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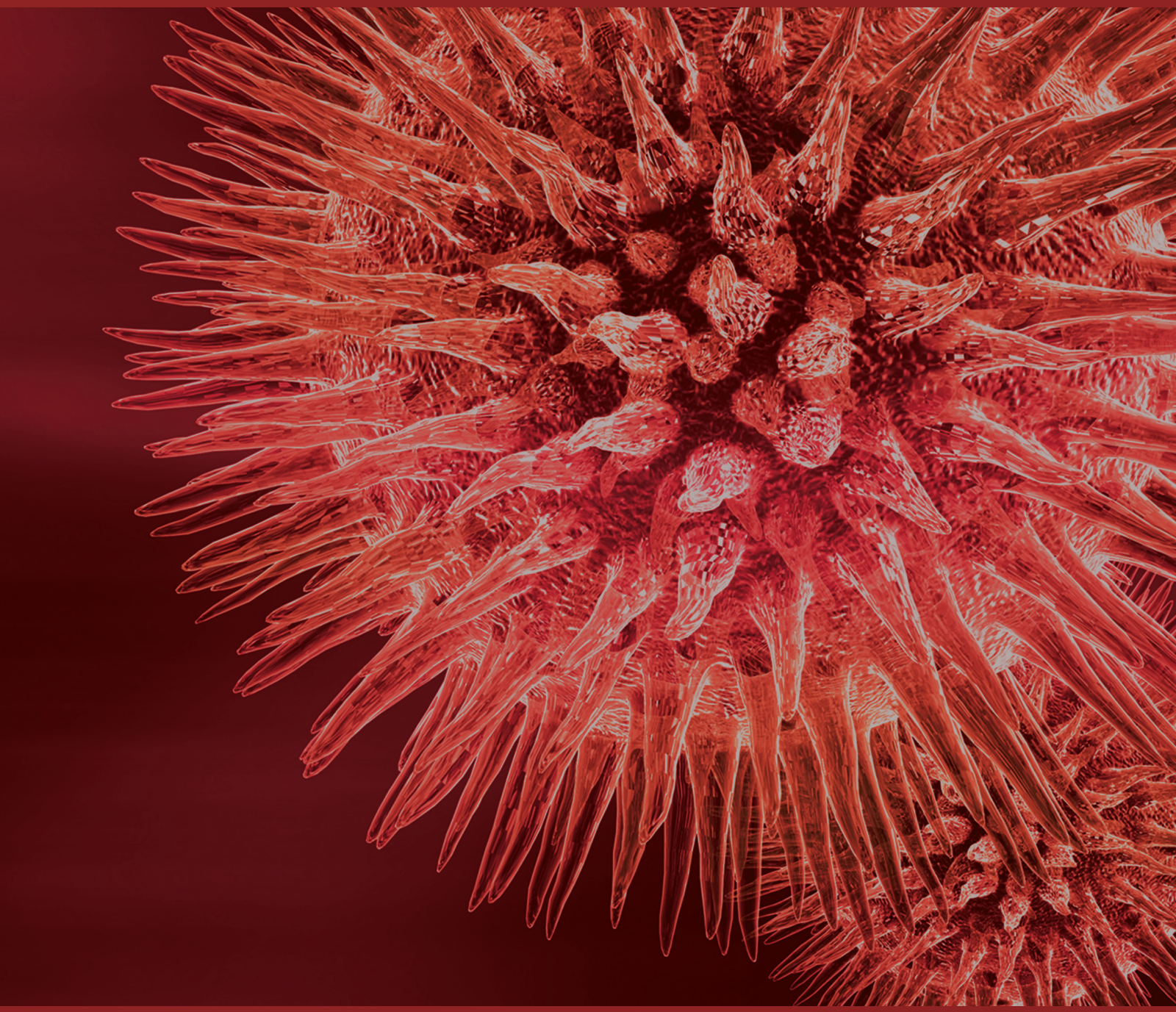
Guest Editors: E. K. Tan, Amit K. Srivastava, W. David Arnold, Mahendra P. Singh, and Yiying Zhang



