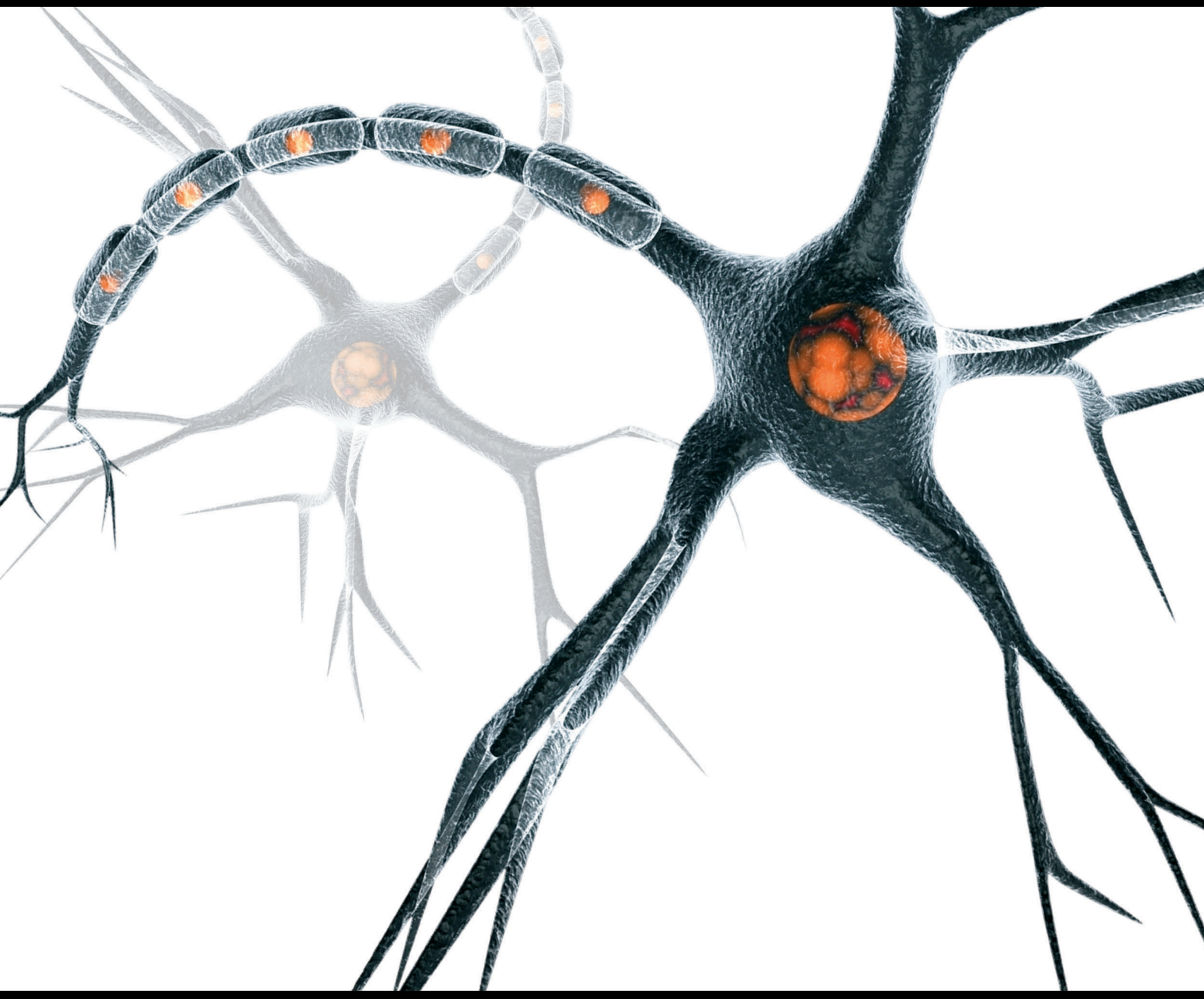
Neural Plasticity

Neuroplasticity in the Pathology of Neurodegenerative Diseases

Lead Guest Editor: Jolanta Dorszewska
Guest Editors: Wojciech Kozubski, Wioletta Waleszczyk, Matthew Zabel, and Kevin Ong



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Editorial

Neuroplasticity in the Pathology of Neurodegenerative Diseases

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The phenomenon of neuroplasticity was first described about 50 years ago. Neuroplasticity is a primary property of the nervous system that has been widely observed in both physiological and pathological conditions. Neuroplasticity in physiological conditions includes developmental plasticity, learning and memory, compensatory plasticity, and repair of the adult brain. Neuroplasticity in pathological conditions include plasticity after injury and removal of brain tumor, stroke, epilepsy, and neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and Huntington's disease. The current special issue is primarily concerned with the pathogeneses of common neurodegenerative diseases and their effects on neuroplasticity.

It is known that the central changes that occur during a stroke vary widely. Regeneration after ischemic injury and recovery of lost functions can be attributed to the plasticity of neurons which enable their ability to reorganize and rebuild the brain. These neuroplastic changes which include gene expression and possession of specific genetic variants are influenced by environmental factors and epigenetic mechanisms. Neuro-, glio-, and angiogenesis processes, molecular changes occurring after stroke such as synaptic plasticity and axon growth, are discussed in the article by J. Dąbrowski et al.

Many rare diseases are due to disorders at the genetic level such as Waardenburg syndrome (WS). WS is one of the most common forms of deafness with heterogeneity of loci and alleles and variable expression of clinical features. In this issue, W. Li et al. described fourteen mutations in Chinese patients with WS, including c.808C>G, c.117C>A, c.152T>G, c.803G>T, c.793-3T>G, and c.801delT on *PAX3*; c.642_650delAAG on *MITF*; c.122G>T and c.127C>T on *SOX10*; c.230C>G and c.365C>T on *SNAI2*; and c.481A>G, c.1018C>G, and c.1015C>T on *EDNRB*. Importantly, three CNV variants were *de novo* and five *EDNRB* mutations were first associated with WS type 1 heterozygous, with a detection rate of 22.2%. These studies suggest that *EDNRB* mutations should be considered as another common pathogenic gene in WS type 1. More information can be found in the articles by W. Li et al.

At the cellular level, synaptic plasticity plays an important role in maintaining neuronal connections. Synaptic plasticity leads to changes in the structure of synapses and dendritic spines, synaptogenesis, and axonal modification. Disturbed contact between neurons leads to senile changes and elderly diseases such as dementia. Common causes of dementia include Alzheimer's disease, vascular dementia, Parkinson's disease dementia, dementia with Lewy

bodies, and frontotemporal dementia. These diseases are characterized by impaired cognitive function as well as personality and emotional changes, including psychopathological disorders. The clinical picture may also include other neurological symptoms such as parkinsonism.

Neuroplasticity at molecular and cellular levels occurs as short-term plasticity (STP), long-term potentiation (LTP), and long-term potentiation depression (LTD). Activation of the NMDA receptor especially in the CA1 region of the hippocampus is necessary for the induction of LTP, even though LTP may be induced in almost all brain structures. GABAergic inhibitory transmission is another important molecular process involved in synaptic plasticity, learning, and memory. Abnormal GABAergic transmission is involved in the pathophysiology of fragile X syndrome (FXS), an inherited neuropsychological disease caused by silencing of the *fmr1* gene and deficiency in the mental retardant protein fragile X (FMRP). Patients with FXS show neuronal changes that lead to severe intellectual disability. More information can be found in the articles by F. Gao et al.

Molecular factors responsible for synaptic transmission disorders include β-amyloid deposition; tau aggregation forming neurofibrillary tangles; α-synuclein accumulation; growth factors such as BDNF, NGF, and GDNF impaired levels; and disorders of the immune system. Experimental studies using glial cell lines designed to secrete high levels of neurotrophic factor (human ARPE-19 cells) implanted in the rat striatum showed a beneficial effect on GDNF distribution throughout the striatum and their neuroprotection. It seems that GDNF has effective action not only in experimental animals but also in patients with Parkinson's disease, stroke, epilepsy, and Huntington's disease. More information may be found in the article by D. F. Emerich et al.

Another molecular factor involved in the regulation of various processes in neurons and synaptic plasticity is glycogen-3β synthase kinase (GSK-3β). Altered GSK-3β expression or deletion in mice may cause behavioral and cognitive abnormalities. Disturbed GSK-3β activity may lead to the development of synaptic plasticity dysfunction and development of neuropsychiatric and neurodegenerative disorders. For more information on GSK-3β contribution to neuropsychiatric and neurodegenerative disorders, refer to the article by T. Jaworski et al.

Among the factors affecting synapse plasticity and neurogenesis, astroglia need to be considered. Astrocytes are the most numerous glial cells and play a key role in brain function. Yet little research has been done on their role in neurodegeneration. Research done so far on experimental models indicates the importance of cross signaling pathways between astrocytes and dopaminergic neurons. In Parkinson's disease, astrocytes are involved in neuronal survival and viability. More information on the neuroprotective potential for normalizing astrocyte function in the animal model of Parkinson's disease is available in the article by L. Udovin et al.

Neuroplasticity not only rebuilds brain function but also repairs developmental disorders and is responsible for learning and memory. It appears in response to various internal and external insults and factors, including age-dependent neurodegenerative diseases and pharmacological treatment.

It is known that the repair of brain damage depends on the degree and extent of central lesions. This is an important consideration in the neurorehabilitation and neuropsychological treatment of patients with central nervous system disorders like Alzheimer's disease. Recent studies suggest that intensive and cognitively demanding physical exercise programs are capable of inducing plastic brain changes in Parkinson's disease. The article by E. Palasz et al. elaborates on physical training that provides protection for dopaminergic neurons in rodent parkinsonism models produced by neurotoxins.

We should also mention innovative therapeutic methods to improve neuroplasticity such as deep brain stimulation (DBS). DBS is a recognized therapy for Parkinson's disease. There is evidence that DBS exerts dopaminergic neuroprotection in animal models with disease-modifying effects. Rodent studies have shown an increase in cholinergic neurotransmitters, hippocampal neurogenesis, synaptic plasticity, and reduction of β-amyloid plaques with DBS. The effectiveness of DBS is currently being evaluated in patients with mild Alzheimer's disease 65 years of age and older.

Finally, with better understanding of brain plasticity mechanisms in neurodegeneration, more effective therapies and improvement in the quality of life for neurological patients can be achieved.

Conflicts of Interest

The editors declare that they have no conflicts of interest regarding the publication of this special issue.

Authors' Contributions

The authors of the Editorial are guest coeditors of this special issue.

Jolanta Dorszewska
Wojciech Kozubski
Wioletta Waleszczyk
Matthew Zabel
Kevin Ong

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Review Article

Role of Astrocytic Dysfunction in the Pathogenesis of Parkinson's Disease Animal Models from a Molecular Signaling Perspective

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Guest Editor: Matthew Zabel

Despite the fact that astrocytes are the most abundant glial cells, critical for brain function, few studies have dealt with their possible role in neurodegenerative diseases like Parkinson's disease (PD). This article explores relevant evidence on the involvement of astrocytes in experimental PD neurodegeneration from a molecular signaling perspective. For a long time, astrocytic proliferation was merely considered a byproduct of neuroinflammation, but by the time being, it is clear that astrocytic dysfunction plays a far more important role in PD pathophysiology. Indeed, ongoing experimental evidence suggests the importance of astrocytes and dopaminergic neurons' cross-linking signaling pathways. The Wnt-1 (wingless-type MMTV integration site family, member 1) pathway regulates several processes including neuron survival, synapse plasticity, and neurogenesis. In PD animal models, Frizzled (Fzd) neuronal receptors' activation by the Wnt-1 normally released by astrocytes following injuries leads to β-catenin-dependent gene expression, favoring neuron survival and viability. The transient receptor potential vanilloid 1 (TRPV1) capsaicin receptor also participates in experimental PD genesis. Activation of astrocyte TRPV1 receptors by noxious stimuli results in reduced inflammatory response and increased ciliary neurotrophic factor (CNTF) synthesis, which enhances neuronal survival and differentiation. Another major pathway involves IκB kinase (IKK) downregulation by ARL6ip5 (ADP-ribosylation-like factor 6 interacting protein 5, encoded by the cell differentiation-associated, JWA, gene). Typically, IKK releases the proinflammatory NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) molecule from its inhibitor. Therefore, by downregulating NF-κB inhibitor, ARL6ip5 promotes an anti-inflammatory response. The evidence provided by neurotoxin-induced PD animal models guarantees further research on the neuroprotective potential of normalizing astrocyte function in PD.

1. Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disease following Alzheimer's disease. It is characterized by loss of dopaminergic neurons in the midbrain [1, 2] and bradykinesia, rigidity, and tremor as main clinical symptoms. Regularly, patients also display nonmotor symptoms like cognitive impairment, mood disorders, sleep alterations, dysautonomia, and hallucinations [1].

Typical, though not only, histopathological changes are the progressive loss of the nigrostriatal dopaminergic pathway and hence of the striatal dopaminergic tone [2]. Over the last 40 years, administration of the amino acid precursor of dopamine L-DOPA (L-3,4-dihydroxy-L-phenylalanine) to parkinsonian patients has been considered the most effective symptomatic treatment [3].

Abnormal accumulation of misfolded protein aggregates [4] as the Lewy bodies, made of α-synuclein [5], appears to be

one of the physiopathological hallmarks of the disease. One major target of α-synuclein is Rab-1 (a member of the Ras superfamily of monomeric G proteins, Rab GTPase family), a key molecular switch of the endoplasmic reticulum-Golgi traffic pathway [6]. The α-synuclein accumulation-induced endoplasmic reticulum stress is likely a leading disruptive mechanism, responsible for the so-called "unfolded protein response" adaptive reaction [7], cytoprotective when moderate but deleterious when highly activated [8, 9]. Accumulation of α-synuclein may also originate abnormal synaptic connectivity or synaptopathy at nigrostriatal pathways and intrastriatal interneuronal connections, presumably most apparent at the initial stages of the disease.

Notwithstanding the fact that astrocytes are the most abundant glial subtype and are critical for brain function, only a few studies have historically focused on their putative role in neurodegenerative diseases like PD. Recently, however, several studies have reported that genes known to have a causative role in PD are expressed in astrocytes and have important roles in their function [10], suggesting that astrocyte dysfunction may be relevant for PD development. Furthermore, α-synuclein aggregates in astrocytes contributing to such dysfunction [11].

This review aims at summarizing the evidence for astrocyte participation in experimental PD genesis, the probable neuroprotective effect of molecules like GDNF (glial-derived neurotrophic factor), MANF (mesencephalic astrocyte-derived neurotrophic factor), and CNTF (ciliary neurotrophic factor), and the involved pathological cascades described so far, illustrating the potential use of these findings in developing new-generation neuroprotective agents. Following PubMED searches performed using "Parkinson's Disease, astrocytes, molecular signaling" strings, relevant papers published in English or Spanish before January 1, 2018, were included, while reference sections were also scrutinized out of these publications for new studies.

2. Role of Astrocyte Dysfunction in the Genesis of Experimental Parkinson's Disease

The glia accounts for over 50% of brain cells, comprising various cell subtypes, of which astrocytes are the most abundant [12, 13]. Although astrocytes were documented 100 years ago, relatively few studies have been designed to dig into their role in neurological disorders and diseases over time. Astrocytes can be both helpful and harmful in PD [14, 15], and a key aspect of PD pathophysiology is neuroinflammation in the central nervous system (CNS), for long considered a downstream response to dopaminergic neuronal death, definitely including the concurrence of reactive astrocytes [16, 17]. However, ongoing evidence suggests that astrocytes have a role in setting up PD pathophysiology. Astrocytes may have neuroprotective effects by producing factors like the glial cell line-derived neurotrophic factor (GDNF) [18], the mesencephalic astrocyte-derived neurotrophic factor (MANF) [19], and the ciliary neurotrophic factor (CNTF) [20]. Recently, a relative increase in the astrocytic level of senescence markers, inflammatory cytokines, and metalloproteinases was observed on postmortem substantia nigra specimens of five PD patients compared with five controls, illustrating astrocytes' relevance in PD [21]. Furthermore, astrocytes and fibroblasts developed senescent phenotypes when exposed to the neurotoxin paraquat in human cell cultures, and conversely, neurodegeneration was attenuated in response to paraquat in a senescent astrocyte-selectively depleted mouse model [21].

This section reviews evidence from a molecular signaling perspective about the participation of astrocytes in the genesis of experimental PD and the involved molecular cascades.

2.1. Wnt/β-Catenin Signaling Cascade. The Wnt1 (wingless-type MMTV (mouse mammary tumor virus) integration site family, member 1) pathway has emerged as an essential signaling cascade regulating differentiation, neuron survival, axonal extension, synapse formation, neurogenesis, and many other processes in developing and adult tissues [22]. Little is known on the role of Wnt agonists in the midbrain [23]. In healthy human progenitor-derived astrocytes (PDAs), β-catenin leads to modulation of genes relevant to regulating aspects of glutamate neurotransmission [24]. However, the expression of Wnt components in adult astrocytes [25, 26] and the identification of activated midbrain astrocytes as candidate components of Wnt1 signaling suggest that astrocytes may be relevant sources of Wnt1 [27]. Using the proneurotoxin MPTP- (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-) lesioned mouse model, 92 mRNA species molecular profiling in the midbrain revealed a specific, robust, and persistent increase in the expression of the canonical Wnt1 agonist, but not of Wnt3a or Wnt5a, during MPTP-induced dopaminergic degeneration [28]. The activated astrocytes rescued mesencephalic dopaminergic neurons from MPP+-induced tyrosine hydroxylase-positive (TH^+) neuron toxicity promoting dopaminergic neurogenesis through Wnt1/β-catenin signaling activation [28]. Further evidence supports that the Wnt signaling system may be reinforced following injury in the adult CNS [29]. Likewise, some studies suggest that Wnt/β-catenin activation reduces neurodegeneration in mouse models of Alzheimer's disease [30, 31].

Growing evidence endorses the critical participation of Wnt1 in PD genesis. The neuroprotective effects of the Wnt pathway could be blocked by a Wnt1 antibody [28], and also, the Wnt1-targeted interfering RNA-induced Wnt1 depletion in midbrain astrocytes resulted in a substantial decrease in TH^+ neuron survival upon serum deprivation and 6-OHDA or MPP+ treatment in neuron-astrocyte cocultures [32].

Furthermore, the Fzd-1 immunofluorescent signal largely increased in the rescued TH^+ neurons in dopaminergic DA neurons cocultured with midbrain astrocytes, oppositely to the dramatic Fzd-1 receptor downregulation observed in purified neurons, either in vitro or in vivo, following the neurotoxic insult [32].

Interestingly, exogenous activation of Wnt signaling with a specific GSK-3β (glycogen synthase kinase 3) antagonist sharply amplified astrocyte-induced DA neuroprotection in MPP+-treated astroglia-neuron cocultures. Glial inserts or Wnt1 direct addition to purified DA neurons just before

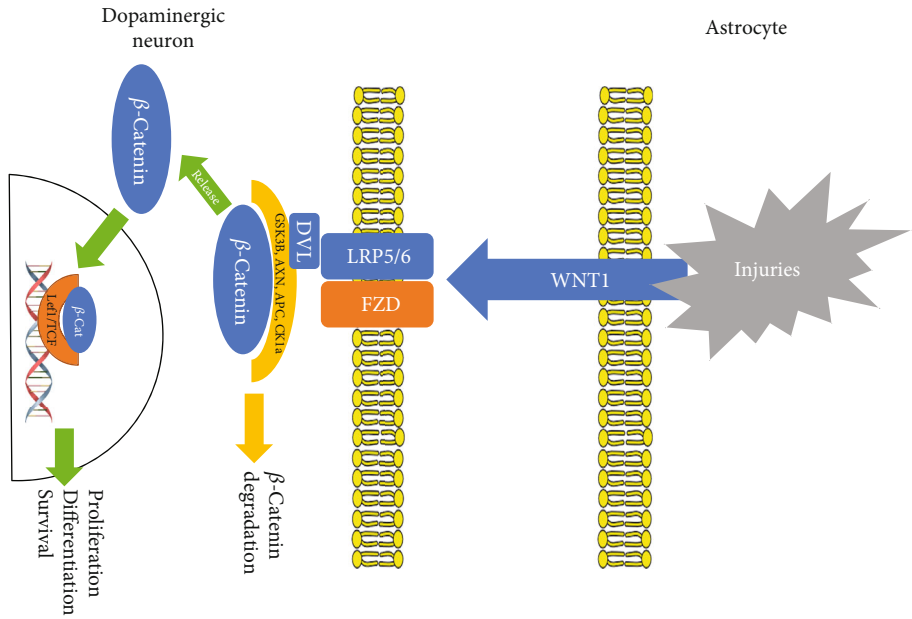

Figure 1: Wnt/β-catenin signaling cascade in Parkinson's disease. Upon insult, Wnt/β-catenin activation stimulates neurogenesis in mouse models of Parkinson's and Alzheimer's diseases. In mammalians, the signaling pathway is activated when the Wnt ligand binds to its Frizzled (FZD) receptor. The protein complex of FZD, LRP5/6, CK1, and GSK-3β marks β-catenin protein for degradation in the proteasome. Unless β-catenin undergoes degradation, it will be translocated to the nucleus to regulate the proliferation and survival of dopaminergic neurons.

MPP+ insult largely conferred neuroprotection, which was blocked by a Wnt1 antibody or the Wnt antagonist Fzd-1-cysteine-rich domain, supporting the critical role of Wnt1 in dopaminergic neuron survival [28]. Over and above, pharmacological inhibition of GSK-3β activity increased neuroblasts' population and promoted their migration towards the rostral migratory stream and the lesioned striatum in PD animal models [33]. Inhibiting GSK-3β enhanced dendritic arborization and survival of the granular neurons and stimulated neural stem cell-to-neuronal phenotype differentiation in the hippocampus of PD animal models. Figure 1 summarily illustrates the Wnt/β-catenin/Fdz-1 pathway.

2.2. Transient Receptor Potential Vanilloid 1 (TRPV1). Transient receptor potential vanilloid 1 (TRPV1), the capsaicin receptor, is involved in nociception, is highly expressed in sensory neurons [34], and may also modulate neuronal function in other brain areas [35], control motor behavior [36], and regulate neuroinflammation [37]. The TRPV1 channel is expressed in neuronal and nonneuronal cells, where it is involved in the regulation of neurotransmitter release, and postsynaptically, where it influences neurotransmitter signaling [38]. In astrocytes, TRPV1 channels are responsible for Ca^{2+} entry from the extracellular space, accounting for nearly 20% of total Ca^{2+} events occurring in hippocampal astrocytes. Besides, the TRPV1 channels have been linked to some forms of long-term potentiation of glutamatergic transmission and GABAergic transmission regulation [39, 40]. Capsaicin-mediated activation of TRPV1 on astrocytes increases CNTF endogenous synthesis in vivo, increasing dopaminergic neuron viability through activation of the CNTF receptor alpha subunit (CNTFRα) and preventing neurodegeneration after MPP+ and 6-OHDA administration in PD rat models [41, 42]. Activation of TRPV1 in a PD rat model was recently associated with a reduced expression of the TNF-α and interleukin-1β proinflammatory cytokines, the reactive oxygen species/reactive nitrogen species (ROS/RNS) generated by NADPH oxidase at the microglia, and the inducible nitric oxide synthase or reactive astrocyte-derived myeloid peroxidase [43]. The relevance of this pathway to PD is further supported by the increased TRPV1 and CNTF levels in $GFAP^+$ (glial fibrillary acidic protein-positive) astrocytes and CNTFRα on dopaminergic neurons found in PD patients [41]. The TRPV1-CNTF pathway is summarized in Figure 2.

2.3. The JWA Gene (ADP-Ribosylation-Like Factor 6 Interacting Protein 5). Oxidative damage has been considered a primary pathogenic mechanism of nigral dopaminergic neuronal cell death in PD [44]. At the molecular level, both DNA damage and abnormal activation of the known mediator of tissue damage and inflammation NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) have been implicated in oxidative damage [45]. The NF-κB protein complex exists as a cytoplasmic p50/p65 heterodimer which binds to the IκB inhibitory subunit [46]. The activation of NF-κB is mediated by the upstream IκB kinase (IKK), a heterotrimer made of 2 catalytic subunits, IKKα and IKKβ, and the NF-κB essential modulator regulatory IKKγ subunit [47]. Exposure to various stimuli like oxidative stress, proinflammatory cytokines, and growth factors induces IKK phosphorylation, leading to IκB polyubiquitination and proteasomal degradation. In turn, IκB degradation induces NF-κB translocation to the nucleus, where NF-κB binds to its cognate DNA sequences and its coactivators to ultimately regulate gene expression [48].

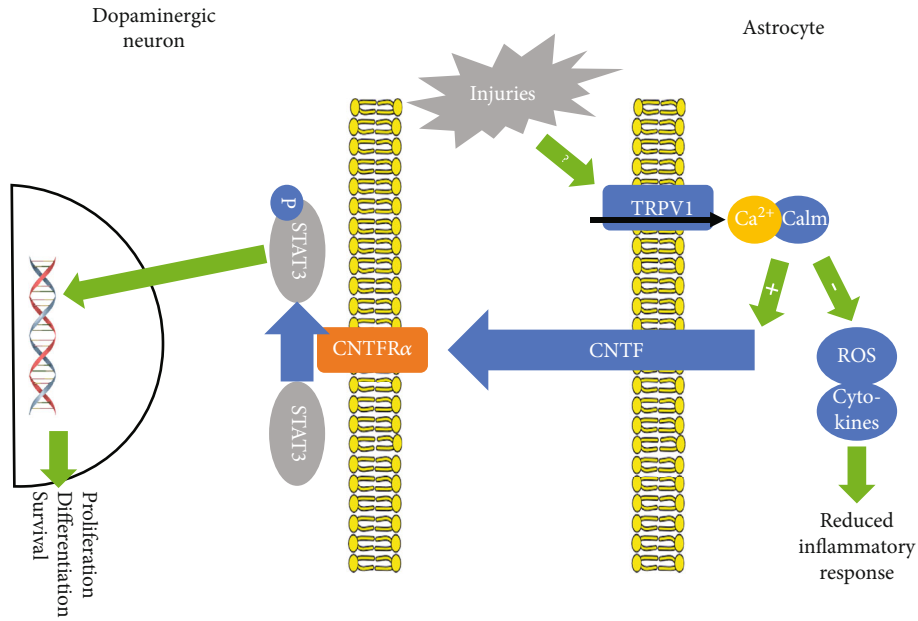

Figure 2: TRPV1-CNTF signaling cascade in PD. Capsaicin-mediated stimulation of TRPV1 through activation of CNTFRα and the STAT pathway increases dopaminergic neuron viability in PD rat models. Activation of TRPV1 has also been associated with a reduced expression of the proinflammatory cytokines and reactive oxygen species/reactive nitrogen species in a PD rat model. TRPV1: transient receptor potential vanilloid 1 channel; CNTFRα: ciliary neurotrophic factor receptor α subunit.

The ARL6ip5 (ADP-ribosylation-like factor 6 interacting protein 5) or JWA gene codes for a novel microtubule binding protein regulating cancer cell migration via MAPK cascades [49] and mediating leukemic cell differentiation [50, 51]. It is also a key regulator of base excision repair of oxidative stress-induced DNA damage by XRCC1 (X-ray repair cross-complementing 1) stability regulation [52, 53]. Miao and colleagues reported that JWA knockout (KO) astrocytes showed NF-κB pathway activation in dopaminergic neurons and neurodegeneration [54], suggesting JWA downregulation of the NF-κB signaling pathway [54]. Indeed, JWA downregulated the expression of IKKβ inhibiting NF-κB signaling pathway activation [54]. Figure 3 summarizes the JWA/NF-κB pathway.

2.4. Nrf2-ARE Pathway in Parkinson's Disease. Free radicals, regularly produced at physiological levels, are required for signaling and plasticity in the healthy brain. However, oxidative stress appears when their production exceeds the cellular antioxidant defense. High levels of free radicals are neurotoxic leading to pathological processes and cell death in time. Oxidative stress has been associated with neuronal death and involved in the pathogenesis of multiple chronic neurodegenerative diseases including Alzheimer's disease, PD, Huntington's disease, amyotrophic lateral sclerosis, and neurological illnesses [55, 56]. The nuclear factor erythroid 2-related factor 2 (Nrf2) antioxidant response element (ARE) is a key in the Nrf2 antioxidant system pathway upregulating an array of antioxidant and detoxifying enzymes. Currently, as Nrf2 is considered a possible therapeutic target for treating oxidative stress-related disorders, some studies have targeted Nrf2 to confer neuroprotection in PD [55, 56]. The Nrf2 factor counteracted PD-related neuronal cell death through the expression of cytoprotective genes with anti-inflammatory and antioxidant properties. Data from postmortem PD human brains and Nrf2 knockout mice indicate an association between Nrf2-ARE pathway dysfunction and PD pathogenesis [57]. An Nrf2 deficiency increases MPTP sensitivity and exacerbates vulnerability to 6-OHDA both in vitro and in vivo. Transplants of astrocytes overexpressing Nrf2 were protected from 6-OHDA-induced damage in the living mouse [58–61]. In postmortem brains of PD patients, p62 (nucleoporin p62) and NQO1 (NAD(P)H dehydrogenase [quinone] 1) were found partially sequestered in Lewy bodies, indicating that Nrf2 compromised neuroprotective capacity [62]. Also, Nrf2 activation by dimethyl fumarate protected the substantia nigra neurons against α-synuclein toxicity in a murine PD model, an effect not evident in Nrf2-knockout mice [62, 63]. The activation of Nrf2 upregulated brain heme oxygenase-1 (HO-1) and NQO1 and prevented MPTP-induced neuronal death in the substantia nigra [62, 63]. Likewise, Nrf2-ARE pathway activation by siRNA (small double-stranded interfering RNAs) knockdown of Keap1 (Kelch-like ECH-associated protein 1) reduced oxidative stress partially protecting from MPTP neurotoxicity [64]. Some studies suggest that Nrf2 activation in glial cells may be required to exert its protective effects in PD and PD models [60, 61]. However, glial Nrf2 nuclear translocation in the substantia nigra was not found in PD brains [65], and in vitro studies show that neuronal Nrf2 activation, even in the absence of glia, induces neuroprotection against oxidative damage triggered by parkinsonism-inducing neurotoxins [61, 66–70]. Uric acid activated the Nrf2-ARE pathway by increasing mRNA and the expression of Nrf2 and three Nrf2-responsive genes and inhibited oxidative stress in MPTP-treated mice

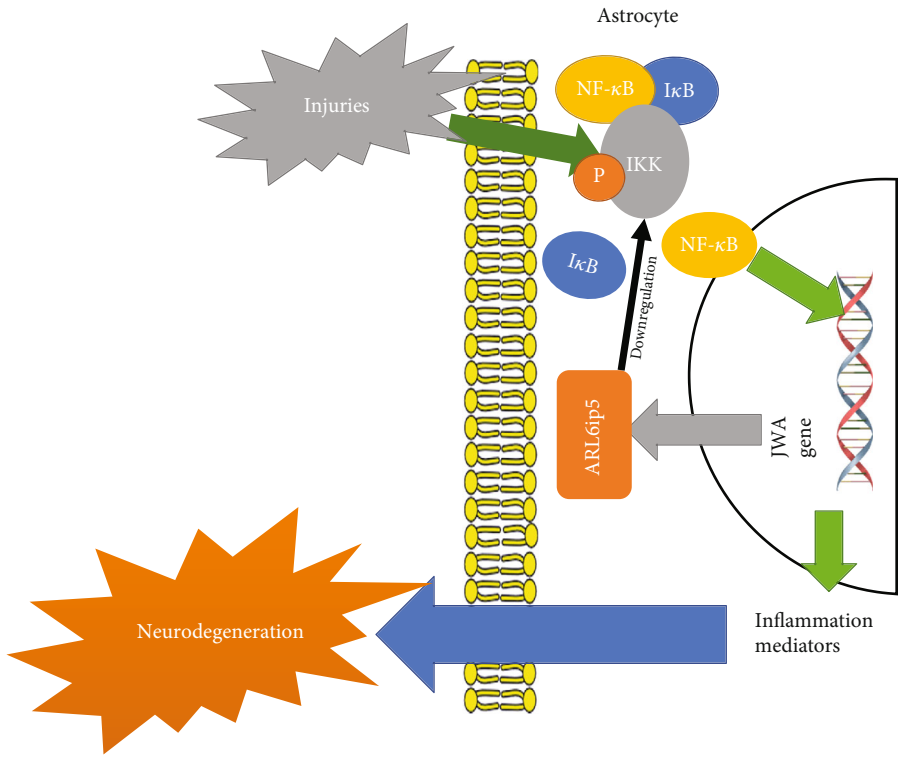

Figure 3: NF-κB/JWA/ARL6ip5 signaling pathway in PD. Both DNA damage and abnormal activation of the known mediator of tissue damage and inflammation NF-κB have been implicated in oxidative damage. The ARL6ip5 downregulates IKKβ expression inhibiting NF-κB signaling pathway activation. NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells; ARL6ip5: ADP-ribosylation-like factor 6 interacting protein 5.

improving behavioral performance and cognition. It also increased TH^+ dopaminergic neurons and decreased $GFAP^+$ astrocytes in the substantia nigra [71]. Astrocyte contribution to neuroprotection and the underlying neuroprotective mechanisms are yet to be studied. Glutathione secretion from astrocytes was increased following Nrf2-ARE activation in vitro [55]. Figure 4 summarizes the Nrf2-ARE pathway.

2.5. Other Pathways Associated with Astrocyte Dysfunction in PD. This section briefly reviews other less convincingly supported pathways as to their involvement in PD-related astrocytic dysfunction.

The toxic dopamine quinones resulting from cytosolic mismanagement of dopamine excess can perpetuate dopaminergic dysfunction in PD [72]. They can be competitively antagonized by other cysteine-rich molecules including superoxide dismutase, glutathione, and metallothioneins (MTs). They are a family of ubiquitous low-weight proteins, of which MT1 appears to be expressed in astrocytes in response to mechanical or toxic neuronal injury [43, 72, 73]. Interestingly, MT1 attenuated neurotoxin-induced neuronal death both in vivo and in vitro [72–74].

In humans, the deglycase DJ-1 protein is encoded by the PARK7 gene, whose mutation causes one of the hereditary forms of PD [75]. Interestingly, its overexpression in reactive astrocytes has been reported in sporadic cases of PD [75], suggesting a pathophysiological role in PD. Indeed, DJ-1 overexpression reduced rotenone neurotoxicity in neuron-astrocyte cocultures, whereas the opposite was found after DJ-1 deletion [75, 76]. Its exact mechanism of action in astrocytes remains elusive, some data suggesting an effect on mitochondrial function that in turn might favor the release of paracrine-acting molecules [76, 77].

The enzymatic protein thrombin plays a key role in the coagulation cascade and is upregulated upon CNS damage [78, 79]. In normal conditions, thrombin activates the protease-activated receptor (PAR) subtypes PAR-1, PAR-3, and PAR-4 [78, 79], although it may bind to PAR-2 at high concentration [79]. To date, the four known PAR subtypes are associated with G proteins and determine multiple cellular responses [78, 79].

Ishida and colleagues studied the presence of the thrombin-PAR pathway in human samples of the substantia nigra pars compacta [78]. The thrombin precursor prothrombin and the PAR-1 were observed only in astrocytes, expressed at a higher level for the latter along with a higher density of thrombin-positive vessels in PD brain specimens compared with controls. In astrocyte cultures, PAR-1 activation by thrombin increased GDNF and glutathione peroxidase expression, albeit not inflammatory molecules like IL-1b, IL-6, IL-8, and MCP-1 and the nerve growth factor level.

There is conflicting evidence on the neuroprotective potential of GDNF from astrocytic origin [80]. Pretreatment with GDNF attenuated neuronal death in dopamine-depleted corpus striatum [80–82] while the GDNF level in brain tissue from PD patients was comparable to that

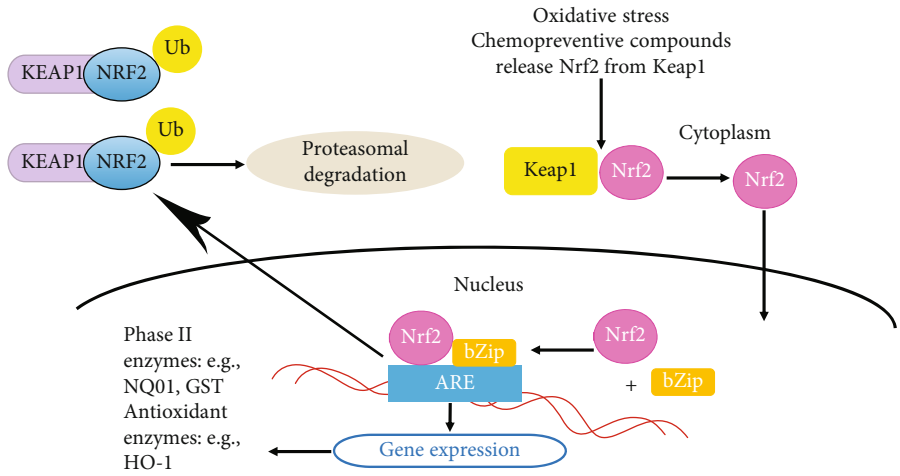

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Figure 4: The Nrf2-ARE signaling pathway in Parkinson's disease. Under unstressed conditions, oxidative molecules like ROS and RNS activate the protective antioxidant pathway, dissociating the cytosolic Nrf2/Keap1 complex. The Nrf2 factor translocates to the nucleus where associated with bZip proteins trigger the expression of several homeostatic genes with the ARE sequence in their promoters, including SOD, HO-1, GST, and NQO1. Upon inactivation, Nrf2 is sequestered by Keap1 and targeted for ubiquitination and proteasomal degradation. Nrf2: nuclear factor (erythroid-derived 2)-related factor 2; Keap1: Kelch-like ECH-associated protein 1; bZip: basic region leucine zipper (bZip) transcription factors; SOD: superoxide dismutase; HO-1: heme oxygenase-1; GST: glutathione S-transferase; NQO1: NAD(P)H: quinone oxidoreductase-1.

found in control patients and higher in the nigrostriatal dopaminergic region [83].

3. Restoring Astrocyte Function as a Preventive Strategy against Dopaminergic Neurodegeneration in Parkinson's Disease

As hereinabove discussed, astrocytic dysfunction may largely contribute to dopaminergic neurodegeneration [10, 16, 17]. The astrocytes release Wnt1 which may lengthen dopaminergic neuron survival by activating Fzd-1 receptors [84]. Addition of Wnt1 to purified DA neurons prevented MPP+ neurotoxicity [28], likely disclosing a promising neuroprotective therapy in PD and warranting clinical studies which, at present, are lacking in this regard.

Capsaicin-mediated activation of astrocytic TRPV1 is followed by CNTF release and CNTFRα activation on dopaminergic neurons whose viability increases [41]. Indeed, pretreatment with capsaicin 0.5 mg/kg largely reduced dopaminergic neurons' death and improved behavioral outcomes in MPTP-lesioned mice [43], while treatment with TRPV1 antagonists capsazepine and iodine-resiniferatoxin reversed both effects. Similar results were observed in 6-OHDA-lesioned mice [41, 42]. Capsaicin increased superoxide dismutase and catalase levels and decreased lipid peroxidation in the brain, suggesting an antioxidant effect [42].

Knocking down JWA in astrocytes has also been related to DA neurodegeneration, likely by NF-κB disinhibition [85]. Provided that NF-κB is a potent proinflammatory molecule [85], even though neuroprotection following exogenous JWA or related compounds administration has not been reported, a new experimental PD model unrelated to dopaminergic neurotoxins may stem out of the above.

Large evidence supporting astrocyte involvement in the genesis of experimental PD comes from cell culture studies sparing any interaction with the glia which is functional in the brain.

The potential neuroprotective effect of GDNF was studied by inducing its expression in astrocytes through vector transfection in 6-OHDA- and MPTP-treated rats and mice, respectively [80]. Overexpression of GDNF prevented neurotoxicity, namely, neuronal death and behavioral abnormalities, even up to 14 weeks after transfection when astrocytic activation and astrogliosis were observed in the MPTP model [80].

Silibinin or silybin is the major active constituent of the standardized extract of the milk thistle seeds known as silymarin with potential hepatoprotective and antineoplasic effects [86, 87] which showed neuroprotective effects in MPTP-treated mice [88]. Silibinin also reduced glial activation, dependent on extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) stress response kinase activation [89, 90]. Accordingly, in vitro studies demonstrated that silibinin suppressed astroglial activation inhibiting ERK and JNK phosphorylation in primary astrocytes following MPP+ treatment [88].

Loss of Nrf2-mediated transcription exacerbated vulnerability to the neurotoxin 6-hydroxydopamine (6-OHDA) in a Parkinson mice model and N27 rat dopaminergic neuronal cell line. Also, astrocytes overexpressing Nrf2 transplantation induced the Nrf2-ARE pathway protecting from 6-OHDA-induced damage in the living mouse [61]. On the other hand, Keap1 siRNA administration in striatum primary astrocytes upregulated the Nrf2-ARE pathway, protected from oxidative stress, and modestly spared from MPTP-induced dopaminergic terminal damage [64]. Uric acid also exerted a neuroprotective effect improving behavior and cognition in

Table 1: Potentially neuroprotective molecules upon astrocytic behavior modification.

Molecule	Proposed mechanism of action	Tested PD models
Capsaicin	Activation of TRPV1 in astrocytes	MPTP (mouse), 6-OHDA (rat)
GDNF (vector transfection)	GDNF overexpression in astrocytes	MPTP (mouse), 6-OHDA (rat)
Silibinin	Suppression of astrocyte activation (via ERK/JNK phosphorylation)	MPTP (mouse)

GDNF: glial cell line-derived neurotrophic factor; MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; TRPV1: transient receptor potential vanilloid 1 channel; ERK/JNK: extracellular signal-regulated kinase/c-Jun N-terminal kinase.

MPTP mice, increased TH^+ dopaminergic neurons, and decreased $GFAP^+$ astrocytes in the substantia nigra [71]. All in all, experimental evidence supports a key role for astrocytes in the Nrf2-ARE and neuroprotection. The Nrf2-ARE pathway poses as a promising therapeutic target for reducing or preventing cell death in PD.

Table 1 summarizes astrocyte-interacting drugs with possible neuroprotective effects.

4. Conclusion

Despite the fact that astrocytes, the most abundant glial cells, are critical for brain function, their role in PD was long considered a byproduct of neuroinflammation. However, the bulk of ongoing evidence suggests that astrocyte dysfunction might occupy a central position in the genesis of experimental PD [14, 15].

Three main pathways contributing to PD development involving astrocytes could be identified. Firstly, noxious stimuli increase Wnt1 synthesis in astrocytes [27, 28] modifying gene expression in DA neurons upon Fzd receptor activation and β-catenin nuclear translocation [32]. Secondly, noxious stimuli and perhaps inflammation too stimulate astrocytic TRPV1 reducing oxidative species generation, releasing CNTF [41, 42], modifying gene expression, and improving dopaminergic neuron survival and viability [41]. Last but not least, the JWA gene induces astrocytic ARL6ip5 synthesis, which inhibits IKKβ lowering the level of the active NF-κB level [54], a potent inductor of inflammatory responses. The relevance of other pathways involving metallothioneins, DJ-1 protein, thrombin, and GDNF is less clear, though might turn out as equally important.

The pursuit of neuroprotective strategies in PD is a top priority as once and again negative results have been obtained so far [91]. The pathways herein discussed disclose interesting targets to be explored in this regard. Certain molecules like capsaicin [43] and silibinin [88] have shown unquestionably interesting effects in rodent PD models. They are naturally found in chili peppers and cardum, respectively; they have sometimes been used for therapeutic purposes. Needless to say that before clinical trials in PD may be envisaged, studies in primate PD models are needed. Results are hitherto encouraging, and more data are hopefully coming forth in the near future. Overexpression of GDNF by vector transfection has also shown some efficacy in rodent models [80] contrasting with the lack of clinical benefit after intraputaminal or intracerebroventricular infusions of GDNF in PD patients [92, 93]. Nevertheless, an eventual benefit from GDNF infusion might be limited by its reach to and bioavailability at the site of interest, making drug delivery a crucial aspect of GDNF therapy worth exploring.

Knocking out JWA increased NF-κB activity in DA neurons [54] presumably depicting a new PD model, eventually surpassing the limitations of neurotoxin PD models which do not accurately reproduce full PD pathophysiology [94]. The JWA knockout mouse developed a PD-like phenotype with selective loss of dopaminergic neurons in the substantia nigra pars compacta and monoaminergic neurotransmitter level in the corpus striatum [85]. Constitutive expression of NF-κB, a known promoter of inflammatory responses, participates in neurogenesis, neuritogenesis, and plasticity while inducible NF-κB expression leads to glial proinflammatory responses, neuronal proapoptotic responses and death, vascular inflammation, and increased endothelial permeability [95]. Inducing experimental inflammation, a PD hallmark [96], might advantageously reproduce the whole spectrum of the disease bearing other brain areas compromised. Further research is warranted to fully characterize this plausible new model.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

References

[1] K. R. Chaudhuri and A. H. Schapira, "Non-motor symptoms of Parkinson's disease: dopaminergic pathophysiology and treatment," *The Lancet Neurology*, vol. 8, no. 5, pp. 464–474, 2009.

[2] O. Hornykiewicz, "Dopamine (3-hydroxytyramine) and brain function," *Pharmacological Reviews*, vol. 18, no. 2, pp. 925–964, 1966.

[3] S. Fahn, "The history of dopamine and levodopa in the treatment of Parkinson's disease," *Movement Disorders*, vol. 23, Supplement 3, pp. S497–S508, 2008.

[4] C. Soto, "Unfolding the role of protein misfolding in neurodegenerative diseases," *Nature Reviews. Neuroscience*, vol. 4, no. 1, pp. 49–60, 2003.

[5] M. G. Spillantini, M. L. Schmidt, V. M. Y. Lee, J. Q. Trojanowski, R. Jakes, and M. Goedert, "Alpha-synuclein in Lewy bodies," *Nature*, vol. 388, no. 6645, pp. 839-840, 1997.

[6] A. A. Cooper, A. D. Gitler, A. Cashikar et al., "α-Synuclein blocks ER-Golgi traffic and Rab1 rescues neuron loss in Parkinson's models," *Science*, vol. 313, no. 5785, pp. 324–328, 2006.

[7] D. Ron and P. Walter, "Signal integration in the endoplasmic reticulum unfolded protein response," *Nature Reviews. Molecular Cell Biology*, vol. 8, no. 7, pp. 519–529, 2007.

[8] G. Mercado, P. Valdes, and C. Hetz, "An ERcentric view of Parkinson's disease," *Trends in Molecular Medicine*, vol. 19, no. 3, pp. 165–175, 2013.

[9] C. Hetz and B. Mollereau, "Disturbance of endoplasmic reticulum proteostasis in neurodegenerative diseases," *Nature Reviews. Neuroscience*, vol. 15, no. 4, pp. 233–249, 2014.

[10] H. D. E. Booth, W. D. Hirst, and R. Wade-Martins, "The role of astrocyte dysfunction in Parkinson's disease pathogenesis," *Trends in Neurosciences*, vol. 40, no. 6, pp. 358–370, 2017.

[11] Z. A. Sorrentino, B. I. Giasson, and P. Chakrabarty, "α-Synuclein and astrocytes: tracing the pathways from homeostasis to neurodegeneration in Lewy body disease," *Acta Neuropathologica*, vol. 138, no. 1, pp. 1–21, 2019.

[12] S. Herculano-Houzel, "The human brain in numbers: a linearly scaled-up primate brain," *Frontiers in Human Neuroscience*, vol. 3, p. 31, 2009.

[13] A. Verkhratsky, M. V. Sofroniew, A. Messing et al., "Neurological diseases as primary gliopathies: a reassessment of neurocentrism," *ASN Neuro*, vol. 4, no. 3, 2012.

[14] M. S. Beausoleil, E. B. Schulze, D. Goodale, C. O. Postenka, and A. L. Allan, "Deletion of the thrombin cleavage domain of osteopontin mediates breast cancer cell adhesion, proteolytic activity, tumorgenicity, and metastasis," *BMC Cancer*, vol. 11, no. 1, p. 25, 2011.

[15] P. M. Rappold and K. Tieu, "Astrocytes and therapeutics for Parkinson's disease," *Neurotherapeutics*, vol. 7, no. 4, pp. 413–423, 2010.

[16] T. M. Dawson, H. S. Ko, and V. L. Dawson, "Genetic animal models of Parkinson's disease," *Neuron*, vol. 66, no. 5, pp. 646–661, 2010.

[17] J. B. Koprich, C. Reske-Nielsen, P. Mithal, and O. Isacson, "Neuroinflammation mediated by IL-1β increases susceptibility of dopamine neurons to degeneration in an animal model of Parkinson's disease," *Journal of Neuroinflammation*, vol. 5, no. 1, p. 8, 2008.

[18] L. Aron and R. Klein, "Repairing the parkinsonian brain with neurotrophic factors," *Trends in Neurosciences*, vol. 34, no. 2, pp. 88–100, 2011.

[19] Y. Shen, A. Sun, Y. Wang et al., "Upregulation of mesencephalic astrocyte-derived neurotrophic factor in glial cells is associated with ischemia-induced glial activation," *Journal of Neuroinflammation*, vol. 9, no. 1, p. 254, 2012.

[20] M. J. McGregor, M. Cohen, C. R. Stocks-Rankin et al., "Complaints in for-profit, non-profit and public nursing homes in two Canadian provinces," *Open Medicine*, vol. 5, no. 4, pp. e183–e192, 2011.

[21] S. J. Chinta, G. Woods, M. Demaria et al., "Cellular senescence is induced by the environmental neurotoxin paraquat and contributes to neuropathology linked to Parkinson's disease," *Cell Reports*, vol. 22, no. 4, pp. 930–940, 2018.

[22] V. Dounin, A. Constantinof, H. Schulze, T. T. Bachmann, and K. Kerman, "Electrochemical detection of interaction between Thioflavin T and acetylcholinesterase," *Analyst*, vol. 136, no. 6, pp. 1234–1238, 2011.

[23] R. de la Fuente-Fernández, M. Schulzer, L. Kuramoto et al., "Age-specific progression of nigrostriatal dysfunction in Parkinson's disease," *Annals of Neurology*, vol. 69, no. 5, pp. 803–810, 2011.

[24] S. D. Narasipura, L. J. Henderson, S. W. Fu, L. Chen, F. Kashanchi, and L. al-Harthi, "Role of β-catenin and TCF/LEF family members in transcriptional activity of HIV in astrocytes," *Journal of Virology*, vol. 86, no. 4, pp. 1911–1921, 2012.

[25] J. D. Cahoy, B. Emery, A. Kaushal et al., "A transcriptome database for astrocytes, neurons, and oligodendrocytes: a new resource for understanding brain development and function," *The Journal of Neuroscience*, vol. 28, no. 1, pp. 264–278, 2008.

[26] T. Kuwabara, J. Hsieh, A. Muotri et al., "Wnt-mediated activation of NeuroD1 and retro-elements during adult neurogenesis," *Nature Neuroscience*, vol. 12, no. 9, pp. 1097–1105, 2009.

[27] E. M. Toledo and N. C. Inestrosa, "Activation of Wnt signaling by lithium and rosiglitazone reduced spatial memory impairment and neurodegeneration in brains of an APPswe/PSEN1ΔE9 mouse model of Alzheimer's disease," *Molecular Psychiatry*, vol. 15, no. 3, pp. 272–285, 2010.

[28] B. Marchetti, "Wnt/β-catenin signaling pathway governs a full program for dopaminergic neuron survival, neurorescue and regeneration in the MPTP mouse model of Parkinson's disease," *International Journal of Molecular Sciences*, vol. 19, no. 12, article 3743, 2018.

[29] F. Osakada, S. Ooto, T. Akagi, M. Mandai, A. Akaike, and M. Takahashi, "Wnt signaling promotes regeneration in the retina of adult mammals," *The Journal of Neuroscience*, vol. 27, no. 15, pp. 4210–4219, 2007.

[30] H. Faghfoury, J. Baruteau, H. Ogier de Baulny, J. Häberle, and A. Schulze, "Transient fulminant liver failure as an initial presentation in citrullinemia type I," *Molecular Genetics and Metabolism*, vol. 102, no. 4, pp. 413–417, 2011.

[31] H. G. Schulze, R. B. Foist, K. Okuda, A. Ivanov, and R. F. B. Turner, "A model-free, fully automated baseline-removal method for Raman spectra," *Applied Spectroscopy*, vol. 65, no. 1, pp. 75–84, 2011.

[32] F. L'Episcopo, M. F. Serapide, C. Tirolo et al., "A *Wnt1* regulated *Frizzled-1*/*β-catenin* signaling pathway as a candidate regulatory circuit controlling mesencephalic dopaminergic neuron-astrocyte crosstalk: therapeutical relevance for neuron survival and neuroprotection," *Molecular Neurodegeneration*, vol. 6, no. 1, p. 49, 2011.

[33] S. Singh, A. Mishra, S. Bharti et al., "Glycogen synthase kinase-3β regulates equilibrium between neurogenesis and gliogenesis in rat model of Parkinson's disease: a crosstalk with Wnt and notch signaling," *Molecular Neurobiology*, vol. 55, no. 8, pp. 6500–6517, 2018.

