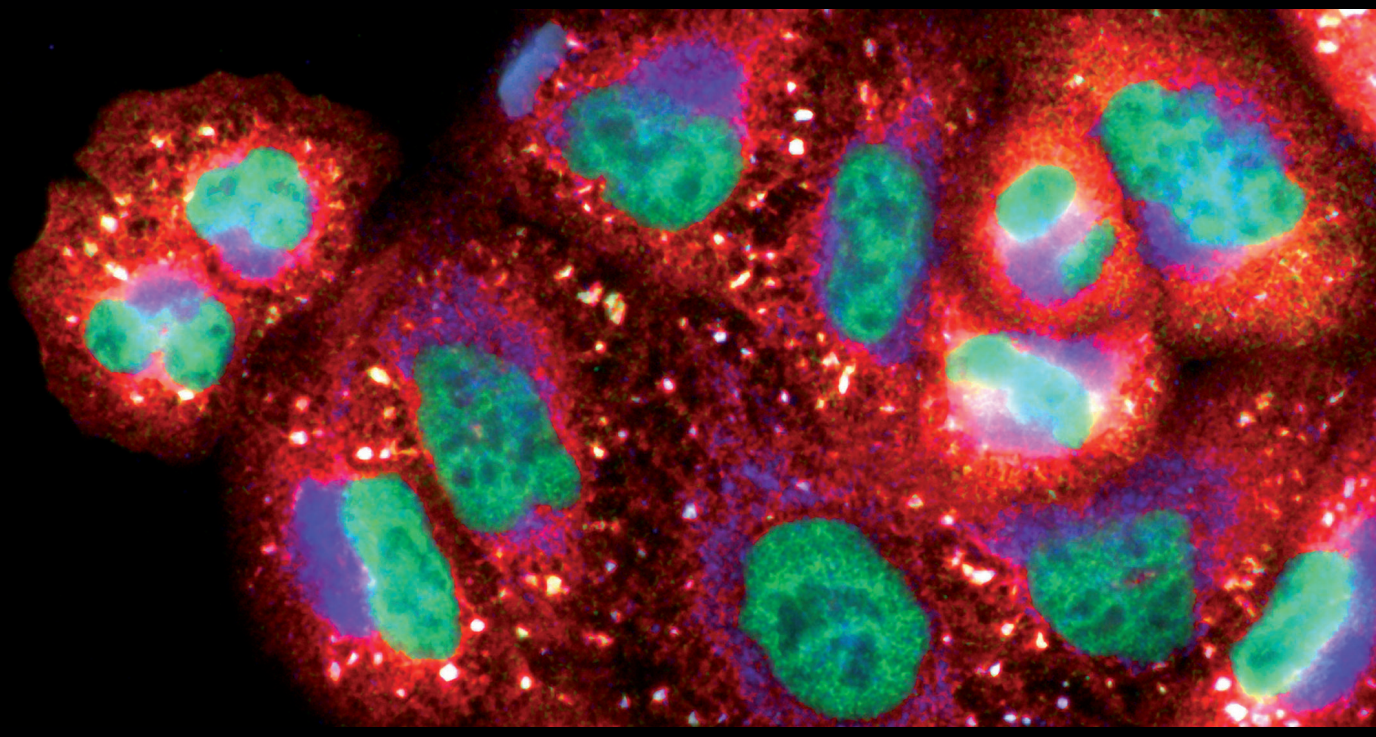
Oxidative Medicine and Cellular Longevity

Neurodegeneration, Mitochondrial Dysfunction, and Oxidative Stress

Guest Editors: Emilio L. Streck, Grzegorz A. Czapski, and Cleide Gonçalves da Silva



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Editorial

Neurodegeneration, Mitochondrial Dysfunction, and Oxidative Stress

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Mitochondria are intracellular organelles that play a crucial role in energy metabolism. Most cell energy is obtained through mitochondrial metabolic pathways, especially the Krebs cycle and electron transport chain which is the main site for production of reactive oxygen species such as superoxide, hydrogen peroxide, and hydroxyl radicals. Brain tissue is highly sensitive to oxidative stress due to its high oxygen consumption, iron and lipid contents, and low activity of antioxidant defenses. Thus, energy metabolism impairment and oxidative stress are important events that have been related to the pathogenesis of diseases affecting the central nervous system.

In the present issue, the pathogenesis of common Alzheimer's (AD) and Parkinson's (PD) diseases is addressed in five papers. Mondragón-Rodríguez et al. proposed that phosphorylated tau protein could play the role of potential connector and that a combined therapy involving antioxidants and check points for synaptic plasticity during early stages of the disease could become a viable therapeutic option for AD treatment. This paper is accompanied by a study by T. Rohn that explores the potential role that the triggering receptor expressed on myeloid cells 2 (TREM2) normally plays and how loss of function may contribute to AD pathogenesis by enhancing oxidative stress and inflammation within the central nervous system. Additionally, O. Myhre et al. review the possible impact of environmental exposures in metal dyshomeostasis and inflammation in AD and PD. Furthermore, T. Omura et al. explore recent studies on the mechanism of endoplasmic reticulum stress-induced neuronal death related to PD, focusing on the involvement of human ubiquitin ligase HRD1 in the prevention of neuronal death as well as a potential therapeutic approach for PD based on the upregulation of HRD1. Lastly, S. Matsuda et al. showed a concise overview on the cellular functions of the mitochondrial kinase PINK1 and the relationship between Parkinsonism and mitochondrial dynamics, with particular emphasis on a mitochondrial damage response pathway and mitochondrial quality control.

Three of the papers deal with aspects of oxidative stress implicated in the pathogenesis of neurodegenerative diseases. W. Liu et al. reviewed the current literature on the effects of oxidative stress due to exhaustive training on uncoupling protein 2 (UCP2) and Bcl-2/Bax in rat skeletal muscles. A.-M. Enciu et al. explore the possibility of oxidative-induced molecular mechanisms of blood-brain barrier disruption and tight junction protein expression alteration, in relation to aging and neurodegeneration. Moreover, M. Tajes et al. suggest that peroxynitrite induces cell death and is a very harmful agent in brain ischemia.

A. Hosseini and M. Abdollahi review the pathogenesis of diabetic neuropathy with a focus on oxidative stress and introduced therapies dependent or independent of oxidative stress. A. Sekigawa et al. review the currently available evidence that neither mitochondria nor leucine-rich repeat

kinase 2 (LRRK2) was present in the swellings of mice expressing P123H β-synuclein, suggesting that α- and β-synucleins might play differential roles in the mitochondrial pathology of α-synucleinopathies. The paper by P. F. Schuck et al. showed that *trans*-glutaconic acid is toxic to brain cells *in vitro*, by causing alterations in cell ion balance and probably neurotransmission, as well as oxidative stress in rat cerebral cortex. To complete the issue, M. J. Rodríguez et al. discuss the mitochondrial K_{ATP} channel as a new target to control microglia activity, avoid its toxic phenotype, and facilitate a positive disease outcome. Fortes et al. showed that 5TIO1 can protect the brain against neuronal damage regularly observed during neuropathologies. These papers are accompanied by a review by Z. Yu et al. on how the neuroglobin's neuroprotection is related to mitochondria function and regulation.

By compiling these papers, we hope to enrich our readers and researchers with respect to mitochondrial dysfunction, energy metabolism impairment, and oxidative stress in the pathophysiology of neurodegenerative diseases.

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Research Article

Nitro-Oxidative Stress after Neuronal Ischemia Induces Protein Nitrotyrosination and Cell Death

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Ischemic stroke is an acute vascular event that obstructs blood supply to the brain, producing irreversible damage that affects neurons but also glial and brain vessel cells. Immediately after the stroke, the ischemic tissue produces nitric oxide (NO) to recover blood perfusion but also produces superoxide anion. These compounds interact, producing peroxynitrite, which irreversibly nitrates protein tyrosines. The present study measured NO production in a human neuroblastoma (SH-SY5Y), a murine glial (BV2), a human endothelial cell line (HUVEC), and in primary cultures of human cerebral myocytes (HC-VSMCs) after experimental ischemia *in vitro*. Neuronal, endothelial, and inducible NO synthase (NOS) expression was also studied up to 24 h after ischemia, showing a different time course depending on the NOS type and the cells studied. Finally, we carried out cell viability experiments on SH-SY5Y cells with H_2O_2, a prooxidant agent, and with a NO donor to mimic ischemic conditions. We found that both compounds were highly toxic when they interacted, producing peroxynitrite. We obtained similar results when all cells were challenged with peroxynitrite. Our data suggest that peroxynitrite induces cell death and is a very harmful agent in brain ischemia.

1. Introduction

Ischemic stroke, the most common type of stroke, is an acute vascular accident caused by blockage in a brain vessel, which yields to a lack of oxygen supply that dramatically affects the brain parenchyma and brain vasculature. The tissue surrounding the ischemic lesion where neurons are still alive, but their viability that is highly compromised, called the penumbra area, is a major target for ischemic stroke treatments [1, 2].

After ischemia, nitric oxide (NO) is released by endothelial cells to recover blood perfusion [3]. The NO is produced by the NO synthases (NOS), a family of enzymes coded by genes located in different chromosomes [3]. The major regulator of the vascular tone is the endothelium, through the endothelial NOS (eNOS). Diffusion of NO

to vascular smooth myocytes produces myorelaxation in a cyclic, guanosine monophosphate-dependent manner [4]. The neuronal (nNOS) and inducible (iNOS) enzymes are expressed in neurons, glial cells, and vascular myocytes [5–12] and may contribute to NO production in ischemic processes. Moreover, a burst of free radicals such as superoxide anion ($O_2^{\bullet-}$) [13, 14] is produced after the ischemic event. This scenario could be very harmful because superoxide anion has a high affinity for NO, higher than for the superoxide dismutase [15, 16], and both compounds react with each other and produce peroxynitrite ($ONOO^-$) [17]. Peroxynitrite has a gaseous nature that allows it to spread around the surrounding tissue to the ischemic focus. Peroxynitrite provokes protein nitrotyrosination, an irreversible chemical process consisting in the addition of a nitro group (NO_2) to the tyrosine residues to generate 3-nitrotyrosine [18, 19]. This posttranslational modification is pathological because it usually impairs the physiological function of the proteins [20, 21]. Evidence of protein nitrotyrosination in ischemic stroke has been reported [22].

The present study analyzed the role of the different cell types from brain parenchyma and brain vessels in NO production. Protein expression and mRNA levels of the different NOS were assessed. Moreover, we studied the effects of peroxynitrite on the viability of the different brain cells.

2. Materials and Methods

2.1. Cell Cultures. Human neuroblastoma cells (SH-SY5Y) were grown in DMEM (Gibco) supplemented with 15% fetal bovine serum (FBS; Gibco) and antibiotics (100 units/mL penicillin and 10^{-6} μg/mL streptomycin; Gibco). HUVECs were grown in M-199 medium (Gibco) supplemented with 10% FBS, 3.2 mM glutamine (Sigma), and antibiotics. Murine microglial cells (BV2) were grown in RPMI (Gibco) supplemented with 10% FBS and antibiotics. Primary cultures of HC-VSMCs were produced from cerebral arteries (basilar) obtained from autopsies of 4 individuals (55.3 ± 5.6 years) and utilized up to the ten passages [23]. Procedure was approved by the ethics committee of the Institut Mar d'Investigació Mèdica and the Universitat Pompeu Fabra (IMIM-IMAS-UPF). Briefly, pieces of tunica media were incubated with 0.1% collagenase type IV (Sigma) for 35 min at 37°C and cultured to allow HC-VSMC migration to the flask surface. Cells were grown in DMEM with 4.5 g/L glucose (Sigma), 25 mM HEPES (Gibco), 10% FBS, 2.5 μg/mL amphotericin B (Gibco), 100 units/mL penicillin, and 10^{-6} μg/mL streptomycin. Myocytes were characterized by immunostaining with mouse anti-smooth muscle α-actin antibody (Ab; Sigma). Cells were used up to ten passages.

2.2. Mouse Embryo Hippocampal Cell Cultures. Hippocampal cells were isolated from 18-day-old CB1 mouse embryos. Procedure was approved by the ethics committee of the IMIM-IMAS-UPF. Hippocampi were aseptically dissected and trypsinized. Cells were seeded in DMEM plus 10% horse serum (Gibco) into 1% poly-L-lysine (Sigma) coated coverslips (5×10^4 cells/cover). After 2 h, medium was removed, and Neurobasal medium containing 1% B27 supplement (Gibco) plus antibiotics was added. Glial proliferation was avoided by adding 2 μM cytosine arabinoside (Sigma) at day 3 for 24 h. Cultured hippocampal cells were used for the experiments on day 7, when they were considered to be mature neurons [24].

2.3. Human Brain Study. The human cortex sample was obtained from an autopsy of a patient who had a cortical ischemic stroke (Servei d'Anatomia Patològica, Hospital del Mar, Barcelona). The sample was fixed in formalin and embedded in paraffin. Procedure was approved by the ethics committee of the Institut Municipal d'Investigació Mèdica and the Universitat Pompeu Fabra (IMIM-IMAS-UPF).

2.4. In Vitro Ischemia. Ischemia was induced by an oxygen-glucose deprivation protocol (OGD). Briefly, the culture medium was replaced with a glucose-free balanced saline solution containing 116 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 1 mM NaH_2PO_4, 26.2 mM $NaHCO_3$, and 1.8 $CaCl_2$, incubated for 1 h in a nitrogen chamber at 37°C. The OGD medium was then replaced by culture medium, and cells were incubated for up to 12 h or 24 h after OGD under standard culture conditions.

2.5. Free Radical Production. SH-SY5Y cells were seeded on 1.5% gelatin-coated 35 mm coverslips (1×10^5 cells/well) for image analysis with a Leica TCS SP confocal microscope. SH-SY5Y cells were seeded in a 96-well plate (1×10^3 cells/well) for fluorescence quantification with a Fluostar Optima Microplate Reader (BMG Labtech). In both cases, cells were subjected to OGD with the OGD medium plus 5 μM 2,7-dichlorofluorescin diacetate (DCF-DA; Sigma). The OGD medium was replaced by culture medium plus 5 μM DCF-DA, cells were incubated in standard culture conditions, and experimental analysis was completed within 1 h.

2.6. NO Assay. HUVEC, SH-SY5Y, BV2, and HC-VSMC cells were seeded on T-25 flasks (approximately 5×10^6 cells/flask) and subjected to OGD. Control media, 12 h post-ischemic media and 24 h postischemic media were collected, and NO was measured using a nitrate/nitrite colorimetric assay kit (Cayman).

2.7. Immunofluorescence. Hippocampal cells (4×10^4 cells/well) were seeded on 1.5% gelatin coated, 12 mm coverslips. Cells were challenged for 1 h with OGD and maintained in standard culture conditions up to 24 h. Cells were fixed and incubated for 2 h at room temperature (RT) with 1 : 500 antinitrotyrosine polyclonal Ab (Chemikon), followed by incubation with 1 : 500 Alexa Fluor 488 goat anti-rabbit polyclonal Ab (Dako) for 1 h at RT. Nuclei were stained in blue with To-pro 3. The human cortex sample was cut at 3 μm, deparaffinized at 70°C for 1 h, and washed with decreasing concentrations of ethanol. Antigen retrieval was performed with proteinase K at 40 μg/mL in a 1 : 1 glycerol and TE buffer solution. Immunostaining was performed with 1 : 200 mouse monoclonal anti-NTyr Ab (Cayman Chem) for 2 h

at RT, followed by 1 : 1000 Alexa555-bound anti-mouse as secondary Ab (Dako) overnight at 4°C. Sections were stained with To-pro 3 to identify the nuclei. All the coverslips were mounted with Mowiol. Images were taken with a Leica TCS SP confocal microscope and analyzed with Leica confocal software (Leica).

2.8. Protein Identification by Western Blot. Cell cultures were lysed on ice with a solution containing 1 M Tris-HCl, 1% Nonidet P-40, 150 mM NaCl, 5 mM EDTA, 1 mM sodium orthovanadate, 1 mM dithiothreitol, pH 7.4, and a protease inhibitor cocktail (Roche). Protein concentration was determined by Bio-Rad protein assay. Protein samples were analyzed using 3–8% Tris-acetate gels (Invitrogen) for NOS detection. Gels were run at 150 V for 1 h and transferred to nitrocellulose membranes (Millipore) at 100 V for 2 h. Membranes were blocked in Tween 20-Tris buffer solution (100 mM Tris-HCl, 150 mM NaCl, and pH 7.5; 0.1% Tween, 5% milk) and incubated for 2 h at 25°C with 1 : 1,000 anti-nNOS (Santa Cruz Biotechnology), anti-iNOS (Santa Cruz Biotech.), and anti-eNOS (Santa Cruz Biotech.) Abs. Peroxidase-conjugated donkey anti-rabbit and anti-mouse Abs (Amersham Bioscience) were used as secondary Abs at 1 : 5,000 for 1 h at 25°C. Bands were visualized with Super Signal (Pierce) and Amersham Bioscience Hyperfilm ECL kit.

2.9. RNA Isolation and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Analysis. Total RNA from cell cultures was isolated using Trizol reagent (Invitrogen), following the manufacturer instructions. Briefly, 1 μg of RNA was applied to RT-PCR using the OneStep RT-PCR Kit (Qiagen, Hamburg, Germany). The *iNOS*-specific primers for mice were 5′-CCATCACTGTGTTCCCCC-3′ and 5′-AAGGTGGCAGCATCCCC-3′ (Genbank accession number: NM_010927). The *iNOS*-specific primers for humans were 5′-CTGCTTGAGGTGGGCGG-3′ and 5′-GTGACTCTGACTCGGGACGCC-3′ (NM_000625). The *eNOS*-specific primers were 5′-CAAGTATGCCACCAACCGGG-3′ and 5′-ACTGAAGGGGGCTGCGG-3′ (NM_000603). The *nNOS*-specific primers were 5′-GAGAAGGAGCAGGGGGGG-3′ and 5′-CACATTGGCTGGGTCCCC-3′ (NM_000620). These primers were used to amplify the different *NOS*. Hypoxanthine phosphoribosyltransferase- (*hPRT*-) specific primers (5′-GGCCAGACTTTGTTGGATTTG-3′ and 5′-TGCGCTCATCTTAGGCTTTGT-3′; NM000194) were used as positive control. Negative control was performed in the absence of oligonucleotide primers. Results were analyzed with Image Gauge software (Fuji Photo Film Co.).

2.10. Cell Viability Assay. Cells were seeded in 96-well plates at a density of 8,000 cells/100 μL per well and treated with 3-morpholinosydnonimine hydrochloride (SIN-1), sodium nitroprussiate (SNP), and/or H_2O_2, as described in the corresponding figures. Cells were incubated for 6 or 24 h at 37°C, and cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction method. Absorbances at 540 and 650 nm were determined in a Microplate Reader (Bio-Rad) and expressed as percentage of control.

2.11. Statistical Analysis. Data were expressed as the mean ± SEM of the values from the number of experiments indicated in the corresponding figures. Data were analyzed using one-way ANOVA with Bonferroni *post hoc* analysis.

3. Results

3.1. Ischemia Induces an Increase in Nitrotyrosination, Reactive Oxygen Species (ROS), and NO Production in Brain Cells. A strong labeling for nitrotyrosination was observed in the cortex from a stroke patient, not only in the brain parenchyma but also in the tunica intima and media of the blood vessel (Figure 1(a)). Neurons are very sensitive to oxidative stress. Therefore, we analyzed the protein nitrotyrosination after ischemia in mature mouse hippocampal neurons (Figure 1(b)). We assayed the effect of ischemia by subjecting the hippocampal neurons to OGD for 1 h, followed by reoxygenation. Neurons were maintained in a growth medium containing glucose for 24 h. We observed that hippocampal neurons subjected to ischemia had high levels of nitrotyrosination. The presence of nitrotyrosinated proteins demonstrated the existence of nitro-oxidative stress resulting from ischemia.

To study free-radical production after ischemia, we exposed human neuroblastoma cells (SH-SY5Y) to OGD. After 1 h, SH-SY5Y cells showed a burst in free radical production, measured by DCF oxidation (Figure 1(c), right) and visualized by confocal microscopy (Figure 1(c), left).

In addition to neurons, other brain cell types are found in the ischemic area; all cell types could contribute to the damage by producing NO. After the ischemic challenge, microglia and endothelial cells showed an increase in NO production (Figure 1(d)) that persisted up to 24 h. Endothelial cells and microglia were the highest NO producers in both baseline conditions and after ischemia; SH-SY5Y and brain vascular myocytes had no effect on NO release due to ischemia (Figure 1(d)).

3.2. Protein and mRNA Levels of NOS Types Are Modified in Brain Cells after Ischemia. NO production is dependent on the expression and activity of the different NOS. For this reason we examined the effect of ischemic insult on the protein and mRNA levels of *nNOS*, *eNOS,* and *iNOS*. In SH-SY5Y cells, we studied nNOS (Figure 2(a)). At 24 h after ischemia, we observed a significant reduction in nNOS protein ($P < 0.05$), but *nNOS* mRNA levels were not significantly affected (Figure 3(a)).

The main responsibility for the NO burst occurring after brain ischemia has classically been attributed to microglial cells. We studied the behavior of iNOS protein and mRNA in microglial cells (BV2) challenged with *in vitro* ischemia (Figure 2(b)). We found that iNOS is present in non-stimulated control microglial cells. Moreover, iNOS expression increased significantly at 12 h after the ischemic challenge ($P < 0.005$). The analysis of *iNOS* mRNA revealed, once

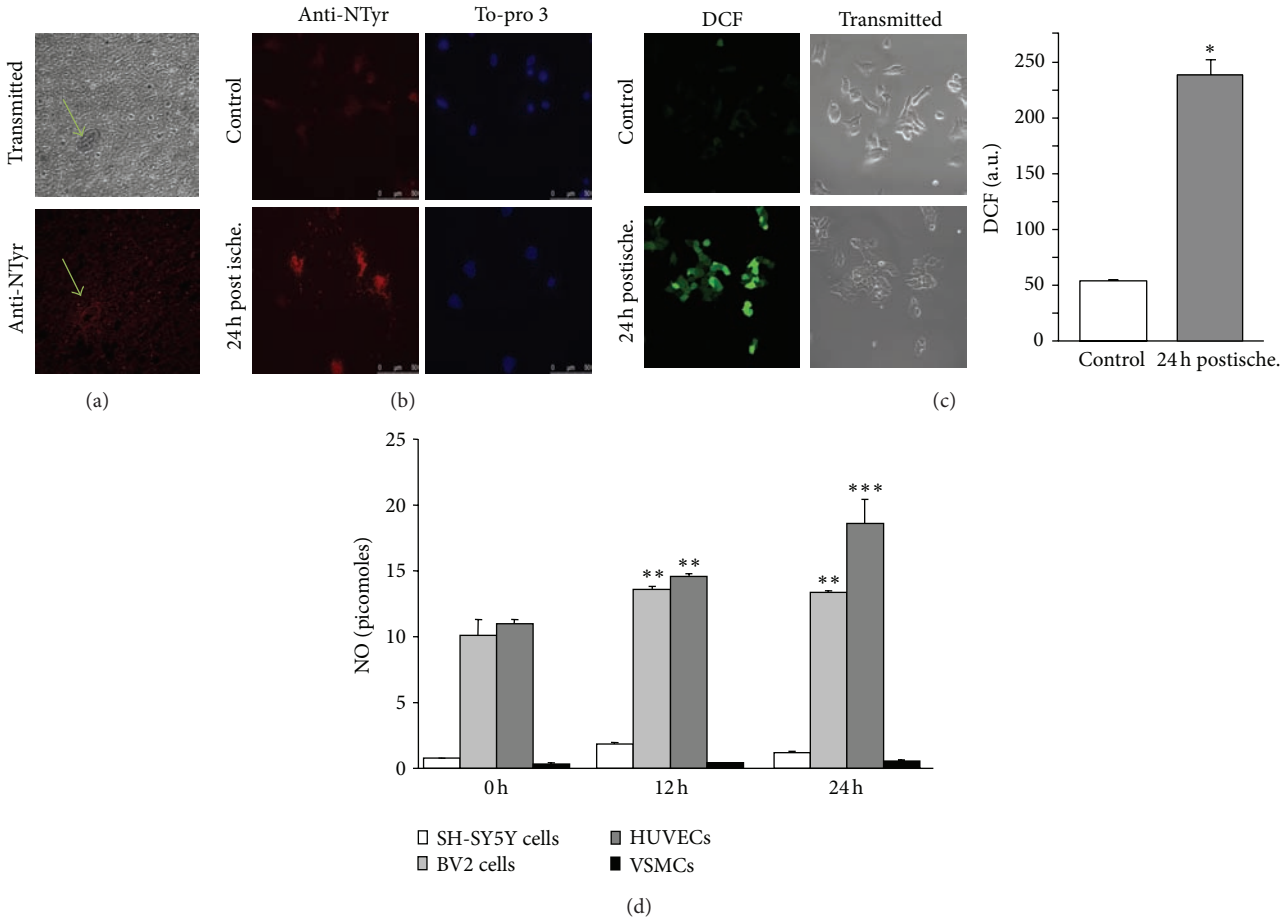

Figure 1: *In vitro* ischemia induces nitro-oxidative stress. (a) A representative image of nitrotyrosination (bottom image, stained in red) and bright field (top image) in a section of cortex from a stroke brain analyzed by immunohistochemistry. The arrows indicate blood vessels. (b) Primary hippocampal cells were subjected to *in vitro* ischemia followed by reperfusion with normal medium for 24 h. Nitrotyrosine (red staining) was demonstrated by immunostaining. Nuclei are stained in blue with To-pro 3. (c) Human neuroblastoma cells were subjected to *in vitro* ischemia reperfused with normal medium for 24 h. Free radical production was detected by DCF fluorescence and quantified. The mean fluorescence of DCF represents the levels of ROS. Data are mean ± SEM values of 4 independent experiments. $^{*}P < 0.05$ versus control. (d) NO production (expressed in picomoles) was measured in neurons, microglia, endothelial, and vascular smooth muscle cells challenged with *in vitro* ischemia and later reoxygenated with normal growing medium containing glucose at 0, 12, and 24 h. Data are mean ± SEM values of 4 independent experiments. $^{**}P < 0.01$; $^{***}P < 0.001$ versus controls at 0 h.

again, a drop in the *iNOS* mRNA levels at 12 h ($P < 0.05$) that was normalized 24 h after the ischemic insult (Figure 3(b)).

Regarding the expression of eNOS in endothelial cells (HUVEC), we did not see any significant change in the protein content after *in vitro* ischemia (Figure 2(c)), whereas the mRNA levels are upregulated after the ischemic challenge ($P < 0.05$; Figure 3(c)).

The iNOS and nNOS expression has been reported in systemic rat vascular smooth muscle cells [25–29]. Neither iNOS protein nor *iNOS* mRNA was detected in myocytes (primary cultures of HC-VSMC) in basal conditions or after ischemia (data not shown); nNOS protein and mRNA were clearly identified (Figure 2(d)). nNOS protein is constitutively present in cerebral vascular myocytes. We did not observe any difference in *nNOS* (Figure 2(d)) and mRNA levels (Figure 3(d)) following exposure to OGD medium.

3.3. $ONOO^-$ Induces Cell Death. We used a NO donor (SNP) to demonstrate that NO alone is not toxic to human neuroblastoma cells at high physiological concentrations (Figure 4(a)), tested up to 1.5 mM SNP (Figure 4(a)). However, when SNP was applied together with nontoxic concentrations of H_2O_2 (an oxidative stress source when metabolized by cells), we obtained neurotoxicity due to the formation of $ONOO^-$ at 10 ($P < 0.05$) and 20 ($P < 0.05$ and $P < 0.005$) μM H_2O_2, even with the lowest SNP concentration (0.5 mM), which is closer to the physiological concentrations obtained in NO burst production.

The $ONOO^-$ donor (SIN-1) was assayed at increasing concentrations in the different cell types (Figure 4(b)). The threshold to cause cytotoxicity was at 1 mM SIN-1 in endothelial cells and neuroblastoma cells. Myocytes and microglial cells were more susceptible to $ONOO^-$, with the cytotoxicity threshold at 100 μM SIN-1.

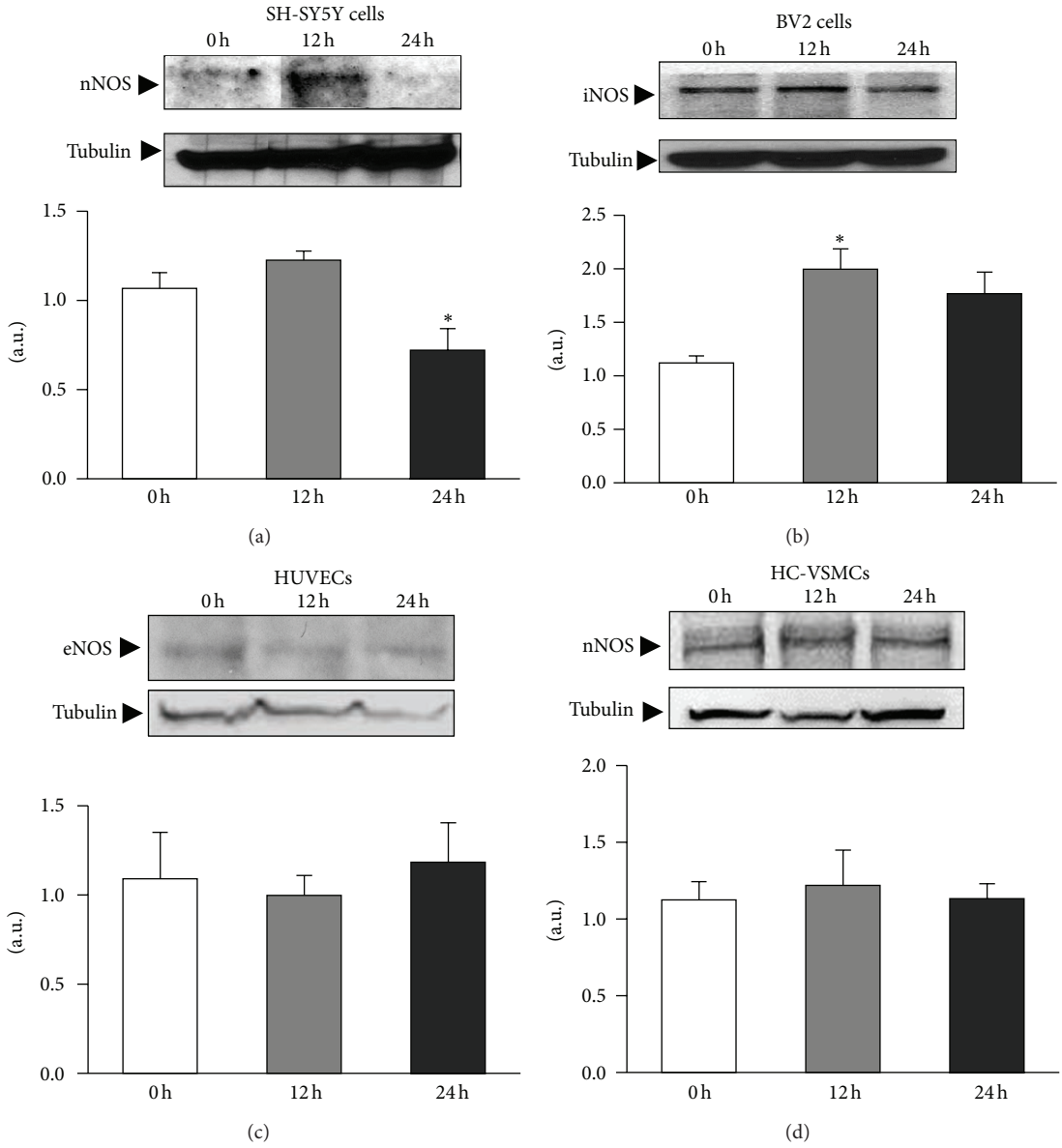

Figure 2: NO is produced in different cell types by the different NOS types. Cells were challenged with ischemia and iNOS, eNOS, and nNOS protein levels were studied immediately at time 0, 12, and 24 h after the ischemic challenge. Densitometric analysis of the bands quantified NOS expression relative to tubulin in all cell types. Data are mean ± SEM values of 6 experiments for microglia and 3 experiments for neurons, endothelial cells, and myocytes. $^{*}P < 0.05$ versus controls at 0 h.

4. Discussion

Stroke generates a cascade of molecular events as a consequence of arrested blood supply [30]. Immediately after the first minutes of ischemic cerebral damage, the activity of eNOS becomes elevated in an attempt to improve blood supply [31]. Our data demonstrate that NO is released after *in vitro* ischemia in brain cells. The NO production is activated up to 24 h after ischemia, mainly in endothelial and microglial cells. At first it might constitute a homeostatic response to increase blood perfusion, but studies performed in iNOS and nNOS knockout (KO) mice suggest that the activation of these two NOS after ischemia is highly harmful to brain cells [32, 33].

The regulation of NO levels depends on the levels and expression of the different NOS. The activity of constitutive NOS (eNOS and nNOS) is regulated by intracellular calcium that increases in response to ischemic challenge. iNOS is regulated at the transcriptional level because it lacks the regulatory arm activated by Ca^{2+}-calmodulin. Its transcription is activated by various stimuli, including LPS [34, 35].

We found significantly increased protein levels of iNOS in BV2 cells at 12 h after *in vitro* ischemia, and baseline levels were not totally recovered at 24 h. The regulation of mRNA expression for *iNOS* (BV2 cells) showed a decrease in mRNA levels at 12 h after ischemia, which correlates with high protein expression. The high production of NO by microglial

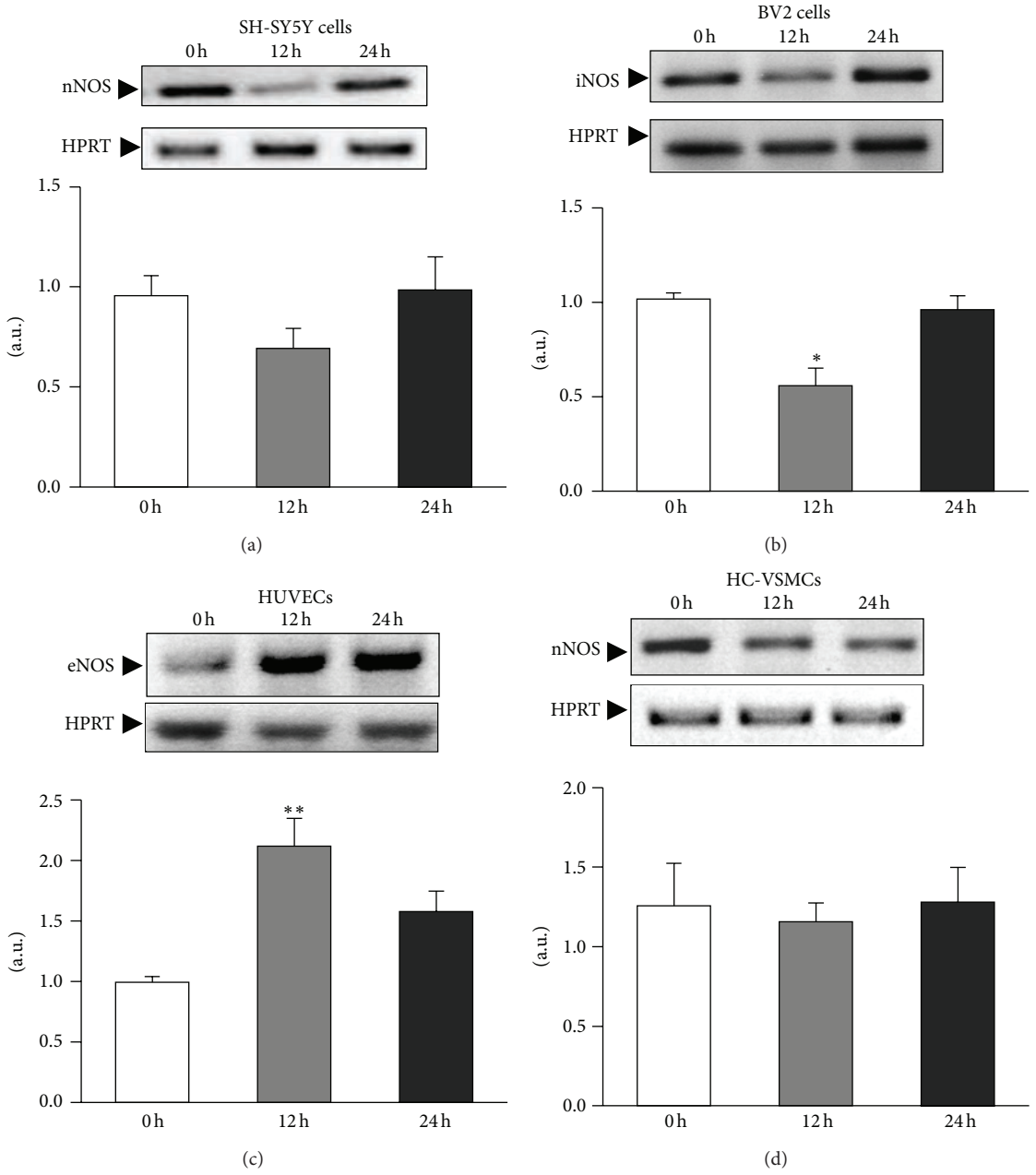

Figure 3: Differential expression of mRNA NOS types occurs in different cell types. Cells were challenged with ischemia, and *iNOS*, *eNOS*, and *nNOS* mRNA expression were studied immediately at time 0, 12 and 24 h after the ischemic challenge. The expression of mRNA was assessed by semiquantitative RT-PCR, and bands were quantified by *HPRT* densitometric analysis in all cell types. Data are mean ± SEM values of 3 experiments in all cell types. $^{*}P < 0.05$; $^{**}P < 0.01$ versus controls at 0 h.

cells after an ischemic challenge would be related to the nonspecific response of these cells to activation, as occurs with other immune cells [36] before phagocytosis of the cellular debris produced by postischemia necrosis [37]. This NO will not contribute to vasodilation but will mostly react with superoxide anion to form peroxynitrite. The iNOS KO mice had less neuronal death after ischemic stroke [32].

Postischemic protein levels (eNOS in HUVECs and nNOS in SH-SY5Y and HC-VSMCs) had not changed at 24 h. The *eNOS* mRNA was overexpressed at 12 h, which strongly suggests an inhibitory translational control of eNOS expression after ischemia. In one study, neuronal death increased in eNOS KO mice after a stroke [38], suggesting a protective role for this enzyme that is likely due to its direct control of blood flow. On the other hand, *nNOS* mRNA levels in SH-SY5Y and HC-VSMCs did not change during postischemic observation.

Oxidative stress plays a key role in ischemic-reperfusion situations [13] because mitochondrial dysfunction leads to a burst in free radical production that cannot be scavenged

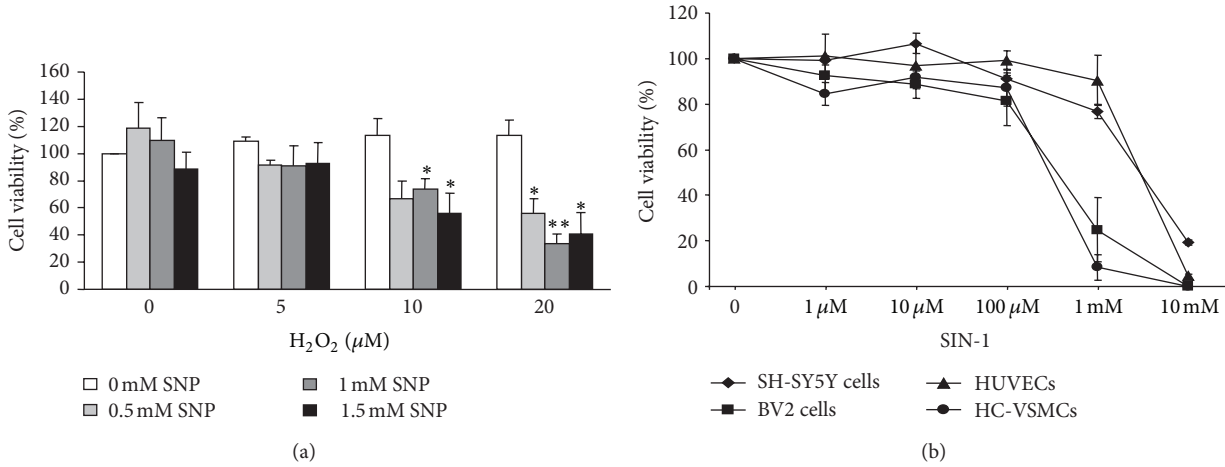

FIGURE 4: $ONOO^-$ induces cell death. (a) Human neuroblastoma cells were treated with increasing concentrations of a NO donor (SNP) and H_2O_2 (free radical source). Cells were incubated for 6 h, and cell viability was assayed by MTT reduction. Data are expressed as percentage of control cells. Data are mean ± SEM values of 7 experiments. $^*P < 0.05$; $^{**}P < 0.01$ versus controls without SNP. (b) Cells were challenged with increasing concentrations of the $ONOO^-$ donor SIN-1, and cell viability was assayed by MTT reduction.

by the constitutive antioxidant cellular defense systems [39]. The large amount of free radicals produced during ischemia not only scavenges NO but also transforms it into the toxic $ONOO^-$ [17], which yields to the protein nitrotyrosination and cell death observed in the present study. Although $ONOO^-$ is a short lived molecule [40], its ability to diffuse through biological membranes can spread its harmful effects into neighboring cells and tissues. This critical process defines survival or death in the penumbra area. Consequently, we analyzed the effects of NO, H_2O_2, and NO plus H_2O_2 on neuronal survival. Neurotoxicity was only induced when cells were challenged with NO plus H_2O_2, which will produce peroxynitrite. Furthermore, we assayed increasing concentrations of a peroxynitrite donor, SIN-1, on all the brain cell types. We found a similar pattern of cell viability for all the cell types at low micromolar concentrations but a major cytotoxicity for glial cells and HC-VSMCs at 1 mM SIN-1. This effect may be related to a lower antioxidant defense. Further work is needed to investigate the possible protective role of antioxidants in the prevention of ischemic damage [41].

5. Conclusion

Our work demonstrates that brain ischemia induces nitro-oxidative stress that produces protein nitrotyrosination. The high production of peroxynitrite after ischemia will cause neuronal death and dramatically affect the survival of other brain cells.

Nonstandard Abbreviations Used

Ab: Antibody
BSA: Bovine serum albumin
BV2: Mouse microglial cells
eNOS: Endothelial nitric oxide synthase
FBS: Fetal bovine serum
HC-VSMC: Human cerebral vascular smooth myocytes
hPRT: Hypoxanthine phosphoribosyltransferase
HUVEC: Human umbilical vein endothelial cells
iNOS: Inducible nitric oxide synthase
MTT: 3-(4,5-Dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide
NO: Nitric oxide
NOS: Nitric oxide synthase
O_2^-: Superoxide anion
OGD: Oxygen-glucose deprivation
$ONOO^-$: Peroxynitrite anion
RT: Room temperature
RT-PCR: Reverse transcriptase-polymerase chain reaction
SH-SY5Y: Human neuroblastoma cells
SIN-1: 3-Morpholinosydnonimine hydrochloride.

Conflict of Interests

The authors indicate no financial conflict of interests.

Acknowledgments

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Review Article

Phosphorylation of Tau Protein as the Link between Oxidative Stress, Mitochondrial Dysfunction, and Connectivity Failure: Implications for Alzheimer's Disease

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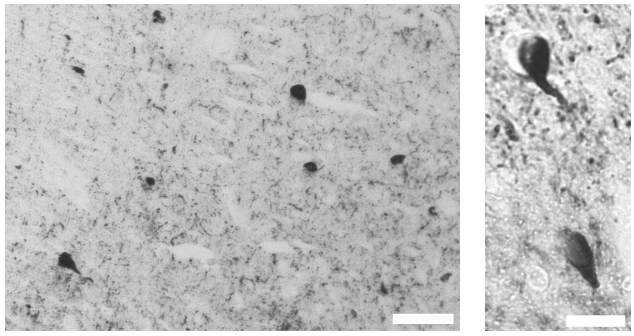
Alzheimer's disease (AD) is defined by the concurrence of abnormal aggregates composed of phosphorylated tau protein and of abnormal cellular changes including neurite degeneration, loss of neurons, and loss of cognitive functions. While a number of mechanisms have been implicated in this complex disease, oxidative stress remains one of the earliest and strongest events related to disease progression. However, the mechanism that links oxidative stress and cognitive decline remains elusive. Here, we propose that phosphorylated tau protein could be playing the role of potential connector and, therefore, that a combined therapy involving antioxidants and check points for synaptic plasticity during early stages of the disease could become a viable therapeutic option for AD treatment.

1. Introduction

Oxidative stress is the damage resulting from reactive oxygen species (ROS) that breach oxidant defences [1]. It has been found to play a crucial role during the development of many pathophysiological conditions including cancer, diabetes, cardiovascular disease, and neurodegenerative disorders [2–4]. Data has shown that oxidative stress is present in increased proportions during aging, similar to the increased susceptibility seen during Alzheimer's disease (AD) [5]. It has been strongly linked to neuronal dysfunction and ultimately to neuron death. It has also been suggested as a central mediator of toxicity [6], and the list will grow as bibliography is added to the discussion. In sum, evidence that links AD and oxidative stress is vast and continues to grow; however, a detailed mechanism leading from one event to the other remains elusive. In this regard, our group suggests that under degenerative conditions, the capacity of cells to maintain redox balance decreases resulting in mitochondrial dysfunction, metabolic dysfunction, deregulation of metal homeostasis, and alterations in the cell cycle [7–10]. Additional studies have shown that reactive oxygen species generated by mitochondria regulates p53 activity, which in turn regulates cell cycle progression and DNA repair and, in cases of irreparable DNA damage, executes programmed cell death [11]. This series of events certainly contributes to the classical fibril aggregations seen during AD, the neurofibrillary tangles (NFTs), mainly composed of phosphorylated tau protein (Figure 1). The role of aggregated tau protein during AD goes further: tau accumulation



(a) (b)

Figure 1: Neurofibrillary tangles are the classical hallmark of AD. Immunohistochemistry technique that evidences the most typical NFT that appears around affected areas (a, b). Here phosphorylated tau protein is the main element in the NFT aggregate scale bars 100 and 20 μm, respectively.

within the neuronal cytoplasm is associated with impaired axonal transport of mitochondria between the cell nucleus and synapse, which leads to severe energy dysfunction and imbalance in the generation of reactive oxygen species (ROS) and nitrogen species (RNS) [12, 13], all together leading to synaptic failure, another classical hallmark of AD and neurodegeneration [14]. Without discussion and according to current data, we can conclude that oxidative stress, protein deposition, and synaptic failure are crucially involved during neurodegeneration and specifically during AD; however, the order in which they appear during the progression and the identity of the mechanism that links them remains under extensive study. Addressing this point, evidence supports the hypothesis that mitochondrial and metallic abnormalities are direct precursors of oxidative stress during the early stages of AD [7, 15, 16]. In the same context we have proposed that deposition of tau protein could be a consequence of early posttranslational modifications like abnormal phosphorylation events [17]. In addition, we recently found that those abnormal phosphorylation events could be the link between synaptic failure and tau pathology [18], but more importantly, that those events could be occurring during early stages of AD (unpublished data). Therefore, taking this background into account, the purpose of this brief essay is not to discuss the effects of the disease, but rather to focus on chronology and mechanism of early events. With this in mind, the final objective is to identify the common mechanisms that could link all the events and therefore become an attractive therapeutic target for AD treatment.

2. Mitochondria and Oxidative Stress: The Relationship

Since the brain is characterized by high energy consumption, mitochondria become crucial as an energy source. The role of mitochondria goes further; they are known for mediating anabolic/catabolic processes in several cell types, as well as for controlling a wide range of cellular processes, including cell proliferation and aging [19]. Unfortunately, under certain conditions, the production of free radicals [hydrogen peroxide (H_2O_2), hydroxyl (·OH), and superoxide (O_2^-·)], in which the brain is especially vulnerable, is an undesirable consequence [1, 20]. The reactive oxygen species, generated by mitochondria, have many targets such as lipids, protein, RNA, DNA, and mitochondrial DNA (mtDNA), which, due to the lack of histones, becomes a vulnerable target of oxidative stress [16]. Clearly, evidence supports the close dependence between mitochondria and brain activity, and because of this, it is therefore not surprising that oxidative stress as a product of mitochondria activity is related to neurodegeneration and, specifically, to AD. In this regard, during AD, significant reduction of intact mitochondria, as well as a reduction in microtubules, was found [21]. Oxidative stress markers, mtDNA deletion, and abnormalities in mitochondrial structure in the vascular walls of AD cases were also found to be increased [22, 23]. Changes in mitochondrial enzymes, mitochondrial structure, localization, and mobility are all involved in AD. In addition, markers for mitochondrial fission and fusion, which impair mitochondrial function, leading to energy hypometabolism and elevated reactive oxygen species production, are altered in models of AD [24]. It has been also reported that the neurons exhibiting increased oxidative damage in AD are coincident with striking and significant increase in cytochrome oxidases and mtDNA [25]. In sum, mitochondria contribute to AD, mainly through an oxidative stress-dependent mechanism with many targets in which tau is not an exception. Although the mechanism that links oxidative stress and tau has yet to be disclosed, recent data suggest that phosphorylation of tau protein could be the potential connector.

3. Phosphorylation of Tau Protein during AD

Formation of intracellular neurofibrillary tangles is one of the pathological hallmarks that characterize Alzheimer's disease (Figure 1). According to current AD hypotheses, (a) tau becomes abnormally phosphorylated, (b) dissociates from microtubules, and (c) aggregates into neurofibrillary tangles (NFTs) [26, 27]. Tau has more than 45 phosphorylation sites, most of which are located in the proline-rich region (P-region) (residues 172–251) and the C-terminal tail region (C-region) (residues 368–441) [28]. Tau phosphorylation at both of these regions affects its capacity to interact with microtubules [29]. Importantly, it is well documented that phosphorylation could contribute and enhance tau polymerization [30]. Beyond the vast and growing evidence that relates phosphorylation and aggregation during AD, we have found that phosphorylation of tau protein is probably the earliest event that occurs during tau abnormal processing in AD and other tau pathologies [17, 31, 32]. Additionally, we have found that phosphorylated tau plays a crucial role during synaptic plasticity, specifically during long-term depression (LTD) [18]. Interestingly, we reported that phosphorylation of tau protein is the key mechanism that regulates such a tau synaptic function [18, 33, 34]. So far, phosphorylation of

tau protein seems to be an early phenomenon that relates to synaptic transmission. It remains to be determined, however, whether the phosphorylation of tau protein is an event that follows synaptic failure or if synaptic failure follows abnormal tau phosphorylation. The answer is certainly not straightforward, as it seems that both events could have a previous event. This is where oxidative stress could be a critical participant. Emerging evidence suggests that diffuse phosphorylated tau could be the result of endoplasmic reticulum stress, and vice versa, creating a pathological feedback loop [35]. We have also found that levels of tau phosphorylation are altered under oxidative stress conditions (unpublished data). Due to the multiple targets for oxidative stress, it is not surprising that it could affect tau phosphorylation levels. However, the fact that phosphorylation events in tau protein and synaptic transmission could be connected by oxidative stress becomes crucial.

4. Tau Phosphorylation during Synaptic Transmission

Memory storage is probably one of the most intriguing questions currently being studied in the neuroscience community. Although the answer seems far from being fully addressed, growing evidence is helping to understand this complex and fascinating mechanism. At the cellular and molecular level, long-lasting synaptic plasticity is gaining acceptance as a model for memory storage. Synaptic strength can be long-lasting enhanced (long-term potentiation, LTP) or long-lasting depressed (long-term depression, LTD), and these changes can persist from seconds to hours and days [36, 37]. LTD is mediated by persistent presynaptic and postsynaptic changes. Importantly, such changes could suggest structural modifications that at present are not well understood. One of the best described mechanisms for LTD comprises N-methyl-D-aspartate (NMDA) receptor activation at many different synapses in the brain. This allows the entrance of Ca^{2+} to the cell and leads, through a Ser/Thr protein phosphatase cascade (GSK3β, PP1, Fyn, etc.), to removal of AMPA receptors from the postsynaptic membrane. The mechanism goes further, with caspase-3 being required for AMPA receptor endocytosis and LTD induction, in which cytochrome-C release from mitochondria is necessary for the activation of caspase-3 [38, 39]. At this point, the data establish the role for mitochondria during synaptic depression, but what could link this phenomenon to AD development? We recently showed that endogenous tau is found at postsynaptic sites where it interacts with the PSD95-NMDA receptor complex. That NMDA receptor activation leads to a selective phosphorylation of specific sites in tau, regulating the interaction of tau with Fyn and the PSD95-NMDA receptor complex, suggesting that abnormal NMDA receptor overexcitation could lead to abnormal tau phosphorylation, and therefore affecting synaptic transmission [18, 33, 34]. Overall, the data discussed here suggest that synaptic transmission is an indirect link between mitochondria and tau phosphorylation.

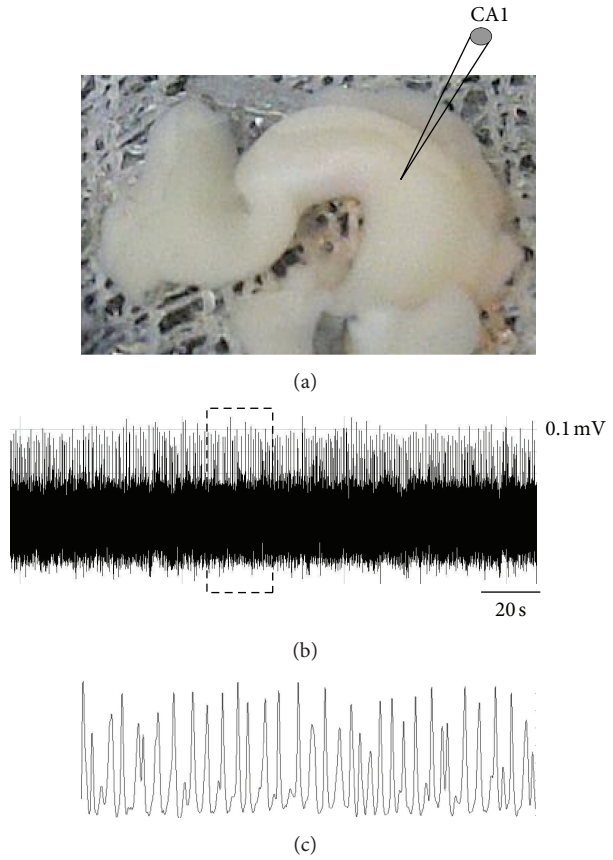


Figure 2: Dendritic recording of theta oscillations *in vitro*. Continuous recording (CA1 pyr layer) in the complete septohippocampal preparation (a). Voltage-dependent theta oscillation in pyramidal cell dendrites *in vitro* (b); see magnification in (c). The local field potential (i.e., the extra cellular potential measured in that brain area) shows robust and sustained oscillations at 2–4 Hz. This frequency is the theta band.

