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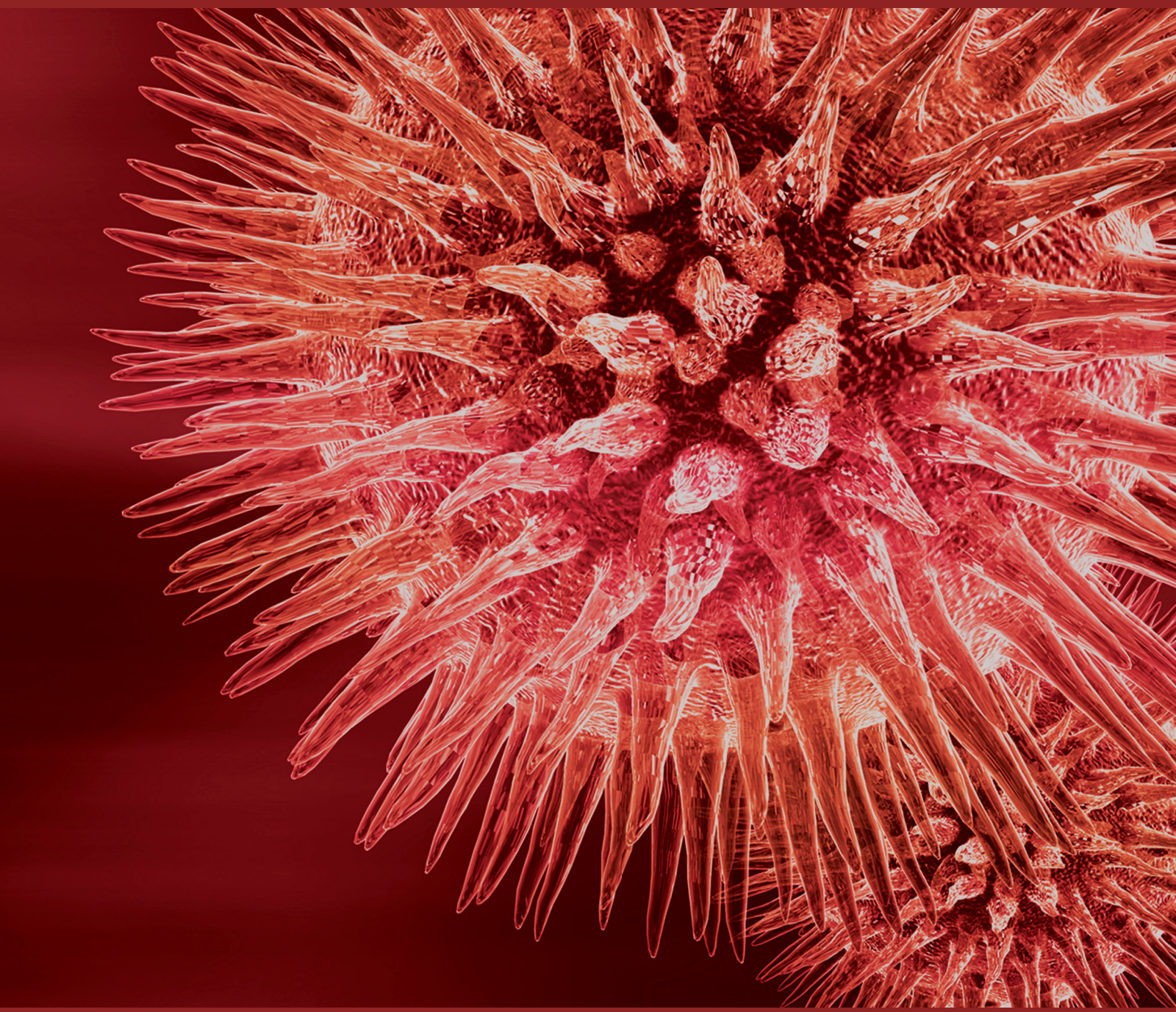
Neurodegeneration: Etiologies and New Therapies 2016

Guest Editors: Eng King Tan, Amit K. Srivastava, W. David Arnold, Mahendra P. Singh, and Yiying Zhang



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Editorial

Neurodegeneration: Etiologies and New Therapies 2016

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In this special issue, we have 9 articles that highlight diverse biochemical, immunological, molecular, and neuroimaging techniques used to decipher the pathophysiological mechanisms underpinning neurodegeneration in various cellular and animal models. The common diseases are covered, including a review on the therapeutic options in Alzheimer's disease and a discussion on the factors influencing homocysteine levels in Parkinson's disease. Of particular interest in this issue is an article on the entity of "chemo brain," a condition that overlaps between oncology and neurology. "Chemo brain" is a common term used to describe thinking and memory problems that can occur after cancer treatment. Here the authors demonstrate PET evidence of the effect of donepezil on cognitive performance in an animal model. We hope this special issue will generate further interest and debate on the pathoetiology of neurodegenerative diseases and provide a platform to generate impetus to further identify and validate new therapeutic options.

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Research Article

Edited Magnetic Resonance Spectroscopy Detects an Age-Related Decline in Nonhuman Primate Brain GABA Levels

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Recent research had shown a correlation between aging and decreasing Gamma-aminobutyric acid (GABA), the primary inhibitory neurotransmitter in the brain. However, how GABA level varies with age in the medial portion of the brain has not yet been studied. The purpose of this study was to investigate the GABA level variation with age focusing on the posterior cingulate cortex, which is the "core hub" of the default mode network. In this study, 14 monkeys between 4 and 21 years were recruited, and MEGA-PRESS MRS was performed to measure GABA levels, in order to explore a potential link between aging and GABA. Our results showed that a correlation between age and GABA+/Creatine ratio was at the edge of significance ($r = -0.523$, $p = 0.081$). There was also a near-significant trend between gray matter/white matter ratio and the GABA+/Creatine ratio ($r = -0.518$, $p = 0.0848$). Meanwhile, the correlation between age and grey matter showed no significance ($r = -0.028$, $p = 0.93$). Therefore, age and gray matter/white matter ratio account for different part of R-squared (adjusted R-squared = 0.5187) as independent variables for predicting GABA levels. Adjusted R-squared is about 0.5 for two independent variables. These findings suggest that there is internal neurochemical variation of GABA levels in the nonhuman primates associated with normal aging and structural brain decline.

1. Introduction

As we age, the brain undergoes gradual functional and cognitive declines; aging is related to neurochemical alterations in the central nervous system and cortical disruptions. Γ-Aminobutyric acid (GABA) is the primary inhibitory neurotransmitter in the brain and plays an integral role in metabolism and neuronal activity. GABA dysfunction is implicated diverse physiological and neurological diseases, such as schizophrenia [1], ADHD [2]. Various studies report cognitive deficiencies associated with aging, like motor decision speed [3] and the tendency for cognitive failures [4]. Aging has also been demonstrated to be related to cortical network disruption in structures such as precuneus, retrosplenial, and posterior cingulate cortices [5]. Moreover, previous research has shown aging influenced default mode network (DMN), which is an essential neuronal network underlying brain functions [6]. The most relevant and recent research [7] used MEGA-PRESS for GABA detection localized on human frontal and parietal cortex. Their results demonstrated age-related GABA+ decline trend in those two cortical areas. In the current study, we focused on the posterior cingulate cortex, which is the "core hub" of default mode network [8]. We hypothesized that the increasing age would associate with the decreasing GABA+ concentration in the posterior cingulate cortex of nonhuman primates.

Although some previous studies reported age-related GABA declines [9–11], comprehensive research investigating age-related changes in GABA concentrations has not been conducted yet to the best of our knowledge. One of the advantages of using nonhuman primates as the subject is that the monkeys never develop Alzheimer disease, which means that they are the ideal subject for normal aging research.

GABA detection is comparatively challenging as its concentration is an order of magnitude lower than that of other metabolites. Therefore, using standard single-voxel techniques is not practical due to the overlap of GABA and other metabolites mainly Creatine (Figure 1), whose amount is much larger than GABA around 3.0 ppm. Meanwhile, another vital characteristic of MRS spectrum, spin-spin

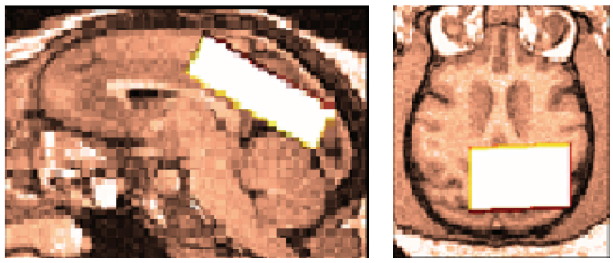

Figure 1: MRS VOI position: the volume of interest was placed in the posterior cortical cortex, without including the splenium of the corpus callosum. The voxel location is indicated by the white rectangle shown on axial and sagittal views.

coupling (J-coupling), provided the crucial clue for disentangling the GABA signal from other metabolites. The so-called multiplets in MR spectrum stem from the neighboring spin interaction. Although the broader footprint and lower peak intensity caused by J-coupling lead to the difficulty of GABA detection, the coupling of GABA signal around 3.0 ppm with the signals at 1.9 ppm differentiates itself from the other primary signals at 3.0 ppm, which are not coupled with the signal at 1.9 ppm. The edited GABA detection took advantage of this interaction by applying a frequency-selective (edited) pulse on 1.9 ppm, influencing the GABA signal on 3.0 ppm indirectly, while the other signals on 3.0 ppm are not affected.

Consequently, the difference between edited pulse ON at 1.9 ppm and edited pulse OFF version will illustrate merely the signals influenced by the edited ON pulse. The resulting edited spectrum filters out majority signal at 3.0 ppm, which is not coupling with the signal at 1.9 ppm. Among many of the spectral editing methods, MEscher-GArwood Point RESolved Spectroscopy (MEGA-PRESS) is widely used for GABA quantification due to its simplicity of implementation. As the name implies, MEGA-PRESS adds two frequency-selective editing pulses on the PRESS single-voxel MRS experiment.

2. Methods

2.1. Subjects. Sixteen aging Rhesus monkeys (*Macaca mulatta*) that were part of ongoing studies of healthy aging at Boston University in June to November of 2012 were imaged after anesthetizing. All monkeys (age: range 4–21, mean = 15.5 ± 6.07 years) had complete medical and birth records and were in good health.

2.2. Ethical Statement. This project followed the rules of Animal Care Program of Boston University, Boston, MA.

2.3. MRI and MRS Acquisition. All subjects were scanned on a 3 T scanner (Philips "Achieva" TX Best, The Netherlands) using an eight-channel phased array head coil receiver. T1-weighted three-dimensional Fast Field Echo images were obtained with the following parameters: TR = 250 ms, TE = 3.163 ms; slice thickness = 0.6 mm; matrix 256 × 256 pixels; field of view (FOV) = 220 × 220 mm; and flip angle = 90°. The volume of interest (VOI) with a size of 10 × 30 × 35 mm^3 was chosen in the medial brain, focusing on the posterior cingulate cortex. The median sagittal plane acted as a reference slice for voxel localization: the VOI was arranged to keep away from the ventricles and skull, as they might influence the result of MRS. The region of interest was set to be aligned with the shape of the corpus callosum and superior to the splenium of the corpus callosum. The medial plane was positioned large enough to cover the PPC area.

2.4. MRS Setting. The GABA concentration was measured using an MEGA-PRESS sequence [12], and the parameters are as the following: TR/TE = 1500/68 ms; 16 ms editing pulses alternating at 1.9 and 7.5 ppm to separate the GABA molecule from other chemicals. The deficiency of standard single-voxel techniques had been improved by utilizing an editing pulse (EDIT-ON) at GABA spins at 1.9 ppm to separate the GABA signal from the other metabolites like Creatine at 3.0 ppm, while EDIT-OFF referred to the pulse applied to somewhere else other than 3.0 ppm. The difference between the EDIT-ON and EDIT-OFF spectra yields those peaks affected by the editing pulses. Because the contribution of macromolecules (MM) could not be excluded in every respect as restrained by the fitting limitations, in the following part of this manuscript, the signal detected at 3 ppm is labeled as GABA+ instead of GABA, indicating the potential occurrence of these other compounds. GABA+ and Glx concentration were quantified from the MEGA-PRESS specialized tool Gannet v2.0 [2], which fit the GABA+ model into Gaussian distribution. GABA+ qualification was calculated with the area under the curve, and GABA+ levels were gauged via GABA+/Creatine ratio. Creatine (Cr) performs an adequate reference because of the common location of original of the GABA+ and Cr signals, which comes from the EDIT-OFF MEGA-PRESS spectra.

2.5. VOI Localization and VOI Coregistration. VOI was placed in posterior cingulate cortex, in a relatively large area as shown in Figure 1. The voxel has been put in the PCC with an aim to encompass as much of the localized activity as possible, but while also avoiding the scalp/muscle/fatty tissue. Given the need to achieve sufficient signal-to-noise and also some degree of regional specificity, placement of MRS volume of interest can be a difficulty.

Volume of interest (VOI) was coregistered to the structural images via developed Matlab tool called *mrsvoi* to confirm the accuracy of VOI creation; this method could create more reliable mask comparing Gannet's Coregister tool. The grey matter (GM) fraction was defined as the GM volume to the white matter (WM) volume in VOI. Other gray matter indexes are the ratio between GM volume and the whole brain volume and the ratio between GM volume and the volume of VOI.

2.6. The Brain Components Segmentation. In general, we followed the BET-FLIRT-FAST pipeline from FSL package (Oxford University, Oxford, UK) [13]. BET (Brain Extraction Tool) erases all the areas outside of brain from a whole brain image, such as T1 or T2 models [14]. FLIRT (FMRIB's Linear

Image Registration Tool) is the automatic tool for linear (affine) brain image registration [13]. The second step, FLIRT, is not required for human brain imaging processing, but it is an essential step for monkey brain imaging processing before FAST, due to the variability of the monkey brain. FLIRT could improve the accuracy of image template registration and then enhance the following processing step. Lastly, FAST (FMRIB's Automated Segmentation Tool) divides a structural brain image into different tissue types (grey matter, white matter, CSF, etc.), while also correcting for bias field or RF inhomogeneities [15]. To be more specific, we did the imaging analysis as the following: The T1-weighted brain images were skull-stripped via BET (Brain Extraction Tool) from FSL package [13]. We opted to use the Rhesus Macaque Atlases from Wisconsin ADRC imaging group because of already having an extensive group of users and also used 3 mm FWHM as smoothing for creating each monkey's templates [16]. The structural T1 and grey-prior templates from this template set were firstly rotated according to the orientation of the monkey samples; then the brain image registration tool FLIRT (FMRIB's Linear Image Registration Tool) was used to produce customized templates considering the variation of monkeys' brain. At last, the T1-weighted monkey brain images were segmented as gray matter (GM), white matter (WM), or cerebrospinal fluid (CSF) by FAST (FMRIB's Automated Segmentation Tool) [15] from FSL package, while standardizing to the customized template produced from FLIRT.

2.7. GABA+ Measurement Qualification. One of the examples of the GABA+ spectrum is shown in Figure 2. The quality control of Gannet is standard deviation of residual, and the baseline of references modeling served as the vital foundation of success modeling GABA+ concentration. Three monkeys' GABA+ spectra were excluded from further statistical analysis due to low model fitting as the Fit Error (Cr) excesses 45% in those cases (the definition of Fit Error is the standard deviation of the residuals expressed as a percentage of the signal height). The remaining monkeys' GABA+ concentration ranged from 0.06 to 0.1 (mean = 0.08, sd = 0.01).

2.8. Statistical Analysis. The relationships between variables (e.g., age; gray matter in VOI; GABA+/Cr) were examined via Pearson Product Correlation ($p < 0.05$, uncorrected). All group differences were tested on unpaired 2-tailed t-tests ($p < 0.05$, uncorrected). Analyses were performed with R (R version 3.0.3).

3. Results

3.1. The Outlier Examination. Two outlier subjects were excluded from further analysis based on Weisberg t-test, which is a robust outlier test at small values. The GABA+/Creatine ratio of one monkey is much higher than the others (0.14 compared to mean of all other monkeys: 0.0784); another monkey had an aberrant value of gray matter (2.26E+04 compared to an average of all other monkeys: 18953). Besides the two outliers, one monkey has two records of GABA+ excitation: the second time scan was 15 days later than the first scan. We chose the version from the same terminal as other monkies (longitudinal terminal instead of DSI terminal).

3.2. Group Difference Comparisons. We grouped the monkeys into two cohorts based on their age (young: 7.5 (±) 2.38; old: 19.5 (±) 0.76). The VOI placement and spectral fit for GABA and Glx were not different between two groups. The GABA/Cr ratio difference between old group (0.08 (±) 0.01) and young group (0.09 (±) 0.01) had a clear tendency to significance ($p = 0.05834$, t-test, Figure 3). On the other hand, the amount of gray matter in VOI was not that different between two groups ($p = 0.9074$, t-test). Besides GABA+, we also measured Glx (Glutamate + Glutamine) since it contains Glutamate, which is the dominant excitatory neurotransmitter. The Glx level was not different between two cohorts ($p = 0.25$, t-test).

3.3. Correlations and Linear Regression Models. After examining the Pearson Product Correlation between all the factors, GABA+/Creatine ratio, respectively, reached a considerable trend toward significance with age ($r = -0.523$, $p = 0.081$ in Figure 4) and gray matter in VOI voxel ($r = -0.553$, $p = 0.06$ in Figure 5). Meanwhile, the correlation between age and GM/VOI showed no significance ($r = -0.028$, $p = 0.93$ in Figure 6). The relationship between those variables implies age and gray matter are related to GABA/Creatine ratio but not related to each other. This result suggested a general linear model using both age and gray matter as independent variables for predicting GABA/Creatine ratio (Table 1). The adjusted R-squared is about 0.5 for two independent variables, while satisfying all the hypotheses of linear regression.

The correlation between Glx/Creatine and GABA+/Creatine was not significant ($r = 0.0196$, $p = 0.9516$), as well as Glx/Creatine and age ($r = -0.273$, $p = 0.3906$). We also utilized GABA+/Glx ratio as a reference, yet GABA+/Glx was not significantly correlated with any essential factors, such as GM/VOI and age.

4. Discussions

Using Creatine as a normalizing factor for GABA quantification could cause confounding effect on the result. One of the recent meta-analysis studies [17] reported that Creatine increases with age, which implies in our case GABA+/Cr ratio would be influenced by the Creatine variation. Since no unsuppressed water spectra were collected, it was not possible to normalize GABA+ to the unsuppressed water signal. For clarifying this point, we examined the Pearson Product Correlation between GABA+ and age ($r = -0.53$, $p = 0.07275$). The correlation coefficient did not change that much compared to the correlation between age and GABA+/Cr ratio; at least in our case Creatine was not that influential as it might be. Moreover, Creatine was not related to the increasing age ($r = -0.21$, $p = 0.45$). Glx level was not significantly related to age either ($r = 0.32$, $p = 0.3091$).

With regard to the question of whether the main result (the trend towards a correlation between GABA+/Cr and age)

Table 1: A general linear model uses both age and *GM/VOI* as independent variables for predicting GABA+ level (multiple regression analysis ($N = 12$), dependent variable: GABA+/creatine ratio).

Independent variables	Estimate	Std. error (S.E.)	t value	Pr(>|t|)
(Intercept)	0.1777298	0.0321091	5.535	$p < 0.001$
Age	−0.0011562	0.0004191	−2.759	0.022156
GM/VOI	−0.1837662	0.0713761	−2.575	0.029960

Residual standard error: 0.008431 on 9 degrees of freedom.
Multiple R-squared: 0.6062, adjusted R-squared: 0.5187.
F-statistic: 6.927 on 2 and 9 DF, p value: 0.01509.

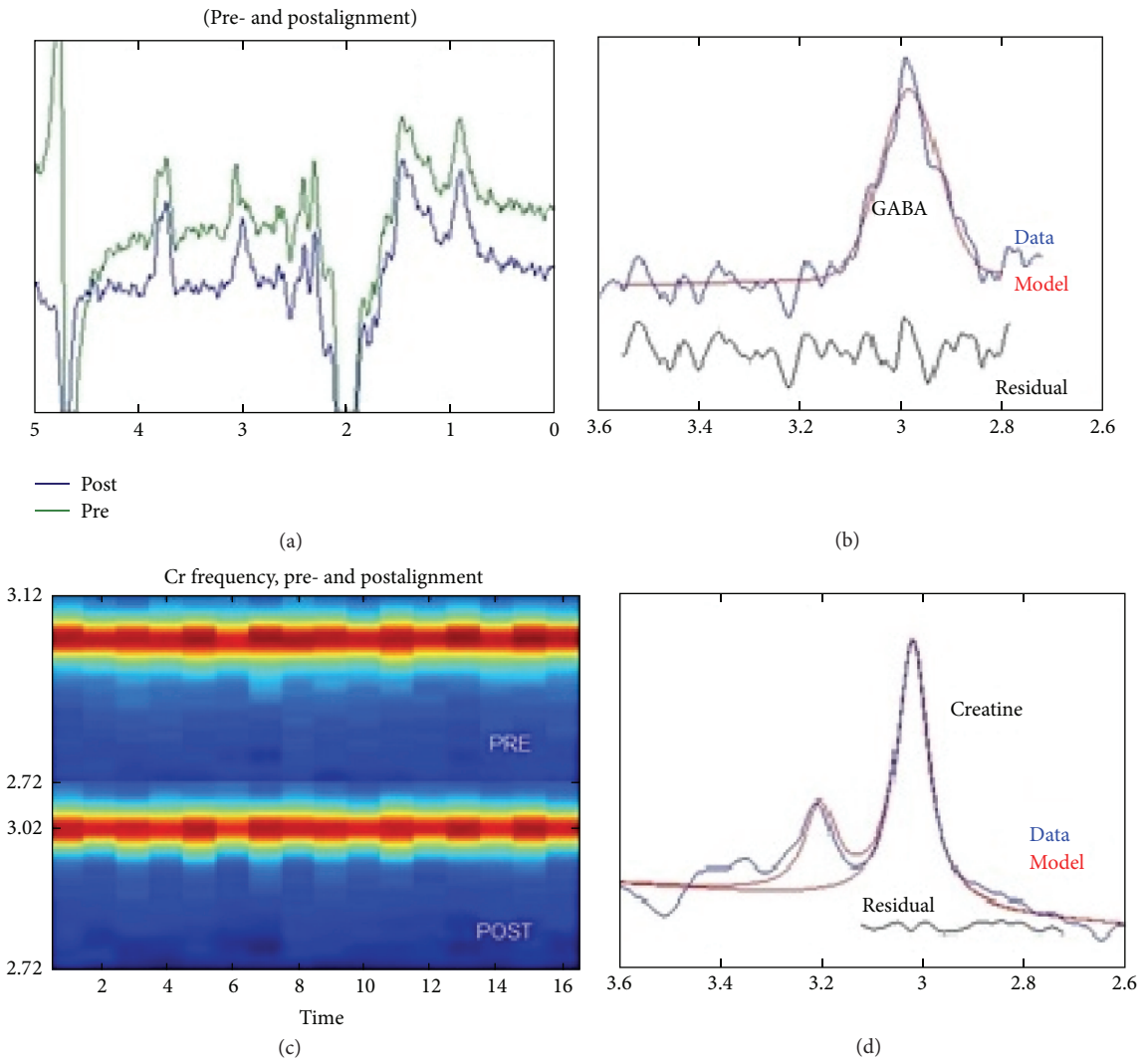

Figure 2: The original GABA+ spectrum editing results: (a) the processed GABA-edited difference spectrum (EDIT-OFF − EDIT-ON = DIFF), which is the primary output of the *GannetLoad* module. Green spectrum is the one *before* frequency and phase correction, while the blue line is the one *after* frequency and phase correction. (b) Model fitting of GABA+ and Creatine spectrum peak: this plot represents the GABA+ signal modeling (*GannetLoad* output). The blue line is the actual GABA-edited spectrum while the red one is the model of best fit (using a simple Gaussian model by default). The residual is the black curve below the modeling plot. (c) Cr signal variation through the whole experiment; the y-axis represents the frequency of Cr signal (ppm); the intensity registers with a color scale, so the Cr signal appears as "hot" stripe through the plot. The lower half line should be more evenly horizontal compared with the upper half strip due to the frequency and phase correction. (d) The bottom panel is the Cr signal from the OFF spectrum.

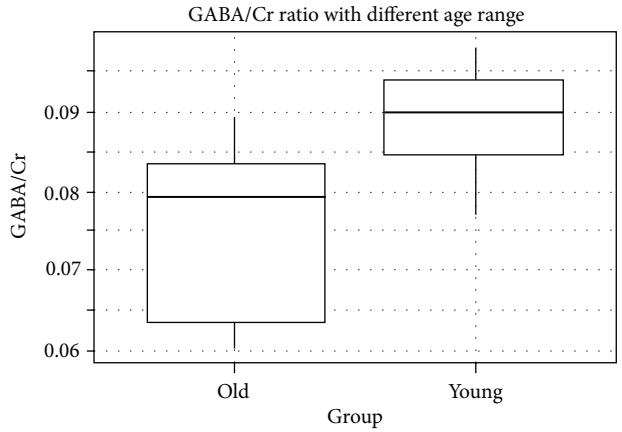

Figure 3: Box plot of GABA levels grouped by age. ns: not significant; t-test. Abbreviations: GABA, G-aminobutyric acid; Cr, Creatine.

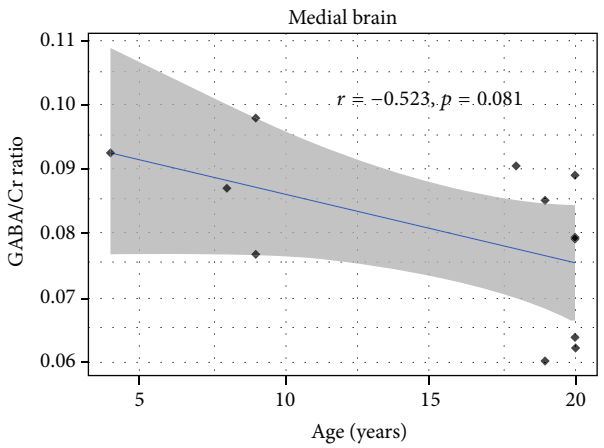

Figure 4: The correlation between GABA/Cr ratio and age.

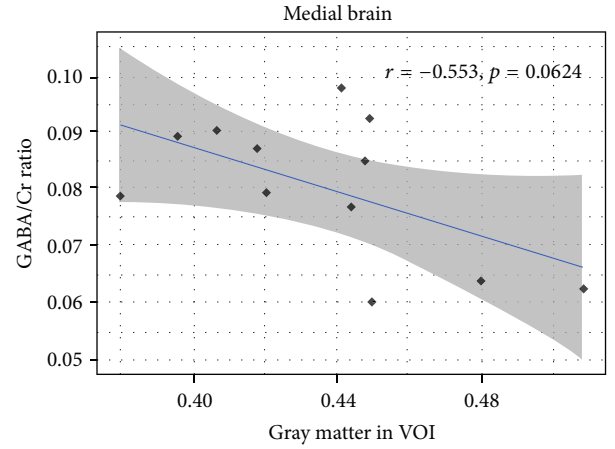

Figure 5: The correlation between GABA/Cr ratio and VOI GM.

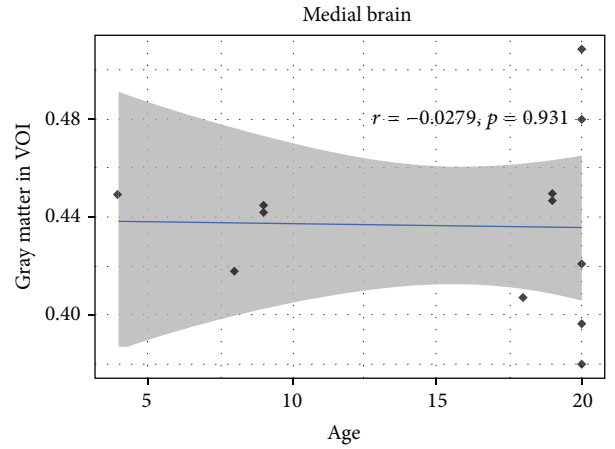

Figure 6: The correlation between age and GM/VOI.

is driven by GABA+ differences with aging or by Cr changes with age, the lack of a significant correlation between Glx/Cr and age provides indication that the reported correlation between GABA+/Cr and age is real (and driven by GABA+ rather than Creatine).

The discrepancy between our results and Haga et al.'s may come from the following: (1) The subject pool in Haga et al. study was the aging human brains while our subjects are non-human primate brains. (2) Most data of this meta-analysis came from the frontal region, while in this study the volume of interest concentrated on the posterior cingulate cortex. After all, it is difficult to compare GABA+/Cr ratios between this and the previous studies, as different measurements and parameters, as well as VOI setting, were utilized.

The most substantial finding of this study was a decreasing trend of GABA+ levels with increasing age and increasing gray matter-white matter ratio in the medial brain region of healthy monkeys ranging from 4 to 21 years old. Moreover, age and gray matter tissue fraction do not correlate with each other, which means the variation of age is related to GABA+ concentration in medial portions of the brain. The group comparison results also support this idea.

G-Aminobutyric acid (GABA), the primary inhibitory neurotransmitter in neural system, unexpectedly had not frequently been studied due to the measurement limitation. Recently, various reliable MRS protocols rendered the possibility to measure the GABA in brain reliably [18].

Many researchers suggest that the GABA reduction might be the cause of various cognitive disorders such as memory loss, one of the aging syndromes [19, 20]. However, to the best of our knowledge, only a handful of MRS studies have scrutinized direct age-related changes in GABA concentrations, and most of them were human and other animal models [7]. Although some studies found no correlation between GABA concentration and aging [21], they suffered from the low statistical power, and those findings were not the primary purpose of those studies. Other studies found there was little age dependency of GABA+ [22], however, but they concentrated on the anterior cingulate cortex instead of the posterior cingulate cortex. Many studies realized the GABA concentration in ACC associated with default mode network

(DMN), which implied the connection with posterior cingulate cortex (PCC), the core hub of DMN. However, so far the current study is the first research concentrating on posterior cingulate cortex (PCC) as a more direct measurement of the relation between GABA and DMN.

The major inhibitory interneurons are engaged in GABA and therefore presumably the GABA reduction with aging implies *GABAergic neuron* loss during aging [23]. This study offers little insight, as GABAergic neuronal detection is likely not to be applicable on a general brain segmentation level. Another possibility is *GABAergic neuron* dysfunction; if it is due to dysfunction, either GABA synthesis itself or enzymatic deficiency in Glutamate-Glutamine cycling may be involved.

Aging gives rise to degeneration of brain functions such as learning and memory. Aging has also been illustrated to be related to various brain structural and functional changes. The posterior cingulate cortex (PCC) became the focus of aging research as it serves as the central node for DMN (especially in aging condition). Previous studies indicated metabolic reductions in PCC in early Alzheimer's disease [24]. Moreover, the crucial role of PCC had been showed by previous PET study that the metabolic activity in the posterior cingulate cortex is higher than any other cortical areas in resting-state functional MRI [25]. Additionally, the discriminating high inherent signal alternation appeared compared to other general DMN regions in low-frequency signal exposure [26]. Although the importance of the PCC for aging DMN has been well explored, to date, the relationship between GABA+ concentration and the PCC has not been explored on nonhuman primates. Our results are not overly surprising that decline of GABA+ concentration in the PCC associates with aging based on the previous studies implying this possibility.

5. Limitation

Our study suffers from several limitations. One of the most well-known deficiencies of MEGA-PRESS is that macromolecule signals could not be excluded from the editing pulse method and then the GABA signals are merged with macromolecule signals. For our study, the pure GABA detection is technically impossible. Another issue from the methodological aspect is that relatively large voxel size ($3 \times 3 \times 3\,\text{cm}^3$) is required and the specific region information might be missing. Lastly, the protocol of this study did not contain nonsuppressed water scans; as a result, we could only use metabolite as internal standard rather than using water as a reference. In sum, the results of this study suggest brain GABA+/Cr ratio decreases with age and GM fraction in the medial brain, focusing on the posterior cingulate cortex. Further studies may be able to associate aging and GABA concentration based on DMN functions and aid in the development of new clinical outcomes.

Competing Interests

None of the authors have conflict of interests.

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Research Article

Myoinositol Attenuates the Cell Loss and Biochemical Changes Induced by Kainic Acid Status Epilepticus

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Identification of compounds preventing or modifying the biochemical changes that underlie the epileptogenesis process and understanding the mechanism of their action are of great importance. We have previously shown that myoinositol (MI) daily treatment for 28 days prevents certain biochemical changes that are triggered by kainic acid (KA) induced status epilepticus (SE). However in these studies we have not detected any effects of MI on the first day after SE. In the present study we broadened our research and focused on other molecular and morphological changes at the early stages of SE induced by KA and effects of MI treatment on these changes. The increase in the amount of voltage-dependent anionic channel-1 (VDAC-1), cofilin, and caspase-3 activity was observed in the hippocampus of KA treated rats. Administration of MI 4 hours later after KA treatment abolishes these changes, whereas diazepam treatment by the same time schedule has no significant influence. The number of neuronal cells in CA1 and CA3 subfields of hippocampus is decreased after KA induced SE and MI posttreatment significantly attenuates this reduction. No significant changes are observed in the neocortex. Obtained results indicate that MI posttreatment after KA induced SE could successfully target the biochemical processes involved in apoptosis, reduces cell loss, and can be successfully used in the future for translational research.

1. Introduction

Epilepsy is a heterogeneous syndrome characterized by recurrent and spontaneous seizures. Approximately 1% of the population in the world suffers from epilepsy. However, 20%–30% of the patients are refractory to therapies using currently available antiepileptic drugs (AEDs) [1]. Current epilepsy therapy is symptomatic using AEDs. This therapy suppresses seizures but does not prevent or cure epilepsy. Thus, treatment strategies that could interfere with the process leading to epilepsy (epileptogenesis) would have significant benefits over the current approach [1–3] and will be of great importance for epilepsy treatment. Unfortunately, at present, there is no drug which could fulfill these demands and effectively prevent the process of epileptogenesis in humans. The alternative goal for epileptogenesis treatment would be disease modification, which means that although a treatment may not prevent the occurrence of a disease, it may nevertheless modify the natural course of the disease [1]. Disease modification after epileptogenic brain insults may affect the development of spontaneous seizures in that the seizures, if not prevented, are less frequent and less severe [1].

Some native plants of the Ranunculaceae family (to which plant *Aquilegia vulgaris* belongs) are widely used in Chinese and Tibetan folk medicine as antiepileptic and soporific medicaments [4]. In our early studies we discovered that water extract of *Aquilegia vulgaris* contains compounds which act on γ-aminobutyric acid- (GABA-) A receptors; namely, it completely inhibits ^{3}H-muscimol (a GABA-A receptor agonist) binding to rat brain membranes and also increases ^{3}H-flunitrazepam (a specific ligand for the GABA-A receptor benzodiazepine site) binding by approximately a factor of two [4]. γ-Aminobutyric acid (GABA) is the main inhibitory neurotransmitter of the mammalian central

nervous system (CNS) and several antiseizure medicines act through the GABA system [5]. GABA itself is unable to penetrate the blood-brain barrier and systemic intraperitoneal administration of GABA is not accompanied by anticonvulsive activity [6]. Therefore it was hypothesized that water extract from *Aquilegia vulgaris* contains active components other than GABA itself. In the next series of our experiments compounds were identified, which inhibit ^{3}H-muscimol binding to rat brain membranes and increase ^{3}H-flunitrazepam binding in *in vitro* system. High performance liquid chromatography and subsequent gas chromatography coupled with mass spectrometry identified two main active compounds of this extract: (1) myoinositol (MI) and (2) oleamide—sleep inducing lipid [4]. MI was not expected to influence ^{3}H-muscimol binding, but we experimentally confirmed that MI is the compound of the fraction inhibiting ^{3}H-muscimol binding [4].

In later studies we revealed that MI pretreatment significantly decreases the severity of seizures induced either by pentylenetetrazole (PTZ) or by kainic acid (KA) [7, 8]. In the next series of experiments initially we induced the status epilepticus (SE) by KA and then tried MI daily treatment. Using such design of experiment antiepileptogenic properties of compound could be explored (see for review [1]). We found out that MI treatment during 28 days attenuates biochemical changes underlying the process of epileptogenesis. Namely, KA induced epileptogenesis is accompanied by a strong decrease in the amount of GLUR1 subunit of AMPA-glutamate receptors and calcium-calmodulin dependent protein kinase II (CaMKII) in the hippocampus, which are nearly completely reversed by daily treatment of MI [9]. Our recent data indicated that MI treatment, utilizing the same design of experiment, restores the normal level of gamma-2 subunit of GABA-receptors' amount (mainly found in synapses and participating in phasic inhibitions), which is drastically reduced on the 28th day after KA treatment [10]. MI treatment demonstrated no specific effect on expression levels of GLUR1, CaMKII, or GABA-A receptor subunits 28–30 h after KA induced SE [9, 10]. Nevertheless it is highly plausible that some other biochemical processes are affected by MI treatment at early stages of KA induced SE and epileptogenesis. In the present series of experiments we focused on mitochondrial proteins, enzymatic marker of apoptosis, and also evaluated the cell loss in hippocampus after KA induced SE and MI and diazepam treatment.

The rationale of these experiments was as follows: it is well documented that KA increases mitochondrial dysfunction and apoptosis in neurons of hippocampus (reviewed in [11]). We have shown that MI pretreatment before KA induced SE exerts strong neuroprotective effect on hippocampal cell loss during the process of epileptogenesis and preserves the structure of neurons, synapses, and mitochondria on the 14th day after treatment [12]. We were interested in exploring whether MI treatment after KA induced SE influenced the processes of apoptosis, mitochondrial markers, and cell loss at early stages, namely, on the 1st day after KA administration.

We have addressed these questions by studying (i) changes in the amount of the following proteins: voltage-dependent anionic channel- (VDAC-) 1, VDAC-2, I subunit of cytochrome oxidase c (CO-I), and cofilin; (ii) alterations of the caspase-3 activity; and (iii) hippocampal cell loss.

The above-mentioned proteins were selected for the following reasons. (i) Both VDAC-1 and VDAC-2 are considered as a major regulator of cell death (e.g., for recent reviews see [13–15]). (ii) CO-I is one of the catalytic subunits of the cytochrome c oxidase enzyme complex and is expressed in mitochondria. This enzyme is central to energy metabolism [16], and mitochondria play a role in many aspects of neuronal function and neuropathologies including epilepsy [17–19]. (iii) Cofilin is one of the proteins upregulated during the neuronal death [20, 21] and mitochondrial translocation of cofilin is an early step in apoptosis induction [22]. (iv) Caspase-3 is the main downstream effector caspase that cleaves the majority of the cellular substrates in apoptotic cells [23].

MI significantly reduces the seizure score and seizure duration of KA and PTZ induced convulsions [7, 8] and also inhibits ^{3}H-muscimol binding to GABA-A receptors *in vitro* [4]. Thus it is possible that MI effects on biochemical processes of epileptogenesis, observed in our previous experiments [9, 10], were mediated by its action on GABA-A receptors and/or anticonvulsant properties of MI, other than its action on GABA-A receptors. To evaluate this possibility in our present experiments we included a group of animals treated with anticonvulsant and GABA-A receptor agonist diazepam.

2. Materials and Methods

2.1. KA Induced SE. Male Wistar rats of 2.5–3 months of age received a single intraperitoneal injection of KA (10 mg/kg, Sigma) dissolved in saline. After injection each animal was placed into an individual plastic cage for observation for 4 hours. Seizures were scored according to a modified Racine scoring system from 0 to 6 [9, 10, 24, 25].

Thirty-two animals with seizures of grades 4-5 were selected. Those animals exhibited seizures at least for 60 minutes—which is enough to induce epilepsy for this type of treatment [1]. Six KA treated animals died within the first day and experiments were continued on 26 animals; 8 rats were used for morphological studies and 18 for biochemical experiments.

Twenty-two control rats received saline injections and after that were treated in the same way as KA treated animals; 18 animals for biochemical experiments and 4 animals for morphological studies were selected. To summarize 36 animals were used in biochemical experiments and 12 for morphological studies.

2.2. Biochemical Experiments. Eighteen rats treated with KA and 18 rats treated with saline (see above) were divided into six group. Four hours following KA treatment and SE development the 18 animals were divided into three groups 6 in each: the first group received a saline (0.9% NaCl sterile solution) injection (1 mL/kg, KA + SAL group), the second group MI (30 mg/kg) injection (KA + MI group), and the third group diazepam (5 mg/kg) injection (KA + DIAZ group). Each of these groups received the same injections at

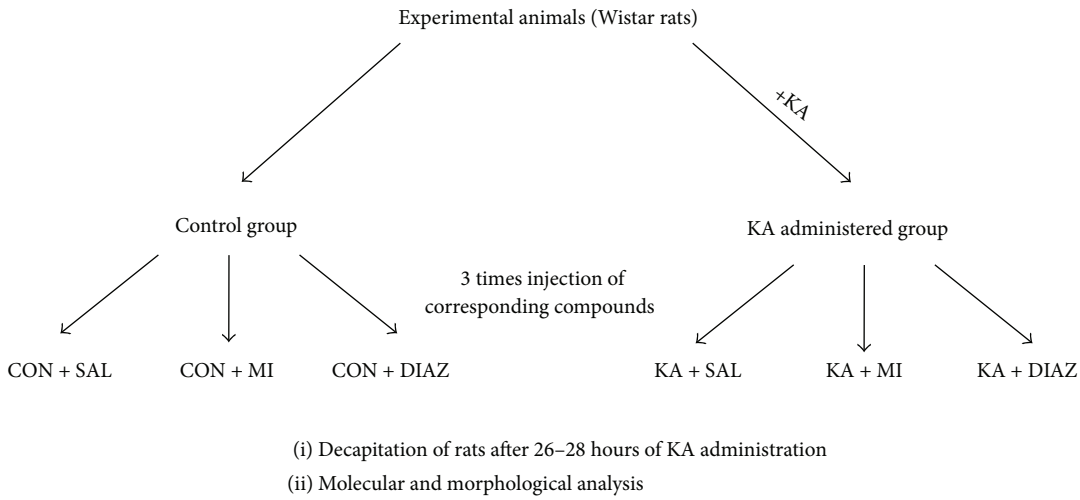

Figure 1: The diagram of experiment design.

the end of the first day and in the morning of the following day. The total amount of injections was 3. 6 h later after the last injection the rats were decapitated (time corresponding to the 28–30 h following KA treatment).