[34] M. J. Gunthorpe and A. Szallasi, "Peripheral TRPV1 receptors as targets for drug development: new molecules and mechanisms," *Current Pharmaceutical Design*, vol. 14, no. 1, pp. 32–41, 2008.

[35] J. A. Kauer and H. E. Gibson, "Hot flash: TRPV channels in the brain," *Trends in Neurosciences*, vol. 32, no. 4, pp. 215–224, 2009.

[36] R. Gonzalez-Aparicio and R. Moratalla, "Oleoylethanolamide reduces L-DOPA-induced dyskinesia via TRPV1 receptor in a mouse model of Parkinson s disease," *Neurobiology of Disease*, vol. 62, pp. 416–425, 2014.

[37] E. S. Park, S. R. Kim, and B. K. Jin, "Transient receptor potential vanilloid subtype 1 contributes to mesencephalic dopaminergic neuronal survival by inhibiting microglia-originated oxidative stress," *Brain Research Bulletin*, vol. 89, no. 3-4, pp. 92–96, 2012.

[38] N. Stella, "Cannabinoid and cannabinoid-like receptors in microglia, astrocytes, and astrocytomas," *Glia*, vol. 58, no. 9, pp. 1017–1030, 2010.

[39] E. Shigetomi, O. Jackson-Weaver, R. T. Huckstepp, T. J. O'Dell, and B. S. Khakh, "TRPA1 channels are regulators of astrocyte basal calcium levels and long-term potentiation via constitutive D-serine release," *The Journal of Neuroscience*, vol. 33, no. 24, pp. 10143–10153, 2013.

[40] E. Shigetomi, X. Tong, K. Y. Kwan, D. P. Corey, and B. S. Khakh, "TRPA1 channels regulate astrocyte resting calcium and inhibitory synapse efficacy through GAT-3," *Nature Neuroscience*, vol. 15, no. 1, pp. 70–80, 2012.

[41] J. H. Nam, E. S. Park, S. Y. Won et al., "TRPV1 on astrocytes rescues nigral dopamine neurons in Parkinson's disease via CNTF," *Brain*, vol. 138, no. 12, pp. 3610–3622, 2015.

[42] Z. Zhao, J. F. Wang, L. L. Wang et al., "Capsaicin protects against oxidative insults and alleviates behavioral deficits in rats with 6-OHDA-induced Parkinson's disease via activation of TRPV1," *Neurochemical Research*, vol. 42, no. 12, pp. 3431–3438, 2017.

[43] Y. C. Chung, J. Y. Baek, S. R. Kim et al., "Capsaicin prevents degeneration of dopamine neurons by inhibiting glial activation and oxidative stress in the MPTP model of Parkinson's disease," *Experimental & Molecular Medicine*, vol. 49, no. 3, article e298, 2017.

[44] M. S. Yoo, H. S. Chun, J. J. Son et al., "Oxidative stress regulated genes in nigral dopaminergic neuronal cells: correlation with the known pathology in Parkinson's disease," *Brain Research. Molecular Brain Research*, vol. 110, no. 1, pp. 76–84, 2003.

[45] H. Xu, H. Jiang, J. Wang, and J. Xie, "Rg1 protects the MPP +-treated MES23.5 cells via attenuating DMT1 up-regulation and cellular iron uptake," *Neuropharmacology*, vol. 58, no. 2, pp. 488–494, 2010.

[46] S. Mitchell, J. Vargas, and A. Hoffmann, "Signaling via the NFκB system," *Wiley Interdisciplinary Reviews: Systems Biology and Medicine*, vol. 8, no. 3, pp. 227–241, 2016.

[47] M. S. Hayden, A. P. West, and S. Ghosh, "NF-κB and the immune response," *Oncogene*, vol. 25, no. 51, pp. 6758–6780, 2006.

[48] J. S. Tilstra, A. R. Robinson, J. Wang et al., "NF-κB inhibition delays DNA damage–induced senescence and aging in mice," *The Journal of Clinical Investigation*, vol. 122, no. 7, pp. 2601–2612, 2012.

[49] H. Chen, J. Bai, J. Ye et al., "JWA as a functional molecule to regulate cancer cells migration via MAPK cascades and F-actin cytoskeleton," *Cellular Signalling*, vol. 19, no. 6, pp. 1315–1327, 2007.

[50] S. Huang, Q. Shen, W. G. Mao et al., "JWA, a novel signaling molecule, involved in the induction of differentiation of human myeloid leukemia cells," *Biochemical and Biophysical Research Communications*, vol. 341, no. 2, pp. 440–450, 2006.

[51] S. Huang, Q. Shen, W. G. Mao et al., "JWA, a novel signaling molecule, involved in all-trans retinoic acid induced differentiation of HL-60 cells," *Journal of Biomedical Science*, vol. 13, no. 3, pp. 357–371, 2006.

[52] R. Chen, W. Qiu, Z. Liu et al., "Identification of *JWA* as a novel functional gene responsive to environmental oxidative stress induced by benzo[*a*]pyrene and hydrogen peroxide," *Free Radical Biology & Medicine*, vol. 42, no. 11, pp. 1704–1714, 2007.

[53] S. Wang, Z. Gong, R. Chen et al., "JWA regulates XRCC1 and functions as a novel base excision repair protein in oxidative-stress-induced DNA single-strand breaks," *Nucleic Acids Research*, vol. 37, no. 6, pp. 1936–1950, 2009.

[54] S. H. Miao, H. B. Sun, Y. Ye et al., "Astrocytic JWA expression is essential to dopaminergic neuron survival in the pathogenesis of Parkinson's disease," *CNS Neuroscience & Therapeutics*, vol. 20, no. 8, pp. 754–762, 2014.

[55] J. A. Johnson, D. A. Johnson, A. D. Kraft et al., "The Nrf2-ARE pathway: an indicator and modulator of oxidative stress in neurodegeneration," *Annals of the New York Academy of Sciences*, vol. 1147, no. 1, pp. 61–69, 2008.

[56] A. R. Vasconcelos, N. B. dos Santos, C. Scavone, and C. D. Munhoz, "Nrf2/ARE pathway modulation by dietary energy regulation in neurological disorders," *Frontiers in Pharmacology*, vol. 10, p. 33, 2019.

[57] K. U. Tufekci, E. Civi Bayin, S. Genc, and K. Genc, "The Nrf2/ARE pathway: a promising target to counteract mitochondrial dysfunction in Parkinson's disease," *Parkinson's Disease*, vol. 2011, article 314082, 14 pages, 2011.

[58] N. C. Burton, T. W. Kensler, and T. R. Guilarte, "In vivo modulation of the Parkinsonian phenotype by Nrf2," *Neurotoxicology*, vol. 27, no. 6, pp. 1094–1100, 2006.

[59] C. A. Colton, "Heterogeneity of microglial activation in the innate immune response in the brain," *Journal of Neuroimmune Pharmacology*, vol. 4, no. 4, pp. 399–418, 2009.

[60] P. C. Chen, M. R. Vargas, A. K. Pani et al., "Nrf2-mediated neuroprotection in the MPTP mouse model of Parkinson's disease: critical role for the astrocyte," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 8, pp. 2933–2938, 2009.

[61] R. J. Jakel, J. A. Townsend, A. D. Kraft, and J. A. Johnson, "Nrf2-mediated protection against 6-hydroxydopamine," *Brain Research*, vol. 1144, pp. 192–201, 2007.

[62] I. Lastres-Becker, A. J. García-Yagüe, R. H. Scannevin et al., "Repurposing the NRF2 activator dimethyl fumarate as therapy against synucleinopathy in Parkinson's disease," *Antioxidants & Redox Signaling*, vol. 25, no. 2, pp. 61–77, 2016.

[63] A. Jazwa, A. I. Rojo, N. G. Innamorato, M. Hesse, J. Fernández-Ruiz, and A. Cuadrado, "Pharmacological targeting of the transcription factor Nrf2 at the basal ganglia provides disease modifying therapy for experimental parkinsonism," *Antioxidants & Redox Signaling*, vol. 14, no. 12, pp. 2347–2360, 2011.

[64] T. P. Williamson, D. A. Johnson, and J. A. Johnson, "Activation of the Nrf2-ARE pathway by siRNA knockdown of Keap1 reduces oxidative stress and provides partial protection from MPTP-mediated neurotoxicity," *Neurotoxicology*, vol. 33, no. 3, pp. 272–279, 2012.

[65] C. P. Ramsey, C. A. Glass, M. B. Montgomery et al., "Expression of Nrf2 in neurodegenerative diseases," *Journal of Neuropathology and Experimental Neurology*, vol. 66, no. 1, pp. 75–85, 2007.

[66] Y. P. Hwang and H. G. Jeong, "The coffee diterpene kahweol induces heme oxygenase-1 via the PI3K and p38/Nrf2 pathway to protect human dopaminergic neurons from 6-hydroxydopamine-derived oxidative stress," *FEBS Letters*, vol. 582, no. 17, pp. 2655–2662, 2008.

[67] E. L. MacKenzie, P. D. Ray, and Y. Tsuji, "Role and regulation of ferritin H in rotenone-mediated mitochondrial oxidative stress," *Free Radical Biology & Medicine*, vol. 44, no. 9, pp. 1762–1771, 2008.

[68] M. Niso-Santano, R. A. González-Polo, J. M. Bravo-San Pedro et al., "Activation of apoptosis signal-regulating kinase 1 is a

key factor in paraquat-induced cell death: modulation by the Nrf2/Trx axis," *Free Radical Biology & Medicine*, vol. 48, no. 10, pp. 1370–1381, 2010.

[69] T. Satoh, N. Harada, T. Hosoya, K. Tohyama, M. Yamamoto, and K. Itoh, "Keap1/Nrf2 system regulates neuronal survival as revealed through study of keap1 gene-knockout mice," *Biochemical and Biophysical Research Communications*, vol. 380, no. 2, pp. 298–302, 2009.

[70] C. J. Wruck, M. Claussen, G. Fuhrmann et al., "Luteolin protects rat PC 12 and C6 cells against MPP^+ induced toxicity via an ERK dependent Keapl-Nrf2-ARE pathway," in *Neuropsychiatric Disorders An Integrative Approach*, M. Gerlach, J. Deckert, K. Double, and E. Koutsilieri, Eds., vol. 72 of Journal of Neural Transmission. Supplementa, pp. 57–67, Springer, Vienna, 2007.

[71] T. T. Huang, D. L. Hao, B. N. Wu, L. L. Mao, and J. Zhang, "Uric acid demonstrates neuroprotective effect on Parkinson's disease mice through Nrf2-ARE signaling pathway," *Biochemical and Biophysical Research Communications*, vol. 493, no. 4, pp. 1443–1449, 2017.

[72] I. Miyazaki, M. Asanuma, Y. Kikkawa et al., "Astrocyte-derived metallothionein protects dopaminergic neurons from dopamine quinone toxicity," *Glia*, vol. 59, no. 3, pp. 435–451, 2011.

[73] I. Miyazaki, M. Asanuma, S. Murakami et al., "Targeting 5-HT_{1A} receptors in astrocytes to protect dopaminergic neurons in Parkinsonian models," *Neurobiology of Disease*, vol. 59, pp. 244–256, 2013.

[74] A. Y. Shih, D. A. Johnson, G. Wong et al., "Coordinate regulation of glutathione biosynthesis and release by Nrf2-expressing glia potently protects neurons from oxidative stress," *The Journal of Neuroscience*, vol. 23, no. 8, pp. 3394–3406, 2003.

[75] S. J. Mullett and D. A. Hinkle, "DJ-1 deficiency in astrocytes selectively enhances mitochondrial complex I inhibitor-induced neurotoxicity," *Journal of Neurochemistry*, vol. 117, no. 3, pp. 375–387, 2011.

[76] S. J. Mullett and D. A. Hinkle, "DJ-1 knock-down in astrocytes impairs astrocyte-mediated neuroprotection against rotenone," *Neurobiology of Disease*, vol. 33, no. 1, pp. 28–36, 2009.

[77] N. J. Larsen, G. Ambrosi, S. J. Mullett, S. B. Berman, and D. A. Hinkle, "DJ-1 knock-down impairs astrocyte mitochondrial function," *Neuroscience*, vol. 196, pp. 251–264, 2011.

[78] Y. Ishida, A. Nagai, S. Kobayashi, and S. U. Kim, "Upregulation of protease-activated receptor-1 in astrocytes in Parkinson disease: astrocyte-mediated neuroprotection through increased levels of glutathione peroxidase," *Journal of Neuropathology and Experimental Neurology*, vol. 65, no. 1, pp. 66–77, 2006.

[79] B. M. M. Kremers, H. ten Cate, and H. M. H. Spronk, "Pleiotropic effects of the hemostatic system," *Journal of Thrombosis and Haemostasis*, vol. 16, no. 8, pp. 1464–1473, 2018.

[80] A. Drinkut, Y. Tereshchenko, J. B. Schulz, M. Bähr, and S. Kügler, "Efficient gene therapy for Parkinson's disease using astrocytes as hosts for localized neurotrophic factor delivery," *Molecular Therapy*, vol. 20, no. 3, pp. 534–543, 2012.

[81] Y. M. Ding, J. D. Jaumotte, A. P. Signore, and M. J. Zigmond, "Effects of 6-hydroxydopamine on primary cultures of substantia nigra: specific damage to dopamine neurons and the impact of glial cell line-derived neurotrophic factor," *Journal of Neurochemistry*, vol. 89, no. 3, pp. 776–787, 2004.

[82] T. Nakagawa and J. P. Schwartz, "Gene expression profiles of reactive astrocytes in dopamine-depleted striatum," *Brain Pathology*, vol. 14, no. 3, pp. 275–280, 2004.

[83] M. Mogi, A. Togari, T. Kondo et al., "Glial cell line-derived neurotrophic factor in the substantia nigra from control and parkinsonian brains," *Neuroscience Letters*, vol. 300, no. 3, pp. 179–181, 2001.

[84] F. L'Episcopo, C. Tirolo, N. Testa et al., "Reactive astrocytes and Wnt/β-catenin signaling link nigrostriatal injury to repair in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine model of Parkinson's disease," *Neurobiology of Disease*, vol. 41, no. 2, pp. 508–527, 2011.

[85] R. Wang, X. Zhao, J. Xu et al., "Astrocytic JWA deletion exacerbates dopaminergic neurodegeneration by decreasing glutamate transporters in mice," *Cell Death & Disease*, vol. 9, no. 3, article 352, 2018.

[86] S. S. Raza, M. M. Khan, M. Ashafaq et al., "Silymarin protects neurons from oxidative stress associated damages in focal cerebral ischemia: a behavioral, biochemical and immunohistological study in Wistar rats," *Journal of the Neurological Sciences*, vol. 309, no. 1-2, pp. 45–54, 2011.

[87] P. Yaghmaei, K. Azarfar, M. Dezfulian, and A. Ebrahim-Habibi, "Silymarin effect on amyloid-β plaque accumulation and gene expression of APP in an Alzheimer's disease rat model," *Daru Journal of Pharmaceutical Sciences*, vol. 22, no. 1, p. 24, 2014.

[88] Y. Lee, H. J. Chun, K. M. Lee, Y. S. Jung, and J. Lee, "Silibinin suppresses astroglial activation in a mouse model of acute Parkinson's disease by modulating the ERK and JNK signaling pathways," *Brain Research*, vol. 1627, pp. 233–242, 2015.

[89] T. G. Son, H. R. Park, S. J. Kim et al., "Senescence marker protein 30 is up-regulated in kainate-induced hippocampal damage through ERK-mediated astrocytosis," *Journal of Neuroscience Research*, vol. 87, no. 13, pp. 2890–2897, 2009.

[90] G. P. McLennan, A. Kiss, M. Miyatake et al., "Kappa opioids promote the proliferation of astrocytes via Gbetagamma and beta-arrestin 2-dependent MAPK-mediated pathways," *Journal of Neurochemistry*, vol. 107, no. 6, pp. 1753–1765, 2008.

[91] A. E. Lang and A. J. Espay, "Disease modification in Parkinson's disease: current approaches, challenges, and future considerations," *Movement Disorders*, vol. 33, no. 5, pp. 660–677, 2018.

[92] A. E. Lang, S. Gill, N. K. Patel et al., "Randomized controlled trial of intraputamenal glial cell line-derived neurotrophic factor infusion in Parkinson disease," *Annals of Neurology*, vol. 59, no. 3, pp. 459–466, 2006.

[93] J. G. Nutt, K. J. Burchiel, C. L. Comella et al., "Randomized, double-blind trial of glial cell line-derived neurotrophic factor (GDNF) in PD," *Neurology*, vol. 60, no. 1, pp. 69–73, 2003.

[94] V. Jackson-Lewis, J. Blesa, and S. Przedborski, "Animal models of Parkinson's disease," *Parkinsonism & Related Disorders*, vol. 18, Supplement 1, pp. S183–S185, 2012.

[95] R. H. Shih, C. Y. Wang, and C. M. Yang, "NF-kappaB signaling pathways in neurological inflammation: a mini review," *Frontiers in Molecular Neuroscience*, vol. 8, p. 77, 2015.

[96] Q. Wang, Y. Liu, and J. Zhou, "Neuroinflammation in Parkinson's disease and its potential as therapeutic target," *Translational Neurodegeneration*, vol. 4, no. 1, p. 19, 2015.

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Review Article

GSK-3β at the Intersection of Neuronal Plasticity and Neurodegeneration

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In neurons, Glycogen Synthase Kinase-3β (GSK-3β) has been shown to regulate various critical processes underlying structural and functional synaptic plasticity. Mouse models with neuron-selective expression or deletion of GSK-3β present behavioral and cognitive abnormalities, positioning this protein kinase as a key signaling molecule in normal brain functioning. Furthermore, mouse models with defective GSK-3β activity display distinct structural and behavioral abnormalities, which model some aspects of different neurological and neuropsychiatric disorders. Equalizing GSK-3β activity in these mouse models by genetic or pharmacological interventions is able to rescue some of these abnormalities. Thus, GSK-3β is a relevant therapeutic target for the treatment of many brain disorders. Here, we provide an overview of how GSK-3β is regulated in physiological synaptic plasticity and how aberrant GSK-3β activity contributes to the development of dysfunctional synaptic plasticity in neuropsychiatric and neurodegenerative disorders.

1. Neuronal Plasticity

Neural plasticity is an ability of the brain to adapt in response to normal developmental processes, experience, or injury. It covers such modifications in the brain structures as growth of new neurons, the formation of new networks, and change within existing networks, that is, changes in synaptic strengths, resulting in modifications in function and behavior.

2. Synaptic Plasticity

Reversible modification of synaptic strength underlies synaptic plasticity and is activity dependent. Synaptic strength can either be enhanced in a process of long-term potentiation (LTP) or depressed in long-term depression (LTD), and it affects both pre- and postsynaptic sides. LTP is triggered by the intense activation of the NMDA receptor producing a signaling cascade that causes the recruitment of AMPA receptors into the postsynaptic membrane, whereas LTD is triggered by weaker and prolonged activation of NMDA receptors leading to the removal of postsynaptic AMPA receptors [1]. Majority of the excitatory synapses are located on dendritic spines, and their growth following LTP and elimination following LTD are two opposite facts accompanying the bidirectional plasticity of excitatory transmission. Formation of new spines, as well as their morphological modifications in the adult brain, constitutes the structural bases of neuronal plasticity. The dynamic changes of dendritic spine morphology reflect changes in synaptic strength according to its use or disuse. It should be noted, however, that other forms of synaptic plasticity exist which add to the complexity of glutamatergic synapses [2].

On the other hand, inhibitory synaptic transmission driven by the interaction of GABA and ionotropic GABAA receptors constitutes a major form of inhibitory synaptic transmission. Loss of synaptic stability caused by improper excitatory/inhibitory balance and trafficking of synaptic receptors as well as abnormal density and morphology of dendritic spines may lead to the disruption of neuronal

circuits resulting in neuropsychiatric disorders. The underlying mechanisms remain to be elucidated, but they depend essentially on kinase-dependent signaling pathways [3, 4].

3. Glycogen Synthase Kinase-3

Glycogen Synthase Kinase-3 (GSK-3) is a serine/threonine protein kinase that was first discovered for its role in glycogen synthesis [5]. Later on, extensive studies have implicated GSK-3 in the regulation of many critical cellular processes with over 40 different proteins identified as phosphorylation targets for GSK-3 [6].

GSK-3 exists as two isozymes, GSK-3 alpha (α) and GSK-3 beta (β), both of which are encoded by distinct genes [7]. They split from the common ancestor at the emergence of vertebrates, while birds lost GSK-3α in the evolution [8]. GSK-3α and β share 85% amino acid sequence similarity, including 98% sequence identity within their catalytic domains [7]. Despite their structural similarity, GSK-3α and GSK-3β are not functionally identical because the beta isozyme is indispensable in development [9, 10]. In mammals, both GSK-3 isozymes are ubiquitously expressed in all tissues [7], but they are most abundant in the adult brain where they are crucial for its function [11].

GSK-3 is unique among other kinases because it is constitutively active in quiescence cells under resting conditions [12, 13]. The extracellular signals such as growth factors, neurotransmitters and hormones initiate signaling pathways, which cause the reduction of GSK-3 enzymatic activity by dynamic serine phosphorylation of GSK-3. This inhibitory regulation is achieved by a rapid and reversible N-terminal phosphorylation of Ser21 for GSK-3α and Ser9 for GSK-3β, which creates a pseudosubstrate that binds to the GSK-3 catalytic domain and prevents access of substrates to the GSK-3 active site [12, 14–17].

Phosphorylation and thus inhibition of GSK-3α/β is carried out by multiple kinases, including Akt/PKB and protein kinases A (PKA) and C (PKC) [6]. In contrast, the dephosphorylation of the N-terminal serine residue by the serine/threonine protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A) results in the activation of GSK-3 [6, 13, 15, 16].

In contrast, the positive regulation of GSK-3 is achieved by tyrosine phosphorylation: Tyr279 in GSK-3α and Tyr216 in GSK-3β. Tyrosine phosphorylation in GSK-3 occurs cotranslationally by autophosphorylation or is executed by different tyrosine kinases [18–21].

In the mouse brain, GSK-3β exists as three phosphoisotopes: double phosphorylation at Ser9 and Tyr216, single phosphorylation at Tyr216, and the nonphosphorylated isotype, the active form, i.e., phosphorylated at Tyr216 with little Ser9 phosphorylation predominating [22]. In neurons, changes in membrane electrical potential or insulin-like growth factor (IGF) treatment affect GSK-3β activity by dynamic PI3K/Akt-mediated phosphorylation and PP2A/PP2B-mediated dephosphorylation of Ser9 [23], while phospho-Tyr216 level remains unchanged [22].

Two independently regulated pools of GSK-3 exist in the cell: the Wnt signaling pathway (Figure 1(a)) and the PI3K/Akt signaling pathway (Figure 1(b)). In the Wnt signaling pathway, in the absence of extracellular Wnt ligands or the presence of Wnt negative modulators such as extracellular protein Dickkopf-1 (DKK1), the transcriptional coactivator β-catenin is phosphorylated by GSK-3 in a complex composed of the tumor suppressor adenomatous polyposis coli (APC) and the scaffolding protein Axin. Subsequently, phosphorylated β-catenin is targeted for proteasome-dependent degradation. In the presence of extracellularly secreted Wnt proteins, Frizzled receptor and the low-density lipoprotein-related protein 5 and 6 (LRP5/6) receptors are activated [24]. This event leads to the recruitment of Dishevelled mammalian homolog Dvl1, resulting in the destabilization of the Axin-APC-GSK-3β protein complex and its sequestration into multivesicular bodies (MVB) [25]. GSK-3 inactivation allows for β-catenin stabilization and facilitates gene expression by the TCF/LEF transcription factors.

In the phosphoinositide 3-kinase (PI3K)/Akt pathway, growth signals activate the catalytic subunit of PI3K, which phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP2) to produce phosphatidylinositol-3,4,5-trisphosphate (PIP3) and activates phosphoinositide-dependent protein kinase-1 (PDK-1). PDK-1 phosphorylates and thus activates the recruited serine-threonine kinase Akt/protein kinase B. Akt/PKB phosphorylates GSK-3 to inhibit its activity [6, 12, 15].

GSK-3 controls many neuronal functions by phosphorylating protein substrates involved in the regulation of gene transcription, metabolism, apoptosis, and cytoskeletal dynamics (Figure 1(b)). To ensure the proper execution of these actions, GSK-3 activity must be accurately controlled by the interplay of phosphorylation, localization, and sequestration by GSK-3-interacting proteins [6, 26, 27].

4. GSK-3 Function in the Developing and Adult Brain

4.1. Neuronal Progenitors: Proliferation and Differentiation. Neural progenitor proliferation and differentiation are regulated by multiple extracellular signals and intracellular signaling mechanisms in which GSK-3 is implicated. Early in neural development, GSK-3 functions to regulate neural progenitor self-renewal, homeostasis, and apical-basal polarity via β-catenin, Notch, FGF, and Wnt signaling [28].

Establishing neuronal polarity is a consequence of the reorganization of cytoskeletal elements after the local activation of symmetry-breaking signals. GSK-3 is a key regulator of neuronal polarity and microtubule-cytoskeleton reorganization [29, 30]. These functions are controlled by GSK-3-mediated phosphorylation of microtubule-associated proteins (MAPs), such as collapsin response mediator protein-2 (CRMP-2) [31], adenomatous polyposis coli (APC) [32], Tau [33], microtubule-associated protein 1B (MAP1B) [34], Doublecortin (DCX) [35], end-binding 1 (EB1) [36], and cytoplasmic linker-associated proteins (CLASPs) [37], and subsequent regulation of cytoskeletal dynamics. For example, APC and CLASPs promote microtubule stability and, upon phosphorylation by GSK-3, they dissociate from and destabilize microtubules [37, 38]. Therefore, polarized deposition of polarity proteins underlies asymmetric cell division which is

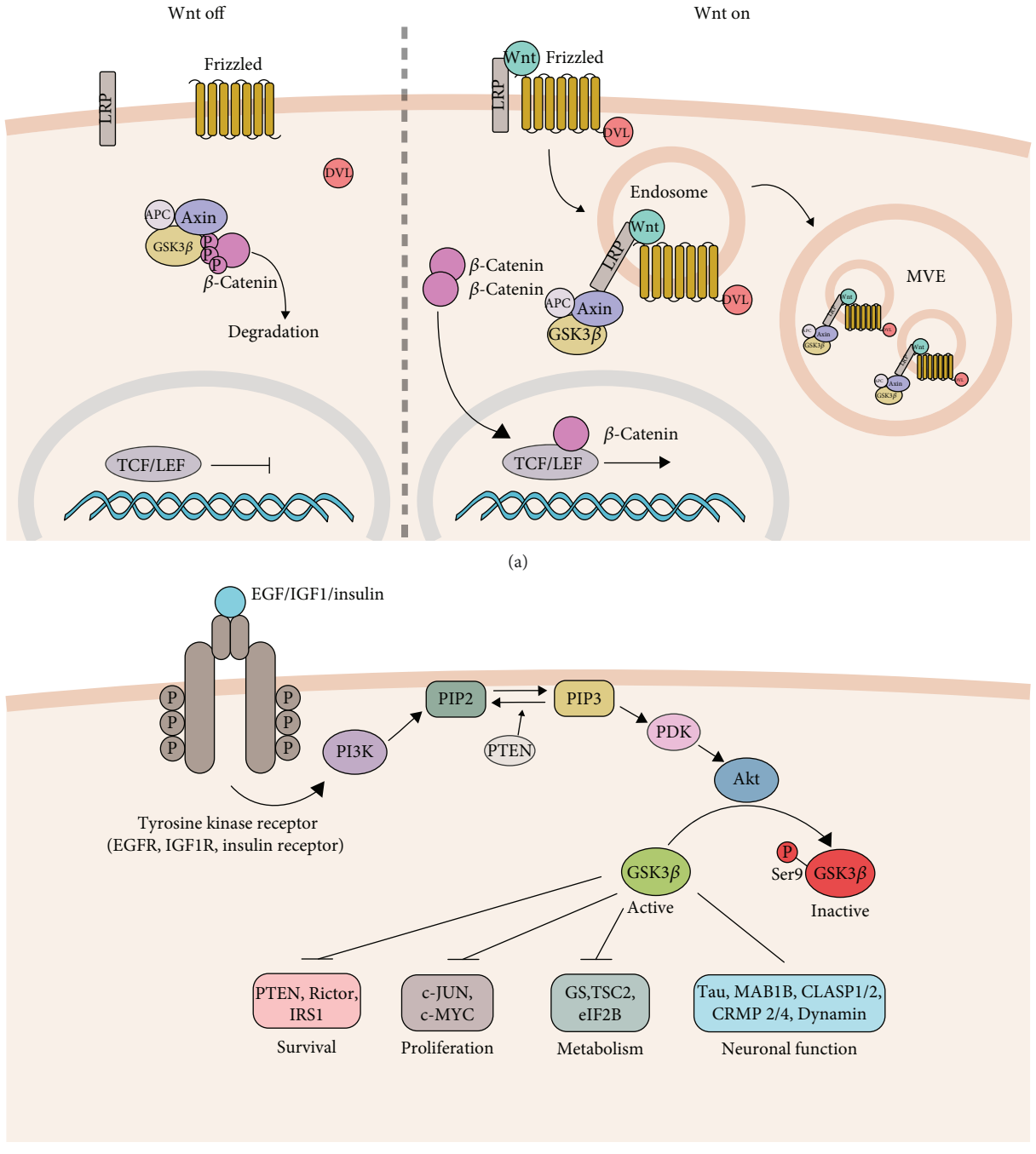

Figure 1: Molecular mechanisms of GSK-3β regulation. (a) The Wnt canonical pathway. In the absence of Wnt, β-catenin is degraded within a destruction complex composed of Axin, APC, and GSK-3β proteins. Following Wnt binding to Frizzled and LRP5/6 receptors, Dvl is recruited resulting in the sequestration of the destruction complex within the MVB. This allows β-catenin to accumulate, translocate to the nucleus, and subsequently induce gene expression via the TCF/LEF transcription factors. (b) The PI3K/Akt pathway. The activation of PI3K following the stimulation of Tyrosine Kinase Receptor leads to the production of PIP3. Akt kinase is recruited and is activated upon phosphorylation at Thr308 and Ser473 by PDK1 and mTORC2, respectively. The signal is terminated following PIP3 dephosphorylation by PTEN phosphatase. Akt kinase phosphorylates and inhibits GSK-3β activity by a reversible phosphorylation at Ser9. An incomplete list of the GSK-3β substrates and cellular processes that it regulates is shown.

necessary for the neurogenic division of neural progenitors. Indeed, polarized apical deposition of polarity proteins, including APC, EB1, and cadherin, is disrupted in GSK-3α/β-deleted developing cortex [28].

4.2. Neuronal Migration. Following differentiation of progenitors into neurons, GSK-3 signaling is crucial to neuronal migration. For example, removal of GSK-3α and GSK-3β in cortical excitatory neurons leads to the failure of radial

migration in the cortex [39]. GSK-3 regulates neuronal migration by phosphorylating key microtubule regulatory proteins such as APC and other microtubule-associated proteins to rearrange the intracellular cytoskeleton. As mentioned before, APC is a microtubule-associated protein and is important for microtubule-based cytoskeleton dynamics [40]. When GSK-3 is inactive, APC stabilizes microtubules at the leading edge of migrating neurons [38]. When GSK-3 becomes active, it binds to and phosphorylates APC causing its dissociation from microtubules [41].

Other studies have implicated other GSK-3 interacting proteins, including β-catenin and DISC1, in neuronal migration [42–46]. DISC1/GSK-3 interaction may be particularly important for determining the transition of neural progenitor self-renewal to neuronal migration because GSK-3 binds to DISC1 during the embryonic stage (E14) when neural progenitor proliferates but dissociates from DISC1 during later embryonic stages (E18) when neuronal migration takes place [46].

4.3. Neuronal Morphology and Synaptic Development. Several lines of evidence implicate GSK-3 in the regulation of different aspects of neuronal morphogenesis, including axon growth, dendritic branching, and synaptic development. Pharmacological inhibition of GSK-3 decreases the rate of axon elongation, increases the size of growth cones [47], and disturbs polarity, leading to the formation of multiple axon-like processes in hippocampal neurons [48, 49]. Likewise, genetic elevation of GSK-3β activity causes shrinkage of dendrites, whereas GSK-3β inhibition enhances dendritic growth *in vivo* [50]. Another study showed that neurons with deleted GSK-3 exhibit markedly abnormally oriented basal dendrites [39].

GSK-3 also contributes to the regulation of synapse morphology and formation in mature, postmitotic neurons (Figure 2(a)). Deletion of the GSK-3β gene in the cortex and hippocampus causes a reduction of spine density, loss of persistent spines, and reduced stabilization of new spines, accompanied by a decrease of AMPAR-dependent miniature excitatory postsynaptic currents [51]. Accordingly, overexpression of GSK-3β alters dendritic branching and reduces the number of the functional synapses of dentate gyrus granule neurons [52]. A recent study showed that GSK-3β is involved in the maturation of dendritic spines, because genetically elevating GSK-3β activity increases the number of thin spines, whereas removal of the GSK-3β gene increases the number of stubby spines in the dentate gyrus neurons [53]. Likewise, pharmacological inhibition of GSK-3β decreases the number of mature spines favouring an accumulation of immature types [54].

4.4. Neurotransmission. GSK-3α and β are present within the synapse because they were detected in the synaptosomal fraction which consists of pre- and postsynaptic termini [55]. More specifically, an electron microscopic study showed GSK-3β labelling of postsynaptic densities in a subset of dendritic spines [56].

GSK-3 plays an important role in synaptic plasticity at GABAergic as well as at glutamatergic synapses. At GABAergic synapses, active GSK-3β decreases inhibitory synaptic strength [50] by phosphorylating the scaffolding protein gephyrin [57].

At glutamatergic synapses, GSK-3β regulates the interaction between two major forms of synaptic plasticity: NMDA-dependent LTP and LTD (Figure 2(b)). During LTP, the activation of NMDA receptors causes the inhibition (by Ser9 phosphorylation) of GSK-3β activity via the PI3K/Akt pathway, whereas the action of PP1 in LTD causes an increase of GSK-3β activity [58]. Thus, GSK-3β is crucial for the initiation of NMDA-induced LTD in hippocampal neurons.

Molecular mechanisms requiring the modulation of GSK-3 Ser21/9 phosphorylation, during experimental LTP or LTD, are crucial for learning and memory [55, 58, 59]. The phosphorylation of GSK-3β at Ser9 increases following the training of mice in hippocampus-dependent cognitive tasks, i.e., inhibitory avoidance and novel object recognition task [59]. Furthermore, LTP is impaired, whereas LTD is facilitated, in two different transgenic mice overexpressing active GSK-3β [55, 59]. These LTP deficits can be reversed by treatment with lithium, a GSK-3 inhibitor [55]. Accordingly, removal of GSK-3β in dentate gyrus excitatory neurons inhibits hippocampal synaptic transmission and reduces levels of NMDAR and AMPAR receptors, postsynaptic PSD93 and drebrin, and presynaptic synaptophysin proteins causing impairments in spatial and fear memories [60].

Furthermore, GSK-3 contributes to NMDA and AMPA receptor trafficking and function in cortical neurons [61, 62]. GSK-3 causes internalization of NMDARs and forms a complex between AMPARs, thereby affecting the expression of LTD. AMPA receptor mobilization is important for LTD to occur. A critical step in this process is the destabilization of PSD-95 by GSK-3β [63].

In addition to postsynaptic actions, GSK-3 also participates in presynaptic functions in developing and mature synapses [64]. For example, high GSK-3 activity reduces glutamate release from the presynapse causing impairments in LTP [55, 65]. Additionally, retrieval of synaptic vesicles at the presynapse by endocytosis requires the regulation of dynamin 1 by GSK-3 [66]. Moreover, GSK-3β negatively regulates synaptic vesicle fusion events via interfering with Ca(2+)-dependent SNARE complex formation which is required for efficient neurotransmitter release [67]. These observations show that GSK-3 is crucial for synapse assembly and function, although the GSK-3 synaptic phosphoproteome has not been described yet. Overall, GSK-3 regulates neuronal excitation/inhibition balance. Dysregulated excitatory/inhibitory control has been reported in different neuropsychiatric disorders.

5. Implications of GSK-3 Dysregulation

5.1. GSK-3 Knockout and Transgenic Mouse Models. The dysfunction of GSK-3 signaling pathways is associated with the pathogenesis of different neurological and neuropsychiatric disorders. Several mouse models lacking or overexpressing GSK-3α or β have been generated that mimic pathological conditions observed in different neuropsychiatric and neurological

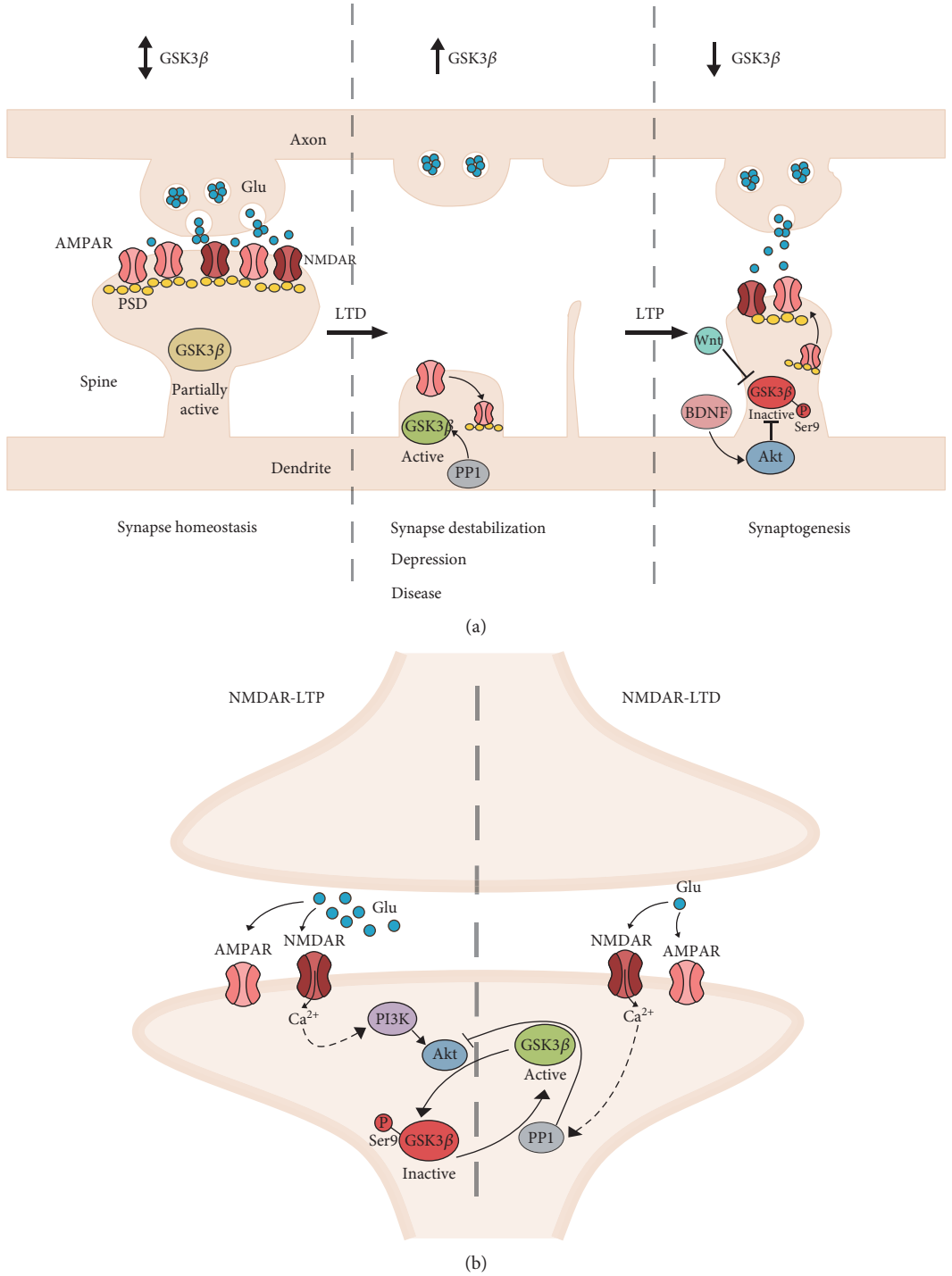

Figure 2: GSK-3β at glutamatergic synapse. (a) Role of GSK-3β in the structural plasticity of glutamatergic synapse. (Left) Under normal conditions, synapse function is maintained by homeostatic mechanisms that depend on the cycling of glutamate receptors within the synapse. Transient changes in GSK-3β activity will support molecular mechanisms required for these processes. (Middle) Synaptic destabilization following LTD or chronic stress decreases synaptic density and causes synapse atrophy. High GSK-3β activity is required for pre- and postsynaptic molecular mechanisms to support the occurrence of LTD. Increased GSK-3β activity has been reported in different neurological and neuropsychiatric disorders. (Right) Following LTP stimuli, GSK-3β is inhibited to enable synaptic growth. LTP stimuli also increase BDNF and Wnt proteins which act to inhibit GSK-3β during LTP. (b) GSK-3β determines the direction of NMDA receptor-mediated plasticity. (Right) During LTD, activation of PP1 causes dephosphorylation and thus activation of GSK-3β by the Ser9 mechanism. Simultaneously, active PP1 inhibits Akt preventing Ser9 phosphorylation of GSK-3β. During LTP, the activation of NMDA receptors stimulates the PI3K-Akt pathway, which phosphorylates and inhibits GSK-3β activity to prevent the induction of LTD.

disorders. These mice recapitulate pathological conditions with aberrant GSK-3 activity and thereby point at GSK-3 as a critical regulator of different physiological neurological processes.

Total removal of GSK-3β is lethal in late embryonic development due to liver apoptosis or heart defects [9, 10]. Removal of only one GSK-3β allele causes behavioral abnormalities, including aggressive behaviors, increased anxiety, and memory deficits, in GSK-3β heterozygous (+/-) mice [68, 69].

In contrast, homozygous mice lacking GSK-3α are viable but male mice are infertile [70]. They show minor abnormalities in brain anatomy, such as an altered neuronal architecture of the hippocampus [70] or a lower number of Purkinje cells in the cerebellum [71]. These two mouse strains show minor neurobehavioral abnormalities such as reduced exploratory activity, increased anxiety, and decreased social motivation and associative memory [70, 71].

Postnatal neuronal specific GSK-3β knockout mice (GSK-3$\beta^{n-/-}$) together with GSK-3α mice (GSK-3$\alpha^{n-/-}$) were developed based on the Cre/loxP system to omit the developmental problems of GSK-3β deficiency [72]. Neurological examination showed that GSK-3$\beta^{n-/-}$ mice have reduced dentate gyrus volume [73] and decreased stability of dendritic spines [53], while GSK-3$\alpha^{n-/-}$ mice have a reduced-size CA1 pyramidal blade and pre- and postsynaptic deficits [70], suggesting distinct synaptic functions of GSK-3 isozymes in the adult brain.

Transgenic mice that overexpress human GSK-3β employ the S9A mutant form of the kinase to prevent its inhibitory phosphorylation [74]. The thy1 gene promoter employed drives the expression of GSK-3β(S9A) postnatally in neurons. This transgenic mouse displays a twofold higher GSK-3β level and activity relative to wild-type mice. Consequently, increased tau phosphorylation is evident, but only in older GSK-3β(S9A) mice. These mice have decreased brain weight and volume counterbalanced by a higher cortical neuronal density and decreased size of their cell bodies and of their somatodendritic compartments [75]. The decreased brain size was further confirmed in a recent study showing a decreased dentate gyrus volume in GSK-3β(S9A) mice [73]. Biochemical analysis showed increased brain-derived neurotrophic factor (BDNF) and Akt1 levels in the hippocampus and decreased levels of PPP2R3A (PP2A regulatory subunit) and GSK-3α in the striatum in GSK-3β(S9A) mice [76]. Furthermore, overexpression of GSK-3β was shown to result in the differential expression of a large number of proteins, including the downregulation of MAP2 [77]. Despite increased tau phosphorylation and decreased hippocampus volume, GSK-3β(S9A) mice display normal memory in the Morris water maze test [74]. However, follow-up studies demonstrated impairments in hippocampal-dependent, species-typical behavioral tasks [73] and passive inhibitory avoidance [59]. Furthermore, GSK-3β(S9A) mice show hyperactivity and lower immobility time in the forced swim test (FST) which recapitulate symptoms of schizophrenia or the manic phase of bipolar disorder [76].

These mouse studies show that while GSK-3β is important during development, in the adult brain both GSK-3 isozymes have important nonredundant functions in the regulation of learning, memory, and behavior, which may result from similar but not the same spectrum of protein substrates in neurons [78].

Altogether, a delicate balance of GSK-3β activity is important for the regulation of different aspects of neuronal plasticity at the developmental stage as well as in adulthood. Not surprisingly, the dysregulation of GSK-3β activity may have deleterious consequences leading to brain disorders.

5.2. Alzheimer's Disease. Alzheimer's disease (AD) is characterized by a progressive loss of episodic memory and by cognitive and behavioral impairments and ultimately death. Synaptic dysfunction and hence memory impairments come early in the disease process. Histopathological hallmarks at postmortem analysis are extracellular senile plaques made up of amyloid-β (Aβ) protein and intracellular neurofibrillary tangles (NFTs) composed of hyperphosphorylated tau protein. Since its initial discovery as a tau protein kinase [79], GSK-3β is considered to be essential to AD pathogenesis [80]. It plays a fundamental role in pathological events such as Tau phosphorylation, Aβ formation, neurotoxicity, neuritic dystrophy, impaired cognition, neuronal survival, and neurodegeneration [74, 81–84]. Increased levels of GSK-3 have been reported in brains from AD patients compared to age-matched control samples [85]. Furthermore, a spatial and temporal pattern of increased active GSK-3β expression correlates with the progression of neurofibrillary tangles (NFT) composed of hyperphosphorylated forms of Tau, Aβ formation, inflammatory markers, and neurodegeneration [86]. Accordingly, increased GSK-3β activity has been used to replicate neuronal dysfunctions in mouse models of AD [74, 84]. GSK-3β has been shown to be the major tau kinase *in vivo* [74], and it phosphorylates at least 36 residues in tau protein [87]. Furthermore, comparative phenotypic analysis of two bigenic mouse lines APP.V717I-tau.P301L and GSK-3β.S9A-tau.P301L reveals that amyloid or GSK-3β leads to a similar tau phosphorylation pattern and NFT accumulation [84]. Additionally, Aβ has been shown to activate GSK-3β signaling *in vitro* [88]. Altogether, GSK-3β is the mediator of amyloid action on tau phosphorylation and neurodegeneration in AD.

It should be noted that changes in the GSK-3β kinase activity, besides being involved in the regulation Aβ or tau phosphorylation, will negatively affect synaptic plasticity essential for learning and memory [55, 58, 59, 65]. For example, overexpression of GSK-3β in transgenic mice impairs memory [89, 90]. Pharmacologically balancing normal levels of GSK-3β activity recues memory deficits [90, 91]. This GSK-3β-induced cognitive impairment is mediated by tau protein because the genetic deletion of tau as well as GSK-3β inhibition blocks Aβ-induced impairments of LTP [92]. Furthermore, the genetic deletion of tau in GSK-3β-overexpressing mice ameliorates memory deficits [93].

5.3. Parkinson's Disease. Parkinson's disease (PD), the second most common neurodegenerative disease, is a chronic movement disorder resulting from the progressive loss of dopaminergic neurons in the substantia nigra pars compacta, leading

to pathological and clinical abnormalities, including bradykinesia (slowness and minimal movement), rigidity, resting tremor, and postural instability. Additional symptoms include cognitive decline, depression, anxiety, and sleep disturbances resulting from neurodegeneration in the cortex and brainstem [94, 95]. The loss of dopaminergic neurons and thus decreased dopamine levels in the striatum is accompanied by an intracellular buildup of alfa-synuclein inclusions called Lewy bodies (LB) and hyperphosphorylated tau [96].

Evidence for GSK-3β involvement in PD comes from genetic studies in which single-nucleotide polymorphisms (SNPs) in the *GSK-3β* gene (rs334558 and rs6438552) are associated with PD [97]. The T allele (rs6438552) alters the GSK-3β splicing pattern resulting in the augmentation of GSK-3β activity [97]. Other studies in different populations have also linked SNPs in the *GSK-3β* gene to PD [98–100].

Accordingly, an increased GSK-3β expression has been reported in postmortem PD brains [101]. Furthermore, GSK-3β colocalizes with α-Synuclein in the Lewy bodies (LBs) [101]. *In vitro* GSK-3β phosphorylates α-Synuclein at Ser129 facilitating its toxic misfolding, aggregation, and accumulation leading to the degeneration of dopaminergic neurons [102]. Furthermore, GSK-3β contributes to Tau pathology associated with PD [102], corroborating the genetic data [100, 103]. Specifically, in a cell model of PD alpha-synuclein, pSer396/404-Tau and pGSK-3β coimmunoprecipitate following MPP(+) treatment [104]. Moreover, GSK-3β inhibitors prevent MPP(+)-induced death, increased α-synuclein accumulation, and pTau formation [104]. Studies from animal models demonstrated that in mice expressing a constitutively active, human GSK-3β(S9A) mutated form, levels of p-α-synuclein-S129 and pTau (S396/404) rise in TH+ dopaminergic neurons along with animal aging [102]. In α-synuclein A53T mutant mice, elevated levels of α-synuclein together with increased levels of pTau (pSer202, 396/404) and the active form of pGSK-3β (pTyr216) were detected in the striatum by western blot analysis; all of these components were also found to aggregate together, as confirmed by immunohistochemical stainings [105].

In line with these results, GSK-3β inhibitors were considered to counteract the degeneration of dopaminergic neurons. Accordingly, chronic treatment with lithium prevented the degeneration of dopaminergic neurons in the mouse model of PD [106]. Likewise, more specific GSK-3β inhibitors such as indirubin-3$'$-oxime and AR-A014418 suppress the loss of dopaminergic neurons and restore dopamine concentration [107].

Cautiously, human study demonstrated that chronic lithium treatment itself can induce parkinsonian pathological features, including impaired motor coordination accompanied by neuronal loss in the basal ganglia [108]. Therefore, considerations such as designing specific GSK-3 inhibitors, preventing their side effects, and determining optimum levels of GSK-3β inhibition have to be taken into account in planning GSK-3-based therapeutic strategies.

5.4. Lithium: GSK-3 Inhibitor. For many years, lithium has been used as a mood stabilizer in the treatment of mental disorders, including bipolar disorder, schizophrenia, and depression. Despite that many molecular targets have been identified, lithium is best known as a GSK-3 inhibitor [109, 110]. Lithium directly inhibits GSK-3α and GSK-3β [109] both in cells [110] and in the brain *in vivo* [111] at an IC50 of 2 mM, which is slightly higher than the therapeutic concentration of 0.5-1.5 mM [109]. The direct mechanism by which Li^+ ions inhibit GSK-3 is that they compete for the binding of magnesium, which is a cofactor of different kinases, including GSK-3 [112]. Lithium can also indirectly inhibit GSK-3 by activating the Akt kinase or by disrupting the β-arrestin complex [113–115].

A large number of studies on the effects of lithium confirmed that GSK-3 is associated with different diseases, including fragile X syndrome (FXS) and schizophrenia. Lithium or the specific pharmacological modulation of GSK-3 activity has been shown to correct behavioral deficits in mouse models of these diseases [116, 117]. This highlights GSK-3 as a valid target of lithium; however, it must be noted that lithium is a nonspecific GSK-3 inhibitor (it inhibits many other kinases) with high *in vivo* toxicity.

5.5. Fragile X Syndrome. Patients with FXS have intellectual disability. Fragile X syndrome (FXS) results from the lack of expression of the functional fragile X mental retardation protein (FMRP) due to the expansion of CGG triplets resulting in the overmethylation of the gene promoter. FMRP is an RNA-binding protein that controls cellular mRNA translocation.

Since mRNA translocation towards dendrites and local translation play a pivotal role in neuronal function, FXS is characterized by several behavioral and brain structural abnormalities. Mice lacking the FMRP expression (FMRP KO mice), which model FXS, display similar characteristics as patients with FXS. FMRP KO mice exhibit impaired structural synaptic plasticity characterized by an increased dendritic spine length and number, accompanied by a reduced maturation of spines, as compared to control mice [118–120]. Indeed, other reports showed that FMRP plays a role in the normal maturation of synaptic connections [118, 121]. In addition, FX mice display distinct functional synaptic alternations such as enhanced metabotropic glutamate receptor- (mGluR-) dependent long-term depression (LTD) in the hippocampal CA1 neurons. Interestingly, further research showed aberrant mGluR signaling to GSK-3 in FX mice, and lithium treatment normalized increased mGluR-dependent LTD at CA1 synapses in these mice [122].

GSK-3 inhibition following the administration of lithium or more specific inhibitors in these mice led to corrections of multiple functional and structural FX-related phenotypes, such as normalization of hyperactive locomotor and social behaviors and improvement of passive avoidance learning as well as normalization of dendritic spine length and density and synaptic transmission [116, 123].