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Editorial

Neurodegeneration: Etiologies and New Therapies

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Neurodegeneration refers to the progressive loss of structure or function of neurons and it can lead to devastating neurological conditions such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), and amyotrophic lateral sclerosis (ALS). Neurodegeneration is frequently multifactorial in origin though aging and genetic and environmental factors are thought to play a significant role. The relative contribution of genes and environmental factors has been debated. For AD and PD, both familial (monogenic and complex inheritance) and sporadic forms exist while some like HD are purely genetic in nature.

The key pathological hallmarks of neurodegenerative diseases include oxidative stress, proteasomal impairment, mitochondrial dysfunction, and accumulation of abnormal protein aggregates. Advances in the field of etiology and therapy are mainly based on understanding of basic biochemical and molecular events underlying degeneration in human postmortem brain specimens and animal models. Molecular imaging and the emergence of complex bioinformatics tools have provided important insights [1].

This special issue on neurodegeneration provides a platform for critical reviews on recent advances and original articles that offer significant insights into biochemical and molecular aspects of neurodegeneration with the potential of identifying novel therapeutic targets (such as synthetic/naturally occurring agents/extracts).

Oxidative stress, mitochondrial dysfunction, and gliosis are found to play critical functions in the process of neurodegeneration [2]. Epileptic seizures can vary from brief and undetectable reaction to prolonged and vigorous shaking. While the contributors of epilepsy are relatively mysterious, brain injury leading to neurodegeneration could also contribute to epileptic episodes. S. Puttachary et al. review the role of seizures-induced oxidative stress in neurodegeneration along with spontaneous recurrent seizures in temporal lobe epilepsy. They highlight the contribution of the mitochondrial dysfunction, gliosis, and neurodegeneration in temporal lobe epilepsy and alleviating effects of endogenous antioxidant pathways.

Although HD is an autosomal genetic disorder, development of a fully reliable rodent model that mimics all symptomatic and pathological traits of disease is still a challenge. Y. Mazurová et al. allude to the histopathological changes in the brain of transgenic HD rats. This paper emphasizes how glial cells could play a unique role and transgenic rats could be used as a valid model for diagnostic and therapeutic interventions.

The cardinal challenge in neurodegeneration is to either slow down or reverse disease progression. Extracts of naturally occurring agents theoretically may have less toxicity compared to synthetic drugs. F. Ghahremanitamadon et al. demonstrate the shielding efficacy of *Borago officinalis* extract in amyloid β-peptide (25–35)-induced oxidative stress and behavioral deficits. They demonstrate the usefulness of *Borago officinalis* extract in reducing memory impairment.

Another major difficulty is to effectively deliver an active or pro drug across the blood-brain barrier. J. Hou et al. describe an in vivo microdialysis of (N-[2-(4-hydroxy-phenyl)-ethyl]-2-(2,5-dimethoxy-phenyl)-3-(3-methoxy-4-22hydroxy-phenyl)-acrylamide) penetration through the blood-brain barrier in normal and 6-hydroxydopamine-induced parkinsonism. The findings could potentially lead to clinical trials of bioactive molecules.

Diagnosis and delineation of neurodegeneration are still complicated. A. P. Patterson et al. highlight the relevance of in vivo optical imaging systems in neurodegeneration. The authors draw attention to the contribution of imaging tools along with fluorescent and bioluminescent molecules, in the diagnosis and monitoring of neurodegenerative diseases.

The cause and effect of inflammation in neurodegeneration are still unknown. Y. Chao et al. provide a concise review of the involvement of inflammatory factors and immune system in the pathogenesis of PD and discuss the potential therapeutic targets to regulate immune response in the disease. In another review article, Y. Lee et al. described the role of microglia in pathogenesis of ischemic stroke and several therapeutic approaches to modulate microglial response.