Eighteen control animals after saline injections were divided also into three groups 6 in each: the first group received a saline injection (1 mL/kg, CON + SAL group), the second group MI (30 mg/kg) injection (CON + MI group), and the third group diazepam (5 mg/kg) injection (CON + DIAZ group). The time schedule of the remaining treatment and decapitation was exactly the same as for KA treated animals (see above). The diagram of experimental design is provided in Figure 1. During the whole experiment, the rats were housed in cages with free access to water and food. All experiments were carried out at the I. Beritashvili Center of Experimental Biomedicine and were performed in compliance with approved institutional animal care guidelines.

After decapitation, two regions—hippocampus and neocortex—were removed from each brain and immediately frozen in dry ice. The number of animals used was estimated to be the minimum required for adequate statistical analysis [9, 10]. All biochemical experiments were carried out in "blind" manner; samples were then coded and all subsequent procedures were performed without knowledge of the rats' experimental history.

2.3. Electrophoresis and Immunoblotting. The tissue samples were rapidly homogenized in 20 mM Tris-HCl (pH 7.4), 0.32 M sucrose, 1 mM ethylenediaminetetraacetic acid, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 0.5 mM ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid, and a cocktail of protease inhibitors (Sigma P8340). One-fourth of the whole homogenate was saved for the determination of caspase-3 activity and the three-quarters (remaining part) were centrifuged at 1000 ×g for 10 min. The supernatant was further centrifuged at 15,000 ×g for 20 min. The pellet was washed once and is referred to as the P2 mitochondrial-membrane fraction. This fraction was dissolved in 5% sodium dodecyl sulfate (SDS) solution.

In all fractions, the protein concentration was determined in quadruplicate using a micro bicinchoninic acid protein assay kit (Pierce). Aliquots containing 30 μg of protein and of equal volume were applied to the gels. SDS gel electrophoresis and Western blotting were carried out as described previously [9, 10]. After the protein had been transferred onto nitrocellulose membranes, the membranes were stained with Ponceau S solution to confirm the transfer and the uniform loading of the gels.

For the detection and quantification of selected proteins the following antibodies were used: (i) for VDAC-1 (Abcam, ab15895); (ii) for VDAC-2 (Abcam, ab37985); (iii) for cofilin (Abcam, ab 42824); (iv) and for CO-I (CO-I; Molecular Probes, A6403). As cofilin was measured in the P2 mitochondrial-membrane fraction it is referred to as M-cofilin.

Due to the close proximity of molecular weights of the VDAC-1 and VDAC-2 separate electrophoresis/Western immunoblottings were carried out for quantification of these proteins. Standard immunochemical procedures were carried out using peroxidase-labeled secondary antibodies and Super-Signal West Pico Chemiluminescent substrate (Pierce) [9, 10]. The optical densities of bands corresponding to the VDAC-1, VDAC-2, M-cofilin, and CO-I were measured using LabWorks 4.0 (UVP). The autoradiographs were calibrated by including in each gel four standards of P2 mitochondrial-membrane fraction from the brain of untreated rats. Each standard contained 15–60 μg of total protein. Optical density was proportional to the amounts of VDAC-1, VDAC-2, M-cofilin, and CO-I (see Figure 3). To obtain the data given in Figures 4 and 5 the optical density of each band from the experimental sample was divided by the optical density which, from the calibration of the same autoradiograph, corresponded to 30 μg of total protein in the standard [9, 10]. Data expressed in this way will be referred to as "relative amount" of protein.

Data from experimental stained protein bands were not normalized with respect to actin or any other housekeeping protein because it cannot be guaranteed that such proteins are

unaffected by KA or other treatment [26–28] (for discussion of the unreliability of normalization to housekeeping proteins see [29]). Instead, we controlled loading by Ponceau S staining and calibrated all gels with the same protein standards (see above and also [30]).

2.4. Caspase-3 Activity. Caspase-3 enzymatic activity was assayed using a colorimetric caspase-3 assay kit (Sigma-Aldrich, Cat. CASP-3-C) according to the manufacturer's instructions. This kit measures the activity of caspase-3, one of the critical enzymes of apoptosis, and includes a specific inhibitor for precise measurement of caspase-3 activity. The enzyme activity was measured in the total homogenate fractions of rat hippocampus and neocortex separately.

Each tissue sample was assayed in three parallel measurements without caspase-3 inhibitor and in three measurements with inhibitor. Plate wells contained precise amounts of homogenate proteins in the range of 10–12 micrograms and incubated for 2 h at 37°C with caspase-3 peptide substrate, with or without enzyme inhibitor. The amount of *p*-nitroaniline (pNA) released in the assay was measured using a spectrophotometer (OD 405 nm) and the concentration was determined by the standard curve. These values were subtracted from the values obtained without the inhibitor. The enzyme activity is expressed as nanomoles of pNA released per minute per 1 mg of tissue homogenate protein.

2.5. Cell Count. Three groups of rats were used to perform cell count: CON + SAL, KA + SAL, and KA + MI. Each group consisted of 4 rats. Animals were deeply anesthetized with ketamine (100 mg/kg) and then perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Excised brains were postfixed at 4°C in the same fixative for another 24 h and then cryoprotected in a 30% sucrose-solution. For Nissl staining 15 μm coronal sections were cut on a cryostat (Microm HM 500 M). Every 6th section was collected and mounted on a poly-L-lysine coated glass slides. The slides were left to dry and rehydrated with 100% alcohol, 95% alcohol, and distilled water. Subsequently, the sections were stained in 0.1% Cresyl violet (Sigma-Aldrich, Cat. number C504) solution. The sections were then differentiated into 95% ethyl alcohol, dehydrated in 100% alcohol, and rinsed in xylene. Finally, the sections were mounted and observed under a light microscope (Leica DM LB). Cell counting in hippocampal CA1, CA3, and dentate gyrus fields was conducted blindly. For this purpose the systematic random sampling was employed. The 2-dimensional counting grid (250 μm × 250 μm) at the magnification 400x was used. Totally 10–12 sections from each level within experimental and control animals were selected (35 randomly chosen ranges of visions at the same site of all sections from each animal).

2.6. Statistical Analysis

2.6.1. Seizure Grades and Seizure Duration Comparison. The seizure grades between three different groups of KA treated rats used in biochemical experiments and two groups of rats used in morphological studies were compared with Mann-Whitney test. The same groups of rats were compared by total duration of seizures; in this case Student's-*t* test was used.

2.6.2. Changes in Protein Amounts and Caspase-3 Activity. Data for each protein and caspase-3 activity were analyzed separately by two-way ANOVA with the following factors: experimental condition (CON + SAL, CON + MI, and CON + DIAZ; KA + SAL, KA + MI, and KA + DIAZ) and brain region (hippocampus and neocortex). Planned comparisons were made between all groups (CON + SAL, CON + MI, and CON + DIAZ; KA + SAL, KA + MI, and KA + DIAZ) at a defined region (hippocampus or neocortex).

2.6.3. Cell Count. Data for cell counts was analyzed by two-way ANOVA with the following factors of experimental condition (CON + SAL, KA + SAL, and KA + MI) and hippocampus subfield (CA1, CA3, and dentate gyrus). Planned comparisons were made between these groups at a defined hippocampus subfield (e.g., CA1).

All statistical tests were two-tailed and all significant differences are reported.

3. Results

3.1. Seizure Grade and Duration. The seizure grades in three groups of KA treated rats used in biochemical experiments were compared with each other and no significant differences were found between them (Mann-Whitney test—KA + SAL versus KA + MI, $W = 39.0$, $p = 1.0$; KA + SAL versus KA + DIAZ, $W = 42.0$, $p = 0.64$; and KA + MI versus KA + DIAZ, $W = 42.0$, $p = 0.64$). The medians for KA + SAL and KA + MI groups were 5.0 and for KA + DIAZ group 4.5.

The two groups of rats used in morphological studies consisted of 4 animals and by the seizure grade they were not significantly different from each other (Mann-Whitney test—KA + SAL versus KA + MI, $W = 16.0$, $p = 0.61$). The median for KA + Sal was 4.5 and for KA + MI 5.0.

The groups of rats for biochemical and morphological experiments were not significantly different from each other by total duration of seizures (see Figure 2).

Approximately 4.5 h after KA treatment all groups of rats were video-monitored by infrared cameras till decapitation. No recurrent seizures were observed in any group of rats.

3.2. Immunostaining. Anti-VDAC-1 antibodies bound to a band of molecular weight 30 kDa, anti-VDAC-2 stained a protein band with molecular weight 31 kDa, anti-CO-I antibodies reacted with a protein band of molecular weight 57 kDa, and anti-cofilin antibodies stained a protein band of molecular weight 17 kDa (Figure 3). All of these weights corresponded to the expected size of the target proteins.

Four standards (15, 30, 45, and 60 μg of total protein) were applied to each gel. For these standards the optical densities of the immunostained bands (for VDAC-1, VDAC-2, CO-I, or M-cofilin) were plotted against the amounts of protein; in all these standards, least-squares regression showed a significant fit to a straight line (Figure 3).

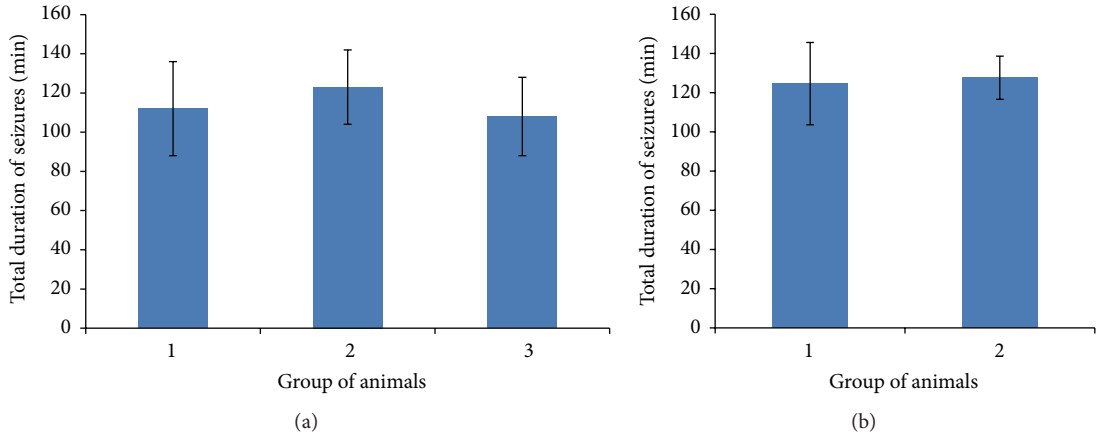

Figure 2: Total duration of seizures (min) in various group of animals. The duration of observable seizure activity was measured for each animal and mean values ± standard error of the mean are provided for each group. (a) Groups of animals used in biochemical experiments; (b) groups of animals used in morphological studies. 1: KA + SAL; 2: KA + MI; and 3: KA + DIAZ. Groups were compared using a two-tailed t-test. No significant differences were found ($p > 0.6$ for each comparison).

Thus the used antibodies are specifically reacting against target proteins and measured optical densities are quantitatively reflecting the amounts of proteins.

3.2.1. VDAC-1. Two-way ANOVA revealed a significant effect of experimental condition on the amount of VDAC-1 ($F_{5,71} = 5.42$, $p < 0.0001$). The effect of the factor-region was not significant, but the interaction between these two factors was significant ($F_{5,71} = 7.75$, $p < 0.0001$). The significant differences between the different groups of animals were found only in the hippocampus. The mean amount of VDAC-1 in KA + SAL group was significantly higher as compared to all control groups (KA + SAL versus CON + SAL, $t = 7.24$, $p < 0.0001$; KA + SAL versus CON + MI, $t = 5.95$, $p < 0.0001$; and KA + SAL versus CON + DIAZ, $t = 9.40$, $p < 0.0001$; for all comparisons df = 10) and also significantly higher as compared to KA + MI group ($t = 8.47$, $p < 0.0001$, and df = 10). The KA + DIAZ group was indistinguishable from KA + SAL group and the mean amount of VDAC-1 significantly exceeded the mean amounts of the control groups (KA + DIAZ versus CON + SAL, $t = 4.59$, $p = 0.001$; KA + DIAZ versus CON + MI, $t = 3.90$, $p = 0.003$; and KA + DIAZ versus CON + DIAZ, $t = 5.71$, $p < 0.0001$; for all comparisons df = 10) and also was significantly higher as compared to KA + MI group ($t = 4.71$, $p = 0.001$, and df = 10). The mean value of VDAC-1 in KA + MI group was not different from the corresponding values in any control group. Thus KA induced increase in VDAC-1 amount is abolished by MI posttreatment but is not changed by diazepam treatment (see Figure 4).

In neocortex no significant differences were found.

3.2.2. VDAC-2. No significant changes were revealed in ANOVA and no differences were significant between the groups.

3.2.3. CO-I. There was not any significant effect of factors experimental conditions or region in ANOVA on the amounts of CO-I and no differences were significant between the groups.

3.2.4. M-Cofilin. For the amount of M-cofilin the effects of both factors (experimental condition and region) were significant ($F_{5,71} = 4.84$, $p = 0.001$, and $F_{1,71} = 25.06$, $p < 0.0001$, resp.) and interaction between these factors was also significant ($F_{5,71} = 4.80$, $p = 0.001$). These effects in ANOVA were due to the changes in hippocampus. The mean amount of M-cofilin in the KA + SAL treated groups of hippocampus was significantly higher as compared to all other groups except the KA + DIAZ group (KA + SAL versus CON + SAL, $t = 3.02$, $p = 0.013$; KA + SAL versus CON + MI, $t = 3.00$, $p = 0.013$; KA + SAL versus CON + DIAZ, $t = 3.24$, $p = 0.009$; and KA + SAL versus KA + MI, $t = 5.49$, $p < 0.0001$; for all comparisons df = 10). The changes in KA + DIAZ group were essentially the same as in KA + SAL group and the mean amount of M-cofilin in KA + DIAZ group significantly exceeded the mean amounts of other groups (KA + DIAZ versus CON + SAL, $t = 2.63$, $p = 0.025$; KA + DIAZ versus CON + MI, $t = 2.62$, $p = 0.026$; KA + DIAZ versus CON + DIAZ, $t = 2.86$, $p = 0.017$; and KA + DIAZ versus KA + MI, $t = 5.25$, $p < 0.0001$; for all comparisons df = 10). Treatment of KA group with MI not only prevents the increase of the M-cofilin (see comparisons above) but also significantly decreases the level of protein as compared to all control groups (KA + MI versus CON + SAL, $t = 6.08$, $p < 0.0001$; KA + MI versus CON + MI, $t = 4.36$, $p = 0.001$; and KA + MI versus CON + DIAZ, $t = 4.69$, $p = 0.001$; for all comparisons df = 10) (Figure 5).

No differences were significant in neocortex.

3.3. Caspase-3. Two-way ANOVA revealed a significant effect of experimental condition and region on the changes of caspase-3 activity ($F_{5,71} = 3.55$, $p = 0.007$, and $F_{1,71} = 9.53$, $p = 0.003$, resp.). The interaction between these two factors was also significant ($F_{5,71} = 4.79$, $p = 0.001$). The changes in

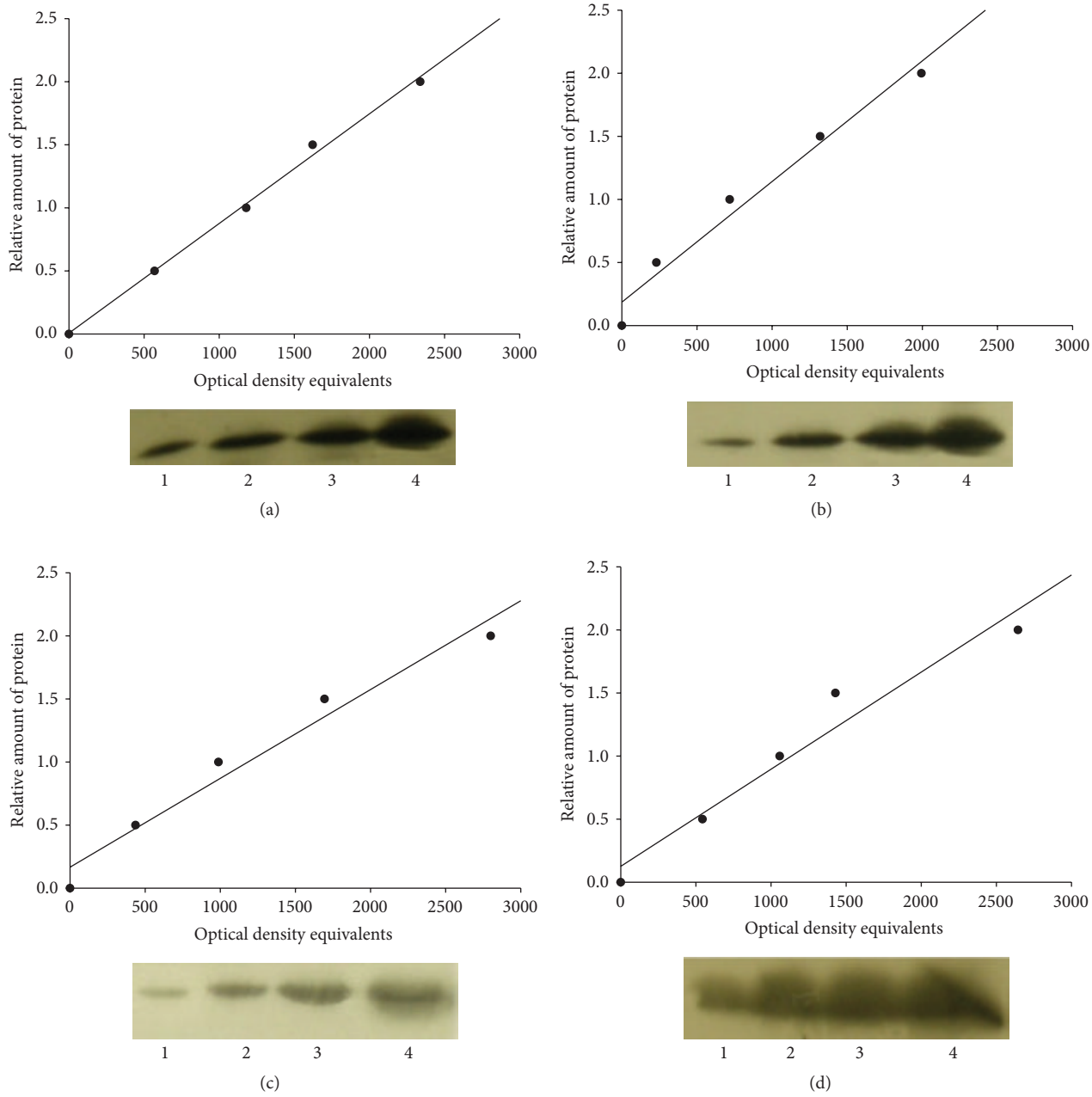

Figure 3: Sample films and calibration plots for (a) VDAC-1; (b) VDAC-2; (c) CO-I; and (d) M-cofilin. Bottom panels: sample radiographs; top panels: calibration plots (lines fitted by linear least-squares regression).

caspase-3 activity are analogous to the changes of the amount of VDAC-1 and M-cofilin. The highest activity of the enzyme is observed in the hippocampus of KA + SAL and KA + DIAZ groups. KA + SAL group significantly exceeds all control as well as KA + MI groups (KA + SAL versus CON + SAL, $t = 2.72$, $p = 0.021$; KA + SAL versus CON + MI, $t = 2.73$, $p = 0.021$; KA + SAL versus CON + DIAZ, $t = 3.70$, $p = 0.004$; and KA + SAL versus KA + MI, $t = 3.03$, $p = 0.013$; for all comparisons df = 10) (Figure 6).

The mean amount of caspase-3 activity in KA + DIAZ is significantly higher as compared to CON + DIAZ group ($t = 2.93$, $p = 0.015$, and df = 10) and as compared to KA + MI group ($t = 2.49$, $p = 0.032$, and df = 10), whereas the differences with CON + SAL and CON + MI groups are significant only on one-tailed t-test ($t = 2.04$, $p = 0.035$, and $t = 2.00$, $p = 0.037$, resp.; for both cases df = 10) (Figure 6).

No differences were significant between neocortex samples.

3.4. Cell Number in Hippocampal Subfields. There was a strong effect of experimental condition and subfield on the number of neurons in hippocampus in two-way ANOVA (training condition factor $F_{2,35} = 142.76$, $p < 0.0001$; subfield factor $F_{2,35} = 67.84$, $p < 0.0001$). The interaction

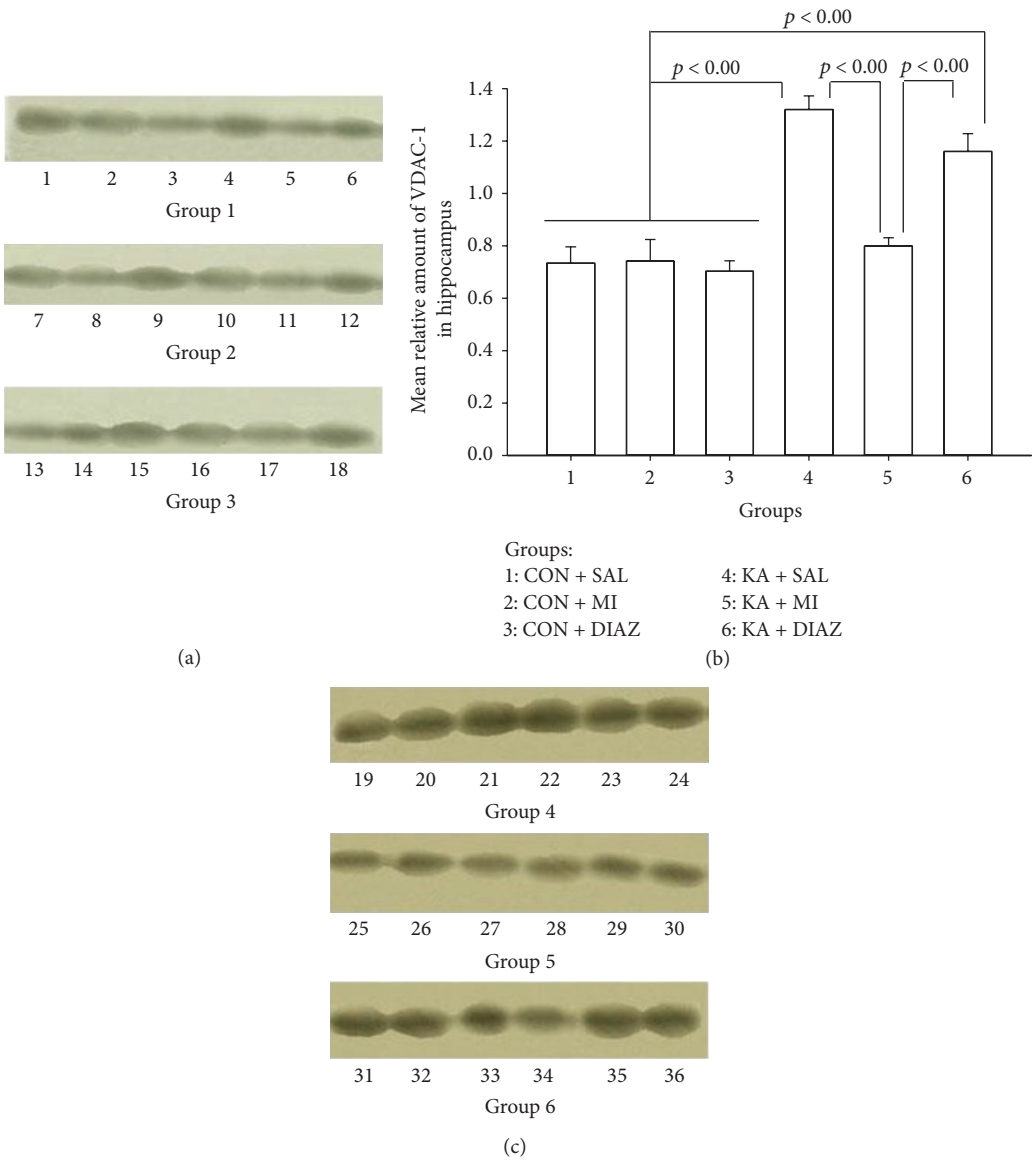

Figure 4: Representative Western blot autoradiograph of P2 mitochondrial-membrane fraction for VDAC-1, (a) and (c), and mean relative amounts of VDAC-1 (b) from the hippocampus of 6 different groups of rats. (a) and (c) Each lane was derived from a single sample. Lanes 1–6: CON + SAL; lanes 7–12: CON + MI; lanes 13–18: CON + DIAZ; lanes 19–24: KA + SAL; lanes 25–30: KA + MI; and lanes 31–36: KA + DIAZ. (b) Error bars represent the standard errors of the means. KA + SAL and KA + DIAZ groups significantly exceed all control groups as well as KA + MI group ($p < 0.000$). The mean amount of VDAC-1 in the KA + MI group is not significantly different from the means of any of the control groups and the means of control groups differ significantly from each other. Groups of rats: 1: CON + SAL; 2: CON + MI; 3: CON + DIAZ; 4: KA + SAL; 5: KA + MI; and 6: KA + DIAZ.

between these two factors was also significant ($F_{4,35} = 34.28$, $p < 0.0001$). The planned comparison between the groups of subfields was carried out separately.

3.4.1. CA1. The KA treatment significantly reduces the number of neurons by more than 30% as compared to control rats ($t = 17.90$, $p = 0.000$, and df = 6). In the KA + MI group the reduction of cell number is less (<20%) but still significantly different from control group ($t = 6.58$, $p = 0.001$, and df = 6). The difference between the KA + MI and KA + SAL groups is also significant ($t = 5.43$, $p = 0.002$, and df = 6). Thus MI treatment significantly reduces the KA induced neuronal cell loss in the CA1 subfield of hippocampus (Figure 7).

3.4.2. CA3. The significant decrease in neuronal cell number after KA induced SE as compared to control group is also observed in CA3 subfield (>20%; $t = 21.41$, $p < 0.0001$, and df = 6). In KA + MI group the decrease is also significant ($t = 11.32$, $p < 0.0001$, and df = 6), but less (15%). The difference between the KA + SAL and KA + MI group is significant

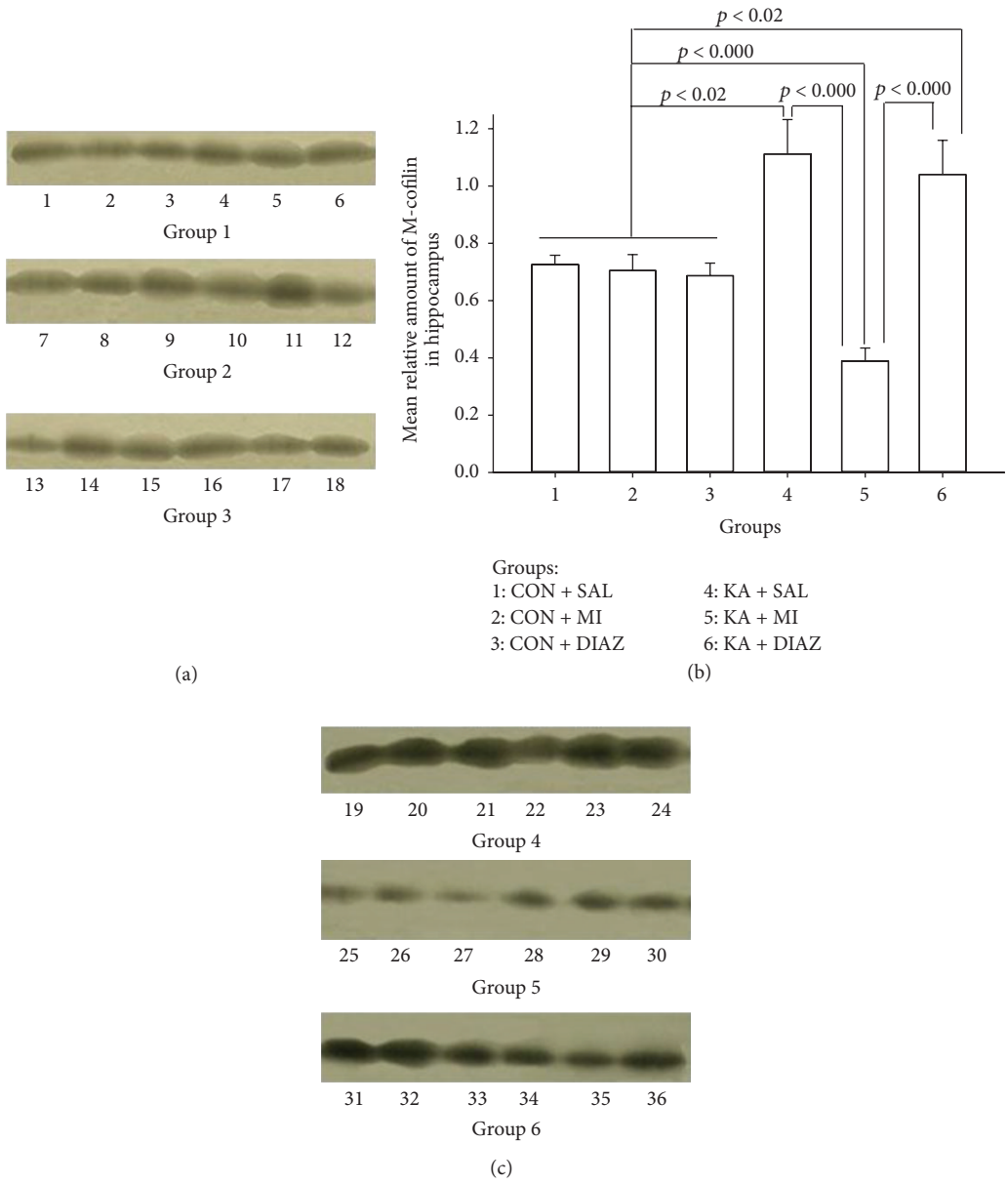

Figure 5: Representative Western blot autoradiograph of P2 mitochondrial-membrane fraction for M-cofilin, (a) and (c), and mean relative amount of M-cofilin (b) from the hippocampus of 6 different groups of rats. (a) and (c) Each lane was derived from a single sample. Lanes 1–6: CON + SAL; lanes 7–12: CON + MI; lanes 13–18: CON + DIAZ; lanes 19–24: KA + SAL; lanes 25–30: KA + MI; and lanes 31–36: KA + DIAZ. (b) Error bars represent the standard errors of the means. KA + SAL and KA + DIAZ groups significantly exceed all control groups ($p < 0.002$) as well as KA + MI group ($p < 0.000$). The mean amount of M-cofilin in the KA + MI group is significantly lower as compared to the means of any of the control groups ($p < 0.000$). The means of control groups do differ significantly from each other. Groups of rats: 1: CON + SAL; 2: CON + MI; 3: CON + DIAZ; 4: KA + SAL; 5: KA + MI; and 6: KA + DIAZ.

($t = 5.33$, $p = 0.002$, and df = 6), indicating that MI also exerts neuroprotective effect in CA3 subfield of hippocampus (Figure 7).

3.4.3. Dentate Gyrus. There are no significant differences between the groups; however the rank order of the means is the same as for CA1 and CA3 subfields: CON + SAL > KA + MI > KA + SAL.

4. Discussion

In our earlier studies we demonstrated that long-term (28 days), daily treatment of rats with MI after KA induced SE prevents some biochemical processes of epileptogenesis [9, 10]. These biochemical processes included changes in different subunits of GABA-A receptors, GLUR1 subunit of AMPA-glutamate receptors and α-subunit of CaMKII [9, 10]. However MI had no influence on these biochemical processes

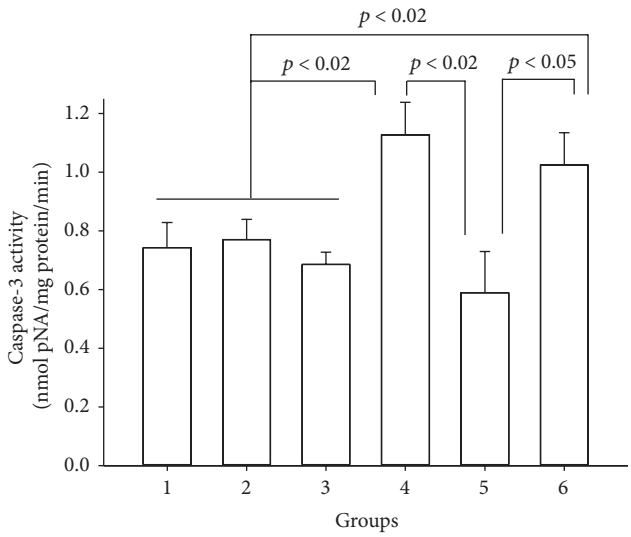

Figure 6: The mean amount of caspase-3 activities in the hippocampus of different experimental groups of rats. Error bars represent the standard errors of the means. KA + SAL and KA + DIAZ groups significantly exceed all control groups ($p < 0.002$) as well as KA + MI group ($p < 0.002$ and $p < 0.005$, resp.). The mean amount of enzyme activity in the KA + MI group does not significantly differ from the means of any of the control groups and the mean of control groups does significantly differ from each other. Groups of rats: 1: CON + SAL; 2: CON + MI; 3: CON + DIAZ; 4: KA + SAL; 5: KA + MI; and 6: KA + DIAZ.

one day after SE [9, 10] suggesting that some other molecular processes are affected at early phases of MI treatment. In our previous experiments MI was applied before KA induced SE and neuroprotective properties of MI on hippocampal cell loss and structure of neurons were elucidated [12]. The major goal of our research is to find the ways to prevent processes of epileptogenesis or to modify it since the process is already initiated. Therefore the main interest of present studies was to investigate MI effects after KA induced SE, especially at its early phases (1 day). Based on our previous data [12] we have hypothesized that MI could prevent the neuronal loss and processes of apoptosis even when the treatment starts after SE and obtained data supports this suggestion.

The spontaneous recurrent seizures after KA induced SE start after 7–10 days [31]. Therefore with our experimental design (1 day after SE) it was impossible to detect which experimental animals would develop recurrent seizures. However, in our experiments, we included only those rats which displayed seizures at least for 60 min during first 4 h after KA treatment that should be enough for the development of epilepsy [1]. Indeed our unpublished observation indicates that more than 90% of such rats develop recurrent seizures.

Do the observed changes reflect biochemical processes of epileptogenesis? One of the major challenges to the epilepsy research community indeed has been to determine which of the molecular changes after SE contribute to epileptogenesis, which are compensatory, and which are noncontributory [32]. Our results have mainly revealed molecular changes associated with apoptosis and cell loss. Could be these changes unequivocally associated with the processes of epileptogenesis? The effect of seizures on neuronal death and the role of seizure-induced neuronal death in acquired epileptogenesis have been debated for decades (e.g., for reviews see [33–35]). According to the hypothesis of "*recapitulation of development*" a loss of synaptic input from the dying neurons is a critical signal to induce axonal sprouting and synaptic-circuit reorganization [33]. According to the *"neuronal death pathway" hypothesis*, the biochemical pathways causing programmed neurodegeneration, rather than neuronal death *per se*, are responsible for or contribute to epileptogenesis [33]. Thus we could only suggest that reported molecular changes and the effects of MI could be linked to the epileptogenesis.

4.1. VDACs. VDACs are the most abundant proteins of mitochondrial outer membrane through which a continuous bidirectional transport of ATP, ADP, NADH, and ions and general metabolite flux take place. Mitochondrial outer membrane is responsible for maintaining mitochondrial integrity, which may otherwise lead to cellular dysfunctions, including cessation of ATP synthesis, dysregulation of Ca^{2+} homeostasis, release of cytochrome c, and apoptosis [36, 37]. There are three isoforms of VDAC in eutherian mammals. VDAC-1 is characterized by the highest expression levels in most cell types and is considered to possess proapoptotic properties [36, 37], whilst VDAC-2 and VDAC-3 are considered to be positive regulators of ferroptosis, but not of apoptosis [15].

The obtained data point out to significant changes in VDAC-1, but not in VDAC-2 after KA induced SE in the hippocampus of rats. The mean amount of VDAC-1 is increased in KA + SAL and KA + DIAZ groups as compared to all control and also to KA + MI groups. The latter group does not differ from control groups. Thus, MI treatment 4 h later after KA injection abolishes increase of VDAC-1 in the hippocampus. We suggest that increase of VDAC-1 in KA + SAL and KA + DIAZ groups should be the indicator of increased apoptosis, and MI treatment prevents this process.

4.2. CO-I. We have not found any significant changes in the expression of CO-I. The cytochrome c oxidase is the marker of the mitochondrial complex IV and the CO-I is encoded by mitochondrial DNA. It was also shown that KA induced SE within 3 hours of KA administration produces decrease in the activity of respiratory complex I, but not of complex IV [38].

The increase in VDAC-1, but not changes in CO-I and VDAC-2, indicates that changes in VDAC-1 are specific for mitochondria after KA induced SE. Thus mitochondria are undergoing specific changes after KA treatment.

4.3. M-Cofilin. Cofilin is a well-known regulator of actin filament nonequilibrium assembly and disassembly (e.g., for review see [39]). Dephosphorylation of cofilin is linked to subsequent increase of cofilin-actin binding and actin

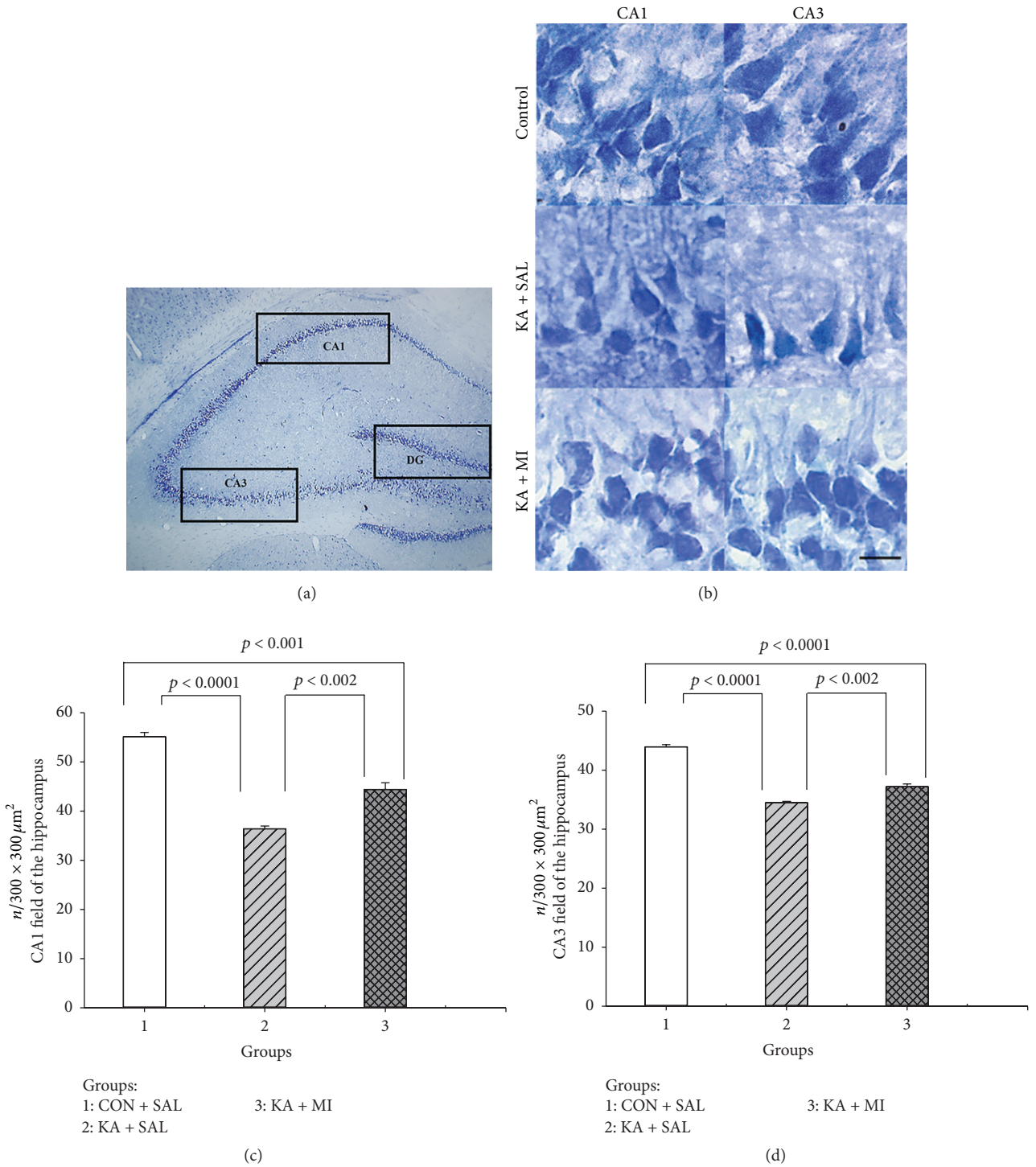

FIGURE 7: The view of hippocampus subfields, where cell count was performed (a). Hippocampal CA1 (left) and CA3 (right) subfields of CON + SAL, KA + SAL, and KA + MI treated animals (b). Mean numbers of neurons in the CA1 (c) and CA3 (d) subfields of the hippocampus in three different groups of animals. (b) The photomicrographs demonstrate the obvious decrease of neuronal cells in CA1 (a) and CA3 (b) subfields of hippocampus as a result of KA + SAL treatment and partial rescue of the cells in KA + MI group. (c) and (d) Mean number of neurons (number of cell counts per counting frame area ($250 \times 250\ \mu m^2$)) in the CA1 and CA3 subfields of the hippocampus. Error bars represent the standard errors of the means. In the CA1 subfield the mean number of cells in KA + SAL groups is significantly less as compared to CON + SAL group ($p < 0.000$) as well as compared to KA + MI group ($p = 0.001$). The CON + SAL group also significantly exceeds the KA + MI group ($p = 0.002$). In the CA3 subfield of hippocampus the mean number of cells in CON + SAL is significantly higher as compared to KA + SAL as well as compared to KA + MI group (for both comparisons $p < 0.0001$). KA + MI group significantly exceeds the KA + SAL group ($p = 0.002$). Scale bar = 15.

depolymerization. This is one of the cellular mechanisms leading to dendritic spine loss in the pilocarpine model of SE [40]. The second function of cofilin is not related to the regulation of actin assembly and involves translocation to the mitochondria and induction of apoptosis [22, 39]. In the present study we studied the changes in the amount of M-cofilin in the mitochondria-plasma membrane—P2 fraction. The selection of this fraction for present experiments had another reason too—in our previous experiments we compared hippocampal P2 fraction protein extracts of KA + SAL and KA + MI groups of rats using 2-dimensional electrophoresis. One of the proteins which was drastically upregulated in KA + SAL group as compared to KA + MI group was identified by mass spectrometry as cofilin (unpublished data).