5.6. Schizophrenia. Schizophrenia is a widespread mental disorder, characterized by progressive functional decline and lifelong disability. Common symptoms are typically

categorized into positive (hallucinations and delusions), negative (disruption of normal emotions and behavior), and cognitive (disruption of executive performance and memory). People with schizophrenia often have additional mental health problems such as anxiety or depression. Schizophrenia is thought to be caused by a combination of environmental and genetic factors.

Genetic studies have supported the association between *AKT1* genetic variants and schizophrenia [124, 125], suggesting that impaired AKT/GSK-3 signaling contributes to the pathogenesis of schizophrenia [125, 126]. AKT1 protein level is significantly reduced in the hippocampus and frontal cortex in postmortem brain samples. Consequently, the activity of the major AKT1 target—GSK-3—is altered in patients with schizophrenia [125]. Additionally, *GSK-3β* promoter polymorphism rs3755557 that results in a higher promoter activity [127] is associated with schizophrenia in the Chinese population [128].

A recent study showed that increased GSK-3β activity early in development predisposes to altered synaptic plasticity, dendritic spine loss, and cognitive disability in a rat neurodevelopmental model of schizophrenia [54]. Accordingly, chronic treatment with antipsychotics such as clozapine, risperidone, or haloperidol increases the inhibitory phosphorylation of GSK-3β in the rat prefrontal cortex and striatum [129, 130].

Dysregulated dopamine neurotransmission is thought to underlie schizophrenia pathophysiology as dopamine D2 receptor antagonists are antipsychotic drugs. Akt/GSK-3 signaling is important for dopamine D2 receptor function, because mice lacking GSK-3β have an impaired function of the striatal D2 receptor [131]. Molecularly, the D2 receptor stimulates the formation of a signaling complex made up of β-arrestin-2, Akt, and PP2A—the latter inactivates Akt by the dephosphorylation of its regulatory Thr308 residue [132]. Accordingly, the regulation of Akt by dopamine is impaired in mice devoid of β-arrestin-2 [132]. Akt inhibition is known to activate GSK-3, suggesting that GSK-3 signaling is involved in the regulation of dopamine-dependent locomotor behavior. Likewise, pharmacological or genetic abolishing of GSK-3 activity decreases dopamine-dependent locomotor behavior [133].

5.7. Major Depressive Disorder. Major depressive disorder (MDD) is the most frequent psychiatric disorder with a prevalence of 17% in the general population, although gender disproportion exists [134]. MDD negatively affects personal life and general health. The most widely used animal model of depression is the chronic unpredictable mild stress (CUMS) model in rats. CUMS results in the augmentation of GSK-3β activity [135–137]. Accordingly, lithium and specific GSK-3β inhibitors ameliorate cognitive deficits induced by CMS [135–137].

One of the associated symptoms of MDD are disturbances in the hypothalamic–pituitary–adrenal axis (HPA axis) connected with an incorrect response of the glucocorticoid receptor to chronic stress [138]. Chronic administration of corticosterone that models depression in mice impairs synaptic plasticity and upregulates GSK-3β activity—both of which are ameliorated by the administration of an antidepressant drug [139].

The *GSK-3β* gene may have a role in determining regional grey matter (GM) volume differences in MDD. Analysis of single-nucleotide polymorphisms (SNPs) of *GSK-3β* with regional GM volume differences in patients with MDD showed the most significant association for rs6438552 [140]. In a different study, the activating allele T of the functional polymorphism rs334558 was significantly associated with remission in MDD [141].

5.8. Bipolar Disorder. Bipolar affective disorder is characterized by manic episodes that are interspersed with depression. Inadequate serotonin (5HT) neurotransmission may be a key factor driving depression. Evidence suggests that increased serotonergic activity following the administration of antidepressants inhibits GSK-3β in the brain by the Ser9 mechanism [142]. Thus, GSK-3β may not be properly inhibited in conditions of decreased 5HT levels in depression. Indeed, lower phosphorylated GSK-3β Ser9 levels were detected in platelets of patients with schizophrenia [143]. Indeed, animal studies provide further support that overactive GSK-3 contributes to depression. Transgenic mice with GSK-3β overexpression show increased locomotor activity as seen in the manic phase of bipolar disorder [76]. Furthermore, the administration of the GSK-3β peptide inhibitor, ATP competitive inhibitor, and lithium and the genetic reduction of GSK-3β in GSK-3$\beta^{+/-}$ mice produce antidepressant behavioral effects, such as decreased immobilization time in FST, which is indicative of depressive behavior [69, 144, 145].

5.9. Epilepsy. Epilepsy, which is estimated to affect over 50 million people worldwide, comprises a group of neurological diseases characterized by epileptic seizures resulting from an excessive neuronal activity [146]. In addition to seizures, epilepsy is usually associated with cognitive impairments. Epilepsy frequently accompanies various mental conditions, such as autism spectrum disorders or schizophrenia. Development of epilepsy, known as epileptogenesis, may take months or even years following brain injury, stroke, brain tumors, brain infections, or birth defects, whereas a small proportion of the cases are due to genetic mutations [147, 148]. Epileptogenesis can be reproduced in animal models using electrical or chemical kindling with pentylenetetrazole (PTZ), whereas the status epilepticus is induced by kainic acid (KA) or pilocarpine [149]. Even though extensive research shows that GSK-3 contributes to brain excitability and seizure-induced pathology, the existing data are conflicting [150–153]. For example, GSK-3β phosphorylation at Ser9 was reported to increase or decrease in brain tissue extracted from epileptic patients [154, 155]. Furthermore, kainic acid- (KA-) triggered epileptogenesis was shown to either increase or inhibit GSK-3β activity [152, 156]. Acute PTZ injection rapidly increases GSK-3β Ser9 phosphorylation and PTZ-induced kindling also gradually increases phosphorylation at Ser9 [53, 151], whereas pilocarpine-induced seizures transiently inactivate GSK-3β [150]. Pharmacological studies aimed at elucidating the role of GSK-3β inhibition in epilepsy showed a neuroprotective effect of

GSK-3β inhibition against glutamate-induced toxicity *in vitro* and *in vivo* [157]. Accordingly, the GSK-3β inhibitor TDZD-8 protects against seizure-induced damage [152]. Consistently, a recent study reported the anticonvulsant properties of two distinct GSK-3 inhibitors (Indirubin and BIO-acetoxime) in three different animal models of epilepsy: the PTZ-treated zebrafish, the pilocarpine rat model for limbic seizures, and the 6 Hz refractory seizure mouse model [158]. In contrast, lithium was shown to exert proconvulsive [159] or anticonvulsive effects [160].

More complexity comes from recent animal studies. It was shown that genetically increasing as well as decreasing the activity of GSK-3β exacerbated seizure-induced brain damage after KA injection into the amygdala [161]. In a different study, GSK-3β decreased the susceptibility to kainic acid-induced epileptiform discharges and the progression of kainic acid-induced epileptogenesis [162]. Similarly, the neuronal deficiency of GSK-3β exacerbated the magnitude and severity of PTZ-induced seizures in GSK-3$\beta^{n-/-}$ mice (with postnatal neuronal deficiency) [53].

Regardless of these discrepancies, GSK-3β is considered an important contributor to the development of epilepsy.

6. Conclusions

Evidence convincingly shows that GSK-3β is critically involved in various aspects of brain function starting from early brain development, to distinct aspects of its function in the adult such as proper synaptic development and neurotransmission. GSK-3β is regulated at multiple levels and precise balance of its activity is important to execute its functions in neurons. Not surprisingly dysregulation of GSK-3β activity either in the early development or in the adulthood may predispose to neuropsychiatric and neurological disorders. GSK-3β is thus a relevant target for treatment of these diseases. Few GSK-3 inhibitors are currently undergoing clinical trials for various disorders such as progressive supranuclear palsy, Alzheimer's disease or cancer [163]. Pharmacological targeting of this kinase, however, may be problematic because of its involvement in different signaling pathways as well as because of overlapping functions with GSK-3α isozyme. Therefore, generating novel inhibitors with increased specificity, designing co-treatments and preventing side effects are of importance in pharmacological targeting of GSK-3.

Conflicts of Interest

All authors declare that they have no conflict of interest.

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References

[1] R. Malinow and R. C. Malenka, "AMPA receptor trafficking and synaptic plasticity," *Annual Review of Neuroscience*, vol. 25, no. 1, pp. 103–126, 2002.

[2] J. Lisman, "Glutamatergic synapses are structurally and biochemically complex because of multiple plasticity processes: long-term potentiation, long-term depression, short-term potentiation and scaling," *Philosophical Transactions of the Royal Society B: Biological Sciences*, vol. 372, no. 1715, article 20160260, 2017.

[3] R. Gao and P. Penzes, "Common mechanisms of excitatory and inhibitory imbalance in schizophrenia and autism spectrum disorders," *Current Molecular Medicine*, vol. 15, no. 2, pp. 146–167, 2015.

[4] P. Penzes, M. E. Cahill, K. A. Jones, J. E. VanLeeuwen, and K. M. Woolfrey, "Dendritic spine pathology in neuropsychiatric disorders," *Nature Neuroscience*, vol. 14, no. 3, pp. 285–293, 2011.

[5] N. Embi, D. B. Rylatt, and P. Cohen, "Glycogen synthase kinase-3 from rabbit skeletal muscle. Separation from cyclic-AMP-dependent protein kinase and phosphorylase kinase," *European Journal of Biochemistry*, vol. 107, no. 2, pp. 519–527, 1980.

[6] R. S. Jope and G. V. W. Johnson, "The glamour and gloom of glycogen synthase kinase-3," *Trends in Biochemical Sciences*, vol. 29, no. 2, pp. 95–102, 2004.

[7] J. R. Woodgett, "Molecular cloning and expression of glycogen synthase kinase-3/factor A," *EMBO Journal*, vol. 9, no. 8, pp. 2431–2438, 1990.

[8] L. T. Alon, S. Pietrokovski, S. Barkan et al., "Selective loss of glycogen synthase kinase-3α in birds reveals distinct roles for GSK-3 isozymes in tau phosphorylation," *FEBS Letters*, vol. 585, no. 8, pp. 1158–1162, 2011.

[9] K. P. Hoeflich, J. Luo, E. A. Rubie, M. S. Tsao, O. Jin, and J. R. Woodgett, "Requirement for glycogen synthase kinase-3β in cell survival and NF-κB activation," *Nature*, vol. 406, no. 6791, pp. 86–90, 2000.

[10] R. Kerkela, L. Kockeritz, K. MacAulay et al., "Deletion of GSK-3β in mice leads to hypertrophic cardiomyopathy secondary to cardiomyoblast hyperproliferation," *The Journal of Clinical Investigation*, vol. 118, no. 11, pp. 3609–3618, 2008.

[11] H. B. Yao, P. C. Shaw, C. C. Wong, and D. C. C. Wan, "Expression of glycogen synthase kinase-3 isoforms in mouse tissues and their transcription in the brain," *Journal of Chemical Neuroanatomy*, vol. 23, no. 4, pp. 291–297, 2002.

[12] C. Sutherland, I. A. Leighton, and P. Cohen, "Inactivation of glycogen synthase kinase-3β by phosphorylation: new kinase connections in insulin and growth-factor signalling," *Biochemical Journal*, vol. 296, no. 1, pp. 15–19, 1993.

[13] J. R. Woodgett, "Regulation and functions of the glycogen synthase kinase-3 subfamily," *Seminars in Cancer Biology*, vol. 5, no. 4, pp. 269–275, 1994.

[14] H. Eldar-Finkelman, R. Seger, J. R. Vandenheede, and E. G. Krebs, "Inactivation of glycogen synthase kinase-3 by epidermal growth factor is mediated by mitogen-activated protein kinase/p90 ribosomal protein S6 kinase signaling pathway in NIH/3T3 cells," *Journal of Biological Chemistry*, vol. 270, no. 3, pp. 987–990, 1995.

[15] C. A. Grimes and R. S. Jope, "The multifaceted roles of glycogen synthase kinase 3β in cellular signaling," *Progress in Neurobiology*, vol. 65, no. 4, pp. 391–426, 2001.

[16] V. Stambolic and J. R. Woodgett, "Mitogen inactivation of glycogen synthase kinase-3β in intact cells via serine 9

phosphorylation," *Biochemical Journal*, vol. 303, no. 3, Part 3, pp. 701–704, 1994.

[17] F. Zhang, C. J. Phiel, L. Spece, N. Gurvich, and P. S. Klein, "Inhibitory phosphorylation of glycogen synthase kinase-3 (GSK-3) in response to lithium," *Journal of Biological Chemistry*, vol. 278, no. 35, pp. 33067–33077, 2003.

[18] K. Hughes, E. Nikolakaki, S. E. Plyte, N. F. Totty, and J. R. Woodgett, "Modulation of the glycogen synthase kinase-3 family by tyrosine phosphorylation," *EMBO Journal*, vol. 12, no. 2, pp. 803–808, 1993.

[19] L. Kim, J. Liu, and A. R. Kimmel, "The novel tyrosine kinase ZAK1 activates GSK3 to direct cell fate specification," *Cell*, vol. 99, no. 4, pp. 399–408, 1999.

[20] P. A. Lochhead, R. Kinstrie, G. Sibbet, T. Rawjee, N. Morrice, and V. Cleghon, "A chaperone-dependent GSK3β transitional intermediate mediates activation-loop autophosphorylation," *Molecular Cell*, vol. 24, no. 4, pp. 627–633, 2006.

[21] Q. M. Wang, C. J. Fiol, A. DePaoli-Roach, and P. J. Roach, "Glycogen synthase kinase-3 beta is a dual specificity kinase differentially regulated by tyrosine and serine/threonine phosphorylation," *Journal of Biological Chemistry*, vol. 269, no. 20, pp. 14566–14574, 1994.

[22] A. Krishnankutty, T. Kimura, T. Saito et al., "In vivo regulation of glycogen synthase kinase 3β activity in neurons and brains," *Scientific Reports*, vol. 7, no. 1, p. 8602, 2017.

[23] Y. I. Lee, M. Seo, Y. Kim et al., "Membrane depolarization induces the undulating phosphorylation/dephosphorylation of glycogen synthase kinase 3β, and this dephosphorylation involves protein phosphatases 2A and 2B in SH-SY5Y human neuroblastoma cells," *Journal of Biological Chemistry*, vol. 280, no. 23, pp. 22044–22052, 2005.

[24] M. Wehrli, S. T. Dougan, K. Caldwell et al., "*arrow* encodes an LDL-receptor-related protein essential for Wingless signalling," *Nature*, vol. 407, no. 6803, pp. 527–530, 2000.

[25] V. F. Taelman, R. Dobrowolski, J. L. Plouhinec et al., "Wnt signaling requires sequestration of glycogen synthase kinase 3 inside multivesicular endosomes," *Cell*, vol. 143, no. 7, pp. 1136–1148, 2010.

[26] B. W. Doble and J. R. Woodgett, "GSK-3: tricks of the trade for a multi-tasking kinase," *Journal of Cell Science*, vol. 116, no. 7, pp. 1175–1186, 2003.

[27] S. Frame and P. Cohen, "GSK3 takes centre stage more than 20 years after its discovery," *Biochemical Journal*, vol. 359, no. 1, Part 1, pp. 1–16, 2001.

[28] W. Y. Kim, X. Wang, Y. Wu et al., "GSK-3 is a master regulator of neural progenitor homeostasis," *Nature Neuroscience*, vol. 12, no. 11, pp. 1390–1397, 2009.

[29] H. Jiang, W. Guo, X. Liang, and Y. Rao, "Both the establishment and the maintenance of neuronal polarity require active mechanisms," *Cell*, vol. 120, no. 1, pp. 123–135, 2005.

[30] S. Etienne-Manneville and A. Hall, "Cdc42 regulates GSK-3β and adenomatous polyposis coli to control cell polarity," *Nature*, vol. 421, no. 6924, pp. 753–756, 2003.

[31] T. Yoshimura, Y. Kawano, N. Arimura, S. Kawabata, A. Kikuchi, and K. Kaibuchi, "GSK-3beta regulates phosphorylation of CRMP-2 and neuronal polarity," *Cell*, vol. 120, no. 1, pp. 137–149, 2005.

[32] F. Q. Zhou, J. Zhou, S. Dedhar, Y. H. Wu, and W. D. Snider, "NGF-induced axon growth is mediated by localized inactivation of GSK-3beta and functions of the microtubule plus end binding protein APC," *Neuron*, vol. 42, no. 6, pp. 897–912, 2004.

[33] W. H. Stoothoff and G. V. W. Johnson, "Tau phosphorylation: physiological and pathological consequences," *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, vol. 1739, no. 2–3, pp. 280–297, 2005.

[34] N. Trivedi, P. Marsh, R. G. Goold, A. Wood-Kaczmar, and P. R. Gordon-Weeks, "Glycogen synthase kinase-3 phosphorylation of MAP1B at Ser1260 and Thr1265 is spatially restricted to growing axons," *Journal of Cell Science*, vol. 118, no. 5, pp. 993–1005, 2005.

[35] P. M. Bilimoria, L. de la Torre-Ubieta, Y. Ikeuchi, E. B. Becker, O. Reiner, and A. Bonni, "A JIP3-Regulated GSK3 /DCX Signaling Pathway Restricts Axon Branching," *Journal of Neuroscience*, vol. 30, no. 50, pp. 16766–16776, 2010.

[36] T. Watanabe, J. Noritake, M. Kakeno et al., "Phosphorylation of CLASP2 by GSK-3 regulates its interaction with IQGAP1, EB1 and microtubules," *Journal of Cell Science*, vol. 122, no. 16, pp. 2969–2979, 2009.

[37] A. Akhmanova, C. C. Hoogenraad, K. Drabek et al., "Clasps are CLIP-115 and -170 associating proteins involved in the regional regulation of microtubule dynamics in motile fibroblasts," *Cell*, vol. 104, no. 6, pp. 923–935, 2001.

[38] J. Zumbrunn, K. Kinoshita, A. A. Hyman, and I. S. Näthke, "Binding of the adenomatous polyposis coli protein to microtubules increases microtubule stability and is regulated by GSK3β phosphorylation," *Current Biology*, vol. 11, no. 1, pp. 44–49, 2001.

[39] M. Morgan-Smith, Y. Wu, X. Zhu, J. Pringle, and W. D. Snider, "GSK-3 signaling in developing cortical neurons is essential for radial migration and dendritic orientation," *Elife*, vol. 3, article e02663, 2014.

[40] A. I. M. Barth, H. Y. Caro-Gonzalez, and W. J. Nelson, "Role of adenomatous polyposis coli (APC) and microtubules in directional cell migration and neuronal polarization," *Seminars in Cell & Developmental Biology*, vol. 19, no. 3, pp. 245–251, 2008.

[41] N. Asada and K. Sanada, "LKB1-Mediated Spatial Control of GSK3 and Adenomatous Polyposis Coli Contributes to Centrosomal Forward Movement and Neuronal Migration in the Developing Neocortex," *Journal of Neuroscience*, vol. 30, no. 26, pp. 8852–8865, 2010.

[42] A. Chenn and C. A. Walsh, "Increased neuronal production, enlarged forebrains and cytoarchitectural distortions in beta-catenin overexpressing transgenic mice," *Cerebral Cortex*, vol. 13, no. 6, pp. 599–606, 2003.

[43] D. Zechner, Y. Fujita, J. Hülsken et al., "β-Catenin signals regulate cell growth and the balance between progenitor cell expansion and differentiation in the nervous system," *Developmental Biology*, vol. 258, no. 2, pp. 406–418, 2003.

[44] C. A. Mutch, N. Funatsu, E. S. Monuki, and A. Chenn, "Beta-catenin signaling levels in progenitors influence the laminar cell fates of projection neurons," *Journal of Neuroscience*, vol. 29, no. 43, pp. 13710–13719, 2009.

[45] Y. Mao, X. Ge, C. L. Frank et al., "Disrupted in schizophrenia 1 regulates neuronal progenitor proliferation via modulation of GSK3β/β-catenin signaling," *Cell*, vol. 136, no. 6, pp. 1017–1031, 2009.

[46] K. Ishizuka, A. Kamiya, E. C. Oh et al., "DISC1-dependent switch from progenitor proliferation to migration in the

developing cortex," *Nature*, vol. 473, no. 7345, pp. 92–96, 2011.

[47] R. Owen and P. R. Gordon-Weeks, "Inhibition of glycogen synthase kinase 3β in sensory neurons in culture alters filopodia dynamics and microtubule distribution in growth cones," *Molecular and Cellular Neuroscience*, vol. 23, no. 4, pp. 626–637, 2003.

[48] J. J. Garrido, D. Simón, O. Varea, and F. Wandosell, "GSK3 alpha and GSK3 beta are necessary for axon formation," *FEBS Letters*, vol. 581, no. 8, pp. 1579–1586, 2007.

[49] A. Gartner, X. Huang, and A. Hall, "Neuronal polarity is regulated by glycogen synthase kinase-3 (GSK-3) independently of Akt/PKB serine phosphorylation," *Journal of Cell Science*, vol. 119, no. 19, pp. 3927–3934, 2006.

[50] Y. Rui, K. R. Myers, K. Yu et al., "Activity-dependent regulation of dendritic growth and maintenance by glycogen synthase kinase 3β," *Nature Communications*, vol. 4, no. 1, 2013.

[51] S. M. Ochs, M. M. Dorostkar, G. Aramuni et al., "Loss of neuronal GSK3β reduces dendritic spine stability and attenuates excitatory synaptic transmission via β-catenin," *Molecular Psychiatry*, vol. 20, no. 4, pp. 482–489, 2015.

[52] M. Llorens-Martín, A. Fuster-Matanzo, C. M. Teixeira et al., "GSK-3β overexpression causes reversible alterations on postsynaptic densities and dendritic morphology of hippocampal granule neurons in vivo," *Molecular Psychiatry*, vol. 18, no. 4, pp. 451–460, 2013.

[53] I. Kondratiuk, S. Łęski, M. Urbańska et al., "GSK-3β and MMP-9 cooperate in the control of dendritic spine morphology," *Molecular Neurobiology*, vol. 54, no. 1, pp. 200–211, 2017.

[54] B. Xing, Y. C. Li, and W. J. Gao, "GSK3β hyperactivity during an early critical period impairs prefrontal synaptic plasticity and induces lasting deficits in spine morphology and working memory," *Neuropsychopharmacology*, vol. 41, no. 13, pp. 3003–3015, 2016.

[55] C. Hooper, V. Markevich, F. Plattner et al., "Glycogen synthase kinase-3 inhibition is integral to long-term potentiation," *European Journal of Neuroscience*, vol. 25, no. 1, pp. 81–86, 2007.

[56] E. Perez-Costas, J. C. Gandy, M. Melendez-Ferro, R. C. Roberts, and G. N. Bijur, "Light and electron microscopy study of glycogen synthase kinase-3β in the mouse brain," *PLoS One*, vol. 5, no. 1, p. e8911, 2010.

[57] S. K. Tyagarajan, H. Ghosh, G. E. Yevenes et al., "Regulation of GABAergic synapse formation and plasticity by GSK3 -dependent phosphorylation of gephyrin," *Proceedings of the National Academy of Sciences*, vol. 108, no. 1, pp. 379–384, 2011.

[58] S. Peineau, C. Taghibiglou, C. Bradley et al., "LTP inhibits LTD in the hippocampus via regulation of GSK3β," *Neuron*, vol. 53, no. 5, pp. 703–717, 2007.

[59] I. Dewachter, L. Ris, T. Jaworski et al., "GSK3ß, a centre-staged kinase in neuropsychiatric disorders, modulates long term memory by inhibitory phosphorylation at Serine-9," *Neurobiology of Disease*, vol. 35, no. 2, pp. 193–200, 2009.

[60] E. Liu, A. J. Xie, Q. Zhou et al., "GSK-3β deletion in dentate gyrus excitatory neuron impairs synaptic plasticity and memory," *Scientific Reports*, vol. 7, no. 1, p. 5781, 2017.

[61] P. Chen, Z. Gu, W. Liu, and Z. Yan, "Glycogen synthase kinase 3 regulates N-methyl-D-aspartate receptor channel trafficking and function in cortical neurons," *Molecular Pharmacology*, vol. 72, no. 1, pp. 40–51, 2007.

[62] J. Wei, W. Liu, and Z. Yan, "Regulation of AMPA receptor trafficking and function by glycogen synthase kinase 3," *Journal of Biological Chemistry*, vol. 285, no. 34, pp. 26369–26376, 2010.

[63] C. D. Nelson, M. J. Kim, H. Hsin, Y. Chen, and M. Sheng, "Phosphorylation of threonine-19 of PSD-95 by GSK-3β is required for PSD-95 mobilization and long-term depression," *Journal of Neuroscience*, vol. 33, no. 29, pp. 12122–12135, 2013.

[64] K. J. Smillie and M. A. Cousin, "The role of GSK3 in presynaptic function," *International Journal of Alzheimer's Disease*, vol. 2011, article 263673, pp. 1–8, 2011.

[65] L. Q. Zhu, S. H. Wang, D. Liu et al., "Activation of glycogen synthase kinase-3 inhibits long-term potentiation with synapse-associated impairments," *Journal of Neuroscience*, vol. 27, no. 45, pp. 12211–12220, 2007.

[66] E. L. Clayton, N. Sue, K. J. Smillie et al., "Dynamin I phosphorylation by GSK3 controls activity-dependent bulk endocytosis of synaptic vesicles," *Nature Neuroscience*, vol. 13, no. 7, pp. 845–851, 2010.

[67] L. Q. Zhu, D. Liu, J. Hu et al., "GSK-3 inhibits presynaptic vesicle exocytosis by phosphorylating P/Q-type calcium channel and interrupting SNARE complex formation," *Journal of Neuroscience*, vol. 30, no. 10, pp. 3624–3633, 2010.

[68] T. Kimura, S. Yamashita, S. Nakao et al., "GSK-3β Is Required for Memory Reconsolidation in Adult Brain," *PLoS One*, vol. 3, no. 10, p. e3540, 2008.

[69] W. T. O'Brien, A. D. Harper, F. Jové et al., "Glycogen synthase kinase-3 haploinsufficiency mimics the behavioral and molecular effects of lithium," *Journal of Neuroscience*, vol. 24, no. 30, pp. 6791–6798, 2004.

[70] H. Maurin, B. Lechat, I. Dewachter et al., "Neurological characterization of mice deficient in GSK3α highlight pleiotropic physiological functions in cognition and pathological activity as Tau kinase," *Molecular Brain*, vol. 6, no. 1, p. 27, 2013.

[71] O. Kaidanovich-Beilin, T. V. Lipina, K. Takao et al., "Abnormalities in brain structure and behavior in GSK-3alpha mutant mice," *Molecular Brain*, vol. 2, no. 1, p. 35, 2009.

[72] T. Jaworski, I. Dewachter, B. Lechat et al., "GSK-3α/β kinases and amyloid production in vivo," *Nature*, vol. 480, no. 7376, pp. E4–E5, 2011.

[73] I. Kondratiuk, H. Devijver, B. Lechat, F. Van Leuven, L. Kaczmarek, and R. K. Filipkowski, "Glycogen synthase kinase-3beta affects size of dentate gyrus and species-typical behavioral tasks in transgenic and knockout mice," *Behavioural Brain Research*, vol. 248, pp. 46–50, 2013.

[74] K. Spittaels, C. Van den Haute, J. Van Dorpe et al., "Glycogen Synthase Kinase-3β Phosphorylates Protein Tau and Rescues the Axonopathy in the Central Nervous System of Human Four-repeat Tau Transgenic Mice," *Journal of Biological Chemistry*, vol. 275, no. 52, pp. 41340–41349, 2000.

[75] K. Spittaels, C. van den Haute, J. van Dorpe et al., "Neonatal neuronal overexpression of glycogen synthase kinase-3β reduces brain size in transgenic mice," *Neuroscience*, vol. 113, no. 4, pp. 797–808, 2002.

[76] J. Prickaerts, D. Moechars, K. Cryns et al., "Transgenic mice overexpressing glycogen synthase kinase 3beta: a putative model of hyperactivity and mania," *Journal of Neuroscience*, vol. 26, no. 35, pp. 9022–9029, 2006.

[77] K. Tilleman, I. Stevens, K. Spittaels et al., "Differential expression of brain proteins in glycogen synthase kinase-3 transgenic mice: a proteomics point of view," *Proteomics*, vol. 2, no. 1, pp. 94–104, 2002.

[78] M. P. M. Soutar, W.-Y. Kim, R. Williamson et al., "Evidence that glycogen synthase kinase-3 isoforms have distinct substrate preference in the brain," *Journal of Neurochemistry*, vol. 115, no. 4, pp. 974–983, 2010.

[79] D. P. Hanger, K. Hughes, J. R. Woodgett, J. P. Brion, and B. H. Anderton, "Glycogen synthase kinase-3 induces Alzheimer's disease-like phosphorylation of tau: generation of paired helical filament epitopes and neuronal localisation of the kinase," *Neuroscience Letters*, vol. 147, no. 1, pp. 58–62, 1992.

[80] A. Takashima, "GSK-3 is essential in the pathogenesis of Alzheimer's disease," *Journal of Alzheimer's Disease*, vol. 9, no. s3, pp. 309–317, 2006.

[81] T. Jaworski, I. Dewachter, C. M. Seymour et al., "Alzheimer's disease: old problem, new views from transgenic and viral models," *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, vol. 1802, no. 10, pp. 808–818, 2010.

[82] D. Muyllaert, D. Terwel, P. Borghgraef, H. Devijver, I. Dewachter, and F. Van Leuven, "Transgenic mouse models for Alzheimer's disease: the role of GSK-3β in combined amyloid and tau-pathology," *Revue Neurologique*, vol. 162, no. 10, pp. 903–907, 2006.

[83] D. Muyllaert, A. Kremer, T. Jaworski et al., "Glycogen synthase kinase-3β, or a link between amyloid and tau pathology?," *Genes, Brain and Behavior*, vol. 7, pp. 57–66, 2008.

[84] D. Terwel, D. Muyllaert, I. Dewachter et al., "Amyloid Activates GSK-3β to Aggravate Neuronal Tauopathy in Bigenic Mice," *American Journal of Pathology*, vol. 172, no. 3, pp. 786–798, 2008.

[85] J. J. Pei, T. Tanaka, Y. C. Tung, E. Braak, K. Iqbal, and I. Grundke-Iqbal, "Distribution, levels, and activity of glycogen synthase kinase-3 in the Alzheimer disease brain," *Journal of Neuropathology and Experimental Neurology*, vol. 56, no. 1, pp. 70–78, 1997.

[86] K. Leroy, A. Boutajangout, M. Authelet, J. R. Woodgett, B. H. Anderton, and J. P. Brion, "he active form of glycogen synthase kinase-3? is associated with granulovacuolar degeneration in neurons in Alzheimer's disease," *Acta Neuropathologica*, vol. 103, no. 2, pp. 91–99, 2002.

[87] D. P. Hanger, H. L. Byers, S. Wray et al., "Novel phosphorylation sites in tau from alzheimer brain support a role for casein kinase 1 in disease pathogenesis," *Journal of Biological Chemistry*, vol. 282, no. 32, pp. 23645–23654, 2007.

[88] A. Takashima, K. Noguchi, G. Michel et al., "Exposure of rat hippocampal neurons to amyloid β peptide (25–35) induces the inactivation of phosphatidyl inositol-3 kinase and the activation of tau protein kinase I/glycogen synthase kinase-3β," *Neuroscience Letters*, vol. 203, no. 1, pp. 33–36, 1996.

[89] J. J. Lucas, F. Hernández, P. Gómez-Ramos, M. A. Morán, R. Hen, and J. Avila, "Decreased nuclear beta-catenin, tau hyperphosphorylation and neurodegeneration in GSK-3beta conditional transgenic mice," *EMBO Journal*, vol. 20, no. 1, pp. 27–39, 2001.

[90] F. Hernández, J. Borrell, C. Guaza, J. Avila, and J. J. Lucas, "Spatial learning deficit in transgenic mice that conditionally over-express GSK-3β in the brain but do not form tau filaments," *J. Neurochem.*, vol. 83, no. 6, pp. 1529–1533, 2002.

[91] T. Engel, P. Goñi-Oliver, J. J. Lucas, J. Avila, and F. Hernández, "Chronic lithium administration to FTDP-17 tau and GSK-3? overexpressing mice prevents tau hyperphosphorylation and neurofibrillary tangle formation, but preformed neurofibrillary tangles do not revert," *Journal of Neurochemistry*, vol. 99, no. 6, pp. 1445–1455, 2006.

[92] O. A. Shipton, J. R. Leitz, J. Dworzak et al., "Tau protein is required for amyloid -induced impairment of hippocampal long-term potentiation," *Journal of Neuroscience*, vol. 31, no. 5, pp. 1688–1692, 2011.

[93] E. G. de Barreda, M. Pérez, P. G. Ramos et al., "Tau-knockout mice show reduced GSK3-induced hippocampal degeneration and learning deficits," *Neurobiology of Disease*, vol. 37, no. 3, pp. 622–629, 2010.

[94] M. Selikhova, D. R. Williams, P. A. Kempster, J. L. Holton, T. Revesz, and A. J. Lees, "A clinico-pathological study of subtypes in Parkinson's disease," *Brain*, vol. 132, no. 11, pp. 2947–2957, 2009.

[95] C. H. Williams-Gray, J. R. Evans, A. Goris et al., "The distinct cognitive syndromes of Parkinson's disease: 5 year follow-up of the CamPaIGN cohort," *Brain*, vol. 132, no. 11, pp. 2958–2969, 2009.

[96] C. A. Davie, "A review of Parkinson's disease," *British Medical Bulletin*, vol. 86, no. 1, pp. 109–127, 2008.

[97] J. B. J. Kwok, M. Hallupp, C. T. Loy et al., "GSK3B polymorphisms alter transcription and splicing in Parkinson's disease," *Annals of Neurology*, vol. 58, no. 6, pp. 829–839, 2005.

[98] J. Infante, I. García-Gorostiaga, P. Sánchez-Juan et al., "Synergistic effect of two oxidative stress-related genes (heme oxygenase-1 and GSK3β) on the risk of Parkinson's disease," *European Journal of Neurology*, vol. 17, no. 5, pp. 760–762, 2010.

[99] K. Kalinderi, L. Fidani, Z. Katsarou, J. Clarimón, S. Bostantjopoulou, and A. Kotsis, "GSK3β polymorphisms, MAPT H1 haplotype and Parkinson's disease in a Greek cohort," *Neurobiology of Aging*, vol. 32, no. 3, pp. 546.e1–546.e5, 2011.

[100] I. García-Gorostiaga, P. Sánchez-Juan, I. Mateo et al., "Glycogen synthase kinase-3β and tau genes interact in Parkinson's and Alzheimer's diseases," *Annals of Neurology*, vol. 65, no. 6, pp. 759–761, 2009.

[101] M. Nagao and H. Hayashi, "Glycogen synthase kinase-3beta is associated with Parkinson's disease," *Neuroscience Letters*, vol. 449, no. 2, pp. 103–107, 2009.

[102] J. J. Credle, J. L. George, J. Wills et al., "GSK-3β dysregulation contributes to Parkinson's-like pathophysiology with associated region-specific phosphorylation and accumulation of tau and α-synuclein," *Cell Death & Differentiation*, vol. 22, no. 5, pp. 838–851, 2015.

[103] J. Simón-Sánchez, C. Schulte, J. M. Bras et al., "Genome-wide association study reveals genetic risk underlying Parkinson's disease," *Nature Genetics*, vol. 41, no. 12, pp. 1308–1312, 2009.

[104] T. Duka, V. Duka, J. N. Joyce, and A. Sidhu, "α-Synuclein contributes to GSK-3β-catalyzed Tau phosphorylation in Parkinson's disease models," *FASEB Journal*, vol. 23, no. 9, pp. 2820–2830, 2009.

[105] J. Wills, J. Credle, T. Haggerty, J. H. Lee, A. W. Oaks, and A. Sidhu, "Tauopathic changes in the striatum of A53T α-synuclein mutant mouse model of Parkinson's disease," *PLoS One*, vol. 6, no. 3, p. e17953, 2011.

[106] M. B. H. Youdim and Z. Arraf, "Prevention of MPTP (N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) dopaminergic neurotoxicity in mice by chronic lithium: involvements of Bcl-2 and Bax," *Neuropharmacology*, vol. 46, no. 8, pp. 1130–1140, 2004.

[107] W. Wang, Y. Yang, C. Ying et al., "Inhibition of glycogen synthase kinase-3β protects dopaminergic neurons from MPTP toxicity," *Neuropharmacology*, vol. 52, no. 8, pp. 1678–1684, 2007.

[108] D. Lecamwasam, B. Synek, K. Moyles, and K. Ghose, "Chronic lithium neurotoxicity presenting as Parkinson's disease," *International Clinical Psychopharmacology*, vol. 9, no. 2, pp. 127–130, 1994.

[109] P. S. Klein and D. A. Melton, "A molecular mechanism for the effect of lithium on development," *Proceedings of the National Academy of Sciences*, vol. 93, no. 16, pp. 8455–8459, 1996.

[110] V. Stambolic, L. Ruel, and J. R. Woodgett, "Lithium inhibits glycogen synthase kinase-3 activity and mimics wingless signalling in intact cells," *Current Biology*, vol. 6, no. 12, pp. 1664–1669, 1996.

[111] J. R. Muñoz-Montaño, F. J. Moreno, J. Avila, and J. Diaz-Nido, "Lithium inhibits Alzheimer's disease-like tau protein phosphorylation in neurons," *FEBS Letters*, vol. 411, no. 2–3, pp. 183–188, 1997.

[112] W. J. Ryves and A. J. Harwood, "Lithium inhibits glycogen synthase kinase-3 by competition for magnesium," *Biochemical and Biophysical Research Communications*, vol. 280, no. 3, pp. 720–725, 2001.

[113] H. Eldar-Finkelman and A. Martinez, "GSK-3 inhibitors: preclinical and clinical focus on CNS," *Frontiers in Molecular Neuroscience*, vol. 4, p. 32, 2011.

[114] L. Freland and J. M. Beaulieu, "Inhibition of GSK3 by lithium, from single molecules to signaling networks," *Frontiers in Molecular Neuroscience*, vol. 5, p. 14, 2012.

[115] R. S. Jope, "Lithium and GSK-3: one inhibitor, two inhibitory actions, multiple outcomes," *Trends in Pharmacological Sciences*, vol. 24, no. 9, pp. 441–443, 2003.

[116] A. V. Franklin, M. K. King, V. Palomo, A. Martinez, L. L. McMahon, and R. S. Jope, "Glycogen synthase kinase-3 inhibitors reverse deficits in long-term potentiation and cognition in fragile X mice," *Biological Psychiatry*, vol. 75, no. 3, pp. 198–206, 2014.

[117] M. K. King and R. S. Jope, "Lithium treatment alleviates impaired cognition in a mouse model of fragile X syndrome," *Genes, Brain and Behavior*, vol. 12, no. 7, pp. 723–731, 2013.

[118] T. A. Comery, J. B. Harris, P. J. Willems et al., "Abnormal dendritic spines in fragile X knockout mice: maturation and pruning deficits," *Proceedings of the National Academy of Sciences*, vol. 94, no. 10, pp. 5401–5404, 1997.

[119] S. A. Irwin, B. Patel, M. Idupulapati et al., "Abnormal dendritic spine characteristics in the temporal and visual cortices of patients with fragile-X syndrome: a quantitative examination," *Am. J. Med. Genet.*, vol. 98, no. 2, pp. 161–167, 2001.

[120] S. A. Irwin, M. Idupulapati, M. E. Gilbert et al., "Dendritic spine and dendritic field characteristics of layer V pyramidal neurons in the visual cortex of fragile-X knockout mice," *American Journal of Medical Genetics*, vol. 111, no. 2, pp. 140–146, 2002.

[121] I. J. Weiler and W. T. Greenough, "Synaptic synthesis of the Fragile X protein: possible involvement in synapse maturation and elimination," *American Journal of Medical Genetics*, vol. 83, no. 4, pp. 248–252, 1999.

[122] C. H. Choi, B. P. Schoenfeld, A. J. Bell et al., "Pharmacological reversal of synaptic plasticity deficits in the mouse model of Fragile X syndrome by group II mGluR antagonist or lithium treatment," *Brain Research*, vol. 1380, pp. 106–119, 2011.

[123] Z. H. Liu, D. M. Chuang, and C. B. Smith, "Lithium ameliorates phenotypic deficits in a mouse model of fragile X syndrome," *International Journal of Neuropsychopharmacology*, vol. 14, no. 05, pp. 618–630, 2011.

[124] S. N. Bajestan, A. H. Sabouri, M. Nakamura et al., "Association of AKT1 haplotype with the risk of schizophrenia in Iranian population," *American Journal of Medical Genetics Part B: Neuropsychiatric Genetics*, vol. 141B, no. 4, pp. 383–386, 2006.

[125] E. S. Emamian, "AKT/GSK3 signaling pathway and schizophrenia," *Frontiers in Molecular Neuroscience*, vol. 5, 2012.

[126] E. S. Emamian, D. Hall, M. J. Birnbaum, M. Karayiorgou, and J. A. Gogos, "Convergent evidence for impaired AKT1-GSK3β signaling in schizophrenia," *Nature Genetics*, vol. 36, no. 2, pp. 131–137, 2004.

[127] C. Dobson-Stone, P. Polly, M. S. Korgaonkar et al., "GSK3B and MAPT polymorphisms are associated with grey matter and intracranial volume in healthy individuals," *PLoS One*, vol. 8, no. 8, p. e71750, 2013.

[128] M. Li, Y. Mo, X. J. Luo et al., "Genetic association and identification of a functional SNP at GSK3β for schizophrenia susceptibility," *Schizophrenia Research*, vol. 133, no. 1–3, pp. 165–171, 2011.

[129] H. Alimohamad, L. Sutton, J. Mouyal, N. Rajakumar, and W. J. Rushlow, "The effects of antipsychotics on β-catenin, glycogen synthase kinase-3 and dishevelled in the ventral midbrain of rats," *Journal of Neurochemistry*, vol. 95, no. 2, pp. 513–525, 2005.

[130] N. Kozlovsky, S. Amar, R. H. Belmaker, and G. Agam, "Psychotropic drugs affect Ser9-phosphorylated GSK-3β protein levels in rodent frontal cortex," *International Journal of Neuropsychopharmacology*, vol. 9, no. 03, pp. 337–342, 2006.

[131] R. Gomez-Sintes, A. Bortolozzi, F. Artigas, and J. J. Lucas, "Reduced striatal dopamine DA D2 receptor function in dominant-negative GSK-3 transgenic mice," *European Neuropsychopharmacology*, vol. 24, no. 9, pp. 1524–1533, 2014.

[132] J. M. Beaulieu, T. D. Sotnikova, S. Marion, R. J. Lefkowitz, R. R. Gainetdinov, and M. G. Caron, "An Akt/β-Arrestin 2/PP2A signaling complex mediates dopaminergic neurotransmission and behavior," *Cell*, vol. 122, no. 2, pp. 261–273, 2005.

[133] J. M. Beaulieu, T. D. Sotnikova, W. D. Yao et al., "Lithium antagonizes dopamine-dependent behaviors mediated by an AKT/glycogen synthase kinase 3 signaling cascade," *Proceedings of the National Academy of Sciences*, vol. 101, no. 14, pp. 5099–5104, 2004.

[134] N. Craddock and L. Forty, "Genetics of affective (mood) disorders," *European Journal of Human Genetics*, vol. 14, no. 6, pp. 660–668, 2006.

[135] R. Silva, A. R. Mesquita, J. Bessa et al., "Lithium blocks stress-induced changes in depressive-like behavior and hippocampal cell fate: the role of glycogen-synthase-kinase-3β," *Neuroscience*, vol. 152, no. 3, pp. 656–669, 2008.

[136] F. Higuchi, S. Uchida, H. Yamagata et al., "Hippocampal microRNA-124 enhances chronic stress resilience in mice," *Journal of Neuroscience*, vol. 36, no. 27, pp. 7253–7267, 2016.

[137] Q. Mao, X. Gong, C. Zhou et al., "Up-regulation of SIRT6 in the hippocampus induced rats with depression-like behavior via the block Akt/GSK3β signaling pathway," *Behavioural Brain Research*, vol. 323, pp. 38–46, 2017.

[138] A. G. Moraitis, T. Block, D. Nguyen, and J. K. Belanoff, "The role of glucocorticoid receptors in metabolic syndrome and psychiatric illness," *Journal of Steroid Biochemistry and Molecular Biology*, vol. 165, pp. 114–120, 2017.

[139] H. Weina, N. Yuhu, H. Christian, L. Birong, S. Feiyu, and W. Le, "Liraglutide attenuates the depressive- and anxiety-like behaviour in the corticosterone induced depression model via improving hippocampal neural plasticity," *Brain Research*, vol. 1694, pp. 55–62, 2018.

[140] B. Inkster, T. E. Nichols, P. G. Saemann et al., "Association of GSK3β polymorphisms with brain structural changes in major depressive disorder," *Archives of General Psychiatry*, vol. 66, no. 7, pp. 721–728, 2009.

[141] A. Levchenko, I. S. Losenkov, N. M. Vyalova et al., "The functional variant rs334558 of GSK3B is associated with remission in patients with depressive disorders," *Pharmacogenomics and Personalized Medicine*, vol. Volume 11, pp. 121–126, 2018.

[142] X. Li, W. Zhu, M. S. Roh, A. B. Friedman, K. Rosborough, and R. S. Jope, "In vivo regulation of glycogen synthase kinase-3β (GSK3β) by serotonergic activity in mouse brain," *Neuropsychopharmacology*, vol. 29, no. 8, pp. 1426–1431, 2004.

[143] A. S. Ferreira, N. R. B. Raposo, P. C. Sallet et al., "Lower phosphorylated glycogen synthase kinase-3B levels in platelets of patients with schizophrenia: increment by olanzapine treatment," *European Archives of Psychiatry and Clinical Neuroscience*, vol. 265, no. 2, pp. 167–170, 2015.

[144] O. Kaidanovich-Beilin, A. Milman, A. Weizman, C. G. Pick, and H. Eldar-Finkelman, "Rapid antidepressive-like activity of specific glycogen synthase kinase-3 inhibitor and its effect on β-catenin in mouse hippocampus," *Biological Psychiatry*, vol. 55, no. 8, pp. 781–784, 2004.

[145] T. D. Gould, H. Einat, R. Bhat, and H. K. Manji, "AR-A014418, a selective GSK-3 inhibitor, produces antidepressant-like effects in the forced swim test," *International Journal of Neuropsychopharmacology*, vol. 7, no. 4, pp. 387–390, 2004.

[146] R. S. Fisher, J. H. Cross, J. A. French et al., "Operational classification of seizure types by the International League Against Epilepsy: position paper of the ILAE Commission for Classification and Terminology," *Epilepsia*, vol. 58, no. 4, pp. 522–530, 2017.

[147] E. M. Goldberg and D. A. Coulter, "Mechanisms of epileptogenesis: a convergence on neural circuit dysfunction," *Nature Reviews Neuroscience*, vol. 14, no. 5, pp. 337–349, 2013.

[148] M. Pandolfo, "Genetics of epilepsy," *Seminars in Neurology*, vol. 31, no. 05, pp. 506–518, 2011.

[149] A. J. Becker, "Review: Animal models of acquired epilepsy: insights into mechanisms of human epileptogenesis," *Neuropathology and Applied Neurobiology*, vol. 44, no. 1, pp. 112–129, 2018.

[150] C. Y. Lee, T. Jaw, H. C. Tseng, I. C. Chen, and H. H. Liou, "Lovastatin modulates glycogen synthase kinase-3β pathway and inhibits mossy fiber sprouting after pilocarpine-induced status epilepticus," *PLoS One*, vol. 7, no. 6, p. e38789, 2012.

[151] W. J. Huang, F. F. Tian, J. M. Chen et al., "GSK-3β may be involved in hippocampal mossy fiber sprouting in the pentylenetetrazole-kindling model," *Molecular Medicine Reports*, vol. 8, no. 5, pp. 1337–1342, 2013.

[152] M. Bhowmik, R. Khanam, N. Saini, and D. Vohora, "Activation of AKT/GSK3β pathway by TDZD-8 attenuates kainic acid induced neurodegeneration but not seizures in mice," *Neurotoxicology*, vol. 46, pp. 44–52, 2015.

[153] G. Gangarossa, S. Sakkaki, P. Lory, and E. Valjent, "Mouse hippocampal phosphorylation footprint induced by generalized seizures: focus on ERK, mTORC1 and Akt/GSK-3 pathways," *Neuroscience*, vol. 311, pp. 474–483, 2015.

[154] D. M. Talos, L. M. Jacobs, S. Gourmaud et al., "Mechanistic target of rapamycin complex 1 and 2 in human temporal lobe epilepsy," *Annals of Neurology*, vol. 83, no. 2, pp. 311–327, 2018.

[155] C. Liu, J. Russin, C. Heck et al., "Dysregulation of PINCH signaling in mesial temporal epilepsy," *Journal of Clinical Neuroscience*, vol. 36, pp. 43–52, 2017.

[156] S. Goodenough, S. Conrad, T. Skutella, and C. Behl, "Inactivation of glycogen synthase kinase-3β protects against kainic acid-induced neurotoxicity in vivo," *Brain Research*, vol. 1026, no. 1, pp. 116–125, 2004.

[157] S. Kelly, H. Zhao, G. Hua Sun et al., "Glycogen synthase kinase 3β inhibitor Chir025 reduces neuronal death resulting from oxygen-glucose deprivation, glutamate excitotoxicity, and cerebral ischemia," *Experimental Neurology*, vol. 188, no. 2, pp. 378–386, 2004.

[158] N. Aourz, A.-S. K. Serruys, J. N. Chabwine et al., "Identification of GSK-3 as a potential therapeutic entry point for epilepsy," *ACS Chemical Neuroscience*, vol. 10, no. 4, pp. 1992–2003, 2018.

[159] M. Bellesi, L. Passamonti, M. Silvestrini, M. Bartolini, and L. Provinciali, "Non-convulsive status epilepticus during lithium treatment at therapeutic doses," *Neurological Sciences*, vol. 26, no. 6, pp. 444–446, 2006.

[160] S. Shukla, S. Mukherjee, and P. Decina, "Lithium in the treatment of bipolar disorders associated with epilepsy: an open study," *Journal of Clinical Psychopharmacology*, vol. 8, no. 3, pp. 201–204, 1988.

[161] T. Engel, R. Gómez-Sintes, M. Alves et al., "Bi-directional genetic modulation of GSK-3β exacerbates hippocampal neuropathology in experimental status epilepticus," *Cell Death & Disease*, vol. 9, no. 10, p. 969, 2018.

[162] M. Urbanska, P. Kazmierska-Grebowska, T. Kowalczyk et al., "GSK3β activity alleviates epileptogenesis and limits GluA1 phosphorylation," *EBioMedicine*, vol. 39, pp. 377–387, 2019.

[163] A. P. Saraswati, S. M. Ali Hussaini, N. H. Krishna, B. N. Babu, and A. Kamal, "Glycogen synthase kinase-3 and its inhibitors: potential target for various therapeutic conditions," *European Journal of Medicinal Chemistry*, vol. 144, pp. 843–858, 2018.

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Research Article

Neuroplasticity and Neuroprotective Effect of Treadmill Training in the Chronic Mouse Model of Parkinson's Disease

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Physical training confers protection to dopaminergic neurons in rodent models of parkinsonism produced by neurotoxins. The sparing effect of physical training on dopaminergic neurons can be tested with training applied during chronic MPTP treatment, while the neurorestorative effect when training is applied after completing such treatment. In this study, the effect of the onset of training respective to chronic MPTP treatment was specifically addressed. Three groups of mice were injected with 10 doses of MPTP (12.5 mg/kg/injection) over 5 weeks. The first group remained sedentary; the second one underwent early onset training, which started 1 week before commencing MPTP treatment, continued throughout 5 weeks of treatment and 4 weeks thereafter; the third group underwent late-onset training of the same length and intensity as the former group, except that it started immediately after the end of MPTP treatment. Two groups served as controls: a saline-injected group that remained sedentary and saline-injected group, which underwent the same training as the early and late-onset training groups. Both early and late-onset physical training saved almost all nigral and VTA dopaminergic neurons, prevented inflammatory response, and increased the BDNF and GDNF levels to a similar extent. From these results one may conclude that early and late-onset training schedules were equipotent in their neuroprotective effect and that the mechanism of neuroprotection was similar. The sparing effect of early onset training may be satisfactorily explained by assuming that the increased level of BDNF and GDNF prevented the degeneration of dopaminergic neurons. To explain a similar number of dopaminergic neurons detected at the end of the early and late-onset training, one should additionally assume that the former training schedule induced neurogenesis. Results of this study support the view that physical activity may be neuroprotective even at a more advanced stage of PD and justify starting physical activity at any point of the disease.