Neurodegenerative processes are complex and clinical and pathological correlation is often hard to demonstrate. A multiprong research approach evaluating potential molecular targets, the most effective way to deliver potential pharmacological agents to the brain, and the appropriate design of clinical trials that can monitor and evaluate the efficacy of novel drugs will be vital. In this light, we hope this special issue can provide impetus for investigators to take on these challenges with the ultimate hope of finding a cure for many of the devastating neurological diseases.

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Amit K. Srivastava
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Review Article

Integrating Retrogenesis Theory to Alzheimer's Disease Pathology: Insight from DTI-TBSS Investigation of the White Matter Microstructural Integrity

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Microstructural abnormalities in white matter (WM) are often reported in Alzheimer's disease (AD) and may reflect primary or secondary circuitry degeneration (i.e., due to cortical atrophy). The interpretation of diffusion tensor imaging (DTI) eigenvectors, known as multiple indices, may provide new insights into the main pathological models supporting primary or secondary patterns of WM disruption in AD, the retrogenesis, and Wallerian degeneration models, respectively. The aim of this review is to analyze the current literature on the contribution of DTI multiple indices to the understanding of AD neuropathology, taking the retrogenesis model as a reference for discussion. A systematic review using MEDLINE, EMBASE, and PUBMED was performed. Evidence suggests that AD evolves through distinct patterns of WM disruption, in which retrogenesis or, alternatively, the Wallerian degeneration may prevail. Distinct patterns of WM atrophy may be influenced by complex interactions which comprise disease status and progression, fiber localization, concurrent risk factors (i.e., vascular disease, gender), and cognitive reserve. The use of DTI multiple indices in addition to other standard multimodal methods in dementia research may help to determine the contribution of retrogenesis hypothesis to the understanding of neuropathological hallmarks that lead to AD.

1. Introduction

Alzheimer's disease (AD) is one of the most prevalent neurodegenerative disorders in the elderly which is estimated to affect tens of millions of people worldwide [1]. A recent study estimates that dementia shall affect over 81 million individuals worldwide by 2040 [2]. The progression of clinical-pathological correlations of AD can be understood in terms of disconnection syndromes and functional distributed networks underlying cognitive abilities [1, 3].

The investigation of AD neuropathology and its relation to cognitive decline, previously restricted to postmortem studies, has developed substantially with the advent of neuroimaging techniques in the last decades. Early neuroimaging studies on AD were focused on volumetric based morphometric techniques and region of interest investigations (ROI), which were performed through image registration and smoothing [4]. Progressively, conventional ROI approaches have been replaced by whole brain diffusor tensor imaging (DTI) analysis, which offers higher accuracy for white

matter (WM) registration between subjects. DTI enables the definition of major WM tracts and their trajectories and also WM microstructure [1], thus providing a comprehensive investigation of brain circuitry integrity. DTI is sensitized to the random motion of water molecules as they interact within tissues, thus reflecting characteristics of their immediate structural surroundings. One of the most frequently employed DTI methods is the tract-based spatial statistics (TBSS) [5], which represents an effort to overcome some limitations of conventional ROI method [6, 7], including tract misalignment and variance effects in brain atrophy and partial volume estimations [8].

The evidence base gathered by DTI investigations in the last decade has helped to better define the pathological cascade underlying AD [6]. Diffusion studies on AD were primarily focused on the pattern of lesions distribution, the localization of DTI changes, the distribution of disrupted networks, and the nature of microstructural pathology. Regardless of DTI sensitivity in assessing WM microstructural changes, differences in diffusion patterns across clinical groups may be challenging to interpret [1]. Several studies reported DTI changes in the parahippocampus, hippocampus, posterior cingulum, and splenium even at the MCI stage [9–13]. Widespread areas of DTI abnormalities may also be observed in AD. It has been estimated that the whole brain may present a mass reduction of nearly 3-4% per year [14].