5. From Single Neuron to Network Function: The Chronologic Point of View

We have discussed data that tends to support the hypothesis of interconnection between oxidative stress and phosphorylation events in tau protein, events that mainly occurred during early stages of the neurodegeneration process; however, the early stage of synaptic failure has yet to be disclosed. In this regard, early memory changes, as the most related AD mark becomes key. Unsurprisingly, clinical AD symptoms are preceded by important network alterations. At this level, collective actions of neurons can be studied by recording theta oscillations in the brain [40]. Oscillatory activity in the theta range (3–12 Hz) is a dominant, synchronous signal in the hippocampus that has been shown to be present particularly during exploratory activity (Figure 2). Interestingly, it is now known that theta activity is an essential component of spatial memory and that its disruption leads to severe memory impairment [41]. Although the mechanism remains to be revealed, it is known that NMDA receptors, along with other receptors and transmitters, play a crucial role in theta

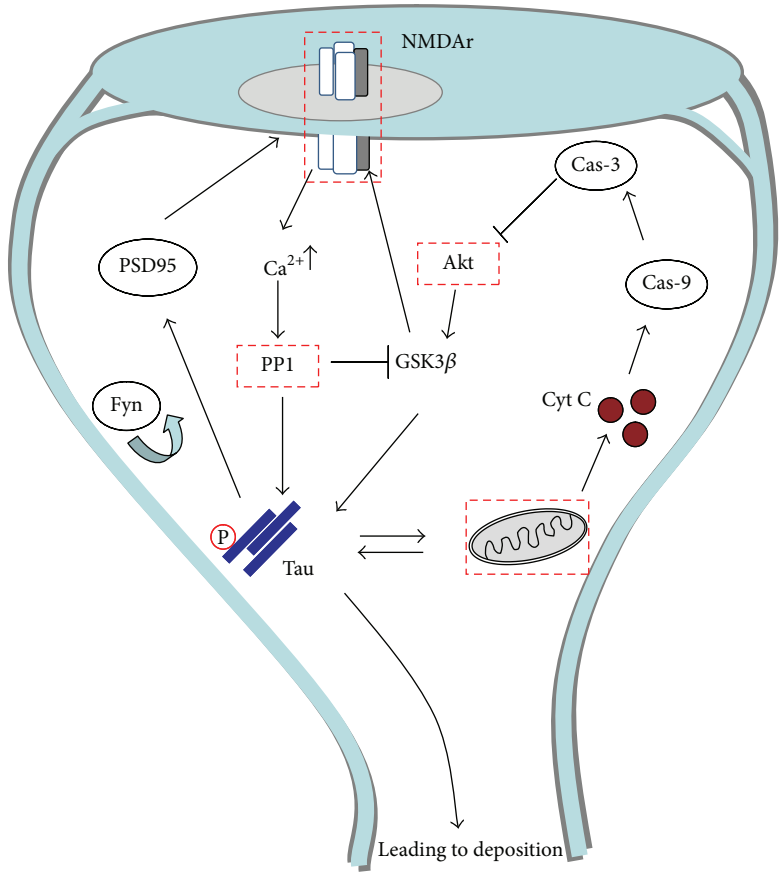


Figure 3: The relationship between synaptic transmission, tau, and mitochondria. The intricate and close relationship between mitochondria and synaptic check points makes them potential therapeutic targets (red squares). Phosphorylated tau protein acts as a central connector between mitochondria and synaptic formations. Calcium (Ca^{2+}) enters via NMDA receptors and this leads to activation of protein phosphatase 1 (PP1), a key enzyme in synaptically-induced LTD. PP1 can dephosphorylate GSK3β, leading to the activation of tau and NMDA receptor. Tau contributes to NMDA activation through the PSD95-Fyn complex. Importantly, GSK3β is also activated by Akt through mitochondrial pathways, suggesting a fine regulatory mechanism. During neurodegeneration, oxidative stress affects levels of tau phosphorylation and GSK3β; this breaks the fine balance that controls memory formation, therefore leading to synaptic failure.

oscillations [42]. Theta oscillations have been observed in numerous cortical structures: the subicular complex, entorhinal cortex, perhinal cortex, cingulated cortex, and the amygdale, among other structures present in the hippocampus [42]. Beyond the hippocampus as the main theta generator structure, the medial septum-diagonal band of Broca (MS-DBB) has also been proposed to act as a theta generator [43]. Recently, by using the complete septo-hippocampal preparation [44] from AD transgenic model (Figure 2), we found that synaptic connectivity between the MS-DBB and hippocampus is affected when compared to controls, but, more importantly, this event is present without evident cytopathology (unpublished data). Although the mechanism that links single molecular events and network connectivity remains under extensive study, this data, along with existing data, places synaptic transmission as an early phenomenon in which phosphorylated tau through NMDA receptors is certainly playing a crucial role with profound implications for network function involved in neurodegeneration.

6. Discussion and Perspectives

The main idea in this essay was not to discuss the role or relationship between oxidative stress and synaptic failure with AD, but rather to discuss the potential mechanistic relationship between them. In this regard, we propose that phosphorylated tau protein could be playing the role of a potential connector. Phosphorylation of tau protein seems to be the mechanism that regulates the physiological role of the protein at the synaptic terminal. Here, our data suggests that a fine balance of phosphorylation levels determines whether tau protein contributes to synaptic formation or neurodegeneration (Figure 3). But what could be causing such deregulation? Data lends support to the notion that oxidative stress is critically involved. Oxidative stress models, either *in vitro* or *in vivo*, are characterized by the presence of abnormally phosphorylated tau, in this direction, through inhibition of glutathione synthesis in M17 neuroblastoma cells, increased levels of tau phosphorylated were reported [45]. In null mice lacking superoxide dismutase whose

phenotype is characterized by mitochondrial dysfunction and oxidative stress, abnormally phosphorylated tau was also found [46]. In sum, data tends to suggest that oxidative stress caused at synaptic terminals by mitochondria could be affecting downstream targets that have direct repercussion in tau phosphorylation levels, for example, GSK3β. Interestingly, altered tau can create a pathological feedback loop with mitochondria. This loop therefore creates a series of undesirable events for the neuron: (a) tau is no longer able to bind to PSD95, (b) tau does not recruit Fyn kinase that is crucial for NMDA receptor activation, and therefore synaptic formation is affected. Instead, tau is capable of affecting mitochondria that, in turn, will cause more damage to the neuron (Figure 3). At the end, the combination of all these events will result in spine retraction and synaptic failure. Therefore, therapeutic strategies that focus on early events, such as those described here, rather than on consequences such as protein deposition, will certainly offer a better possibility of success, as we previously discussed [31, 33]. Overall, our data, along with current data, suggests that tau abnormal phosphorylation could be a consequence of oxidative stress, with this relationship having critical repercussions for synaptic transmission. Therefore, a combined therapy that considers antioxidants and check points for synaptic plasticity during early stages of the disease could offer new hope (Figure 3).

Abbreviations

ROS: Reactive oxygen species
AD: Alzheimer's disease
NFTs: Neurofibrillary tangles
LTD: Long-term depression
NMDA: N-Methyl-D-aspartate receptor.

Disclosure

George Perry is, or has in the past been, a paid consultant for and/or owns equity or stock options in Neurotez Pharmaceuticals, Panacea Pharmaceuticals, Takeda Pharmaceuticals, and Voyager Pharmaceuticals. The authors have no other relevant affiliations or financial involvements with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the paper apart from those disclosed. Procedures for mice recordings were performed according to protocols and guidelines approved by the McGill University Animal Care Committee and the Canadian Council on Animal Care.

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Review Article

Targeting Microglial K_{ATP} Channels to Treat Neurodegenerative Diseases: A Mitochondrial Issue

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Neurodegeneration is a complex process involving different cell types and neurotransmitters. A common characteristic of neurodegenerative disorders is the occurrence of a neuroinflammatory reaction in which cellular processes involving glial cells, mainly microglia and astrocytes, are activated in response to neuronal death. Microglia do not constitute a unique cell population but rather present a range of phenotypes closely related to the evolution of neurodegeneration. In a dynamic equilibrium with the lesion microenvironment, microglia phenotypes cover from a proinflammatory activation state to a neurotrophic one directly involved in cell repair and extracellular matrix remodeling. At each moment, the microglial phenotype is likely to depend on the diversity of signals from the environment and of its response capacity. As a consequence, microglia present a high energy demand, for which the mitochondria activity determines the microglia participation in the neurodegenerative process. As such, modulation of microglia activity by controlling microglia mitochondrial activity constitutes an innovative approach to interfere in the neurodegenerative process. In this review, we discuss the mitochondrial K_{ATP} channel as a new target to control microglia activity, avoid its toxic phenotype, and facilitate a positive disease outcome.

1. An Imbalanced Four-Partite Synapse Crosstalk

The diversity of clinical phenotypes of neurodegenerative diseases share common neuropathological features that underlie significant modifications of the physiological glia-neuronal crosstalk. Over time, this leads to neuronal damage, specific pathway dysfunctions, and neurological disability [1]. Initially, the four-partite synapse, that is, the presynaptic and postsynaptic neurons, astrocytes, and microglia, presents a diversity of new communications that render and maintain a chronic neuroinflammation [1–3] with activation of various well-coordinated adaptive mechanisms to avoid or reverse damage [1, 3]. In fact, this imbalanced four-partite synapse crosstalk underlies the disease pathogenesis, and its progression—and also brain aging—reflects at each moment the contributions of each cell type. Because of that, higher perturbations are associated with significant molecular and cellular changes that lead to a progressive chronic neurodegenerative process from which return to physiological conditions is impossible [4]. As such, the disease progression represents the dynamic communicative process between all the synaptic participants in which the equilibrium between damaging and neuroprotective signals favors permanently and increasingly neuronal damage and synaptic loss.

The pivotal role of these four cell types in disease has yielded a huge amount of information to explain the dysfunction or disruption of neural circuits. At the present time, the working theories attribute key roles to protein misfolding, excitotoxicity, and mitochondrial dysfunction, this last one related to integrity, bioenergetics, calcium homeostasis, or reactive oxygen species (ROS) generation. In fact, all these elements are interdependent, and all theories consider the participation of the same four-partite synapse organization,

in which microglia, as a sensor and effector of CNS immune function, directly influence the disease initiation, progression, and outcome.

2. Microglia and Neurodegeneration

2.1. Misfolded Protein Diseases. Neurodegenerative diseases can be classified as misfolded protein diseases (MPD) because each one presents specific misfolded proteins. These proteins share no common sequence nor common structural identity between them and include the Protease-resistant Prion Protein, Polyglutamine, Amyloid-β, Tau protein, α-synuclein, superoxide dismutase 1 (SOD1), TAR DNA-binding protein 43 (TDP-43), or fused in Sarcoma (FUS) [5, 6]. These misfolded proteins reveal a failure to adopt a proper protein folding due to an enhanced production of abnormal proteins or to a perturbation of cellular function and aging under the effects of ROS [7, 8]. In addition, their insufficient clearance also reflects a chronic impaired microglia autophagy resulting in accumulation of protein aggregates, cell damage, and progressive death [1, 9]. For example, in amyotrophic lateral sclerosis (ALS), the misfolded proteins like SOD1 released initially from motor neurons activate microglia, and the ensuing neuronal injury depends upon a well-orchestrated dialogue between motor neurons and microglia [10]. Phagocytic microglial cells are very efficient scavengers but with a limited capacity towards α-synuclein aggregates [11, 12] characteristics for Lewy body disorders (LBDs), in particular for Parkinson's disease. The same microglia phagocytosis triggers the release of proinflammatory cytokines, chemokines, and ROS, which may further promote neuronal dysfunction and degeneration and misfolded protein overload [13, 14]. In addition, microglia may also participate in the LBDs pathophysiology due to variations of their human leukocyte antigen region [15]. Thus, in all the different situations, the protein aggregates initiate and maintain a chronic activation of microglia as a constitutive element of MPD with their subsequent direct participation in neural damage through chronic ROS formation and cytokine secretion [16–18].

2.2. Excitotoxicity. Regarding excitotoxicity, the alterations of synaptic glutamate and calcium homeostasis lead to neuronal and glia glutamate receptor overactivation, culminating in neuronal death and progressive neural circuit dysfunctions [19, 20]. In this situation, three main compensatory responses are activated to avoid glutamate-induced damage [21]. Thus, the direct activation of the retaliatory systems, based on a coordinated increase of GABA, taurine, and adenosine signaling, will reduce glutamate receptor activation [22, 23], and the rapid adaptation of calcium homeostasis with the neuronal and astroglial formation of calcium precipitates will reduce the increased calcium signaling associated with glutamate [3, 24], and the reduction of glutaminase activity of the astrocyte-neuron glutamate/glutamine cycle will reduce glutamate level in the presynaptic neuron and synaptic glutamate activation [25]. Microglial cells are the glial cell type least susceptible to excitotoxicity, because they mostly express glutamate receptors when they are reactive. Injured neurons would directly activate microglia [26] by the release of a diversity of factors like galectin-3, [27], cystatin C, chemokine (C-X3-C motif) ligand 1 (CX3CL1) [28], or the danger-associated molecular pattern (DAMP) ligands that bind to Toll-like receptors (TLR) [29]. The benefits of such inflammation may be substantial to help avoid further neuronal damage if exposure to excitotoxicity is limited in time or intensity [25, 30]. Otherwhise, microglia effects will be deleterious through chronic ROS formation and cytokines secretion and directly related to the neurodegenerative process. With aging, this process will be potentiated by the senescent affectation of microglia, which changes cell morphology and functions [31–33]. In fact, cumulative evidence indicates a direct pathogenic role of senescent microglia in degenerative CNS diseases [34–36].

2.3. Energy Demand and Microglia Reaction. Microglia do not constitute a unique cell population but rather, present a range of phenotypes [1, 37] closely related to the evolution of the lesion process [33]. In a dynamic equilibrium with the lesion microenvironment, these phenotypes range from the well-known proinflammatory activation state to a neurotrophic one directly involved in cell repair and extracellular matrix remodeling [38], with a diversity of intermediary mixed phenotypes that present or not a phagocytic activity [39]. All these adaptative phenotypes relate directly to the evolution of the lesion, and variations in the expression of toxic, protective factors, and phagocytic activities greatly determine the outcome of the tissue [33, 37, 40].

In addition, microglia-like neurons present a high-energy demand, for which the lack of a mitochondrial DNA (mtDNA) repair system determines mtDNA cumulative defects with aging. As said previously, in these conditions, senescent microglia become increasingly dysfunctional and participates in the direct development of neurodegeneration [40, 41]. As in other cells, calcium signaling governs the communication between cytosol and mitochondria [42]. In macrophages, the phagocytic response represents a burst of ROS formation through an increased activity of the NADPH oxidase and also of mitochondria. The mitochondria ROS (mtROS) are potentiated by the translocated tumor necrosis factor receptor-associated factor 6 (TRAF6) [43] and its interaction with the ubiquitinated protein evolutionarily conserved signaling intermediate in Toll pathways (ECSIT) [44]. ECSIT associates with the oxidative phosphorylation complex I components and facilitates the assembly of the mitochondrial electron transport chain [44]. Thus, in macrophages, TRAF6 translocation induces the juxtaposition of phagosomes and mitochondria and potentiates mtROS formation and energy production [45]. A similar TRAF6 recruitment to mitochondria that engages ESCIT on the mitochondria surface is also observed in the same cells upon lipopolysaccharide (LPS, a TLR4 agonist) treatment [43].

In microglia, recruitment of cytoplasmic TRAF6 modulates LPS-evoked cytokine release [46]. The great similarity between peripheral and central immune systems, in particular between macrophages and microglia, makes a similar action of TRAF6 in microglia activated by LPS possible. If true, the mitochondria respiratory burst rending increased

energy and ROS production in response to the elevated calcium required for the adoption of a specific microglia phenotype would also depend on TRAF6 translocation and interaction with ECSIT. And how calcium variations and TRAF6 interact to modulate their microglia effects should help identify the molecular pathways linked to the adoption of a phenotype or another one.

In parallel, a defect in the astrocyte-neuron crosstalk does not ensure sufficient neuronal supply of glucose, lactate, and oxygen, leading to increased neuronal damage, probably impossible to effectively repair [47]. This damage results in subsequent switch in microglia phenotype from an initial neuroprotective one to a final proinflammatory one and to the disease onset and progression [1].

Thus, whether microglia adopt a phenotype that will exacerbate tissue injury or one to promote brain repair and phagocytosis is likely to depend on the diversity of signals from the lesion environment and of the microglia response capacity [48, 49]. At each moment, microglia effectiveness to adapt to the changing synaptic signals and phagocytic needs to rapidly remove cellular debris depends on its accurate adaptation to match its immediate energy demand. This implies that to ensure and maintain an activated stage and adapt constantly their expression and function to a determined phenotype, microglia have to correspond permanently with a balanced ATP availability from aerobic glycolysis [50]. However, like in astrocytes, in reactive microglia, part of glucose renders ATP and lactate through anaerobic glycolysis. Thus, in the postulated four-partite synapse, lactate will be shuttling to neurons not only from astrocytes but also from microglia.

Mitochondrial bioenergetics depends on a proper stimulation of mitochondrial oxidative phosphorylation to produce enough ATP to the cell and on the same mitochondria morphology [41, 51]. This requires a constant dynamic and rapid response of its array of individual mitochondria to adapt their shape and size to the microglia energy demand. Thus, via the combined fusion and fission events that are mediated by large guanosine triphosphatases, they improve their number and optimize their regional networks in order to deliver at each moment the needed ATP [52]. In microglia, a higher number of mitochondria mark the transition from microglia resting state to the activated one, as shown by an increased expression of the mitochondrial peripheral benzodiazepine receptor (PBR) labeled with (R)-PK11195 [53]. Both transmission electron microscopy (TEM) and atomic force microscopy (AFM) procedures evidenced that this mitochondrial PBR complex functions as a pore, allowing the translocation of cholesterol and other molecules through the inner mitochondrial membrane [54]. Presently, the (R)-PK11195 ligand is commonly used to quantify microglia in brain sections from excitotoxic damage like stroke, brain trauma, epilepsy, or chronic neurodegenerative disorders [38, 55, 56] and also for PET detection as an in vivo marker of active disease [57].

The mtDNA is arranged in nucleoprotein complexes evenly distributed along the mitochondrial network [58]. Stimulation of mitochondrial fusion by mitofusin 1 and 2 and OPA1 maximizes oxidative phosphorylation because the fused mitochondria share and homogenize the content of all their compartments. Thus, when the mtDNA mutation load is minor, integration of defective mitochondria through fusion complementation increases their oxidative capacity by sharing RNA components and proteins, because the lack of a functional component in one mitochondria can be complemented by the presence of the component in another one [59]. If the mutation load is major, fission activation by Drp1 and Fis 1 increases the number of mitochondria and maintains their physiological functions removing the defective parts by mitophagy [60, 61]. Otherwise, abnormal mitochondrial network dynamics develop, rendering dysfunctional cells as a direct participant of the degenerative process. Presently, this physiopathological mechanism has not yet been evidenced in microglia. The known diseases associated with defects in mitochondria fusion-fission factors, like the OPA1-linked autosomal dominant optic atrophy, are genetic disorders, and not specific of microglia [62]. However, the blockade of glutamate-induced toxicity by antioxidants combined with inhibitors of glutamate uptake argues for a key role of ROS production in excitotoxicity [63]. Also, the importance of some inflammatory-independent neurodegenerative mechanisms associated with mitochondria dysfunction and oxidative stress in multiple sclerosis reinforces the possible participation of defective microglia function in the disease [64]. Thus, unraveling molecular mechanisms amenable to prevent or reverse defective microglia mitochondrial networks would open new possibilities to control neurodegenerative disorders.

The rise in cytosol calcium signaling associated with activation of glutamate and cytokine receptors of microglia match microglia cell energy demand with mitochondria supply. Upon activation, microglia express glutamine synthase (GS) and the two transporters EAAT-1 and -2 for glutamate and glutamine synaptic extrusion [65]. In mitochondria, glutamate and glutamine render oxoglutarate for the Krebs cycle and ammonium. At high concentration, ammonium contributes to the generation of superoxide, which after reaction with NO forms the highly reactive peroxynitrite [66]. The progressive reduction of neuronal glutaminase activity that parallels the switch from the apoptotic to the necrotic neuronal death [25] underlies a similar displacement of the glutamate/glutamine cycle toward a reduced glutamate formation and a net glutamine output [25]. So, the highest glutamine level present in necrosis renders more ammonium and more ROS production in microglia—adopting a mostly proinflammatory phenotype—damaging the surrounding cells and also their mitochondria network. This will cause microglia dysfunctions that may result in apoptosis [66].

Finally, stimulation of the mitochondrial calcium uniporter (MCU), located in the inner membrane (MIM), promotes an active mitochondria calcium uptake [67]. Calcium moves down the MIM electrochemical gradient and, in the matrix, stimulates the rate limiting enzymes—pyruvate, isocitrate, and oxoglutarate dehydrogenases and also the ATP synthase—to render increased amounts of ATP and also of ROS [67–70]. Thus, whenever the cause of a major energy demand, a defective protein that does not allow a proper coordinated fission and fusion process induces

a mitochondria dysfunction. This will cause a reduced cytosol ATP availability, an increased ROS production, major mtDNA dysfunctions, and a calcium dyshomeostasis, all of them resulting in a definitive loss of mitochondria network integrity and cell abnormalities [41, 71]. In view of the abovementioned theories, which all consider the participation of cellular and molecular multidirectional synaptic interactions, identification of therapeutic targets related to disease pathogenesis for the rationale design of treatments remains quite elusive. Modulation of microglia response by controlling their mitochondrial activity constitutes an innovative approach to interfere in the neurodegenerative process. The identification of a mitochondrial K_{ATP} channel expressed in human and rodents is a new target to control microglia activity, avoid their toxic phenotype, and facilitate a positive disease outcome.

3. K_{ATP} Channels in the CNS

K_{ATP} channels play important roles in many cellular functions by coupling cell metabolism to electrical activity with the participation of glucokinase. First detected in cardiac myocytes, they have been found in pancreatic β cells, skeletal and smooth muscle, neurons, pituitary, and tubular cells of the kidney [72–74]. In these tissues, K_{ATP} channels couple electrical activity to energy metabolism by regulating potassium fluxes across the cell membrane when glucose is available in sufficient conditions [75]. Increased energy metabolism leads to channel closure, membrane depolarization, and electrical activity. Conversely, metabolic inhibition opens K_{ATP} channels and suppresses electrical activity [76, 77].

Plasmalemmal K_{ATP} channels are assembled as a hetero-octameric complex [78, 79] from two structurally distinct subunits: the pore forming inwardly rectifying potassium channel (Kir) subunit 6.1 or 6.2 and the regulatory sulphonylurea receptor (SUR). SUR, like all other ATP-Binding Cassette transporters, contains two transmembrane domains and two cytoplasmic ones (Nucleotide Binding Folds 1 and 2). Its N-terminal transmembrane domain mediates SUR-Kir6 interactions [80]. While ATP inhibits the K_{ATP} channel by direct binding to the cytoplasmic Kir6 domains [81], activators like Mg-nucleotides [82] potassium channel openers (KCOs) and inhibitors like sulfonylurea drugs [83] bind SUR to modulate the channel. K_{ATP} channels play a multitude of functional roles in the organism. In endocrine cells, they play an important role in hormone release, including insulin from pancreatic β cells [84] and glucagon from pancreatic α cells [85]. Epithelial cells of blood vessels also express K_{ATP} channels, where they are involved in the control of blood flow and cerebrovascular processes [86].

In the brain, neuronal expression of K_{ATP} has been described in the substantia nigra, the neocortex, hippocampus, and hypothalamus [72, 74]. In these areas, K_{ATP} channels modulate electrical activity and neurotransmitter release [87], protect against seizures [88], and play an essential role in the control of glucose homeostasis [82]. The expression of K_{ATP} channels has also been suggested in microglia [89]. As discussed in Section 4, our previous studies showed that reactive microglia increase their expression of the K_{ATP}-channel components Kir6.1, Kir6.2, SUR1, and SUR2B after brain pathologies such as stroke and Alzheimer's disease (AD) [90–92].

Finally, K_{ATP} channels have also been described in the mitochondria, located on the inner membrane of these organelles where they play a crucial role in the maintenance of mitochondrial homeostasis and of the proton gradient involved in the respiratory chain [93, 94].

3.1. K_{ATP} Channel Gating and Pharmacology. The octameric structure of the K_{ATP} channel with four inhibitory ATP-binding sites per channel (one on each Kir6.2 subunit) and eight stimulatory Mg-nucleotide-binding sites on SUR [95] represents a complex regulation by nucleotides. The same for the channel kinetics, with a large number of kinetic states, as its activity reflects at each moment the result of the nucleotide effects at each site. Several endogenous ligands bind the K_{ATP} channel subunits: ATP (with or without Mg^{2+}) inhibits the Kir6.2 subunits, and phosphatidylinositol-4,5-bisphosphate activates them; sulphonylureas inhibit the SUR subunits, and KCO drugs activate them. In addition, in the presence of Mg^{2+}, ATP and ADP can activate the channel through interaction with the nucleotide-binding folders of SUR [83]. Inhibition by ATP binding to Kir6.2 and activation by Mg-nucleotides is probably the first physiological regulatory mechanism [82].

In recent years, K_{ATP} channels have attracted increasing interest as targets for drug development. Their pivotal role in a plethora of physiological processes has been underscored by recent discoveries linking potassium channel mutations to various diseases. The second generation of KCOs or potassium channel blockers, with an improved in vitro or in vivo selectivity, has broadened the chemical diversity of K_{ATP} channel ligands. However, despite this significant progress, a lot of work remains to be done to fully exploit the pharmacological potential of K_{ATP} channels and their KCOs or potassium channel blocker ligands. Sulfonylureas that bind K_{ATP} channel are oral hypoglycemic agents widely used in the treatment of type II diabetes mellitus [96]. For example, glibenclamide binds with subnanomolar or nanomolar affinity and is a potent inhibitor of SUR1-regulated channel activity [97]. SUR1-regulated channels are exquisitely sensitive to changes in the metabolic state of the cell, responding to physiologically meaningful changes in intracellular ATP concentration by modulating channel open probability [98].

On the other hand, considering the unique role that K_{ATP} channels play in the maintenance of cellular homeostasis, the KCOs family adds its potential in promoting cellular protection under conditions of metabolic stress to the already existing pharmacotherapy. Preclinical evidence indicates a broad therapeutic potential for KCOs in hypertension, cardiac ischemia, asthma, or urinary incontinence to mention a few. For example, diazoxide (7-chloro-3-methyl-4H-1,2,4-benzothiadiazine 1,1-dioxide) is a small molecule well known to activate K_{ATP} channels in the smooth muscle of blood vessels and pancreatic β cells by increasing membrane permeability to potassium ions. Diazoxide binds with similar

affinities to SUR1 and SUR2B on the interplay between SUR and Kir subunits, a site different of the other KCOs binding sites [83, 99], and its hyperpolarization of cell membranes prevents calcium entry via voltage-gated calcium channels, resulting in vasorelaxation and the inhibition of insulin secretion [100]. For all this, diazoxide has been approved and used since the 1970s for treating malignant hypertension and hypoglycemia in most European countries, the USA, and Canada [101, 102].

4. The Mitochondrial K_{ATP} Channel

More than 20 years ago, a putative mitochondrial K_{ATP} (mitoK_{ATP}) channel was proposed, both functionally and molecularly distinct from the one in the plasma membrane [103]. Initially, functional mitoK_{ATP} channels were thought to be composed in brain of Kir6.1 or Kir6.2, and SUR2B [104–106] and diazoxide or nicorandil have been proposed as specific mitoK_{ATP} channel openers, whereas 5-hydroxydecanoate would specifically block this channel type [107–111].

Some authors have questioned the existence of the mitoK_{ATP} channel since they failed to detect its existence in isolated heart and brain mitochondria [112, 113], and the specificity of diazoxide and 5-hydroxydecanoate has been questioned [114, 115]. According to these authors, the lack of specific effects of diazoxide could indicate that mitoK_{ATP} channels are not present in mitochondria and that the pharmacological effects of diazoxide and 5-hydroxydecanoate are caused by interaction with other pharmacological or unspecific targets (see [116] for a review). However, one must consider that the variable findings can depend on minor differences in mitochondrial isolation procedures, since the channel may be inactivated during isolation of mitochondria, or a cofactor that needs to be present may be lost [113]. Moreover, the nonspecific effects of diazoxide are observed in vitro when very high concentrations are used (higher than 50 μM). Since in rat heart mitochondria, diazoxide is found to open mitochondrial K_{ATP} channels with a half-maximal saturation of 2.3 μM [94]. These concentrations are in excess compared with those described to promote mitochondrial K_{ATP} channel opening [117, 118].

Putative tissue heterogeneity of mitoK_{ATP} channel expression must also be taken into account to explain the specificity of diazoxide actions. For instance, expression of K_{ATP} channel components was not detected in resting microglia of rat and human brain, whereas activated cells in ischemic rats and AD patients presented strong labeling for SUR and Kir components in the cytoplasm and plasmalemma [90–92]. In this line, diazoxide prevents apoptosis of epithelial cells in the aorta of diabetic rats compared to controls [119]. Thus, in physiological conditions, very low expression of mitoK_{ATP} channels in cardiac tissue or neurons may render cells refractory to low diazoxide concentrations, whereas these tissues may increase the channel expression in pathological conditions.

Within this controversy, a major problem is that the molecular identity of mitoK_{ATP} has not yet been determined. A recent study has presented strong proteomic and pharmacological evidence of kcnj1 as a pore-forming subunit of mitoK_{ATP} channels [120]. This result supports the existence of specific mitoK_{ATP} channels and should help to explain the tissue specificity of different KCOs and clarify why some authors found mitoK_{ATP} channels sensitive to drugs that do not affect the plasmalemmal K_{ATP} one [121].

Because SUR1-regulated channels are exquisitely sensitive to changes in the metabolic state of the cell and that microglia are permanently sensing the environment, the expression of K_{ATP} channels in activated microglia will couple cell energy to membrane potential and microglia response with the adoption of a specific phenotype to the surrounding signals. This channel expression may then be critical in determining, at least in part, microglia participation in the pathogenic process.

4.1. MitoK_{ATP} Channels in Neurodegeneration. Our laboratory has described the expression of K_{ATP} channels in microglia [1, 90, 91], which control the release of a diversity of inflammatory mediators, such as nitric oxide (NO), interleukine-6, or TNF-α [122]. In a rat model of neurodegeneration and in postmortem samples of patients with AD, we also reported that activated microglia strongly expressed K_{ATP} channel SUR components [90] and that reactive microglia increase their expression of the K_{ATP} channel components Kir6.1, Kir6.2, SUR1, and SUR2B after brain insults [92, 122]. In this context, controlling the extent of microglial activation may offer prospective clinical therapeutic benefits for inflammation-related neurodegenerative disorders. We and other authors have documented that pharmacological activation of K_{ATP} channels can exert neuroprotective and anti-inflammatory effects on the brain against ischemia, trauma, and neurotoxicants [123–126].

Diazoxide has evidenced prevention of rotenone-induced microglial activation and production of TNF-α and prostaglandin E2 in cultured BV2 microglia [126]. Also, diazoxide inhibited in vitro NO, TNF-α, interleukin-6 production, and inducible nitric oxide synthase expression by LPS-activated microglia. In mitochondria isolated from these cells, diazoxide also alleviated rotenone-induced mitochondrial membrane potential loss [126], for which mitoK_{ATP} channels participate in the regulation of microglial proinflammatory activation.

In vivo studies have confirmed that diazoxide exhibits neuroprotective effects against rotenone, along with the inhibition of microglial activation and neuroinflammation, without affecting cyclooxygenase-2 expression and phagocytosis [126]. When tested in an experimental autoimmune encephalomyelitis (EAE) murine model of multiple sclerosis, 1 mg/kg oral diazoxide ameliorated EAE clinical signs but did not prevent disease. A significant reduction in myelin and axonal loss accompanied by a decrease in glial activation and neuronal damage was observed. In this model, diazoxide did not affect the number of infiltrating lymphocytes positive for CD3 and CD20 in the spinal cord [122]. Finally, diazoxide has been tested in the triple transgenic mouse model of AD (3xTg-AD) that harbors three AD-related genetic loci: human PS1M146V, human APPswe, and human tauP301L

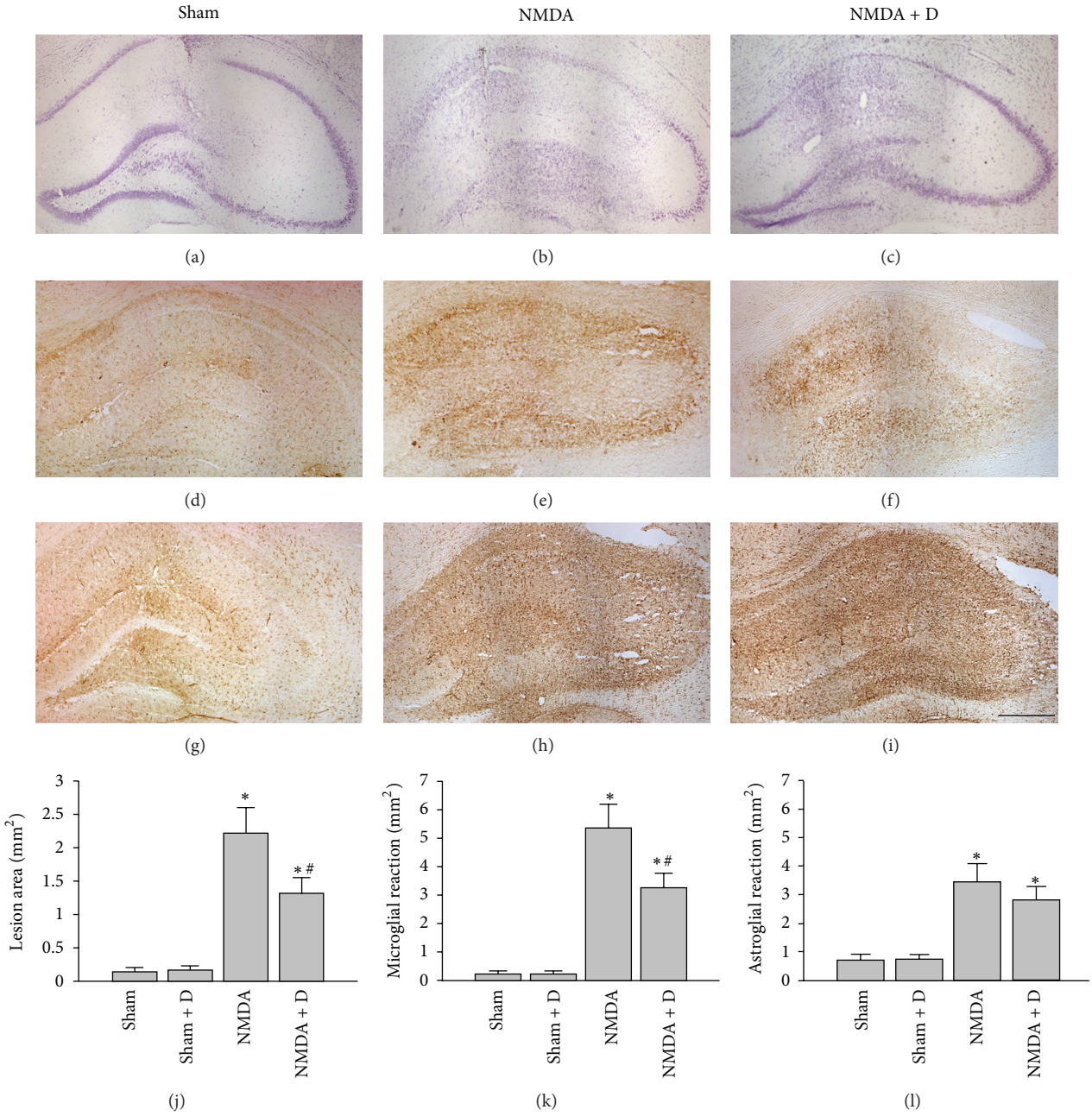

Figure 1: Effect of diazoxide treatment on the NMDA-induced hippocampal lesion. Microphotographs of Nissl-stained sections of a rat hippocampus 15 days after the injection of 0.5 μL of (a) vehicle, (b) 40 mM NMDA, and (c) 40 mM NMDA and treated with 1 mg/kg/day diazoxide p.o. Note that treatment with diazoxide decreased NMDA induced hippocampal lesion. (d) Isolectin B4 histochemistry (IB4) staining of microglia in the hippocampus of sham rats, (e) NMDA-lesioned rats, and (f) NMDA rats treated with diazoxide. Note that treatment with diazoxide decreased the area of enhanced IB4 staining. GFAP immunostaining of the astrocytes in the hippocampus of (g) sham rats, NMDA-lesioned rats (h), and NMDA rats treated with diazoxide (i). Histograms show the quantification of the diazoxide effects in the area of lesion (j), area of microgliosis (k), and area of astrogliosis (l) in the hippocampus of NMDA-lesioned rats. Sham refers to rats injected with vehicle (50 mM PBS, pH 7.4), NMDA refers to rats injected with 0.5 μL of 40 mM NMDA in the hippocampus, and NMDA + D refers to NMDA-injected rats treated with 1 mg/kg/day diazoxide p.o. from postlesion day 5 to 15. Stereotaxic coordinates were −3.3 mm and 2.2 mm from bregma and −2.9 mm from dura [25]. All animals were manipulated in accordance with the European legislation (86/609/EEC), $N = 6$ rats/group. Scale bar 1 mm. $^{*}P < 0,05$ compared to sham, $^{\#}P < 0,05$ compared to NMDA, LSD (posthoc test).

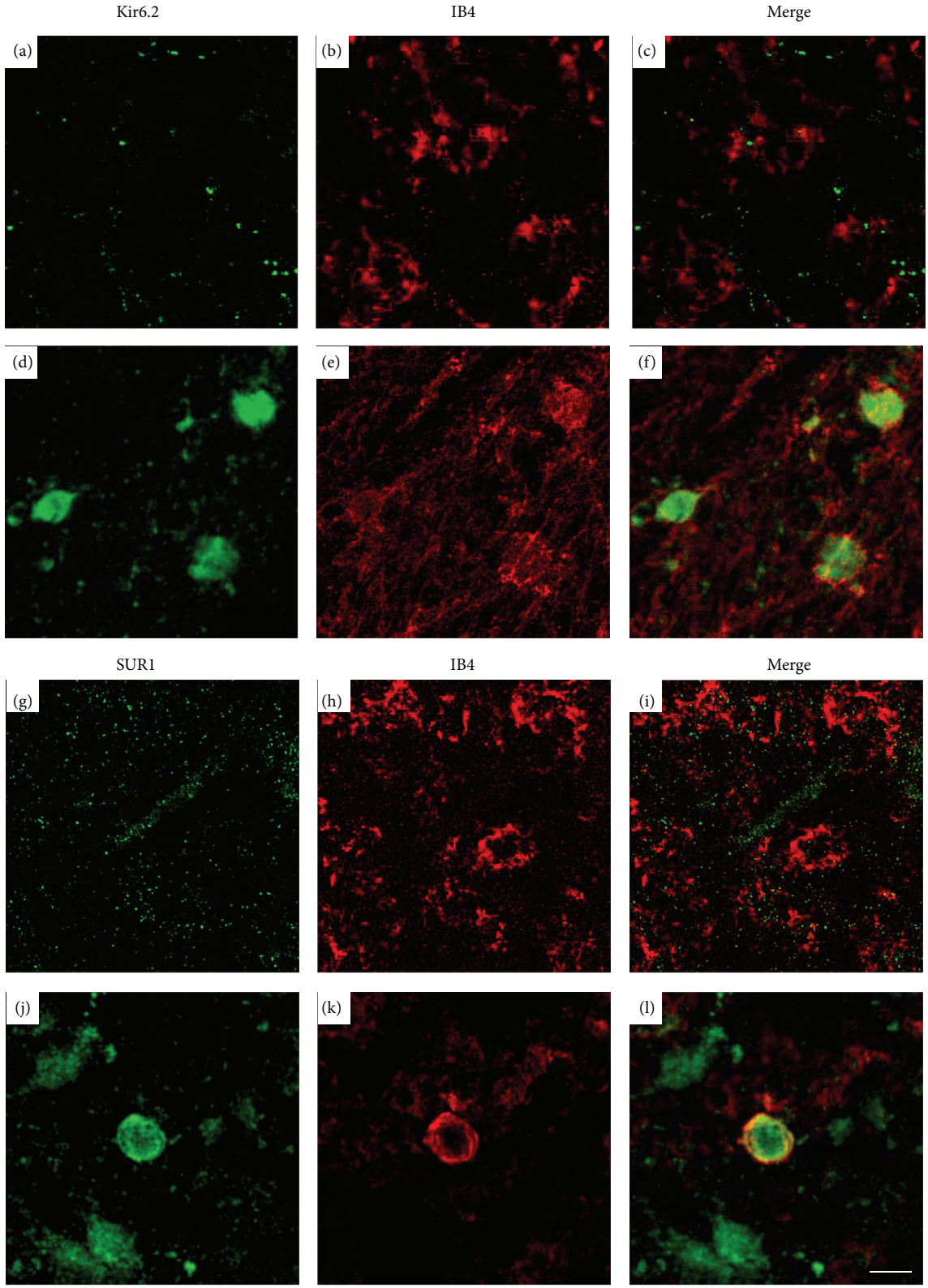

Figure 2: Expression of K_{ATP} channel components SUR1 and Kir6.2 in activated IB4-positive cells into the core of the hippocampal lesion (Bregma −3.3). ((a)–(f)) Confocal photomicrographs of hippocampal sections immunostained with IB4 and anti-Kir6.2 antibodies in control ((a)–(c)) and NMDA-lesioned rats ((d)–(f)). ((g)–(l)) Confocal photomicrographs of hippocampal sections immunostained with IB4 anti-SUR1 antibodies in control ((g)–(i)) and NMDA-lesioned rats ((j)–(l)). Note that reactive amoeboid microglia stained with IB4 show specific immunostaining with anti-Kir6.2 and anti-SUR1 antibodies in the cell membrane but also in the cytoplasm. For lesion details, see legend of Figure 1. Scale bar 10 μm.

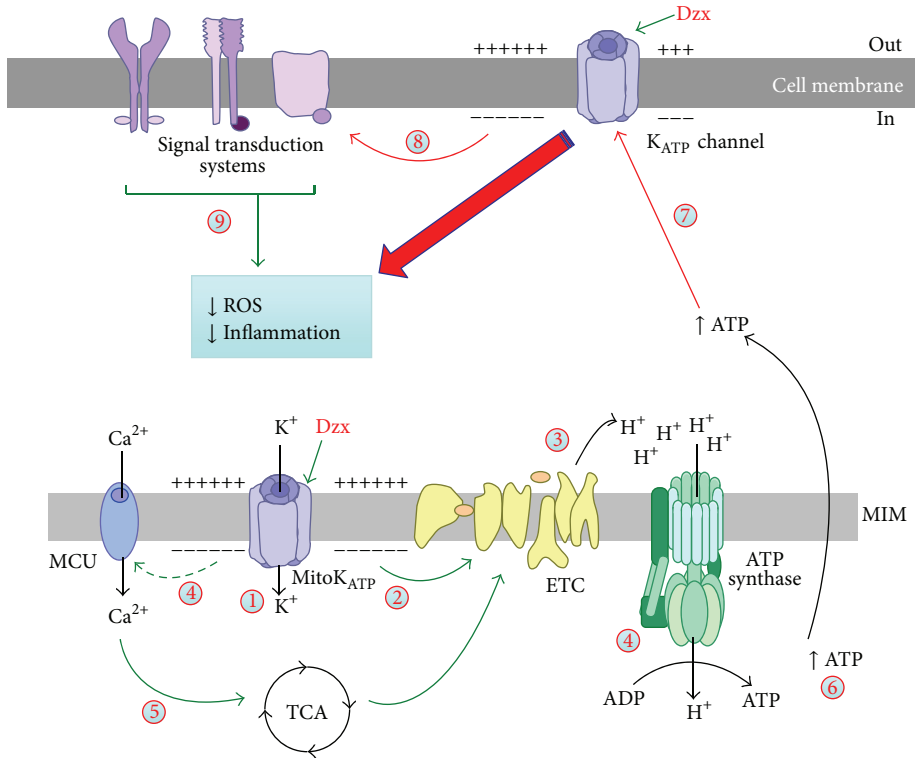

FIGURE 3: Diazoxide modifies microglial reactivity in the brain. Drawing of the effects of mitochondrial K_{ATP} ($mitoK_{ATP}$) channel opening in the energy metabolism and its consequences in the microglia reaction during neurodegeneration. (1) Diazoxide activates $mitoK_{ATP}$ channels, which (2) depolarizes the mitochondrial internal membrane (MIM), and (3) induces translocation of H^+ by the electron transport chain (ETC) that enhances both ATP synthesis and activation of the mitochondrial calcium uniporter (MCU) (4). Calcium in the mitochondria activates dehydrogenases of the tricarboxylic acid cycle (TCA) (5) that also enhances ATP production (6). ATP closes the K_{ATP} channel from the plasma membrane (7), while diazoxide opens the channel. As a result, the cell response to activation signals decreases (8), leading to a reduction of the ROS generation and the inflammatory response (9) of reactive microglia (see the text for details).

[127], and 3xTgAD mice treated with 10 mg/kg/day diazoxide for 8 months exhibited improved performance in the Morris water maze test and decreased accumulation of Aβ oligomers and hyperphosphorylated Tau in the cerebral cortex and hippocampus [128]. In turn, exposition to Aβ oligomers increases the neuronal expression of K_{ATP} channels. With time, this increase is specific for Kir6.1 and SUR2 components [129], which suggest that Aβ oligomers induce differential regulations of K_{ATP} subunit neuronal expression. Exposed to Aβ oligomers, the continuous oxidative stress results in neuronal severe mitochondria dysfunction [130, 131], and the expression changes of K_{ATP} channel components may reflect neuronal attempts to resist the insult in accordance with the metabolic state.

The chronic glutamate-mediated overexcitation of neurons is a newer concept that has linked excitotoxicity to neurodegenerative processes in ALS, Huntington's disease, AD, and Parkinson's disease [19]. Such a chronic excitotoxic process can be triggered by a dysfunction of glutamate synapses, due to an anomaly at the presynaptic, postsynaptic, or astroglial levels [21]. The contribution of excitotoxicity to the neurodegenerative process can be reproduced by the microinjection of low doses of glutamate agonist into the rodent brain. Due to the high affinity of ionotropic glutamate receptors for their specific agonists, such as N-methyl-D-aspartate (NMDA), these drugs injected in non-saturable conditions can trigger calcium-mediated excitotoxicity in several rat brain areas and induce an ongoing neurodegenerative process [55, 56, 132, 133].

For example, any microinjection of NMDA in the rat hippocampus triggers a persistent process that leads to progressive hippocampal atrophy with a widespread neuronal loss and a concomitant neuroinflammation [133]. This paradigm also mimics the alterations of other neurotransmitter systems and neuromodulators found in human neurodegeneration [22].

This neurodegenerative NMDA-induced hippocampal process is also attenuated by diazoxide oral treatment, which ameliorated microglia-mediated inflammation and reduced neuronal loss (Figure 1). In this model, anti-SUR1 and anti-Kir6.2 antibodies immunostained both the plasmalemma membrane and the perinuclear space of amoeboid reactive microglia (Figure 2), suggesting that microglial activation involves expression and translocation of SUR1 from its internal reservoir toward the cell surface and the mitochondria. Increased expression and translocation of the K_{ATP} channel to the cell surface were also detected by specific binding of fluorescently tagged glibenclamide (glibenclamide BODIPY

FL; green fluorescence) to SUR1 in rat primary microglial cultures [92].

Therefore, the expression of mitoK$_{ATP}$ channels by activated microglia indicates that KCOs, such as diazoxide, could be used as therapeutic agents to treat inflammatory processes of neurodegenerative diseases such as multiple sclerosis, Parkinson's disease, or AD.

4.2. MitoK$_{ATP}$ Channels Modulate Microglia Reaction. As explained, depending on the nature of the damage, microglial activation may take place, initially expressing neuroprotective signals to help avoid further neuronal death, but then changing progressively to an inflammatory phenotype [33]. The neuroprotective effects of diazoxide are proposed to modulate the inflammatory microglial activation, without affecting cyclooxygenase-2 expression and phagocytosis [122, 126]. The mechanism of action of diazoxide and other KCOs has not been completely elucidated yet. However, the diazoxide-mediated neuroprotection is supposed to be mediated by its interactions with mitochondria, which are the main ATP-generating sites in microglial cells. In chronically reactive microglia, diazoxide increases potassium flux into the mitochondrial matrix [117, 118] by activation of mitoK$_{ATP}$ channels, which depolarize the MIM and preserve mitochondrial structural and functional integrity [132]. Indeed, cells treated with diazoxide demonstrate a favorable energetic profile with limited damage following stress challenges [108, 134], and mitoK$_{ATP}$ channel opening promotes translocation of H^+ through the MIM, underscoring the protonophoric uncoupling and enhancing ATP synthesis (Figure 3). As a proposed mechanism, mitoK$_{ATP}$ channels opening serves to maintain constant volume and avoid an excessive mitochondrial contraction that is deleterious for electron transport [117, 118]. Thus, mitoK$_{ATP}$ channel opening may prevent respiratory inhibition due to matrix contraction that would otherwise occur during high rates of ATP synthesis. The increased H^+ gradient also constitutes the energy source for calcium transport through the MCU towards the mitochondrial matrix [67]. This calcium controls the malate/aspartate transport and dehydrogenases from the tricarboxylic acid cycle, which finally results in increased production of NADH, the electron donor of the respiratory chain. As a result, diazoxide increases the ATP/ADP ratio in the mitochondria and cytoplasm [135] of reactive microglia. ATP closes the K$_{ATP}$ channels from the plasma membrane preventing electrical activity and modifying the cell response to tissue injury. However, specific action of diazoxide on plasmalemma K$_{ATP}$ channel must not be discarded, and the activity of K$_{ATP}$ channel in the membrane will result from a compromise between the ATP-mediated inhibition and the diazoxide-induced opening (Figure 3). As a result, the cell response to tissue injury includes a decreased synthesis of proinflammatory molecules and suppression of mitochondria-derived ROS [126] that result in a maintenance of the mitochondria network integrity and of the phagocytic activity.

Thus, control of mitochondria activity by mitoK$_{ATP}$ channel opening decreases the microglia cytotoxic activity and prevents overactivation of these cells during neurodegeneration. This therapeutic approach may keep the microglia inflammatory activity under the cytotoxic threshold throughout the course of the disease, avoiding amplification of the progressive neuronal loss over time and facilitating a positive disease outcome.

Disclosure

NM and MJR have applied for a PCT application (application no. PCT/EP2011/050049). The other authors report no disclosure.

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Review Article

Diabetic Neuropathy and Oxidative Stress: Therapeutic Perspectives

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Diabetic neuropathy (DN) is a widespread disabling disorder comprising peripheral nerves' damage. DN develops on a background of hyperglycemia and an entangled metabolic imbalance, mainly oxidative stress. The majority of related pathways like polyol, advanced glycation end products, poly-ADP-ribose polymerase, hexosamine, and protein kinase c all originated from initial oxidative stress. To date, no absolute cure for DN has been defined; although some drugs are conventionally used, much more can be found if all pathophysiological links with oxidative stress would be taken into account. In this paper, although current therapies for DN have been reviewed, we have mainly focused on the links between DN and oxidative stress and therapies on the horizon, such as inhibitors of protein kinase C, aldose reductase, and advanced glycation. With reference to oxidative stress and the related pathways, the following new drugs are under study such as taurine, acetyl-L-carnitine, alpha lipoic acid, protein kinase C inhibitor (ruboxistaurin), aldose reductase inhibitors (fidarestat, epalrestat, ranirestat), advanced glycation end product inhibitors (benfotiamine, aspirin, aminoguanidine), the hexosamine pathway inhibitor (benfotiamine), inhibitor of poly ADP-ribose polymerase (nicotinamide), and angiotensin-converting enzyme inhibitor (trandolapril). The development of modern drugs to treat DN is a real challenge and needs intensive long-term comparative trials.

1. Introduction

A conduction problem arising in peripheral nerves is called peripheral neuropathy. Depending on the cause, the damage may appear in the axons or the myelin sheaths. The involved neurons may be afferent (sensory), efferent (motor), or both. The size of affected axons is an important issue, since sometimes only the small C unmyelinated and the A-delta fibers are affected. If these are damaged, symptoms move forward to pain sensors in the skin and autonomic neurons. Damage to large sensory fibers, which are the A-alpha and A-beta fibers, causes deficits in the proprioception and vibration sensation that results in muscle-stretch reflexes [1].

Diabetic neuropathy (DN), a microvascular complication of diabetes, comprises disorders of peripheral nerve in people with diabetes when other causes are ruled out. Diabetic peripheral neuropathy (DPN) is associated with considerable mortality, morbidity, and diminished quality of life [2]. The prevalence of neuropathy in diabetic patients is about 30%, whereas up to 50% of patients will certainly develop neuropathy during their disease [3]. In fact, against estimated universal prevalence of diabetes of 472 million by 2030, DPN is likely to affect 236 million persons worldwide causing lots of costs [4]. DPN can be broadly divided into generalized polyneuropathies and focal/multifocal varieties [5, 6]. The generalized form can be further classified into typical and atypical in terms of difference in onset, course, associations, clinical manifestations, and pathophysiology. The typical DPN is a chronic, symmetrical length-dependent sensorimotor polyneuropathy (DSPN) and the most common presentation of the peripheral nervous system damage by diabetes [7]. Therefore, considering the widespread of DN, it is vital to investigate details of its pathophysiology and therapeutic strategies. DN develops on a background of hyperglycemia and associated metabolic imbalances mainly oxidative stress. Hyperglycemia-induced overproduction of

free radicals has been recognized as the source of further complications. Studies in the recent years have identified major pathways that are linked to DN, such as stimulated polyol, advanced formation of glycation end products, and other cascades of stress responses [8]. Since oxidative stress leads to such a major influence in the development of DN, in this paper we have highlighted the evidence linking DN, oxidative stress, and its consequences.

Despite efforts to make an early diagnosis and to stop the progression of DN, currently very few drugs are available to cure this disease and the others only provide symptomatic relief. Meanwhile, current goal of treatment of DN is to increase the functionality and quality of life and to diminish pain. In the present review, therapies on the horizon based on oxidative stress have been criticized.

2. Methods

Databases of PubMed, Google Scholar, Web of Science, Embase, Scopus, and DARE were searched up to 30 November 2012, for all relevant studies with DN. The search terms were diabetic neuropathy, oxidative stress, mechanisms, and current and new treatments without limiting search elements. All of relevant human (Table 1) and animal (Table 2) studies were included.

3. Clinical Features of DN

The most common form of DN is DSPN that accounts for a large proportion of all peripheral nerve manifestations attributed to diabetes, although, some physicians use the terms DSPN and DN interchangeably. Poor control of blood glucose is an important risk factor for the development of DN starting in the toes and gradually progressing proximally. Once it is diagnosed in the lower limbs, it may develop to the upper limbs with sensory loss [4]. For example, a patient with painful sensory neuropathy due to diabetes might first complain of burning or itchy sensations or even pain in the feet that is called paresthesias. The symptoms distribute on a so-called "glove and stocking" manner, as it starts from the longest axons. The unmyelinated nerve endings in the epidermis are degenerated first [2]. Neuropathic pain is the most disabling symptom observed in around one-third of patients with DN and about 20% of all diabetic patients. Painful DN deleteriously influences quality of life, sleep, mood, and the ability to work [4].

4. Pathogenesis of DN: Interaction of Oxidative Stress with Other Physiological Pathways

Although development of DN is multifactorial and the exact pathogenic mechanism is yet to be understood, a number of theories can be described. The current belief is that hyperglycemia, activation of polyol, advanced glycation end products (AGEs), hexosamine, diacylglycerol/protein kinase C (PKC), oxidative stress, nitric oxide, and inflammation all play key roles in DN. Based on evidence, oxidative stress is involved in all the above pathways (see Figure 1). These mechanisms are described one by one in the next sections of this paper, and then a conclusion is made.

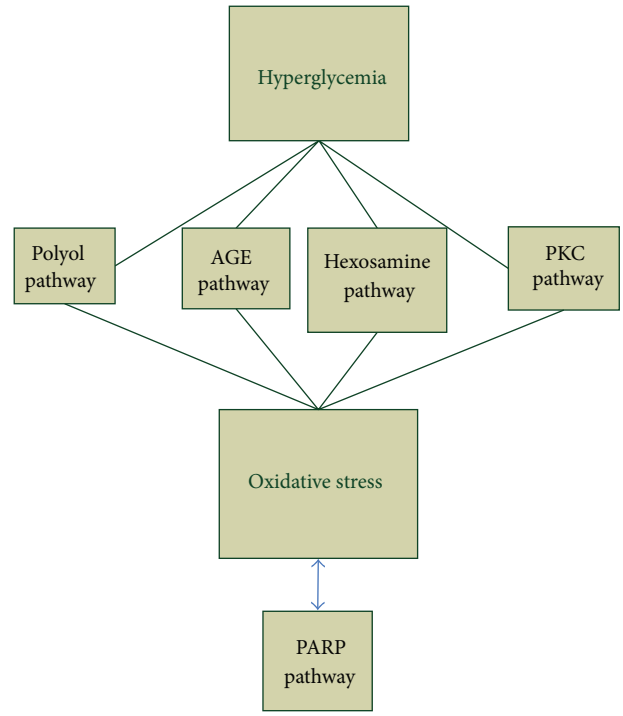

Figure 1: Interaction of oxidative stress with other physiological pathways in DN.

