerebral palsy children	The therapeutic effect of CIMT is independent of age and gender, but its effect on muscle tone and protective extension is yet to be investigated [48].
Atefehaboutorabi et al. (2017)	Efficacy of ankle foot orthoses types on walking in children with cerebral palsy: a systematic review	Specific types of orthosis improve ankle and knee range of motion, walking speed, and stride length in CP children. They reduce energy expenditure and were found to be effective in improving stride length, speed of walking, single limb support, and gait symmetry; it also helped in decreasing energy expenditure of hemiplegic CP as compared with the barefoot condition. Further studies are required for better evidence regarding this [74].
Alexendermaclntosh et al. (2019)	Biofeedback interventions for individuals with cerebral palsy: a systematic review	Biofeedback interventions will help improve movement patterns in cerebral palsy children; however, poor quality and quantity studies are hindering finding the actual efficacy of the technique [65].
Ana Paula Salazar et al. (2019)	Neuromuscular electrical stimulation to improve gross motor function in children with cerebral palsy: a meta-analysis	NMES improves gross motor function in children with CP. However, it was found effective to improve GMFM-sitting and standing dimensions but not GMFM-walking dimensions; however, the literature found was of low quality [75].
Anna Tevelde (2019)	Early diagnosis and classification of cerebral palsy: an historical perspective and barriers to an early diagnosis	A timeline of calls for early diagnosis is described in the paper. Reduction of age for diagnosis and factors that cause difficulty in diagnosis are evaluated [76].
Cihanuyanik et al. (2022)	Brainy home: a virtual smart home and wheelchair control application powered by brain-computer interface	BCI is being used as a smart technology in normal settings; however, it is very rarely used in disabled populations. This study is done in a virtual setup among the disabled population and reveals that it will be an effective means of improving communication and functional status among this population shortly in the real world [77].
David Graham (2019)	Current thinking in the health care management of children with cerebral palsy	This paper focuses on early diagnosis and treatment to be an effective management strategy for CP. It also discusses various techniques of management like oromotor stimulation, deep brain stimulation, and functional classification of CP children [37].

Table 8: Continued.

Author (year)	Title	Summary
Ewelina Matusiak-Wieczorek et al. (2019)	The influence of hippotherapy on the body posture in a sitting position among children with cerebral palsy	Hippotherapy has a positive effect on head position, arm function, and trunk control in mild cerebral palsy children. The study concluded that hippotherapy has a positive influence on body posture and the function of different body structures in sitting positions [78].
Eli Kinney-Lang et al. (2022)	Advancing brain-computer interface applications for severely disabled children through a multidisciplinary national network: summary of the inaugural pediatric BCI Canada meeting	BCI helps children with disabilities to communicate with their thoughts, and those who are cognitively sound children can benefit from this new technology. However, as this is a young field, very fewer studies are done, so it is recommended that various researches using BCI shall be conducted in different populations and different geographical locations [79].
Hussein ZA et al. (2019)	Effect of simultaneous proprioceptive-visual feedback on the gait of children with spastic diplegic cerebral palsy	A 3 times/week treatment for 2 months with simultaneous proprioceptive and visual feedback resulted in significant differences in spatial and temporal parameters of gait; however, there were fewer effects on kinetic gait parameters [80].
Hongyuchen et al. (2016)	A review of wearable sensor systems for monitoring body movements of neonates	The study focuses mainly on the use of wearable sensors for body movements for detecting body movements in very young children as movements in babies give an idea about the level of brain development and brain damage [81].
Irene Mall et al. (2017)	Functional electrical stimulation of the ankle dorsiflexors during walking in spastic cerebral palsy: a systematic review	FES can be a helpful adjunct as a replacement for orthosis in lower limbs of cerebral palsy children though it needs extensive studies in the future for improving the state of spastic muscles [82].
Iona Novak et al. (2017)	Early, accurate diagnosis and early intervention in cerebral palsy: advances in diagnosis and treatment	Diagnosis and prediction of cerebral palsy age have been reduced from 12 months to as low as 5 months using various advances like HINE, PQAGM, and MRI [83].
Jakub Mlodawsk et al. (2019)	Cerebral palsy and obstetric-neonatological interventions	Various strategies to prevent brain damage before birth were discussed along with a focus on risk factors during delivery and after delivery. Indications of hypothermia are explained in detail [84].
Jing Zhang (2017)	Multivariate analysis and machine learning in cerebral palsy research	This study briefs out the use of machine learning and multivariate analysis in predicting developing brain damage. Its future implications include detecting and management of cerebral palsy using the same [85].
Joao Pedroproenka (2017)	Serious games for upper limb rehabilitation: a systematic review	The study found that computer games are an emerging technique to improve upper limb function in cerebral palsy children; however, extensive search is required to improve its efficacy [86].
Jessica Rose et al. (2017)	Artificial walking: technologies to improve gait in cerebral palsy: multichannel neuromuscular stimulation	The study focuses on the use of neuromuscular electrical stimulation as it has much more benefits than traditional medical and surgical techniques to improve the walking pattern of hypertonic CP children [87].
Jun Wang et al. (2018)	Effect of suspension exercise training on motor and balance functions in children with spastic cerebral palsy	Motor functions and balance improve with suspension exercise training in plastic cerebral palsy [88].
Jyoti Upadhyay (2020)	Cerebral palsy: aetiology, pathophysiology, and therapeutic interventions	Common causative agents of cerebral palsy before, during, and after delivery are discussed, and the use of a new technology called electrical stimulation to improve muscle strength along with deep brain stimulation is on the verge of therapy for CP patients [8].
Li Hua Jin et al. (2020)	The effect of robot-assisted gait training on locomotor function and functional capability for daily activities in children with cerebral palsy: a single-blinded, randomized cross-over trial	RAGT improves walking ability and improves the activities of daily living. Better effects were seen in children who can walk with support [89].

Table 8: Continued.

Author (year)	Title	Summary
Michael T Clarke et al. (2016)	Augmentative and alternative communication for children with cerebral palsy	This study throws light on the use of AAC strategies using sign language and various other ways to improve communication and language among speech and learning impaired cerebral palsy children [20].
Masahito Mihara et al. (2016)	Review of functional near-infrared spectroscopy in neurorehabilitation	This review concluded that NIRS is an emerging investigation tool and needs further follow-ups to use it as a therapeutic modality [90].
Mary M Rodgers et al. (2019)	Wearable technologies for active living and rehabilitation: current research challenges and future opportunities	Wearable technologies are being used to improve functional status. However, it is used for a short period in all the evident studies. This study urges to find the efficacy of these devices when used for a longer duration. There are certain barriers in this context such as the ability to use and comfort status of the wearable [91].
Mahindra Rana et al. (2017)	A systematic review on etiology, epidemiology, and treatment of cerebral palsy	Various classifications of CP are discussed. Factors that lead to brain damage and their prevention along with recent technological advances are focused on [14].
Moshe Stavsky et al. (2017)	Cerebral palsy—trends in epidemiology and recent development in prenatal mechanisms of disease, treatment, and prevention	The occurrence of CP has been stable for the last two decades, and the paper has discussed various strategies to prevent brain damage in the developing period. They also discussed the effects of various recent techniques [7].
Neha A. Parikh et al. (2019)	Early detection of cerebral palsy using sensorimotor tract biomarkers in very preterm infants	The use of biomarkers to evaluate the presence of brain damage is an effective technique as discussed in this paper [5].
Peter Wilson et al. (2016)	Integrating new technologies into the treatment of CP and DCD	The study discussed the effect of advanced techniques like virtual reality and its impacts on developmental disorders like CP and DCD [92].
Petra Karlsson (2022)	Brain-computer interface is a potential access method for communication in non-verbal children with cerebral palsy: a state-of-the-art review	This study focuses on the fact that BCI is a promising technology that is emerging very rapidly in the literature, and cerebral palsy children will be largely benefited from this technological advancement.
Qi Wang et al. (2017)	Interactive wearable systems for upper body rehabilitation: a systematic review	Wearable systems are important for various neurological disorders. They use sensors, accelerometers, and inertial measurement units for measuring improvement parameters in the upper limb [92].
Rocco Salvatore Calabro et al. (2016)	Robotic gait rehabilitation and substitution devices in neurological disorders: where are we now	This study has discussed in detail various robotic rehabilitation techniques that are used for various neurological cases including cerebral palsy to improve their walking abilities [93].
Shahshanchen et al. (2016)	Toward pervasive gait analysis with wearable sensors: a systematic review	This paper focuses on wearable sensors for the evaluation of gait kinetics and kinematics as the existing technologies like OGA and force plates are costly and need expertise [94].
Sonika Agarwal et al. (2021)	Cerebral palsy and rehabilitative care: the role of home-based care and family-centered approach	Home-based physiotherapy programs, telemedicine, and video monitoring of home-based therapies are found to be effective among these children [95].
Stanislava Klobucká et al.	Effect of robot-assisted gait training on motor functions in adolescent and young adult patients with bilateral spastic cerebral palsy: a randomized controlled trial	The robot-assisted gait training regimen is more effective than conventional therapy in terms of improvements in gross motor functions in adolescent and adult patients with bilateral spastic CP [96].
Tony W. Wilson et al. (2016)	Neuroimaging with magnetoencephalography: a dynamic view of brain pathophysiology	This study focuses on the use of MEG in processing neural information of the brain and to find out the abnormal neural information processing in cerebral palsy cases along with other neurological cases. It also implicates future studies in this context [97].

Table 8: Continued.

Author (year)	Title	Summary
Wei-Peng Teo (2016)	Does a combination of virtual reality, neuromodulation, and neuroimaging provide a comprehensive platform for neurorehabilitation? – A narrative review of the literature	The study focuses on the use of VR in combination with various other recent technologies for improving its effect on CP children. The study urges larger studies in the same context [98].
Yupigchen et al. (2018)	Effectiveness of virtual reality in children with cerebral palsy: a systematic review and meta-analysis of randomized controlled trials	The review found VR to be an effective technology in comparison to other techniques to improve movement in cases of brain damage [68].
Chen and Howard (2016)	Effects of robotic therapy on upper-extremity function in children with cerebral palsy: a systematic review	Various components of hand function improved using the robotic therapy; however, more studies are required with larger cohorts [99].
Zeannajadavji et al. (2021)	Can children with perinatal stroke use a simple brain-computer interface?	BCI is an emerging and promising technology that can help individuals with brain damage. Future studies are directed toward the effect of BCI among unilateral stroke due to early brain damage [100].
Zhong-Yue Lv et al. (2020)	Progress in clinical trials of stem cell therapy for cerebral palsy	Stem cells from human umbilical cord blood are the most common followed by bone marrow, fetal brain, adipose, and peripheral blood. Autologous stem cells are preferred for children with CP. Greater improvement was seen in younger children between 10 months and 10 years old. Lumbar puncture and intravenous injection are mostly used to insert the stem cells. Gross motor function and cognition abilities improve better with this treatment [101].

are specifically used for cerebral palsy children and many others that can be of importance. Some services of these apps are correcting foot deformities of CP children by producing an auditory signal during altered biomechanics in foot placement and risk assessment of hip dysplasia in CP children by health care specialists [25].

4. Conclusion

Injury to the developing brain before, during, and after birth causes various symptoms to evolve in a child; the condition is referred to as cerebral palsy. It affects the normal movement in different parts of the body along with problems such as abnormal resistance to movements, the attitude of the body, and movement and activity limitation, accompanied by various sensory disturbances along with perception, cognition, communication, behavior, epilepsy, and secondary musculoskeletal problems. The causes may be prenatal, perinatal, and postnatal. Certain risk factors are identified for damaging the brain that also include the health condition of the mother before conception. The prevalence of CP is found to be stable in various epidemiological studies owing to different preventative, neonatal care, and postnatal strategies. Brain damage during the developing period or immediately after birth can be detected by gathering information from the mother during events that have occurred before or during delivery and certain early signs in the child. Diagnostic tools are now available for the detection of cerebral palsy as early as less than 5 months. Based on the heterogeneity of the condition, various associated problems arise in the child along with motor disturbances which need to be assessed and managed by a multidisciplinary team of specialists. Along with the traditional management approaches of cerebral palsy, many emerging techniques are making advancements in research that have future scopes of advancement to give a pleasant and functional life to the affected children. A new view of cerebral palsy etiology is the recent development of genetic studies on cerebral palsy as there are cases found which have no prenatal, natal, or postnatal explanations. Research on these areas may be beneficial to find a correlation between genetics and cerebral palsy. Apart from this, artificial intelligence also directs future study and rehabilitation strategies to improve the functional status of cerebral palsy children.

Data Availability

The data underlying the results presented in the study are available within the manuscript.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Review Article

Targeting Molecular Mediators of Ferroptosis and Oxidative Stress for Neurological Disorders

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With the acceleration of population aging, nervous system diseases including Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), anxiety, depression, stroke, and traumatic brain injury (TBI) have become a huge burden on families and society. The mechanism of neurological disorders is complex, which also lacks effective treatment, so relevant research is required to solve these problems urgently. Given that oxidative stress-induced lipid peroxidation eventually leads to ferroptosis, both oxidative stress and ferroptosis are important mechanisms causing neurological disorders, targeting mediators of oxidative stress and ferroptosis have become a hot research direction at present. Our review provides a current view of the mechanisms underlying ferroptosis and oxidative stress participate in neurological disorders, the potential application of molecular mediators targeting ferroptosis and oxidative stress in neurological disorders. The target of molecular mediators or agents of oxidative stress and ferroptosis associated with neurological disorders, such as reactive oxygen species (ROS), nuclear factor erythroid 2–related factor-antioxidant response element (Nrf2-ARE), n-acetylcysteine (NAC), Fe^{2+}, NADPH, and its oxidases NOX, has been described in this article. Given that oxidative stress-induced ferroptosis plays a pivotal role in neurological disorders, further research on the mechanisms of ferroptosis caused by oxidative stress will help provide new targets for the treatment of neurological disorders.

1. Introduction

As a novel mode of regulated cell death (RCD), ferroptosis was firstly proposed by Dixon in 2012 [1]. Ferroptosis is an iron-dependent and nonapoptotic cell death mode, which is caused by excessive accumulation of ROS and imbalance of cell lipid oxide metabolism. In 2018, the Nomenclature Committee on Cell Death (NCCD) advised defining ferroptosis as RCD caused by oxidative stress in the cell microenvironment which can be regulated by glutathione peroxidase 4 (GPX4). Moreover, ferroptosis can be inhibited by iron chelators, iron intake inhibitors, and lipophilic antioxidants [2, 3]. Recent studies have shown that ferroptosis is obviously different from traditional programmed cell death. It has distinctive morphological features and biochemical features [4], such as morphological changes of mitochondrial, accumulation of iron, and lipid reactive oxygen species (ROS). At present, it is believed that the main cause of ferroptosis to cell death is the inactivation of the cellular antioxidant system, which includes the inhibition of cystine/glutamate antiporter system (system Xc^-) and GPX4, disrupted iron homeostasis, and lipid peroxidation. Ultimately, the decrease of antioxidant capacity and the accumulation of intracellular lipid ROS lead to oxidative cell death [5].

Although there are many studies about ferroptosis, the mechanism of ferroptosis is not clear yet. Ferroptosis was identified to involve in oxidative stress-induced cell death [6]. During the process of ferroptosis, cellular overaccumulation of ROS eventually generates oxidative stress and cell death. According to current research, the possible mechanisms of ferroptosis include oxidative stress, lipid peroxidation, iron metabolism, and other mechanisms to be explored. The process of oxidative stress in ferroptosis is complex that is regulated by a variety of regulatory factors.

Neurological disorders have become the major leading cause of disability and the second leading cause of death

worldwide [7]. Over the past few decades, due to the lack of effective precaution and treatment, the mortality and the disabled owing to neurological diseases have increased, particularly in low-income and middle-income countries [8]. The death and disability caused by neurological disorders have caused a huge burden on families and countries. Meanwhile, it has become a serious social problem. In order to alleviate the burden, there are increasing studies on how to treat or prevent the process of neurological disorders.

Mounting evidence has shown that oxidative stress-induced ferroptosis plays a significant role in neurological disorders [3]. Oxidative stress is both an important process of ferroptosis and the pathogenesis of neurological disorders. Hence, oxidative stress has become a critical linking between ferroptosis and neurological disorders. Up to now, the inhibitors and activators of ferroptosis [9–14], as well as other involved signaling pathways [15–17], have been widely studied. Furthermore, the mediators of oxidative stress might become a potential therapeutic target for the treatment of neurological disorders by regulating the process of ferroptosis. In this review, we will briefly summarize the mechanisms of ferroptosis, and highlight the role of targeting molecular mediators of oxidative stress in neurological disorders, so as to provide an option for the therapeutic application of ferroptosis in neurological disorders.

2. The Mechanism of Ferroptosis

Ferroptosis is a unique kind of nonapoptotic-regulated cell death, which is distinct from apoptosis, autophagy, and necrosis in morphology, biochemistry, and genetics. Iron metabolism and lipid peroxidation are two essential events in ferroptosis [2]. Additionally, system Xc^- and GPX4 are considered to be the primary signaling pathways [18]. Here, we summarize the main mechanisms involved in the process of ferroptosis, such as oxidative stress, lipid peroxidation, iron metabolism, and the signaling pathways (Figure 1).

2.1. Oxidative Stress in Ferroptosis. Oxidative stress was formulated in 1985 [19], which is an important cause leading to neurological disorders that mainly arise from the imbalance between depletion of antioxidants and production of peroxides [20]. Ferroptosis is a unique, oxidative stress-induced cell death pathway characterized by glutathione depletion and lipid peroxidation. Based on current research, ferroptosis can be regulated by system Xc^- [21]. System Xc^- can exchange glutamate and cystine inside and outside of the cell. Glutathione is an important free radical scavenger and antioxidant in vivo, which can be categorized as either reduced (GSH) or oxidized (GSSG). Intracellular cystine can be converted into reduced GSH by a series of biochemical reactions. GPX4 is a member of the glutathione peroxidase family which can convert GSH to GSSG, and GSH/GSSG constitutes an antioxidant system and provides reducing equivalents to eliminate oxidative species [22]. GPX4 is a redox enzyme and can also inhibit ferroptosis by decreasing the level of lipid peroxides. GPX4 plays a crucial role in reducing reactive aldehydes (PUFAs-OOH) to their alcohol form (PUFAs-OH) which can reduce the content of ROS [18].

ROS are some of the most common oxidants in cells. ROS includes superoxide ($O_2^-\bullet$), hydrogen peroxide (H_2O_2), lipid peroxides (ROOH), or the corresponding hydroxyl (HO•) and peroxyl radicals (ROO•) [23]. Accumulation of ROS can lead to oxidative stress, causing oxidative stress-induced lipid peroxidation. What is worse, lipid peroxidation may further generate ROS or degrade into reactive compounds capable of crosslinking DNA and proteins [24]. Oxidative stress-induced lipid peroxidation eventually leads to ferroptosis. Meanwhile, ferroptosis can be induced by oxidative stress directly (Figure 1).

2.2. Iron Metabolism in Ferroptosis. Metabolism is essential for the biochemical process in cells and goes awry in many diseases. Iron metabolism is an important mechanism of ferroptosis [2]. Iron is a significant trace element in the body [25]. Abnormal distribution and content of iron in the body can affect the normal physiological processes. Fe^{2+} is absorbed by the intestine or degraded by erythrocyte and then can be oxidized to Fe^{3+} by ceruloplasmin [26]. Fe^{3+} can combine with transferrin (Tf) on cytomembrane to form Fe^{3+}-Tf, which can be endocytosed into cells to form a complex with membrane protein TF receptor (Tfr) [26]. The complex enters the endosome and is divided into TF-TFR and Fe^{3+} [26]. Fe^{3+} is then reduced to Fe^{2+} by the six-transmembrane epithelial antigen of the prostate 3 (STEAP3), and Fe^{2+} is stored in the unstable iron pool (LIP) and ferritin [26]. Nevertheless, under pathological conditions, iron homeostasis is destroyed, and the overwhelming production of Fe^{2+} exceeds the limit, breaking cell homeostasis [27]. Excess Fe^{2+} can combine with H_2O_2, reducing H_2O_2 to hydroxyl radical (OH·) by Fenton reaction [28, 29], which was first described by Fenton [30], who in 1894 reported on the oxidation of malic acid by hydrogen peroxide in the presence of ferrous ions.

2.3. Role of Mitochondria in Ferroptosis. Mitochondria, through the tricarboxylic acid cycle (TCA) and electron transport chain (ETC) activity, are the principal sources of cellular energy production [31]. Meanwhile, ROS can be produced during the process of TCA which is an important cause of ferroptosis-induced lipid peroxides [32]. In addition, mitochondrial morphological changes are considered to be an important basis for the diagnosis of ferroptosis [1]. The change of mitochondrial membrane potential is also an important discovery in the study of ferroptosis [33, 34]. To date, abnormal mitochondrial architecture including mitochondrial fragmentation, rupture of mitochondrial outer membrane, and mitochondrial shrinkage, as well as vanished mitochondrial cristae, is regarded as the typical morphological characteristic of ferroptosis [1]. In summary, mitochondria play a significant role in ferroptosis [35], and molecular research targeting mitochondria is of great significance to intervene in the development of ferroptosis (Figure 1).

2.4. Other Mechanisms. As a novel form of cell death, the mechanism of ferroptosis is complex. Ferroptosis is regulated by numerous pathways and implicated in many

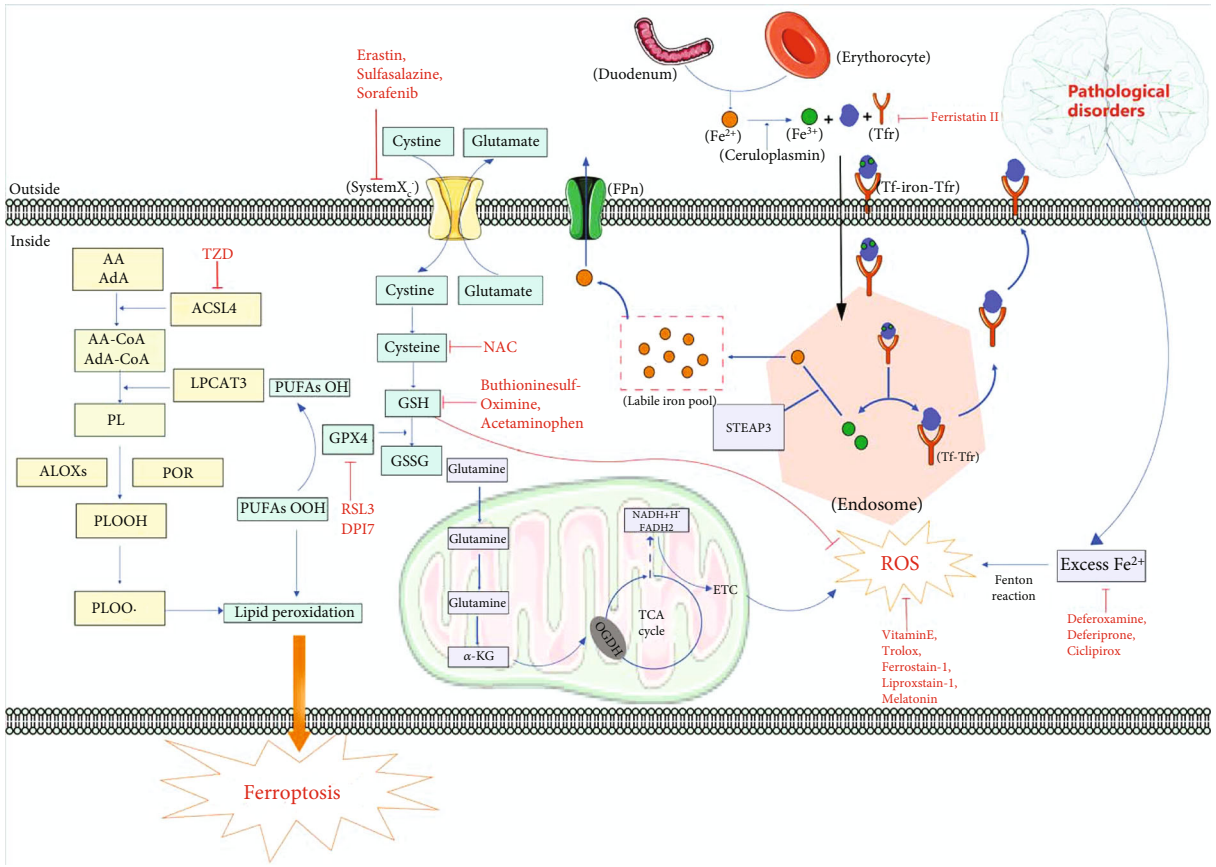


Figure 1: A schematic summary of ferroptosis mechanisms in neurological disorders. Lipid peroxidation and iron homeostasis are currently recognized as important mechanisms affecting ferroptosis. In cells, under the action of ACSL4, PRO, and ALOX, PUFA generates PLOOH through a series of biochemical reactions, which then generates PLOO, leading to lipid peroxidation and eventually ferroptosis. GPX4 can reduce PLOOH to PLOH to inhibit lipid peroxidation. In addition, GPX4 is regulated by the cofactor GSH. When GSH is exhausted, GPX4 will be inactivated. Glutamate and cystine generate GSH through systemX_C^- and GSH can be oxidized to GSSG. Mitochondria generate ROS through ETC in TCA cycle, which leads to oxidative stress and eventually ferroptosis. Fe^{2+} is oxidized to Fe^{3+} after being absorbed in the duodenum. Fe^{3+} enters the cell by combining with Tf and Tfr to form a complex. Iron ions decomposed from the endosome can leave the cell through FPN protein on the cell membrane, and other iron ions enter the unstable iron pool. Tf-Tfr complex leaves the cell for the next cycle. Under pathological conditions (neurological disorders), excessive Fe^{2+} will participate in Fenton reaction to produce a large amount of ROS, which will lead to ferroptosis. Abbreviations: AA: arachidonic acid; AdA: adrenic acid; ACLS4: acyl-CoA synthetase long chain family member 4; ALOXs: lipoxygenases; CoA: coenzyme A; DPI7: diphenyleneiodonium chloride7; ETC: electron transport chain; FPn: ferroportin; GPX4: glutathione peroxidase 4; GSH: glutathione; GSSG: oxidized glutathione; LPCAT3: lysophosphatidylcholine acyltransferase 3; OGDH: oxoglutarate dehydrogenase; PL: phospholipid; POR: cytochrome p450 oxidoreductase; PLOOH: phospholipid hydroperoxides; PUFA: polyunsaturated fatty acids; RSL3: (1S,3R)-RSL3; ROS: reactive oxygen species; STEAP3: STEAP family member 3; Tf: transferrin; Tfr: transferrin receptor; TZD: thiazolidinediones; TCA: tricarboxylic acid.

diseases. In addition to the mechanisms mentioned above, there are other possible mechanisms which have been proposed, such as the antioxidant mechanisms which include GPX4 pathway [36], FSP1 pathway [37], and other pathways. FSP1-NADPH-CoQ pathway [38] and nuclear factor erythroid 2-related factor 2 (Nrf2) pathway are potential regulatory pathways of ferroptosis [39]. Hence, the mechanism of ferroptosis remains to be explored.

3. Ferroptosis in Neurological Disorders

In recent years, the incidence of neurological disorders increases substantially. Because of the lack of effective treatment, sequelae caused by neurological disorders have brought an enormous burden on society. Ferroptosis has been proved to be closely related to the occurrence and development of various neurological disorders [40–43]. Therefore, figuring out the role of ferroptosis in neurological disorders will provide an important basis for the treatment of diseases (Figure 1, Table 1).

3.1. Ferroptosis in Parkinson's Disease. Parkinson's disease (PD), also called paralysis agitans, is the second most common neurodegenerative disorder worldwide. It is typical of neuronal death in the substantia nigra pars compacta (SNpc), which adjusts motor function. PD causes clinical symptoms such as resting tremor, rigidity, bradykinesia, postural instability, and other motor symptoms [44–46].

Table 1: Ferroptosis-induced effects or altered physiology in neurological disorders.

Object	Effect/physiological changes	Reference
Alzheimer's disease (AD) model	Induce neuronal death and memory impairment	[80]
Rat corticostriatal brain slices	Induce the oxidative destruction of PUFA	[94]
Cuprizone model	Induce oligodendrocyte loss and demyelination	[176]
In vivo TBI model/mice model with TBI	Induce inflammation and neuronal death	[177]
Transient cerebral ischemia model	Induce neuronal death	[178]
Type 1 diabetes rat model	Induce cognitive dysfunctions	[179]
Lund human mesencephalic cells/ mice model with PD	Induce dopaminergic cell death	[180]
Mitochondrion	Shrinkage of mitochondria with enhanced mitochondrial membrane density, mitochondrial volume reduction, vanishing of mitochondria crista, outer mitochondrial membrane rupture	[181]

However, its basic mechanism is not completely clear. Currently therapeutic arsenal mainly includes the application of the dopamine precursor levodopa (L-DOPA), dopamine metabolism inhibitors, and dopamine agonists [33], which only offer symptomatic relief. There still are not effective treatments for PD, so novel targets to improve therapeutic and diagnostic methods for PD patients are urgently needed. Iron is considered to be an important target of neurodegenerative diseases. Besides, iron metabolism is closely related to the pathogenesis of neurodegenerative diseases [34, 47]. Researchers found that patients with PD suffered from GSH depletion, ROS elevation, and lipid peroxidation [48–50]. Ferritin heavy chain 1 (FTH1), a main iron storage protein, can affect intracellular iron metabolism and then trigger ferroptosis. As an important ferroptosis-related protein, FTH1 is differentially expressed in rats with PD compared with normal rats. Overexpression of *FTH1* can reduce the effect of ferroptosis in PD-related cells [51]. These findings indicate that ferroptosis may be used as a therapeutic target for PD [52, 53]. Neuroimaging and postmortem examination report that much iron accumulation in substantia nigra (SN) results in an increase in iron content in the residual dopaminergic neurons [54]. α-Synuclein causes iron cytotoxicity by acting on mitochondria in the neurodegeneration of PD. A study found that cyclosporine A, a blocker of mitochondrial permeability transition pore (mPTP), could prevent iron-induced mitochondrial damage and cell death. Furthermore, knocking down α-synuclein expression by siRNA can play a similar role [55]. α-Synuclein is the main component of Lewy bodies and is abundantly expressed in the nervous system, which is strongly related to PD's pathophysiology [56]. Besides, it has been recently shown that α-synuclein causes lipid peroxidation by producing ROS, leading to increased calcium influx and consequent cell death [57]. Ferroptosis inhibitors like ferrostatin or iron chelators [58] can inhibit cell death caused by the above method, supporting the hypothesis that ferroptosis participates in this process and may harbor therapeutic potential. Studies have also shown that ferroptosis occurs earlier than apoptosis in the development of PD and ferric ammonium citrate (FAC)-induced ferroptosis was dependent on p53, not MAPK signaling pathway [54].

3.2. Ferroptosis in Alzheimer's Disease. Alzheimer's disease (AD) is one of the most common neurodegenerative diseases worldwide. Pathological features of AD include cerebral atrophy, intraneuronal accumulation of hyperphosphorylated tau in neurofibrillary tangles, extracellular deposition of amyloid-β peptide in senile plaques, oxidative stress, chronic inflammation, and loss of neurons and synapses [59]. There is evidence that iron excess and homeostasis disorder can lead to neurodegeneration of AD [60, 61]. The increase of brain iron content in patients with AD has been confirmed in many studies [62–67]. When brain iron regulation in AD patients is disturbed, excess Fe^{2+} can not only produce hydroxyl radicals in Fenton reaction but also induce neuroinflammation, resulting in oxidative stress and neurodegeneration mediated by ferroptosis [68–72]. The research found that the increase of ferritin light chain (FTL) is related to the decrease of GPX4 level in AD which indicates that dysfunctional ferritin will reduce the antioxidant capacity of brain and the level of glutathione will decrease in AD patients [73]. Increased light subunit (xCT) expression in cells of patients with AD [74] and xCT can be upregulated by Nrf2 [75, 76]. Furthermore, mice with a conditional deletion of GPX4 show cognitive impairment and hippocampal degeneration similar to patients with AD and have the neurodegenerative characteristics of ferroptosis. Simultaneously, these symptoms can be improved by ferroptosis inhibitors [77], which provide a basis for the association between AD and ferroptosis. Recently, researchers observed that tau can stabilize ferroportin (FPN1) which is the only iron export channel found in mammalian cells [78, 79]. FPN decreased significantly in the brain tissue of AD patients. According to MMSE (mini-mental state examination) scores, FPN is involved in the cognitive impairment and brain atrophy of AD [80]. In patients with AD and a mouse model of AD, NADPH oxidases 4 (NOX4) causes lipid peroxidation and promotes ferroptosis of astrocytes via the damage of mitochondrial metabolism in AD [81]. Bhatia et al. [82] summarized a variety of multitarget targeted ligands with iron chelation properties, which is potentially useful in AD. Ashraf et al. [83] summarized the clinical cases involving iron chelators, antioxidants, NAC, and selenium in the treatment of AD and provided a feasible basis for the treatment of AD.

These studies have shown that ferroptosis plays an important role in AD, in which a variety of regulatory factors are involved, and provide many potential therapeutic targets for the treatment of AD.

3.3. Ferroptosis in Huntington's Disease. Huntington's disease (HD) is an autosomal dominant, late-onset, and fatal neurodegenerative disorder [84], which is caused by an abnormal CAG repeat in the huntingtin gene [85]. It is characterized by highly selective striatal injury, leading to dance-like movement, progressive dementia, and dystonia. In 2001, Pigeon et al. [86] first identified the association between hepcidin and iron homeostasis. Studies found that hepcidin could promote ferroptosis through iron metabolism [87] and could prevent erastin-induced ferroptosis by degrading Fpn [88]. Qian et al. [89] summarized the therapeutic potential of hepcidin in neurodegenerative diseases, including HD, indicating that there is a link between ferroptosis and HD. A study found that there is an accumulation of toxic iron in neurons of mouse model with HD and the symptoms of HD can be alleviated by deferoxamine, indicating that the accumulation of iron may contribute to the neurodegenerative process [90]. Studies have shown that HD patients will cause oxidative stress and neurotoxicity to neurons in the striatum, resulting in neuronal death, and eventually leading to motor and cognitive impairment [91]. HD patients have shown higher levels of plasma lipid peroxidation and lower GSH levels [92]. Oxidative stress and lipid peroxidation are important mechanisms leading to ferroptosis. In 2017, Cardoso et al. [93] proposed that as a key factor in regulating ferroptosis, GPX4 could provide protective mechanisms against neurodegeneration. Skouta et al. [94] found that ferroptosis inhibitor Fer-1 and its derivatives can prevent cell death in HD brain slice model. Mi et al. [85] summarized the evidence of ferroptosis involved in HD in different animal models or human patients. It revealed the close connection between ferroptosis and HD which would provide an important potential target for the treatment of HD.

3.4. Ferroptosis in Anxiety and Depression. With the development of society, the incidence rate of anxiety and depression is increasing year by year. The research on the mechanism and the treatment of anxiety and depression have also attracted extensive attention. Jiao et al. [95] found that the contents of total iron and ferrous ion in the hippocampus of chronic unpredictable mild stress (CUMS) model mice increased, and GPX4, FTH1, ACSl4, and COX-2 also changed significantly, which proved the evidence of ferroptosis in the hippocampus of the depression mouse model. Previous research reported that sodium hydrosulfide (NaHS) can alleviate the depressive and anxiety-like behaviors induced by type 1 diabetes mellitus (T1DM) [96]. Wang et al. [97] revealed that NaHS reduced ferroptosis in the prefrontal cortex (PFC) of the T1DM mouse model by reducing iron deposition and oxidative stress, increasing the expression of GPX4 and SLC7A11, thus alleviating the anxiety-like and depression-like behavior of the mouse model with T1DM. Moreover, there is increasing evidence that oxidative stress has close contact with anxiety and depression [98, 99]. As a free radical scavenger [100], Edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one, EDA) can improve depression and anxiety-like behavior, as well as inhibit oxidative stress and neuroinflammation in a mouse model of depression, and knocking down of GPX4 expression in CSDs mouse model can inhibit the therapeutic effect of EDA on depression and anxiety [101]. The study has shown that GPX4 can affect the role of EDA in mouse models of anxiety and depression, which suggests that GPX4-mediated ferroptosis may be a potential mechanism affecting anxiety and depression.