On the microstructural level, WM abnormalities in AD may be interpreted as myelin breakdown and axonal damage [15]. Different pathological models have been suggested to account for these microstructural alterations: retrogenesis and Wallerian degeneration. Retrogenesis assumes primary white matter atrophy through myelin breakdown and axonal damage [15–18]. It has been suggested that fibers more susceptible to neurodegeneration due to the retrogenesis process are those with small-diameter corticocortical axons [19–21], namely, from the temporal lobe and neocortical areas. Conversely, the Wallerian degeneration assumes secondary white matter atrophy due to cortex degeneration [22]. Evidence favouring the Wallerian degeneration or the retrogenesis remains disputed [23]. For instance, neuronal disruption at predementia stages may not solely account for Wallerian degeneration and there are anatomical regions where the retrogenesis hypothesis might better explain WM atrophy (Figure 1). Moreover, the corpus callosum may be susceptible to AD and, depending on its anatomical localization, DTI changes would be associated either with retrogenesis or Wallerian degeneration [8]. Previous studies reported a correlation between gray matter (GM) temporal atrophy and the reduced volume of CC posterior segments [15], while in others cortical atrophy failed to show an association with anterior CC fibers [15]. In fact, it has been also demonstrated that the genu of the CC is a region where fibers myelinate later in neurodevelopment [24]; this region contains the highest density of small diameter fibers, whereas fibers of the splenium of the CC myelinate earlier on life [24].

The overarching aim of this comprehensive review is to summarize the main etiological mechanisms associated with AD neuropathology, based on the most recent alternative explanation, the retrogenesis hypothesis. The contribution of DTI studies to the understanding of the retrogenesis model is critically analyzed, as well as the clinical significance of the main DTI proxies (described in detail below) for the interpretation of neuropathological mechanisms involved in neuronal disruption, namely, axonal damage and myelin breakdown [25] (Figure 1). Additionally, patterns of neurodegeneration will be discussed in relation to risk factors, progression of lesions along disease course, DTI changes, and the influence of retrogenesis model on regional tracts. Finally, the interaction between late-myelinating alterations, amyloid deposition, and vascular factors in AD is reviewed.

2. Methods

A review of the literature was performed from 2004 to 2014 through searches in the electronic databases PubMed (http://www.ncbi.nlm.nih.gov/pubmed/), Institute for Scientific Information Web of Knowledge (http://www.isiknowledge.com), and EMBASE (http://www.embase.com), using the following terms: "retrogenesis," "diffusion tensor imaging," "DTI," "Alzheimer's disease," "mild cognitive impairment," "Wallerian degeneration," and "neuropathology." We also hand-searched articles cited in the selected papers, so that publications missed by the electronic research could be added. Inclusion criteria were as follows: original articles written in English and focusing on AD and MCI and DTI studies carried out through TBSS with non-FA indices (see further explanation below) in addition to FA calculation. Reviews and case reports were excluded from this review; studies using clinical constructs other than AD and MCI were also excluded from this study.

2.1. Pathological Mechanisms Underlining Retrogenesis and Wallerian Degeneration Model. Evidence based on human neuropathological studies suggests that the brain regions most metabolically active in AD might be also the most capable to respond to mitogenic stimulus and, consequently, those with highest vulnerability to degenerate [16]. One useful terminology for characterizing the pattern of neuronal vulnerability for retrogenic process is the arboreal entropy. According to this model, the greater the neuroprotection, the less vulnerable the myelin and axon. Conversely, neuronal fibers may be attacked from their inside by neurofibrillary and neurotubular changes secondary to hyperphosphorylation, which ultimately may lead to axonal injury and myelin loss [16].