Our present data indicate significant increase of M-cofilin in KA + SAL and KA + DIAZ groups as compared to all control groups as well as to KA + MI group. MI treatment after KA induced SE not only prevents the increase in the amount of M-cofilin, but also significantly reduces it as compared to control groups. We suggest that decrease of M-cofilin in P2 mitochondrial-membrane fraction reflects the reduced amount of protein translocated to mitochondria and consequently inhibits the KA induced processes of apoptosis. This suggestion is also supported by another series of our results (see below).

4.4. Caspase-3 Activity. Caspase-3 activity is significantly upregulated in the hippocampus of KA + SAL and KA + DIAZ groups as compared to control and KA + MI groups. As in the previous cases (VDAC-1, M-cofilin) MI significantly prevents this increase. Caspase-3 cleaves the majority of cellular substrates in apoptotic cells and is considered to be the main downstream effector caspase. Thus obtained data convincingly demonstrates intensification of apoptosis after KA induced SE in hippocampus and inhibitory effect of MI treatment on it.

What could be the possible causal relationship between VDAC-1, M-cofilin, CO-1, and caspase-3? In our experiments KA treatment induces increase in VDAC-1, M-cofilin, and caspase-3 activity, with no changes in CO-1 amount. Increase in the amount of VDAC-1 and M-cofilin could lead to the changes in mitochondrial permeability transition pore, with increased escape of cytochrome c to cytoplasm and activation of caspase cascade, including caspase-3. It is interesting to note that the same types of changes were observed in rat brain with endurance treadmill training—this exercise decreases the amounts of VDAC-1, cofilin, and caspase-3 alongside with other markers of oxidative stress and apoptotic signaling, whereas no changes were found in CO-I [41].

4.5. Cell Number Changes. For VDAC-1, M-cofilin, and caspase-3 activity changes after KA induced SE and MI treatment effects were observed only in the hippocampus. The same is true for CaMKII, GLUR1, and GABA-A receptor subunits' changes in our previous studies [9, 10]. In the present study, according to molecular changes, KA + SAL group did not differ from KA + DIAZ group and therefore cell count studies were carried only in the hippocampus and only in three groups of animals: CON + SAL, KA + SAL, and KA + MI.

KA induced SE is accompanied by a significant cell number decrease in the CA1 and CA3 subfields of hippocampus as compared to control group. In the KA + MI group for both subfields the neuronal cell number was significantly higher as compared to KA + SAL group, but also significantly less as compared to CON + SAL group. Thus, MI posttreatment after KA induced SE decreases the cell death significantly, though not till the control level. In the DG no significant changes were found; however the rank order of the means was CON + SAL > KA + MI > KA + SAL. It is possible that the same processes take place in DG as in the CA1 and CA3 subfields, but the magnitude of changes is much smaller and our approach could not detect them on a significant level.

MI is characterized by anticonvulsant properties and this inositol could partially reduce the seizure grade and duration of PTZ or KA induced convulsions [7, 8]. The influence of inositol on molecular changes after SE could be simply mediated by antiseizure activity of compound and/or its action on GABA-A receptors. To elucidate this possibility in present experiments we have included additional two groups of animals which were treated by GABA-A receptor agonist and anticonvulsant diazepam. In our experiments we started to administer MI 4 h after KA injection. At this time point behavioural seizures are already absent. The first administration is followed by 2 more injections. Taking into account that behavioural seizures are already finished and administration of MI took place 3 times we decided to use moderate doses of diazepam in control groups. If diazepam treatment had revealed similar effects as MI, we would have assumed that inositol influence was due to its action on GABA-A receptors and/or its anticonvulsant properties. KA + DIAZ group is identical to KA + SAL group and we propose that the influence of MI is mediated by its other mode of action (discussed below).

It should be emphasized that the same intensity of MI administration to the group of rats not treated by KA does not produce any significant changes in the amounts of studied proteins or enzyme activity. Thus, MI mediates specific influence on molecular machinery induced by KA treatment and we consider these changes as the markers of the rescue from apoptosis and neurodegeneration.

In conclusion, our obtained biochemical and morphological data for the first time indicates that MI posttreatment inhibits KA induced cell death apoptosis and related molecular processes in the hippocampus of rats. As well as in our previous studies [9, 10] effects of MI treatment are not observed in neocortex and thus the MI action should be specific for hippocampus.

It is well documented that alteration in MI deposition may play a role in a number of neuropathological conditions including epilepsy, either as a physiologically important osmolyte or as a precursor molecule for phosphoinositide synthesis (reviewed in [42, 43]). Lithium is characterized by proconvulsant effects, which has been attributed to its ability to block the action of inositol monophosphatase, and by concomitant decrease of free inositol. These proconvulsant effects of lithium can be reversed by MI [44, 45].

Soman is an organophosphorus nerve agent that acts as an irreversible inhibitor of acetylcholinesterase. One of the consequences of soman action is convulsive seizures. The strong decrease in the MI levels during 72 h takes place after soman induced seizures. Combined ketamine and atropine treatment shortly after soman injection prevents the decrease in MI level [46].

Experiments on KA induced seizures have shown that Na^+/MI cotransporter is upregulated in various parts of hippocampus shortly after seizures [47]. In temporal lobe epilepsy patients' seizure focus has an increased level of MI, whereas areas of seizure spread have a lowered level of MI [48]. Gene coding for myoinositol monophosphatase 2 is likely to be febrile seizure susceptibility gene [49]. Thus, changes in MI metabolism are involved in different types of epilepsy and MI system could be important target for successful therapeutic approaches.

We think that MI effects on VDAC-1 and M-cofilin amounts and on caspase-3 activity alterations after KA induced SE could not account for direct action of inositol on the processes of gene expression, protein cleavage, or enzyme inhibition. The general target of MI action could be the normalization of disturbed cellular condition mediated by its osmolyte properties [50, 51]. During intense neuronal excitation (such as seizures) massive influx of Na^+, Ca^{2+}, and Cl^- takes place, which leads to water inward flow and cellular swelling [52, 53]. Under such conditions normal enzyme functioning is significantly disturbed [54]. To compensate this disturbed condition neuronal cells accumulate high concentrations of small organic osmolytes, which do not perturb the functioning of the enzymes and could restore the normal cellular conditions [50, 51]. We suggest that for the normalization of hippocampal cell functioning, after KA insult, higher amounts of MI (or some other osmolytes) are required and MI administration in our experiments supplies these required amounts. MI is actively transported via Na^+/MI cotransporter (see [47]) and at least partially rescues hippocampal cells from apoptosis and other types of cell death.

According to our data MI long-term treatment prevents biochemical changes of epileptogenesis [9, 10]. Present findings point to one of the earlier effects of MI action, namely, the inhibition of apoptosis and attenuation of neuronal cell loss.

Obtained results indicate the early mechanisms of MI action on the processes summoned by KA induced SE and shed a new light on unexpected activities of inositol. The results of the present work could pave the way for the enhancement of neuroprotective and antiapoptotic features of MI and support the potential development of new and effective antiepileptic drugs.

Competing Interests

The authors declare that they have no competing interests.

Acknowledgments

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Research Article

Sodium Tanshinone IIA Sulfonate Attenuates Scopolamine-Induced Cognitive Dysfunctions via Improving Cholinergic System

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Sodium Tanshinone IIA sulfonate (STS) is a derivative of Tanshinone IIA (Tan IIA). Tan IIA has been reported to possess neuroprotective effects against Alzheimer's disease (AD). However, whether STS possesses effect on AD remains unclear. This study aims to estimate whether STS could protect against scopolamine- (SCOP-) induced learning and memory deficit in Kunming mice. Morris water maze results showed that oral administration of STS (10 mg/kg and 20 mg/kg) and Donepezil shortened escape latency, increased crossing times of the original position of the platform, and increased the time spent in the target quadrant. STS decreased the activity of acetylcholinesterase (AChE) and increased the activity of choline acetyltransferase (ChAT) in the hippocampus and cortex of SCOP-treated mice. Oxidative stress results showed that STS increased the activity of superoxide dismutase (SOD) and decreased the levels of malondialdehyde (MDA) and reactive oxygen species (ROS) in hippocampus and cortex. In addition, western blot was carried out to detect the expression of apoptosis related proteins (Bcl-2, Bax, and Caspase-3). STS upregulated the protein expression of Bcl-2 and downregulated the proteins expression of Bax and Caspase-3. These results indicated that STS might become a promising therapeutic candidate for attenuating AD-like pathological dysfunction.

1. Introduction

Alzheimer's disease (AD) is one of the most prevalent neurodegenerative diseases characterized as progressive impairment of cognition and affective disorder. Amyloid plaques, neurofibrillary tangles, and neuronal death are regarded as the basic pathological abnormalities in AD [1]. Although the exact mechanisms of AD remain unclear, abundant studies indicated that oxidative stress and cholinergic dysfunction were important in the procession of causing AD [2]. Oxidative stress has been reported to lead to cell death via apoptosis [3] and degeneration of cholinergic nervous system, which result in impairments of cognition and memory [4]. Besides, Nathan et al. found that reduction of acetylcholine (ACh) level caused by disorders of some neurotransmitters occurred in AD patients [5]. Previous studies have clearly indicated that enhanced level of ACh leads to functional improvement of central cholinergic synapses and protection of neuronal degeneration [6]. Elevation of ACh achieved by inhibiting acetylcholinesterase (AChE), metabolizing enzyme of ACh, could improve the cholinergic dysfunction of AD [7]. Several AChE inhibitors, tacrine, donepezil, galantamine, rivastigmine, and memantine, have proven to improve cognitive deficits. However, these drugs are not ideal for clinical use due to their side effects, such as hepatotoxicity and adverse gastrointestinal effects [8, 9]. Therefore, it is critical to discover alternative drugs with cholinomimetic and antioxidative activities for the treatment of AD [10].

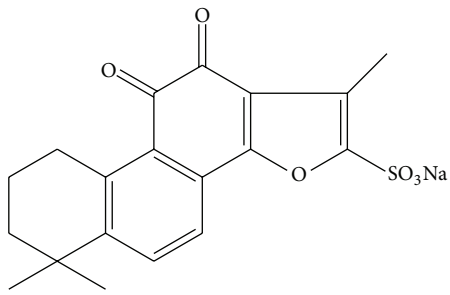

Figure 1: Molecular structure of STS.

Sodium Tanshinone IIA sulfonate (STS, structure shown in Figure 1) is a derivative of Tanshinone IIA (Tan IIA), which was extracted from *Salvia miltiorrhiza* [11]. Both STS and Tan IIA have well-established cardioprotective effect on cardiovascular injury [12]. In addition, Tan IIA also possesses neuroprotective activity against neural dysfunction [13]. A recent report suggested that Tan IIA can inhibit amyloid formation, disassemble Aβ fibrils, and protect SH-SY5Y and PC12 cells from Aβ-induced toxicity [14, 15]. Moreover, it has been proven that the protective effects of Tan IIA against memory deficits induced by streptozotocin (STZ) are by attenuating oxidative damage and improving central cholinergic neurotransmission [16]. Some studies show that STS and Tan IIA might have similar pharmacological actions, such as antioxidative stress [13, 17], anti-inflammation [17, 18], and antiapoptosis [19, 20]. Thus, we speculate that STS also has the protective effect on neurons.

In the present study, we evaluate the effect of STS (10 mg/kg and 20 mg/kg) on scopolamine- (SCOP-) induced learning and memory impairment in Kunming mice. Donepezil, a reversible AChE inhibitor, was employed as a positive reference drug for the treatment of cognitive deficits. Our study found that STS could obviously ameliorate cognitive impairment through attenuating oxidative damage and improving central cholinergic neurotransmission. Meanwhile, STS could protect against the apoptosis induced by SCOP in the hippocampus and cortex. These results demonstrated that STS could be served as a promising therapeutic candidate drug for AD.

2. Materials and Methods

2.1. Materials. Sodium Tanshinone IIA sulfonate (99.5%) was obtained from Loki's Pharmaceuticals (Beijing, China). Scopolamine hydrobromide injection (Guangzhou Baiyun Mountain Mingxing Pharmaceutical Co., Ltd., Guangzhou, China) was purchased from Guangzhou Pharmaceuticals Corporation (Guangzhou, China). Kits used for detection of reactive oxygen species (ROS), malondialdehyde (MDA), superoxide dismutase (SOD), choline acetyltransferase (ChAT), and acetylcholinesterase (AChE) were purchased from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Primary antibodies (Bcl-2, Caspase-3) were purchased from Cell Signaling Technology, Inc. Anti-Bax antibody was purchased from Santa Cruz Biotechnology, Inc. Anti-β-actin was purchased from Sigma-Aldrich. Secondary antibodies (horseradish peroxidase conjugated anti-rabbit IgG and anti-mouse IgG) were purchased from Cell Signaling Technology, Inc. All other reagents were of the highest grade available commercially.

2.2. Animal and Treatment. Male Kunming mice (KM, weighing 35–40 g) were purchased from the Experimental Animal Center of Guangzhou University of Chinese Medicine (Guangzhou, China). They were maintained on standard laboratory conditions with free access to water and food. Procedures for animal experiments were conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals as adopted and promulgated by the United States National Institutes of Health. Mice were randomly divided into five groups: vehicle control group (CON, 0.9% saline, $n = 10$), scopolamine group (SCOP, $n = 10$), low dose STS group (STSL, SCOP 3 mg/kg + STS 10 mg/kg, $n = 10$), high dose STS group (STSH, SCOP 3 mg/kg + STS 20 mg/kg, $n = 10$), and Donepezil group (DON, SCOP 3 mg/kg + ARI 3 mg/kg, $n = 10$). Mice were treated with saline, STS, and Donepezil, respectively, by gavage, once per day for two weeks. SCOP was injected from the eighth day for one week (intraperitoneally, IP). The SCOP was injected 0.5 h before the Morris water maze test.

2.3. Morris Water Maze Test. The Morris water maze test was similar to the method of Morris [21], with minor modifications [22]. The water maze equipment (Guangzhou Feidi Biology Technology Co., Ltd., Guangzhou, China) consisted of a black circular pool, a black platform, and a record system. The water (30 cm in depth; temperature: 22–26°C) in pool (diameter: 120 cm; height: 40 cm) was dyed with nontoxic soluble black colored. And the pool was divided into four equal quadrants. The black escape platform (diameter: 10 cm, 1.5 cm below the water surface) was placed in the center of one of the pool quadrants. The learning and memory ability of mice was detected by the Morris water maze test in a dark room. Mice were given a place navigation test for four consecutive days. For each daily trial, there were four sequential training trials beginning with placing the animal in the water facing the wall of the pool with drop location changing for each trial; then the record system starts to record the time. The escape latency was recorded at the end. If the mouse failed to find the platform within 60 s, it would be guided to the platform by the trainer and to remain there for 10 s; its escape latency would be recorded as 60 s. On the fifth day, the mice were allowed to swim freely in the pool for 60 s without the platform. The times of crossing through the original platform position and the time spent in the target quarter were measured, which indicated the degree of memory consolidation.

2.4. Measurement of AChE and ChAT Activity. All mice were anesthetized and decapitated after the Morris water maze

test immediately; hippocampus and cortex were carefully dissected from brains for examination. All the processes were performed on ice-cold plate. Tissues were rapidly stored at −80°C. The hippocampus and cortex tissues were homogenized with ice-cold saline. The homogenate was centrifuged at 12,000 ×g for 10 min at 4°C. The supernatant was used to detect the activity of ChAT and AChE according to the manufacturer's instructions by using Universal Microplate Spectrophotometer (Bio-Rad, Hercules, CA, USA).

2.5. ROS Production. The hippocampus and cortex tissues were homogenized with ice-cold saline and centrifuged at 12,000 ×g for 10 min at 4°C. The supernatant was used to detect the levels of ROS. ROS were measured using the redox-sensitive fluorescent dye, DCFH-DA. Conversion of nonfluorescent DCFH-DA to fluorescent dichlorofluorescein (DCF) in the presence of ROS was measured on a microplate reader. Fluorescence emission intensity of DCF (538 nm) was measured in response to 485 nm excitation. The level of intracellular ROS was expressed as a percentage of control cultures incubated in DCFH-DA.

2.6. MDA, SOD, and GSH-Px Assays. The hippocampus and cortex tissues were homogenized with ice-cold saline and centrifuged at 12,000 ×g for 10 min at 4°C. The supernatant was used to detect the levels of MDA and the activity of SOD according to the manufacturer's instructions by using Universal Microplate Spectrophotometer (Bio-Rad, Hercules, CA, USA).

2.7. Western Blot Analysis. The hippocampus and cortex tissues were homogenized and lysed in ice-cold RIPA buffer (containing 1 : 100 PMSF, 1 : 100 inhibitor proteases and phosphatases cocktail) for 15 min. The lysate was centrifuged at 12,000 ×g for 10 min at 4°C. The same amount of protein (30 μg) was separated by SDS-PAGE analysis gel. Then the separated protein migrated to PVDF membranes and was blocked in 5% skim milk that dissolved in Tris-buffered saline-Tween-20 (TBST) for 1 h at room temperature. The membranes containing the protein were incubated with rabbit anti-Bax (1 : 1,000, number SC-526, Santa Cruz, Barbara, CA, USA), rabbit anti-Bcl2 (1 : 1,000, number 2876s, Cell Signaling Technology, Boston, MA, USA), rabbit anti-Caspase-3 (1 : 1,000, number 9662, Cell Signaling Technology, Boston, MA, USA), and mouse anti-β-actin (1 : 1,000, number A5441, Sigma-Aldrich, St. Louis., MO, USA) overnight at 4°C. Then the membrane was incubated with horseradish peroxidase conjugated anti-rabbit (number 7074s, Cell Signaling Technology, Boston, MA, USA) or anti-mouse (number 7076s, Cell Signaling Technology, Boston, MA, USA) IgG antibody (1 : 1,000) for 1 h at room temperature. The membrane was visualized by using a superenhanced chemiluminescence reagent (ECL; Applygen Technologies Inc., Beijing, China).

2.8. TUNEL Staining. Sections were washed in xylene and rehydrated through a graded series of ethanol and double-distilled water. Then, the sections were washed in PBS and incubated with 50 μL TUNEL reaction mixture for 1 h at 37°C in the dark. Further incubation with 50 μL converter-POD was performed at 37°C for 30 min. The sections were then rinsed with PBS and stained with DAB substrate for 10 min at room temperature. TUNEL staining was performed using the In Situ Cell Death Detection kit (Roche Diagnostics GmbH, Mannheim, Germany). Images were analyzed by using a light microscope and LEICA QWin Plus (Leica Microsystems, Wetzlar, Germany).

2.9. Statistical Analysis. Experimental values were given as means ± SD. The statistical analysis between two groups would be evaluated with Student's unpaired t-test. Statistical analysis of the data among multigroups was performed using the SPSS 19.0 statistical software. Two-way analysis of variance (ANOVA) was applied to analyze difference in data of biochemical parameters among the different groups, followed by Dunnett's significant post hoc test for pairwise multiple comparisons. Differences were considered as statistically significant at $p < 0.05$.

3. Results

3.1. Effects of STS on Learning and Memory of SCOP-Treated Mice. As shown in Figure 2(a), the time for mice to find the hidden platform was declined progressively during the four training days. In contrast to vehicle control group, intraperitoneal injection with SCOP remarkably increased the period of time to find the hidden platform. However, pretreatment with low (10 mg/kg) and high dose (20 mg/kg) of STS and Donepezil obviously shortened escape latency when contrasted to SCOP group. The corresponding swimming paths of each group on the fourth trail day were shown in Figure 2(b). SCOP group presented a chaotic and longer swimming path, which were improved by STS and Donepezil. On the fifth day, the probe trial was performed by removing the platform and allowing the mice to swim freely to estimate their spatial-working memory (Figures 2(c)–2(e)). SCOP group presented a longer latency (20.2 ± 2.3 s), a fewer times (2.9 ± 1.6) crossing the position of the removed platform, and a fewer times (13.4 ± 4.1 s) spent in the target quadrant, which were ameliorated by STS and Donepezil. These results demonstrated that treatment with STS remarkably reversed the cognition deficits, which was induced by SCOP.

3.2. Effect of STS on the Activity of AChE and ChAT in SCOP-Treated Mice. To illuminate the potential mechanism of STS in ameliorating cognition deficiency caused by SCOP, the activities of cholinergic marker enzymes were detected. As shown in Figure 3(a), SCOP caused a remarkable increase of AChE activity in both hippocampus (1.43 ± 0.19%) and cortex (1.41 ± 0.17%), suggesting that the dysfunction of cholinergic nervous system may facilitate the process of cognitive impairment, while the treatment with STS and Donepezil significantly decreased the AChE activity. As shown in Figure 3(b), the activity of ChAT in SCOP-treated

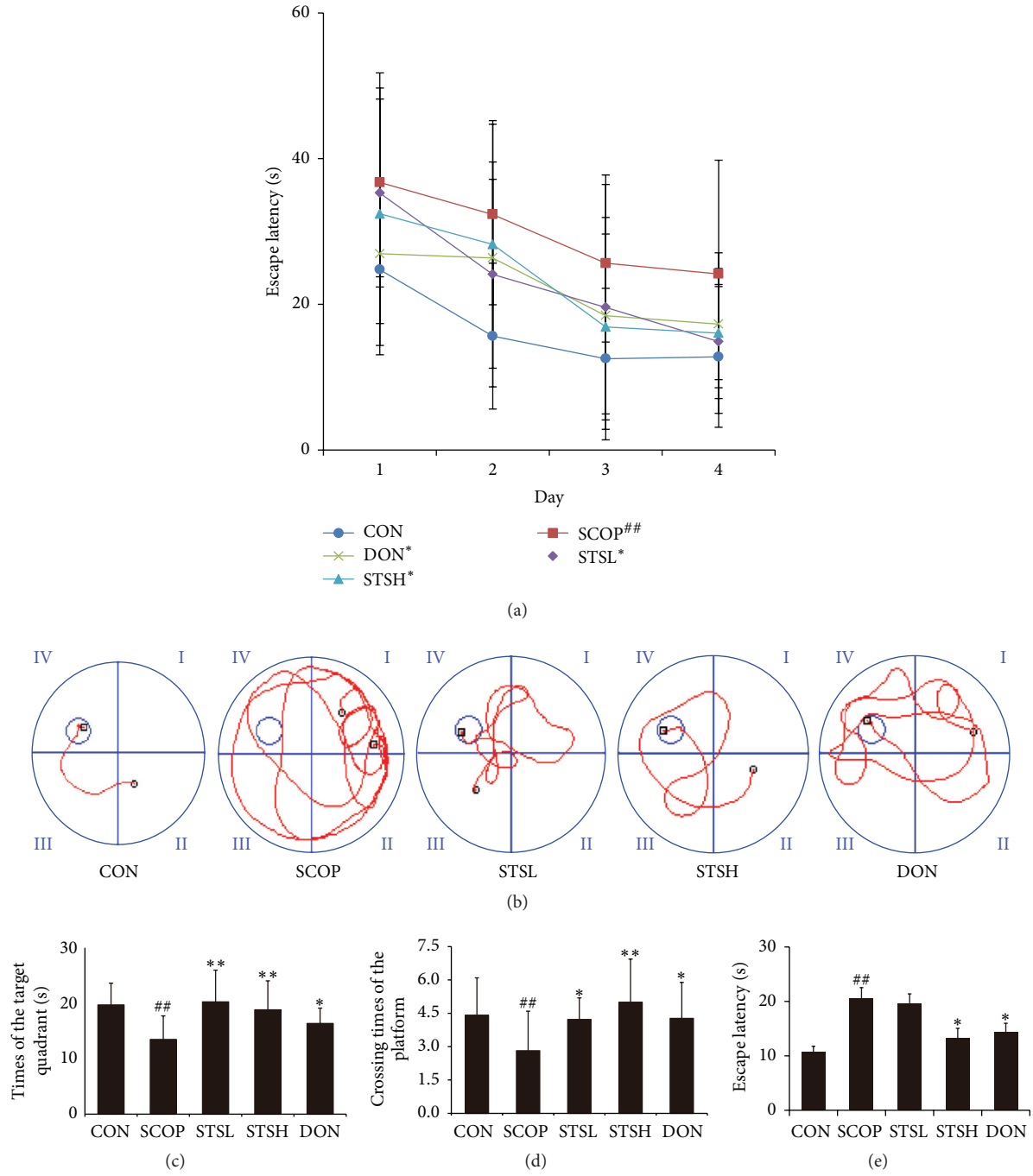

FIGURE 2: Effects of STS on learning and memory of SCOP-treated mice. (a) Escape latency of four consecutive days' test. (b) The swimming paths of respective groups on fourth day. (c) Escape latency of finding the hidden platform in the probe trial. (d) Crossing times of the target platform in the probe trial. (e) Time spent in the quadrant of the platform in the probe trial. CON: vehicle control; SCOP: scopolamine; STSL: scopolamine + STS (10 mg/kg); STSH: scopolamine + STS (20 mg/kg); DON: scopolamine + Donepezil. Data represent mean ± SEM ($n = 10$ per group). $^{\#\#}p < 0.01$ versus vehicle control group; $^{*}p < 0.05$ and $^{**}p < 0.01$ versus SCOP-treated group.

group was decreased sharply in both hippocampus (0.48 ± 0.07%) and cortex (0.27 ± 0.09%), whereas STS enhanced the activity of ChAT significantly. Thus, STS could protect against SCOP-induced dysfunction of cholinergic marker enzymes.

3.3. Effect of STS on the Oxidative Stress Status in SCOP-Treated Mice. Oxidative stress status was also determined in both hippocampus and cortex of SCOP-treated mice (Figures 4(a)–4(c)). ROS (4 ± 0.41% or 2.96 ± 0.05%) and MDA

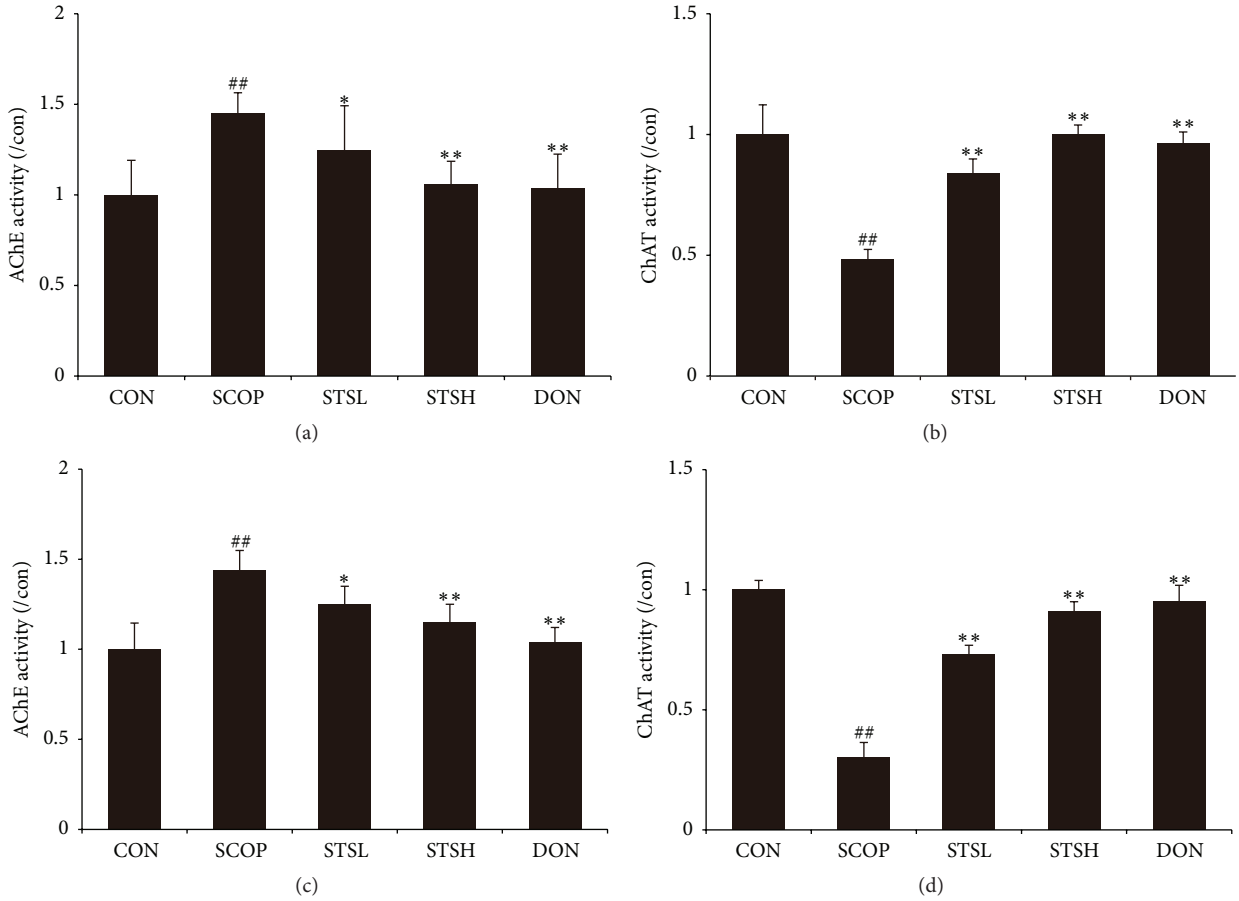

Figure 3: Effect of STS on the activity of AChE and ChAT in SCOP-treated mice. The supernatant of hippocampus homogenate was used for the assay of (a) AChE and (b) ChAT activities. The supernatant of cortex homogenate was used for the assay of (c) AChE and (d) ChAT activities. Data represent mean ± SEM ($n = 10$ per group). CON: vehicle control; SCOP: scopolamine; STSL: scopolamine + STS (10 mg/kg); STSH: scopolamine + STS (20 mg/kg); DON: scopolamine + Donepezil. $^{\#\#}p < 0.01$ versus vehicle control group; $^{*}p < 0.05$ and $^{**}p < 0.01$ versus SCOP-treated group.

(3.02 ± 0.44% or 2.52 ± 0.17%) levels were robustly increased, while activity of SOD (0.38 ± 0.11% or 0.47 ± 0.16%) was suppressed in SCOP group. Both STS and Donepezil decreased the MDA and ROS levels and increased the SOD activity.

3.4. Effect of STS on the Protein Expressions of Bax, Bcl2, and Caspase-3 in SCOP-Treated Mice. As shown in Figures 5(a)–5(c), intraperitoneal injection with SCOP remarkably increased the proapoptotic proteins Bax (2 ± 0.12% or 1.82 ± 0.08%) and cleaved Caspase-3 (1.44 ± 0.07% or 2.87 ± 0.12%) expression and decreased the expression of Bcl-2 (0.48 ± 0.1% or 0.94 ± 0.02%) in both hippocampus and cortex. Both STS and Donepezil significantly upregulated the Bcl-2 expression and downregulated the Bax and cleaved Caspase-3 expressions when compared to SCOP group. These results demonstrated that STS could protect against SCOP-induced memory deficit through antiapoptosis.

3.5. Effect of STS on Neuronal Apoptosis in the Hippocampus. As shown in Figure 6, TUNEL-positive cells were stained deep brown in the hippocampus. Compared with vehicle control mice, the neuronal apoptosis in the hippocampus of SCOP-treated mice was prominently increased. STS and Donepezil markedly attenuated the neuronal apoptosis in SCOP-treated mice. These results indicated that STS could protect against SCOP-induced neuronal apoptosis.

4. Discussion

In this study, a classical AD-like model induced by scopolamine was employed to evaluate the protective effect of STS [11]. We found that STS administration (10 mg/kg and 20 mg/kg) could improve SCOP-induced learning and memory impairment in Kunming mice. Meanwhile, STS could obviously improve central cholinergic neurotransmission and attenuate oxidative damage. In addition, STS could protect against SCOP-induced apoptosis in hippocampus and cortex.

AD is one of the most ordinary neurodegenerative diseases. Progressive impairment of cognition and affective disorder were considered as typical symptoms of AD [23].

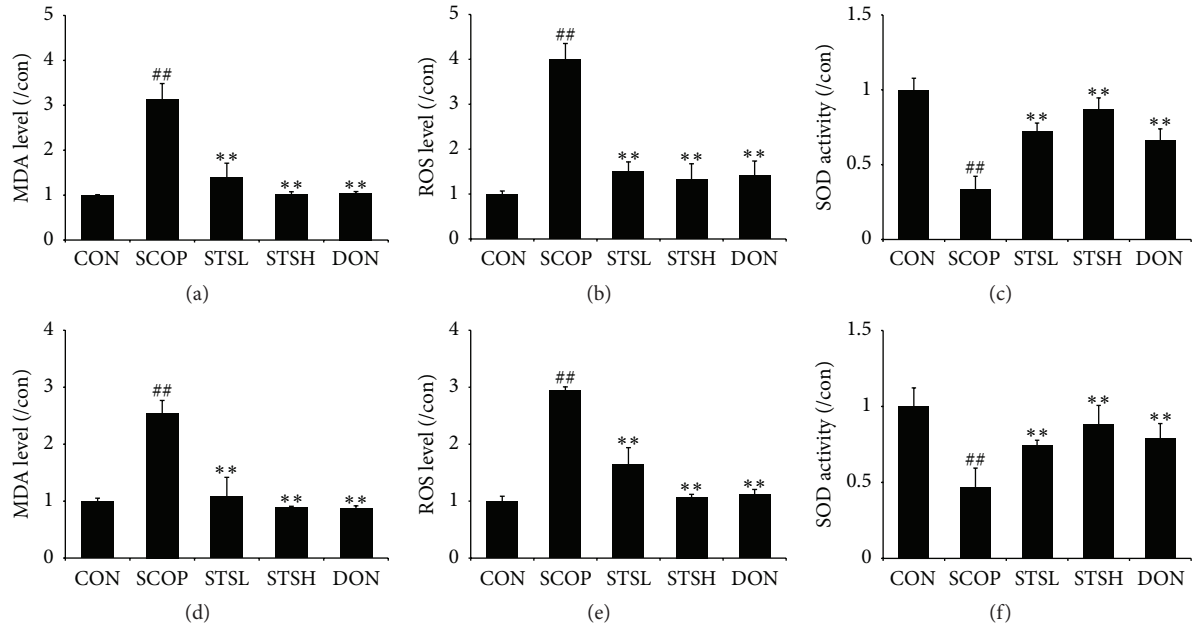

Figure 4: Effect of STS on the oxidative stress status in SCOP-treated mice. The supernatant of hippocampus homogenate was used for the assay of (a) ROS and (b) MDA levels and (c) SOD activity. The supernatant of cortex homogenate was used for the assay of (d) ROS and (e) MDA levels and (f) SOD activity. Data represent mean ± SEM ($n = 10$ per group). CON: vehicle control; SCOP: scopolamine; STSL: scopolamine + STS (10 mg/kg); STSH: scopolamine + STS (20 mg/kg); DON: scopolamine + Donepezil. $^{\#\#}p < 0.01$ versus vehicle control group; $^{**}p < 0.01$ versus SCOP-treated group.

The cognitive deficiency associated with AD is considered to be primarily related to disorder of cholinergic neurotransmission in the cerebral hippocampus and cortex [23, 24]. Various factors can induce brain impairment by influencing the synthesis, release, and uptake of acetylcholine (ACh) [25]. Scopolamine, an anticholinergic drug, can block ACh receptors and lead to a significant increase of acetylcholinesterase (AChE) level in the hippocampus and cortex [26]. The SCOP-induced amnesic model has been widely used as a pharmacological model of memory dysfunction [27]. We employed the Morris water maze test to measure the memory deficits of SCOP-treated mice. Result showed that SCOP group had a longer latency, a fewer times crossing the area of the hidden platform, and a fewer times spent in the quadrant of the original platform, which demonstrated that an amnesia model induced by SCOP was successfully established.

AChE is an important regulatory enzyme that rapidly hydrolyzes ACh, while ChAT is an enzyme that associated with the synthesis of ACh [28]. During neurotransmission, ACh is released from the presynaptic neuron into the synaptic cleft and binds to ACh receptors on the postsynaptic membrane, relaying the signal from the nerve. AChE, also located on the postsynaptic membrane, terminates the signal transmission by hydrolyzing ACh. The alterations in the membrane can be a decisive factor in changing the conformational state of the AChE molecule [25]. The results of increasing activity of AChE and decreasing activity of ChAT in the cortex and hippocampus of SCOP-treated mice were consisted with previous studies [26, 27]. Therefore, protecting cholinergic system from functional degeneration and sustaining the normal activity of ChAT and AChE might be serviceable against SCOP-induced amnesia.

STS is a sulfonated product of Tan IIA [11]. According to previous studies, STS and Tan IIA both have cardioprotective effect on cardiovascular injury. In addition, they also possess other similar pharmacological functions, such as antioxidative stress [13, 17], anti-inflammation [17, 18], and antiapoptosis [19, 20]. Interestingly, Liu et al. found that Tan IIA could remarkably ameliorate cognitive impairment in STZ-treated mice. The effects might be mediated by ameliorating the damage of cholinergic system and attenuating oxidative damage. Thus, we speculate that STS could also improve cholinergic system dysfunction. In this study, Morris water maze results showed that low dose (10 mg/kg) and high dose (20 mg/kg) of STS and Donepezil obviously shortened the escape latency, improved the swimming path, and presented more times crossing the position of the removed platform and more time spent in the target quadrant in the probe trial. Meanwhile, STS and Donepezil also significantly decreased the AChE activity and increased the ChAT activity. These results demonstrated that STS could protect against the cholinergic system dysfunction.

Imbalance between reactive oxygen species (ROS) generation and removal has been known to be involved in neuronal damage [29]. ROS can cause extensive damage to lipids, proteins, and DNA, leading to change in structure and function of neural cells in the brain [30]. Malondialdehyde (MDA) is an indicator of lipid peroxidation [31]. SOD is an antioxidant enzyme, inducing increased free radical generation [32]. In this study, treating with SCOP robustly increased ROS and

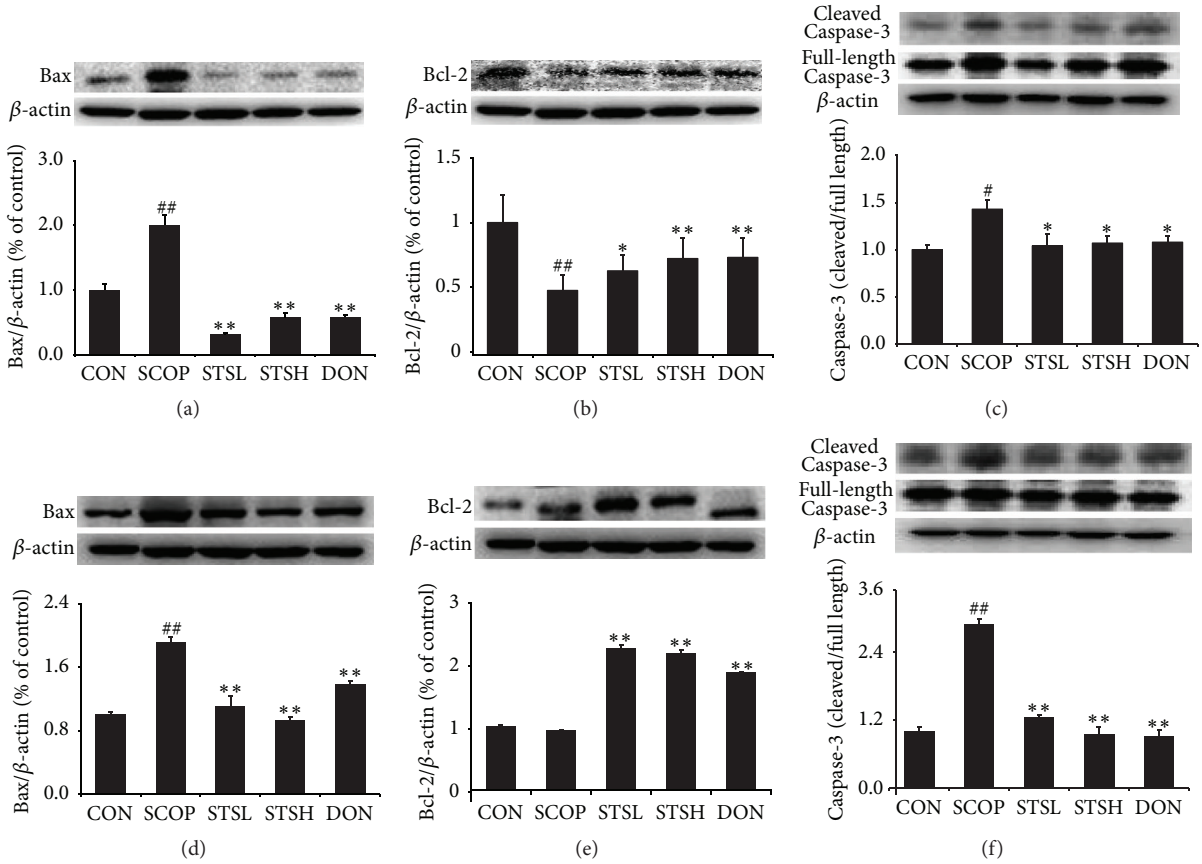

Figure 5: Effect of STS on the protein expressions of Bax, Bcl2, and Caspase-3 in SCOP-treated mice. Proteins expression of (a) Bax, (b) Bcl-2, and (c) Caspase-3 was detected in hippocampus. Proteins expression of (d) Bax, (e) Bcl-2, and (f) Caspase-3 was detected in cortex. CON: vehicle control; SCOP: scopolamine; STSL: scopolamine + STS (10 mg/kg); STSH: scopolamine + STS (20 mg/kg); DON: scopolamine + Donepezil. $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ versus vehicle control group; $^{*}p < 0.05$ and $^{**}p < 0.01$ versus SCOP-treated group.

MDA levels and suppressed the activity of SOD. Evidence shows that STS could be used as an antioxidant to validly inhibit the formation of reactive oxygen radicals [33] and eliminate lipid-free radicals [34]. The present study showed that STS conspicuous increased SOD activity and reduced MDA and ROS levels in both hippocampus and cortex. These results indicated that STS could upregulate oxidation tolerance in hippocampus and cortex.

Apoptosis is considered to be one of the main causes of neurodegeneration. Oxidative stress can lead to the consecutiveness response apoptosis straightly [35, 36]. Caspase-3, which belongs to the subgroup of Caspase protease in the Caspase family, is the terminal executing enzyme in apoptosis. And it can cut the structural protein of cells and lead to apoptosis [37]. Bcl-2 can be extracted from B cell lymphoma and present a distinct capacity of antiapoptosis [38]. In contrast to Bcl-2, Bax protein exerts the opposite effect of promoting cell apoptosis [39]. Therefore, we can determine cell survival according to relative ratio of proapoptotic protein Bax and antiapoptotic protein Bcl-2. Meanwhile, several neurodegenerative diseases are associated with activation of apoptosis. Our results showed that STS remarkably downregulated the apoptotic index Bax/Bcl2 and cleaved Caspase-3 expressions in hippocampus and cortex of SCOP-treated mice. In addition, TUNEL staining showed that STS markedly attenuated the neuronal apoptosis in SCOP-treated mice. These results suggested that the protective effect of STS against SCOP-induced injury is related to inhibiting the damage induced by free radicals and consecutive apoptosis.