3.5. Ferroptosis in Stroke. Stroke is mainly divided into ischemic stroke and intracerebral hemorrhage (ICH) stroke, the former accounting for a higher proportion [102]. Zhang et al. [103] summarized the multiple roles of ferroptosis in stroke. Jin et al. [104] reviewed some neuroprotectants that show protective effects in stroke models. These substances have been recently validated as ferroptosis inhibitors. These researches indicate that ferroptosis plays a key role in the progression and toxicity of stroke. Chen et al. [105] analyzed and identified ferroptosis-related differentially expressed genes (DEGs) in ischemic stroke by bioinformatics, which provided more evidence for the important role of ferroptosis in ischemic stroke. NAC is considered as a potential therapeutic agent of ferroptosis. Recently, studies have found that NAC can reduce neuronal death and improve functional recovery in mouse models with ICH by inhibiting ferroptosis [106]. Clinical trials and experimental studies have reported that treatment with natural compounds could reduce oxidative stress in ischemic stroke. [107]. Therefore, reducing the production of ROS during reperfusion is the key to the treatment of ischemic stroke. Guan et al. found that natural product carvacrol can increase the expression of GPX4 to inhibit ferroptosis, protecting the cognitive function of mice with ischemia-reperfusion injury [108]. In addition, Cui et al. revealed that ACSL4 can exacerbate ischemic stroke by promoting ferroptosis-induced brain injury and neuroinflammation [109]. The incidence rate of ICH is low, but the deformity and mortality rates are significantly higher than ischemic stroke [110]. ICH can lead to the rupture of blood vessels in the brain and release red blood cells to produce a large amount of free iron and ROS, which result in oxidative stress and ultimately neuronal damage [111]. Iron chelator deferoxamine (DFO) can effectively alleviate ICH-induced neuronal injury in mice [112], which also provides evidence for the above process. The same characteristics of ferroptosis were observed in the animal model of ICH [113]. Furthermore, selenium can drive adaptive transcription against ferroptosis to protect neurons and provide direction for the treatment of stroke [114].

3.6. Ferroptosis in TBI. As one of the acute central nervous system (CNS) trauma [115], traumatic brain injury (TBI) is a major cause of death and disability worldwide [116]. Owing to the difficulty of treatment and serious sequelae, TBI has always been a huge problem in the medical field and caused a huge burden on families and society [41]. Analyzing the mouse TBI model [117] and clinical cases with TBI [118], abnormal regulation of ferroptosis after TBI was found both in experimental model and clinical cases.

Interestingly, Xie et al. found that the content of iron in damaged cortical cells increased, the function of iron metabolism was impaired, lipid ROS accumulation was increased, and mitochondrial atrophy was enhanced. The above symptoms can be alleviated by intraventricular administration of ferroptosis inhibitor Fer-1 in a mouse model with TBI [119], which proves that ferroptosis is one of the causes of TBI. Previous studies have shown that mir-212-5p is highly expressed in the brain [120] and is associated with a variety of neurological diseases [77, 119, 121, 122]. The use of mir-212-5p in the CCI mouse model can target PTGS2 to reduce ferroptosis and protect the brain [123]. Moreover, Cheng et al. [124] found that the use of iron uptake inhibitor ferristatin II in mouse model with TBI can inhibit the formation of iron proteasomes and alleviate TBI injury in brain. For a long time, due to the lack of clear understanding, the treatment of TBI is very difficult. These studies provide a potential theoretical basis for the treatment of TBI.

4. Mediators of Oxidative Stress in Neurological Disorders

Oxidative stress can spread, which aggravates the production of ROS. Due to the high oxygen demand and high energy demand of the brain, but its antioxidant capacity is weak, it is more vulnerable to oxidative stress [125], which will lead to various neurological diseases. Therefore, inhibiting or weakening oxidative stress might effectively reduce the damage to the nervous system. Studies for targeting molecular mediators of oxidative stress are considerable and will provide effective treatment for neurological disorders (Figure 2).

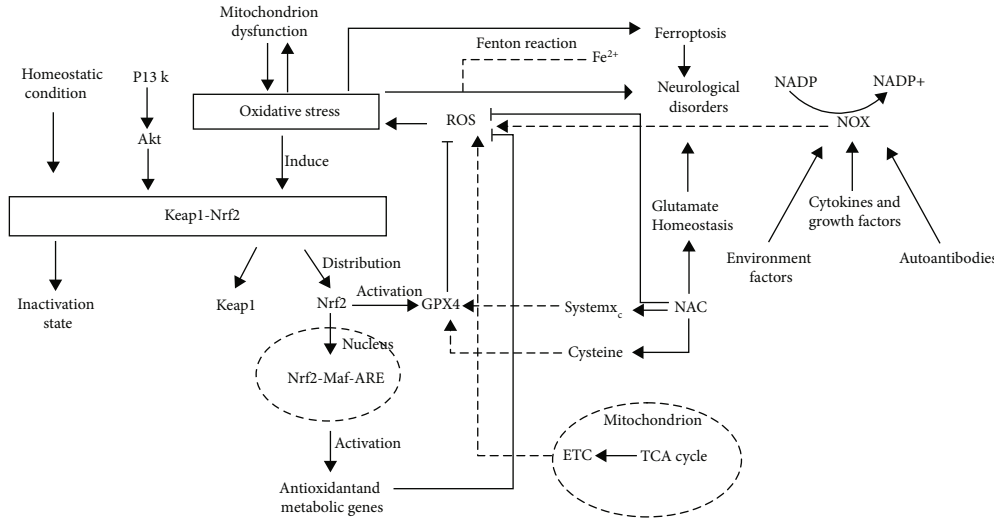
4.1. Nrf2-ARE-Mediated Oxidative Stress. Nuclear factor erythroid 2–related factor (Nrf2) is a transcription factor belonging to the basic leucine zipper (bZIP) transcription factor family that can participate in the regulation of oxidative stress. The antioxidant capacity of Nrf2 works by inducing glutathione biosynthesis [126]. Studies found that Nrf2 can play a neuroprotective role in central nervous system (CNS) diseases [127–129]. In addition to the transcriptional regulation of Nrf2, current hot research directions for the activity of Nrf2 are Kelch-like ECH-associated protein 1 (Keap1)/Nrf2/antioxidant response element (ARE) pathway. Keap1/Nrf2/ARE can be divided into two parts, one in the cytoplasm and the other in the nucleus. Normally, Keap1 and Nrf2 bind in the cytoplasm and are in an inactive state. If they are not activated all the time, Nrf2 will be ubiquitinated and then degraded. Under certain stimulation, the binding of keap1-nrf2 will be unstable. Nrf2 will be released, transferred to the nucleus, combined with ARE, activate the transcription of downstream genes, and then translate a series of related proteins, which can induce various antioxidant enzymes, including heme oxygenase 1 (HO-1), NAD (P) H: quinone oxidoreductase 1 (NQO1), superoxide dismutase (SOD), glutathione peroxidase (GPX), and catalase (CAT). These enzymes can play physiological functions [130]. Targeted regulators of Nrf2 are of great significance in the treatment of oxidative stress-induced injury in neurological disorders. Nrf2 plays a neuroprotective role by regulating the antioxidant system of cells. The regulation will have an impact on glutathione (GSH), thioredoxin (TXN), NADPH, and various other ROS-related enzymes [131, 132].

Keap1/Nrf2/ARE pathway has been proved to be an important antioxidant target. Keap1 can perceive ROS levels through cysteine-rich regions [133–135]. In the steady state, Nrf2 in the cytoplasm can be degraded by Keap1, and Nrf2 increases rapidly under stress and transfers to the nucleus for transcriptional activity [136]. Mitoquinone (MitoQ) is a mitochondrial-targeted antioxidant and can cause a sharp decline in Keap1 in cells, prompting more Nrf2 to be transmitted to the nucleus to produce an antioxidant response. The effect was confirmed in subarachnoid hemorrhage (SAH) animal model experiments [137]. Nrf2 can also play a protective role as a therapeutic target in astrocytes. There are seven functional domains of Nrf2 (neh1-neh7) that regulate its transcriptional activity and stability [138]. Kelch-like ECH-associated protein 1 (Keap1) is an inhibitor of Nrf2 [139, 140] and interacts with Nrf2 through neh2 domain [141]. Early studies believed that Keap1 inhibition was the main mechanism to activate astrocytes. Recent studies have found that mild oxidative stress (nonlethal oxidative stress) can also activate Nrf2 independently through neh5 transactivation domain [142]. In addition to the various regulatory factors mentioned above, there are other Keap1/Nrf2 pathway regulators such as microRNA-592 (miR-592) [143], eriodictyol [144], and tripartite motif 16 (TRIM16) [145]. These regulatory factors can act on Keap1/Nrf2/ARE pathway to play the role of antioxidant stress, so as to treat or alleviate neurological disorders (Figure 2).

4.2. NADPH- and NOX-Mediated Oxidative Stress. Nicotinamide adenine dinucleotide-phosphate (NADPH) is a coenzyme, which is the product of the final electron acceptor NADP + after receiving electrons. It participates in a variety of biochemical reactions as a hydrogen transmitter in vivo. Studies have confirmed that NADPH can resist oxidative stress and improve energy metabolism, so as to play a neuroprotective role in stroke [146]. NADPH oxidases (NOXs) have 7 kinds of subtypes.

H_2O_2 can interact with mutant huntingtin in the brain of HD patients [147]. The activity of NOX can be measured by lucigenin-enhanced luminescence method [148]. The heterozygous huntingtin (HTT) and Cd can induce cytotoxicity which will cause NOXs-mediated oxidative stress. As an exogenous antioxidant and NOX inhibitor, apocynin can block the process of oxidative stress in the process of HD [149]. So far, there are still few studies on the role of NOX in the process of HD. As a potential therapeutic target of HD, the mechanism of NOX and its way of alleviating HD patients deserve further exploration.

Studies found that the cerebellum is an important participant in mental illness and is affected by oxidative stress. Schiavone et al. found that inhibition of NOX during brain maturation can prevent the development of psychotic behavioral dysfunction. Administration of ketamine to the postpartum mouse model can enhance oxidative stress and reduce IL-10 in this region [150]. In recent years, many compounds that inhibit NOX have been studied for the



Molecule	Target	Action	Effect on oxidative stress
Nrf2	Antioxidantand metabolic genes	Gene activation	Suppress
	GPX4	Activation	Suppress
NAC	Cysteine	Precursor of L-Cysteine	Suppress
	GPX4	Precursor of GPX4	Suppress
	ROS	Scavenge free radicals	Suppress
	Metal ions	Chelator	Suppress
NOX	ROS	Free radical formation	Induce
Fe^{2+}	ROS	Free radical formation	Induce

Figure 2: The top schematic is a summary of oxidative stress mechanisms in neurological disorders. Under homeostatic conditions, Nrf2 binds with Keap1 and remains inactive. Nrf2-keap1 is also regulated by PI3K-Akt pathway. Under pathological conditions (neurological disorders) cause oxidative stress, Nrf2 and Keap1 will be separated. Nrf2 enters the nucleus and combines with MAF and ARE to activate antioxidant metabolic genes and play an antioxidant role. In addition, Nrf2 can activate GPX4 and be inhibited by Keap1. Both TCA cycle in mitochondria and Fenton reaction involving Fe^{2+} can cause oxidative stress by producing ROS. NAC, as a precursor of GPX4 and L-cysteine, affects oxidative stress through systemX_C^- and GPX4. In addition, NAC can interfere with glutamate homeostasis and participate in the occurrence of a variety of nervous system diseases. NOX can produce ROS and cause oxidative stress under the action of a variety of regulatory factors. The bottom table is a summary of several molecules that affect oxidative stress in neurological disorders, including their action targets, action modes, and action results. Abbreviations: Akt: protein kinase B; ARE: antioxidant response element; ETC: electron transport chain; GPX4: glutathione peroxidase 4; Keap1: Kelch-like ECH-associated protein 1; Maf: musculoaponeurotic fibrosarcoma oncogene homolog; NAC: N-acetylcysteine; NOX: nicotinamide adenine dinucleotide phosphate oxidases; NADP: nicotinamide adenine dinucleotide phosphate; Nrf2: NF-E2-related factor 2; PI3K: phosphatidylinositol-3-kinase; ROS: reactive oxygen species; TCA: tricarboxylic acid.

treatment of neurological disorders, and Ganguly et al. [191] reviewed the relationship between NOX and AD, and summarized drug trials of NOX inhibitors in Alzheimer's disease and its precursor conditions. The results displayed that the activity of NOX increased in the brain of mouse models with neurodegenerative diseases and AD patients [192-194]. However, the effects of NOX inhibitors and their precursors are not satisfactory, and the therapeutic results of these naturally obtained compounds for AD are quite variable, including berberine and blueberry-derived polyphenols. Because of the complexity of neurological disorders, the study of NOX inhibitors can provide a new direction for the treatment of neurological disorders (Figure 2).

4.3. Other Mediators and Potential Therapeutic Agents. In this part, we mainly summarize three common regulators of targeted oxidative stress in neurological disorders. In addition to the above three common regulators, other regulators can be used to treat or relieve the symptoms of neurological disorders, such as PINK1, Ca^{2+}, Sigma-1 receptor (sig-1R), peroxidase (prx), toll-like receptors (TLRs), and alarmins/c-jun N-terminal kinase (JNK) [151]. PINK1 is a mitochondrial-targeted E3 ubiquitin ligase, whose deficiency can lead to mitochondrial damage, oxidative stress, and exert neuroprotective effects by activating Parkin. Ca^{2+} can consume GSH to regulate cell death [152]. Sigma-1 receptor (sig-1R) [153, 154] is a mitochondrial endoplasmic

reticulum chaperon that can regulate cell pathophysiological processes. Peroxidase (prx) can scavenge H_2O_2 to regulate redox signal transduction and play a protective role [155]. In addition, Toll-like receptors (TLRs) are also closely related to the injury of a variety of neurological disorders and can promote microglia to release neuroprotective agents through conduction [156]. Anfinogenova et al. [157] described the role of alarmins/c-jun N-terminal kinase (JNK) signaling transduction in cerebrovascular inflammation and summarized the therapeutic strategies of intracellular anti-JNK, which provided ideas for the strategies of JNK in the treatment of neurological disorders.

As an important regulator of oxidative stress, N-acetylcysteine (NAC) is a synthetic derivative of the endogenous amino acid L-cysteine and a precursor of GSH. GSH is a key factor in the clearance of ROS. Studies have shown that NAC mainly relies on GSH to exert its indirect antioxidant capacity, but this capacity is weak [158–160]. The direct antioxidant capacity of NAC depends on its nucleophilic free sulfhydryl group [158]. A large amount of preclinical evidence shows that NAC can play a therapeutic role in CNS by regulating glutamate homeostasis [161]. Glutamate is the main excitatory neurotransmitter in the nervous system. Its overexcitation will lead to neuronal damage [162]. Extracellular glutamate is regulated by glutamate transporter-1 (GLT1), glutamate aspartate transporter (GLAST), and the systemXc^- to maintain homeostasis. Experiments have confirmed that NAC can activate systemXc^- [163, 164] and induce the expression of GLT1 [165].

As a precursor of GSH, NAC plays a beneficial role in the pathology and sequelae of TBI. Previous studies have shown that the administration time of NAC affects the therapeutic effect of NAC on TBI. Previous studies have shown that the administration time of NAC affects the therapeutic effect of NAC on TBI. In the 3-day TBI model, NAC administration 15-60 minutes after the injury can reduce the inflammatory response [166, 167], and NAC administration 12 hours after controlled cortical impact (CCI)-induced injury can reduce the level of ROS by increasing GSH [168], and oral NAC treatment within 24 hours after injury can alleviate the sequelae of TBI, such as confusion, headache, and abnormal sleep [169]. Recent studies have also found that the use of antioxidant dual therapy targeting antioxidant stress has a significant effect on the symptoms of TBI and improves the functional outcome [170]. For example, the use of NAC and sulforaphane (SFN) dual therapy can reduce TNF, IL, and other neuroinflammatory markers. In addition, the dual therapy of NAC + SPF has also been proved to be effective in alleviating epilepsy after SE [171]. In recent studies, c57bl/6 (wild type, WT) mice and CCL5 knockout (CCL5-KO) mice were used to induce mild brain injury and establish a weight drop model [172], which is a novel TBI model that can simulate the full spectrum of human TBI, mainly focusing on simulating diffuse brain injury. In the weight drop model, NAC can significantly alleviate the symptoms of decreased memory and learning ability after trauma and increase the level of Gpx1 in the hippocampus of mice [173]. With the application of nanomaterials in biomedicine, it is found that embedding NAC eluted poly (d,l-lactide-co-glycolide) (PLGA) nanofibers into scaffolds can effectively improve the shelf life of drugs and reduce systemic side effects. At the same time, it can give full play to the neuroprotective effect of NAC and the cell proliferation of nanosystems [174], which provides a broad prospect for the application of nanosystems in nerve repair. For example, neutral hydroxyl-terminated polyamide (PAMAM) dendrimers have shown great potential as nanocarriers in multiple brain injury models [175].

5. Conclusion

In this review, we summarize the relevant mechanisms of ferroptosis and some regulators of targeted oxidative stress in the treatment of neurological disorders. In the process of searching the literature, we find that oxidative stress and neurological disorders are closely related to the role of mitochondria (Figure 2), but the connection is very complex and lacks a systematic and clear mechanism. Ferroptosis may be a key mechanism to study the connection (Figure 1). For future research on neurological disorders, the role of mitochondria and the mechanism of ferroptosis will be the focus and hotspot of research. In addition, in view of the harmfulness of neurological disorders and the complexity of oxidative stress and ferroptosis, future research on the molecular mediators targeting oxidative stress and ferroptosis should pay more attention to the underlying mechanisms, so as to provide a theoretical basis for the treatment of neurological disorders.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Authors' Contributions

Jing Li, Bowen Jia, and Ying Cheng contributed equally to this work.

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Research Article

CXCR4/CX43 Regulate Diabetic Neuropathic Pain via Intercellular Interactions between Activated Neurons and Dysfunctional Astrocytes during Late Phase of Diabetes in Rats and the Effects of Antioxidant N-Acetyl-L-Cysteine

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Growing evidence suggests that the interactions between astrocytes and neurons exert important functions in the central sensitization of the spinal cord dorsal horn in rodents with diabetes and neuropathic pain (DNP). However, it still remains unclear how signal transmission occurs in the spinal cord dorsal horn between astrocytes and neurons, especially in subjects with DNP. Chemokine CXC receptor 4 (CXCR4) plays critical roles in DNP, and connexin 43 (CX43), which is also primarily expressed by astrocytes, contributes to the development of neuropathy. We thus postulated that astrocytic and neuronal CXCR4 induces and produces inflammatory factors under persistent peripheral noxious stimulation in DNP, while intercellular CX43 can transmit inflammatory stimulation signals. The results showed that streptozotocin-induced type 1 diabetic rats developed heat hyperalgesia and mechanical allodynia. Diabetes led to persistent neuropathic pain. Diabetic rats developed peripheral sensitization at the early phase (2 weeks) and central sensitization at the late phase (5 weeks) after diabetes induction. Both CXCR4 and CX43, which are localized and coexpressed in neurons and astrocytes, were enhanced significantly in the dorsal horn of spinal cord in rats undergoing DNP during late phase of diabetes, and the CXCR4 antagonist AMD3100 reduced the expression of CX43. The nociceptive behavior was reversed, respectively, by AMD3100 at the early phase and by the antioxidant N-acetyl-L-cysteine (NAC) at the late phase. Furthermore, rats with DNP demonstrated downregulation of glial fibrillary acidic protein (GFAP) as well as upregulation of c-fos in the spinal cord dorsal horn at the late phase compared to the controls, and upregulation of GFAP and downregulation of c-fos were observed upon treatment with NAC. Given that GFAP and c-fos are, respectively, makers of astrocyte and neuronal activation, our findings suggest that CXCR4 as an inflammatory stimulation protein and CX43 as an intercellular signal transmission protein both may induce neurons excitability and astrocytes dysfunction in developing DNP.

1. Introduction

Diabetic neuropathy affects hundreds of million people in the world each year, and the incidence has increased dramatically over the past two to three years [1]. Diabetic neuropathic pain (DNP) refers to the clinical manifestation of diabetic peripheral neuropathy. In addition, approximately 60% of patients suffer from persistent allodynia and hyperalgesia. The clinical manifestations of DNP in patients are similar to other types of neuropathic pain, while the burning and prickling feeling is more prolonged, and the mechanism of pathogenesis is more complicated. At present, effective treatment is lacking in the clinic; and, the efficacy of tight glycemic control and the administration of ion channel-targeting drugs (e.g., gabapentin and oxcarbazepine) for the treatment of DNP are not convincing. The involvement of peripheral and central neurons could not fully explain the functional and molecular regulatory mechanisms of DNP [1].

The level of chemokine receptor CXC receptor 4 (CXCR4) is increased in the peripheral neurons of diabetic patients with neuropathy, which demonstrates a similar and crucial role for CXCR4 in the neurons of sufferers with DNP [2, 3]. CXCR4 refers to a G protein-coupled s receptor, which is primarily denoted in neurons of the spinal cord dorsal horn and dorsal root ganglion (DRG). It binds chemokine CXC motif ligand 12 (CXCL12), which can also be recognized to be stromal cell-derived factor-1 (SDF-1). [4–8]. Recently, we and others have identified that CXCR4 is strongly linked to the various responses to noxious stimuli, such as DNP and neuropathic pain triggered by sciatic nerve ligation, in the regions of DRG and spinal cord dorsal horn in rodents [9–12]. Several studies report that intraperitoneal or intrathecal administration of the AMD3100, a selective CXCR4 antagonist, can reverse nociceptive behaviors caused by partial nerve ligation-induced neuropathic pain [9, 11, 13]. However, clinically, in patients with DNP, oral administration of neuronal ion channel inhibitors could not effectively reverse mechanical allodynia and thermal hyperalgesia, suggesting the underlying involvement of glial cells in the central sensitization of the central nervous system (CNS) [14, 15].

Besides, it has also been suggested by numerous researches that the regulation of the plasticity of the central neurons (central sensitization) and primary sensory neurons (peripheral sensitization) of patients with DNP is attributable to the communication between glia and neurons [16–19]. The function and energy homeostasis of astrocytes, the most abundant type of glia in CNS, plays critical roles in maintaining the homeostasis of CNS [20]. Under basal conditions, connexin 43 (CX43), a major component of the gap junctions, is primarily expressed by astrocytes and maintains the normal shape and function of astrocytes [21, 22]. CX43 can modulate metabolism and mediate the interaction between astrocytes and neurons in the CNS [21, 23, 24]. Studies have recognized that activated astrocytes may be attributable to the central sensitization and the process of neuropathic pain in rodent models of pain, such as neuropathic pain induced by spinal nerve ligation [25–27]. However, the functional roles of CX43 hemichannels in DNP are unknown. As a result, the current work attempted to assess the expression of and the interaction between CX43 and CXCR4 in astrocytes and neurons in the spinal cord dorsal horn of DNP in rats, aiming to establish a theoretical basis for effective applications of preventive and/or therapeutic regimens for DNP.

2. Materials and Methods

2.1. Research Animals. The Laboratory Animal Unit (LAU) of University of Hong Kong provided male Sprague-Dawley (SD) rats (220-250 g), which were housed at 25°C with a 12/12 hours light-dark cycle (lights on at 7 am). In this work, the approval of animal study protocols was obtained by the Committee on the Use of Live Animals in Teaching and Research (CULATR) of the University of Hong Kong (Permit Number: 3995-16/3995-15) as well as the Laboratory Animal Welfare and Ethics Committee of Army Medical University (AMUWEC2020413). Apart from that, the protocol aimed to minimize the use of rats and avoid the animals from suffering.

2.2. DNP Model. A diabetic neuropathic pain model was produced by streptozotocin (STZ) (Sigma, Cat#: S0130) injection through the tail vein as previous description [28, 29]. Briefly, the anesthetization of animals was performed with the mixture of ketamine (67.7 mg/kg) and xylazine (6.77 mg/kg) delivered via intraperitoneal injection. Diabetes was induced in rats via the single intravenous injection of 65 mg/kg STZ, which was dissolved with citrate buffer (pH 4.5). Besides, after the completion of STZ injection, the levels of blood glucose were assessed with a glucometer (OneTouch Ultra) 72 hours. Besides, rats which had a blood glucose level higher than 16.7 mmol/L were considered to be diabetic, which can also be included in the ensuring study. At one week after inducing diabetes and onwards, the paw withdrawal threshold (PWT) as well as the paw withdrawal latency (PWL) were evaluated with the purpose of evaluating heat hyperalgesia as well as mechanical allodynia.

2.3. Experimental Protocol. This study conducted two types of research to firstly investigate the localization and expression levels of CXCR4 in the spinal cord dorsal horn and then to detect the impacts of the CXCR4 antagonist AMD3100 (Sigma, A5602) and the treatment impacts of N-acetyl-L-cysteine (NAC) (Sigma, A7250) on DNP. In the first part of study, animals were classified to two groups ($n = 6$/group) at random: (1) the control group (saline group, i.v. injection of saline) and (2) diabetes group (Dia): rats were injected with STZ. For the second part study, the rats were randomly allowed to one of the four groups ($n = 6$, per group): (1) the saline control group: the injection of nondiabetic rats were performed with saline (i.p.); (2) Dia group: untreated diabetic group; (3) AMD3100-treated diabetic group (AMD group): rats with diabetes were administered with AMD3100 (5 mg/kg/d, i.p.) for three consecutive days at 2 weeks or at 5 weeks after the establishment of type 1 diabetes following the injection of STZ. Diabetic rats were euthanized either at 2 weeks or at 5 weeks after the completion of AMD3100 treatment and related tests. (4) NAC-treated diabetic rats (NAC group): NAC was given daily via drinking water to rats with diabetes at a dosage of 1.5 mg/kg/d. As previously described, the chemical NAC was diluted in the drinking water and was given for 4 consecutive weeks beginning from week 1 after intravenous STZ

injection [30]. Three consecutive days of intraperitoneal injection of AMD3100 in rats has been shown to effectively inhibit CXCR4 [31]. Behavioral tests at baseline and the behavioral changes over time during the course of treatment were assessed before and after intravenous STZ injection. The timeline of the experimental protocol in rats is shown in Figure 1.

2.4. Behavioral Test. Before intravenous injection of STZ, heat hyperalgesia and mechanical allodynia were evaluated by behavioral tests as the baseline. At the same time, the rats' behavioral changes were examined at week 2 and week 5 after the establishment of diabetes following STZ administration. As we and others reported previously, bilateral hind paw withdrawals were considered a positive reaction for the establishment of DNP in an animal model [10, 32].

2.5. Mechanical Allodynia. As described previously [10, 33, 34], the PWT of mechanical allodynia was examined by adopting a specific apparatus for Von Frey test (IITC/Life Science, Inc., USA). In short, rats with individually assigned numbers were positioned separately in the plexiglass boxes with a metal mesh floor and were also permitted a quarter hour for the animals with the purpose of adapting to the experimental environment. With a blunted probe, hind paws were vertically and carefully stimulated to elicit positive paw withdrawal. The rest was repeated for 3 times in each paw, and the value was averaged. In addition, the mean was recorded to be the mechanical threshold.

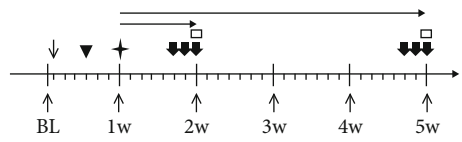
2.6. Thermal Hyperalgesia Test. Using a Hargreaves apparatus (Ugo Basile, Varese, Italy), thermal hyperalgesia was measured. Before the test, rats in each group were individually placed in cages for 15 minutes to adapt to the environment. A free heat source was placed below the plexiglass plate to give a noxious heat stimulus, and the PWL was recorded. The maximum PWL of 20 s and 5-minute intervals in each trial were set to avoid excessive noxious heat source stimulation, and the mean value of 3 consecutive trials was calculated for each hind paw.

2.7. Tissue Preparation. The euthanization of rats was performed with overdose sodium pentobarbital (100 mg per kilogram of body weight). The L3-L5 spinal cord at both sides were obtained at two time points, respectively, at early phase (2 weeks) and at late phase (5 weeks). After being quickly removed, the tissue samples were frozen in liquid nitrogen and also housed at -80°C in order to carry out western blot assays. In terms of immunofluorescence assay, rats were initially perfused with normal saline with the application of 4% paraformaldehyde (PFA) (Sigma, Cat#: 8187151000). Subsequently, the L3-L5 segments of the spinal cord were then postfixed in 4% PFA and stayed for the whole night at 4°C as well as dehydrated in 30% sucrose solution.

2.8. Primary Cell Cultures. Using a protocol previously detailed, spinal cord neuron cultures were prepared from postnatal day 1 SD rats (Centre for Comparative Medicine Research, the University of Hong Kong) [35]. Briefly, isolated spinal cords from 4 to 6 SD rats were rapidly minced and rinsed three times with the Dulbecco's Modified Eagle Medium and Nutrient Mixture F-12 (DMEM/F12, GIBCO, Cat#: A4192001), and digested for a duration of 25 minutes at 37°C in a solution containing 2 mg/ml papain (Sigma, Cat#: P3152) and 1 μg/ml DNAse (Sigma, Cat#: 260913) in neurobasal medium (GIBCO, 21103049). The termination of enzyme reaction was made by the supplementation of 10% fetal bovine serum (FBS) (GIBCO, Cat#:16140063) with DMEM/F12. Tissue pieces were triturated gently in 1 ml cold culture medium (DMEM/F12 added with 10% FBS). Then, the resulting suspension was filtered using the 70 μm cell strainer (BD Falcon, Cat#: 352350) and be centrifuged at 1,000 rpm for a duration of 5 minutes. In addition, the cells were seeded into a culture flask as well as placed in 37°C aseptic incubators with 5% CO_2. After 40 minutes, flipped the culture flask, then aspirated the cell suspension, resuspended in DMEM/F12 medium, and plated at 10,000 cells/ml density in a confocal 35-mm petri dish (Solarbio, YA0572). After 4 to 6 hours, when the cells were almost attached to the petri dish, the medium was changed to neurobasal (Invitrogen, Cat#: 21103049) culture medium supplemented with B27 (Invitrogen, Cat#: 17504044). Twice each week, half of the culture medium was substituted with fresh medium. Using an OLYMPUS microscope BX46, the viability of neurons was confirmed after being placed for 5 days in culture.

Spinal cord astrocyte culture was conducted following a protocol described previously [36]. Briefly, isolated and minced spinal cord tissues from 4 to 6 SD rats were digested with 0.125% trypsin (Sigma, Cat#: T4049) solution and were stayed for a duration of 15 minutes at 37°C, and the tissues were then gently triturated in DMEM/F12 containing 10% FBS. With a 70 μm cell strainer, the resulting suspension was filtered. Then, the cells were resuspended in DMEM/F12 culture medium which was housed at 37°C in aseptic incubators with 5% CO_2. After 24 hours, the cells, at a density of 10,000 cells, were seeded in 35-mm confocal petri dishes in DMEM/F12 culture medium without FBS. Twice each week, half of the culture medium was substituted with fresh medium. Astrocyte viability was confirmed using an OLYMPUS microscope BX46 after being placed in culture for 5 days.

2.9. Western Blot Analysis. The harvested spinal cord tissue was homogenized using a Polytron homogenizer (Kinematica, Switzerland) using ice-cold lysis buffer in the volume of in 100 μl and for a duration of 1 hour. The centrifugation of lysate was further performed at 12,000 rpm for a quarter hour at 4°C. After extraction and centrifugation, the assessment of protein concentrations was made by adopting the Bradford Protein Assay Kit (Bio-Rad, USA). In addition, proteins at the amounts of 30 μg per sample were divided using 12.5% SDS-polyacrylamide gels and were then transferred to polyvinylidene fluoride (PVDF) membranes. After being blocked with 5% nonfat milk that was dissolved in Tris-buffered saline with Tween (TBST) for 1 hour under the condition of chamber temperature, the membranes were then raised with rabbit anti-CXCR4 (1 : 1000, Abcam, ab124824), mouse anti-CX43 (1 : 1000, Sigma, C8093), mouse anti-GFAP (1 : 1000, Cell Signaling, Cat#: 3670), and mouse anti-c-fos (1 : 1000, Abcam, ab208942) antibodies overnight under the condition of 4°C. Thereafter, the membranes were rinsed for 3 times using TBST



↓ Intravenous STZ, 65mg/Kg ✦ Daily oral NAC, 1.5 mg/Kg/d

▼ Measure blood sugar levels ↑ Von frey/hargreaves once time a week

AMD3100 was administrated for 3 consecutive days by intraperitoneal injection at 2 weeks or 5 weeks followed by electronic Voin frey and hargreaves test.

Rats were sacrified at 3 hours after last time intraperitoneal injection AMD3100/saline.

NAC was dissolved in drinking water for 1 or 4 consecutive weeks starting at one week after STZ-induced diabetes.

Figure 1: Timeline of experiment protocol in SD rats. BL: baseline; w: week.

(10 min each), before being nurtured with horseradish peroxidase (HRP)-linked antirabbit IgG and antimouse IgG secondary antibodies (1 : 2000, Cell Signaling, A0545/SAB5600195) for an hour under the condition of room temperature. In addition, the bands of proteins were visualized by incubating the membranes in ECL solution (Bio-Rad, Cat#: 1705061) for 1 minute and subject to X-ray films (Kodak, USA) in the dark room. Apart from that, we adopted the ImageJ software (the National Institutes of Health, USA) for assessing optical density.

2.10. Immunofluorescence Staining. As described previously, immunofluorescence staining of spinal cord slices was performed [10]. In brief, 20 μm-thick slices of spinal cord L3-L5 were cut by using a Cryotome (Thermo, USA). The permeabilization of sections was made by incubation with 0.25% Triton X-100 for a duration of 15 minutes and subsequently was blocked with the concentration of 10% bovine serum albumin (BSA) (Sigma, Cat#: 10735108001) for a duration of 1 hour under the condition of the room temperature. Subsequently, the slices of the spinal cord dorsal horn at L3-L5 were incubated with combinations of the rabbit anti-CXCR4 (1 : 200, Abcam, ab124824) and the mouse anti-c-fos (1 : 200, Abcam, ab208942), rabbit anti-CXCR4 and mouse anti-GFAP (1 : 200, Cell Signaling, Cat#: 3670), rabbit anti-CXCR4 and mouse anti-CX43 (1 : 200, Sigma, C8093), or mouse anti-CX43 (1 : 200, Sigma, C8093) antibodies overnight at 4°C. In addition, after three washes (10 minutes each time), slices were illuminated with a mixture of Alexa Fluor 488-conjugated antimouse (1 : 500, Abcam, ab150113) and Alexa Fluor 568-conjugated antirabbit (1 : 500, Abcam, ab175703) antibodies or Alexa Fluor 568-conjugated antirabbit antibody alone for the duration of 1 hour at room temperature. Subsequently, the nuclei subsequently stained using DAPI (Cell Signaling, Cat#: 8961). In addition, images were captured with the use of an LSM 710 laser scanning confocal microscope (Zeiss) and were then explored by adopting the ImageJ software.

Immunofluorescence staining of primary cultured astrocytes and neurons in confocal 35-mm petri dishes was carried out as the description presented in vivo. Briefly, cultured cells were fixed by using 4% PFA for the duration of 30 minutes at 37°C and permeabilized using 0.1% Triton X-100 for a quarter hour. Next, we blocked the cells with 1-hour exposure to 10% BSA in PBS. Subsequently, the astrocytes were incubated with a combination of rabbit anti-CXCR4 (1 : 200, Abcam, ab124824) and mouse anti-CX43 (1 : 200, Sigma, C8093) or rabbit anti-CXCR4 (1 : 200, Abcam, ab124824) and mouse anti-GFAP (1 : 200, Cell Signaling, Cat#: 3670) antibodies. Primary neurons were incubated with a combination of rabbit anti-CXCR4 (1 : 200, Abcam, ab124824) and mouse anti-CX43 (1 : 200, Sigma, C8093) or rabbit anti-CXCR4 (1 : 200, Abcam, ab124824) and mouse anti-NeuN (1 : 200, Abcam, ab104224) antibodies through the whole night at 4°C. On the next day, secondary antibodies, a mixture of Fluor 488-conjugated antimouse (1 : 500, Abcam, ab150113) and Alexa Fluor 568-conjugated antirabbit (1 : 500, Abcam, ab175703) antibodies were added to the petri dishes and were then incubated for a duration of an hour at chamber temperature, and the nuclei were stained with DAPI, with images being analyzed using the confocal laser microscope (LSM 800, Zeiss).

2.11. Assays for the Antioxidant Enzymes Glutathione Peroxidase (GSH-Px) and Superoxide Dismutase (SOD) and the Lipid Peroxidation Product Malondialdehyde (MDA). In this study, the GSH-Px (S0057S, Beyotime Institute of Biotechnology, Nantong, Jiangsu, China), MDA (S0131S, Beyotime Institute of Biotechnology, Nantong, Jiangsu, China), and SOD (S0109, Beyotime Institute of Biotechnology, Nantong, Jiangsu, China) Detection Kits were, respectively, employed to measure GSH-Px (nm/mgprot), MDA (nmol/mgprot), and SOD (U/mgprot) levels in the spinal cord tissues. The assay procedures followed the respective supplier's instructions to calculate the content or activity of the samples.