Myelin may be a living, metabolically active part of the neuronal axon, with a membrane running through it, which is an extension of the cell (axonal membrane). Mitogenic activation is involved in cell plasticity and there is consistent evidence showing that mitogenic pathways in neurons are erroneously activated early during AD [26]. Distinct mechanisms may be associated with such mitogenic pathways [26], including hypoxia and β-amyloid deposition [27, 28], deficiency of vitamin B12 levels or folate, increased serum homocysteine levels, and increased serum methylmalonic acid levels [29, 30], even though their interaction in the myelin degeneration awaits further elucidation (for a thorough review see Arendt [26]). Atherosclerosis and

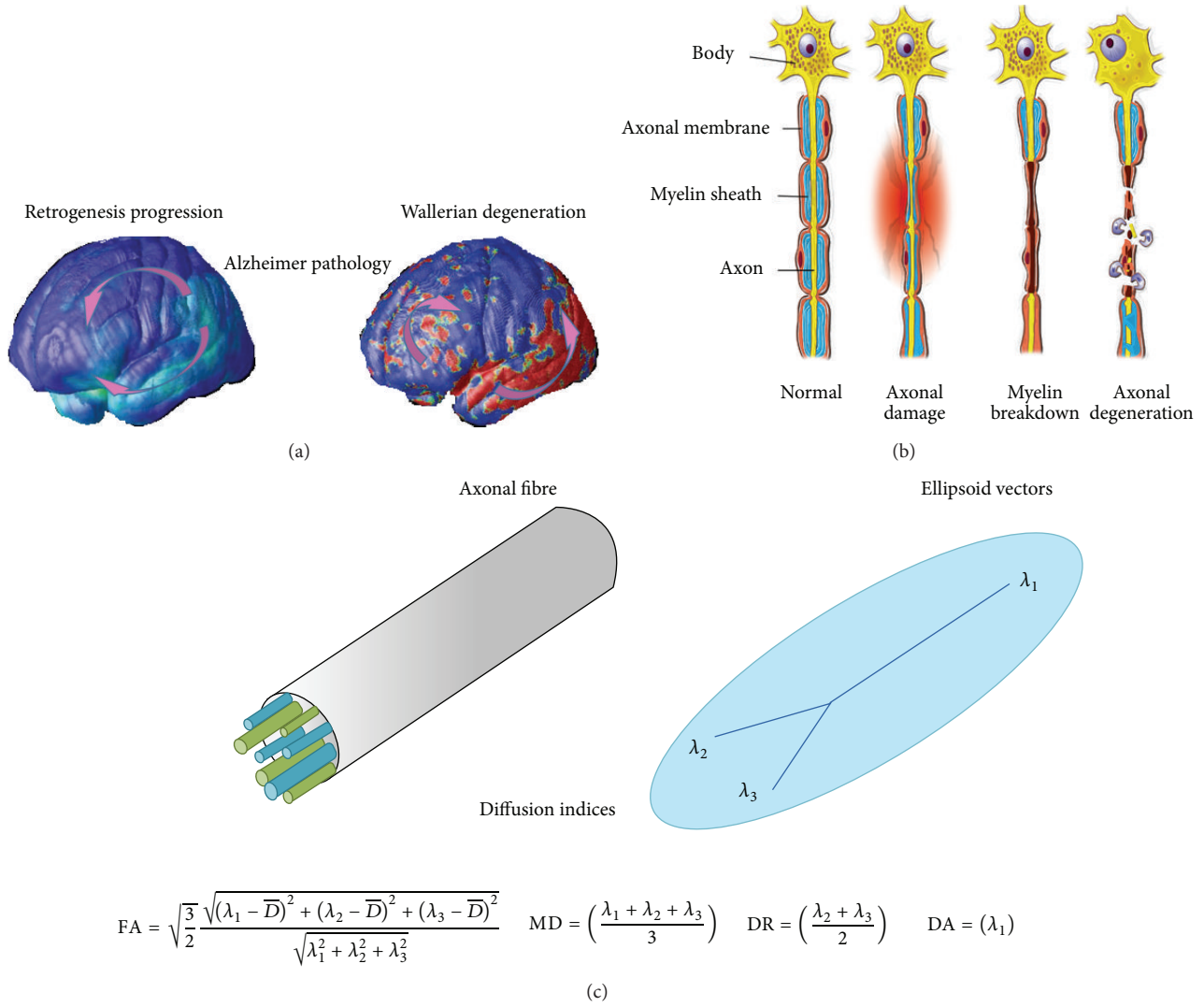
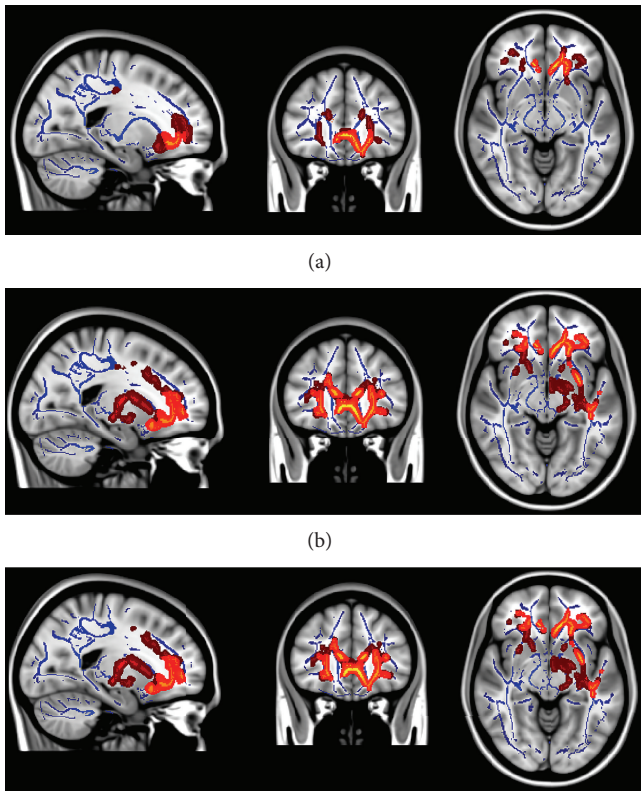