In conclusion, our study suggested that the cognitive-protecting activities of STS on SCOP-induced memory impairment might result from its effect of improving the cholinergic nervous system and antioxidative stress. However, the effect of STS on other AD pathological symptoms, such as synaptic degeneration, neuroinflammation, and neurite degeneration, remains unclear, which is worthy to investigate in the future.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

Qing-Qing Xu and Yi-Jun Xu contributed equally to this paper.

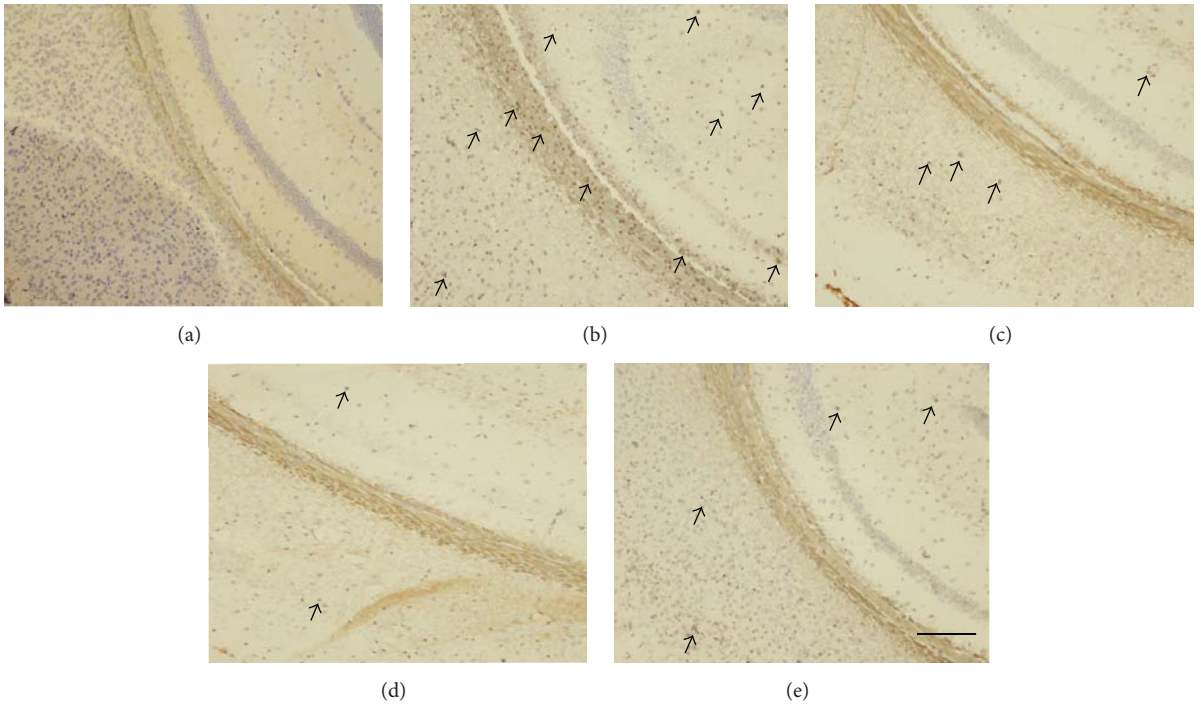

Figure 6: TUNEL staining in the hippocampus of SCOP-treated mice. (a) Vehicle control; (b) scopolamine; (c) scopolamine + STS (10 mg/kg); (d) scopolamine + STS (20 mg/kg); (e) scopolamine + Donepezil. Black arrows showed the neuronal apoptosis. Scale bar: 100 μm.

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Research Article

PET Evidence of the Effect of Donepezil on Cognitive Performance in an Animal Model of Chemobrain

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A considerable number of patients with breast cancer complain of cognitive impairment after chemotherapy. In this study, we showed that donepezil enhanced memory function and increased brain glucose metabolism in a rat model of cognitive impairment after chemotherapy using behavioral analysis and positron emission tomography (PET). We found that chemotherapy affected spatial learning ability, reference memory, and working memory and that donepezil improved these cognitive impairments. According to PET analysis, chemotherapy reduced glucose metabolism in the medial prefrontal cortex and hippocampus, and donepezil increased glucose metabolism in the bilateral frontal lobe, parietal lobe, and hippocampus. Reduced glucose metabolism was more prominent after treatment with doxorubicin than cyclophosphamide. Our results demonstrated the neural mechanisms for cognitive impairment after chemotherapy and show that cognition was improved after donepezil intervention using both behavioral and imaging methods. Our results suggested that donepezil can be employed clinically for the treatment of cognitive deficits after chemotherapy.

1. Introduction

A considerable number of patients with breast cancer complain of cognitive impairment after chemotherapy. Cognitive deficits in such patients consist of hippocampus-dependent memory and executive functions associated with the frontal lobe. Similar cognitive impairment was also shown in animal models [1–3]. Chemotherapy-associated cognitive dysfunction was referred to as chemobrain. Selamat and colleagues reported that awareness of cognitive changes was dependent on the healthcare and cultural context. To overcome chemobrain, various interventions such as cognitive training, methylphenidate, and erythropoietin have been performed [4–8]. Chemobrain was also found when patients with B-cell non-Hodgkin lymphomas were given rituximab, cyclophosphamide, doxorubicin, vincristine and prednisone, vincristine and rituximab, or vincristine and bendamustine [9]. Chiaravalloti's group reported that brain glucose metabolism in patients with Hodgkin disease was affected after diagnosis and during chemotherapy treatment [10]. Ponto's group reported that breast cancer survivors treated with chemotherapy may manifest long-term changes in brain glucose metabolism indicative of subtle frontal hypometabolism [11]. Simó's group reported a long-term decrease in gray matter and white matter volume in chemotherapy-treated patients [12].

Donepezil, a reversible acetylcholinesterase inhibitor, improves cognition for the patients with Alzheimer's disease and stroke traumatic brain injury and even for normal older adults [13–16]. However, to the best of our knowledge, there was no report on the effect of donepezil to chemobrain. In the present study, we investigated the effect of donepezil on memory function and brain glucose metabolism in a rat chemobrain model using behavioral test and positron emission tomography (PET).

2. Materials and Methods

2.1. Animal and the Experimental Design. Female Sprague-Dawley rats weighting 250–280 g each were used. The animals were allowed to acclimatize themselves for at least 7 days prior to the experimentation. The animals were housed in individual cages under light-controlled conditions (12/12 h light/dark cycle) and at 23°C room temperature. Food and water were made available. This experimental protocol was approved by an Institutional Review Committee for the Use of Human or Animal Subjects or the procedures are in compliance with at least the Declaration of Helsinki for human subjects or the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication number 85-23, revised 1985), the UK Animals Scientific Procedures Act 1986, or the European Communities Council Directive of 24 November 1986 (86/609/EEC). The rats were allowed to adapt to their environment at least 1 week before the experiments.

Chemobrain rat model was constructed using doxorubicin or cyclophosphamide because doxorubicin or cyclophosphamide was widely used in chemotherapy. Trimethyltin chloride (C3H9ClSn) (TMT) was also used as a control of cognitive dysfunction group for the comparison with chemobrain model. TMT is a potent neurotoxicant that selectively induces neuronal death in both human and animal limbic system and in particular in the hippocampal formation.

Rats were divided into six groups of ten individuals as follows: normal control group (group 1, $n = 10$), control group of cognitive dysfunction group induced by TMT (group 2, $n = 10$), cyclophosphamide-treated group (group 3, $n = 10$), doxorubicin-treated group (group 4, $n = 10$), cyclophosphamide-treated and donepezil administered group (group 5, $n = 10$), and doxorubicin-treated and donepezil administered group (group 6, $n = 10$). To generate a rat model of chemotherapy, 100 mg/kg of cyclophosphamide was intraperitoneally (IP) injected for groups 3 and 4. 4 mg/kg of doxorubicin was IP injected for groups 4 and 6 once a week for 3 weeks. For donepezil intervention groups, 5 mg/kg of donepezil was IP administrated every day for 3 weeks. 8 mg/kg of TMT was injected to induce cognitive dysfunction group for group 2.

2.2. Behavioral Study. The Morris water maze test was performed to evaluate spatial learning ability and reference memory [17]. The swimming pool of the Morris water maze was a circular water tank 200 cm in diameter and 35 cm deep. It was filled to a depth of 21 cm with water at 23. A platform 15 cm in diameter and 20 cm in height was placed inside the tank with its top surface being 1.5 cm below the surface of the water. The pool was surrounded by many cues that were external to the maze. A CCD camera was equipped with a personal computer for the behavioral analysis. Each rat received four daily trials. For 4 consecutive days, the rats were tested with three acquisition tests. They also received retention tests on the 5th day. For the acquisition test, the rat was allowed to search for the hidden platform for 180 s and the latency to escape onto the platform was recorded. The animals were trained to find the platform that was in a fixed position for 4 days for the acquisition test, and then, for the retention test (at the 5th day), they received a 1 min probe trial in which the platform was removed from the pool. The intertrial interval time was 1 min. The performance of the test animals in each water maze trial was assessed by a personal computer for the behavioral analysis (S-mart program, Spain). Passive avoidance test was also performed to assess explicit memory function [18].

2.3. FDG PET. Siemens Inveon PET was used in this study. Regional cerebral glucose metabolism in the same groups of rats was measured using F-18 fluorodeoxyglucose (FDG) PET after behavioral test. The FDG PET scan was performed to assess cerebral glucose metabolism for chemobrain and the effect of donepezil interventions compared to normal control. Before PET scanning, the rats were fasted for at least 8 h, after they were anesthetized with 2% isoflurane in 100% oxygen (Forane Solution; Choong Wae Pharma, Seoul, South Korea). During PET scanning, the temperature of rats was kept at 36°C with heating pads. Heart rates were monitored using a BioVet system (M2M Imaging Corp., USA). After injection of FDG (18.5 MBq/100 g body weight), 40 min of emission PET data was acquired. Transmission PET data were acquired for 15 min after emission PET scan. Emission list-mode data were sorted into 3D sinograms and reconstructed using 3D reprojection (3DRP) algorithms. No filter was applied. The image matrix was $256 \times 256 \times 159$, the pixel size $0.155 \times 0.155\ mm^2$, and the slice thickness 0.796 mm.

Voxel-wise statistical analysis was performed to identify the regional differences between groups using SPM 8 (http://www.fil.ion.ucl.ac.uk/spm/). For SPM analysis, the brain region of interest was extracted and a study-specific rat brain template was constructed. Individual PET data were spatially normalized onto the rat brain template. Spatial normalization for individual PET was performed using affine and nonlinear transformations. The voxel size of spatially normalized images was $0.3 \times 0.3 \times 0.3\ mm^3$. 3 mm of Gaussian smoothing kernel was applied for enhancing the signal to noise ratio. Count normalization was also performed. Two sample t-tests were used to identify regional differences between groups with a threshold of $P < 0.05$ (uncorrected).

3. Results

Figures 1(a) and 1(b) show the result of the Morris water maze test after administration of each chemotherapeutic agent, chemobrain rats needed more time to acquire information, and memory retention was impaired. After donepezil intervention these impairments were partially rescued, indicating that while chemotherapeutic agents affected spatial learning ability and reference memory, donepezil facilitated their recovery.

Figures 1(c) and 1(d) show the result of the passive avoidance test. The result shows that response times decreased after administration of each chemotherapeutic agent, and cerebral glucose metabolism was partially recovered after administration of donepezil for both the doxorubicin and cyclophosphamide induced chemobrain. This suggests that explicit working memory was impaired after chemotherapy

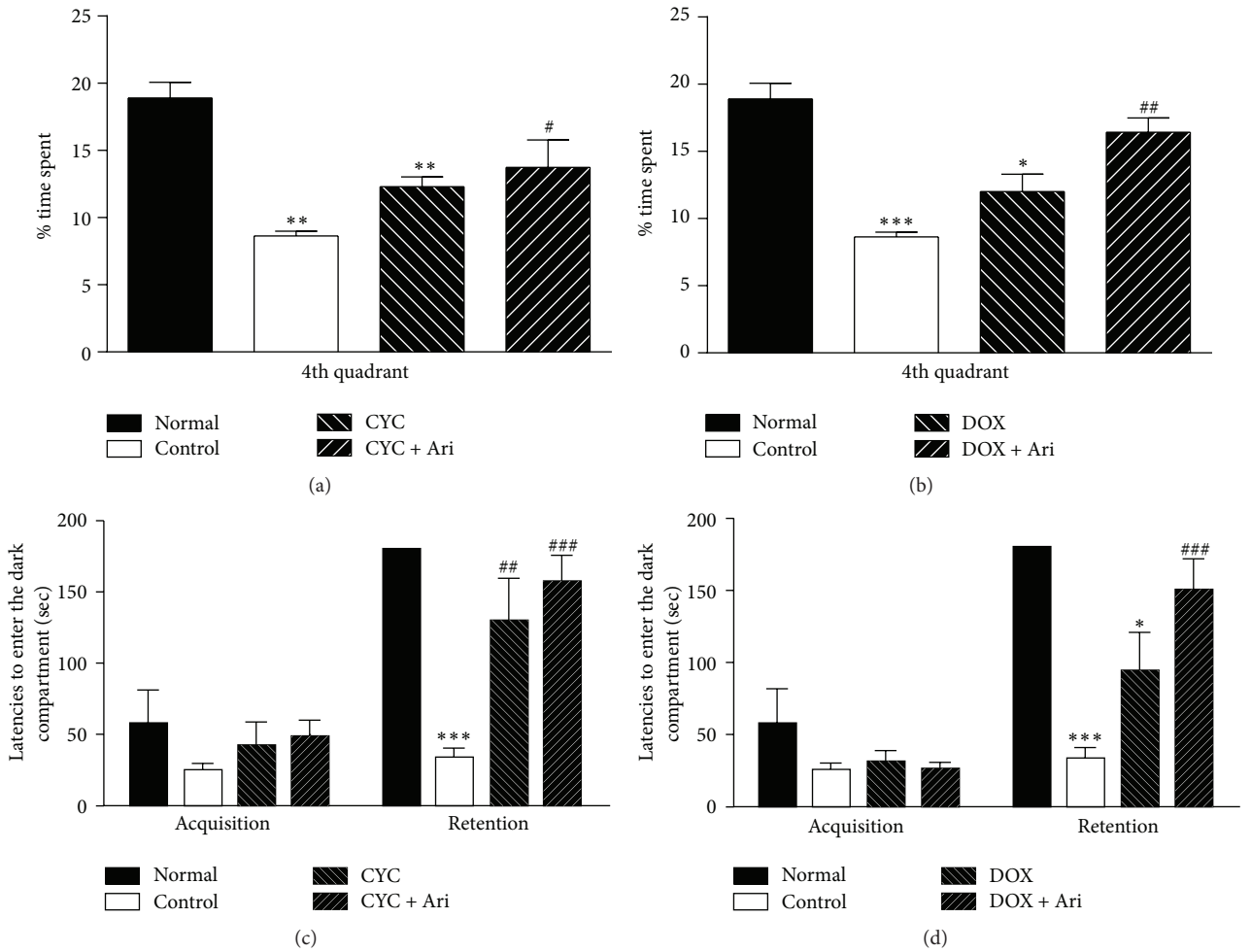


Figure 1: Learning and memory assessed by performance on the Morris water maze (a, b) and passive avoidance test (c, d). Time spent on the platform or 4th quadrant of the Morris water maze was recorded for the retention test (a, b). Latencies to step on the platform in the acquisition and retention trials of the passive avoidance test (c, d). Each value is represented as mean ± SEM. Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ *versus* the naive group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ *versus* the TMT group, respectively.

treatment; however, donepezil intervention could improve the cognitive function. Recovery of cognitive function after administration of donepezil was more evident for doxorubicin induced chemobrain group compared to cyclophosphamide induced chemobrain group.

Figure 2(a) and Supplementary Figure 1a, in Supplementary Material available online at http://dx.doi.org/10.1155/2016/6945415, show the result of decrease of cerebral glucose metabolism after chemotherapy with cyclophosphamide in the region of bilateral medial prefrontal cortices ($P < 0.05$).

Figure 2(b) and Supplementary Figure 1b show that the result of cerebral glucose metabolism was decreased in the region of left medial prefrontal cortex and bilateral hippocampus after doxorubicin chemotherapy when compared with the normal untreated group ($P < 0.05$).

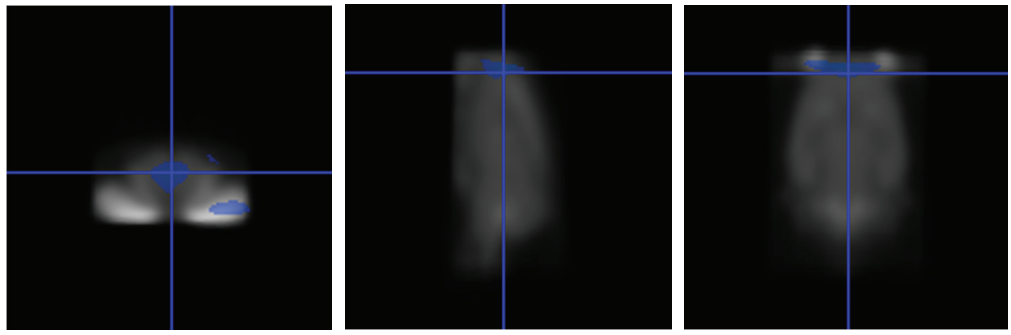
Figure 2(c) and Supplementary Figure 1c show the increase of glucose metabolism in the region of the bilateral medial prefrontal cortices, bilateral hippocampi (L > R), bilateral medial hippocampi, and bilateral parietal cortices after intervention with donepezil for cyclophosphamide-treated group ($P < 0.05$).

Figures 2(d) and 1(d) show the increase of glucose metabolism in the region of bilateral global area of the cortices including the bilateral frontal, bilateral parietal, bilateral temporal, and bilateral hippocampi in the doxorubicin-treated group after donepezil intervention ($P < 0.05$).

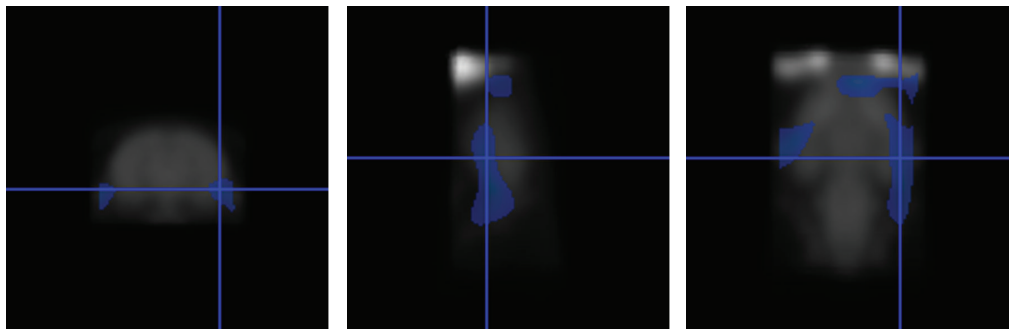
Histochemical analysis demonstrated that the number of neurons in the hippocampus and the expression of choline acetyltransferase in the hippocampus decreased after each chemotherapeutic treatment, and donepezil intervention expedited the restoration of these, although these differences were not statistically significant (Supplementary Figure 2).

4. Discussion

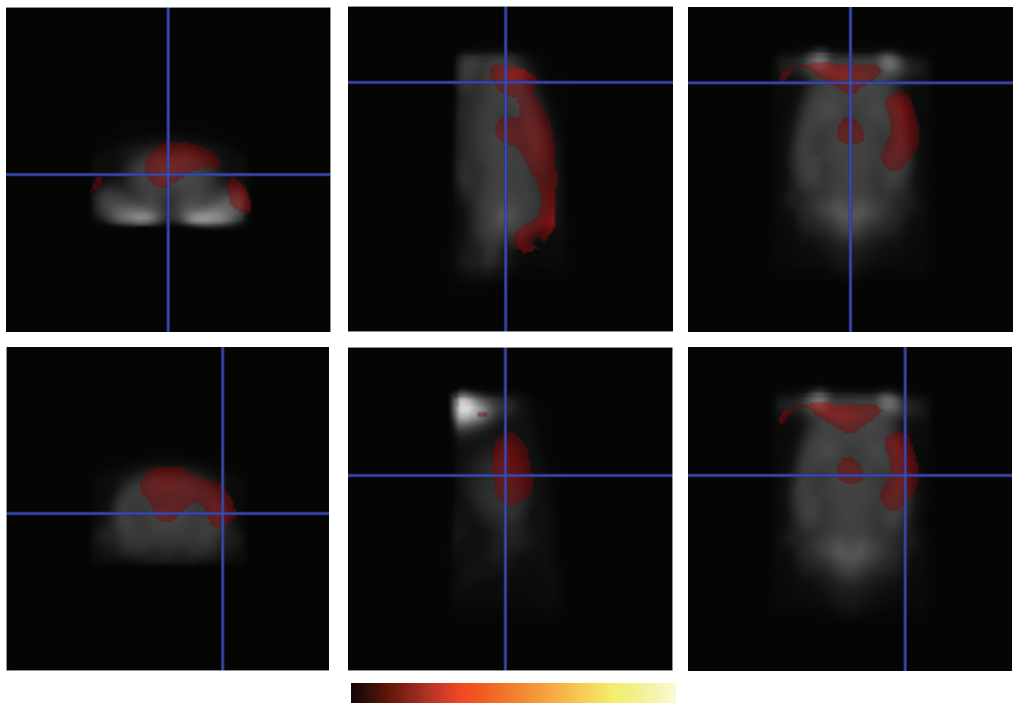
More than half (up to 75%) of the patients with cancer complain of cognitive impairment during or after treatment of their cancer. Breast cancer survivors in particular suffer



(a)



(b)



(c)

Figure 2: Continued.

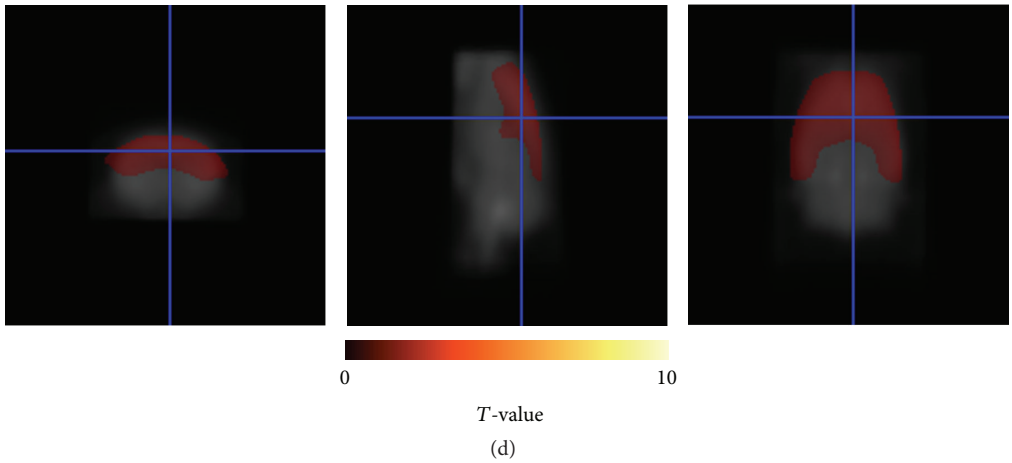


Figure 2: Brain regions showing decreased (blue) or increased (red) FDG brain uptake. T-map was overlaid on the rat brain template. Decrease in brain glucose metabolism after chemotherapy at a threshold of $P < 0.05$, uncorrected (a) in the bilateral medial prefrontal cortices for the cyclophosphamide-treated group and (b) in the left medial prefrontal cortex and bilateral hippocampi for the doxorubicin-treated group compared to normal control. Increase of brain glucose metabolism after donepezil intervention at a threshold of $P < 0.05$, uncorrected (c) in the bilateral medial prefrontal cortices, bilateral hippocampi (L > R), bilateral medial hippocampi, and bilateral parietal cortices for the cyclophosphamide-treated group and (d) in the bilateral global area of the cortices including the frontal, parietal, and temporal cortices and bilateral hippocampi for the doxorubicin-treated group, when compared with the respective chemotherapy-treated groups without donepezil intervention.

from such impairment because survival after breast cancer is prolonged compared with other cancers. Longitudinal studies and meta-analyses have revealed evidence for chemotherapy-induced cognitive deficits in a subgroup of patients with breast cancer [1, 19]. The cognitive impairment ranged over diverse domains of cognition including working memory, executive function, attention, and processing speed [2]. A number of studies have used animal models of chemotherapy to understand the mechanisms underlying these cognitive changes. Cognitive impairment in patients with cancer after chemotherapy was considered significant with the increase of the survival rates of patients after cancer treatment.

Donepezil was widely used for the improvement of cognition in clinics for Alzheimer's disease, stroke, traumatic brain injury, and normal older adults. Donepezil is a reversible acetylcholinesterase inhibitor, which can facilitate the synaptic supply of acetylcholine, enhance cholinergic neural pathways, and consequently improve cognitive function [13, 14, 16, 20, 21].

In this present study, behavioral analysis revealed that chemotherapy affected spatial learning ability, reference memory, and working memory and that donepezil improved these cognitive impairments. According to PET results, chemotherapy reduced glucose metabolism in the medial prefrontal cortex and hippocampus, and donepezil increased glucose metabolism in the bilateral frontal cortices, parietal cortices, and hippocampus. Decreased glucose metabolism was more prominent after doxorubicin treatment than cyclophosphamide treatment.

In comparison with glucose metabolism change in patients after chemotherapy, results of the present study correlate with those of earlier study in that glucose metabolism decreased in the region of prefrontal cortex for rat model and glucose metabolism decreased in the region of bilateral orbitofrontal cortex in patients. It requires caution to interpret these findings because there might be significant difference between species and different chemotherapy regimens [10].

Our results revealed cognitive improvement after donepezil intervention using both behavioral and imaging methods. This suggests that donepezil could be used in clinics for the treatment of cognitive deficits after chemotherapy. The cerebral glucose metabolic responses after donepezil intervention in the present study correspond with those reported by previous studies. Using MRI, these studies reported increased activation in the bilateral prefrontal areas, inferior frontal lobes, and the left inferior parietal lobe after donepezil administration to patients with stroke [22]. They also reported selective increases of brain glucose metabolism after donepezil treatment in patients with Alzheimer's disease [23] and increased brain glucose metabolism after donepezil administration to rhesus monkeys [24].

Our results demonstrate that chemotherapy impairs cognitive function and brain glucose metabolism and that donepezil enhances these features in a rat model of chemotherapy. The present results concur with those of earlier studies, in which cyclophosphamide and/or doxorubicin affected passive avoidance learning, novel object recognition, and memory retention [25, 26], although other studies have reported no impairment after cyclophosphamide [27] or doxorubicin [28] administration. With respect to the changes in brain metabolism and structure after chemotherapy, we previously reported decreased brain glucose metabolism in the temporal lobe and cingulate gyrus after chemotherapy in patients with breast cancer [29]. Other studies have reported cerebral white matter changes [30] in the frontal

and temporal lobes; gray matter changes [31] in the frontal and temporal lobes, cerebellum, and thalamus; and gray and white matter changes [32] in the prefrontal cortex, parahippocampal gyrus, and precuneus after chemotherapy. The mechanism of cognitive improvement after donepezil intervention is thought to involve modulation of cognitive function by restoring the shortage of intracerebral cholinergic neurotransmitters through inhibition of acetylcholine hydrolysis [33]. Acetylcholine plays a role as a modulator of the cortex in task-related cerebral plasticity and is essential for learning, memory, language, and attention [14, 22]. The result in our present study was well in accordance with those of a recent study [34] that reported a reduction in cognitive impairment after donepezil intervention in a mouse chemotherapy model; however, they employed a different chemotherapeutic regimen and their study was confined to behavioral examination.

The result in this present study collectively shows the effect of donepezil in a rat chemobrain model using behavioral and imaging analysis at the same time.

5. Conclusion

Our results suggest that donepezil may improve cognitive function and brain glucose metabolism after cognitive impairment in a rat chemotherapy model. Further clinical investigation is warranted to administer donepezil for cognitive deficits after chemotherapy.

Disclosure

The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the paper. Hye-Young Joung's current address is Asan Institute for Life Sciences, Asan Medical Center.

Competing Interests

The authors declare that there are no competing interests regarding the publication of this paper.

Authors' Contributions

Ilhan Lim and Hye-Young Joung are co-first authors and equally contributed to this work.

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Review Article

Therapies for Prevention and Treatment of Alzheimer's Disease

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Alzheimer's disease (AD) is the most common cause of dementia associated with a progressive neurodegenerative disorder, with a prevalence of 44 million people throughout the world in 2015, and this figure is estimated to double by 2050. This disease is characterized by blood-brain barrier disruption, oxidative stress, mitochondrial impairment, neuroinflammation, and hypometabolism; it is related to amyloid-β peptide accumulation and tau hyperphosphorylation as well as a decrease in acetylcholine levels and a reduction of cerebral blood flow. Obesity is a major risk factor for AD, because it induces adipokine dysregulation, which consists of the release of the proinflammatory adipokines and decreased anti-inflammatory adipokines, among other processes. The pharmacological treatments for AD can be divided into two categories: symptomatic treatments such as acetylcholinesterase inhibitors and N-methyl-D-aspartate (NMDA) receptor antagonists and etiology-based treatments such as secretase inhibitors, amyloid binders, and tau therapies. Strategies for prevention of AD through nonpharmacological treatments are associated with lifestyle interventions such as exercise, mental challenges, and socialization as well as caloric restriction and a healthy diet. AD is an important health issue on which all people should be informed so that prevention strategies that minimize the risk of its development may be implemented.

1. Introduction

Alzheimer's disease (AD) is an age-related, progressive, and irreversible neurodegenerative disorder characterized by cognitive and memory impairment, and it is the most common cause of dementia in older adults. The estimated prevalence of this disease in 2015 was 44 million people throughout the world and it is estimated that this figure will double by 2050 [1]. Most people with AD (over 95%) have sporadic or late-onset AD (LOAD), a multifactorial disease in which environmental factors and genetic predisposition contribute to the pathology [2]. The other form of AD, familial or early-onset AD (EOAD), corresponds to less than 5% of the AD population and is due to mutations in any of the three following genes: (a) the amyloid precursor protein (APP) gene on chromosome 21, (b) presenilin 1 (PSEN-1) gene on chromosome 14, and (c) presenilin 2 (PSEN-2) gene on chromosome 1 [3–5]. The classification of AD is based on clinical criteria including medical history, physical examination, laboratory tests, neuroimaging, and neuropsychological evaluation [6].

2. Pathogenesis and Clinical Features in AD

The neuropathological features of both forms of AD are characterized by the abnormal extracellular accumulation of amyloid-β peptide (Aβ) in amyloid plaques and tau protein aggregated in intracellular neurofibrillary tangles (NFTs). There are epidemiological, clinical, and experimental data that sustain several hypotheses of AD pathogenesis: (1) the amyloid cascade hypothesis proposes that the accumulation of Aβ as neuritic plaques, diffuse plaques, or oligomeric forms in the brain is the main pathogenic event [7]; Aβ plaques are composed primarily of Aβ peptides generated by the amyloidogenic pathway [1]. The amyloidogenic pathway produces amyloid peptides of 39–43 amino acids that are proteolytically derived from the sequential enzymatic action of β- and γ-secretases on amyloid precursor protein (APP) distributed in the neuron membrane [8, 9] while the nonamyloidogenic pathway produces nontoxic αAPP fragments that are generated by α-secretase action [5]; (2) the tau hypothesis suggests hyperphosphorylation of tau as the primary event [10]; (3)

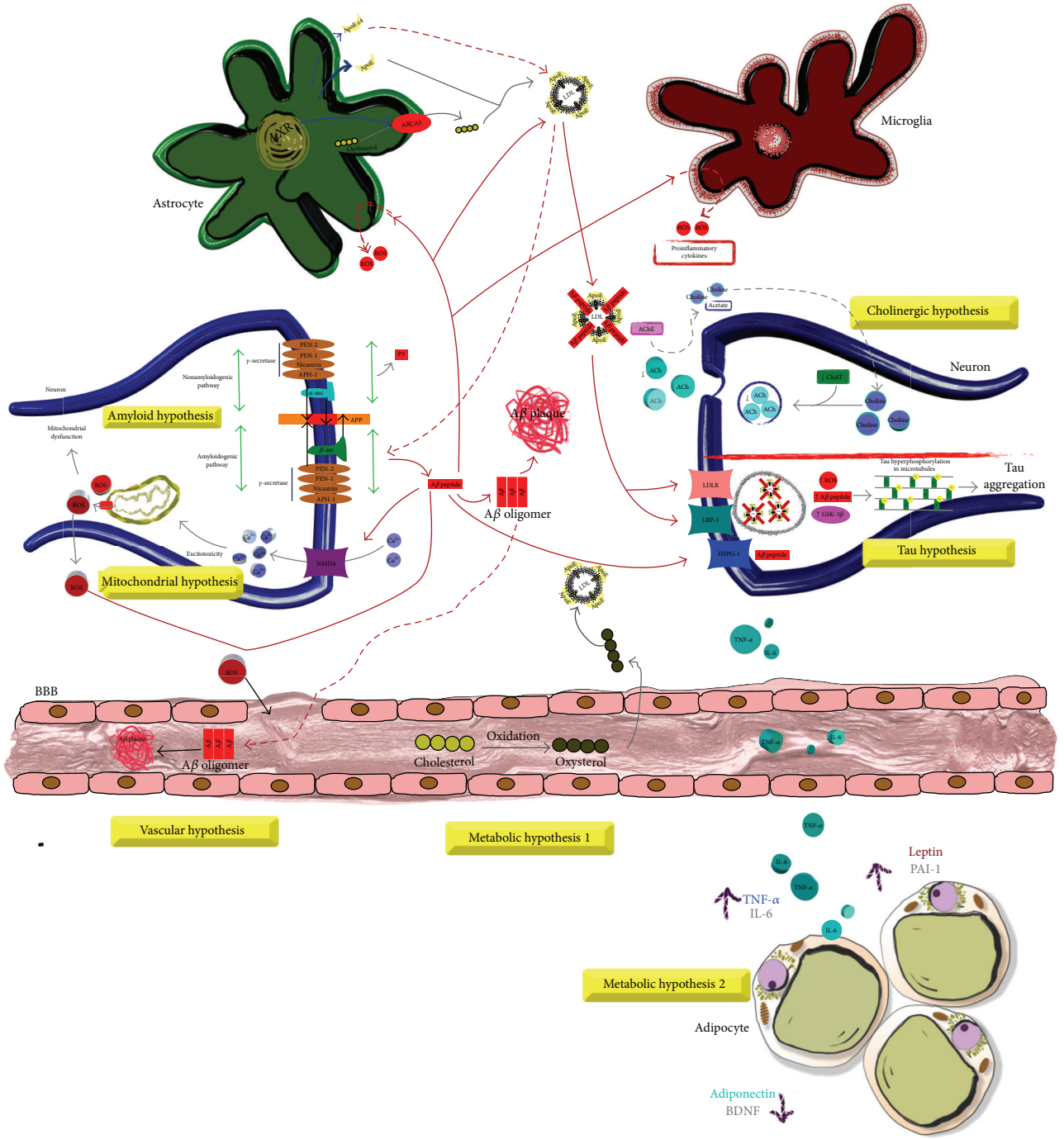

Figure 1: The etiology of AD has been classified in different hypotheses.

the cholinergic hypothesis proposes that there is a reduction in the activity of choline acetyltransferase and acetylcholine levels in areas such as the cerebral cortex [11]; (4) the mitochondrial cascade hypothesis points to impairment of brain mitochondria as the first pathogenic event leading to neurodegeneration [6]; (5) the metabolic hypothesis holds that the disease is caused by changes in metabolic processes such as obesity, diabetes, and hypercholesterolemia [12]; finally, (6) the vascular hypothesis presents the reduction of cerebral blood flow as the main characteristic [13] (Figure 1).

The clinical diagnosis of AD requires a neuropsychological evaluation according to the criteria of the Diagnostic and Statistical Manual of Mental Disorders (DSM-V criteria) and of the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS/ADRDA criteria).

Also, with the progression of the disease, laboratory tests such as oxidative stress products, Aβ levels, oxysterols including 24- and 27-hydroxycholesterol, and proinflammatory cytokines in blood and CSF [6, 7, 14], along with neuroimaging studies such as Magnetic Resonance Imaging (MRI) and Positron Emission Tomography (PET), should be performed [15]. The diagnosis is "probable AD" if cognitive impairment is shown in neuropsychological tests or "possible diagnosis of AD" if there are some positive results of biochemical and neuroimaging tests [2, 16]. It is important to note that, in most cases, but not always, impairment of cognitive domains in which the clinical diagnosis is AD correlates with the neuropathological features of *postmortem* brains with AD [2].

The disease is characterized by pathological changes, including hypometabolism [17], blood-brain barrier (BBB) disruption [13], oxidative stress, mitochondrial impairment [18], and neuroinflammation [19], which can be generated by several metabolic disorders considered strong risk factors for AD. The inflammatory response by activated microglia and astrocytes leading to the production of cytokines and reactive oxygen species (ROS) with associated neuronal damage is another important feature of AD pathogenesis [2].

2.1. Risk Factors for LOAD. To minimize the possibility of a future with a high percentage of people with AD, it is necessary to determine which are the factors that influence this disease. In recent years, a significant number of epidemiological studies related to the definition of risk factors for AD have been published. Risk factors for LOAD are classified as susceptibility genes and environmental factors [16]. LOAD has a strong genetic component, namely, apolipoprotein E (ApoE), the most widely studied genetic risk factor for AD. ApoE is produced by the liver, macrophages, and the central nervous system (CNS) [20]. In the CNS, it is produced by astrocytes and microglia; however, neuronal expression of ApoE can be induced in response to stress or neuronal damage under certain pathological conditions (stressors and injurious agents) [21].

The main metabolic and nongenetic risk factors include hypercholesterolemia [22, 23], obesity [24, 25], hyperhomocysteinemia [2], hypertension [26], and type 2 diabetes mellitus (T2DM) [27, 28].

2.1.1. Genetic Susceptibility to LOAD. Apolipoproteins are a family of proteins involved in lipid homeostasis, which bind and transport lipids through the lymphatic and circulatory systems [29]. It has been shown that ApoE has a strong relationship with the pathogenesis of LOAD [21]. ApoE is a glycoprotein of 299 amino acids and its structure varies depending on genetic polymorphisms [30]. The three major ApoE isoforms differ from each other by amino acid substitutions at positions 112 and 158 where the wild-type ε3 allele is Cys112 and Arg158, while the ApoE ε2 allele carries the Cys112 and Cys158 polymorphism, and the ApoE ε4 allele contains Arg112 and Arg158 [31]. A deficiency in ApoE can result in modifications in its structure and function [32], and an alteration of the function of ApoE results in an increase of plasma levels of cholesterol and triglycerides [29].

The ApoE ε4 allele is the most important genetic risk factor [29] and it was probably first identified as a risk factor for LOAD, by the initialization and acceleration of Aβ accumulation in the brain [33]. There are numerous studies that have replicated this association in different ethnic groups including African Americans [34], Latinos [35], Asians [36], and Caucasians [37, 38]. One study of the Chinese Han population showed that both the ApoE ε4 allele and the CYP17A1 rs743572 allele (a key regulatory enzyme in the steroidogenic pathway) increase the risk of LOAD [36]. Furthermore, a strong association has been reported between the ApoE ε4 allele and dementia due to AD pathology, but not with vascular dementia [39]; however, another study produced conflicting results showing that the ApoE ε4 allele has a strong relationship with vascular dementia through chronically degenerating white matter in the brain [40]. The mechanisms underlying the association between vascular risk factors and white matter damage are not fully understood.

Genome-wide association studies (GWAS) have identified polymorphisms in several genes that are associated with AD risk, including ABCA7 (which transports substrates across cell membranes), CLU (a stress-activated chaperone protein that functions in apoptosis, complement regulation, lipid transport, membrane protection, and cell-cell interactions), CR1, CD33 (involved in clathrin-independent receptor-mediated endocytosis), CD2AP (implicated in cytoskeletal reorganization and intracellular trafficking), EPHA1, BIN1 (involved in regulating endocytosis and trafficking, immune response, calcium homeostasis, and apoptosis), PICALM (involved in clathrin assembly), and MS4A (associated with the inflammatory response) [41].

2.1.2. Metabolic and Nongenetic Risk Factors for LOAD

(1) Hypercholesterolemia. High serum and plasma cholesterol levels have been suggested as risk factors for AD [42, 43]. In the adult brain, primary cholesterol synthesis occurs in astrocytes and in lesser proportion in neurons; cholesterol is transported into the brain by local high density lipoproteins (HDL) [22]. Low-density lipoprotein (LDL) levels are elevated in cardiovascular diseases and increased oxidation and nitration-related systemic modifications are observed in LDL (oxLDL) in hypercholesterolemia [44].

In an experimental cell-based study, cholesterol distribution within membrane is seen to have effects on APP metabolism, trafficking of APP, activities of β-, γ-, and α-secretases, and Aβ synthesis [45, 46]. The mechanism by which cholesterol deregulates Aβ metabolism has not yet been elucidated, but several studies suggest that changes in cholesterol levels alter the cell membrane [44] due to impairment of lipid rafts, which are membrane microdomains focused on protein trafficking [47], signal transduction [48], and neurotransmission [47, 49]. The γ-secretase cleavage of APP, the final step in Aβ peptide production, occurs in these cholesterol-rich lipid rafts [44].

In a recent study, it was suggested that inhibition of cholesterol biosynthesis, using AY9944, which blocks the final step of cholesterol biosynthesis, reduces γ-secretase activity

associated with the generation of Aβ peptides [50]. Moreover, low cholesterol levels increase α-secretase activity on APP [51], promoting neuroprotection by increasing levels of αAPP fragments, which are involved in neurotrophic functions [52]. In another study, it was reported that plasma cholesterol levels in AD patients were elevated by about 10% compared to control subjects [53], though these levels have been linked to the burden of ApoE [54].

The brain is capable of metabolizing cholesterol excess to oxysterols, which are the product of cholesterol oxidation [23]. Several studies have reported some oxysterols including 6-cholesten-5α-hydroperoxide, 7-oxocholesterol (7-ketocholesterol), 7β-OHC (7β-hydroxycholesterol), 7-dehydrocholesterol, 27-OHC (27-hydroxycholesterol), and 25-OHC (25-hydroxycholesterol) [14, 44, 55]. The intermediates 24-OHC and 27-OHC are commonly found in the plasma of patients with AD; thus, these metabolites are very promising as biomarkers in AD patients [56].