2.12. Statistical Analysis. Data are denoted as mean ± the standard deviation (SD). Besides, the same time point values of PWT and PWL among groups were analyzed by the application of one-way ANOVA with Tukey's post hoc test, while comparisons of the changes of PWT and PWL over time were assessed by paired t-test. For molecular data, the results were explored by adopting one-way ANOVA based on Tukey's post hoc test. In addition, the SPSS 19.0 (USA) and the GraphPad Prism 7.0 (USA) statistical software were

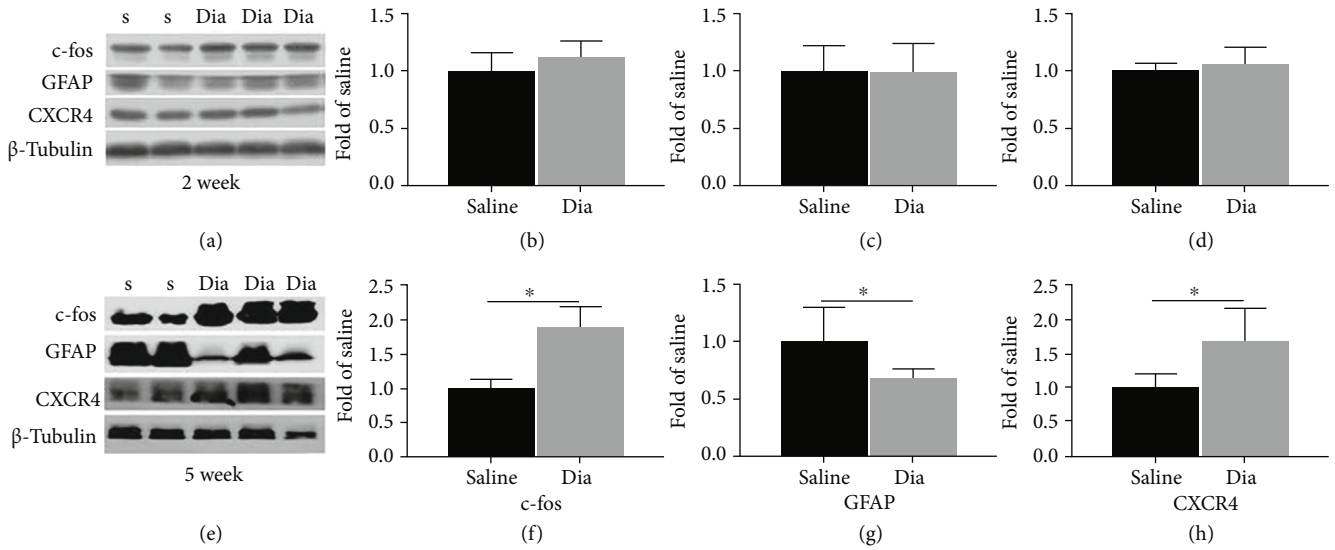


Figure 2: Streptozotocin (STZ)-induced DNP in rats enhanced spinal cord dorsal horn CXCR4 and c-fos and decreased GFAP at 5 weeks (e–h) yet not at 2 weeks (a–d) of diabetes. (a, e) c-fos, GFAP, and CXCR4 expression in the spinal cord, according to western blotting, at 2 and 5 weeks after intravenous STZ-induced diabetes (Dia) and at 2 and 5 weeks after injection of saline (saline control group). (b–d, f–h) Quantification of c-fos, GFAP, and CXCR4 levels in the spinal cord. Apart from that, western blot results can be shown to be means ± SD. $*P < 0.05$ in relative to saline group, $n = 6$/group. S = saline; Dia = diabetes.

applied to perform data analyses. A P value less than 0.05 was considered to show statistically significant difference.

3. Results

3.1. Changes of c-fos, CXCR4, and GFAP Protein Expression at the L3-L5 Level Spinal Cord of Diabetic Rats at 2 Weeks and 5 Weeks of the Disease. As shown in Figure 2, the protein levels of the proinflammatory CXCR4 in the spinal cord was significantly increased at week 5 (late phase), but not at week 2 (early phase) of diabetes, which is in line with our previous report [10]. However, the expression of the astrocytic marker GFAP and the neuronal activation marker c-fos in the spinal cord dorsal horn of rats with DNP in the early and late phases of diabetes has not been explored previously. As shown in Figure 2, our current study demonstrated that the protein levels of spinal cord c-fos and CXCR4 were significantly enhanced at 5 weeks (Figures 2(f) and 2(h), $P < 0.05$) but not at 2 weeks (Figures 2(b) and 2(d)). In particular, in the late phase (5 weeks), GFAP protein contents (Figure 2(g)) decreased significantly in rats with DNP, which is in contrast to the observations in rats in other models of neuropathic pain (e.g., neuropathic pain trigged by chronic constriction injury) [37].

3.2. Localization and Expression of CXCR4 in the Spinal Cord Dorsal Horn and in Primary Cultured Astrocytes and Neurons. In terms of control and diabetic rats, immunofluorescence staining of the L3-L5 spinal cord sections confirmed that the expression of CXCR4 mainly existed in neurons and resided partly in astrocytes (Figures 3(a) and 3(b)). The expression of CXCR4 was notably enhanced in diabetic rats in relative to that in the control rats ($P < 0.01$, saline vs. Dia, Figure 3(e)). Furthermore, the immunostaining intensity of c-fos was significantly enhanced in rats with DNP ($P < 0.05$, saline vs. Dia, Figure 3(c)). The coexpression of CXCR4 and c-fos marked active neurons was also obviously enhanced in diabetic rats in comparison with that in the control rats ($P < 0.001$, saline vs. Dia, Figure 3(f)). Notably, immunostaining intensity of GFAP was notably reduced in DNP rats ($P < 0.01$, saline vs. Dia, Figure 3(d)), whereas the coexpression of CXCR4 and GFAP-marked astrocytes were notably enhanced in the spinal dorsal horn of rats with DNP ($P < 0.001$, saline vs. Dia, Figure 3(g)). The results of primary cultured neurons and astrocytes consolidated that CXCR4 was colocalized with GFAP-marked astrocytes and NeuN-marked neurons (Figures 4(a) and 4(b)). Collectively, the obtained data indicate that CXCR4 can be denoted in both neurons and astrocytes in the spinal cord dorsal horn and that the expression of CXCR4 is enhanced in neurons and astrocytes in rats with DNP.

3.3. Heat Hyperalgesia and Mechanical Allodynia Development in Diabetic Rats and the Treatment Effects with AMD3100 or NAC at 2 and 5 Weeks of Diabetes. To explore the heat hyperalgesia and mechanical allodynia development in rats undergoing DNP and the impacts of treatment with AMD3100 or NAC, mechanical allodynia and heat hyperalgesia were evaluated as did in our previous DNP model [10]. The baseline values of PWL and PWT did not show the difference among groups (Figures 5(a) and 5(b), (BL) $P > 0.05$). Besides, 14 days after the establishment of diabetes, the PWT and PWL values were notably lowered in the diabetic rats in comparison with rats in the nondiabetic control group (Figures 5(a) and 5(b), $P < 0.05$, Dia vs. saline), while AMD3100 treatment reversed the reductions in PWT and PWL seen in the untreated diabetic group (Figures 5(a) and 5(b), $P < 0.05$, Dia vs. AMD). However, NAC did not obviously affect the PWT and PWL values in rats after 2 weeks of diabetes (Figures 5(a) and 5(b), $P > 0.05$, Dia

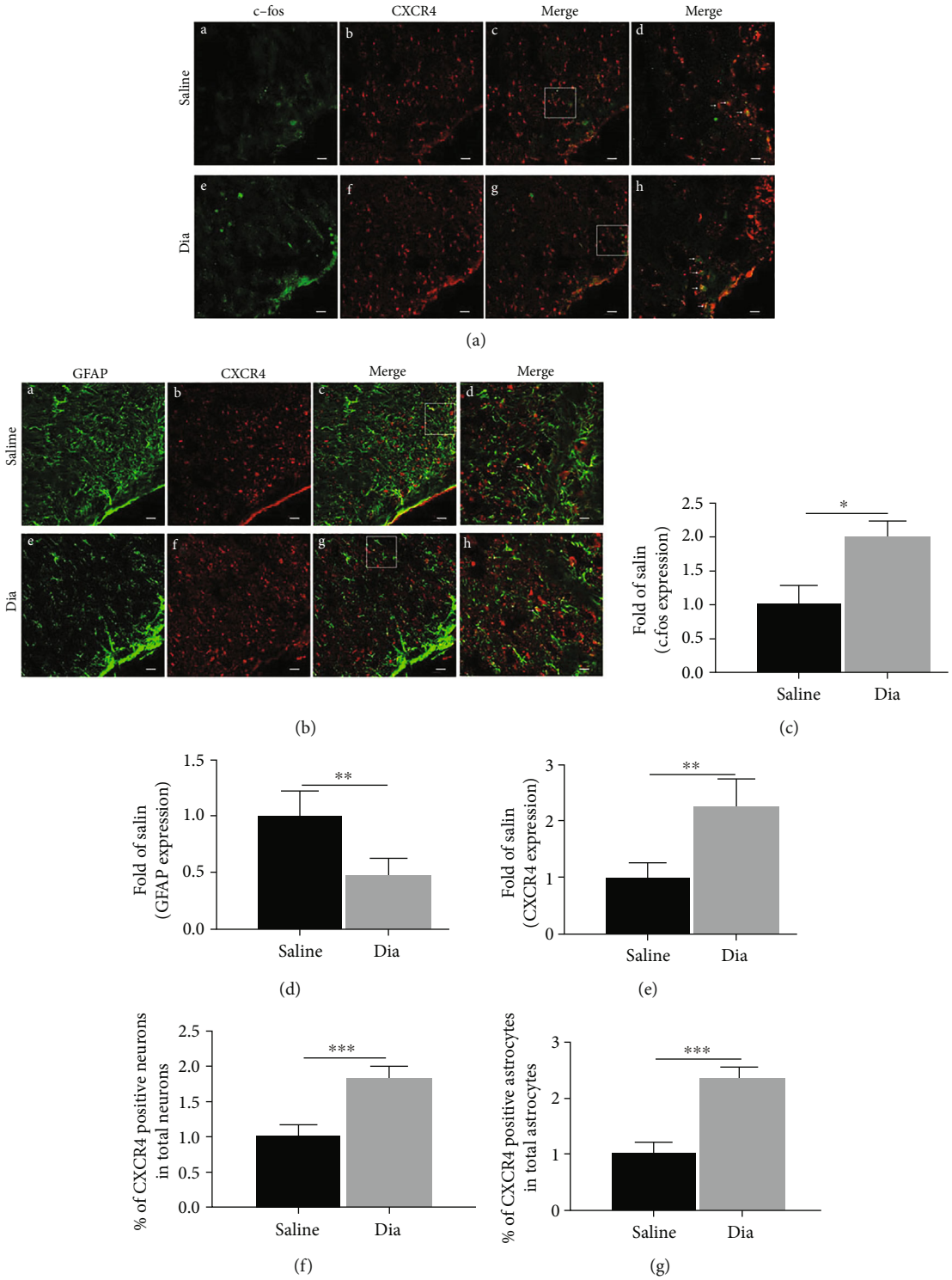


Figure 3: Confocal images showed coexpression and distribution of CXCR4 and GFAP, CXCR4 and c-fos, and the expression of c-fos, CXCR4, and GFAP in the L3-L5 spinal cord dorsal horn in rats with STZ-induced DNP at 5 weeks of diabetes and in control rats receiving saline at 5 weeks. (a, b) Double-immunostaining for c-fos (green (a) (A and E)), GFAP (green (b) (A and E)), and CXCR4 (red, B and F) in the spinal dorsal horn after STZ-induced diabetes at 5 weeks. C and G were merged images of A and B or E and F separately (original magnification: 200×, scale bar A–C, E–G 20 μm). Merged and enlarged images were shown in D and H (original magnification: 400×, scale bar 10 μm). Immunostaining for c-fos (a) (A, E), GFAP (b) (A, E), and CXCR4 (B, F) of the spinal cord dorsal horn was determined at 5 weeks after STZ-induced diabetes. Besides, quantitative analysis of c-fos (c), GFAP (d), and CXCR4 (e) of the intensities at 5 weeks. Quantification of the coexpression of CXCR4 and c-fos (f) and CXCR4 and GFAP (g). All data are indicated to be means ± SD. $*P < 0.05$, $**P < 0.01$, and $*** P < 0.001$ in relative to saline group. $n = 6$/group. .

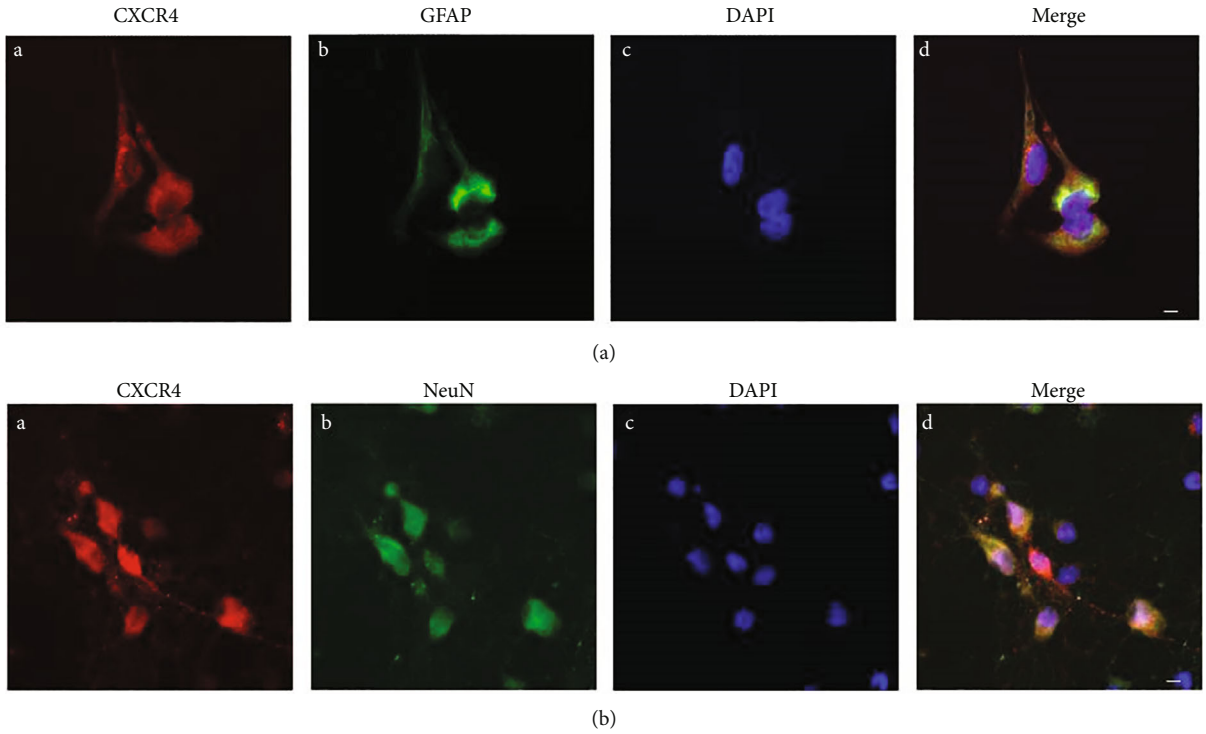


Figure 4: Representative confocal images presented co-expression of CXCR4 and GFAP, CXCR4 and NeuN in primarily cultured astrocytes and neurons, respectively. (a, b) Double-immunostaining for CXCR4 (red, A), GFAP (astrocytic marker, green (a) (B)), NeuN (neuronal marker, green (b) (B)), DAPI (nuclear marker, blue, C). (a) (D) and (b) (D) were merged A, B, and C images (original magnification: 400×, scale bar 10 μm, $n = 6$/group).

vs. NAC). At 5 weeks of diabetes, 4 weeks of continuous NAC treatment that was initiated at 1 week of diabetes induction significantly increased both PWT and PWL in diabetic rats to levels comparable to those of the nondiabetic rats (Figures 5(a) and 5(b), $P < 0.05$, Dia vs. NAC). However, unexpectedly, AMD3100 intraperitoneal injection at 5 weeks of diabetes did not have a significant effect on PWT or PWL (Figures 5(a) and 5(b), $P > 0.05$, Dia vs. AMD). Furthermore, the PWT and PWL values were obviously reduced from 2 weeks to 5 weeks as compared to baseline in diabetic group. And PWT and PWL were not significantly changed at 2 weeks compared to baseline in the AMD group but significantly decreased at 5 weeks, suggesting a positive treatment result in early phase (Figures 5(c) and 5(d), $P < 0.05$, BL vs. 2 weeks, 5 weeks).

3.4. Changes of the c-fos, CXCR4, and GFAP Proteins in the L3-L5 Spinal Cord and the Treatment Effects of AMD3100 or NAC at 2 and 5 Weeks of Diabetes. Based on the behavioral results, we further examined the expressions of c-fos, CXCR4, and GFAP proteins in the spinal cord of the animals in the four experimental groups. Notably, in the 2-week diabetic group (Dia), the spinal cord expression levels of c-fos, CXCR4, and GFAP did not present obvious difference from that in the nondiabetic control group (Figure 6(a)), which were not consistent with the behavioral results of PWT and PWL (Figures 5(a) and 5(b)). As a previous study demonstrated, in STZ-induced diabetic rats, the proinflammation proteins TNF-α and CXCR4 were both enhanced at the initial phase (2 weeks) of diabetes in the DRG but did not change in the spinal cord dorsal horn [10]. Therefore, peripheral sensitization of DNP rats occurred at an early stage, and prolonged peripheral sensitization might trigger central sensitization of the spinal cord dorsal horn [10]. AMD treatment significantly hindered the expression of CXCR4 and c-fos in the spinal cord (Figures 6(b) and 6(d), $P < 0.05$, Dia vs. AMD) at 2 weeks. GFAP protein expression increased at an earlier phase following AMD3100 treatment (Figure 6(c), $P < 0.05$, Dia vs. AMD), but no remarkable changes were seen at the later phase (Figures 6(f)–6h, $P > 0.05$, Dia vs. AMD). Furthermore, at 5 weeks, the expression of c-fos and CXCR4 in the spinal cord could be reduced by daily oral administration of NAC (Figure 6(f) and 69h), $P < 0.05$, Dia vs. NAC), whereas GFAP protein (Figure 6(g), $P < 0.05$, Dia vs. NAC) was increased. Nevertheless, CXCR4, c-fos and GFAP protein did not alter significantly in diabetic rats at 2 weeks nor did NAC have a significant impact on CXCR4, c-fos, and GFAP protein levels at this stage (Figures 6(b)–6(d), $P > 0.05$, Dia vs. NAC). The above data demonstrated that dysfunctional astrocytes might promote and maintain central sensitization and exert an essential function in the late phase of STZ-induced diabetic rats. Moreover, daily oral administration of NAC improved nociceptive behaviors in the late stage (5 weeks of diabetes) as assessed by PWT and PWL and significantly reduced the expression of CXCR4 in the spinal cord dorsal horn of rats with DNP (Figure 5, $P < 0.05$, Dia vs. NAC; Figure 6(h), $P < 0.05$, Dia vs. NAC).

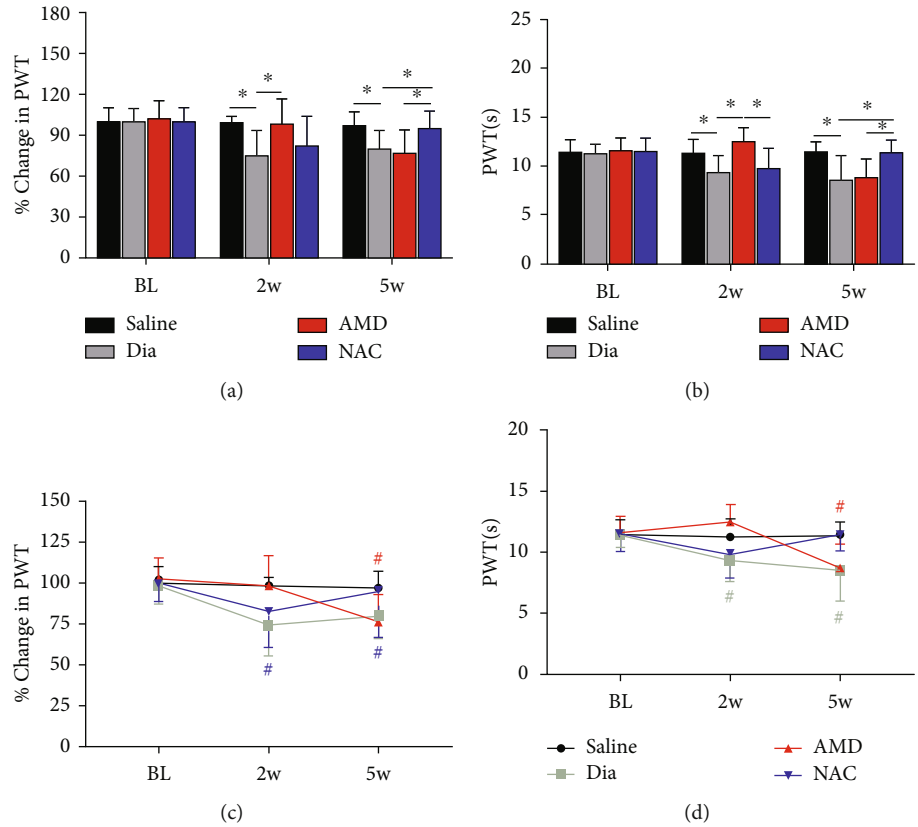


Figure 5: Variations of PWT (a) and PWL (b) in untreated diabetic rats (Dia) and in nondiabetic control (saline) or diabetic rats exposed to treatment with AMD or NAC and variations of PWT (c) and PWL (d) from baseline in the saline, Dia, AMD, or NAC group. PWT (a) and PWL (b) were notably decreased in STZ-induced diabetic rats and increased in AMD-treated diabetic rats but not in NAC-treated diabetic rats at 2 weeks, while PWT and PWL were significantly increased in diabetic rats receiving oral NAC by 5 weeks as identified by electronic Von Frey and Hargreaves test. In addition, PWT (c) and PWL (d) were reduced from 2 weeks to 5 weeks compared to baseline in Dia group, and PWT (c) and PWL (d) were obviously lowered in 5 weeks in comparison with baseline in the AMD group. All findings are shown to be means ± SD, $n = 6$/group, $*P < 0.05$. $^{\#}P < 0.05$ vs. baseline. BL = baseline; w = week; PWT = paw withdraw threshold; PWL = paw withdrawal latencies; Dia = diabetes; NAC = N-acetyl-L-cysteine; AMD = AMD3100.

3.5. NAC Inhibited the Accumulation of Oxidation Products. We further examined the antioxidant effect of NAC. As shown in Figure 7, a significant rise was found in MDA contents of spinal cord at 5 weeks after STZ-induced diabetes (Figure 7(b), $P < 0.001$, Dia vs. saline) accompanied by reduced activities of SOD and GSH-Px (Figures 7(a) and 7(c), $P < 0.001$, Dia vs. saline). Furthermore, NAC treatment significantly prevented the above changes in MDA, SOD, and GSH-Px expression (Figures 7(a)–7(b), $P < 0.001$, Dia vs. NAC). As shown in Figure 7(b), AMD3100 moderately yet obviously reduced the accumulation of MDA ($P < 0.05$, Dia vs. AMD); however, it did not increase the antioxidant enzymes SOD and GSH-Px ($P > 0.05$, Dia vs. AMD). These results illustrated that NAC enhanced the ability to combat against oxidative damage and increased endogenous antioxidant capacity, and AMD3100 only demonstrated the ability to inhibit oxidative damage in the spinal cord at late phase (week 5) after STZ-induced diabetes.

3.6. Expression and Localization of CX43 in the Spinal Cord, and Coexpression of CXCR4 and CX43 in Spinal Cord Dorsal Horn and in Primary Culture Astrocytes/Neurons. Numerous researches have demonstrated that astrocytes exert essential functions in energy metabolism, not only in providing nutritional support for neurons in the CNS but also in maintaining the dynamic balance of the microenvironment [16, 38, 39]. CX43, a hemichannel protein that primarily exists in astrocyte membranes and is permeable to Ca^{2+}, IP_3, ATP, cAMP, etc. [40–42], acts as a "bridge" channel for intercellular connections. To elucidate the cause and effect of astrocyte dysfunction on the central sensitization of rats with DNP in the late phase of diabetes, this study further explored the levels of CX43 hemichannel protein in the L3-L5 spinal cord dorsal horn. Contrary to our expectation, as shown in Figures 8(a) and 8(c), CX43 expression in diabetic rats increased significantly at the late phase, and either AMD3100 intraperitoneal injection or daily oral administration of NAC reversed this increase (Figures 8(b) and 8(d)). Furthermore, immunostaining confirmed that the proinflammatory protein CXCR4 and hemichannel protein CX43 were coexpressed in the spinal cord dorsal horn, both in rats with DNP and in saline-treated nondiabetic control rats (Figures 9(a) and 9(b)). With the purpose of further exploring the coexpression of these

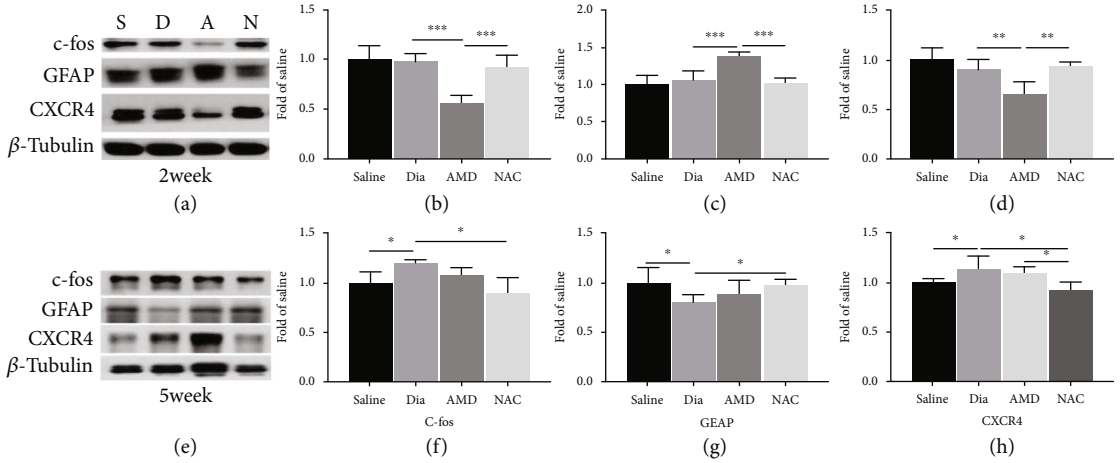


Figure 6: Correlative proinflammatory protein CXCR4 and neuronal marker protein c-fos of the spinal cord in rats with DNP was inhibited, and astrocytic marker protein GFAP was activated by intraperitoneal AMD3100 at 2 weeks and by daily oral NAC treatment at 5 weeks of diabetes. (a, e) Western blot demonstrated the protein expression of c-fos, GFAP, and CXCR4. (b–d, f–h) Quantitative analysis of c-fos, GFAP, and CXCR4 comparing with β-tubulin in spinal dorsal at 2 weeks and 5 weeks. In addition, all the obtained data can be shown to be means ± SD. $*P < 0.05$, $n = 6$/group.

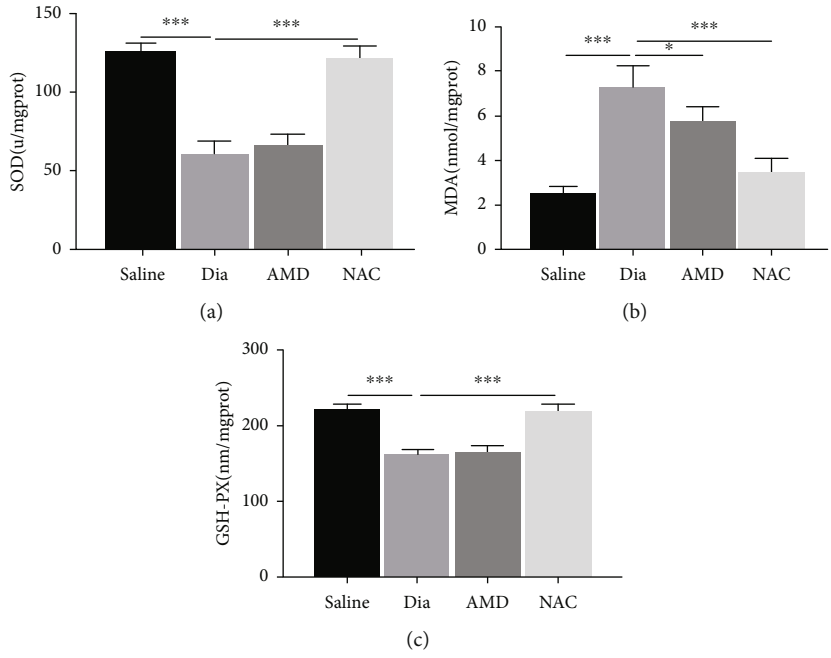


Figure 7: Changes of antioxidant enzymes (SOD and GSH-PX) and lipid peroxidation product malondialdehyde (MDA) in the spinal cord in rats undergoing DNP at 5 weeks of diabetes and the treatment effects of NAC and AMD. (a–c) The activity of SOD (a) and the contents of MDA (b) and GSH-Px (c) in rat spinal cord at 5 weeks of diabetes. Besides, all the obtained data are denoted to be means ± SD. $*P < 0.05$, $**P < 0.01$, and $*** P < 0.001$, $n = 6$/group.

proteins in the spinal cord dorsal horn, we used immunostaining to locate the CXCR4 and CX43 proteins in primary cultured neurons and astrocytes. Immunostaining showed that CXCR4 and CX43 were coexpressed in both primary neurons and astrocytes (Figures 9(c) and 9(d)). All these results suggested that activated CX43 and CXCR4 could affect the development of DNP in STZ-induced type 1 diabetic rats via dysfunctional astrocytes and activated neurons in the spinal cord dorsal horn at the late phase of diabetes.

4. Discussion

The current study revealed that neuronal activation and astrocyte dysfunction may be mediated by upregulated CXCR4 and

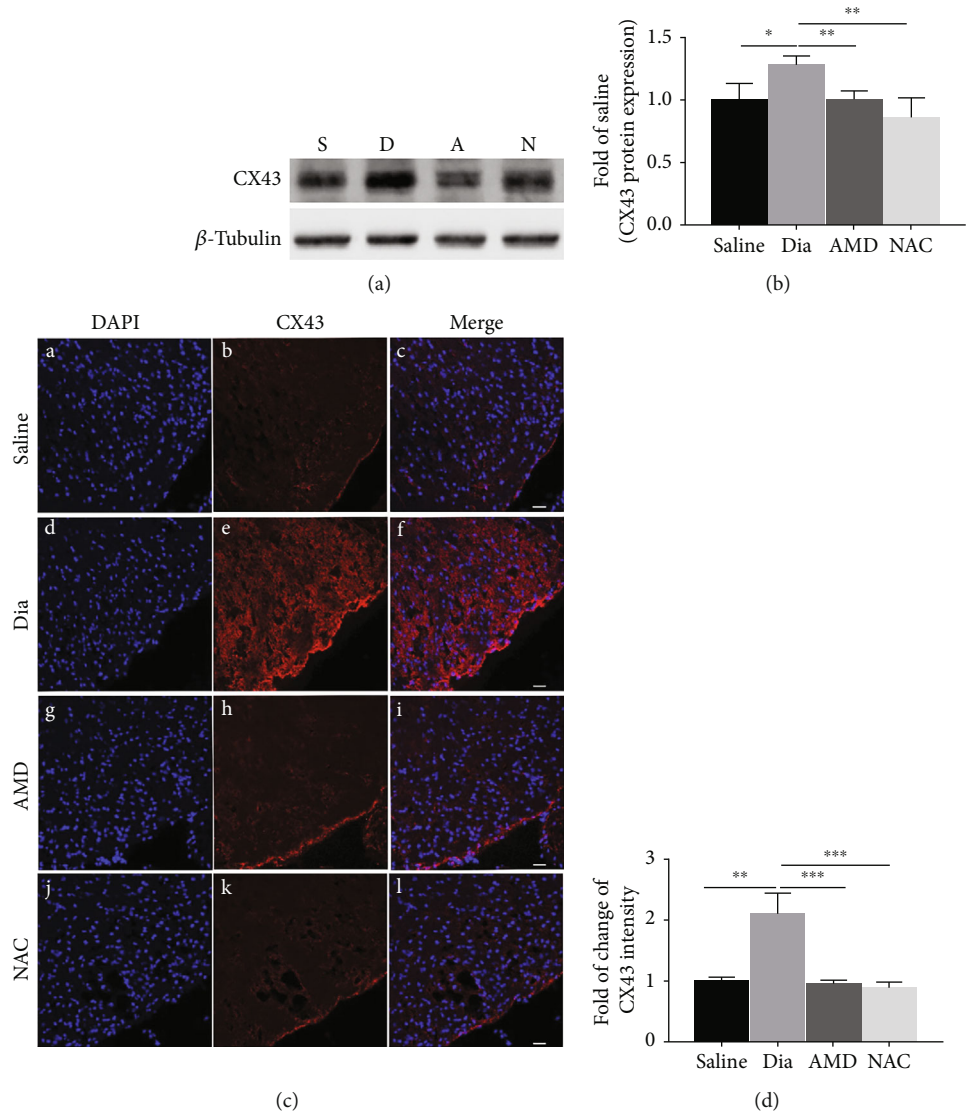


Figure 8: DNP was associated with significantly added expression of CX43 in the spinal cord dorsal horn of STZ-induced diabetic rats at 5 weeks of the disease, which could be inhibited by intraperitoneal AMD3100 and NAC. (a) Western blot and (c) confocal images showed the protein expression of CX43 in the spinal cord dorsal horn. (b) Quantitative analysis of CX43 comparing with β-tubulin in the spinal dorsal at 5 weeks. All the data are demonstrated to be means ± SD. $*P < 0.05$, $**P < 0.01$, and $*** P < 0.001$, $n = 6$/group. C, F, I, and M were merged images A and B, D and E, G and H, and J and K, separately. (d) Semiquantification of CX43 immunofluorescence intensity. $n = 6$/group (original magnification: 200×, scale bar 20 μm).