1. Introduction

Since most of the symptoms of Parkinson's disease (PD) are caused by a deficiency of dopamine (DA) in the brain, many drugs are designed to temporarily supplement the level of this neurotransmitter or mimic its action. Currently available treatments are only symptom targeting, their effectiveness decreases with the progression of the disease, and they have many side effects. All these facts highlight the need to seek new treatments supporting the pharmacological therapy of PD. One nonpharmacological approach that supports PD patients is physical exercise. Physical activity alleviates and slows down the development of PD symptoms as demonstrated by improvement in muscle strength, balance, speed and length of stride, daily activities, general wellbeing, and the extended time of independence [1–7]. However, these may be unspecific effects of physical activity, and question arises whether this activity is also a disease-modifying factor. This is suggested by the epidemiological studies, which demonstrated an inverse relationship between physical exercise and the risk of the occurrence and development of PD [8, 9].

The experimental data show that the goal-based exercise and aerobic training can enhance brain plasticity, which plays a key role in improving motor and cognitive functions in people with PD [10]. Current knowledge about the

mechanism of the neuroprotective properties of physical training on the functional state of dopaminergic neurons is based on data obtained from animal studies. Many of these studies suggest that exercise can prevent or slow down neurodegenerative processes and rebuild disturbed signaling pathways [11–13]. The neuroprotective effect of physical training is associated with the activation of the neurotrophin signaling pathways [14, 15], synaptogenesis [16], angiogenesis [17] and neurogenesis strengthening [18, 19], reduction of inflammatory processes [20–22], and stabilization of calcium homeostasis [23]. Physical activity prevents the loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) and also increases the DA level and the sensitivity of its receptor [24, 25]. In the literature, there are a number of reports that physical exercise leads to increased expression of endogenous trophic factors and reduced expression of proinflammatory markers, thereby reducing the vulnerability of dopaminergic neurons to oxidative stress and death. The study performed by Lau et al. [26] has revealed that the neuronal and behavioral recovery produced by exercise in the chronic 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of PD was associated with an improved mitochondrial function and an increase in the brain region-specific levels of brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF). In turn, Zhao et al. [27] have shown that vibration training could significantly increase the number of nigrostriatal dopaminergic neurons and the levels of striatal DA and BDNF in the MPTP mice. Using the 6-hydroxydopamine (6-OHDA) model of PD in rats, Tajiri et al. [25] have demonstrated the effects of forced treadmill training on the nigrostriatal dopaminergic projection, neurotrophic factors level, and motor skills of experimental animals.

Recent evidence suggests that physical activity can lead to the reduction of inflammatory processes in the brain of PD patients in the course of neurodegenerative diseases [28]. In a progressive MPTP mouse model of PD, Sconce et al. [20] have noticed recovery of motor skills, increased vesicular monoamine transporter 2 (VMAT2) expression, decreased glycosylated dopamine transporter (DAT) expression, reduced levels of vesicular glutamate transporter 1 (VGLUT1), glutamate transporter-1 (GLT-1), and lower levels of the inflammatory marker, component 3 of the nuclear factor of activated T-cells (NFATc3), and of the astrocytic marker, glial fibrillary acidic protein (GFAP), in MPTP/exercised mice as compared to MPTP mice without exercise. In turn, a four-week swimming training attenuated motor and cognitive impairment, prevented the increase in reactive oxygen species (ROS) and interleukin 1 beta (IL-1β) levels, glutathione S-transferase (GST), and glutathione reductase (GR) activity, prevented the inhibition of glutathione peroxidase (GPx) activity, restored the levels of DA and its metabolites in the striatum of mice administered with 6-OHDA [21]. Similarly, Jang et al. [29] have noticed that 8-week treadmill training after chronic MPTP administration in mice restores motor coordination abilities, increases tyrosine hydroxylase (TH) level in the striatum and SNpc, and decreases α-synuclein expression in the striatum resulting in downregulation of toll-like receptor 2 (TLR2) signaling molecules such as myeloid differentiation primary response 88 (MyD88), tumor necrosis factor receptor-associated factor 6 (TRAF6), and phosphorylated transforming growth factor-β-activated protein kinase 1 (p-TAK1).

In animal models of PD, the neuroprotective effect of physical exercise was examined when training was applied before, during, and after parkinsonism inducing treatment. In addition, these studies have used an acute [25, 30–33] or chronic regime of neurotoxin administration [34]. The studies of Ahmad et al. [34] and Lau et al. [26] demonstrated that physical exercise applied during application of the MPTP neurotoxin prevents, at least partly, the loss of TH-positive nigrostriatal neurons and likely those in the ventral tegmental area (VTA), while the same dose and duration of neurotoxin administration causes moderate loss of these neurons in sedentary mice. Studies on the restorative effect of exercise applied after neurotoxin application show disparate results: from no effect to partial or complete recovery of the number of TH-positive nigrostriatal neurons.

Although these data suggest that physical exercise is effective regardless of whether it starts, there are no data that compare the effectiveness of different physical training onsets. Thus, the aim of this study was to examine precisely the effect of only one factor: the timing of exercise application with respect to the intoxication period. For this purpose, the effects of treadmill training applied before, during, and after induction of the chronic model of PD were compared with the effects of treadmill training of the same intensity and duration applied after completing the MPTP treatment. We applied the chronic PD model in mice [34] in which the induction of parkinsonism takes 5 weeks, during which time 10 injections of MPTP are administered. Such treatment causes neurological deficits resembling PD. Unlike the most commonly used acute and subacute MPTP treatments, after which neurological and behavioral deficits soon spontaneously reverse, the effects of this chronic PD model last at least 6 months. To elucidate the role of exercise alone, training was applied in non-MPTP-treated mice. In order to gain insight into the mechanisms of neuroprotection and neurorestoration, the levels of neurotrophic factors and markers of inflammation were examined. To our knowledge, such comparison of the effect of exercise timing relative to MPTP treatment has not been performed in one and the same study yet.

2. Methods

2.1. Animals and Treatments. All animal experimental procedures used in this study were approved by the First Warsaw Local Ethics Committee for Animal Experimentation and carried out in accordance with the Polish Law on the Protection of Animals and National Institute of Health's Guide for Care and Use of Laboratory Animals (publication no. 85-23, revised 1985) and the European Union Council Directive (63/2010/EU). Studies were performed on 12-week-old male C57BL/6J mice purchased from the Medical University of Bialystok (Poland) and delivered to the Nencki Institute of Experimental Biology 1 month prior to experiments. The

Table 1: Experimental groups used in the study.

Group	Description of the treatment	Number of mice (n)	Treadmill training	The total duration of the experiment (weeks)
C (Control)	Saline (s.c.) and DMSO (i.p.) injections	15	**NO**	10
CTT (Control + treadmill training)	Saline (s.c.) and DMSO (i.p.) injections, 10 weeks of treadmill training	11	**YES**	10
M (MPTP)	MPTP (s.c.) and probenecid (i.p.) injections	15	**NO**	10
METT (MPTP + early onset treadmill training)	MPTP (s.c.) and probenecid (i.p.) injections 10 weeks of treadmill training started 1 week before the intoxication	13	**YES**	10
MLTT (MPTP + late-onset treadmill training)	MPTP (s.c.) and probenecid (i.p.) injections 10 weeks of treadmill training started immediately after the intoxication	13	**YES**	16

mice were housed at a 12 : 12 h dark light cycle, with constant temperature and humidity (23 ± 1°C, 55 ± 5%), and had free access to both food and water. For the chronic paradigm of MPTP administration, mice received 10 subcutaneous (s.c.) doses of MPTP (12.5 mg/kg in saline; Santa Cruz Biotechnology, Cat. No. sc-206178; Axon Medchem, Cat. No. 1075) in combination with intraperitoneal (i.p.) injections of probenecid (250 mg/kg in dimethyl sulfoxide, DMSO; Sigma-Aldrich, Cat. No. P8761) for 5 weeks [34]. Control mice received saline and DMSO injections only. Mice were subdivided into a control group (C, $n = 15$), control treadmill training group (CTT, $n = 11$), MPTP-injected group (M, $n = 15$), MPTP-injected trained group, which started treadmill training 1 week before the induction of parkinsonism (METT, MPTP + early onset treadmill training, $n = 13$), and MPTP-injected trained group, which started treadmill training immediately after the induction of parkinsonism (MLTT, MPTP + late-onset treadmill training, $n = 13$). Table 1 presents the experimental groups used in the study. Animals from the groups assigned to treadmill training performed exercises 40 min/day, 5 days/week for 10 weeks. The 40-minute long treadmill protocol consisted of 5 steps: 5 minutes at 10 cm/s, 5 minutes at 15 cm/s, 20 minutes at 20 cm/s, 5 minutes at 25 cm/s, and 5 minutes at 20 cm/s [34].

2.2. Immunohistochemistry. The brains of five animals from each group were used for immunohistochemical staining. The animals were deeply anesthetized with Vetbutal and transcardially perfused with cold (4°C) phosphate-buffered saline (PBS) containing 5 IU of heparin per 1 ml of buffer, followed by 4% paraformaldehyde (PVA) in PBS and 5% glycerol with 2% DMSO in PBS. The brains were removed, placed for 1 hour (h) in 4% PVA, and then immersed for cryoprotection in 10% glycerol with 2% DMSO (24 h) and subsequently in 20% glycerol with 2% DMSO (24 h). Forty μm thick frozen sections were washed in PBS (3 × 5 min), incubated with 1% H_2O_2 for 30 min at room temperature (RT) to block endogenous peroxidases, and blocked for 1 h at RT in a 5% normal serum solution (NRS). Sections were then incubated with primary antibody diluted in PBS containing 1% bovine serum albumin (BSA), 0.3% Triton X, 5% NRS for 1 h at RT, and overnight at 4°C. In the subsequent step, sections were washed in PBS (3 × 5 min) and incubated for 1 h at RT with secondary antibody diluted to working concentrations in PBS with 5% NRS, 1% BSA, and 0.3% Triton X-100 (see Table 2). Incubation with fluorescent antibodies was carried out in the dark to prevent photobleaching. When 3,3′-diaminobenzidine (DAB, 0.025%) was used as a chromogen, visualization was preceded by a 1-hour incubation at RT with Vectastain ABC kit (Vector Laboratories). Sections were mounted onto slides using UltraCruz® Aqueous Mounting Medium with 4′,6-diamidyno-2-fenyloindol (DAPI, Santa Cruz Biotechnology) or DePeX (SERVA Electrophoresis GmbH) for fluorescent and enzymatic labels, respectively.

2.3. Densitometric Analysis of Dopaminergic Neurons. Sections were imaged using a Nikon Eclipse Ni-E microscope. The computerized densitometric image analysis (NIS-Elements BR4.30.00, Nikon Instruments) of TH-immunoreactive (-ir) neurons was performed in SNpc (bregma −3.15 to −3.51) and VTA (bregma −2.79 to −3.07) identified in accordance with the Mouse Brain Stereotaxic Atlas [35]. Regions of interest were outlined using the software's X-Y plotting system that measures the square area (mm^2) of the marked frame, and TH-ir neurons were counted at 400x magnification. The following criteria were used in the quantitative analysis: neuronal somata were TH-ir, and the cell nucleus and the proximal segment of one or two dendrites was well visible within the counting frame. These criteria enabled the exclusion of noncomplete remnants of neurons. Cell counts per section were then corrected with Abercrombie's formula [36], and the packing density of dopaminergic neurons was calculated as a function of the rostrocaudal level and of location within the VTA and SNpc by using the obtained cell counts and the square area of the marked frames in each analyzed section.

2.4. Enzyme-Linked Immunosorbent Assay. Mice were sacrificed by cervical dislocation, the brains were rapidly removed, and the striatum and midbrain containing substantia nigra (SN) were dissected. Tissue samples were weighed and homogenized in 20 volumes of ice-cold homogenization buffer to wet tissue weight. After centrifugation, protein

Table 2: List of antibodies, their suppliers, and dilutions used in this study.

Primary antibody	Supplier	Primary antibody dilution	Secondary antibody	Supplier	Secondary antibody dilution
Antityrosine hydroxylase (TH, AB152)	Merck	1 : 1000	Biotinylated Goat Anti-Rabbit IgG Antibody (BA-1000)	Vector Laboratories	1 : 200
Antivesicular monoamine transporter 2 (VMAT 2, sc-15314)	Santa Cruz Biotechnology	1 : 200	Biotinylated Goat Anti-Rabbit IgG Antibody (BA-1000)	Vector Laboratories	1 : 200
Antiglial cell-derived neurotrophic factor (GDNF, sc-328)	Santa Cruz Biotechnology	1 : 500	Biotinylated Goat Anti-Rabbit IgG Antibody (BA-1000)	Vector Laboratories	1 : 200
Anti-brain-derived neurotrophic factor (BDNF, sc-20981)	Santa Cruz Biotechnology	1 : 500	Biotinylated Goat Anti-Rabbit IgG Antibody (BA-1000)	Vector Laboratories	1 : 200
Anti-CD11b (MCA711G)	Bio-Rad	1 : 200	Goat anti-Rat IgG (H+L) Cross-Adsorbed Antibody, Alexa Fluor 488 (A-11006)	ThermoFisher	1 : 1000
Antiglial fibrillary acidic protein (GFAP, Z0334)	Dako	1 : 1000	F(ab')2-Goat anti-Rabbit IgG (H + L) Cross-Adsorbed Antibody, Alexa Fluor 488 (A-11070)	ThermoFisher	1 : 1000
Anti-ionized calcium binding adaptor molecule 1 (Iba1, ab5076)	Abcam	1 : 500	Rabbit Anti-Goat IgG Antibody, HRP conjugate (AP106P)	Merck	1 : 500

concentrations in the supernatants were determined using Protein Assay Dye Reagent Concentrate (Bio-Rad). Enzyme-linked immunosorbent assay (ELISA) was used to quantify IL-1β (ab100705, Abcam), GFAP (NS830, Merck), BDNF (CYT306, Merck), and GDNF (e0043m, EIAab) concentrations in the brain regions of interest. All steps of quantification were performed according to the manufacturer's recommendations. Standards and samples were added to the microtiter plate in triplicate, the optical density of each well was determined at 450 nm (Thermo LabSystems, Multiskan RC Microplate Reader), and protein concentrations were calculated from the standard curve. Results were expressed in pg/ml for BDNF, GDNF, and Il-1β or in ng/ml for GFAP.

2.5. Statistical Analysis. All data sets are expressed as group mean ± SD. A comparison between experimental groups was carried out with 1-way or 2-way analysis of variance (ANOVA) followed by post hoc comparison using Newman-Keuls test. The differences between groups were considered statistically significant for $p \leq 0.05$ or below. Statistical analysis was performed using STATISTICA 12 software (StatSoft Polska, http://www.StatSoft.pl).

3. Results

3.1. The Effect of Chronic MPTP Administration and Physical Training on Dopaminergic Neurons Plasticity. TH is the first and rate-limiting enzyme involved in the biosynthesis of catecholamines from tyrosine. From this point, TH is considered as a useful marker of dopaminergic neurons. In order to calculate the number of TH-ir neurons, the brain sections of animal groups, C ($n = 5$), CTT ($n = 4$), M ($n = 5$), METT ($n = 3$), and MLTT ($n = 3$), were used for anti-TH immunohistochemical staining. The focus was placed on the SNpc and VTA, structures where the dopaminergic neurons are located. A substantial reduction in TH staining intensity was observed in both SNpc (Figure 1(a)) and VTA (Figure 1(b)) of MPTP-treated mice (M group) when compared to any other examined group. In addition, a computer-assisted densitometric analysis of packing density of dopaminergic neurons in these structures was performed separately for the left and right hemisphere. In all experimental groups, there were no significant differences in the mean number of TH-ir neurons between the two hemispheres, therefore Figures 1(c) and 1(d) present averaged data of both hemispheres. The densitometric analysis showed a 65% decrease in the number of TH-positive neurons in the SNpc (Figure 1(c); two-way ANOVA, $p < 0.05$) and a 45% decrease in the VTA (Figure 1(d); two-way ANOVA $p < 0.001$) in animals injected with MPTP compared to the control group. Reduction in the number of dopaminergic neurons in the SNpc and VTA of MPTP-treated mice is comparable to the extent of degeneration of dopaminergic neurons observed in the human brain of PD patients. In turn, in both MPTP groups with treadmill training, there was an increase in the number of TH-positive neurons in the SNpc and VTA compared to MPTP sedentary animals. Mice that started training on the treadmill 1 week before the induction of parkinsonism (METT group) showed a 50% increase in TH-positive neurons in the SNpc and 42% in the VTA, while mice that started training on the treadmill shortly after the induction of parkinsonism (MLTT group) showed 55% increase in the number of dopaminergic neurons in the SNpc and 38% in the VTA compared to sedentary MPTP animals (group M).

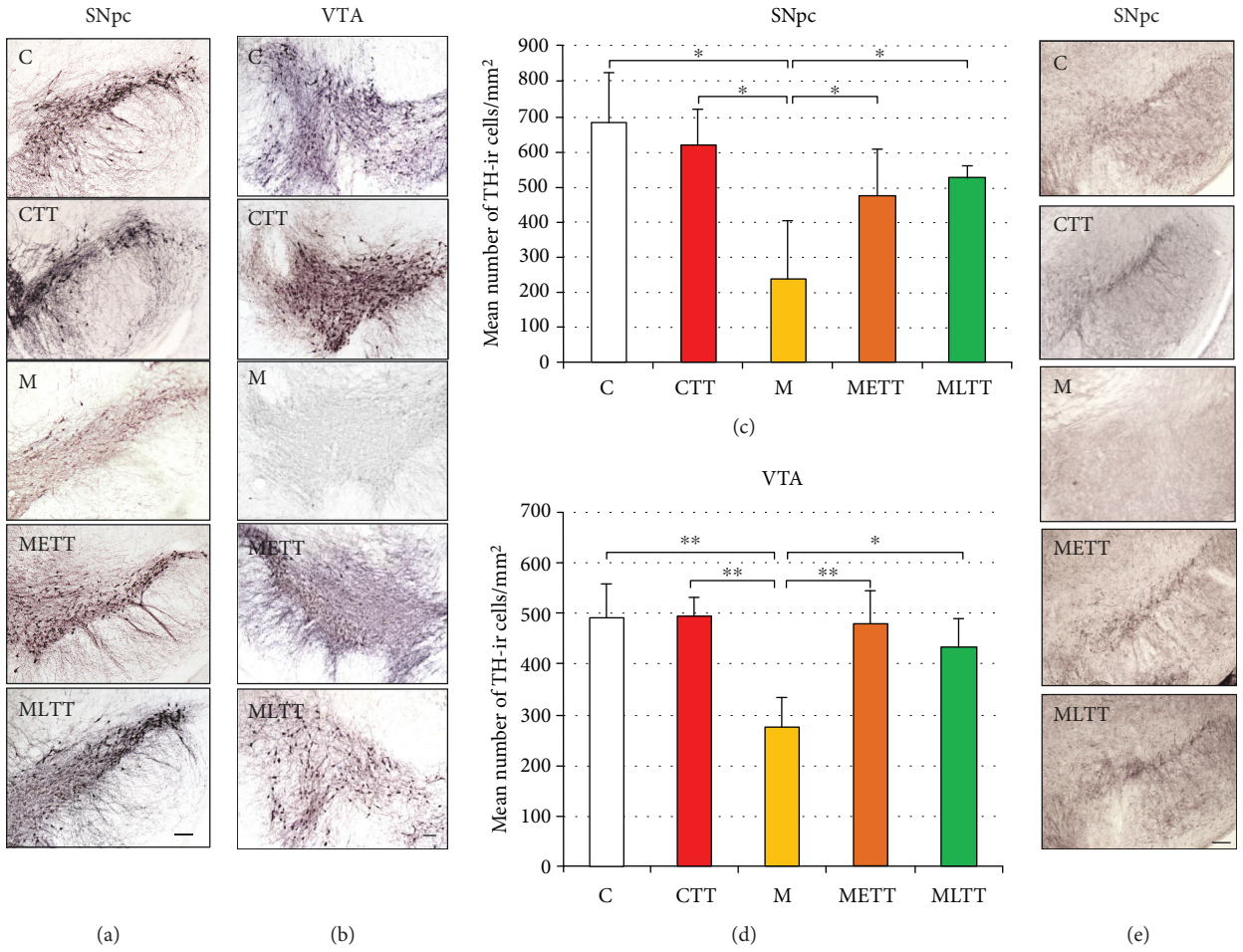

Figure 1: Packing density of TH-immunoreactive neurons in the midbrain regions in the five experimental groups. Representative microphotographs of TH immunohistochemical staining in the substantia nigra pars compacta (SNpc) (a) and ventral tegmental area (VTA) (b), mean packing density of TH-immunoreactive neurons in the SNpc (c) and VTA (d), and microscopic images of VMAT2 immunohistochemical staining in the SNpc (e). C: control; CTT: control + treadmill training; M: treatment with MPTP; METT: MPTP treatment + early onset treadmill training; MLTT: MPTP treatment + late-onset treadmill training group. Scale bar: 100 μm. Statistical comparisons were performed with two-way ANOVA followed by Newman-Keuls *post hoc* test; $^{**}p < 0.001$, $^{*}p < 0.05$.

A 10-week treadmill training either used before (METT group) or after (MLTT group) MPTP administration prevented the loss of TH-ir cells in both SNpc and VTA (METT vs. CTT group; MLTT vs. CTT group, Figures 1(c) and 1(d)). There was no difference in the number of TH-ir cells in both analyzed structures (METT vs. MLTT group, Figures 1(c) and 1(d)) depending on whether the treadmill training started before or after MPTP administration. Neuronal function was also assessed using anti-VMAT2 staining. VMAT2, which in addition to TH is considered to be one of the useful markers of dopaminergic neurons, pumps cytosolic dopamine into synaptic vesicles in an ATP-dependent way. A lower intensity of immunostaining against this protein was found in the SNpc in the M group as compared to the other experimental groups (Figure 1(e)) thus confirming the results obtained for anti-TH staining.

3.2. The Effect of Chronic MPTP Administration and Physical Training on the Level of BDNF and GDNF. Neurotrophins, such as BDNF and GDNF, that play an important role in the differentiation, trophic support, and survival of dopaminergic neurons are considered to contribute to the neuroprotective effect of exercise [37–39].

The impact of physical training and MPTP administration on BDNF and GDNF expression was explored using immunohistochemistry staining against these proteins in the SNpc and an ELISA assay in the midbrain and in the striatum. The immunohistochemical staining in the SNpc showed a significant increase in staining intensity against BDNF (Figure 2(a)) and GDNF (Figure 2(b)) in the METT and MLTT groups as compared to the CTT group as well as to the C group. Additionally, anti-GDNF staining showed a slight increase in intensity in the M group.

ELISA assay revealed significant upregulation of BDNF concentration in the midbrain both in the METT and MLTT groups as compared to the other experimental groups (Figure 2(c), $p < 0.001$) and higher concentration of GDNF in the METT and MLTT groups in comparison to the M group (Figure 2(d), $p < 0.05$). In the striatum, statistical analysis revealed that 10 weeks of treadmill training

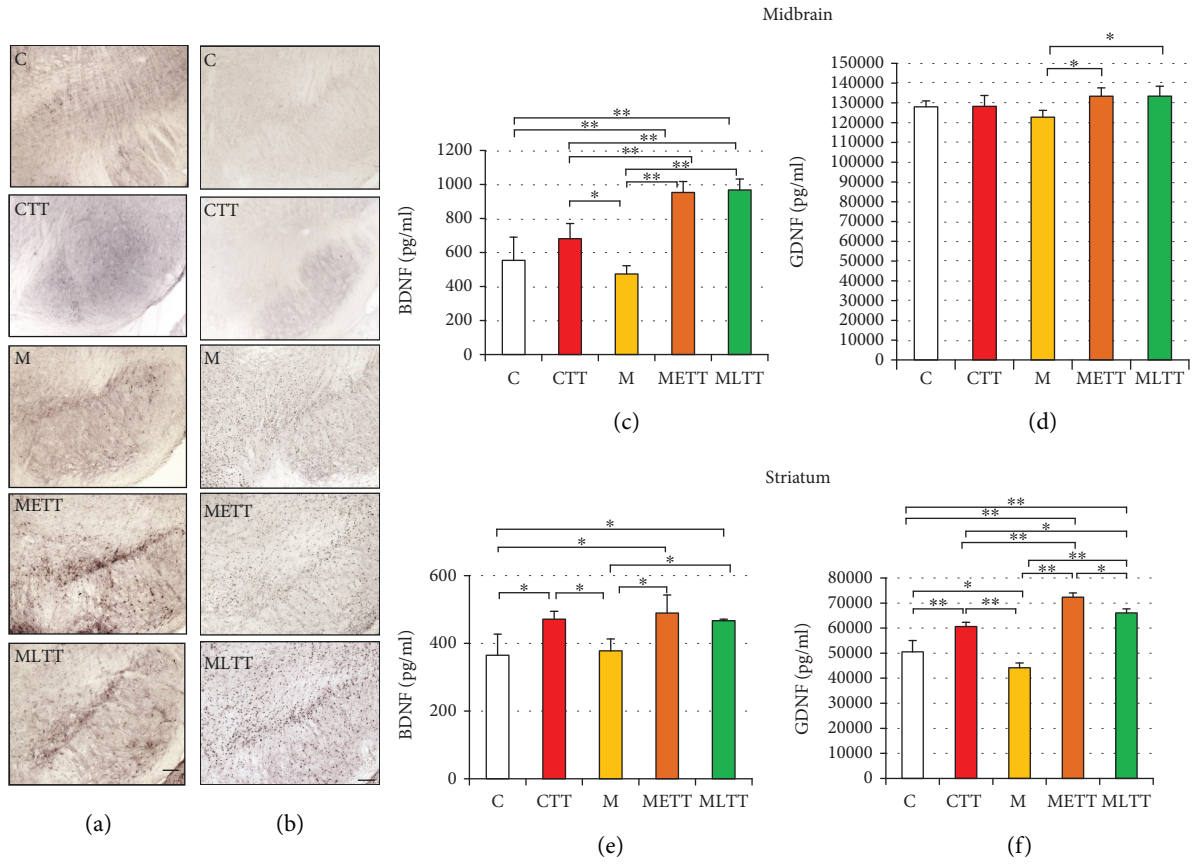

Figure 2: Effect of MPTP and treadmill training on the brain-derived neurotrophic factor (BDNF) and glial cell-derived neurotrophic factor (GDNF) level in the midbrain and striatum. Immunohistochemical staining against BDNF (a) and GDNF (b) in the substantia nigra pars compacta (SNpc), quantitative analysis of BDNF and GDNF in the midbrain (c, d), and striatum (e, f). C: control; CTT: control + treadmill training; M: treatment with MPTP; METT: MPTP + early onset treadmill training; MLTT: MPTP + late-onset treadmill training group. Scale bar: 100 μm. Statistical comparisons were performed with one-way ANOVA followed by Newman-Keuls *post hoc* test; $^{**}p < 0.001$, $^{*}p < 0.05$.

increased the concentration of BDNF in the CTT group as compared to the C and M groups (Figure 2(e), $p < 0.05$). The physical training accompanied by MPTP administration (METT and MLTT groups) increased also the BDNF concentration in comparison with the C and M groups (Figure 2(e), $p < 0.05$). In respect to GDNF, an increase in its concentration in the CTT group as compared to the C and M groups (Figure 2(f), $p < 0.001$) and opposite direction of GDNF concentration change between groups C and M was observed (Figure 2(f), $p < 0.05$). In addition, GDNF concentrations in both METT and MLTT groups were significantly higher than in other experimental groups (Figure 2(f)). The highest concentration of GDNF was found in the METT group; it was even significantly ($p < 0.05$) higher than the GDNF concentration in the MLTT group (Figure 2(f)).

3.3. Reactivity of Astrocyte and Microglia in Response to Chronic MPTP Administration and Physical Training. Glial cells play an important role in maintaining homeostasis of the central nervous system (CNS); however, under certain conditions, long-term stimulation of microglia may activate astrocytes leading to chronic neuroinflammation or even to neurodegeneration. Several studies have highlighted the role of glial cells in the toxic mechanism of MPTP [40–42].

To focus on the neuroinflammatory profile induced by MPTP treatment and hypothetical neuroprotective properties of treadmill training on the functioning of dopaminergic neurons, an immunohistochemical analysis of GFAP, Iba1, integrin CD11b in the SNpc and VTA, and immunoenzymatic analysis of GFAP and Il-1β in the midbrain and striatum were performed. Microscopic analysis showed a significant increase in the intensity of staining against GFAP (Figures 3(a) and 3(d)), Iba1 (Figures 3(b) and 3(e)), and CD11b (Figures 3(c) and 3(f)) in the SNpc (Figures 3(a)–3(c)) and VTA (Figures 3(d)–3(f)) in the M group compared with the C group. It seems that only in the M group, there was an increase in the number of microglia as a result of their proliferation (Figures 3(b), 3(c), 3(e), and 3(f)). At the same time, the morphological transformation of resting microglia into activated cells which resembled amoeboid phagocytic cells was visible in the MPTP-treated group (Figures 3(b) and 3(e) insertions, M group). The mobilization of microglia in mice with induced parkinsonism was observed both for anti-Iba1 and anti-CD11b staining. The higher intensity of

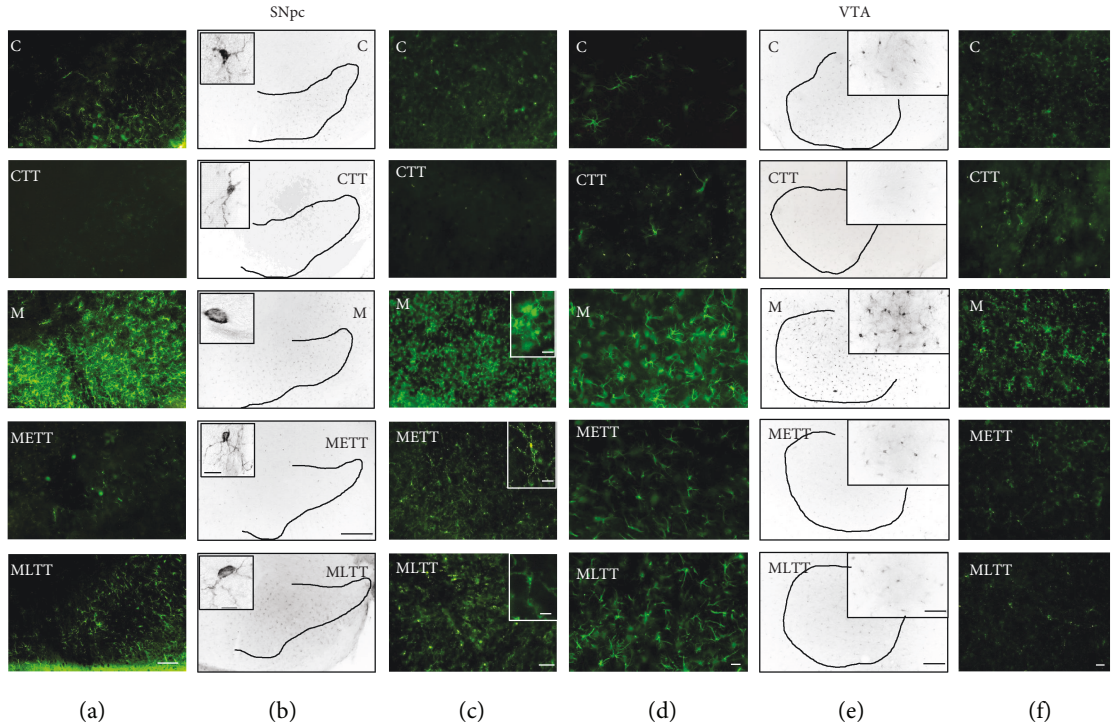

Figure 3: Immunohistochemical staining against the glial fibrillary acid protein (GFAP) (a, d), ionized calcium binding adaptor molecule 1 (Iba1) (b, e), and integrin CD 11b (c, f) in the SNpc (a, b, c) and VTA (D, E, F). C: control; CTT: control + treadmill training; M: treatment with MPTP; METT: MPTP + early onset treadmill training; MLTT: MPTP + late-onset treadmill training group. Scale bar: 200 μm (a); 400 μm and 10 μm (insertions) (b); 50 μm and 10 μm (insertions) (c); 100 μm (d, f). 400 μm and 100 μm (insertions) (e).

staining against GFAP (Figures 3(a) and 3(d)) in mice exposed to the MPTP neurotoxin indicates that increased activation of astrocytes may contribute to the inflammatory response to dopaminergic neurons injury. It seems that in the METT and MLTT groups, physical training mitigates the proinflammatory response of microglia and astrocytes both in the SNpc and in the VTA (Figures 3(a)–3(f)).

Quantitative ELISA assessment confirmed data from the microscopic analysis and revealed a significant increase in the level of GFAP and a proinflammatory cytokine Il-1β both in the midbrain (Figure 4(a), $p < 0.001$ for GFAP and Figure 4(b), $p < 0.05$ for Il-1β) and in the striatum (Figures 4(c) and 4(d), $p < 0.05$ for GFAP and Il-1β) in the M group as compared to the rest of the experimental groups. The level of GFAP and Il-1β in groups subjected to intoxication and training did not differ significantly from the level of both proteins in the sedentary and training control groups (Figures 4(a) and 4(d)).

4. Discussion

The present work compared the effect of long-term physical activity initiated before and after parkinsonism induction on the number of midbrain dopaminergic neurons, the level of neurotrophic factors, and inflammatory process in dopaminergic structures in a chronic MPTP mouse model of PD.

4.1. The Neuroprotective Effects of Treadmill Training on Dopaminergic Neurons. It has long been recognized that physical activity tends to have a positive effect in humans in the context of motor skills and proper functioning of the CNS, including the influence on cognitive functions. There is also an increasing body of evidence that physical exercises help recovery and lessen the risk of CNS damage and development of neurodegenerative diseases in animal models. Although there are reports in the literature on the neuroprotective effects of various forms of physical exercise of different duration and intensity on the state of dopaminergic neurons, there is still no definite answer to the question of which form of physical activity gives the best results. In the conducted study, modified forced treadmill training described by Ahmad et al. [34], and then also verified by Pothakos et al. [43] and Lau et al. [26], was implemented. Such training schedule, in contrast to voluntary training or the enriched environment, provides more comparable experimental conditions. The present study tested the effectiveness of two treadmill training schedules: the first one (i) started one week before the beginning of neurotoxin treatment, lasted throughout the 5 weeks of neurotoxin administration, and was continued 4 weeks after neurotoxin treatment (preceding training), while the second one (ii) started immediately after 5 weeks of neurotoxin treatment and lasted 10 weeks (follow-up training). It was found that 10 weeks of exercise, applied both before and after induction of parkinsonism, effectively protects dopaminergic neurons in the SNpc and VTA against the toxic action of MPTP.

It has been shown in animal models of PD that physical exercise is neuroprotective when applied before, during,

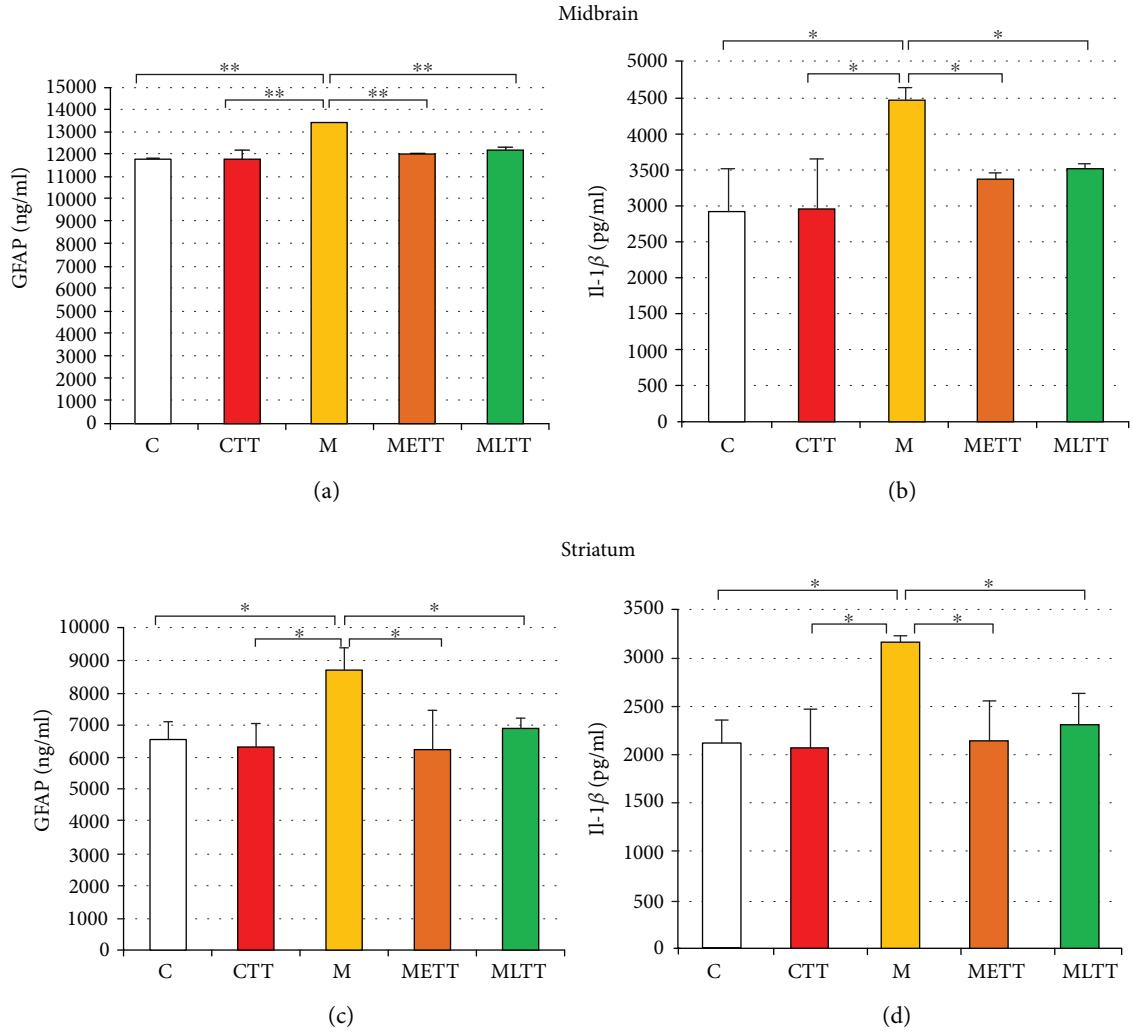

FIGURE 4: The quantitative analysis of glial fibrillary acid protein (GFAP) (a, c) and interleukin 1 beta (Il-1β) (b, d) concentration in the midbrain (a, b) and striatum (c, d). C: control; CTT: control + treadmill training; M: treatment with MPTP; METT: MPTP + early onset treadmill training; MLTT: MPTP + late-onset treadmill training group. Statistical comparisons were performed with one-way ANOVA, followed by Newman-Keuls *post hoc* test; $^{**}p < 0.001$, $^{*}p < 0.05$.

and after parkinsonism inducing treatment. This means that physical exercise may have preventive, protective, or restorative effects, respectively. The preventive effect of exercise was shown by Gerecke et al. [31] who found that intensive exercise on the running wheel performed by mice for 3 months before acute administration of MPTP completely prevented the loss of dopaminergic neurons in the SNpc and that less intensive exercise or/and performed for a shorter time produced only partial neuroprotection or no protection at all. However, complete neuroprotection was not accompanied by a complete recovery of DA level in the striatum. Fisher et al. [30] found that 30 days of treadmill training in C57 BL/6J mice applied 4 days after acute MPTP administration resulted in a significant downregulation of striatal DAT in the MPTP-treated exercised mice compared to MPTP-treated nonexercised mice but in no significant difference in the TH protein levels. No observation on the number of DA nigrostriatal neurons has been reported. Kintz et al. [32] found that 37 days of exercise starting 5 days after acute MPTP administration did not increase DA striatal level reduced by this neurotoxin. Zhao et al. [27] have shown that vibration training in mice, applied after 1 week of MPTP treatment and lasting 4 weeks, brought the number of nigrostriatal dopaminergic neurons almost to the level noted in control mice and significantly above that in MPTP-treated nontrained mice. A similar result was obtained for striatal DA level. Jang et al. [33] found that 6 weeks of treadmill training applied four weeks after one week lasting MPTP treatment (25 mg/kg daily) resulted in a number of TH-positive neurons almost equal to that in control mice and in the recovery of the TH and DAT levels. Also, in a rat model, Tajiri et al. [25] found significant preservation of TH-positive fibers in the striatum and TH-positive neurons in the SNpc caused by 4 weeks of treadmill training applied 24 hours after a 6-OHDA lesion of right striatum of female rats.

In order to examine the bona fide neuroprotective role of exercise, training should be applied concomitantly with

induction of parkinsonism. Such possibility is offered by the chronic PD model in mice, in which induction of parkinsonism takes 5 weeks, during which time 10 injections of MPTP are administered. Such treatment causes neurological deficits showing many features resembling PD [44]. Ahmad et al. [34], using this chronic model of PD, have demonstrated that the treadmill training starting one week before, continued over 5 weeks of MPTP treatment and 4 or 12 weeks thereafter, protected dopaminergic neurons in the VTA. Shorter training produced a significant although a small increase in the number of VTA TH-positive cells comparing to sedentary MPTP mice, whereas longer training returned their number to that observed in the nonparkinsonian control mice. Pothakos et al. [43] using a similar chronic model and starting treadmill training one week before MPTP treatment, maintaining it during 5 weeks of intoxication and 8-12 weeks thereafter, found neither reduction in depletion of striatal DA nor sparing of TH-positive neurons in the SNpc in MPTP-treated exercising mice vs. MPTP-treated sedentary ones. Of possible importance, the difference between these two studies was that the MPTP dose in the latter study was twice that used in the former one, i.e., 12.5 vs. 25 mg/kg/injection. The significance of the dose seems to be confirmed by Lau et al. [26], who used the same chronic model and 18-week treadmill training starting 1 week before the commencement of MPTP treatment. In contrast to Pothakos et al. [43], they observed a significant though incomplete recovery of the number of TH-positive neurons in the SNpc and, similarly, a significant though incomplete restoration of the striatal DA level when compared to that in control mice. The difference in MPTP dosing seems to be reflected by the loss of TH-positive neurons, with a dose of 15 mg MPTP/kg/injection used in Lau et al.'s study [26]; 55% of these neurons were lost vs. 72% in the study of Pothakos et al. In Al-Jarrah et al.'s study [45], four weeks of aerobic training following 5 weeks of chronic MPTP treatment with 25 mg/kg/injection and 1 week of pretraining resulted in a minimal rise of nigrostriatal TH and DA in mice as compared to sedentary MPTP-treated ones. Koo et al. [46] have investigated the effects of 8 weeks of progressive treadmill exercise applied 2 weeks after the completion of chronic MPTP treatment. Despite using the 25 mg/kg/injection dose of MPTP, i.e., the same as Pothakos et al. [43] and Al-Jarrah et al. [45], these investigators found a significantly reduced dopaminergic neuron loss in the SNpc and similar restitution of the TH and DAT level in MPTP-treated exercising mice. Of note is the 50% loss of TH-positive neurons in sedentary MPTP-treated mice, closer to that observed by Lau et al. [26] who used 15 mg/kg/injection than to that noted by Pothakos et al. [43] who applied 25 mg/kg/injection.

However, there are also studies in which there was no effect of physical training on TH-positive cell number, despite a marked improvement in motor performance [20, 43, 47]. The inability to observe the neuroprotective effect of physical activity on dopaminergic neurons may be due to insufficient time needed to restore the proper level of dopaminergic neuronal markers or to the experimental design, e.g., the animals used (species, strain, and age), the applied therapeutic intervention method (treadmill vs. wheel running, brief vs. continual, low vs. high intensity, and light vs. dark cycle), the nature of neurotoxin administration, and the type and dose of the neurotoxin used [48].

Although there are a number of reports in the literature about the protective effect of exercise on the reduction of parkinsonian symptoms in animals, there are no data comparing the effectiveness of physical exercise applied before and after PD induction. The present study evaluated the effect of physical exercise depending on the time of its application: before or after induction of parkinsonism. The comparison of both training schedules in one experiment, in which elements of the procedure were comparable, convincingly demonstrated that both early onset and late-onset physical training exert a beneficial effect on the survival and/or restoration of midbrain dopaminergic neurons, as well as on the expression of trophic factors and the degree of inflammation in the brain in response to the toxic action of MPTP. It is very intriguing that the training applied after intoxication was as advantageous as the training preceding the induction of parkinsonism. Emphasizing this point may bring about substantial progress in the clinical treatment of PD patients with physical exercises as a complementary therapy.

4.2. Training on the Treadmill Raises the Level of Neurotrophins. Since the reduction of the BDNF [49] and GDNF [50] levels in PD brains has been observed, a new concept has emerged that an increase in the concentration of neurotrophic factors, which are considered to be capable of supporting neuronal survival, may be neuroprotective in PD [51, 52]. In recent years, a number of clinical trials have been carried out by directly injecting neurotrophins into the brain and implanting genetically modified neurotrophin-producing cells or gene vectors to protect dopaminergic neurons [53]. It seems, however, that less invasive methods, such as long-term physical exercise, may also be neuroprotective and neurorestorative and lead to increased levels of endogenous neurotrophic factors.

In the present study, the effect of 10 weeks of treadmill training, starting both before and after MPTP administration, resulted in an increase of BDNF concentration in the midbrain and in the striatum compared to MPTP-treated sedentary mice. Furthermore, an increase in BDNF in the midbrain in both MPTP groups with treadmill training was also observed, compared to sedentary control and control with treadmill training, and in the striatum compared to sedentary control. It seems that the effect of training was potentiated by MPTP treatment. This phenomenon is absent in the striatum, wherein training, irrespective of whether accompanied by MPTP treatment or not, increases significantly and to a similar degree, the BDNF levels in CTT, METT, and MLTT groups.

Changes in the GDNF levels in the striatum strongly resemble those of the BDNF levels in the midbrain. There is also evident potentiation of the effect of training by MPTP treatment. However, late-onset training caused a somewhat smaller, though statistically significant, rise of the GDNF level than the early onset one. The GDNF levels in the midbrain are slightly elevated by early onset and late-onset

training and significantly higher only in comparison with sedentary MPTP-treated mice. These observations correspond with those presented by Lau et al. [26]. In their study, the effects of chronic treatment with MPTP were compared in sedentary vs. early trained mice, i.e., 18 weeks of training starting 1 week before the 5 weeks of MPTP (15 mg/kg/injection) treatment. As in our study, this kind of training significantly rises BDNF in the SNpc and GDNF in the striatum as compared to control and mice chronically treated with MPTP. Training also raises the striatal BDNF levels, but the increase does not attain statistical significance, and also increases the nigral GDNF level slightly but significantly above that in sedentary chronically MPTP-treated mice. Qualitatively, the results of Lau et al. and ours agree. Moreover, in our study, in contrast to Lau et al.'s study, there is a training control group; thus, the effects of exercise alone can be elucidated.

An increased midbrain level of BDNF, induced by training, may have a protective role on dopaminergic neurons as normalization of the striatal TH and DA level was observed in parkinsonian MPTP mice [26]. In turn, Tajiri et al. [25], using rats with 6-OHDA lesion, also demonstrated an effect of treadmill training on the upregulation of the BDNF and GDNF level in the striatum of the exercise group. Available literature provides several additional arguments supporting the thesis of the key role played by BDNF and GDNF in dopaminergic neuroprotection. Boger et al. [54] have observed an accelerated and age-related decrease in TH immunostaining in the SN in GDNF+/- mice as compared to control animals. In turn, Gerecke et al. [55] have demonstrated that BDNF+/− mice allowed 90 days of unrestricted exercise were not protected from MPTP-induced dopaminergic neuron loss in the SNpc, while control mice allowed 90-day exercise demonstrated complete protection against MPTP-induced neurotoxicity. Furthermore, blocking the action of BDNF, using tropomyosin receptor kinase B (TrkB) antagonist, suppressed exercise-induced protection against lipopolysaccharide- (LPS-) induced damage to dopaminergic neurons [56]. A similar conclusion was made by Real et al. [57], who found that the neuroprotective effect of physical training in the 6-OHDA rat model was not observed when a blocker of BDNF receptors was used. These results suggest that physical activity may be neuroprotective and may reduce the sensitivity of dopaminergic neurons to toxins by activating signaling cascades triggered by the increased availability of BDNF and GDNF.

4.3. Physical Training Attenuates the Inflammatory Process Induced by Chronic MPTP Administration. Although the etiology of Parkinson's disease is not fully understood, chronic inflammation plays an important role in the development of PD. On the other hand, it is not yet clear whether inflammation is a primary or secondary phenomenon that is a consequence of neuronal death [58]. Characteristic features of neuroinflammation include activated microglia and reactive astrocytes known to produce cytokines, chemokines, prostaglandins (PG), protein complement cascades, ROS, and reactive nitrogen species (RNS) [59]. The immunofluorescence staining against GFAP, in the presented study, showed an increased number of astrocytes in SNpc and VTA in MPTP sedentary mice as compared to all other groups. GFAP concentration was also verified using the ELISA method. The ELISA results were consistent with the immunohistochemical GFAP staining, i.e., the highest GFAP concentration was observed in the MPTP group. These observations concerned both midbrain and striatum. On the other hand, treadmill training caused reduced GFAP concentration in both MPTP-trained groups (METT and MLTT), comparable to the concentration of GFAP observed in the control groups (C and CTT). Similar results were described by Sconce et al. [20], who showed an increased GFAP level in the SN in animals treated with an increasing dose of MPTP for 4 weeks. The difference in results obtained by Sconce et al. and those presented in this study was that the group treated with MPTP and exercised had an elevated GFAP level compared to the control group; however, it was still much lower than in the MPTP sedentary animals. Furthermore, staining against CD11b and Iba1, markers of microglia, also showed higher intensity in SNpc and VTA in MPTP mice without treadmill training compared with the results obtained in controls and both MPTP groups with treadmill training. Additionally, higher magnification of anti-Iba1 staining in the SNpc indicated a change of microglial cell phenotype from resting to amoeba-like proinflammatory shape.

It seems that during the development of neurodegenerative diseases, a proinflammatory way of activation plays a pivotal role. The mutual activation of microglia and astrocytes depends mainly on the inflammatory cytokines secreted by them or the interaction of their receptors [60]. In addition, it was shown that cytokines secreted from activated microglia can lead to activation of astrocytes, which are cytotoxic to neurons, and these reactive astrocytes potentiate ongoing inflammation [60, 61]. Il-1β and/or TNF-α, key proinflammatory cytokines, are considered to be implicated in the early signaling pathways leading to astrogliosis [62]. Although Il-1β is secreted mainly by activated microglia [60], reactive astrocytes also show Il-1β expression [63]. In the present study, it was found that concentration of Il-1β was elevated in the MPTP sedentary group, both in the midbrain and in the striatum, while Il-1β concentration in MPTP mice with early and late training was similar to that observed in the control groups. These results suggest that MPTP administration leads to activation of the proinflammatory phenotype of glial cells and that physical training alleviates this activation and may inhibit the inflammatory process within the brain. The hypothesis that the inflammatory process is involved in the degeneration of dopaminergic neurons and is associated with the administration of MPTP and that treadmill exercise inhibits the activation of microglial cells in treated mice was previously demonstrated by another research groups [64].

Both subtypes of glial cells, astrocytes and microglia, may be activated in two different ways, resulting in the formation of proinflammatory (classical M1 activation) or anti-inflammatory (alternative M2 activation) response. Alternatively stimulated microglia show increased expression of cytokines recognized as anti-inflammatory, such as IL-10, TGF-β, IGF-1, NGF, and BDNF [65]. Astrocytes, like microglia, also secrete anti-inflammatory agents into the

environment, including neurotrophic factors (e.g., GDNF, BDNF, and MANF (mesencephalic astrocyte-derived neurotrophic factor)), which stimulate the survival and revive damaged dopaminergic neurons [66]. In addition, in *in vitro* conditions, it was shown that endogenous IL-1β may induce gene expression, synthesis, and secretion of GDNF [67]. It is possible that physical training applied in MPTP mice with parkinsonism launches an alternative neuroprotective activation of microglia rather than reduces proinflammatory glial activation. Such neuroprotective activation could result from increased synthesis of trophic factors induced by prolonged physical exertion. Then, there is no proinflammatory proliferation and activation of glial cells because dopaminergic neurons, protected by neurotrophins, do not degenerate and do not send signals that mobilize the inflammatory response.

4.4. Equipotent Neuroprotective Effect of Early Onset and Late-Onset Training. To our knowledge, this is the first study in which the impact of the timing of physical training on the neuroprotective effect observed in a murine model of PD has been precisely addressed. This was achieved by using the same murine strain, dose, and mode of neurotoxin administration (i.e., chronic treatment with 10 injections of 12.5 mg/kg/injection of MPTP) and the same kind, duration, and intensity of physical effort but different onset of training: the training started either 1 week before and continued over the 5-week period of MPTP treatment and beyond (for 4 weeks) or the training started immediately after the conclusion of the neurotoxin treatment and also lasted 10 weeks.