Figure 1: (a) Wallerian degeneration occurs as a secondary product of gray matter loss, while retrogenesis hypothesis outlines the degeneration of late-myelination fibers in neocortical areas. The Wallerian degeneration model postulates a posterior-anterior gradient of fibre degeneration (right side, arrows); the normal myelinisation occurs throughout the first life decades, beginning at dorsal brain and reaching neocortical areas at end stages (right side, arrows). According to the retrogenesis model, neocortical fibers are those more likely to suffer early degeneration by AD; (b) myelin breakdown and axonal damage are one of the key pathological mechanisms underlying white matter microscopic lesions (b). (c) A projection of the ellipse onto the three main axes (λ_1, λ_2, λ_3) or eigenvectors. The main DTI indices of fractional anisotropy (FA) and axial (DA), radial (DR) and mean (MD) diffusivity are based on the eigenvector calculations (bottom).

cerebrovascular disease are other risk factors associated with AD, which have been primarily associated with myelin disruption. Therefore, the entire retrogenesis process implicated in AD neuropathology may comprise myelin, as well as the neuronal reactivation of mitogenic factors. The process of myelination is now known to continue well into the latter portion of life [17, 31]. Possibly, myelin plays a role not only in the conduction of electrical impulses in the neuron, but also in protection and maintenance of the oligodendroglia, myelin, and axonal relationship [17, 31]. Accordingly, early-myelination neurons may become increasingly more thickly myelinated across the years. Consequently, the most recently affected and, as a result, most thinly myelinated brain regions may be the most vulnerable to injury.