CX43 in the spinal cord dorsal horn of STZ-induced diabetic rats at the late phase of the disease which may represent a mechanism of DNP development. We dissected the potential mechanism of DNP using several approaches: (1) in diabetic rats, the STZ-induced DNP model could persistently elicit the upregulation of CXCR4 in both neurons and astrocytes in the spinal cord dorsal horn (from 2 weeks to 5 weeks). (2) Activated neurons and dysfunctional astrocytes could be observed in the spinal cord dorsal horn of rats with DNP at late phase diabetes (5 weeks) yet not in the early phase (2 weeks). (3) Intraperitoneal administration of AMD3100 could reverse the thermal hyperalgesia and mechanical allodynia of STZ-induced DNP in rats and inhibited CXCR4 protein expression in the spinal cord dorsal horn in the early phase, but not in the late phase of DNP. Comparatively, NAC given via daily oral administration to rats with DNP maintained the behavioral responses as evaluated by PWT and PWL at a level that was comparable to that seen in the nondiabetic control rats and downregulated CXCR4 in the late phase of diabetes. (4) The expression of the hemichannel protein CX43, which mediates neuron-glia interactions, was notably upregulated in the spinal cord dorsal horn of rats with DNP in the late phase of diabetes, and intraperitoneal administration of AMD3100 inhibited the upregulation of CX43 and attenuated symptoms of DNP, which further illustrates the interactions between dysfunctional astrocytes and activated neurons in the development of DNP. (5) Coexpression of CX43 and CXCR4 was confirmed in the spinal cord dorsal horn of

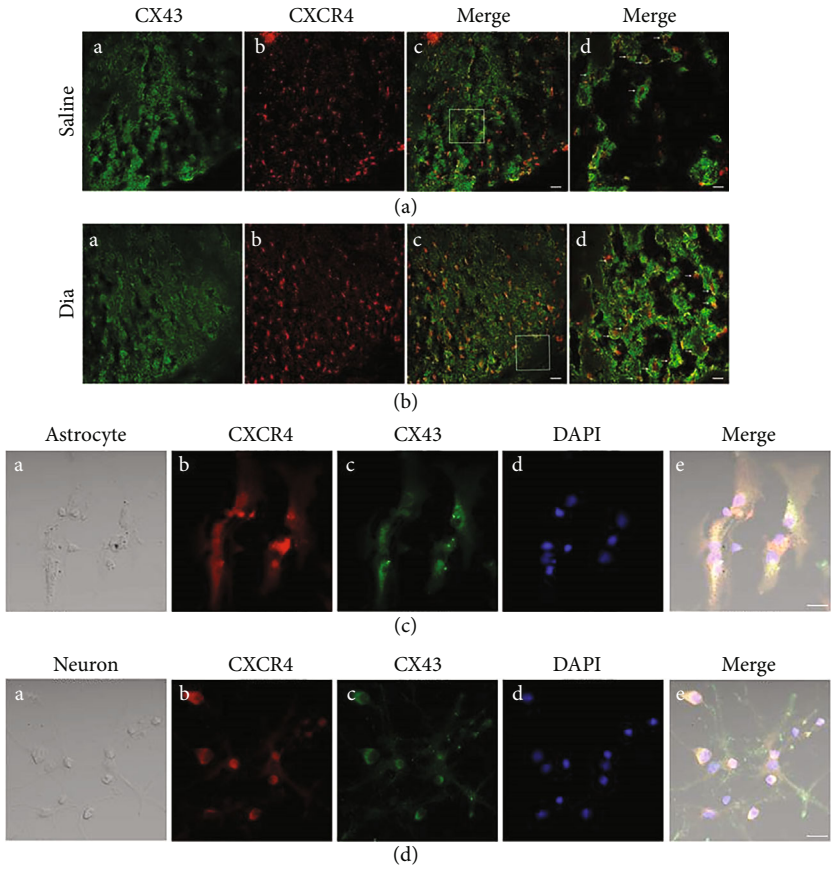


Figure 9: Confocal images show coexpression of CXCR4 and CX43 in the spinal dorsal horn of rats after STZ-induced DNP at 5 weeks of diabetes and in saline control rats (a, b), and in primary culture astrocytes (c) and neurons (d). (a) and (b) co-expression of CX43 and CXCR4 of spinal dorsal cord were shown in confocal images. (a) (A, B) and (b) (A, B) were double-immunostaining for CX43 (green, A) and CXCR4 (red, B). (a) (C) and (b) (C) were merged images A and B (original magnification: 200×, scale bar 20 μm). Merged and enlarged images were shown in (a) (D) and (b) (D) (original magnification: 400×, scale bar 10 μm, n = 6/group). (c) (A) and (d) (A) were bright field images of astrocytes and neurons, respectively. (c, d) Double-immunostaining for CXCR4 (red, B), CX43 (green, C), DAPI (blue, D). (c) (E) and (d) (E) were merged images A, B, C, and D (original magnification: 400×, scale bar 20 μm, $n = 6$/group).

DNP rats, as well as in the primary cultures of astrocytes and neurons isolated from neonatal nondiabetic rats.

4.1. CXCR4 in the Spinal Cord Dorsal Horn Astrocytes and Neurons in the Early- and Late-Phase Diabetic Rats with Neuropathic Pain. Intravenous administration of STZ has been considered a more reliable and reproducible method to induce diabetic rats with neuropathic pain than intraperitoneal administration of STZ [43]. The dose of intravenous STZ at 65 mg/kg induces type 1 diabetes in rats reproducibly [9, 10, 29]. Notably, as previous studies pointed out, heat hyperalgesia and mechanical allodynia occurred in the first week in STZ-induced diabetic rats [10, 44]. CXCR4 as well as its ligand SDF-1 were found to be upregulated in the spinal cord dorsal horn of animals with neuropathic pain triggered by partial sciatic nerve ligation (PSNL)- and in chronic postischemia (CPIP)-induced neuropathic pain [9, 11]; however, both of them are inhibited by AMD3100. Therefore, we specifically assessed the spinal cord dorsal horn CXCR4 variations in rats at the early and late phases of diabetes. Our previous study revealed that the CXCR4 was enhanced only in the DRG neurons at week 2 (early phase of diabetes) after STZ injection [10]. Furthermore, CXCR4 protein was enhanced in both the DRG and the spinal cord dorsal horn at week 5 (late phase of diabetes), which was consistent with the upregulation of proinflammatory protein expression in other neuropathic pain models (e.g., cancer pain model, nerve ligation) [45, 46]. This result suggested that persistent peripheral inflammatory stimulation induced central sensitization in DNP.

Accumulating studies indicated that astrocytes exert essential functions in the maintenance of neuropathic pain. Especially, reactive astrocytes have been revealed in some animal models of neuropathic pain such as pain triggered by chronic constriction injury (CCI), suggesting that reactive astrocytes could modulate neuropathic pain. Our finding revealed an obvious decrease of GFAP expression in the spinal cord dorsal horn of diabetic rats, showing consistence with previous studies [47–49]. This pathological change of astrocytes in DNP was different from reactive astrocytes that was characterized by increased GFAP expression [9, 36]. GFAP, as the main intermediate filament protein in mature

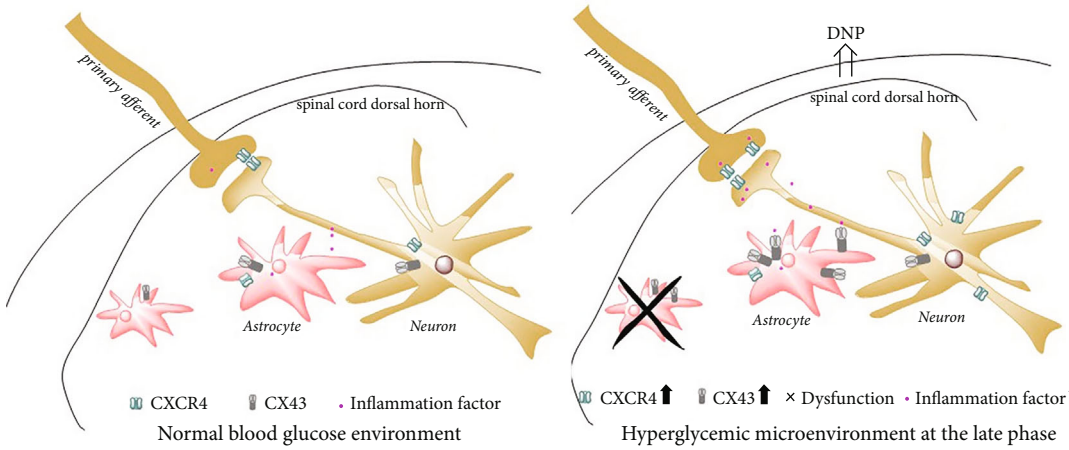


Figure 10: Schematic hypothesis of neuronal-astrocytic CXCR4 and CX43-mediated diabetic neuropathic pain. STZ-induced type 1 diabetes resulted in persistent upregulation of CXCR4 and CX43 in astrocytes and neurons. It is shown that persistent increase of CXCR4 may cause activated neuron excitability and CX43 may mediate intercellular inflammation signal transmission, then dysfunctional astrocytes cannot counter the inflammatory factors at late phase of diabetes, and DNP occurs and exacerbates.

astrocytes, also serves as a critical constituent of the cytoskeleton in astrocytes during the development, and its decreased expression could result in the failure of astroglial support, impaired homeostatic function of astrocytes, and the abnormal synaptic transmission [50]. This pathological change of astrocytes has been identified in various neurological disorders such as neuropsychiatric disorders, addictive disorders, epilepsy, and neurodegenerative degeneration [50], which may also be a critical pathogenesis of DNP. In addition, astrocytes form large intercellular networks to provide nutritional support for neurons and maintain the dynamic balance of the CNS [51]. Therefore, the dysfunction of astrocytes also resulted in impaired function of glutamate uptake and exacerbated glutamate excitotoxicity [52, 53]. Extracellular glutamate increase has been shown to cause aberrant synaptic signaling that cause neuronal excitotoxicity and death [53]. Our findings demonstrated that c-fos as a rapid and transient marker of activated neurons [54–56] was notably elevated in the spinal cord of rats at the late phase of diabetes. To summarize, significantly decreased astroglia density and activated neurons were exhibited in the spinal cord dorsal horn of rats undergoing DNP at the late phase of diabetes.

The release of TNF-α, NF-κB, IL-6, and other proinflammatory factors can be mediated by CXCR4 in the spinal cord dorsal horn, thus increasing neuronal excitability and aggravates central sensitization [57]. In our current work, we found that CXCR4 was mainly present in neurons (Figures 3(a) and 3(b)) and partly in astrocytes, which is in consistence with results of previous researches by us and others [6, 9]. To further investigate the critical role of CXCR4, rats with DNP were intraperitoneally injected with the CXCR4 antagonist AMD3100 at the early and late phases [31]. Intraperitoneal injection of AMD3100 reversed thermal hyperalgesia and mechanical allodynia and downregulated CXCR4 in the spinal cord dorsal horn of rats undergoing DNP at the early phase (Figures 4 and 6(a)) as was reported in other neuropathic pain models [9, 58]. However, AMD3100 did not reverse nociceptive behaviors nor did it regulate the relevant proteins in DNP rats at the late phase. It is speculated that the cascade amplification effect caused by the excessive production of inflammatory factors containing SDF-1 and TNF-α may be mediated by CXCR4 stimulation in the spinal cord dorsal horn at the late phase. We evaluated only short-term effects of AMD3100 treatment because of its short half-life (3.6 h) [59]. Another interesting finding is that daily oral administration of NAC, which could reduce nociceptive responses of thermal hyperalgesia and mechanical allodynia in STZ-induced diabetic rats, downregulated CXCR4 expression in DNP rats at the late phase (Figures 4 and 6(b), Figures 3(c)–3(e)). In our previous and current researches, we found that antioxidant NAC treatment reduced oxidative stress (Figure 7) and restored normal autophagic function in STZ-induced diabetic rats [30, 60]. However, daily oral administration of NAC was ineffective in DNP rats in the early phase. A possible explanation is that the treatment duration of one week was too short, and as such, it was not yet effective in the early phase. Similarly, injection of NAC inhibited and reversed chronic inflammatory pain, but it did not reduce nociceptive behavior in the formalin-induced acute pain model in mice [61]. According to current study, CXCR4 exerts an essential role in the peripheral sensitization of STZ-induced diabetic rats with DNP at the early phase. Meanwhile, a persistent increase in CXCR4 and high blood glucose may further exacerbate astrocyte dysfunction at the late phase of diabetes.

4.2. Connexin 43 Hemichannel and CXCR4 Release in Astrocytes and Neurons in the Late-Phase Diabetic Neuropathic Pain. CX43 acts as a channel for intercellular connections and allows transmission of ions or small molecule neurotransmitters, which plays a crucial role in neuropathic pain [41, 62–64]. Previous studies showed that CX43 existed in astrocytes [21, 22], while several publications have demonstrated that CX43 existed in neurons [65–67]. Our findings support that the expression of CX43 existed in not only primary cultured astrocytes but also in neurons (Figures 9(c) and 9(d)). In addition, CX43 acts as a channel for intercellular connections and maintains late-phase neuropathic pain [63]. Consistently, our study demonstrated that CX43 was obviously added in the

spinal cord dorsal horn of adult rats with DNP in the late phase of diabetes (Figures 8(a)–8(d)). In particular, rats with DNP treated with AMD3100 significantly decreased the expression of CX43 in the spinal dorsal horn. In addition, the finding that the expression of CX43 is regulated by CXCR4 during neuropathic pain is supported by studies conducted in other neuropathic pain models such as nerve ligation [68]. It is speculated that elevation in the expression of proinflammatory CXCR4 occurred in rats with DNP at the late phase of diabetes, and it subsequently induced increases in CX43 expression. CXCL12, a ligand of CXCR4, is likely involved in stimulating the activation and phosphorylation of CX43 via PI3K/Akt pathway, as evidenced in other studies [69, 70]. Furthermore, CXCR4 and CX43 were coexpressed in the spinal cord dorsal horn of STZ-induced DNP rats in the late phase (Figures 9(a) and 9(b)). This suggests that inflammatory or harmful substance-stimulated signals may have been amplified via transmission through CX43 and subsequently facilitated the initiation and the maintenance and exacerbation of DNP. Further study is needed to quantify and locate CX43 in the spinal cord dorsal horn of rodents with DNP and to consolidate its relationship with CXCR4 in the development and progression of DNP.

5. Conclusion

We report unique roles of CXCR4 and CX43 in cell excitability and neurotransmission mediated by dysfunctional astrocytes and activated neurons in the spinal cord of rats with DNP in rats, as summarized in Figure 10. According to results of the present work, the CXCR4/CX43 signaling pathway might play the role of a promising therapeutic target to alleviate DNP. In the late phase, irreversible damage to neurons may be mediated by dysfunctional astrocytes of the spinal cord dorsal horn, which may generate an excitatory neuronal membrane potential and induce an abnormal sense of pain. This dysfunction could be caused by either neuronal damage or inadequate protection of astrocytes, which still requires further exploration.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

This study is claimed to show no conflicts of interest.

Authors' Contributions

ZY and CJ considered and guided the experiments. ZD, F-TT, C-YH, L-YP, L-DY, CY, and TJ performed the experiments. H-XY performed the statistical analyses of the data. ZD wrote the manuscript. X-ZY and CJ proofread and revised the manuscript.

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Review Article

Mesenchymal Stem Cell Therapy: A Potential Treatment Targeting Pathological Manifestations of Traumatic Brain Injury

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Traumatic brain injury (TBI) makes up a large proportion of acute brain injuries and is a major cause of disability globally. Its complicated etiology and pathogenesis mainly include primary injury and secondary injury over time, which can cause cognitive deficits, physical disabilities, mood changes, and impaired verbal communication. Recently, mesenchymal stromal cell- (MSC-) based therapy has shown significant therapeutic potential to target TBI-induced pathological processes, such as oxidative stress, neuroinflammation, apoptosis, and mitochondrial dysfunction. In this review, we discuss the main pathological processes of TBI and summarize the underlying mechanisms of MSC-based TBI treatment. We also discuss research progress in the field of MSC therapy in TBI as well as major shortcomings and the great potential shown.

1. Introduction

More than 50 million people worldwide suffer from traumatic brain injury (TBI) annually, creating a significant burden on society and families [1]. It has also been shown that TBI is associated with an increased incidence of common neurodegenerative diseases such as Alzheimer's disease (AD) [2, 3] and Parkinson's disease [4, 5]. Severe TBI can trigger a long-term neurodegenerative process leading to pathological features and clinical manifestations similar to those of neurodegenerative diseases such as AD, structural destruction of neurons and functional impairment, and memory and cognitive decline, which in turn affect speech and motor systems [6]. TBI refers to the physical damage to brain tissue caused by a violent blow to the head. The primary injury results from direct mechanical injury. The secondary injury is characterized by diffuse axonal injury and inflammation that can protect tissues from pathogens and remove cell debris; however, severe cases can lead to neurodegeneration and secondary neuron death [7–9]. The secondary injury is a progressive process that lasts from hours to days, which means that therapeutic interventions can be administered at this stage to avoid progressive nerve cell death and enhance functional recovery after brain trauma. TBI may disrupt the blood–brain barrier (BBB) to cause neurochemical, metabolic, and cellular changes [10–12] and activate microglia and astrocytes.

The activation of microglia and astrocytes leads to the removal of cellular debris, restoration of the BBB, and production of neurotrophic factors [13]. However, inflammatory cells, such as neutrophils, are recruited to accelerate the inflammatory response and cause damage to peripheral tissues [14, 15]. The adult brain undergoes limited remodeling to compensate for tissue damage after TBI [16]. Therefore, new treatments for TBI can be developed by

elucidating brain tissue remodeling and internal repair processes.

Over the past few decades, treatment for TBI has always been a focus of attention. Three main options are commonly used to treat TBI: hypothermic therapies reduce intracranial pressure, decrease inflammatory responses, and lower cerebral metabolic rate [17]. Surgical therapies remove most of the skull bone by debridement decompression to reduce intracranial pressure and remove hematomas [18]. Pharmacological therapies reduce active bleeding, nourish the nerves, are anti-inflammatory, and include erythropoietin [19], tranexamic acid [20], and recombinant interleukin-1 receptor antagonist [21, 22].

The latest studies have shown that mesenchymal stromal cells (MSCs) have great potential in treating TBIs due to their anti-inflammatory and antiapoptotic properties and the ability to generate new nerves. Similarly, extracellular vesicles (EVs) released by MSCs cross the BBB and promote endogenous angiogenesis and neurogenesis, reduce inflammation, and facilitate cognitive and sensorimotor recovery after TBI. Taken together, this suggests that MSCs may be a promising cell-free therapy for TBI [23]. In this review, we summarize the possible molecular or cellular mechanisms of MSCs as a therapeutic approach in TBI pathology. At the same time, the prospect of cellular therapy, represented by MSCs and exosome-based, cell-free therapy, is analyzed to demonstrate its therapeutic potential.

2. TBI-Based Functional Features of MSCs

To date, 125 clinical trials have been conducted using MSCs for neurological diseases [24], including TBI treatment. The administration of autologous bone marrow MSCs (BM–MSCs) to patients during the subacute phase of TBI resulted in improved neurological function in 40% of patients [25]. A stem cell is a type of cell that is not highly differentiated and has the potential to regenerate various tissues, organs, and the human body. These cells can be classified into totipotent stem cells, multipotent stem cells, and unipotent stem cells according to different differentiation potentials. Stem cells can be induced to proliferate and differentiate into corresponding tissues and organs under appropriate conditions, which is of extraordinary significance in clinical treatment. MSCs are multipotent stem cells with self-renewal and multidifferentiation abilities [26]. These cells are widely found in a variety of tissues throughout the body and can be isolated from many sources, including BM [27], synovial membrane, skeletal muscle [28], adipose tissue [29], and peripheral blood [30]. MSCs can differentiate into mesodermal cells and tissues in different microenvironments [31]. Such cells have the advantages of easy access, low immunogenicity, regenerative potential even after freezing, and the ability to migrate to the lesion [32]. These characteristics make MSCs a promising regenerative treatment for brain trauma. Initially, the therapeutic efficacy of MSCs was thought to be based on their ability to differentiate and replace damaged cells. However, recent studies have revealed that the repair of damaged tissues is mainly through cell–cell interactions, paracrine effects, and the release of EVs [33, 34]. In a rat model of TBI, intravenously administered BM-MSCs can penetrate the BBB and increase trophic factors in the brain [35]. They can also selectively migrate to injured areas of brain tissue and differentiate into neurons and astrocytes [36]. Promoting axonal remodeling in the brain and angiogenesis and glial cell growth at the site of injury can accelerate the internal repair process while achieving the goal of promoting neuroprotection, neurorepair, and restoration of motor function.

Exosomes are small vesicles with a 50-200 nm diameter containing RNA, mRNA, DNA, and biologically active substances such as proteins and lipids [37]. Released from numerous cells, exosomes play a key role in intercellular signal transduction in physiological or pathological processes [38]. BM-MSC-derived exosomes reduce neuroinflammation by releasing anti-inflammatory cytokines and affecting the apoptosis of activated T cells [39, 40]. It was found that MSCs promoted neurological recovery in a rat model of TBI [41]. Even the exosomes secreted by MSCs under hypoxic conditions can delay neuronal degeneration and promote neural recovery [42]. Studies have shown that EVs have low immunogenicity and the ability to stimulate neurovascular repair, characteristics similar to those of MSCs. Compared to MSCs, EVs are more stable and equally capable of crossing the BBB. The use of EVs reduces safety issues associated with the administration of live cells, such as microvascular obstruction and abnormal growth of transplanted cells [43]. In addition, they have the advantages of being free of ethical problems, are less invasive, and show low tumorigenicity [44], which has extraordinary significance for their wide range of applications. The cell source of exosomes can be clonally selected to ensure their standardization and reproducibility, making the industrial production of exosomes more promising [45]. However, proteomic analysis revealed differences between human MSC-derived exosomes isolated from BM, adipose, and human umbilical cord perivascular cells [46]. More studies are therefore needed to determine the best choice of MSCs for exosomes to be used in TBI treatment.

In short, MSCs and their secreted exosomes are promising candidates for TBI treatment. Many clinical studies are underway to determine the optimal route and time of administration and dosage of MSCs and exosomes, which are popular directions for future research.

3. The Role of MSCs in Treating TBI

3.1. Mitochondrial Dysfunction and Transfer. Mitochondria not only produce adenosine triphosphate (ATP) for various metabolic activities but are also involved in regulating cell death. In TBI-related neurological injury, secondary injury is mainly caused by mitochondrial dysfunction [47]. Damaged mitochondria trigger a chain of pathological events [48], such as excitotoxicity, increased reactive oxygen species (ROS) production, oxidative stress, mitochondrial DNA damage, and mitophagy [49], leading to decreased cellular energy production [47] and apoptosis.

Due to the prevalence of mitochondrial dysfunction in TBI, one potential therapeutic target is to improve mitochondrial function. Numerous studies have focused on mitochondria as therapeutic targets for acute brain injury in recent years. For example, therapies that reverse mitochondrial uncoupling, increase mitochondrial antioxidant production, or inhibit mitochondrial permeability transition pores (MPTP) have been investigated [50]. Neuroprotective therapies have also been identified as promising therapies. Reperfusion strategies, hemoglobin management, and therapeutic (induced) hypothermia do well in neuroprotective therapy [51]. As a new mechanism of stem cell therapy, MSC-derived mitochondrial transplantation has achieved promising results [52]. A series of preclinical studies and clinical trials have shown that MSCs can transfer mitochondria to damaged cells via various routes [34], replace defective mitochondria, or compensate for their dysfunction [53]. Mitochondrial transfer protects cells from damage and apoptosis by increasing mitochondrial membrane potential, restoring aerobic respiration, or reducing inflammation. As previously mentioned, neurogenic inflammation is a pathological manifestation of TBI [52]. Studies have shown that MSCs moderate secondary injury due to inflammation [54]. Mitochondria can be transferred between MSCs and immune cells, including macrophages and T cells [55], regulating their functions and changing cytokine expression profiles. Morrison et al. reported that MSCs could donate mitochondria to host macrophages, leading to suppressed cytokine production, increasing M2 macrophage marker expression, and enhanced macrophage phagocytosis [56].

Furthermore, in rats, transferred mitochondria have been shown to enhance angiogenesis and improve functional recovery of the brain microvascular system [57]. In the process of neuronal apoptosis involved in mitochondria, B-cell lymphoma 2 (Bcl-2) family proteins are proapoptotic factors and promote mitochondrial membrane permeability [58]. An apoptotic cascade is triggered, and caspases (including caspase-3) are activated, resulting in caspase-dependent DNase proteolysis and internucleosomal DNA fragmentation [59]. Mitochondrial transfer from MSCs can decrease apoptosis rates in recipient cells and improve cell survival [60] by regulating the Bcl-2-associated X protein (Bax)/Bcl-2 ratio and decreasing caspase-3 expression [61]. The protective effect of mitochondrial transfer therapy on nerves and the restoration of spinal cord function are apparent. Research has indicated that mesenchymal multipotent stromal cells can supply mitochondria to damaged astrocytes [62]. The transfer of MSC-derived mitochondria to oxidant-damaged neurons may help increase neuronal survival and improve metabolism [63]. In a spinal cord injury rat model, mitochondria can be transferred from BM-MSCs to injured motor neurons to significantly improve locomotor functions six weeks after injury [64].

A growing body of research suggests that intercellular mitochondrial transfer between MSCs and target cells occurs through tunneling nanotubes (TNTs) [65], microvesicles [66], EVs, gap junctions, and cytoplasmic fusion [67, 68]. At present, the formation of TNTs is the most widely accepted theory. TNTs are a type of nanotube that can transport substances directly between cells, including proteins, ions, RNA, organelles, viruses, and cytosol [69]. Thus, although mitochondrial transfer is directed and mostly one-way transportation [52], it can also manifest as bidirectional transportation [70], meaning that MSCs may exchange mitochondria with other types of cells. The regulation of mitochondrial transport directionality remains to be studied further. Mitochondria also play a regulatory role in the renewal and differentiation of MSCs. In other words, a bidirectional interaction exists between mitochondria and MSCs.

In addition, mitochondrial transfer therapy has other potential dangers. Transferred mitochondria support tumor progression by providing energy to cancer cells [71] and increasing drug resistance [72]. A tumor-induced inflammatory response leads to the production of chemokines, which attract MSCs to the site of inflammation. Due to good differentiation capabilities, MSCs can differentiate into cancer-induced fibroblasts. Such fibroblasts play a role in immune regulation thus promoting the growth and migration of cancer cells. Studies have shown that MSCs can transport mitochondria to breast cancer cells and glioblastoma stem cells to promote tumor growth. Studies have found that MSCs transfer cytoplasmic content but not mitochondria to cancer cells and may lead to chemotherapy resistance in cancer cells. However, the specific mechanism of mitochondrial transport between MSCs and other cells is still unclear. Therefore, in some cases, mitochondrial transfer should be suppressed. It is worth mentioning that the source and status of MSCs also affect mitochondrial transfer. The mitochondrial transferability of MSCs isolated from different tissue sources varies. The therapeutic effects of damaged or aged MSCs are limited and unsuitable for stem cell therapy. In inflammatory environments, the formation of TNTs is inhibited, thus affecting the transport of mitochondria from MSCs to damaged cells. Therefore, the MSC source should also be considered.

3.2. Oxidative Stress. Oxidative stress is a disorder in the generation and removal of ROS, a double-edged sword. While causing some damage, ROS also stimulate repair. When excess radicals are produced, repair processes are impaired, leading to oxidative stress and cell death through apoptosis or necrosis [73]. Studies have shown that during secondary TBI injury [74], free radical production and oxidative damage are influential in neuronal structures (e.g., axons). After axon injury, excessive Ca^{2+} influx can cause mitochondrial dysfunction and ROS overproduction [75]. ROS can destroy the integrity of cell membranes and cause cell damage through lipid peroxidation, protein, and DNA oxidation and the inhibition of mitochondrial electron transport chains. ROS can also activate microglia in the brain to release inducible nitric oxide synthase (iNOS) and cytokines, leading to inflammation and cell death [76].

Meanwhile, due to a lack of introns and its proximity to the source of ROS, mitochondrial DNA (mtDNA) is liable to

oxidative damage. This may lead to decreased respiratory function and promote ROS production—a vicious cycle that eventually induces apoptosis [77]. ROS are also known to trigger the mitochondrial apoptosis cascade through interaction with the permeability transition pore complex protein [78]. The importance of oxidative stress in mitochondrial dysfunction and neuronal death after acute brain injury cannot be ignored and suggests that targeted therapy is promising.

Many studies have shown that MSCs can protect brain tissue from severe damage by inhibiting oxidative stress. In a TBI mouse model, overexpression of specific genes, such as that for superoxide dismutase 2, *in vitro* can enhance the antioxidant effect of MSCs and improve their therapeutic effect [79]. Histone deacetylase 1 (HDAC1) promotes stromal cell self-renewal and disease recovery by enhancing histone acetylation [80]. Silencing HDAC1 in MSCs attenuates oxidative stress and neuroinflammation, thus improving its therapeutic effect [81]. *In vitro* studies have found that mitochondria from MSCs are reduced in mouse neurons following hydrogen peroxide exposure [63]. Transferring mitochondria from MSCs to neurons impaired by oxidative stress may contribute to the preservation of posttraumatic neurons and restore their function. In addition, it has been shown that MSCs can mitigate the effects of oxidative stress in the central nervous system by changing the activity of ascorbic acid and catalase [82]. MSCs can also increase expression of the antiapoptotic gene, *Bcl-2*, and decrease the level of superoxide anion, thereby protecting brain tissue [83]. Olfactory mucosa MSCs are helpful in antioxidative stress and neuroprotection by upregulating *SPCA1* expression, reducing Ca^{2+} overload and Golgi edema and lysis, therefore, playing a significant role in combatting oxidative stress and facilitating neuroprotection [84]. In addition, exosomes produced by MSCs can increase ATP production, reduce oxidative stress, and activate the phosphoinositide 3-kinase/Akt pathway [85], which is of great value for the application of exosomes in TBI treatment. MSC-derived EVs inhibit proinflammatory responses and reduce oxidative stress and fibrosis in *in vivo* models [86]. The above results show that MSCs play a significant role as antioxidants in treating TBIs.

In addition, during oxidative stress, astrocyte-derived exosomes transport neuroprotective apolipoprotein D to neurons to improve the neuronal survival rate [87]. Meanwhile, astrocyte-derived exosomes protect hippocampal neurons after TBI by activating the nuclear factor erythroid 2-related factor 2 signaling pathway in animal models to prevent TBI-induced oxidative stress and neuronal apoptosis [88]. Recent studies have shown that micro (mi)RNAs within astrocyte exosomes are different under proinflammatory and oxidative stress conditions versus the resting state [89]. This has important implications for future studies on the potential role of miRNAs in cellular communication, inflammation, and exosome therapy for TBI.

3.3. Neuroinflammation. Neuroinflammation is associated with secondary TBI injury [90]. TBI leads to neuronal damage and damages the integrity of the BBB. Immune cells invade and activate glial cells such as microglia and astrocytes [91, 92]. Microglia polarize to the M1 (proinflammatory) phenotype, and expression of the surface protein cluster of differentiation 14 (CD14) is promoted, which is a sign of acute inflammation caused by TBI. Glial cells continuously release inflammatory mediators, such as interleukin- (IL-) 1, IL-6, tumor necrosis factor- (TNF-) α, and other cytokines, to attract more peripheral macrophages and neutrophils across the leaky BBB, consequently converting inflammation from the acute to chronic phase [93]. At the same time, neurons and microglia are damaged, and cellular adhesion molecules and matrix metalloproteinases are secreted in addition to immune cells. The persistent TBI-induced inflammation can result in neuronal loss and cerebral edema [94] and lead to degenerative diseases such as AD [95]. TBI can also cause peripheral inflammation, mainly in the spleen and thymus, which may lead to multiple organ dysfunction and even death. Studies have shown that plasma levels of inflammatory molecules begin to rise 6 hours after TBI and continue to increase [96]. The release of these inflammatory molecules, including TNF-α, IL-6, and ROS, promotes systemic diseases such as cancer, atherosclerosis, and diabetes. Neuroprotective and anti-inflammatory drugs are potential therapies for TBI. Many preclinical studies and clinical trials have demonstrated that MSCs can regulate the inflammatory microenvironment, thus decreasing inflammation and immune reactions to promote tissue repair [97, 98]. The therapeutic effects of MSCs regarding neuroinflammation are achieved through paracrine factors [99]. Following implantation, MSCs cross the BBB, migrate to the site of injury, and release trophic factors to recover neuronal structure and function [100]. MSCs regulate innate and adaptive immune cells by releasing soluble factors to enhance anti-inflammatory pathways at the site of injury [101]. A study in a TBI rat model showed that MSCs decreased the number of microglia and other inflammatory cells, reduced the production of proinflammatory cytokines, and increased anti-inflammatory cytokines to inhibit TBI-induced inflammatory responses. MSCs enhance TNF-stimulated gene 6 expression, which suppresses the NF-κB signaling pathway [102]. When BM-MSCs were administered seven days after TBI, a 50% reduction in interferon-γ and TNF-α expression was observed, as well as an increase in neurogenesis and a significant decrease in BBB permeability, edema, microglial activation, and norepinephrine levels [103, 104]. A recent study of 20 patients with severe TBI showed that after successful intravenous MSC treatment, the percentage of neutrophils in the blood decreased significantly to normal levels, and the production of IL-6, C-reactive protein, TNF-α, and ROS also decreased. It is suggested that MSC therapy restricts the accumulation of immune cells and systemic inflammatory cytokines at the injured site. In addition, compared with the control group, the Glasgow score and Health Stroke Scale of the group treated with MSCs increased starting on the seventh day post-TBI. This proved that MSC therapy contributed to the

recovery of motor function and consciousness in patients with TBI [96].

Moreover, MSCs inhibit phagocytosis and stimulate microglial polarization to the more neuroprotective and anti-inflammatory M2 phenotype, thereby ameliorating functional deficits in rats with TBI [105]. Studies have shown that proteins in BM-MSC-exosomes injected into C57BL/6 male mice can downregulate iNOS and upregulate CD206 and arginase-1, resulting in polarization of microglia/macrophages and inhibition of early neuroinflammation in TBI [106]. MSCs can also suppress T cell proliferation and monocyte differentiation, thus affecting dendritic cell functions and increasing the production of IL-10 [100].

Many studies have shown that infusion is a common method of drug administration in stem cell therapy. Intranasal secretome administration has been assessed as a noninvasive and efficient route of administration that targets cells to the brain [107]. Administering autologous BM–MSCs by lumbar puncture has also been shown to be a safe and efficient cell therapy [25]. Focal intracerebral transmission of MSCs may be more suitable for focal injury as this will directly target areas of inflammation [105]. Compared to monotherapy, combination therapy that includes regulatory T cells and MSCs enhances potency and significantly attenuates inflammation after TBI [108]. Combined BM-MSCs and the peroxisome proliferator-activated receptor gamma agonist, pioglitazone [109] or propranolol [103] can also enhance anti-inflammatory effects. Several studies have shown that intravenous injection of BM mononuclear cells followed by MSCs improves cognitive function in patients with TBI [96]. A few studies showed that the reaction of host immune cells to the transplanted MSCs may be harmful [110]. Therefore, more research is needed to understand the long-term impact of stem cell therapy.

3.4. Apoptosis. In addition to trauma-induced primary damage to the BBB and neuronal death, neuronal and oligodendrocytic apoptosis is a marker of secondary brain injury [111]. After TBI, significant nerve cell death can be found in the hippocampus. The cell fragments released from the damaged site can activate an immune response from microglia and astrocytes, resulting in the release of inflammatory factors and result in neuroinflammation. The release of TNF-α can activate the caspase-3 signaling pathway and induce neuronal apoptosis. Neuronal apoptosis is dependent on the opening of MPTP and the release of cytochrome C [50, 112]. Cytochrome C forms an apoptosome in the cytosol by interacting with the protein cofactor, apoptotic protease activating factor-1, to trigger an apoptotic cascade. The complex activates procaspase-9 and induces a caspase-9-dependent intrinsic pathway [113]. Subsequently, caspase-3 and other caspases are activated resulting in caspase-dependent DNase proteolysis and internucleosomal DNA fragmentation [59]. Mitochondrial pathway-induced apoptosis can be ameliorated by mitochondrial transfer of MSCs. The paracrine mechanism of MSCs promotes angiogenesis and is anti-inflammatory and antiapoptotic [114]. Many studies have shown that MSCs can improve neuronal survival and cure brain injury by interfering with the apoptotic pathway [100]. An experiment by Mettang et al. found elevated levels of the proapoptotic mediators, Bax and Bad, in a closed head injury model of TBI [115]. A recent study showed that the neurological function of C57BL/6 male mice treated with 30 μg protein equivalent of BM-MSC-exosomes was significantly improved compared to control mice. The expression of the proapoptotic protein, Bax, was inhibited, while the expression of the antiapoptotic protein, Bcl-2, was enhanced. Injection of MSC-EVs into 3-day-old Wistar rats decreased nerve cell death, white matter microstructure destruction, and glial cell proliferation induced by lipopolysaccharides [116]. MSCs can also downregulate caspase-3, promote the production of antioxidants, and secrete neurotrophic factors such as neurotrophin-3 [117]. In addition, various nutritional factors secreted by MSCs can inhibit endothelial cell apoptosis [118] and promote the formation of new capillary branches in injured brain tissue [119]. Angiogenic paracrine factors of MSCs include human vascular endothelial growth factor, transforming growth factor-β1, monocyte chemotactic protein-1, and IL-6 [120]. These factors have been proved to reduce apoptosis and injury volume and improve motor and cognitive impairment in patients. The paracrine effects of MSCs play an essential role in regulating apoptosis pathways, thereby improving neuronal survival rate, promoting angiogenesis, repairing nerve injury, and maintaining the physiological functions of the brain.