4.1. Hyperglycemia. Excess intracellular glucose is processed by increased flux via one or more glucose metabolism pathways, and thus prolonged hyperglycemia results in progress of chronic complications of diabetes including DN.

4.2. Role of Polyol. Hyperglycemia results in elevated intracellular glucose in nerves, leading to saturation of the normal glycolytic pathway. Extra glucose lightens up the polyol pathway and that produces sorbitol and subsequently fructose by aldose reductase and sorbitol dehydrogenase, respectively. Increased polyol flux causes intracellular hyperosmolarity by an accumulation of impermeable sorbitol and compensatory efflux of other osmolytes such as myoinositol, taurine, and adenosine. In turn, shortage of myoinositol results in exhaustion of phosphatidylinositol and withdraws creation of adenosine triphosphate (ATP). All these processes result in a reduced activity of Na^+/K^+-ATPase and PKC, impaired axonal transport and structural breakdown of nerves, and finally presents itself as abnormal action potential. Aldose reductase-mediated reduction of glucose to sorbitol is associated with consumption of NADPH, and since NADPH is required for regeneration of reduced glutathione (GSH), this directly contributes to oxidative stress. In addition, formation of fructose from sorbitol promotes glycation, depletes NADPH, and increases AGEs which all result in major redox imbalance (see Figure 2) [9, 10].

Table 1: Current pharmacotherapy in DN.

NNT	Study outcome	Treatment duration	Study design	Daily dose (mg)	Trial size	Trial	Drug used	Drug class
—	Amitriptyline > placebo	2 × 6 wk	Crossover	Up to 150 mg	29	Max [15]	Amitriptyline	Antidepressants: TCAs:
2.1	Amitriptyline > placebo	2 × 6 wk	Crossover	≤150 mg	29	Max et al. [16]	Amitriptyline	
2.2	Amitriptyline = desipramine > placebo	2 × 6 wk	Crossover	Amitriptyline: 105 mg; desipramine: 111 mg	38	Max et al. [17]	Amitriptyline and desipramine	
—	Amitriptyline > maprotiline > placebo	4 wk	Crossover	75 mg	37	Vrethem et al. [18]	Amitriptyline and maprotiline	
—	Amitriptyline > placebo	2 × 6 wk	Crossover	25–75 mg	24	Morello et al. [19]	Amitriptyline	
—	Clomipramine > desipramine > placebo	6 wk	Crossover	Desipramine: 200 mg and clomipramine: 75 mg (in extensive metabolisers). 50 mg of both drugs (in poor metabolisers)	19	Sindrup et al. [20]	Desipramine and clomipramine	
	Desipramine > placebo	6 wk	Crossover	201 mg	20	Max et al. [21]	Desipramine	
—	Imipramine > placebo	5 + 5 wk	Crossover	100 mg	12	Kvinesdal et al. [22]	Imipramine	
—	Combination > placebo	8 wk	Crossover	Nortriptyline: 10 mg; fluphenazine: 0.5 mg	18	Gomez-Perez et al. [23]	Nortriptyline and fluphenazine	
—	Citalopram > placebo	2 × 3 wk	Crossover	40 mg	15	Sindrup et al. [24]	Citalopram	SSRI:
—	Venlafaxine + gabapentin > placebo in patients who do not respond to gabapentin	2 × 8 wk	Parallel	—	11 and 42	Simpson [25]	Venlafaxine and gabapentin	SNRIs
5.2 for venlafaxine and 2.7 for imipramine.	Venlafaxine > imipramine > placebo	4 wk	Crossover	Venlafaxine: 225 mg; imipramine: 150 mg	29	Sindrup et al. [26]	Venlafaxine versus imipramine	
4.5	Venlafaxine > placebo	6 wk	Parallel	150–225 mg	244	Rowbotham et al. [27]	Venlafaxine	
—	Venlafaxine > placebo	8 wk	Parallel	75–150 mg	60	Kadiroglu et al. [28]	Venlafaxine	
11 (60 mg group); 5 (120 mg group)	Duloxetine > placebo	12 wk	Parallel	60, 120 mg	348	Raskin et al. [29]	Duloxetine	
4.3 (60 mg group); 3.8 (120 mg group)	Duloxetine > placebo	12 wk	Parallel	20, 60, 120 mg	457	Goldstein et al. [30]	Duloxetine	
6.3 (60 mg group); 3.8 (120 mg group)	Duloxetine > placebo	12 wk	Parallel	60, 120 mg	334	Wernicke et al. [31]	Duloxetine	
5.2 and 4.9 (duloxetine 60 mg once daily and 60 mg BID, resp.)	Duloxetine > placebo	3 × 12 wk	Parallel	60 mg	1024	Kajdasz et al. [32]	Duloxetine	

Table 1: Continued.

NNT	Study outcome	Treatment duration	Study design	Daily dose (mg)	Trial size	Trial	Drug used	Drug class
3.6 (300 mg group); 3.3 (600 mg group)	Pregabalin (300, 600 mg) > placebo	5 wk	Parallel	75, 300, 600 mg	338	Lesser et al. [33]	Pregabalin	
3.9	Pregabalin > placebo	8 wk	Parallel	300 mg	146	Rosenstock et al. [34]	Pregabalin	
4.2 (600 mg group)	Pregabalin (600 mg) > placebo	6 wk	Parallel	150, 600 mg	246	Richter et al. [35]	Pregabalin	
3.6	Flexible and fixed > placebo	12 wk	Parallel	Flexible: 150, 300, 450, 600 mg; fixed: 300, 600 mg	338	Freynhagen et al. [36]	Pregabalin	
6.3 (600 mg group)	Pregabalin (600 mg) > placebo	12 wk	Parallel	150, 300, or 600 mg	395	Tölle et al. [37]	Pregabalin	
—	Pregabalin > placebo	13 wk	Parallel	600 mg	167	Arezzo et al. [38]	Pregabalin	
4.04 (600 mg group); 5.99 (300 mg group); 19.06 (150 mg group)	150, 300, 600 mg TID > placebo; 600 mg BID > placebo	5 to 13 wk	Parallel	150, 300, 600 mg administered TID or BID	—	Freeman et al. [39]	Pregabalin	
4	Gabapentin > placebo	8 wk	Parallel	Titrated from 900 to 3600 mg	165	Backonja et al. [40]	Gabapentin	
—	Gabapentin = placebo	2 × 6 wk	Crossover	900 mg	40	Gorson et al. [41]	Gabapentin	
—	Sodium valproate > placebo	4 wk	Parallel	600–1200 mg	52	Kochar et al. [42]	Sodium valproate	
—	Sodium valproate > placebo	16 wk	Parallel	500 mg	39	Kochar et al. [43]	Sodium valproate	Anticonvulsants:
—	Sodium valproate = placebo	4 wk	Crossover	1500 mg	31	Otto et al. [44]	Sodium valproate	
4	Lamotrigine > placebo	6 wk	Parallel	Titrated from 25 to 400 mg	59	Eisenberg et al. [45]	Lamotrigine	
—	Lamotrigine = placebo	19 wk	Parallel	200, 300, 400 mg	360	Vinik et al. [46]	Lamotrigine	
—	Lamotrigine = amitriptyline	6 wk	Crossover,	Lamotrigine: 25, 50, 100 mg twice daily; amitriptyline: 10, 25, 50 mg at night time	53	Jose et al. [47]	Lamotrigine and amitriptyline	
—	Carbamazepine > placebo	2 wk	Crossover	200–600 mg	30	Rull et al. [48]	Carbamazepine	
—	Oxcarbazepine > placebo	16 wk	Parallel	300 mg titrated to a maximum dose of 1800 mg	146	Dogra et al. 2005 [49]	Oxcarbazepine	
7.9 (1200 groups); 8.3 (1800 groups)	Oxcarbazepine > placebo (1200, 1800 mg groups)	16 wk	Parallel	600, 1200, 1800 mg	347	Beydoun et al. [50]	Oxcarbazepine	
—	Oxcarbazepine = placebo	16 wk	Parallel	1200 mg	141	Grosskopf et al. [51]	Oxcarbazepine	
—	Lacosamide > placebo	—	Parallel	400 mg	94	Rauck et al. [52]	Lacosamide	
—	Lacosamide (400 mg group) > placebo	18 wk	Parallel	200, 400, 600 mg	—	Wymer et al. [53]	Lacosamide	
3.1	Tramadol > placebo	6 wk	Parallel	210 mg	131	Harati et al. [54]	Tramadol	
4.3	Tramadol > placebo	2 × 4 wk	Crossover	200–400 mg	45	Sindrup et al. [55]	Tramadol	
—	Tramadol/acetaminophen > placebo	8 wk	Parallel	Tramadol: 37.5 mg; acetaminophen: 325 mg	311	Freeman et al. [56]	Tramadol/ acetaminophen	
—	Oxycodone > placebo	6 wk	Parallel	10–100 mg	159	Gimbel et al. [57]	Oxycodone	Opioids:
2.6	Oxycodone > placebo	4 wk	Crossover	10–80 mg	45	Watson et al. [58]	Oxycodone	
—	Oxycodone + gabapentin > placebo + gabapentin	12 wk	Parallel	Oxycodone: 10–80 mg + gabapentin: 100–3600 mg	338	Hanna et al. [59]	Oxycodone	
—	Morphine + gabapentin > morphine > gabapentin > placebo	4 × 4 wk	Crossover	120, 60 mg morphine + 2400 mg gabapentin, 3600 mg gabapentin	57	Gilron et al. [60]	Morphine	

Table 1: Continued.

NNT	Study outcome	Treatment duration	Study design	Daily dose (mg)	Trial size	Trial	Drug used	Drug class
—	Capsaicin > vehicle	8 wk	Parallel	0.075% capsaicin	252	Anonymous et al. [61]	Capsaicin	Topical medications:
—	Capsaicin > vehicle	8 wk	Parallel	0.075% capsaicin	—	Scheffler et al. [62]	Capsaicin	
—	Capsaicin > vehicle	8 wk	Parallel	0.075% capsaicin four times a day	22	Tandan et al. [63]	Capsaicin	
—	Capsaicin > vehicle	8 wk	Parallel	0.075% capsaicin four times a day		Anonymous et al. [64]	Capsaicin	
—	Isosorbide > placebo	2 × 4 wk	Crossover	30 mg	22	Yuen et al. [65]	Isosorbide dinitrate spray	
—	Glyceryl > placebo	2 × 4 wk	Crossover	—	48	Agrawal et al. [66]	Glyceryl trinitrate spray	
4.4	Lidocaine > placebo	4 wk	Crossover	5% lidocaine patch	40	Meier et al. [67]	Lidocaine patch	
—	Lidocaine significantly improved pain and quality of life	3 wk study with a 5 wk extension	Open label, flexible dosing	5% lidocaine patch	56	Barbano et al. [68]	Lidocaine patch	
—	Mexiletine > placebo	10 wk	Crossover	10 mg	16	Dejgard et al. [69]	Mexiletine	Anesthetics/ antiarrhythmics:
	Mexiletine > placebo	3 wk	Parallel	675 mg	216	Oskarsson et al. [70]	Mexiletine	
—	Mexiletine = placebo	3 wk	Parallel	600 mg	29	Wright et al. [71]	Mexiletine	
4	Dextromethorphan > placebo	2 × 6 wk	Crossover	Mean 381 mg	14	Nelson et al. [72]	Dextromethorphan	NMDA antagonists:
3.2	Dextromethorphan > placebo	2 × 9 wk	Crossover	400 mg	19	Sang et al. [73]	Dextromethorphan	
3.03 at 12 weeks	Botulinum toxin > placebo	24 wk	Parallel	Intradermal of subtype A (20–190 units) into the painful area	29	Ranoux et al. [74]	Botulinum toxin	Other drugs:
—	Botulinum toxin > placebo	12 × 12 wk	Crossover	50 units of subtype A in 1.2 mL 0.9% saline given intradermally into each foot, each injection 4 U subtype A	18	Yuan et al. [75]	Botulinum toxin	
—	Improved pain and nerve fiber regeneration	2 × 52 wk	Parallel	500 and 1,000 mg, three times per day	—	Sima et al. [76]	Acetyl-L-carnitine	
—	α-lipoic acid = placebo	28 wk	Parallel	600 mg	509	Ziegler et al. [77]	α-lipoic acid	
—	α-lipoic acid ≥ placebo with clinically meaningful degree	3 wk	Parallel	600 mg	1258	Ziegler et al. [78]	α-lipoic acid	

NNT: number needed to treat; TCAs: tricyclic antidepressants; SSRI: selective serotonin reuptake inhibitor; SNRIs: serotonin norepinephrine reuptake inhibitors; NMDA: N-methyl-D-aspartate; TID: three times daily; BID: twice daily.

Table 2: New therapeutic approaches for DN. DRG: dorsal root ganglion neuron.

Endpoint	Study populations	Compound	Study
Improvement of peripheral nerve function	Diabetic rats	Salvianolic acid A	Yu et al. [79]
Improvement of DN	Animal model of T2D	High-fat diet with menhaden oil	Coppey et al. [80]
Improvement of DN	Patients with T2D and neuropathy	Tai Chi exercise	Ahn and Song [81]
Improvement of DN	T2DM patients	Beraprost sodium	Shin et al. [82]
Improvement of DN	STZ-diabetic rats	Anandamide	Schreiber et al. [83]
Improvement of peripheral nerve function	Mouse model of DPN	Thymosin $\beta 4$	Wang et al. [84]
Improvement of chronic pain, including PDN	Rat model of STZ-induced PDN	Gastrodin	Sun et al. [85]
Prevention of progression of DN	Patients enrolled in the aldose reductase inhibitor-diabetes complications	Epalrestat	Hotta et al. [86]
Improvement of DN	STZ-diabetic rats	Gliclazide with curcumin	Attia et al. [87]
Improvement of DN	STZ-diabetic rats	Bone marrow-derived mononuclear cells	Naruse et al. [88]
Neuroprotection effect	In vitro model of high glucose-treated DRG neurons in culture	Galanin	Xu et al. [89]
Improvement of DN	—	Baicalein	Yorek [90]
Improvement of neuropathic pain	Animal models of neuropathic pain	Brazilian armed spider venom toxin Tx3-3	Dalmolin et al., [91]
Neuroprotection effect Improvement of DN	STZ-diabetic rats	Magnesium-25 carrying porphyrin-fullerene nanoparticles	Hosseini et al. [92, 93]
Maintaining health in diabetes	STZ-diabetic rats	Phosphodiesterase inhibitors	Milani et al. [94]
Improve transplant outcome and graft function in diabetes	Isolated rat pancreatic islets	IMOD	Larijani et al. [95]
Improve islet transplantation in diabetes	Isolated rat pancreatic islets	Cerium and yttrium oxide nanoparticles	Hosseini and Abdollahi [96, 97]

4.3. Role of Advanced Glycation End Products (AGEs). Hyperglycemia accelerates generation of AGEs via attachment of reactive carbohydrate groups to proteins, nucleic acids, or lipids. These groups tend to damage the biological task of proteins, which as a result affects cellular function. Extracellular AGEs also bind to the receptor of AGE (RAGE) and initiate inflammatory flows, activate NADPH oxidases, and generate oxidative stress. Long-term inflammatory responses upregulate RAGE and stimulate nuclear factor kappa B (NFκB). Collectively, the biochemical damage induced by AGEs results in diminished neurotrophic support, impaired nerve blood flow, disrupted neuronal integrity, and impaired repair mechanisms (Figure 2) [3, 10].

4.4. Role of Hexosamine. The hexosamine, an additional factor, is implicated in the pathology of diabetes-induced oxidative stress and its complications. Fructose-6 phosphate, a metabolic intermediate of glycolysis, is shifted from the glycolytic pathway to the hexosamine way where fructose-6 phosphate is converted to uridine diphosphate-N-acetylglucosamine (UDPGlcNAc). Then, the UDPGlcNAc attaches to the serine and threonine residues of transcription factors. Hyperglycemic conditions create additional flux through hexosamine pathway that ultimately result in an increased activation of Sp1, a transcription factor implicated in diabetic complications. Activation of Sp1 leads to overexpression of transforming growth factor-$\beta 1$ (TGF-$\beta 1$) and plasminogen activator inhibitor-1 (PAI-1). The PAI-1 is upregulated by both hexosamine and PKC pathways. Collectively, activation of hexosamine pathway is implicated in multiple metabolic derangements in diabetes (see Figure 2) [9].

4.5. Activation of Diacylglycerol Protein Kinase C. Hyperglycemia stimulates formation of diacylglycerol, which then activates PKC. The PKC is an important element in function of nerves and pathogenesis of DN. Activation of PKC initiates an intracellular signaling cascade such as overexpression of PAI-1, NF-κB, and TGF-β. It also increases the production of extracellular matrix and cytokines. Furthermore, it enhances contractility, permeability, and vascular endothelial cell proliferation such as motivation of cytosolic phospholipase A2 and inhibition of Na^+/K^+ ATPase (Figure 2) [9, 11]. PKC has a unique structural feature that according to redox status of cell facilitates its regulation. An antioxidant can react with catalytic domain to inhibit its activity, while the prooxidants

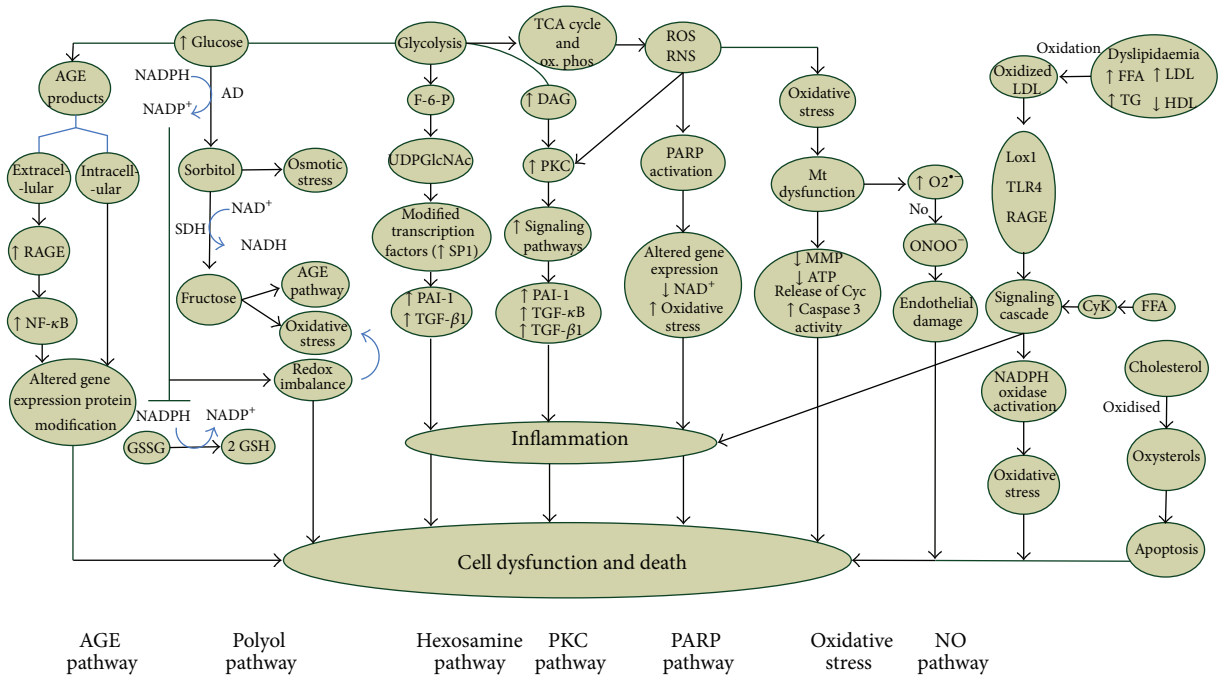

Figure 2: Mechanisms of diabetic neuropathy. The AGE and polyol pathways directly alter the redox capacity of the cell either through depletion of necessary components of glutathione recycling or by direct formation of ROS. The hexosamine, PKC, and PARP pathways indicate damage through expression of inflammation proteins. Dyslipidaemia with high incidence in T2D also linked to DN, and several underlying mechanisms have been identified. AGEs: advanced glycation end products; RAGEs: receptor for advanced glycation end products; NF-κB: nuclear factor kappa B; AD: aldose reductase; SDH: sorbitol dehydrogenase; GSH: glutathione; GSSG: oxidized glutathione; F-6-P: fructose-6 phosphate; UDPGlcNAc: uridine diphosphate-N-acetylglucosamine; PAI-1: plasminogen activator inhibitor-1; TGF-β1: transforming growth factor-β1; DAG: diaceylglycerol; PKC: protein kinase C; ROS: reactive oxygen species; RNS: reactive nitrogen species; PARP: poly ADP-ribose polymerase; Mt: mitochondria; MMPs: mitochondrial membrane potentials; Cyc: cytochrome c; NO: nitric oxide; LDL: low-density lipoprotein; LOX1: oxidised LDL receptor 1; TLR4: toll-like receptor 4; FFA: free fatty acids; TG: triglycerides; HDL: high-density lipoprotein; CyK: cytokine.

react with regulatory domain to stimulate its activity. On activation, PKC triggers stress genes that phosphorylate transcription factors and thus alter the balance of gene expression resulting in oxidative stress [8].

4.6. Role of Oxidative Stress, Apoptosis, and Poly ADP-Ribose Polymerase. The generation of free radicals is a major factor in development of DN through increased glycolytic process. Oxidative stress and reactive oxygen species (ROS) link the physiological mediators and metabolic initiators implicated in progressive nerve fiber damage, dysfunction, and loss in DN. Simultaneous with generation of free radicals during the glycolytic process, oxidative stress harms mitochondrial DNA, proteins, and membranes [9, 12]. In fact, mitochondrial damage takes place due to surplus formation of ROS or reactive nitrogen species (RNS). Hyperglycemia induces mitochondrial changes such as release of cytochrome C, activation of caspase 3, altered biogenesis and fission, which all lead to a programmed cell death. Reduced mitochondrial action potentials (MMP) with modest ATP are resulted from thrilling entrance of glucose. This process results in surplus transport of oxidant electrons into the mitochondria. Neurotrophic support such as neurotrophin-3 (NT-3) and nerve growth factor (NGF) are also reduced by mitochondrial injury. It should be noted that axons are disposed to hyperglycemic hurt owing to their large content of mitochondria [12]. Oxidative stress in conjunction with hyperglycemia activates poly ADP-ribose polymerase (PARP) which further cleaves nicotinamide adenine dinucleotide (NAD^+) to nicotinamide and ADP-ribose residues. This process continues by a link to nuclear proteins and results in changes of gene transcription and expression, NAD^+ depletion, oxidative stress, and diversion of glycolytic intermediates to other pathogenic pathways such as PKC and AGEs (Figure 2) [9]. Collectively, the polyol, AGEs, PKC, hexosamine, and PARP, all contribute to neuronal damage together. The AGEs and polyol pathways openly modify the redox capacity of the cell either through weakening of necessary components of glutathione recycling or by direct construction of ROS. The hexosamine, PKC, and PARP pathways are representatives of damage mediated through expression of inflammatory proteins [9].

4.7. Nitric Oxide Deficiency/Impaired Endothelial Function. Vascular factors include impaired nerve perfusion, hypoxia, and nerve energetic defects that are all implicated in the pathogenesis of DN. Nerve blood flow is reduced in DN perhaps mediated via nitric oxide (NO). Overproduction of

superoxide anion by the mitochondrial electron transport chain in DN leads to binding of this anion to NO to form the strong oxidant peroxynitrite which is right lethal to endothelial cells. Endothelial cells also produce NO that acts as a vasodilator and antagonizes thrombosis. The NO also defends against inflammation by adjusting (Na^+/K^+)-ATPase or inhibiting the production of potent vasoconstrictor peptide endothelin (ET)-1 [13, 14]. In addition, hyperhomocysteinemia is associated with impairment of endothelial function, providing a mechanism for its possible involvement in DN. There is a synergistic effect between AGEs and homocysteine in initiation of endothelial damage (Figure 2) [13].

4.8. Inflammation. The nerve tissues in diabetes undergo a proinflammatory process that presents symptoms and develops neuropathy. In addition, inflammatory agents such as C-reactive protein and tumor necrosis factor-α (TNF-α) are present in the blood of both type 1 diabetes (T1D) and type 2 diabetes (T2D) patients. The levels of C-reactive protein and TNF-α correlate with the incidence of neuropathy. Production of the initiating inflammatory mediators such as TNF-α, TGF-β, and NF-κB results from several glucose-induced pathways. Cyclooxygenase-2 (COX-2) is an important enzyme that is upregulated by NF-κB in diabetic peripheral nerves and consecutively generates prostaglandin E2 and ROS that trigger NF-κB. Inducible nitric oxide synthase (iNOS) is an additional inflammatory enzyme which is regulated by NF-κB. Similar to COX-2, iNOS either induces NF-κB or is induced by it. This gives the impression that chronic NF-κB activation is in the center of all the inflammatory elements operating in DN. Subsequent to ischemia reperfusion, an extensive and modest infiltration of macrophages and granulocytes takes place in diabetic peripheral nerves. The cytokines which are induced by NF-κB in Schwann cells, endothelial cells, and neurons lead to absorption of macrophages in the diabetic nerves. Macrophages promote DN via a variety of mechanisms, including making of cytokines, ROS, and proteases, which all result in cellular oxidative damage and myelin breakdown. Excessive macrophage recruitment impairs regeneration of nerves in DN [9, 12].

4.9. Growth Factors. Neurotrophic factors play roles in the development, maintenance, and survival of neuronal tissue. In DN, the Schwann cells are damaged and neurons disintegrate, and the growth factors such as NGF, NT-3 and insulin-like growth factors (IGFs) are affected [9, 11].

5. Differences in the Pathophysiology of T1D and T2D in DN

As noted, hyperglycemia is a fundamental factor in DN. Dyslipidemia and changes in insulin signaling come after hyperglycemia in T2D. Levels of both insulin and C-peptide are reduced in patients with T1D while the neuronal insulin sensitivity is reduced in T2D. Therefore, the circumstances of disease in T1D and T2D are different, and this affects the efficacy of some medications [3].

5.1. Dyslipidemia. Dyslipidemia with high incidence in T2D is linked to DN. Free fatty acids have systemic effects such as release of inflammatory cytokine from adipocytes and macrophages. Plasma lipoproteins, especially low-density lipoproteins (LDLs), can be modified by glycation or oxidation where then binds to extracellular receptors comprising the oxidized LDL receptor 1(LOX1), toll-like receptor 4(TLR4), and RAGEs that activate NADPH oxidase. All these result in oxidative stress. Additionally, cholesterol can be simultaneously oxidized to oxysterols to cause neuronal apoptosis (Figure 2) [3].

5.2. Impaired Insulin Signaling. Insulin has neurotrophic effects and promotes neuronal growth and the survival while it is not involved in uptake of glucose into the neurons. Reduction of this neurotrophic signaling due to insulin resistance (T2D) or insulin deficiency (T1D) contributes to the pathogenesis of DN. In neurons, insulin resistance occurs by inhibition of the PI3K/Akt signalling pathway. Disruption of this pathway leads to mitochondrial dysfunction, oxidative stress, and dysfunction or death of the nerve. Tight glucose control is not as efficacious in patients with T2D, whereas it can reduce neuropathy in patients with T1D. This divergence is most likely related to differences in the underlying mechanisms in terms of hyperglycemia, dyslipidemia, and insulin resistance in T1D and T2D [3].

6. Treatment of DN

6.1. Control of Hyperglycemia. As discussed above, hyperglycemia and/or insulin deficit and their concomitant actions are principally involved in the pathogenesis of DN. Thus, glycemic control gives the impression to be the most effective treatment to delay onset of DN and slowing its progress [14]. By contrast with the results obtained from patients with T1D, glucose control produces less definitive effect in patients with T2D. Therefore, despite the similarities between T1D and T2D, there are dissimilarities in the mechanisms and complications. It seems that glucose control is the only disease-modifying therapy for DN, as uncontrolled diabetes results in a pronounced oxidative stress that can be reversed if patients attain glycemic control. According to assumption that oxidative stress may mediate vascular, microvascular, and specific tissue complications in diabetes, antioxidant therapy remains a vital therapy [3]. Pain management is the other buttress of treatment for DN that can largely improve the quality of patients' life. As shown in Table 1, over the past two decades, enormous efforts have been made by doing randomized placebo-controlled trials to find effective treatments for DN. Based on these studies, several classes of drugs are considered to be effective for the treatment of DN, and also given the pathogenesis of DN, other drugs have been suggested as disease modifying all based on oxidative stress (Figure 3).

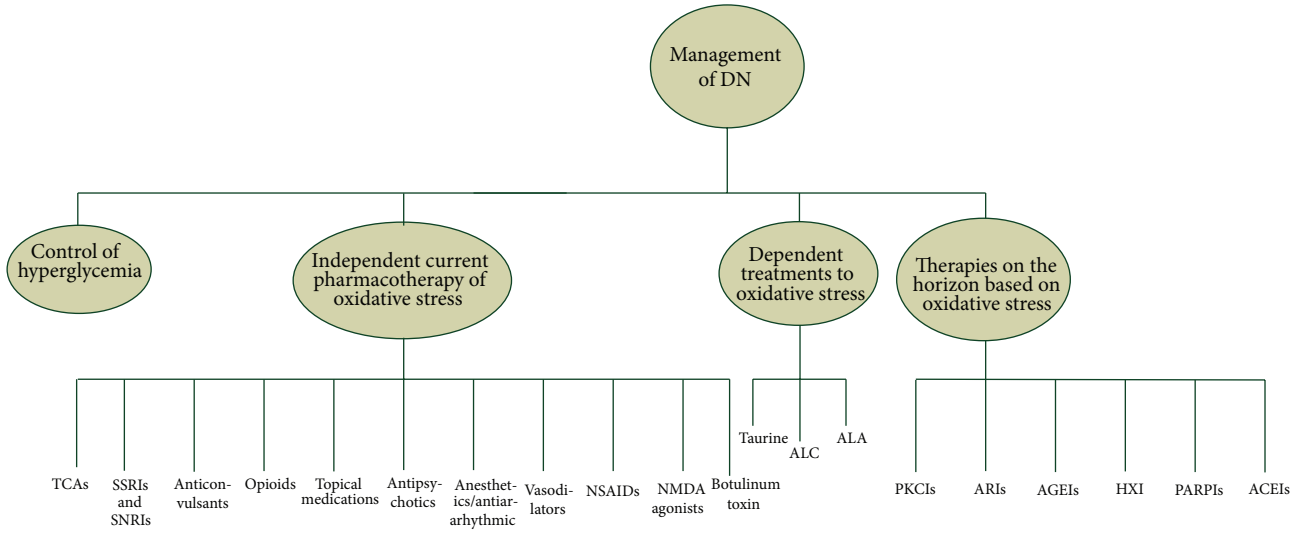

Figure 3: Algorithm for treatment of DN pain. TCAs: tricyclic antidepressants; SSRIs: selective serotonin reuptake inhibitors; SNRIs: serotonin norepinephrine reuptake inhibitors; NSAIDs: nonsteroidal anti-inflammatory drugs; ALC: acetyl-l-carnitine; ALA: α-lipoic acid; PKCIs: protein kinase C inhibitors; ARIs: aldose reductase inhibitors; AGEIs: advanced glycation end product inhibitors; GF: growth factor; PARPIs: poly ADP-ribose polymerase inhibitors; ACEIs: angiotensin converting enzyme inhibitors; HXIs: hexosamine pathway inhibitors.

6.2. Current Pharmacotherapy of DN Independent of Oxidative Stress

6.2.1. Tricyclic Antidepressants (TCAs). The TCAs are recommended as the first-line therapy to relief pain of DPN for many years, even though they are not specifically endorsed for it. TCAs besides affecting catecholamines, they inhibit sodium and calcium channels, adenosine and N-methyl-D-aspartate (NMDA) receptors on the way to suppress neuronal hyperexcitability. TCAs have many side effects, principally anticholinergic effects [98, 99]. A meta-analysis based on number needed to treat (NNT) for TCAs resulted in the scores of 2 to 3 with a number needed to harm (NNH) of 14.7 [100].

6.2.2. Selective Serotonin Reuptake Inhibitors (SSRIs) and Serotonin Norepinephrine Reuptake Inhibitors (SNRIs). Because of the relative high rate of adverse effects and several contraindications of TCAs, the SSRIs can be considered for those who do not tolerate TCAs. The SSRIs inhibit presynaptic reuptake of serotonin but not norepinephrine, and unlike TCAs, they lack postsynaptic receptor blocking effects and quinidine-like membrane stabilization. There is limited evidence showing a beneficial role for SSRIs, as they have not been licensed for the treatment of DN pain [14, 99, 101]. Antidepressants with dual selective inhibition of serotonin and norepinephrine (SNRIs) such as duloxetine and venlafaxine are better. SNRIs block the noradrenaline and 5-HT transporters and inhibit monoamine reuptake from the synaptic cleft into the presynaptic terminal which finally result in inhibition of excitatory impulse and pain perception [98, 101–103].

6.2.3. Anticonvulsants. Gabapentin and pregabalin should be used as first-line treatment for DPN pain if there is inadequate response or contraindications to TCAs. Pregabalin and gabapentin bind to the alpha2-delta subunit of the calcium-sensitive channels on the presynaptic neuron and modulate neurotransmitter release [99, 100]. Based on the new guidelines, pregabalin is recommended for the treatment of painful DN [102, 103]. Sodium valproate is probably effective in lessening pain and should be considered for the treatment of painful DN, but its adverse effects are high. For instance, it can worsen glycemic control and weight gain. Lamotrigine, oxcarbazepine and lacosamide should probably not be considered for the treatment of painful DN. Topiramate also lacks adequate support to be used in DN [103].

6.2.4. Opioids. Opioids by interacting with receptors located on neuronal cell membranes prevent neurotransmitter release at the presynaptic nerve terminal and reduce pain [14]. Controlled-release oxycodone, tramadol, and morphine can be exampled [99]. A meta-analysis indicated an NNT of 2.6 for oxycodone, 3.9 for tramadol, and 2.5 for morphine [100] in the treatment of painful DN [103].

6.2.5. Topical Medications. Current topical treatments for DPN pain include capsaicin cream and lidocaine 5% patches. This combination stimulates C fibers to release, and subsequently deplete substance P. Capsaicin application causes degeneration of epidermal nerve fibers as a mechanism of its analgesic effect, and thus caution for its use in insensitive diabetic foot is needed [14]. In a 2009 pooled data analysis, the calculated NNT for capsaicin was 5.7 [53]. Lidocaine blocks voltage-gated channels in damaged nerves [99]. There have been small effective trials with lidocaine. A randomized controlled trial (RCT) in 2003 revealed an NNT of 4.4 for it [67]. Topical clonidine is probably not effective and should not be considered [103].

6.2.6. Antipsychotics. Few atypical antipsychotics have been introduced for painful DN. The newer, less dopamine selective antipsychotics was supported by some animal studies consistent with clinical trials [104]. These compounds may induce negative metabolic effects such as weight gain and insulin resistance [105].

6.2.7. Anesthetics/Antiarrhythmic. These agents are all potent voltage-gated sodium channel antagonists and perhaps are less used in DN [105]. Mexiletine, lidocaine, and tocainide have all shown benefits for painful DN in smaller RCTs. Although tocainide has positive effects, but caution is needed for its cardiotoxicity [105]. Mexiletine has not been found effective [103]. The easiest method to incorporate the utility of the anesthetic/antiarrhythmics in patients with painful DN is the use of 5% lidocaine patches [105].

6.2.8. Vasodilators. Isosorbide dinitrate nasal spray and glyceryl trinitrate transdermal patches have shown positive effects in painful DN [105].

6.2.9. Nonsteroidal Anti-Inflammatory Drugs (NSAIDs). In patients with acute painful peripheral neuropathy, simple analgesics such as NSAIDs may provide pain control by modulating the nociceptive and inflammatory pain pathways [14].

6.2.10. NMDA Antagonists. Dextromethorphan, a low affinity NMDA receptor blocker, has been effective in relieving pain and improving quality of life in patients with DN [103].

6.2.11. Botulinum Toxin. This toxin not only works on the acetylcholine channels but also involves other mechanisms. Existing studies lack large number of subjects, and more is required to overcome doubt and debate about efficacy of this toxin [105].

6.3. Treatments Dependent on Oxidative Stress. The strongest sign for the role of oxidative stress in DN is the studies that report positive effects of antioxidants in both animal models and patients. Although, it is impossible to review all the antioxidants that can be effective to prevent or delay the onset of DN, some can be listed such as acetyl-L-carnitine, taurine, β-carotene, free amino acids, vitamin E, curcumin, ascorbic acid, and lipoic acid [8].

6.3.1. Taurine. Taurine is an antioxidant with effects on neuronal calcium signaling. Taurine improves electrophysiological parameters and nerve blood flow and exhibits analgesic properties in patients with DN [14].

6.3.2. Acetyl-L-Carnitine. Acetyl-L-carnitine (ALC), the acetylated ester of the amino acid L-carnitine, as an antioxidant has shown significant reduction in pain of patients with DN [14].

6.3.3. Alpha Lipoic Acid. D-L-α-lipoic acid (ALA) is a potent antioxidant, which has been extensively evaluated in subjects with DN and has shown good effects [14]. But based on new guidelines, there is insufficient evidence to recommend it in treatment of DN [103].

6.4. Nonpharmacologic Agents. Percutaneous electrical nerve stimulation is probably effective in reducing pain and improving sleep in patients who have painful DN. Exercise and acupuncture lack evidence of efficacy in the treatment of painful DN. Low-intensity laser therapy, Reiki therapy, and electromagnetic field treatment (such as magnetized shoe insoles) are probably not effective and should not be considered [103].

6.5. Therapies on the Horizon Based on Oxidative Stress. Since constant tight glucose control is difficult and still a challenge in most cases, additional therapies that target the hyperglycemia-induced complications are now under attention of researchers. To this end, coping against some known pathways that are activated as a consequence of increased oxidative stress and glucose flux is deemed effective to control DN. These pathways and inhibition of them are reviewed below.

6.5.1. Protein Kinase C Inhibitors. It is thought that activation of PKC through hyperglycemia-induced oxidative stress contributes to the generation of free radicals that result in diabetic microvascular complications [14]. Some of the PKC inhibitors such as ruboxistaurin have been shown to exhibit antioxidant effects. Ruboxistaurin has been particularly successful in reducing the progress of DN, and it is pending to get official approval [106].

6.5.2. Aldose Reductase Inhibitors (ARIs). As stated earlier, hyperglycemia-mediated activation of the polyol pathway can produce oxidative stress that may underlie DN. Aldose reductase is a key enzyme in pathogenesis of DN. ARIs drop the flux of glucose through the polyol or sorbitol pathways resulting in a reduction of intracellular accumulation of sorbitol and fructose [14]. The effects of fidarestat (a novel ARIs) on nerve conduction and the subjective symptoms of DN provided evidence that this treatment can control DN with concomitant reduction in oxidative stress markers [107]. Similarly long-term treatment with epalrestat, an ARI, can effectively delay the progress of DN and improve the symptoms, particularly in subjects with limited microangiopathy and good glycemic control [108]. Treatment with ranirestat (ARIs) also appears to have an effect on motor nerve function in mild to moderate diabetic sensorimotor polyneuropathy (DSP), but the results of this study did not show a statistically significant difference in sensory nerve function relative to placebo [109].

6.5.3. Advanced Glycation End Product Inhibitors (AGEIs). Accumulation of AGEs and activation of AGE receptors lead to oxidative stress and microvascular damage in DN. Benfotiamine, a derivative of thiamine (vitamin B1), reduces tissue AGEs formation and oxidative stress in subjects with DN with varying doses and duration of treatment

[14, 110]. Also aspirin, because of its antioxidant capacity, and aminoguanidine, a free radical scavenger, can inhibit this pathway and reduce oxidative stress in DN [9].

6.5.4. Hexosamine Pathway Inhibitors. As described above, diabetes-induced oxidative stress stimulates hexosamine pathway that implicates in the pathology of DN. Benfotiamine decreases flux through hexosamine pathway resulting in lower oxidative stress. This agent can reduce the pain associated with DN [9].

6.5.5. Inhibition of PARP. As discussed above, PARP activation involves in the pathogenesis of DN, and its inhibition lightens numerous experimental pathologic conditions connected with oxidative stress in DN. Nicotinamide has been shown to act as a PARP inhibitor and an antioxidant in animals that improves complications of early DN [9].

6.5.6. Angiotensin Converting Enzyme Inhibitors. Angiotensin II is a potent vasoconstrictor with proinflammatory properties, which especially in the absence of NO causes thrombosis. It also stimulates intercellular adhesion molecules and vascular adhesion molecules (VCAMs). The role of angiotensin converting enzyme inhibitors in DN is probably through inhibition of angiotensin II. In this regard, trandolapril has shown a small but significant improvement in some complication of DN [14].

7. New Therapeutic Approaches for DN

Despite relative lack of success of interventional agents to reverse or slow established DN, there is still hope to find some good agents. We have summarized a few of new approaches in Table 2 [79–97]. Hopefully, some reviews in the recent years have proven positive effects of natural antioxidants and herbal products such as *Satureja* species and *Urtica dioica* in diabetes and its complications [111–116].

8. Conclusion

In the present review, we tried to elaborate the pathogenesis of disease with a focus on oxidative stress and introduced therapies dependent or independent of oxidative stress. Diabetes can injure peripheral nerves in various distributions, and DSPN is the most common presentation in diabetes, which lead to substantial pain, morbidity, and impaired quality of life. Social and health-care costs linked with DN are high. DN develops on a background of hyperglycemia and associated metabolic imbalance. Numerous biochemical mechanisms of neurovascular and nerve damage have been identified in DN, but excessive production of ROS or oxidative stress is thought to be a common etiologic factor.

Treatment of DN always begins with optimizing glycemic control and then control of pain. Regarding role of oxidative stress and consequential factors in pathogenesis of DN, observing positive results with inhibitors of key pathways at the preclinical level is not surprising, but final decision will be made on the basis of clinical trials. If oxidative stress is assumed only as an ancillary player in DN, then antioxidants should be supplemented with conventional treatments. Development of new drugs to treat DN still remains a challenge that needs intensive long-term comparative trials.

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Review Article

Endoplasmic Reticulum Stress and Parkinson's Disease: The Role of HRD1 in Averting Apoptosis in Neurodegenerative Disease

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Endoplasmic reticulum (ER) stress has been known to be involved in the pathogenesis of various diseases, particularly neurodegenerative disorders such as Parkinson's disease (PD). We previously identified the human ubiquitin ligase HRD1 that is associated with protection against ER stress and its associated apoptosis. HRD1 promotes the ubiquitination and degradation of Parkin-associated endothelin receptor-like receptor (Pael-R), an ER stress inducer and causative factor of familial PD, thereby preventing Pael-R-induced neuronal cell death. Moreover, upregulation of HRD1 by the antiepileptic drug zonisamide suppresses 6-hydroxydopamine-induced neuronal cell death. We review recent progress in the studies on the mechanism of ER stress-induced neuronal death related to PD, particularly focusing on the involvement of HRD1 in the prevention of neuronal death as well as a potential therapeutic approach for PD based on the upregulation of HRD1.

1. Introduction

The endoplasmic reticulum (ER), an organelle found in the cells of eukaryotes, plays a key role in protein synthesis, glycosylation, and folding [1]. ER stress caused by glucose starvation, hypoxia, disruption of calcium homeostasis, or oxidative stress leads to the accumulation of unfolded or misfolded proteins. This induces cellular physiologic protective responses termed as the unfolded protein response (UPR). However, during prolonged ER stress, unfolded proteins may stimulate specific proapoptotic pathways through the activation of the transcription factor C/EBP homologous protein (CHOP) and cysteine proteases caspase-4/12 [2–6].

The UPR includes the repression of protein synthesis via phosphorylation of the α subunit of the eukaryotic initiation factor 2α (eIF2α), which is promoted by the activation of protein kinase RNA-like ER kinase (PERK) [7], and degradation of the unfolded proteins by ER-associated degradation (ERAD) (Figure 1). An additional key UPR pathway is the promotion of appropriate protein folding through induction of ER chaperones via activation of the activating transcription factor 6 (ATF6), PERK, and inositol-requiring enzyme-1 (IRE1) (Figure 1) [8]. ATF6 is cleaved by proteases under ER stress, yielding an active cytosolic ATF6 fragment p50 that migrates to the nucleus and activates ER chaperones [9]. PERK dimerization and transautophosphorylation lead to the phosphorylation of eIF2α, reducing the overall frequency of mRNA translation initiation. However, ATF4 mRNA is preferentially translated in the presence of phosphorylated eIF2α. ATF4 activates the transcription of ER chaperones [10, 11]. Furthermore, autophosphorylation and oligomerization of IRE1 activate IRE1 endoribonuclease, resulting in X-box

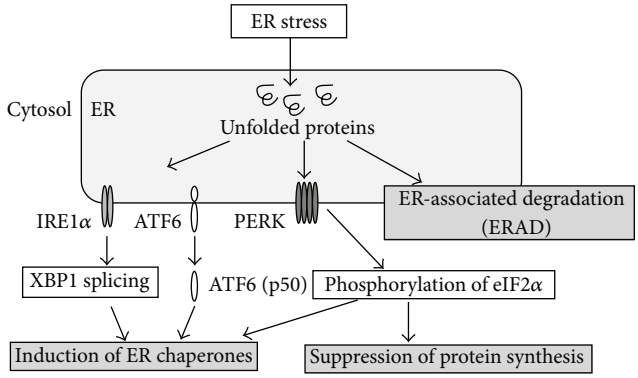

Figure 1: Cellular responses to ER stress. ER stress due to hypoxia and other factors results in the accumulation of unfolded proteins that trigger the UPR. The UPR is composed of three pathways: induction of ER chaperones by the activation of IRE1α-XBP1, ATF6, and PERK-eIF2α, inhibition of protein synthesis by the phosphorylation of PERK-eIF2α, and ERAD.

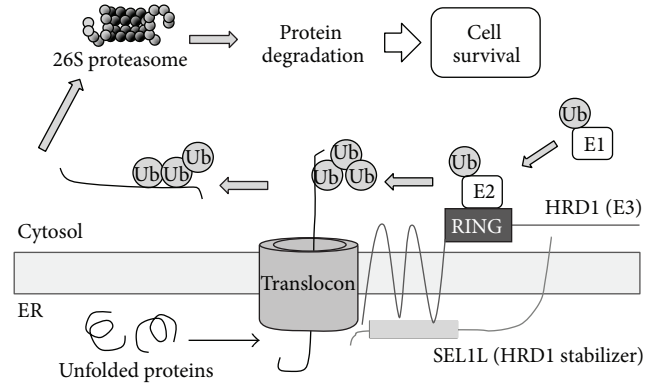

Figure 2: Mechanism of the ERAD system. Unfolded proteins are retrotranslocated from the ER to the cytosol through the translocon. Substrates are then polyubiquitinated by E1, E2, E3, and other components and are subsequently degraded by the 26S proteasome, resulting in cell survival. HRD1 and SEL1L are components of the ERAD system that colocalize in the ER and interact with one another. HRD1 has E3 activity, and SEL1L regulates E3 activity and HRD1 stability.

binding protein 1 (XBP1) mRNA cleavage and splicing. The transcription factor XBP1 regulates genes responsible for ERAD, as well as genes associated with protein folding [12].

During ERAD, unfolded proteins are retrotranslocated to the cytosol from the ER via the translocon; polyubiquitinated by the ubiquitin-activating enzyme (E1), ubiquitin conjugating enzyme (E2), ubiquitin ligase E3, and other components; and finally degraded by the 26S proteasome (Figure 2). The RING finger domain of E3 plays a particularly important role in the ubiquitination of unfolded proteins, mediating the transfer of ubiquitin from E2 to substrates [13, 14].

ER stress has been proposed as a possible molecular mechanism underlying the onset of diabetes mellitus [15–17], rheumatoid arthritis [18, 19], and neurodegenerative diseases such as Parkinson's disease (PD) [20–23] and Alzheimer's disease (AD) [24]. This discovery of a link between ER stress and disease onset indicates that unfolded proteins play a role in the etiology of many of the most prevalent diseases. It has been suggested that therapeutic drug targeting and other interventions aimed at disrupting the ER stress cycle in such diseases would provide a useful treatment strategy [25–27].

Here we review recent evidence for the involvement of ER stress in PD. Furthermore, we describe the suppressive roles of the ubiquitin ligase HRD1 in ER stress-induced neuronal death and propose a new approach for the treatment of PD focusing on HRD1.

2. PD and ER Stress

PD is the most common movement disorder, particularly in the elderly, and is the second most common neurodegenerative disease. It is characterized by motor symptoms including bradykinesia, rigidity, resting tremor, and postural instability. The pathological hallmark of PD is the loss of dopaminergic neurons in the substantia nigra pars compacta (SNC), resulting in a reduction of the dopamine content in the striatum [28, 29].

Cases of PD are mostly sporadic, and it is estimated that only approximately 5–10% of patients exhibit monogenic forms of the disease [30]. The genes involved in autosomal recessive PD are *Parkin* (*PARK2*), *PTEN-induced putative kinase 1* (*PINK1*; *PARK6*), *DJ-1* (*PARK7*), and *ATP13A2* (*PARK9*), whereas the genes involved in autosomal dominant PD are *α-synuclein* (*PARK1/4*), *leucine-rich repeat kinase 2* (*LRRK2*; *PARK8*), and *ubiquitin carboxy-terminal hydrolase L1* (*UCHL-1*; *PARK5*) [31]. In addition, a genome-wide association study in individuals of Japanese and European ancestries found that *LRRK2* and *α-synuclein* are common risk factors for sporadic PD [32, 33]. LRRK2 and α-synuclein are substrates for ERAD-related E3, C-terminus of Hsp70-interacting protein (CHIP), and Parkin, demonstrating that disturbances in the ERAD system are relevant to the onset of PD [34–37]. Based on these reports, it is presumed that ER stress is a causative factor of PD.

Parkin is an E3 containing two RING finger motifs that bind one or more ubiquitin molecules, thereby targeting the substrate for proteasomal degradation [37]. Through its E3 activity, Parkin degrades its own substrate, the misfolded Parkin-associated endothelin receptor-like receptor (Pael-R) and, thus, suppresses cell death caused by the accumulation of Pael-R [20]. However, Parkin mutation results in the loss of E3 activity, which can cause the accumulation of unfolded Pael-R and finally ER stress-induced cell death [34]. Therefore, ER stress that occurs as a result of the accumulation of unfolded Pael-R is suggested to be one of the pathophysiological mechanisms underlying autosomal recessive PD [38, 39].

Pael-R also accumulates in the core of Lewy bodies in sporadic PD [40]. However, Parkin-deficient mice exhibit no significant changes in either dopaminergic neurodegeneration or in the accumulation of any Parkin substrates [41–43]; this contrasts with the results of a study showing that

Parkin knockout/Pael-R transgenic mice exhibit progressive loss of dopaminergic neurons [44]. These reports suggest that other E3s are capable of degrading accumulated Pael-R in the absence of Parkin, as a compensatory mechanism for the maintenance of cellular homeostasis.

Two recent studies highlight the roles of PINK1 and Parkin in PD. PINK1 and Parkin work together to regulate mitochondrial fission [45, 46]. In particular, autophosphorylation of PINK1 reportedly recruits Parkin to damaged mitochondria and Parkin then initiates the mitochondrial degradation; however, these events are averted in PD by mutations of PINK1 [47, 48]. These key findings demonstrate the importance of E3 Parkin in the onset of PD.

3. HRD1 and PD

We previously identified HRD1, a human homolog of yeast Hrd1p/Der3p [49]. Hrd1p/Der3p is a RING finger domain-containing E3 localized to the ER and is involved in ERAD and ubiquitination of HMG-CoA reductase (Hmg2p) [50, 51]. We also identified the HRD1-stabilizer SEL1L, a human homolog of Hrd3p [52, 53]. We demonstrated that HRD1 has E3 activity, mRNA and protein levels of HRD1 are upregulated in response to ER stress, and HRD1 inhibits ER stress-induced cell death [53]. Furthermore, it has been reported that HRD1-SEL1L complex components OS-9 and GRP94 are responsible for delivering substrate [54], indicating that HRD1 ubiquitinates substrates cooperatively with SEL1L, OS-9, and various other ERAD-related components; it is believed that Derlin-1, XTP3-B, or other molecules may also play similar delivery roles in the complex [54, 55], but detailed functional analysis of such molecules remains unclear. Our studies demonstrated that HRD1 expression is reduced by the knockdown of SEL1L [49].

Based on the above-mentioned findings, it is presumed that overexpressed HRD1 degrades many unfolded proteins, resulting in the inhibition of cell death caused by ER stress. Thus, we searched for endogenous HRD1 substrates related to ER stress and focused on Pael-R, because of its importance in causing ER stress [56].

Because human HRD1 has been reported to be expressed in the brain by RT-PCR—ELISA studies [57], we examined the localization of HRD1 in the murine brain and demonstrated that it is expressed in the SNC, particularly in dopaminergic neurons [56]. It has been reported that Pael-R is also expressed in SNC dopaminergic neurons [20]. Thus, we hypothesized and demonstrated that HRD1 and Pael-R exist in correlation with one another; HRD1 and Pael-R colocalize in the ER in dopaminergic SH-SY5Y cells, and HRD1 interacts with unfolded Pael-R. Furthermore, we demonstrated that Pael-R is ubiquitinated and degraded by HRD1 and that Pael-R-induced cell death is suppressed by the overexpression of HRD1 (Figure 3).

Moreover, we demonstrated that HRD1 is expressed in neurons, but not in glial cells, of the murine brain, and that this ligase is also expressed in the pyramidal cell layer of the hippocampus, globus pallidus, striatum, and Purkinje cells of the cerebellar cortex, in addition to the SNC dopaminergic neurons. It has been reported that these regions are injured in various neurodegenerative disorders, particularly in motor dysfunctions such as PD, Huntington's disease, spinocerebellar ataxia, and prion diseases [28, 29, 58–60]. Therefore, it is plausible that HRD1 may be associated with the onset of other motor dysfunctions.

A detailed functional analysis revealed that in addition to the RING finger domain, HRD1 contains a proline-rich domain involved in interaction with Pael-R, as well as a transmembrane domain [56, 61]. The transmembrane domain of HRD1 transports Pael-R from the ER to the cytosol and is also needed to stabilize HRD1 itself [61]. We previously reported that SEL1L stabilizes HRD1 [49], and, more recently, Fonseca et al. [62] reported Wolfram syndrome 1 protein as another HRD1 stabilizer. Therefore, as HRD1 was not able to interact with SEL1L or other components without the transmembrane domain, we assume that HRD1 had lost its stability.

4. Treatment Strategies for PD Involving ER Stress

Based on the above-mentioned findings, we propose the following therapeutic strategies for PD involving ER stress: (i) the promotion of appropriate protein folding to avoid ER stress or (ii) the upregulation of HRD1 or its related components to promote the degradation of unfolded proteins (Figure 4). With respect to (i), it has been reported that chemical chaperones or molecular chaperone inducers promote the appropriate folding of proteins [63–65]. We similarly reported that the chemical chaperone 4-phenyl butyrate (4-PBA) or its derivatives promote the correct folding of unfolded Pael-R and suppress the cell death caused by the accumulation of Pael-R [66, 67]. In addition, 4-PBA improves motor deterioration in human α-synuclein A30P/A53T double-transgenic mice [68] and prevents memory deficits and decreases amyloid β in AD transgenic mice [69]. Furthermore, the molecular chaperone inducer Bip inducer X (BIX) prevents ER stress-induced neuronal death [63]. Based on these reports, the acceleration of appropriate protein folding using chemical chaperones or BIX is considered to be useful for the treatment of PD and other neurodegenerative disorders caused by ER stress.