It was found that both the early onset and late-onset training (1) almost completely preserved the number of dopaminergic neurons in SNpc and VTA, (2) increased to a similar degree the BDNF level in the midbrain and the GDNF level in the striatum, and (3) entirely prevented inflammatory response evoked by chronic MPTP treatment. A possible explanation of the neuroprotective effect of late-onset training should account for the greater number of dopaminergic neurons in late-onset training MPTP-treated mice than in sedentary MPTP-treated mice. Their number should have been similarly reduced in both groups at the end of 5 weeks of chronic MPTP treatment as these groups were treated in the same way during this period. It may be assumed that there was a further decline in the number of dopaminergic neurons in sedentary MPTP-treated mice, during the 4 weeks, from the termination of MPTP treatment to brain isolation. During the same 4 weeks, physical training induced preservation or reduced decline of dopaminergic neurons in late-onset training MPTP-treated mice. This decline of dopaminergic neurons in the former group and their preservation in the latter group would account for the difference in the number of these neurons between groups.

Preservation of dopaminergic neurons might be due to increased midbrain level of BDNF, what in turn prevents the degeneration of these neurons and restrains inflammatory response, as reflected by the diminished level of inflammatory markers in late-onset training MPTP-treated group. This hypothesis implies that degeneration of neurons progresses also after the termination of MPTP treatment. Subsistence of the inflammatory process 4 weeks after the cessation of neurotoxin treatment was confirmed in our study by a significantly higher level of inflammatory markers in sedentary MPTP-treated mice. This is in agreement with others' observations that such degeneration continues 3 weeks after MPTP/probenecid treatment and is accompanied by a significant inflammatory response [68].

Training-induced preservation of dopaminergic neurons may not account for the fact that in early onset training MPTP-treated group, the number of dopaminergic neurons was similar to that in the late-onset training MPTP group. If training exerted its neuroprotective effect already during MPTP treatment, then the number of dopaminergic neurons after cessation of this treatment should be higher in the early onset training MPTP group than that in the late-onset training MPTP group. The sparing effect would be present during 4 weeks following the termination of MPTP treatment, as both groups trained at that time, but preservation alone could not result in a similar number of dopaminergic neurons. Adding increase in number of dopaminergic neurons would explain their similar number in both groups. It is known that spontaneous regeneration of dopaminergic neurons occurs in acute MPTP mice models [69, 70]. The chronic treatment with MPTP, such as applied in this study, is claimed to prevent this regeneration [68]; however, it is possible that also in such model of parkinsonism training leads to recovery by inducing neurogenesis [25, 33] or by restoring neurotransmitter phenotype [71]. In order to make recovery a more plausible explanation of our observations, one can postulate that the time window for this process starts with some delay after the end of MPTP treatment, thus allowing for an increase in a number of dopaminergic neurons during the last 6 weeks of training of late-onset MPTP-treated mice. If neurogenesis and/or restoration of neurotransmitter phenotype takes place, the importance of continued physical activity in PD patients should be strongly underpinned.

5. Conclusions

This study supports the view that physical effort is neuroprotective also after the neurotoxic assault. Furthermore, it suggests a similar mechanism of neuroprotection of early and late-onset physical training. Although the actual sequence of events remains to be elucidated, it seems that neurotrophic factors prevent neurodegeneration and in this way prevent the appearance of the inflammatory response. These results underscore the conviction that physical activity may be neuroprotective also at a more advanced stage of PD and justify the rationale of starting physical activity at any point of the disease, as long as it is feasible.

Data Availability

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

Conflicts of Interest

No conflicts of interest, financial or otherwise, are declared by the authors.

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References

[1] I. Miyai, Y. Fujimoto, Y. Ueda et al., "Treadmill training with body weight support: its effect on Parkinson's disease," *Archives of Physical Medicine and Rehabilitation*, vol. 81, no. 7, pp. 849–852, 2000.

[2] H. W. Berendse and H. J. Groenewegen, "Organization of the thalamostriatal projections in the rat, with special emphasis on the ventral striatum," *Journal of Comparative Neurology*, vol. 299, no. 2, pp. 187–228, 1990.

[3] J. L. Bergen, T. Toole, Elliott RG 3rd, B. Wallace, K. Robinson, and C. G. Maitland, "Aerobic exercise intervention improves aerobic capacity and movement initiation in Parkinson's disease patients," *NeuroRehabilitation*, vol. 17, no. 2, pp. 161–168, 2002.

[4] M. A. Hirsch, T. Toole, C. G. Maitland, and R. A. Rider, "The effects of balance training and high-intensity resistance training on persons with idiopathic Parkinson's disease," *Archives of Physical Medicine and Rehabilitation*, vol. 84, no. 8, pp. 1109–1117, 2003.

[5] I. Reuter, S. Mehnert, P. Leone, M. Kaps, M. Oechsner, and M. Engelhardt, "Effects of a flexibility and relaxation programme, walking, and Nordic walking on Parkinson's disease," *Journal of Aging Research*, vol. 2011, Article ID 232473, 18 pages, 2011.

[6] T. Herman, N. Giladi, L. Gruendlinger, and J. M. Hausdorff, "Six weeks of intensive treadmill training improves gait and quality of life in patients with Parkinson's disease: a pilot study," *Archives of Physical Medicine and Rehabilitation*, vol. 88, no. 9, pp. 1154–1158, 2007.

[7] A. Nadeau, E. Pourcher, and P. Corbeil, "Effects of 24 wk of treadmill training on gait performance in Parkinson's disease," *Medicine and Science in Sports and Exercise*, vol. 46, no. 4, pp. 645–655, 2014.

[8] H. Chen, S. M. Zhang, M. A. Schwarzschild, M. A. Hernan, and A. Ascherio, "Physical activity and the risk of Parkinson disease," *Neurology*, vol. 64, no. 4, pp. 664–669, 2005.

[9] F. Yang, Y. Trolle Lagerros, R. Bellocco et al., "Physical activity and risk of Parkinson's disease in the Swedish National March Cohort," *Brain*, vol. 138, no. 2, pp. 269–275, 2015.

[10] G. M. Petzinger, B. E. Fisher, S. McEwen, J. A. Beeler, J. P. Walsh, and M. W. Jakowec, "Exercise-enhanced neuroplasticity targeting motor and cognitive circuitry in Parkinson's disease," *Lancet Neurology*, vol. 12, no. 7, pp. 716–726, 2013.

[11] E.-T. Ang, Y.-K. Tai, S.-Q. Lo, R. Seet, and T.-W. Soong, "Neurodegenerative diseases: exercising towards neurogenesis and neuroregeneration," *Frontiers in Aging Neuroscience*, vol. 2, 2010.

[12] M. F. Almeida, R. S. Chaves, C. M. Silva, J. C. S. Chaves, K. P. Melo, and M. F. R. Ferrari, "BDNF trafficking and signaling impairment during early neurodegeneration is prevented by moderate physical activity," *IBRO Reports*, vol. 1, pp. 19–31, 2016.

[13] M. F. Almeida, C. M. Silva, R. S. Chaves et al., "Effects of mild running on substantia nigra during early neurodegeneration," *Journal of Sports Sciences*, vol. 36, no. 12, pp. 1363–1370, 2017.

[14] S. F. Sleiman, J. Henry, R. al-Haddad et al., "Exercise promotes the expression of brain derived neurotrophic factor (BDNF) through the action of the ketone body β-hydroxybutyrate," *eLife*, vol. 5, 2016.

[15] C. Phillips, M. A. Baktir, M. Srivatsan, and A. Salehi, "Neuroprotective effects of physical activity on the brain: a closer look at trophic factor signaling," *Frontiers in Cellular Neuroscience*, vol. 8, p. 170, 2014.

[16] P. Ambrogini, D. Lattanzi, S. Ciuffoli, M. Betti, M. Fanelli, and R. Cuppini, "Physical exercise and environment exploration affect synaptogenesis in adult-generated neurons in the rat dentate gyrus: possible role of BDNF," *Brain Research*, vol. 1534, pp. 1–12, 2013.

[17] C. Lopez-Lopez, D. LeRoith, and I. Torres-Aleman, "Insulin--like growth factor I is required for vessel remodeling in the adult brain," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 101, no. 26, pp. 9833–9838, 2004.

[18] H. van Praag, T. Shubert, C. Zhao, and F. H. Gage, "Exercise enhances learning and hippocampal neurogenesis in aged mice," *Journal of Neuroscience*, vol. 25, no. 38, pp. 8680–8685, 2005.

[19] K. van der Borght, D. É. Kóbor-Nyakas, K. Klauke et al., "Physical exercise leads to rapid adaptations in hippocampal vasculature: temporal dynamics and relationship to cell proliferation and neurogenesis," *Hippocampus*, vol. 19, no. 10, pp. 928–936, 2009.

[20] M. D. Sconce, M. J. Churchill, R. E. Greene, and C. K. Meshul, "Intervention with exercise restores motor deficits but not nigrostriatal loss in a progressive MPTP mouse model of Parkinson's disease," *Neuroscience*, vol. 299, pp. 156–174, 2015.

[21] A. T. R. Goes, L. C. Souza, C. B. Filho et al., "Neuroprotective effects of swimming training in a mouse model of Parkinson's disease induced by 6-hydroxydopamine," *Neuroscience*, vol. 256, pp. 61–71, 2014.

[22] C. C. Real, P. C. Garcia, and L. R. G. Britto, "Treadmill exercise prevents increase of neuroinflammation markers involved in the dopaminergic damage of the 6-OHDA Parkinson's disease model," *Journal of Molecular Neuroscience*, vol. 63, no. 1, pp. 36–49, 2017.

[23] W. Chen, D. Qiao, X. Liu, and K. Shi, "Treadmill exercise improves motor dysfunction and hyperactivity of the corticostriatal glutamatergic pathway in rats with 6-OHDA-induced Parkinson's disease," *Neural Plasticity*, vol. 2017, Article ID 2583910, 11 pages, 2017.

[24] G. M. Petzinger, D. P. Holschneider, B. E. Fisher et al., "The effects of exercise on dopamine neurotransmission in Parkinson's disease: targeting neuroplasticity to modulate basal ganglia circuitry," *Brain Plasticity*, vol. 1, no. 1, pp. 29–39, 2015.

[25] N. Tajiri, T. Yasuhara, T. Shingo et al., "Exercise exerts neuroprotective effects on Parkinson's disease model of rats," *Brain Research*, vol. 1310, pp. 200–207, 2010.

[26] Y.-S. Lau, G. Patki, K. Das-Panja, W.-D. Le, and S. O. Ahmad, "Neuroprotective effects and mechanisms of exercise in a chronic mouse model of Parkinson's disease with moderate

neurodegeneration," *European Journal of Neuroscience*, vol. 33, no. 7, pp. 1264–1274, 2011.

[27] L. Zhao, L. X. He, S. N. Huang et al., "Protection of dopamine neurons by vibration training and up-regulation of brain-derived neurotrophic factor in a MPTP mouse model of Parkinson's disease," *Physiological Research*, vol. 63, no. 5, pp. 649–657, 2014.

[28] M. Gleeson, N. C. Bishop, D. J. Stensel, M. R. Lindley, S. S. Mastana, and M. A. Nimmo, "The anti-inflammatory effects of exercise: mechanisms and implications for the prevention and treatment of disease," *Nature Reviews Immunology*, vol. 11, no. 9, pp. 607–615, 2011.

[29] Y. Jang, J. H. Koo, I. Kwon et al., "Neuroprotective effects of endurance exercise against neuroinflammation in MPTP-induced Parkinson's disease mice," *Brain Research*, vol. 1655, pp. 186–193, 2017.

[30] B. E. Fisher, G. M. Petzinger, K. Nixon et al., "Exercise-induced behavioral recovery and neuroplasticity in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-lesioned mouse basal ganglia," *Journal of Neuroscience Research*, vol. 77, no. 3, pp. 378–390, 2004.

[31] K. M. Gerecke, Y. Jiao, A. Pani, V. Pagala, and R. J. Smeyne, "Exercise protects against MPTP-induced neurotoxicity in mice," *Brain Research*, vol. 1341, pp. 72–83, 2010.

[32] N. Kintz, G. M. Petzinger, G. Akopian et al., "Exercise modifies α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor expression in striatopallidal neurons in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-lesioned mouse," *Journal of Neuroscience Research*, vol. 91, no. 11, pp. 1492–1507, 2013.

[33] Y. Jang, I. Kwon, W. Song, L. M. Cosio-Lima, and Y. Lee, "Endurance exercise mediates neuroprotection against MPTP-mediatedParkinson's disease via enhanced neurogenesis, antioxidant capacity, and autophagy," *Neuroscience*, vol. 379, pp. 292–301, 2018.

[34] S. O. Ahmad, J. H. Park, L. Stenho-Bittel, and Y. S. Lau, "Effects of endurance exercise on ventral tegmental area neurons in the chronic 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine and probenecid-treated mice," *Neuroscience Letters*, vol. 450, no. 2, pp. 102–105, 2009.

[35] G. Paxinos and K. Franklin, *The Mouse Brain in Stereotaxic Coordinates*, Academic Press, Elsevier, 2013.

[36] M. Abercrombie, "Estimation of nuclear population from microtome sections," *The Anatomical Record*, vol. 94, no. 2, pp. 239–247, 1946.

[37] M. E. Afzalpour, H. T. Chadorneshin, M. Foadoddini, and H. A. Eivari, "Comparing interval and continuous exercise training regimens on neurotrophic factors in rat brain," *Physiology & Behavior*, vol. 147, pp. 78–83, 2015.

[38] E. Pałasz, A. Bąk, A. Gąsiorowska, and G. Niewiadomska, "The role of trophic factors and inflammatory processes in physical activity-induced neuroprotection in Parkinson's disease," *Postepy higieny i medycyny doswiadczalnej*, vol. 71, no. 1, pp. 713–726, 2017.

[39] Z. C. Baquet, P. C. Bickford, and K. R. Jones, "Brain-derived neurotrophic factor is required for the establishment of the proper number of dopaminergic neurons in the substantia nigra pars compacta," *Journal of Neuroscience*, vol. 25, no. 26, pp. 6251–6259, 2005.

[40] G. Chandra, A. Roy, S. B. Rangasamy, and K. Pahan, "Induction of adaptive immunity leads to nigrostriatal disease progression in MPTP mouse model of Parkinson's disease," *Journal of Immunology*, vol. 198, no. 11, pp. 4312–4326, 2017.

[41] J. Schwenkgrub, M. Zaremba, I. Joniec-Maciejak, A. Cudna, D. Mirowska-Guzel, and I. Kurkowska-Jastrzębska, "The phosphodiesterase inhibitor, ibudilast, attenuates neuroinflammation in the MPTP model of Parkinson's disease," *PLoS One*, vol. 12, no. 7, article e0182019, 2017.

[42] R. J. Smeyne, C. B. Breckenridge, M. Beck et al., "Assessment of the effects of MPTP and paraquat on dopaminergic neurons and microglia in the substantia nigra pars compacta of C57BL/6 mice," *PLoS One*, vol. 11, no. 10, article e0164094, 2016.

[43] K. Pothakos, M. J. Kurz, and Y.-S. Lau, "Restorative effect of endurance exercise on behavioral deficits in the chronic mouse model of Parkinson's disease with severe neurodegeneration," *BMC Neuroscience*, vol. 10, no. 1, p. 6, 2009.

[44] L. M. L. De Lau and M. M. B. Breteler, "Epidemiology of Parkinson's disease," *The Lancet Neurology*, vol. 5, no. 6, pp. 525–535, 2006.

[45] M. al-Jarrah, K. Pothakos, L. Novikova et al., "Endurance exercise promotes cardiorespiratory rehabilitation without neurorestoration in the chronic mouse model of parkinsonism with severe neurodegeneration," *Neuroscience*, vol. 149, no. 1, pp. 28–37, 2007.

[46] J.-H. Koo, J.-Y. Cho, and U.-B. Lee, "Treadmill exercise alleviates motor deficits and improves mitochondrial import machinery in an MPTP-induced mouse model of Parkinson's disease," *Experimental Gerontology*, vol. 89, pp. 20–29, 2017.

[47] S. J. O'Dell, N. B. Gross, A. N. Fricks, B. D. Casiano, T. B. Nguyen, and J. F. Marshall, "Running wheel exercise enhances recovery from nigrostriatal dopamine injury without inducing neuroprotection," *Neuroscience*, vol. 144, no. 3, pp. 1141–1151, 2007.

[48] M. J. Zigmond and R. J. Smeyne, "Exercise: is it a neuroprotective and if so, how does it work?," *Parkinsonism & Related Disorders*, vol. 20, Supplement 1, pp. S123–S127, 2014.

[49] D. W. Howells, M. J. Porritt, J. Y. F. Wong et al., "Reduced BDNF mRNA expression in the Parkinson's disease substantia nigra," *Experimental Neurology*, vol. 166, no. 1, pp. 127–135, 2000.

[50] N. B. Chauhan, G. J. Siegel, and J. M. Lee, "Depletion of glial cell line-derived neurotrophic factor in substantia nigra neurons of Parkinson's disease brain," *Journal of Chemical Neuroanatomy*, vol. 21, no. 4, pp. 277–288, 2001.

[51] C. W. Cotman and N. C. Berchtold, "Exercise: a behavioral intervention to enhance brain health and plasticity," *Trends in Neurosciences*, vol. 25, no. 6, pp. 295–301, 2002.

[52] A. Hennigan, R. M. O'Callaghan, and Á. M. Kelly, "Neurotrophins and their receptors: roles in plasticity, neurodegeneration and neuroprotection," *Biochemical Society Transactions*, vol. 35, no. 2, pp. 424–427, 2007.

[53] L. Hou, W. Chen, X. Liu, D. Qiao, and F. M. Zhou, "Exercise-induced neuroprotection of the nigrostriatal dopamine system in Parkinson's disease," *Frontiers in Aging Neuroscience*, vol. 9, 2017.

[54] H. Boger, L. Middaugh, P. Huang et al., "A partial GDNF depletion leads to earlier age-related deterioration of motor function and tyrosine hydroxylase expression in the substantia nigra," *Experimental Neurology*, vol. 202, no. 2, pp. 336–347, 2006.

[55] K. M. Gerecke, Y. Jiao, V. Pagala, and R. J. Smeyne, "Exercise does not protect against MPTP-induced neurotoxicity in BDNF happloinsufficent mice," *PLoS One*, vol. 7, no. 8, article e43250, 2012.

[56] S. Y. Wu, T. F. Wang, L. Yu et al., "Running exercise protects the substantia nigra dopaminergic neurons against inflammation-induced degeneration via the activation of BDNF signaling pathway," *Brain, Behavior, and Immunity*, vol. 25, no. 1, pp. 135–146, 2011.

[57] C. C. Real, A. F. B. Ferreira, G. P. Chaves-Kirsten, A. S. Torrão, R. S. Pires, and L. R. G. Britto, "BDNF receptor blockade hinders the beneficial effects of exercise in a rat model of Parkinson's disease," *Neuroscience*, vol. 237, pp. 118–129, 2013.

[58] M. G. Tansey and M. S. Goldberg, "Neuroinflammation in Parkinson's disease: its role in neuronal death and implications for therapeutic intervention," *Neurobiology of Disease*, vol. 37, no. 3, pp. 510–518, 2010.

[59] S. Vivekanantham, S. Shah, R. Dewji, A. Dewji, C. Khatri, and R. Ologunde, "Neuroinflammation in Parkinson's disease: role in neurodegeneration and tissue repair," *International Journal of Neuroscience*, vol. 125, no. 10, pp. 717–725, 2015.

[60] L.-W. Chen, M.-H. Dong, F. Kuang, J.-T. Liu, J.-Q. Zhang, and Y. Bai, "Microglia and astroglia: the role of neuroinflammation in lead toxicity and neuronal injury in the brain," *Neuroimmunology and Neuroinflammation*, vol. 2, no. 3, pp. 131–137, 2015.

[61] M. Neal and J. R. Richardson, "Epigenetic regulation of astrocyte function in neuroinflammation and neurodegeneration," *Biochimica et Biophysica Acta - Molecular Basis of Disease*, vol. 1864, no. 2, pp. 432–443, 2018.

[62] A. R. Little and J. P. O'Callaghan, "Astrogliosis in the adult and developing CNS: is there a role for proinflammatory cytokines?," *Neurotoxicology*, vol. 22, no. 5, pp. 607–618, 2001.

[63] S. S. Choi, H. J. Lee, I. Lim, J. I. Satoh, and S. U. Kim, "Human astrocytes: secretome profiles of cytokines and chemokines," *PLoS One*, vol. 9, no. 4, article e92325, 2014.

[64] M. Swiątkiewicz, M. Zaremba, I. Joniec, A. Członkowski, and I. Kurkowska-Jastrzębska, "Potential neuroprotective effect of ibuprofen, insights from the mice model of Parkinson's disease," *Pharmacological Reports*, vol. 65, no. 5, pp. 1227–1236, 2013.

[65] K. Łabuzek, E. Skrudlik, B. Gabryel, and B. Okopień, "Anti-inflammatory microglial cell function in the light of the latest scientific research," *Annales Academiae Medicae Silesiensis*, vol. 69, pp. 99–110, 2015.

[66] P. L. McGeer and E. G. McGeer, "Glial reactions in Parkinson's disease," *Movement Disorders*, vol. 23, no. 4, pp. 474–483, 2008.

[67] A. Saavedra, G. Baltazar, and E. P. Duarte, "Interleukin-1β mediates GDNF up-regulation upon dopaminergic injury in ventral midbrain cell cultures," *Neurobiology of Disease*, vol. 25, no. 1, pp. 92–104, 2007.

[68] G. E. Meredith, S. Totterdell, J. A. Potashkin, and D. J. Surmeier, "Modeling PD pathogenesis in mice: advantages of a chronic MPTP protocol," *Parkinsonism & Related Disorders*, vol. 14, pp. S112–S115, 2008.

[69] Y. Mitsumoto, A. Watanabe, A. Mori, and N. Koga, "Spontaneous regeneration of nigrostriatal dopaminergic neurons in MPTP-treated C57BL/6 mice," *Biochemical and Biophysical Research Communications*, vol. 248, no. 3, pp. 660–663, 1998.

[70] D. S. Rothblat, J. A. Schroeder, and J. S. Schneider, "Tyrosine hydroxylase and dopamine transporter expression in residual dopaminergic neurons: potential contributors to spontaneous recovery from experimental parkinsonism," *Journal of Neuroscience Research*, vol. 65, no. 3, pp. 254–266, 2001.

[71] A. D. Cohen, M. J. Zigmond, and A. D. Smith, "Effects of intrastriatal GDNF on the response of dopamine neurons to 6-hydroxydopamine: time course of protection and neurorestoration," *Brain Research*, vol. 1370, pp. 80–88, 2011.

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Research Article

Widespread Striatal Delivery of GDNF from Encapsulated Cells Prevents the Anatomical and Functional Consequences of Excitotoxicity

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Methods. Human ARPE-19 cells engineered to secrete high levels of the glial cell line-derived neurotrophic factor (GDNF) were encapsulated into hollow fiber membranes. The devices were implanted into the rat striatum 1 week prior to striatal quinolinic acid injections. Animals were evaluated using a battery of validated motor tests, and histology was performed to determine the extent of GDNF diffusion and associated prevention of neuronal cell loss and behavioral deficits. *Results*. Encapsulated cell-based delivery of GDNF produced widespread distribution of GDNF throughout the entire implanted striatum. Stereological estimates of striatal neuron number and volume of lesion size revealed that GDNF delivery resulted in near complete neuroprotection. *Conclusions*. Delivery of neurotrophic molecules such as GDNF using encapsulated cells has reached a technological point where clinical evaluation is justified. Because GDNF has been effective in animal models of Parkinson's disease, stroke, epilepsy, and Huntington's disease, among other debilitating neurodegenerative diseases, encapsulated cell-based delivery of GDNF might represent one innovative means of slowing the neural degeneration seen in a myriad of currently untreatable neurological diseases.

1. Introduction

Treating neurodegenerative diseases is an urgent challenge. Neurotrophic factors are attractive therapeutic candidates because they can enhance neuronal functioning, are neuroprotective, and have the potential to reverse ongoing neurodegeneration that causes neurological deficits. While neurotrophic factors have been consistently effective in animal models [1–8], clinical development and evaluation has been limited. A major reason for the delayed development of effective neurotrophic therapies has been the inability to deliver them across the blood-brain barrier (BBB) directly to target sites in a stable, controlled, and continuous manner [9–13]. Several strategies are under development to optimize the diffusion and spread of trophic factors into the brain tissue. These include direct brain infusion [14], various gene therapy approaches [7, 8], cell therapies [15], and biomaterial-based drug-delivery systems [15]. Each approach has its own advantages and limitations, but none have yet produced significant enough efficacy to justify widespread clinical evaluation.

Here, we describe a novel means of delivering very high concentrations of neurotrophic factors directly to the site of neuronal damage using an encapsulated cell therapy technology [16, 17]. Cells are enclosed in a semipermeable capsule, which is then implanted into the brain. The capsule membrane allows oxygen and nutrients to enter and nourish the encapsulated cells while also allowing the therapeutic

molecule of interest to leave the capsule and diffuse into the surrounding brain tissue. Immunological reactions to the encapsulated cells are reduced because the semipermeable membrane prevents elements of the host immune system from gaining access to the cells, thereby protecting against rejection. Indeed, even under xenograft conditions, the cells within the capsule remain viable without the need for immunosuppression. Furthermore, using human cells as the delivery vehicle further reduces the chances of immunological reactions. The cell line was produced using a transposon-based gene expression system resulting in high protein secretion 1-2 orders of magnitude higher than that used in previous cell encapsulation studies [18–20]. The cells were encapsulated within devices containing an optimized cell scaffolding previously shown to promote long-term cell viability in both animal models [21, 22] and recent human clinical trials in Alzheimer's patients [23]. In this study, we tested the hypothesis that encapsulated cell-based delivery of the neurotrophic molecule glial cell line-derived neurotrophic factor (GDNF) could result in widespread, but targeted, delivery of biologically active GDNF to the striatum. GDNF-secreting devices were implanted into the rodent striatum prior to quinolinic acid (QA) lesions. Comprehensive histological analysis and neurological testing revealed that GDNF was distributed throughout the striatum to exert a potent, essentially complete, neuroprotective effect. Together with previous demonstrations of long-term, controlled, safe, and targeted delivery of GDNF in small and large animal models, these data provide ongoing support for continued clinical development of this approach.

2. Methods and Materials

2.1. Subjects. Adult male Sprague-Dawley rats (Harlan Laboratories), ~3 months old and weighing 225-250 grams, were housed in groups of 4 in a temperature- and humidity-controlled colony room maintained on a 12-hour light/dark cycle. Food and water were available *ad libitum* throughout the experiment. All experimentation was conducted in accord with National Institutes of Health guidelines.

2.2. Cell Culture. ARPE-19 cells were cultured using standard plating and passaging procedures in T-175 flasks with growth medium; DMEM+glutamax (1x) was supplemented with 10% fetal bovine serum (Gibco). Routine culture consisted of feeding the cells every 2-3 days and passaging them at 70-75% confluence. Cells were incubated at 37°C, 90% humidity, and 5% CO_2.

2.3. Cell Line Establishment. Human GDNF cDNA optimized for human cell line expression was produced by Invitrogen, Denmark, and subsequently cloned to replace NGF in the expression vector pT2.CAn.hNGF [21], resulting in the plasmid pT2.CAn.hoG. ARPE-19 cells were transfected with this vector using the Sleeping Beauty (SB) transposon system as previously described [21, 22]. Briefly, cells were cotransfected with the plasmid pT2.CAn.hoG and the SB vector pCMV-SB-100x. As the SB vector does not contain a eukaryotic selection marker cassette, it is only transiently expressed. The transient expression window allows for the active, transposase-mediated integration of the SB transposon, i.e., the inverted repeat SB substrate sequences and the sequences contained within these repeats, including the GDNF expression and neomycin antibiotic resistance cassettes. Clones were selected using G418 (Sigma-Aldrich, Copenhagen, Denmark), and single colonies were expanded and isolated based on their GDNF release levels.

2.4. Device Fabrication. Cells were encapsulated into hollow fiber membranes manufactured from 7 mm segments of polyethersulfone membrane (Akzo, Germany) internally fitted with filaments of polyethylene terephthalate yarn scaffolding for cell adhesion. Prior to filling, cultured cells were dissociated and suspended in HE-SFM at a density of 8,333 cells/μl. 6 μl of cell solution (5×10^4 cells in total) was injected into each device using a custom-manufactured automated cell-loading system. Devices were kept in HE-SFM at 37°C and 5% CO_2 for either 3 weeks (low-dose group) or 12 weeks (high-dose group) prior to surgical implantation.

Previous studies [24] used scanning electron microscopy (SEM) to examine the morphology of these membranes. SEM cross sections of the polyethersulfone membrane confirmed that the membrane possessed a typical isoreticulated morphology with a relatively dense, thin outer skin and an open, much thicker macroporous substructure. Measures of membrane cross sections revealed an inner diameter of 481 μm, an outer diameter of 663 μm, and a corresponding wall thickness of approximately 90 μm.

2.5. Surgery. Rats were anesthetized with isoflurane (3-4%) and placed into a stereotaxic instrument (Stoelting Inc.). A midline incision was made in the scalp, and a hole drilled for the unilateral placement of a device (7 mm in length) into the striatum using a stainless-steel cannula mounted to the stereotaxic frame. The coordinates for implantation with respect to the Bregma were as follows: AP: 0.0, ML: 3.2, and DV: -7.5. After placement of the device, the cannula was withdrawn and the skin sutured closed.

One week following device implantation, the rats were anesthetized with isoflurane and positioned in a stereotaxic frame for the injection of the QA (225 nmol). A 28-gauge Hamilton syringe was connected to the stereotaxic frame and lowered into the previously implanted striatum at the following coordinates with respect to the Bregma: AP: 1.0, ML: 2.6, and DV: -5.0. The QA was infused in a volume of 1 μl per site over 5 minutes. The needle was left in place for an additional two minutes to allow the QA to diffuse from the injection site then removed and the skin sutured closed. The treated rats were allotted to 3 experimental groups: QA lesion only ($n = 8$), QA+GDNF low-dose ($n = 8$), and QA +GDNF high-dose ($n = 6$).

2.6. Neurological Evaluation. Using a validated battery of tests [25], the rats were evaluated to provide a behavioral measure of the extent of the lesion as well as the magnitude of benefit provided by the GDNF implants. All tests were conducted 24 hours prior to device implantation (baseline), 24 hours prior to QA injection (prelesion), and again 2 and

4 weeks postlesion. All testing was performed in a dim light testing room and the individual tests included (in order of testing) the following.

2.6.1. Cylinder Test of Spontaneous Forelimb Use. The rats were placed individually in an acrylic cylinder (20 cm in diameter and 40 cm in height), and left and right forepaw contacts with the wall of the cylinder were quantified. Twenty total forepaw contacts were required to complete each testing session.

2.6.2. Spontaneous Forelimb Placing Use. The forelimb placing test assessed the rat's ability to make directed forelimb movements in response to sensory stimuli. The rats were held with their limbs hanging unsupported and the length of their body parallel to the edge of a table. They were then raised and their whiskers were stimulated by brushing each side against the edge of the table. In naïve rats, this elicits a same-side forelimb response by placing the paw on the top of the table. Each rat received 10 consecutive trials with each forelimb.

2.6.3. Stepping Test. Rats were placed on a flat surface and their hind legs gently lifted by raising their tail upward leaving the forelimbs resting on the table surface. The animal was pulled steadily backward, 1 meter over 30 seconds, and the adjusting steps were recorded for each forepaw.

2.7. GDNF ELISA. GDNF secretion from cell-loaded devices was confirmed prior to implantation and again following retrieval from the brain. Immediately following retrieval, all devices were incubated at 37°C in HE-SFM. Media samples (4-hour incubation) were collected the next day to quantify GDNF release using a commercially available kit (DuoSet® for human GDNF; R&D Systems, Minneapolis, MN).

2.8. Immunohistochemistry. Rats were deeply anesthetized and transcardially perfused with 200 ml of 0.9% ice-cold saline. Following saline perfusion, the rats were decapitated and the devices were removed and processed for GDF secretion as described below. The brains were placed into Zamboni's fixative for 1 week and then transferred to 25% sucrose for 48 hours. Frozen, 40 μm thick coronal sections throughout the striatum and substantia nigra were cut and saved. An immunoperoxidase labeling method was used to visualize the volume of GDNF distribution in the rat striatum and substantia nigra while NewN-immunoreactive neurons were assessed in the striatum only. Endogenous peroxidase was quenched by 20-minute incubation in 0.1 M sodium periodate, and background staining was blocked by 1-hour incubation in a solution containing either 2% bovine serum albumin or 5% normal horse serum. Tissue sections were immunostained for GDNF (R&D Systems, AF-212-NA; 1 : 500) and NeuN (Millipore, MAB377; 1 : 1000) overnight at room temperature. After 6 washes, the sections were sequentially incubated for 1 hour in biotinylated horse anti-goat IgG (Vector Laboratories; 1 : 200) for GDNF and horse anti-mouse IgG (Vector Laboratories; 1 : 200) for NeuN followed by the *Elite* avidin-biotin complex (Vector Laboratories; 1 : 500) for 75 minutes. The immunohistochemical reaction was completed with 0.05% 3,3′-diaminobenzidine, 0.005% H_2O_2, and 0.05 M nickel (II) sulfate. Sections were mounted on gelatin-coated slides, dehydrated through graded alcohol, cleared in xylene, and cover-slipped with Cytoseal (Richard-Allan Scientific, Kalamazoo, MI).

2.9. Quantification. Optical fractionator unbiased sampling was used to estimate the total number of NeuN-immunoreactive neurons within the striatum [26, 27]. In each rat, we evaluated equispaced sections throughout the striatum length from its most anterior extent (Bregma + 2.2 mm) to the caudal level of the optic chiasm (Bregma − 1.3 mm). The striatum was outlined through a 1.25x objective using the Stereo Investigator software (MicroBrightField, VT), and the total number of NeuN-immunopositive neurons within the striatum was calculated for each animal. The Cavalieri estimator [26–28] was used to assess the volume of the striatum, the extent of QA lesion, and the GDNF distribution. Serial coronal sections extending throughout the striatum were sampled as described above using a 100×100 μm point grid with a 10x objective. The volume of the QA lesion was assessed by quantifying the extent of the absent area, whereas GDNF distribution was quantified by measuring the spreading of GDNF immunoreactivity, and the effects of QA injections and GDNF treatments on the striatal neural population were assessed by counting NeuN-immunostained neurons. Quantification of the relative optical density (OD) of striatal GDNF immunoreactivity was performed using an Olympus microscope coupled to a computer-assisted morphometry system (Scion Image 1.63; NIH), as described previously [27]. The GDNF immunostaining throughout the striatum was identified and manually outlined. The OD was then automatically measured by using the NIH image software. To account for differences in background staining intensity, background OD measurements in each section were taken from corpus callosum-lacking GDNF immunoreactive profiles and then subtracted from the OD of each GDNF-stained striatum to provide a final OD value.

3. Results

3.1. GDNF ELISA. GDNF secretion from cell-loaded devices was confirmed prior to implantation and again following retrieval from the brain. In all animals, the implanted devices were easily retrieved with no host tissue adhering to the capsule wall, they were removed intact, and there was no evidence that any capsule broke either in situ or during the retrieval procedure. All implants were located centrally within the striatum. Dorsally, the devices extended through the corpus callosum, the overlying cortex, and extended ventrally to approximately the level of the anterior commissure. Prior to implantation, device secretion corresponded to 163 ± 5.0 ng/24 hours and 1056 ± 194.0 ng/24 hours for the GDNF low- and GDNF high-dose groups, respectively. Following the 4-week implant, GDNF levels were elevated relative to preimplant values reaching secretion levels of 893.9 ± 205.6 ng/24 hours in the low-dose group and 2046.2 ± 381.0 ng/24 hours in the high-dose group.

3.2. Histology. Immunohistochemical staining of GDNF secreted from encapsulated cells revealed robust distribution

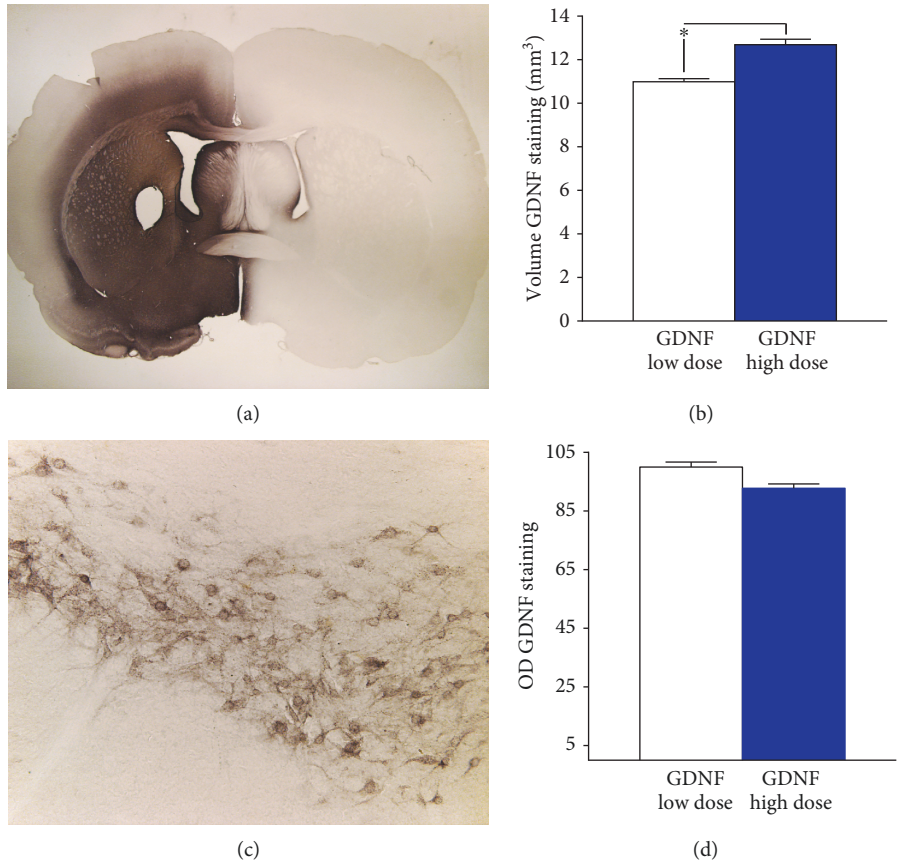

Figure 1: Widespread distribution of GDNF in the rat striatum. Photomicrographs of widespread GDNF immunoreactivity in the striatum of rats implanted with the lower dose GDNF-secreting device (a) and associated immunoreactivity in the substantia nigra (c). Histograms of the mean (±SEM) of the volume (b) and optical density (d) of GDNF staining. $^{*}p < 0.05$.

of the growth factor in all animals. Intense GDNF immunoreactivity was seen throughout the striatum and into the globus pallidus and ventral pallidum. Immunoreactivity was also observed in the corpus callosum and the overlying cortex adjacent to the implant site (Figure 1(a)). Neurons within the pars compacta of the substantia nigra stained positive for GDNF, consistent with the retrograde transport of the protein (Figure 1(c)). The volume of striatal GDNF was 10.99 $\pm$ 1.1 mm^3 for the low-dose group and 12.69 $\pm$ 1.24 mm^3 for the high-dose group (Figure 1(b)). In these cases, the volume of GDNF distribution accounted for approximately 82% and 90% of the total striatal volume, respectively. Quantitative measures of the OD of GDNF staining confirmed the high levels of GDNF although no differences were noted between the low- and high-dose GDNF groups (Figure 1(d)).

Immunohistochemical staining for NeuN confirmed that QA injections produced a marked spherical-shaped lesion that encompassed much of the striatum at the level of the injection (Figure 2(a)). Quantitative estimates of neuronal numbers confirmed both the magnitude of the QA lesion and the robust neuroprotection induced by GDNF. While neuronal numbers were decreased approximately 80% in QA-lesioned animals, this loss was largely prevented by GDNF with animals exhibiting a modest 15% and 6% neuronal loss in the low- and high-dose GDNF groups, respectively (Figure 2(b)). Lesion volume determinations showed a similar pattern with large volumetric losses induced by QA that were significantly prevented by GDNF (Figure 2(c)). QA injections produced a lesion that occupied approximately 65% of the total striatal volume. In contrast, treatment with GDNF resulted in a robust neuroprotective effect that manifests as small lesions encompassing only 4-5% of the implanted striatum (Figure 2(d)).

3.3. Behavioral Function. QA produced a significant loss in body weight that was attenuated by treatment with GDNF (Figure 3). While nontreated animals lost >20% of their initial weight and did not begin to regain weight for approximately 1 week postlesion, the GDNF-treated animals exhibited only a transient loss of weight lasting for approximately 24 hours that was then followed by a typical pattern of weight gain thereafter. The loss in weight was attenuated by both doses of GDNF, and this benefit was slightly more pronounced, but not statistically significant, in the high-dose group.

Tests of forelimb function in the cylinder, placing, and stepping tests confirmed that all groups of rats displayed normal forelimb use prior to the initiation of the study (preimplant; Figures 4(a)–4(c), respectively). Implantation of GDNF devices did not impact performance on any of the behavioral tasks. In contrast, the QA lesion produced significant behavioral deficits in all 3 tests. This effect was

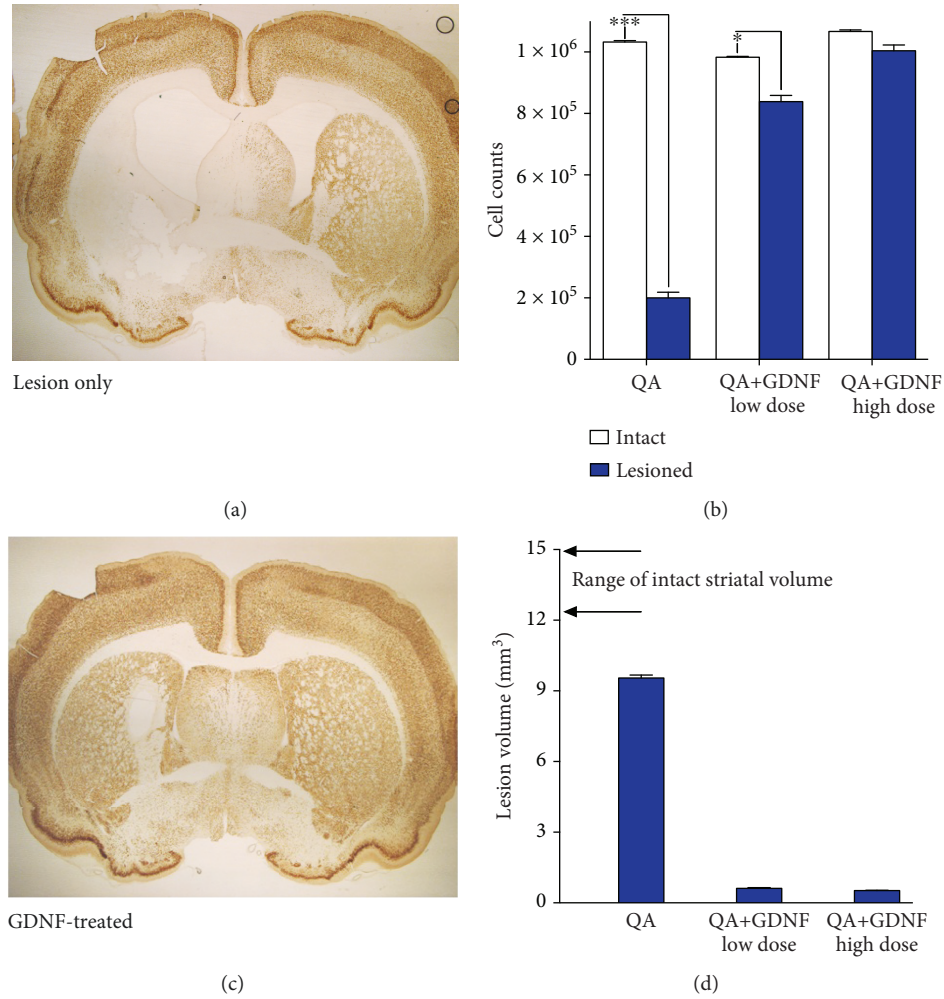

Figure 2: Protection of striatal neurons against QA toxicity. Photomicrographs of NeuN immunoreactivity illustrating an extensive loss of striatal neurons (a) that is largely prevented by prior implantation with the lower dose GDNF-secreting device (c). Quantification of striatal neurons (b) confirmed the extensive neuroprotection induced by GDNF with greater benefits observed in those animals receiving the higher dose implants. Quantification of the striatal volume further illustrated both the extensive nature of the QA lesion and the robust neuroprotection in GDNF-treated animals (d). $^{*}p < 0.05$; $^{***}p < 0.001$.

consistent and did not vary when the animals were tested at 2 versus 4 weeks postlesion. Relative to the intact forelimb, performance on the contralateral, impaired forelimb was decreased 69%-73%, 82%, and 49-56% (p's < 0.05) in these control animals on the cylinder, placing, and stepping tests, respectively. In contrast, the GDNF-treated rats displayed a notable, dose-related improvement in performance with the higher dose of GDNF resulting in a virtually complete recovery of behavior.

4. Discussion

Every year millions of people worldwide are diagnosed with a neurodegenerative disorder that is ultimately fatal. Unfortunately, despite significant stepwise advances in our understanding of the underlying causes of many of these diseases, effective treatments have yet to be developed. This is particularly true for treatments capable of slowing or even reversing the insidious and progressive nature of many degenerative diseases. Despite identifying trophic proteins that protect and/or augment the function of targeted populations of neurons in multiple animal models of human diseases, the dream of translating preclinical success to the clinic has not yet been realized.

While many factors play a role in the difficulty of translating preclinical work into an effective therapy, an overriding issue across therapies is delivering proteins to the brain at therapeutic levels. Direct brain delivery is usually required due to the remarkably effective protective physiology and anatomy of the blood-brain barrier (BBB) that restricts entry of the majority of systemically delivered molecules [29]. Attempting to bypass this barrier by directly injecting drugs and proteins into the brain tissue also tends to be ineffective because poor diffusion from a point source of infusion reducing exposure of the targeted tissue and limiting any therapeutic impact [13]. These problems are compounded by the fact that systemically delivered drugs, in particular proteins, are large charged molecules that tend to be unstable and have a

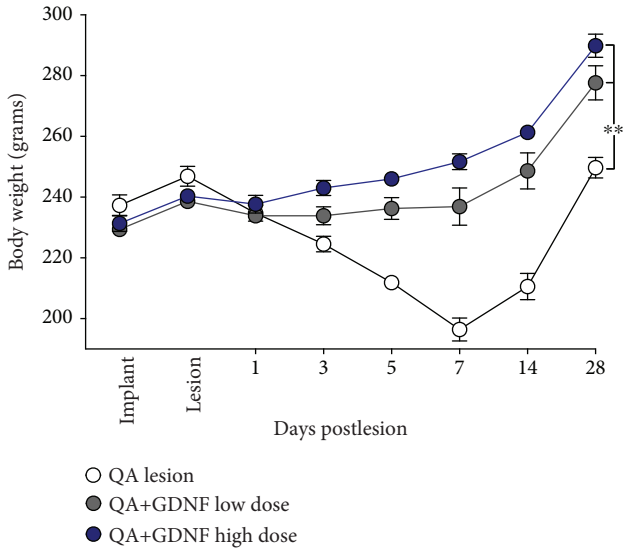

Figure 3: GDNF prevents the loss in body weight following QA lesion. QA alone produces a significant loss in body weight postlesion that is prevented by GDNF. While a trend towards greater benefit was observed with the higher secreting devices, this effect did not reach overall statistical significance. $^{**}p < 0.01$.

high propensity to aggregate and misfold that renders them ineffective and potentially toxic. Even if a protein can cross the BBB, it will be widely distributed and mistargeted throughout all brain parenchyma elevating the possibility for serious side effects such as those seen with mistargeting of proteins including the glial cell line-derived neurotrophic factor.

Here, we describe the use of polymer-encapsulated cells as a platform technology approach that has matured over the past 25 years and has now emerged as a viable therapeutic option capable of providing targeted, long-term, continuous, de novo synthesized delivery of very high levels of therapeutic molecules that can be distributed over significant portions of the brain [30]. In this approach, cells are enclosed or "encapsulated" within a capsule that has a semipermeable outer wall or membrane that can be implanted directly into the desired brain region [31–33]. The capsule wall morphology can be controlled to provide a pore structure that allows oxygen and nutrients to enter and nourish the cells while simultaneously providing a route for cell-secreted proteins, small molecules, antibodies, etc. to diffuse from the capsule and into the adjacent brain tissue. Immunological reactions that would typically occur against unencapsulated cells are prevented because the same porous structure that permits bidirectional flow eliminates entry of damaging elements of the host immune system into the capsule. Using human-derived cells even further eliminates any potential immunological reactions against the encapsulated cells. Of note, the present study used 2 different doses of GDNF obtained by simply extending the duration that the encapsulated cells were maintained in culture prior to implantation. We chose this pragmatic method only as a means of demonstrating a potential dose difference over the short duration of this study recognizing that such an approach would be insufficient for longer term dose differentiation as the encapsulated cells would simply reach capacity and equilibrate. Several more reliable and precise techniques of dose control could be used in clinical development including modifying the numbers of implants, changing the size of the devices including both the diameter and length, obliterating the center of the devices to alter the numbers of cells encapsulated, or selecting clonal cell lines with different secretion rates.

Intrastriatal injections of QA have been used as a model of Huntington's disease (HD) because the resulting excitotoxic lesion produces morphological changes similar to those seen in HD [34]. Trophic factors including GDNF and its family member neurturin have shown promise in animal models of several different neurodegenerative disorders, including HD [35–45]. The use of trophic factors has some unique appeal as a potential therapeutic in HD because genetic testing permits the identification of mutated gene carriers destined to suffer from HD [46]. Accordingly, identifying the genetic marker provides the potential opportunity to intercede prior to the development of symptoms secondary to neuronal degeneration. Still, the acute onset of toxicity produced by QA does not adequately capture the genetically driven onset and progression of neurodegeneration seen in the human disease, and the studies described here should be augmented with data obtained from studies using genetic mouse models of HD.

Ultimately, to be feasible, several essential prerequisites would need to be satisfied to treat the chronic and progressive nature of diseases such as HD. Here, we demonstrate that encapsulated cell technology can be used to provide widespread and targeted delivery of high levels of GDNF. These studies are enabled by the development of cell lines produced using a transposon-based gene expression system resulting in high GDNF secretion together with optimized cell scaffolding and membranes shown to promote long-term cell viability in vivo [21–23]. Additional studies have shown that the increases in striatal GDNF are persistent and stable for at least 6 months in the minipig putamen and 14 months in the rat striatum (the longest time point examined). The widespread GDNF diffusion was associated with pronounced behavioral protection and preservation of striatal anatomy as measured by sparing of NeuN-positive neurons and preservation of the striatal volume. While encouraging, subsequent studies should provide a more detailed analysis of striatal neuronal subtypes including more precise indices of individual neuron morphology and function. While not shown here, we have recently published several papers in animal models [31, 32], including excitotoxic lesions [33], that used GFAP immunohistochemistry to confirm the lack of inflammatory response following trophic factor delivery for longer periods of time than demonstrated in the current studies. Immunohistochemistry was also used to confirm GDNF receptor engagement. GDNF signals through a multicomponent receptor, first binding the GDNF family receptorα1 (GFRα1) with the resulting complex recruiting the transmembrane receptor kinase Ret or the neural cell adhesion molecule (NCAM) to initiate downstream signaling pathways. We found that treatment with GDNF dramatically increased the receptor expression and also highly increased RET phosphorylation [32].