4. Comparison of Existing Therapies

With its severe and complex secondary pathologies, TBI has greatly affected patients' quality of life and brought a medical burden onto their families and society. In the past few decades, traditional treatments, such as hypothermic therapies, surgery, and drug therapies, have been the main treatments for TBI. Medical interventions, such as drug and hypothermia therapies, are usually considered for patients with mild to moderate TBI. Invasive surgical treatment is needed for extra-axial hematoma, concussions, and brain edema. However, limitations exist with traditional treatments. Specifically, the focus is on the relief of physiological symptoms to maintain quality of life. However, treatment efficacy is limited and is more likely to cause secondary trauma. In addition, the pain of long-term sequelae and lifelong disability cannot be prevented. In recent years, stem cell therapy has become more popular. Stem cell transplantation can prevent or reverse damage at the biochemical and cellular levels and relies on endogenous healing mechanisms to restore brain function. For elderly patients with TBI, a combination of cell transplantation and other treatments, such as cooling and electrical stimulation, may be needed to promote brain repair. Stem cell therapy may be more effective in promoting neuronal regeneration in young people [121]. Stem cells can be divided into hematopoietic stem cells, MSCs, neural stem cells (NSCs), epithelial stem cells, and skin stem cells. Recent studies have shown that various stem cells can treat neural damage after TBI, including MSCs, NSCs,

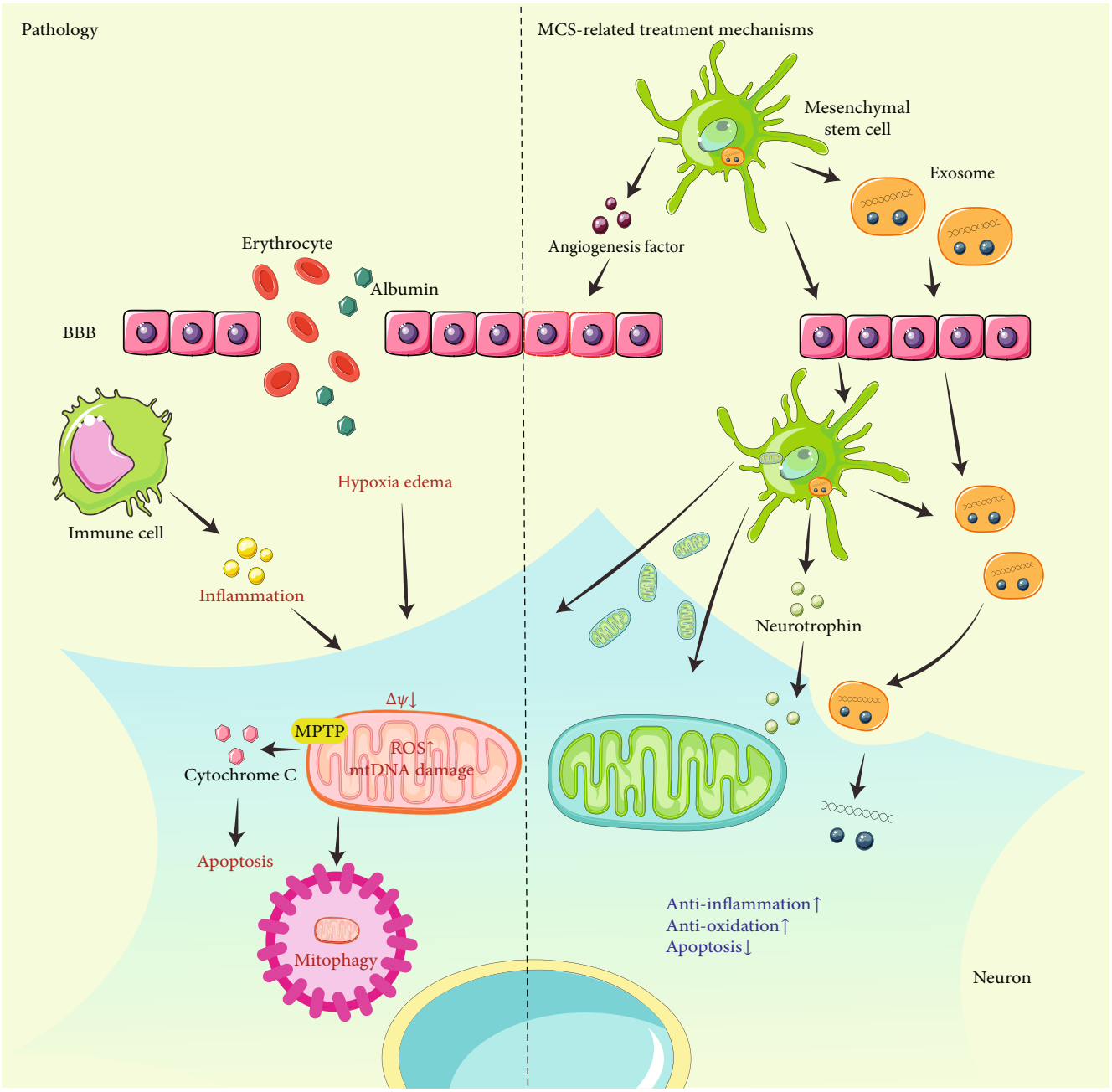

Figure 1: TBI pathology and MSC-related treatment mechanisms. When TBI occurs, the BBB is disrupted, leading to a series of responses such as hypoxia, edema, and release of inflammatory factors by immune cells. Excessive production of ROS in neurons and alteration of mitochondrial membrane potential results in a variety of pathological processes, such as oxidative stress, mtDNA damage, mitophagy, and apoptosis, some of which will eventually lead to cell death. Furthermore, they also enable MPTP to open instantly promoting the above pathological processes. When we use MSCs to treat TBI, MSCs and their released exosomes can cross the BBB stably and release various cytokines, such as neurotrophic factors and vascular regeneration factors, to promote nerve and blood vessel repair and regeneration. MSCs can also deliver healthy mitochondria to neurons through TNTs to enhance the anti-inflammatory, antioxidant, and antiapoptotic abilities of neurons and eventually improve their functions. BBB: blood-brain barrier; MSC: mesenchymal stromal cell; MPTP: mitochondrial permeability transition pores; mtDNA: mitochondrial DNA; ROS: reactive oxygen species; TBI: traumatic brain injury; TNT: tunneling nanotubes.

multipotent adult progenitor cells, and endothelial progenitor cells. Of these, MSCs have the most significant therapeutic potential because of the ease of isolation, low immunogenicity, and ability to differentiate into various tissue lineages, including brain cells [122]. However, several limitations still exist for MSC transplantation. Contamination is probable during the culture and treatment of MSCs, and *in vitro* cultured cells are prone to mutation. Cell transplantation may also lead to the transmission of foreign pathogens. In addition, MSC transplantation may provide energy for cancer cells and promote tumor growth and metastasis. The initiation and regulation of mitochondrial transfer from MSCs are not clear. Additionally, the probability of allogeneic immune rejection cannot be ignored. Therefore, it is particularly important to improve the safety of MSC therapy.

5. Conclusion and Future Perspectives

The treatment of TBI has received much attention due to its high morbidity and complex secondary cascades. Oxidative stress, neuroinflammation, neuronal apoptosis, and mitochondrial dysfunction are all classic pathological manifestations of TBI. In recent years, MSC transplantation has been investigated as a therapeutic approach due to its ability to repair damaged brain tissue in TBI models. Transplanted MSCs can pass through the BBB and migrate to damaged brain tissue to play a therapeutic role through multidirectional differentiation, paracrine effects, and the release of EVs. Apart from secreting nutritional factors to exert anti-inflammatory effects and promote angiogenesis, MSCs can also transfer mitochondria to damaged neurons via TNTs (Figure 1). Compared with traditional therapies, MSC treatment can directly improve TBI-induced pathological changes and promote recovery of neurological function. However, the efficacy and safety of MSCs as a potential therapy for TBI remain controversial. Available preclinical studies have shown that the excellent repairability of MSC may sometimes be translated into oncogenic ability. The potential risk of an immune response by the host's own immune cells to MSCs is unclear. In addition, the appropriate timing of drug administration, more efficient routes of administration, reliable cell sources, and methods of cell culture, storage, and transportation are all worthy of discussion. Insufficient clinical trials have been conducted to demonstrate a direct therapeutic effect of MSC therapy on the pathological manifestations of TBI. In a series of clinical studies on stroke, despite the fact that MSCs isolated from different tissues were effective in treating this disease, disparities in efficacy existed between trials. Although transporting MSCs through the intracerebral pathway is most effective, it is also the most invasive. In contrast, the intravenous route is the least invasive and reaches the least number of MSCs in the damaged brain tissue. Therefore, it may be challenging to obtain stable cells, deliver MSCs accurately through a safe delivery method, and obtain stable efficacy of MSC therapy for TBI. MSC therapy can be optimized in several ways. For example, genetically modified MSCs can be the basis for the next generation of cell-based therapies for TBI. In addition, compared to monotherapy, combination therapy with other drugs can enhance the effectiveness of treatment. Furthermore, the use of MSC-derived exosomes can avoid several problems associated with cell transplantation. However, further preclinical and clinical studies are needed to discover the therapeutic potential of MSCs.

Conflicts of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Authors' Contributions

K.Z., Y.J., D.S., and X.Z. made substantial contributions to the conception and design of the study. K.Z., Y.J., B.W., T. L., D.S., and X.Z. participated in drafting the article. K.Z. and Y.J. created the figure. All authors gave the approval of the final manuscript. Kaige Zhang and Yiming Jiang contributed equally to this work and shared first authorship.

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Research Article

circHtra1/miR-3960/GRB10 Axis Promotes Neuronal Loss and Immune Deficiency in Traumatic Brain Injury

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Circular RNAs (circRNAs) are abundant in the brain and contribute to central nervous system diseases; however, the exact roles of circRNAs in human traumatic brain injury (TBI) have not been established. In this study, we used a competing endogenous RNA (ceRNA) chipset as well as *in vitro* and *in vivo* assays to characterize differentially expressed circRNAs in TBI. We detected 3035 differentially expressed circRNAs in the severe TBI group, 2362 in the moderate group, and 433 in the mild group. A ceRNA network was constructed. The circRNA has_circ_0020269 (circHtra1) was significantly upregulated after brain insults and was correlated with the severity of injury. circHtra1 inhibited cell proliferation and promoted apoptosis, and its knockdown reversed these effects. Further analyses revealed that circHtra1 functions as a miR-3960 sponge and increases the expression of *GRB10*, which is involved in NK cell infiltration after TBI. circHtra1 was identified as a target of the IGF-1/ADAR1 axis. Reduced expression of ADAR1 (involved in A-to-I editing) after brain insults upregulated circHtra1. Our results show that circHtra1 promotes neuronal loss by sponging miR-3960 and regulating GRB10 and apoptosis during brain insults. In addition, A-to-I editing could regulate circRNA expression profiles after TBI, and circHtra1 is a potential therapeutic target.

1. Introduction

Traumatic brain injury (TBI) is associated with neurodegeneration, cognitive impairment, and psychiatric disorders, representing an enormous burden on modern society [1]. However, exact molecular and pathological changes in TBI are not clear. Noncoding RNAs have recently become novel targets for both mechanistic and therapeutic studies [2, 3]. However, few studies have evaluated tissue- or developmental stage-specific expression patterns of circular RNAs (circRNAs) and their regulatory effects in TBI. circRNAs are highly expressed in the brain, are specifically related to neuronal and synaptic function, and have been identified as independent biomarkers [4]. This type of RNA constitutes a large class of posttranscriptional regulators, some of which can act as ceRNAs by inhibiting miRNAs in the brain [5]. For instance, ciRS-7 acts as a miR-7 sponge, leading to the increased expression of miR-7 target genes, particularly in neocortical neurons and tumor cells as well [6].

Recently, Jiang et al. found a series of circRNAs widely distributed in the cortex of mice with TBI [7]. However, studies of circRNA profiles as well as their diagnostic and therapeutic value in human TBI are limited. Accordingly, we used a competing endogenous RNA (ceRNA) chipset to evaluate circRNAs and target genes in blood samples from humans with TBI and constructed a ceRNA network. A new circRNA, circHtra1, was identified, and its relationship with clinical features was assessed in both *in vitro* and *in vivo* brain injury models. Our findings highlight the important role of the circHtra1-miR-3960-GRB10 axis and its potential therapeutic value for TBI.

2. Results

2.1. Differentially Expressed circRNAs in the Blood of Humans with TBI. Differentially expressed circRNAs between patients with severe, moderate, and mild TBI and healthy controls were identified. As visualized by a volcano plot, there were 1612 differentially expressed circRNAs between the severe group and the healthy control group ($P < 0.05$, fold change > 2) (Figure 1), including 859 upregulated circRNAs and 753 downregulated circRNAs. The five most significantly upregulated circRNAs were hsa_circ_0020273, hsa_circ_0093014, HSA_CIRCpedia_152345, hsa_circ_0020269, and hsa_circ_0064339, with logFC values of 3.7–4.3. The five most significantly downregulated circRNAs were hsa_circ_0061796, hsa_circ_0129469, HSA_CIRCpedia_35578, hsa_circ_0070423, and hsa_circ_0116097, with logFC values ranging from -3.9 to -3.1 (Table S3).

In the moderate TBI group, 287 circRNAs were upregulated and 370 circRNAs were downregulated. In the mild group, 41 circRNAs were upregulated, and 67 circRNAs were downregulated. These results suggest an obvious trend of increase in the total number of altered circRNAs with increase in the severity of brain injury. Altered circRNAs were clearly separated into two clusters in a heatmap, indicating that samples had good intragroup consistency, and circRNAs expressed in the TBI group were significantly different from those in the healthy control group (Figure 1(c)).

2.2. GO and KEGG Analyses. A GO enrichment analysis of target genes of differentially expressed circRNAs was performed to evaluate alterations in molecular functions (MF), biological processes (BP), and cellular components (CC). The target genes of circRNAs in the severe TBI group were mainly enriched for the adaptive immune response, neutrophil degranulation, and defense response to virus (in the BP category, Figure 1(d)).

We also performed a KEGG pathway enrichment analysis and generated a bubble chart of the top 30 pathways related to altered target genes, including Th1 and Th2 cell differentiation, T cell receptor signaling pathway, and antigen processing and presentation (Figure 1). Target genes were mostly involved in T cell pathways and immunity in both moderate and severe TBI. In particular, the immune response was within the top three most highly enriched pathways in both the severe TBI and moderate TBI groups.

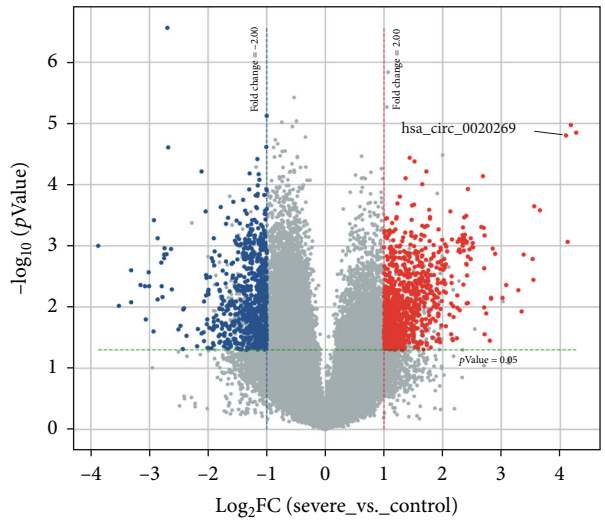
2.3. Establishment of a ceRNA Network. Using Cytoscape, relationships between individual circRNAs, miRNAs, and mRNAs were determined based on Pearson's coefficient coefficients. After rigorous selection, a circRNA–mRNA coexpression network was constructed based on the 200 most significant circRNA-associated ceRNA pairs. Several ceRNA pairs with high ceRNA scores and energy values were chosen to form a circRNA–miRNA–mRNA network (Figure S1). In particular, circRNA_0020269, CIRCpedia_36273, circRNA_0116394, CIRCpedia_152529, and circRNA_0099010 pairs showed competitive binding to dozens of miRNAs, such as hsa-miR-10400-5p, and hsa-miR-3960.

To evaluate whether the circRNAs could impact pathways by acting as ceRNAs, one core circRNA, circRNA_0020269, was further investigated. A circRNA–miRNA–mRNA interaction network derived from circRNA_0020269 and five miRNAs was analyzed further (Figure S1). The circRNA_0020269 was predicted to act as a ceRNA for four miRNAs.

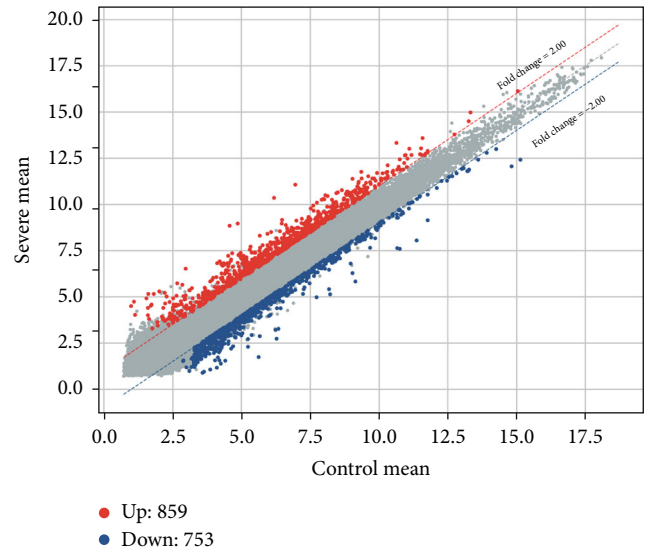
2.4. si-circHtra1 (circRNA_0020269) Facilitates Neuronal Maturation and Proliferation. Because plasma circHtra1 was upregulated in TBI, we next investigated its effect on primary cultured neurons. si-circHtra1 was used to knock down the expression of circHtra1, using si-NC as a control. As determined by CCK-8 assays, primary cultured neurons transfected with si-circHtra1 showed higher cell proliferation than that in the control (Figures 2(a) and 2(b)). Similarly, transfection with si-circHtra1 significantly increased MAP2 and β-tubulin expression in cell culture ($P < 0.05$, Figures 2(c)–2(e)). In contrast, circHtra1 overexpression inhibited CCK-8 expression in primary neurons and reduced MAP2 and β-tubulin levels (Figures 2(f)–2(j)), while si-circHtra1 or circHtra1 overexpression did not alter *Htra1* mRNA level (Figure S1 A&B).

2.5. Circular RNA circHtra1 Facilitates Neuronal Death via the miR-3960/GRB10 Axis. To determine whether circHtra1 functions as a miRNA sponge, as suggested by the ceRNA network analysis, we assessed the sequence of *circHtra1* using miRanda and circBase and identified four candidate miRNAs (miR-1908, miR-3960, miR-4665-3p, and miR-10400-5p; Figures 3(a)–3(c)). The cellular location of circHtra1 was investigated by fluorescence in situ hybridization (FISH), revealing dominant expression in the cytoplasm (Figure 3(d)). AGO2 is essential for miRNA silencing of gene expression by forming the RNA-induced silencing complex (RISC). We predicted that AGO2 could bind to circRNAs and miRNAs (based on predicted relationships between circHtra1 and AGO2 by CircInteractome). Accordingly, we performed an RNA immunoprecipitation (RIP) assay to pull down RNA transcripts that bind to AGO2 in cultured neurons. Indeed, endogenous circHtra1 was efficiently pulled down by anti-Ago2 (Figures 3(e) and 3(f)). To further test whether circHtra1 could sponge miRNAs, an miRNA pull-down assay was performed using biotin-coupled miRNA mimics (miR-1908, miR-3960, miR-4665-3p, and miR-10400-5p). Interestingly, circHtra1 was only efficiently enriched by miR-3960 but not by the other three miRNAs (Figures 3(b) and 3(c)). To confirm the interaction, we performed a luciferase assay for miR-3960 and circHtra1. Luciferase intensity decreased after cotransfection with the wild-type (WT) luciferase reporter and miR-3960 mimics, while the mutant luciferase reporter did not have the same effect (Figure 3(c)).

2.6. GRB10 Is a Direct Target of miR-3960 and Is Positively Regulated by circHtra1. Our results demonstrated that circHtra1 can bind directly to miR-3960 and act as an mRNA sponge. We next identified miR-3960 target genes. Using RNA22 v2, *GRB10* was a predicted target of miR-3960, and



(a)



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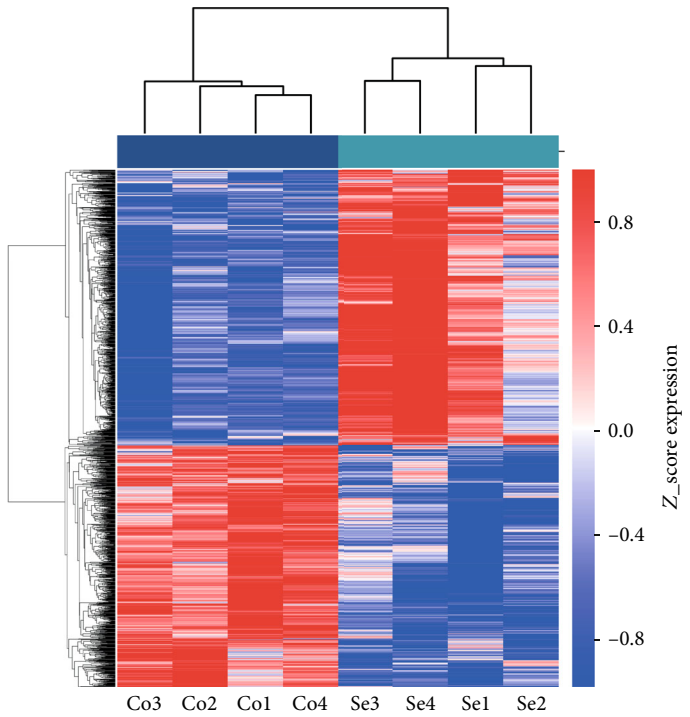
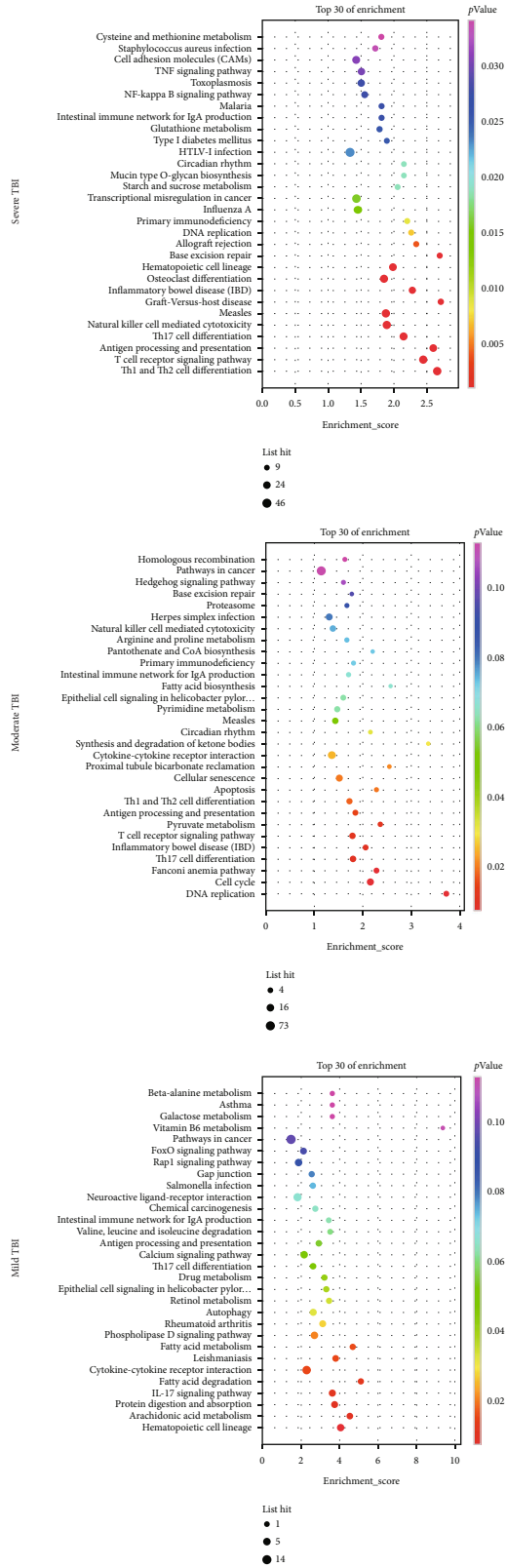


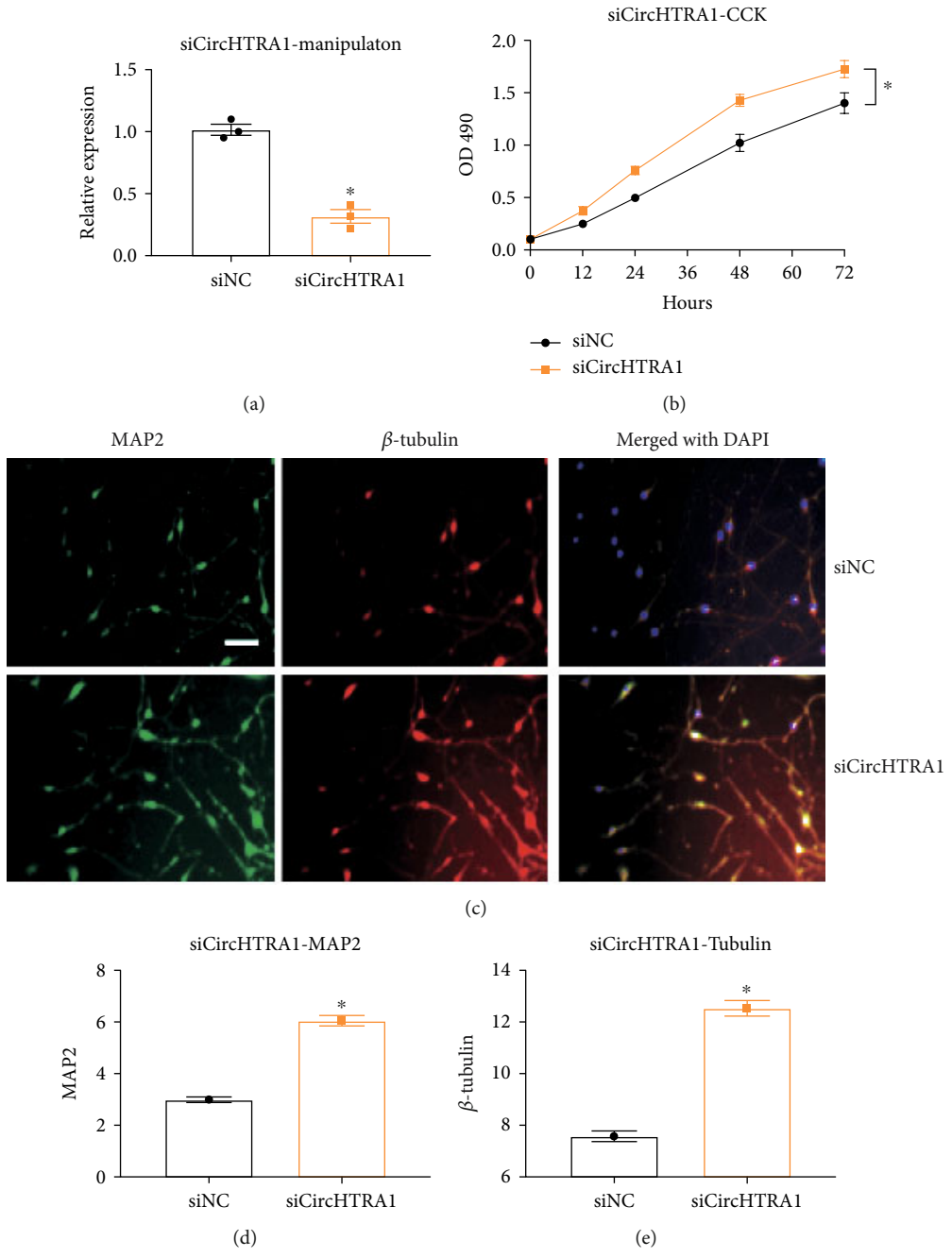
(c)

Figure 1: Continued.



(d)

Figure 1: Volcano plots, clustering analysis, and functional enrichment analysis of circRNAs and target genes. (a, b) Volcano and scatter plots revealed that 3035 circRNAs were differentially expressed between the severe group and the control group, and four severe TBI groups clustered together. (c) Top 30 KEGG pathway results visualized by bubble plots for TBI groups. (d) Upper panel: related target genes in severe TBI. Middle panel: related target genes in moderate TBI. Lower panel: related target genes in mild TBI.



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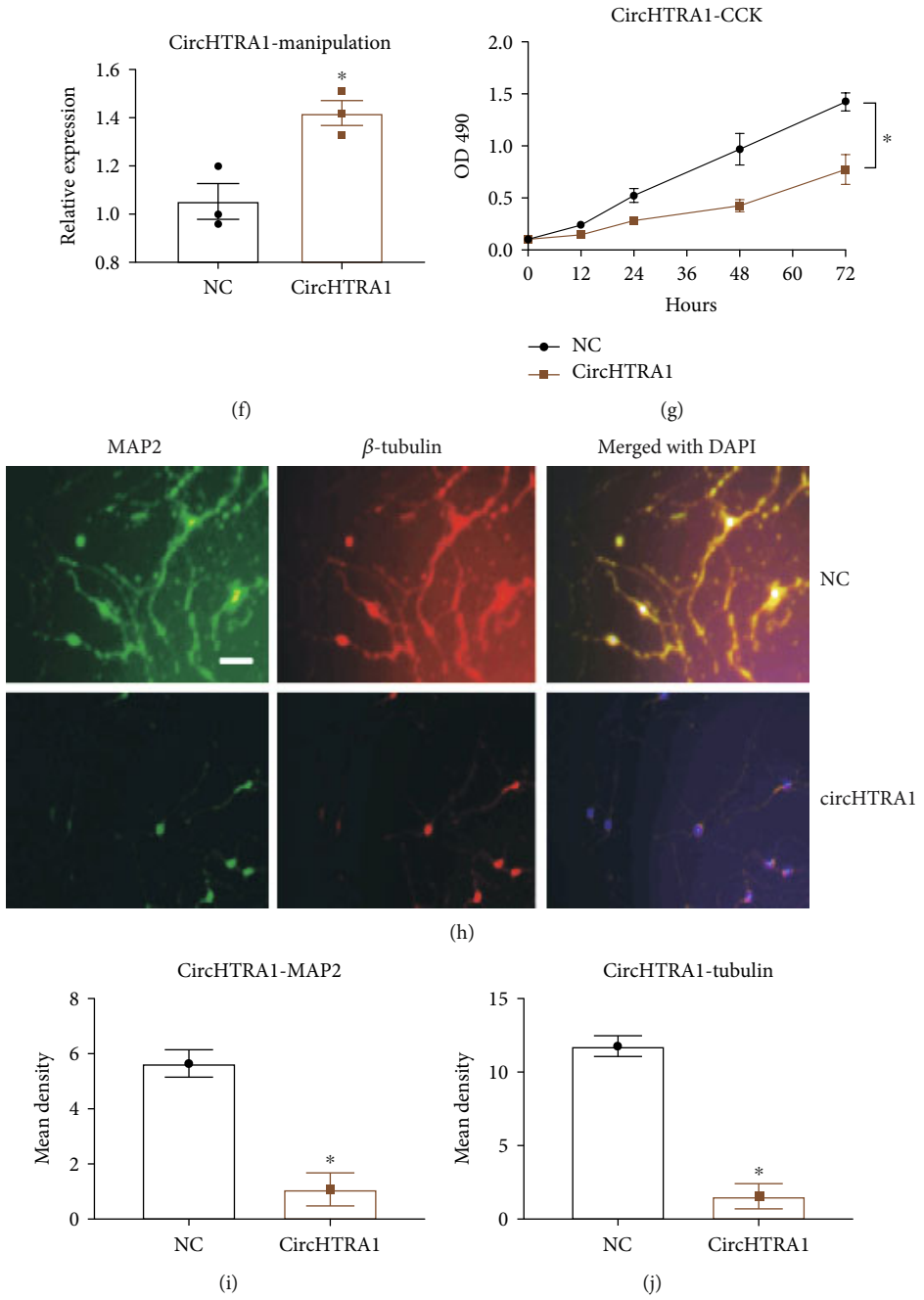
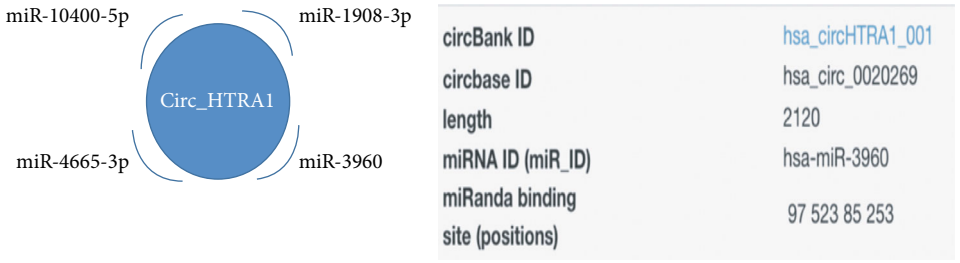


Figure 2: Effects of circHtra1 on neuronal proliferation. (a) Expression of circHtra1 in si-circ was verified. (b) After primary cortical neurons were transfected with si-NC or si-circHtra1, a CCK-8 assay was applied to assess cell proliferation. (c) Effect of si-circHtra1 on neuronal maturation (green: MAP2; red: β-tubulin, merged with DAPI). (d, e) Quantification of mean densities of MAP2 ($t = 9.496$, $df = 4$) and β-tubulin ($t = 12.14$, $df = 4$). Scale bar represents 200 μm. $^*P < 0.05$ (compared to the si-NC group); effects of circHtra1 on neuronal proliferation. (f) Verification of circHtra1 overexpression. (g) Primary cortical neurons were transfected with NC or circHtra1. (h) Effect of circHtra1 on neuronal proliferation (green: MAP2; red: β-tubulin, merged with DAPI). (i, j) Quantification of mean densities of MAP2 ($t = 7.964$, $df = 4$) and β-tubulin ($t = 9.245$, $df = 4$). Scale bar represents 200 μm. $^*P < 0.05$ (compared to the NC group).

the potential binding sites are listed in Figure 3(g). Candidate target genes were selected based on bioinformatics predictions and mRNA coexpression in TBI. To investigate the miR-3960 target genes in neurons and their correlations with circHtra1 levels, cortical neurons were transfected with NC mimics, miR-3960 mimics, mutant miR-3960, or circHtra1 or cotransfected with circHtra1 and miR-3960 mimics or its mutant form. Overexpression of miR-3960 (but not mutant miR-3960) decreased *GRB10* mRNA level; thus, *GRB10* was a target of miR-3960 in neurons (Figure 3(h)). Furthermore, circHtra1 also increased the mRNA level of *GRB10*, and circHtra1-induced upregulation



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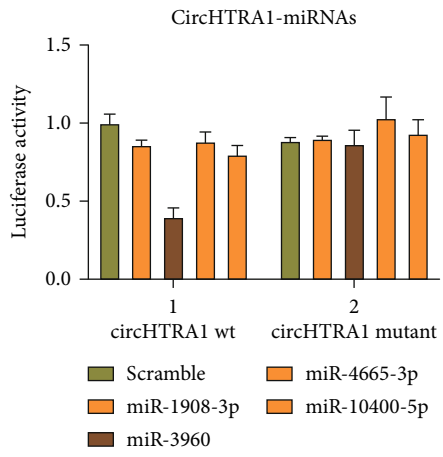

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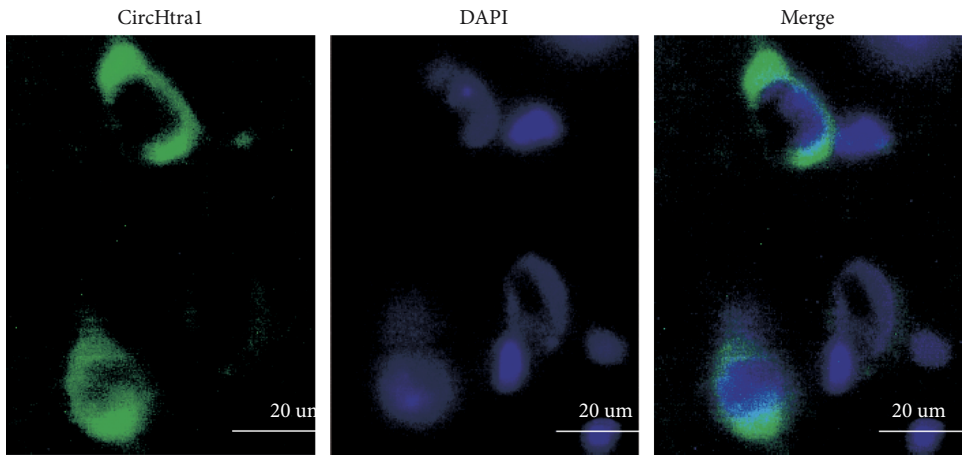

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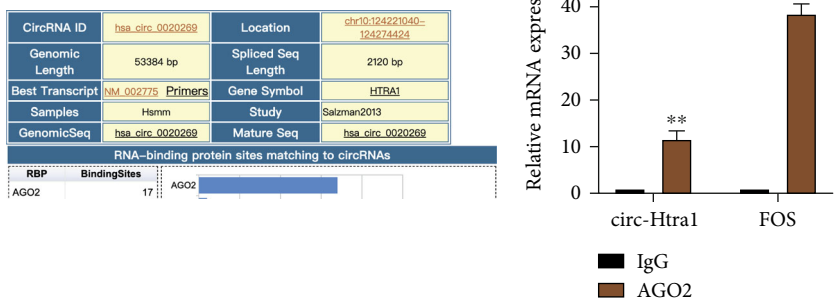
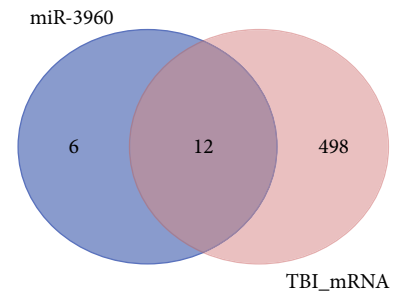