Regarding treatment strategy (ii), we have been trying to identify chemicals that promote the expression of HRD1 proteins; through this research, we identified the antiepileptic drug zonisamide as an upregulator of HRD1 [70]. Zonisamide has recently been shown to improve the cardinal symptoms of PD and is approved in Japan for use as a low-dose adjunctive therapy for PD patients [71, 72]. However, the molecular mechanisms through which zonisamide suppresses the progression of PD remain unclear. We have demonstrated that a low concentration of zonisamide suppresses neuronal cell death caused by 6-hydroxydopamine-induced ER stress [70]. Zonisamide upregulates the HRD1 protein, without upregulating HRD1 mRNA, through a mechanism involving SEL1L. It upregulates expression of SEL1L mRNA and protein, resulting in the stabilization of HRD1 protein and followed by an increase in the HRD1 protein level. In contrast,

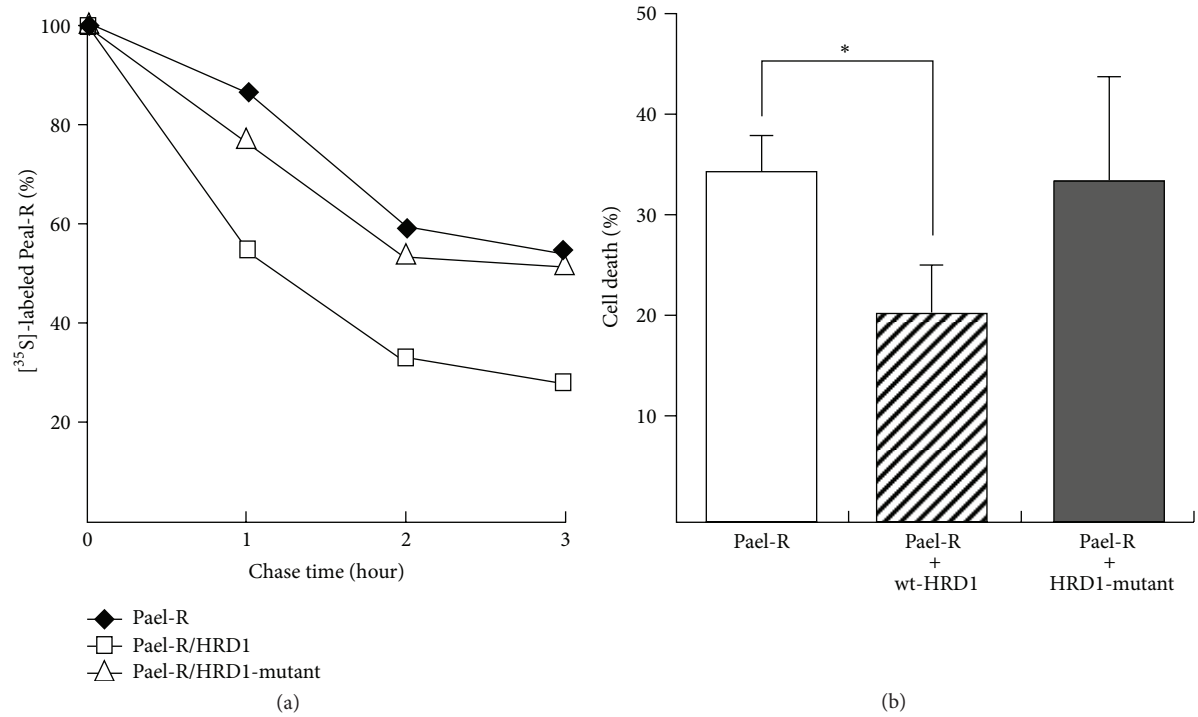

Figure 3: HRD1 degrades unfolded Pael-R and suppresses Pael-R-induced cell death. (a) Neuro2a cells were transiently transfected with Pael-R and HRD1, or HRD1-mutant. At 36 h after transfection, cells were pulse-labeled with [^{35}S]-methionine/cysteine and chased for the indicated times. The levels of [^{35}S]-labeled Pael-R are plotted relative to the amount present at time 0. (b) HEK293 cells (control) and HEK293 cells expressing HRD1 or HRD1-mutant were transiently transfected with Pael-R. The surviving cells were stained with crystal violet. The percentage of cell death was calculated as follows: 100 – ((optical density for assay/optical density for control well) × 100). The results obtained from each cell transfected with Pael-R were compared with those obtained from cells transfected with control vector. The results are expressed as the means ± S.D. of three independent experiments performed in duplicate. Statistical analysis was performed using Student's *t*-test ($^{*}P <$ 0.01 versus normal) [56].

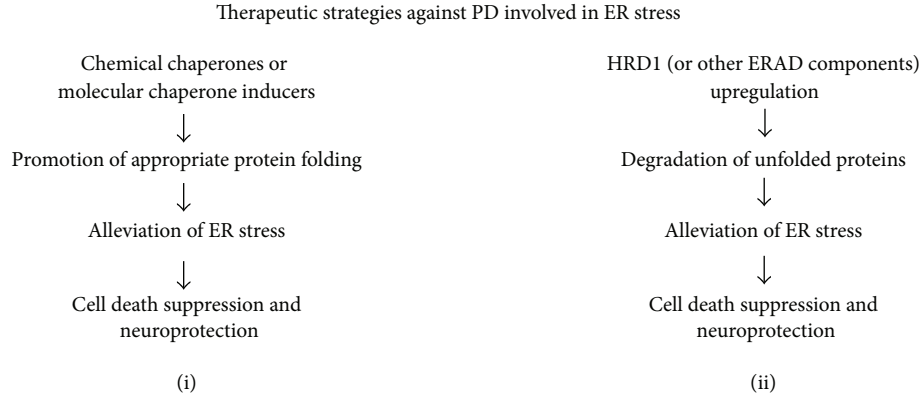

Figure 4: Therapeutic strategies for PD involving ER stress. (i) Addition of chemical chaperones (e.g., 4-PBA and tauroursodeoxycholic acid) or molecular inducers of ER chaperones (e.g., BIX); these molecules promote the appropriate folding of proteins and suppress the accumulation of unfolded proteins and ER stress-induced cell death, resulting in the prevention of neurodegeneration in PD, and (ii) the upregulation of ubiquitin ligase HRD1, its stabilizer SEL1L, or other ERAD components; HRD1 and its components promote the degradation of unfolded proteins and suppress ER stress-induced cell death, resulting in the prevention of neurodegeneration in PD.

knockdown of SEL1L downregulates HRD1 and suppresses the protective effect of zonisamide against ER stress. These findings indicate that zonisamide may activate SEL1L to act as an HRD1 stabilizer, and the resulting upregulated HRD1 proteins repress 6-hydroxydopamine-induced cell death [70].

5. Conclusions

We reviewed the involvement of ER stress in the etiology of PD, the critical role of HRD1 as a ubiquitin ligase in ERAD, and a therapeutic strategy against PD based on HRD1.

PD has recently been reported to be a multifactorial neurodegenerative disease; therefore, it is important to approach its treatment from different angles, including environmental factors, oxidative stress, and mitochondrial dysfunction, in addition to ER stress [73–75]. We also described important findings demonstrating the involvement of HRD1 in the degradation of the amyloid precursor protein and the subsequent reduction of amyloid β, a possible factor in the pathogenesis of AD [24].

Based on these findings, we propose that HRD1 has a variety of substrates underlying protein conformational diseases, including PD and AD, and speculate that the molecules that activate HRD1 may have therapeutic potential for the treatment of neurodegenerative disorders. If ER stress is indeed one of the causes of neurodegenerative diseases, it is possible that this approach represents a common neuroprotective strategy that can be exploited for the treatment of neurodegenerative disorders in general.

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Review Article

Metal Dyshomeostasis and Inflammation in Alzheimer's and Parkinson's Diseases: Possible Impact of Environmental Exposures

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A dysregulated metal homeostasis is associated with both Alzheimer's (AD) and Parkinson's (PD) diseases; AD patients have decreased cortex and elevated serum copper levels along with extracellular amyloid-beta plaques containing copper, iron, and zinc. For AD, a putative hepcidin-mediated lowering of cortex copper mechanism is suggested. An age-related mild chronic inflammation and/or elevated intracellular iron can trigger hepcidin production followed by its binding to ferroportin which is the only neuronal iron exporter, thereby subjecting it to lysosomal degradation. Subsequently raised neuronal iron levels can induce translation of the ferroportin assisting and copper binding amyloid precursor protein (APP); constitutive APP transmembrane passage lowers the copper pool which is important for many enzymes. Using *in silico* gene expression analyses, we here show significantly decreased expression of copper-dependent enzymes in AD brain and metallothioneins were upregulated in both diseases. Although few AD exposure risk factors are known, AD-related tauopathies can result from cyanobacterial microcystin and β-methylamino-L-alanine (BMAA) intake. Several environmental exposures may represent risk factors for PD; for this disease neurodegeneration is likely to involve mitochondrial dysfunction, microglial activation, and neuroinflammation. Administration of metal chelators and anti-inflammatory agents could affect disease outcomes.

1. Introduction

Degenerative brain disorders among the elderly constitute a growing burden for the affected individuals, their families, and for the society. The mechanisms behind Alzheimer's and Parkinson's diseases (AD and PD) are still unclear. Although symptom-relieving drugs exist, drugs halting their progression are still lacking [1–5]. Our brains are continuously exposed to a broad spectrum of chemicals from various sources; these may react or interfere with biomolecules, or they may bioaccumulate (e.g., mercury, nanoparticles, and pesticides and persistent organic pollutants (POPs)), causing changes in brain function. In addition, an endogenous production of metabolic byproducts including free radicals inflict damages which constantly need to be repaired. Whereas repeated single chemical exposures may be associated with PD [6–8], convincing evidence is lacking that single chemical exposures (e.g., copper, mercury, or POPs) are causal for AD; several previously suspected agents (e.g., aluminium from antiperspirants and pans) have been written off [9]. However, there is strong support for the involvement of metal dyshomeostasis, inflammation, and oxidative damages to biomolecules in both diseases [10–13]. AD is a slowly progressing disease for which age is the most important risk factor [1, 2], and factors regulating metal homeostasis and inflammation known to change with age may be causally related to both AD and PD. Microglia activation is seen in multiple sclerosis [14], stroke [15], traumatic brain injury [16], Creutzfeldt-Jakob disease [17], in addition to PD and AD.

In this paper, AD metal dyshomeostasis and potential AD-causative cyanobacterial neurotoxins present in water and foods are discussed. We propose a neuronal metal dyshomeostasis mechanism mediated by the systemic iron regulatory peptide hepcidin and discuss therapeutic use of metal chelators and possible preventive measures. For PD, putative roles of chemical and nanoparticle exposure, metal dyshomeostasis and inflammation are discussed.

2. Alzheimer's Disease (AD)

AD is the most common form of dementia, characterized histopathologically by extracellular amyloid-beta (Aβ) plaques and intraneural fibrillar tangles (microtubule-associated tau protein aggregates) along with synaptic and neuronal losses. These pathological processes are initiated in the entorhinal cortex and hippocampus for unknown reasons and spread as the disease progresses, resulting in cortex and hippocampal shrinkage, dementia, personality changes, and death. Late-onset (>60 years) sporadic AD accounts for most cases and has been suggested to result from complex interactions among multiple genetic, epigenetic, and environmental factors [2] where food composition, physical, and mental activities appear important [25], but for which chemical exposure risk factors other than possibly smoking [25] have been difficult to identify. Individuals carrying the mutant apolipoprotein E4 (apoE4) allele are at increased risk for late-onset AD which is believed to be related to apoE's role in Aβ clearance [2, 26]. Early-onset (<60 years) autosomal dominant genetic inheritage accounts for less than 1% of all AD cases [2] and affects individuals carrying mutations in three genes: the amyloid precursor protein (APP), and presenilin 1 and 2 (γ-secretase components). Unlike this genotype, a rare APP mutation positioned close to the site where β-secretase cleaves APP into amyloid-β leads to reduced formation of Aβ peptide monomers and protection against AD [27]. Aβ peptides (ca. 90% Aβ_{40} and 10% Aβ_{42}, of which Aβ_{42} is considered to be more toxic) are formed through sequential intramembranous and extraneuronal proteolytic processing of the transmembranal APP by β- and γ-secretases (Figure 1). Aβ has been suggested to act as an antioxidant when present as a monomer but seems to lose this function when aggregated into oligomers or plaques, then becoming a reactive oxygen species (ROS) generator [28]. The high prevalence of sporadic AD, with an incidence of approximately one person out of twenty over the age of 65 suffering from Alzheimer's disease [29], has yet not been explained by single exposure factors. To some surprise, a moderate alcohol consumption appears to be protective against dementia [30], possibly due to anti-inflammatory effects [31].

In the light of new knowledge, we hypothesize that the iron and inflammation responsive hormone hepcidin could cause metal dyshomeostasis and oxidative stress in AD. We therefore performed *in silico* gene analyses of proteins regulating metal homeostasis and compared AD cases with unaffected elderly. Regarding environmental exposure risks, recent studies suggest that highly potent neurotoxins from food and drink contaminated with cyanobacteria can induce AD-resembling pathologies. Food constituents (e.g., antioxidants) can also protect against AD and much effort is spent on developing treatments, including drugs. These topics are discussed below.

2.1. Metal Dyshomeostasis in AD. For both AD and PD, numerous studies support a dysregulated metal (iron, copper, and zinc) brain homeostasis and metal catalysed oxidative damages [10–13]. A recent meta-analysis study on reported AD (versus aged controls) brain metal levels found no support for elevated neocortex iron, copper, or zinc levels, but significantly decreased neocortex copper levels when considering quantitative (metal content per wet weight tissue) analyses [32]. The same study also found a significant publication bias, with papers reporting increased iron levels were much more frequently cited than those reporting no change or decreased levels [32]. Still there is convincing support of the notion that certain Aβ-plaques forms contain iron, copper, and zinc [22–24], and individual intracellular or brain regional metal levels may also differ. Whereas Aβ-plaque associated iron and copper ions can redox cycle and produce ROS [11], zinc does not, but has been reported to be a particularly good Aβ-plaque aggregator [24, 33] and a tau hyperphosphorylation inducer [34]. Zinc may also inhibit APP's iron-export ferroxidase activity [18]. Approximately 20–40% of cognitively normal elderly people also show evidence of significant brain Aβ-plaque depositions [35] suggesting that not all plaque forms are toxic. Another recent meta-analysis found significantly increased serum copper levels among AD patients versus controls [36]. Positive outcomes dominate when metal chelators were administrated therapeutically in AD animal models and in clinical human AD studies, see below.

2.2. Are Hepcidin and APP Causal for Decreased Copper Levels in AD Cortex Neurons? In the brain, the complex processes regulating metal delivery to the various cell types, metal storage and export mechanisms, are yet not fully understood. In aging, a mild systemic inflammation [37] and elevated iron levels in some organs (brain [38–40], skeletal muscle [41, 42], and liver [41]) is commonly seen, while iron levels decrease in bone marrow (related to anaemia) [43]. Human brain iron levels increase with age to reach a relative steady state at around 55 years in neurologically normal subjects [32]. Hepcidin, a master regulator of body iron metabolism, is an evolutionary conserved antimicrobial-like peptide hormone expressed in several organs including brain [20, 44] as part of the innate (nonspecific) immune system in response to pathogens [45] including lipopolysaccharide (LPS) [44]. Hepcidin restricts iron availability for microbes by its binding to the only known cellular iron exporter ferroportin in host cell membranes which causes internalization and lysosomal ferroportin degradation (Figure 1) [19]. Liver hepcidin expression can be induced via the bone morphogenetic protein (BMP6) pathway by intracellular iron or through the JAK-STAT3 pathway by the cytokine interleukin 6 (IL-6; possibly also other cytokines) [46, 47]. Brain hepcidin mRNA increases with aging in mice [20]. The expression of the iron exporter ferroportin is induced by iron (and

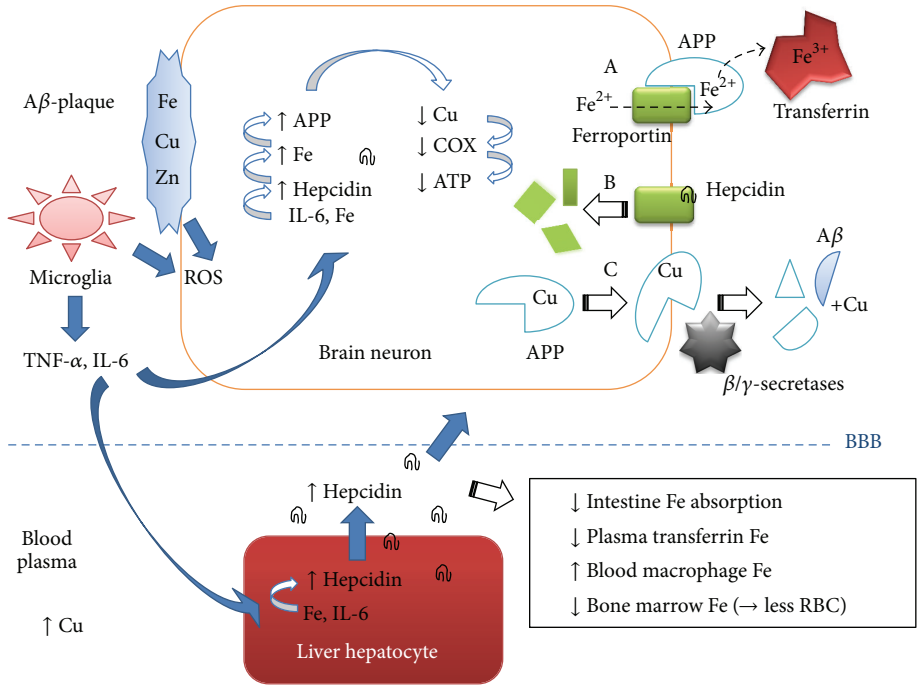

Figure 1: Putative linkage between cytokine (IL-6) and iron (Fe) induced hepcidin production with APP-mediated copper (Cu) lowering in the AD brain. Both liver and brain cells can produce the iron regulatory peptide hepcidin which may cross the blood-brain barrier (BBB). (A) In neurons, plasma membrane localized ferroportin exports ferrous iron (Fe^{2+}) which is oxidized extracellularly by means of the ferroportin-collaborating amyloid precursor protein (APP) which has ferroxidase activity [18] and which loads ferric iron (Fe^{3+}) into transferrin. (B) Hepcidin binding to ferroportin causes its internalization and lysosomal breakdown, preventing iron export [19, 20]. In response, iron levels in individual neurons may increase during aging, initiating APP-mRNA iron responsive constitutive translation of APP [21] which contains a copper binding domain. (C) APP travels to the plasma membrane and is cleaved by secretases to form short peptides, of which the Aβ peptide can form plaques containing metal ions [22–24]. Lowered neuronal copper levels, for which the cellular pool is low, can affect vital copper enzymes negatively (e.g., mitochondrial respiratory ATP producing COX, Cu/Zn-SOD, etc.). Aβ-plaque attacking microglia release various cytokines including IL-6 and ROS, that along with ROS generated from Aβ-plaque associated redox-cycling metals (e.g., Fe and Cu ions) inflict free radical damage to neurons. Liver hepcidin production affects iron metabolism in several organs (see text).

heme) through an mRNA iron regulatory element (IRE) [48]. However, in experiments studying neuronal cell (substantia nigra) produced hepcidin [49] or injected hepcidin [50], hepcidin was found to downregulate ferroportin expression, suggesting that hepcidin (over iron) dominantly controls ferroportin. Systemic increased plasma hepcidin lowers transferrin-bound plasma iron by inhibiting iron release from several organs (also liver). Reticuloendothelial cells (macrophages) accumulate iron through constant red blood cell phagocytosis; with increased hepcidin less iron passes on towards the bone marrow for red blood cell synthesis by hematopoietic cells [46], which may cause age-related anaemia [51]. Also iron absorption from food in the gut is blocked since hepcidin prevents enterocyte-mediated iron export into the blood system. It is presently unclear if liver produced hepcidin reaches the brain, but several peptides are known to cross the blood-brain barrier (BBB). It is suggested that hepcidin in the brain mainly originates from other organs (unpublished data) [52]. The same study found that hepcidin was absent in microglia but colocalized with ferroportin in neurons and astrocytes [52]. Hepcidin's inhibition of ferroportin [19] may increase intracellular neuronal iron levels which can induce APP-mRNA translation due to an IRE stem loop in the 5′-untranslated region [21].

APP is a multifunctional metalloprotein containing a copper binding domain (not localized in its Aβ part) [53]. APP was found to possess ferroxidase (oxidizes Fe^{2+} into Fe^{3+}) activity assisting in plasma membrane Fe^{2+}-export by ferroportin, counteracting iron accumulation and oxidative stress [18]. APP's ferroxidase activity has been shown to take place on the extracellular plasma membrane side, where APP (in interaction with ferroportin) loads Fe^{3+} into blood transferrin [18] (Figure 1). Ceruloplasmin also has ferroxidase activity, but this protein is normally not expressed in cortical neurons [54]. APP may therefore be the sole iron-exporting ferroxidase in neurons [18].

In neuronal supporting astrocytes, cellular copper export is mediated by the copper transporting P-type ATPase ATP7A, which translocates from the trans-Golgi network to the plasma membrane in the presence of elevated copper [55]. Free intracellular copper is bound to metallothioneins or is stored in vesicular copper pools, but copper levels are generally low, much lower than the iron pool which includes cytosolic and mitochondrially stored iron in the

form of ferritin. We hypothesize that constitutive expression of APP may be responding to elevated iron levels in individual neurons, with the purpose of assisting iron export by ferroportin through the plasma membrane. However, since ferroportin may have been internalized by hepcidin, and since APP has a copper binding domain [53], constitutive APP transmembranal passage and extracellular proteolysis may reduce the already relatively low intracellular copper pool, resulting in suboptimal copper levels in individual neurons. In support of this (see also Section 2.3), overexpression of the APP in transgenic mice resulted in significantly lower copper levels, but the iron levels remained unaltered [56]. Enzymes requiring copper include cytoplasmic Cu/Zn-superoxide dismutase (intracellular SOD1, extracellular SOD3), tyrosinase, cytochrome c oxidase (COX), ceruloplasmin, dopamine beta-hydroxylase, hephaestin, lysyl oxidase, peptidylglycine alpha-amidating monooxygenase (PAM), and amine oxidases, of which several are important for neurological functioning [57]. COX is vital for mitochondrial respiratory ATP production, and fewer well-functional mitochondria may induce apoptosis. Significantly decreased COX activities (but not respiratory complexes I + III, II + III activities) in AD temporal cortex and hippocampus were observed [58]. Decreased SOD1 activity and increased cerebrospinal fluid copper levels were noted in several neurodegenerative diseases [59].

In addition to the described cellular challenges, microglial cells recognize and attack Aβ-plaques, starting a vicious circle of ROS (e.g., superoxide, $O_2^{\bullet -}$; hydrogen peroxide, H_2O_2; and hypochlorous acid, HOCl) and cytokine (e.g., IL-6, IL-1β, and tumour necrosis factor-alfa, TNF-α) release [60] (Figure 1). ROS is also produced from extracellular iron and copper ions attached to Aβ-oligomers and plaques [23, 24], and peroxides react with loosely attached redox-cycling intracellular iron ions attached to biomolecules (e.g., nucleic acids, lipids, and proteins) inflicting oxidative damage. Elevated intracellular ROS levels can also be sensed by transcription factors such as heme oxygenase-1 (HO-1) [61] and also nuclear factor-kappa B (NF-κB) [62], which can lead to further production of inflammatory cytokines also in neurons. Notably, microglia also have a protective role by mediating clearance of Aβ through phagocytosis [63].

Negatively charged nucleic acids attract loosely attached iron ions inflicting ROS mediated damages through Fenton chemistry [64, 65]. In particular, oxidative RNA damages, commonly observed in AD [10, 66, 67], may cause erroneous protein translations or truncations leading to dysfunctional proteins [66]. Observation of significantly less protein in AD frontal cortex has been reported [68]. Reactive byproducts from lipid peroxidation include the intermediates 4-hydroxy-2,3-nonenal (HNE), 4-oxo-2-nonenal (ONE), and acrolein, that react with macromolecules forming alkylative adducts [12, 69]. Metal-catalysed oxidation of amino acids generates protein carbonyls [70, 71] which may hinder proper protein function. Not all forms of alkylative and oxidative lesions are well repaired.

2.3. In Silico Analysis of Genes Involved in Brain Copper/Iron Homeostasis Pathway. We wanted to investigate how the expression patterns of genes involved in brain copper/iron homeostasis pathway are modulated in neurologically normal (healthy) elderly and AD-affected individuals. To do so, we performed a search in the publicly available gene expression database NCBI Gene Expression Omnibus (GEO) [72]. We used the microarray data from Liang et al. (2008) [73] and Liang et al. (2007) [74] (GEO accession number GSE5281), which are comprehensive genome-wide gene expression studies of samples collected from six brain regions that are either histopathologically or metabolically relevant to AD: hippocampus (HIP), entorhinal cortex (EC), middle temporal gyrus (MTG), posterior cingulate cortex (PC), superior frontal gyrus (SFG), and primary visual cortex (VCX) [73, 74]. For more detailed description of sample collection, experimental design and flow, we refer the reader to the original studies [73, 74]. We downloaded the raw microarray data (GSE5281) deposited in the GEO database [72]. The raw microarray data were reanalyzed using J-Express v2009 as described previously [75, 76]. From the dataset we selected 61 genes involved in brain copper/iron homeostasis pathway. The processed intensities of the selected 61 genes were log2-transformed. Figure 2 shows unsupervised hierarchical clustering analysis of these 61 genes, and the results were visualized in a dendrogram using the MeV v4.7 software [77]. By visual inspection of the heatmap (Figure 2), we observed that samples from AD-affected individuals clustered close to each other in one branch while samples from normal elderly individuals clustered in the other branch. Two-class, unpaired SAM (Significance Analysis of Microarray) [78] analysis was conducted in order to identify genes whose mean expression level is significantly different between AD-affected and normal elderly individuals control samples. Twenty-one genes were differentially expressed (false discovery rate, FDR < 10%). Eleven of these genes were overexpressed in AD-affected individuals and underexpressed in normal elderly individuals, whereas 10 genes were underexpressed in AD-affected individuals and overexpressed in normal elderly individuals (Table 1). Genes overexpressed in AD-affected individuals include *FTHL17, SLC40A1, AOC3, MT1E, HEPH, MT1X, MT1H, MT1F, MT1G, MT2A,* and *MT1M* (Table 1), and most of these genes clustered close to each other in one branch (Figure 2), while *COX6A1, COX6C, HMOX2, COX11, SOD1, PAM, ATOX1, COX5B, COX6B1,* and *COX7B* were underexpressed (Table 1), and clustered together in another branch (Figure 2). Notably, brain tissue is composed of several types of cells (astrocytes, neurons, microglia, and endothelial cells, etc.) where neuron supporting astrocytes numerically dominate, but the results show that crucial copper-dependent enzymes (COX, SOD1, and PAM) and ATOX1 (a copper-chaperone protein) are underexpressed in AD brains. Amine oxidase (copper containing 3), however, was overexpressed in AD brains for unclear reasons. Moreover, in AD brains, several metallothioneins (small copper and zinc binding proteins) are overexpressed, as well as iron binding ferritin and ferroportin (SLC40A1). Hephaestin (contains copper but functions as an iron ferroxidase in collaboration with ferroportin) was overexpressed in AD brains. Tight transcriptional control of the Cu/Fe homeostasis pathway involved genes in the brain is very important, and

Table 1: Genes ($n = 21$) involved in brain metal homeostasis pathways for which expression is significantly changed in AD.

Gene symbol	Description	SAM score (d)*	Fold up- or downregulation
MT1M	Metallothionein 1M	6.7	2.50
MT2A	Metallothionein 2A	5.3	2.00
MT1G	Metallothionein 1G	4.8	2.30
MT1F	Metallothionein 1F	4.6	2.30
MT1H	Metallothionein 1H	4.4	2.00
MT1X	Metallothionein 1X	4.2	1.80
HEPH	Hephaestin	4.1	2.00
MT1E	Metallothionein 1E	4.0	1.80
AOC3	Amine oxidase, copper containing 3 (vascular adhesion protein 1)	3.8	2.90
SLC40A1	Solute carrier family 40 (iron-regulated transporter), member 1	3.0	1.50
FTHL17	Ferritin, heavy polypeptide-like 17	2.2	1.60
COX6A1	Cytochrome c oxidase subunit VIa polypeptide 1	−3.3	−1.43
COX6C	Cytochrome c oxidase subunit VIc	−3.7	−1.67
HMOX2	Heme oxygenase (decycling) 2	−3.7	−1.67
COX11	COX11 cytochrome c oxidase assembly homolog (yeast)	−4.3	−1.43
SOD1	Superoxide dismutase 1, soluble	−4.5	−2.00
PAM	Peptidylglycine alpha-amidating monooxygenase	−4.6	−1.67
ATOX1	ATX1 antioxidant protein 1 homolog (yeast)	−4.6	−2.00
COX5B	Cytochrome c oxidase subunit Vb	−5.2	−1.67
COX6B1	Cytochrome c oxidase subunit VIb polypeptide 1 (ubiquitous)	−5.3	−2.00
COX7B	Cytochrome c oxidase subunit VIIb	−5.8	−2.00

*Significantly differentially expressed genes between AD-affected cases and controls, FDR < 10% for GSE5281 microarray date, and unlogged fold up- and downregulated genes ($n = 21$). Eleven genes were overexpressed and 10 genes were underexpressed in the samples from AD-affected cases in comparison to age-matched unaffected controls.

dysregulation of these genes might affect the Cu/Fe balance, resulting in potential damage to brain function. The gene expression profiles constituted by these altered genes can be used to identify candidate genes to be associated with AD, which might contribute to early detection of this complex disorder. Identifying AD-predictive genes may uncover gene products with mechanistic properties relevant to AD; this should be pursued in future studies.

2.4. Cyanobacterial Toxins-Potential Tauopathy and AD Risk Factors. Cyanobacteria and their potential impact on human health are an emerging public health issue that has received increasing scientific interest resulting in new research [79–81]. While it has been confirmed that cyanobacteria produce toxins (e.g., microcystins, MCs [80], and chemicals resembling amino acids [81]) that are potentially capable of causing neurological disorders in humans, many questions remain unanswered regarding the identification and quantification of cyanobacteria and cyanotoxins in the environment and to what degree they translate into health risks.

Several freshwater cyanobacteria produce MCs that irreversibly inhibit serine/threonine-specific protein phosphatases [83] and have caused morbidities in animals and humans [84, 85]. In a severe human MC intoxication 1996 in Caruaru, Brazil, patients developed signs of acute neurotoxicity, for example, deafness, tinnitus, intermittent blindness, as well as subsequent hepatotoxicity [86]. In conjunction with some animal studies this suggests that MCs are potent neurotoxins acting by inducing caspase activity, chromatin condensation, and microtubule tau hyperphosphorylation [79, 83]. In one study, the effect of MCs on neurite degeneration has been analyzed with confocal microscopy; neurite length was determined using image analysis [83]. MC induced significant neurodegeneration already at 0.5 μM MC-LF (Figure 3) and neuronal apoptosis was significantly increased by the MC variants MC-LF and MC-LW at higher concentrations (≥ 3 μM). Moreover, sustained hyperphosphorylation of the tau protein with all MC congeners was found [83]. The concentration and congener-dependent mechanisms observed suggest that low concentrations of MC-LF and MC-LW can induce subtle neurodegenerative effects, reminiscent of Alzheimer's disease type human tauopathies. Such effects should be taken more seriously with regard to potential human health effects, than the apical cytotoxicity (apoptosis or necrosis) demonstrated at high MC concentrations [83]. It has been shown that MC-LR (see Figure 3) treated hippocampi showed alterations in proteins involved in cytoskeleton, neurodegenerative disease, oxidative stress, apoptosis, and energy metabolism; three proteins related to neurodegenerative disease, septin5, α-internexin, and α-synuclein, were identified to be altered by MC-LR exposure. It was found that MC-LR induced inhibition of protein phosphatases and abnormal hyperphosphorylation of the neural microtubule associated protein tau [79]. This was found to lead to impairment of learning and memory, accompanied by severe damage and neuronal apoptosis in the hippocampal

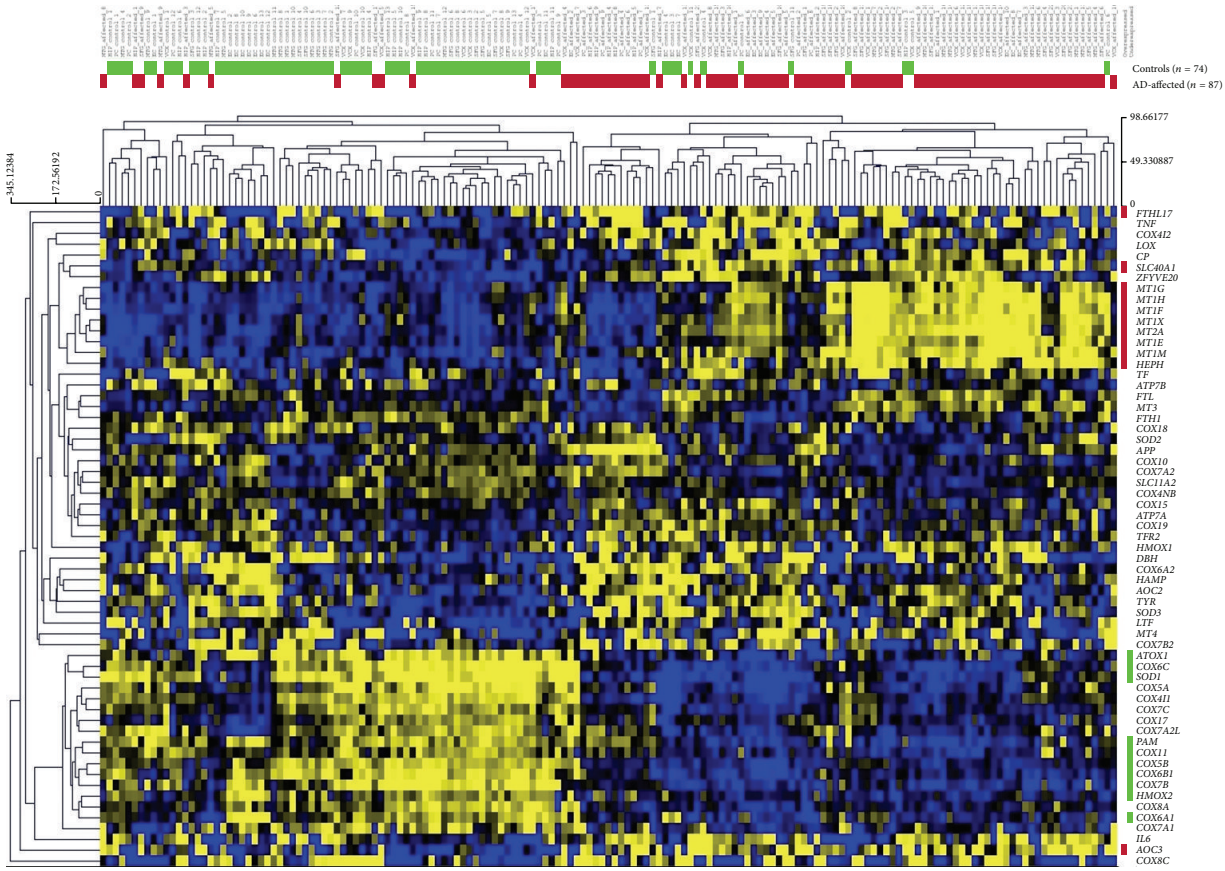

Figure 2: Unsupervised hierarchical clustering analysis of 61 selected genes involved in brain copper/iron homeostasis, comparing gene expression profiles of AD and unaffected age-matched controls. Unsupervised hierarchical clustering analysis (complete-linkage and Manhattan distance similarity measurement) is based on similarities in gene expression. The 21 SAM-identified genes are color coded (right side of figure) based on the group they belong to: red color code represents 11 overexpressed genes, while green color code represents 10 underexpressed genes. The horizontal red color bar indicates AD-affected samples (n = 87), whereas the green bar indicates normal elderly controls (n = 74).

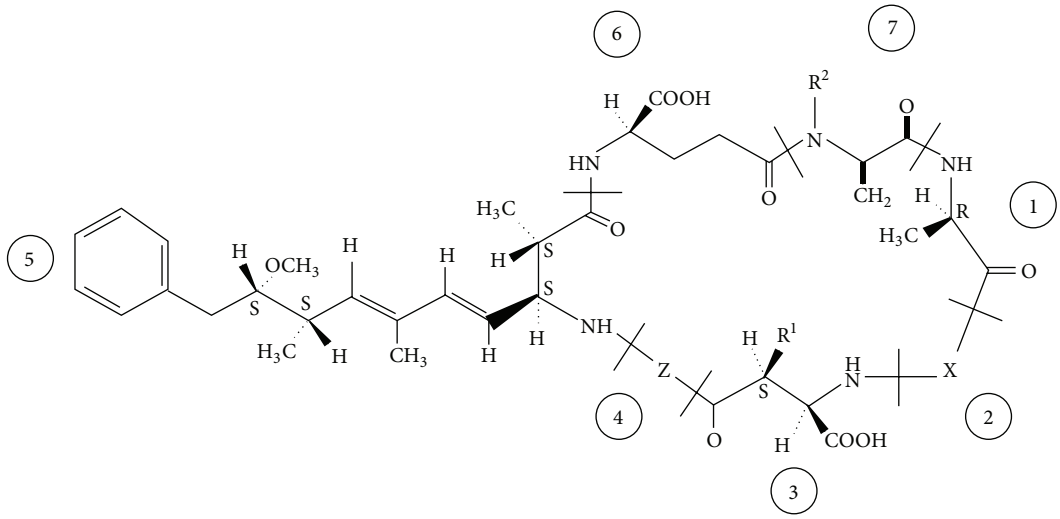

Figure 3: General "cyclo–(D-Ala^1–X^2–D–MeAsp^3–Z^4–Adda^5–D–Glu^6–Mdha^7)" structure of microcystins (MCs), showing the most frequently found variations. X and Z are variable L-amino acids (in MC-LR, MC-LF and MC-LW, X = L-Leucine (L) and Z = arginine (R), phenylalanine (F) or tryptophan (W)); R^1 and R^2 are H (demethylmicrocystins) or CH_3; D-MeAsp is D-*erythro*-β-methylaspartic acid and Adda is (2*S*,3*S*,8*S*,9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6 dienooic acid; Mdha is N-methyldehydroalanine (Dha = dehydroalanine). From [82].

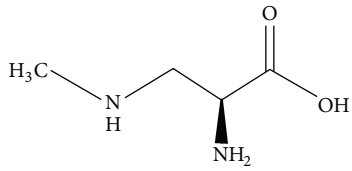

Figure 4: β-Methylamino-L-alanine (BMAA).

CA1 regions of rats. Thus, MC-LR was found to induce tau hyperphosphorylation, spatial memory impairment, neural degenerative changes, and apoptosis, suggesting that this cyanotoxin may contribute to Alzheimer's disease in humans [79].

Cyanobacteria also produce beta-methylamino-L-alanine (BMAA; Figure 4), a nonprotein amino acid produced by several cyanobacteria including *Spirulina* that can be misincorporated into protein chains within human neurons, causing proteins to misfold and form aggregates within the cells [87] and is thought to lead to neurofibrillary tangles, an indication of neurodegenerative disease. Whereas a tie between cyanobacteria and human health is rather well accepted [80], at this point there is interestingly also a possible tie between cyanobacterial toxins and risk of progressive neurodegenerative disease (BMAA was identified in neuroproteins from AD and amyotrophic lateral sclerosis (ALS) brains [88]), an issue which is rarely discussed.

Exposure to cyanobacteria toxins can occur by drinking untreated water from a lake, pond, reservoir, or spring with a cyanobacterial bloom, or when ingesting contaminated foods (e.g., crops watered with untreated water) [89]. Also aerosol inhalation from blooming waters may pose a health risk, for example, when watering the lawn. MC intoxication frequently occurs in conjunction with toxic cyanobacterial blooms in water reservoirs (lakes and ponds) used for drinking water or recreational purposes. It has also been suggested that nutritional supplements from cyanobacteria (blue-green algae) such as *Spirulina* and *Aphanizomenon* may not only be beneficial as they may contain MCs which constitute a group of cyanobacterial toxins with more than 100 congeners [90]. Since cyanobacteria blooms occur all over the world, the possible implication of the cyanobacteria for health problems might be severe. Globally the most frequently found cyanobacterial toxins in blooms from fresh and brackish waters are the cyclic peptide toxins of the microcystin and nodularin family [82]. Large numbers of the human population are therefore at risk to be exposed to these toxins with adverse health effects.

2.5. Treatment of and Protection from AD. Removing pathogenic Aβ peptides to prevent, and possibly reverse, aggregation is the most sought after method for slowing or delaying the onset of AD. Development of AD-modifying drugs include amyloidtargeting monoclonal antibodies (mAbs), β- and γ-secretase inhibitors, anti-amyloid vaccines, and tau-based therapies [3]. In principle, Aβ targeting mAbs should penetrate the blood-brain barrier and recognize various forms of Aβ, guiding Aβ clearance into the blood stream for liver degradation. However, some recent clinical mAbs phase III trials have failed [3]. Secretase inhibitors are intended to decrease APP cleavage and lower the amount of Aβ peptides formed, and, vaccines can be used to boost the immune system to recognize Aβ. Tau-based therapies are reported to moderate downstream effects due to Aβ-plaque build-up [3].

The hepcidin-mediated copper lowering mechanisms discussed above suggest that locally active antagonists interfering with hepcidin's binding to neuronal cell's ferroportin would be expected to promote neuronal ferroportin export of iron, decreasing undesired APP-mediated copper export as well as iron-mediated oxidative damages intracellularly. Ways of downregulating hepatic hepcidin expression (e.g., siRNA) could be attempted, but lowered systemic hepcidin levels will also result in increased intestinal iron absorption. Membrane-permeable metal chelators capable of washing out toxic metals may be effective [91–94], and *in vitro*, Aβ deposits can be dissolved using metal chelators [22]. Lowering systemic iron levels by controlled phlebotomy has been suggested as an AD therapeutic [95]. A clinical study administrating deferoxamine (a nonmembrane-permeable strong Fe^{3+}-chelator that to some degree is taken up into cells by endocytosis, but which does not cross the BBB readily) slowed the clinical progression of dementia associated with AD [96]. For a patient suffering from an iron metabolism disorder, treatment for 10 months with the iron chelator deferoxamine decreased brain iron stores, prevented progression of the neurological symptoms, and reduced plasma lipid peroxidation [97]. Many issues confront the use of chelators including metal saturation. The compounds' ability to reach the intended site is affected by metabolic instability, and toxicity is an important issue (administration of copper chelators to dissolve Aβ-plaques could further lower neuronal copper levels, etc). Current clinical oral administration of lipophilic membrane permeable iron chelators includes deferiprone (produced by ApoPharma) and deferasirox (Novartis). These are today mainly used for treatment of thalassemia major and anaemia, respectively; only a few reports concern their uses in relevant neurodegenerative situations [98]. Molecular modifications may be needed for improved BBB passage and stability [99]. For efficient iron removal from the brain, it has been suggested that the lipophilic chelator-iron (III) complex should have a molecular mass less than 500 Da and be uncharged for cell membrane and BBB permeability [93]. The copper/zinc chelator clioquinol (CQ) [100] was suggested to inhibit Aβ accumulation due to its ability to remove metals from the brain; however, CQ is now considered toxic. This led to the development of the analogue PBT2, a potential therapeutic compound for AD which reduced cerebrospinal fluid (CSF) $A\beta_{42}$ and improved cognition without serious adverse effects, according to a phase II clinical trial [101]; the compound is being further investigated. Other chelators tested in AD models having showed positive effects include the iron chelator M30 [92], the copper chelators JKL-169 [102] and IQM-622 [103], and the calcium/copper/zinc chelators DP-109 and DP-460 [104, 105]. On the contrary, a clinical trial of D-penicillamine, a copper chelator, was unable to produce any clinical improvement in AD patients and resulted in

toxicities [106]. Nanoparticle-based chelators that can cross the blood-brain barrier are also being developed [107].

Antioxidants from food such as flavonoids, carotenoids, tocopherols, and selenium have been suggested to protect against AD [94, 108–111]. Interestingly, the occurrence of AD in India was found to be just one quarter of that in the USA which may be related to intake of the plant phenolic antioxidant curcumin possessing iron binding capacity [112]. Curcumin is present in roots of the turmeric plant used as spice in various foods including curry [108, 113]. Natural antioxidants have also showed protective effects against PD [114], and can also lower microglial cytokine production [115]. Also food compounds not expected to act as antioxidants may protect against neurodegeneration. One example is caffeine (present in coffee) which has been suggested to be a potent neuroprotector [116, 117].

3. Parkinson's Disease (PD)

PD is today recognized as the second most common neurodegenerative disorder after AD. The disease is known to cause motor dysfunctions in addition to autonomic and cognitive deficits [118]. PD is characterized by the accumulation of α-synuclein and selective and progressive loss of dopaminergic neurons in the substantia nigra (SN) and the degeneration of projecting nerve fibres in the striatum [119, 120]; however, its true aetiology still remains unknown. α-Synuclein aggregation is thought to appear early in disease development and to spread across the nervous system as the disease progresses [121], and early neuroimmune activation by oligomerized α-synuclein has been speculated to precede neuronal degeneration. There are probably both environmental and genetic (familial) causative factors for developing PD. About 5–10% of patients are known to have monogenic forms of the disease; for those, mutations in at least 13 loci and 9 genes have been reported to correlate with PD [122]. However, exposure to environmental factors may play a more significant role than genetic mutations in the vast majority of PD patients (sporadic PD) [123, 124]. This is supported by an etiology study of twins with PD [124], where it was found that genetic factors do not play a major role in causing typical PD; no genetic component was evident when the disease began after the age of 50. On the other hand, genetic factors appear to be important when the disease began at or before the age 50 [124]. It is then tempting to speculate that aging may play a predominant role in the etiology of PD but in combination with environmental toxins.

Hypotheses on the loss of dopaminergic neurons in PD suggest that mitochondrial dysfunction may be involved in neuronal death [125, 126], through mechanisms involving oxidative stress and impaired energy metabolism [127, 128]. Inhibition of complex I can lead to ROS, lipid peroxidation, protein dysfunction, and a decrease in reduced glutathione: all hallmark features of postmortem PD [129]. Moreover, chemically induced mitochondrial dysfunction and cellular lesions can activate inflammatory pathways. Below we discuss the role of inflammation in the context of environmental risk factors for the development of PD. We also present data from *in silico* analysis of published transcription data of PD compared to normal brains.

3.1. Inflammation in PD. Inflammation in the brain has recently garnered much interest due to clinical investigations, which show an increase in activated microglia and high levels of inflammatory factors in the nigrostriatal system from postmortem analyses of PD patients [130]. In some cases, the onset of PD has been reported to be associated with head trauma or encephalitis, also suggesting that an inflammatory component is involved in the disease process [131]. Levels of proinflammatory cytokines are elevated in PD patients compared with healthy subjects, and it has been shown that these cytokines can contribute to dopaminergic cell death *in vivo* [132, 133]. Indeed, several recent studies have demonstrated an important inflammatory component in PD pathogenesis. Injection of lipopolysaccharide (LPS), an endotoxin produced by gram-negative bacteria, into the SN [134] or striatum [135] induces inflammation in the brain, and this has been used as a tool to produce animal models of PD. As reported by Zhou and coworkers in 2012, a single intracerebroventricular injection of LPS led to activation of microglia in the hippocampus, striatum, and in the SN, followed by phospho-α-synuclein expression and abnormal motor behaviour [134]. Interestingly, these results indicate that microglia are activated for several months after a single, low dose injection of LPS in the rat, which eventually results in progressive and selective damage to dopaminergic neurons in the SN.

Although PD and AD have distinct pathologies, they also share some similarities. Both underlying pathologies present different forms of protein aggregates that appear to be causally linked to their respective diseases [151]. The pathological hallmark of AD involves misfolding and aggregation of Aβ-plaques and neurofibrillar tangles, mainly composed of the protein tau, whereas PD inclusions (Lewy bodies) are largely composed of intracytoplasmic aggregates of α-synuclein. However, aggregations of Aβ and α-synuclein are not unique to AD and PD; the pathologies are often found to coexist in patients with each of these conditions [152, 153]. α-Synuclein has been reported to activate microglia in the SN pars compacta of mice, which precipitates dopaminergic degeneration [154]. *In vitro* observations have suggested that α-synuclein can have a direct effect on microglial activation [155]. An increased susceptibility of dopaminergic cells to LPS-induced toxicity is seen in neurons overexpressing the human form of α-synuclein, and the generated inflammatory response further leads to aggregation of α-synuclein in nigral neurons [156]. Furthermore, Aβ deposition may be deleterious not only in AD but also in PD, and this Aβ effect is associated with inflammatory processes. Inflammation can promote Aβ deposition that can in turn lead to ROS formation, neuronal cell death [157], glia cell activation, and subsequent release of proinflammatory cytokines [158, 159]. Hochstrasser et al. (2013) showed that Aβ significantly decreased the number of, for example, hydroxylase-positive dopaminergic neurons, and that anti-inflammatory drugs partially counteracted the Aβ-induced neuronal decline through the suppression of glial cell activation [160]. Recent data have

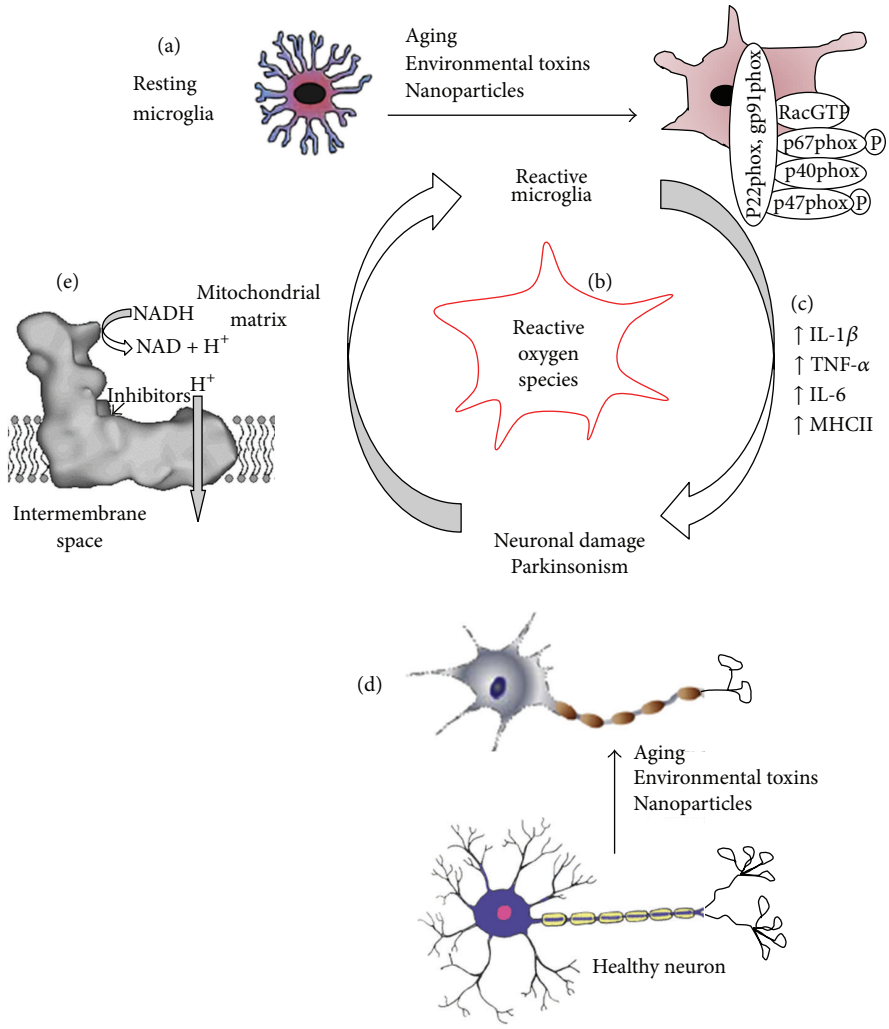

FIGURE 5: Chemical stress and aging can alter microglia reactivity and activation of the phagocytic NADPH oxidase complex. (a) Stimulation of microglia induces the parallel activation of oxidase components within the cytoplasm. This activation causes the conversion of Rac into an active GTP-bound form and the phosphorylation of p47phox and p67phox. These subunits then translocate to the membrane where they interact with p22phox and gp91phox (NOX) to initiate ROS production (b) [180]. Excessive or prolonged inflammation (e.g., IL-1β, IL-6, TNF-α) (c) and ROS resulting from increased microglial activation may contribute to neuronal damage (d). In addition, chemicals can damage complex I in mitochondria ((e) figure adapted from [181]) and induce deleterious changes to neurons in SN by ROS formation and ATP depletion. The figure is adapted with modifications from [168].

indicated that Aβ is slightly decreased in the cerebral spinal fluid of PD patients and Aβ can interact with α-synuclein to accelerate cognitive decline [161]. Further investigations on the relation between α-synuclein and Aβ may provide new insights into the molecular mechanisms of the pathogenesis of PD and suggest potential new approaches to its treatment.

3.2. Environmental Toxins, Microglia, and Neurodegenerative Diseases. In the brain, microglia perform dynamic cellular functions that include synaptic plasticity [162], cleaning of cellular debris, wound healing through alternative activation [163, 164], and innate immune defence. Microglial cells comprise approximately 10–12% of the cells in the brain and dominate in the grey matter, with particularly high concentrations in the hippocampus, hypothalamus, basal ganglia, and SN [165–167]. In general, age-related priming of microglia in the brain plays a role in the development of age-related inflammatory diseases [168], as shown by a progressive increase in the expression of microglial MHCII in rodents and primates [169]. A variety of noxious compounds released from inappropriately activated microglia, including ROS, reactive nitrogen species (RNS; e.g., nitric oxide, $NO^{\bullet}$, and peroxynitrite, $ONOO^{-}$), proinflammatory cytokines (e.g., TNFα, IL-1β, and IL-6), and prostaglandins may be important mediators of dopaminergic cell death [170–172], which may propagate into a disease state [173]. Stressed dopaminergic neurons are reported to cause microglial activation by releasing stimulatory signalling molecules such as α-synuclein, neuromelanin, and matrix metalloproteinase-3 [174–176]. Also,

Table 2: Environmental toxins linked to neuronal diseases.

Environmental toxins (linked to brain disease)	Implicated disease/condition in humans or in animal studies	References
Cyanobacterial toxins (MCs, BMAA)	Tau hyperphosphorylation in AD (MC) and protein misfolding (BMAA)	[79, 83, 87]
Smoking	Dementia, AD	[25]
Paraquat	Cell death dopaminergic neurons (Bcl-2 induced) and PD possibly by mitochondrial dysfunction and oxidative stress	[6, 136, 137]
Rotenone	Oxidative stress, potent inhibitor of mitochondrial complex I, nigrostriatal cell death (Bcl-2 induced) and PD	[8, 138]
Dieldrin	PD	[139]
Diesel exhaust particles (air pollution)	PD	[140]
Lindane	PD	[141]
Mancozeb	PD	[142]
Maneb	Oxidative stress, cell death (Bcl-2 induced), and PD	[6, 142]
3-nitropropionate	Inhibits succinate dehydrogenase; striatal degeneration; Huntington's disease	[143, 144]
Trichloroethylene	Mitochondrial dysfunction in striatum and PD	[145]
1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP)	Oxidative stress, cell death (Bcl-2 induced), and PD	[7]
Insecticides (Lorsban, Dursban, or other chlorpyrifos products)	PD	[146]
Metals (e.g., manganese, copper)	PD	[147–149]
Nanoparticles in air emissions	Brain functional deficits?	[150]

cytokines, such as TNFα, IL-1β [177], IL-4 [178] and IL-13 [179], are reported to initiate microglial NADPH oxidase activation and $O_2^{\bullet-}$ generation.