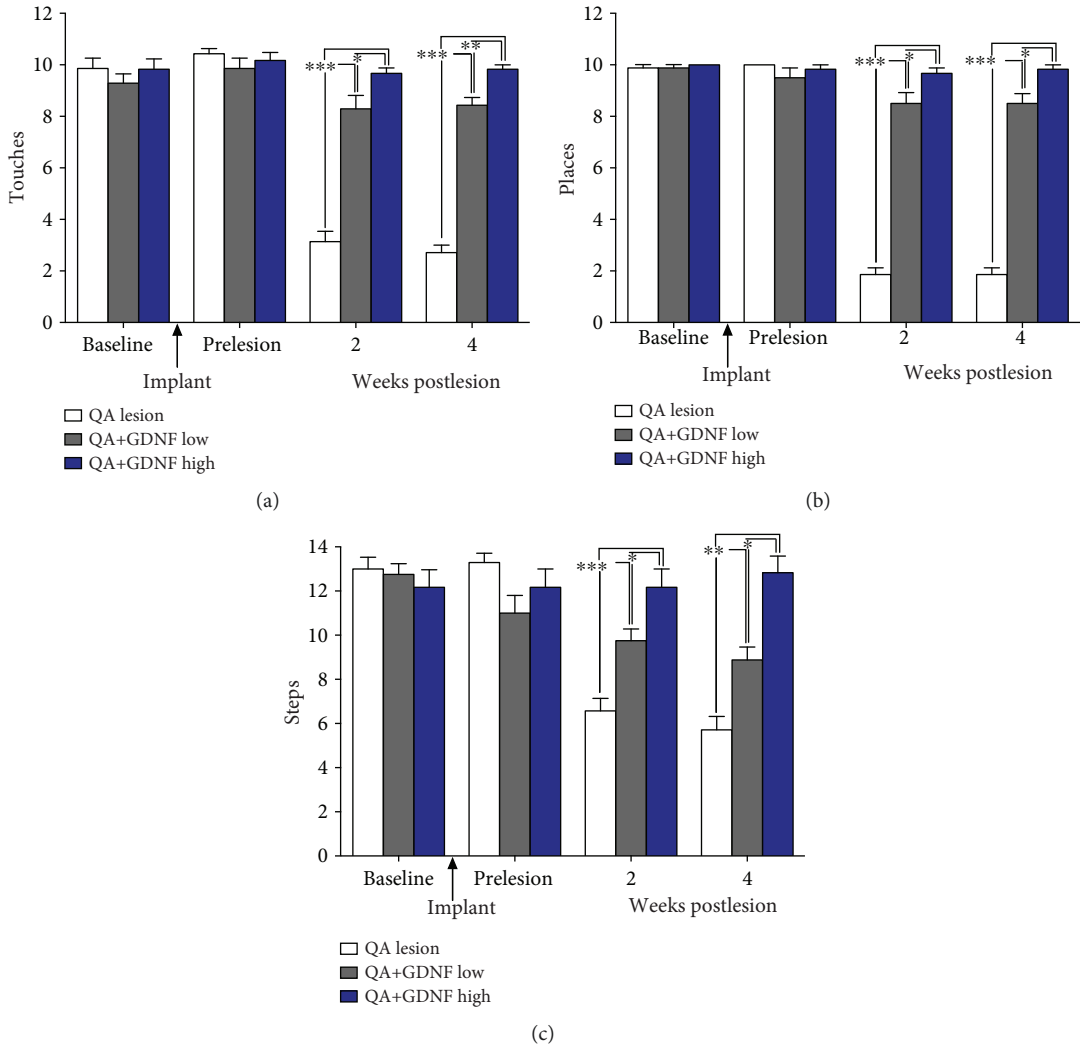

Figure 4: Performance in all the cylinder, placing, and stepping tests is significantly impaired at 2 and 4 weeks following intrastriatal injections of QA. In contrast, performance on each of these tests is preserved, in a dose-related manner, by EC-GNDF implants with the higher dose-treated animals performing comparably to presurgery levels (preimplantation and prelesion). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

Formal, GLP-compliant safety/toxicology studies conducted at Gloriana Therapeutics (unpublished data) have also confirmed the safety and tolerability of this approach by demonstrating that minipigs receiving bilateral implants of clinical-sized GDNF devices show no changes in food consumption/weight gain or behavior, no changes in blood chemistries, no production of anti-GDNF antibodies, and no surgery- or GDNF-related histopathological alterations were noted when the brains and peripheral organs were examined by a board-certified neuropathologist.

In conclusion, the encapsulated cell-based delivery system described here represents a greatly improved version of previous encapsulated cell-based systems to deliver trophic factors to the brain and to our knowledge is the first cellular delivery system capable of establishing the essential prerequisites of sustained, targeted, long-term delivery of sufficient quantities of GDNF to the CNS. As such, this approach represents a potentially novel and effective treatment for HD and other chronic neurodegenerative diseases.

Data Availability

The experimental data used to support the findings of this study are included within the article.

Conflicts of Interest

LUW and DFE are employees of Gloriana Therapeutics Inc., a for-profit biotechnology company which is developing the encapsulated cell technology to treat CNS diseases. All other authors declare that they have no conflicts of interest.

Authors' Contributions

DFE and LUW were involved in the design and conceptualization of the studies. DFE was responsible for all surgical aspects of the studies, and JHK and YC conducted all the anatomical aspects of the studies. GP was responsible for

statistical analyses. CT and BB were paid consultants at the time of these studies and provided general technical support.

References

[1] L. Wang, S. Muramatsu, Y. Lu et al., "Delayed delivery of AAV-GDNF prevents nigral neurodegeneration and promotes functional recovery in a rat model of Parkinson's disease," *Gene Therapy*, vol. 9, no. 6, pp. 381–389, 2002.

[2] R. Grondin, Z. Zhang, Y. Ai, D. M. Gash, and G. A. Gerhardt, "Intracranial delivery of proteins and peptides as a therapy for neurodegenerative diseases," in *Peptide Transport and Delivery into the Central Nervous System*, L. Prokai and K. Prokai-Tatrai, Eds., vol. 61 of Progress in Drug Research, pp. 101–123, Birkhäuser, Basel, 2003.

[3] E. Dowd, C. Monville, E. M. Torres et al., "Lentivector-mediated delivery of GDNF protects complex motor functions relevant to human Parkinsonism in a rat lesion model," *The European Journal of Neuroscience*, vol. 22, no. 10, pp. 2587–2595, 2005.

[4] J. S. Zheng, L. L. Tang, S. S. Zheng et al., "Delayed gene therapy of glial cell line-derived neurotrophic factor is efficacious in a rat model of Parkinson's disease," *Molecular Brain Research*, vol. 134, no. 1, pp. 155–161, 2005.

[5] Y. Miyoshi, Z. Zhang, A. Ovadia et al., "Glial cell line-derived neurotrophic factor-levodopa interactions and reduction of side effects in parkinsonian monkeys," *Annals of Neurology*, vol. 42, no. 2, pp. 208–214, 1997.

[6] D. M. Gash, Z. Zhang, A. Ovadia et al., "Functional recovery in parkinsonian monkeys treated with GDNF," *Nature*, vol. 380, no. 6571, pp. 252–255, 1996.

[7] S. Palfi, L. Leventhal, Y. Chu et al., "Lentivirally delivered glial cell line-derived neurotrophic factor increases the number of striatal dopaminergic neurons in primate models of nigrostriatal degeneration," *The Journal of Neuroscience*, vol. 22, no. 12, pp. 4942–4954, 2002.

[8] J. H. Kordower, M. E. Emborg, J. Bloch et al., "Neurodegeneration prevented by lentiviral vector delivery of GDNF in primate models of Parkinson's disease," *Science*, vol. 290, no. 5492, pp. 767–773, 2000.

[9] J. H. Kordower, S. Palfi, E. Y. Chen et al., "Clinicopathological findings following intraventricular glial-derived neurotrophic factor treatment in a patient with Parkinson's disease," *Annals of Neurology*, vol. 46, no. 3, pp. 419–424, 1999.

[10] A. E. Lang, S. Gill, N. K. Patel et al., "Randomized controlled trial of intraputamenal glial cell line-derived neurotrophic factor infusion in Parkinson disease," *Annals of Neurology*, vol. 59, no. 3, pp. 459–466, 2006.

[11] J. G. Nutt, K. J. Burchiel, C. L. Comella et al., "Randomized, double-blind trial of glial cell line-derived neurotrophic factor (GDNF) in PD," *Neurology*, vol. 60, no. 1, pp. 69–73, 2003.

[12] T. B. Sherer, B. K. Fiske, C. N. Svendsen, A. E. Lang, and J. W. Langston, "Crossroads in GDNF therapy for Parkinson's disease," *Movement Disorders*, vol. 21, no. 2, pp. 136–141, 2006.

[13] M. F. Salvatore, Y. Ai, B. Fischer et al., "Point source concentration of GDNF may explain failure of phase II clinical trial," *Experimental Neurology*, vol. 202, no. 2, pp. 497–505, 2006.

[14] P. F. Morrison, R. R. Lonser, and E. H. Oldfield, "Convective delivery of glial cell line–derived neurotrophic factor in the human putamen," *Journal of Neurosurgery*, vol. 107, no. 1, pp. 74–83, 2007.

[15] D. Shi, G. Chen, L. Lv et al., "The effect of lentivirus-mediated TH and GDNF genetic engineering mesenchymal stem cells on Parkinson's disease rat model," *Neurological Sciences*, vol. 32, no. 1, pp. 41–51, 2011.

[16] O. Lindvall and L. U. Wahlberg, "Encapsulated cell biodelivery of GDNF: a novel clinical strategy for neuroprotection and neuroregeneration in Parkinson's disease?," *Experimental Neurology*, vol. 209, no. 1, pp. 82–88, 2008.

[17] D. F. Emerich, G. Orive, C. Thanos, J. Tornoe, and L. U. Wahlberg, "Encapsulated cell therapy for neurodegenerative diseases: from promise to product," *Advanced Drug Delivery Reviews*, vol. 67-68, pp. 131–141, 2014.

[18] M. D. Lindner, S. R. Winn, E. E. Baetge et al., "Implantation of encapsulated catecholamine and GDNF-producing cells in rats with unilateral dopamine depletions and parkinsonian symptoms," *Experimental Neurology*, vol. 132, no. 1, pp. 62–76, 1995.

[19] D. F. Emerich, M. Plone, J. Francis, B. R. Frydel, S. R. Winn, and M. D. Lindner, "Alleviation of behavioral deficits in aged rodents following implantation of encapsulated GDNF-producing fibroblasts," *Brain Research*, vol. 736, no. 1-2, pp. 99–110, 1996.

[20] H. Kishima, T. Poyot, J. Bloch et al., "Encapsulated GDNF-producing C2C12 cells for Parkinson's disease: a pre-clinical study in chronic MPTP-treated baboons," *Neurobiology of Disease*, vol. 16, no. 2, pp. 428–439, 2004.

[21] L. Fjord-Larsen, P. Kusk, D. F. Emerich et al., "Increased encapsulated cell biodelivery of nerve growth factor in the brain by transposon-mediated gene transfer," *Gene Therapy*, vol. 19, no. 10, pp. 1010–1017, 2012.

[22] L. Fjord-Larsen, P. Kusk, J. Tornøe et al., "Long-term delivery of nerve growth factor by encapsulated cell biodelivery in the Göttingen minipig basal forebrain," *Molecular Therapy*, vol. 18, no. 12, pp. 2164–2172, 2010.

[23] L. U. Wahlberg, G. Lind, P. M. Almqvist et al., "Targeted delivery of nerve growth factor via encapsulated cell biodelivery in Alzheimer disease: a technology platform for restorative neurosurgery," *Journal of Neurosurgery*, vol. 117, no. 2, pp. 340–347, 2012.

[24] N. Saito, A. Washio, K. Miyashita et al., "Effects of polymer encapsulated glial cell line-derived neurotrophic factor secreting cells on odontoblast-like cell survival," *Journal of Regenerative Medicine*, vol. 6, no. 3, p. 3, 2017.

[25] J. Tornøe, M. Torp, J. R. Jørgensen et al., "Encapsulated cell-based biodelivery of meteorin is neuroprotective in the quinolinic acid rat model of neurodegenerative disease," *Restorative Neurology and Neuroscience*, vol. 30, no. 3, pp. 225–236, 2012.

[26] R. T. Bartus, C. D. Herzog, Y. Chu et al., "Bioactivity of AAV2-neurturin gene therapy (CERE-120): differences between Parkinson's disease and nonhuman primate brains," *Movement Disorders*, vol. 26, no. 1, pp. 27–36, 2011.

[27] Y. Chu, G. A. Morfini, L. B. Langhamer, Y. He, S. T. Brady, and J. H. Kordower, "Alterations in axonal transport motor proteins in sporadic and experimental Parkinson's disease," *Brain*, vol. 135, no. 7, pp. 2058–2073, 2012.

[28] H. J. G. Gundersen and E. B. Jensen, "The efficiency of systematic sampling in stereology and its prediction," *Journal of Microscopy*, vol. 147, no. 3, pp. 229–263, 1987.

[29] W. M. Pardridge, "The blood-brain barrier: bottleneck in brain drug development," *NeuroRX*, vol. 2, no. 1, pp. 3–14, 2005.

[30] L. Fjord-Larsen, P. Kusk, M. Torp et al., "Encapsulated cell biodelivery of transposon-mediated high-dose NGF to the Göttingen mini pig basal forebrain," *The Open Tissue Engineering and Regenerative Medicine Journal*, vol. 5, no. 1, pp. 35–42, 2012.

[31] C. Falcicchia, G. Paolone, D. F. Emerich et al., "Seizure-suppressant and neuroprotective effects of encapsulated BDNF-producing cells in a rat model of temporal lobe epilepsy," *Molecular Therapy - Methods & Clinical Development*, vol. 9, pp. 211–224, 2018.

[32] G. Paolone, C. Falcicchia, F. Lovisari et al., "Long-term, targeted delivery of GDNF from encapsulated cells is neuroprotective and reduces seizures in the pilocarpine model of epilepsy," *The Journal of Neuroscience*, 2019.

[33] A. Nanobashvili, E. Melin, D. Emerich et al., "Unilateral ex vivo gene therapy by GDNF in epileptic rats," *Gene Therapy*, 2018.

[34] S. Ramaswamy, J. L. McBride, and J. H. Kordower, "Animal models of Huntington's disease," *ILAR Journal*, vol. 48, no. 4, pp. 356–373, 2007.

[35] J. R. Pineda, N. Rubio, P. Akerud et al., "Neuroprotection by GDNF-secreting stem cells in a Huntington's disease model: optical neuroimage tracking of brain-grafted cells," *Gene Therapy*, vol. 14, no. 2, pp. 118–128, 2007.

[36] J. Alberch, E. Pérez-Navarro, and J. M. Canals, "Neuroprotection by neurotrophins and GDNF family members in the excitotoxic model of Huntington's disease," *Brain Research Bulletin*, vol. 57, no. 6, pp. 817–822, 2002.

[37] S. Marco, E. Pérez-Navarro, E. Tolosa, E. Arenas, and J. Alberch, "Striatopallidal neurons are selectively protected by neurturin in an excitotoxic model of Huntington's disease," *Journal of Neurobiology*, vol. 50, no. 4, pp. 323–332, 2002.

[38] E. Gratacòs, E. Pérez-Navarro, E. Tolosa, E. Arenas, and J. Alberch, "Neuroprotection of striatal neurons against kainate excitotoxicity by neurotrophins and GDNF family members," *Journal of Neurochemistry*, vol. 78, no. 6, pp. 1287–1296, 2001.

[39] E. Pérez-Navarro, E. Arenas, J. Reiriz, N. Calvo, and J. Alberch, "Glial cell line-derived neurotrophic factor protects striatal calbindin-immunoreactive neurons from excitotoxic damage," *Neuroscience*, vol. 75, no. 2, pp. 345–352, 1996.

[40] S. Ramaswamy, J. L. McBride, I. Han et al., "Intrastriatal CERE-120 (AAV-neurturin) protects striatal and cortical neurons and delays motor deficits in a transgenic mouse model of Huntington's disease," *Neurobiology of Disease*, vol. 34, no. 1, pp. 40–50, 2009.

[41] S. Ramaswamy, J. L. McBride, C. D. Herzog et al., "Neurturin gene therapy improves motor function and prevents death of striatal neurons in a 3-nitropropionic acid rat model of Huntington's disease," *Neurobiology of Disease*, vol. 26, no. 2, pp. 375–384, 2007.

[42] J. L. McBride, S. Ramaswamy, M. Gasmi et al., "Viral delivery of glial cell line-derived neurotrophic factor improves behavior and protects striatal neurons in a mouse model of Huntington's disease," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 24, pp. 9345–9350, 2006.

[43] D. M. Araujo and D. C. Hilt, "Glial cell line-derived neurotrophic factor attenuates the excitotoxin-induced behavioral and neurochemical deficits in a rodent model of Huntington's disease," *Neuroscience*, vol. 81, no. 4, pp. 1099–1110, 1997.

[44] D. M. Araujo and D. C. Hilt, "Glial cell line-derived neurotrophic factor attenuates the locomotor hypofunction and striatonigral neurochemical deficits induced by chronic systemic administration of the mitochondrial toxin 3-nitropropionic acid," *Neuroscience*, vol. 82, no. 1, pp. 117–127, 1998.

[45] J. L. McBride, M. J. During, J. Wuu, E.-Y. Chen, S. E. Leurgans, and J. H. Kordower, "Structural and functional neuroprotection in a rat model of Huntington's disease by viral gene transfer of GDNF," *Experimental Neurology*, vol. 181, no. 2, pp. 213–223, 2003.

[46] The Huntington's Disease Collaborative Research Group, "A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes," *Cell*, vol. 72, no. 6, pp. 971–983, 1993.

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Research Article

New Genotypes and Phenotypes in Patients with 3 Subtypes of Waardenburg Syndrome Identified by Diagnostic Next-Generation Sequencing

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Background. Waardenburg syndrome (WS) is one of the most common forms of syndromic deafness with heterogeneity of loci and alleles and variable expressivity of clinical features. *Methods*. The technology of single-nucleotide variants (SNV) and copy number variation (CNV) detection was developed to investigate the genotype spectrum of WS in a Chinese population. *Results*. Ninety WS patients and 24 additional family members were recruited for the study. Fourteen mutations had not been previously reported, including c.808C>G, c.117C>A, c.152T>G, c.803G>T, c.793-3T >G, and c.801delT on *PAX3*; c.642_650delAAG on *MITF*; c.122G>T and c.127C>T on *SOX10*; c.230C>G and c.365C>T on *SNAI2*; and c.481A>G, c.1018C>G, and c.1015C>T on *EDNRB*. Three CNVs were de novo and first reported in our study. Five *EDNRB* variants were associated with WS type 1 in the heterozygous state for the first time, with a detection rate of 22.2%. Freckles occur only in WS type 2. Yellow hair, amblyopia, congenital ptosis, narrow palpebral fissures, and pigmentation spots are rare and unique symptoms in WS patients from China. *Conclusions*. *EDNRB* should be considered as another prevalent pathogenic gene in WS type 1. Our study expanded the genotype and phenotype spectrum of WS, and diagnostic next-generation sequencing is promising for WS.

1. Introduction

Waardenburg syndrome (WS) is a rare genetic disorder with a reported frequency estimated to be approximately 1 : 40000 in the general population but is present in approximately 3% of all patients with congenital deafness [1, 2]. The disorder is characterized by the presence of pigmentation abnormalities, including depigmented patches of the skin and hair, heterochromia iridis, and sensorineural hearing impairment. Dystopia canthorum, musculoskeletal abnormalities of the limbs, or Hirschsprung disease, are used for the clinical classification. Four subtypes of WS have been described based on the clinical manifestations. Six genes are involved in this inherited disorder, including *PAX3* (encoding the paired box 3 transcription factor), *MITF* (microphthalmia-associated transcription factor), *SOX10* (encoding the Sry box 10 transcription factor), *EDNRB* (endothelin receptor type B), *EDN3* (endothelin 3), and *SNAI2* (SNAIL homolog 2). WS type 1 (OMIM # 193500) was first described by Waardenburg [3]. Dystopia canthorum, an outward displacement of the

inner canthus of the eyes, is the most penetrant feature of WS type 1. *PAX3* mutations account for the majority of WS type 1 cases. Features of WS type 2 (OMIM #) show marked interfamilial and intrafamilial variability. There are 3 genes linked to WS type 2, namely, *MITF*, *SOX10*, and *EDNRB*. However, pathogenic genes cannot be detected in 70% of WS type 2 cases [4]. WS type 3 is associated with limb deformities together with the symptoms observed in type 1. *PAX3* mutations have also been found in the heterozygous or homozygous state in WS type 3 (OMIM # 148820) [5]. WS type 4 (OMIM # 277580), also called the Shah-Waardenburg syndrome, is characterized by the association of deafness, depigmentation, and intestinal aganglionosis (called Hirschsprung disease (HD)). The endothelin pathway (endothelin 3 (*EDN3*), endothelin receptor type B (*EDNRB*), and Sry box 10 (*SOX10*)) was found to be involved in WS type 4 [6, 7].

It has been determined that pathogenic mutations were not and still cannot be detected in a considerable number of WS cases. Other types of variations may exist, given the limitations of conventional detection technology. Copy number variation (CNV) is a new topic of increasing interest in genetic research. In addition, CNV has been reported to be associated with WS [8–10]. Recently, one study addressed the molecular etiology investigation of WS in individuals mostly from southeastern Brazil by sequential Sanger sequencing of all coding exons of the 6 WS-associated genes, followed by CNV detection by multiplex ligation-dependent probe amplification (MLPA) of the *PAX3*, *MITF*, and *SOX10* genes, and revealed novel pathogenic mutations [11]. Traditional sequencing methods are designed for point mutation detection without considering the possibility of CNV. Exon capture sequencing for simultaneous SNV and CNV detection in WS has been developed in our study.

To date, there is no large cohort on the genotype and phenotype spectrum of WS patients from China. In this study, we aimed to investigate the genetic etiology and phenotype differences using target exon capture of 6 known causative genes in WS.

2. Materials and Methods

2.1. Patients and Family Members. The patients diagnosed with WS were examined occasionally at the Otology Clinic and training schools for deaf and mute individuals in China from September 2006 to February 2018. There were 114 participants, including sporadic WS cases and 18 families (Figure 1, Table 1). All the WS patients were clinically evaluated by at least one otologist. The clinical signs and symptoms of the 90 patients diagnosed with WS are presented in Table S1. Two hundred randomly selected normal hearing individuals were included in this study. Blood samples (4-6 ml) were extracted from the peripheral veins of all the participants for DNA extraction. The ethics committee of Xiangya Hospital, Central South University, had approved this study, and signed informed consents were obtained from each of the subjects or their guardians.

2.2. Clinical Evaluation. A comprehensive clinical history was collected by questionnaire and telephone inquiry. The audiological examinations consisted of otoscopy, pure-tone audiometry (PTA) or auditory brainstem response (ABR), immittance, and distortion product otoacoustic emission (DPOAE). Special attention was given to the color of the skin, hair, and irises and other developmental defects. Assessment of dystopia canthorum on the basis of ocular measurements was described by Farrer et al. [12].

2.3. Exon Capture Sequencing for Simultaneous SNV and CNV Detection in WS. The primer sequences for 6 WS-related genes were designed for the target regions (Table S2), and the regions of interest were captured and enriched. PCR amplification was divided into two rounds. The first round of PCR amplification was to amplify the 6 relevant WS genes. The amplification region includes the promoter regions (~500 bp), 5′untranslated region (5′UTR), coding regions, splice sites (~8 bp), and 3′untranslated region (3′UTR) (Table 2). The multiplex PCR amplification system can amplify 20-30 gene fragments at the same time. Each sample requires approximately 4 multiplex PCRs to complete the first round of enrichment. The second round of PCR added the 4 multiplex PCR products above into a mixture by amplifying the universal sequence. Indexes were added to distinguish between different samples. After these two rounds of amplification were finished, the WS sequencing library was constructed. Bidirectional sequencing validation of the target segments was performed by 2x 250 bp sequencing with an Illumina MiSeq Sequencer. The average effective sequencing depth for each sample was 300x, with all bases having greater than 20x sequencing depth (Figure 2)

The CNV detection technique utilizes ligase to hybridize and ligate the region of interest. Then, different lengths of the ligated products corresponding to the loci were obtained by introducing nonspecific sequences of different lengths to the ends of the ligation probes and performing a ligation reaction. The PCR product was amplified by fluorescently labeled universal primers. The amplified products were separated by fluorescence capillary electrophoresis and analyzed by electrophoresis. The peak height at each site was analyzed

2.4. Bioinformatics Analysis. After mapping WS-related gene sequences to the DNA samples, the results were aligned with the reference genome. Sequencing data quality was assessed through the sequencing depth of each fragment of each sample. Inconsistent sites were detected by comparison with the reference genome, which is called SNV calling. Then, SNVs were functionally annotated and functional candidate sites were determined.

2.5. Sanger Sequencing for the Segregation of Candidate Variants. DNA samples of affected siblings and their available first-degree relatives were collected for segregation analysis of candidate variants. For validation and segregation analyses, the primers were designed to amplify the regions flanking the variant as previously described [4, 13]. Segregation analysis by Sanger sequencing was also performed in 200 ethnically matched individuals with normal hearing. These included variants annotated as nonsense mutations, splicing mutations disrupting either a splice donor or acceptor site,

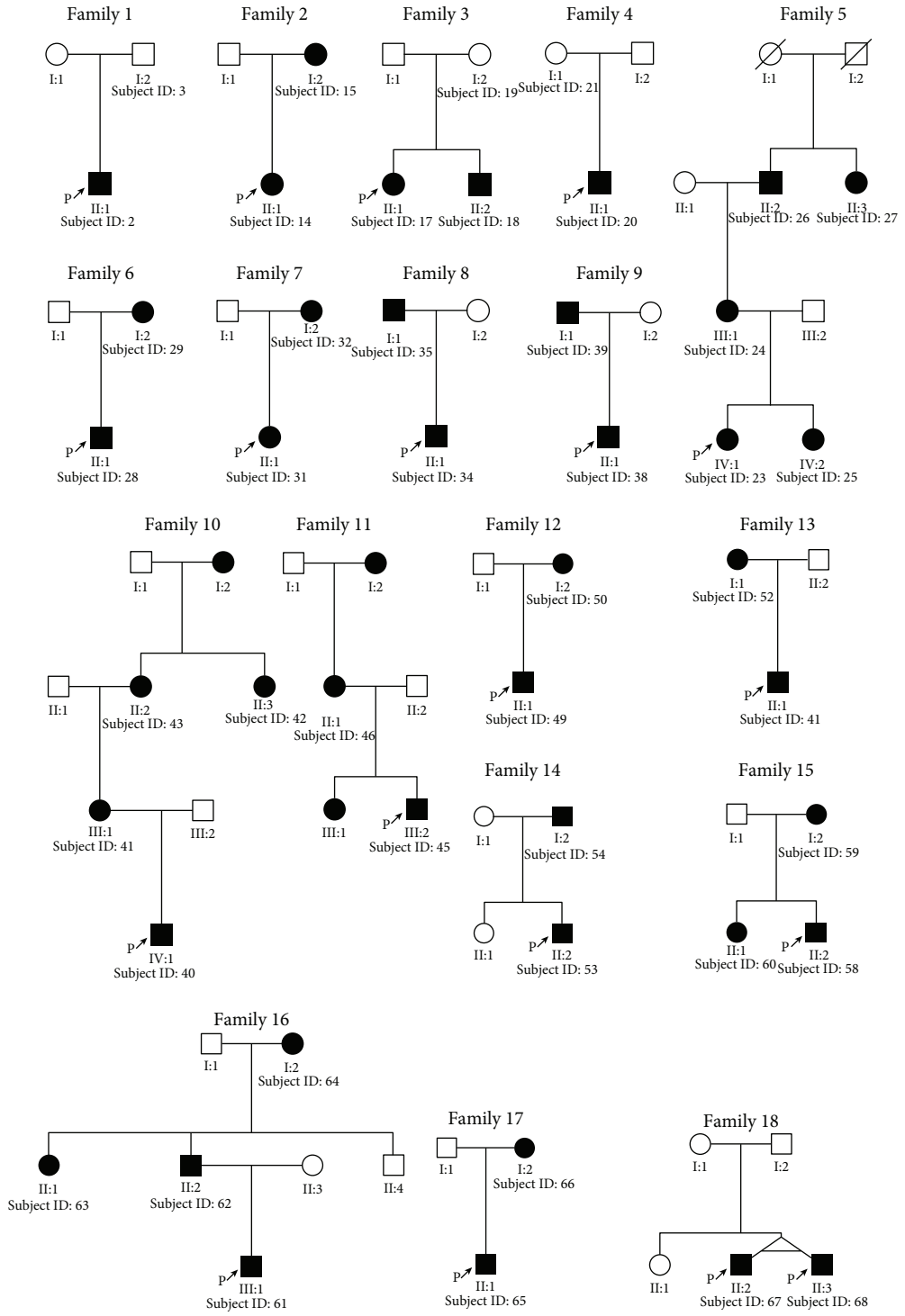

Figure 1: Eighteen WS families with at least 2 DNA samples and clinical information collected in the study. Family ID and Subject ID were added to the individuals with DNA samples. A table with all data for family cases was shown in Table 1.

frameshift or non-frameshift-causing InDels, and missense mutations predicted as damaging by at least one of the following methods: SIFT, Polyphen2_HVAR, Polyphen2_HDIV, MutationTaster, and CADD [14–16]. Nonsynonymous SNVs with a SIFT score < 0.05, Polyphen2_HVAR score ≥ 0.047, Polyphen2_HDIV ≥ 0.0453, MutationTaster score > 0.85, or CADD score > 15 were considered significantly deleterious. To sort potentially deleterious variants from benign polymorphisms, Perl scripts were used to filter the SNVs against those in the 1000 Genomes and esp6500si_all databases. We also tested all the variants for their allele frequencies in the Exome Aggregation Consortium (ExAC) (http://exac.broadinstitute.org/) to further support the pathogenicity of the new variants detected. The SNV

Table 1: All data for family cases.

Family ID	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17		18
Subject ID	2.3.	14.15.	17.18.19.	20.21.	23.24.25.26.27.	28.29.	31.32.	34.35.	38.39.	40.41.42.43.	45.46.	49.50.	51.52.	53.54.	58.59.60.	61.62.63.64.	65.66.		67.68.
Gene	*PAX3*	*SOX10*	*SOX10*	*SOX10*	*MITF*	*MITF*	NA	*PAX3*	*PAX3*	*MITF*	NA	*PAX3*	*PAX3*	*PAX3*	NA	*MITF*	*PAX3*	*EDNRB*	*MITF*
Mutation	c.238C>G	c.743_744delAG	c.254G>A	c.122G>T	c.763C>T	c.328 C>T	—	c.784C>T	c.232G>A	c.944_946del	—	c.808C>G	c.117C>A	c.452-2A>G	—	c.1013+1G>A	c.793-3T>G	c.469A>G	c.1022_1023delTC
Protein mutation	p.H80D	p. E248Afs*32	p.W85*	p.G41V	p. R255*	p.R110*	—	p.R262*	p.V78M	NA	—	,p.R270G	p.N39K	—	—	NA	NA	p.I157V	p.L341Rfs*18

NA in gene: the molecular etiology of cases that remained unexplained. NA in protein mutation: protein changes that cannot be described.

Table 2: WS-associated genes and their PCR target regions.

Gene name	NM_accession	Exon number	Gene length	mRNA length	Exon length
PAX3	NM_181457.3	8	97284	2032	478 (268)|coding+3′UTR,215,166,206,135,130,236,466|coding+5′UTR
SNAI2	NM_003068.4	3	3764	2112	1312 (183)|coding+3 ′UTR,546,254|coding+5′UTR
MITF	NM_000248.3	9	31738	4472	156|coding+5′UTR,228,84,96,118,75,76,148,3491 (402)|coding+3′ UTR
EDNRB	NM_000115.3	8	80049	4282	2854 (136)|coding+3′UTR,109,134,150,205,113,534|coding+5′ UTR,183|5′UTR
SOX10	NM_006941.3	4	12221	2862	1887 (705)|coding+3′UTR,269,512|coding+5′UTR,194|5′UTR
EDN3	NM_207034.1	5	25549	2619	421|coding+5′UTR,313,177,46,1662 (129)|coding+3′UTR

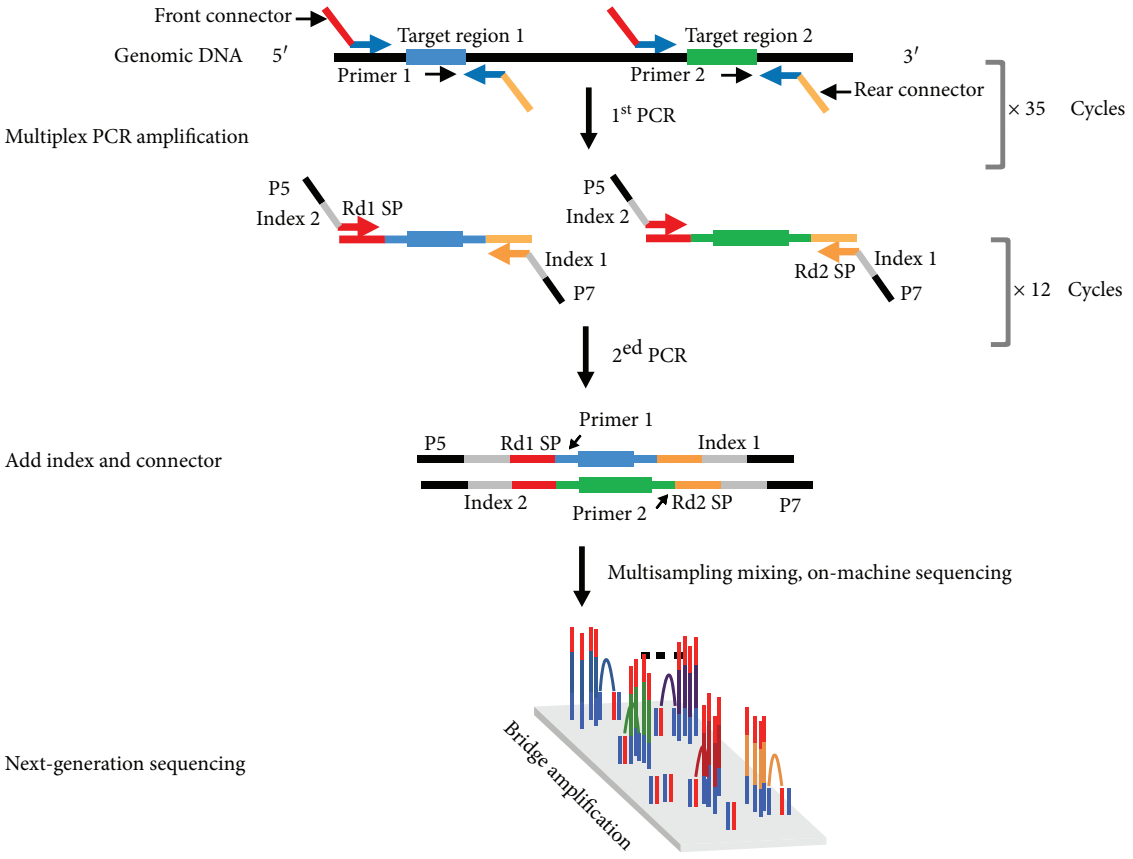

Figure 2: Multiple PCR target enrichment and next-generation Sequencing of WS-related genes.

recorded in the population database with a minor allele frequency of <1/100000 in the population from the database was considered disease causing and therefore remained.

3. Results

Most proteins associated with the known WS genes are involved in melanocyte migration and neural crest and inner ear cell development. The clinical symptoms of WS result from neural crest embryonic cell defects. Sensorineural hearing loss is one of the most common signs and symptoms (93.3%, 84/90) in the 90 cases meeting the diagnostic criteria for WS. Auditory function is variable within and between families, ranging from normal to profound deafness. Bilateral deafness is much more common than unilateral, as only one case in our study was found to have unilateral hearing loss. The reported prevalence of temporal bone abnormalities varies from 0 to 50% [17]. Vestibular aqueduct dilatation, together with Waardenburg syndrome, was discovered in 2 cases (Subjects 113 and 114) in our study. No vestibular dysfunctions were found in any of the participants. A white forelock was present in 15.6% (14/90) of all 90 cases. Interestingly, 4 patients of Chinese Han race WS patients presented with yellow hair before reaching 3 years old. All reported hair color in WS patients was prematurely graying and turning white, but the normal hair color in the Chinese

Han race is black. Hypoplastic iridis, particularly brilliant blue eyes, was present in 86.7% (78/90) of WS patients. The heterochromia was observed to be complete or segmental. The following prevalent phenotype included freckles on the face (20.0%, 18/90), which seems to be a special clinical sign in the Chinese population, given its high occurrence rate. The rate of hypopigmented skin lesions was more rare than that in other populations [18], with only 3 in 90 cases (3.3%) being found. Pigmentation spots (2.2%, 2/90) on the skin might be a special subtype of skin pigmentation disturbances. Other rare and unreported phenotypes, including amblyopia, congenital ptosis, and narrow palpebral fissures, were recorded to accompany WS, but no clear evidence has been found showing that these phenotypes relate to neural crest embryonic cell defects at present (Figure 3).

The subtypes of WS were defined on the basis of the presence or absence of additional symptoms. WS type 1 was characterized by dystopia canthorum and WS type 2 with no additional features. Type 4 was also called Shah-Waardenburg syndrome, Hirschsprung disease included. Twenty-seven cases were diagnosed as WS type 1, 57 as WS type 2, and 6 as WS type 4. There were no WS type 3 cases recruited. WS types 1 and 2 were more frequent than type 4. The diagnostic next-generation sequencing in all 114 participants revealed 119 variants (Table S3); however, only 90 cases were diagnosed as WS according to the widely accepted diagnostic criteria [19]. Of the 90 WS patients, 49 were considered causative in WS and recorded in the Human Gene Mutation Database (HGMD) with a known disease mutation detection rate of 53.3% (49/90). Fourteen unreported mutations were detected and selected for further bioinformatics pathogenicity analysis (Table 3). Three CNVs discovered are listed separately in Table 4. Figure 4 shows the position in the protein domains of the new SNVs detected in our study. WS type 2 is mainly caused by heterozygous pathogenic variants in genes *MITF* (24/57, 42.1%), *PAX3* (10/57, 17.5%), and *SOX10* (9/57, 15.8%). Homozygous deletions in *SNAI2* were also reported to associate with WS type 2 cases [20]. One variant was detected in our research, which indicated that *SNAI2* mutations seem to be rare in WS type 2 among Eastern and Western populations. In contrast with the reported findings, *EDN3* mutations were also detected in the WS type 2 cases (Subjects 72, 76, and 104) in our study. Even after extensive analysis of the 6 known genes, a percentage of the WS type 1 and type 2 cases, 14.8% (4/27) and 26.3% (15/57), respectively, remained molecularly unexplained (Figure 4). No WS type 3 cases were recruited in our study. The *EDNRB*, *EDN3*, and *SOX10* mutations [21, 22] are related to WS type 4, and close to 80% of the WS type 4 cases were found to be caused by mutations in *SOX10* in our study.

Two CNVs and 3 SNVs, including c.110_219del110bp in *MITF*, the whole-gene deletion in *SOX10*, c.801delT on *PAX3*, c.642_650delAAG on *MITF*, and c.127C>T on *SOX 10*, resulted in a truncated protein with a premature termination, which were loss-of-function (LoF) mutations and therefore considered to be cause of disease. The last CNV meant a duplicate sequence from promoter 2 to exon 1 on *MITF*, which might be one of the reasons for a gain-of-function (GoF) mechanism. Therefore, protein function is activated. The expression or degradation of proteins resulted in increased protein dosage. All the remaining missense substitutions were selected for pathogenicity prediction and population frequency, and the forecast results and data are shown in Table 3.

4. Discussion

WS is a genetic disorder with locus heterogeneity and variable expressivity of clinical features [23]. WS type 1 and type 2 are conspicuously differentiated by the presence or absence of dystopia canthorum among populations [18]. In this present study, we have identified mutations in *PAX3* in cases (Subjects 2, 4, 6, 49, 51, 54, 90, 91, 98, and 104) without dystopia canthorum, typical WS type 2 characteristic; conversely, Subjects 67, 68, and 110 with the clinical feature of WS type 1 (with the presence of dystopia canthorum) were found to carry *MITF* variants. Thus, it is difficult to establish a link between a genotype and a classical WS phenotype in our cohort. This implies that other factors including interactions between genes, gene-environment interactions, and ethnic background may modulate WS phenotypes.

Overall, 55.5% (15/27) of the cases of WS type 1 are caused by pathogenic variants in *PAX3*. A recent study suggests that homozygous mutation in *EDNRB* can cause the WS type 1 phenotype [24], whereas in our study, heterozygous mutations in *EDNRB* (Subjects 48, 65, 66, 85, 108, and 111) were related to WS type 1 for the first time, with a detection rate of 22.2% (6/27).

Six pathogenic genes have been associated with the clinical manifestations so far. Overall, 81 pathogenic or likely causative variants were associated with WS in our sample of 90 WS patients, including the probands and their available family members, and consisting of 27 variants located in *MITF* (33.3%, 27/81), 25 in *PAX3* (30.9%, 25/81), 15 in *SOX10* (18.5%, 15/81), 9 in *EDNRB* (11.1%, 9/81), 3 in *EDN3* (3.7%, 3/81), and 2 in *SNAI2* (2.5%, 2/81).

In the group of 27 participants with WS type 1, 15 were found to carry mutations in *PAX3*, which corresponds to a detection rate of 55.5% (15/27). In the review by Pingault et al. [18], it is stated that 90% of WS type 1 patients have mutations in the *PAX3* gene. On the other hand, in the sample from Caucasian [25], mutations in *PAX3* were detected in 33.6% of 119 patients with clinical suspicion of WS. In the latter study by Bocángel et al. [11], 11 variants were located in *PAX3* (57.9%). Our data were very similar to the results of Bocángel et al. [11]. Genetic analyses in a study by Morimoto et al. [24] revealed that the proband had a missense mutation (p.R319W) in the *EDNRB* gene. In our study, 4 *PAX3*-negative WS type 1 patients (Subjects 48, 85, 108, and 111) were found to carry at least one novel *EDNRB* heterozygous mutation, suggesting that *EDNRB* is the second most prevalent pathogenic gene and should be considered for screening analysis in WS type 1 patients.

Three mutations in *MITF* were linked to WS type 1, and 10 variants in *PAX3* were related to WS type 2. Dystopia

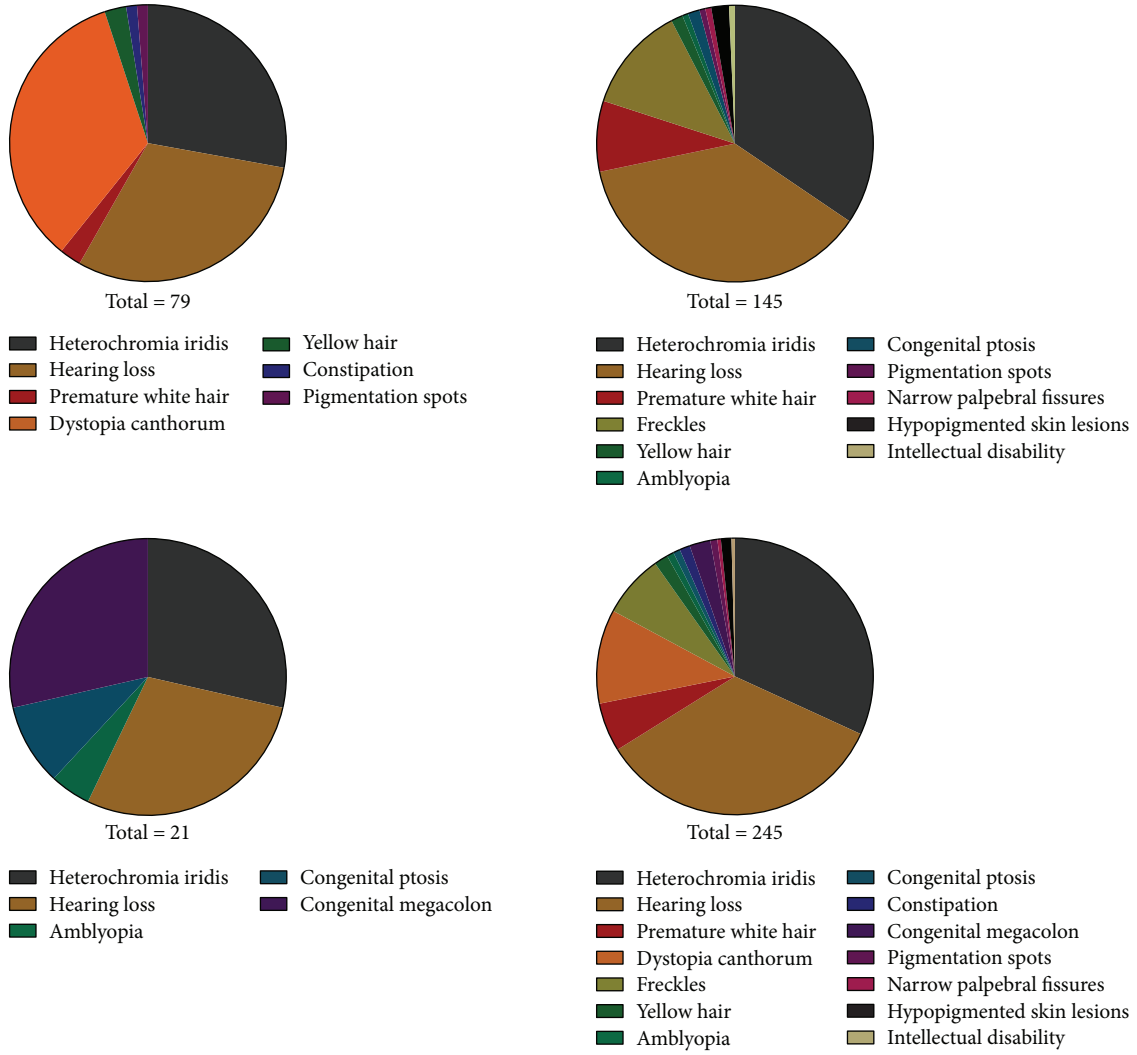

Figure 3: The proportion of phenotypes detected in WS types 1, 2, and 4. Dystopia canthorum was the most frequent sign in WS type 1 (100%, 27/27), followed by sensorineural hearing loss (88.9%, 24/27), heterochromia iridis (81.5%, 22/27), hair hypopigmentation (14.8%, 4/27), constipation (1/27, 3.7%), and pigmentation spots (1/27, 3.7%). In WS type 2, sensorineural deafness (94.7%, 54/57) and heterochromia iridis (87.7%, 50/57) were still the most common clinical signs and symptoms, followed by freckles (31.6%, 18/57), hair hypopigmentation (24.6%, 14/57), hypopigmented skin lesions (5.3%, 3/57), and congenital ptosis (3.5%, 2/57). Amblyopia (1.8%, 1/57), congenital ptosis (1.8%, 1/57), and narrow palpebral fissures (1.8%, 1/57) are rare and unique symptoms in WS type 2 in the Chinese population.

canthorum is considered as the most reliable feature for WS type 1 classification due to its very high penetrance [19]. In fact, Asian individuals generally have a wider and lower nasal root than European and American individuals, suggesting that the classification criteria cannot be applied to all populations and molecular genetic testing may be a complementary tool for establishing the diagnosis. In the group of 57 individuals with WS type 2, mutations in 4 other causative subtype genes are 51.1% (24/47) in *MITF*, 19.1% (9/47) in *SOX10*, 6.4% (3/47) in *EDN3*, and 2.0% (1/47) in *SNAI2*. Compared to Pingault et al.' s review [18], in which the overall detection rate in WS type 2 is approximately 50%, our detection technology (73.7%, 42/57) has absolute advantages. *SNAI2* and *EDN3* mutations in WS type 2 are not observed frequently, which is similar to our data. Three CNVs overlapping the *MITF* and *SOX10* genes were found in 3 patients (Subjects 9, 22, and 33).

It is also remarkable that known disease mutations were inherited from unaffected fathers in Families 1 (Subjects 2 and 3), 3 (Subjects 17, 18, and 19), and 4 (Subjects 20 and 21). The c.238C>G in *PAX3* is a known disease-causing mutation. It is puzzling is that there are no WS-related clinical manifestations appearing in Subject 3 who harbors the same mutation. The results found in our study greatly expanded the database of hotspot and novel mutations in WS types 1, 2, and 4. PAX3 is a transcription factor expressed during embryonic development [26] and four structural motifs, paired domain, octapeptide sequence, homeodomain, and a Pro-Ser-Thr-rich COOH terminus, were included in PAX3. The 6 mutations in

Table 3: Summary of the new SNV results of the molecular screening of WS-related genes, including location of mutations, pathogenicity predictions, and population data.

Subject ID	WS type	Chromosome location	Gene	Mutation	Protein mutation	Pathogenicity prediction					Population frequency			Inheritance status
						SIFT_pred	Polyphen2_HVAR_pred	Polyphen2_HDIV_pred	MutationTaster_pred	CADD13_PHRED	1000g_EAS	ExAC_EAS	esp6500si_all	
49	2	Chr2(q36.1)	*PAX3*	c.808C>G	p.R270G	NA	D	NA.	D	32	—	—	—	Novel
51	2	Chr2(q36.1)	*PAX3*	c.117C>A	p.N39K	T	D	D	D	25.9	—	—	—	Novel
109	1	Chr2(q36.1)	*PAX3*	c.808C>G	p.R270G	D	D	B	D	32	—	—	—	Novel
112	1	Chr2(q36.1)	*PAX3*	c.152T>G	p.L51R	D	D	D	D	29.5	—	—	—	*De novo*
65,66	1	Chr2(q36.1)	*PAX3*	c.793-3T>G	NA	NA	NA	NA	D	12.22	—	—	—	Novel
113,114	1	Chr2(q36.1)	*PAX3*	c.803G>T	p.S268I	D	D	D	D	33	—	—	—	*De novo*
113,114	1	Chr2(q36.1)	*PAX3*	c.801delT	p. F267Lfs*17	T	NA	P	NA	NA	—	—	—	*De novo*
7,8	2	Chr3(P13)	*MITF*	c.642_650delAAG	NA	NA	NA	NA	NA	NA	—	—	—	*De novo*
20	4	Chr22(q13.1)	*SOX10*	c.122G>T	p.G41V	T	B	B	D	15.9	0.0109	0.0082	0.000077	*De novo*
102	4	Chr22(q13.1)	*SOX10*	c.127C>T	p.R43X	D	NA	B	A	35	—	—	—	*De novo*
76	2	Chr22(q13.1)	*SOX10*	c.122G>T	p.G41V	D	B	D	D	15.9	0.0109	0.0082	0.000077	*De novo*
76	2	Chr8(q11..21)	*SNAI2*	c.230C>G	p. S77C	D	B	P	D	21.3	0.004	0.0045	—	*De novo*
17	2	Chr8(q11..21)	*SNAI2*	c.365C>T	p.A122V	D	P	D	D	25.2	0.001	0.0015	—	*De novo*
85	1	Chr13(q22..3)	*EDNRB*	c.481A>G	p.K161E	T	P	B	D	23.3	—	—	—	*De novo*
92	1	Chr13(q22..3)	*EDNRB*	c.1018C>G	p.H340D	D	D	D	D	32	—	—	—	*De novo*
111	1	Chr13(q22..3)	*EDNRB*	c.1015C>T	p.L339F	T	D	D	D	23.2	—	—	—	*De novo*

Novel refers to variants that were absent in 200 control subjects; de novo refers to variants that were absent in the parents and 200 control subjects. "NA" means "not applicable." "—" means none. SIFT D: deleterious (sift ≤ 0.05); T: tolerated (sift > 0.05). Polyphen2_HVAR D: probably damaging (≥0.909); P: possibly damaging (0.447 ≤ pp2_hvar ≤ 0.909); B: benign (pp2_hvar ≤ 0.446). Polyphen2_HDIV_pred D: probably damaging (≥0.957); P: possibly damaging (0.453 ≤ pp2_hdiv ≤ 0.956); B: benign (pp2_hdiv ≤ 0.452). MutationTaster_pred A: disease_causing_automatic; D: disease_causing; N: polymorphism; P: polymorphism_automatic. CADD13_PHRED D: CADD_Phred > 15; InDel is not applicable. "EAS" means East Asian populations.

Table 4: Summary of new structure variation (SV) or CNV-detected results of the molecular screening of WS-related genes, including location of mutations and population data.

Subject ID	WS type	Chromosome location	Gene	Mutation	Genomic position		Population frequency		
					Start	End	1000g_EAS	ExAC_EAS	esp6500si_all
9	2	Chr3(P13)	*MITF*	c.110_219del110bp	110	219	—	—	—
22	2	Chr3(P13)	*MITF*	Duplication of exons 01 and 02	Promoter2	Exon01	—	—	—
33	2	Chr22(q13.1)	*SOX10*	Large fragment deletions including the whole *SOX10* gene	Promoter2	Exon04	—	—	—

"—" means none.

PAX3, c.808C>G, c.117C>A, c.152T>G, c.793-3T>G, c.803G>T, and c.801delT, are located in the highly conserved domain of PAX3. Alterations in this domain may lead to a decrease in DNA binding affinity or a change in DNA binding specificity. MITF is also a transcription factor. A basic helix-loop-helix zipper motif is vital for the survival and development of melanocytes. The *MITF* mutation, c.642_650 delAAG, results in a premature termination codon, and the mutant protein is void of functional domains. The variant likely results in disease through the mechanism of haploinsufficiency. SOX10 is a member of the group E SOX genes. A central high-mobility group (HMG) domain and a C-terminal transactivation domain were included in the protein [27]. In this study, we identified two novel *SOX10* mutations, c.122G>T and c.127C>T, as associated with WS type 2 in the Chinese population. As described in Table 3, c.122G>T in *SOX10* was not considered causative due its frequency, which was >1/10000 in the population database of ExAC_EAS. In addition, c.122G>T in *SOX10* can be observed in Figure 4 that this mutation affects an amino acid residue located outside the high-mobility group box domain of SOX10. More specifically, the c.122G>T variant in *SOX10* (Subjects 20 and 21, Figure 1) was inherited from the unaffected mother. The c.127C>T variant on *SOX10* was regarded as likely causative, because of its in silico predictions of pathogenicity and it is absent in 200 normal controls and with no frequency in population databases.