(e) (f)

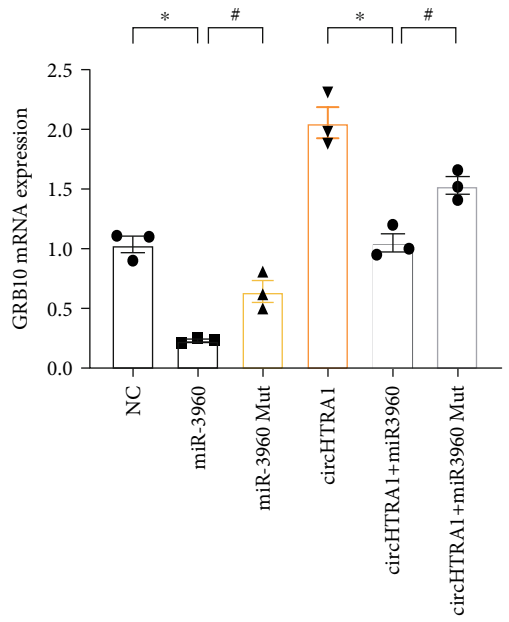
Figure 3: Continued.

miR Name	transcript name	leftmost position of predicted target site	folding energy (in -Kcal/mol)	heteroduplex	p value
hsa_miR_3960 MIMAT0019337	GRB10	313	-19.50	ACGACG-ATTTGCTGCTGTG | || |:::|:|:|: GGGGGCGGAGGCGGCGGCGG	2.04E-1
hsa_miR_3960 MIMAT0019337	GRB10	540	-13.20	TTTTTGCACTCCTTCTTTGTT :::::|| |||| :| ::|:: GGGGGCG-GAGGCGGCGGCGG	9.89E-2
hsa_miR_3960 MIMAT0019337	GRB10	1348	-17.60	CTAGAGGCTCCTCCTCTGCT |: | |||| || |:|| GGGGGCGGAGGCGGCGGCGG	9.89E-2
hsa_miR_3960 MIMAT0019337	GRB10	2147	-18.30	CTCTC-TCTCACACGCCTCT |:|:| :||| |||| |: GGGGGCGGAGGCGGCGGCGG	7.17E-2

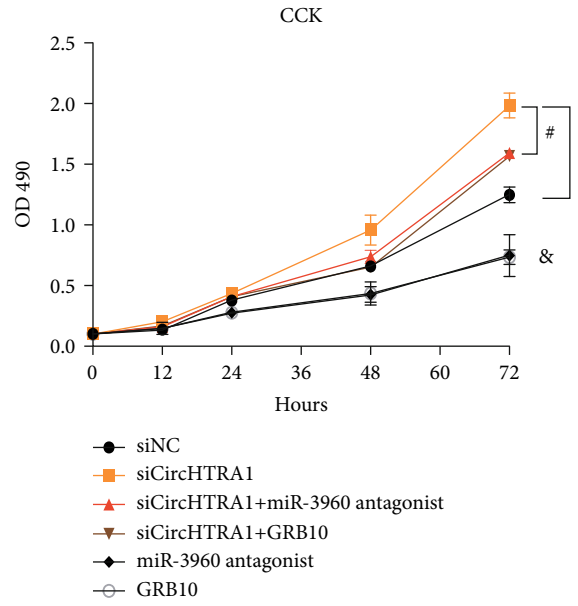
(g)



(h)



(i)

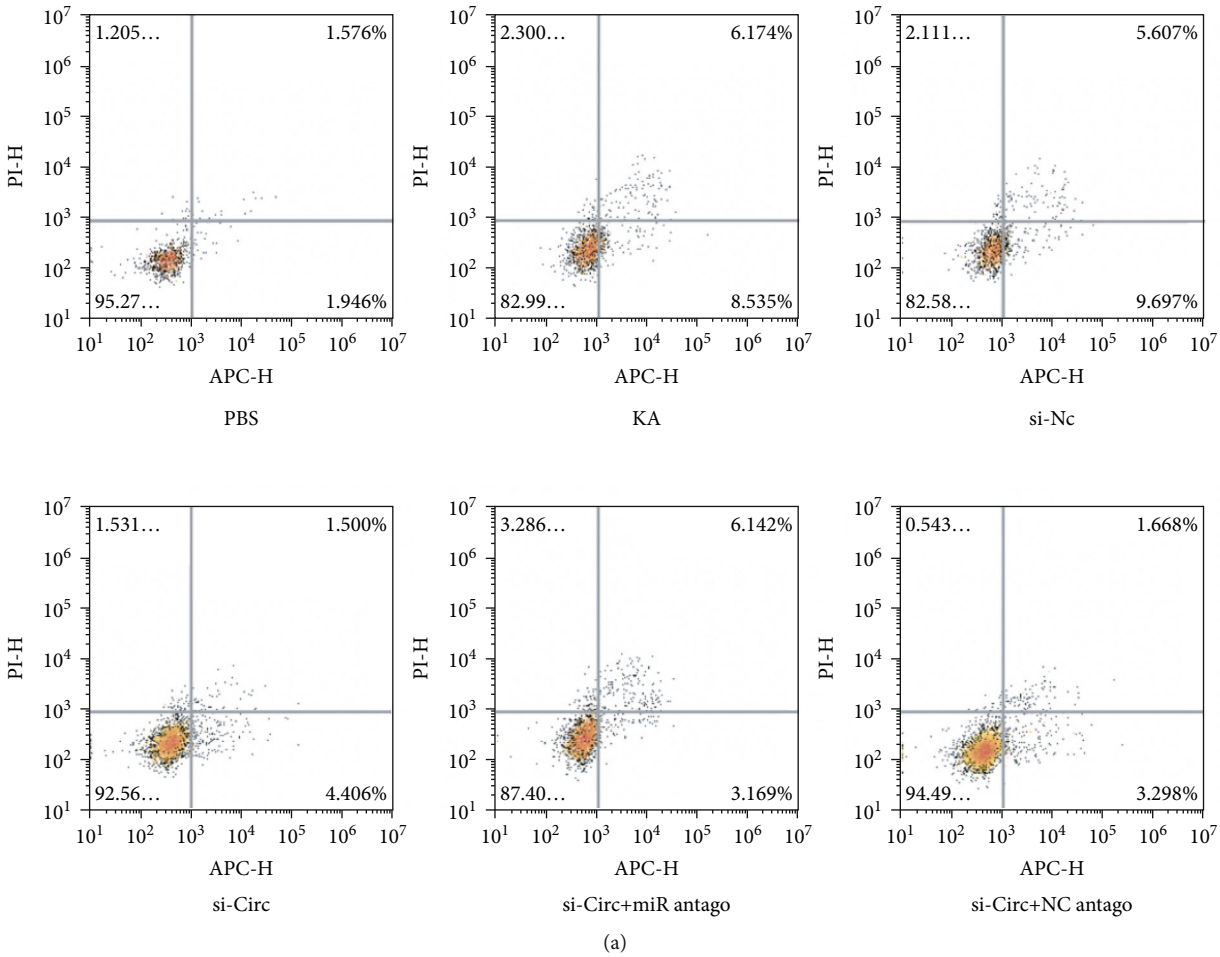


(j)

Figure 3: circHtra1 binds to miR-3960 and GRB10 in neurons. (a) Based on the interactome prediction, four miRNAs were predicted to bind to circHtra1. (b) The complementary site between circHtra1 and miR-3960 is shown. (c) Primary cultured neurons were cotransfected with each miRNA mimic and the wild-type circHtra1 reporter or mutant circHtra1 reporter. The *y*-axis shows the luciferase intensity. The scrambled mimic was used as a control. (d) RNA FISH for circHtra1 detection in primary cultured neurons. Nuclei were stained with DAPI (blue), and circHtra1 appears green. circHtra1 was dominantly located in the cytoplasm. Scale bars represent 20 mm. (e, f) circHtra1 is predicted to interact with AGO2 (a miRNA binding protein), as supported by a RIP assay. (g, h) miR-3960 could bind to Htra1 in three positions, as predicted by miRanda. (i) After primary cultured neurons were transfected with NC, miR-3960, miR mutant, circHtra1, circHtra1+miR-3960, and circHtra1+miR mutant, mRNA levels of *htra1* were assessed in primary cultured neurons $t = 11.57$, $df = 4$; $t = 4.503$, $df = 4$; $t = 6.680$, $df = 4$; and $t = 4.563$, $df = 4$, respectively. (j) After transfection with si-NC, si-circHtra1, or si-circHtra1+miR-3960 antagonist, si-circHtra1+Htra1, miR-3960 antagonist and Htra1, CCK-8 levels were assessed. *, $^{\#}$, $^{\&}P < 0.05$.

of *GRB10* was attenuated by miR-3960 mimics (Figure 3(i)). Additionally, a CCK-8 test showed that si-circHtra1 promoted neuronal proliferation, which was blocked by an miR-3960 antagonist or GRB10 overexpression. When primary cultured neurons were transfected with si-NC, si-circHtra1, or si-circHtra1+miR-3960 antagonist and miR-3960 antagonist, cell proliferation was significantly lower in the si-circHtra1+ miR-3960 antagonist group than in the si-circHtra1 group ($P < 0.05$, Figure 3(j)), and this effect was partially blocked by treatment with mutant miR-3960. These results indicate that circHtra1 promotes cell death by sponging miR-3960.

2.7. si-circHtra1 Has Neuroprotective Effects in TBI. Using the Human Assembly (GRCh37/hg19) on the UCSC Genome Browser, we searched chr10:124221040–124274424 (53385 bp) and found that circHtra1 was conserved in rhesus macaques, mice, dogs, and elephants (Figure S2A). Therefore, we used a mouse model to explore the effect of circHtra1 *in vivo*. AAVs transfected



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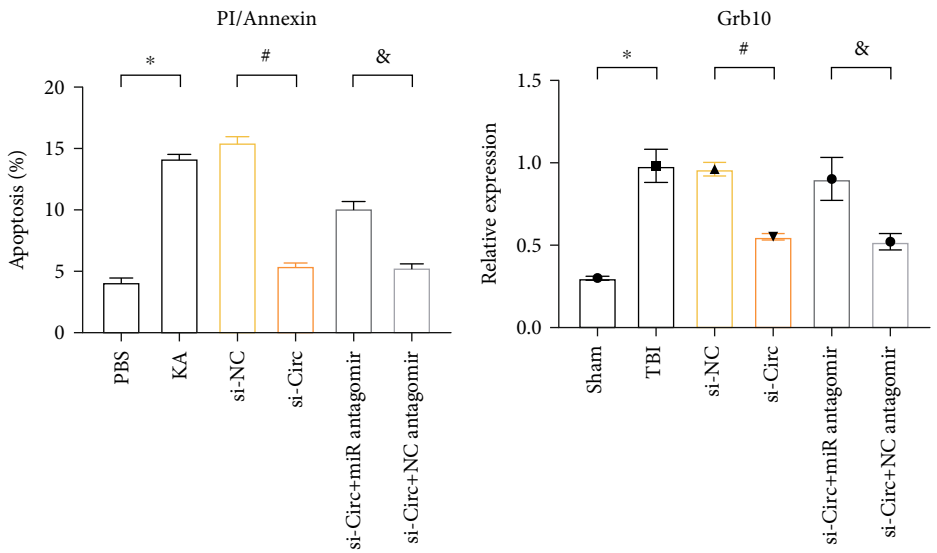

(b) (c)

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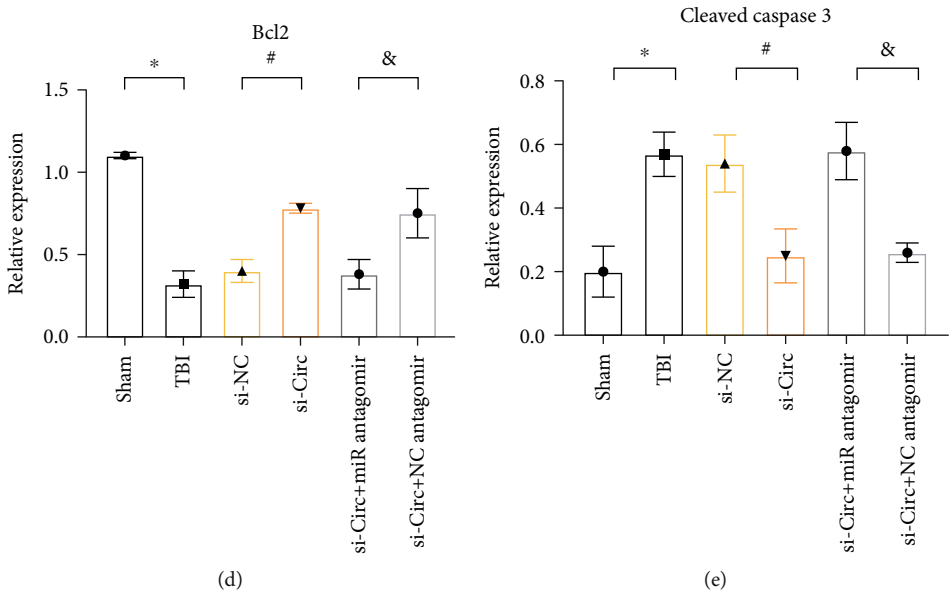


FIGURE 4: si-circHtra1 inhibits apoptosis in TBI. (a) Apoptosis of neurons in primary cortical neurons (expressed as percentages). (b) Apoptosis quantification in each group. (c–f) Protein levels of GRB10, cleaved caspase-3, and Bcl-2 determined by ELISA $n = 3$. Compared with the sham group, $^{*}P < 0.05$; compared with the si-NC group, $^{\#}P < 0.05$; and compared with the siRNA+miR antagomir group, $^{\&}P < 0.05$.

with si-NC or si-circHtra1 and mixed with miR-3960 antagomir or NC antagomir were injected subcutaneously into lateral ventricles of mice with TBI. HE staining showed that knocking down the expression of circHtra1 markedly decreased the brain injury in both the cortex and hippocampus *in vivo* (Figure S2B). We performed behavioral tests, including analyses of the neurological severity score (NSS) and paw grasping ability, to assess the motor function of mice with TBI. si-circHtra1 could reduce motor dysfunction in mice with TBI, as reflected by a lower NSS and grasping score. The neuroprotective effect of si-circHtra1 was significantly blocked in the si-NC and si-circ mixed with miR-3960 antagomir groups ($P < 0.05$, Figure S2C). Taken together, these findings indicate that circHtra1 promoted neuronal loss and motor impairment in brain insults *in vivo*.

2.8. Downregulation of circHtra1 Reduces the Number of Annexin-Positive Cells, GRB10, and Cleaved Caspase-3 in a Mouse Model of TBI. Both Htra1 and GRB10 affect the Wnt and β-catenin pathways to regulate apoptosis and neurodegeneration. [8] We investigated the effect of circHtra1 on apoptotic markers. A PI/Annexin assay was performed to assess apoptosis *in vitro*. As shown in Figures 4(a) and 4 (b), the proportion of Annexin+ cells in the KA group was significantly higher than that in the PBS group ($P < 0.05$). There was no obvious difference in Annexin+ cells between the KA group and siRNA-NC group ($P > 0.05$). The number of Annexin-positive cells in the si-Circ group was lower than that in the siRNA-NC group, and this reduction was partially blocked by the miR-3960 antagomir. The rate of apoptosis in the si-circ+NC antagomir group was lower than that in the si-circ+miR-3960 antagomir group ($P < 0.05$). We further separate the early and late apoptosis between different groups (Figure S1). We found the si-circ is able to prevent both early and late apoptosis after TBI; however, si-circ combined with miR antagomir could partly block the effects of si-circ on early apoptosis.

Expression levels of GRB10, cleaved caspase-3, and BCL-2 in the ipsilateral cortex of mice with TBI were further confirmed by ELISA. As shown in Figures 4(c)–4(e), GRB10 and cleaved caspase-3 levels were elevated in TBI, with reduced BCL-2 expression compared with those in control mice ($P < 0.05$). There were no differences in the expression levels of GRB10, cleaved caspase-3, and BCL-2 among the TBI, si-NC, and si-circ+miR-3960 antagomir groups ($P > 0.05$). GRB10 and cleaved caspase-3 levels were lower, and BCL-2 levels were higher in the si-circHtra1 group than those in the negative control group ($P < 0.05$). Compared with levels in the si-circ+miR-3960 antagomir group, GRB10 and cleaved caspase-3 expression levels were lower in the si-circ+NC antagomir group, while BCL-2 levels were higher ($P < 0.05$).

2.9. IGF-1 Reduces circHtra1 via ADAR1. IGF-1 has been shown to reduce the HtrA1 expression by enhancing its protease susceptibility [9]. However, the exact effects of IGF-1 on Htra1 and circRNA profiles after brain insults remain unclear. Since astrocytic IGF-1 has a neuroprotective effect [10], we further evaluated the effects of IGF-1 on circHtra1, ADAR1, and GRB10. We have recently demonstrated that IGF-1 could regulate ADAR1 expression in excitotoxicity [11]. After treatment with KA, neurons have reduced ADAR1 expression and higher calcium loads, leading to neuronal death [11]. Coculture with astrocytes can reverse this process in an IGF-1-dependent manner, as an IGF-1R antagonist could block the effect of IGF-1 on ADAR1

expression. ADAR1 also influences the expression of circRNAs [4]. Therefore, we postulated that IGF-1 has an effect on both circHtra1 and GRB10 via ADAR1.

First, we confirmed the interaction between IGF-1 and Htra1 by Co-IP. Consistent with previous findings [9], we found that Htra1 could be immunoprecipitated by IGF-1 in HEK cells (Figure 5(a)), and this effect could be blocked by AG1024 (an IGF-1R antagonist, which has a much higher binding affinity with IGF-1). We have previously shown that KA treatment could reduce ADAR1 expression via IGF-1. In this study, IGF-1 increased ADAR1 expression after KA, and this was reversed by AG1024 (Figures 5(c) and 5(d)). Next, we evaluated the effect of IGF-1 on circHtra1. We found that KA increased the expression of circHtra1, and both IGF-1 and ADAR1 reduced its expression levels; these effects were also blocked by AG1024 or ADAR1 siRNA (Figure 5(b)). To verify the sponge effect of circHtra1, we further looked at the effects of IGF-1 and ADAR1 on GRB10 expression. Again, we found that KA increased GRB10 expression, and both IGF-1 and ADAR1 reduced GRB10 expression; these effects were also blocked by AG1024 or ADAR1 siRNA (Figures 5 (e) and 5(f)). Accordingly, the effect of IGF-1 on GRB10 expression was consistent with the results of our previous chipset analysis of the PI3K-Akt pathway [10]. To further confirm the effect of ADAR1 on the expression of Grb10, we integrated the single-cell sequencing data from GEO which showed the expression of Grb10 and Htra1 were dominantly in stromal cells (Figure S3).

2.10. Clinical Significance of circHtra1 in TBI. Next, we investigated circRNA expression in TBI. We identified the top 10 most highly increased circRNAs and found that circHtra1 expression increased with TBI severity (Figure S3A&B). We further evaluated Htra1 and circHtra1 expression levels in patients with TBI and found that both increased with the severity of injury (Figure S3C&E). Htra1 expression in TBI was further verified using GSE data (GDS4911/1386884_at), wherein Htra1 expression increased after 12 hours of brain injury and returned to baseline levels at 48 hours in a mouse model of TBI (Figure S3D). Furthermore, circHtra1 and Htra1 expression levels were positively correlated with each other ($r^2 = 0.37496$, $P = 0.01$; Figure S3F). These results indicated that circHtra1 is a biomarker for TBI.

2.11. GRB10 And NK Cell Immune Infiltration in TBI. In our GO and KEGG pathway enrichment analyses, the immune response was the top altered pathway in moderate and severe TBI, and it has recently been reported that impaired NK cells in patients with TBI are correlated with the severity of injury [12]. We first evaluated immune infiltration in the plasma of patients with TBI based on our ceRNA chipset using CIBERSORT and QUANTISEQ (Figures 6(a) and 6 (b)). We found that the NK cell percentage was lower in moderate and severe TBI than in mild cases Figures 6(c) and 6(d)). Furthermore, more severe TBI cases corresponded with a much lower NK percentage than those in the mild and moderate groups ($P < 0.05$). Plasma NK cells are positively associated with the GCS score ($R^2 = 0.4251$, $P < 0.01$, Figures 6(e) and 6(f)) [12]. GRB10 regulates NK cells [13]. In both severe and moderate TBI, GRB10 expression levels were much higher than those in mild TBI and in the control group. Importantly, GRB10 expression was negatively correlated with the GCS score ($R^2 = 0.4251$, $P = 0.01$; Figures 6(g) and 6(h)). Furthermore, we performed FACS analyses to evaluate the proportion of NK cells (CD56-positive and CD3-negative, upper-left quadrants of Figures 6(i)–6(k)). As the severity of brain injury increased, the population of NK cells in plasma of patients with TBI decreased. This can also be reflected by the CIBERSORT result (both numbers of resting NK and activated NK cells were reduced in TBI compared to the healthy controls, Figure 6(l)). We further applied the sc-seq in TBI patients plasma and showed that consistent with the decreased NK cells in TBI, the cell interaction between NK and monocytes was reduced as well (Figures 6(m) and 6(n)).

3. Discussion

Several recent studies have established the important roles of circRNAs in various central nervous system (CNS) diseases, such as Alzheimer's disease (AD), Parkinson's disease (PD), ischemic brain injury, and neurotoxicity [3, 5, 14]. They may exert critical biological functions as microRNA (sponges), or by regulating protein function. However, the exact role of circRNAs in TBI has not been deeply determined. We therefore characterized circRNA expression profiles in human TBI by a chipset analysis. We found that the total number of altered circRNAs increased as the severity of brain injury increased, indicating that gene editing is more highly impaired in severe TBI.

3.1. circHtra1 Is a Biomarker for TBI. In GO and KEGG functional enrichment analyses, target genes of altered circRNAs were highly enriched in the cytosol and were related to the inflammatory response and innate immunity (which may reflect their sponging function). According to the KEGG pathway analysis of the host genes for circRNAs in our study, the most enriched pathways were Th1 and Th2 cell differentiation, T cell receptor signaling pathway, and antigen processing and presentation, suggesting that an immune deficiency is critically involved in TBI.

Previous studies have focused on the circRNA profiles in mouse cortex after TBI, and up to now, almost no studies have investigated the circRNA expressions in human TBI. The five most highly upregulated circRNAs in our study were hsa_circ_0020273, hsa_circ_0093014, HSA_CIRCpedia_152345, hsa_circ_0020269, and hsa_circ_0064339. Interestingly, three of these were produced from Htra1. Further analyses revealed that circHtra1 is a promising biomarker for the severity of TBI. The circHtra1 expression level was remarkably higher after brain insults *in vitro* and *in vivo*, and its upregulation was positively correlated with Htra1 expression and the GCS score in TBI. In addition, the knockdown or overexpression of circHtra1 significantly reduced or facilitated cell loss in primary cultured neurons. With respect to the underlying mechanism, circHtra1 promoted neuronal loss by sponging miR-3960, thereby

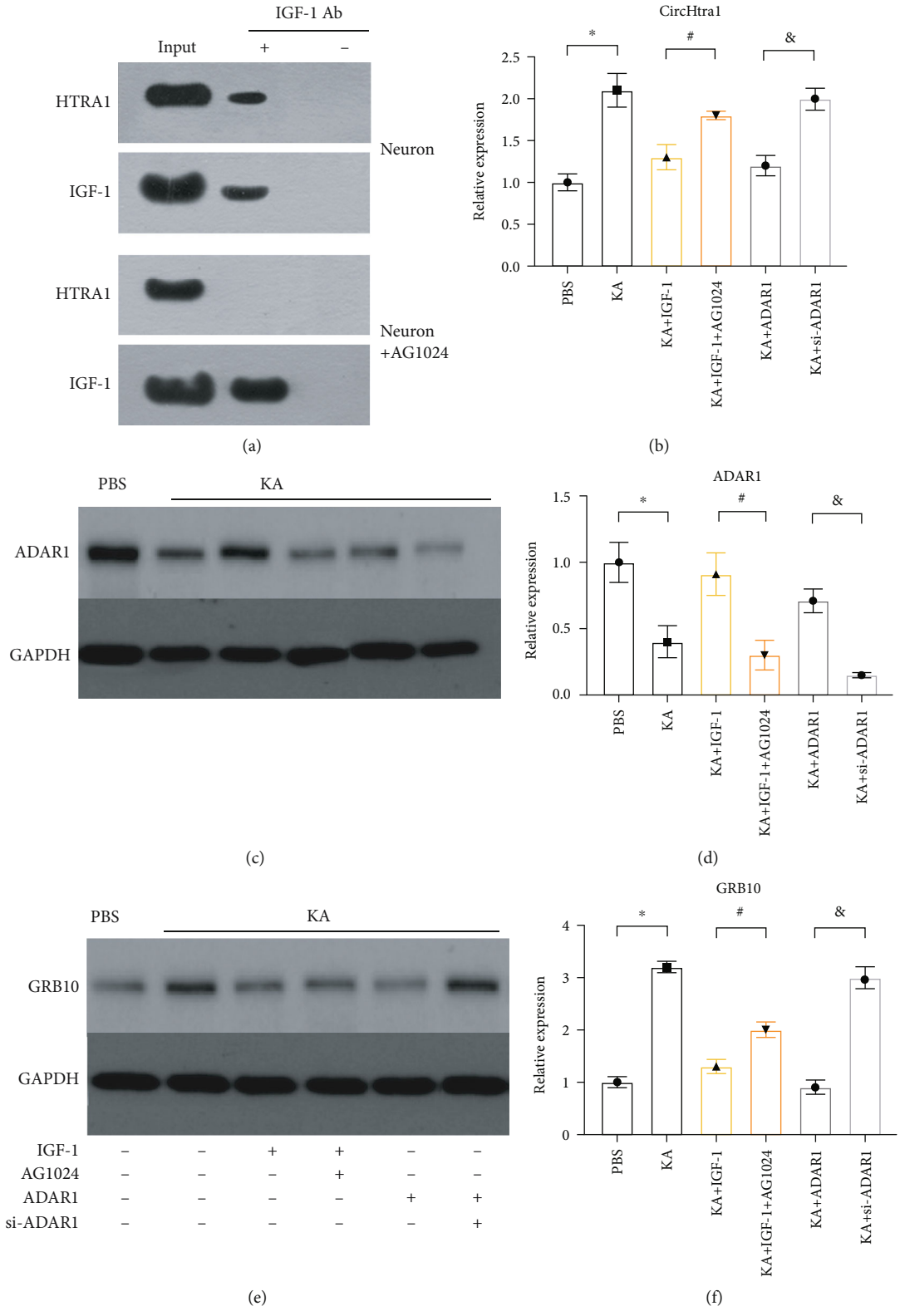
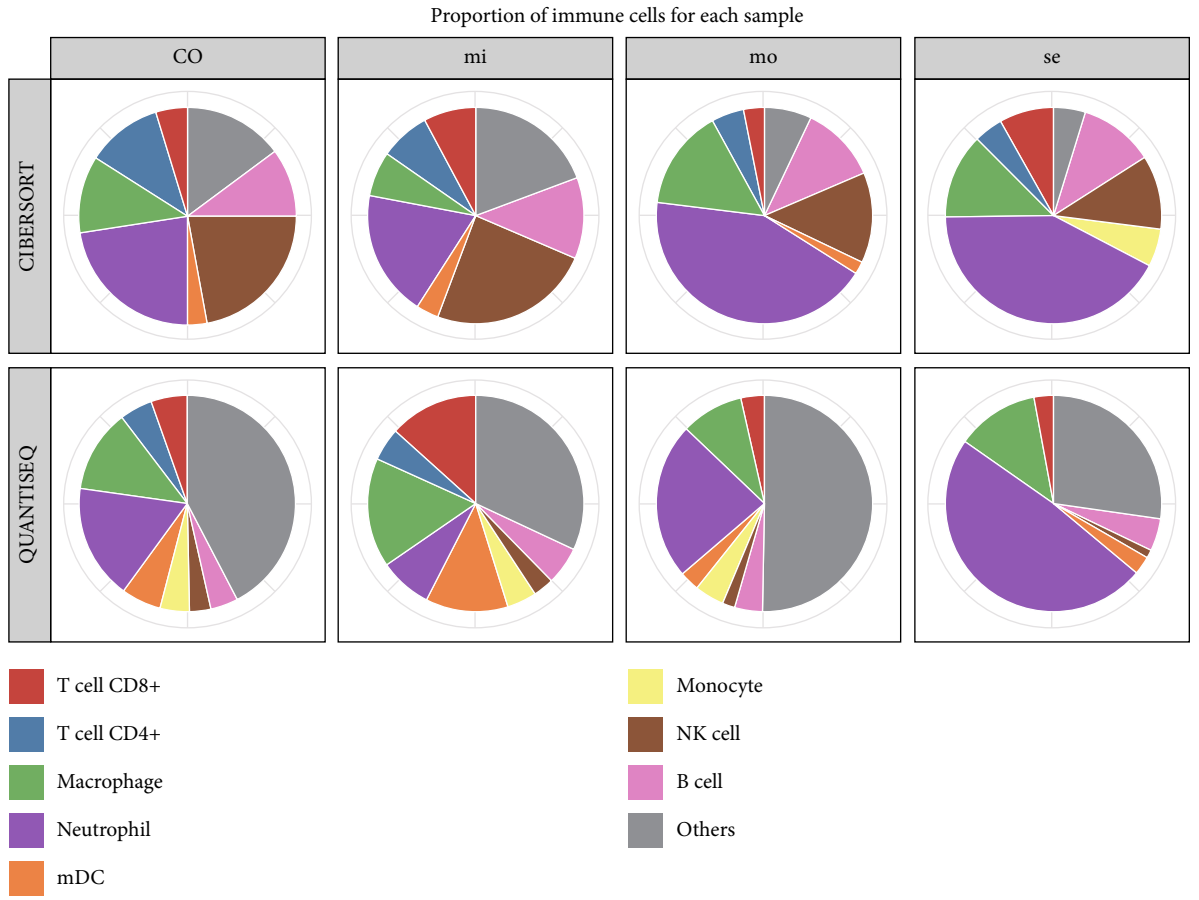
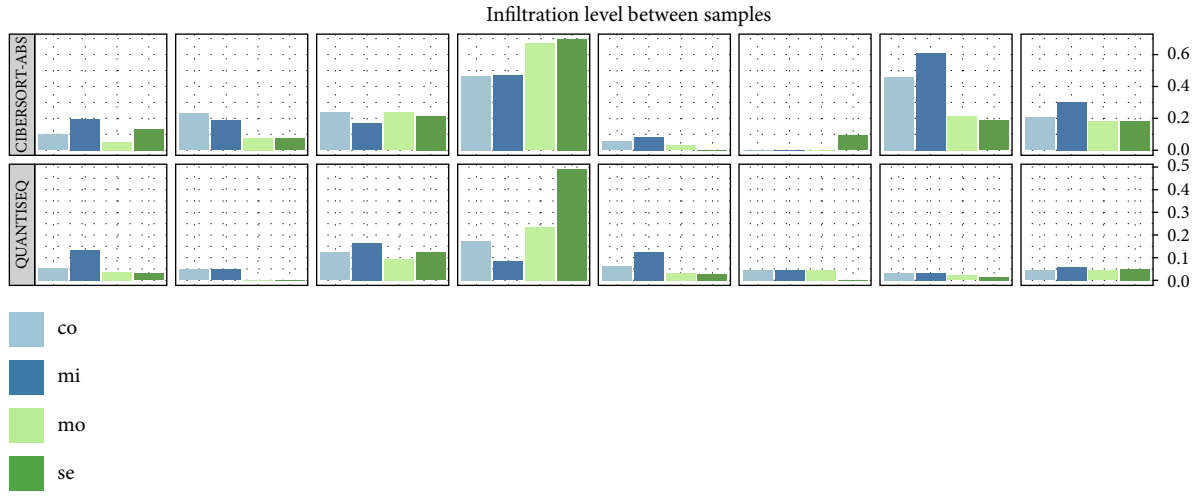


Figure 5: IGF-1 reduces circHtra1 via ADAR1. (a) IGF-1 is immunoprecipitated with htra1 in HEK cells, and this effect is blocked by AG1024. (b) circHtra1 expression in neurons treated with KA, IGF-1, ADAR1, and their inhibitors ($t = 4.919$, 3.162, and 8.503, respectively, $df = 4$). (c, d) Expression of ADAR1 in neurons treated with KA, IGF-1, ADAR1, and their inhibitors ($t = 3.123$, 3.142, and 6.074, respectively, $df = 4$). (e, f) Expression of GRB10 in neurons treated with KA, IGF-1, ADAR1, and their inhibitors ($t = 11.242$, 2.162, and 7.230, respectively, $df = 4$). $^{*, \#, \&}P < 0.05$.