Microglia express the phagocyte NADPH oxidase [182, 183], composed of four regulatory cytosolic components (p47phox, p67phox, p40phox, and rac proteins) and a transmembrane flavocytochrome heterodimer consisting of the p22phox and the gp91phox (NOX2) catalytic subunit (Figure 5). NOX2 redox signalling has been shown to play an essential role in the microglial proinflammatory response and associated neurotoxicity, and there is evidence for ROS involvement in regulation of NOX2 subunits Rac1 and P47phox [136, 184]. Environmental toxins (including paraquat, rotenone, dieldrin, diesel exhaust particles, lindane, mancozeb, maneb, in addition to some metals; Table 2) have been reported to reach the brain and activate microglial NADPH oxidase to produce ROS and induce mitochondrial dysfunction. Continued neuronal damage and rotenone have been shown to synergistically activate NOX2 and amplify the microglial proinflammatory response to LPS and neurotoxicity *in vitro* [8, 138]. It has recently been shown that 1-methyl-4-phenyl-1,2,5,6-tetrahydro-pyridine (MPTP) intoxication is accompanied by a strong microglia response which is characterized by the release of inflammatory molecules that are believed to further drive inflammation-mediated degeneration of DA neurons [185]. Interestingly, paraquat is shown to be a potent inducer of microglial activation; both *in vivo* [186] and *in vitro* [187] studies suggest that this is the primary mechanism of dopaminergic neurotoxicity. Rotenone is known to inhibit the transfer of electrons from iron-sulphur centres in complex I to ubiquinone (coenzyme Q10). *In vitro* experiments indicate that rotenone is selectively toxic to dopaminergic neurons only in the presence of microglia [138]. Thus, altogether these studies show that triggering of microglial NADPH oxidase activation include both environmental toxins and central nervous system (CNS) disease pathways, suggesting that the microglial NADPH oxidase may be a promising target for PD treatment, especially in delaying the progression of PD.

Epidemiological studies have suggested exposure to various pesticides as risk factors for PD. These include the fungicide maneb which contains manganese [188], paraquat, and other herbicides or other pesticides [189, 190]. Chronic occupational exposure to manganese or copper, individually, or in combination with lead, iron and copper, is associated with PD [147]. In PD patients, redox-active iron and copper are considered to be an important factor in the pathology and progression of PD due to its ability to generate free radicals and to promote redox reactions. Accumulation of iron is frequently observed in brain areas linked to PD [191]. The brain regions responsible for motor functions seem to have more iron than nonmotor related regions also explaining why movement disorders such as PD are often associated with iron loading [192]. High content of copper and zinc is also present in the cerebrospinal fluid of patients affected by PD [193]; the concentrations of these metals were up to threefold higher than control levels. A detailed review of Cu, Zn, and Fe related to neurodegeneration in PD can be found in Kozlowski et al. (2012) [194].

Recent studies have revealed a possible link between nanoparticle (NP) exposure, and neuronal inflammation and disease. Silver (Ag) particles are one of the most used NP in consumer products; exposure has been suggested to induce learning and memory deficits in rats, possibly through oxidative stress-induced pathological changes in the hippocampus and attenuation of long-term potentiation [198]. It has previously been shown that a single intravenous exposure of rats to AgNP caused time- and size-dependent accumulation of

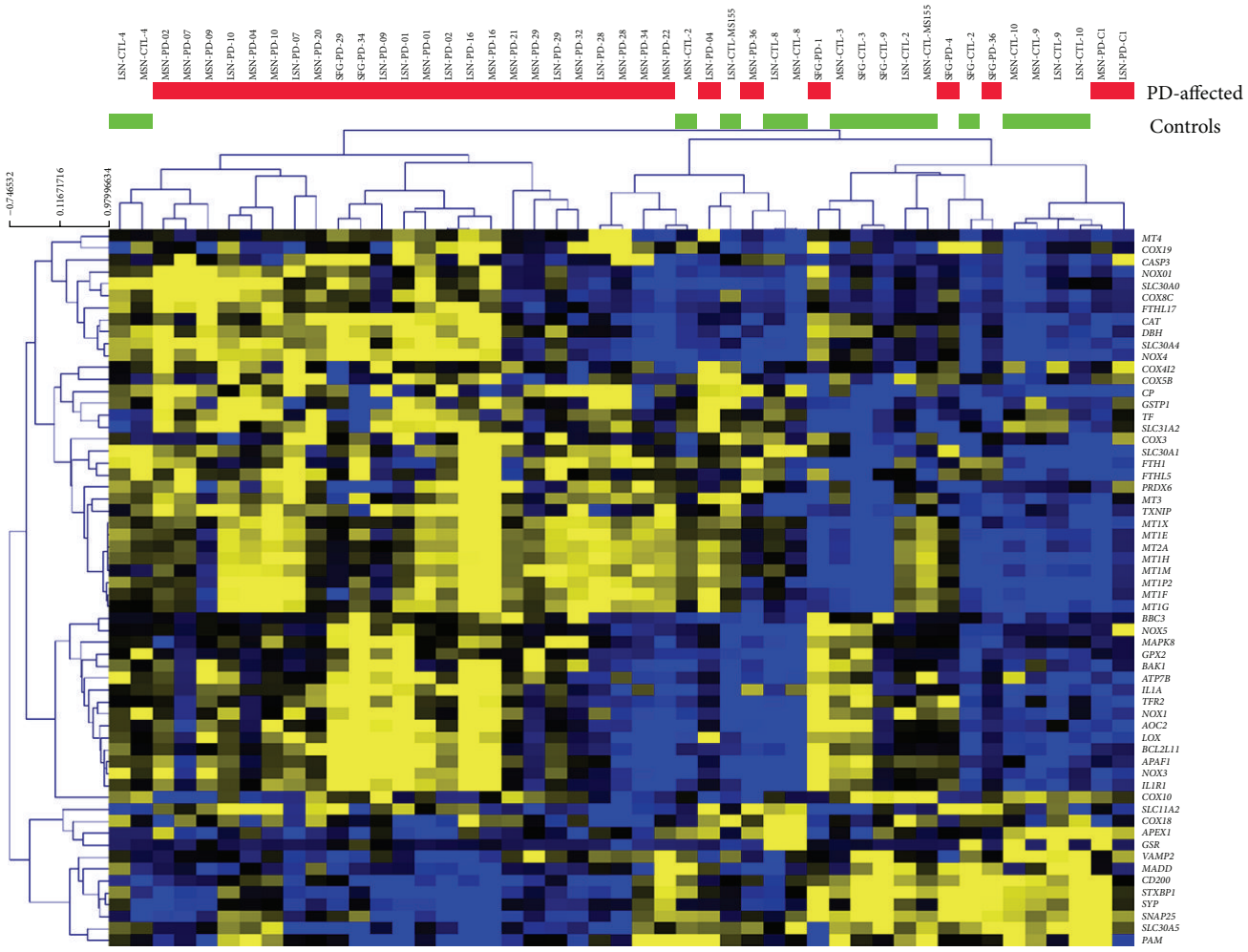

FIGURE 6: Unsupervised hierarchical clustering analysis (complete-linkage and Manhattan distance similarity measurement) of the expression of 60 selected genes in PD. The clustering analysis is based on similarities in gene expression. The yellow color code represents overexpressed genes ($n = 47$), while blue color code represents underexpressed genes ($n = 13$). The horizontal red color bar indicates PD-affected samples ($n = 31$) and the green bar indicates normal elderly controls ($n = 16$).

NPs in the brain [199]. Further, AgNP induced DNA-strand breaks, apoptosis, necrosis, and decreased proliferation in different cell models [200, 201]. AgNPs may disturb the neurotransmitter signaling in the brain [202], and according to the authors such disturbances are linked to conditions such as PD and AD, and have been implicated in motor, cognitive, and affective functions. In general, the observed effects of NP in the brain can be categorized as inflammation and oxidative stress, which may be responsible for cognitive deficits and other diseases [203]. Recently, several types of inorganic NPs have been shown to activate microglia and damage neurons *in vitro* [204]. While the accumulation of NPs in brain has been clearly demonstrated, less information is available concerning potential contribution of health-related outcome(s) from NP exposure in airborne emissions, particularly in modest polluted areas. Elder et al. (2006) showed increases in proinflammatory mediators and markers of oxidative stress and immune cell activation (e.g., TNF-α), and SOD in rat brain regions where manganese accumulated following manganese oxide NP exposure [205]. These findings suggest that NP exposure resulted in CNS oxidative stress and inflammation. Although mechanisms driving NP-induced CNS pathology are poorly understood, new evidence suggests that microglial activation may be a key component, with an important contribution of conditions that predispose the individual to oxidative stress [206]. However, further research should address the mechanisms of toxicity and the risk of the general population for developing PD after NP exposure. Motor, cognitive, and behavioral functions have been assessed in healthy children and elderly residents in Valcamonica, Italy, and an increased prevalence of Parkinsonism was observed as being associated with the manganese levels in the deposited dust from ferroalloy airborne emissions [148]. Inflammation of the olfactory bulb and deficits in olfaction has been observed in Mexican children residing in highly polluted areas [207]. Olfactory loss is an early finding in both AD and PD and precedes cognitive and motor symptoms [208, 209]. α-Synuclein neuronal aggregation and accumulation of

Table 3: Genes ($n = 18$) involved in brain metal homeostasis and neurodegeneration for which expression is significantly changed in PD.

Gene symbol	Description	SAM score (d)*	Fold up- or downregulation	Fold change[a]	Fold change[b,c]
MT1G	Metallothionein 1G	1.7	1.94	0.90	0.82
MT1F	Metallothionein 1F	1.6	1.87	0.88	0.55
MT1M	Metallothionein 1M	1.5	1.73	1.10	0.92
MT1P2	Metallothionein 1 pseudogene 2	1.5	1.79	0.43	0.57
MT1H	Metallothionein 1H	1.5	1.65	0.69	0.79
MT1E	Metallothionein 1E	1.5	1.64	0.62	0.58
MT1X	Metallothionein 1X	1.4	1.56	0.67	0.73
BCL2L11	BCL2-like 11 (apoptosis facilitator)	1.4	2.05	0.29	0.61
MT2A	Metallothionein 2A	1.3	1.51	0.46	0.81
SLC30A1	Solute carrier family 30 (zinc transporter), member 1	1.3	1.38	0.47	0.37
MT4	Metallothionein 4	1.2	3.51	0.29	0.22
CAT	Catalase	1.1	2.00	0.30	0.40
TF	Transferrin	1.0	1.62	0.68	0.74
SNAP25	Synaptosomal-associated protein, 25 kDa	−1.3	−1.50	−1.50	−1.90
SYP	Synaptophysin	−1.3	−2.04	−0.51	−0.53
CD200	CD200 molecule	−1.5	−1.90	−0.62	−1.10
STXBP1	Syntaxin binding protein 1	−1.8	−1.90	−1.00	−0.71
SLC30A9	Solute carrier family 30 (zinc transporter), member 9	−1.9	−1.79	−0.69	−0.63

* Significantly differentially expressed genes between PD-cases and controls, FDR < 10% for GSE8397 microarray date and unlogged fold up- and downregulated genes ($n = 18$). Thirteen genes were overexpressed and 5 genes were underexpressed in the samples from PD-affected cases in comparison to age-matched unaffected controls. The microarray dataset is from (a) microarray dataset from Moran et al., 2006 [195] with GEO accession number, GSE8397; (b) microarray dataset from Zhang et al. 2005 [196] with accession number GSE20168; (c) microarray dataset from Lesnick et al. 2007 [197] with GEO accession number GSE7621. All three microarray datasets are reanalyzed in the Parkinson's disease database (Park DB: http://www2.cancer.ucl.ac.uk/Parkinson_Db2/index.php).
[a,b,c] Are log-transformed fold change differences between PD-cases and controls from Park DB.

3-nitrotyrosine and 8-hydroxydeoxyguanosine, biomarkers of oxidative stress, were also detected in nuclei of these children's brainstem [150]. That NPs can significantly enhance the rate of protein fibrillation adds a new important aspect to NP's role in neurodegeneration [207].

3.3. In Silico Analysis of Genes Involved in Parkinson's Disease. To further elucidate the mechanisms for degeneration of the SN in PD patients, we performed a search in the publicly available databases and in the literature for genes involved in brain metal homeostasis pathways, neurodegenerative disorders related genes (in particular PD), oxidative phosphorylation, oxidative stress, and apoptosis pathways related genes. We selected 138 genes from these pathways. To investigate whether the expression patterns of those genes are modulated in neurologically normal elderly and PD individuals, we downloaded the microarray data (GSE8397) deposited in the GEO database [72] where tissue samples from PD-cases were compared to controls [195]. Whole genome expression profiling of studies of 47 tissue samples collected from brain regions relevant to PD were analyzed for PD-cases and controls: lateral substantia nigra (LSN), medial substantia nigra (MSN), and superior frontal gyrus (SFG) [195]. For more detailed description of sample collection, experimental design and flow, we refer the reader to the original studies [195]. From the GSE8397 dataset, we identified 60 genes (out of our 138 gene list) whose mean expression level is differentially expressed between PD-cases and controls using two-class, unpaired SAM (FDR < 10%) [78]. The processed intensities of the 60 genes were mean-centered and log2-transformed. Figure 6 shows unsupervised hierarchical clustering analysis of these 60 genes, and the results were visualized in a dendrogram using MeV v4.8 software [77]. By visual inspection of the heatmap (Figure 6), we observed that samples from PD-cases clustered close to each other in one branch while samples from controls clustered in the other branch. We further compared the identified 60 genes with gene expression datasets available in the Parkinson's disease database (Park DB: http://www2.cancer.ucl.ac.uk/Parkinson_Db2/index.php) in order to identify key genes with consistent expression profiles across several datasets. Of the 60 genes, we selected 18 genes showing significant differences across three different datasets (Table 3) [195–197].

Genes consistently overexpressed in PD-cases include *MT1E, MT1F, MT1G, MT1H, MT1 M, MT1P2, MT1X, MT2A, MT4, BCL2L11, CAT, SLC30A1,* and *TF*, while *CD200, SLC30A9, SNAP25, STXBP1,* and *SYP* were underexpressed (Table 3). Noticeably, in the PD brains, several metallothioneins are overexpressed, as well as iron binding transferrin. Dysregulation of these genes might disturb or compensate a dysregulated metal homeostasis. Downregulation of proteins in SN or prefrontal cortex associated with synaptic functions in neurons, such as synaptosomal-associated protein (SNAP25), synaptophysin (SYP), and syntaxin binding

protein 1 (STXBP1), indicates synaptic functional disturbance and neuronal loss. Decreased CD200 expression indicates loss of neuronal regulation of microglia, and increase of CAT expression level may indicate oxidative stress. Interestingly, several genes (*NOX1, NOX3, NOX4,* and *NOX5*) coding proteins in the NADPH oxidase complex are overexpressed (Figure 6) in the PD-cases which may indicate formation of ROS and proinflammatory mediators in the SN. Altogether the expression profile constituted by these genes may serve as a basis for identification of PD-predictive genes that may explain the underlying molecular mechanisms associated with PD. This should be pursued in future studies.

4. Concluding Remarks

More studies are needed to investigate hepcidin's role in the brain, focusing on aging, metal dyshomeostasis, and inflammation in degenerative diseases. However, to unravel the disease aetiologies of AD and PD in relation to metal storage and shuttling between the various types of brain cells (e.g., astrocytes, neurons, endothelial cells, and microglia) new studies are required. These should, however, have high resolution and the capability to analyse protein (and/or gene) expressional and functional changes of individual cell types during disease progression. The role of combined chemical exposures for development of neuronal diseases needs to be elucidated in future studies.

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Research Article

Neurotoxic Effects of *trans*-Glutaconic Acid in Rats

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trans-Glutaconic acid (*t*GA) is an unsaturated C5-dicarboxylic acid which may be found accumulated in glutaric aciduria type I, whose pathophysiology is still uncertain. In the present work it was investigated the *in vitro* effect of increasing *t*GA concentrations on neurochemical and oxidative stress parameters in rat cerebral cortex. We observed that Na^+, K^+-ATPase activity was reduced by *t*GA, but not creatine kinase, respiratory chain complex IV, and ATP synthase activities. On the other hand, *t*GA significantly increased lipid peroxidation (thiobarbituric acid-reactive species levels and spontaneous chemiluminescence), as well as protein oxidative damage (oxidation of sulfhydryl groups). *t*GA also significantly decreased nonenzymatic antioxidant defenses (TRAP and reduced glutathione levels). Our data suggest that *t*GA may be neurotoxic in rat brain.

1. Introduction

Glutaryl-CoA dehydrogenase deficiency (OMIM: number 231670), also known as glutaric aciduria type I, is an autosomal recessive metabolic disorder due to a blockade in the catabolic pathway of the amino acids lysine, hydroxylysine, and tryptophan. This disease was first described by Goodman et al. [1] and is biochemically characterized by tissue accumulation of predominantly glutaric acid and, to a lesser degree, of 3-hydroxyglutaric and *trans*-glutaconic (*t*GA) acids [2, 3]. Clinically, affected patients present macrocephaly, progressive dystonia, and dyskinesia, symptoms that are apparent within the first year of life [4, 5]. Degeneration of caudate and putamen following encephalopathic crises and frontotemporal atrophy at birth is also commonly seen in these patients [6, 7]. Children presenting residual glutaryl-CoA dehydrogenase activity of up to 30% (low excretors) present low or undetectable excretion of glutaric acid, but the neurological signs may be found similar in these individuals, regardless of the amount of glutaric acid excreted [8–11].

The primary cause of neurological alterations in glutaric acidemia type I is still not defined. It has been demonstrated that brain tissue exposure to the main accumulating metabolites, glutaric, and 3-hydroxyglutaric acids results in excitotoxicity [12–15], oxidative stress induction [16–18], and/or disruption of energy homeostasis [12, 19–24]. Furthermore, it has been recently showed that lysine administration to a knockout mice model of glutaric aciduria type I (*Gcdh*−/−) results in oxidative damage and energy impairment in the brain of these animals, as well as in compromised neurodevelopmental and cognitive behavior [25–28]. On the other hand, very little is known regarding the direct toxicity of *t*GA. In this scenario, and it has been demonstrated that *t*GA is able to inhibits glutamate decarboxylase activity in rat and rabbit brains, human mitochondrial NAD(P)(+)-dependent malic enzyme, and dopamine beta-monooxygenase activities in purified preparations, as well as inducing mitochondrial permeability transition in rat liver mitochondrial preparations, provoking apoptosis in immature oligodendrocytes,

and reducing cell viability of primary cultures of mice cerebral neocortical neurons [29–34].

Therefore, the aim of the present work was to investigate the *in vitro* effects of *t*GA on various neurochemical parameters in cerebral cortex of young rats. The investigated parameters were the values of Na^+, K^+-ATPase, creatine kinase (CK), complex IV and ATP synthase activities, carbonyl and sulfhydryl content, thiobarbituric acid-reactive substances (TBA-RS) levels, spontaneous chemiluminescence, reduced glutathione (GSH) concentrations, and total nonenzymatic antioxidant capacity of the tissue (TRAP) in cerebral cortex of young rats.

2. Material and Methods

2.1. Reagents. All chemicals were purchased from Sigma (St. Louis, MO, USA). *t*GA was dissolved in the day of the experiments in the incubation medium used for each technique, and the solution had its pH adjusted to 7.4. The final concentrations of *t*GA in the medium ranged from 0.01 to 1.0 mM.

Parallel experiments were always carried out with negative controls (blanks) in the presence or absence of *t*GA and also with or without cortical supernatants in order to detect artifacts caused by this organic acid in the assays. Therefore, any interference of *t*GA on the reactions used to measure the biochemical parameters would be identified.

2.2. Animals. Thirty-day-old male Wistar rats obtained from the Central Animal House of the Departamento de Bioquímica, Federal University of Rio Grande do Sul (UFRGS), were used. Rats were kept with dams until weaning at 21 days of age. The animals had free access to water and to a standard commercial chow and were maintained on a 12 : 12 h light/dark cycle in an air-conditioned constant temperature ($22 \pm 1^{\circ}$C) colony room. The "Principles of Laboratory Animal Care" (NIH publication number 80-23, revised 1996) were followed in all experiments and the Ethics Committee for Animal Research of UFRGS, Porto Alegre, Brazil, approved the experimental protocol. All efforts were made to minimize the number of animals used and their suffering.

2.3. Tissue Preparation and Incubation for Oxidative Stress Parameters. On the day of the experiments the animals were killed by decapitation without anesthesia, and the brain was rapidly excised on a Petri dish placed on ice. The olfactory bulbs, brain stem, medulla, cerebellum, hippocampus, corpus callosum, and striatum were discarded, and the cerebral cortex was peeled away from the subcortical structures, weighed and homogenized in 10 volumes (1 : 10, w/v) of 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM KCl. Homogenates were centrifuged at 750 ×g for 10 min at 4°C to discard nuclei and cell debris [35]. The pellet was discarded, and the supernatant, a suspension of mixed and preserved organelles, including mitochondria, was separated and incubated in 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM KCl at 37°C for one hour with *t*GA at concentrations of 0.01, 0.1, or 1 mM. Controls did not contain this metabolite in the incubation medium. Immediately after incubation, aliquots were taken to measure the values of carbonyl and sulfhydryl content, TBA-RS levels, spontaneous chemiluminescence, GSH concentrations, and TRAP.

2.4. Preparation of Synaptic Plasma Membrane from Rat Cerebral Cortex. Cerebral cortex was homogenized in 10 volumes of 0.32 mM sucrose solution containing 5.0 mM HEPES and 1.0 mM EDTA. The homogenate was preincubated at 37°C for 1 h in the absence or presence of 0.01, 0.1, or 1 mM *t*GA. Membranes were prepared afterwards according to the method of Jones and Matus [36] using a discontinuous sucrose density gradient consisting of successive layers of 0.3, 0.8, and 1.0 mM. After centrifugation at 69,000 ×g for 2 h, the fraction at the 0.8–1.0 mM sucrose interface was taken as the membrane enzyme preparation.

2.5. Tissue Preparations for Determination of Respiratory Chain Complex IV and Creatine Kinase Activities. For the determination of the activities of the respiratory chain complex IV, cerebral cortex was homogenized (1 : 20, w/v) in SETH buffer (250 mM sucrose, 2.0 mM EDTA, 10 mM Trizma base, and 50 $UI \cdot mL^{-1}$ heparin), pH 7.4. The homogenates were centrifuged at 800 ×g for 10 min, and the supernatants were kept at −70°C until being used for enzyme activity determination. For total creatine kinase activity determination, the cerebral cortex was homogenized (1 : 10 w/v) in isosmotic saline solution [37].

2.6. Mitochondrial Preparations. Mitochondrial fractions prepared according to Cassina and Radi [38] were used for the determination of ATP synthase activity, TBA-RS levels, and sulfhydryl content.

2.7. Determination of Na^+, K^+-ATPase Activity. Na^+, K^+-ATPase activity was evaluated according to Tsakiris and Deliconstantinos [39]. Released inorganic phosphate (Pi) was measured by the method of Chan et al. [40]. Enzyme-specific activity was expressed as nmol Pi released$\cdot min^{-1} \cdot$mg $protein^{-1}$.

2.8. Determination of Bioenergetics Parameters. The activity of respiratory chain complex IV was determined according to Rustin et al. [41] slightly modified, as described in details in a previous report [42]. ATP synthase activity was measured in mitochondrial preparations from cerebral cortex, according to Rustin et al. [41]. Complex IV and ATP synthase activities were calculated as nmol$\cdot min^{-1} \cdot$mg $protein^{-1}$.

CK activity was measured in total homogenates according to Hughes [43] with slight modifications, as described by Schuck et al. [37]. Results are expressed as μmol of creatine$\cdot min^{-1} \cdot$mg $protein^{-1}$.

2.9. Determination of Protein Carbonyl Formation Content. Protein carbonyl content, a marker of oxidized proteins, was measured spectrophotometrically according to Reznick and Packer [44]. The results were calculated as nmol of carbonyls

groups·mg of protein^{-1}, using the extinction coefficient of 22,000 × 106 nmol·mL^{-1} for aliphatic hydrazones.

2.10. Sulfhydryl (Thiol) Group Oxidation. This assay was performed according to Aksenov and Markesbery [45]. The protein-bound sulfhydryl content is inversely correlated to oxidative damage to proteins. Results were reported as nmol TNB·mg of protein^{-1}.

2.11. Determination of TBA-RS Levels. TBA-RS was determined according to the method of Esterbauer and Cheeseman [46]. A calibration curve was performed using 1,1,3,3-tetramethoxypropane, and each curve point was subjected to the same treatment as supernatants. Some experiments were performed in the absence or presence of reduced glutathione (GSH; 100 μM), melatonin (100 μM), the nitric oxide synthase inhibitor N$^{\omega}$-nitro-L-arginine methyl ester (L-NAME; 500 μM), trolox (5 μM), or a combination of catalase (Cat; 10 mU·mL^{-1}) plus superoxide dismutase (SOD; 10 mU·mL^{-1}). TBA-RS values were calculated as nmol of TBA-RS·mg protein^{-1}.

2.12. Spontaneous Chemiluminescence. Samples were assayed for spontaneous chemiluminescence in a dark room by the method of Gonzalez-Flecha et al. [47]. Results were calculated as cpm·mg protein^{-1}.

2.13. Determination of TRAP. TRAP, representing the total nonenzymatic antioxidant capacity of the tissue, was determined by measuring the chemiluminescence intensity of luminol induced by 2,2′-azo-bis-(2-amidinopropane) (ABAP) according to the method of Lissi et al. [48]. TRAP values were expressed as nmol of trolox·mg of protein^{-1}.

2.14. GSH Levels Quantification. GSH levels were measured according to Browne and Armstrong [49]. Some experiments were performed in the presence or absence of melatonin (100 μM), L-NAME (500 μM), trolox (5 μM), or Cat (10 mU·mL^{-1}) plus SOD (10 mU·mL^{-1}). Calibration curve was prepared with standard GSH (0.01–1 mM), and the concentrations were calculated as nmol·mg protein^{-1}.

The oxidation of a commercial solution of GSH (200 μM) was also tested by exposing this solution to 1 mM *t*GA for one hour in a medium devoid of brain supernatants. After *t*GA exposition, 7.4 mM *o*-phthaldialdehyde was added to the vials, and the mixture was incubated at room temperature during 15 minutes.

2.15. Protein Determination. Protein was measured by the method of Lowry et al. [50] using bovine serum albumin as standard.

2.16. Statistical Analysis. Results are presented as mean ± standard deviation. Assays were performed in duplicate, and the mean was used for statistical analysis. Data was analysed using one-way analysis of variance (ANOVA) followed by the post hoc Duncan multiple range test when F was significant.

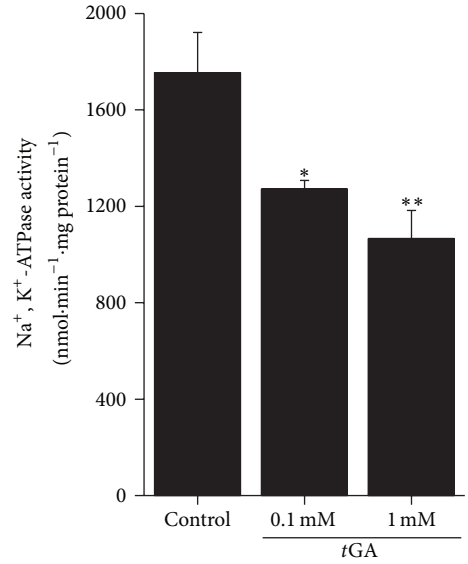

Figure 1: *In vitro* effect of *trans*-glutaconic acid (*t*GA) on Na$^+$, K$^+$-ATPase activity in rat cerebral cortex of 30-day-old rats. The experiments were performed in triplicate, are plotted as mean ± S.E.M. ($n = 4$), and are expressed as nmol·mg protein^{-1}. $^*P < 0.05$ and $^{**}P < 0.01$ compared to control group (Duncan multiple range test).

Differences between groups were rated significant at $P < 0.05$. All analyses were carried out in an IBM-compatible PC computer using the Statistical Package for the Social Sciences (SPSS) software.

3. Results

*t*GA inhibits Na$^+$, K$^+$-ATPase activity in synaptic plasma membranes from rat cerebral cortex.

Initially, we tested the influence of *t*GA on Na$^+$, K$^+$-ATPase activity in synaptic plasma membranes prepared from cerebral cortex. Figure 1 shows that the incubation of *t*GA with purified synaptic membrane preparations caused a significant inhibition of Na$^+$, K$^+$-ATPase activity.

3.1. tGA Does Not Interfere on Important Parameters of Brain Bioenergetics. We also investigated the effect of *t*GA on important parameters of brain bioenergetics. It is observed in Table 1 that the presence of up to 1.0 mM *t*GA in the incubation medium does not disturb creatine kinase, respiratory chain complex IV, and ATP synthase activities in rat cerebral cortex.

3.2. Protein Oxidative Damage Is Induced by tGA. We then evaluated the effect of *t*GA on carbonyl formation and sulfhydryl oxidation in cortical homogenates in order to evaluate protein oxidative damage. We found that *t*GA provoked a significant decrease of sulfhydryl content at 0.1 and 1.0 mM in cortical homogenates (Figure 2(a)), although it did not affect protein carbonyl content. Furthermore, sulfhydryl content was not altered by *t*GA in brain mitochondrial preparations (Table 2).

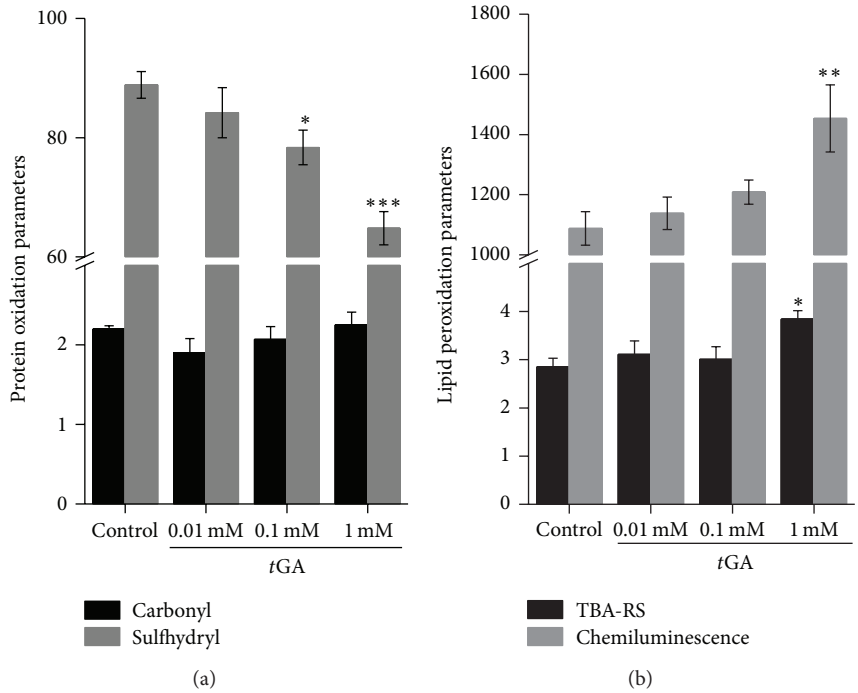

Figure 2: *In vitro* effect of *trans*-glutaconic acid (*t*GA) on oxidative damage parameters (a) carbonyl and sulfhydryl content and (b) TBA-RS levels and spontaneous chemiluminescence in cerebral cortex of 30-day-old rats. The experiments were performed in triplicate and are plotted as mean ± S.E.M. (n = 5-6). Carbonyl and sulfhydryl content and TBA levels are expressed as nmol·mg protein^{-1}; chemiluminescence values are expressed as cpm·mg protein^{-1}. $^{*}P < 0.05$; $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to control group (Duncan multiple range test).

Table 1: *In vitro* effect of *trans*-glutaconic acid (*t*GA) on creatine kinase (CK), respiratory chain complex IV, and ATP synthase activities in cerebral cortex of 30-day-old rats.

Group	CK	Complex IV	ATP synthase
Control	11.28 ± 0.20	114.72 ± 7.42	476.00 ± 31.23
0.1 mM *t*GA	10.66 ± 0.78	116.46 ± 2.57	—
1 mM *t*GA	11.04 ± 1.18	107.14 ± 3.29	508.75 ± 15.47

The experiments were performed in triplicate, and data represent mean ± standard error of the mean (n = 4-5). It was not observed any difference among groups (ANOVA and Student' t-test). CK: μmol · min^{-1} · mg of protein^{-1}; complex IV and ATP synthase: nmol · min^{-1} · mg of protein^{-1}.

Table 2: *In vitro* effect of *trans*-glutaconic acid (*t*GA) on thiobarbituric acid-reactive species (TBA-RS) levels and sulfhydryl (SH) content in mitochondrial preparations from cerebral cortex of 30-day-old rats and glutathione (GSH) content in a commercial GSH preparation.

Group	TBA-RS	SH	GSH
Control	6.44 ± 0.59	55.98 ± 2.30	2941 ± 144.3
1 mM *t*GA	6.16 ± 0.40	56.22 ± 3.59	2640 ± 70.35

The experiments were performed in triplicate, and data represent mean ± standard error of the mean (n = 4-5). It was not observed any difference between groups (Student' t-test for paired samples). TBA-RS and SH: nmol · mg of protein^{-1}; GSH: fluorescent unities.

3.3. tGA Induces Lipid Peroxidation. The influence of *t*GA on the lipid peroxidation parameters TBA-RS levels and spontaneous chemiluminescence was investigated. Figure 2(b) shows that *t*GA at 1 mM significantly increased TBA-RS levels in cortical homogenates. Similar results were obtained for spontaneous chemiluminescence.

We also tested the effect of the antioxidants GSH (100 μM), melatonin (100 μM), L-NAME (200 μM), trolox (5 μM), or a combination of Cat plus SOD (10 mU mL^{-1} each) on *t*GA-elicited increase of TBA-RS levels (Figure 3). These antioxidants were coincubated with 1.0 mM of the organic acid and brain cortical homogenates for 60 min, after which the levels of TBA-RS were measured. We observed that GSH and melatonin fully prevented *t*GA-induced increased lipid peroxidation, whereas the combination of Cat plus SOD partially prevented such effects. Trolox and L-NAME did not prevent *t*GA effect.

It was also observed that *t*GA did not affect TBA-RS levels in brain mitochondrial preparations (Table 2).

3.4. Nonenzymatic Antioxidant Defenses Are Decreased by tGA. We assessed the *in vitro* effect of *t*GA on cortical GSH levels and TRAP. We found that this organic acid significantly decreased GSH levels and TRAP at the highest tested concentration (1.0 mM) (Figure 4). Furthermore, *t*GA-induced effect was totally prevented by the free-radical scavengers melatonin and trolox and by the combination of Cat plus SOD, but not by L-NAME (Figure 3).

We then investigated whether *t*GA-induced GSH levels decrease was secondary to a direct oxidative attack. We

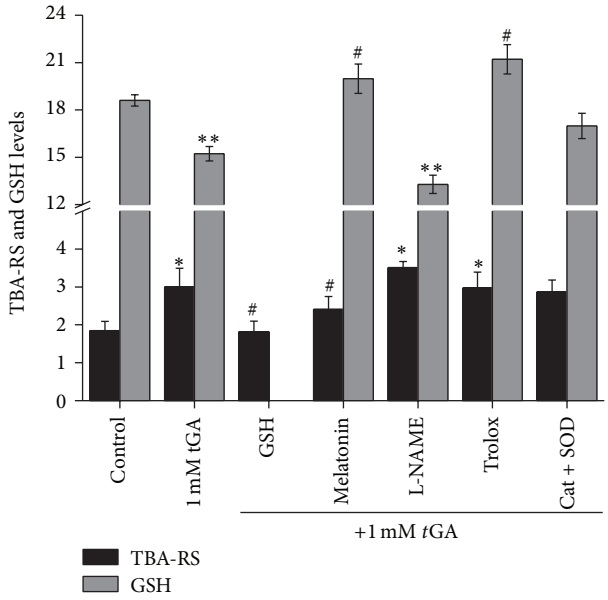

Figure 3: *In vitro* effect of the antioxidants GSH (100 μM), melatonin (100 μM), L-NAME (500 μM), trolox (5 μM), and catalase (Cat) plus superoxide dismutase (SOD; 10 mU·mL^{-1} each) on *trans*-glutaconic- (*t*GA-) induced alterations on thiobarbituric-acid reactive substances (TBA-RS) and glutathione (GSH) levels in cerebral cortex of 30-day-old rats. Cortical homogenates were preincubated for 15 min with the antioxidants before the addition of the 1 mM *t*GA. The experiments were performed in triplicate, are plotted as mean ± S.E.M. (n = 4-5), and are expressed as nmol·mg protein^{-1}. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to control group; $^{\#}P < 0.05$ compared to *t*GA group (Duncan multiple range test).

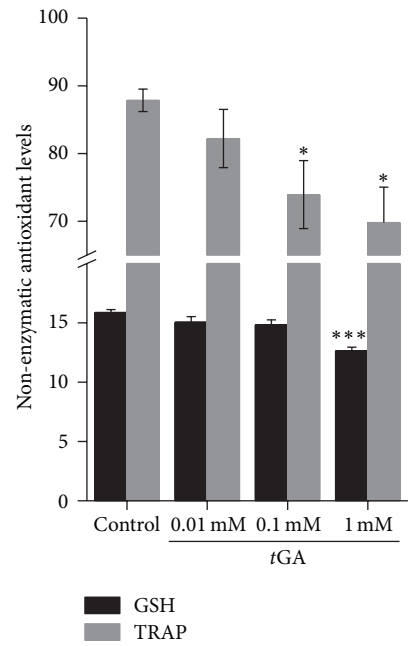

Figure 4: *In vitro* effect of *trans*-glutaconic acid (*t*GA) on the nonenzymatic antioxidant markers *total*-radical trapping antioxidant potential (TRAP) and glutathione (GSH) levels in rat cerebral cortex of 30-day-old rats. The experiments were performed in triplicate, plotted as mean ± S.E.M. (n = 5-6), and are expressed as nmol·mg protein^{-1}. $^{*}P < 0.05$ and $^{***}P < 0.001$ compared to control group (Duncan multiple range test).

therefore exposed a commercial solution of GSH (200 μM) to 1.0 mM *t*GA for 60 min in the absence of cortical supernatants. It was observed that this organic acid *per se* did not oxidize highly purified GSH (Table 2).

4. Discussion

*t*GA, or (*E*)-pentene-1,5-dioic acid, is an unsaturated C5-dicarboxylic acid that is found in patients affected by glutaric aciduria type I. Its role on this organic aciduria pathogenesis is under debate, since there are patients affected by glutaric aciduria type I who do not present *t*GA accumulation [3]. On the other hand, several *t*GA toxic effects have already been reported [29–34].

Herein we present data demonstrating that Na^+, K^+-ATPase activity, a crucial enzyme for maintaining ion homeostasis and membrane potential in cells [51], was inhibited by *t*GA at 0.1 and 1.0 mM concentrations. Inhibition of Na^+, K^+-ATPase activity has been described in various diseases, including cerebral ischemia [52, 53], neurodegenerative [54], and metabolic disorders [55, 56]. Furthermore, studies have indicated that Na^+, K^+-ATPase inhibition may result in cellular death by activating apoptotic cascades and neuronal damage probably due to amplification of potassium homeostasis impairment [57].

Considering that this enzyme is present at high concentrations in brain cellular membranes and consumes about 40–50% of the ATP generated [58], our next step was to evaluate the effects of *t*GA on some important bioenergetics parameters in order to identify whether ATP deficit could contribute to Na^+, K^+-ATPase activity inhibition caused by this organic acid. It was observed that *t*GA does not affect the activities of respiratory chain complex IV, creatine kinase, and ATP synthase. However, at this point, it should be mentioned that effects of *t*GA on glycolysis Krebs cycle enzyme activities, and the other respiratory chain complexes activities cannot be ruled out.

It was further investigated the *in vitro* effect of *t*GA on oxidative stress parameters, since Na^+, K^+-ATPase activity is highly dependent of critical sulfhydryl groups present in its catalytic site, rendering this enzyme activity highly susceptible to oxidative damage [59, 60]. We showed that *t*GA *in vitro* induced oxidative damage to proteins, as observed by sulfhydryl content decrease, and to lipids, since it increased TBA-RS levels and spontaneous chemiluminescence. Interestingly, carbonyl content was not altered by the presence of *t*GA in the incubation medium, suggesting that particularly sulfhydryl groups are prone to protein oxidative damage by this organic acid. On the other hand, light emitted in the spontaneous chemiluminescence assay mainly arises from oxidized lipids due to an increase in reactive oxygen or nitrogen species production, and TBA-RS levels reflects the amount of malondialdehyde formation, an end product of

membrane fatty acid peroxidation [61]. In this scenario, it is tempting to speculate that *t*GA oxidized lipids from cell and organelle membranes, which could lead to an impairment of membrane fluidity. Consequently, integral proteins such as Na^+, K^+-ATPase could also be affected, having their functioning impaired.

Furthermore, it was observed that GSH and melatonin fully prevented *t*GA-induced increased lipid peroxidation, since these compounds prevented TBA-RS increase elicited by this organic acid, whereas the combination of Cat plus SOD only partially prevented such effects. On the other hand, trolox (a hydrophilic vitamin E analogue) and L-NAME did not prevent *t*GA effect. It is speculated that lipid oxidation induced by *t*GA occurs due to an increase of reactive oxygen species.

We then assessed nonenzymatic antioxidant defenses by measuring GSH levels and TRAP and observed that both parameters were decreased by *t*GA *in vitro*. Since these parameters are suitable to evaluate the capacity of a tissue to prevent and respond to oxidative damage [35, 61, 62], it is likely that *t*GA impairs rat cortical antioxidant defenses. Moreover, *t*GA-induced effect on GSH levels was totally prevented by the free-radical scavengers melatonin and trolox and by the combination of Cat plus SOD, but not by L-NAME, suggesting that mainly peroxyl, alkoxyl, and hydroxyl radicals were involved in the reduction of GSH levels provoked by *t*GA. Considering that *t*GA was not able to oxidize a commercial GSH solution in the absence of brain supernatants in the incubation medium, it is unlikely that this organic acid is *per se* a direct oxidant agent, corroborating the idea that it probably provoked lipid and protein oxidative damage by increasing free radical generation.

Since the alterations elicited by *t*GA on TBA-RS and sulfhydryl content in whole homogenates, which contain the whole cell machinery, were not observed when this organic acid was supplemented to mitochondrial preparations, it is feasible that oxidative stress induced by *t*GA was probably mediated by cytosolic mechanisms. For instance, some putative mechanisms could involve xanthine oxidase, cytosolic NADPH oxidase, lysosomes, peroxisomes, and others [63].

Oxidative stress is an imbalance between the total tissue antioxidant (enzymatic and nonenzymatic) defenses and reactive species generation in cell leading to a deleterious cell condition [61]. In this study, we showed evidences that *t*GA induces oxidative stress in cerebral cortex *in vitro*, since it increased oxidative damage and decreased antioxidant defenses. It should be emphasized that the brain is highly susceptible to damage induced by increased reactive species generation, since it has low cerebral antioxidant defenses as compared to other tissues [64]. Furthermore, it may be also speculated that the inhibition of Na^+, K^+-ATPase activity elicited by *t*GA reported in the present study could be secondary to increased oxidation of critical sulfhydryl groups at the catalytic site of the enzyme.

Taken together, our results provide evidences that *t*GA is toxic to brain cells *in vitro*, by causing alterations in cell ion balance, and probably neurotransmission, as well as oxidative stress in rat cerebral cortex. We cannot at this point establish whether our data have a pathophysiological significance for glutaric aciduria type I. *t*GA accumulates in urine for excretion [65, 66], and excretion of this acid may become prominent, exceeding that of 3-hydroxyglutaric acid, during episodes of ketosis [4, 67]. Some studies indicated that the diagnostic relevance of *t*GA is limited [68], since *t*GA excretion in urine may be inconsistently found. However, it should be highlighted that even glutaric acid, known to be the major accumulating in glutaric aciduria type I, may be excreted at discrete or even normal concentrations (low excretor patients), as above mentioned.

5. Conclusion

Considering that the main signs and symptoms of affected patients are neurological [3], and in case the present findings are confirmed *in vivo* in animal experiments and also in tissues of patients accumulating *t*GA, it may be speculated that *t*GA toxicity could collaborate to the brain damage characteristic of glutaric aciduria type I affected patients.

Conflict of Interests

The authors declare that there are no possible conflict of interests.

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Review Article

Mitochondrial Mechanisms of Neuroglobin's Neuroprotection

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Neuroglobin (Ngb) is an oxygen-binding globin protein that has been demonstrated to be neuroprotective against stroke and related neurological disorders. However, the underlying mechanisms of Ngb's neuroprotection remain largely undefined. Mitochondria play critical roles in multiple physiological pathways including cell respiration, energy production, free radical generation, and cellular homeostasis and apoptosis. Mitochondrial dysfunction is widely involved in the pathogenesis of stroke and neurodegenerative diseases including Alzheimer's, Parkinson's, and Huntington's diseases. Accumulating evidence showed that elevated Ngb level is associated with preserved mitochondrial function, suggesting that Ngb may play neuroprotective roles through mitochondria-mediated pathways. In this paper we briefly discuss the mitochondria-related mechanisms in Ngb's neuroprotection, especially those involved in ATP production, ROS generation and scavenging, and mitochondria-mediated cell death signaling pathways.

1. Introduction

Neurological disorders including stroke, brain trauma, and age-related neurodegeneration are leading causes of death and severe long-term disability among adults in the USA and many other countries. The impact of these diseases is devastating in terms of loss of life, decreased life quality for survivors and their families. However, effective therapies are still lacking. To date the only approved therapy against stroke is the thrombolytic therapy using tissue plasminogen activator (tPA), which, however, is markedly limited due to its narrow time window and severe side effect such as hemorrhage [1, 2]. For neurodegeneration diseases such as Alzheimer's disease (AD), currently only symptomatic treatments are available, whereas disease-modifying treatments are still under development [3].

Recently, activation of endogenous neuroprotective mechanisms has emerged as a more promising strategy for the development of therapeutics against these neurological disorders. Neuroglobin (Ngb) is an oxygen-binding globin protein that was identified in 2000 [4]. Ngb has been demonstrated to be an endogenous neuroprotective molecule, as Ngb gene expression inversely correlated with the severity of histological and functional deficits in stroke and other related neurological disorders in both *in vitro* cell culture and *in vivo* animal models [5–9]. However, the underlying mechanisms of Ngb's neuroprotection remain largely not clarified [10, 11]. Mitochondria are key intracellular organelles that play prominent roles in energy metabolism, calcium homeostasis [12], redox signaling, reactive oxygen species (ROS) generation, and apoptosis-programmed cell death [13]. Neurons are particularly dependent on mitochondria because of their high energy demands [14], thus mitochondria dysfunction is correlated with a wide range of neurological disorders. Early studies have shown that Ngb expression is confined to metabolically active, oxygen-consuming cell types [15], therefore suggesting a functional relationship between Ngb and mitochondria. In this paper we briefly summarize the mechanisms of Ngb's neuroprotection that are related to mitochondria function and regulation.

2. Roles and Mechanisms of Ngb in Mitochondrial Function Related to Neurological Disorders

Accumulating evidence has demonstrated the neuroprotective roles of Ngb in a wide range of neurological disorders. For example, an *in vitro* study showed that antisense-mediated knockdown of Ngb rendered cortical neurons more vulnerable to hypoxia, whereas Ngb overexpression driven by CMV promoter in pcDNA vector, which yielded about 2-fold Ngb protein increase, protected cultured neurons against hypoxia [16]. Similar effect was observed in neuroblastoma cell line SH-SY5Y that Ngb overexpression by pEGFP-Ngb transfection (over 100-fold Ngb protein level increase, possibly due to low basal Ngb level) enhanced cell survival under anoxia or oxygen/glucose deprivation (OGD) [17]. In Ngb-transgenic animal studies, Ngb overexpression with over 2.7-fold Ngb level increase driven by CMV promoter [5] or a much higher Ngb level increase by chicken β-actin promoter [6] both ameliorated the severity of histological and functional deficits in mouse stroke models. Furthermore, antisense oligonucleotide-mediated endogenous Ngb knockdown deteriorated the outcome of focal cerebral ischemia in rats [8].

Ngb overexpression is also protective against beta-amyloid-induced neurotoxicity and Alzheimer phenotype *in vivo* in Ngb and APP (amyloid precursor protein) double-transgenic mice [7]. Furthermore, our study showed that Ngb overexpression (about 2.7-fold Ngb increase in Ngb-Tg mouse) protects retinal ganglion cells (RGC) against ocular hypertension and glaucomatous damage in mouse [9]. In these studies, mitochondrial functions including ATP production, ROS generation, and cell survival/death signaling have been significantly affected by the alteration of Ngb expression, which might be part of the mechanisms of Ngb's neuroprotection.

2.1. Ngb and Mitochondrial ATP Production. Although Ngb is an O_2-binding protein, O_2 transportation and supply to neurons may not be an important function of Ngb due to the high O_2 binding rate and low O_2 dissociation rate of Ngb, plus the relatively low level of Ngb protein in the brain (<1 μM) [18, 19]. Nevertheless, the O_2-binding property implies that Ngb may play a role in O_2 sensing and cellular energy metabolism, that is, ATP production. The molecular machinery required for ATP production is mitochondrial electron transport chain (ETC), which is an assembly of electron donors and acceptors, namely, complex I, II, III, and IV, that reside in the cristae and matrix of mitochondria. ATP production starts from citric acid cycle using pyruvate as the substrate. The coenzymes nicotinamide adenine dinucleotide (NAD) and flavin adenine dinucleotide (FAD) [20] are reduced during the citric acid cycle, yielding NADH and FADH2, respectively, which supply electron and energy to ETC. The electron is passed along the cascade of complex I–IV and protons are pumped across the inner membrane to establish a proton gradient. The mitochondria membrane potential (MMP) generated by oxidative phosphorylation (OXPHOS) across the inner membrane is a vital feature of mitochondria and plays essential roles in mitochondrial functions such as ATP and ROS production [21]. ATP is finally synthesized by ATP synthase when the chemiosmotic gradient drives the phosphorylation of ADP [22].

Stroke is associated with great ATP loss. The drastic reduction in blood flow within the ischemic core area leads to a shortage in O_2 and glucose supply and eventually reduced ATP production. ATP level of the ischemic core area falls markedly during the first 5 min or so of arterial occlusion and then stabilizes at levels approximately 15–30% of nonischemic tissue for at least the first 2 h of focal ischemia [23–25]. A similar pattern of energy alteration happens in the penumbral area, but the drop of ATP content is less severe. The ATP loss reflects the impaired mitochondrial oxidative metabolism [24] and is consistent with the increased lactate level in the penumbra area, since lactate is the fermentation product of pyruvate. Consistent with the reduced ATP level following ischemia, the capacity of mitochondria respiration is considerably decreased in both the core tissue and penumbra area [26, 27]. Reperfusion can transiently restore the mitochondrial respiratory function, which is then decreased at later times [26, 28]. The possible mechanisms that cause the failure of mitochondrial respiration include oxidative stress and cytochrome c (Cyt c) release from mitochondria to cytosol [29, 30].

Early experimental observations have suggested a close link between Ngb and mitochondria. Ngb is highly expressed in retina, and its distribution in photoreceptors correlates with the subcellular localization of mitochondria, that is, the plexiform layers and the inner segment which consume most of the retinal oxygen [31]. Later studies further showed the roles of Ngb in maintaining mitochondria function in response to insults. For example, Ngb overexpression by plasmid (pcDNA3-Ngb) transfection (2–8 fold Ngb protein increase) promoted cell survival against beta-amyloid toxicity and attenuated beta-amyloid-induced mitochondrial dysfunction in cultured PC-12 cells [32]. Ngb overexpression by chicken β-actin promoter in Ngb transgenic mouse also eliminated hypoxia-induced mitochondrial aggregation and neuron death [33]. Our lab has demonstrated that Ngb overexpression (about 2.7-fold Ngb protein increase in Ngb transgenic mouse) improved mitochondrial function in cultured mouse cortical neurons [34]. We showed that the rate of decline of ATP level was significantly ameliorated by Ngb overexpression at early time points after hypoxia/reoxygenation. In another study, elevation of Ngb expression by Ngb plasmid (pDEST40-Ngb) transfection (about 4-fold Ngb protein increase) in SH-SY5Y cell line led to a significant decrease in oxidative stress caused by H_2O_2 and an increase in the intracellular ATP level [35]. Furthermore, Ngb overexpression by plasmid (pDEST40-Ngb) transfection increased cell viability and inhibited hypoxia/reoxygenation-induced ATP loss in cultured human neuronal cells [36]. All of these data suggested a function of Ngb in preserving mitochondrial ATP production, either through preserving general mitochondrial function or specific influence on mitochondrial respiration; however, the exact mechanisms remain undefined.

Ngb protein was mainly localized in cytosol [37], which is supportive of its role in O_2 binding and sensing and involvement in cell signaling, for example, its possible function as guanine-nucleotide-dissociation inhibitor (GDI) [38]. However, accumulating evidence has revealed that Ngb is not only localized in cytosol, but also is closely associated with mitochondria. For example, Hundahl et al. detected subcellular localization of Ngb in neuronal cytosol, mitochondria and nucleus using immunocytochemistry and electron microscopy [39]. Additional evidence includes the observed colocalization of Ngb with neuronal nitric oxide synthase (nNOS), an indirect suggestion of Ngb's association with mitochondria since nNOS is present in both cytosol and mitochondria [40]. We recently performed yeast two hybrid assay to identify Ngb-binding proteins, and one of them is cytochrome c1 (Cyc1), a subunit of mitochondria complex III [41]. In support of this finding, our recent study clearly demonstrated that Ngb is physically localized in the mitochondria of primary cultured cortical neurons using Western blot, immunocytochemistry, and immunoelectron microscopy, although the mitochondrial Ngb is only a small portion (~10% of total Ngb), and the major portion of Ngb is in cytosol (~90%) [42]. In this study, the specificity of anti-Ngb antibody was validated by preabsorption with recombinant Ngb and secondary antibody alone to ensure the reliability of our results. Additionally, a very recent study further confirmed that Ngb is localized inside the mitochondria of retinal neurons [43]. The authors treated the mitochondria fractions with proteinase K (PK) and showed that significant amount of Ngb was insensitive to PK-induced proteolysis, therefore indicating that Ngb was truly integrated into the mitochondria. They further showed that Ngb knockdown can reduce the activities of mitochondrial complexes I and III [43]. These studies suggest that Ngb might play a role in ATP production through binding with respiratory complexes and affecting their activities.

Despite the previous evidence for Ngb's subcellular localization and association with mitochondria, Hundahl et al. [44] recently raised a critical point that a fully validated anti-Ngb antibody is essential in detecting Ngb's subcellular localization and function, and the most reliable validation method is to use Ngb-null mice, which is only available a short time ago [45]. In this regard, great cautions should be taken when trying to interpret the previously reported subcellular localization of Ngb detected by immunostaining. The physical and functional associations of Ngb with mitochondria may not be conclusive so far but should be further investigated using fully validated anti-Ngb antibody.

2.2. Ngb and Mitochondrial ROS Generation, Oxidative Stress. In addition to the putative role of Ngb in ATP production, it is possible that Ngb may also be involved in another aspect of mitochondria respiration, that is, ROS (reactive oxygen species) production. This hypothesis was supported by the fact that Ngb can bind to nitric oxide (NO) besides O_2 [46]. ROS was produced at the end of mitochondrial respiration when a portion of electrons leak to oxygen through complex I and III, generating superoxide radical anion (superoxide anion, $O_2^{\bullet -}$) [47]. The rate of superoxide anion production increases when the electron carriers harbor excess electrons, for example when oxidative phosphorylation is inhibited under pathological conditions. Superoxide anion can be converted to H_2O_2 by manganese superoxide dismutase (MnSOD) or CuZnSOD in the intermembrane space. A series of reactive species could be further derived from superoxide anion and H_2O_2, including reactive hydroxyl radical ($OH^{\bullet}$) and carbonate radical anion ($CO_3^{\bullet -}$), which altogether make up a family of reactive oxygen species (ROS) [48]. Another reactive species, peroxynitrite ($ONOO^-$), is produced by the reaction of superoxide anion with nitric oxide (NO) *in vivo* [49] or synthesized from hydrogen peroxide and nitrite [50] and can react with other molecules to make additional types of reactive nitrogen species (RNS). Rapid increase of ROS has been demonstrated in ischemic stroke, both during ischemia and reperfusion [51, 52]. ROS can initiate damage to nucleic acids, proteins, and lipids in both mitochondria and cytosol [53]. Proteins were damaged by ROS through oxidation or nitration of various amino acids side chains, generating glutamate and aminoadipic semialdehydes [54]. Moreover, several enzymes in the ETC have been shown to be inhibited by ROS, resulting in compromised ATP synthesis [55].