(2) Hyperhomocysteinemia. Increased levels of homocysteine depend on several factors such as age, genetics, lifestyle, and sex [57]. The causes of this risk factor in the population are multiple and include both nongenetic and genetic mechanisms. Deficiency of vitamin B12, folate, and pyridoxine may be responsible for hyperhomocysteinemia in the general population [58]. Pharmacological data show that homocysteine stimulates lipid accumulation [57], inflammatory processes, and N-methyl-D-aspartate receptor (NMDA) activation [59]. NMDA receptors have been shown to mediate downstream effects of the Aβ peptide in AD models and the pharmacological inhibition of this receptor's activity deletes the pathological effect of Aβ [60, 61].

(3) Hypertension. Several studies have linked hypertension to brain atrophy and the generation of NFTs; therefore, an association between hypertension and AD is conceivable [62]. However, this association is complex and differs with age. It has been shown that high blood pressure in middle age is associated with an increased risk of AD [63], while other studies found no association between hypertension in the elderly and dementia [26, 64].

(4) Obesity. Obesity is a precursor condition for numerous disorders, including hypercholesterolemia, cardiovascular disease, metabolic syndrome, and type 2 diabetes mellitus (T2DM) [17]. This is due to changes in lifestyle, for example, low levels of physical activity, an unbalanced diet, and overnutrition, leading to inflammatory and oxidative stress processes, altering the metabolic pathways necessary for homeostasis [24].

There are several studies linking obesity to increased cognitive decline and AD risk [12, 65, 66] and to central nervous system inflammation [67, 68] through an increase in proinflammatory cytokines [69]. Studies in both human and animal models suggest that particular dietary constituents may be important in modulating AD risk [70]. For example, a diet rich in fatty acids is associated with obesity and thus with a higher risk of AD [71, 72]. It was recently reported that a high-fat diet causes damage similar to that observed in Alzheimer's pathology, such as potentiation of β-secretase processing of APP [73], cognitive impairment [74], and mitochondrial damage associated with insulin resistance [75].

Numerous studies have suggested that obesity in midlife is related to a greater risk of subsequent dementia [76, 77], while in a meta-analysis of longitudinal studies it was reported that obesity in late life is not always associated with AD [66]. In contrast, a recent cohort study reported that midlife obesity (measured as body mass index or BMI) reduces dementia risk [78]. The BMI is an obesity index and some studies have shown an association between BMI and AD, with a significant increase in risk for obese individuals [76]; however, adiposity may be a more important factor and predictor of AD risk than BMI [70].

Adiposity is defined as an increase in total body mass by adipose tissue alterations [79]. Notably, the effect of adiposity on AD incidence has been associated with the consequences of chronic hyperinsulinemia on the blood-brain barrier [80]; thus, it is known that midlife obesity is one of the main factors contributing to the development of type 2 diabetes [79]. It is known that adipose tissue produces regulatory molecules called adipokines, which have autocrine, paracrine, and exocrine effects [77]. Adipokine dysregulation has been correlated with AD, producing changes in proinflammatory adipokines such as an increase in TNF-α, interleukin 6 (IL-6), and leptin; a decrease in anti-inflammatory adipokines such as adiponectin; decreased brain derived neurotrophic factor (BDNF); and increased plasminogen activator inhibitor-1 (PAI-1) and angiotensin (AGT) [81, 82]. Adipokines are able to cross the BBB and activate their specific receptors in central nervous system regions such as the hippocampus [81]. The most important adipokines mentioned in the literature with regard to AD are leptin and adiponectin.

Leptin is a 16 kDa adipocyte-derived hormone that is secreted in proportion to the adipose stores, with high circulating plasma levels in obesity [83] resulting in leptin resistance, which boosts tau phosphorylation, in turn increasing Alzheimer pathology [84]. In physiological conditions, leptin generates a reduction of body weight by suppressing appetite or increasing energy balance. Several studies have demonstrated that leptin has beneficial effects through the modulation of memory by regulating both long-term potentiation and synaptic plasticity [79]. In a study of peripheral blood adipokines in patients with AD, it was reported that low leptin levels increase AD risk [85]. However, other studies have reported that high leptin levels are associated with a high risk of dementia [86]. Existing data provide evidence that obesity may interfere with the neuroprotective effect of leptin on the brain, possibly by leptin resistance [87]. Leptin resistance is induced by several defects involving the leptin receptor, in BBB transport, cellular transduction, the induction of feedback inhibitors, and biological systems with changes in cellular networks [84]; overactive leptin receptor signaling results in its intense phosphorylation via the (Janus kinase) JAK-2/STAT-3 pathway, which improves suppression of cytokine signaling 3 (SOCS-3) expression [88]. SOCS-3 is a feedback inhibitor of leptin signaling and has been associated with leptin resistance in obesity. Interestingly, leptin signaling

downstream effectors such as adenosine monophosphatase-activated protein kinase (AMPK) and mammalian target of rapamycin (mTOR) regulate the activity of glycogen synthase kinase 3β (GSK-3β) and BACE1, which are involved in tau phosphorylation and Aβ genesis [12, 89].

In addition to its effects on the AMPK pathway, leptin signaling results in the activation of the energy sensor sirtuin-1 (SIRT1), reducing the acetylation of the p65 subunit of NF-κB and leading to a reduction of tau phosphorylation and of $A\beta_{1-40}$ production [89–91]. Moreover, it has been reported that AMPK and SIRT brain activity is inhibited by prolonged states of positive energy balance, as in obesity [92]. Leptin, as a trophic factor, activates different cascades and, for example, is critical in spine formation in hippocampal neurons; in culture, this is promoted through the phosphorylation of calcium/CaM-dependent kinase γ (CaMKI γ and β-pix), which are required for the trafficking of transient receptor potential-canonical (TrpC1/3) channels to the membrane [93]. Hence, leptin resistance and aging resulting in low levels of this protein are correlated with cognitive impairment and participation by other cytokines.

The adipokine adiponectin is a 30 kDa protein released by adipose tissue and the most abundant adipokine in plasma [94]. Unlike leptin, it is inversely correlated with fat mass; that is, circulating adiponectin levels decrease with increasing adiposity [83]. Adiponectin is a sensor of insulin and can induce body weight loss [95] and insulin resistance, with high levels of tumor necrosis factor (TNF) in plasma and adipose tissue [96]. Adiponectin has been found to modulate some brain functions such as memory and to produce a neuroprotective effect on hippocampal neurons [97, 98]. Furthermore, adiponectin levels are inversely associated with insulin resistance, obesity, type 2 diabetes, and AD. The mechanisms involving adiponectin in the brain are still unclear; however, many epidemiological studies have reported that, for both AD patients and T2DM patients, plasma adiponectin levels are significantly lower than those of healthy individuals [98], although a recent study did not produce the same results [99].

Adiponectin and TNF-α inhibit each other's production in adipose tissue, and adiponectin does the same with IL-6, in addition to modulating inflammatory responses, inhibiting the NF-κB-induced pathway [81, 82]. An increase of NF-κB activity in inflammatory status promotes aspartyl protease β-site APP-cleaving enzyme (BACE1) synthesis, thus enhancing APP cleavage, and Aβ genesis where low adiponectin levels are associated with AD [82, 100].

The increase in proinflammatory adipokine TNF-α levels promotes neuroinflammation as well as inhibition of neuronal proliferation and differentiation [101]. The mechanism involved in these neurotoxic effects is as follows: increase in the production of other cytokines such as IL-6 and leptin, increase of AMPA receptor activity as well as secretases by the nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor (IκB) degradation pathway, and overactivation of NF-κB, which may stimulate APP production [81, 102].

The interleukin IL-6, also associated with AD, induces inflammation, inhibition of neurogenesis, and decrease of synaptic plasticity in the hippocampus by triggering neuroinflammation via STAT3, exerting cAMP response element-binding (CREB) protein downregulation by Akt inhibition or by the activation of transcription factors, which compete for a limited pool of coactivators such as STAT-1, c-Jun, and NF-κB, promoting hypocholinergic signaling [103–105].

A member of the neurotrophin family, BDNF, is expressed in adult neurons at high levels throughout the CNS [106] and a decrease thereof has been reported in obesity [107]. Low levels of BDNF have been related to many diseases such as AD because such levels induce a decrease in mitochondrial biogenesis, neuronal survival, and plasticity associated with a deficiency in the TrkB signaling receptor by different pathways: MAPK, a regulator of neuronal differentiation and maturation; PI3K, important in neuronal survival and synaptic protein formation; and PLCγ, which induces the release of intracellular Ca^{2+} related to synaptic plasticity [108]. Decreased BDNF also induces apoptosis in neurons by the inhibition of the antiapoptotic family Bcl-2 and the expression of the proapoptotic proteins Bax and Bad, promoting mitochondrial biogenesis [109].

The adipokines related to vascular health are PAI-1 and AGT, which are involved in cell migration and are related to learning and memory process. PAI-1 can be produced by adipocytes, microglia, and astrocytes; the increase of this adipokine in obesity is related to inflammation (an AD risk factor) and fibrinolysis. Moreover, PAI-1 has also been discussed as regards its possible neuroprotective effect associated with the MAPK/ERK pathway [81].

AGT is produced by the liver and white adipocyte tissue in order to increase blood pressure. Increased AGT has been reported in obesity and is considered a risk factor for AD because it increases blood pressure and inflammation [110].

(5) Type 2 Diabetes Mellitus (T2DM). Type 2 diabetes mellitus (T2DM) is another prevalent disease associated with obesity and aging, and it is considered an independent risk factor for AD [111]. T2DM and obesity are diseases that affect millions of people worldwide [28, 112]. T2DM is characterized by hyperglycemia resulting in production of increased hepatic glucose, impairment of insulin production by pancreatic β-cells, and insulin resistance [27]. Glucose is the only required source of energy for neurons and any disruption in glucose metabolism leads to compromised neuronal functions [113].

The proposed scenarios between diabetes and dementia are numerous; they include vascular lesions, inflammation, oxidative stress, elevated end products of glycolysis, insulin resistance, abnormal insulin receptor signaling, and degradation of insulin and its relation to Aβ protein deposits [114, 115]. Interestingly, both pathologies present amyloidogenesis that forms Aβ plaques [28]. High glucose levels and insulin resistance have a likely impact on oxidative stress pathways and neuroinflammatory signals in the brain, thereby connecting diabetes to neurodegeneration [112]. Furthermore, many researches sustain the hypothesis that AD responds to neuronal pathogenic energy imbalance produced by impairment in the function of glucose [116].

Insulin is a relevant molecule in the regulation of metabolism and energy expenditure. In the brain, insulin

is considered a paracrine/autocrine effector, binding to insulin receptors (IRs) and activating the IR substrate (IRS) in two canonical pathways, the phosphoinositide-3 kinase (PI3K)/Akt and the Ras/mitogen-activated kinase cascades [117]. Central insulin is considered to regulate structural and functional aspects of synapses, and neuron-specific insulin receptor knockout (NIRKO) in mice, producing defects in Akt-Foxo3, an insulin signal, develops insulin resistance and causes increased activation of GSK-3β and tau hyperphosphorylation [118, 119]. Insulin resistance impairs IR/PI3K/Akt/mTOR insulin signaling, promoting decreased GLUT4, AMPA, and NMDAR exportation to the membrane. Taken together, all these events result in glutamate neurotransmission and long-term potentiation (LTP) dysfunction and tau hyperphosphorylation [117]. It is also important to note that Aβ peptide can bind to the IR and also to ApoE; ApoE itself binds to the IR, and the different interactions between ApoE isoforms suggest that the ApoE ε4 genotype could lead to earlier impairment of brain insulin signaling [120].

(6) Gastrointestinal Microbiota. Microbiota composition changes have been related as factor risk for several diseases such as obesity, atherosclerosis [121], and T2DM [122] in addition to gastrointestinal disease. More recently, microbiota has been involved with AD due to the possible infectious etiologies of neurogenerative diseases [123]. In this context, previous studies reported associations between *Chlamydophila pneumoniae* [124] as well as herpes simplex virus infection and AD [125]. The possible mechanisms that link microbiota to AD include (1) interactions between the gut microbiota and the CNS in a "microbiota-gut-brain axis" [123], which modify immune response, enhancing response to cerebral Aβ [126], (2) microbiota that could promote prion-like behavior of amyloid proteins leading to neurodegeneration [127], and (3) microbiota changes during aging such as the increase in the proportion of Bacteroidetes to Firmicutes as well as the reduction of bifidobacterial counts [128], which decrease the synthesis of proinflammatory cytokines [129]. Finally, epidemiological links between oral bacteria and AD have been reported [121], due to the increase of TNF-α production [130]. Interestingly, oral and gut microbiota can be modified by diet.

3. Pharmacological Treatment

Alzheimer's disease requires precise diagnosis, early if possible, and adequate etiological treatment, and, as an incurable age-related neurodegenerative disorder, its particular pathophysiology needs to be considered. The therapeutic options have focused on ameliorating the symptoms as well as reducing the rate of progression of damage, although this has not significantly reversed the disease, so prevention is a better solution for this public health problem [4, 131].

The toxic conformations of Aβ or tau in the brain are thought to spread the disease, and blocking the generation of these peptides may be part of useful treatments. Nevertheless, the current treatments of this disease are based on cholinesterase inhibitors and a glutamate antagonist, providing only symptomatic relief, while evidence for the complexity and multicausality of this dementia is recognized in basic and clinical studies [132]. Efforts in etiology-based treatment are currently underway in clinical trials, as well as complement preventive treatments such as physical activity, proper diet, cognitive stimulation, and the management of comorbidity [133].

3.1. Symptomatic Treatment

3.1.1. Acetylcholinesterase Inhibitors. It is well known that acetylcholine (ACh) plays a crucial role in mediating learning and memory [134]. Furthermore, direct interaction between Aβ and cholinergic systems has been proposed, with negative feedback to the production of the peptide; it has been suggested that the alteration in this negative feedback loop and abnormal accumulation of Aβ reduced cholinergic transmission effectiveness, focused on alpha-7 nicotinic acetylcholine receptors [135, 136].

On this basis, effective treatment for AD is achieved with cholinesterase inhibitors, which corresponds well to Davies and Maloney's early cholinergic deficit hypothesis (1976) explaining AD pathophysiology. Tacrine, donepezil, rivastigmine, galantamine, xanthostigmine, para-aminobenzoic acid, coumarin, flavonoid, and pyrroloisoxazole analogs have been developed and studied for the treatment of AD. Rivastigmine, donepezil, and galantamine are the approved drugs that promote higher ACh levels and improve the brain's cholinergic function by inhibiting the enzyme acetylcholinesterase which degrades the neurotransmitter [137–139]. In general, acetylcholinesterase inhibitors (except tacrine) are well tolerated and adverse effects are dose-related [4]. The acetylcholinesterase inhibitor ladostigil (TV3326) is in phase II clinical trials and it also produces antidepressant effects for the inhibition of monoamine oxidases A and B [140].

3.1.2. N-Methyl-D-aspartate Receptor (NMDA) Antagonist. Glutamate-mediated excitotoxicity is known to result in calcium overload and mitochondrial dysfunction, with increased nitric oxide generation, which can be detrimental to cells, forming high levels of oxidants and eliciting neuronal apoptosis. This overstimulation can be blocked by NMDA receptor antagonists such as memantine, which was approved in 2003 by the Food and Drug Administration (FDA) for the treatment of moderate-to-severe AD, with a marginal beneficial effect on cognition in mild-to-moderate AD [4, 141, 142].

Memantine can protect neurons by attenuating tau phosphorylation through a decrease in glycogen synthase kinase 3β (GSK-3β) activity. This noncompetitive glutamatergic NMDA receptor antagonist can be administered alone or in combination with an acetylcholinesterase inhibitor [143], although there may be few significant favorable changes in the combination therapy [137].

3.1.3. Other Neurotransmitter Systems. Muscarinic and nicotinic ACh receptors are also considered targets for AD treatment, although selectivity of the agonists has been a problem outcome in clinical trials. EVP-6124 is currently in phase II trial [131].

Based on the cholinergic hypothesis and NMDA glutamate participation in AD, it is natural to consider the different

neurotransmitter networks, particularly of the hippocampus. Serotonin receptors are expressed in areas of the CNS involved in learning and memory. The inhibition of 5-HT_6 serotonin receptors was shown to promote acetylcholine release, and some compounds are in various stages of clinical research, considered as possible treatments for mild-to-moderate AD [140].

Histamine receptors, particularly H_3 receptors, are also present in large amounts in memory- and cognition-related structures in the brain. It seems that H_3 receptor antagonists may improve cholinergic neurotransmission. Phase I and II studies with H_3 antagonists are currently being conducted [139].

3.2. Etiology-Based Treatment. As indicated above, ApoE $\varepsilon 4$ is the major genetic risk factor for sporadic AD (the major risk factor is age), although, for disease-modifying treatment based on the amyloid cascade hypothesis, efforts are targeting secretase modulation and amyloid binders, as well as targeting kinases involved in the hyperphosphorylation of tau protein [131, 132, 140].

3.2.1. Secretase Inhibitors. APP is first cleaved either by α-secretase or by β-secretase enzymes, and the resulting fragments are processed by γ-secretase. The proposal of the "overactivation" of β- and γ-secretases, or age-related decreased α-secretase processing, has led to the use of inhibitors for this amyloidogenic pathway [144].

Several metalloproteinases have been studied with α-secretase activity. The upregulation with gemfibrozil (PPAR-α agonist) of the α-secretase "A disintegrin and metalloproteinase" 10 (ADAM10) has been proposed as a good strategy for the prevention of Aβ generation [145]; melatonin also stimulates the nonamyloidogenic processing of APP through positive transcriptional regulation of ADAM10 and ADAM17 [146] and stimulation with serotonin 5-HT_4 receptor agonists regulates α-secretase activity [147]. Overexpression of matrix metalloproteinase 9 (MMP-9, another α-secretase) also prevents cognitive deficits displayed by the transgenic AD mouse model harboring five familial AD-related mutations (5xFAD) [148].

The transmembrane aspartyl protease BACE1 has inhibitors proposed with a molecular docking-based approach for the inaccessible catalytic center that initially led to unsuccessful trials [131, 149, 150]. BACE1 plays an important role in the metabolism of myelination proteins; however, its inhibition displays less severe side effects than other ADAM proteases. Few compounds have reached clinical trials, the most promising being Merck Sharp & Dohme's MK-8931 (Verubecestat) and Eli Lilly/Astra-Zeneca's AZD3293 (LY3314814), in phase II/III trials NCT01739348 and NCT02245737, respectively [139, 140]. Flavonols and flavones, especially myricetin and quercetin, have exhibited very good cell-free BACE1 inhibitory effects [131].

γ-secretase is a transmembrane multisubunit protease complex, composed of presenilin 1, nicastrin, anterior pharynx defective-1 (APH-1), and presenilin 1 enhancer-2 (PEN-2), and it is involved in the proteolysis of many intramembranous signaling proteins. There have been many studies with γ-secretase inhibitors, which induced significant side effects, including gastrointestinal disorders and increased risk of skin cancer. One of its substrates is Notch protein, which regulates cell proliferation, differentiation, and growth; γ-secretase Notch-sparing inhibitors were designed, although results in clinical trials are not very promising [139]. It seems that α-secretase activity is needed to prevent Aβ peptide formation and its age-related downregulation may be compensated by dietary changes including several antioxidants that activate the promoter of the ADAM proteases involved; γ-secretase activity is also needed before β-secretase reaches APP in order to prevent Aβ, since evidence for genetic defects in γ-secretase (PSEN-1 and PSEN-2) as major risk factors for familial AD is conclusive. This may explain why the use of γ-secretase inhibitors has failed in early trials but modulators of this complex have better expectations.

The toxicity of γ-secretase inhibitors depends on other signaling pathways activated by other cleaved receptors, including Notch receptor [150]. It has been shown that a γ-secretase inhibitor, but not a γ-secretase modulator, induces defects in BDNF axonal trafficking and signaling [151]. These modulators have effects on the Aβ cleaving site generator without affecting other cleaving sites of the complex [152, 153].

Hypercholesterolemia is considered a risk factor, as stated above, and also the participation of cholesterol in secretase activity has been discussed, but it is more important to recall that many acidic steroids are γ-secretase modulators that selectively decrease Aβ42; cholestenoic acid, a cholesterol metabolite, is one of these endogenous modulators [154]; the regulation of these endogenous metabolites may be involved in obesity-induced AD (including other risk factors such as dyslipidemia and metabolic syndrome).

3.2.2. Amyloid Binders. The deposition of Aβ in AD is concentration-dependent; increased amyloidogenic processing of APP and inefficient removal of peptides may be involved in the pathology. There is reduced activity of Aβ-degrading enzymes, such as neprilysin, an insulin-degrading enzyme, as well as the ApoE determinant, which correlates well with the proposal of AD as a metabolic disorder [140].

Preventing the formation of Aβ extracellular neuritic (senile) plaques is one of the targets for disease-modifying treatment in AD, although there is evidence of correlation with Aβ biomarkers and cognitive deficits, previous to senile plaques. Inhibitors of Aβ aggregation have reached clinical trials [140]. In addition, amyloid-β-directed immunotherapy includes several biological products involving probable sequestration of soluble monomeric Aβ (solanezumab) or microglia-mediated clearance (bapineuzumab, crenezumab, gantenerumab, aducanumab, and BAN2401) currently in clinical trials [132]. However, active and passive immunization may involve side effects with neuroinflammation, which is considered in itself to explain the pathophysiology of AD, and anti-inflammatory agents for treatment of AD might be considered as well.

3.2.3. Anti-Aβ Aggregation Compounds. In recent decades, research has focused on developing therapies in which the Aβ peptide formation or its aggregation is prevented. Among

the small molecule inhibitors of Aβ aggregation in clinical trials are tramiprosate (phase III), clioquinol (phase II), scyllo-inositol (phase II), and epigallocatechin-3-gallate (phase II/III); although these drugs have achieved stabilization of the Aβ monomers, they have important side effects [155]. Also, synthetic β-sheet breaker peptides of the iAβ5p sequence such as azetidine-2-carboxylic acid, 3-phenyl azetidine-2-carboxylic acid, β-proline, and β-sulfonylproline modulate the cell damage caused by the Aβ exposure by preventing fibril formation and they have shown improved results with regard to spatial memory [156, 157]. Stemazole has been shown to protect SH-SY5Y cells from toxicity induced by Aβ *in vitro*, reducing Aβ aggregation [158]. Likewise, compounds such as curcumin, T718MA, and SK-PC-B70M protect neurons from Aβ-induced toxicity [156].

3.2.4. Tau Therapies. Prevention of aggregates of paired, helically twisted filaments of hyperphosphorylated tau in neurofibrillary tangles is one of the targets of this therapy. Immunotherapy has been developed; AADvac1 was the first vaccine in clinical trials, and ACI-35 (another liposomal-based vaccine) trials have begun [139].

Inhibitors of the phosphorylation of tau proteins such as tideglusib, an irreversible GSK-3β inhibitor, have been tested with no statistically significant benefits [139]; cyclin-dependent kinase 5 (CDK5), which is also involved in the hyperphosphorylation of tau proteins, has been considered as a possible drug target [140].

Several molecules have been shown to act as good inhibitors of tau aggregation and are in clinical trials. Among these drugs, methylene blue (MB) and its metabolites azure A and azure B are able to promote protein degradation and inhibit caspase-1 and caspase-3 activity [159]. Similarly, leucomethylthioninium with a suitable counterion (LMTX in phase III clinical trials) and methylthioninium chloride or MTC (phase II clinical trial) have been shown to reduce tau aggregation and reverse behavioral deficits in transgenic mouse models [160] and to slow the progression of the disease in patients with AD [10]. However, the exact mechanisms by which LMTX and MTC generate neuroprotective effects *in-vivo* are not completely understood. Other promising inhibitors of tau aggregation are N-phenylamines, anthraquinones, phenylthiazolyl-hydrazides, rhodanines, benzothiazoles, and phenothiazines [161].

3.2.5. Other Therapies. As an age-related pathology, AD is correlated with other chronic-degenerative disorders, and coordinated therapies are needed. A type 3 diabetes hypothesis of AD has been developed, and intranasal insulin is included as a possible treatment for the disease, due to its ability to penetrate the brain-blood barrier [139].

Elevated low-density lipoprotein (LDL) concentration increases the risk of developing AD but the use of statins as a protective treatment is controversial [162, 163]. Dyslipidemia and obesity are considered causative factors in relation to other pathologies such as metabolic syndrome, which includes atherogenic dyslipidemia and central obesity, hyperglycemia and insulin resistance, hypertension, and a prothrombotic state and a proinflammatory state [164, 165]. Statins may prevent dementia due to their role in cholesterol reduction, although there is evidence that statins given in late life to people with a risk of vascular disease do not prevent cognitive decline or dementia [166]. There is a reduction in cholesterol levels in the diabetic brain, as well as in neuron-derived cholesterol content, which affects receptor signaling [167], so the use of statins in AD treatment should be with consideration to the early management of the disease.

In addition, drugs used in the treatment of type II diabetes mellitus may have a neuroprotective effect in AD. Amylin and glucagon-like peptide-1 receptor agonist are also under study as AD treatments [139].

Finally, the mitochondrial cascade hypothesis includes oxidative stress, a state of lost balance with overproduction of oxidative free radicals as well as reactive oxygen species (ROS) and reactive nitrogen species (RNS) [141, 168]. This imbalance also includes the participation of immune cells and NO signaling, and preventive treatment with antioxidants (see Nonpharmacological Treatments) and anti-inflammatory drugs is considered [169]. What is certain is that prevention is our best strategy for AD, with efforts to prevent obesity and chronic-degenerative disorders.

4. Nonpharmacological Treatments

Nonpharmacological treatments are important for the prevention of AD or as adjuvants in other treatments. AD prevention strategies can be divided into two groups, the first associated with lifestyle and the second with diet and chemical compounds.

4.1. Lifestyle. Lifestyle strategies include physical activity, mental challenges, energy restriction, and socialization as preventive factors in AD [170]. Physical activity such as aerobic exercise was associated with the reduction of AD deficits in a cohort study [171]. This was not consistent with studies that considered a small number of cases [172].

Exercise was reported to enhance hippocampal neurogenesis [173, 174] and learning in aging rodents [175]. The three mechanisms proposed in order to explain the exercise neuroprotective effect of exercise are (1) the release of neurotrophic factors such as BDNF and insulin-like growth factor (IGF-1), nerve growth factor (NGF), and vascular endothelial growth factor (VEGF) [138, 176] from neurons in synaptic activity, which stimulates neurogenesis and synaptic neural plasticity through the stimulation of CREB transcription factor; (2) the reduction of free radicals in the hippocampus as well as the increase in superoxide dismutase and endothelial nitric oxide synthase [176]; (3) peripheral signals that help to support the demands of active neuronal networks such as BDNF release in addition to energy restriction on the brain [109, 177–179].

It has been proposed that mental challenges may protect against cognitive decline and probably against AD [180]. Computer courses and psychoeducation have moderate beneficial effects [181]. Stimulation by cognitive activities has been associated with an increase in neuronal density, which provides brain reserve and plasticity [170].

The relation between caloric restriction and brain motivation is important since many years ago humans needed to

obtain their food by killing wild animals and often vigorous exercise was required [182]. In different AD mouse models treated with food and caloric restriction, a decrease in phosphorylated tau and amyloid-β was observed in the brain. The possible mechanism may be associated with SIRT1, a protein with nicotinamide adenine dinucleotide-dependent deacetylase or adenosine diphosphate-ribosyltransferase activity [183], because its increase was reported in p25 CK mice with characteristics similar to AD. In addition, SIRT I stimulation by resveratrol induces neuronal death protection. SIRT1 levels also increase with NADp *in vitro*, and SIRT induces an increase of α-secretase and a decrease of β amyloid deposition in primary cultures in a mouse model of AD [184]. The relationship between hunger and neuroprotection was induced by ghrelin in a mouse model of AD; the results indicated improved cognition in the water maze test and a decrease in amyloid-β levels and inflammation [185].

Socialization is important to mental and physical human development and a lack thereof induces loneliness, which has been associated with various diseases such as depression, alcohol abuse, obesity, diabetes, hypertension, AD, and cancer [186].

4.2. Diet and Chemical Substances. Dietary supplements for prevention of AD were studied with vitamins such as B6, B12, folates, and E, C, and D vitamins. Vitamin B studies produced mixed results; on one hand, a two-year treatment with homocysteine and vitamin B in 271 patients indicated a significant difference compared to placebo in whole brain atrophy [187, 188], whereas other reports indicate different results [189, 190]. It has been proposed that folic acid has neuroprotective activity through an epigenetic mechanism that inhibits amyloid-β peptide accumulation. Studies with 2000 IU of vitamin E did not indicate a protective effect for AD with three years of treatment [191], nor with the combined treatment with vitamin C [192]. Additionally, vitamin D supplementation improves cognitive performance [193].

With regard to the intake of chemical substances, the results in alcohol studies indicate an association between the prevention of AD and low levels of red wine consumption [194] due to its polyphenols composition, whereas drinking alcohol frequently was associated with a risk of dementia [195]. Different molecules have been proposed for their neuroprotective effect, including glucosamine, omegas 3 and 6 which induce interleukins or prostaglandins for inflammatory responses [196], and antioxidants such as β-carotene and lycopene 6 [197].

Other studies of chemical substances related to possible protection against neuropsychiatric disorders such as AD were those related to the intake of plants and their secondary metabolites: flavonoids, alkaloids, or terpenoids [198, 199]. Flavonoids are considered safe [200] and their neuroprotection was confirmed in 90 people treated with flavanol [201]. Flavonoids also inhibit acetylcholinesterase and improve memory [202], in addition to inhibiting glutamate release [203].

Resveratrol is a polyphenol found in various plants, especially berries, peanuts, and red grapes, as well as in red wine [204]. This polyphenol has shown various biological activities such as antioxidant, anti-inflammatory, phytoestrogen, vasodilator, cardioprotective, and anticarcinogenic activities, while many studies have proposed resveratrol as a molecule with therapeutic potential in neurodegenerative diseases such as AD [205]. Neuroprotective functions of resveratrol in the pathogenesis of AD have been evaluated through different mechanisms of action. The neuroprotective effects of resveratrol have been associated with the modulation of transcription factors NF-κB, cAMP, p53, and cyclins, as well as an increase in BDNF among others related to mitochondrial biogenesis, oxidation of fatty acids, and suppression of proinflammatory molecules [206].

One mechanism by which resveratrol generates anti-inflammatory effects is by proinflammatory cytokine activity inhibition (IL-1β, IL-6, and TNF-α) and prostanoid synthesis, principally prostaglandin E2 (PGE2) [207]. Furthermore, it has been shown that resveratrol has the ability to activate sirtuin, particularly SIRT1, leading to the protection of neurons from apoptotic processes and oxidative stress [208]; it has also been demonstrated that activation of SIRT1 induced by resveratrol reduced NF-κB signaling pathway activation in glial cells exposed to Aβ [209]. Under normal cellular conditions, SIRT1 is activated by AMPK, and then activated SIRT1 is deacetylated to transcription factors such as peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1-α), which translocates to the nucleus and interacts with peroxisome proliferator-activated receptor (PPAR-γ) to enhance gene expression, which promotes cell survival and the proper functioning of mitochondria [206, 210].

Another flavonoid is luteolin which has been reported to exhibit significant action in AD prevention associated with its antioxidant, anti-inflammatory, and microglia-inhibiting effects along with improved spatial memory [211]. Luteolin also inhibits multiple transduction signals such as NF-κB, PKC, STAT3, and intracellular calcium [212, 213].

The Mediterranean diet may improve neuroprotection because it is based on low intake of saturated fatty acids, but high consumption of unsaturated fatty acids, as well as vegetables, legumes, fruits, fish, and olive oil, along with polyphenols such as oleuropein aglycone (OLE), which interfere with amyloid aggregation, and reduced the LDL cholesterol levels. The monosaturated fatty acids have been reported with antioxidant and anti-inflammatory effects, as well as endothelial function improvement and less cognitive decline, whereas polysaturated fatty acids are important in neuronal membrane integrity and function; omega 3 was related to gene expression that might influence the inflammatory process, nerve membranes neuroplasticity, and synaptic transmission [214–218]. Another diet related to neuroprotection against neurodegenerative diseases is the Asiatic diet, because it includes high levels of green tea consumption, the antioxidant curcumin, and the dietary supplement *Gingko biloba*, considered to be a protector against memory decline, due to its antioxidant effect and the decrease of Aβ aggregation; it is necessary to increase the research in order to know its toxic effects [138]. On the other hand, the western diet is considered as a risk factor for AD because it is characterized by excessive consumption of sugar and animal

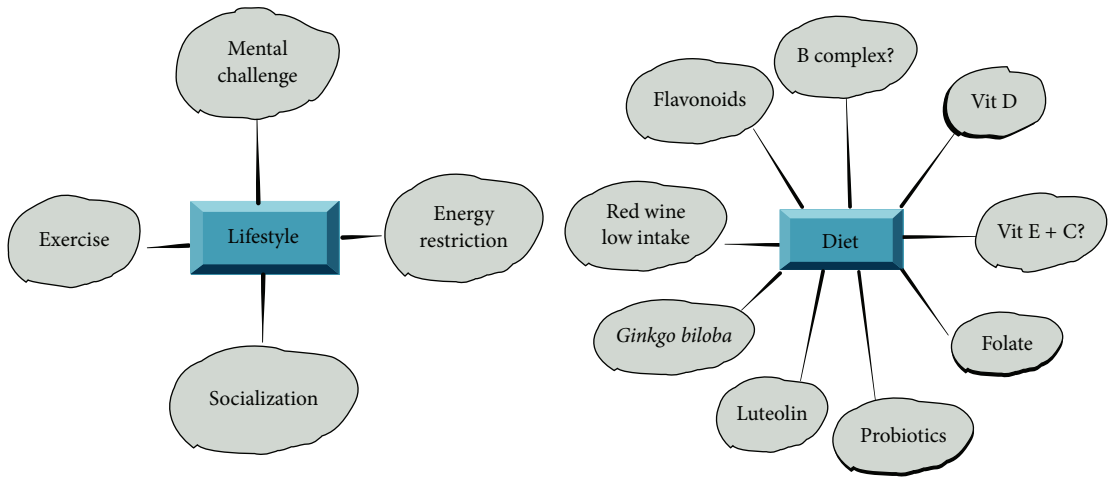

Figure 2: Nonpharmacological treatments.

products, with a higher content of saturated fats, which negatively affect cognitive function, Aβ-deposition, and oxidative stress [218].

OLE is an important compound with neuroprotective effects because it interferes with amylin, tau, and Aβ peptide aggregation and toxicity *in vitro*, studied by behavioral, biological, biophysical, biochemical, and electrophysiological techniques. OLE also has pharmacological activities such as cardioprotective, antioxidant, anticancer, antimicrobial, and antiviral effects; this compound also prevents low-density lipoprotein oxidation and platelet aggregation, inhibiting eicosanoid production, and it produces an effect against metabolic syndrome: obesity and type 2 diabetes and hepatic steatosis induced by a high-fat diet in mice, probably associated with WNT expression downregulation of the inhibitor genes and toll-like receptor and of the proinflammatory cytokines. OLE also downregulates several transcription factors and their target genes involved in adipogenesis and upregulation genes such as β-catenin [214].

Another nonpharmacological treatment could be the intake of probiotics due to their reduction of the proinflammatory cytokines associated with gut microbiota changes during aging [128] (Figure 2). Probiotics administration in the elderly may improve gut health and boost anti-inflammatory activity [219], since the "microbiota-gut-brain axis" can decrease the neuroinflammatory process. Furthermore, the beneficial effects of probiotics in AD have been associated with their production of metabolites by fermentation, including short-chain fatty acids (SCFAs) such as propionic and butyric acids [220]. A recent study reported a neuroprotective effect of *Clostridium butyricum* which restored brain levels of butyrate in a mouse model of vascular dementia [221]. Probiotics increase intestinal barrier integrity by activating epithelial cells protecting against pathogens [222]. In addition, previous work showed downregulation of TNF-α levels and an increase in IL-10 production resulting from the administration of *Lactobacillus rhamnosus* [223]. It is important to note that the intake of probiotics, such as *Lactobacillus plantarum*, may also induce behavioral changes, through monoamine neurotransmitter augmentation [224].

AD is a multifactorial disease and the combination of two or more nonpharmacological treatments for prevention is important, in addition to pharmacological treatments.

AD should be considered at early ages in order to avoid risk factors, and, for elderly individuals, increasing treatment to a combination of two or more nonpharmacological treatments strengthens prevention. In cases where dementia is present, it is important to improve treatments by adding lifestyle and dietary changes. AD research needs to be further developed in order to propose new molecules for therapy and prevention.

Competing Interests

The authors declare that they have no competing interests.

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Research Article

Homocysteine Levels in Parkinson's Disease: Is Entacapone Effective?

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Plasma homocysteine (Hcy) levels may increase in levodopa-treated patients with Parkinson's disease (PD) as a consequence of levodopa methylation via catechol-O-methyltransferase (COMT). Results from previous studies that assessed the effect of COMT inhibitors on levodopa-induced hyperhomocysteinemia are conflicting. We aimed to evaluate the effects of levodopa and entacapone on plasma Hcy levels. A hundred PD patients were enrolled to the study and divided into three treatment groups (group I: levodopa and/or dopamine agonists; group II: levodopa, entacapone, and/or a dopamine agonist; and group III: dopamine agonist alone). We measured the serum B12, folic acid, and Hcy levels in all patients. There were no statistically significant differences between groups in terms of modified Hoehn and Yahr stages, Unified Parkinson's Disease Rating Scale II/III, Standardized Mini-Mental Test scores, and serum vitamin B12 and folic acid levels. Plasma median Hcy levels were found above the normal laboratory values in groups I and II, but they were normal in group III. However, there was no statistically significant difference in plasma Hcy levels between groups. Our results showed that levodopa treatment may cause a slight increase in the Hcy levels in PD compared with dopamine agonists and that COMT inhibitors may not have a significant effect on preventing hyperhomocysteinemia.

1. Introduction

High levels of homocysteine (Hcy) are a known risk factor for vascular diseases and dementia in the general population [1, 2]. Plasma Hcy levels may increase as a result of genetic and acquired causes [3]. In terms of the genetic causes, a gene mutation exists that encodes the methylenetetrahydrofolate reductase (MTHFR) enzyme and is commonly encountered in the general population [3]. Plasma Hcy levels can also be affected by severe metabolic disorders, vitamin B12 and folic acid deficiency, and the use of vitamins and certain medications [3].

An increase in plasma Hcy levels has been reported in Parkinson's disease (PD) patients who were using levodopa. Total Hcy concentrations in the cerebrospinal fluid were also higher following levodopa therapy than before treatment and than in controls [4]. The catalysis of levodopa with the catechol-O-methyltransferase (COMT) enzyme results in the formation of S-adenosylhomocysteine (SAH), which hydrolyses to form Hcy [3].

Previous studies have indicated a relationship between Hcy and DNA damage, apoptosis, excitotoxicity, and oxidative stress, which are of great importance in neurodegeneration [3, 5]. Experimental studies have demonstrated that Hcy can be neurotoxic and excitotoxic to the substantia nigra. Furthermore, Hcy may be associated with dyskinesia, which is an indicator of possible neurodegeneration due to the disruption of the balance of striatal activity [6, 7]. Following *in vitro* and *in vivo* observations on the toxic effects of Hcy on dopaminergic neurons in the substantia nigra, some authors have suggested that hyperhomocysteinemia associated with levodopa can play a role in the progression of PD and the development of motor complications. Dyskinesia and motor fluctuations may be due to the toxic effects of Hcy [7, 8].

COMT inhibitors have been widely administrated to control motor complications such as "wearing off" in treatment of PD. Evidence of COMT inhibitors decreasing plasma Hcy levels that have been increased by levodopa in rats has had a pioneering influence on studies performed with COMT inhibitors on humans [9]. However, the results of studies

performed to date have varied. While some studies have shown that COMT inhibitors can reduce plasma Hcy levels [10–12], this effect has not been demonstrated in others [13, 14]. Zesiewicz et al. examined 5 studies arising from Europe and USA and reported that the ability of COMT inhibition to reduce or prevent hyperhomocysteinemia induced by levodopa in PD patients may be attributed to differences in the vitamin status of the study participants. In addition, authors also indicated that, in patients with low or low-normal folic acid levels, levodopa administration is associated with a greater increase in Hcy and concomitant entacapone administration is associated with greater reduction in Hcy [15].

In this study, we evaluated the effects of various treatment options on plasma Hcy levels in idiopathic PD and investigated whether the addition of entacapone (a COMT inhibitor) to the treatment contributed to a reduction in plasma Hcy levels.

2. Methods

2.1. Subjects. For this prospective study, we enrolled one hundred patients (54 men, 54%) diagnosed with idiopathic PD according to the UK Parkinson's Disease Society Brain Bank Criteria [16]. The average age of the patients was 70 years (range 40–89). Patients with a severe metabolic disorder, a history of vitamin use, or secondary Parkinsonism were excluded from the study.

2.2. Study Design and Protocol. We recorded the following demographic data for all patients: age at disease onset, duration of disease, treatment regimes, levodopa dosage and duration of use, and entacapone dosage and duration of use. Then, we divided patients into three groups according to the type of treatment received.

Group I consisted of patients treated with levodopa and/or a dopamine agonist: 15 patients with levodopa alone and 43 patients with levodopa and dopamine agonists (17 pramipexole, 15 ropinirole, and 11 piribedil). The median duration of dopamine agonist treatment was 4 years (range 1–20).

Group II consisted of patients treated with levodopa, entacapone, and/or a dopamine agonist: 5 patients with levodopa and entacapone and 25 patients with levodopa, entacapone, and dopamine agonists (10 pramipexole, 10 ropinirole, and 5 piribedil). The median duration of dopamine agonist treatment was 7 years (range 1–24). Group II patients were treated with levodopa and entacapone simultaneously (levodopa-entacapone combined preparation).

Group III consisted of patients treated with a dopamine agonist alone (6 pramipexole, 4 ropinirole, and 2 piribedil). The median duration of dopamine agonist treatment was 2.5 years (range 1–12).

In previous studies, the effects of dopamine agonists on Hcy levels could not be demonstrated despite the fact that levodopa has been shown to increase plasma Hcy levels [10]. In this regard, we compare the plasma Hcy values of patients within groups I and II to patients within group III.