(a)



(b)

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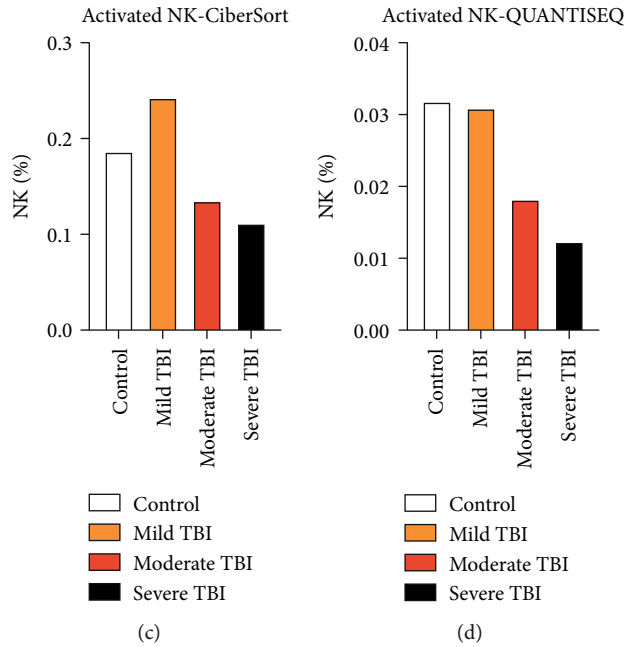
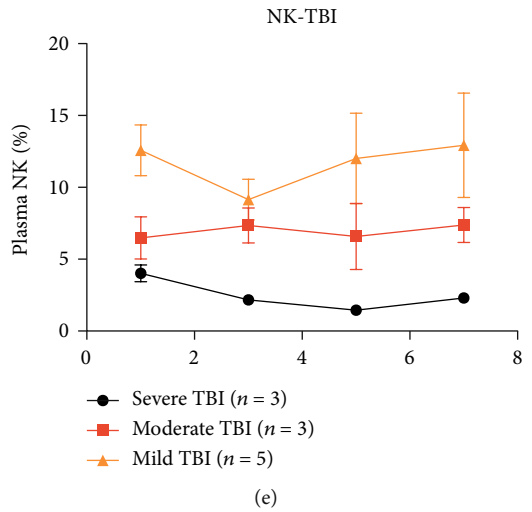
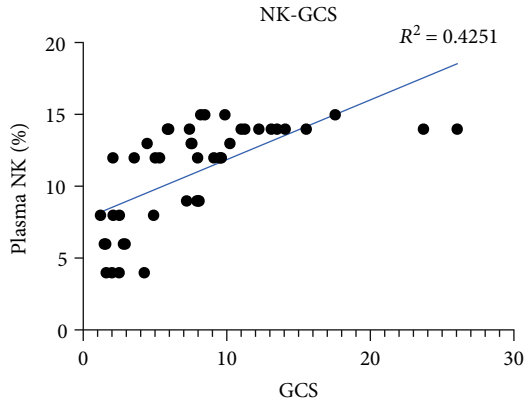


(c) (d)



(e)



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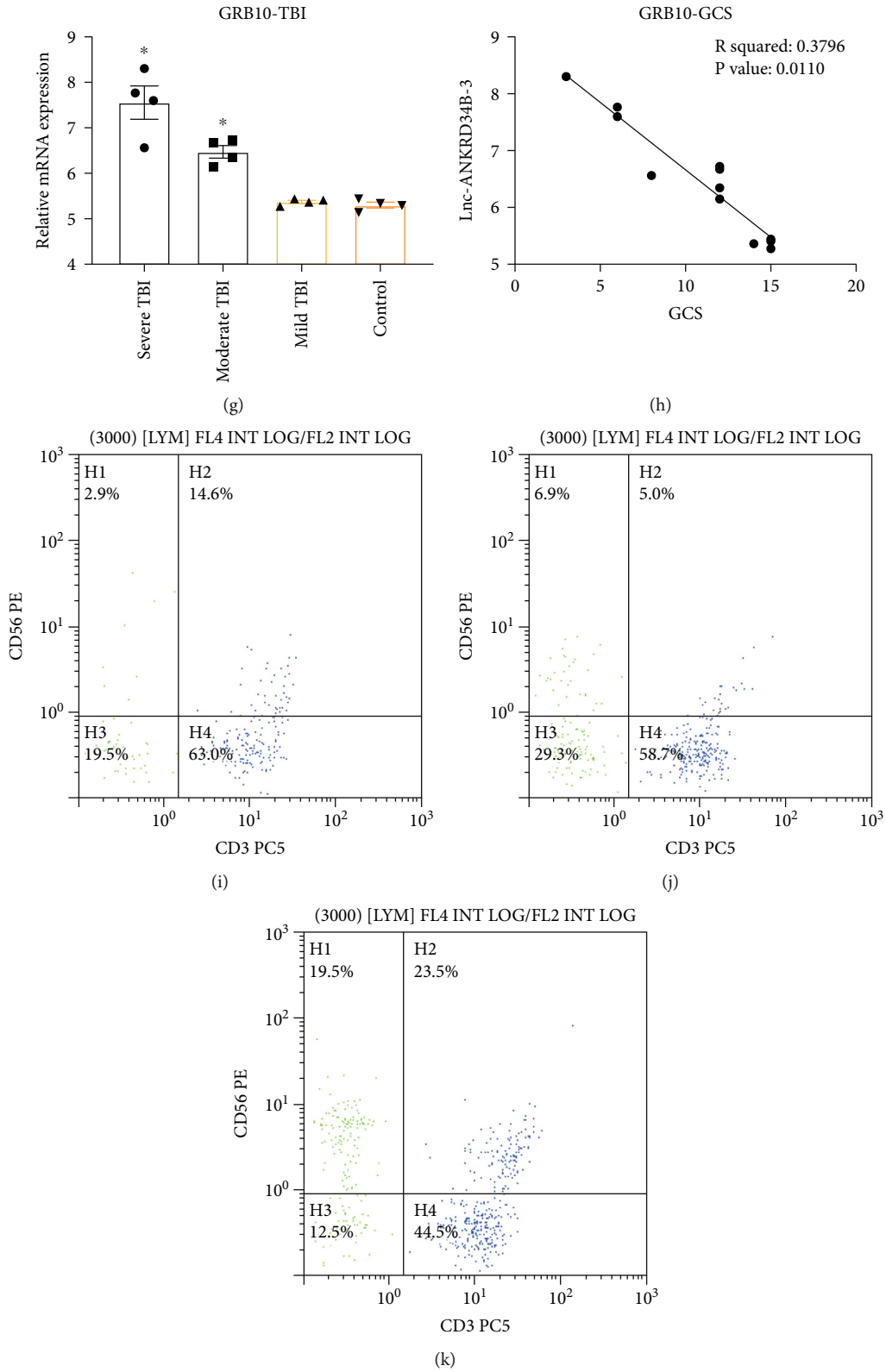

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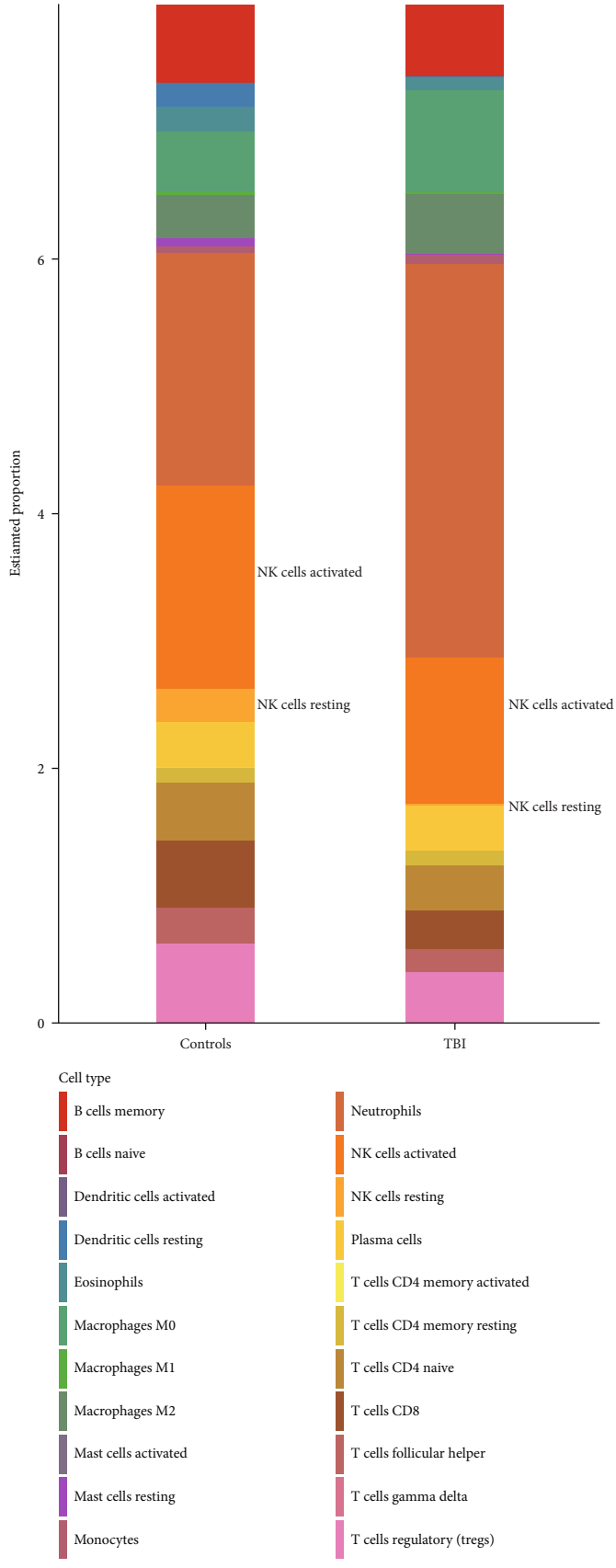

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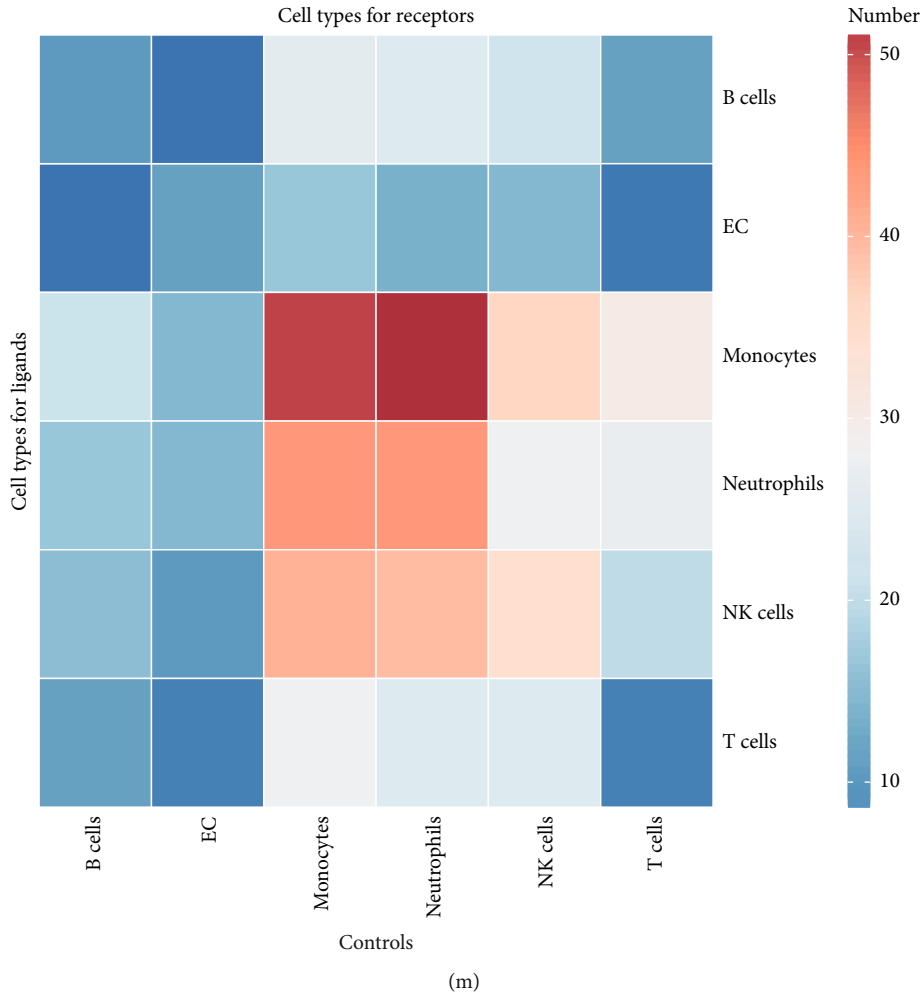

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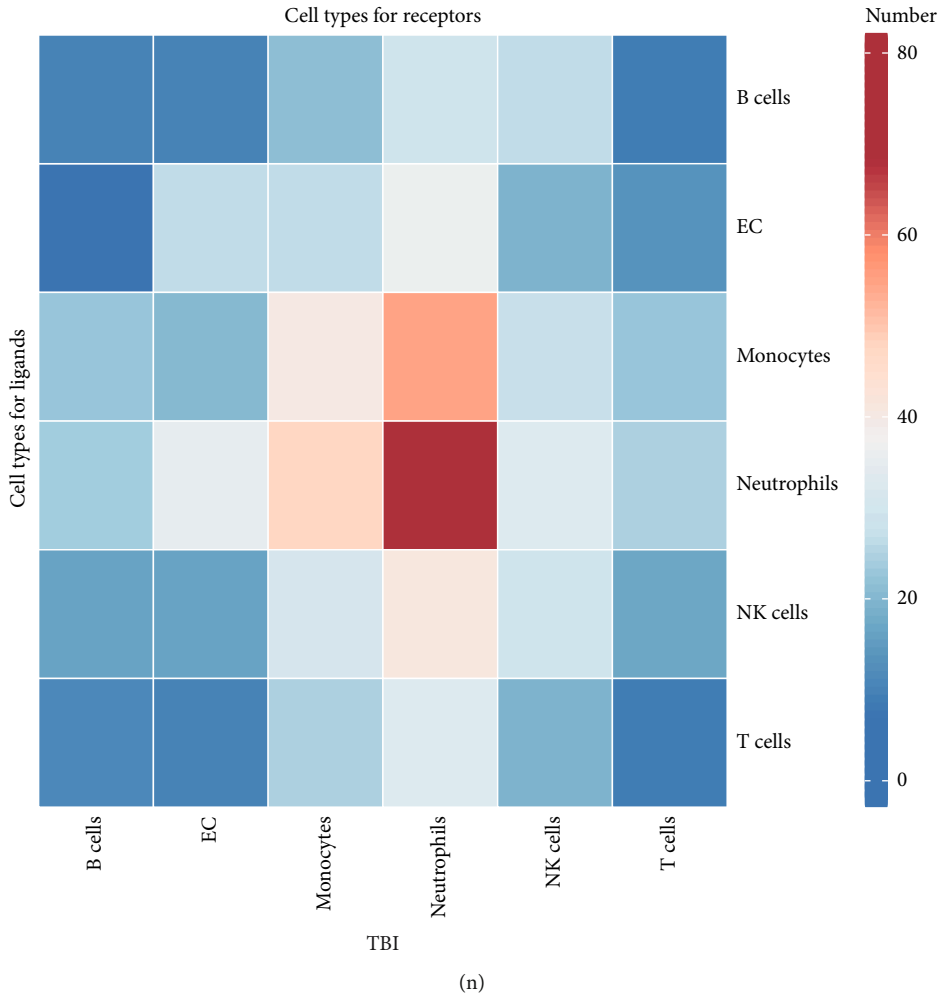

Figure 6: Plasma NK cell reduction in TBI. (a, b) CIBERSORT and QuanTIseq were used to assess immune infiltration in TBI. (c, d) Quantification of results shown in panels (a) and (b), showing that the percentage of NK cells decreases in moderate or severe TBI. (e, f) The percentage of NK cells is lower in severe TBI than in the mild group and is positively correlated with plasma NK (%). (g, h) The expression of GRB10 in TBI, $F(3, 12) = 16.839$ and its correlation with GCS. (i–k) The relative plasma NK ratio (reflected by CD3-CD56+) in severe TBI (i); moderate TBI (j), and mild TBI (k). (l) The CIBERSORT method shows the immune cells distribution in plasma of TBI patients and healthy controls. (m, n) The cell communication in the blood from sc-seq data in healthy controls (m) and TBI patients (n).

increasing GRB10 expression. Considering the stable circular structure and enrichment in the CNS, circHtra1 is a potential therapeutic target for TBI.

Of note, *htra1*, which has an IGF domain, was predicted to be competitively regulated by circRNA_0020269. The knockdown *of HtrA1* activates *PI3K/Akt* signaling in A549 cells, which indicates that Htra1 might be a downstream of IGF-1 signaling [15]. *In vivo* studies have also shown that the knockdown of *HtrA1* promotes tumorigenesis [15]. *HtrA1* also reduces Wnt signaling by binding to β-catenin and decreases the rate of cell proliferation [8]. Consistent with previous results, our Annexin and PI staining and analyses of apoptosis markers revealed that circHtra1 knockdown efficiently inhibited apoptosis, including both early and late stage of apoptosis and this effect was partially blocked by miR-3960.

3.2. circHtra1 Is Affected by A-to-I Editing in TBI. A-to-I editing is increased during brain development [16]. In addition, the RNA editor ADAR1 could regulate neural fate and the expression levels of circRNAs in the CNS [4]. However, the regulating role of ADAR1 on circRNAs after brain injury has not been investigated previously. We therefore studied the role of ADAR1 in the biogenesis of neuronal circRNAs and found that increased circHtra1 after brain insults corresponded with reduced ADAR1 expression. This is consistent with our previous *in vitro* assays showing that excitotoxic injury reduced ADAR1 expression and affected calcium hemostasis. Astrocytic IGF-1 could reverse these pathologies. We further demonstrated that IGF-1 regulates circHtra1 expression (Figure S4A-D). However, the underlying mechanisms are unclear, and the regulatory effects might be mediated by the effects of IGF-1 on exosome release.

In both *in vitro* and *in vivo* analyses, we found that si-circHtra1 has a neuroprotective role, as evidenced by increased neuronal proliferation and improved motor function in a mouse model of TBI. RNAi delivery has been used as a therapeutic strategy in brain insults; however, circRNA interference has rarely been evaluated in clinical studies. Considering the stable structure and expression of circRNAs in the brain, these could become potential targets for clinical applications. Although circRNAs act mostly as miRNA sponges and adjust the expression of downstream mRNAs, they also bind directly to proteins or are translated into peptides [17]. Therefore, our understanding of the functions of circHtra1 could be enhanced by further RNA purification-(CHIRP-) sequencing or RIP-sequencing analyses.

3.3. circHtra1 Is Associated with Immune Deficiency in TBI. Inflammation and the immune response are vital mechanisms in secondary brain injury [18, 19]. Htra1 promotes inflammation and macrophage infiltration [20]. NK cells, another important immune cell subset, are rapidly recruited and promote recovery after TBI, leading to reduction in NK cell numbers in the peripheral blood [12]. Recent evidence suggests, however, that NK cells are detrimental after hemorrhagic injury [21]. The reason for these contradictory functions of NK cells remains unclear. In addition, NK cell-mediated cytotoxicity deserves further attention, as it is associated with specific circRNAs that are rarely detected in non-TBI samples. NK cells are thought to be the first line of defense for immune monitoring and exert a critical role in anti-infection therapy. circRNAs contribute to the immune response by promoting NK cell activity and upregulating NK-mediated immune responses. For example, circARSP91 enhances the cytotoxicity of NK cells in liver cancer cells [22]. Furthermore, recent research has demonstrated that hypoxia-triggered circ-0000977 inhibition promotes the killing effect of NK cells by regulating hypoxia-inducible factor 1-alpha (HIF1α). This axis regulates the HIF1α-mediated immune escape of PC cells by NK cell activity [23]. However, the direct relationship between circRNAs and NK cells and the interaction between cancer cells and NK cells have not been evaluated. Similarly, we did not study the relationship between NK cells and neurons directly. Future studies of the mechanisms by which circRNAs modulate NK cell activity are needed for the development of strategies to mediate the immune response via NK cells.

Recently, Deng et al. found that plasma GRB10 levels are correlated with NK cell counts and identified GRB10 and E2F3 as biomarkers for osteoarthritis and their association with immune infiltration [13]. NK cell alterations have also been reported in TBI; the percentage of NK cells in the peripheral blood is correlated with GCS and Glasgow Outcomes Scores (GOS) in patients with TBI [12]. These findings are consistent with those of our study, indicating that GRB10 may be involved in NK cell reduction peripherally in TBI events. The mechanisms underlying NK cell reduction and its biological consequences remain to be elucidated. Several factors could result in alterations of NK cells in TBI. First, NK cells might penetrate the damaged blood–brain barrier, thereby reducing their expression peripherally and entering the brain. Alternatively, increased susceptibility to infection could be due to immunodepression in patients with TBI, consistent with our results for the percentage of peripheral NK cells in TBI. The exact relationship between GRB10 and NK cells, among other immune cells (i.e., Th1 or Th2), also has a critically important role in the immune response after TBI, requiring further studies. It is also necessary to study the crosstalk between neurons and NK cells to investigate their interaction effects in CNS.

Some limitations of this work need to be addressed in future studies. First, the sample size was relatively limited (12 patients with TBI and 4 healthy controls). Although we were able to obtain the positive results from such experiments, as a biomarker, circHtra1 needs to be validated using a larger sample size (more than 50 patients in each group). However, as for the chipset study, these preliminary findings with a smaller sample are acceptable like RNA-sequencing data. Second, the effect of circRNAs might be multifaceted. It mostly exerts its role as a miRNA sponge to regulate the target gene expression; meanwhile, it can also directly bind to mRNAs and proteins and sometime translate to peptides as well [17]. Interestingly, circRNA could also directly act as a protein sponge, which is very similar to miRNA function. [24] The circRNA-protein interaction might also be affected by their binding site and tertiary structure, which is very useful for future pharmaceutical design. [17] The multiple mechanisms of circHtra1 need to be further explored in TBI session in future studies as well. Third, the spatial expression patterns of circRNAs in patients with TBI might be different from those in healthy controls; spatial transcriptomics analyses are necessary to address this point. However, poly A enrichment method for spatial transcriptomics are currently not able to investigate the circRNA expression and our results clearly emphasize the role of circRNAs in immune infiltration and suggest that circHtra1 is a candidate biomarker for assessing the neuroimmune response and for predicting outcomes.

4. Conclusion

Analyses of the regulatory mechanisms underlying the circRNAs discovered in this study are expected to be valuable for the diagnosis and treatment of TBI. Overall, we obtained a comprehensive circRNA expression profile based on blood samples from patients with TBI. Aberrantly expressed circHtra1 might regulate cell proliferation and the immune response in the injury cascade post-TBI via IGF-1, which is associated with genetic editing, and by sponging miRNAs (Figure S4E). Our results provide novel research directions related to the neuro-endocrine-immune system aimed at the development of effective TBI therapies.

5. Materials and Methods

5.1. Sample Collection. Peripheral human blood was prospectively obtained and transferred into PAX RNA Tubes (BD, Shanghai, China) within one day post-TBI [25]. Patients with TBI were recruited in 2019 based on brain contusions on initial head CT findings. The study protocol was

approved by the local Ethics Committee in Shanghai Pudong New Area People's Hospital (20170223-001 on March 7, 2017). Patients with TBI were classified into groups according to the GCS score: severe group (GCS 3–8), moderate group (GCS 9–12), and mild group (GCS 13–15). Patients who were 18–65 years old with a closed brain injury were included. The exclusion criteria were as follows: (1) severe complication with a thoracic or abdominal injury, (2) serious previous diseases (such as thrombocytopenia and cancer), and (3) family refused to undergo blood collection. Clinical information for patients is listed in Table S1.

5.2. Microarray Information. The Agilent Human lncRNA Microarray 2019 (4 ∗ 180 k, design ID: 086188) was used in this experiment, and data for the 16 samples were analyzed by OE Biotechnology Co., Ltd. (Shanghai, China).

5.3. Gene Microarray. Total RNA was quantified by the NanoDrop ND-2000 (Thermo Scientific, Carlsbad, CA, USA), and the RNA integrity was assessed using the Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). The RNA was manipulated according to the manufacturer's protocols. In brief, total RNA was transcribed to cDNA, synthesized into cRNA, and labeled with Cyanine-3-CTP. The labeled cRNAs were hybridized onto the microarray chipset. After being washed, the chipset was scanned using the Agilent Scanner G2505C (Agilent Technologies).

5.4. NK Cells Tested by FACS. To count NK cells in the plasma of patients with TBI, peripheral blood mononuclear cells (PBMCs) were separated from human blood samples. Then, PBMCs were centrifugated at 2000 rpm for 30 minutes at room temperature (23°C). The samples were further tagged with CD3 antibody and fluorescein CD56 antibody (BD Biosciences Pharmingen, CA, USA) for 30 minutes at room temperature. Both monoclonal antibodies were obtained from Invitrogen (CD56 monoclonal antibody, MEM-188 and CD3 monoclonal antibody, OKT3). Human NK cells were gated as CD3-CD56+ lymphocytes for further analysis. FlowJo (Ashland, OR, USA) was used to count the number of NK cells.

5.5. Data Analysis. The thresholds for the identification of differentially expressed genes (DEGs) were set at fold change > 2.0 and $P < 0.05$. DEGs were further filtered by a volcano plot. Next, GO and KEGG enrichment analyses of circRNA target genes were performed. GSE data (GSE24047) were used to confirm the differential expression of Htra1 after TBI [26].

5.6. ceRNA Network Construction. The miRanda algorithm was used to predict ceRNA interactions. If the expression levels of a circRNA and mRNA were positively correlated, the RNAs were included in the ceRNA analysis. If expressed miRNAs were negatively correlated with both of the above circRNAs and showed complementary binding to both, these miRNAs were identified as competitively inhibited targets of the circRNAs. According to these criteria, the top 200 significant interacting circRNA–mRNA pairs were used to generate a circRNA–mRNA network based on the core circRNAs, and a competing endogenous RNA network was constructed based on the miRNAs shared by the most significant circRNA–mRNA pairs.

5.7. sc-RNA-seq Analysis. We applied the 10X Genomics sc-seq in the blood of 12 TBI patients and four healthy controls. The single-cell transcriptome dataset GSE110746 was downloaded from the Gene Expression Omnibus (GEO) database. The chipset data from TBI patients was previously reported and used as an external verification here. R (v. 4.0.2) was used for bioinformatical analysis.

The Seurat package was used for the sc-RNA seq study [13]. The dimension of data was reduced by PCA and t-SNE. Marker genes for different clusters were identified using the Seurat package. All clusters were annotated using the SingleR package with a mouse dataset [14], and cell communication analysis was performed using the Cell-Phone package.

5.8. Reagents and Antibodies. The rabbit polyclonal antibody Htra1 (55011-1-AP) and the rabbit polyclonal antibody IGF-1 (PA5-27207) were purchased from Thermo Fisher. Goat β-actin antibody (ab8227) and rabbit anti-ADAR1 antibody (bs-2168R; Bioss, Woburn, MA, USA), rabbit polyclonal to GRB10 (ab125583; Abcam, Cambridge, UK), mouse monoclonal to Bcl-2 (ab692; Abcam), and rabbit monoclonal to anti-cleaved caspase-3 (EPR21032; Abcam) were used to evaluate apoptosis in mice with TBI. A mouse monoclonal MAP2 antibody (ab11267; Abcam) and rabbit monoclonal β-tubulin antibody (ab201831, Abcam) were utilized to evaluate cell proliferation. AG1024 (121767) was obtained from Sigma-Aldrich (St. Louis, MO, USA), and negative control siRNA (AM4611) was obtained from Invitrogen.

5.9. Primary Cortical Neuronal Cultures and Coculture with Astrocytes. Primary cultures of cortical neurons and astrocytes were performed as described previously [10]. Excitotoxicity was introduced by KA treatment of neurons at 1 nmol/L, and the coculture system was described previously [11]. For pharmaceutical intervention, cells were treated with 1 μM IGF-1R inhibitor, si-circHtra1, miR-3960 antagonist, or si-ADAR1. Human embryonic kidney (HEK) 293T cells were cultured in Dulbecco's modified Eagle's medium with D-glucose and 10% fetal bovine serum. For Htra1 overexpression, 3×10^6 HEK 293T cells were transfected with the Htra1-HA plasmid constructed by amplifying genomic cDNA according to the manufacturer's instructions (pcDNA3.1/N-HA vector; Clontech, Oxon, UK).

5.10. Mouse TBI Model, qRT-PCR, Western Blotting, and Immunofluorescence Staining. Lateral FPI surgery was performed on 6- to 8-week-old male C57-B6 mice as described previously [10, 27]. The qRT-PCR primers are listed in Table S2. Relative expression levels were calculated with the following formula: $2^{-\Delta\Delta Ct}$, $\Delta\Delta Ct = (Ct_{\text{target gene}} - Ct_{\beta\text{-actin}})$ TBI $- (Ct_{\text{target gene}} - Ct_{\beta\text{-actin}})$ control. Experiments were repeated at least three times. The relative expression of MAP2 and β-tubulin was determined by the average

optical density of the fluorescence area. MAP2 is dendritic marker for neurons, while tubulin is an early marker for the maturation of neurons. Therefore, we used these two markers as representative marker for the maturation of neurons.

5.11. Plasmid, siRNAs, miRNA Mimic, Inhibitor, Transient Transfection, and Construction of Stable Cell Lines. Plasmid-mediated circRNA overexpression and knockdown vectors were obtained from OE Biotech (Shanghai, China). siRNAs targeting circRNAs were obtained from GenePharma (Suzhou, China), the miR-3960 mimic was obtained from RiboBio (Guangzhou, China), and the lentiviral expression vector for the miR-3960 inhibitor and the control plasmid were obtained from GeneCopoeia (Rockville, MD, USA). For stable transfection, puromycin was used to select cells with stable expression of circHtra1 and the negative vector.

5.12. CCK-8 Assay. Each group of cells was adjusted to 1,000 cells per well. Then, 10 mL of CCK-8 solution (Beyotime Biotechnology, Haimen, China) was added to the cell dish after 24 hours. The blank control had only CCK-8 solution. The absorbance (OD) value of each well was read at 490 nm every 24 hours for 3 days.

5.13. RIP Assay. The RIP assay was performed using the EZ-Magna RiP Kit (Millipore, Billerica, MA, USA). Cells were lysed in lysis buffer and further incubated with magnetic beads together with human anti-Ago2 (Millipore) or normal human IgG control (Millipore). The IP RNAs were extracted with TRIzol and assessed by qRT-PCR.

5.14. Co-IP Assay. For Co-IP of Htra1 and IGF-1, HEK 293T-derived wild-type or Htra1 (1 g) was incubated with human IGF-1 (P5502-1 mg; Beyotime) at 4°C for 2 hours. HtrA1 was immunoprecipitated for overnight incubation. To visualize IGF-1, the bottom half of the PVDF membrane was probed with an IGF-1 monoclonal antibody.

5.15. Luciferase Reporter Assay. A luciferase assay was performed as previous reported [28]. Primary cultured neurons (5×10^4 cells per well) were added to a 96-well plate and incubated for 1 day. The related plasmids were transfected using Lipofectamine 3000 (Thermo Fisher Scientific). After 2 days of transfection, luciferase signals were assessed by a luciferase assay (E1980; Promega, Madison, WI, USA). The binding sites of GRB10 and miR-3960 were predicted using RNA22 v2 [29]. In addition, the sequence of circHtra1 containing the putative or mutant putative binding sites for miR-1908-3p, miR-3960, miR-3665-3p, and miR-10400-5p was separately cloned into the pmirGLO vector (Promega). The pmirGLO-circHtra1-WT reporter and pmirGLO-circHtra1-MUT reporter were cotransfected into cells with miRNA mimics, miR-NC, and other miRNA mimics with Lipofectamine 3000. On the third day, a luciferase reporter assay was performed.

5.16. HE Staining and PI/Annexin FACS Assay. HE and PI/Annexin staining following the conventional protocol was used to assess the level of apoptosis in the ipsilateral cortex and hippocampus of mice with TBI. All samples were observed under a microscope (Nikon, Tokyo, Japan) and analyzed by FACS (Navios, Beckman Coulter, Brea, CA, USA).

5.17. Assessments of Motor Function in Mice with TBI. Motor function was evaluated at 0 (baseline), 1, 3, and 7 days after TBI using the NSS method [30]. Briefly, the test includes forelimb flexion, lateral push, forelimb and hindlimb placement, vestibulomotor function, and motor performance on a balance beam. Neuromuscular functions are scored 0, 1, or 2. Vestibulomotor functions are scored 0–6. Complex neuromotor functions are scored 0–5.

For the paw grasp, grip strength of mice in all groups was evaluated before and after TBI surgery. Neuromuscular function was tested in mice with ipsilateral and contralateral paw grip strength. This was scored by two researchers blinded to the groups on a three-point scale, where one is normal, two is impaired, and three is severely impaired.

5.18. Statistical Analysis. All data are presented as means ± standard error of the mean. GraphPad Prism 8.3.1 (USA) was used for statistical analyses. Differences among more than two groups were analyzed by one-way ANOVA and LSD tests. Otherwise, Student's *t*-tests were applied for two-group comparison. Repeated one-way ANOVA was used to analyze the CCK assay results and behavioral assay results. Spearman's correlation analysis was used to assess correlations between two parameters, such as those between GRB10 and NK cell frequencies and GCS. $P < 0.05$ was considered significant.

Abbreviations

ADAR1: Adenosine deaminase acting on RNA
BP: Biological processes
CC: Cellular components
circRNA: Circular ribonucleic acid
ceRNA: Competitive endogenous RNA
DEGs: Differential expression genes
GCS: Glasgow Coma Scale
GSK3β: Glycogen synthesis kinase
GH: Growth factor
HEK: Human embryonic kidney
IGF-I: Insulin-like growth factor 1
IGF-1R: Insulin-like growth factor 1 receptor
KA: Kainic acid
lncRNAs: Long noncoding RNAs
MAPK: Mitogen-activated protein kinase
MS: Mass spectroscopy
MAP: Microtubule-associated protein
MoCA: Montreal Cognition Assessment
METTL1: Methyltransferase-like 1
MF: Molecular functions
MWM: Morris water maze
NK: Natural killer
PBMCs: Peripheral blood mononuclear cells
MTT: Thiazolyl blue tetrazolium bromide
TBI: Traumatic brain injury
WT: Wildtype.

Data Availability

The dataset supporting the conclusions of this article are available from the corresponding author.

Ethical Approval

Experiments were performed under ethical guidelines (20170223-001) and handled according to institutionally approved procedures.

Conflicts of Interest

The authors declare that they have no competing interests.

Authors' Contributions

PZ and JW designed the whole study. DBR and LS performed *in vitro* and *in vivo* experiments. ZCK collected peripheral blood and did FACS analysis. PZ, YSZ, and JW analyzed the data, did the statistics, and wrote the paper. All authors read and approved the final manuscript.

Acknowledgments

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Supplementary Materials

Figure S1: the ceRNA network for circular RNA-miRNA-mRNA. Figure S2: treatment with si-circHtra1 significantly prevents the TBI-associated injury and motor deficits. Figure S3: quantitative PCR of circHtra1 and Htra1 level in whole blood from TBI patients. Figure S4: the cellular expression of Grb10 and Htra1 level in the ADAR1 KO cell line. Table S1: clinical characteristics of patients with traumatic brain injury. Table S2: list of all primer sequences used in real-time PCR experiment. Table S3: top five upregulated and downregulated circRNAs in severe TBI. *(Supplementary Materials)*

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Research Article

Sorting Nexin 5 Plays an Important Role in Promoting Ferroptosis in Parkinson's Disease

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Parkinson's disease (PD) is a common neurodegenerative disease in the elderly, which is related to brain iron metabolism disorders. Ferroptosis is a newly discovered iron-dependent programmed cell death mode, which has been considered an essential mechanism of PD pathogenesis in recent years. However, its underlying mechanisms have not been fully understood. In the present study, the PD rat model and PD cell model were induced by 6-hydroyxdopamine (6-OHDA). The results showed that the expression of Sorting Nexin 5 (SNX5) and the level of ferroptosis will increase after treatment with 6-OHDA. Consistent with these results, ferroptosis inducer erastin synergistically reduced the expression of glutathione peroxidase 4 (GPX4) and increased the expression of SNX5 in the PD cell model, while ferroptosis inhibitor ferrostatin-1 (Fer-1) inhibited the decrease of GPX4 and the increase of SNX5 in the PD cell model. Knockdown of SNX5 in PC-12 cells could reduce intracellular lipid peroxidation and accumulation of Fe^{2+} and significantly inhibit the occurrence of ferroptosis. In conclusion, the present study suggested that SNX5 promotes ferroptosis in the PD model, thus providing new insights and potential for research on the pharmacological targets of PD.

1. Introduction

Parkinson's disease (PD) is the second-largest neurodegenerative disease affecting the global population [1]. From 2005 to 2015, the global death toll from PD increased by about 40%, and the incidence of PD increased faster than other nervous system diseases. In our country, the incidence of PD is 17% over the age of 65 [2]. PD is characterized by a decrease in the number of dopaminergic neurons and an abnormal accumulation of α-synuclein [3, 4]. The decrease of dopamine will lead to tremor, bradykinesia, rigidity, posture balance disorder, and other clinical motor symptoms [5, 6]. However, no cure has been found for PD. Therefore, there is an urgent need to develop new targets to improve the diagnosis and treatment of PD patients.

Iron ions are essential for normal brain development and cognitive function, and iron-dependent enzymes and ferritin are necessary for synapses, myelin formation, and neurotransmitter production and transformation [7, 8]. However, iron is an oxidant, and excessive deposition can also cause severe damage to the cell. Excessive free iron causes oxidative nitrification stress, inflammation, and excitatory toxicity, resulting in cell damage and neurodegeneration [9]. The consequences of metabolic disorders of iron ions may be the main cause of PD. Relevant studies have shown that ferroptosis participates in the pathogenesis of PD. Iron

overload and lipid peroxidation are the main characteristics of ferroptosis [10]. Ferroptosis is related to glutathione peroxidase 4 (GPX4) activity [11–13]. However, the mechanism of ferroptosis in PD remains unclear.

In the previous work, the difference in the expression of Sorting Nexin 5 (SNX5) between the PD rat model and the normal rat was determined by iTRAQ protein sequencing analysis [14]. SNX5 is one of the most important proteins in the endosome sorting component retromer, which is mainly expressed in the endosome and is involved in the identification, transport, and unloading of substances between various organelles in the cell [15, 16]. The content of SNX5 in cells affects the endocytosis sorting and other component transportation of cells [16, 17]. It has been reported that the SNX family regulates protein homeostasis in nerve cells, and incorrect protein transport is related to the development of many neurodegenerative diseases, including PD [18]. However, the relationship between SNX5 and ferroptosis in PD has not been explored and deserves further study.

In this study, we investigated the specific mechanism of SNX5 in the appearance of PD. The results confirmed that the knockdown expression of SNX5 in the PD cell model could reduce the level of lipid peroxidation and inhibit the occurrence of ferroptosis. Therefore, SNX5 had the effect of promoting ferroptosis in PD, thus providing new insights and potential for research on the pharmacological targets of PD.

2. Materials and Methods

2.1. Animal. A total of 36 male Sprague-Dawley (SD; 180 g-200 g; 6-8 weeks of age) rats were purchased from Guangdong Zhiyuan Biomedical Technology Co. LTD., and animal certificates were provided (no. 110322210101368063). All animal experiments are permitted by the Animal Ethics Committee of Guangzhou University of Chinese Medicine (no. 20220330002). And all study conducted following the guidelines for the care and use of experimental animals of the National Institutes of the health of the United States and provided free access to food and water. The rats were divided into three groups (control group, sham group, and model group), with 12 rats in each group.