The SNAIL-related zinc-finger transcription factor SNAI2 (Slug) is a member of the SNAIL family of zinc-finger TFs that share an evolutionarily conserved role in mesoderm formation [28]. SNAI2 is expressed in migratory neural crest cells (NCCs) and is indispensable for melanoblast survival or migration. We had detected 2 variants (c.230C>G and c.365C>T) in *SNAI2* in the cohort of WS participants that were not considered causative due to their frequency (>1/10000) in the population database. Therefore, *SNAI2* has a minor involvement in WS in the Chinese population. The endothelins are a group of three peptides (ET1, ET2, and ET3) [29]. In vertebrates, Ednrb (encoding the endothelin receptor type B (ETB)) is first expressed at the dorsal tip of the neural tube, then in NCCs in both dorsoventral and dorsolateral pathways [30]. The c.469A>G, c.553G>A, c.481A>G, c.1015C>T, and c.1018C>G mutations in *EDNRB* were detected in patients with WS type 1 in the present study in the Chinese population for the first time. Six cases were found to carry c.49G>A in *EDN3* that has been linked to WS type 4 in the HGMD database.

The clinical manifestations of WS vary widely between different populations. In the present study, heterochromia iridis and sensorineural deafness were the most frequent features of both WS type 1 and WS type 2. Freckles were not observed in WS type 1 patients but were present in 31.6% (18/57) of type 2 patients, which might be a preidentified indicator between types 1 and 2 in Chinese populations. The present study and those of Silan et al. [31] and Tamayo et al. [32] were the primary screening programs in the institutionalized deaf populations in China, Turkey, and Colombia. In general, the distribution of WS type 2 is more common than that of type 1, which was in agreement with Silan et al. [31] and Tamayo et al. [32]. However, the majority of reports [33] presented more cases of WS type 1. We were looking for WS cases in hospitals or schools for deaf and mute individuals. Hearing loss is more common in WS type 2, which might be the reason resulting in the consequence. A white forelock was reported and estimated to be present in at least one-third of both WS type 1 and 2 cases [18]. However, the proportion of this phenotype in our population was much lower, which is different from the deaf populations in Turkey [31] and Colombia [32] and could be considered a difference among different populations. Ethnic migration may be the reason underlining this difference. Hypopigmented and depigmented patches on the skin can also be seen in previous reports from other populations [31, 32, 34]. Other associated symptoms described, such as cleft/lip palate, spina bifida, and musculoskeletal anomalies, were not found in the present study. In contrast, some specific signs recorded in our study are rare and were not reported in other populations, such as yellow hair, amblyopia, congenital ptosis, and narrow palpebral fissures.

Three cases (Subjects 70, 72, and 100) with genotypes linked to WS type 4 have type 2 phenotype characteristics in the present study. The observation of different phenotypes in Family 2 with the same mutations may be due to heterochromia penetrance associated with a *SOX10* mutation (Subjects 14 and 15). The presence versus absence of WS features in *MITF* (Subjects 28 and 29, Family 6) also argues for the influence of the genetic background, which supports the hypothesis that there is an interplay between genetic and environmental factors.

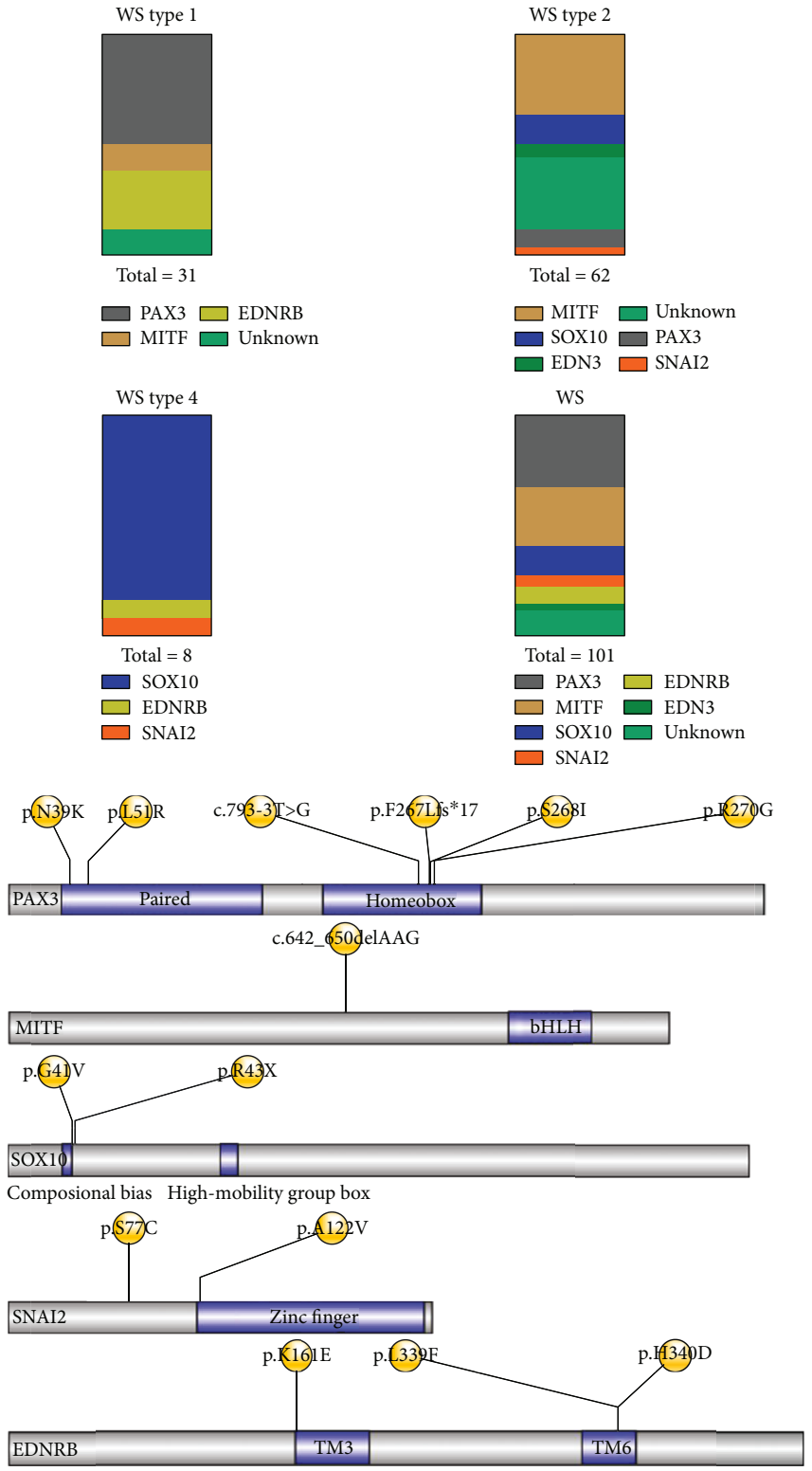

Figure 4: The proportion of genotypes detected in WS types 1, 2, and 4 and the position in the protein domains of the new SNVs detected in our study.

Wildhardt et al. [25] had screened *PAX3*, *MITF*, and *SOX10* CNVs by MLPA and detected *PAX3* and *MITF* in the search for pathogenic variants. However, other associated genes were not included in their cohort. Bocángel et al. carried out the molecular investigation of WS by sequential Sanger sequencing of all 6 coding exons; then, CNV detection by MLPA of *PAX3*, *MITF*, and *SOX10* genes in selected cases followed [11]. Detection by repeated Sanger sequencing

would be time-consuming and expensive. Captured sequencing, also called diagnostic next-generation sequencing, has specifically targeted regions. Compared with whole-exome sequencing, the cost is greatly reduced and the depth of coverage can reach to 300x. The target region capture system in our study was designed to amplify and detect 6 WS pathogenic genes in one reaction system. The observations in our study have also proved that this technology would be a promising tool to identify the molecular etiology in WS. However, the molecular etiology of a variable fraction of cases remained unexplained [18, 35], indicating that noncoding regions should also be included for molecular analysis. Moreover, it is possible that novel disease-causing genes are implicated in WS. Zazo Seco et al. [36] identified mutations in *KITLG* in WS families. Whole-exome sequencing and even whole-genome sequencing will fully reveal candidate and novel genes in molecularly unexplained cases of WS.

Data Availability

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

Conflicts of Interest

The authors declare no conflict of interest.

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Supplementary Materials

Supplementary 1. Table S1: clinical signs and symptoms of the 90 WS cases.

Supplementary 2. Table S2: the primer sequences of 6 WS-related genes.

Supplementary 3. Table S3: SNVs detected in the 114 cases.

References

[1] A. L. Dourmishev, L. A. Dourmishev, R. A. Schwartz, Mph, and C. K. Janniger, "Waardenburg syndrome," *International Journal of Dermatology*, vol. 38, no. 9, pp. 656–663, 1999.

[2] A. Zaman, R. Capper, and W. Baddoo, "Waardenburg syndrome: more common than you think!," *Clinical Otolaryngology*, vol. 40, no. 1, pp. 44–48, 2015.

[3] P. Waardenburg, "A new syndrome combining developmental anomalies of the eyelids, eyebrows and nose root with pigmentary defects of the iris and head hair and with congenital deafness," *American Journal of Human Genetics*, vol. 3, no. 3, pp. 195–253, 1951.

[4] H. Chen, L. Jiang, Z. Xie et al., "Novel mutations of PAX3, MITF, and SOX10 genes in Chinese patients with type I or type II Waardenburg syndrome," *Biochemical and Biophysical Research Communications*, vol. 397, no. 1, pp. 70–74, 2010.

[5] B. Wollnik, T. Tukel, O. Uyguner et al., "Homozygous and heterozygous inheritance of PAX3 mutations causes different types of Waardenburg syndrome," *American Journal of Medical Genetics*, vol. 122A, no. 1, pp. 42–45, 2003.

[6] Y. Akutsu, K. Shirai, A. Takei et al., "A patient with peripheral demyelinating neuropathy, central dysmyelinating leukodystrophy, Waardenburg syndrome, and severe hypoganglionosis associated with a novel SOX10 mutation," *American Journal of Medical Genetics Part A*, vol. 176, no. 5, pp. 1195–1199, 2018.

[7] K. Inoue, M. Khajavi, T. Ohyama et al., "Molecular mechanism for distinct neurological phenotypes conveyed by allelic truncating mutations," *Nature Genetics*, vol. 36, no. 4, pp. 361–369, 2004.

[8] N. Falah, J. E. Posey, W. Thorson et al., "22q11.2q13 duplication including SOX10 causes sex-reversal and peripheral demyelinating neuropathy, central dysmyelinating leukodystrophy, Waardenburg syndrome, and Hirschsprung disease," *American Journal of Medical Genetics Part A*, vol. 173, no. 4, pp. 1066–1070, 2017.

[9] A. Hemmi, K. Okamura, R. Tazawa et al., "Waardenburg syndrome type IIE in a Japanese patient caused by a novel non-frame-shift duplication mutation in the SOX10 gene," *The Journal of Dermatology*, vol. 45, no. 5, pp. e110–e111, 2018.

[10] D. Wang, G. F. Ren, H. Z. Zhang, C. Y. Yi, and Z. J. Peng, "A de novo 2q35-q36.1 deletion incorporating IHH in a Chinese boy (47,XYY) with syndactyly, type III Waardenburg syndrome, and congenital heart disease," *Genetics and Molecular Research*, vol. 15, no. 4, 2016.

[11] M. A. P. Bocángel, U. S. Melo, L. U. Alves et al., "Waardenburg syndrome: novel mutations in a large Brazilian sample," *European Journal of Medical Genetics*, vol. 61, no. 6, pp. 348–354, 2018.

[12] L. A. Farrer, K. S. Arnos, Asher JH Jr et al., "Locus heterogeneity for Waardenburg syndrome is predictive of clinical subtypes," *American Journal of Human Genetics*, vol. 55, no. 4, pp. 728–737, 1994.

[13] Y. Liu, L. Wang, Y. Feng et al., "A new genetic diagnostic for enlarged vestibular aqueduct based on next-generation sequencing," *PLoS One*, vol. 11, no. 12, article e0168508, 2016.

[14] I. A. Adzhubei, S. Schmidt, L. Peshkin et al., "A method and server for predicting damaging missense mutations," *Nature Methods*, vol. 7, no. 4, pp. 248-249, 2010.

[15] P. C. Ng and S. Henikoff, "SIFT: predicting amino acid changes that affect protein function," *Nucleic Acids Research*, vol. 31, no. 13, pp. 3812–3814, 2003.

[16] J. M. Schwarz, C. Rödelsperger, M. Schuelke, and D. Seelow, "MutationTaster evaluates disease-causing potential of sequence alterations," *Nature Methods*, vol. 7, no. 8, pp. 575-576, 2010.

[17] C. Madden, M. J. Halsted, R. J. Hopkin, D. I. Choo, C. Benton, and Greinwald JH Jr, "Temporal bone abnormalities associated with hearing loss in Waardenburg syndrome," *Laryngoscope*, vol. 113, no. 11, pp. 2035–2041, 2003.

[18] V. Pingault, D. Ente, F. Dastot-le Moal, M. Goossens, S. Marlin, and N. Bondurand, "Review and update of mutations causing Waardenburg syndrome," *Human Mutation*, vol. 31, no. 4, pp. 391–406, 2010.

[19] X. Z. Liu, V. E. Newton, and A. P. Read, "Waardenburg syndrome type II: phenotypic findings and diagnostic criteria," *American Journal of Medical Genetics*, vol. 55, no. 1, pp. 95–100, 1995.

[20] M. Sánchez-Martín, A. Rodríguez-García, J. Pérez-Losada, A. Sagrera, A. P. Read, and I. Sánchez-García, "SLUG (SNAI2) deletions in patients with Waardenburg disease," *Human Molecular Genetics*, vol. 11, no. 25, pp. 3231–3236, 2002.

[21] L. Jiang, H. Chen, W. Jiang et al., "Novel mutations in the SOX10 gene in the first two Chinese cases of type IV Waardenburg syndrome," *Biochemical and Biophysical Research Communications*, vol. 408, no. 4, pp. 620–624, 2011.

[22] J. Song, Y. Feng, F. R. Acke, P. Coucke, K. Vleminckx, and I. J. Dhooge, "Hearing loss in Waardenburg syndrome: a systematic review," *Clinical Genetics*, vol. 89, no. 4, pp. 416–425, 2015.

[23] H. Zhang, H. Chen, H. Luo et al., "Functional analysis of Waardenburg syndrome-associated PAX3 and SOX10 mutations: report of a dominant-negative SOX10 mutation in Waardenburg syndrome type II," *Human Genetics*, vol. 131, no. 3, pp. 491–503, 2012.

[24] N. Morimoto, H. Mutai, K. Namba, H. Kaneko, R. Kosaki, and T. Matsunaga, "Homozygous EDNRB mutation in a patient with Waardenburg syndrome type 1," *Auris Nasus Larynx*, vol. 45, no. 2, pp. 222–226, 2018.

[25] G. Wildhardt, B. Zirn, L. M. Graul-Neumann et al., "Spectrum of novel mutations found in Waardenburg syndrome types 1 and 2: implications for molecular genetic diagnostics," *BMJ Open*, vol. 3, no. 3, article e001917, 2013.

[26] A. L. DeStefano, L. A. Cupples, K. S. Arnos et al., "Correlation between Waardenburg syndrome phenotype and genotype in a population of individuals with identified PAX3 mutations," *Human Genetics*, vol. 102, no. 5, pp. 499–506, 1998.

[27] K. K. Chan, C. K. Y. Wong, V. C. H. Lui, P. K. H. Tam, and M. H. Sham, "Analysis of SOX10 mutations identified in Waardenburg-Hirschsprung patients: differential effects on target gene regulation," *Journal of Cellular Biochemistry*, vol. 90, no. 3, pp. 573–585, 2003.

[28] C. Cobaleda, M. Pérez-Caro, C. Vicente-Dueñas, and I. Sánchez-García, "Function of the zinc-finger transcription factor SNAI2 in cancer and development," *Annual Review of Genetics*, vol. 41, no. 1, pp. 41–61, 2007.

[29] A. S. McCallion and A. Chakravarti, "EDNRB/EDN3 and Hirschsprung disease type II," *Pigment Cell Research*, vol. 14, no. 3, pp. 161–169, 2001.

[30] D. M. Parichy, E. M. Mellgren, J. F. Rawls, S. S. Lopes, R. N. Kelsh, and S. L. Johnson, "Mutational analysis of endothelin receptor b1 (rose) during neural crest and pigment pattern development in the zebrafish Danio rerio," *Developmental Biology*, vol. 227, no. 2, pp. 294–306, 2000.

[31] F. Silan, C. Zafer, and I. Onder, "Waardenburg syndrome in the Turkish deaf population," *Genetic Counseling*, vol. 17, no. 1, pp. 41–48, 2006.

[32] M. L. Tamayo, N. Gelvez, M. Rodriguez et al., "Screening program for Waardenburg syndrome in Colombia: clinical definition and phenotypic variability," *American Journal of Medical Genetics Part A*, vol. 146A, no. 8, pp. 1026–1031, 2008.

[33] E. Pardono, Y. van Bever, J. van den Ende et al., "Waardenburg syndrome: clinical differentiation between types I and II," *American Journal of Medical Genetics*, vol. 117A, no. 3, pp. 223–235, 2003.

[34] M. H. Sham, V. C. Lui, B. L. Chen, M. Fu, and P. K. Tam, "Novel mutations of SOX10 suggest a dominant negative role in Waardenburg-Shah syndrome," *Journal of Medical Genetics*, vol. 38, no. 9, pp. 30e–330, 2001.

[35] N. Bondurand, F. Dastot-le Moal, L. Stanchina et al., "Deletions at the SOX10 gene locus cause Waardenburg syndrome types 2 and 4," *American Journal of Human Genetics*, vol. 81, no. 6, pp. 1169–1185, 2007.

[36] C. Zazo Seco, L. Serrão de Castro, J. W. van Nierop et al., "Allelic mutations of KITLG, encoding KIT ligand, cause asymmetric and unilateral hearing loss and Waardenburg syndrome type 2," *American Journal of Human Genetics*, vol. 97, no. 5, pp. 647–660, 2015.

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Review Article

Brain Functional Reserve in the Context of Neuroplasticity after Stroke

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Stroke is the second cause of death and more importantly first cause of disability in people over 40 years of age. Current therapeutic management of ischemic stroke does not provide fully satisfactory outcomes. Stroke management has significantly changed since the time when there were opened modern stroke units with early motor and speech rehabilitation in hospitals. In recent decades, researchers searched for biomarkers of ischemic stroke and neuroplasticity in order to determine effective diagnostics, prognostic assessment, and therapy. Complex background of events following ischemic episode hinders successful design of effective therapeutic strategies. So far, studies have proven that regeneration after stroke and recovery of lost functions may be assigned to neuronal plasticity understood as ability of brain to reorganize and rebuild as an effect of changed environmental conditions. As many neuronal processes influencing neuroplasticity depend on expression of particular genes and genetic diversity possibly influencing its effectiveness, knowledge on their mechanisms is necessary to understand this process. Epigenetic mechanisms occurring after stroke was briefly discussed in this paper including several mechanisms such as synaptic plasticity; neuro-, glio-, and angiogenesis processes; and growth of axon.

1. Introduction

According to the new definition of stroke by the AHA/ASA from 2013, it includes any objective evidence of permanent brain, spinal cord, or retinal cell death due to a vascular cause [1]. In clinical terms, stroke is diagnosed when neurologic deficit in a form of speech, visual disturbance, muscle weakness, or cerebellar dysfunction lasts more than 24 h. In case of symptoms lasting for a shorter period of time, transient ischemic attack (TIA) is diagnosed provided without focus of ischemia in neuroimaging exams [2]. Terms utilizing duration of neurologic symptoms are currently being

redefined with use of high-tech imaging methods such as magnetic resonance imaging (MRI) with implementation of diffusion-weighted imaging (DWI) where early ischemic lesions demonstrate increased water level in echo-planar imaging [3]. Pathophysiology definition of ischemic stroke occurs when the blood flow to an area of the brain is interrupted, resulting in some degree of permanent neurological damage [4]. The common pathway of ischemic stroke is lack of sufficient blood flow to perfuse cerebral tissue, due to narrowed or blocked arteries leading to or within the brain. Ischemic strokes can be subdivided into thrombotic and embolic strokes [5]. It is estimated that stroke is the second cause of death after cardiovascular disease and cancer in both low - and high-income countries [6]. Furthermore, ischemic strokes constitute approximately 80% of all strokes [7]. Ischemic strokes can be subdivided into thrombotic and embolic strokes [8]. It is emphasized that pharmacological actions aiming at limiting the area of damage should also include maintaining protective functions of neurons and endothelial cells of vessels composing neurovascular units [9]. Stroke management changed significantly what constitutes natural course of modern stroke units, better medical care, and more targeted motor and speech rehabilitation engaged in the early stage [10]. Increasingly fibrinolytic treatment with recombinant tissue plasminogen activator (rt-PA) and embolectomy are used [11, 12]. There is no commonly accepted therapy targeted on neuroplasticity [13]. During the last decades, researchers searched for indicators of ischemic stroke and neuroplasticity in order to determine effective diagnostics, prognostic assessment, and therapy [14, 15]. Interest of biomarkers has begun since introduction of thrombolytic treatment possible to administer up to 4.5 h from onset of symptoms and in individual cases up to 6 h after fulfilling inclusion and exclusion criteria towards standards of management in acute ischemic phase—according to the American Heart Association (AHA)/American Stroke Association (ASA) [16].

1.1. Neuroplasticity. The brain is a complex network of various subsets of cells that have the ability to be reprogrammed and also structurally rebuild [17]. The main point of neuroplasticity is capability of stimulation by a variety of stimuli for modulation of brain activity [18]. Brain compensates damages through reorganization and creation of new connections among undamaged neurons [19]. After ischemia of cells, oxygen deprivation in neurons cascades destruction in focus of infarction being formed lasts for many hours, usually leading to progression of damage [20].

1.2. Future Approach. Future research will be focused on markers of brain damage and could aid in understanding mechanisms disturbing plasticity. One of these may be inflammatory reaction initiated immediately after stroke leading to neuron damage but also possibly demonstrating neuroprotective activity [21]. The scientists from the University of California, Harvard University, and Federal Polytechnic in Zurich provided that after injury of the spinal cord exists the increased expression on genes leading to growth of damaged axons in mice and rats [22].

1.3. Focus of Ischemia: Pathology. Ischemic stroke occurs as a result of two primary pathological processes including oxygen loss and interruption in glucose supply to specific brain regions [23]. Inhibition of energy supplies leads to dysfunction of neurotransmission [24]. It was observed that disturbance of neuron functions occurs when cerebral flow decreases to 50 ml/100 g/min [25]. Irreversible damage occurs when cerebral blood flow decreases consecutively to 30 ml/100 g/min [26]. The level and duration of decreased flow are associated with increasing probability of irreversible neuron damage [27]. In an event of blood flow arrest in cerebral tissue, neuronal metabolism is disturbed after 30 seconds, whereas in consecutive minutes of oxygen deficiency, cascade reaction begins, eventually leading to brain infarction [28, 29]. Among occurring reactions included are as follows: local dilation of vessels, circulatory disturbances in vessels, local swelling, and necrosis [30]. Alternations on the neuronal level lead to disturbed functional activity of cells and their apoptosis [31]. These disturbances originate from dysfunction of Na^+/K^+ATPase leading to depolarization of neuronal membrane, releasing excitatory neurotransmitters and opening of calcium (Ca^{2+}) channels [32]. Secondary damage of neurons and cell organelles and further dysregulation of cellular metabolism occur [33]. In this case, Ca^{2+} ions spread intracellularly through channels gated with potential or receptors that may be additionally induced by several neurotransmitters in excitotoxicity mechanism [34, 35]. More delayed processes accompanying stroke are related to the neuroinflammatory process and cellular apoptosis initiated within a number of minutes after ischemic attack and may last for even several weeks and months [36]. These events may lead to delayed death of neurons and are subject of vast research concerning neuroprotective theories and agents [37]. Complex background of events following an ischemic episode hinders successful design of effective therapeutic strategies. Current research is directed at neuroprotective and proregenerative therapies which may aid recovery of lost functions by neurons after an ischemic episode. Studies have proven that regeneration after stroke and recovery of lost functions may be assigned to neuronal plasticity understood as the ability of the brain to reorganize and rebuild as an effect of changed environmental conditions [38]. It is well known that ischemic stroke triggers inflammatory cascade through activation of numerous cell mediators. Ischemia leads to accumulation of glutamate (Glu) in extracellular space and excitotoxicity [39]. In ischemic tissue, reactive oxygen species are generated and blood-brain barrier (BBB) integration is significantly disturbed [40]. Microglia are the first line of cells reacting on damage and primary source of proinflammatory cytokines and chemokines [41, 42]. Their release causes local activation of microglia, intensification of cell adhesion, and mobilization of leukocytes [43]. Increased oxidative stress and cytokine activation contribute to further intensification of inflammatory process including regulation of matrix metalloproteinase (MMP) from astrocytes and microglia leading to BBB dysfunction and eventually death of neurons [44]. Ageing decreases capabilities of neurons for functional plasticity in a healthy brain [45]. Regaining lost functions may

be explained by neuronal plasticity and decreased ability for reorganization possibly being a significant factor causing a worse functional result in elderly patients [46]. For better understanding of neuroplasticity, tracking of genetic changes influencing it is needed [47]. As many neuronal processes influencing neuroplasticity depend on expression of particular genes and genetic diversity possibly influencing its effectiveness, knowledge on their mechanisms is necessary to understand this process. Epigenetic mechanisms occurring after stroke will be briefly discussed in this paper. Background of epigenetic changes is characterized by several mechanisms such as synaptic plasticity; neuro-, glio-, and angiogenesis processes; and growth of axon. Each of these processes is modified molecularly through DNA methylation, histone modification, and microRNA (miRNA) actions.

2. Neuronal Plasticity

Synaptic plasticity is achieved through improvement of communication in synaptic connections between existing neurons and is fundamental for retaining neuronal networks [48]. Its very important information surrounding the focus of ischemia is the existing area name penumbra. Immediately following the event, blood flow and therefore oxygen transport are reduced locally, leading to hypoxia of the cells near the location of the original insult. This can lead to hypoxic cell death (infarction) and amplify the original damage from the ischemia; however, the penumbra area may remain viable for several hours after an ischemic event due to the collateral arteries that supply the penumbral zone. As time elapses after the onset of stroke, the extent of the penumbra tends to decrease; therefore, in the emergency department, a major concern is to protect the penumbra by increasing oxygen transport and delivery to cells in the danger zone, thereby limiting cell death. The existence of a penumbra implies that salvage of the cells is possible. There is a high correlation between the extent of spontaneous neurological recovery and the volume of penumbra that escapes infarction; therefore, saving the penumbra should improve the clinical outcome [49]. Epigenetic regulation, which involves DNA methylation and histone modifications, plays a critical role in retaining long-term changes in postmitotic cells. Accumulating evidence suggests that the epigenetic machinery might regulate the formation and stabilization of long-term memory in two ways: a "gating" role of the chromatin state to regulate activity-triggered gene expression and a "stabilizing" role of the chromatin state to maintain molecular and cellular changes induced by the memory-related event. The neuronal activation regulates the dynamics of the chromatin status under precise timing, with subsequent alterations in the gene expression profile.

2.1. DNA Methylation. In the study of Levenson and Sweatt in 2005, they proved that DNA methyltransferase enzyme family (DNMT) is important for synaptic plasticity [50]. Inhibition of DNMT activity causes long-term blockade of hippocampus potentiation and leads to decreased methylation of protein promoters called reelin, brain-derived neurotrophic factor (BDNF), and other genes participating in synaptic plasticity. Increased excitability within the penumbra is associated with dynamic regulation of DNA methylation [51, 52]. One of the most interesting phenomena is the process of active demethylation of gene promoter regions of BDNF through growth arrest and DNA-damage-inducible beta (GADD45B) protein activity. The role of GADD45B as a key DNA demethylation coordinator is mostly based only on in vivo experiments; however, it is difficult to distinguish active from passive demethylation of DNA. N-Methyl-D-aspartate (NMDA) agonism is found to induce expression of GADD45B mRNA and BDNF, at the same time reducing mRNA expression [53]. Ma et al. in 2009 documented that BDNF IXa is demethylated by GADD45b in mice [54]. Although there are regulatory differences between human BDNF IXabcd and mouse BDNF IXa [55], there also exist several similarities. In vivo and in vitro human BDNF IXabcd and mouse BDNF IXa are similarly induced by neuronal activity [56].

2.2. Histone Modifications. Histone modifications protruding from the nucleosome core are acetylated or deacetylated. It is an epigenetic mechanism for controlling gene expression. A very important epigenetic mechanism for controlling gene expression is posttranslational modification of histones. In that modification, the rest of lysine at the N-terminus are acetylated or deacetylated. The function of histone lysine deacetylase (HDAC2) is not limited to long-term synaptic potentiation; it also includes creation of memory in the hippocampus [57]. In anatomical terms, inhibition of HDAC2 significantly increases creation of dendritic bridges in neurons of the hippocampus. It is now evident that integration and regulation of epigenetic modifications allow for complex control of gene expression necessary for long-term memory formation and maintenance. Dynamic changes in DNA methylation and chromatin structure are the result of well-established intracellular signaling cascades that converge on the nucleus to adjust the precise equilibrium of gene repression and activation [58].

2.3. miRNA. miRNAs are endogenous, noncoding RNAs that take part in the posttranscriptional regulation of gene expression mainly by binding to the 3-untranslated region of messenger RNAs (mRNAs). A few of miRNAs which are isolated from brain play an important role in synaptic plasticity. They also take important part in learning and memory function [59].

Activity-regulated cytoskeleton-associated protein (ARC) gene is an important regulator of synaptic plasticity. Its expression is decreased in the ischemic cortex and significantly increased in the tissue cortex surrounding ischemic focus shortly after stroke, probably as an effect of Glu release and activation of neurons [60]. ARC expression is regulated by multiple miRNAs and ectopic miRNA expression in hippocampal neurons and by inhibition of the endogenous miRNAs in neurons. Frisén in 2016 proved that during in vitro neuronal development, there is an inverse relationship between ARC mRNA expression and expression of ARC-targeting miRNAs. Thus, at DIV10, expressions of

miR-19a, miR-34a, miR-326, and miR-193a were decreased while ARC mRNA was elevated [61].

3. Neuro-, Glio-, and Angiogenesis

Taking into consideration that synaptic plasticity is achieved through improvement of communication in synapses between existing neurons, the terms neuro-, glio-, and angiogenesis refer to development and formation of new neurons and blood vessels in the brain [62–64]. Recently, it has been proved that formation of new neurons is not limited to the time before birth [65, 66]. However, in order for neurogenesis to occur, one condition must be fulfilled, that is, presence of stem cells and progenitor cell and special types of cells in the dentate gyrus, in the hippocampus, and possibly in the prefrontal cortex which will become completely equipped neuron with axons and dendrites [67, 68]. New neurons can migrate to distant areas of the brain to fulfill important and previously lost functions [69]. Neuronal death is a strong stimulant for neurogenesis after ischemic stroke [70, 71]. Ischemic event is followed by increased formation of cells from these regions and alteration of migration pathways toward damaged area [72–74]. The majority of cells die and very few participate in this process [75]. Recently, researchers use the denomination of "neurovascular unit." The neurovascular unit involves connection of neurons with blood vessels and involves growth factors influencing neurogenesis which indirectly affect angiogenesis [76]. Neurogenesis and angiogenesis occur after ischemic stroke. It is modulated by DNA methylation, histone modification, and miRNA actions. The formation of long-term memory involves a series of molecular and cellular changes, including gene transcription, protein synthesis, and synaptic plasticity dynamics [77].

3.1. DNA Methylation. Methylation silences gene expression in a variety of ways, one of which is recruitment of specific binding proteins to an element of promoter [78]. The family of methyl-CpG-binding domain (MBD) binding proteins include MBD1-4 and methyl-CpG-binding protein 2 (MECP2). The scientists observed increase of MBD1 and MECP2 after 24 hours of stroke, and expression of MBD2 increases after 6 h from ischemia [79]. All mentioned proteins have regulatory functions in neurogenesis process [80]. DNA methylation was recognized in the past as a highly stable gene silencing method. At present, evidence suggests that methylation states may be more dynamic than it was previously assumed [81]. Growth arrest and DNA damage 45 (GADD45) proteins are significant elements of active cytosine (Cys) residue demethylation process [82]. The process is mediated by DNA repair pathway. GADD45 may function through feedback of necessary enzymatic process in which demethylation could lead to increased expression of specific genes significant for neuroplasticity.

3.2. Histone Modifications. Particular elements of polycomb-group proteins participate in neurogenesis [83]. Formation of oligodendrocytes is also transformed during stroke with histone deacetylase. It is already in the acute phase of stroke that oligodendrocyte progenitor cells (OPC) in the white matter of penumbra demonstrate increased protein expression of HDAC1 and HDAC2 along with increased proliferation [84]. What is more particular, HDAC isoforms may have diverse impact on cell maturation [85]. In their study, Wang et al. in 2012 demonstrated that valproic acid (VPA), a strong histone deacetylase inhibitor, has impact on regaining functions after stroke. That acid additionally increases the density of blood vessels thus improving cerebral blood flow to the ischemic hemisphere 14 days after stroke [86]. It was also demonstrated that VPA mediates in regeneration through promoting neuronal diversity in hippocampus progenitor cells [87].

3.3. miRNA. The role of miRNA was widely recognized as a regulator in neurogenesis. As previously stated, miR-124 is important in the acute phase of stroke. This is a ligand Jagged1 (JAG1) targeting as a neuronal determinant in the normal subventricular zone (SVZ) [88]. That miRNA influences repair after stroke through regulation of behavior of progenitor cells. In the brain with ischemia, miR-124 is reduced in the SVZ for 7 days after stroke that corresponds with time of significant neurogenesis [89]. Another miRNA transcript potentially important for brain repair after stroke is miR-9 [90]. Its loss inhibits proliferation in human neuronal progenitor cells and intensifies migration of these cells after transplantation to the ischemic brain [91].

4. Axon Growth

The growth of the axon becomes the main requirement for plasticity and recovery of lost functions. The axon regrowth depends on several neurobiological modifications such as the level of myelination and synapse formation. Despite the ability of axons to grow by altering the extracellular and intracellular substances, dedifferentiation in which axons are responsible for recovering functions from those that are functionally silent is still a matter of discussion. In this case, the intuitive translation relation between anatomical and functional regeneration is questioned [92]. In ischemic stroke as well as in brain injury, the area of brain damage is characterized by the formation of glial scar, in which growth inhibitors are upregulated; preventing the effective regeneration of this scar is characterized by significant upregulation of proteoglycans, preventing the effective regeneration of axons. In the close proximity to the glial scar, however, there is a cortical area that is characterized by the expression of many growth-promoting factors that allow axonal growth [93]. One of the main components of glial scars is extracellular matrix proteins known as CSPG, which consist of protein chains and glycosaminoglycans (GAGs). CSPGs are present in the developing and also adult central nervous system, but their expression significantly increases after injury. Reactive astrocytes are responsible for the production and secretion of many CSPGs after injury, and their increased expression is observed for many months. Two reasons for the failure of the CNS regeneration are extrinsic inhibitory molecules and poor internal growth ability [94].

4.1. DNA Methylation. Descriptions of the mechanism of DNA methylation in axon growth regulation after stroke are based on published postinjury models; we do not have any models of ischemia [95]. Therefore, recreation of ischemic conditions is difficult. An important role in promoting axon number growth has been recently attributed to proline-rich protein (SPRR1) released after axotomy [96]. High concentration of SPRR1 is released in the cortex of ischemic focus in the initial phase of stroke.

4.2. Histone Modifications. SPRR1 may be induced by hypomethylating agents and its expression may be modulated by histone modification [97]. Similarly to the impact of 5-azacytidine on keratinocytes, SPRR1 expression is increased in these cells after treatment with an HDAC inhibitor such as sodium butyrate. Nowadays, we do not have examinations on the human brain [98]. Growth-Associated Protein 43 (GAP43) consists of protein related with a growth cone promoting growth of axons through regulation of cytoskeleton organization with protein kinase C signaling. That expression is strongly induced in the ischemic cortex after ischemic stroke [99]. According to Yuan et al. in 2001, administration of VPA may induce expression of GAP43 as well as of other growth proteins simultaneously promoting regeneration of axons [100].

4.3. miRNA. The most important role in the growth of axon is played by miRNA [101, 102]. The role of miR-9, whose level is reduced in the ischemic white matter, is best known. Therefore, miR-9 is released in primary axons of the neuron cortex of a developing brain. miR-9 replicates microtubule-associated protein 1B (MAP1B) connected with a cytoskeleton [103]. That inhibition occurs through RNA interference resulting not only in a significantly increased length of axon but also in a decreased pattern of branches. As in the case of two previous processes, the issue of growth of axon requires further research.

5. Discussion

Neuroplasticity is a widespread phenomenon in the function of the nervous system. Spontaneous recovery is the norm in the early poststroke period. Cortical reorganization is common and necessary for postbrain injury recovery. Representations of sensory and motor cortical areas may be modified by the inflow of environmental stimulation during learning and memory processes. Physiotherapy strategies used during recovery process affect the spontaneous neuroplasticity. After stroke, the main functional dysfunctions are aphasia and hemiplegia. Regarding the dynamic changes of a clinical picture of a patient after an ischemic episode, multiplicity, and diversity of pathology, the doctors, physiotherapists, and speech therapists do not have a universal procedure or concept.

A correct therapy depends on the actual deficit and patient necessity [104, 105]. Neural plasticity allows progress of the central nervous system under the influence of variable conditioning environment, learning, and memorization; the new abilities and adaptation into changes happen inside and outside of entourage and activity compensatory process after ischemia. It happens because of a neuron's property enabling overlap indicating changes in the neuronal system in response to organism's needs and challenge of reality [106]. Daily activity, learning, and training have a main influence on brain function. Developing right connections through axons, projections, synapse, and chemical transmitter is an ongoing intricate process with different intensities all throughout the human life. His course determines the information written in the DNA. Genetic predisposition is modified as a result of experience; throughout human life, through environmental changes, the number of synaptic pathways can rise. Many new emerging neuronal cells succumb apoptosis—programmed and irreversible autodestruction—and pruning. The elements of neurons, for example, mitochondria in apoptotic bodies, are removed by macrophages or absorbed through familiar cells. Overproduction of neurons is necessary to obtain an appropriate number of synaptic pathways that kill these cells who cannot create connection functional active [107].

A properly carried out treatment achieves skills by allowing rehabilitation to move beyond the walls of the hospital or home, and this contributes to the functional independence of patients.

Researchers and therapists are still looking for a new possibility of impact of the neuronal system; it will contribute in the future to the functional progress and usage capacity of the mechanisms of neuronal plasticity in the case of his damage [108].

In many sciences, it was confirmed a fact that in regular methodical learning, we can considerably increase intellectual capacity and correct memory, concentration, and logical thinking, and in the case of neurological disorder by targeting the process of neural compensatory plasticity, we can obtain significant improvement of disturbed performance. The effects of neural plasticity depends on the clinical factor, age, intellect, and education of patient. In well-educated people, there exists cognitive reserve of the brain, which may have an impact on the recovery process [109].

A small group of scientists studied Albert Einstein's brain in search of special abilities in the structure of the neuronal system. They compared with another four brains from other people who died at the same age. They discovered in the brain of Albert Einstein a difference in the cytoarchitecture when compared with brains of other people. They found out a higher ratio of astrocytes to neurons in the cerebral cortex parietal lobe in the left hemispheres. Glial cells enable provision of nutritional substances to the brain through connection with the blood vessel, from which we conclude that astroglia can be a ground for neural plasticity [110]. Among many methods of streamlining patients with hemiplegia, we use proprioceptive neuromuscular facilitation (PNF), neurodevelopmental treatment (NDT)/Bobath, constraint-induced movement therapy (CIMT), training oriented on top of approach task-oriented training, neuromuscular arthroskeletal plasticity (NAP), and occupational therapy based on the aim with the rule SMART—specified, measured, attractive, real, and timely. We must select every time a therapy which adapts into individual needs and ability

of patient [111]. Neural plasticity allows progress of the central nervous system under the influence of variable conditioning environment, learning, and memorization and the new abilities and adaptation into changes happen inside and outside of entourage and activity compensatory process after ischemia. It happens because of the neuron's property enabling overlap indicating changes in the neuronal system in response to the organism's needs and challenge of reality [112, 113].

According to the above considerations and analyses, it should be indicated that an issue of ischemic stroke not only constitutes individual physical and social impairments but also represents significant financial burden for the global health care systems concerning professionally active people in productive age [114–116]. Patients after an ischemic episode frequently become dependent on institutional organization [117]. Return to daily living and professional activity is hindered, often impossible, for these patients, leading to dependence on the closest relatives [118, 119]. Necessity to help a disabled person causes dysregulation of social and professional life of careers [120]. Optimally, clinical experience should be combined with search for new forms of brain functional reserve [121]. Recovery after stroke is a complex phenomenon. In a study of anti-inflammatory strategies that have been effective for recovery in experimental stroke, Liguz-Lecznar and Kossut described that the most important aspect of therapies targeting the immune system will be regulating the balance between the neurotoxic and neuroprotective effects of inflammatory state components [122]. Clinical experience, awareness of the scale of the problem, and molecular research may be used in combination with each other. It may be assumed that combination of new therapies with neurologic rehabilitation could be a new trend in the treatment of patients after stroke. Another stroke-related issue concerns the substantial prevention. The stroke prevention should consist of complex medical and political issues [123]. We strongly believe that issues discussed in this study should allow better understanding of physiological background and other social aspects of escalating problem of stroke indicating future research directions.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

References

[1] S. Hatano, "Experience from a multicentre stroke register: a preliminary report," *Bulletin of the World Health Organization*, vol. 54, no. 5, pp. 541–553, 1976.

[2] A. G. Sorensen and H. Ay, "Transient ischemic attack: definition, diagnosis, and risk stratification," *Neuroimaging Clinics of North America*, vol. 21, no. 2, pp. 303–313, 2011.

[3] J. Liang, P. Gao, Y. Lin, L. Song, H. Qin, and B. Sui, "Susceptibility-weighted imaging in post-treatment evaluation in the early stage in patients with acute ischemic stroke," *Journal of International Medical Research*, vol. 47, no. 1, pp. 196–205, 2018.

[4] R. L. Sacco, S. E. Kasner, J. P. Broderick et al., "An updated definition of stroke for the 21st century: a statement for healthcare professionals from the American Heart Association/American Stroke Association," *Stroke*, vol. 44, no. 7, pp. 2064–2089, 2013.

[5] T. D. Musuka, S. B. Wilton, M. Traboulsi, and M. D. Hill, "Diagnosis and management of acute ischemic stroke: speed is critical," *Canadian Medical Association Journal*, vol. 187, no. 12, pp. 887–893, 2015.

[6] D. Della-Morte, F. Guadagni, R. Palmirotta et al., "Genetics of ischemic stroke, stroke-related risk factors, stroke precursors and treatments," *Pharmacogenomics*, vol. 13, no. 5, pp. 595–613, 2012.

[7] L. Brewer, F. Horgan, A. Hickey, and D. Williams, "Stroke rehabilitation: recent advances and future therapies," *QJM*, vol. 106, no. 1, pp. 11–25, 2012.

[8] M. Chouchane and M. R. Costa, "Cell therapy for stroke: use of local astrocytes," *Frontiers in Cellular Neuroscience*, vol. 6, 2012.

[9] R. Khatib, A. M. Jawaada, Y. A. Arevalo, H. K. Hamed, S. H. Mohammed, and M. D. Huffman, "Implementing evidence-based practices for acute stroke care in low- and middle-income countries," *Current Atherosclerosis Reports*, vol. 19, no. 12, p. 61, 2017.

[10] K. Gache, H. Leleu, G. Nitenberg, F. Woimant, M. Ferrua, and E. Minvielle, "Main barriers to effective implementation of stroke care pathways in France: a qualitative study," *BMC Health Services Research*, vol. 14, no. 1, 2014.

[11] T. R. Lawson, I. E. Brown, D. L. Westerkam et al., "Tissue plasminogen activator (rt-PA) in acute ischemic stroke: outcomes associated with ambulation," *Restorative Neurology and Neuroscience*, vol. 33, no. 3, pp. 301–308, 2015.

[12] A. J. Yoo and T. Andersson, "Thrombectomy in acute ischemic stroke: challenges to procedural success," *Journal of Stroke*, vol. 19, no. 2, pp. 121–130, 2017.

[13] H. Kaur, A. Prakash, and B. Medhi, "Drug therapy in stroke: from preclinical to clinical studies," *Pharmacology*, vol. 92, no. 5-6, pp. 324–334, 2013.

[14] G. C. Jickling and F. R. Sharp, "Blood biomarkers of ischemic stroke," *Neurotherapeutics*, vol. 8, no. 3, pp. 349–360, 2011.

[15] E. Burke and S. C. Cramer, "Biomarkers and predictors of restorative therapy effects after stroke," *Current Neurology and Neuroscience Reports*, vol. 13, no. 2, p. 329, 2013.

[16] W. J. Powers, A. A. Rabinstein, T. Ackerson et al., "2018 guidelines for the early management of patients with acute ischemic stroke: a guideline for healthcare professionals from the American Heart Association/American Stroke Association," *Stroke*, vol. 49, no. 3, pp. e46–e110, 2018.

[17] M. Götz and S. Jarriault, "Programming and reprogramming the brain: a meeting of minds in neural fate," *Development*, vol. 144, no. 15, pp. 2714–2718, 2017.

[18] P. Voss, M. E. Thomas, J. M. Cisneros-Franco, and E. de Villers-Sidani, "Dynamic brains and the changing rules of neuroplasticity: implications for learning and recovery," *Frontiers in Psychology*, vol. 8, 2017.

[19] M. P. Lin and D. S. Liebeskind, "Imaging of ischemic stroke," *Continuum: Lifelong Learning in Neurology*, vol. 22, no. 5, pp. 1399–1423, 2016.

[20] M. Sumer, I. Ozdemir, and O. Erturk, "Progression in acute ischemic stroke: frequency, risk factors and prognosis," *Journal of Clinical Neuroscience*, vol. 10, no. 2, pp. 177–180, 2003.

[21] S. C. Cramer, M. Sur, B. H. Dobkin et al., "Harnessing neuroplasticity for clinical applications," *Brain*, vol. 134, no. 6, pp. 1591–1609, 2011.

[22] J. Anrather and C. Iadecola, "Inflammation and stroke: an overview," *Neurotherapeutics*, vol. 13, no. 4, pp. 661–670, 2016.

[23] N. M. Robbins and R. A. Swanson, "Opposing effects of glucose on stroke and reperfusion injury: acidosis, oxidative stress, and energy metabolism," *Stroke*, vol. 45, no. 6, pp. 1881–1886, 2014.

[24] C. Xing, K. Arai, E. H. Lo, and M. Hommel, "Pathophysiologic cascades in ischemic stroke," *International Journal of Stroke*, vol. 7, no. 5, pp. 378–385, 2012.

[25] H. Jaffer, V. B. Morris, D. Stewart, and V. Labhasetwar, "Advances in stroke therapy," *Drug Delivery and Translational Research*, vol. 1, no. 6, pp. 409–419, 2011.

[26] N. Mitsios, J. Gaffney, P. Kumar, J. Krupinski, S. Kumar, and M. Slevin, "Pathophysiology of acute ischaemic stroke: an analysis of common signalling mechanisms and identification of new molecular targets," *Pathobiology*, vol. 73, no. 4, pp. 159–175, 2006.

[27] O. Y. Bang, J. L. Saver, J. R. Alger et al., "Determinants of the distribution and severity of hypoperfusion in patients with ischemic stroke," *Neurology*, vol. 71, no. 22, pp. 1804–1811, 2008.

[28] C. Xiao and R. M. Robertson, "Timing of locomotor recovery from anoxia modulated by the white gene in Drosophila," *Genetics*, vol. 203, no. 2, pp. 787–797, 2016.

[29] I. M. Macrae and S. M. Allan, "Stroke: the past, present and future," *Brain and Neuroscience Advances*, vol. 2, article 2398212818810689, 2018.

[30] J. A. Stokum, V. Gerzanich, and J. M. Simard, "Molecular pathophysiology of cerebral edema," *Journal of Cerebral Blood Flow and Metabolism*, vol. 36, no. 3, pp. 513–538, 2016.

[31] D. Radak, N. Katsiki, I. Resanovic et al., "Apoptosis and acute brain ischemia in ischemic stroke," *Current Vascular Pharmacology*, vol. 15, no. 2, pp. 115–122, 2017.

[32] M. J. Kim, J. Hur, I. H. Ham et al., "Expression and activity of the na-k ATPase in ischemic injury of primary cultured astrocytes," *The Korean Journal of Physiology & Pharmacology*, vol. 17, no. 4, pp. 275–281, 2013.

[33] L. Watts, R. Lloyd, R. Garling, and T. Duong, "Stroke neuroprotection: targeting mitochondria," *Brain Sciences*, vol. 3, no. 4, pp. 540–560, 2013.

[34] S. Ding, "Ca2+ signaling in astrocytes and its role in ischemic stroke," *Advances in Neurobiology*, vol. 11, pp. 189–211, 2014.

[35] T. W. Lai, S. Zhang, and Y. T. Wang, "Excitotoxicity and stroke: identifying novel targets for neuroprotection," *Progress in Neurobiology*, vol. 115, pp. 157–188, 2014.

[36] S. Vidale, A. Consoli, M. Arnaboldi, and D. Consoli, "Postischemic inflammation in acute stroke," *Journal of Clinical Neurology*, vol. 13, no. 1, pp. 1–9, 2017.

[37] A. Majid, "Neuroprotection in stroke: past, present, and future," *ISRN Neurology*, vol. 2014, Article ID 515716, 17 pages, 2014.

[38] C. Alia, C. Spalletti, S. Lai et al., "Neuroplastic changes following brain ischemia and their contribution to stroke recovery: novel approaches in neurorehabilitation," *Frontiers in Cellular Neuroscience*, vol. 11, 2017.

[39] A. Brassai, R. G. Suvanjeiev, E. G. Bán, and M. Lakatos, "Role of synaptic and nonsynaptic glutamate receptors in ischaemia induced neurotoxicity," *Brain Research Bulletin*, vol. 112, pp. 1–6, 2015.

[40] A. E. Sifat, B. Vaidya, and T. J. Abbruscato, "Blood-brain barrier protection as a therapeutic strategy for acute ischemic stroke," *The AAPS Journal*, vol. 19, no. 4, pp. 957–972, 2017.

[41] Y. B. Lee, A. Nagai, and S. U. Kim, "Cytokines, chemokines, and cytokine receptors in human microglia," *Journal of Neuroscience Research*, vol. 69, no. 1, pp. 94–103, 2002.

[42] R. Guruswamy and A. ElAli, "Complex roles of microglial cells in ischemic stroke pathobiology: new insights and future directions," *International Journal of Molecular Sciences*, vol. 18, no. 3, 2017.

[43] A. R. Patel, R. Ritzel, L. D. McCullough, and F. Liu, "Microglia and ischemic stroke: a double-edged sword," *International Journal of Physiology, Pathophysiology and Pharmacology*, vol. 5, no. 3, pp. 73–90, 2013.

[44] S. E. Lakhan, A. Kirchgessner, D. Tepper, and A. Leonard, "Matrix metalloproteinases and blood-brain barrier disruption in acute ischemic stroke," *Frontiers in Neurology*, vol. 4, 2013.

[45] S. N. Burke and C. A. Barnes, "Neural plasticity in the ageing brain," *Nature Reviews Neuroscience*, vol. 7, no. 1, pp. 30–40, 2006.

[46] M. J. Spriggs, C. J. Cadwallader, J. P. Hamm, L. J. Tippett, and I. J. Kirk, "Age-related alterations in human neocortical plasticity," *Brain Research Bulletin*, vol. 130, pp. 53–59, 2017.

[47] K. M. Pearson-Fuhrhop and S. C. Cramer, "Genetic influences on neural plasticity," *PM&R*, vol. 2, 12 Supplement 2, pp. S227–S240, 2010.

[48] J. D. Power and B. L. Schlaggar, "Neural plasticity across the lifespan," *Wiley Interdisciplinary Reviews: Developmental Biology*, vol. 6, no. 1, p. e216, 2017.

[49] J. V. Guadagno, C. Calautti, and J.-C. Baron, "Progress in imaging stroke: emerging clinical applications," *British Medical Bulletin*, vol. 65, no. 1, pp. 145–157, 2003.

[50] J. M. Levenson and J. D. Sweatt, "Epigenetic mechanisms in memory formation," *Nature Reviews Neuroscience*, vol. 6, no. 2, pp. 108–118, 2005.

[51] K. Schiene, C. Bruehl, K. Zilles et al., "Neuronal hyperexcitability and reduction of GABAA-receptor expression in the surround of cerebral photothrombosis," *Journal of Cerebral Blood Flow and Metabolism*, vol. 16, no. 5, pp. 906–914, 1996.

[52] J. U. Guo, Y. Su, C. Zhong, G. L. Ming, and H. Song, "Emerging roles of TET proteins and 5-hydroxymethylcytosines in active DNA demethylation and beyond," *Cell Cycle*, vol. 10, no. 16, pp. 2662–2668, 2011.

[53] D. K. Ma, J. U. Guo, G. L. Ming, and H. Song, "DNA excision repair proteins and Gadd45 as molecular players for active DNA demethylation," *Cell Cycle*, vol. 8, no. 10, pp. 1526–1531, 2009.