We used the "modified Hoehn and Yahr" (mHY) scale to evaluate the PD stage of each patient and the "Unified Parkinson's Disease Rating Scale" (UPDRS) Parts II and III, performed at the "on" period, to evaluate the severity of disease symptoms [17, 18]. Global cognitive status was evaluated using the "Standardized Mini-Mental Test" (SMMT).

For all patients, blood samples were taken from the peripheral vein and they were pooled into EDTA vacuum tubes in the morning after 12 hours of fasting and at 12-hour drug-free periods. Plasma Hcy levels were measured using the "high performance liquid chromatography with fluorescence detection" method according to Vester and Rasmussen [19]. The normal range for Hcy is considered between 5 and 15 μmol/L [20]; however, evidence from large epidemiological studies indicates that Hcy levels lower than 15 μmol/L are also associated with higher risk of vascular events [21–23]. Therefore, plasma levels for hyperhomocysteinemia were set higher than 12 μmol/L, although plasma levels of Hcy above the 20 μmol/L are also associated with greater risk.

Serum B12 and folic acid levels were analysed by automated electrochemiluminescent immunoassay (Elecsys 2010, Roche Diagnostics, Basel, Switzerland) [24]. Normal values range from 156 to 698 pg/mL for serum B12 and 3.1 to 17.5 ng/mL for serum folic acid.

All participants in this study provided informed consent. The study was carried out according to the Helsinki Declaration and was approved by the institutional ethics committee.

2.3. Statistical Analysis. Statistical analyses were performed using the SPSS Statistics Program (version 19, IBM, Chicago, IL). Initially, the normality of variables was studied. Analysis using the Kolmogorov-Smirnov test showed that the distribution of variables, except for folic acid, was not consistent with a normal distribution. We used nonparametric tests for the analysis of variables that were inconsistent with a normal distribution and parametric tests for the analysis of variables that were consistent with a normal distribution. The Chi-square test was used to study gender-related differences. The Mann-Whitney U test was used to analyse differences between subgroups of male/female populations with respect to plasma Hcy levels.

3. Results

Demographic data, clinical characteristics, and serum vitamin B12, folic acid, and Hcy levels are shown in Table 1.

We found statistically significant differences between treatment groups regarding age, disease duration, and age at disease onset ($p = 0.04$, $p = 0.01$, and $p = 0.002$, resp.). Specifically, group II patients (levodopa, entacapone, and/or dopamine agonist) were younger than group I patients (levodopa and/or dopamine agonist). Furthermore, while the disease duration was significantly longer in group II than in group III patients (dopamine agonist only), the age at disease onset was significantly lower in group II than that in both group I and group III patients. There were no differences between groups in terms of mHY stages, UPDRS Part II/III scores, and SMMT scores (Table 2).

Additionally, there were no statistically significant differences in serum B12 and folic acid levels between groups. While plasma median Hcy levels were above normal laboratory values (14 μmol/L) in group I and II patients, they

Table 1: Demographic data, clinical characteristics, and serum vitamin B12, folic acid, and homocysteine levels of patients*.

	(n = 100)
Age (years)	70 (40–89)
Male/female	54 (54%)/46 (46%)
Duration of PD (years)	5 (1–24)
Age of onset (years)	65 (32–86)
UPDRS II score	10 (2–36)
UPDRS III score	15 (6–43)
mHY stage	2 (1–5)
SMMT score	24 (10–30)
Hcy (μmol/L)	14.45 (5.18–54.3)
Vitamin B12 (pg/mL)	263.5 (58–990)
Folic acid (ng/mL)	9.29 ± 3.44

*Data reported as median (minimum–maximum) except folic acid values. Folic acid values reported as mean ± SD.
PD: Parkinson's disease; UPDRS: Unified Parkinson's Disease Rating Scale; mHY: modified Hoehn and Yahr staging scale; SMMT: Standardized Mini-Mental Test; Hcy: homocysteine.

were normal in group III patients. However, there was no statistically significant difference in plasma Hcy levels between groups. Similarly, there was no statistically significant difference between group I and II patients in terms of the treatment dosages and duration of levodopa (Table 2).

We found plasma Hcy levels were statistically higher in men for all patients (p = 0.001). Intragroup plasma Hcy levels of male/female patients were also compared and there was no statistically significant difference between group I and group II; group I and group III; and group II and group III regarding plasma Hcy levels among men (p = 0.71, p = 0.16, and p = 0.62, resp.). Similarly there was no statistically significant difference between group I and group II; group I and group III; and group II and group III regarding plasma Hcy levels among women (p = 0.69, p = 0.58, and p = 0.56, resp.). No relationship was found for serum vitamin B12 and folic acid levels regarding gender among all patients and treatment subgroups.

4. Discussion

In our study, we found that there were no differences in Hcy values between PD patients treated with entacapone and levodopa and patients treated with levodopa without entacapone. Our results indicated that COMT inhibitors may not have an effect on preventing the potential development of high levels of Hcy due to levodopa.

Hyperhomocysteinemia is a known risk factor for atherosclerotic vascular diseases and dementia [1, 2] and may develop from genetic or acquired causes [3]. Genetic mutations in the C677T allele encoding the MTHFR enzyme are causes of hyperhomocysteinemia, a condition frequently encountered in the general population [3]. Acquired causes include severe metabolic disorders, vitamin B12 and folate deficiency, and the use of vitamins and certain medications [3].

In 1995, Allain et al. were the first to determine that Hcy levels were higher in PD patients than those in healthy subjects [25]. Following this, Müller et al. showed that the Hcy levels of patients treated with long-term levodopa were higher than those in patients who had never used levodopa [26]. In PD patients, plasma Hcy levels may increase because of chronic levodopa therapy. The catabolism of levodopa with the COMT enzyme results in SAH, which rapidly hydrolyses and eventually forms Hcy [3].

Some studies have shown that the combination of levodopa/decarboxylase inhibitors with the COMT inhibitor entacapone lowers the levels of Hcy in rats [9]. While experimental studies have shown that COMT inhibitors may lower Hcy levels, the results of *in vivo* prospective studies with this objective have varied.

Valkovič et al. studied three groups of PD patients treated with the following: (1) levodopa; (2) both levodopa and entacapone; and (3) dopamine agonists. The mean plasma Hcy levels were higher in the levodopa group; in addition, the levodopa group was more prone to B12 hypovitaminosis compared with the other groups [10].

Lamberti et al. studied plasma Hcy levels in treatment groups of levodopa and levodopa-entacapone combination and found that the Hcy levels of both groups were higher than those in the control group, but the folate level was lower in the levodopa group. By statistical analysis, they also found that the lower Hcy levels of the levodopa-entacapone combination group compared with the levodopa group were related to the use of entacapone rather than the effect of folate [11].

In a study performed with four groups of patients (levodopa, levodopa-entacapone combination, dopamine agonist, and control), Zoccolella et al. determined that the levodopa increased plasma Hcy, while the COMT inhibitors such as entacapone effectively reduced this increase [12].

Nevrly et al. compared Hcy levels in patients who used long-term levodopa with Hcy levels in patients who never used levodopa and started the levodopa-entacapone combined treatment. By week 8, there was no increase in the Hcy levels of the patients undergoing combined therapy. Based on this result, they concluded that combination therapy might protect against increases in Hcy in the early stages of PD [27].

Müller and Muhlack showed that while acute levodopa treatment increases plasma Hcy, entacapone prevents this increase. This study highlighted the fact that the effect of entacapone on the Hcy level was acute and that this should be taken into consideration in Hcy measurements [28].

However, while some studies suggest that high levels of Hcy due to levodopa therapy may be prevented with COMT inhibitors, other studies do not support such findings.

O'Suilleabhain et al. concluded that entacapone had no effect on plasma Hcy [13]. Following this study, in a multicentre, open-ended study of 169 patients, patients receiving levodopa therapy were switched to levodopa-entacapone therapy for at least 4 weeks. The results revealed that there was no difference in plasma Hcy levels before and after entacapone use and that entacapone therapy was ineffective in reducing plasma Hcy [14].

In a 6-week, randomized, double-blind, placebo-controlled study, Postuma et al. investigated the effect of 1 mg of

Table 2: Demographic data, clinical characteristics, and serum vitamin B12, folic acid, and homocysteine levels of patients with PD according to treatment groups*.

	Group I ($n = 58$)	Group II ($n = 30$)	Group III ($n = 12$)	p
Age (years)	70 (40–89)	65.5 (42–84)	69.5 (53–77)	0.04†
Male/female	32 (59.3%)/26 (56.5%)	16 (29.6%)/14 (30.4%)	6 (11.1%)/6 (13%)	0.94
Duration of the PD (years)	5 (1–20)	9.5 (1–24)	3 (1–12)	0.01‡
Age of onset (years)	65 (35–86)	57 (32–78)	67 (52–72)	0.002§
UPDRS II score	11 (3–36)	12 (3–26)	8 (2–16)	0.07
UPDRS III score	15 (6–43)	17 (6–35)	14 (6–30)	0.27
mHY stage	2 (1–5)	2 (1–5)	1.5 (1–3)	0.23
SMMT score	24 (10–30)	25 (15–29)	26 (17–28)	0.46
Hcy (μmol/L)	15.1 (6.3–50)	15.2 (5.18–36)	12.6 (7.92–54.3)	0.30
Vitamin B12 (pg/mL)	268.1 (100–662)	262.5 (147.9–990)	295 (58–768)	0.80
Folic acid (ng/mL)	9.38 ± 2.87	8.67 ± 3.67	10.73 ± 5.02	0.26
LD dose (mg/day)	300 (100–1000)	400 (150–1000)	(—)	0.30
Duration of LD medication (years)	2.5 (1–20)	5.5 (1–20)	(—)	0.27
COMTI dose (mg/day)	(—)	800 (400–1400)	(—)	(—)
Duration of COMTI medication (years)	(—)	2 (1–10)	(—)	(—)

*Data reported as median (minimum–maximum) except folic acid values. Folic acid values reported as mean ± SD.
†Statistical difference was significant between group I and group II ($p < 0.05$).
‡Statistical difference was significant between group II and group III ($p < 0.05$).
§Statistical difference was significant between group II and both of the other groups ($p < 0.05$).
PD: Parkinson's disease; UPDRS: Unified Parkinson's Disease Rating Scale; mHY: modified Hoehn and Yahr staging scale; SMMT: Standardized Mini-Mental Test; Hcy: homocysteine; LD: levodopa; COMTI: catechol-O-methyl transferase inhibitors.

folate/500 mg of vitamin B12 and entacapone on serum Hcy in 35 PD patients who were receiving levodopa treatment; entacapone was found to be ineffective compared with placebo and folate/vitamin B12 was found to be effective in reversing the levodopa-related hyperhomocysteinemia [29].

Zesiewicz et al. examined the effect of entacapone use on the elevation of plasma Hcy levels due to levodopa use in PD in a meta-analysis consisting of studies from Europe and the USA. The studies by Lamberti, Zoccolella, and Valkovič showed entacapone use decreased plasma Hcy levels as a positive outcome, but the studies by Ostrem and O'Suilleabhain did not. Zesiewicz et al. underlined patients of three studies showed a decreasing effect of entacapone with low vitamin levels. On the other hand, patients in Ostrem and O'Suilleabhain's study group had normal vitamin levels. Zesiewicz et al. concluded normal or higher vitamin levels may help to metabolize Hcy more and the positive outcome of the European studies could be a result of vitamin levels instead of COMT inhibition [15].

In our study, while there was no difference in plasma Hcy levels between levodopa and levodopa-entacapone combination, Hcy levels were not statistically different in patients who received dopamine agonist alone compared with other groups. However, apart from the group III patients receiving dopamine agonist alone, median Hcy levels in groups I and II were slightly above normal values. These results suggested that levodopa therapy might increase Hcy levels but not significantly; however, concomitant use of entacapone with levodopa might not reduce Hcy levels. In our study, vitamin B12 and folic acid values were normal in all the three groups and there was no difference between groups regarding vitamin B12 and folic acid values. It is possible that levodopa did not cause a significant increase in Hcy levels in our patients because their vitamin levels were normal. As suggested in other studies, the main cause of hyperhomocysteinemia may be low vitamin B12 and/or folic acid levels rather than levodopa treatment [15].

In this study, we found that the duration of PD was shorter in patients treated with dopamine agonist than patients treated with levodopa and entacapone. This result may be explained since dopamine agonists are the first choice of treatment in the earlier period of PD. In addition, when the disease duration advances, progression may occur; therefore, levodopa and COMT inhibitors may take place in the treatment regimen. Data showed that the UPDRS II and III scores were lower in group III than in groups I and II but without statistical significance.

We also found that the onset age was younger in the group treated with levodopa and entacapone compared with patients treated with levodopa and with the group treated with the dopamine agonist alone. This may be because when the age at the disease onset was earlier, more disease progression and motor fluctuations may appear in the advanced disease; therefore, levodopa and COMT inhibitors usually were administered in this group.

Some studies have suggested a correlation between hyperhomocysteinemia and male gender, but this correlation has not been demonstrated in other studies [5, 30–32]. We

compared plasma Hcy levels between men and women and found that plasma Hcy levels were higher in men, but we could not find any statistical difference between treatment subgroups of male patients and similarly in female subgroups. In addition, there was no difference according to gender distribution in the subgroups in our study. Therefore, gender may not have been an important factor on plasma Hcy levels in the results of this study.

5. Conclusions

The results of our study indicated that, in PD patients, levodopa therapy may cause slight increases in plasma Hcy levels, though not a significant increase, when compared with dopamine agonists. Furthermore, the results did not support the finding that COMT inhibitors have a preventive effect on hyperhomocysteinemia, which may be associated with levodopa therapy.

Competing Interests

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

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Research Article

Metformin Alleviated Aβ-Induced Apoptosis via the Suppression of JNK MAPK Signaling Pathway in Cultured Hippocampal Neurons

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Both diabetes and hyperinsulinemia are confirmed risk factors for Alzheimer's disease. Some researchers proposed that antidiabetic drugs may be used as disease-modifying therapies, such as metformin and thiazolidinediones, although more evidence was poorly supported. The aim of the current study is to investigate the role of metformin in Aβ-induced cytotoxicity and explore the underlying mechanisms. First, the experimental results show that metformin salvaged the neurons exposed to Aβ in a concentration-dependent manner with MTT and LDH assay. Further, the phosphorylation levels of JNK, ERK1/2, and p38 MAPK were measured with western blot analysis. It was investigated that Aβ increased phospho-JNK significantly but had no effect on phospho-p38 MAPK and phospho-ERK1/2. Metformin decreased hyperphosphorylated JNK induced by Aβ; however, the protection of metformin against Aβ was blocked when anisomycin, the activator of JNK, was added to the medium, indicating that metformin performed its protection against Aβ in a JNK-dependent way. In addition, it was observed that metformin protected the neurons via the suppression of apoptosis. Taken together, our findings demonstrate that metformin may have a positive effect on Aβ-induced cytotoxicity, which provides a preclinical strategy against AD for elders with diabetes.

1. Introduction

In the world, over 35 million people are suffering from Alzheimer's disease (AD), and the number of patients is estimated to be more than 100 million by 2050 [1]. Unfortunately, no medications have been testified to be effective for AD by the US Food and Drug Administration to date [2]. As an irreversible neurodegenerative disease, the research hotspot for AD has turned to its preclinical stage [3]. By now, AD is featured by progressive memory loss and a gradual impairment in cognitive function, finally resulting in premature death of the individual usually during 3–9 years after diagnosis [4, 5]. The neuropathological characteristics of AD include the existence of extracellular senile plaques with amyloid-β (Aβ) protein, neurofibrillary tangles with intracellular and abnormally phosphorylated tau protein, and a sharp loss of neurons and synapses [6–8]. Based on the confirmed pathological changes, the "amyloid cascade hypothesis" is most acceptable view currently. Therefore, strategies to decrease Aβ production, increase Aβ removal, or reduce Aβ-induced cytotoxicity are considered as effective to Alzheimer's disease.

Nowadays, the scientists have laid more and more emphasis on the relationship between AD and diabetes, and diabetes has absolutely been considered as a risk factor for AD [9]. Type 2 diabetes was one of the strongest risk factors of AD, which increases the risk level to double compared with people without this disease [10, 11]. Metformin (N′,N′-dimethylbiguanide) is acceptably used as the first-line medicine for the therapy of type 2 diabetes [12]. Metabolic dysfunction is one of the vital pathological features of AD, too. Moreover, a new study suggests the balance of metabolism ameliorates cognitive function. Metformin is one of FDA approved drugs,

which is potentially applied in alleviating metabolic dysfunction in AD [13]. As an effective medication aimed at AD and diabetes both, metformin deserves a further exploration.

The trials of metformin on AD treatment have been applied and testified to be successful. Hettich et al. reported that metformin decreased the hyperphosphorylation of TAU protein, BACE1 protein expression, and the production of Aβ peptides both *in vivo* and *in vitro*; thereafter metformin will be valuable for therapy against AD via interfering with both pathological hallmarks of AD [14]. However, Picone et al. reported metformin increased APP and presenilin levels, at the same time, induced oxidative stress, mitochondrial damage, and cytochrome C release, which resulted in an aggregation of AD [15]. The paradoxical results may be caused by different experimental results, but due to the large population suffering from both AD and diabetes, further exploration should be carried out, so as the mechanism of metformin. In the current study, we set up a cytotoxicity cellular model with Aβ exposure and observed the effect of metformin on it. Strikingly, we confirmed the therapeutic value of metformin on Aβ-induced cytotoxicity, suggesting metformin may be a promising agent of clinical treatment for both AD and diabetes.

2. Method

2.1. Hippocampal Neurons Culture and Treatments. Pregnant Wistar rats were bought from the animal facility of the Peking University. All experimental animals were approved by the Institutional Animal Care and Use Committee (IACUC). Hippocampal neurons were cultured as the previous method [16]. The neurons were used for experiments on about 10 days after being plated. For the Aβ incubation studies, freshly diluted oligomer Aβ1–42 (Sigma-Aldrich) was added to the hippocampal cultures, and electrophysiological measurements were performed after 24 h.

2.2. Thiazolyl Blue Tetrazolium Bromide (MTT) Assay. The cell viability of cultured neurons was evaluated with a MTT assay. Neurons cultured in 96-well plates were incubated with MTT solution at 37°C for 4 h. Then, dimethyl sulfoxide (DMSO, 100 μL/well) was used to dissolve the formazan crystals; cell viability was measured with the percentage of MTT decrease compared with that under the control conditions.

2.3. Lactate Dehydrogenase (LDH) Release Assay for Cell Death. Cell death was measured by LDH leakage. The medium of the cultured neurons in 6-well plates was gathered to evaluate the LDH release with an LDH assay kit by the manufacturer's instructions.

2.4. JC-10 Staining. The mitochondrial membrane potential is one of the hallmarks of apoptosis, which is detected with JC-10 mitochondrial membrane potential assay kit (AAT Bioquest, Sunnyvale, USA). The medium was withdrawn and the neurons were exposed under JC-10 for 30 min at 37°C in an incubator. These neurons were rinsed twice with ice-cold PBS, and then the fluorescence intensity of JC10 was observed via fluorescence microscopy. Decreased fluorescence of the neurons was considered as the mitochondrial membrane potential collapsed.

2.5. Western Blot Analysis. To determine the phosphorylation level of ERK1/2, JNK and p38, whole-cell extracts were got and fractionated with SDS-PAGE. After electrophoresis, we electrotransferred the proteins onto the nitrocellulose membranes, and blotted them with primary antibody for phospho-Thr202/Tyr204 ERK1/2 (Cell Signaling Technology, CST, Danvers, MA, USA), ERK1/2 (CST), phospho-Thr183/Tyr185 JNK (CST), JNK (CST), phospho-Thr180/Tyr182 p38 (Abcam Technology, Cambridge, MA, USA), p38 (Abcam), and the corresponding secondary antibodies. The Enhanced Chemiluminescence (ECL) kit (GE Healthcare, UK) was selected to represent the signals.

2.6. Statistical Analysis. Data are presented as mean ± SE. Statistical analysis is performed by one-way analysis of variance and followed by all pairwise multiple comparison procedures with Bonferroni test (sigmastat 10.0). $p < 0.05$ is confirmed to be statistically significant.

3. Results

3.1. Metformin Alleviates Aβ-Induced Cytotoxicity in a Concentration-Dependent Manner. Hippocampal neurons were injured by Aβ at different concentrations for 24 hours. Neuronal death was increased after Aβ exposure compared with that of the control group at concentrations ranging from 20 to 400 μM Aβ using MTT and LDH assay (Figures 1(a) and 1(b)). The neuronal viability was decreased from the dose of 200 μM ($^*p < 0.05$, $n = 6$), and when Aβ dose was increased to over 200 μM, the viability decreased significantly ($^*p < 0.01$, $n = 6$). Considering we are not sure of the role of metformin in Aβ-induced cytotoxicity, 200 μM Aβ was chosen to set up the cellular model. Although metformin is testified to play a role in AD, its tone and mechanism are poorly known and required to be explored. We addressed the viability and LDH release of the hippocampal neurons treated with naive Aβ 200 μM or metformin treatment at different concentrations. At this dose of 100 mM, metformin suppressed the neuronal injuries by 200 μM Aβ ($p < 0.05$, $n = 6$), while the effect of 1 or 10 mM metformin against Aβ-induced injuries was not distinct (Figures 1(c) and 1(d)), which suggested that the protective effect of metformin was dose-dependent.

3.2. Metformin Reversed Aβ-Induced Hyperphosphorylation of JNK in the Hippocampal Neurons. As we observed, metformin salvaged the neurons exposed to Aβ; however, the mechanism was still obscure and required to be explored further. Mitogen-activated protein kinases (MAPK) are an evolutionarily conserved signal-transduction family, in which the serine/threonine kinases are unique to eukaryotes. JNK is an important member of this family and involved in numerous stimuli, including excitotoxic stress and DNA damage [17]. Moreover, it has been reported that the activation of JNK will always result in the proapoptotic signaling activation by inhibiting the antiapoptotic proteins, such as Bcl-xL and

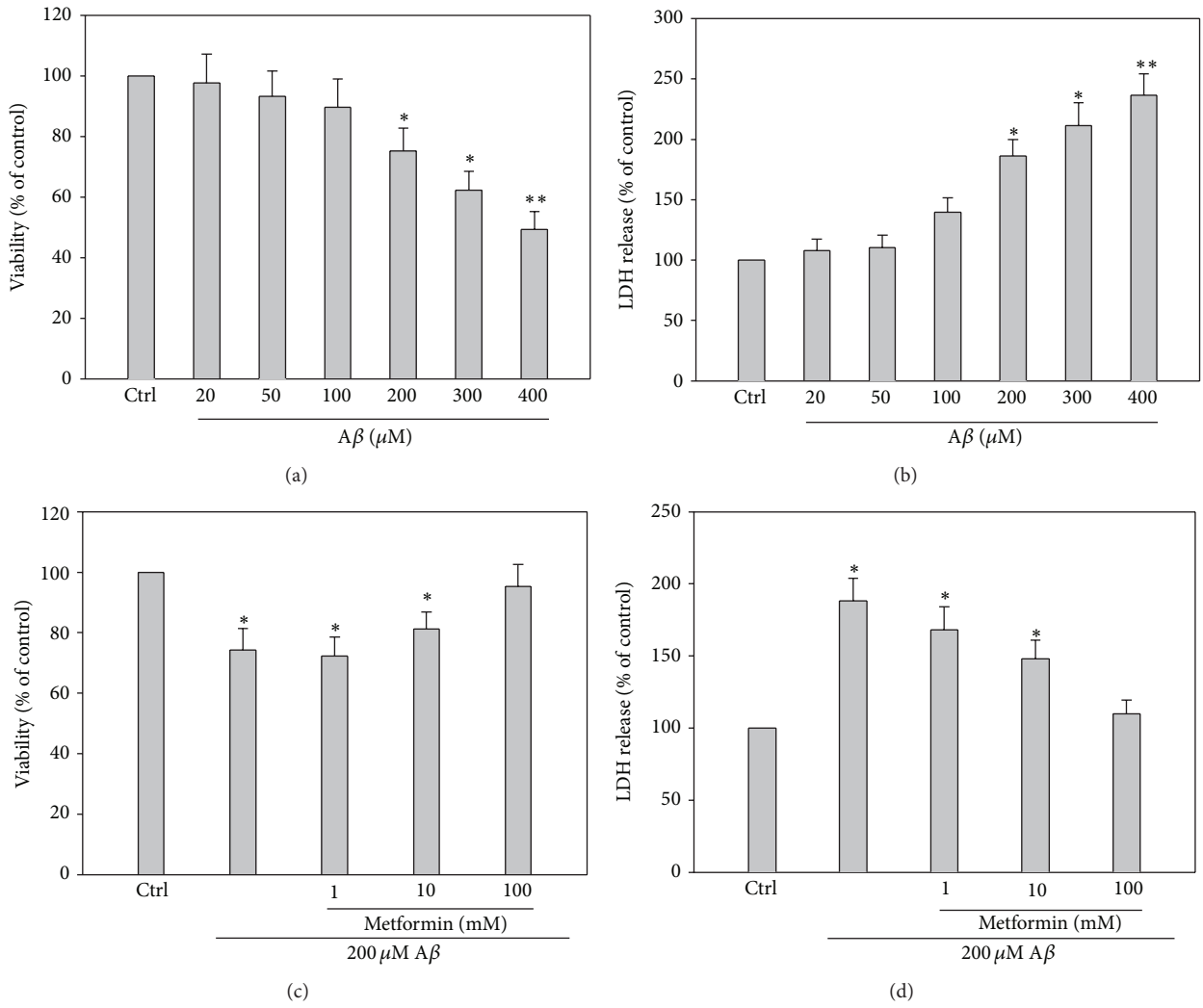

Figure 1: Concentration-dependent cytotoxicity of cultured hippocampal neurons induced by exposure under different Aβ concentrations from 20 to 400 μM. Metformin alleviated Aβ-induced cytotoxicity also in a dose-dependent manner. (a) and (b) Quantitative analysis of MTT and LDH release assay showed that Aβ decreased cell viability from 50 to 200 μM ($^{*}p < 0.05$, $^{**}p < 0.01$ versus that of control, $n = 6$ per group). (c) and (d) Quantitative analysis of MTT and LDH release assay represented that metformin pretreatment decreased injuries induced by 200 μM Aβ in a concentration-dependent way. As shown in (c) and (d), 100 mM metformin can increase the neuronal viability distinctively.

Bcl2 [18]. Recently, JNK signaling pathway has been considered as "a therapeutic target for Alzheimer's disease" because of its vital role in etiology analysis of Alzheimer's disease [19]. Increased phosphorylated JNK (pJNK) and a positive colocalization with Aβ were revealed in human postmortem brain samples of AD patients [20, 21]. In addition, the activation of JNK was reported to be positively related with cognitive decline, a marker of AD [22]. To determine via which signaling pathway metformin performs its protection against Aβ exposure, we immunoprecipitated ERK1/2, JNK, and P38 and used western blotting to evaluate their activation with the phosphorylation levels. There was a significant difference only in the phosphorylation level of JNK between the experimental groups under naive Aβ exposure ($p < 0.05$, $n = 5$). The results indicated that Aβ exposure increased the phosphorylation of JNK but not ERK1/2 or p38 (Figures 2(a), 2(b), and 2(c); the presentative blots are not shown in this paper). Further, when metformin was added before Aβ exposure, the hyperphosphorylation of JNK was blocked, suggesting JNK played a vital role in Aβ-induced cytotoxicity and metformin-involved protection.

3.3. Metformin Decreased Aβ-Induced Cytotoxicity in a JNK-Dependent Way. Although we investigated the role of metformin against Aβ-induced cytotoxicity via JNK signaling pathway, it still needs to elucidate the key role of JNK, which may be an indicator of strategic target. Metformin has been reported to play its role via a lot of signaling pathways. We added anisomycin, the activator of JNK, to the medium, to investigate the protection of metformin under this condition.

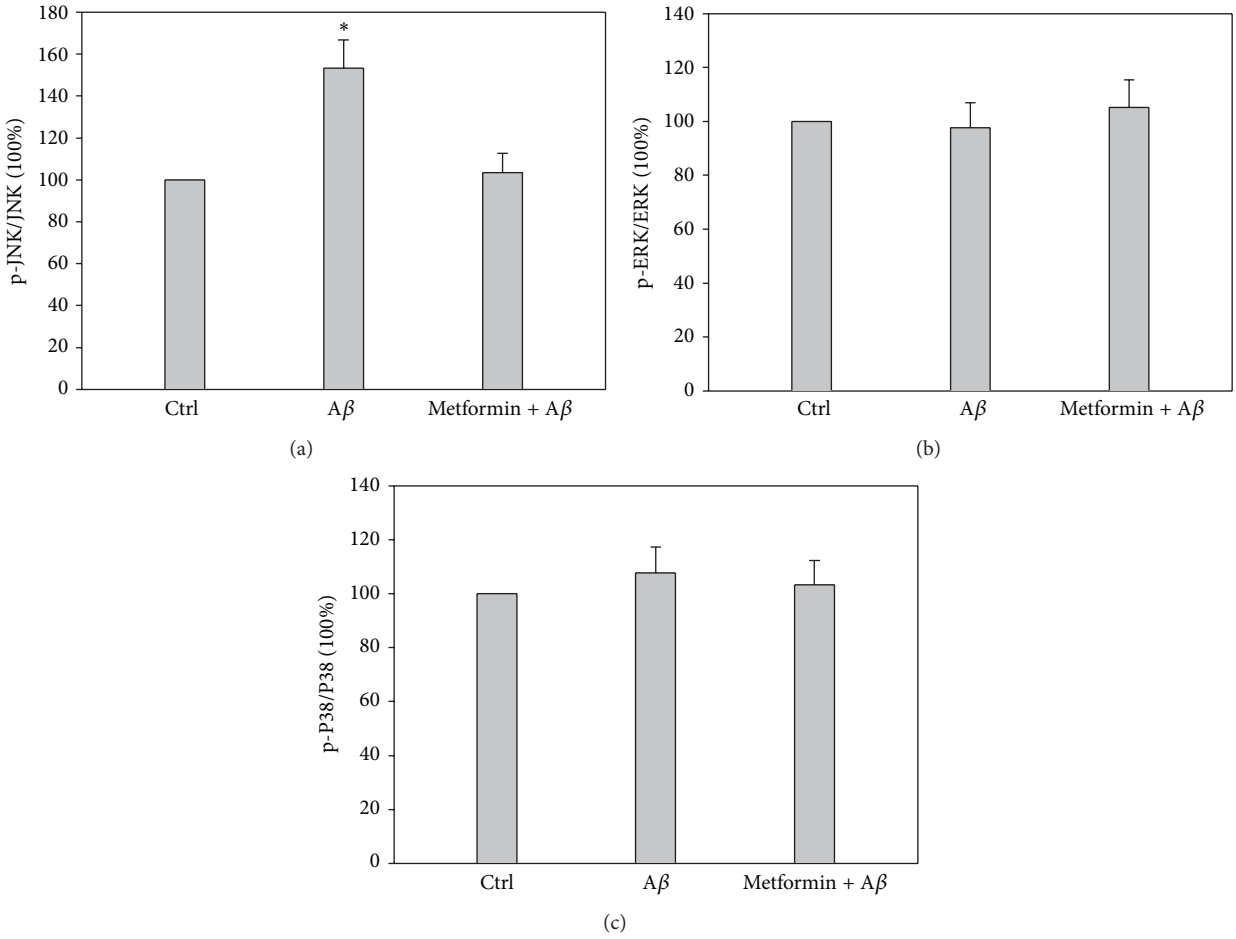

Figure 2: Metformin rescued Aβ- (200 μM) induced hyperphosphorylation of MAPK JNK. (a), (b), and (c) Quantitative analysis of western blot assay showed that Aβ increased the phosphorylation level of JNK, which was reserved by metformin ($^{*}p < 0.05$, $n = 5$), while there was no significant difference in the phosphorylation level of ERK1/2 or P38 between the experimental groups ($p > 0.05$, $n = 5$).

Interestingly, metformin failed to protect hippocampal neurons with the interference of anisomycin (Figures 3(a) and 3(b)). Based on this result, we conclude that JNK-involved signaling pathway is the key target of metformin against Aβ-induced cytotoxicity, although we still cannot decide which stage of AD 200 μM Aβ is corresponding to by now.

3.4. Metformin Decreased Aβ-Induced Cytotoxicity through the Inhibition of Apoptosis. To date, three kinds of programmed cell death have been confirmed according to the morphological criteria, including apoptotic cell death (type I), autophagic cell death (type II), and necrosis or cytoplasmic cell death (type III). Based on the correlation between JNK and apoptotic cell death, we further observed the mitochondrial membrane potential of the cultured neurons with JC-10 staining, which is an acceptable indicator of apoptosis. The statistical analysis results showed that Aβ increased the apoptosis of cultured hippocampal neurons, which was reversed by metformin ($p < 0.05$, $n = 3$, Figure 4; the statistical results are not shown in this paper). When β-lapachone, the activator of apoptosis, was added to the medium, the protective effect of metformin was blocked, suggesting metformin performed its influence by decreasing the cellular apoptosis. Taken together, JNK-involved signaling pathway contributes to 200 μM Aβ-induced cytotoxicity, which can be reversed by metformin treatment.

4. Discussion

As a devastating neurodegenerative disorder, emphasis is laid on Alzheimer's disease by worldwide scientists in both prevention and treatment strategies. In the past years, many factors including aging, genetic, and environmental ones have been confirmed to contribute to the development and progression of AD. Although drugs for AD have been clinically tested, the therapeutic effects are poor by now, suggesting further researches are still required. Epidemiological studies intensely indicate that metabolic defects result in the functional modifications involved in cerebral aging and in AD pathogenesis. The dysfunction of cerebral glucose metabolism in early stages of AD is testified [23], and the molecular

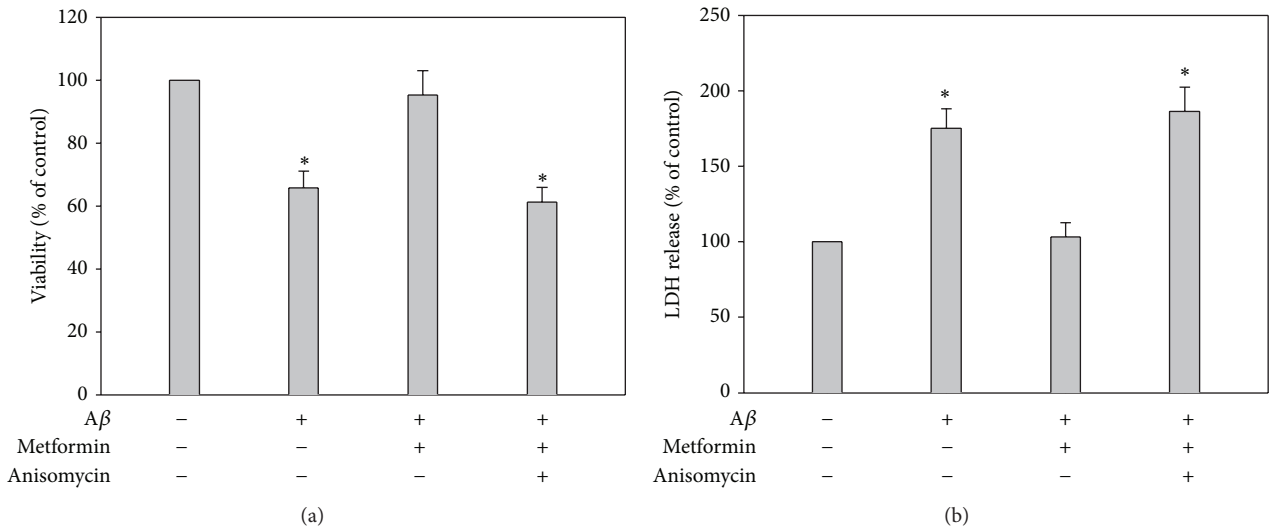

FIGURE 3: Metformin salvaged Aβ-induced cytotoxicity via MAPK JNK signaling pathway. (a) and (b) Quantitative analysis of MTT and LDH represented that Aβ increased the phosphorylation of JNK, which could be reserved by metformin treatment ($^{*}p < 0.05$, $n = 5$). In addition, anisomycin treatment blocked metformin-involved protection, suggesting the depression of JNK had a vital role in the effect of metformin.

markers of insulin resistance is found to colocalize with tau inclusions in AD brain [24], indicating that the insulin-involved pathway may be a vital element to the AD pathophysiological cascade [25].

More and more evidence provides that diabetes mellitus (DM) is more than a common syndrome, which is considered as a risk factor for AD in the elderly. The high prevalence of DM and AD in the elderly population and their close correlation urgently call for a proper concomitant pharmacotherapy according to FDA approval. Metformin is a biguanide which has multipurpose influences on metabolism, by increasing insulin-sensitization, glucose uptake and the activation of AMP activated protein kinase, and so on. To date, the effect of metformin is focused on in the strategy for both DM and AD. Although more and more attentions have been paid to the usage of metformin in AD treatment, its tone and mechanism are still controversial and required to be explored further.

Many trials have been performed with metformin treatment on AD, although its effects are still controversial. Chen et al. reported that metformin increased Aβ efflux across the blood-brain barrier under diabetic context; then hippocampal Aβ1–40 or Aβ1–42 and neuronal apoptosis were significantly decreased, which ameliorated memory impairment [26]. Hettich et al. reported that metformin decreased the phosphorylation of tau at AD-relevant phosphosites and the protein expression of beta-secretase (BACE1) by interfering with an mRNA-protein complex [14]. However, some other scientists supported different insights about the influence of metformin on AD treatment. Picone et al. addressed that metformin activated NF-κB by translocating it from the cytoplasm to the nucleus where NF-κB increased APP and Pres 1 transcription, suggesting metformin promoted the progression of AD [15]. Chen et al. found that metformin significantly increased the production of both intracellular and extracellular Aβ species via the upregulation of BACE1 [27]. The paradoxical results may result from different experimental conditions, such as the concentration and treatment duration. But, due to the big population with AD and diabetes and the extensive usage of metformin, its effect requires a thorough exploration. Based on previous studies and our present results, we conclude that the dose and delivery time of metformin are vital for its effect. Moreover, our delivery time is before Aβ exposure, which reveals its prevention against AD. The decreased apoptosis by metformin shed a light on its application for AD strategy.

An increasing number of lines have put forward a strong correlation between JNK signaling pathway and Alzheimer's disease. Aβ increased the activation of JNK [22, 28], which is involved in the following cell injury and death. However the related mechanism is still obscure. Yenki et al. revealed that the inhibition of phosphorylated JNK decreased Aβ-induced endoplasmic reticulum stress and increased prosurvival mitochondrial proteins [29]. Mohammadi et al. found JNK inhibitor performed protection against Aβ through the reduction of autophagy and then alleviated memory deficit induced by Aβ [30]. In addition, Paquet et al. proposed JNK in human fluids may be used as one of the potential surrogate markers to appraise cell death and clinical prognosis for AD [31]. Some phosphorylation sites of JNK have been addressed. Triaca et al. reported that NGF modulated APP phosphorylation via the alteration in phosphorylation levels of the p54 JNK [32]. In particular, the phosphorylation of JNK at Thr-183/Tyr-185 is confirmed to be an early event in AD [33]. In our present study, we find metformin decreases neuronal apoptosis via suppressing the activation of JNK at the Thr-183/Tyr-185 sites, which enriched the understanding of both metformin and Aβ-induced cytotoxicity. To our knowledge, JNK-involved pathway also participates in many physiological processes in our body, limiting the application of JNK inhibitor for AD. So, as the importance of JNK in AD,

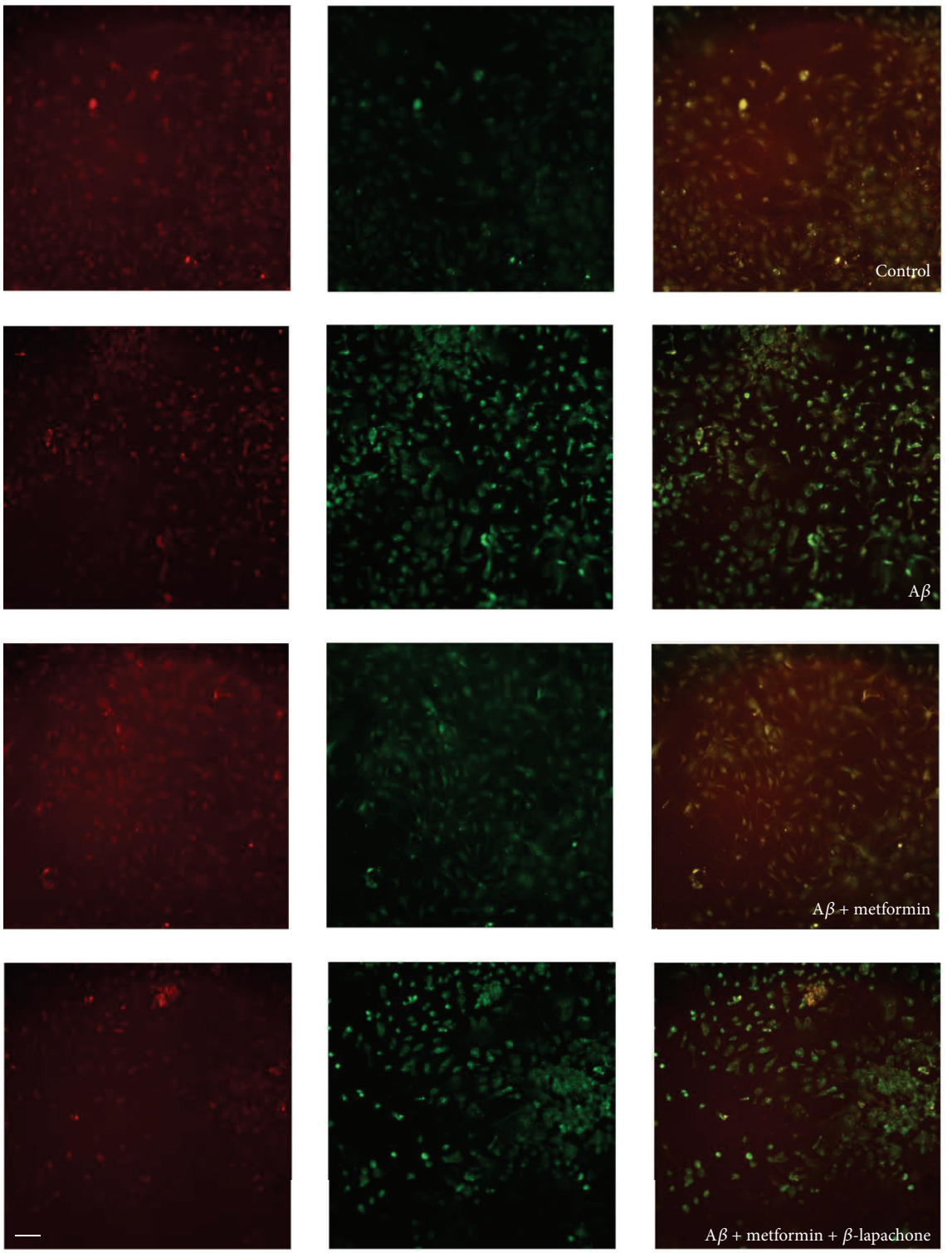

Figure 4: Metformin decreased Aβ-induced cytotoxicity by suppression of apoptosis. Representative results of JC-10 staining. Quantitative analysis of fluorescence intensity showed that Aβ increased the phosphorylation level of JNK, which was reserved by metformin ($^{*}p < 0.05$, $n = 5$). However, when β-lapachone, the activator of apoptosis, was added into the medium, the metformin-involved protection was blocked, indicating metformin played its role via decreasing apoptosis; scale bars, 50 μm.

metformin may be of therapeutic value for preventing AD. Besides, in the future study, the detailed interaction site between metformin and JNK deserves a thorough exploration.