2.2. PD Rat Model Establish. The PD rat model was established by injecting 6-hydroyx dopamine (6-OHDA) (H116-5MG, Sigma-Aldrich, MO, USA) into the one-sided medial forebrain bundle (MFB) target of the rat [19]. Dissolve 6-OHDA powder in 0.2% ascorbic acid to prepare 8 mg/ml 6-OHDA solution. Rats were anesthetized by intraperitoneal injection of 30 mg/kg of 3% sodium pentobarbital. After rats were anesthetized, their heads were shaved and fixed on the brain stereotaxic frame. Then, the rat head skin was sterilized with 75% alcohol, and the rat head skin was cut with scissors to find the location of the anterior fontanel, which was then used as the origin for positioning. The rats of the model group were injected with 25 μg 6-OHDA at MFB (A/P = 4.4, M/L = 1.2, and D/V = 7.8 mm). When using the cranial drill, avoid damaging the brain of rats due to excessive force, and the microsyringe was used to slowly insert to the brain. Before awakening, rats should use a thermal insulation pad to keep the rats' body temperature at 36.5°C. In order to prevent infection, penicillin (100,000 units) was injected intraperitoneally after the operation. The experimental operation in the sham group was exactly the same as that in the model group, except that 6-OHDA was replaced with an equal volume of saline solution. The rats in the control group were raised normally without any treatment.

2.3. Behavioral Test. The spontaneous rotation behavioral test was performed 28 days after the establishment of the PD rat model. After intraperitoneal injection of apomorphine (APO, 017-18321, Wako Pure Chemical Industries, Osaka, Japan) at 0.5 mg/kg, the rats were placed on a circular platform with a diameter of 50 cm. Rotations were manually counted 10 minutes after the APO injection. Record the number of left-hand rotations for 30 minutes. The model rats with a rotational speed over 7 turns/minutes were considered suitable for further analysis.

2.4. Immunofluorescence. Brain perfusion was performed on three rats in each group, followed by paraffin embedding and sectioning. Firstly, paraffin wax dewaxing with xylene for 15 minutes and hydrated with ethanol (anhydrous ethanol, 95% ethanol, 90% ethanol, and 70% ethanol for 10 minutes each). Secondly, antigen repair by completely immersing the tissue slides in 0.01 M citric acid buffer, boiling for 6 minutes, and then cooling to room temperature, repeating for 2 times. Then, sections were washed 1 time in PBS and permeabilized with an immunostaining permeabilization buffer with saponin (P0095-100 ml, Beyotime, Shanghai, China) for 10 minutes and were blocked with 10% goat serum (C0265, Beyotime, Shanghai, China) for 20 minutes at room temperature. After blocking, the sections were incubated overnight with tyrosine hydroxylase (TH, 1 : 2000, 25859-1-AP, Proteintech, Wuhan, China). On the next day, sections were washed in PBS and incubated with secondary antibody (1 : 1000, ab150077, Abcam, MO, USA) for 1.5 h at 37°C and washed in PBS. The nucleus was stained with DAPI (1 : 1000, C1002, Beyotime, Shanghai, China) for 5 minutes at room temperature and then washed with PBS. At last, the sections were sealed with antifade mounting medium (P0126-5 ml, Beyotime, Shanghai, China) and imaged by Cytation 5™ Cell Imaging Multi-Mode Reader (BioTek, VT, USA).

2.5. Cell Culture and Reagents. PC-12 cells were obtained from the Chinese Academy of Sciences. Cells were cultured in RPMI 1640 medium (C11875500BT, Gibco, CA, USA) containing 10% fetal bovine serum (XC6936T, Guangzhou, China) and 1% Penicillin-Streptomycin (15140122, Gibco, CA, USA). Erastin (HY-15763, NJ, USA) and ferrostatin-1 (fer-1, HY-100579, NJ, USA) were purchased from MedChem Express.

2.6. Cell Viability Assay. The MTS assay kit (G3580, Promega, WI, USA) was used to determine cell viability. PC-12 cells (1×10^4/well) were plated in flat-bottomed 96-well plates. Discarding the cell culture medium after 24 hours of

6-OHDA stimulation, MTS was added to each well and incubated for 2 hours at room temperature. A microplate reader was used to record the OD values at a wavelength of 490 nm (BioTek, VT, USA). Live-death cell staining was detected using the calcein-AM/PI test kit (R37601, Thermo, CA, USA). PC-12 cells (1×10^4/well) were plated in black-walled porous 96-well plates and then treated with gradient concentrations of 6-OHDA for 24 hours. Then, cells were washed before being stained for 20 minutes with 5 M PI and 5 M calcein-AM and imaged by Cytation 5™ Cell Imaging Multi-Mode Reader.

2.7. Western Blot Analysis. The cells were lysed in RIPA buffer (89900, Thermo, CA, USA) and centrifuged at 12,000 rpm for 15 minutes at 4°C to obtain the supernatant. The pierce BCA protein assay kit (23227, Thermo Scientific, CA, USA) was used to determine the protein content according to the manufacturer's protocol. The total protein (30 μg) of the sample was separated using 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membrane was blocked for 1 hour at room temperature with 5% skimmed milk powder before overnight incubation with primary antibodies such as SNX5 (1 : 1000, 17918-1-AP, Proteintech, Wuhan, China), FTH1 (1 : 1000, 4393S, Cell Signaling Technology, MA, USA), GPX4 (1 : 1000,52455S, Cell Signaling Technology, MA, USA), HRP-conjugated β-actin (1 : 2000, HRP-60008, Proteintech, Wuhan, China), and TH (1 : 2000, 25859-1-AP, Proteintech, Wuhan, China). Rinse three times with TBST and incubate with HRP-labelled secondary antibody (1 : 1000, ab6721, Abcam, MO, USA) for one hour at room temperature. Immunoblotting Western HRP substrate (WBKLS0500, Millipore, MA, USA) was used to visualize the bands. Image J software was used to assess the density of the bands. All of the data comes from at least three different experiments.

2.8. Cell Transfection. GenePharma discovered and produced siRNAs that target SNX5 (m-Pack1999, RIBOBIO, Guangzhou, China). The sequence of siRNA was as follows: siRNA#1, 5′GGATGACTTCTTTGAGCAA 3′; siRNA#2, 5′ GCACAAAGGCCCTAATTGA3′; siRNA#3, 5′ GGAA GAGAGTGGCAGCATT3′. Lipofectamine 3000 (Lipi3000, L3000015, Invitrogen, CA, USA) was used to transfect the siRNA to the cells according to the manufacturer's protocol. Lipo3000 and siRNA were diluted for in OptiMEM (31985070, Gibco, CA, USA) and mixed gently; then, the mixture was added to the cell cultures.

2.9. Determination of Reduced Glutathione (GSH)/Oxidized Glutathione (GSSG) Activity. In order to detect the ratio of GSH/GSSG in the cell and animal, the GSH/GSSG Assay Kit (S0053, Beyotime, Shanghai, China) was performed according to the manufacturer's protocol. PC-12 cells (2.5×10^6/well) were cultured in a 6 cm culture dish, and then, the culture media was withdrawn after a 24-hour treatment with 40 μM 6-OHDA. Three rats in each group were anesthetized, and substantia nigra (SN) was collected in the unconscious state of the rats.

2.10. Determination of Malondialdehyde (MDA). In order to detect the concentration of MDA in the cell and animal, the Lipid Peroxidation MDA Assay Kit (S0131S, Beyotime, Shanghai, China) was performed according to the manufacturer's protocol. Cells and tissues were lysed by IP-lysis (87787, Thermo Scientific, CA, USA) for 20 minutes at 4°C. After sample preparation, protein concentration can be measured with the pierce BCA protein assay kit to facilitate subsequent calculation of MDA content in tissues or cells per unit protein weight.

2.11. Intracellular Ferrous Ion Imaging. The FeRhonox-1 staining kit (SCT030, Goryo Chemical, Sapporo, Japan) was used to determine the distribution of Fe^{2+} in the cell according to the manufacturer's protocol. PC-12 cells (1×10^4/well) were cultured in the black-walled porous 96-well plates overnight. The cell culture medium was withdrawn after the cells had been treated with 6-OHDA for 24 hours, and the cells were washed two times with PBS. Then, the cell media was supplemented with 1 μM FeRhoNox-1 and imaged by Cytation 5™ Cell Imaging Multi-Mode Reader.

2.12. Statistical Analysis. The experimental data are presented as the mean ± SEM. The figures were produced by GraphPad Prism 8.0 (GraphPad Software, San Diego, CA). The comparison between multiple groups was analyzed by One-way analysis of variance followed by SNK multiple comparisons test as the post hoc test. $p < 0.05$ was considered to indicate a statistically significant difference.

3. Results

3.1. The Expression of TH Significantly Decreased in the PD Rat Model. After 28 days of modeling, the rats were intraperitoneal injection of APO to observe the number of laps to determine whether the modeling was successful. Twelve rats turned >7 times/min and reached the modeling standard (Figure 1(a)). TH is the signature protein of dopaminergic neurons in the nervous system. The IF result showed that the expression of TH in the SN (Figure 1(b)) and the striatum (Figure 1(c)) was significantly decreased in the 6-OHDA-treated hemisphere compared with the contralateral hemisphere in the model group. While the expression of TH in the control group and the sham group was symmetric.

3.2. The Level of Ferroptosis and the Expression of SNX5 Were Increased in the PD Rat Model. A growing number of studies have demonstrated that ferroptosis is an essential mode of dopamine neuron death. To determine whether 6-OHDA can induce ferroptosis in the PD rat model, we examined the expression of GPX4, the concentration of MDA, and the ratio of GSH/GSSG. As shown in Figure 2(a), the expression of TH and GPX4 was decreased significantly in the model group. The concentration of MDA increased significantly in the model group, which represents the level of lipid peroxides was increased in the model group (Figure 2(b)). In addition, glutathione depletion and the decrease of GPX4 activity can lead to increased lipid peroxidation. Therefore, the ratio of GSH/GSSG

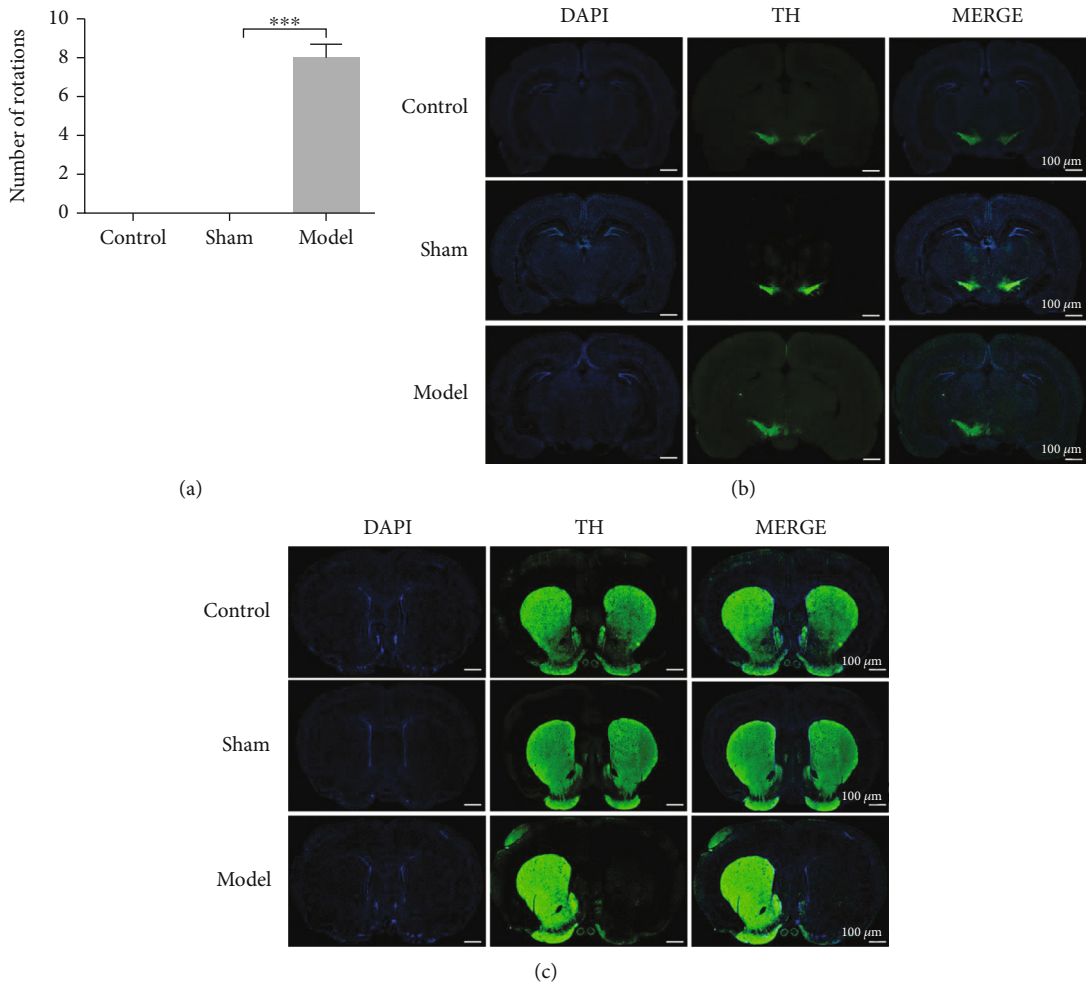

Figure 1: The expression of TH significantly decreased in the PD rat model. (a) The changes in the rotation number of rats after injection of APO. The changes of the control, sham, and model groups were compared. (b) Immunofluorescence was performed on the SN of the rat in the control, sham, and model groups. (c) Immunofluorescence was performed on the striatum of the rat in the control, sham, and model groups. Data were expressed as mean ± S.D.; $n \geq 3$ per group for all the studies ($^{***}p < 0.05$).

decreases significantly in the model group (Figure 2(c)). The expression of SNX5 was significantly increased in the model group (Figure 2(a)), which indicated that an abnormal increase of SNX5 induced by 6-OHDA contributes to the formation of PD pathology.

3.3. The Expression of SNX5 and the Level of Ferroptosis Were Increased in the PD Cell Model. The result of MTS has the similarly trend as the expression of TH (Figure 3(a)) and live-death cell staining (Figure 3(b)), 6-OHDA shows significant cytotoxicity at high concentrations, and the IC50 value was 40 μM (Figure 3(c)). Based on the above experimental results, we chose 40uM 6-OHDA as the optimal stimulation concentration for the next experiment. Our results also showed that the expression of TH and GPX4 was significantly decrease in the model group, while the expression of SNX5 was significantly increase (Figure 4(a)). To explore the level of ferroptosis in the model group, we examined the level of lipid peroxidation and the ratio of GSH/GSSG. The results showed that the concentration of MDA increased significantly (Figure 4(b)), while the ratio of GSH/GSSG decreased significantly in the model group (Figure 4(c)), which was consistent with the results in the PD rat model and suggested that 6-OHDA could induce ferroptosis. In summary, the experimental results demonstrated that SNX5 expression was upregulated in the model group compared to the control group, suggesting that SNX5 is involved in developing PD pathogenesis.

3.4. The Effects of Erastin and Fer-1 on PD Cell Model. The above experiments showed that 6-OHDA induced the onset of ferroptosis in PC-12 cells and changes in the corresponding indexes. In this study, the effects of erastin and fer-1 on the PD cell model were explored. The results showed that the ferroptosis inducer erastin enhanced the role of 6-OHDA in the cells. The expression of TH and GPX4 was decreased more, and the expression of SNX5 was increased more in cells treated with the combination of erastin and 6-OHDA than in cells treated with only 6-OHDA (Figure 5(a)). However, all changes in indicators due to ferroptosis are redeemed by the ferroptosis inhibitor fer-1. Compared with the cells treated with 6-OHDA alone, the

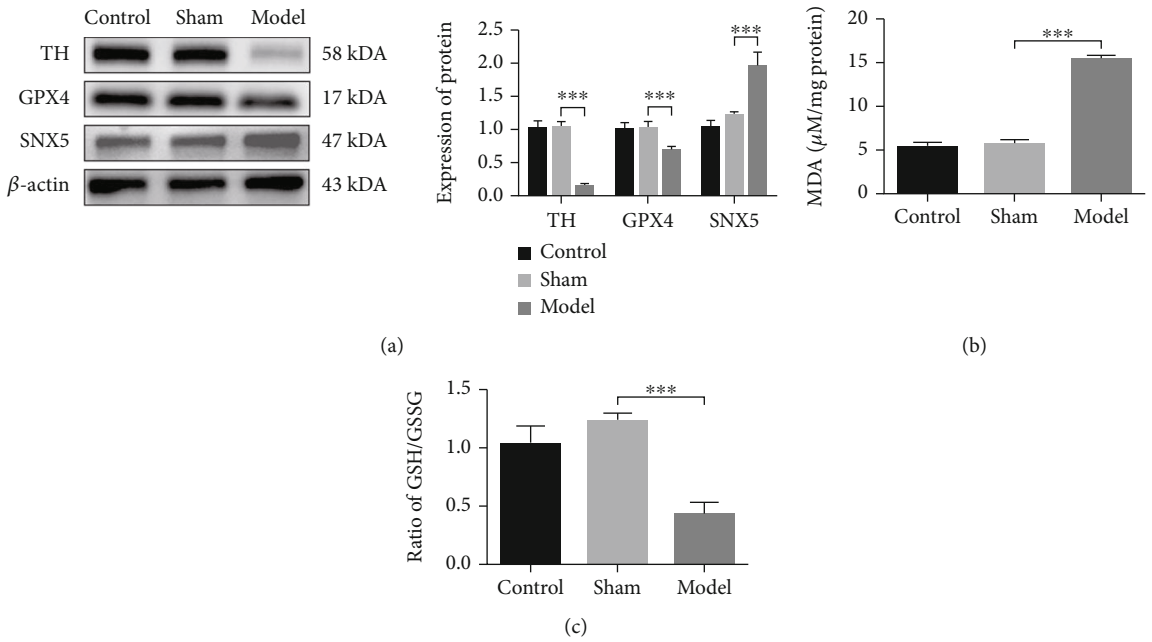

Figure 2: The level of Ferroptosis and the expression of SNX5 were increased in the PD rat model. (a) Western blot analysis of the expression of TH, GPX4, and SNX5. (b) The concentration of GSH/GSSG in SN of the PD rats was detected by MDA Assay. (c) The ratio of GSH/GSSG in SN of the PD rats was detected by GSH/GSSG-GLO Assay. Data were expressed as mean ± S.D.; $n \geq 3$ per group for all the studies ($^{***}p < 0.05$).

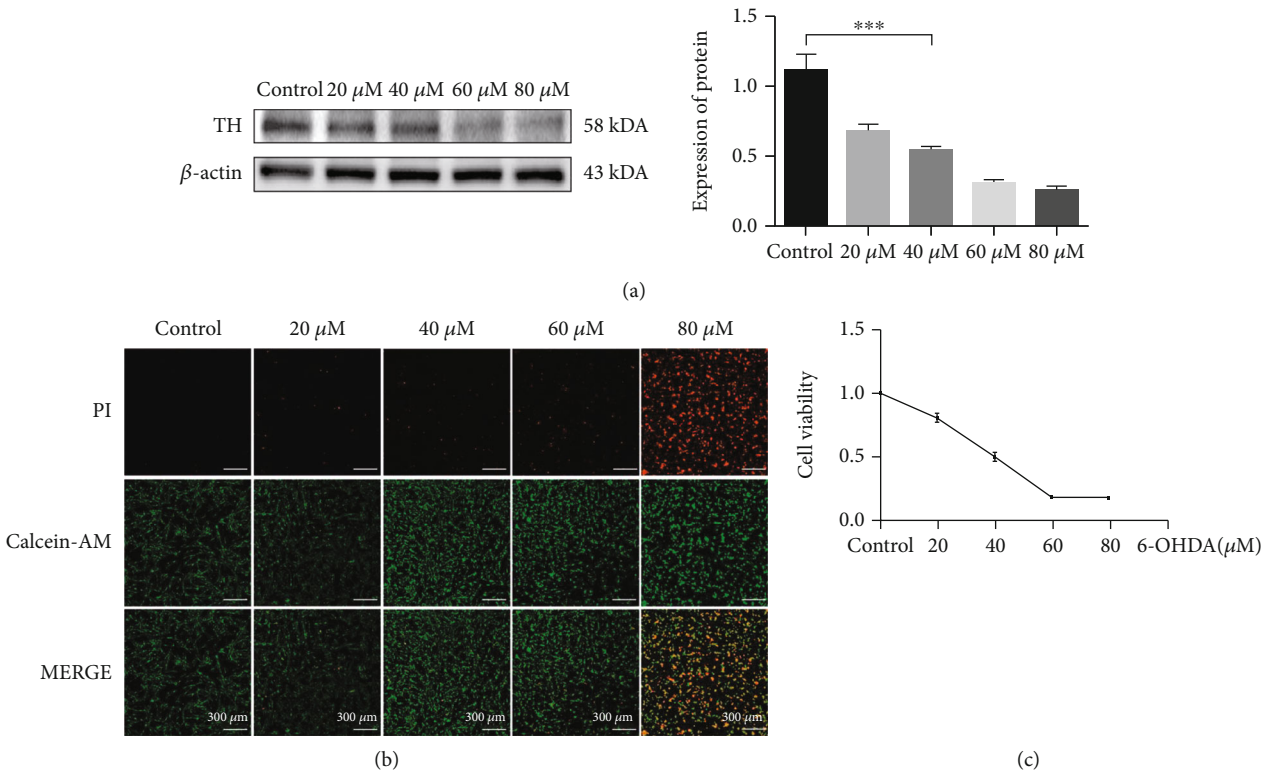

Figure 3: Screening the optimal stimulation concentration of the 6-OHDA to establish the PD cell model. (a) Stimulation of PC-12 cells with 20, 40, 60, and 80 μM concentration of 6-OHDA for 24 hours. The expression of TH was detected by Western blotting. (b) PC-12 cells were treated with 20, 40, 60, 80 μM concentration of 6-OHDA for 24 hours, and live and dead cells were detected by immunofluorescence, live cells by red PI, and dead cells by green calcein-AM. Observation of cellular changes with fluorescence microscopy. (c) PC-12 cells were treated with 20, 40, 60, 80 μM concentration of 6-OHDA for 24 hours, cell viability was evaluated by the MTS assay ($^{***}p < 0.05$).

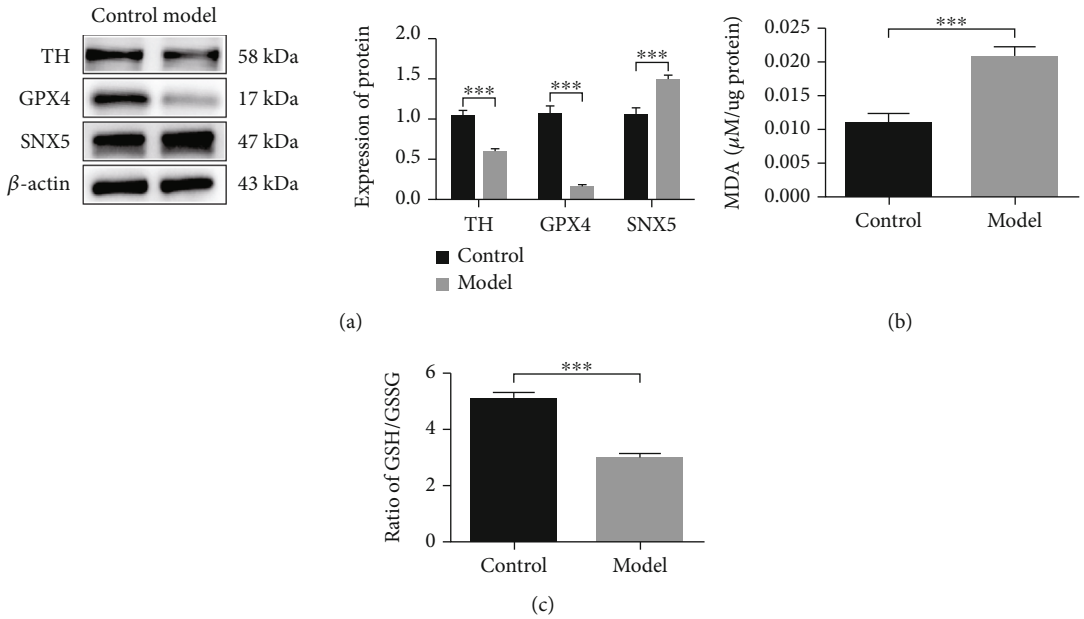

Figure 4: The expression of TH and GPX4 was decreased, while the expression of SNX5 was increased in the PD cell model. (a) PC-12 cells were untreated or treated with 40 μM 6-OHDA for 24 h. The expression of TH, GPX4, and SNX5 was detected by Western blotting. (b) MDA Assay was used to detect the concentration MDA in PC-12 cells was induced by 6-OHDA. (c) GSH/GSSG ratio in vitro PD was detected by GSH/GSSG Assay. Data were expressed as mean ± S.D.; three independent replicates of the experiment ($^{***}p < 0.05$).

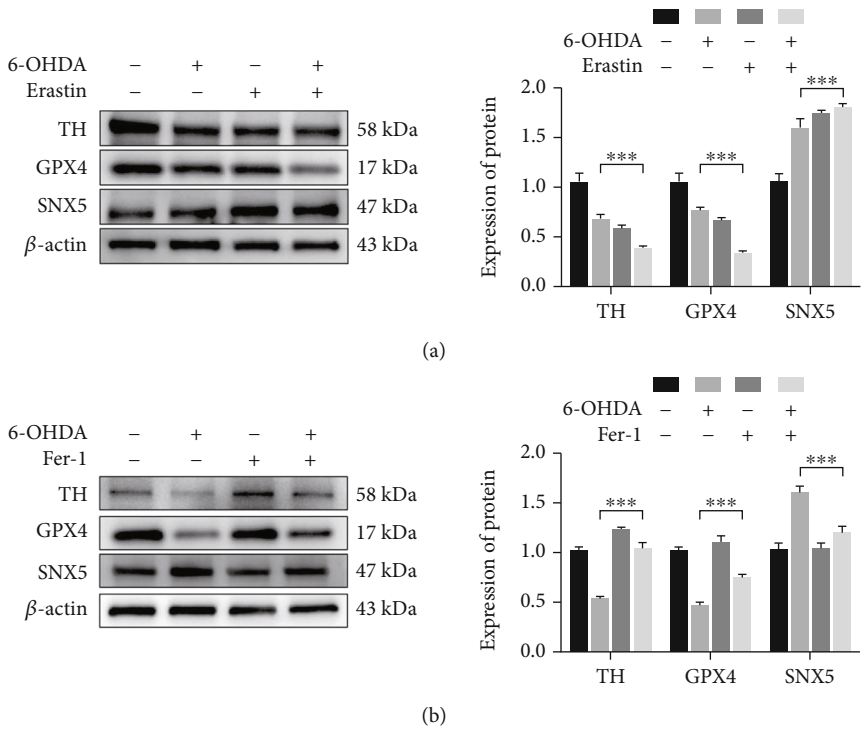

Figure 5: The effects of erastin and fer-1 on PD cell model. PC-12 cells were induced with 40 μM 6-OHDA in combination with an inducer (1 μM erastin) or inhibitor (2.5 μM fer-1) of ferroptosis for 24 h. The expression of GPX4, TH, and SNX5 was detected by Western blotting (a) and (b). Data were expressed as mean ± S.D.; three independent replicates of the experiment ($^{***}p < 0.05$).

expression of TH and GPX4 did not decrease, and the expression of SNX5 did not increase in the cells treated with the combination of 6-OHDA and fer-1, indicating that the level of ferroptosis in the PD cells was inhibited by fer-1 (Figure 5(b)). In conclusion, these results further demonstrate that 6-OHDA can induce ferroptosis in PC-12 cells,

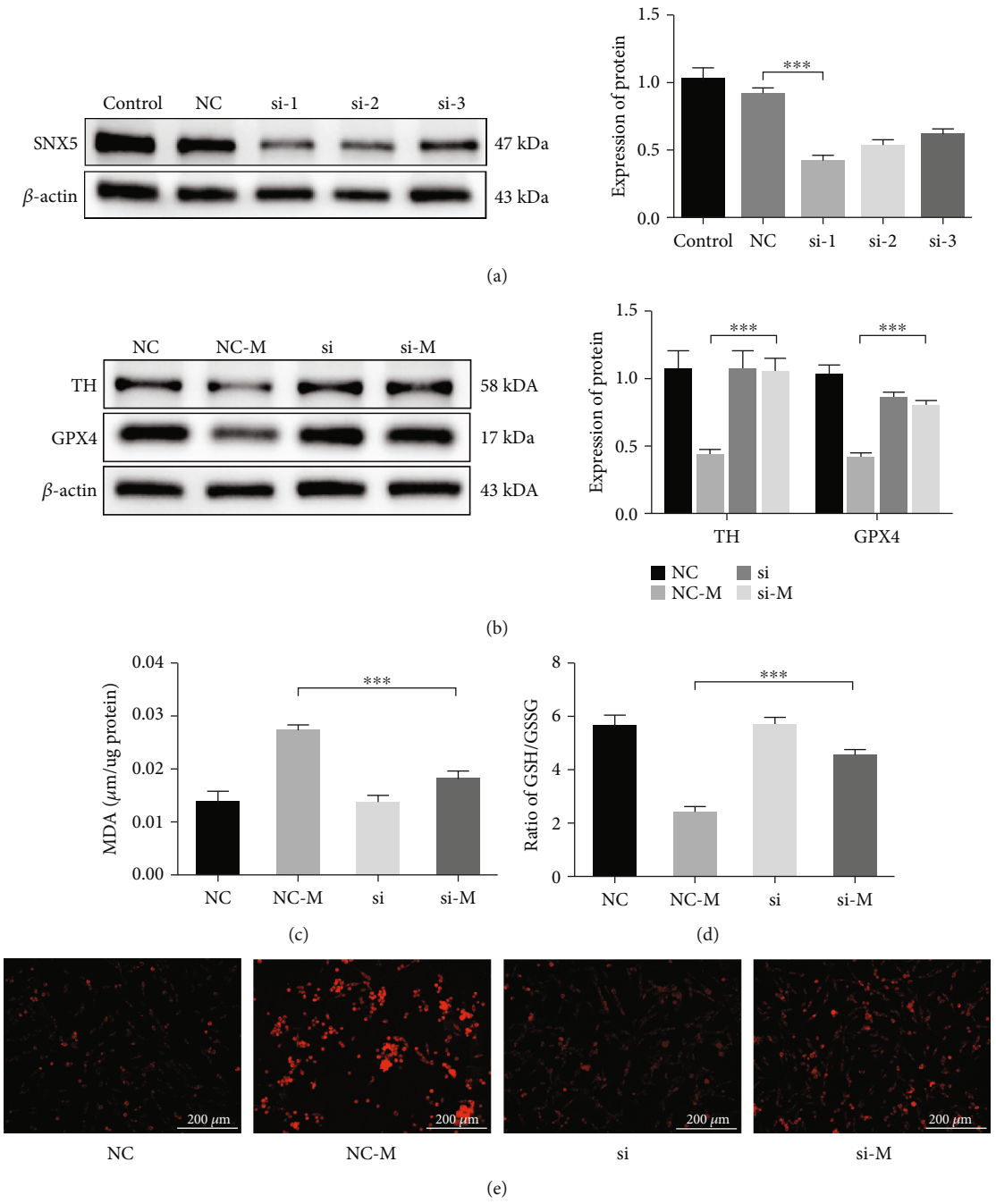

Figure 6: SNX5 promotes ferroptosis in the PD cell model. (a) Infection of PC-12 cells by conventional siRNA and screening the infected fragment with the best knockdown effect by Western blotting. PC-12 cells were infected with SNX5 transfected fragments before being treated with 40 μM 6-OHDA for 24 h. The expression of TH and GPX4 was analyzed using Western blotting (b), and the effect of SNX5 knockdown on MDA (c), GSH/GSSG (d), and Fe^{2+}(e) was detect by assay kit. Data were expressed as mean ± S.D.; three independent replicates of the experiment ($^{***}p < 0.05$).

and the expression of SNX5 will be upregulated when cells undergo ferroptosis.

3.5. SNX5 Promotes Ferroptosis in the PD Cell Model. To further validate the role of SNX5 in PD pathology and ferroptosis, we used siRNA interference fragments to knockdown the expression of SNX5 in the cell and then used 6-OHDA to stimulate the cell. Screening by experiment, the si-1 was the most efficient sequence for silencing (Figure 6(a)). After silencing SNX5 in PC-12 cells, administration of 6-OHDA stimulation, we found that knockdown the expression of SNX5 in the SI-M group can increase the expression of TH and GPX4 significantly compared to the NC-M group (Figure 6(b)). At the same time, the concentration of MDA decreased significantly, and the ratio of GSH/GSSG increased significantly in the SI-M group compared with

the NC-M group (Figures 6(c) and 6(d)). In addition, the ferrous imaging results also confirmed that the SI-M group could result in reduced Fe^{2+} accumulation in the PD cell model (Figure 6(e)). These data further suggest that SNX5 plays a vital role in PD pathology and ferroptosis. In PD cell models, abnormal aggregation of SNX5 may affect the process of ferroptosis onset, leading to the accumulation of toxic lipid peroxides and reactive oxygen species (ROS) and finally causing ferroptosis of cells.

4. Discussion

This study determined that SNX5 is abnormally overexpressed in the PD model, and it can promote ferroptosis. In the previous work, the result shows that the expression of SNX5 increases significantly in the PD rat model by iTRAQ protein sequencing, and the high expression of SNX5 is driven by superenhancer [14]. However, the influence of SNX5 on PD and ferroptosis is not clear. SNX5, as the core protein of the endosomal sorting component retromer, significantly affects the morphology of the Golgi complex [20, 21]. The dimer formed by SNX5 is responsible for recruiting retromer into the endosome and is an essential protein involved in intracellular and intracellular material transport and signal transduction [16, 22]. In recent years, genomics animal and cell biology have shown that retromer-mediated protein transport defects in endosomal sorting are closely related to PD pathogenesis [15, 23, 24]. SNXs play a variety of roles in different aspects of endosomal transport, including the transport of endosomes to the trans-Golgi network, circulation to the cell surface, and endocytosis [25]. Retromer is also required for endosomatic circulation of iron transporter DMT1 [26], suggesting that endosomal sorting mediates part of the intracellular iron ion transport process.

In this study, the PD model was established by 6-OHDA, the PD pathology was enhanced, and the expression of SNX5 was increased significantly in the PD model. Besides, the ratio of GSH/GSSG and the expression of GPX4 in the model group were decreased, while the concentration of MDA was increased, suggesting that ferroptosis appeared in the PD model. Then, the ferroptosis inducer erastin and the ferroptosis inhibitor fer-1 were used to stimulate PC-12 cells. Erastin, as a classical ferroptosis inducer, can accelerate ROS accumulation in PC-12 cells by directly decreasing GSH activity to induce ferroptosis [27, 28]. In the experiment shown in Figure 5, the effects of 6-OHDA and erastin on ferroptosis were similar, and erastin can promote PD cell damage. Moreover, fer-1, as an inhibitor of lipid ROS production, can alleviate ferroptosis to a certain extent [29, 30]. When 6-OHDA and fer-1 are used in combination, fer-1 can inhibit the decrease of GPX4 and the increase of SNX5 in the PD cell model. Fer-1 could rescue PD cell model ferroptosis to a certain extent, suggesting that PD cells appear in ferroptosis and SNX5 is related to the appearance of ferroptosis. After the expression of SNX5 was inhibited by siRNA in PC-12 cells, the expressions of TH and GPX4 in the SI-M group were increased, the concentration of MDA was decreased, and the ratio of GSH/GSSG was increased significantly compared with the NC-M group. Compared with the SI group, the SI-M group showed no significant difference. In addition, the knockdown of SNX5 in PC-12 cells improved the intracellular accumulation of Fe^{2+}. The results showed that the knockdown of SNX5 had a protective effect on PD and ferroptosis, indicating that SNX5 could promote ferroptosis in PD. However, the specific mechanism of how SNX5 regulates ferroptosis in PD is uncertain and needs to be further explored. Because previous studies have shown that SNX5 is one of the most important proteins in the endosome, which participates in the transport of multiple substances in the cell, including iron transport, SNX5 may influence the occurrence of ferroptosis by affecting iron transport.

5. Conclusion

In conclusion, this study demonstrated that abnormally high expression of SNX5 promotes ferroptosis in PD. The discovery of SNX5 also provides new insights into ferroptosis in PD, new diagnostic markers for PD diagnosis, and is expected to become a new therapeutic target for PD.

Data Availability

The data and materials produced during the study can be obtained from the corresponding authors on reasonable request.

Ethical Approval

All animal experiments are permitted by the Animal Ethics Committee of Shenzhen PKU-HKUST Medical Center (no. 2021656) and conducted following the guidelines for the care and use of experimental animals of the National Institutes of the health of the United States.

Conflicts of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Authors' Contributions

HJ and WP completed the experiment together and wrote the manuscript. HZ and WC designed the experiment and analyzed the data. FY, ZL, and HQ conduct the animal experiments. MZ and DC conceived and designed the experiment. All authors have read and approved the final draft. Zifeng Huang, Jiajun Han, and Peipei Wu contributed equally to this work.

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