Under normal physiological conditions, ROS was maintained at a safe level by enzymatic or nonenzymatic antioxidant machineries. Major ROS-scavenging enzymes include the superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), and peroxiredoxins (Prx), while nonenzymatic antioxidants include ascorbic acid (vitamin C), tocopherols (vitamin E), and glutathione (GSH) [56]. Interestingly, ROS can act as signaling molecules to regulate the expression of antioxidant genes, providing a feedback regulation mechanism for ROS levels. For example, in cultured mouse muscle cell line, H_2O_2 exposure led to both gene transcription and enzymatic activity increase for antioxidant genes SOD, GPx, and CAT [57]. In addition, another study showed that antioxidant enzymes GPX and heme oxygenase-1 (HO-1) were upregulated by repetitive ischemia/reperfusion (I/R) in mouse heart, and this upregulation was dependent on ROS [58].

Ngb has been proposed to have a ROS scavenging function. Our previous study showed that Ngb overexpression (~2.7-fold increase in Ngb transgenic mouse) significantly reduced the generation of superoxide anion after hypoxia/reoxygenation in primary cultured mouse cortical neurons compared to wild-type-controls [34]. Additionally, other than O_2 binding, Ngb can also bind NO [59] and can protect against NO-induced neurotoxicity [60], suggesting that Ngb may neutralize the neurotoxic effects of reactive nitrogen species (RNS), which may be another mechanism of Ngb's neuroprotection. Furthermore, Ngb overexpression (~2.6-fold increase in Ngb transgenic mouse) is associated with significantly reduced ROS/RNS production and lipid peroxidation in the CA1 region and reduced CA1 neuronal injury in a mouse model of ischemia-reperfusion injury [61]. However, the mechanisms of this effect remain unclear. It might be ascribed to direct binding between Ngb and these reactive species or the interaction between Ngb and mitochondrial respiration chain components, such as mitochondrial complex III, which was demonstrated by our

recent studies [11, 42], but it could also be an indirect effect through a general improvement of mitochondrial function. A recent study using recombinant human Ngb (rhNgb) confirmed that Ngb has a direct antioxidant capacity and can efficiently scavenge a variety of free radicals, including the [2,2′-azino-di-(3-ethyl-benzthiazoline-6-sulfonic acid)] (ABTS) cation, superoxide anion, hydrogen peroxide, and hydroxyl radical [62]. The capacity of rhNgb in scavenging superoxide anion was less but comparable with equal amount of vitamin C (Vc) (from 2.5–12.5 μg/mL) but far superior than GSH. Furthermore, rhNgb's capacity in scavenging hydrogen peroxide was even higher than Vc at 10 μg/mL [62].

Antioxidant treatments have been investigated as potential therapeutics for stroke. The compounds with ROS scavenging capabilities such as lipoic acid and the glutathione precursor, NAC (n-acetyl-cysteine), were reported to reduce infarct volume in animal stroke models [63–65]. Other potent free radical scavengers include NXY-059, edaravone, and resveratrol, which have been proved to protect against stroke and brain trauma in animal models and are being tested in clinical trials [66–69]. Similarly, ATP restoration could also be targeted, maybe indirectly, for developing therapies against stroke. For example, nicotinamide was protective in a mouse model of ischemia reperfusion by providing a reserve of NAD+ and restoring ATP level [70]. Another study showed that histone deacetylase (HDAC) inhibitors protect mouse optic nerve from OGD-induced damage, partially through preserving ATP levels [71]. Additionally, coenzyme Q10 (CoQ10), the electron acceptor of complex I and II in ETC, is neuroprotective in a rat model of cerebral ischemia, probably through conserving ATP production and antioxidant property [72]. Ideally, it would be more efficient for a treatment to target more than one protection mechanisms. In this regard, since Ngb has multiple protective mechanisms including preserving ATP and scavenging ROS, the development of endogenous Ngb upregulation strategy might be a potentially more effective therapy against neurological disorders, which warrants further investigation [11].

2.3. Mitochondria-Mediated Cell Death Signaling and Ngb. Other than the roles of Ngb in preserving mitochondrial ATP production and scavenging ROS, Ngb has also been hypothesized to be a signaling molecule. For example, it was found that ferric human Ngb (met-Ngb) binds to the GDP-bound state of G protein α subunit (Gα) and exerts guanine-nucleotide dissociation inhibitor (GDI) activity [38]. Ferric Ngb inhibits the exchange of GDP for GTP, thus prevents the Gα subunit from binding to the G$\beta\gamma$ complex and activates the downstream signal transduction pathway, which is protective against oxidative stress [38, 73].

Mitochondria play key roles in cell death and survival signaling in response to injuries. The direct effectors of mitochondria membrane disruption include a group of prodeath Bcl-2 family proteins such as Bax, Bak, Bid, Bim, Bad, and PUMA, among others. Bax and Bak directly cause mitochondrial membrane disruption via channel formation in mitochondrial outer membrane. Bid and PUMA function in facilitating Bax and Bak channel formation, whereas Bad and Bim inhibit prosurvival Bcl-2 and Bcl-xL [74].

After mitochondria membrane disruption, proapoptotic molecules such as Cyt c and apoptosis-inducing factor (AIF) are released into cytosol and initiate caspase-dependent and -independent cell death, respectively. Released Cyt c in cytosol initiates the assembly of apoptosome by binding with Apaf 1, which in turn activates caspase 9. Caspase 9 goes on to activate caspase 3 and caspase 7 [75]. In caspase-independent apoptosis, AIF translocates into nucleus, where it initiates the chromosomes condensation and DNA fragmentation, the key step of apoptosis [76]. Other proapoptotic proteins released from mitochondria include procaspases, EndoG, Smac/DIABLO, and Omi/HtrA2 [77].

Mitochondrial ROS is also actively involved in cell death signaling pathways. The early evidence for the involvement of mitochondrial ROS in cell death arose from the study of TNF-α-induced cytotoxicity [78]. Mounting evidence later from studies using antioxidants or ROS-overproduction approaches has demonstrated the central roles of ROS in cell death signaling pathways, including programmed cell death (PCD) [79].

Ngb may play a regulatory role in neuronal signaling pathways in response to insults such as hypoxia. Khan et al. [33] have shown that Ngb overexpression in primary neuron culture from Ngb-transgenic mouse diminished hypoxia-induced microdomain polarization and mitochondrial aggregation, the early responses of neurons to death stimuli. Subsequently, Cyt c is released from mitochondria to cytosol, which is generally believed to be caused by mitochondrial permeability transition pore (mPTP) opening [80], followed by activation of caspase-dependent or -independent apoptosis pathways. Studies in our lab have shown that Ngb overexpression by AAV-Ngb transduction (about 4-fold Ngb level increase) is correlated with reduced mPTP opening and decreased Cyt c release in primary cultured mouse cortical neurons after oxygen/glucose deprivation (OGD) and reoxygenation (unpublished data). This suggests an inhibitory effect of Ngb in OGD-induced mPTP opening, which could be one of the mechanisms of Ngb neuroprotection. The reduced Cyt c release by Ngb overexpression may be partially attributed to the inhibition of mPTP opening by Ngb. However, other mechanisms may also be involved. For example, an *in vitro* biochemistry study showed that ferrous Ngb can rapidly reduce ferric Cyt c, converting ferric Cyt c to ferrous Cyt c [81]. Since Cyt c released from mitochondria is predominantly in the ferric form [82], and only ferric Cyt c, but not ferrous Cyt c, was reported to be active in initiating apoptosis [83], thus ferrous Ngb may prevent apoptosis initiation by reducing ferric Cyt c. Furthermore, computational modeling confirmed that the binding of Ngb to Cyt c and the subsequent redox reaction can block caspase 9 activation [84, 85]. It is possible that Ngb-Cyt c binding is also causative for decreased Cyt c release from mitochondria, which remains to be further investigated.

It should be emphasized that the redox state of Ngb not only is critical in regulating Cyt c-mediated apoptosis but also may have significant implications in other functions of Ngb. For example, ferrous Ngb is more favorable in NO scavenging than ferric Ngb [46]. It is therefore important to maintain the redox cycling of Ngb. Although an NAD(P)H-dependent

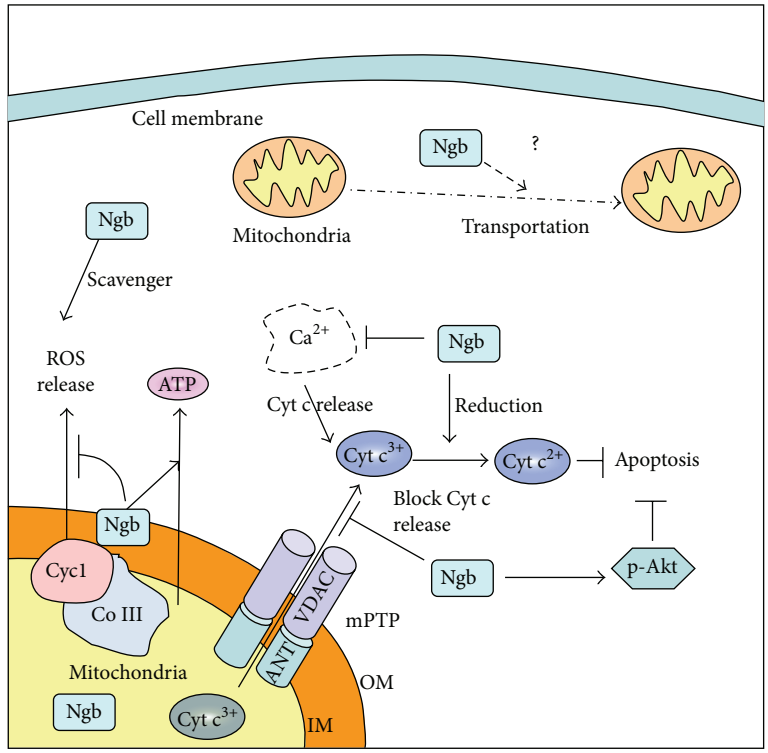

Figure 1: Potential mitochondrial mechanisms of Ngb neuroprotection. Ngb may be neuroprotective by preserving mitochondrial ATP production and scavenging ROS. Ngb may bind to VDAC, inhibit mPTP opening after OGD, and then block Cyt c release from mitochondria and the subsequent apoptosis. Ferrous Ngb can convert ferric Cyt c to ferrous Cyt c and thus prevent ferric Cyt c-induced apoptosis initiation. Ngb may ameliorate injury-induced calcium influx, therefore inhibiting calcium-induced amplification of Cyt c release and apoptosis. Ngb may also inhibit apoptosis by activating p-Akt. Finally, Ngb might have some effect in mitochondria transportation. OM: mitochondria outer membrane and IM: mitochondria inner membrane.

Ngb-reductase activity has been detected in human brain tissue homogenates [86], the enzyme(s) responsible for this activity has not been identified [87]. More advanced study about the Ngb reductase system is highly warranted in the future, which would greatly enhance our understanding of the regulation mechanisms of Ngb function.

Calcium is a key signaling molecule for many cellular functions including apoptosis [88]. A major source of cytosolic calcium is endoplasmic reticulum (ER) [89]. Calcium is involved in regulating mitochondrial morphology and release of proapoptotic proteins. Upon death stimuli, calcium can be released from ER and fluxed into mitochondria, resulting in mitochondria swelling and fragmentation and subsequent Cyt c release [90]. Interestingly, it was reported that Cyt c released from mitochondria at the initiation of apoptosis can translocate into ER and bind to inositol (1,4,5) trisphosphate receptor ($InsP_3R$); this binding leads to more calcium release from ER and increased cytosolic calcium level, which in turn results in coordinate release of Cyt c from all mitochondria and amplifies the apoptosis signal [91]. Ngb may also play a role in apoptosis by regulating cytosolic calcium level in response to death stimuli. It has been reported that Ngb overexpression by plasmid transfection (pDEST40-Ngb) significantly blocked hypoxia/reoxygenation-induced cytosolic calcium level increase in cultured neuronal cell lines [36]. This effect could be either through regulating membrane transporters or calcium release from ER, which is worth being further investigated.

Furthermore, Ngb may prevent apoptosis by indirectly modulating apoptosis regulators. For example, Ngb overexpression by plasmid (pDEST40-Ngb) transfection in SH-SY5Y cells protects against H_2O_2 injury by sustained activation of mito-K_{ATP} channel and Akt phosphorylation [35]. Phosphorylated Akt (p-Akt) inhibits the release of AIF and Cyt c, thereby inhibiting apoptosis [92], and thus the effect of Ngb in p-Akt may be another way of Ngb in regulating apoptosis. The possible involvements of Ngb in ATP production, ROS scavenging, and mitochondria-mediated apoptosis signaling were summarized in Figure 1.

A lot of studies have shown that strategies targeting apoptosis are neuroprotective in various animal stroke models [77]. For example, specific inhibitors for caspase-3 and caspase-9 ameliorated brain tissue loss and improved neurological outcomes in rat or mice stroke models [93–95]. It is reasonable to assume that a strategy targeting upstream regulators of mitochondria-mediated cell death pathway would provide better neuroprotection than targeting downstream regulators. One important upstream regulator is c-Jun N-terminal kinase (JNK). JNK can phosphorylate the scaffolding protein 14-3-3 and lead to the translocation of Bax

into mitochondria [96], which further results in inhibition of prosurvival Bcl-xL and Bcl-2 [97]. JNK inhibition using pharmacological inhibitor [96] or the small peptide inhibitor D-JNKI-1 [98] has shown prolonged neuroprotection for up to 14 days of reperfusion in animal models of focal ischemia. Based on the potential effect of Ngb in inhibiting apoptosis, targeting apoptosis inhibition by Ngb upregulation may be a more effective strategy for treatment of stroke and related neurological disorders, since Ngb may confer neuroprotection via multiple mechanisms including preserving ATP and scavenging ROS as well. A possible strategy for this purpose is to screen for endogenous Ngb upregulating compounds as potential therapies against brain injuries including stroke [11].

3. Molecular Interactions between Ngb and Mitochondria

Other than the proteins involved in normal mitochondrial functions, such as the protein components of electron transfer chain (ETC), mitochondria also harbor numerous proteins that are originally localized in cytoplasm but are translocated to mitochondria in response to death stimuli. Some of these are apoptosis signaling proteins like the prodeath Bcl-2 family members [74]. Additionally, emerging data show that mitochondria also host endogenous neuroprotective molecules such as Ngb, which might also contribute to the neuroprotection of Ngb [42].

We previously described that Ngb plays important roles in mitochondrial functions such as ATP production, ROS generation, and apoptosis signaling. To further dissect the molecular mechanisms of Ngb neuroprotection, our laboratory recently performed a screening for the protein interaction partners of mouse Ngb using yeast two-hybrid assay. We identified several Ngb-binding proteins, including Na/K ATPase beta 1, cytochrome c1 (Cyc1), ubiquitin C, voltage-dependant anion channel (VDAC), and a few more [41]. Interestingly, among these Ngb-binding proteins, VDAC and Cyc1 are mitochondrial proteins that are biologically important for neuronal function and survival. Cyc1 is a subunit of the mitochondria complex III, which is critical for mitochondrial ATP production and the generation of superoxide anion [99]. Cyc1 also plays pathological roles in response to oxidative stress [100] and regulates hypoxia-inducible-factor-1 (Hif1) activation induced by hypoxia. VDAC is a critical regulator of mitochondria permeability transition pore (mPTP) opening [101]. As a support for Ngb binding with mitochondrial proteins, our recent study clearly demonstrated that Ngb can be localized in mitochondria, and this localization was increased by OGD/reoxygenation [42]. Furthermore, Ngb overexpression is correlated with increased mitochondrial distribution of Ngb, suggesting the mitochondrial localization of Ngb may be important for neuroprotection. The mitochondrial localization of Ngb was further confirmed by another recent study in retinal neurons [43]. However, the detailed function of Ngb in mitochondria and its binding with mitochondrial proteins requires further investigation. The binding of Ngb with Cyc1 and complex III might be one of the mechanisms of Ngb's role in mitochondrial respiration and ATP production. Ngb binding with VDAC might have some effect in regulating mPTP, as we have found that Ngb overexpression can inhibit OGD-induced mPTP opening and subsequent Cyt c release (unpublished data). These potential mechanisms of Ngb neuroprotection were summarized in Figure 1.

4. Putative Involvement of Ngb in Mitochondrial Dynamics?

Mitochondria are remarkably dynamic organelles as they undergo repeated fission and fusion to interchange their contents. Mitochondria are also actively transported to subcellular sites where a high level of energy is required. Moreover, the quality of mitochondria is maintained through mitophagy in which defective mitochondria are selectively degraded. Dysfunction in mitochondrial dynamics is widely implicated in neurodegenerative diseases such as Parkinson's [102]. Since Ngb plays a role in mitochondrial energy production and can affect mitochondrial aggregation induced by hypoxia [33], it may be worth investigating the possible roles of Ngb in mitochondrial dynamics.

4.1. Brief Introduction of Mitochondrial Dynamics. Proper fission and fusion are required for maintaining normal mitochondrial function. Mitochondria fission is mediated by two key proteins, dynamin-related protein 1 (Drp1) and Fis1 [103], while mitochondrial fusion requires two families of dynamin-like proteins, Mfn1/Mfn2 and OPA1 [104]. Emerging evidence has linked mitochondria fission/fusion defects with neurodegenerative diseases [105, 106]. Inhibition of mitochondrial fission by knockdown of Drp1 or overexpression of Mfn1 mitigated NO-induced neuronal cell death, suggesting a role of mitochondrial fission in neuron death [106]. A recent study revealed that Drp1 and Opa1 were both upregulated in the ischemic penumbra but decreased in the ischemic core area after transient middle cerebral artery occlusion (tMCAO) in mice [107], suggesting a continuous mitochondrial fission and fusion in the salvageable ischemic penumbra.

It is crucial for mitochondria to transport to subcellular regions such as presynaptic terminals where high energy is demanded [108]. Mitochondria are transported along microtubule tracks, which are driven by ATP-dependent "motor" proteins, mainly the kinesin family members and dynein [109, 110]. Kinesins do not directly bind with mitochondria but through the adaptor proteins including TRAK1, TRAK2 and MIRO1, MIRO2 [111, 112]. Mitochondrial transport and spatial distribution in neurons are directly correlated with synaptic activity and neuron viability [113]. For example, hypoxia/reoxygenation in cultured cortical neurons impaired mitochondrial movement and morphology [114].

Proper and timely degradation of damaged and aged mitochondria is crucial for mitochondrial quality control. Dysfunctional mitochondria are cleared by mitophagy, a process that selectively eliminates mitochondria by autophagy [115]. The key mediators of mitophagy include Parkin

and PINK1 (PTEN-induced putative kinase 1) [116–118]. It was recently reported that ischemic preconditioning induced Parkin translocation to mitochondria and increased ubiquitination in cardiomyocytes, therefore promoting mitophagy [119]. Parkin knockout abolished the ischemic preconditioning-induced mitophagy and the cardioprotection effect as well [119]. Mitophagy is also involved in neurodegeneration. One example is in degenerating Purkinje neurons, a common feature of inherited ataxias in humans and mice, mitophagy was found to be abnormally enhanced, suggesting an important role of the properly regulated mitophagy in neuronal function [120].

4.2. Potential Involvement of Ngb in Mitochondria Dynamics? One early study showed that Ngb overexpression can eliminate hypoxia-induced mitochondrial aggregation [33]. Mitochondrial aggregation is an event upstream of Cyt c release from mitochondria during apoptosis, and mitochondria transportation is very likely to be involved in this process [121], and thus the study by Khan et al. [33] suggested that Ngb may also play a role in mitochondria transportation under hypoxic/ischemic conditions. As an indirect supporting evidence of Ngb's role in mitochondria transport, Antao et al. found that Ngb overexpression can ameliorate H_2O_2-induced actin condensation, suggesting a potential function of Ngb in maintaining cell membrane integrity [35]. Since actin cytoskeleton is required for short-term mitochondrial movement and mitochondrial immobilization in neurons [122], this data further suggested an indirect role of Ngb in mitochondrial transportation. Further studies will be valuable to investigate the function of Ngb in mitochondrial dynamics.

5. Summary

In summary, Ngb is an endogenous neuroprotective molecule against stroke and related neurological disorders, but the neuroprotection mechanisms remain largely undefined. Mitochondria are key players in neuronal death and survival determination in stroke and related neurological disorders, affecting multiple pathophysiological processes including energy metabolism, cellular homeostasis, and cell death signaling pathways. It has been demonstrated that Ngb preserves mitochondria ATP production, reduces ROS generation, and participates in mitochondria-mediated cell death signaling. However, the detailed molecular interactions between Ngb and mitochondrial proteins remain to be further elucidated, which will be beneficial in understanding the mechanisms of Ngb's neuroprotection and development of Ngb and mitochondria-targeted therapeutics against stroke and related neurological disorders.

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Review Article

Diversity of Mitochondrial Pathology in a Mouse Model of Axonal Degeneration in Synucleinopathies

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There is mounting evidence for a role of mitochondrial dysfunction in the pathogenesis of α-synucleinopathies such as Parkinson's disease (PD) and dementia with Lewy bodies (DLB). In particular, recent studies have demonstrated that failure of mitochondrial quality control caused by loss of function of the PTEN-induced kinase 1 (PINK1, PARK6) Parkin (PARK2) pathway may be causative in some familial PD. In sporadic PD, α-synuclein aggregation may interfere with mitochondrial function, and this might be further exacerbated by leucine-rich repeat kinase 2 (LRRK2). The majority of these findings have been obtained in *Drosophila* and cell cultures, whereas the objective of this paper is to discuss our recent results on the axonal pathology of brains derived from transgenic mice expressing α-synuclein or DLB-linked P123H β-synuclein. In line with the current view of the pathogenesis of sporadic PD, mitochondria abnormally accumulated in α-synuclein/LRRK2-immunopositive axonal swellings in mice expressing α-synuclein. Curiously, neither mitochondria nor LRRK2 was present in the swellings of mice expressing P123H β-synuclein, suggesting that α- and β-synuclein might play differential roles in the mitochondrial pathology of α-synucleinopathies.

1. Introduction

The high energy consumption in neural activities in brains makes mitochondria important in neurons as regulators of energy metabolism (as ATP producers), the NAD^+/NADH ratio, and endogenous production of reactive oxygen species (reviewed in [1]). Mitochondria are also pathologically important since abnormal mitochondria are associated with several neurological and neurodegenerative diseases, as well as with normal aging in brains [1–3]. Most importantly, mitochondrial dysfunction is a hallmark of the pathogenesis of Parkinson's disease (PD). This is because dopaminergic neurons in the substantia nigra are chronically exposed to oxidative stress caused by high levels of iron and autooxidation of dopamine. Thus, mitochondria are gradually damaged in aging, leading to enhanced oxidative stress and stimulation of neuronal dysfunction and degeneration. Mitochondria may also be acutely susceptible to environmental toxins such as drugs (e.g., 1-methyl-4-phenyl-1,2,3,4-tetrahydropyridine), herbicides, and pesticides [4]. Consistent with the idea that mitochondria are critical in neurodegeneration, complex I deficiency has been observed in postmortem PD brain and in peripheral tissues in PD patients, including platelets, lymphoblasts, muscle, and fibroblasts [5]. Furthermore, high levels of mtDNA deletions have been observed in dopaminergic neurons from the substantia nigra of postmortem human brains from elderly individuals and idiopathic PD patients [6].

Besides this circumstantial evidence, recent genetic studies of PD risk factors have unambiguously shown the central role of mitochondria in the pathogenesis of PD [7]. In early onset of familial PD, several autosomal recessive factors,

such as Parkin, Pink1, and DJ-1, are clearly involved in mitochondrial pathophysiology, and loss of function of these factors might be causative for PD. On the other hand, aggregation of α-synuclein (αS) may interfere with mitochondria in sporadic PD, and mitochondrial dysfunction might be further exacerbated by dysregulation of leucine-rich repeat kinase 2 (LRRK2). However, these findings have mainly been obtained in *Drosophila* and cell cultures, and the role of mitochondrial dysfunction in the mammalian brain is still elusive. In this paper, we briefly review recent progress in genetic findings for both familial and sporadic PDs, and we give a perspective on our recent findings on the axonal pathology in transgenic mice brains expressing αS or DLB-linked P123H β-synuclein (βS) [8].

2. Role of PINK1 and Parkin in Mitochondrial Pathology and Neurodegeneration in Familial PD

Since the discovery of αS in a case of rare familial PD, mutations in at least 16 PD-genetic loci (PARK) have been linked to the pathogenesis of PD [1]. Among these, characterization of products for several autosomal recessive genes, including PINK-1 (PARK6), DJ-1 (PARK7), and Parkin (PARK2), has greatly accelerated research on the mitochondrial pathology of familial PD. DJ-1 may function as a redox-sensitive chaperone as well as a sensor of oxidative stress and apparently protects neurons against oxidative stress and cell death [9]. Pink-1 encodes a putative serine/threonine kinase with a mitochondrial targeting sequence [10]. Parkin has been primarily characterized as an E3 ligase in the ubiquitin proteasome system that localizes predominantly to the cytosol, but this molecule also associates with the mitochondrial outer membrane [11].

Recent studies have revealed that PINK1 and Parkin may also play important roles in maintenance of mitochondrial integrity [12, 13]. PINK1 is rapidly degraded in healthy mitochondria but accumulates in membrane potential ($\Delta\Psi$m) deficient mitochondria, where it recruits Parkin to ubiquitinate the damaged mitochondria, which leads to fission and processing for degradation in the lysosome; this mechanism is referred to as "mitophagy." Thus, a prevalent view regarding the pathogenesis of some types of PD (Figure 1) is that PINK1 acts upstream of Parkin in a common pathway that appears to regulate mitochondrial quality and morphology and that dysregulation of the PINK1/Parkin pathway may result in a failure to remove damaged mitochondria, leading to enhanced oxidative stress conditions. These conditions may lead to secondary induction of aggregation of αS [14].

3. Role of α-Synuclein and LRRK2 in the Mitochondrial Pathology of Sporadic PD

Aggregation of αS may play a primary role in the pathogenesis of sporadic PD (Figure 1). In this regard, Hsu and colleagues were the first to show that accumulation of αS could lead to mitochondrial changes that exacerbate oxidative stress and

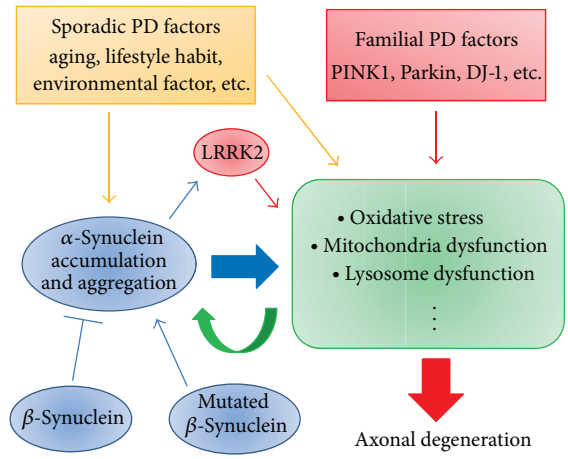

Figure 1: Schematics of the pathogenic mechanism of familial and sporadic α-synucleinopathies. Loss of function of the PINK1-Parkin pathway may be causative in the mitochondrial dysfunctions of some familial PD, while gain of functions of α-synuclein and LRRK2 may play a central role in the pathogenesis of sporadic PD.

neurodegeneration: overexpression of αS in hypothalamic neuronal cells resulted in formation of αS-immunopositive inclusion-like structures and mitochondrial changes accompanied by increased levels of free radicals, all of which were ameliorated by pretreatment with antioxidants such as Vitamin E [15]. More recently, Nakamura and colleagues showed that direct interaction of small oligomeric forms of synuclein with a membrane containing a mitochondrial lipid, cardiolipin, may be important in αS-expressing neurons *in vitro* and *in vivo* [16]. Notably, the effect was specific for synuclein, with more fragmentation of αS than of the β- or γ-isoforms, and was not accompanied by changes in the morphology of other organelles [16].

Evidence has accumulated to suggest that LRRK2 plays a crucial role in the pathology of both familial and sporadic α-synucleinopathies (Figure 1). LRRK2 is a large protein of 2527 amino acids that contain multiple functional domains, including leucine-rich repeats in the N-terminus, a Roc domain and a MAPKKK domain in the central region, and a WD40 domain in the C-terminus [17, 18]. Both αS and LRRK2 (PARK8) are involved in various pathologies such as impairment of cytoskeleton dynamics and dysregulation of the protein degradation system [19]. Mitochondria may also be a common target of αS and LRRK2, since immunoreactivities of LRRK2 are associated with mitochondria and with other membranous and vesicular intracellular structures, including lysosomes, endosomes, and transport vesicles [20]. Furthermore, it has been shown that LRRK2 regulates mitochondrial dynamics by increasing mitochondrial DLP1 (dynamin-like kinase 1) through a direct interaction with DLP1 [21].

In most autopsies, brains with the LRRK2 G2019S mutation have Lewy body pathologies, as opposed to LRRK2 non-G2019S carriers, but there is marked variability in pathological findings, even among carriers with identical mutations,

indicating that additional mechanisms may be critical [22]. Thus, αS and LRRK2 may cooperate with each other to produce diverse pathologies, including mitochondrial dysfunction, but the relationship between these two proteins might be more complicated.

4. Mitochondrial Dysfunction in Axonal Swellings in Mice Models of α-Synucleinopathies

Axonopathy is critical in the early stage of pathogenesis of neurodegenerative diseases, including α-synucleinopathies, and it has been shown that axonal swellings, including globules and spheroids, are characteristic features of axonopathies [23]. Since transgenic (tg) mice expressing αS or DLB-linked P123H βS are characterized by formation of similar axonal globules [24], they were histologically analyzed to compare the pathologies, including mitochondrial function, caused by accumulation of the two different types of synucleins [8].

A recent study suggests that dysfunction of the autophagy-lysosome pathway may be one of the contributors to axonal swellings. Failure to degrade subcellular materials or organelles at distal axons and/or nerve terminals or failure to export these materials by axonal transport has been shown to produce swollen nerve terminals. Such a mechanism might be involved in formation of both αS- and P123H βS-globules. In our recent study, αS-globules in brains of αS tg mice were characterized by autophagosome-like membranous elements and were immunopositive for various minor gangliosides, which is reminiscent of some types of lysosomal storage disease [8]. Consistent with this, lysosomal activity, as assessed by the activities of cathepsins B and D, was significantly decreased in brain extracts of αS tg mice compared with those from non-tg littermates [8]. Similar lysosomal dysfunctions have been observed for P123H βS-globules in brains of P123H βS tg mice [24]. These results suggest that downregulation of the lysosome degradation pathway may be a common mechanism leading to globule formation in αS and P123H βS tg mice.

In contrast to the lysosomal pathology, mitochondria accumulated specifically in αS-globules [8] (Figure 2(a)). Some αS-globules displayed clustering of mitochondria, while others had swollen mitochondria in the peripheral regions. Immunoreactivities of mitochondrial markers such as VDAC1 and cytochrome c were also found in αS-globules (Figure 2(b)). These results suggest that mitochondria clustering might become hyperactive in response to lysosomal dysfunction. Consistent with these findings, αS-globules were associated with oxidative stress, as assessed by staining of 4-HNE and nitrated αS. Conversely, no evidence of mitochondria was obtained in P123H βS-globules; hence oxidative stress (assessed by 4-HNE staining) was less than that in αS-globules [8] (Figure 2(c)).

Notably, LRRK2 was located in αS-globules and may be actively involved in the axonal pathology. The specific accumulation of LRRK2 in αS-globules may corroborate the idea that LRRK2 cooperates with αS in the axonal pathology. Indeed, it has been shown that LRRK2 is crucial for regulation of neurite formation and length [25]. Knockdown of LRRK2 led to long and highly branched neuritic processes, whereas constructs with increased kinase activity exhibited short simple processes in neuronal cultures (or transduced nigrostriatal models) [25]. More recently, LRRK2 R1441G BAC tg mice were shown to have various characteristic axonal pathologies, including large tyrosine hydroxylase-positive spheroid-like structures, dystrophic neuritis, and enlarged axonal endings [26].

5. Summary and Perspective: A Heterogeneity Problem?

As far as we are aware, there has been only one previous description of abnormal mitochondria in the axonal pathology in tg mice expressing prion promoter-driven αS [27]. Thus, the results of our studies in tg mice provide novel information showing that αS-globules derived from αS tg mice and P123H βS-globules derived from P123H βS tg mice have similar (e.g., lysosomal pathology) but distinct characteristics (e.g., mitochondrial alteration and LRRK2 accumulation).

P123H βS may represent a rare cause of familial DLB [28] but might reflect a process in which wild type βS becomes pathogenic in aging and may be involved in axonal pathology (e.g., formation of axonal globules) in sporadic cases of α-synucleinopathies [29, 30]. In support of this view, neurite accumulation of βS has been demonstrated in various synucleinopathies, including PD, DLB, and neurodegeneration with brain iron accumulation, type I [31, 32]. Furthermore, the gracile axonal dystrophy (gad) mouse, which has a naturally deleted ubiquitin carboxy-terminal hydrolase-1 (UCH-L1), is characterized by formation of spheroid bodies in nerve terminals, which are negative for αS but positive for βS or γS [33]. Based on the results of our transgenic studies, one may speculate that the swellings with βS, but not those with αS, might be generally free from mitochondrial pathology and LRRK2 accumulation. Taken together, these results suggest that the synuclein family of peptides might contribute to formation of axonal pathologies through diverse effects on mitochondrial and LRRK2 pathologies.

The diversity of pathology, including mitochondrial pathology, is a hallmark of the pathogeneses of neurodegenerative diseases. However, little attention has been paid to this key issue. We believe that this could be important not only from a mechanistic perspective but also therapeutically. It has generally been believed that mitochondria represent a potential target for development of disease-modifying therapies. Following this logic, clinical trials of antioxidants, including Vitamin E (α-tocopherol), have been controversial for treatment of neurodegenerative disorders [34, 35]. Based on our observations, this could be at least in part due to the heterogeneity of mitochondrial pathologies caused by the synuclein family of peptides. If this view is correct, there is a need to establish a new strategy to overcome such diversity of neuropathologies, including mitochondrial dysfunction, in the treatment of neurodegenerative diseases.

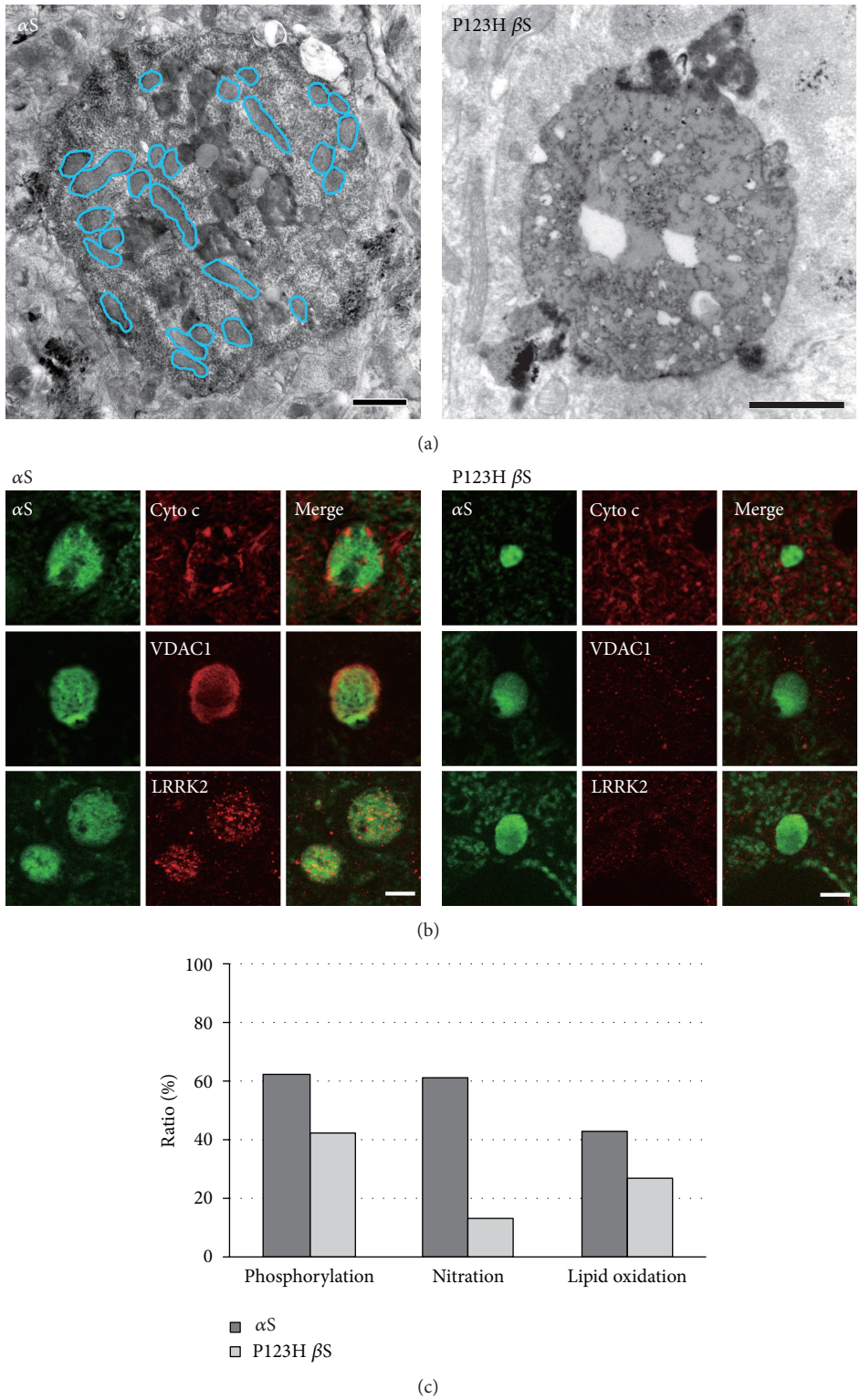

Figure 2: Axonal swellings in two types of synucleinopathy model mice. Immunoelectron microscopic analysis was performed using anti-αS. αS-immunopositive globules (a) were characterized by lysosomal pathologies such as myelinosomes (in αS mice) and lipid droplets (in P123H βS mice). Accumulation of mitochondria was occasionally observed only in αS mice (a: blue). Because P123H βS-immunopositive globules in brains of P123H βS tg mice were immunopositive for αS (~100%), double immunofluorescence analyses of αS tg mice (b: nine left panels) and P123H βS tg mice (b: nine right panels) were performed using αS as a globule identification. In αS tg mice, cytochrome c (b: upper panels) showed punctate patterns, while VDAC1 (b: middle panels) was located diffusely. In P123H βS tg mice, cytochrome c and VDAC1 were all immunonegative. Note that αS-globules were immunopositive for LRRK2 (b: lower panels), whereas P123H βS globules were negative for LRRK2. Quantification of data for phosphorylation of αS, nitration of αS, and lipid oxidation (immunoreactivity for 4-hydroxy-2-nonenal) in the αS-globules of both of synucleinopathy model mice (c). Scale bar = 1 μm for (a); 5 μm for (b). Please see [8] Mol. Brain for detailed information; (reprinted from Mol. Brain, Sekigawa et al., 5 : 34 with permission).

Conflict of Interests

The authors declare no financial conflict of interests.

Acknowledgments

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Research Article

Is Oxidative Stress in Mice Brain Regions Diminished by 2-[(2,6-Dichlorobenzylidene)amino]-5,6-dihydro-4*H*-cyclopenta[*b*]thiophene-3-carbonitrile?

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2-[(2,6-Dichlorobenzylidene)amino]-5,6-dihydro-4*H*-cyclopenta[*b*]thiophene-3-carbonitrile, 5TIO1, is a new 2-aminothiophene derivative with promising pharmacological activities. The aim of this study was to evaluate its antioxidant activity in different areas of mice central nervous system. Male Swiss adult mice were intraperitoneally treated with Tween 80 dissolved in 0.9% saline (control group) and 5TIO1 (0.1, 1, and 10 mg kg^{-1}). Brain homogenates—hippocampus, striatum, frontal cortex, and cerebellum—were obtained after 24 h of observation. Superoxide dismutase and catalase activities, lipid peroxidation and nitrite content were measured using spectrophotometrical methods. To clarify the 5TIO1's mechanism on oxidative stress, western blot analysis of superoxide dismutase and catalase was also performed. 5TIO1 decreased lipid peroxidation and nitrite content in all brain areas and increased the antioxidant enzymatic activities, specially, in cerebellum. The data of Western blot analysis did not demonstrate evidence of the upregulation of these enzymes after the administration of this compound. Our findings strongly support that 5TIO1 can protect the brain against neuronal damages regularly observed during neuropathologies.

1. Introduction

Human cells are constantly exposed to oxidants [1]. There is indisputable evidence that oxidative stress is involved in the pathogenesis of many complex human diseases, including diabetes; cardiovascular, neural, and psychiatric disorders; and in cancer progression [2–4]. These diseases have been associated with alterations in reactive oxygen species (ROSs) [5], reactive nitrogen species (RNSs), and nitric oxide (NO) [6].

Oxidative stress has been a common pathogenic mechanism underlying many major psychiatric disorders, such as anxiety, due to the intrinsic oxidative vulnerability of the brain [7]. Growing evidences have suggested correlation between the imbalance of antioxidant defense mechanism and anxiety-like behavior [8–14]. Therefore, the role and the beneficial effects of antioxidants against various disorders and diseases induced by oxidative stress have received much attention [15], and it becomes important to develop drugs that can possibly exert neuroprotective actions [16, 17].

Among the numerous antioxidants available to cells, sulfur compounds (including cysteine, methionine) and glutathione and their derivatives have been widely studied due to their antioxidant properties [18]. Thereby, sulfur containing heterocycles paved the way for the active research in the pharmaceutical chemistry [19]. Currently, thiophene derivatives

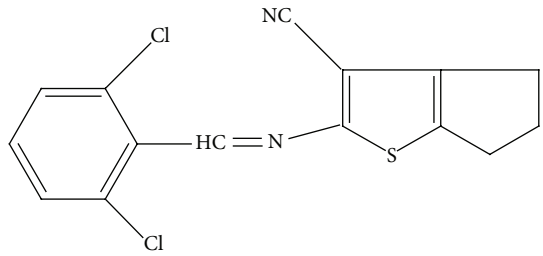

Figure 1: Chemical structure of 5TIO1.

have attracted the interest of the pharmaceutical industry due to its broad pharmacological spectrum, specially, as anxiolytic [20] and antioxidant agents [21].

In this context, the (2-[(2,6-dichlorobenzylidene)amino]-5,6-dihydro-4*H*-cyclopenta [*b*]thiophene-3-carbonitrile), also called 5TIO1 (Figure 1), is a new 2-aminothiophene derivative synthesized via two-step reaction, starting by the Gewald reaction, followed by the condensation with 2,6-dichlorobenzaldehyde, according to previous procedures [22]. 5TIO1 showed anxiolytic-like effect in plus-maze, and light/dark box tests did not change locomotor and coordination activities in open field and rotarod tests, respectively [23].

The aim of this study was to evaluate the antioxidant activity of 5TIO1 in different areas of the central nervous system (CNS) of adult mice by determination of lipid peroxidation level, nitrite content and enzymatic activities of catalase (CAT), and superoxide dismutase (Mn-SOD). To clarify 5TIO1's mechanism on oxidative stress, western blot analysis of Mn-SOD and CAT was also performed in mice brain homogenates 24 h of observation.

2. Materials and Methods

2.1. Animals and Experimental Procedures. Male Swiss adult mice (25–30 g) were used. All animals were maintained at a controlled temperature (25 ± 2°C) under 12 h dark/light cycle with free access to water and food. All experiments were performed according to the Guide for the Care and Use of Laboratory Animals, from the US Department of Health and Human Services, Washington DC, 1985. This protocol was previously approved by the Animal's Ethic Committee at Federal University of Piaui (n° 031/12).

5TIO1 was from Laboratory of Synthesis and Vectorization of Molecules of the State University of Paraiba. It is a yellow crystal, produced in 89% of yield, melting point 159-160°C, R_f 0.54 (n-Hex./AcOEt. 8.5 : 1.5). It was emulsified with 0.05% Tween 80 (Sigma Chem. Co., St. Louis, Mo, USA), dissolved in 0.9% saline (vehicle), and intraperitoneally administered at doses of 0.1, 1 and, 10 mg kg^{-1} (n = 7 per group). Control animals received 0.25 mL of 0.05% Tween 80 dissolved in 0.9% saline (n = 7).

For neurochemical assays, both 5TIO1 and control groups (n = 7) were killed by decapitation 24 h after treatment. Their brains were dissected on ice to remove hippocampus, striatum, frontal cortex, and cerebellum for determination of lipid peroxidation levels, nitrite content, and superoxide dismutase and catalase activities.

Drug dosages of 5TIO1 were determined from dose-response studies (data not shown) and from observation of dose currently used in animal's studies [23–25]. The doses of 5TIO1 used (0.1, 1, and 10 mg/kg) in the present study are not equivalent to those used by humans because rats have different metabolic rates.

2.2. Determination of Lipid Peroxidation and Nitrite Content. In all experimental procedures, 10% (w/v) homogenates of each brain's area were prepared for both groups. Lipid peroxidation in the 5TIO1 groups (n = 7 per group) and control animals (n = 7) was analyzed by measuring thiobarbituric acid reacting substances (TBARSs) in homogenates, as previously described by Draper and Hadley [26]. Briefly, the samples were mixed with 1 mL 10% trichloroacetic acid and 1 mL 0.67% thiobarbituric acid. Then, they were heated in boiling water bath for 15 mins, and then butanol (2 : 1, v/v) was added to the solution. After centrifugation (800 g, 5 mins), TBARSs were determined from the absorbance at 535 nm. The results were expressed in mmol min^{-1} μg protein^{-1}. To determine nitrite content of the control mice (n = 7) and 5TIO1 groups (n = 7 per group), 10% (w/v) homogenates was centrifuged (800 g, 10 mins). The supernatants were collected, and nitric oxide production was determined based on the Griess reaction [27]. Briefly, 100 μL supernatant was incubated with 100 μL of the Griess reagent (1% sulfanilamide in 1% H_3PO_4/0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride/1% H_3PO_4/distilled water, 1 : 1: 1 : 1, v/v/v/v) at room temperature for 10 mins. A_{550} was measured using a microplate reader. Nitrite concentration was determined from a standard nitrite curve generated using $NaNO_2$. The results were expressed in μM.

2.3. Determination of Superoxide Dismutase and Catalase Activities. Each brain area was ultrasonically homogenized in 1 mL 0.05 mM sodium phosphate buffer, pH 7.0. Protein concentration was measured by the Lowry's method [28]. The 10% homogenates were centrifuged (800 g, 20 mins), and the supernatants were used in superoxide dismutase and catalase assays. Superoxide dismutase activity in the 5TIO1 groups (n = 7 per group) and control animals (n = 7) was evaluated by using xanthine and xanthine oxidase to generate superoxide radicals [29]. They react with 2,4-iodophenyl-3,4-nitrophenol-5-phenyltetrazolium chloride to form a red formazan dye. The degree of inhibition of this reaction was measured to assess superoxide dismutase activity. The standard assay substrate mixture contained 3 mL xanthine (500 μM), 7.44 mg cytochrome c, 3.0 mL KCN (200 μM), and 3.0 mL EDTA (1 mM) in 18.0 mL 0.05 M sodium phosphate buffer, pH 7.0. The sample aliquot (20 μL) was added to 975 μL of the substrate mixture plus 5 μL xanthine oxidase. After 1 min, the initial absorbance was recorded, and the timer was started. The final absorbance after 6 mins was recorded. The reaction was followed at 550 nm. Purified bovine erythrocyte superoxide dismutase (Randox Laboratories, Belfast, Northern Ireland, UK) was used under identical conditions to obtain a calibration curve showing the correlation between

Table 1: Determination of the lipid peroxidation levels in hippocampus, striatum, frontal cortex, and cerebellum of mice treated with 5TIO1 at doses 0.1, 1, and 10 mg kg^{-1}.

Groups	TBARS (mmol min^{-1} μg protein^{-1})			
	Hippocampus	Striatum	Frontal cortex	Cerebellum
Vehicle	1.36 ± 0.14	1.34 ± 0.06	1.54 ± 0.04	1.43 ± 0.06
5TIO1 0.1	0.13 ± 0.03[a]	0.13 ± 0.01[a]	0.12 ± 0.02[a]	0.13 ± 0.03[a]
5TIO1 1	0.16 ± 0.01[a]	0.16 ± 0.01[a]	0.15 ± 0.01[a]	0.18 ± 0.03[a,b]
5TIO1 10	0.15 ± 0.01[a]	0.15 ± 0.02[a]	0.15 ± 0.01[a]	0.17 ± 0.03[a,b]

Male mice (25–30 g, 2 month-old) were intraperitoneally treated with doses of 5TIO1 (0.1, 1, and 10 mg kg^{-1}, $n = 7$, 5TIO1 groups) and the control animals with 0.05% Tween 80 dissolved in 0.9% saline ($n = 7$, Vehicle). Results are expressed as means ± SD for the number of animals shown inside in parenthesis. Differences in experimental groups were determined by two-tailed Analysis of Variance (ANOVA). [a]$P < 0.05$ compared to the control group (ANOVA and t-Student-Neuman-Keuls *as post hoc* test); [b]$P < 0.05$ compared to the 5TIO1 0.1 group (ANOVA and t-Student-Neuman-Keuls *as post hoc* test).

Table 2: Nitrite content in hippocampus, striatum, frontal cortex, and cerebellum of mice treated with 5TIO1 at doses 0.1, 1, and 10 mg kg^{-1}.

Groups	Nitrite (μM)			
	Hippocampus	Striatum	Frontal cortex	Cerebellum
Vehicle	95.86 ± 7.67	94.71 ± 3.35	76.57 ± 1.72	82.43 ± 1.99
5TIO1 0.1	5.11 ± 1.16[a]	6.00 ± 1.82[a]	6.49 ± 1.97[a]	6.93 ± 2.25[a]
5TIO1 1	6.36 ± 2.73[a]	4.98 ± 1.51[a]	6.19 ± 3.24[a]	4.81 ± 1.43[a]
5TIO1 10	7.14 ± 2.62[a]	9.04 ± 6.54[a]	8.50 ± 3.33[a]	9.04 ± 4.76[a]

Male mice (25–30 g, 2 month-old) were intraperitoneally treated with doses of 5TIO1 (0.1, 1, and 10 mg kg^{-1}, $n = 7$, 5TIO1 groups) and the control animals with 0.05% Tween 80 dissolved in 0.9% saline (n =7, Vehicle). Results are expressed as means ± SD for the number of animals shown inside in parenthesis. Differences in experimental groups were determined by two-tailed Analysis of Variance (ANOVA). [a]$P < 0.05$ compared to the control group (ANOVA and t-Student-Neuman-Keuls *as post hoc* test).

the inhibition percentage of formazan dye formation and superoxide dismutase activity and the results expressed as U mg protein^{-1}.

Catalase activity in the 5TIO1 groups ($n = 7$ per group) and control animals ($n = 7$) was determined by the method that uses H_2O_2 to generate H_2O and O_2 [30]. The activity was measured by the degree of this reaction. The standard assay substrate mixture contained 0.30 mL H_2O_2 in 50 mL 0.05 mM sodium phosphate buffer, pH 7.0. The sample aliquot (20 μL) was added to 980 μL substrate mixture. The initial absorbance was recorded after 1 min, and the final absorbance after 6 mins. The reaction was followed at 230 nm. A standard curve was established using purified catalase (Sigma, St Louis, MO, USA) under identical conditions. All samples were diluted with 0.1 mmol L^{-1} sodium phosphate buffer (pH 7.0) to provoke a 50% inhibition of the diluent rate (i.e., the uninhibited reaction). Results are expressed as mmol min^{-1} μg protein^{-1} [30, 31].

2.4. Western Blot Analysis. In the immunoblotting assay ($n = 4$ per group), hippocampus, striatum, frontal cortex, and cerebellum homogenates were mixed with protein loading buffer (roti-Load 1, Carl Roth GmbH, Karlsruhe, Germany) according to manufacturer's procedure and placed in a heating bath (95°C) for 5 mins. Proteins were separated using SDS-PAGE (gradient gels from 5% to 25%). The protein amount loaded per lane was 10 Ag. After separation, the proteins were stained with Coomassie Brilliant Blue or transferred to nitrocellulose paper, and unspecific protein binding sites were blocked with blocking buffer (Chemicon International, Hofheim, Germany). The blots were incubated overnight with the primary antibodies against (1) catalase (polyclonal, UBI, Lake Placid, NY, USA, 1 : 1.500) and (2) Mn-SOD (polyclonal, Assayama, Japan, 1 : 800), followed by incubation with horseradish peroxidase-conjugated secondary antibody (goat antirabbit IgG+ peroxidase, Boehringer Mannheim GmbH, Germany, 1 : 1.000). Immunoreactivity was visualized using the ECL detection system (Amersham Pharmacia Biotech, Buckinghamshire, UK). The Western blots did not show the B-actin band, since in our results corrections were made for the variability in protein loading in the gels.

2.5. Statistical Analysis. Results are expressed as means ± SEM for the number of experiments, with all measurements performed in duplicate. The Student-Newman-Keuls test was used for multiple comparison of means of two groups of data. Differences were considered significant at $P < 0.05$. Differences in experimental groups were determined by two-tailed analysis of variance.

3. Results

3.1. Determination of Lipid Peroxidation and Nitrite Content. Tables 1 and 2 show the 5TIO1's effects in lipid peroxidation and nitrite content, respectively, in hippocampus, striatum, frontal cortex, and cerebellum of adult mice. Statistical analysis indicated a decrease superior to 87% in lipid peroxidation and nitrite content in all brain areas for all doses when compared to the control group ($P < 0.05$). There was no dose-dependent effect of 5TIO1, and its scavenging activity was, statistically, the same in all areas for both assays.

Table 3: Catalase activity in hippocampus, striatum, frontal cortex, and cerebellum of mice treated with 5TIO1 at doses 0.1, 1, and 10 mg kg^{-1}.

Groups	Catalase (U μg of protein^{-1})			
	Hippocampus	Striatum	Frontal cortex	Cerebellum
Vehicle	14.22 ± 1.54	19.35 ± 0.46	22.51 ± 0.37	24.61 ± 0.42
5TIO1 0.1	33.62 ± 2.52[a]	20.39 ± 2.79	15.41 ± 2.45[a]	33.62 ± 2.52[a]
5TIO1 1	35.94 ± 2.57[a]	26.36 ± 4.93	29.91 ± 5.54[a,b]	35.94 ± 2.57[a]
5TIO1 10	34.98 ± 3.24[a]	30.52 ± 7.12[a,b]	34.03 ± 3.32[a,b,c]	34.98 ± 3.24[a]

Male mice (25–30 g, 2 month-old) were intraperitoneally treated with doses of 5TIO1 (0.1, 1, and 10 mg kg^{-1}, $n = 7$, 5TIO1 groups) and the control animals with 0.05% Tween 80 dissolved in 0.9% saline ($n = 7$, Vehicle). Results are expressed as means ± SD for the number of animals shown inside in parenthesis. Differences in experimental groups were determined by two-tailed Analysis of Variance (ANOVA). [a]$P < 0.05$ compared to the control group (ANOVA and t-Student-Neuman-Keuls *as post hoc* test); [b]$P < 0.05$ compared to the 5TIO1 0.1 group (ANOVA and t-Student-Neuman-Keuls *as post hoc* test); [c]$P < 0.05$ compared to the 5TIO1 1 group (ANOVA and t-Student-Neuman-Keuls *as post hoc* test).

Table 4: Superoxide dismutase activity in hippocampus, striatum, frontal cortex, and cerebellum of mice treated with 5TIO1 at doses 0.1, 1, and 10 mg kg^{-1}.