Based on our current results, we can draw the following conclusions. First, JNK signaling pathway is important in Aβ-induced cytotoxicity via increasing apoptosis. Second, metformin may protect neurons through suppression of JNK signaling pathway. Third, metformin definitely protects neurons from Aβ exposure in a dose-dependent and preventive way. To aim at the clinical application of metformin for AD, we propose to find proper dose and delivery time. Although our current study only provides the first glimmer of metformin application for AD, it supports a promising strategy for AD treatment.

Competing Interests

The authors declare that they have no competing interests.

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Research Article

Syntaxin 5 Overexpression and β-Amyloid 1–42 Accumulation in Endoplasmic Reticulum of Hippocampal Cells in Rat Brain Induced by Ozone Exposure

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Oxidative stress is a risk factor for Alzheimer's disease and it is currently accepted that oxidative damage precedes the overproduction of A42 peptide. We have reported that ozone causes oxidative stress inducing neurodegeneration in the brain of rats. It is associated with A42 overproduction and intracellular accumulation in hippocampus. Organelles like mitochondria, intracellular membranes, and endoplasmic reticulum have been identified as sites of A42 production and accumulation affecting cellular metabolism. However whether ozone exposure induces overproduction and/or accumulation of A42 in endoplasmic reticulum has not been studied. We evaluated this effect in the endoplasmic reticulum of hippocampal cells of rats exposed chronically to low doses of ozone (0.25 ppm) at 7, 15, 30, 60, and 90 days. The effect of the presence of A42 in endoplasmic reticulum was analyzed evaluating the expression of the chaperone Syntaxin 5. Our results show an accumulation of A42 peptide in this organelle. It was observed by immunofluorescence and by WB in endoplasmic fractions from hippocampal cells of rats at 60 and 90 days of treatment. Significant overexpression of the chaperone Syntaxin 5 at 60 and 90 days of treatment was observed ($^{*}P < 0.05$). These results indicate that the exposure to environmental pollutants could be involved as a risk factor for neurodegenerative processes.

1. Introduction

Oxidative stress is produced as a consequence of pathological, dietary, and environmental factors. Many people living in large cities around the world are affected by environmental pollutants like ozone (O_3) and it has been demonstrated that chronic exposure to ozone (0.025 ppm) similar to that reported in a day of high pollution in Mexico City causes a state of oxidative stress [1]. Oxidative stress is a major risk factor for Alzheimer's disease (AD). Besides, it has been shown that free radicals enhance the amyloid pathology of AD [2].

Several previous studies from our laboratory have shown that the chronic oxidative stress caused by O_3 produces progressive neurodegeneration in rat hippocampi. In addition memory deterioration, motor activity deficits, lipid peroxidation, and mitochondrial dysfunction were also observed in the hippocampi [3–7].

More recently we have shown a direct relationship of βA42 overproduction and intracellular accumulation with oxidative stress [6, 8].

Many reports have determined that the extracellular and intracellular accumulation of βA42 is involved in the development and progression of AD [8]. However, the precise mechanism of βA42 neurotoxicity is not completely understood.

It has been demonstrated that mitochondria, ER, and Golgi apparatus are targets of βA42, while ER dysfunction in AD is well documented [2, 6, 8–12]. However, whether oxidative damage precedes and contributes directly to the ER accumulation of the βA42 peptides remains unclear.

Previous studies have shown that βA42 can bind to different proteins and extracellular and intracellular macromolecules that affect normal neuronal function and it has been proposed that ER dysfunction is a distinctive hallmark

in AD. For example, the presence of βA42 in ER causes ER stress which in turn activates indicators of the unfolded protein response (UPR), a response activated by ER stress. This kind of alterations affects cytoskeleton integrity and leads to cell death by βA42 presence in neurons [13]. Besides, the accumulation of βA42 induces apoptosis by activating different pathways as the crosstalk between ER and mitochondria. On the other hand mitochondrial dysfunction triggers caspases 3, 8, and 12 activation in cybrid cells, indicating that the death receptor and the ER-specific apoptotic pathways are also activated upon exposure to βA42 [14–16].

It is well known that the mitochondrial pathway is one of the principal generators of oxidative stress. Nevertheless, the stress in the ER is involved or may be induced by oxidative stress in AD. For example, disturbances of calcium and ER homeostasis induce an oxidative stress state and potential βA42 aggregation mediated by iron [17].

Our aim in this work was to demonstrate that the chronic exposure of rats to an environmental pollutant like O_3 (0.25 ppm) may cause an imbalance in the production of βA42. Such imbalance may lead to the progressive accumulation of this peptide in the ER, inducing changes in the normal metabolism of the cells.

2. Materials and Methods

2.1. Animal and Animal Care. 72 male Wistar rats weighing 250–300 g were individually housed in acrylic boxes within a clean air box, and food was provided *ad libitum* (NutriCubo, Purina, USA). The control and treated rats were maintained in a temperature-controlled and humidity-controlled environmental bioterium. The animals were maintained and treated in accordance with the Norma Official Mexicana NOM-036-SSA 2-2002 and the Bioethics Committee of the Faculty of Medicine at the National Autonomous University of Mexico.

2.2. General Procedure. The rats were randomly separated into six experimental groups ($n = 12$ per group). Group 1 was exposed daily to a clear airstream free of O_3 for 4 h, and groups 2, 3, 4, 5, and 6 were exposed to O_3 for 7, 15, 30, 60, and 90 days, respectively. The experimental groups were exposed to 0.25 ppm of O_3 for 4 h daily. One of these subgroups was used for immunohistochemical analyses, and the other group was used for cellular fractionation.

2.3. O_3 Exposure. In order to expose them to chronic doses of ozone (0.025 ppm) the animals were placed inside a chamber and the procedure was carried out essentially as described [7].

Two hours after the final exposure to clean air or O_3, the animals from each group were anesthetized with sodium pentobarbital (50 mg/kg i.p.; Sedalpharma, Edo. de México, Mexico) and then decapitated.

The hippocampi of six animals from each group were obtained for Western Blot (WB), and the other three animals were transcardially perfused with 4% paraformaldehyde (Sigma-Aldrich Chemie, Germany) in 0.1 M phosphate buffer (J.T. Baker, NJ; PB, Tecsiquim; pH 7.4) for the immunohistochemistry assays. The postfixation of the brains was made essentially as described previously [1]. Five-micrometer sagittal slices of the brain containing the hippocampus were obtained using a microtome (American Optical), mounted on slides, and stored.

2.4. Subcellular Fractionation. To isolate the endoplasmic reticulum fractions, the hippocampal cells were lysed with a Dounce homogenizer. Microsomes from these cells were isolated using the Endoplasmic Reticulum Isolation Kit (Sigma-Aldrich) according to the manufacturer's instructions. The ER fractions were used immediately for WB assays as described by Hernández-Zimbrón and Rivas-Arancibia [7].

2.5. Western Blot (WB). The production levels of βA42 in the RE fractions were analyzed by gel electrophoresis and WB. The tissue was homogenized, and 50 μg of protein from each sample was boiled and separated on a 4–12% SDS polyacrylamide gel (Invitrogen) for 45 min at 90 volts (V). The proteins were electrophoretically transferred onto PVDF membranes (Sigma-Aldrich, San Luis, MO, USA). The membranes were blocked with 4% fat-free milk in Tris buffer solution (TBS-T) with 0.01% Tween 20 (TBS-T) (Sigma-Aldrich, San Luis, MO, USA) overnight at 37°C. After being blocked, the membranes were incubated individually with the following antibodies: rabbit anti-βA42 (Abcam Inc., Boston, MA, USA) (1 : 2000). The anti-GRP78 antibody (ER marker) was used as loading control. To evaluate the effect of the presence of βA42 in the ER, the expression levels of Syntaxin 5 (Syx5) were evaluated and the antibody goat anti-Syntaxin 5 (Santa Cruz Biotechnology, CA, USA) was used (1 : 200).

The membranes were incubated overnight with gentle shaking at 4°C (Brinkmann OrbMix 110, Brinkmann, Germany). The membranes were rinsed with TBS-T and subsequently incubated for 2 h at room temperature (RT) in TBS-T containing goat anti-rabbit IgG conjugated to the horseradish peroxidase secondary antibody and diluted to 1 : 1000 (Santa Cruz Biotechnology, CA, USA) for 1 h. The immunoreactive bands were detected by chemiluminescence (ECL; General Electric, Santa Clara, CA, USA). The intensity of each band was quantified using an imaging densitometer (model GS-700). The intensities were analyzed using the open access ImageJ software (NIH) as we previously reported.

2.6. Double Immunofluorescence for βA42. Rabbit monoclonal anti-βA42 antibody (obtained from Abcam, MA, USA), Goat polyclonal anti-GRP78 (Santa Cruz Biotechnology, CA, USA), and goat polyclonal anti-Syx5 (Santa Cruz Biotechnology, CA, USA) were used for double IF to detect the βA42 peptide in the ER of hippocampal cells of the rats.

Sagittal sections of each brain containing the hippocampus were paraffin-embedded, treated with a paraffin-removal and heat-retrieval solution (Biocare Medical), and placed in a Decloaking Chamber (Biocare Medical) for 5 min. Then, the sections were rinsed with distilled water and treated with a blocking reagent (PBS-Bovine Serum Albumin (0.04%)) for 1 hour, washed with 0.1 M phosphate saline buffer, and incubated individually for 12 h at 4°C with anti-βA42 (dilution 1 : 200).

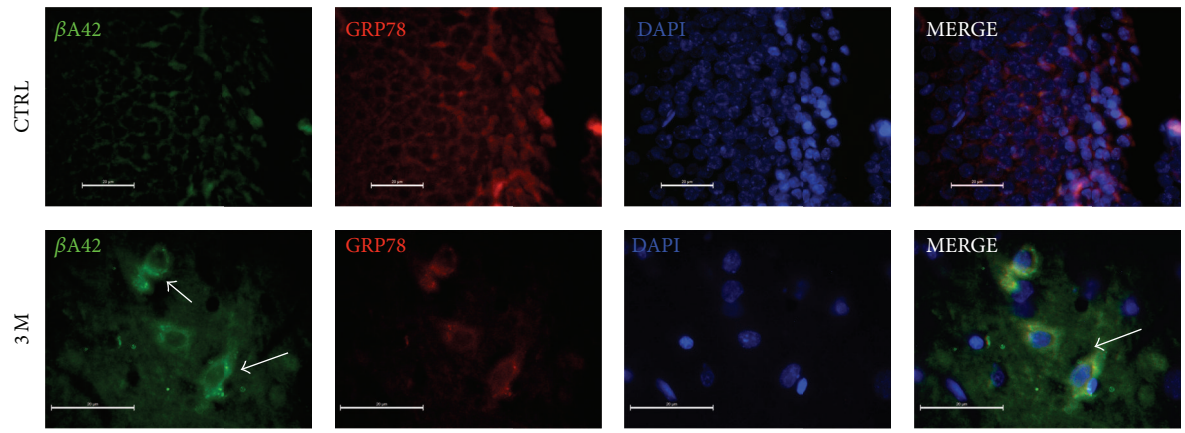

Figure 1: Triple IF to detect beta-amyloid 1–42 in the ER. Five micrometer-thick brain tissue sections form the hippocampi of the control group (CTRL) and the rats exposed to O_3 (0.025 ppm) for 3 months (3 M) are shown. Goat anti-GRP78 was used as ER marker. Rabbit anti-βA42 is shown in green and GRP78 in red. DAPI staining and merge between the red, green, and blue channels are shown (MERGE). The arrows indicate the intracellular βA42 deposition and colocalization in ER. There was no βA42 detection at 7, 15, 30, and 60 days of treatment (data not shown).

For the double immunofluorescence assays (IF), all primary antibodies were incubated overnight at 4°C and then rinsed three times with PBS/Triton (0.03%) and incubated for 2 hours with secondary antibodies. Rabbit monoclonal anti-βA42 and goat polyclonal anti-GRP78 (dilution 1 : 200) were used and visualized with Alexa Fluor 488 goat anti-rabbit IgG (H+L) and Alexa Fluor 594 donkey anti-goat IgG (H+L), respectively. Rabbit monoclonal anti-βA42 and goat polyclonal anti-Syx5 (dilution 1 : 200) were used and visualized with Alexa Fluor 594 goat anti-rabbit IgG (H+L) and Alexa Fluor 488 mouse anti-goat IgG (H+L). All the secondary antibodies were from Molecular Probes, OR, USA. Finally, the slides were rinsed and mounted onto glass slides in Vectashield medium (Vector Laboratories, Burlingame, CA, USA) containing 4′,6-diamino-2-phenylindole (DAPI). Representative brain sections from each group were processed in parallel afterwards and these sections were observed through a Leica DM-LS epifluorescence microscope at 40x and 100x (Leica Microsystems, Wetzlar, GmbH, Germany). The fluorochromes were visualized with their specific filters and analyzed in three channels.

2.7. Statistical Analysis. All of the data are expressed as mean ± SEM. ANOVA analyses followed by Fisher's LSD post hoc, Bonferroni's, and Tukey's tests were used for multiple comparisons. Prism Graph Pad Software was used (Systat Software, Inc., Point Richmond, CA, USA) and differences were considered significant at $P < 0.05$.

3. Results

3.1. βA42 Accumulation in RE Fraction under Oxidative Stress Conditions. We have reported that oxidative stress state caused by O_3 exposure induces intracellular βA42 accumulation as well as mitochondrial accumulation in our neurodegeneration model [6, 7]. To prove the accumulation of this peptide in ER of hippocampal cells of rats treated with low doses of O_3, we performed double IF assays in brain sections derived from both control and experimental groups. The double IF assays showed qualitative increases in the intrareticular accumulation of βA42 in the hippocampal dentate gyrus cells only at 90 days (3 M) of treatment (Figure 1).

3.2. Accumulation of βA42 in ER Isolated Fractions Caused by O_3 Exposure. Next, in order to demonstrate the accumulation of βA42 in the ER fractions, the level of βA42 was quantified by WB and densitometry analyses from the rat hippocampal cells isolated on days 0 (control), 7, 15, 30, 60, and 90 of exposure to O_3. The most representative values of βA42 were obtained. As we can see in Figure 2(a), ~3.5 kDa band for the βA42 monomer was detected only at 90 days of ozone treatment, demonstrating βA42 accumulation in the isolated ER fractions when compared with the control group. The densitometry analysis showed a significant increase of βA42 accumulation in ER fractions (Figure 2(b)).

3.3. Oxidative Stress Causes Overexpression of Syx5 in ER. To demonstrate the possible effect of oxidative stress and beta-amyloid accumulation in ER metabolism, we have performed WB and IF assays to detect changes in the expression levels of Syx5 protein. Syx5 protein has been related with communication processes between mitochondria and ER and described as a chaperone as well. Our WB results showed that ozone exposure alters the expression of Syx5 at 60 and 90 days (Figures 2(c) and 2(d)). Besides, the overexpression of Syx5 was observed by IF (Figure 3(a)) at 90 days and by immunohistochemistry principally at 60 and 90 days of treatment (Figure 3(b)).

4. Discussion

In this study we have demonstrated that O_3 exposure (0.25 ppm) induced a significant increase of Syntaxin 5 and accumulation of βA42 peptide in the ER in cells of the dentate

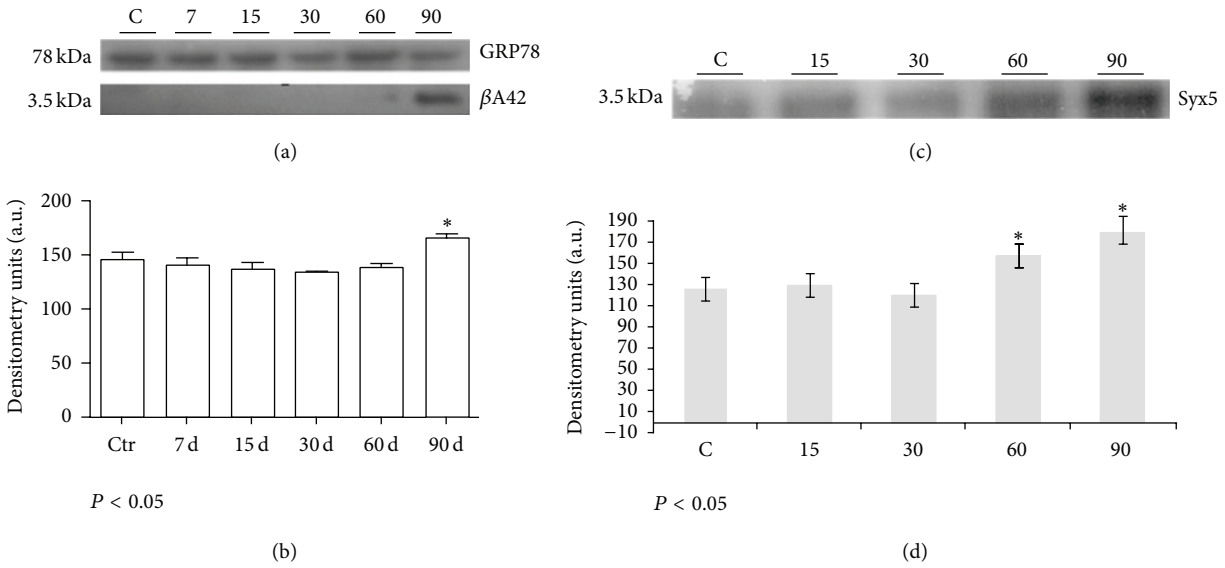

FIGURE 2: Immunodetection of βA42 and Syx5 in the ER protein extracts of rat hippocampi following different O_3 treatments. (a) WB for βA42 in the ER fractions. The ER marker GRP78 was used as loading control and ER isolation control. (b) Densitometry analyses of the WB assays for βA42. βA42 production increased by the end of the ozone treatment. The differences became statistically significant at 90 days of O_3 exposure for βA42; $^*P < 0.05$. (c) WB for Syntaxin 5 from the ER fraction (control, 15, 30, 60, and 90 days). Rabbit anti-Syntaxin 5 was used for immunodetection and was visualized by chemiluminescence. (d) Densitometry analyses of the WB assays. There were significant changes in Syntaxin 5 expression at 60 and 90 days of treatment. The differences between the control and experimental groups were statistically significant; $^*P < 0.05$.

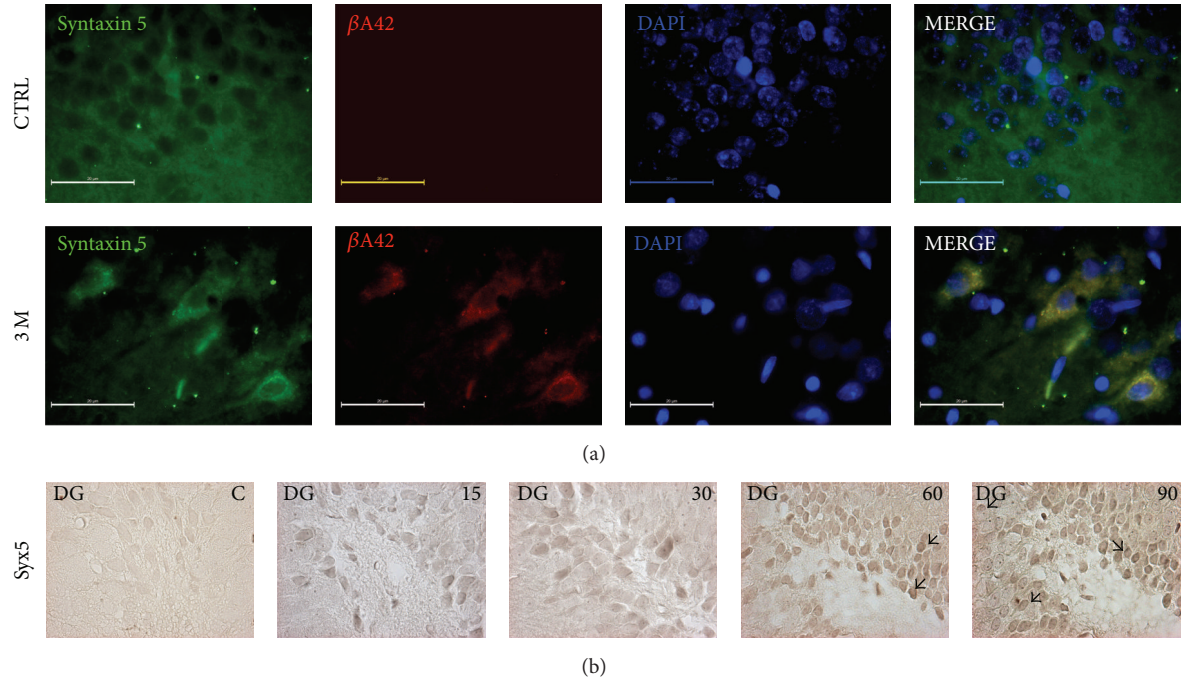

FIGURE 3: Immunodetection of Syx5 and βA42 in the ER. Five micrometer-thick brain tissue sections form hippocampi of the control group (CTRL) and the rats exposed to O_3 (0.25 ppm) for 3 months (3 M) are shown. Rabbit anti-βA42 is shown in red and Syntaxin 5 in green. DAPI staining (DAPI) and merge between red, green, and blue channels are shown (MERGE). The arrows indicate the overexpression of Syx5 and the intracellular βA42 deposition and colocalization in the ER (a). There was no βA42 detection at 7, 15, 30, and 60 days of treatment (data not shown). (b) Immunohistochemistry for Syx5 in brain slides of control (C), 15, 30, 60, and 90 days of treatment showing the overexpression of Syx5 under O_3 treatment.

gyrus. Changes in the expression patterns of the chaperone Syntaxin 5 precede the accumulation of βA42 after the treatment with this environmental pollutant.

AD is a neurodegenerative disease with a complex and progressive pathological phenotype [18]. The βA42 peptide, a hallmark of AD, is produced through the sequential cleavage of APP by β- and γ-secretase [19]. Several reports have demonstrated that the origin and progression of AD are highly correlated with oxidative stress status. Oxidative damage, mitochondrial dysfunction, and age dependent increases of reactive oxygen species (ROS) have been identified as key factors in the development of sporadic AD [20–22]. Besides, oxidative stress may contribute to neuronal degeneration in AD by increasing iron and lipid peroxidation, protein oxidation, and some markers of oxidative stress that are present in the senile plaques in AD [23–25].

Previous results from our laboratory and other groups have demonstrated that the chronic administration of O_3 (0.25 ppm) induces an oxidative stress status, increased lipid peroxidation levels in different brain structures, and morphological and structural changes in neurons and elevates hippocampal superoxide accumulation [26–29].

More recently, we demonstrated that rats exposed to O_3 overproduce βA42, inducing intramitochondrial βA42 accumulation [7]. Moreover, ER oxidative damage has also been well documented in AD. It is well known that the mitochondrial pathway is the main generator of ROS. ER stress is caused by ROS in AD, while the disturbances of calcium and ER homeostasis induce an oxidative stress state which increases Syntaxin 5 and lead to potential βA42 aggregation. However, the precise mechanism that links ER oxidative damage with abnormal βA42 overproduction and accumulation has not been elucidated [30].

Based on our previous results showing mitochondrial accumulation of βA42 we performed assays to demonstrate the effect of ozone exposure in ER. We found an increase of Syntaxin 5 before the accumulation of βA42 in the ER of hippocampal cells. Both effects could be related to ER metabolism disturbance in our progressive neurodegeneration model.

First, we suggest that the overproduction or the increase in βA42 accumulation in hippocampal cells could be related with the increase of ROS production; second, it could be related with the accumulation and processing of APP in these cells. As we have demonstrated before, there is an increase of the amyloidogenic pathway activity in mitochondria [7]. This overactivity and overproduction of beta-amyloid peptide could be associated with the accumulation in ER membranes, mitochondrial membranes, or Mitochondrial Associated Membranes (MAM) as has been suggested by Pereira, 2013 [31–36].

As we have mentioned before, ER is a system formed by continuous membranes. It comprises different architectural shapes of the nuclear envelope, sheet-like structures, containing polyribosomes and smooth tubules present throughout the cell. This organelle is involved in the synthesis, folding, structural maturation, control, and trafficking of integral membrane and secreted proteins in cells. According to its membranous structure, there is evidence demonstrating that MAM are physiological interactions between the ER and mitochondria and have different functions such as lipid transport and synthesis, ER calcium regulation, mitochondrial calcium release, mitochondrial movement and morphology, and protein trafficking.

It has been recently described that PS2 is a protein present in MAM and that the complete γ-secretase complex is present in both ER and MAM [32, 33, 37–40].

In accordance with our results that show the accumulation of βA42 in ER, we could suggest that the βA42 overproduced and PS1 overexpressed in mitochondria may be transported to ER through MAM. The overcleavage of APP by PS1 and PS2 in MAM has been reported in AD and under oxidative stress conditions, as it has been deeply reviewed by [37, 40]. It should be pointed out that the connectivity is increased between ER and mitochondria in AD, and it might be happening in the model present in this work [7, 32, 33]. Another feasible mechanism to explain the βA42 presence in ER is the oxidative stress caused by O_3 exposure *per se*.

The increase in the production and accumulation of beta-amyloid peptide in hippocampal cells may be correlated with the significant increase in PS1 expression and a reduced expression of ADAM9 under oxidative stress conditions. This indicates that low doses of O_3 elicited the overactivation of the amyloidogenic pathway [7].

Then, to demonstrate the effect of the accumulation of βA42 in ER, we evaluate Syntaxin 5, a chaperone directly related with the communication between ER and mitochondria. For example, Syntaxin 5 and Syntaxin 17 facilitate the transport of cholesterol to mitochondria [33, 34].

Syntaxins are a family of vesicular transport receptors involved in membrane traffic through both the constitutive and regulated secretory pathways [41]. One of Syntaxin 5 functions is to shape the ER and is expressed ubiquitously in many cell types and has been located in the ER and Golgi compartment of the early secretory pathways [42]. Besides, Syntaxin 5 is thought to regulate the potential targeting and fusion carrier vesicles at multiple membrane fusion interfaces by affecting the selective combination of the SNARE complex with other SNARE-related proteins (6–9). Some reports have demonstrated that Syntaxin 5 specifically interacts with PS holoproteins, but not with the N-terminal or C-terminal fragments of PS, and the overexpression was shown to upregulate βAPP accumulation in the ER through the Golgi compartments, attenuate accumulation of the C-terminal fragment of βAPP, and reduce Aβ secretion [16].

Our results show that O_3 exposure induces the overexpression of the chaperone Syntaxin 5 at 60 and 90 days of treatment observed by WB (Figures 2(c) and 2(d)) and by IF and IMHQ (Figure 3).

Besides, it has been described that Syntaxin 5 interacts with PSs in ER and the Golgi compartment and its overexpression causes the accumulation of the PS and βAPP as well as the reduction in the level of Aβ secretion [40, 42]. Then we could suggest that the accumulation of Aβ in ER could be in part a consequence of the overexpression of Syntaxin 5 (Figure 4).

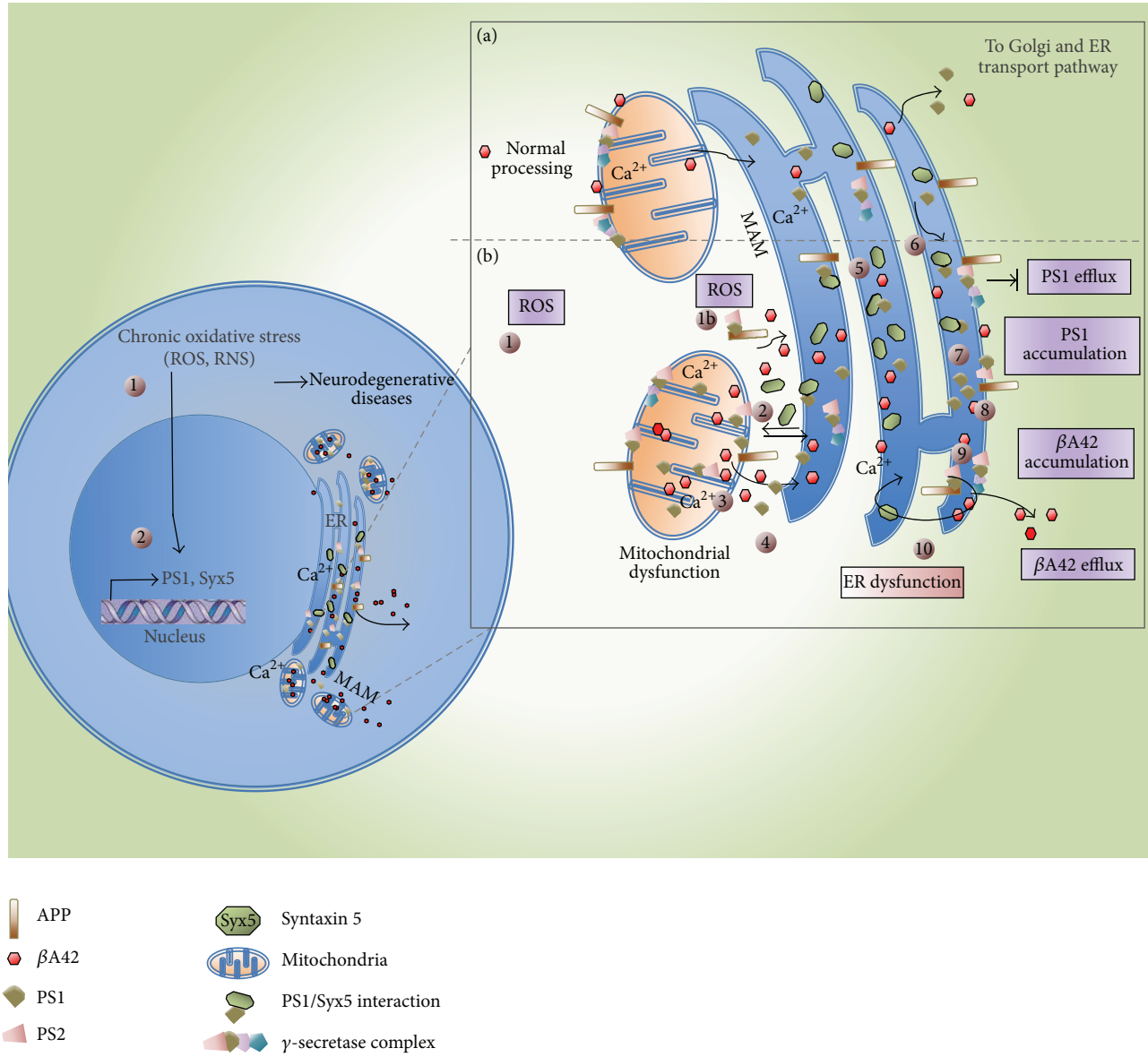

Figure 4: Schematic representation of the suggested mechanisms related with βA42 accumulation in ER. (a) Normal metabolism of mitochondria and ER under no oxidant stress conditions. Normal processing of βA42. (b) Metabolism alterations under oxidant stress conditions. (1) O_3 induces an oxidative stress state causing PS1 and Syx5 overexpression. (1b) ROS induces by itself the overcleavage of APP by PS1 and PS2. (2) PS1 and Syx5 accumulate in mitochondria, MAM, and ER. (3) βA42 accumulation in mitochondria, MAM, and ER. (4) Transport of PS1, Syx5, and βA42 through MAM. (5) Syx5 excess in ER. (6) Interaction Syx5/PS1 blocks the PS1 normal efflux. (7) PS1 accumulation. (8) PS1 transport to γ-secretase complex. (9) Accumulation of βA42 in ER. (10) ER dysfunction.

As we mentioned before, PSs are located in the ER and although they lack proteolytic processing they are active molecules in AD with γ-secretase activity.

The increase of the expression of PS1 in the late compartment of the secretory pathways may lead to higher levels of βA40 and βA42 production [43]. We have reported that there is an overexpression of PS1 in the mitochondria of hippocampal cells at 60 and 90 days of O_3 exposure [7]. We suggest that PS1 overexpressed may be transported to ER through MAM or a different mechanism. Then, PS1 and PS2 in the ER could bind to Syntaxin 5, inhibiting the degradation or transport through the ER associated pathway. In addition, it has been demonstrated that Syntaxin 5 in cooperation with PS alters the production of βA peptides. It affects the processing and/or trafficking of the fragment called βAPP, which arrives at the site where γ-secretase appears.

Additionally, it has been demonstrated that Syntaxin 5 overexpression reduced the concentration of Aβ40 peptide secreted, a fact that supports our results and suggestions [44, 45].

Through the methodological approach that we have used in this work we cannot confirm that there are defects in

the ER-Golgi transport; only we suggest this point based on the bibliographic review. For example, some authors suggest that it may be possible that perturbation of Syx5 isoform expression in neuronal cells cause some changes in Aβ production in the central nervous system. Suga et al. have demonstrated that Syx5 binds to the PS holoprotein in the ER and Golgi compartments of neuronal cells and they modulate the metabolism and trafficking of βAPP. Because PS and βAPP are sorted and processed along the secretory and endocytotic pathways, alterations in the transport machinery could affect the trafficking of these proteins, affecting the generation and secretion of Aβ. Thus, malfunction of this chaperone may cause the accumulation of excess Aβ peptides found in late-onset AD.

Finally, the ER stress and oxidative stress cause changes in the pattern of APP processing in affected neurons, increasing the amount of βA42 peptide. ROS also play a role in cell signaling through a mechanism known as redox signaling. Any alteration in the cellular antioxidant defense system and the increase in ROS lead to redox signaling alterations, which are involved in neurodegenerative processes and directly in the overproduction of βA42 [5–7, 46].

Aβ and oxidative stress together are able to trigger an ER stress response, leading to synaptic and neuronal loss, increasing the levels of markers of the ER stress response (UPR), and activating caspases pathway and apoptosis in cortical neurons [13, 15, 16, 36, 47]. Besides, the presence of Aβ in the ER induces the expression of some chaperones like GRP78 and GRP94, involved in the caspase-12 activation and cell death [48].

5. Conclusion

Cell injury due to ER stress has emerged as a key contributor to the pathophysiology of a wide range of neurodegenerative human diseases like Alzheimer's disease and pathological aging. Additional studies are needed to further investigate the impact of βA42 accumulation in the ER. Nevertheless, the present results help to show that the current levels of environmental pollutants in highly polluted cities might induce and participate in the development of neurodegenerative processes by oxidative stress.

Competing Interests

The authors report no conflict of interests.

Acknowledgments

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Review Article

Intracellular Calcium Dysregulation: Implications for Alzheimer's Disease

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Alzheimer's Disease (AD) is a neurodegenerative disorder characterized by progressive neuronal loss. AD is associated with aberrant processing of the amyloid precursor protein, which leads to the deposition of amyloid-β plaques within the brain. Together with plaques deposition, the hyperphosphorylation of the microtubules associated protein tau and the formation of intraneuronal neurofibrillary tangles are a typical neuropathological feature in AD brains. Cellular dysfunctions involving specific subcellular compartments, such as mitochondria and endoplasmic reticulum (ER), are emerging as crucial players in the pathogenesis of AD, as well as increased oxidative stress and dysregulation of calcium homeostasis. Specifically, dysregulation of intracellular calcium homeostasis has been suggested as a common proximal cause of neural dysfunction in AD. Aberrant calcium signaling has been considered a phenomenon mainly related to the dysfunction of intracellular calcium stores, which can occur in both neuronal and nonneuronal cells. This review reports the most recent findings on cellular mechanisms involved in the pathogenesis of AD, with main focus on the control of calcium homeostasis at both cytosolic and mitochondrial level.

1. Introduction

Alzheimer's Disease (AD) is the most common type of dementia affecting millions of people. According to Alzheimer's Disease International (ADI), as of 2015 people suffering from dementia worldwide accounted for estimated 46.8 million. Approximately 70% of these cases were attributed to AD. This amount will increase to an estimated 74.7 million in 2030 and 131.5 million in 2050, with a parallel rise of healthcare costs. As a matter of fact, global costs of dementia have increased from US$ 604 billion in 2010 to US$ 818 billion in 2015, for a 35.4% increase. The incidence rate for AD grows exponentially with age, with the main onset time observed in people aged over 60, in particular between the age of 70 and 80 [1, 2]. AD has also a sex-related incidence, making women 1.5–3 times more vulnerable than men [3]. It has been widely assumed that the higher risk observed in females is related to the loss of the neuroprotective effect of sex steroid hormones during menopause, resulting in estrogen deficiency in the brain [4–6].

AD is a progressive neurodegenerative disorder leading to severe cognitive, memory, and behavioral impairment [7]. The majority of cases is idiopathic; however a rare variant of AD, known as Familial Alzheimer's Disease (FAD), accounts for a small percentage (1–5%) [2, 8] of all cases. FAD features an autosomal dominant heritability and an early disease onset (<65 years old) [7, 9]. Three genetic mutations have been identified as being responsible for FAD. They involve genes for amyloid precursor protein (APP) on chromosome 21 [10], presenilin 1 (PS1) on chromosome 14 [11], and presenilin 2 (PS2) on chromosome 1 [12]. Both forms of AD share two main pathological hallmarks: the abnormal extracellular accrual and deposition of amyloid-β (Aβ) peptides and the intracellular accumulation of neurofibrillary tangles (NFTs). Aβ peptides are cleaved products of APP obtained via sequential proteolysis by two

membrane-bound endoproteases, aspartyl β-secretase and presenilin-dependent secretase (γ-secretase) [13, 14]. APP can also be cleaved by α-secretase to produce nontoxic fragments, which are thought to antagonize Aβ peptides generation [15]. Aβ is a protein consisting of 39–43 amino acids, and it mainly exists in two isoforms: soluble Aβ_{1-40} (~80–90%) and insoluble Aβ_{1-42} (~5–10%) [15, 16]. In particular, due to a greater tendency to aggregate than Aβ_{1-40}, Aβ_{1-42} seems to be the main pathological isoform [17]. Interestingly, it has been described that soluble Aβ globular oligomers can form along a new aggregation pathway independent of Aβ fibril formation. These globular Aβ oligomers have been found in the brain of patients affected by AD and APP transgenic mice, and they bind specifically to neurons and affect synaptic plasticity, as demonstrated by Barghorn and coworkers [18]. The disturbance afforded by soluble Aβ oligomers has also been supported by evidence showing that they can bind to glutamate receptors (both ionotropic and metabotropic), thereby impairing glutamatergic neurotransmission [19, 20]. It is interesting to underline, however, that APP products and very low concentrations of soluble Aβ can be involved in important physiological processes, such as synapse activity and behavior [21, 22].

As for NFTs, it has been found that their major constituent is the protein tau. Tau is the predominant microtubule-associated protein found in mammalian brain [28]. During early stages of development tau is highly phosphorylated; however phosphorylation decreases with brain aging [29, 30], leading to an unphosphorylated form that binds to microtubules, thereby making them more stable. In AD, tau is aberrantly misfolded and abnormally hyperphosphorylated [7, 13]. Several factors might be involved in tau hyperphosphorylation, including Aβ-mediated caspases activation, Aβ-mediated oxidative stress, chronic oxidative stress, and reduced insulin-like growth factor 1-mediated oxidative stress [31]. Over the course of AD, hyperphosphorylation contributes to the loss of tau physiological functions and it prepares this protein to form neurotoxic aggregates. It has been shown that, in this pathological form, tau can also ectopically enter the somatodendritic compartment where, in conjunction with Aβ oligomers, it promotes excitotoxicity. Additionally, tau phosphorylation can modulate DNA integrity and global changes in transcriptional events [32].

Aβ plaques and NFTs, often referred to as "positive features" [13], occur in specific regions rather than diffusely throughout the brain: in particular hippocampus and cortex are mainly affected [8, 13]. In addition, negative features of AD have also been described, including typical losses of neurons, neuropil, and synaptic elements, that mostly parallel NFTs formation. However, a causative relationship between NFTs and neuronal loss still remains to be clarified [33–40]. Growing evidence supports the involvement of neuroinflammation in AD [41], focusing on its critical role within brain regions where Aβ plaques are mainly distributed. Aβ-deposition renders cells more likely to develop inflammatory responses that involve the production of neuronal and glial cytokines belonging to the Tumor Necrosis Factor-α (TNF-α) superfamily [42]. Interestingly, it has been shown that neutralization of the Tumor Necrosis Factor Related Apoptosis Inducing Ligand (TRAIL) protects human neurons from Aβ-induced toxicity [43]. In this context, *in vitro* experiments conducted using the differentiated human neuroblastoma cell line SH-SY5Y demonstrated that the nonsteroidal anti-inflammatory derivative CHF5074 abrogates neurotoxic effects of both Aβ_{25-35} and TRAIL [44], suggesting a potential role of this drug as neuroprotective agent.

AD patients show symptoms that can be divided into two main categories: cognitive and psychiatric. Cognitive symptoms include loss of long term memory, aphasia, apraxia, and agnosia, while psychiatric symptoms include personality changes, depression, and hallucinations (Alzheimer's Foundation of America, Last Update: January 29, 2016; [8]). AD is a complex multifactorial disorder, neuronal death is a subtle phenomenon, and it is difficult to identify a single cause. The idea that energy/mitochondrial dysfunction and oxidative stress may have a central role in the pathogenesis of AD is widely supported by literature [45–49]. Research on the pathogenesis of AD has recently stressed the role of mitochondria, based on the finding that mutation in APP and tau may directly affect mitochondrial function and dynamics [8], and now it is accepted that the impairment of mitochondrial function may affect other crucial cell signaling pathways, as in calcium signaling. A central role for calcium dysregulation in the pathogenesis of AD has been extensively suggested [7, 50]. This review attempts to clarify connections between mitochondrial pathways impairment and the pathogenesis of AD, drawing attention to the calcium homeostasis deregulation as a potential consequence of mitochondrial function disturbance and to the proteins mainly involved in this process, such as the sodium-calcium exchanger (NCX).