[54] D. K. Ma, M. H. Jang, J. U. Guo et al., "Neuronal activity-induced Gadd45b promotes epigenetic DNA demethylation and adult neurogenesis," *Science*, vol. 323, no. 5917, pp. 1074–1077, 2009.

[55] P. Pruunsild, A. Kazantseva, T. Aid, K. Palm, and T. Timmusk, "Dissecting the human BDNF locus:

bidirectional transcription, complex splicing, and multiple promoters," *Genomics*, vol. 90, no. 3, pp. 397–406, 2007.

[56] P. Pruunsild, M. Sepp, E. Orav, I. Koppel, and T. Timmusk, "Identification of cis-elements and transcription factors regulating neuronal activity-dependent transcription of human BDNF gene," *Journal of Neuroscience*, vol. 31, no. 9, pp. 3295–3308, 2011.

[57] S. Chatterjee, P. Mizar, R. Cassel et al., "A novel activator of CBP/p300 acetyltransferases promotes neurogenesis and extends memory duration in adult mice," *Journal of Neuroscience*, vol. 33, no. 26, pp. 10698–10712, 2013.

[58] J. S. Guan, S. J. Haggarty, E. Giacometti et al., "HDAC2 negatively regulates memory formation and synaptic plasticity," *Nature*, vol. 459, no. 7243, pp. 55–60, 2009.

[59] W. Liu, J. Wu, J. Huang et al., "Electroacupuncture regulates hippocampal synaptic plasticity via miR-134-mediated LIMK1 function in rats with ischemic stroke," *Neural Plasticity*, vol. 2017, Article ID 9545646, 11 pages, 2017.

[60] J. D. Shepherd and M. F. Bear, "New views of Arc, a master regulator of synaptic plasticity," *Nature Neuroscience*, vol. 14, no. 3, pp. 279–284, 2011.

[61] J. Frisén, "Neurogenesis and gliogenesis in nervous system plasticity and repair," *Annual Review of Cell and Developmental Biology*, vol. 32, no. 1, pp. 127–141, 2016.

[62] G. L. Ming and H. Song, "Adult neurogenesis in the mammalian brain: significant answers and significant questions," *Neuron*, vol. 70, no. 4, pp. 687–702, 2011.

[63] T. D. Palmer, J. Ray, and F. H. Gage, "FGF-2-responsive neuronal progenitors reside in proliferative and quiescent regions of the adult rodent brain," *Molecular and Cellular Neurosciences*, vol. 6, no. 5, pp. 474–486, 1995.

[64] S. Rosi, "Neuroinflammation and the plasticity-related immediate-early gene Arc," *Brain, Behavior, and Immunity*, vol. 25, Supplement 1, pp. S39–S49, 2011.

[65] N. Kaneko, M. Sawada, and K. Sawamoto, "Mechanisms of neuronal migration in the adult brain," *Journal of Neurochemistry*, vol. 141, no. 6, pp. 835–847, 2017.

[66] A. Pino, G. Fumagalli, F. Bifari, and I. Decimo, "New neurons in adult brain: distribution, molecular mechanisms and therapies," *Biochemical Pharmacology*, vol. 141, pp. 4–22, 2017.

[67] C. Göritz and J. Frisén, "Neural stem cells and neurogenesis in the adult," *Cell Stem Cell*, vol. 10, no. 6, pp. 657–659, 2012.

[68] P. S. Eriksson, E. Perfilieva, T. Björk-Eriksson et al., "Neurogenesis in the adult human hippocampus," *Nature Medicine*, vol. 4, no. 11, pp. 1313–1317, 1998.

[69] Y.-F. Liu, H.-I. Chen, C.-L. Wu et al., "Differential effects of treadmill running and wheel running on spatial or aversive learning and memory: roles of amygdalar brain-derived neurotrophic factor and synaptotagmin I," *The Journal of Physiology*, vol. 587, no. 13, pp. 3221–3231, 2009.

[70] K. V. Adams and C. M. Morshead, "Neural stem cell heterogeneity in the mammalian forebrain," *Progress in Neurobiology*, vol. 170, pp. 2–36, 2018.

[71] O. Lindvall and Z. Kokaia, "Neurogenesis following stroke affecting the adult brain," *Cold Spring Harbor Perspectives in Biology*, vol. 7, no. 11, article a019034, 2015.

[72] R. J. Felling, M. J. Snyder, M. J. Romanko et al., "Neural stem/progenitor cells participate in the regenerative response to perinatal hypoxia/ischemia," *Journal of Neuroscience*, vol. 26, no. 16, pp. 4359–4369, 2006.

[73] A. Arvidsson, T. Collin, D. Kirik, Z. Kokaia, and O. Lindvall, "Neuronal replacement from endogenous precursors in the adult brain after stroke," *Nature Medicine*, vol. 8, no. 9, pp. 963–970, 2002.

[74] P. Thored, A. Arvidsson, E. Cacci et al., "Persistent production of neurons from adult brain stem cells during recovery after stroke," *Stem Cells*, vol. 24, no. 3, pp. 739–747, 2006.

[75] S. W. Hou, Y. Q. Wang, M. Xu et al., "Functional integration of newly generated neurons into striatum after cerebral ischemia in the adult rat brain," *Stroke*, vol. 39, no. 10, pp. 2837–2844, 2008.

[76] C. Xing, K. Hayakawa, J. Lok, K. Arai, and E. H. Lo, "Injury and repair in the neurovascular unit," *Neurological Research*, vol. 34, no. 4, pp. 325–330, 2012.

[77] J. J. Ohab, S. Fleming, A. Blesch, and S. T. Carmichael, "A neurovascular niche for neurogenesis after stroke," *Journal of Neuroscience*, vol. 26, no. 50, pp. 13007–13016, 2006.

[78] J. S. Guan, H. Xie, and X. Ding, "The role of epigenetic regulation in learning and memory," *Experimental Neurology*, vol. 268, pp. 30–36, 2015.

[79] B. P. Jung, G. Zhang, W. Ho, J. Francis, and J. H. Eubanks, "Transient forebrain ischemia alters the mRNA expression of methyl DNA-binding factors in the adult rat hippocampus," *Neuroscience*, vol. 115, no. 2, pp. 515–524, 2002.

[80] X. Li, B. Z. Barkho, Y. Luo et al., "Epigenetic regulation of the stem cell mitogen Fgf-2 by Mbd1 in adult neural stem/-progenitor cells," *Journal of Biological Chemistry*, vol. 283, no. 41, pp. 27644–27652, 2008.

[81] K. E. Varley, J. Gertz, K. M. Bowling et al., "Dynamic DNA methylation across diverse human cell lines and tissues," *Genome Research*, vol. 23, no. 3, pp. 555–567, 2013.

[82] G. Barreto, A. Schäfer, J. Marhold et al., "Gadd45a promotes epigenetic gene activation by repair-mediated DNA demethylation," *Nature*, vol. 445, no. 7128, pp. 671–675, 2007.

[83] J. Elder, M. Cortes, A. Rykman et al., "The epigenetics of stroke recovery and rehabilitation: from polycomb to histone deacetylases," *Neurotherapeutics*, vol. 10, no. 4, pp. 808–816, 2013.

[84] H. Kassis, M. Chopp, X. S. Liu, A. Shehadah, C. Roberts, and Z. G. Zhang, "Histone deacetylase expression in white matter oligodendrocytes after stroke," *Neurochemistry International*, vol. 77, pp. 17–23, 2014.

[85] M. Haberland, R. L. Montgomery, and E. N. Olson, "The many roles of histone deacetylases in development and physiology: implications for disease and therapy," *Nature Reviews Genetics*, vol. 10, no. 1, pp. 32–42, 2009.

[86] B. Wang, X. Zhu, Y. Kim et al., "Histone deacetylase inhibition activates transcription factor Nrf2 and protects against cerebral ischemic damage," *Free Radical Biology & Medicine*, vol. 52, no. 5, pp. 928–936, 2012.

[87] J. Hsieh, K. Nakashima, T. Kuwabara, E. Mejia, and F. H. Gage, "Histone deacetylase inhibition-mediated neuronal differentiation of multipotent adult neural progenitor cells," *Proceedings of the National Academy of Sciences*, vol. 101, no. 47, pp. 16659–16664, 2004.

[88] Y. Shi, X. Zhao, J. Hsieh et al., "MicroRNA regulation of neural stem cells and neurogenesis," *Journal of Neuroscience*, vol. 30, no. 45, pp. 14931–14936, 2010.

[89] J. Yang, X. Zhang, X. Chen, L. Wang, and G. Yang, "Exosome mediated delivery of miR-124 promotes neurogenesis

after ischemia," *Molecular Therapy - Nucleic Acids*, vol. 7, pp. 278–287, 2017.

[90] X. S. Liu, M. Chopp, R. L. Zhang et al., "MicroRNA profiling in subventricular zone after stroke: miR-124a regulates proliferation of neural progenitor cells through Notch signaling pathway," *PLoS One*, vol. 6, no. 8, article e23461, 2011.

[91] S. E. Khoshnam, W. Winlow, Y. Farbood, H. F. Moghaddam, and M. Farzaneh, "Emerging roles of microRNAs in ischemic stroke: as possible therapeutic agents," *Journal of Stroke*, vol. 19, no. 2, pp. 166–187, 2017.

[92] A. R. Filous and J. M. Schwab, "Determinants of axon growth, plasticity, and regeneration in the context of spinal cord injury," *The American Journal of Pathology*, vol. 188, no. 1, pp. 53–62, 2018.

[93] S. T. Carmichael, "Rodent models of focal stroke: size, mechanism, and purpose," *NeuroRX*, vol. 2, no. 3, pp. 396–409, 2005.

[94] D. Rabinovich, S. P. Yaniv, I. Alyagor, and O. Schuldiner, "Nitric oxide as a switching mechanism between axon degeneration and regrowth during developmental remodeling," *Cell*, vol. 164, no. 1-2, pp. 170–182, 2016.

[95] I. E. Bonilla, K. Tanabe, and S. M. Strittmatter, "Small proline-rich repeat protein 1A is expressed by axotomized neurons and promotes axonal outgrowth," *The Journal of Neuroscience*, vol. 22, no. 4, pp. 1303–1315, 2002.

[96] R. J. Felling and H. Song, "Epigenetic mechanisms of neuroplasticity and the implications for stroke recovery," *Experimental Neurology*, vol. 268, pp. 37–45, 2015.

[97] R. P. Simon, "Epigenetic modulation of gene expression governs the brain's response to injury," *Neuroscience Letters*, vol. 625, pp. 16–19, 2016.

[98] L. I. Benowitz and A. Routtenberg, "GAP-43: an intrinsic determinant of neuronal development and plasticity," *Trends in Neurosciences*, vol. 20, no. 2, pp. 84–91, 1997.

[99] J. Skene, R. Jacobson, G. Snipes, C. McGuire, J. Norden, and J. Freeman, "A protein induced during nerve growth (GAP-43) is a major component of growth-cone membranes," *Science*, vol. 233, no. 4765, pp. 783–786, 1986.

[100] P. X. Yuan, L. D. Huang, Y. M. Jiang, J. S. Gutkind, H. K. Manji, and G. Chen, "The mood stabilizer valproic acid activates mitogen-activated protein kinases and promotes neurite growth," *Journal of Biological Chemistry*, vol. 276, no. 34, pp. 31674–31683, 2001.

[101] H. Chiu, A. Alqadah, and C. Chang, "The role of microRNAs in regulating neuronal connectivity," *Frontiers in Cellular Neuroscience*, vol. 7, 2014.

[102] B. Buller, M. Chopp, Y. Ueno et al., "Regulation of serum response factor by miRNA-200 and miRNA-9 modulates oligodendrocyte progenitor cell differentiation," *Glia*, vol. 60, no. 12, pp. 1906–1914, 2012.

[103] F. Dajas-Bailador, B. Bonev, P. Garcez, P. Stanley, F. Guillemot, and N. Papalopulu, "MicroRNA-9 regulates axon extension and branching by targeting Map1b in mouse cortical neurons," *Nature Neuroscience*, vol. 15, no. 5, pp. 697–699, 2012.

[104] J. Medin, J. Barajas, and K. Ekberg, "Stroke patients' experiences of return to work," *Disability and Rehabilitation*, vol. 28, no. 17, pp. 1051–1060, 2006.

[105] C. M. Stinear, W. D. Byblow, and S. H. Ward, "An update on predicting motor recovery after stroke," *Annals of Physical and Rehabilitation Medicine*, vol. 57, no. 8, pp. 489–498, 2014.

[106] C. Dettmers, U. Teske, F. Hamzei, G. Uswatte, E. Taub, and C. Weiller, "Distributed form of constraint-induced movement therapy improves functional outcome and quality of life after stroke," *Archives of Physical Medicine and Rehabilitation*, vol. 86, no. 2, pp. 204–209, 2005.

[107] J. Liepert, H. Bauder, W. H. R. Miltner, E. Taub, and C. Weiller, "Treatment-induced cortical reorganization after stroke in humans," *Stroke*, vol. 31, no. 6, pp. 1210–1216, 2000.

[108] J. C. Stewart and S. C. Cramer, "Genetic variation and neuroplasticity: role in rehabilitation after stroke," *Journal of Neurologic Physical Therapy*, vol. 41, Supplement 3, pp. S17–S23, 2017.

[109] H. Woldag and H. Hummelsheim, "Evidence-based physiotherapeutic concepts for improving arm and hand function in stroke patients," *Journal of Neurology*, vol. 249, no. 5, pp. 518–528, 2002.

[110] J. A. Colombo, H. D. Reisin, J. J. Miguel-Hidalgo, and G. Rajkowska, "Cerebral cortex astroglia and the brain of a genius: a propos of A. Einstein's," *Brain Research Reviews*, vol. 52, no. 2, pp. 257–263, 2006.

[111] N. S. Ward and L. G. Cohen, "Mechanisms underlying recovery of motor function after stroke," *Archives of Neurology*, vol. 61, no. 12, 2004.

[112] J. Classen, J. Liepert, S. P. Wise, M. Hallett, and L. G. Cohen, "Rapid plasticity of human cortical movement representation induced by practice," *Journal of Neurophysiology*, vol. 79, no. 2, pp. 1117–1123, 1998.

[113] S. R. Belagaje, "Stroke rehabilitation 2017," *Cerebrovascular Disease*, pp. 238–253, 2017.

[114] C. Seneviratne and M. Reimer, "Neurodevelopmental treatment stroke rehabilitation: a critique and extension for neuroscience nursing practice," *Axone*, vol. 26, no. 2, pp. 13–20, 2004.

[115] F. Mu, D. Hurley, K. A. Betts et al., "Real-world costs of ischemic stroke by discharge status," *Current Medical Research and Opinion*, vol. 33, no. 2, pp. 371–378, 2017.

[116] B. M. Kissela, J. C. Khoury, K. Alwell et al., "Age at stroke: temporal trends in stroke incidence in a large, biracial population," *Neurology*, vol. 79, no. 17, pp. 1781–1887, 2012.

[117] M. K. Kapral, A. Laupacis, S. J. Phillips et al., "Stroke care delivery in institutions participating in the Registry of the Canadian Stroke Network," *Stroke*, vol. 35, no. 7, pp. 1756–1762, 2004.

[118] E. Westerlind, H. C. Persson, and K. S. Sunnerhagen, "Return to work after a stroke in working age persons; a six-year follow up," *PLoS One*, vol. 12, no. 1, article e0169759, 2017.

[119] S. Krishnan, M. R. Pappadis, S. C. Weller et al., "Needs of stroke survivors as perceived by their caregivers: a scoping review," *American Journal of Physical Medicine & Rehabilitation*, vol. 96, no. 7, pp. 487–505, 2017.

[120] K. R. Brittain and C. Shaw, "The social consequences of living with and dealing with incontinence–a carers perspective," *Social Science & Medicine*, vol. 65, no. 6, pp. 1274–1283, 2007.

[121] S. E. MacPherson, C. Healy, M. Allerhand et al., "Cognitive reserve and cognitive performance of patients with focal frontal lesions," *Neuropsychologia*, vol. 96, pp. 19–28, 2017.

[122] M. Liguz-Lecznar and M. Kossut, "Influence of inflammation on poststroke plasticity," *Neural Plasticity*, vol. 2013, Article ID 258582, 9 pages, 2013.

[123] V. L. Feigin, B. Norrving, and G. A. Mensah, "Global burden of stroke," *Circulation Research*, vol. 120, no. 3, pp. 439–448, 2017.

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Review Article

Impaired GABA Neural Circuits Are Critical for Fragile X Syndrome

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Fragile X syndrome (FXS) is an inheritable neuropsychological disease caused by silence of the *fmr1* gene and the deficiency of Fragile X mental retardation protein (FMRP). Patients present neuronal alterations that lead to severe intellectual disability and altered sleep rhythms. However, the neural circuit mechanisms underlying FXS remain unclear. Previous studies have suggested that metabolic glutamate and gamma-aminobutyric acid (GABA) receptors/circuits are two counter-balanced factors involved in FXS pathophysiology. More and more studies demonstrated that attenuated GABAergic circuits in the absence of FMRP are critical for abnormal progression of FXS. Here, we reviewed the changes of GABA neural circuits that were attributed to intellectual-deficient FXS, from several aspects including deregulated GABA metabolism, decreased expressions of GABA receptor subunits, and impaired GABAergic neural circuits. Furthermore, the activities of GABA neural circuits are modulated by circadian rhythm of FMRP metabolism and reviewed the abnormal condition of FXS mice or patients.

1. Introduction

Fragile X mental retardation protein (FMRP) is widely expressed in neurons and glia in the brain and acts as an "interactor" regulating ribosome stalling, translational control, and synaptic plasticity in brain circuits [1–3]. FMRP contributes to cognition, emotions, and memory through the referred "interactor" role as well. Fragile X syndrome (FXS) patients are deficient of FMRP due to *fmr1* gene silence caused by a CGG trinucleotide amplification on Xq27.3 in the 5′-UTR on chromosome [4]. According to CGG trinucleotide expansion and clinical symptoms, FM allele mutation-related syndromes could be divided into FXS (>200 repeats) and FXTAS (55–199 repeats) during early diagnosis of FXS. For example, methylation-specific quantitative melt analysis (MS-QMA), respectively, identified methylation mosaicism in an additional 15% and 11% of patients in the Chilean and Australian reports, suggesting the presence of a cryptic FM [5, 6]. Other methods include a variety of polymerase chain reaction (PCR) techniques, such as high polymorphism markers for preimplantation genetic diagnosis (PGD) of FXS [7] and two PCR analyses (PCR screening and PCR premutation) [8]. However, it is difficult to draw a solid criterion due to different inclusive criteria, diagnostic methods, and sample sizes within each study. Although frequencies of clinical characteristics were different between ethnicities, especially in Asian and African people, which provided evidence for genetic counseling [9], FXS is still difficult to be diagnosed on the account of a lack of an obvious phenotype at birth and during prepuberty in clinic.

Previous studies have illustrated that FXS is caused by the alteration at multiple levels from mRNA shuttling to synaptic plasticity and behavioral phenotypes [10]. For example, FMRP regulated proteins in the modulation of synaptic plasticity, which maintain spine shape and dynamics [11–13]. The retardation of FMRP leads to abnormal

group I metabotropic glutamate receptor (mGluR) signaling, together with the loss of AMPA and NMDA receptors [2], although recently clinical trials targeting on mGluR1 failed in FXR patients [14]. Specifically, the enzymes for GABA synthesis and degradation, GABA membrane transporters, and a GABA receptor scaffolding protein are downregulated in the absence of FMRP [15]. Besides, FMRP absence $GABA_A$ receptor $\alpha 1$ and δ subunits were downregulated in *fmr1* gene knockout mouse and *Drosophila* [15–17]. All these studies suggest a perplexing, yet not well understood, link between GABAergic signaling, abnormal neuronal circuits, and dysfunctional behaviors in both FXS animal models and patients. Among all alterations of phenomenal function deficits, dendritic abnormalities are the most evident structural changes in FXS. FMRP regulates neuronal branching as well as dendritic spine morphology and density [18, 19]. However, it remains unclear whether plastic changes of inhibitory circuits may cause abnormal spine morphology in FXS or vice versa. In this review, we summarized mechanisms on the effects of inhibitory synapse alteration from circuits to molecular interaction.

2. Altered GABA Metabolisms in FXS Animal Models and Patients

There have been great progresses in the altered GABA metabolism underlying FXTAS/FXS pathogenesis. Mitochondria provides energy for the cell and the brain using most of the energy among all organs. There is mounting evidence that mitochondrial dysregulation systemically contributes to the decreased cell function, even during the neonatal period of mice, first reported by Rizzo et al. [20]. It is reported that premutated hippocampal neurites contained significantly fewer mitochondria and reduced mitochondria mobility at early stage of differentiation, despite the presence of appreciable FMRP expression [21]. Together, similar significant deficits of mitochondrial dysfunction, induced by Zn levels, were observed in the Zn-rich regions (the hippocampus and cerebellum of premutation carriers), with some of these effects lasting into adulthood [22, 23]. Particularly, in dysregulated GABAergic circuits, mitochondrial dysfunction plays vital role from the aspects of mitochondrial structure, number, membrane permeability, transport, fusion, and fission [21, 24–27]. It is noteworthy that abnormality of mitochondrial structure and function is regulated aberrant expression of microRNAs (miRNA) [28], while few report functions of miRNAs on GABA metabolism in Fragile X syndrome. More work should be needed to illustrate the perplexing role of deregulated miRNA expression profiles within uncommon GABA neural circuits. In a word, abnormalities of mitochondrial dysfunction induced by FMRP deficits altered GABA metabolism, contributing to the etiology of FXS/FXTAS.

In addition, glutamic acid decarboxylase (GAD) or vesicular GABA transporter protein (VGTA) and vesicular glutamate transporter protein (VGLUT) consist of two components of synaptic balance. Increased expression of VGAT relative to VGLUT expression was shown within the medial nucleus of the trapezoid body (MNTB) in FXS [29]. Their mechanisms are necessary to be further explored. In FXS patients, a reduced release of GABA from the GABAergic terminals to the presynaptic $GABA_B$ receptors might induce a decreased inhibition of neurotransmitter spillover, which conversely activated mGluR signaling [30]. One mechanism of modulating GABA release involves the synthesis and mobilization of endocannabinoids. Activation of GroupImGluRs enables mobilization of endocannabinoids in the postsynaptic neuron and negatively modulates GABA release through a mechanism known as depolarization-induced suppression of inhibition (DSI) [31]. Therefore, in consideration of endocannabinoid mobilization in the FXS, it is reported that alterations in eCB signaling could contribute to the cognitive dysfunction associated with FXS [32]. But it only demonstrated DHPG-induced eCB-iLTD, without affecting DSI, at low concentrations. Together, relatively high concentrations of cannabinoids could affect neuropsychiatric disorders via inhibition of monoamine oxidase activity [33]. Therefore, the loss of FMRP may selectively affect specific inhibitory circuits and more evidence is needed in exploring. In the developing and mature brain, it is critical for cortical balance of excitatory and inhibitory neurons to be properly synchronized at behaviorally relevant frequencies. And thus, alteration of mGluR signaling and GABA metabolisms in this specific type of interneuron is likely to have wide-reaching effects in developing and mature cortical networks.

3. Decreased Expression of GABA Receptor Subunits in FXS Models

The anomalous functions of mGluR-dependent synaptic plasticity have been observed in the hippocampus of *fmr1*-KO mice. Activity-dependent synthesis of FMRP in maintaining forms of synaptic plasticity may be induced by augmented mGluR-LTD in hippocampal neurons [34, 35], while the initiation of long-term potentiation (LTP) is a qualitatively different functional consequence of mGluR1-stimulated protein synthesis at the synapses of the hippocampus where LTD can be induced. Besides, the mGluR theory proposes that stimulation of mGluR1 induces local mRNA translation, resulting in protein synthesis that subsequently enhances the internalization of AMPA receptors [36]. This model predicts that in the absence of FMRP, the increased translation of a subset of mRNAs disturbs receptor internalization dynamics and then exaggerates internalization of AMPA receptors and weakens the synapse. Interestingly, $GABA_{B1}$ and $GIRK_2$ internalization also is reported to cause rapid and persistent weakening of $GABA_B$-activated GIRK-mediated ($GABA_B$-GIRK) currents in FXS [37]. Clearly, the fate of internalized $GABA_A$Rs will therefore play a critical role in controlling cell surface receptor levels and hence the efficacy of synaptic inhibition. This may suggest that GABA receptors take internalization process, but its underlying complicated mechanisms still need to be explored. Furthermore, FMRP absence increased steady state surface levels of $GABA_A$Rs, showing a dramatic functional effect of increased surface receptor number. The mechanism underlying post-endocytic $GABA_A$R sorting remains to be demonstrated, and FMRP's particular role in this process is also an area of

active research. The impact of FMRP regulation of $GABA_A$Rs was recently shown in the hypothalamus, causing decreased food intake and loss of body weight [38]. An unresolved issue is whether FMRP acts to promote recycling of $GABA_A$Rs or prevents their lysosomal degradation.

Furthermore, different subunit combination leads to diverse expression patterns of $GABA_A$Rs at specific cell surface. Most surface receptor clusters of $\gamma 2$ receptor subunits are synaptic, while $GABA_A$Rs containing $\alpha 5$ or $\beta 3$ subunit express higher at extrasynaptic. δ subunit is exclusively located outside the synapse at perisynaptic and extrasynaptic locations [39, 40]. For example, it is investigated that tonic $GABA_A$ currents in the subiculum were downregulated in the *fmr1* knockout mouse relative to wild-type animals [41]. These results were associated with reductions in tonic $GABA_A$ receptor subunits. Furthermore, more specific results based on the different GABAR subunits need to be expanded to better identify each function in FXS.

Results from all above pave the way for many interesting avenues of research. First, more work is needed to illustrate the molecular causes of impaired inhibition in FXS. In the *Drosophila* model, limited research available has demonstrated that a $GABA_A$ receptor reduction can lead to behavioral impairments. However, other research from FXS models indicated that the mechanism was likely more complicated and possibly indirect due to not only variable GABA metabolisms but also regional specificity [40, 42, 43]. For example, vision process is modulated by different GABA receptors in spread brain via tonic inhibition, such as temporal cortex, lateral geniculate nucleus (LGN) of the thalamus, and vision cortex [44, 45], while tonic inhibition is mediated via extrasynaptic $\alpha 5$- and δ-containing $GABA_A$Rs [40, 44]. Future research will examine that specific subunits of GABA receptor encode these vision information computations. And it is also worth noting that the role of GABA in the developing CNS is dynamic and variable between brain regions [40, 43, 46]. Another triggering idea is that impaired inhibition comes from activity-dependent synaptic plasticity alteration during developmentally critical periods [43, 47]. Both mouse and *Drosophila* FXS models show impaired critical period plasticity, and early activity is critical for shaping E/I synaptic balance [48–50]. These findings indicate that many mechanisms are to be explored among GABAergic neurons, GABA metabolism, and GABA receptor alteration in FXS.

4. Impaired GABAergic Neural Circuits in FXS

Dysfunctional mGluR1/5 signaling in excitatory synaptic circuitry has been considered as one classic mechanism underlying FXS [51–53]. But a main characteristic of the impairment is usually attributed to a failure in the inhibition of the central set or the need for a supervisory system to be involved in the inhibition of predominant manners. The increased excitability of hippocampal and neocortical circuits in FXS, due to dysregulation of glutaminergic neurons, can in turn disrupt the normal actions of inhibitory GABAergic neurons [32, 54]. It has long been known that FXS models also display reduced function in inhibitory GABAergic circuits [55–57]. Specifically, downregulation of $GABA_A$ receptor subunits influences both the mRNA and protein levels, which would further increase the excitability of limbic and cortical circuits [39].

FMRP is widely expressed in GABAergic neurons [58, 59], and it is also involved in normal interneuron maturation and function modulation [30, 55, 58]. Recently, it was shown that there were lower expressions of several genes involved in GABA metabolism, including *gad1*, *gat1*, and *gat4*, in the brain of both mouse and *Drosophila* FXS models [15, 58, 60]. It is well known that GABAergic neurons can modulate neurotransmitter release in autocrine or paracrine pattern, via presynaptic $GABA_A$ and $GABA_B$ receptors [61–63]. It is indicated that dysfunctional GABAergic neurons affect balance of inhibitory/excitatory circuits particularly during early developmental critical periods, via the role of GABA attenuated regulator in FXS models [62, 64].

For now, GABAergic impairments have been reported in FXS models of *Drosophila*, *zebrafish*, and mouse. And GABAergic signaling is essential for regulating neuronal migration, maturation, and circuit formation. Therefore, defects in the GABAergic system are likely to have profound effects on neuron development and circuit work in FXS. Currently, a better understanding of early developmental changes in GABAergic system in FXS would be reckoned as the key insight into the underpinning of the FXS brain. Also, the relationship between GABAergic systems and mGluRs ones, as well as their overlapping plasticity alteration, is taken as the pivotal basement to strengthen a more comprehensive cognition of FXS.

Besides the deficits in learning and memory in these models, one consistent behavioral abnormality they share is altered circadian rhythm behaviors, which potentially mimics the sleep abnormalities seen in patients with fragile X syndrome. Circadian rhythm describes the approximately 24-hour cycles generated by a master pacemaker located in the suprachiasmatic nuclei (SCN) of the anterior hypothalamus of the mammalians and in the ventral lateral neurons (LNvs) of *Drosophila* [65]. Also, the connections between the SCN and other parts of the system are important for the control of circadian rhythms in the central nervous system [66]. Interestingly, it has been shown that the loss of FMRP and FXR2P results in arrhythmicity resulting from inappropriate neuronal communication within the central nervous system [67]. Additionally, the altered expression of the clock component has been observed in FXS animal models [67, 68]. The upregulation of FMRP increases PER1- and PER2-induced BMAL1–NPAS2 transcriptional activity, suggesting that FMRP is required for regulation of circadian behaviors. Thus, *Drosophila* lacking the *fmr1* gene exhibits altered circadian rhythms. Taken together, these results indicate that fragile X-related proteins might be associated with the induction of abnormal sleep patterns in FXS due to alterations in circadian genes; they may also play a critical role in the regulation of circadian output pathways.

Clinical studies have illustrated that melatonin-dependent signaling pathways can impair vigilance, learning, and memory abilities and may be linked to autistic behaviors such as abnormal anxiety responses [69, 70]. Low melatonin

levels are related with altered GABAergic system [71]. Furthermore, alterations in the circadian clock mechanism due to abnormal melatonin synthesis can affect the function of GABA neural circuits [70, 72]. Recently, studies using animal models of autism have indicated that clock and clock-related genes may interact in the ASD phenotype and studies using *fmr1* KO mice have implicated clock proteins in sleep alterations in FXS [73]. Under dysfunctional FMRP conditions, GABA activity is altered by disruptions in intracellular signaling. Recent studies have proposed the existence of abnormalities in melatonin secretion and circadian patterns in individuals with FXS with ASD that are likely to be due to excessive signaling via GABA [74]. Furthermore, melatonin is helpful for treating the physical alterations of axons and dendritic spines [75, 76]. In addition, other endocrine hormones, such as oxytocin and insulin, participate GABA neuronal function via abnormal biorhythmic patterns. It is reported that oxytocin-mediated GABA excitatory-inhibitory shift during delivery is abolished in FXS model. During delivery and subsequently hippocampal neurons have elevated intracellular chloride levels and elevated gamma oscillations, which suggests the importance of oxytocin-mediated GABAergic inhibition during the process [77]. Similarly, the insulin-producing cells (IPCs) are crucial for normal insulin release and insulin-signaling in the brain and are sufficient to restore normal circadian behavior in the *Drosophila* FXS model [78]. Moreover, IPCs have been demonstrated to receive inputs from multiple neurotransmitters and hormones, including tachykinin, leptin, GABA, and serotonin [79]. But the specific mechanisms deserve further investigation. In brief summary, alteration of GABA inhibition is not simply linked to amplified mGluR signaling, whereas they both are regulated by circadian clock and circadian genes in depth.

Overall, current issues provide much needed *in vivo* evidence for GABAergic circuit impairments in FXS and set the foundation for future work linking molecular to circuit level to behavioral changes. Addressing altered GABAergic circuit function should lead to more effective treatments for FXS patients.

5. Conclusions

In summary, deregulated GABA metabolism, decreased expressions of GABA receptor subunits, and impaired GABAergic neural circuits contribute to abnormal behaviors in FXS. Importantly, it is noteworthy to be studied that circadian clock genes regulate substantial life activities of organism and are related to the process of growth and development in FXS models and patients. Specifically, GABA inhibition is modulated via dysfunctional biorhythmic patterns of endocrine hormones and *fmr1* gene. And better understanding of the GABA neural circuits will support novel therapeutic methods in FXS.

Conflicts of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References

[1] J. D. Richter, G. J. Bassell, and E. Klann, "Dysregulation and restoration of translational homeostasis in fragile X syndrome," *Nature Reviews Neuroscience*, vol. 16, no. 10, pp. 595–605, 2015.

[2] K. Zhang, Y. J. Li, Y. Guo et al., "Elevated progranulin contributes to synaptic and learning deficit due to loss of fragile X mental retardation protein," *Brain*, vol. 140, no. 12, pp. 3215–3232, 2017.

[3] E. Chen, M. R. Sharma, X. Shi, R. K. Agrawal, and S. Joseph, "Fragile X mental retardation protein regulates translation by binding directly to the ribosome," *Molecular Cell*, vol. 54, no. 3, pp. 407–417, 2014.

[4] P. K. Todd, S. Y. Oh, A. Krans et al., "CGG repeat-associated translation mediates neurodegeneration in fragile X tremor ataxia syndrome," *Neuron*, vol. 78, no. 3, pp. 440–455, 2013.

[5] Y. T. Hwang, S. M. Aliaga, M. Arpone et al., "Partially methylated alleles, microdeletion, and tissue mosaicism in a fragile X male with tremor and ataxia at 30 years of age: a case report," *American Journal of Medical Genetics Part A*, vol. 170, no. 12, pp. 3327–3332, 2016.

[6] S. M. Aliaga, H. R. Slater, D. Francis et al., "Identification of males with cryptic Fragile X alleles by methylation-specific quantitative melt analysis," *Clinical Chemistry*, vol. 62, no. 2, pp. 343–352, 2016.

[7] M. Chen, M. Zhao, C. G. Lee, and S. S. Chong, "Identification of microsatellite markers <1 Mb from the *FMR*1 CGG repeat and development of a single-tube tetradecaplex PCR panel of highly polymorphic markers for preimplantation genetic diagnosis of fragile X syndrome," *Genetics in Medicine*, vol. 18, no. 9, pp. 869–875, 2016.

[8] A. P. Amancio, C. A. de O Melo, A. de M Vieira et al., "Molecular analysis of patients suspected of Fragile X syndrome," *Genetics and Molecular Research*, vol. 14, no. 4, pp. 14660–14669, 2015.

[9] C. Charalsawadi, J. Wirojanan, S. Jaruratanasirikul, N. Ruangdaraganon, A. Geater, and P. Limprasert, "Common clinical characteristics and rare medical problems of Fragile X syndrome in Thai patients and review of the literature," *International Journal of Pediatrics*, vol. 2017, Article ID 9318346, 11 pages, 2017.

[10] T. Yang, H. Zhao, C. Lu et al., "Synaptic plasticity, a prominent contributor to the anxiety in Fragile X syndrome," *Neural Plasticity*, vol. 2016, Article ID 9353929, 12 pages, 2016.

[11] T. L. Schmit, J. A. Dowell, M. E. Maes, and M. Wilhelm, "c-Jun N-terminal kinase regulates mGluR-dependent expression of post-synaptic FMRP target proteins," *Journal of Neurochemistry*, vol. 127, no. 6, pp. 772–781, 2013.

[12] F. Niere, J. R. Wilkerson, and K. M. Huber, "Evidence for a fragile X mental retardation protein-mediated translational switch in metabotropic glutamate receptor-triggered arc translation and long-term depression," *The Journal of Neuroscience*, vol. 32, no. 17, pp. 5924–5936, 2012.

[13] J. Steinberg and C. Webber, "The roles of FMRP-regulated genes in autism spectrum disorder: single- and multiple-hit

genetic etiologies," *American Journal of Human Genetics*, vol. 93, no. 5, pp. 825–839, 2013.

[14] A. Mullard, "Fragile X disappointments upset autism ambitions," *Nature Reviews Drug Discovery*, vol. 14, no. 3, pp. 151–3, 2015.

[15] C. L. Gatto, D. Pereira, and K. Broadie, "GABAergic circuit dysfunction in the *Drosophila* Fragile X syndrome model," *Neurobiology of Disease*, vol. 65, pp. 142–159, 2014.

[16] V. Sabanov, S. Braat, L. D'Andrea et al., "Impaired GABAergic inhibition in the hippocampus of *Fmr1* knockout mice," *Neuropharmacology*, vol. 116, pp. 71–81, 2017.

[17] S. Braat, C. D'Hulst, I. Heulens et al., "The GABAA receptor is an FMRP target with therapeutic potential in fragile X syndrome," *Cell Cycle*, vol. 14, no. 18, pp. 2985–2995, 2015.

[18] S. Scotto-Lomassese, A. Nissant, T. Mota et al., "Fragile X mental retardation protein regulates new neuron differentiation in the adult olfactory bulb," *The Journal of Neuroscience*, vol. 31, no. 6, pp. 2205–2215, 2011.

[19] A. Khayachi, C. Gwizdek, G. Poupon et al., "Sumoylation regulates FMRP-mediated dendritic spine elimination and maturation," *Nature Communications*, vol. 9, no. 1, p. 757, 2018.

[20] G. Rizzo, F. Pizza, C. Scaglione et al., "A case of fragile X premutation tremor/ataxia syndrome with evidence of mitochondrial dysfunction," *Movement Disorders*, vol. 21, no. 9, pp. 1541-1542, 2006.

[21] Z. Cao, S. Hulsizer, Y. Cui et al., "Enhanced asynchronous Ca^{2+} oscillations associated with impaired glutamate transport in cortical astrocytes expressing *Fmr1* gene premutation expansion," *Journal of Biological Chemistry*, vol. 288, no. 19, pp. 13831–13841, 2013.

[22] E. Napoli, C. Ross-Inta, S. Wong et al., "Altered zinc transport disrupts mitochondrial protein processing/import in fragile X-associated tremor/ataxia syndrome," *Human Molecular Genetics*, vol. 20, no. 15, pp. 3079–3092, 2011.

[23] E. Napoli, C. Ross-Inta, G. Song et al., "Premutation in the Fragile X mental retardation 1 (*FMR1*) gene affects maternal Zn-milk and perinatal brain bioenergetics and scaffolding," *Frontiers in Neuroscience*, vol. 10, p. 159, 2016.

[24] R. Hagerman, J. Au, and P. Hagerman, "FMR1 premutation and full mutation molecular mechanisms related to autism," *Journal of Neurodevelopmental Disorders*, vol. 3, no. 3, pp. 211–224, 2011.

[25] R. Hagerman, G. Hoem, and P. Hagerman, "Fragile X and autism: intertwined at the molecular level leading to targeted treatments," *Molecular Autism*, vol. 1, no. 1, p. 12, 2010.

[26] E. S. Kaplan, Z. Cao, S. Hulsizer et al., "Early mitochondrial abnormalities in hippocampal neurons cultured from Fmr1 pre-mutation mouse model," *Journal of Neurochemistry*, vol. 123, no. 4, pp. 613–621, 2012.

[27] A. Yao, S. Jin, X. Li et al., "*Drosophila* FMRP regulates microtubule network formation and axonal transport of mitochondria," *Human Molecular Genetics*, vol. 20, no. 1, pp. 51–63, 2011.

[28] R. Richter-Dennerlein, S. Dennerlein, and P. Rehling, "Integrating mitochondrial translation into the cellular context," *Nature Reviews Molecular Cell Biology*, vol. 16, no. 10, pp. 586–592, 2015.

[29] S. E. Rotschafer, S. Marshak, and K. S. Cramer, "Deletion of *Fmr1* alters function and synaptic inputs in the auditory brainstem," *PLoS One*, vol. 10, no. 2, article e0117266, 2015.

[30] M. D. Lange, K. Jüngling, L. Paulukat et al., "Glutamic acid decarboxylase 65: a link between GABAergic synaptic plasticity in the lateral amygdala and conditioned fear generalization," *Neuropsychopharmacology*, vol. 39, no. 9, pp. 2211–2220, 2014.

[31] N. Varma, G. C. Carlson, C. Ledent, and B. E. Alger, "Metabotropic glutamate receptors drive the endocannabinoid system in hippocampus," *The Journal of Neuroscience*, vol. 21, no. 24, article Rc188, 2001.

[32] L. Zhang and B. E. Alger, "Enhanced endocannabinoid signaling elevates neuronal excitability in fragile X syndrome," *The Journal of Neuroscience*, vol. 30, no. 16, pp. 5724–5729, 2010.

[33] Z. Fisar, "Inhibition of monoamine oxidase activity by cannabinoids," *Naunyn-Schmiedeberg's Archives of Pharmacology*, vol. 381, no. 6, pp. 563–572, 2010.

[34] J. Zhang, L. Hou, E. Klann, and D. L. Nelson, "Altered hippocampal synaptic plasticity in the *FMR1* gene family knockout mouse models," *Journal of Neurophysiology*, vol. 101, no. 5, pp. 2572–2580, 2009.

[35] A. J. Iliff, A. J. Renoux, A. Krans, K. Usdin, M. A. Sutton, and P. K. Todd, "Impaired activity-dependent FMRP translation and enhanced mGluR-dependent LTD in Fragile X premutation mice," *Human Molecular Genetics*, vol. 22, no. 6, pp. 1180–1192, 2013.

[36] H. Wang, S. S. Kim, and M. Zhuo, "Roles of fragile X mental retardation protein in dopaminergic stimulation-induced synapse-associated protein synthesis and subsequent α-amino-3-hydroxyl-5-methyl-4-isoxazole-4-propionate (AMPA) receptor internalization," *Journal of Biological Chemistry*, vol. 285, no. 28, pp. 21888–21901, 2010.

[37] S. Lecca, A. Pelosi, A. Tchenio et al., "Rescue of $GABA_B$ and GIRK function in the lateral habenula by protein phosphatase 2A inhibition ameliorates depression-like phenotypes in mice," *Nature Medicine*, vol. 22, no. 3, pp. 254–261, 2016.

[38] B. Liu, L. Li, J. Chen, Z. Wang, Z. Li, and Q. Wan, "Regulation of GABAA receptors by fragile X mental retardation protein," *International Journal of Physiology, Pathophysiology and Pharmacology*, vol. 5, no. 3, pp. 169–176, 2013.

[39] M. Drexel, E. Kirchmair, and G. Sperk, "Changes in the expression of $GABA_A$ receptor subunit mRNAs in parahippocampal areas after kainic acid induced seizures," *Frontiers in Neural Circuits*, vol. 7, p. 142, 2013.

[40] G. Curia, T. Papouin, P. Seguela, and M. Avoli, "Downregulation of tonic GABAergic inhibition in a mouse model of fragile X syndrome," *Cerebral Cortex*, vol. 19, no. 7, pp. 1515–1520, 2009.

[41] N. Zhang, Z. Peng, X. Tong et al., "Decreased surface expression of the δ subunit of the $GABA_A$ receptor contributes to reduced tonic inhibition in dentate granule cells in a mouse model of fragile X syndrome," *Experimental Neurology*, vol. 297, pp. 168–178, 2017.

[42] C. D'Hulst, I. Heulens, N. van der Aa et al., "Positron emission tomography (PET) quantification of $GABA_A$ receptors in the brain of Fragile X patients," *PLoS One*, vol. 10, no. 7, article e0131486, 2015.

[43] S. Kratovac and J. G. Corbin, "Developmental changes in expression of inhibitory neuronal proteins in the Fragile X syndrome mouse basolateral amygdala," *Brain Research*, vol. 1537, pp. 69–78, 2013.

[44] P. H. Frederikse, A. Nandanoor, and C. Kasinathan, "Fragile X syndrome FMRP co-localizes with regulatory targets PSD-95,

GABA receptors, CaMKIIα, and mGluR5 at fiber cell membranes in the eye lens," *Neurochemical Research*, vol. 40, no. 11, pp. 2167–2176, 2015.

[45] J. A. Hirsch, X. Wang, F. T. Sommer, and L. M. Martinez, "How inhibitory circuits in the thalamus serve vision," *Annual Review of Neuroscience*, vol. 38, no. 1, pp. 309–329, 2015.

[46] J. A. C. Broek, Z. Lin, H. M. de Gruiter et al., "Synaptic vesicle dynamic changes in a model of fragile X," *Molecular Autism*, vol. 7, no. 1, p. 17, 2016.

[47] C. A. Doll and K. Broadie, "Activity-dependent FMRP requirements in development of the neural circuitry of learning and memory," *Development*, vol. 142, no. 7, pp. 1346–1356, 2015.

[48] C. A. Doll and K. Broadie, "Impaired activity-dependent neural circuit assembly and refinement in autism spectrum disorder genetic models," *Frontiers in Cellular Neuroscience*, vol. 8, p. 30, 2014.

[49] B. S. Martin, J. G. Corbin, and M. M. Huntsman, "Deficient tonic GABAergic conductance and synaptic balance in the fragile X syndrome amygdala," *Journal of Neurophysiology*, vol. 112, no. 4, pp. 890–902, 2014.

[50] C. J. Westmark, S. C. Chuang, S. A. Hays et al., "APP causes hyperexcitability in Fragile X mice," *Frontiers in Molecular Neuroscience*, vol. 9, p. 147, 2016.

[51] S.-C. Mao, C.-H. Chang, C.-C. Wu, M. J. Orejanera, O. J. Manzoni, and P.-W. Gean, "Inhibition of spontaneous recovery of fear by mGluR5 after prolonged extinction training," *PLoS One*, vol. 8, no. 3, article e59580, 2013.

[52] A. Michalon, A. Bruns, C. Risterucci et al., "Chronic metabotropic glutamate receptor 5 inhibition corrects local alterations of brain activity and improves cognitive performance in fragile X mice," *Biological Psychiatry*, vol. 75, no. 3, pp. 189–197, 2014.

[53] A. S. Pop, B. Gomez-Mancilla, G. Neri, R. Willemsen, and F. Gasparini, "Fragile X syndrome: a preclinical review on metabotropic glutamate receptor 5 (mGluR5) antagonists and drug development," *Psychopharmacology*, vol. 231, no. 6, pp. 1217–1226, 2014.

[54] A. Straiker, K. T. Min, and K. Mackie, "*Fmr1* deletion enhances and ultimately desensitizes CB_1 signaling in autaptic hippocampal neurons," *Neurobiology of Disease*, vol. 56, pp. 1–5, 2013.

[55] T. Nomura, T. F. Musial, J. J. Marshall et al., "Delayed maturation of fast-spiking interneurons is rectified by activation of the TrkB receptor in the mouse model of Fragile X syndrome," *The Journal of Neuroscience*, vol. 37, no. 47, pp. 11298–11310, 2017.

[56] K. Ruby, K. Falvey, and R. J. Kulesza, "Abnormal neuronal morphology and neurochemistry in the auditory brainstem of Fmr1 knockout rats," *Neuroscience*, vol. 303, pp. 285–298, 2015.

[57] L. M. Franco, Z. Okray, G. A. Linneweber, B. A. Hassan, and E. Yaksi, "Reduced lateral inhibition impairs olfactory computations and behaviors in a *Drosophila* model of fragile X syndrome," *Current Biology*, vol. 27, no. 8, pp. 1111–1123, 2017.

[58] J. L. Olmos-Serrano, S. M. Paluszkiewicz, B. S. Martin, W. E. Kaufmann, J. G. Corbin, and M. M. Huntsman, "Defective GABAergic neurotransmission and pharmacological rescue of neuronal hyperexcitability in the amygdala in a mouse model of fragile X syndrome," *The Journal of Neuroscience*, vol. 30, no. 29, pp. 9929–9938, 2010.

[59] S. B. Christie, M. R. Akins, J. E. Schwob, and J. R. Fallon, "The FXG: a presynaptic fragile X granule expressed in a subset of developing brain circuits," *The Journal of Neuroscience*, vol. 29, no. 5, pp. 1514–1524, 2009.

[60] A. El Idrissi, X. H. Ding, J. Scalia, E. Trenkner, W. T. Brown, and C. Dobkin, "Decreased $GABA_A$ receptor expression in the seizure-prone fragile X mouse," *Neuroscience Letters*, vol. 377, no. 3, pp. 141–146, 2005.

[61] F. F. Trigo, A. Marty, and B. M. Stell, "Axonal $GABA_A$ receptors," *The European Journal of Neuroscience*, vol. 28, no. 5, pp. 841–848, 2008.

[62] D. C. Adusei, L. K. K. Pacey, D. Chen, and D. R. Hampson, "Early developmental alterations in GABAergic protein expression in fragile X knockout mice," *Neuropharmacology*, vol. 59, no. 3, pp. 167–171, 2010.

[63] C. D'Hulst, I. Heulens, J. R. Brouwer et al., "Expression of the GABAergic system in animal models for fragile X syndrome and fragile X associated tremor/ataxia syndrome (FXTAS)," *Brain Research*, vol. 1253, pp. 176–183, 2009.

[64] J. Y. Kang, J. Chadchankar, T. N. Vien et al., "Deficits in the activity of presynaptic γ-aminobutyric acid type B receptors contribute to altered neuronal excitability in fragile X syndrome," *The Journal of Biological Chemistry*, vol. 292, no. 16, pp. 6621–6632, 2017.

[65] N. I. Muraro, N. Pirez, and M. F. Ceriani, "The circadian system: plasticity at many levels," *Neuroscience*, vol. 247, pp. 280–293, 2013.

[66] W. J. Schwartz, R. A. Gross, and M. T. Morton, "The suprachiasmatic nuclei contain a tetrodotoxin-resistant circadian pacemaker," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 84, no. 6, pp. 1694–1698, 1987.

[67] J. Zhang, Z. Fang, C. Jud et al., "Fragile X-related proteins regulate mammalian circadian behavioral rhythms," *American Journal of Human Genetics*, vol. 83, no. 1, pp. 43–52, 2008.

[68] S. Xu, M. Poidevin, E. Han, J. Bi, and P. Jin, "Circadian rhythm-dependent alterations of gene expression in *Drosophila* brain lacking fragile X mental retardation protein," *PLoS One*, vol. 7, no. 5, article e37937, 2012.

[69] G. Kulman, P. Lissoni, F. Rovelli, M. G. Roselli, F. Brivio, and P. Sequeri, "Evidence of pineal endocrine hypofunction in autistic children," *Neuro Endocrinology Letters*, vol. 21, no. 1, pp. 31–34, 2000.

[70] J. Won, Y. Jin, J. Choi et al., "Melatonin as a novel interventional candidate for Fragile X syndrome with autism spectrum disorder in humans," *International Journal of Molecular Sciences*, vol. 18, no. 6, 2017.

[71] D. Hodge, T. M. Carollo, M. Lewin, C. D. Hoffman, and D. P. Sweeney, "Sleep patterns in children with and without autism spectrum disorders: developmental comparisons," *Research in Developmental Disabilities*, vol. 35, no. 7, pp. 1631–1638, 2014.

[72] G. J. Bassell and S. T. Warren, "Fragile X syndrome: loss of local mRNA regulation alters synaptic development and function," *Neuron*, vol. 60, no. 2, pp. 201–214, 2008.

[73] E. G. Bechara, M. C. Didiot, M. Melko et al., "A novel function for fragile X mental retardation protein in translational activation," *PLoS Biology*, vol. 7, no. 1, article e16, 2009.

[74] A. Miranda-Paez, S. R. Zamudio, P. Vazquez-Leon, V. Sandoval-Herrera, I. Villanueva-Becerril, and G. Carli, "Effect of melatonin injection into the periaqueductal gray on antinociception and tonic immobility in male rats," *Hormones and Behavior*, vol. 89, pp. 23–29, 2017.

[75] T. Ikeno and R. J. Nelson, "Acute melatonin treatment alters dendritic morphology and circadian clock gene expression in the hippocampus of Siberian hamsters," *Hippocampus*, vol. 25, no. 2, pp. 142–148, 2015.

[76] L. Mendoza-Viveros, C. K. Chiang, J. L. K. Ong et al., "miR-132/212 modulates seasonal adaptation and dendritic morphology of the central circadian clock," *Cell Reports*, vol. 19, no. 3, pp. 505–520, 2017.

[77] R. Tyzio, R. Nardou, D. C. Ferrari et al., "Oxytocin-mediated GABA inhibition during delivery attenuates autism pathogenesis in rodent offspring," *Science*, vol. 343, no. 6171, pp. 675–679, 2014.

[78] R. E. Monyak, D. Emerson, B. P. Schoenfeld et al., "Insulin signaling misregulation underlies circadian and cognitive deficits in a *Drosophila* fragile X model," *Molecular Psychiatry*, vol. 22, no. 8, pp. 1140–1148, 2017.

[79] D. R. Nässel, O. I. Kubrak, Y. Liu, J. Luo, and O. V. Lushchak, "Factors that regulate insulin producing cells and their output in *Drosophila*," *Frontiers in Physiology*, vol. 4, p. 252, 2013.