Groups	Superoxide dismutase (U μg of protein^{-1})			
	Hippocampus	Striatum	Frontal cortex	Cerebellum
Vehicle	2.24 ± 0.41	2.64 ± 0.49	2.20 ± 0.07	3.23 ± 0.35
5TIO1 0.1	3.56 ± 1.63[a]	2.86 ± 0.31[a]	2.52 ± 0.23[a]	3.95 ± 1.84[a]
5TIO1 1	3.14 ± 0.42[a]	2.54 ± 0.89	2.39 ± 0.44[a]	2.81 ± 1.96
5TIO1 10	2.16 ± 0.79	2.42 ± 0.38	2.17 ± 0.34	2.70 ± 1.34

Male mice (25–30 g, 2 month-old) were intraperitoneally treated with doses of 5TIO1 (0.1, 1, and 10 mg kg^{-1}, $n = 7$, 5TIO1 groups) and the control animals with 0.05% Tween 80 dissolved in 0.9% saline ($n = 7$, Vehicle). Results are expressed as means ± SD for the number of animals shown inside in parenthesis. Differences in experimental groups were determined by two-tailed Analysis of Variance (ANOVA). [a]$P < 0.05$ compared to the control group (ANOVA and t-Student-Neuman-Keuls *as post hoc* test).

3.2. Determination of Superoxide Dismutase and Catalase Activities. Table 3 shows the 5TIO1's effects on catalase activity in hippocampus, striatum, frontal cortex, and cerebellum of adult mice. a significantly increase was found in catalase activity in hippocampus (136.43, 152.74, and 145.99% in groups treated with 5TIO1 0.1, 1.0, and 10.0 mg kg^{-1}, resp.) and in cerebellum (36.61, 46.04, and 42.14% in groups treated with 5TIO1 0.1, 1.0, and 10.0 mg kg^{-1}, resp.) in comparison with the control group ($P < 0.05$). In striatum, groups treated with 5TIO1 0.1 and 1.0 increased catalase levels in 5.37% and 36.72%, respectively; but only 5TIO1 10 group augmented significantly (57.72%). In frontal cortex, the catalase activity increased 32.87% and 51.17% in the 5TIO1 1.0 and 10.0 mg kg^{-1} groups, respectively, if compared to the control group, 5TIO1 0.1 and 5TIO1 1.0 groups ($P < 0.05$). Only 5TIO1 at the dose of 0.1 mg kg^{-1} showed a decrease of 31.54% in catalase activity in frontal cortex compared with control group ($P < 0.05$).

Table 4 shows the 5TIO1's effects on superoxide dismutase activity in hippocampus, striatum, frontal cortex, and cerebellum of adult mice. 5TIO1 at the dose of 0.1 mg kg^{-1} showed an increase in superoxide dismutase activity in hippocampus (58.93%), striatum (8.33%), frontal cortex (14.54%), and cerebellum (22.29%) compared to the control group ($P < 0.05$). 5TIO1 at the dose of 1 mg kg^{-1} increased 40.18% and 8.64% SOD activity in hippocampus and frontal cortex, respectively, ($P < 0.05$). On the other hand, in the dose of 10 mg kg^{-1} upregulation of this enzyme activity was not observed.

3.3. Western Blot Analysis. In order to support the results obtained by Mn-SOD and CAT activities in the hippocampus, striatum, frontal cortex, and cerebellum, western blot analysis was also performed. After 24 h of the treatment with 5TIO1 (0.1, 1, and 10 mg kg^{-1}) the total Mn-SOD and catalase activities did not change in comparison with control group. Likewise, 5TIO1 groups did not affect the Mn-SOD and catalase mRNA or protein levels, as tested by immunoblot analyses of hippocampus, striatum, frontal, and cerebellum homogenates of mice treated with 5TIO1 (Figure 2).

Expressed in relative arbitrary units, protein: integrated density value (% control) of Mn-SOD for 0.1, 1, and 10 mg kg^{-1} 5TIO1 groups were, respectively, 89.42 ± 2.48%, 90.58 ± 2.48% and 98.46 ± 2.48% in hippocampus; 100.2 ± 2.09%, 108.4 ± 2.09%, and 97.9 ± 2.09% in striatum; 103.7 ± 3.71%, 106.6 ± 3.71%, and 113.2 ± 3.71% in frontal cortex; and 95.36 ± 4.61%, 111.4 ± 4.61%, and 95.54 ± 3.42% in cerebellum. Analogously, protein levels of catalase for 0.1, 1, and 10 mg kg^{-1} 5TIO1 groups were, respectively, 97.07 ± 2.23%, 97.41 ± 2.23%, and 95.78 ± 1.66% in hippocampus; 97.23 ± 2.24%, 97.05 ± 2.24% and 96.27 ± 1.66% in striatum; 95.94 ± 2.18%, 104.4 ± 2.18% and 95.52 ± 1.62% in frontal cortex; and 105.7 ± 2.38%, 98.34 ± 2.38%, and 98.8 ± 1.77% in cerebellum (Figure 2).

4. Discussion

Oxidative stress, which represents a loss of balance in oxidation-reduction reactions, can dramatically alter neuronal function and has been related to anxiety [7, 10]. Our

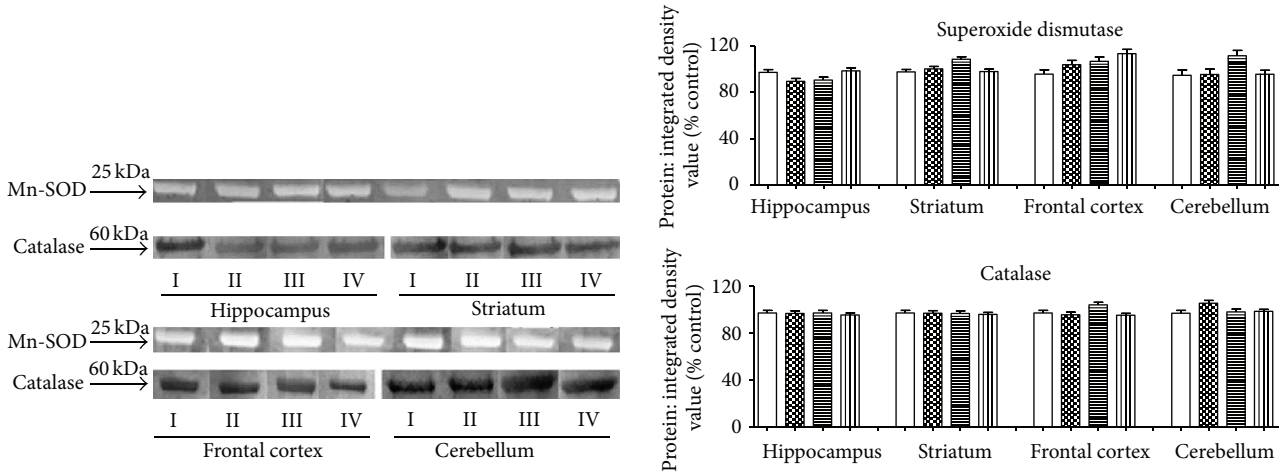

Figure 2: Superoxide dismutase and catalase proteins in hippocampus, striatum, frontal cortex, and cerebellum of mice treated with vehicle and 5TIO1. Male mice (25–30 g, 2 month-old) were intraperitoneally treated with 0.25 mL of 0.05% Tween 80 dissolved in 0.9% saline (vehicle) ($n = 7$, control group), and 5TIO1 groups with 2-[(2,6-dichlorobenzylidene)amino]-5,6-dihydro-4*H*-cyclopenta [*b*]thiophene-3-carbonitrile emulsified in vehicle (0.1, 1, and 10 mg/kg, $n = 7$, 5TIO1 groups). Animals were observed for 24 h and then killed. The protein amount per lane was 10 Ag. Legends: (I) control group; (II) 5TIO1 0.1 mg/kg group; (III) 5TIO1 1 mg/kg group; (IV) 5TIO1 10 mg/kg group. Data are expressed as means ± SD, converted to percentage of control (set at 100%) $n = 4$ experiments; Statistical analysis was by one-way ANOVA and *t*-Student-Neuman-Keuls test as *post hoc* test. There were no significant differences in protein expression of catalase after treatments, although there was a significant difference ($P > 0.05$).

results observed neurochemical alterations after administration of an anxiolytic-like compound, 5TIO1 [23].

The brain is believed to be particularly vulnerable to oxidative stress due to neuronal membrane lipids, rich in highly polyunsaturated fatty acids, undergo rapid lipid peroxidation, and [32–34] with a few antioxidants defenses, such as low activity of catalase and superoxide enzymes [7]. Then, it is important to determine oxidative stress biomarkers which show a change in a biological molecule that has arisen from ROS and/or RNS attack. This concept is applied equally to products derived from lipids, proteins and antioxidant consumption [35].

Lipid peroxidation in a tissue is an index of irreversible biological damage of the cell membrane phospholipid, which, in turn, leads to inhibition of most of the sulfhydryl and some nonsulfhydryl enzymes [3, 36]. As TBARS levels are closely associated with lipid peroxidation [37], our findings demonstrated that 5TIO1 decreased lipid peroxidation in all brain areas, indicating its antioxidant protection.

The nitrite content is also a fundamental oxidative stress' biomarker, since high levels of nitric oxide and their oxidative derivatives, as peroxynitrite, can be toxic, playing an important role in neurodegenerative diseases [38–40]. In normal conditions, there is a steady-state balance between the production of nitric oxide and metabolites (nitrite and nitrate) and their destruction by antioxidant systems [41]. Our results show a decrease in nitrite formation after 5TIO1's administration, suggesting that this substance can prevent RNS formation.

Lipid peroxidation and nitrite content assays suggest that 5TIO1 can be an antioxidant by ROS and RNS scavenging, respectively. These results can be related with 5TIO1's physicochemical properties. Previous research identified sulfur-containing antioxidants as those with the most beneficial therapeutic ratio [42]. 5TIO1 contain a five-membered ring made up of one sulfur as heteroatom. Thus, it is a thiophene derivative. The sulfur atom in this five-membered ring acts as an electron donating heteroatom by contributing with two electrons to the aromatic ring [43]. So, the thiophene and benzene rings turn 5TIO1 into an electron-rich compound, suggesting that 5TIO1's antioxidant property could be linked with the free radicals' conversion into stable products through electron donation.

In normal conditions, there is a balance between oxidative and nitrosative stress and antioxidant actions. The harmful effect of free radicals to the organism induces several defense mechanisms [6, 36, 44]. One of them is the catalytic removal by factors such as catalase, superoxide dismutase, peroxidase, and thiol-specific antioxidants [45]. General protocols are described to measure the antioxidant enzyme activity of superoxide dismutase, catalase, and glutathione peroxidase. The superoxide dismutase converts superoxide radical into hydrogen peroxide and molecular oxygen, whereas the catalase, and peroxidase convert hydrogen peroxide into water. Western blots, activity gels, and activity assays are various methods used to determine protein and activity in both cells and tissues depending on the amount of protein required for each assay [46]. In order to understand 5TIO1's effect under CAT and Mn-SOD activity, we combined a sensitive protein quantification method—Lowry's method [47]—and western blot analysis, which have been the standard method for analyzing differences in protein levels from cells and tissues to compare experimental conditions [48].

Catalase is an enzyme that effectively reacts with H_2O_2 to produce water and molecular oxygen and with H donors (methanol, ethanol, formic acid, or phenols) with peroxidase

activity. Catalase protects cells against H_2O_2 generated inside them. Although catalase is not essential to some cell types under normal conditions, it has an important role in the acquisition of tolerance to oxidative and nitrosative stress during the cellular adaptive response [6]. Superoxide dismutase is an important antioxidant enzyme that rapidly catalyzes the dismutation of superoxide anion ($O^{-\bullet}$) and thus acts as a first line of defense. In the case of superoxide dismutase deficiency or increased superoxide production, it reacts with nitric oxide to produce peroxynitrite ($ONOO^-$), which is a potent nitrosating agent that can cause direct damage to proteins, lipids, and DNA [49].

Our findings suggest higher catalase levels in cerebellum, frontal cortex, striatum, and hippocampus, indicating that this enzyme may exert a protective role in the first two brain regions more specifically over the other areas investigated in animals treated only with vehicle. Alper and coworkers [50] also observed that catalase activity is higher in cerebellum than the cerebral cortex of rodents. While superoxide dismutase activity is higher in cerebellum and striatum, indicating a better activity of this enzyme in these brain areas. So, the highest activity of both enzymes was observed in cerebellum in animals treated with vehicle, this result was similar to Fortunato and coworkers [51]. In our knowledge, this study presents, for the first time, results about oxidative stress in different mice brain areas, making it difficult to compare with other studies, since these data are unpublished for the areas investigated through the modulation of this new compound. So, this work motivates the interest in research and development of new drugs.

The superoxide dismutase activity was positively modulated by 5TIO1 at the dose of $0.1\,mg\,kg^{-1}$ in all brain areas, specially, in hippocampus and cerebellum. However, no significant changes were observed with $10\,mg\,kg^{-1}$. So, a dose-dependent process was not observed, suggesting that in higher doses of 5TIO1, there may be a saturation of this enzymatic activity. Furthermore, it should be noted that the $1\,mg\,kg^{-1}$ increased superoxide activity only in hippocampus and frontal cortex, indicating that 5TIO1 exerts its possible antioxidant effect by Mn-SOD modulation in these areas. Like it was previously mentioned, these unpublished results will contribute significantly to fill the gaps of the causes and consequences of oxidative stress modulated by thiophenic compounds.

In our studies, the total Mn-SOD and catalase activities did not change after treatment with 5TIO1. Data from Western blot analysis did not demonstrate evidence for the upregulation of antioxidant enzymes (Mn-SOD and CAT) after treatment. In addition, data confirmed our hypothesis that occurred only an increase in the enzymatic activities studied, since there was no change in protein contents of Mn-SOD and catalase in treated mice (hippocampus, striatum, frontal cortex, and cerebellum) after 24 h of treatment with 5TIO1.

Moreover, the increased enzymatic activity observed in our study may be due to its allosteric regulation, since no studies have looked at our Western blot analyses that did not change in gene expression of these enzymes. However, the increased activity of the enzymes studied may also have been induced by covalent modification (phosphorylation). Thus, we suggest that during the establishment of oxidative stress in brains, the phosphorylation of these enzymes may play a central role to control neuronal functions involved in the establishment of anxiety disorders.

We clearly showed that 5TIO1 decreased the lipid peroxidation levels and nitrite content and increased the antioxidant enzymatic activities. In our knowledge, these effects of 5TIO1 on oxidative stress observed in brain have not been reported before. Thus, these findings might have important implications for understanding the mechanism of neurodegenerative diseases, promoting new advances in the development of selective, and targeted antiepileptic, antidepressant, and anxiolytic drugs. 5TIO1 can protect the brain against neuronal damages regularly observed during neuropathologies. Further investigation of 5TIO1 effects against necrosis, apoptosis, and/or autophagy observed in brains disorders are in progress to confirm its antioxidant and neuroprotective effects.

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Review Article

Triggers and Effectors of Oxidative Stress at Blood-Brain Barrier Level: Relevance for Brain Ageing and Neurodegeneration

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As fundamental research advances, it is becoming increasingly clear that a clinically expressed disease implies a mixture of intertwining molecular disturbances. Oxidative stress is one of such pathogenic pathways involved in virtually all central nervous system pathologies, infectious, inflammatory, or degenerative in nature. Since brain homeostasis largely depends on integrity of blood-brain barrier (BBB), many studies focused lately on BBB alteration in a wide spectrum of brain diseases. The proper two-way molecular transfer through BBB depends on several factors, including the functional status of its tight junction (TJ) complexes of proteins sealing neighbour endothelial cells. Although there is abundant experimental work showing that oxidative stress associates BBB permeability alteration, less is known about its implications, at molecular level, in TJ protein expression or TJ-related cell signalling. In this paper, oxidative stress is presented as a common pathway for different brain pathogenic mechanisms which lead to BBB dysregulation. We revise here oxidative-induced molecular mechanisms of BBB disruption and TJ protein expression alteration, in relation to ageing and neurodegeneration.

1. Introduction

It has been extensively proven that a large array of neurological diseases and brain ageing itself are associated with oxidative stress [1–3]. Multiple sclerosis, stroke, brain tumours, and neuroinfections are conditions which associate both reactive oxygen species (ROS) aggression and blood brain barrier (BBB) impairment as well-proven pathogenic mechanisms. Relatively recent data documents BBB disruption not only in vascular or inflammatory brain diseases but in neurodegenerative disorders as well, where oxidative stress plays an important role in the pathogenic scenario [4, 5]. Whether oxidative damage is an important and early event in BBB alteration process, it is not established so far.

BBB is the interface between the periphery of circulatory system and central nervous system. The endothelial cells are the primary components of the BBB, responsible for the controlled environment of the brain. These cells lack fenestrations and have increased mitochondrial content, minimal pinocytotic activity, and a low number of caveolae. A 30–40 nm thin basement membrane is found between endothelial and neighbouring glial cells [6, 7]. At the BBB level, paracellular transport is restricted by tight junctions (TJs), allowing a peculiar "sealing" capacity. However, other cell types—pericytes, astrocytes, and neurons—are required for an accurate organization and function of BBB, not necessarily through direct contact with endothelial cells (Figure 1). *Pericytes* are the only cell type to intimately connect with endothelial cells, as they lay embedded within the endothelial basement membrane—a fibrillary structure of collagen IV, laminins, and proteoglycans. Pericytes strengthen the barrier integrity and their loss opens the BBB in an age-dependent

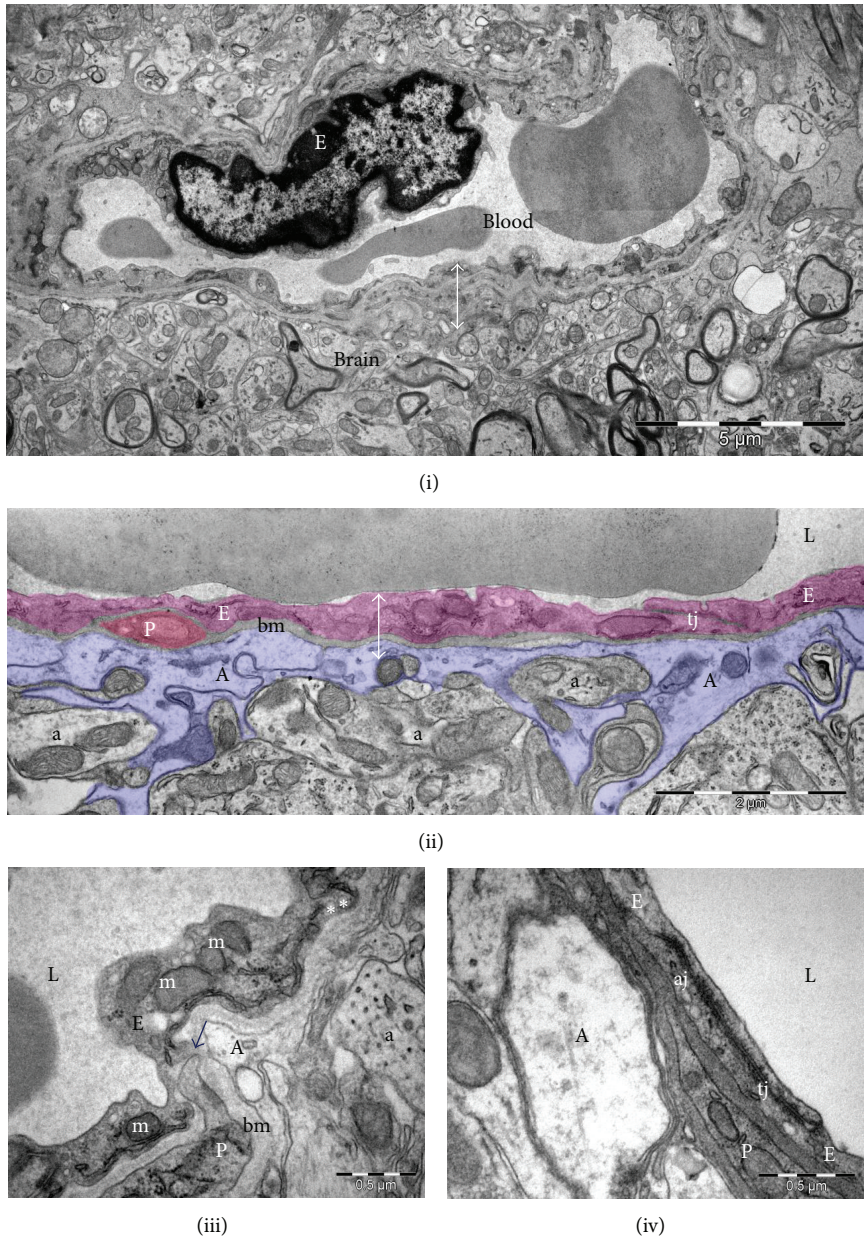

Figure 1: Ultrastructure of blood-brain barrier (↔). (i) Overall electron microscopy image of a cerebral capillary. (ii) Blood-brain barrier components: endothelial cells (E, purple coloured), pericytes (P, brown coloured), basement membrane (bm), and end-feet of astrocytes (A, blue coloured). (iii) Cerebral capillaries have nonfenestrated endothelial cells with numerous mitochondria (m) and rare pinocytotic vesicles (*). Direct membrane-membrane contacts (arrow) often occur between endothelial cells and pericytes. (iv) Tight (tj) and adherens (aj) junctions seal the continuous capillary endothelium. Cerebral capillary lumina (L), axons (a).

manner [8]. Recently, pericytes have been added to the classical *in vitro* 2-cell type model of BBB (coculture of endothelial and astrocytes) [9]. *Astrocytes* are separated from endothelial cells by the basement membrane around which they extend cell processes called end-feet. Hence, no cell-to-cell junctions are involved in this case, but the molecular flow of information between the two cell types is vital for BBB embryonic development [10] and adult life BBB integrity [11]. *In vitro* studies indicate astrocytes as regulators of TJ tightness and polarized distribution of transporters at endothelial level [12]. Furthermore, coculture of astrocytes with epithelial (other than brain endothelial) cells leads to induction of BBB properties [13, 14] and this is now a common practice in *in vitro* BBB models. *Neurons* are not morphologically involved in BBB formation, but numerous myelinated and nonmyelinated axons are found in close proximity of brain capillaries. The current model of brain homeostasis is based on the neurovascular unit, comprising cellular elements of BBB along with the neurons to which they connect into a functional network [15].

The BBB is functionally characterized by highly restrictive transbarrier transport, due to sealing of paracellular pathway

by TJs and low transcytotic traffic through caveolae [10]. Transport of virtually all nondiffusible, nonlipidic molecules is controlled through specific carriers present on both sides of endothelial cells, in a time- and concentration-dependent manner. Consequently, quantification of large protein (albumin, dextran) traffic from blood to nervous tissue is an indicator of "tightness" of cell-to-cell endothelial junctions. A barrier "tightness" or "leakiness" is given by expression and molecular organization of different TJ species, which in the case of BBB are unique. In fact, clusters of densely packed molecules form the interendothelial junctions that contain specific components for both adherens and tight junctions.

The TJ is an intricate macromolecular complex [6, 16] formed by:

(i) integral membrane proteins: claudins (claudin-1, 2, 3, 5, 11, 12, 18), MARVEL (the myelin and lymphocyte protein (MAL) and related proteins for vesicle trafficking and membrane link) proteins (occludin, tricellulin/marvelD2 and marvelD3), junctional adhesion molecules (JAMs), endothelial cell selective adhesion molecule (ESAM), and so forth;

(ii) cytoplasmic proteins: zonula occludens proteins (ZO-1, 2, 3), afadin (AF-6), calcium/calmodulin dependent serine protein kinase (CASK/LIN-2) from membrane-associated guanylate kinase proteins (MAGUK family), actin binding protein (cingulin), small G-proteins (Rho, Rac, Cdc42), ZO-1-associated nucleic acid binding protein (ZONAB), cyclin-dependent kinase-4 (CDK-4), and so forth;

(iii) actin cytoskeleton.

For a long while, the TJ complexes were considered static structures but new data support a dynamic model of barriers and also suggest that regulation of TJ openings and closings may provide sensitive means to modulate barrier function without changing protein expression [17].

In vivo and *in vitro* molecular studies of TJ proteins show that alteration of BBB in neurodegeneration usually cooccurs with modified TJ protein expression. As already mentioned, molecular organization of TJs is responsible for the "leakiness" of the BBB, which physiologically is more tight than other epithelial sites, a fact illustrated by a transepithelial resistance 50 times higher than other epithelia [18]. This peculiarity is not only a consequence of protein composition, but also of cellular sensitivity to microenvironment [19].

Although deleterious effects of oxidative stress on neuronal and glial populations in healthy aged and dementia brains are well stated, less is known about its consequences on endothelial cells, BBB, and tight junction protein expression. Based on the functional concept of neurovascular units, the presumption of BBB alteration in a neuronal/glial oxidative stress microenvironment is a plausible theory, possibly involving oxidative stress-related molecules. Furthermore, age is a certain inductor of BBB alteration, as briefly discussed in the following section; therefore, occurrence of oxidative stress in early stages of neurodegeneration might initiate tight junction impairment.

However, *in vitro* experimental setups used to decipher TJ protein alterations in oxidative environment are very variable in terms of culturing conditions. Most authors acknowledged the need to replicate the results by glial-endothelial cocultures, use of conditioned media, or *in vivo* conditions [20, 21]. Thus, the results are sometimes conflicting or even contradictory (see Table 1).

2. Endothelial Ageing and Tight Junctions in Aged Blood-Brain Barrier

Ageing is an independent factor associated with endothelial dysfunction even in the absence of other cardiovascular risk factors [22]. Aged endothelium showed a defective response to certain vasodilators [23], related to reduced NO-mediated dilatation [24], oxidative stress, and vascular inflammation [25]. Brain vasculature in aged animals showed predisposition to increased oxidative stress, activation of NADPH oxidase [26], and of nuclear enzyme poly(ADP ribose) polymerase (PARP) [27]. Ageing is also associated with increased expression of proinflammatory cytokines in vascular endothelial cells from healthy humans [28] which further favours a prooxidative state. Aged brains show increased matrix metalloproteinase- (MMP-) 2 activity and increased MMP-9 expression upon trauma, along with altered BBB repair mechanisms [29].

Molecular studies of BBB impairment in normal ageing explore only superficially the complexity of underling events, usually addressing only few proteins expression and distribution in one experimental paradigm. Results are generated in animal models and convey towards the conclusion that ageing leads to lower tight junction protein expression and a "leaky" BBB status [30–32].

Cumulative damage to mitochondria and mitochondrial DNA caused by ROS accounts for the mitochondrial theory of aging. In Figure 1(iii), we show a typical EM image of rat BBB, where mitochondria are clearly observed in both endothelial and glial cells. EM assessment of BBB in aged laboratory animals might offer a clue about mitochondria content and morphology in different BBB cell types, considering the large number of mitochondria in cerebral endothelium [8]. However, to our knowledge, there are no reports exploring mitochondrial alteration in aged brain endothelia so far.

Age seems to be a BBB frailty-inducing factor, as aged laboratory animals are more prone to brain oedema formation, ischemic injury, neuronal apoptosis following contusion and earlier onset of neuroinflammation than young littermates [33]. In the same manner, BBB dysfunction in old age was shown to be closely related to white matter lesions and lacunar infarctions [34].

There are several studies to address BBB permeability in aged animals (reviewed in [35]), in different experimental models, such as reproductive senescent mouse females [36], or senescence-accelerated mice [37]. They all led to the same conclusion that BBB permeability is altered in aged brain. Nevertheless, how and why this impairment occurs is not clear, and data regarding occludin and claudins expression and distribution in aged brain are scarce.

Table 1: Expression of tight junction proteins in various cellular models of oxidative stress.

BBB *in vitro* model	Type of experiment	Special conditions	Documentation of BBB permeability increase	Tight junction proteins alterations	Reference
BBMEC monolayers	Hypoxic stress	Glial conditioned-media treatment	Permeability studies with [14]-sucrose	Claudin-1 shows a significant increase following hypoxic stress	[21]
BBMEC monolayers	Hypoxia/reoxygenation	none	TEER measurements and [14]-sucrose transfer across the barrier	Significant increase in expression of occludin, ZO-1, and ZO-2	[131]
Rat GP8/3.9 cells	ROS generating environment by a mixture of xanthine oxidase and hypoxanthine	—	TEER FITC-dextran permeability across the barrier	Decrease of occludin and claudin-5 expression after exposure to oxidative environment	[103]
PBMEC	Hypoxia	Coculture with astrocytes/C6 glioma cells	TEER and passage of [3H]inulin	Decreased ZO-1 immunoreactivity at regions of cell-cell contact	[43]
BMVECs on a 8.0 μm matrigel-based insert	MMPs aggression	Coculture with leukemic cells	40 kDa dextran-FITC flux by flow cytometry analysis	Downregulation of ZO-1, claudin-5, and occludin	[132]
hCMEC/D3 (immortalized human BEC line)	Aβ peptides treatments	—	permeability to the paracellular tracer 70 kD FITC-dextran	Decrease in the occludin level, whereas claudin-5 and ZO-1 were unaffected	[85]
Human BMVEC	Exposure to ROS	—	TEER and monocytes migration studies	Decreased occludin and ZO-1 total content, whereas claudin-5 expression depended on the type of stressor used	[91]

BBMEC: bovine brain microvessel endothelial cells.
TEER: transendothelial electrical resistance.
PBMEC: primary cultures of porcine brain-derived microvascular endothelial cells.
BMVEC: brain microvascular endothelial cells.
ROS: reactive oxygen species.
MMPs: matrix metalloproteinases.

An overall assessment of BBB integrity can be obtained by immunohistochemistry methods, which show the albumin or immunoglobulin abnormal presence in the brain parenchyma, by elevated CSF albumin to plasma albumin ratio, or by increased perivascular enhancement at brain magnetic resonance imaging (MRI). In human aged brains serum protein immunostaining shows a "leaky" BBB which, interestingly enough, is not associated, at molecular level, with significant changes in endothelial expression of TJ proteins [38], and BBB leakage seems to show a wide individual variation [39].

3. Oxidative Stress Inducers at BBB Level

Although oxidative stress has been extensively studied in central nervous system different injuries, not enough data is available yet about its triggers and effectors on BBB. To some extent, as a result of vicious circles generated at molecular levels, it is difficult to separate or clearly indicate the cause and the effect of oxidative stress on BBB.

3.1. Hypoxia. Hypoxia is probably the best documented pathological process that induces BBB opening. It can be studied *in vitro*, by exposure of cell cultures to a mixture of hypoxic gas (95% N_2/5% CO_2; 99% N_2/1% O_2) or to pure NO_2 and *in vivo*, by exposure of animal models to low oxygen air (6–8% O_2) or ligation of cerebral arteries. Permeability may be further assessed by abnormal transport across BBB of large molecules, such as albumin, labelled dextrans, immunoglobulins, or labelled monocyte migration. Proposed mechanisms for altered permeability include increased exposure to free radicals [40] and/or inflammatory cytokines, such as IL-6 and TNF-α [41], activation of MMPs and downregulation of their tissular inhibitors (TIMPs) [42] and induced NOS expression [43], all of them ultimately reflected in the levels of tight junction protein expression.

Opening of BBB in hypoxia/reoxygenation studies is well confirmed in animal models and occurs earlier in aged animals versus young ones [44–46], following a biphasic pattern documented *in vivo* by MRI studies [47, 48].

Hypoxia is known to change BBB permeability and TJ protein expression in cerebral capillaries [49]. Lipid raft-associated occludin oligomeric assemblies were shown to be internalized during hypoxia [50] and ZO-1 and occludin sub-cellular localization correlated with increased paracellular permeability [51]. Reports of claudins expression

during ischemia/reperfusion experiments are, however, contradictory. This can be at least partially explained by different experimental paradigms used in different studies.

3.2. Inflammation, Proinflammatory Cytokines, and Chemokines. Both normal ageing and neurodegenerative disorders are characterized by a degree of neuroinflammation [52]. In the CNS, proinflammatory cytokines are overexpressed as a result of intense/prolonged oxidative stress and are considered marks of neuroinflammation, a well-proven pathogenic mechanism in Alzheimer's disease (AD) and other neurodegenerative conditions. Cytokines, such as IL-1, IL-6, and TNF-α, are increased in plasma and CSF of acute ischemic stroke patients and seem to be associated with increased risk of worsening or recurrence [53–55]. High levels of plasma IL-6, associated with high CRP, seem to be associated with risk of vascular dementia (VaD) [56], and increased levels of IL-6 and TNF-α are also associated with senescence and frailty in old age [57]. Along with other cytokines, growth factors and plasma proteins, IL-1α, IL-8, and TNF-α were proposed as biomarkers able to distinguish AD from controls [58, 59]. A TNF-α inhibitor is reported to improve aphasia in demented patients [60, 61]. Proinflammatory cytokines are important regulators of MMPs and TIMPs expression [41]. In particular, TNF-α-mediated stimulation of MMP expression and synthesis is considered to be an important link between the proinflammatory cytokine network and the local increase of MMP proteolytic activity [41].

Along with TNF-α, IFN-γ has also been repeatedly reported to modify tight junction barrier function in various polarized epithelia [62–64]. Treatment of cell culture with IFN-γ led to decreased protein expression and relocalization of ZO-1 and occludin, occludin and JAM-A [65], in a time and dose-dependent manner. According to Scharl et al., AMP-activated protein kinase (AMPK) in concert with other signals induced by IFN-γ, seems to play a role in mediating reduced epithelial barrier function [66]. Chemokines CCL-2 and CXCL-8 are also reported to be responsible for increased BBB permeability, CCL-2 being produced by both astrocytes and endothelial cells in the late phase of hypoxia/reoxygenation-induced BBB disruption [67].

Some of these cytokines and chemokines appear to exclusively affect the paracellular permeability (e.g., IL-1β and CXCL8), while some others predominantly act to increase transcellular permeability (e.g., TNF-α) [18]. Experimentally induced peripheral inflammation also increases BBB permeability and leads to decreased occludin expression [68] and increased expressions of claudin-3 and 5 [69].

A common experimental animal model used for BBB breakdown in neuroinflammation is the experimental autoimmune encephalomyelitis (EAE), used for the study of multiple sclerosis (MS). An important aspect in the etiopathogeny of MS is loss of immune-privileged environment of the brain and extravasation of leukocytes across the BBB, through chemokine-chemokine receptor interaction. Use of mice with targeted deletions of certain chemokines and their receptors revealed a role for CCL2 and CCR2 in the induction of EAE via effects on infiltrating monocytes [70]. CXCL12 relocation in MS and EAE at the level of the postcapillary venules appears to strongly correlate with the perivascular infiltration of T-cells [71]. CCL19 protein levels in lysates of brain tissue as well as CSF samples were found to be elevated in MS [72]. Regarding the molecular alterations of BBB TJ proteins, in EAE affected mice were noted a coincident loss of both claudin-5 and occludin normal junctional staining patterns [73] and loss of claudin-3 expression that correlated with immune cell infiltration into the CNS and BBB leakiness [74]. Interestingly, although increased expression of claudin-1 in a transgenic EAE mouse model sealed the BBB for paracellular traffic of large molecules, it did not seem to influence immune cell trafficking across the BBB, nor the severity of evolution of the disease [75].

3.3. Beta-Amyloid (Aβ) Peptides and Cerebral Amyloid Angiopathy. AD-related BBB disruption is documented in both animal models [76, 77] and human brains [78]. Aβ peptide, one of AD major pathogenic operators, is considered a strong redox active agent capable of generating peroxide in the presence of metals [79]. Soluble Aβ species have been linked to decreased cytochrome C oxidase activity in the Tg2576 mouse model of AD and were shown to enter the mitochondria and cause a signalling amplification that inactivates SOD-2 and generates additional free radicals [80]. Aβ peptides are known to affect brain small blood vessels by inducement of cerebral amyloid angiopathy (CAA), found in 90% of AD patients and 50% of 90-year-old population [81]. Aβ-loaded capillaries, surrounded by NADPH oxidase-2 (NOX-2)-positive activated microglia are characterized by a dramatic loss of occludin, claudin-5, and ZO-1. Importantly, same brain sections showed abundant vascular expression of the Aβ transporter receptor for advanced glycation end-products (RAGE) [82], that was recently demonstrated to function as a signal transducing cell surface receptor for Aβ1-42, to induce ROS generation from NADPH oxidase [83]. Aβ1-40 perivascular deposition was reported to decrease expression of TJ proteins claudin-1 and claudin-5 and to increase expression of MMP-2 and MMP-9, in both AD brain microvessels and brains of AD transgenic mice [78]. In the neocortex and hippocampus of aged Tg2576 mice, the ratio of occludin to β-actin was reduced by nearly half, when compared to age-matched wild type controls, but also with young transgenic mice [84].

In vitro, in cellular barrier models, Aβ treatment increases endothelial permeability, effect documented for both Aβ1-40 [85] and Aβ1-42 [86], while tight junction protein expression is controversial (Table 1). In cultured endothelial cells, Aβ1-42 induced enhanced permeability by disruption of ZO-1 expression in the plasma membrane and increased intracellular calcium and matrix metalloproteinase (MMP) secretion. Neutralizing antibodies against RAGE and inhibitors of calcineurin and MMPs prevented $A\beta_{1\text{-}42}$-induced changes in ZO-1, suggesting that Aβ-RAGE interactions alter TJ proteins through the Ca^{2+}-calcineurin pathway. Consistent with these *in vitro* findings, Kook et al. found disrupted microvessels near Aβ plaque-deposition areas, elevated RAGE expression, and enhanced MMP secretion in microvessels of AD mouse brains [86].

3.4. Excessive Alcohol Consumption. Excessive alcohol consumption is a known etiologic factor for cognitive impairment and dementia in humans and long-term treatment of adult laboratory rats with 20% ethanol in drinking water ad libitum resulted in cognitive decline, cholinergic dysfunction, and BBB leakage [87]. Ethanol (EtOH) effects are at least partially mediated by ROS, since, in these mice, superoxide production under basal conditions and in the presence of ADP and NAD(P)H, was increased [88]. In laboratory rats, EtOH consumption has previously been reported to associate increased oxidative stress and cytochrome P450-2E1 activation [89]. EtOH-induced activation of MMP-3/9 led to subsequent degradation of BBB proteins, occludin, claudin-5, and ZO-1 [90].

In vitro, EtOH induces ROS generation and ROS-nitrated protein accumulation in BMVEC [91]. At BBB level, EtOH or its metabolite acetaldehyde increases leakage and TJ protein phosphorylation [92]. Similar effects have been reported in other barriers, such as blood-air barrier [93], or other types of TJ-dependent polarized epithelia [94, 95].

4. Mediators of Oxidative Stress and Their Effects on Tight Junction Proteins

4.1. Reactive Oxygen Species. ROS are the main operators of oxidative stress and are responsible for altering protein structure, DNA denaturation, and lipid peroxidation and may act as messengers in redox-signalling systems [49]. In addition to causing cellular oxidative damage to biomolecules, hydroxyl radicals can also react with Aβ, triggering the formation of dityrosine cross-linking between Aβ peptides which leads to enhanced oligomerization and aggregation [96]. In oxidative-inducing conditions, a number of mechanisms have been proposed to trigger ROS generation, with enzymes such as xanthine oxidase, cyclooxygenase, leukocyte NADPH oxidase, and uncoupled endothelial NOS (eNOS) and mitochondria as putative sources [97]. Increased oxidative stress associated with aging further worsens the outcome of a stroke and favours onset of dementia. Common ROS that are deleterious for the vascular endothelium as well are superoxide, hydroxyl radical and hydrogen peroxide, found in concentrations depending of the balance between oxidases, such as NADPH oxidases (Nox enzymes) and superoxide dismutases (SOD). The impact of ROS on BBB function has been documented on SOD deficient mice, in which ischemia/reperfusion experiments demonstrated increased endothelium permeability to large molecules [98]. The hydrogen peroxide is more stable than superoxide, diffuses easily across cell membrane, can stimulate NADPH oxidase in vascular cells and thus further increase levels of superoxide [99].

In *in vitro* models, superoxide and other ROS increase permeability of the BBB in a time- and concentration-dependent manner [100, 101]. The reports on TJ proteins expression yielded contrasting results; however, BBB functionality was altered regardless of experimental paradigm. For instance, Lee et al. reported ROS-induced BBB impairment, quantified by transepithelial electrical resistance (TER) measurements, associated with a slight but significant increase in occludin expression [102], whereas Schreibelt et al. provided evidence that short-term oxidative stress-induced redistribution of occludin and claudin-5, with Western blot evidence of loss of these proteins expression [103].

4.2. Nitric Oxide. NO is a signalling molecule and a potent vasodilator, generated at the BBB level by eNOS, from L-arginine, a process that requires 5,6,7,8-tetrahydro-l-biopterin (BH4) as coenzyme. Apart from the constitutive isoform of NOS, endothelial cells also produce inducible NOS (iNOS), activated by interleukins and TNF-α [104]. Activation of iNOS is long-lasting and leads to an increased production of NO, as compared to constitutive isoform. Transgenic iNOS knockout mice develop brain pathology characteristic of AD (amyloid plaques, tau phosphorylation, and neuronal loss) indicating the NO has a protective role [105]. The peroxinitrite resulted from NO during oxidative stress has neurotoxic effects, similar to other ROS, via lipid peroxidation and DNA damage [106], and its presence is documented in astrocytes, neurons as well as blood vessels of AD brains, both in humans and mouse models of AD [80]. Generation of peroxynitrite from NO and superoxide takes place at a faster rate than the dismutation of superoxide by SOD enzymes and results in the loss of normal NO-mediated signalling. Thus, the local concentration of superoxide is a key determinant of the biological half-life of NO [98].

Interestingly, in certain conditions such as reduced levels of BH4, eNOS itself can produce superoxide, a process referred to as "eNOS uncoupling," in which oxygen becomes terminal electron acceptor instead of L-arginine [107]. NO does not influence the function of BBB during normoxia, but seems to confer protection during ischemia [108].

4.3. Lipid Peroxidation Products. Lipid peroxidation usually designates the oxidative damage of polyunsaturated fatty acids by free radical chain reactions when exposed to O_2 in the presence of trace metal ions. Studies of chain reactions in purified chemical systems show that a single initiation event can oxidatively damage 200 to 400 lipid molecules before two radicals react to eliminate the unpaired electrons and terminate the reaction sequence [109]. Lipid peroxidation causes damage at several levels by generation of various reactive aldehydes, such as 4-hydroxynonenal (4-HNE), that can alter the phospholipid asymmetry of the membrane lipid bilayer, and other products of lipid peroxidation, that can react with mitochondrial enzymes and cause disruption of mitochondrial energetics, increase of free radicals release and further oxidative stress [80]. Aβ peptides exert their oxidative effect on membrane lipids as well and there is a strong correlation between lipid peroxides, antioxidant enzymes, amyloid plaques, and neurofibrillary tangles (NFTs) in AD brains [3]. The composition of brain in phospholipids is unique; therefore, specific intermediates are produced upon lipid peroxidation [110]. These intermediates may diffuse into the blood stream and affect red blood cell membrane, as proven by Skoumalova et al. [111]. Oxidized low-density lipoprotein (ox-LDL), which is a hallmark feature of atherosclerosis acts as a stress signal and plays an influential role in BBB permeability [112]. The mechanisms by which lipid peroxidation affects BBB are not elucidated yet, but it has been proven that

4-HNE increases permeability of an *in vitro* barrier model [113]. In hyperlipemic laboratory mice, lipid peroxidation activates MMP-2/9, which in turn induces RhoA activation, a small GTPase known to phosphorylate TJ proteins and further destabilise BBB [114].

4.4. Matrix Metalloproteinases. Produced by activated microglia, MMPs are responsible for breaking down of endothelial basal lamina of BBB [57]. The main MMPs studied in relation to BBB alteration are MMP-2 and MMP-9, the first being constitutively expressed in CNS and the latter a marker of neuroinflammation [115]. Expression of MMP-9 within 24 h of an ischemic insult has cellular specificity, being primarily confined to the brain endothelium [116]. As already stated above, MMPs activity is balanced by their endogenous inhibitors, the TIMPs. Direct intracerebral injection of MMP-2 results in opening of the BBB with subsequent haemorrhage, effect that can be prevented by co-administration of TIMP-2 [117]. Blocking MMP-2 activation using either a selective inhibitor or a neutralizing antibody demonstrated that this enzyme is responsible for ischemia-induced occludin degradation. Interestingly, claudin-5 seems to be downregulated by different mechanisms, involving caveolin-1 [118]. On the other hand, Bauer et al. argued that hypoxia-induced oedema formation is mediated by MMP-9-dependent TJ rearrangement by a signalling cascade involving trophic factors, such as VEGF [119].

5. Signalling Pathways Affecting Tight Junction Proteins Phosphorylation Status in Oxidative Environments

Several reports of Saitou et al. showed in different experimental models that absence of occludin expression does not disrupt organization and function of TJs [120–122]. Therefore, TJ proteins emerged as possible signalling molecules. Indeed, there are several phosphorylation sites in the C-terminus sequence of occludin and claudins and phosphate addition in these domains increase protein internalization [65]. As a result, phosphorylation promotes an increase in BBB "leakiness." These phosphorylation sites are found within consensus sequences for protein kinase C (PKC) and protein kinase A (PKA) [123] and it was further proven that some PKC isoforms are involved in occludin, claudins, and ZO species phosphorylation in normal and hypoxic conditions. Hypoxia-induced BBB changes involved increased paracellular permeability via a PKC activity-dependent mechanism, in both *in vitro* and *in vivo* conditions [124].

Cytoplasmic relocation of occludin, claudin-1, and ZO-1 were documented in Ras-transformed Madin-Darby canine kidney epithelial cells (MDCK), effect that was specifically reversed by mitogen-activated protein kinase 1 (MEK-1) inhibition [125]. As demonstrated by Wang et al., an occludin mutant lacking the first extracellular loop rescued cells from Raf-1-mediated transformation [126]. Furthermore, different small GTPases, such as Raf-1 [127], Rho, Rac [128], were shown to influence the expression of occludin and claudin-1 in different epithelial models. Moreover, addition of ROS in cell culture media of immortalized rat endothelial brain cells significantly induced transient PKB phosphorylation and subsequent activation, through RhoA activation [103]. Occludin undergoes phosphorylation at Tyr residues during the disruption of TJs by oxidative stress and acetaldehyde [129]. Occludin and claudin-1 protein expression seems to be influenced by Glycogen Synthase Kinase-3 β (GSK-3β) as well, inhibition of this kinase leading to decreased TJ protein levels [130].

6. Conclusions

Oxidative stress has been involved for a long time and by overwhelming scientific data as a main pathogenic event in brain ageing and neurodegeneration. BBB, as crucial gate of brain-blood molecular exchange, seems to be affected by oxidative stress inducers in early stages of different brain diseases. Further studies are needed to understand which is the relationship between ROS deleterious effects on endothelial cells, BBB impairment, and progress of neurodegeneration, and how specific BBB drug targets can be approached in the future.

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Review Article

The Triggering Receptor Expressed on Myeloid Cells 2: "TREM-ming" the Inflammatory Component Associated with Alzheimer's Disease

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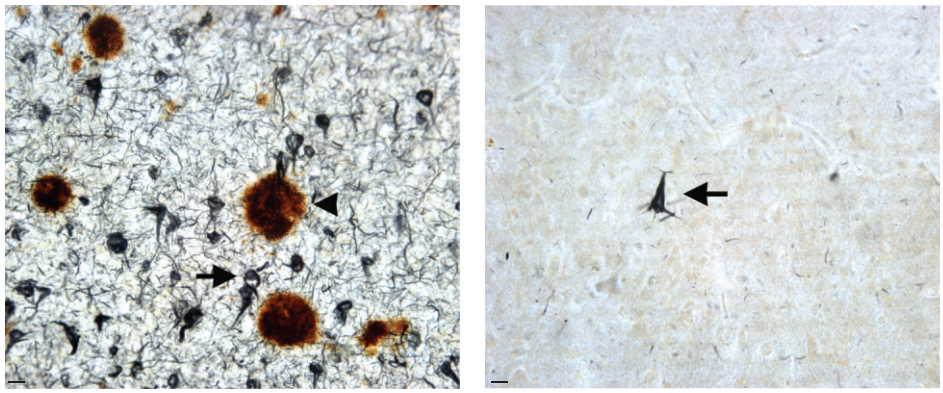
Alzheimer's disease (AD) is an age-related neurodegenerative disorder characterized by a progressive loss of memory and cognitive skills. Although much attention has been devoted concerning the contribution of the microscopic lesions, senile plaques, and neurofibrillary tangles to the disease process, inflammation has long been suspected to play a major role in the etiology of AD. Recently, a novel variant in the gene encoding the triggering receptor expressed on myeloid cells 2 (TREM2) has been identified that has refocused the spotlight back onto inflammation as a major contributing factor in AD. Variants in TREM2 triple one's risk of developing late-onset AD. TREM2 is expressed on microglial cells, the resident macrophages in the CNS, and functions to stimulate phagocytosis on one hand and to suppress cytokine production and inflammation on the other hand. The purpose of this paper is to discuss these recent developments including the potential role that TREM2 normally plays and how loss of function may contribute to AD pathogenesis by enhancing oxidative stress and inflammation within the CNS. In this context, an overview of the pathways linking beta-amyloid, neurofibrillary tangles (NFTs), oxidative stress, and inflammation will be discussed.

1. Defining Alzheimer's Disease

Alzheimer's disease (AD) is classified as a neurodegenerative disorder affecting neurons of the brain that are responsible for memory and higher cognitive functions. The brain consists of over a 100-billion neurons that specialize in the ability to transmit information to other cells, and thus constitute the basic working unit of the brain. Because cortical neurons, in general, do not have the capacity to regenerate, once neurons are lost and symptoms manifest, the process is essentially irreversible. In this manner, Alzheimer's is classified as a progressive neurodegenerative disease that can take anywhere from 5–20 years to run its course. The loss of these neurons is significant with affected individuals losing up to 50% mass of the brain over the course of the disease. The loss of these neurons leads to the symptoms of the disease including memory impairments, difficulties with language, inability to execute motor activities, and the overall decline in cognitive skills [1]. Dementia is the umbrella term describing the symptoms of AD, and AD is by far the leading cause of dementia in the United States, being responsible for over 70% of all known cases of dementia [2]. AD is a multifactorial disorder, whose causes remain largely unknown. Despite extensive research on genetic factors, the vast majority of Alzheimer's cases (>90%) are not directly linked to them [3]. Aging is the most well-established risk factor for the development of sporadic AD with incidence rates showing an exponential growth between the ages of 65 and 85 years, doubling every 5 years [3].

The national numbers on AD are alarming: currently one in eight older Americans has AD making it the sixth leading cause of death in the United States. An estimated 5.4 million Americans have AD, a figure that includes 5.2 million people age 65 and older [1]. Of those with AD, an estimated 4 percent are under the age 65, 6 percent are 64 to 74, 44 percent are 75 to 84, and 46 percent are 85 or older [1]. Of all of the major causes of death in the United States, including stroke, cancer, and heart disease, only Alzheimer's disease has shown a significant increase in mortality during the same time frame (2000–2008).



(a) (b)

Figure 1: The microscopic trouble makers in Alzheimer's disease: senile plaques and neurofibrillary tangles. (a) In AD, widespread accumulation of extracellular beta-amyloid plaques is evident (arrowhead), together with the presence of an abundance of NFTs along with neuropil threads, which are composed principally of modified and aggregated tau (arrow). (b) For comparison purposes, an age-matched control brain is depicted indicating a complete absence of plaques. However, it is not uncommon to find an occasional tangle (arrow) in the normally aged brain although the numbers of tangles is minimal by comparison. Brain sections are representative staining of the hippocampus using an anti-beta-amyloid antibody to label plaques (brown) and anti-PHF-1 (black) to label NFTs. Scale bars are 10 μm.

2. Pathology Associated with AD

AD is diagnosed based upon the extent of senile plaques composed of beta-amyloid and neurofibrillary tangles (NFTs) containing abnormally phosphorylated and truncated tau [4]. The preponderance of research to date suggest a pivotal role for beta-amyloid in the progression of AD, and collectively this concept has coined the beta-amyloid hypothesis [5]. In essence, this hypothesis stipulates that much of the pathology associated with AD is driven by an increased load of beta-amyloid in the brain of AD patients that can occur years before the first symptoms of the disease manifest.

Beta-amyloid is formed following sequential cleavage of the amyloid precursor protein (APP) by two proteases, β-secretase and γ-secretase. Once formed, beta-amyloid has the propensity to self-aggregate into β-sheet structures that deposit extracellularly forming senile plaques (Figure 1). More recently, the beta-amyloid hypothesis has been modified to the "toxic beta-amyloid oligomer" hypothesis to reconcile the apparent lack of correlation between beta-amyloid in plaques and cognitive impairment [6]. This reformulation of the amyloid cascade hypothesis focuses on oligomeric aggregates of beta-amyloid as the prime toxic species causing AD in part because this form of beta-amyloid strongly correlates with the severity of dementia [7, 8]. In addition, this oligomeric form of beta-amyloid is highly toxic and is the trigger for the loss of synapses and neuronal damage [9, 10]. Given the strong support for the amyloid cascade hypothesis, many of the current therapeutic strategies now in clinical trials involve some aspect of modifying beta-amyloid production or clearance [11]. However, despite the overwhelming evidence supporting a role for beta-amyloid in AD, this hypothesis is currently under critical assessment due to the recent clinical trial failures based on the strategy of lowering the beta-amyloid levels in the AD brain [12]. For example, one strategy currently being investigated involves inhibiting gamma-secretase to limit the production of the beta-amyloid peptide derived from APP. One such compound, semagacestat, showed promise in early clinical trials, but a recent phase III trial involving over 2,600 participants was discontinued after failure to demonstrate efficacy. Compared to placebo, patients receiving semagacestat actually did worse both in daily function and cognition and were at higher risk of developing skin cancer [13].

The other major pathological finding in AD is the presence of neurofibrillary tangles (NFTs) (Figure 1) [14]. NFTs are primarily composed of aggregated phosphorylated tau protein and are a clinical feature not just in AD but other diseases that are collectively referred to as "tauopathies" [15]. Tau normally functions to help maintain the stability of the cytoskeleton of neurons by binding to microtubules. However, upon hyperphosphorylation and posttranslational cleavage, tau loses its binding affinity for microtubules, leading to a destabilization of the cytoskeleton and self-assembly of tau into tangles of paired helical filaments (PHFs) [16]. Although not universally accepted, it has been proposed that NFTs may not be a central mediator of disease pathogenesis, but instead, NFTs may actually serve a protective rather than harmful function by providing a compensatory response mounted by neurons against oxidative stress [17].