2. Calcium and AD

Calcium can be considered a ubiquitous intracellular messenger within cells acting as a regulator in multiple physiological functions. As a divalent cation, calcium can bind to several proteins, receptors, and ion channels. All of these properties are of great importance within neurons, where continuous firing of action potentials leads to calcium cycling, and it implies an influx through the calcium channels at the plasma membrane level, intracellular buffering, and an efflux through the calcium plasma membrane transporters. This cycling involves several subcellular compartments and proteins. In particular, two organelles play a major role in calcium buffering, namely, endoplasmic reticulum (ER) and mitochondria, whereas ATPase calcium pump and NCX are the two main systems involved in calcium efflux through the plasma membrane (Figure 1). Perturbation in such delicate balance may have deleterious consequences for cells and in particular for neurons, leading to necrosis and/or apoptosis and subsequently to stroke and neurodegeneration.

2.1. Intracellular Calcium Homeostasis. There is a large body of evidence documenting a connection between calcium homeostasis disruption and the development of neurodegenerative diseases such as Alzheimer's [50]. The involvement

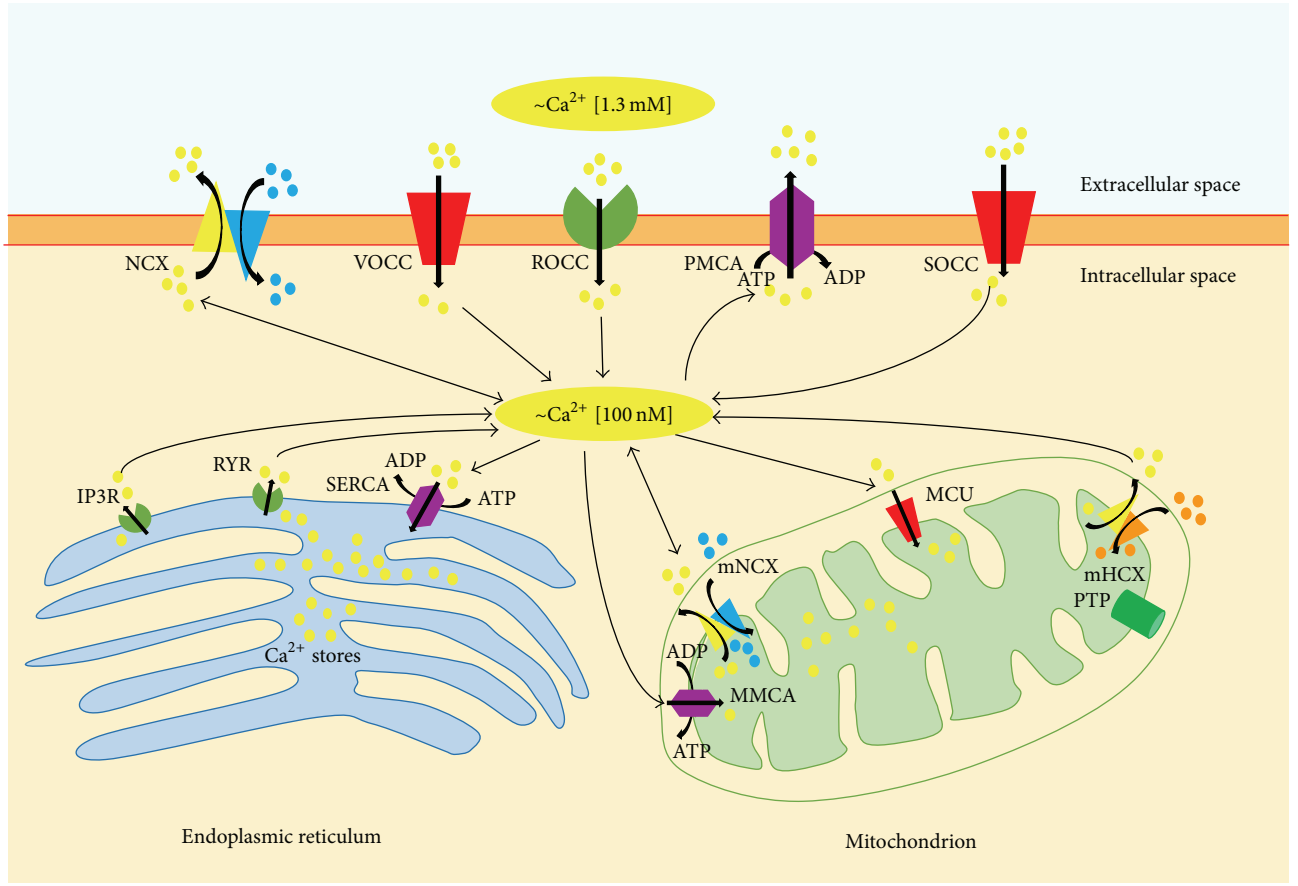

FIGURE 1: Intracellular calcium homeostasis. Intracellular calcium levels are tightly regulated within a narrow physiological range [23]. Cellular calcium influx through the plasma membrane is largely mediated by receptor-operated calcium channels (ROCC), voltage-operated calcium channels (VOCC), store-operated calcium channels (SOCC) and, under exceptional circumstances, the sodium/calcium exchanger (NCX). Under physiological conditions, NCX is mainly involved in calcium efflux; however it can also reverse its mode of operation (reverse mode exchange) thereby contributing to calcium influx, especially during strong depolarization and in the presence of high intracellular sodium concentrations [24]. Calcium may also be released into the cytoplasm from the endoplasmic reticulum, through inositol-1,4,5-trisphosphate (IP3R) and ryanodine receptors (RYR). Different systems operate within the cell to counterbalance the cytosolic calcium increase. Specifically, the plasma membrane calcium pump (PMCA), NCX, and sarcoendoplasmic reticulum calcium ATPase (SERCA) participate in restoring physiological calcium levels. The excess of intracellular calcium can also be taken up by mitochondria through the mitochondrial calcium uniporter (MCU). Calcium can be released back into the cytosol through the activity of mitochondrial NCX (mNCX), which can also reverse its mode of operation allowing the access of calcium ions into the mitochondrial matrix. Recently, the mitochondrial hydrogen/calcium exchanger (mHCX) has been proposed to be an electrogenic 1 : 1 mitochondrial calcium/hydrogen antiporter that drives the uptake of calcium into mitochondria at nanomolar cytosolic calcium concentrations [25]. PTP, permeability transition pore; MMCA, mitochondrial membrane Ca^{2+} ATPase.

of calcium in the pathogenesis of AD has been suggested long time ago by Khachaturian [51], and since then many efforts have been made to clarify this hypothesis [7, 52–56]. Despite the significant progresses made in explaining this theory, several aspects are to be defined. For instance, growing *in vitro* evidence suggests that neuroprotection could be mediated by the restoration of calcium homeostasis. Different calcium channel blockers have been reported to be effective in preventing long- and short-term memory impairment induced by $A\beta_{25-35}$ (the shortest $A\beta$ fragment processed *in vivo* by brain proteases, retaining the toxicity of the full-length peptide [57]) and in decreasing $A\beta$ production, inflammation, and oxidative stress. For example, Rani et al. described the effect of a calcium channel blocker clinically used in angina, in a mouse model of dementia. Interestingly, Morris water maze test, plus maze test and different biochemical analysis, demonstrated the restoration of normal learning and memory functions. Moreover, SCR-1693 (a nonselective calcium channel blocker) has been described to attenuate $A\beta_{25-35}$-induced death in SH-SY5Y cells and to regulate $A\beta$-induced signal cascade in neurons [58–60]. However, the use of calcium channel blockers to

mitigate AD outcomes is still much debated. For example, at least three clinical studies emphasized that elderly people, taking calcium channel blockers as antihypertensive drugs, were significantly more likely to experience cognitive decline than those using other agents [61–63].

At cellular level, it is well documented that abnormal amyloid metabolism induces an upregulation of neuronal calcium signaling, firstly resulting in a decline of memory and then leading to apoptosis [7, 50, 51, 64, 65]. An interesting connection between Aβ, calcium, and AD has been postulated by Arispe and coworkers [66], who suggested that Aβ oligomers can form calcium-permeable channels in membranes. It seems that energy deficits can promote this association, consistently with the observation that neurons with low cytosolic ATP levels showed a pronounced vulnerability to Aβ-induced toxicity [67]. In line with these reports, studies conducted in animal models (i.e., transgenic mice) highlighted an increase in calcium resting levels in the spines and dendrites of pyramidal cortical neurons [68, 69], supporting the hypothesis that calcium-permeable channels can form in the neuronal plasma membrane close to the Aβ plaques, thanks to the high concentration of Aβ oligomers found in these areas [67]. Tau protein is also able to form ion channels in planar lipid bilayer, with lack of ion selectivity and multiple channels conductance, thus contributing to lower membrane potential, dysregulate calcium, depolarize mitochondria, or deplete energy stores [70]. Within neurons, the increase in intracellular calcium levels stimulated by Aβ does not seem to be necessarily sustained by extracellular calcium influx. By using the human neuroblastoma SH-SY5Y cell line, Jensen and coworkers [71] interestingly described that the increase in intracellular calcium levels elicited by the Aβ_{1-42} fragment can occur in the absence of extracellular calcium. Such observation supports the role of calcium release from the ER [72] to the generation of these signals. In addition, they demonstrated that this phenomenon relies only partially on inositol 1,4,5-trisphosphate (IP3) signaling, based on the fact that they observed the calcium mobilizing effect of Aβ_{1-42} when the fragment was applied to permeabilized cells deficient in IP3 receptors (IP3R). Notably, this effect could underpin an additional direct effect of Aβ_{1-42} upon the ER and a mechanism for induction of toxicity by intracellular Aβ_{1-42} [71]. As a matter of fact, ryanodine receptors (RyR) can also contribute to the Aβ-induced calcium release from ER, as described by Ferreiro and coworkers [73, 74]. Exposing rat primary cortical neurons to Aβ_{1-40} or to Aβ_{25-35} peptides, the authors observed an increase in cytosolic calcium levels that was counteracted by either xestospongin C or dantrolene, pharmacological inhibitors of IP3R and RyR, respectively. Once calcium has been mobilized, it can initiate a cascade of events promoting free radicals generation, cytochrome c release from mitochondria, and activation of caspases, culminating in apoptotic cell death [73, 74]. It is worth mentioning that the balance between intracellular calcium levels and ER content involves not only IP3R and RyR, but also the activity of sarcoendoplasmic reticulum calcium ATPase (SERCA), which transports calcium ions from the cytoplasm into the ER (Figure 1). In this regard, Ferreiro and coworkers performed a comparative study by using the selective SERCA blocker thapsigargin [74]. They demonstrated that thapsigargin induced the loss of intracellular calcium homeostasis and the activation of caspase-3, leading to apoptotic cell death, as observed after incubation with Aβ_{1-40} or Aβ_{25-35} peptides. These findings lend support to the hypothesis that intracellular calcium deregulation induced by ER stress may be critical in the neurodegenerative processes triggered by Aβ peptide. Furthermore, the role of SERCA has been also investigated in the context of the FAD. Specifically, it has been proposed that SERCA activity is physiologically regulated by the interaction with presenilin [75], the membrane intrinsic protein that localizes predominantly to the ER membrane, which is responsible for the generation of the Aβ fragment. The finding that the modulation of SERCA activity would alter Aβ production may entail a possible role of the SERCA in the pathogenesis of AD [76].

The alteration of the glutamatergic system may be another important factor causing calcium imbalance in AD. Once released at glutamatergic synapses, glutamate is cleared from the extracellular space by the activity of the high affinity sodium-dependent glutamate transporters (Excitatory Amino Acid Transporters, EAATs) [77], which represent the most prominent system involved in terminating the excitatory signal, recycling the transmitter, and regulating extracellular levels of glutamate. As a result of overproduction and/or impaired clearance from synapses, glutamate may become excitotoxic. In this case, a prolonged exposure to glutamate induces an excessive activation of glutamate receptors, which is associated with a massive calcium influx through the receptor's associated ion channel. The resulting calcium overload is particularly neurotoxic, leading to the activation of several degradation pathways which can have deleterious consequences on the cell fate [78–80]. Marked changes in functional elements of the glutamatergic synapses, such as glutamatergic receptors and transporters, have been described in AD. In 1996, Masliah and coworkers observed a deficit in glutamate transport activity in AD brains, likely occurring at neuronal level [81]. In line with this report, more recent findings suggested that soluble Aβ oligomers can disrupt neuronal glutamate uptake and promote long-term synaptic depression (LTD), a form of synaptic plasticity. In particular, the elegant study by Li and coworkers [82] showed that soluble Aβ oligomers from several sources, including human brain extracts, facilitated electrically evoked LTD in the mouse hippocampal CA1 region, involving both metabotropic and ionotropic glutamate receptors, and high extracellular glutamate levels. Accordingly, neuronal synaptic glutamate uptake was significantly decreased by Aβ. It is interesting to note that Aβ-facilitated LTD was mimicked by the action of the glutamate reuptake inhibitor DL-threo-beta-benzyloxyaspartate (TBOA), confirming that Aβ oligomers ability to perturb synaptic plasticity may rely upon glutamate recycling alteration at the synaptic level. In this regard, a dramatic reduction in the expression of two members of the EAAT family, EAAT1 and EAAT2, has been described at both gene and protein levels in hippocampus and gyrus frontalis medialis of AD patients [83]. Interestingly, in the same regions, glutamate receptors of the kainate type were significantly upregulated, further supporting the hypothesis that

excitotoxic mechanisms can have a role in the pathogenesis of AD [79]. Such upregulation was accompanied by downregulation of the other ionotropic glutamate receptors, namely, N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) receptors. Considering that both NMDA and AMPA receptors are known to mediate long-term potentiation [84, 85], the fundamental molecular mechanism of learning, memory, and cognition, their impairment may be considered a causative factor of the reduced cognitive functions observed in AD patients [83].

Although the observed alterations in intracellular calcium homeostasis in neurons significantly contribute to the pathogenesis of AD, more recent findings suggest that calcium dysregulation occurring in other cell types that support neuronal activity may contribute to degenerative processes [86]. In this regard, Fonseca and colleagues have recently demonstrated that Aβ may imbalance calcium homeostasis in brain endothelial cells with an increase in oxidative stress [87]. Using rat brain microvascular endothelial cells, they showed that the exposure to a toxic dose of Aβ alters ER ability to buffer calcium, and it enhances the mitochondrial and cytosolic response to ATP-stimulated ER calcium release. Although these responses are compensated after a longer exposure to Aβ, the early increase in oxidant levels and the concomitant decrease of antioxidant defenses induce deleterious effects on endothelial cells that undergo apoptosis, contributing to the cerebrovascular impairment observed in AD [87].

Astrocytes are also emerging as active players in AD [88], as highlighted in a recent paper by Dal Prà and coworkers [89]. They suggested an interesting issue concerning Aβ interaction with the Calcium Sensing Receptor (CaSR) [90]. The CaSR is a member of the largest family of cell surface receptors, the G protein-coupled receptors involved in calcium homeostasis. CaSRs expression is ubiquitous within the brain [91], where they are involved in several physiological processes, including synaptic plasticity and neurotransmission [92]. They showed that, in astrocytes, CaSR-Aβ interaction induces a downregulation of CaSR, leading the neighboring neurons to oversecrete *de novo* synthesized Aβ as well as nitric oxide (NO) and the toxic peroxynitrite ($ONOO^-$) [90, 93]. Recently, they have shown that the interaction occurring between Aβ and CaSR in human astrocytes may activate a signaling able to stimulate *de novo* production and secretion of vascular endothelial growth factor (VEGF) [89], whose excessive production can have toxic effects on neurons, astrocytes, and brain–blood barrier [94–97].

In general, the available literature suggests that the prolonged intracellular calcium elevation occurring within brain cells may be a crucial early event in AD pathogenesis, even though the mechanisms have not been fully explained.

In terms of proteins contributing to the calcium homeostasis in the brain, particular attention should be focused on NCX. NCX is a transporter that can move sodium across the membrane in exchange for calcium, operating in either calcium-efflux/sodium-influx mode (forward mode) or calcium-influx/sodium-efflux mode (reverse mode) depending upon the electrochemical ion gradients [24]. Three NCX isoforms have been described, namely, NCX1, NCX2, and NCX3, whose pattern of expression is tissue-specific [98]. Recent reports demonstrated the main role of NCX1 in controlling energy metabolism in several cells types, including neurons and astrocytes [99, 100]. In detail, our group recently reported a functional interaction between NCX1 and the sodium-dependent Excitatory Amino Acid Carrier 1 (EAAC1), at both plasma membrane and mitochondrial level in neuronal, glial, and cardiac models [99, 100]. Notably, we found that NCX1 reverse activity is necessary to restore transmembrane sodium gradient after glutamate entry into the cytoplasm, supporting glutamate utilization as a metabolic substrate that, in turn, enhances ATP production.

The role of NCX isoforms in the pathogenesis of AD is still under investigation. In 1991 Colvin and coworkers [101], measuring NCX activity in cerebral plasma membrane vesicles purified from human postmortem brain tissues of normal, AD, and non-AD origin dementia, identified a transporter altered kinetic in the vesicles of AD patients. The surviving neurons showed an increased NCX activity, leading authors to speculate that this phenomenon could help the surviving neurons to overtake the neurodegenerative process of AD, reinforcing the idea that the increase in intracellular calcium levels can play a major role in the pathogenesis of AD entailing the death of nonsurviving neurons. The hypothesis of an altered activity of NCX in AD patients represents an attractive mechanism that could, at least partially, be accountable for the calcium dysregulation observed in neurodegenerative processes accompanying the pathology [102]. The impairment of NCX activity can be related to the main features of AD. For instance, aggregated Aβ could interact with the hydrophobic surface of NCX, leading to an altered activity of the transporter [103]; however, it cannot be excluded that the observed interaction of Aβ oligomers with the plasma membrane could be *per se* responsible for the alteration of NCX transport properties [103]. The pioneering study of Colvin has inspired further studies that explained the specific role of different NCX isoforms in AD; in this regard, the study by Sokolow and coworkers offered a better understanding of the actual role of NCXs [104]. The analysis of NCX1, NCX2, and NCX3 expression in AD parietal cortex disclosed a specific pattern of expression within nerve terminals. In particular, NCX1 is the main isoform expressed in nerve terminals of cognitively normal patients, while NCX2 and NCX3 seem to be modulated in the parietal cortex in a late AD stage, as NCX2 expression is increased in positive terminals, while NCX3 expression is reduced [104]. Interestingly, the three isoforms colocalize with Aβ, supporting the hypothesis that the NCX activity modulation can be connected to a direct interaction with Aβ; furthermore, in all synaptic terminals containing Aβ, NCX1-3 expression is upregulated [104]. It could be possible that the altered expression of NCX isoforms represents the neurons attempt to counterbalance the Aβ-induced alteration in calcium homeostasis. But, the different pattern observed in NCX isoforms expression can underpin a specific role for each isoform within the neurodegenerative process accompanying AD. In this regard, a specific alteration has been

demonstrated for NCX3 isoform, leading to inactivation. NCX proteins can be inactivated by specific calpain 1 operated cleavage, and this can produce an increase of intracellular calcium levels contributing to the neurodegenerative calcium overload [105, 106]. In AD, the overproduction of Aβ increases calpain-mediated cleavage of NCX3, resulting in a decreased NCX3 activity [107]. Interestingly, the localization of NCX3 in dendrites and astrocytes processes contacting excitatory synapses [108] suggests the major role of NCX3 in regulating calcium current during synaptic activity, which is crucial for normal learning and memory. Therefore, reduced NCX3 activity can strongly contribute to the altered calcium levels associated with neuronal dysfunctions in AD [107].

3. Mitochondria and AD

Mitochondria are essential organelles for both cell survival and death, as they produce the largest part of cellular energy in the form of ATP and they play an active role in apoptosis induction [109, 110]. Mitochondria take part in cellular calcium signaling and act as highly localized buffers, thereby acting in the regulation of cytosolic calcium transient [111–113] (Figure 1). A crucial role in neurodegenerative disorders has been suggested for mitochondria, and AD patients have shown evidence of impaired mitochondrial function [114]. Reddy and coworkers demonstrated the upregulation of genes related to mitochondrial energy metabolism and apoptosis in an AD transgenic mouse model overexpressing a mutant form of APP at different stages of AD progression [115]. Mutant APP and soluble Aβ may enter mitochondria, which generate reactive oxygen species leading to oxidative damage, thereby affecting mitochondrial function. That is why the upregulation of mitochondrial genes could be a compensatory response to mitochondrial dysfunction induced by mutant APP or Aβ [115, 116].

In healthy neurons synaptic activity can be influenced by mitochondrial dynamics, such as fission and fusion events [117]. A number of studies demonstrate that essential proteins for fission and fusion are altered when APP is overexpressed [118, 119]. It has been shown that dynamin-like protein 1 (DLP1) and optic atrophy (OPA1) protein are significantly decreased, whereas levels of fission 1 (Fis1) are significantly increased in cell lines overexpressing APP [119]; this leads to mitochondrial fragmentation and abnormal distribution, which contribute to mitochondrial and neuronal dysfunction [119]. These findings were confirmed by Gan and coworkers [120] that observed significant changes in mitochondria morphology and function in cytoplasmic hybrid (cybrid) neurons, where platelet mitochondria from AD and non-AD human subjects were incorporated into mitochondrial DNA-depleted neuronal cells. They found an impairment of fission/fusion proteins expression and function that was reverted by antioxidant treatment. Interestingly, they showed that oxidative stress negatively affects the extracellular-signal-regulated kinases (ERK) transduction pathway, which alters the expression levels of mitochondrial fission/fusion protein in AD cybrids [120].

Although it was common to focus primarily on Aβ, recently there has been an increasing interest on the role of the hyperphosphorylated form of tau. Hyperphosphorylation can decrease tau binding to microtubules, thereby affecting their stability and axonal transport of organelles, including mitochondria [8, 31]. Recent studies have begun to explore the effect of this altered protein on mitochondrial dynamics. Interesting findings come from the experiments performed by Schulz and coworkers [121] in SH-SY5Y wild-type (wt) and overexpressing P301L mutant tau. They demonstrated that P301L overexpression results in a substantial complex I deficit accompanied by decreased ATP levels and increased vulnerability to oxidative stress. Interestingly, those events were paralleled by pronounced changes in mitochondrial morphology and decreased fusion/fission rates, observed as reduced expression of several fission and fusion proteins such as OPA-1 or DLP- 1 [121]. An imbalance in fission/fusion proteins has also been shown by Manczak and Reddy [122] who demonstrated a physical link between phosphorylated tau and DLP1. The authors concluded that the interaction between phosphorylated tau, DLP1, and Aβ can cause an excessive mitochondrial fragmentation and both mitochondrial and synaptic deficiencies, leading to neuronal damage and cognitive decline [122]. Regardless of its connection with fission/fusion events, the synergistic action of Aβ and tau has been further investigated in a recent study by Quintanilla and colleagues who demonstrated that, in aging neuronal cultures, phosphorylated tau potentiates Aβ-induced mitochondrial dysfunction by affecting mitochondrial membrane potential and increasing oxidative stress [123]. In a previous study, the same group demonstrated that also a truncated form of tau, cleaved at Asp421 by caspases [124], significantly increases oxidative stress response in cortical neurons treated with sublethal concentrations of Aβ [125]. Moreover, interesting results in this field have been obtained by using triple transgenic mice. This model has been obtained by cross-breeding tau transgenic pR5 mice, characterized by tangle formation, and double-transgenic APP152 mice developing Aβ plaques. Only triple transgenic mice, combining both pathologies, at early age (8 months old) showed a reduction of the mitochondrial membrane potential, while at the age of 12 months they showed the strongest defects on oxidative phosphorylation, synthesis of ATP, and reactive oxygen species formation, emphasizing synergistic and age-associated effects of Aβ and tau in perishing mitochondria [126]. Globally, these findings clearly demonstrate that mitochondrial function can be seriously impaired by Aβ and that hyperphosphorylation of tau can enhance the Aβ-induced mitochondrial neuronal damage. Notably, mitochondria are also involved in the maintenance of cellular activities through the contact they establish with ER [127, 128]. Mitochondria-associated ER membranes (MAMs) are intracellular lipid rafts regulating calcium homeostasis and several metabolic pathways, such as glucose, phospholipids, and cholesterol metabolism [127, 129]. The physical interaction between these organelles has been extensively studied, and several MAMs-associated proteins have been identified. A recent research has shown that the contact sites between mitochondria and ER are enriched in PS1 and PS2 [130], components of

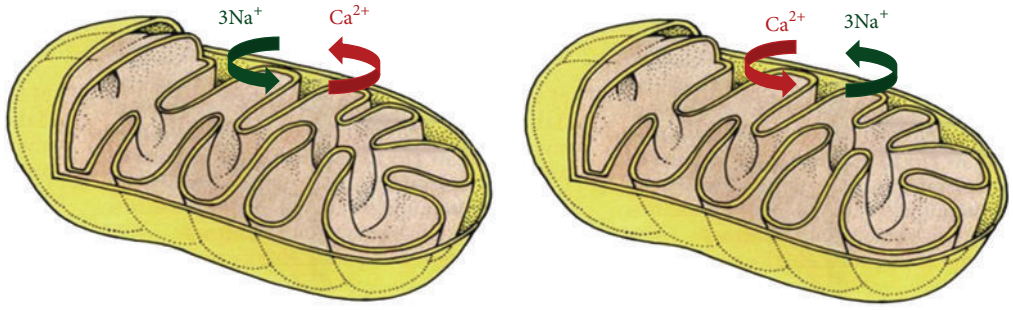

Figure 2: Modes of operation of mNCX. The figure reports the prevalent modes of operation of mNCX. (a) shows the *forward mode* of operation of the exchanger, which is prevalent in physiological conditions. In this mode of operation, mNCX mediates the extrusion of calcium ions from mitochondrial matrix in exchange for sodium ions. (b) shows mNCX *reverse mode* of operation. In this mode of operation, the mitochondrial exchanger mediates the influx of calcium ions into the matrix and the extrusion of sodium ions. The figure has been entirely reproduced from Castaldo et al., 2009 [26], upon written authorization by the editor.

the γ-secretase complex which processes APP to produce Aβ [131]. A large body of evidence indicates PS1 and PS2 mutations as being responsible for the Aβ overproduction by γ-secretase activity leading to FAD [132, 133]. Recently, it has been shown that mutations in PS1, PS2, and APP can upregulate MAMs function and produce a significant increase in ER-mitochondrial connectivity, suggesting that presenilins can negatively regulate this phenomenon [134]. However, the same upregulation in MAMs function and ER-mitochondrial communication has been found in fibroblasts from patients with sporadic AD (SAD), in which there are no mutations in PS1, PS2, and APP structure [134]. This interesting finding suggests that the upregulated function of MAMs, as a common feature in both FAD and SAD, may represent a pathogenic initiator of AD [134]. A recent study by Schreiner and colleagues [135] supports this hypothesis. In this work, the authors determined the production of Aβ in subcellular fractions isolated from mouse brain. They found that a large amount of Aβ was produced at mitochondria-ER contact sites. They postulated that the enhanced Aβ production may perturb mitochondria and mitochondria-ER contact site functions, leading to neurodegeneration and, therefore, to AD [135]. As a matter of fact, the MAMs structure has been postulated to modulate calcium signals and synaptic and integrative activities at neuronal level [127, 136]. In this regard, it has been suggested that MAMs may host important physiological functions related to neuronal integrity, as they have been reported to be uniformly distributed throughout hippocampal neurons and at synaptic level [127]. In particular, two main proteins have been identified as being crucial for MAMs activity and, consequently, for neuronal integrity: phosphofurin acidic cluster sorting protein-2 (PACS-2) and σ1 receptor (σ1R) [127]. These proteins contribute to maintaining MAMs homeostasis. Specifically, PACS-2 is a multifunctional sorting protein controlling ER-mitochondria communication and apoptosis [137], whereas σ1R promotes calcium transport into mitochondria from the ER by interacting with the IP3R [138]. Their knockdown results in neurodegeneration, and this highlights the importance of these proteins in the maintenance of neuronal integrity [127]. Furthermore, exposure to Aβ results in the increase of MAMs-associated proteins expression and of the amount of contact points between ER and mitochondria in different AD models (namely, APP transgenic mice, primary neurons, and AD brain) [127]. In turn, the alteration in MAMs-associated proteins expression can affect calcium homeostasis, which has been considered an underlying and integral component of AD pathology [7, 50, 67]. This issue is further discussed in the following section.

3.1. Role of Mitochondria in Intracellular Calcium Balance. Intracellular calcium dysregulation is a central event in neurodegeneration; it involves plasma membrane transporters and also intracellular organelles, such as mitochondria, thereby creating an endless futile cycle that can have several consequences on neuronal survival [67]. The excess of intracellular calcium is taken up by mitochondrial calcium uniporter (MCU) that, through the large electrochemical gradient across the inner mitochondrial membrane, drives calcium from the cytosol to the mitochondrial matrix [67] (Figure 1). Calcium is then released back into the cytosol through the activity of mitochondrial NCX (mNCX, Figures 1 and 2(a)) [67]. However, mNCX may reverse its mode of operation (Figure 2(b)) from a calcium efflux system to an influx pathway allowing the access of calcium ions into the mitochondrial matrix [26]. Although the molecular identity of mNCX has been extensively researched and strongly debated, our group has provided data showing that plasma membrane NCX (plmNCX) isoforms can contribute to mNCXs. Exploring the subcellular distribution of NCX in the central nervous system by western blot and *in situ* electron microscopy immunocytochemistry in rat neocortex and hippocampus, we observed a large population of neuronal and astrocytic mitochondria expressing NCX1–3 [26, 27] (Figures 3 and 4). Thus, these mitochondrial calcium transporters manage intracellular changes of this "versatile" ion, impacting several cell functions, including cell metabolism. As a matter of fact, the activity of several intramitochondrial dehydrogenases is enhanced by increased mitochondrial calcium

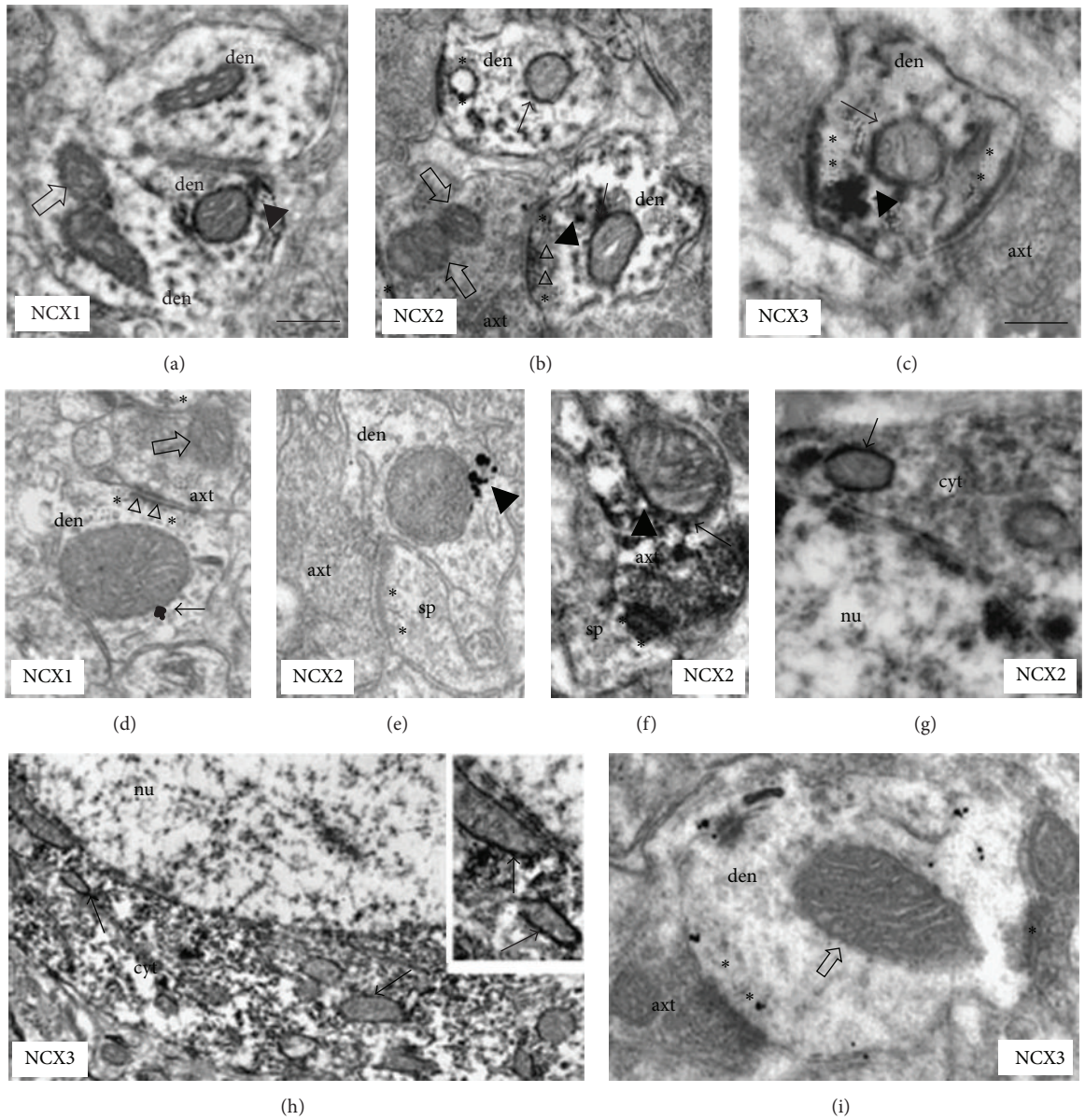

Figure 3: NCXs labeling patterns in neuronal mitochondria ((a)–(i)). (a and d) NCX1-ir mitochondria (arrows) in distal dendrites (CA1 stratum radiatum). ((b) and (e)) NCX2-ir mitochondria (arrows) in hippocampal (b) and neocortical (e) dendrites. (c) NCX3-ir mitochondria in neocortical distal dendrite (arrow). (f) NCX2-positive mitochondrion in a CA1 axon terminal. ((g) and (h)) NCX2 and NCX3-ir mitochondria (arrows) in a cell body (from CA1 pyramidal cell layer); enlarged in the inset in (h), two labeled organelles (arrows) near nuclear envelope. (i) NCX3-ir in neocortical distal dendrite with unlabeled mitochondria (open arrow). In (b) and (d) dendrites are contacted by axon terminals forming asymmetric junction (triangles). In (a) and (e), note the labeling bridging plasma membrane and mitochondrial profile. Open arrows indicate unlabeled mitochondria in dendrites ((a) and (i)) and axon terminals ((b) and (d)). With asterisks the postsynaptic specializations are indicated and the arrowheads show the labeling between mitochondria and plasma membrane. axt, axon terminal; den, dendrite; nu, nucleus; cyt, cytoplasm; sp, dendritic spine. Immunoperoxidase reaction in (a)–(c), (f), (g), and (h) and silver-enhanced immunogold in (d), (e), and (i). Calibration bars: in (a), 0.25 m for (a), (b), (d), (f), and (i); in (a), 0.5 m for inset in (h); in (c), 0.25 m for (c) and (e); in (c), 0.5 m for (g); in (c), 1 m for (h). The figure has been entirely reproduced from Gobbi et al., 2007 [27], upon written authorization by the editor.

levels, thereby stimulating ATP synthesis [139, 140]. The brain is one of the most metabolically active organs in the body. The brain's high energy requirements are mainly due to maintenance and restoration of ion gradients dissipated by signaling processes such as postsynaptic and action potentials, as well as uptake and recycling of neurotransmitters. In AD, the impairment in energy production is one of the factors greatly contributing to the vulnerability of neuronal cells [141]. One of the main works demonstrating the cooperative action of tau and Aβ shows, through a proteomic analysis,

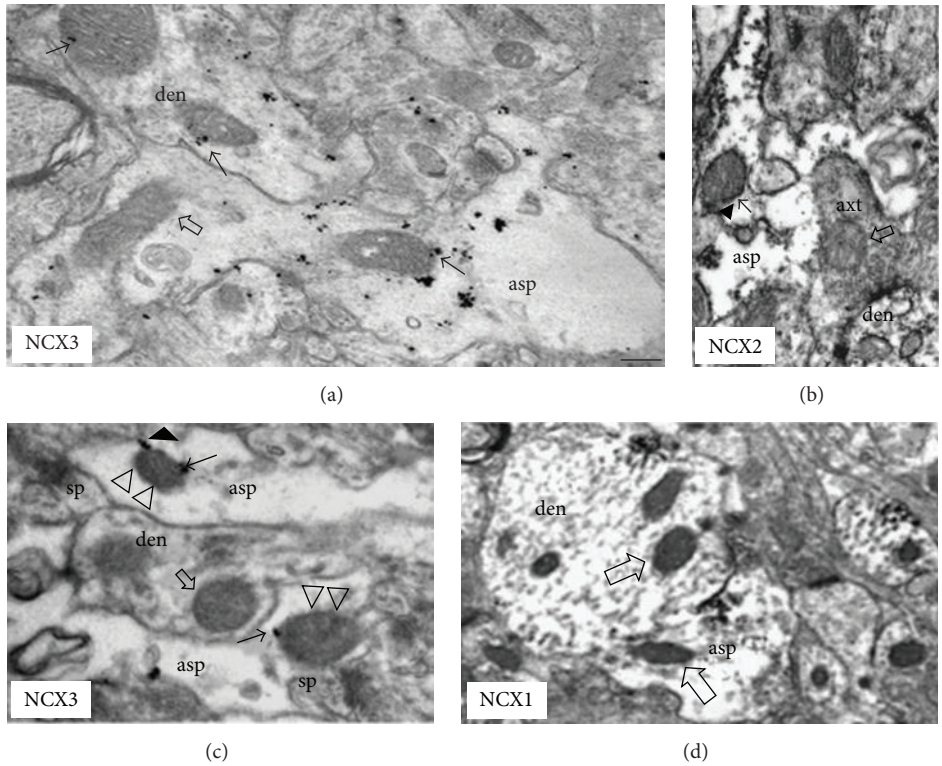

Figure 4: NCXs labeling patterns in astrocytic mitochondria ((a)–(d)). (a) NCX3-expressing mitochondrion (arrow) in neocortical astrocytic process; an adjacent dendrite contains two labeled mitochondria (arrows). (b) NCX2-ir mitochondrion (arrow) in hippocampal glial process. Intense labeling is present on plasma membrane. (c) NCX3-labelled sub-plasma membrane mitochondria (arrows) in two astrocytic processes contacting synaptic structures; labeling between a mitochondrion and the plasma membrane is evident (arrowhead). An unlabeled mitochondrion is localized in a dentritic structure (open arrow). (d) A NCX1-unlabeled mitochondrion (open arrow) in a labeled distal astrocytic process in neocortex. Note some positive distal dendrites with unlabeled mitochondria. Open arrows indicate unlabeled mitochondria; triangles show the mitochondrial labeling near the synaptic membrane. Asp, astrocytic process; den, dendrite; axt, axon terminal; sp, spine apparatus. Immunoperoxidase reaction in (b) and (c) and silver-enhanced immunogold in (a). Calibration bars: in (a), 0.25 m for (a), (b), and (c); in (a), 0.5 m for (d). The figure has been entirely reproduced from Gobbi et al., 2007 [27], upon written authorization by the editor.

that one-third of the deregulated proteins in different AD mouse models is made up of mitochondrial proteins involved in oxidative phosphorylation [126]. Hence, it is tempting to speculate that modulation of mitochondrial calcium transporter activity toward the increase in ATP production could have beneficial effects on neuronal survival during the neurodegenerative processes that characterize AD. In this context, it has been suggested that a partial inhibition of mNCX would lead to an increase of the mitochondrial matrix calcium concentration to a higher physiological steady-state level that could stimulate calcium-sensitive dehydrogenase activity and the rate of ATP synthesis [67, 139, 140]. Therefore, calcium may play a dual role within cells: on the one hand it can help vulnerable neurons increase the rate of ATP synthesis; on the other hand it can be harmful and activate cell death through the induction of the apoptotic pathways [142]. Thus, there must be a critical point representing the boundary between cytoprotective and cytotoxic effect due to the increase in mitochondrial calcium concentration [67]. An increased rate of ATP synthesis can be achieved stimulating the cell in several ways. Recently, our group found that both plmNCX and mNCX can act synergically to sustain the increase in ATP synthesis promoted by glutamate [99, 100]. As reported above, this metabolic response results from a physical and functional interaction between NCX (particularly NCX1) and EAATs, with particular reference to EAAC1, occurring at both plasma membrane and mitochondrial level [99, 100]. The fact that some substrates, such as glutamate, can modulate ATP synthesis may have several implications for AD too, and this can reverse the traditional view of a predominantly harmful effect of this amino acid, towards a benefic role that is able to rescue vulnerable neurons from death. At present, the role of mNCX in AD is still largely unexplored [26]. In an interesting paper, Thiffault and Bennett [143] reported indirect evidence of an involvement of the exchanger in AD. In particular, they showed that cells, lacking endogenous mitochondria and repopulated with mitochondria from AD patients, virtually lack the spontaneous fluctuations in mitochondrial membrane potential ($\Delta\Psi_M$), also called "$\Delta\Psi_M$ flickering," which is normally induced by cyclosporine. It is worth noting that mNCX blockade with CGP-37157 suppresses flickering in control cells, thus recreating a condition similar to the one observed in AD. The role of mNCX in AD is also supported by

the work of Chin and colleagues [144], who observed that Aβ potentiates the increase in cytosolic calcium concentration evoked by nicotine in dissociated rat basal forebrain neurons in a CGP-37157-sensitive way.

4. Conclusions

Dysregulation of intracellular calcium homeostasis has been suggested as a proximal cause of cellular dysfunction during AD, and in this context calcium imbalance has been considered a phenomenon mainly related to the dysfunction of subcellular organelles, such as mitochondria. Functional impairment of calcium-related proteins may play a major role in the pathogenesis of AD. One of the main regulators of intracellular calcium levels, NCX, is emerging as a transporter possibly involved in the nervous system pathophysiology, although its involvement in AD is still poorly investigated. Recent studies conducted by our group [99, 100] show NCX as a key factor in the regulation of cellular metabolism too, acting at both plasma membrane and mitochondrial level. Energy metabolism and intracellular calcium levels are closely related and, therefore, it has been suggested that energy and calcium signaling deficits can be considered the earliest modifiable defects in brain aging [145], including AD. The achievement of an increase in cell metabolism and mitochondrial calcium content through the manipulation of NCX activity may represent a new successful approach to prevent neuronal degeneration and death. However, further studies are needed to support this finding.

Competing Interests

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

Authors' Contributions

Simona Magi and Pasqualina Castaldo equally contributed to this work.

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