3. Mechanisms of Neurodegeneration in AD

According to the beta-amyloid hypothesis, the accumulation and aggregation of beta-amyloid into toxic soluble oligomers is the first step leading to neuronal degeneration in AD [18]. Specifically, an important early molecular step is the lost of synapses, which correlates highly with the initial memory impairment observed in AD [19, 20]. Intensive research over the last two decades has examined potential pathways activated by beta-amyloid aggregates that lead to synaptic dysfunction, NFT formation, and eventual cell death. Figure 2 summarizes some of the major findings on beta-amyloid-induced toxicity that begin with either beta-amyloid activation of apoptotic pathways or promotion of

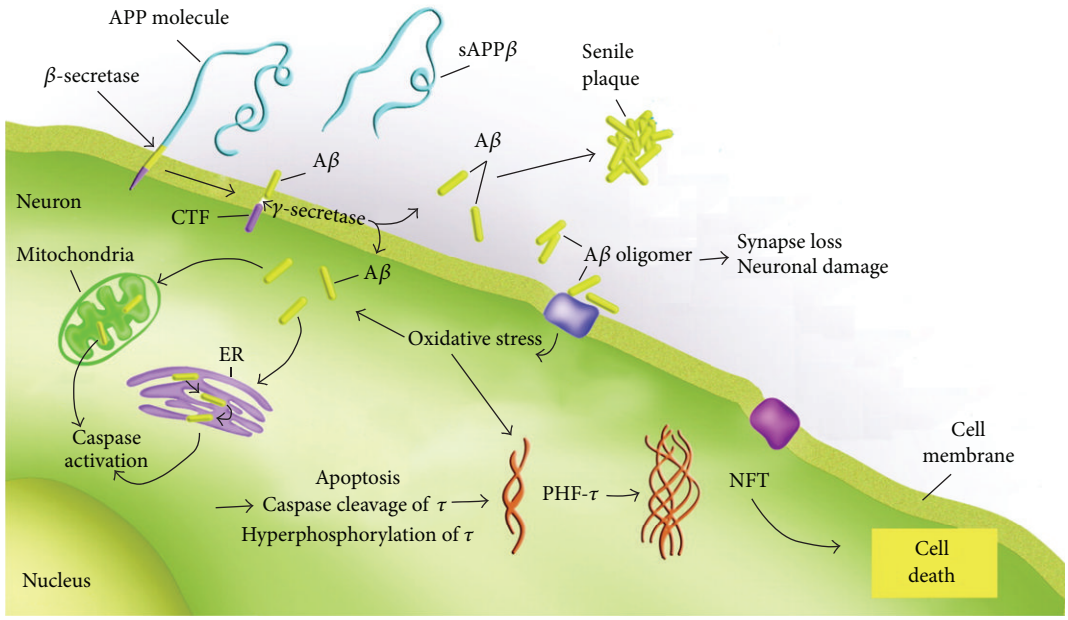


Figure 2: Putative pathway for neurodegeneration in Alzheimer's disease. According to the beta-amyloid hypothesis, the production of beta-amyloid represents the first step in the entire process following the sequential cleavage of APP by β-secretase and γ-secretase. Beta-amyloid in turn may lead to NFT formation and eventual cell death by promoting oxidative stress and caspase activation through initiation of the mitochondrial-mediated pathway of apoptosis. The activation of caspases results in cleavage of critical cellular proteins including tau, leading to its modification and hyperphosphorylation, a key prerequisite for filament formation. See main text for details.

oxidative stress. With regards to apoptosis, since the early nineties, studies have supported a general role for apoptosis in AD [21–23]. In addition, the activation of caspases, including caspase 3, 6, 8, and 9, has been documented in the AD brain [24–30]. In turn, evidence suggests that once activated, caspases may cleave critical cellular proteins in AD including APP, actin, fodrin, glial acidic fibrillary protein, beclin-1, and tau [28, 31–35]. Importantly, several studies have suggested that caspase activation and cleavage of tau may precede and contribute to the formation of NFTs [31, 32, 36].

An interlinking step between beta-amyloid and NFTs could be the promotion of oxidative stress. Oxidative stress either through lipid peroxidation or mitochondrial disruption is an early feature found in AD [37–39]. In addition, oxidative stress may contribute to the activation of apoptosis through both the extrinsic and intrinsic pathway [40, 41]. Finally, tau phosphorylation is upregulated by oxidative stress [42] and tau filaments are modified by products of oxidative stress [43–45]. Oxidative stress also activates several kinases that have been implicated in the hyperphosphorylation of tau including glycogen synthase kinase-3 (GSK3), Jun-N-terminal kinase (JNK), and mitogen-activated protein kinases (MAPKs) [46, 47].

4. Inflammation in AD

Brain inflammation is a pathological hallmark of AD [48, 49]. In this regard, numerous studies have supported a definitive role for inflammation in AD, with a key feature being the presence of activated microglia [50–52] and reactive astrocytes found within senile plaques [53–55]. Epidemiological studies have also pointed to inflammation as central to AD, indicating that the long-term use of anti-inflammatory drugs is linked with reduced risk of developing the disease [56]. Microglia are key players in mediating immune responses in the CNS functioning as the resident macrophages of the CNS and as such contribute to a healthy CNS by attacking and removing potential pathogens and cell debris and by secreting tissue rebuilding factors [57].

Interestingly, the link between the activation of microglia and inflammation may be beta-amyloid. Thus, beta-amyloid is a potent inducer of microglia activation [52, 58–60], and one important role of microglia is to clear beta-amyloid deposits out of the AD brain (for recent review, see [61]).

One potential caveat with inflammation is determining cause and effect. Does AD cause inflammation? does the dysregulation of immune system pathways trigger the disease process? Alternatively, although chronic inflammation may be a driving force in disease pathogenesis, it also may serve as a beneficial response at least early on during the course of AD. Finally, it is possible that inflammation could simply be a byproduct of the disease process and may not effectively alter its course. A recent discovery has now addressed this issue and has unequivocally put inflammation in general and specifically the microglial response center stage.

5. Triggering Receptor Expressed on Myeloid Cells 2 (TREM2)

TREM2 is expressed on the cell membrane of many types of immune cells including macrophages, dendritic cells,

osteoclasts, and microglia [62]. TREM2 is thought to act as a cell surface receptor, and although the endogenous ligand has yet to be identified, it is known that it requires the adaptor protein 12 (DAP12) for the initiation of signaling cascades [63]. Activation of the TREM2 receptor on microglia has two important function consequences: (1) stimulation of phagocytosis activity and (2) decreased microglial proinflammatory responses [64]. Collectively, TREM2 may function to help aid microglia to clear damaged or apoptotic cells and cellular debris and help resolve damage-induced inflammation.

Insights into the important role that TREM2 plays have been deduced from individuals harboring homozygous mutations in the TREM2 gene. Such a mutation leads to The Nasu-Hakola disease, which manifests as a combination of bone cysts and dementia [65]. Affected individuals show progressive inflammatory neurodegeneration with loss of white matter and cystic bone lesions followed by death by the fifth decade of life [66].

Further support for TREM2 being an important mediator in neuroinflammation comes from animal models of multiple sclerosis (MS). Two different studies have reported a protective role of TREM2. In one study, blockade of TREM2 function enhanced disease progression in an experimental murine model of autoimmune encephalomyelitis [67]. In the second study, intravenous application of TREM2-transduced myeloid cells limited tissue destruction and facilitated repair in a murine model of MS [68]. Taken together, these studies highlight a critical role for TREM2 during inflammatory responses in the CNS.

6. TREM2 in Alzheimer's Disease

TREM2 function may affect AD pathology through phagocytosis. In a murine model of AD, Frank et al. demonstrated that TREM2 is upregulated in microglia found at the border of amyloid plaque deposits [69]. Moreover, TREM2 expression has been positively correlated with the phagocytic clearance of beta-amyloid in APP transgenic mice [70]. Given the well-documented role that microglia play in removing beta-amyloid [61], the expression of TREM2 by beta-amyloid plaque-associated microglia may be interpreted as an effort to enhance beta-amyloid clearance and to limit the proinflammatory cytokine expression in response to microglia activation by beta-amyloid itself. Besides clearing beta-amyloid, TREM2 may also function to remove debris and participate in synapse remodeling [71].

The strongest evidence to date supporting a role for inflammation in AD comes from two recent studies demonstrating that TREM2 variants increase the risk for AD approximately 3-fold [72, 73]. These studies indicated that individuals that are heterozygous for several TREM2 mutations (the most common variant being a R47H change) were at a greater risk for AD. In addition, Jonsson and colleagues also showed that elderly carriers of the TREM2 variant who were asymptomatic for AD, nevertheless, performed worse in cognitive exams as compared to noncarriers [72]. It is noteworthy that this particular mutation is extremely rare, only being found at a frequency of 0.63 percent. Compare this to the greatest risk factor for late-onset AD, the apoE4 allele, in which it has been estimated that approximately 40% of AD subjects currently harbor at least one copy of this allele [74].

Interestingly, it is only individuals that are heterozygous for TREM2 that are at risk for AD: homozygous, autosomal recessive mutations for TREM2 result in The Nasu-Hakola disease in some individuals characterized by bone cysts and dementia [75], while resulting in fronttemporal dementia without bone disease in others [76]. Due to the common thread of dementia in these homozygous mutations, it was these initial findings that spurred further research to search for heterozygous mutations in the TREM2 gene in AD subjects.

Given the prophagocytic role of TREM2, loss of phagocytic activity of microglia could represent one mechanism by which the TREM2 mutations increased the risk to develop AD (Figure 3). Because TREM2 functions in microglia to also dampen microglial activation, mutations and loss of function could result in runaway inflammation as well. Based on these results, it is tempting to speculate on the potential pharmacological value that TREM2 agonists might have in AD. Future directions should answer this question as well as the possibility of finding more rare variants that have similar effects, including those in DAP12 and TREM2's intracellular signaling partner.

7. Concluding Remarks

Research in the field of AD has uncovered the detailed molecular mechanisms leading to the hallmark, microscopic lesions consisting of beta-amyloid plaques and NFTs. However, how these lesions lead to neurodegeneration is still under investigation. An important consideration in this paper was the attempt to unify the various potential players that contribute to cell death in AD, including beta-amyloid, NFTs, caspases, oxidative stress, and inflammation. In this regard, it is suggested that beta-amyloid in the form of soluble beta-amyloid oligomers represents the earliest known step in the entire process, setting off a chain of events that ultimately lead to chronic inflammation and neuronal cell death. It has been difficult to assess the cause and effect relationship of inflammation in AD, but the recent discovery of the TREM2 mutations has now put inflammation back on center stage as a process that contributes to disease progression. This is highlighted by the data indicating an approximate 3-fold increase in the risk for AD in individuals harboring heterozygous variants in the TREM2 gene. Because TREM2 functions to modulate the inflammatory immune responses in microglia, mutations in this gene, in turn, could contribute to disease pathogenesis by preventing the clearance of beta-amyloid deposits and/or by enhancing inflammation. However, because the function of TREM2 is still poorly understood, it is difficult to determine how loss of TREM2 function might contribute to the disease process. Future studies examining a direct role for TREM2 in AD should help shed light on this question and provide further support for the role of chronic inflammation in this disease.

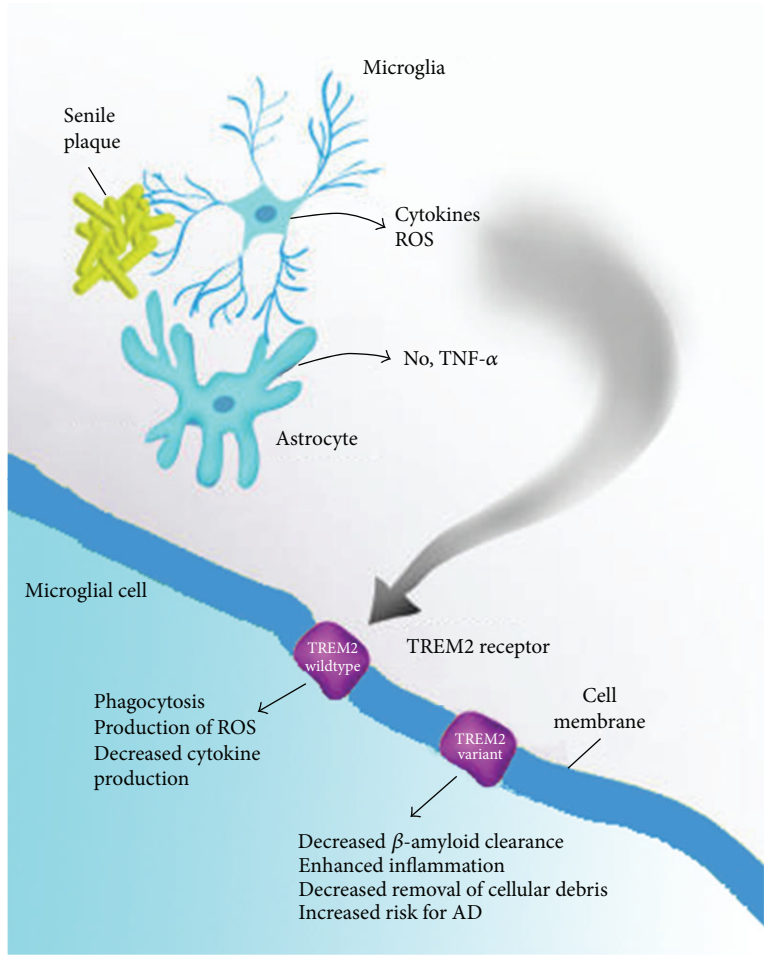


Figure 3: A central role for microglia in Alzheimer's disease is dependent upon a functional TREM2 receptor. Microglia represent one of the three classes of glia cells, whose primary function is to act as a major line of active immune defense in the CNS. In AD, microglia (top) and astrocytes (bottom) function in phagocytosis and in this regard help clear the brain of beta-amyloid deposits and apoptotic cells as well as any cellular debris. The important actions of microglia appear to be mediated through activation of the TREM2 receptor whose few known roles include suppressing inflammation and stimulating phagocytosis. As shown recently, variants in the TREM2 receptor have been discovered, and it has been suggested that the change in sequence leads to a loss of receptor function. It has been hypothesized that the loss of TREM2 activity has two major consequences: (1) decreased ability of microglia to remove extracellular deposits of beta-amyloid and (2) enhanced neuroinflammation. The loss of TREM2 function and altered immune responses by microglia may explain the increased risk for AD for individuals carrying the heterozygous mutations in TREM2.

Abbreviations

AD: Alzheimer's disease
apoE4: Apolipoprotein 4
APP: Amyloid precursor protein
NFTs: Neurofibrillary tangles
PHFs: Paired helical filaments
TREM2: Triggering receptor expressed on myeloid cells 2.

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Review Article

Function and Characteristics of PINK1 in Mitochondria

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Mutations in phosphatase and tensin homologue-induced kinase 1 (PINK1) cause recessively inherited Parkinson's disease, a neurodegenerative disorder linked to mitochondrial dysfunction. Studies support the notion of neuroprotective roles for the PINK1, as it protects cells from damage-mediated mitochondrial dysfunction, oxidative stress, and cell apoptosis. PARL is a mitochondrial resident rhomboid serine protease, and it has been reported to mediate the cleavage of the PINK1. Interestingly, impaired mitophagy, an important autophagic quality control mechanism that clears the cells of damaged mitochondria, may also be an underlying mechanism of disease pathogenesis in patients for Parkinson's disease with the PARL mutations. Functional studies have revealed that PINK1 recruits Parkin to mitochondria to initiate the mitophagy. PINK1 is posttranslationally processed, whose level is definitely regulated in healthy steady state of mitochondria. As a consequence, PINK1 plays a pivotal role in mitochondrial healthy homeostasis.

1. Introduction

Mitochondria play an important role in eukaryotic metabolic processes by serving as cellular energy generators of ATP [1], which are critical for cell survival and for correct cellular functions, and they play an important role in mediating apoptosis and in determining their own destruction called mitophagy [2], an important autophagic control mechanism that clears damaged mitochondria. Mitochondria are also recognized to play an important role in neurodegenerative disorders including multiple sclerosis, Alzheimer's, and Parkinson's diseases, which are characterized by progressive and selective loss of neuronal cell populations [3–5]. Midbrain dopaminergic neurons are susceptible to oxidative stress due to the environment of the dopamine biosynthetic pathways and their low mitochondrial reserve compared to other neuronal populations [6]. Molecular genetics has linked mitochondrial dysfunction to the pathogenesis of Parkinson's disease by the discovery of several inherited mutations in gene products that associate with the mitochondrial function.

The PTEN-induced kinase 1 (PINK1) is a mitochondria-targeted serine/threonine kinase, which is linked to autosomal recessive familial Parkinson's disease [7] (Figure 1). In addition to its protective role against mitochondrial dysfunction and apoptosis, PINK1 is also known to regulate Parkinson's disease-related protein Parkin [7]. The PINK1 recruits the E3 ubiquitin ligase Parkin to mitochondria in order to initiate the mitophagy. In addition, presenilin-associated rhomboid-like serine protease (PARL) can affect the proteolytic processing of the PINK1 [8]. Normal PINK1 localization and stability requires catalytic activity of the PARL. Consequently, PARL deficiency impairs Parkin recruitment to mitochondria, suggesting that PINK1 processing and localization is essential in determining its interaction with Parkin [9]. More than 50 mutations of PINK1 have been mapped throughout the kinase and carboxyl-terminal regulatory domains of PINK1 with various effects on protein stability implicating neuroprotective roles [10, 11]. This paper will provide a concise overview on the cellular functions of the mitochondrial kinase PINK1 and the relationship between parkinsonism and mitochondrial dynamics, particular emphasis on a mitochondrial damage response pathway and mitochondrial quality control.

2. Expression and Characteristics of PINK1

Mutations in PINK1 are the most common cause of recessive familial Parkinsonism [10, 11]. The *PINK1 (phosphatas-e and tensin-homolog- (PTEN-)induced kinase 1)* gene consists of

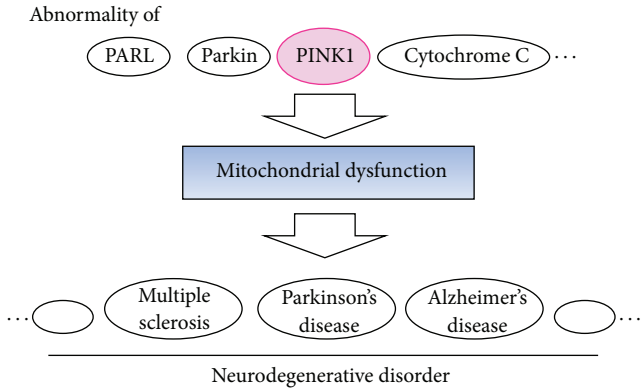

Figure 1: Implication of mitochondrial dysfunction caused by PINK1, Parkin, and so on for neurodegenerative disorders including Parkinson's disease. Abnormality of these molecules may also be a causative factor in the development of mitochondrial dysfunction. There is a relationship between mitochondrial dysfunction and neurodegenerative disorders. Note that some critical molecules have been omitted for clarity.

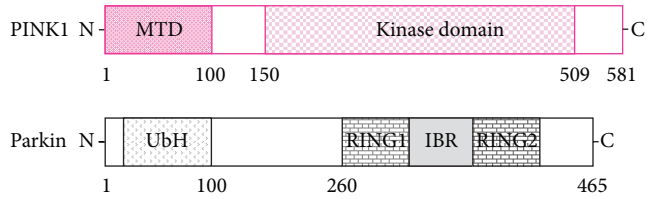

Figure 2: Schematic diagram indicating the domain structures of PINK1 (upper) and Parkin (lower) proteins. The predicted consensual important domain structures for each protein are depicted. MTD: mitochondrial targeting domain, UbH: ubiquitin homology domain, RING1 and RING2: RING finger domain, and IBR: in between RING fingers.

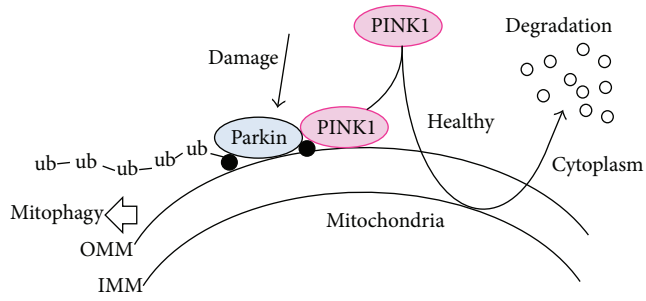

Figure 3: Hypothetical schematic representation of the PINK1 regulatory pathway and Parkin mediated-mitophagy. Under healthy and steady state, PINK1 is degraded within the mitochondria. This may be inhibited by mitochondrial damage, resulting in PINK1 and Parkin accumulation in the outer membrane of mitochondria. Parkin is presumed to ubiquitinate unidentified substrate (black circle), resulting in the induction of mitophagy. Note that some critical pathways have been omitted for clarity. OMM: outer mitochondrial membrane; IMM: inner mitochondrial membrane.

eight exons, encoding a 581-amino acid protein with a predicted molecular mass of 62.8 kilodaltons. Defects in the *PTEN*, which is a tumor suppressor, have been found in cancers arising in a variety of human tissues. *PINK1* mRNA is expressed ubiquitously, but high expression levels are found in the heart, skeletal muscle, testes, and brain [12]. In the brain, higher expression is neuronal in the substantia nigra, hippocampus, and cerebellar Purkinje cells [13]. The PINK1 protein has a central domain with homology to serine/threonine kinases, exhibiting an auto-phosphorylation activity *in vitro* [14]. An amino-terminal mitochondrial-targeting signal domain is sufficient for mitochondrial introduction of PINK1 (Figure 2) [15]. The protein can be found on the outer and inner mitochondrial membrane (Figure 3) [16, 17]. The PINK1 can be processed into at least two shorter forms, which are distributed in both mitochondrial and cytosolic compartments. Physiological PINK1 substrates are localized in the outer mitochondrial membrane or possibly in the cytosol near the mitochondrial surface. The cytoplasmic PINK1 is degraded by proteasome [18]. Adding to the variety of survival functions of PINK1, it has been shown to phosphorylate the mitochondrial heat shock protein 75 kDa (TRAP1), increasing neuronal survival against oxidative stress or heat shock by preventing the release of cytochrome c [19]. The mitochondrial serine protease HtrA2 has been identified to be regulated by PINK1 [20]. Targeted deletion of the HtrA2 causes mitochondrial dysfunction leading to a neurodegenerative disorder with parkinsonian features in mice [20]. The TRAP1 may be a direct substrate for PINK1, which localize primarily in the mitochondrial matrix and at extramitochondrial sites. Whether HtrA2 is a direct PINK1 substrate is somewhat unclear. It is possible that differences in cell viability resulting from PINK1 inactivation may affect HtrA2 through the other kinase such as p38 SAPK [21]. HtrA2 is released from the inter membrane space of mitochondria during apoptosis to the cytosol [22, 23].

PINK1 may also interact with Beclin1, a key proautophagic protein implicated in the pathogenesis of Alzheimer's and Huntington's diseases [24]. Full-length PINK1 interacts with Beclin1 [25], which enhances starvation-induced autophagy. The PARL is a mitochondrial resident rhomboid serine protease and has been reported to mediate the cleavage of PINK1 in mitochondria, which may mediate differential cleavage of PINK1 and phosphoglycerate mutase 5 (PGAM5) depending on the health status of mitochondria [26].

3. PINK1 Function Involved in Mitochondrial Health Status

PINK1 silencing may result in mitochondrial respiratory dysfunction, since PINK1 knockout mice exhibit impaired mitochondrial respiration and decreased activity of oxidative phosphorylation [27]. In addition, the impaired mitochondrial respiration can be exacerbated by exposure of the mitochondria to heat shock [27]. While knockdown studies of endogenous PINK1 indicate a key role for the PINK1 in maintaining the mitochondrial functioning networks, the protective activities of PINK1 depend on its mitochondrial localization. Loss of PINK1 leads to severe alterations

in mitochondrial homeostasis as evidenced by increased mitochondrial reactive oxygen species (ROS) inducing a robust increase in mitochondrial mitophagy [28]. Stable PINK1 silencing may have an indirect role in the mitophagy activation. As proteins with iron sulfur clusters, one of the most ubiquitous redox centers, are sensitive to oxidative stress, prolonged ROS exposure may cause mitochondrial dysfunction [29, 30]. PINK1 has been shown to protect against cell death induced by proteasome inhibition and oxidative damage [31, 32]. Thus, PINK1 has a pivotal role in the mitochondrial quality control via the mitochondrial stabilization, phosphorylation of chaperones, and regulation of the mitophagy. However, imbalanced induction of mitophagic recycling can contribute to neuronal atrophy, neurite degeneration, and neuronal cell death [33]. Excessive rates of mitophagy may prove harmful results [33, 34]. Overexpression of wild-type PINK1 in neuronal cells stabilizes respiring mitochondrial networks through maintaining mitochondrial membrane potential and suppression of the mitophagy [35]. Probably, high levels of cytoplasmic PINK1 may substitute for endogenous protein by phosphorylating substrates at the mitochondrial surface or in the cytoplasm near the mitochondrial surface. In healthy mitochondria, PINK1 is rapidly degraded in a process involving both mitochondrial proteases and the proteasome. The mitochondrial protease PARL can affect the proteolytic processing of PINK1 and normal PINK1 localization, and the stability requires the PARL catalytic activity [17, 36]. The PARL may also mediate differential cleavage of PINK1 depending on the health status of mitochondria. PARL deficiency impairs Parkin recruitment to mitochondria, suggesting that PINK1 processing and localization are important in determining its interaction with the Parkin.

With severe mitochondrial damage, PINK1 facilitates aggregation of depolarized mitochondria through interactions with Parkin protein [37]. In addition, overexpression of full-length PINK1 is required for mitochondrial Parkin recruitment for the mitochondrial aggregation. Besides, transient overexpression of Parkin further augments mitochondrial mitophagy in PINK1 deficient neuronal cells, resulting in cytoprotection and/or restoration of interconnected mitochondrial networks [38]. Many lines evidences indicate interactions of PINK1 with Parkin in promoting mitochondrial health homeostasis [38, 39]. The Parkin can be phosphorylated by PINK1 in its RING finger domain during *in vitro* kinase reactions, which may promote translocation of the Parkin to mitochondria [40]. Furthermore, the phosphorylated Parkin has been reported to facilitate the selective clearance of depolarized mitochondria via mitophagy [41, 42]. Under conditions of PINK1 diminishment or deficiency, it compromises the mitochondrial quality control. Failure of this mitochondrial quality control eventually contributes to cell death. In healthy mitochondria, by the way, PINK1 is rapidly degraded in a process involving both mitochondrial proteases and the proteasome as mentioned above. Loss of either PINK1 or Parkin leads to fragmentation of mitochondria [43, 44]. On the other hand, mitochondrial Parkin promotes the mitophagic degradation of dysfunctional mitochondria [25, 45]. The mitophagic response observed in PINK1 silencing cells could be associated with increased Parkin levels, as endogenous Parkin protein expression is increased in some PINK1 deficient cells [46]. Thus, PINK1 and Parkin could complexly participate in a common mitochondrial protective signaling pathway.

4. Abnormal PINK1 Involved in Neurodegenerative Disease

Intramembrane proteolysis is a conserved mechanism that regulates various cellular processes. The PARL cleaves human PINK1 within its conserved membrane anchor [47], suggesting implication in neurodegenerative disease. Mature PINK1 is then free to be released into the cytosol or into the mitochondrial intermembrane space. Upon depolarization of the mitochondrial membrane potential, the import of PINK1 and PARL-catalyzed processing is blocked, leading to accumulation of the PINK1 precursor [47]. Targeting of this precursor to the outer mitochondrial membrane has been shown to trigger the mitophagy (Figure 3) [48]. The PARL-catalyzed removal of the PINK1 signal sequence in the import pathway may act as a cellular checkpoint for mitochondrial integrity. Interestingly, Parkinson's disease-causing mutations decrease the processing of PINK1 by PARL [49]. Decreased processing of Pink1 may be an implication for the pathogenesis. When mitochondrial import is compromised by depolarization, PINK1 accumulates on the mitochondrial surface, where it recruits the Parkinson's disease-linked Parkin from the cytosol, which in turn mediates the mitophagic destruction of mitochondria (Figure 3) [48, 49]. The importance of PINK1 in mechanisms underlying neurodegeneration is reflected by the neuroprotective properties of the Parkin in counteracting oxidative stress and improvement of mitochondrial function. The involvement of Parkin and PINK1 in mitochondrial dysfunction, oxidative injury, and impaired functioning of the ubiquitin-proteasome system has been investigated in light of Parkinson's disease pathogenesis [48, 49].

A protein kinase microtubule-affinity regulating kinase 2 (MARK2) also plays key roles in several cell processes underlying neurodegenerative diseases, such as Alzheimer's disease, by phosphorylating tau and detaching it from microtubules [50]. MARK2 phosphorylates and activates the PINK1 [51]. Thr-313 is the primary phosphorylation site, a residue mutated to a nonphosphorylatable form in a frequent variant of Parkinson's disease [51]. Mutation of the Thr-313 in PINK1 shows toxic effects with abnormal mitochondrial distribution in neurons. Both MARK2 and PINK1 have been found to colocalize with mitochondria and regulate their transport. So, MARK2 may be an upstream regulator of PINK1, and it regulates the mitochondrial trafficking in neuronal cells. The MARK2-PINK1 cascade provides new insights into the regulation of mitochondrial trafficking in neurons and neurodegeneration in Parkinson's disease. The high temperature requirement A2 (HtrA2) is indirectly phosphorylated and interacts with PINK1 in relation to a signaling pathway [52]. The PINK1-dependent phosphorylation of the HtrA2 enhances its protease activity leading to enhanced survival against oxidative stress [52, 53]. The HtrA2 is also

phosphorylated on activation of the p38 SAPK pathway, occurring in a PINK1-dependent manner [52]. Point mutations in the HtrA2 are a susceptibility factor for Parkinson's disease. However, it has been shown in Drosophila that the HtrA2 is not essential for all the protective functions of PINK1 [54, 55]. Another mitochondrial protease rhomboid-7 has been implicated in posttranslational regulation of both PINK1 and HtrA2 [56, 57]. These protein signaling axes might provide a link between neurodegenerative processes in Alzheimer's and Parkinson's diseases.

5. Perspective

Mitochondrial protein phosphorylation is involved in cell stress-induced programmed cell death such as apoptosis, which also contributes to the regulation of mitochondrial dynamics and mitophagy. Those are significant to maintain mitochondrial quality and ensure cellular homeostasis. PINK1 may function in the first line of mitochondrial quality control, monitoring respiratory chain function [58] and triggering the localized degradation of damaged mitochondrial proteins. In addition, diminishment of PINK1 would have deleterious consequences on mitochondrial function [59]. The PINK1 is a mitochondrial kinase that promotes cell survival, particularly under conditions of oxidative stress. Whether PINK1 levels are enhanced or reduced, strategies to promote selective mitophagy and mitochondrial biogenesis may prove to be effective for multiple forms of neurodegenerative disease. Although the precise physiological substrate of PINK1 is not fully resolved, it is clear that the kinase activity is important in playing roles for many aspects of mitochondrial function [60, 61]. The involvement of PINK1 and Parkin in the mitochondrial dysfunction has now been intensively investigated in Parkinson's disease pathogenesis [62]. These pathological mechanisms are not restricted to the Parkinson's disease, but they might be common characters of various neurodegenerative and neuroinflammatory disorders. It is therefore conceivable that PINK1 and Parkin are also linked to the pathogenesis of other neurological diseases including Alzheimer's disease. The mechanisms by which wild-type PINK1 and Parkin promote interconnected mitochondrial networks may involve different steps in mitochondrial quality control. For example, severe mitochondrial injury may require organelle-level responses including Parkin-facilitated mitochondrial mitophagy. Enhancing pathways that promote mitophagy might also delay age-related diseases by promoting a healthy pool of viable mitochondria in neuronal cells and sustaining energy demands. Future experimental work would be needed to understand the precise mitochondria protective roles of PINK1.

Abbreviations

HtrA2: High temperature requirement protein A2
MARK2: Microtubule affinity-regulating kinase 2
PARL: Presenilin-associated rhomboid-like serine protease
PINK1: PTEN-induced kinase1, phosphatase and tensin homologue-induced kinase 1
PGAM5: Phosphoglycerate mutase 5
PTEN: Phosphatase and tensin homolog
ROS: Reactive oxygen species
SAPK: Stress-activated protein kinase
TRAP1: Tumor necrosis factor receptor-associated protein 1.

Conflict of Interests

The authors declare that they have no financial conflict of interests.

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Research Article

Exhaustive Training Increases Uncoupling Protein 2 Expression and Decreases Bcl-2/Bax Ratio in Rat Skeletal Muscle

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This work investigates the effects of oxidative stress due to exhaustive training on uncoupling protein 2 (UCP2) and Bcl-2/Bax in rat skeletal muscles. A total of 18 Sprague-Dawley female rats were randomly divided into three groups: the control group (CON), the trained control group (TC), and the exhaustive trained group (ET). Malondialdehyde (MDA), superoxide dismutase (SOD), xanthine oxidase (XOD), ATPase, UCP2, and Bcl-2/Bax ratio in red gastrocnemius muscles were measured. Exhaustive training induced ROS increase in red gastrocnemius muscles, which led to a decrease in the cell antiapoptotic ability (Bcl-2/Bax ratio). An increase in UCP2 expression can reduce ROS production and affect mitochondrial energy production. Thus, oxidative stress plays a significant role in overtraining.

1. Introduction

The mechanism of overtraining (OT) remains poorly understood. One common theory considered is glycogen depletion [1]. Our previous study showed that glycogen was not depleted in rat skeletal muscles after continuous exhaustive training, which does not support the glycogen depletion hypothesis [2]. Overtraining can be caused by reduced muscle mitochondrial function, reducing glycogen breakdown and decreasing energy production. Excessive ROS can influence the reduction of mitochondrial function due to continuous exhaustive training. ROS can be associated with overtraining, inducing the opening of the mitochondrial permeability transition pore (MPTP) [3]. Low molecular weight molecules (<1.5 kDa) equilibrate across the inner membrane when the MPTP opens, causing mitochondrial swelling and outer membrane rupture. The opening of the MPTP is considered the "point of no return," after which the myocyte is irreversibly committed to necrotic or apoptotic death pathways [4].

Many pathways can lead to cell apoptosis. One of the mitochondrial-mediated pathways, including the Bcl-2 family, is best characterized and considered critical in regulating apoptosis. In the Bcl-2 family, Bax protein is mainly located in the cytoplasm, which migrates to the outer mitochondrial membrane, forms dimer and oligomer under the apoptosis signal stimulation and combines with the adenine nucleotide translocator of the MPTP complex or voltage-dependant anion channel on the outer mitochondrial membrane. This combination occurs either directly or through the Ca^{2+} released from the endoplasmic reticulum-induced MPTP opening, leading to apoptosis [5]. The main protein inhibiting apoptosis, Bcl-2, anchors to the mitochondria, endoplasmic reticulum, and nuclear envelope of the cytoplasmic side. This action maintains mitochondrial membrane integrity through competitive inhibition of Bax mediated by mitochondrial membrane protein channel formation [6], controlling the opening of PMTP, inhibiting Ca^{2+} transmembrane flow, inhibiting caspase-3 activation, and preventing apoptosis. Apoptosis caused by continuous exhaustive training can result from ROS-induced permeability transition pore opening [7]. A study by Kim et al. on endoplasmic reticulum stress [8] states that Bax inhibitors can reduce ROS accumulation by regulating cytochrome P450 2E1. This suggests that ROS and Bax are closely associated.

UCP2 can regulate ROS generation. Echtay observed that mild uncoupling reduces the mitochondrial production of ROS [9]. ROS are important mediators of tissue damage. A recent study also showed that UCP2 influences apoptosis regulation in different cell systems [10]. The present study investigates the effects of oxidative stress due to exhaustive training on UCP2 and Bcl-2/Bax in rat skeletal muscles. Particularly, this study aims to evaluate the effects of oxidative stress on tissue damage and determine the relationship between oxidative stress and overtraining.

2. Materials and Methods

2.1. Animals. Eighteen 8-week-old female Sprague-Dawley (SD) rats from Shanghai Sino-British Sipper/BK Lab Animal, Ltd. were used. The animals were housed at 25°C with an inverted 12 h light-dark cycle and fed ad libitum. All experiments were approved by the Ethics Committee of Shanghai University of Sport and complied with the National Regulation for Administration of Laboratory Animals. Prior to training, all rats were adapted to treadmill running for one week. The adaptation phase consisted of treadmill running 6 days/week for 5 min at a speed of 10 m/min. At the end of this period, the rats were randomly divided into three groups: the control group (CON), the trained control group (TC), and the exhaustive trained group (ET). Six rats were housed per cage, with the trained animals stored in cages separate from those of untrained animals but in the same room of the animal housing facility.

2.2. Training Protocol. The training protocol was designed to induce a training-to-OT continuum (Table 1) [11]. Both the training volume and intensity were gradually increased in the first six weeks. During the last three weeks, the TC and ET groups were maintained at the same exercise intensity (the same speed and grade); however, the ET group was trained longer until exhaustion. The exhaustion was defined as the point at which the animals failed to get off the shock grid and thus had to be manually returned to the front of the treadmill for three consecutive occasions [12]. The actual training duration of the ET group ranged from 180 min to 200 min in the seventh week and 60 min to 80 min in the eighth and ninth weeks. The training week consisted of six consecutive days of training sessions followed by one rest day. A motorized treadmill with adjustable inclination was used (DSPT202, Qianjiang Technology Company, Hangzhou, China).

2.3. Tissue and Blood Sample Collection. The TC and the ET groups were sacrificed after 36 h after the last training session to avoid acute exercise effects. The control group was sacrificed at the end of the nine weeks. All rats were anesthetized with pentobarbital (40 mg/kg body weight). Blood was rapidly collected from the abdominal aorta; plasma was separated by centrifugation and then stored at −78°C for further analysis [12]. The samples were dissected from the darker side of the red gastrocnemius muscle. Half of the samples were formalin-fixed, whereas the remaining samples were frozen in liquid nitrogen and stored at −78°C.

2.4. Hemoglobin (Hb) Assay. Hb was analyzed by an automatic cytometer (BC-3000, Shenzhen Mindray Bio-Medical Electronics Co., Ltd., Shenzhen, China).

2.5. Mitochondrial Isolation from Skeletal Muscles. The red gastrocnemius muscles were freed of excess fat and connective tissue, finely minced, and washed in a medium containing 20 mM HEPES, 2 mM $MgCl_2$, 120 mM KCl, 5 g/L BSA, with pH of 7.4. The tissue fragments obtained were homogenized with the above medium (1 : 8, w/v). The homogenate was then centrifuged at 600 g for 10 min twice. The resulting supernate was centrifuged at 17000 g for 10 min. The pellet was homogenized with the above medium (1 : 10, w/v) and then centrifuged again at 7000 g for 10 min. The pellet was washed and resuspended in a suspension medium (300 mM sucrose, 0.1 mM EGTA, 2 mM HEPES, pH 7.5) and centrifuged at 3500 g for 10 min. The pellet was resuspended in the suspension medium described above to determine SOD, XOD, and ATPase activities and MDA [13]. The pellet was broken down by sonication (JY92 Ultrasonic Cell Pulverizer, Ningbo Scientz Biotechnology Co., Ltd.) before analysis.

2.6. Protein Concentration. Protein concentrations were determined by Bio-Rad Bradford protein assay.

2.7. Mitochondria Oxidative Stress Markers and ATPase. Mitochondria SOD, XOD, ATPase activities, and MDA concentrations were measured using a commercial kit (Nanjing Jiancheng Bioengineering Institute, China).

2.8. Skeletal Muscle UCP2 Western Blot Analysis. After the red gastrocnemius muscles were homogenized in an ice-cold lysis buffer, whole-tissues homogenates were centrifuged at 14,000 g for 20 min, and the supernates were collected. After quantitation of protein concentration, equal amounts of proteins (30 μg) were electrophoresed on sodium dodecyl sulfate polyacrylamide gel and then transferred onto polyvinyl difluoride. The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline for 1 h at room temperature, and incubated overnight at 4°C with primary antibodies (Wuhan Boster Bio-Engineering Ltd. Co., China). The membranes were then incubated with appropriate horseradish peroxidase-linked secondary antibodies (Pierce, Rockford, IL, USA) for 2 h at room temperature and visualized using an enhanced chemiluminescence detection system (Pierce, Rockford, IL, USA) [14]. Following western blot development, the relative abundance of UCP2 was determined by densitometry. The band intensities of the exposed film were analyzed using Image J software.

2.9. Immunohistochemical Staining. Bcl-2 and Bax from the red gastrocnemius muscle of each group were determined by the streptavidin-biotin complex (SABC) method. The paraffin sections (4 μm) from each group were dehydrated

Table 1: Training protocol.

Weeks	TC group			ET group		
	Speed (m/min)	Grade (%)	Time (min)	Speed (m/min)	Grade (%)	Time (min)
1	15	2	40	15	2	40
2	20	10	50	20	10	60
3	25	10	60	25	10	90
4	30	5	60	30	5	120
5	30	5	60	30	5	120
6	30	8	60	30	8	120
7	35	10	30	35	10	Exhaustion*
8	35	15	30	35	15	Exhaustion*
9	35	15	30	35	15	Exhaustion*

*Exhaustion was defined as the point at which the animals failed to get off the shock grid and had to be manually put back to the front of the treadmill for three consecutive occasions.

in xylene and graded ethanol series and added in order with the primary antibody (rabbit polyclonal antibody, Santa Cruz, USA), biotinylated secondary antibody (goat serum 1 : 100, Wuhan Boster Co., China), SABC reagents, and diaminobenzidine solution (Wuhan Boster Co., China). For negative control, the sections were treated with PBS instead of primary antibody. Ten sample sections from each group were selected for analysis. More than five visual fields were observed per section. The following equation was used: the positive percentage of each protein = protein − positive cells/all cells × 100%.

2.10. Statistical Analysis. Statistical analyses were performed using SPSS 13.0 for Windows (SPSS Inc., Chicago, IL, USA). The results are presented as means ± standard deviation (SD). We used one-way ANOVA followed by least significance difference post hoc test to compare the means of the three groups. If data failed the normality test, the Kruskal-Wallis one-way ANOVA on ranks and Tukey's post hoc test were used. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Body Weight. Both trained groups exhibited a significant decrease in body weight relative to the CON group at the end of the ninth week of training ($P < 0.05$), but no significant difference was indicated between the TC and the ET groups (Table 2).

3.2. Hb, Concentration of MDA, and Activities of XOD, SOD, and ATPase. Hb was significantly decreased in the ET group compared with the CON and the TC groups ($P < 0.001$) (Table 3). MDA concentration was significantly increased in the ET group compared with the CON group ($P < 0.05$). SOD activity was significantly decreased in the ET group compared with the CON group ($P < 0.05$). The SOD/MDA ratio was significantly increased in the TC group compared with the CON group ($P < 0.05$), but the SOD/MDA ratio was significantly decreased in the ET group than that in the CON group ($P < 0.001$). XOD activity was significantly increased in the ET group compared with the CON group ($P < 0.05$).

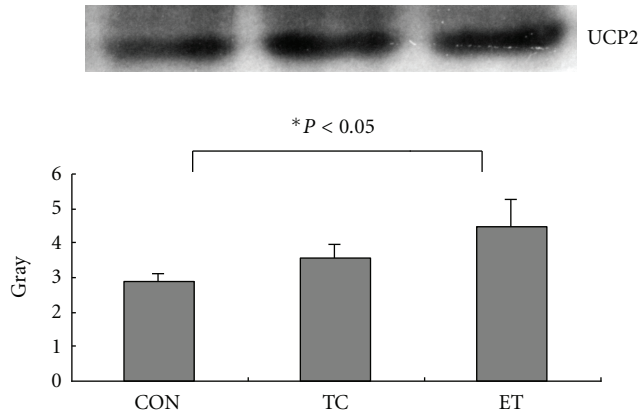


Figure 1: UCP2 protein expression in rat gastrocnemius muscle.

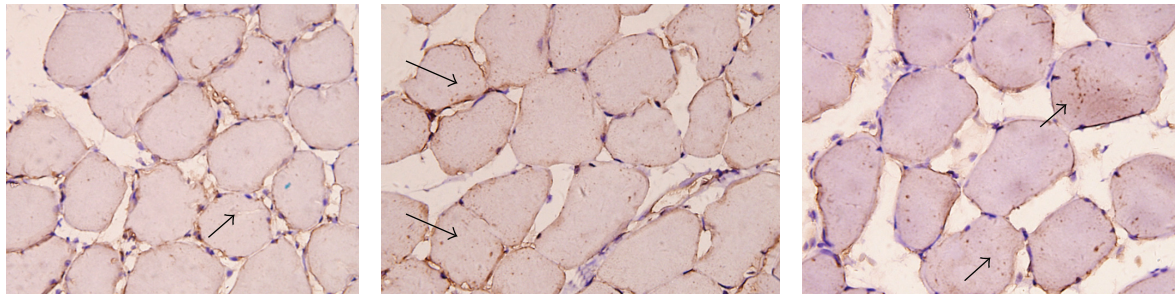
Meanwhile, ATPase activity was significantly decreased in the ET group compared with the CON group ($P < 0.001$).

3.3. Muscle UCP2 Protein Expression. The skeletal muscle UCP2 skeletal muscle UCP2 expression was significantly higher in the ET group than that in the CON group ($P < 0.05$). However, no difference in the UCP2 expression was indicated between the TC and the CON groups (Figure 1).

3.4. Bax and Bcl-2 Protein Expression. Bax protein expression was higher in the ET group than in the CON group (Figure 2). Bcl-2 protein expression was lower in the ET group than in the CON group (Figure 3). The Bcl-2/Bax ratio was significantly ($P < 0.01$) decreased in the ET group compared with the CON group (Table 4).

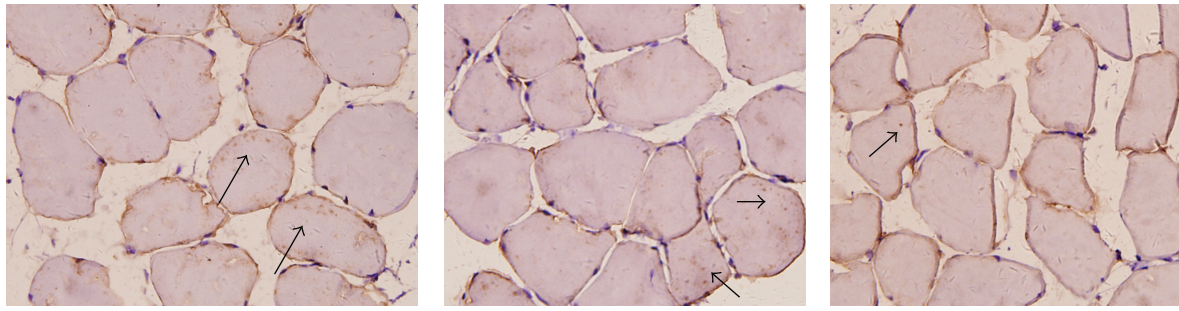
4. Discussion

In the present study, continuous exhaustive training was shown to cause skeletal muscle mitochondrial MDA concentration, and XOD activity significantly increased. However, SOD activity in the rats was suppressed. Continuous exhaustive training damaged the balance between the intracellular



CON (40 × 10) TC (40 × 10) ET (40 × 10)

Figure 2: Positive immunoreactivity of Bax in each group.



CON (40 × 10) TC (40 × 10) ET (40 × 10)

Figure 3: Positive immunoreactivity of Bcl-2 in each group.

Table 2: Body weight change during the training (g).

Week	n	CON	TC	ET
0	6	225.75 ± 5.66	224.40 ± 4.24	228.68 ± 7.39
6	6	284.62 ± 18.19	270.95 ± 18.58	283.17 ± 15.68
9	6	296.75 ± 19.31	266.63 ± 16.41*	268.28 ± 19.53*

*Compared with CON, $^{*}P < 0.05$. Means ± SD.

Table 3: Effect of training on Hb and mitochondrial parameters.

	CON ($n = 6$)	TC ($n = 6$)	ET ($n = 6$)	P value
Hb, g/L	121.9 ± 5.9	119.7 ± 4.5	99.2 ± 8.6^{aa,bb}	< 0.01
SOD, U/mgprot	19.04 ± 2.42	21.12 ± 4.40	15.17 ± 5.56^{b}	< 0.05
MDA, nmol/mgprot	15.52 ± 1.93	14.78 ± 0.82	18.40 ± 3.54^{a,b}	< 0.05
SOD/MDA	1.23 ± 0.12	1.42 ± 0.24^{a}	0.80 ± 0.20^{aa,bb}	< 0.05 < 0.01
XOD, U/mgprot	5.79 ± 0.72	7.49 ± 0.81	8.18 ± 1.20^{a}	< 0.05
ATPase, U/mgprot	12.80 ± 0.45	10.61 ± 0.70	6.95 ± 0.17^{aa}	< 0.01

^{a,aa}Compared with CON: $^{a}P < 0.05$, $^{aa}P < 0.01$; ^{b,bb}compared with TC: $^{b}P < 0.05$, $^{bb}P < 0.01$. Means ± SD.

Table 4: Changes in Bcl-2 and Bax protein expression in rat skeletal muscle.

Group	n	Bcl-2 positive rate (%)	Bax positive rate (%)	Bcl-2/Bax ratio
Con	6	19.29 ± 6.95	12.56 ± 2.99	1.49 ± 0.30
TC	6	17.67 ± 8.23	23.26 ± 6.05*	0.76 ± 0.32*
ET	6	12.22 ± 5.24	28.85 ± 7.75**	0.46 ± 0.23**

*,**Compared with CON: $^{*}P < 0.05$, $^{**}P < 0.001$. Means ± SD.

oxidative and antioxidant factors. The rats simultaneously showed a rapid decrease in motion ability, standing dull hair, decreased locomotor activity, lack of response, and decreased Hb, leading to the OT state [15, 16]. This study found that the Bax expression of promoted apoptosis increased and Bcl-2 expression inhibited apoptosis decreased after continuous exhaustive training. The significant decrease in the ratio of Bcl-2/Bax showed that continuous exhaustive training promoted the apoptosis process. Currently UCP2 is known to regulate ROS concentration and apoptosis. After continuous exhaustive training in our experiments, skeletal muscle mitochondria UCP2 expression increased significantly, which can be related to the increase in the induction of MAD concentration and the reduction in the ratio of Bcl-2/Bax [17].

4.1. Oxidant and Antioxidant. ROS can be produced from numerous sources during exercise. These sources include the mitochondrial electron transport chain and xanthine oxidase system, among others [18]. Our study indicated that after OT mitochondrial MDA concentration and XOD activity increased in rat red gastrocnemius muscles. MDA, which indirectly reflects the degree of ROS on membrane lipid peroxidation, is one of the products of membrane lipid peroxidation. XOD is the main enzyme of the xanthine oxidase pathway. This enzyme is involved in the pathophysiology of ischemia-reperfusion syndrome and can lead to tissue damage after an exhaustive bout of exercise [19], Thus, the increases in XOD activity and MDA concentration indicated that continuous exhaustive training caused by overtraining enhanced tissue damage. On the other hand, SOD activity decreased, suggesting that OT increases ROS generation and inhibits the scavenging ability of ROS [20]. These changes can be related to allosteric downregulation of the enzymes and enzyme inactivation due to overwhelming oxidative stress [21]. Studies suggest that increased oxidative stress influences the pathophysiology of overtraining. The weakened responses of oxidative stress and antioxidant capacity to exercise in the overtraining state can be associated with the inability to exercise effectively and impaired adaptation to exercise [22].

Continuous exhaustive training can significantly affect ROS accumulation. The mitochondrial electron transport chain (ETC) of oxidative phosphorylation is identified as a major site for cellular ROS generation. As electrons pass through the complexes of the ETC, some of these electrons leak to molecular oxygen, thus forming superoxides [23]. When energy consumption in the tissue increased sharply during the exhaustive training, mitochondrial oxygen consumption also increased, and ROS generation rose. Lactic acid concentration increases caused by exhaustive training induce a synergistic effect on ROS production [24].

Mitochondrial ROS production highly depends on the membrane potential generated by the proton gradient formed across the inner mitochondrial membrane [25]. High membrane potential is shown to stimulate ROS production. One view states that in the presence of a large mitochondrial matrix such as in stress situations, $O_2^{\bullet -}$ can be activated in the endometrial stromal side of uncoupling proteins (UCPs). This activation leads to proton transfer and mitochondrial membrane solution coupling, thus reducing ROS generation [26, 27]. ROS production and mitochondrial proton leak are mediated by UCP2. According to feedback loop theory [28], activation of proton leak decreases the mitochondrial membrane potential, thus limiting mitochondrial ROS generation. This effect shows that the UCP2 can reduce ROS generation in oxidative stress and protect the cell from ROS-induced damage. Thus, UCP2 can regulate the concentration of intracellular ROS. Exercise as a stress factor can induce a UCP2 response. Studies showed that a one-time exercise temporarily increases the impression of UCP2b mRNA; endurance exercise shows no such effect [29]. In the present study, UCP2 significantly increased in the ET group after exhaustive training but not in the TC group, which indicates inflammation [30]. Continuous exhaustive training stimulates ROS generation, which results in increased expression of mitochondrial UCP2 protein to protect cells from damage caused by inflammation. Thus, UCP2 is considered an important number in the antioxidant system. The transcription of the UCP2 gene itself is highly inducible under the conditions of oxidative stress [17, 27].

4.2. Uncoupling and Energy Loss. UCP2 can affect mitochondrial energy production when ROS generation is inhibited. Studies found that the ATP content of UCP2-overexpressing islets was reduced by 50% [31] and that ATP stores are reduced by 15% to 30% in UCP2-overexpressing hepatocytes [32, 33]. These findings suggest that increasing mitochondrial proton leak induces a decrease in ATP synthesis and reduces the efficiency of energy metabolism. Bouillaud proposed a metabolic hypothesis in which UCP2 acts through a transport distinct from the proton transport. He asserted that this transport activity decreases the mitochondrial oxidation of glucose-derived pyruvate [34]. These actions increase the influence of UCP2 on cellular metabolism. In the present study, UCP2 expression was significantly increased in the ET group; however, mitochondrial ATP synthase activity was significantly reduced. Thus, OT induced by exhaustive training can decrease ATP synthesis and increase ROS production, enhancing UCP2 expression. UCP2 overexpression mediates ROS production and induces an imbalance between energy metabolism and ROS elimination, which reduces the efficiency of mitochondrial energy metabolism. Mild uncoupling can diminish mitochondrial superoxide production, increasing protection against diseases and tissue damage by a small energy loss [28]. However, a substantial energy loss can occur after continuous exhaustive training. This loss can affect exhaustive training-induced OT. Westerblad and Allen [35] indicate that prolonged increases in ROS are likely to induce posttranslational changes in various proteins, which can directly affect contractile function. Decline in exercise ability may be closely associated with exercise-induced ROS-mediated changes in the ryanodine receptor (RyR1) can cause continuing decreases in sarcoplasmic reticulum (SR) Ca^{2+} release[36].

4.3. UCP2 and Apoptosis. UCP2 does not only suppress mitochondrial ROS; it also regulates apoptosis in various

cell systems [22]. Cultured adult rat cardiomyocytes exposed to free fatty acids were shown to exhibit a dose-dependent increase in apoptosis and a significant increase in UCP2 expression. RNA interference and UCP2 knockdown reduced free fatty acid-induced apoptosis in cardiomyocytes [37]. This finding suggests that an increase in UCP2 expression results in increased apoptosis. A study on A549 cells under hypoxic conditions indicated [38] that UCP2 showed antiapoptotic properties. UCP2 overexpression inhibited ROS accumulation and apoptosis, as well as the release of cytochrome c, and reduced the activation of caspase-9. The aforementioned studies indicate that UCP2 has a regulating effect on ROS and cell apoptosis.

Apoptosis is a complex process involving several cellular proteins. A study suggested that Bcl-2/Bax expression balance determines survival or death following apoptosis [39]. We found that OT led to the elevation of Bax expression and decrease in Bcl-2 expression in muscle tissues. The Bcl-2/Bax ratio was significantly reduced, suggesting that continuous exhaustive training-induced ROS resulting from overtraining could damage cells, leaving the red gastrocnemius muscle with decreased antiapoptotic ability. ROS can activate the early apoptotic signaling pathway MAPK (such as SAPK/JNK, ERK1/2, and p38) [40]. In such a process, ROS can inhibit Bcl-2 expression and promote cell apoptosis. Studies showed that by maintaining mitochondrial membrane integrity, Bcl-2 increases the outflow of protons in the mitochondria and inhibits the decrease in membrane potential. Bax may bind to voltage-dependant anion channel and open a permeability transition pore, inducing a decrease in membrane potential and promoting cell apoptosis [41–43]. Continuous exhaustive training alters the Bcl-2/Bax balance, thus increasing the likelihood of apoptosis.

In summary, exhaustive training increases the ROS in red gastrocnemius muscles, decreases SOD activity, and causes oxidative stress. Extensive ROS production increases UCP2 expression, which modulates the membrane potential and decreases ROS production. However, the increase in UCP2 expression also leads to a decline in mitochondrial energy metabolism. These effects can significantly affect exhaustive training-induced OT. On the other hand, ROS accumulation can increase Bax expression and reduce Bcl-2 expression, promoting cell apoptosis. These behaviors can be related to a pathological change after OT.

4.4. Perspective. The current problem is that under normal circumstances, the ATP/ADP ratio regulates the cytochrome c oxidase (CcO) activity [44]. CcO is the terminal and rate-limiting enzyme of the respiratory chain. When the ATP/ADP ratio increases, ATP allosterically inhibits the CcO enzyme [45]. After continuous exhaustive training, the decreased ATP synthesis in mitochondria results in reduced feedback inhibition of CcO activity, which needs further investigation. CcO inhibition known as the feedback to maintain a low mitochondrial membrane potential $\Delta\Psi m$ and lower ROS levels. Thus, if CcO activity increases, a further increase in ROS levels is promoted, creating the vicious circle phenomenon.

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