There is consistent evidence suggesting that WM alterations could reflect Wallerian degeneration as a secondary product of cortical pathology [22]. The pathological basis for investigating Wallerian degeneration has been largely demonstrated by experimental animal models, such as those with the sciatic nerve of the frog [32]. In fact, amyloid deposition around neuronal cells or neurofibrillary tangles in the cell bodies ultimately leads to degeneration of axons and myelin [33]. Structural changes including the breakdown and dissolution of both the axonal cytoskeleton and myelin and ultimately the elimination of myelin and other debris by Schwann cells and macrophages are pathological events involved in the secondary degeneration, which is in turn induced by amyloid deposition [32, 34, 35]. Conversely,



(a)

(b)

(c)

Figure 2: DTI changes are evidenced in Alzheimer subjects when compared with healthy controls. Overlapping areas of FA decreased/DR increased are indicative of increased diffusion perpendicular to fibre orientation, possibly due to myelin breakdown (yellow-red). These areas can be observed in the corpus callosum (anterior and middle segments), anterior cingulum, and uncinate fasciculus (anterior portion) and remain when adjusting for group differences in gray matter atrophy (a) and white matter burden volume (c). Notes: FA: fractional anisotropy; DR: radial diffusivity.

primary damage to WM tracts has been pointed out by recent studies as an alternative explanation for WM disruption (Figure 2). Interestingly, Aβ deposits around WM vascularity [36] and cellular cytotoxicity provoked by Aβ peptides in oligodendrocytes, the cells responsible for myelin production, have also been reported [37]. Finally, a third mechanism of WM degeneration involving tau has more recently been proposed. The tau protein seems to participate in the integrity and stabilization of axonal cytoskeleton by binding to microtubules [38]. Axonal extensions may become swelled [39] and axonal transport may be disrupted with the functional failure of tau [32, 40]. An important point to be discussed is the relationship of tau production to neuroplasticity. Initial changes in AD may be identified in the entorhinal transitional neuronal networks, which projects through the perforant path to the dental gyrus [19, 41, 42]. Recent studies have demonstrated the mechanism through which tau pathology initially progresses from distal axons to proximal dendrites. Only at later stages may the basal trunk of the dendrites' tree and the body of the neuronal cell be damaged by hyperphosphorylated tau [42]. These events are most likely to be involved in AD pathophysiological cascade.

In summary, the progression of amyloid deposition and hyperphosphorylated tau may hypothetically be linked to the synaptic disconnection of late myelination fibers [42]. Hence, according to the retrogenesis model, small diameter late-myelinating axons of cortical areas would be the earliest and most affected in AD, thereby increasing the susceptibility to amyloid accumulation and hyperphosphorylated tau; conversely, heavily myelinating axons would be less susceptible to AD pathology [17].

2.2. Clinical Basis of DTI. As an indirect measure of various aspects of tissue integrity, DTI signal may be influenced by distinct fiber components, including membrane intactness and myelin density [35, 43]. Diffusivity represented by the water motion in a particular region can thus be altered by ordered structures such as axonal tracts in nervous tissues [44]. Diffusivity oriented by the fiber direction, the so-called anisotropic diffusion, is largely restricted in the GM; an increase in anisotropic diffusion may correlate with myelin sheath content, being a valuable parameter for the investigation of WM microstructure integrity [45]. A representation of the ellipsoid can be computed by sampling the diffusivity along multiple directions spaced on a sphere [46, 47]. DTI uses measures derived from the eigenvectors, represented by eigenvalues, which define the diffusion ellipsoid in every voxel [47]. Axial diffusivity (DA) reflects the diffusion coefficient along the principal eigenvector (λ_1), whereas radial diffusivity (DR) indicates the average diffusion coefficients along the two axes perpendicular to λ_1. Mean diffusivity (MD) is a measure of the total amount of diffusion within a voxel and is computed as an average of all three diffusion axes [47]. Finally, FA is a scalar value between zero and one and it is calculated from the eigenvalues (λ_1, λ_2, λ_3) of the diffusion tensor [47] (Figure 1); FA measures the overall directionality of water diffusion and reflects the complexity of cytoskeleton architecture, which restricts the intra- and extracellular water movement [47]. The relationship between FA and WM microstructure changes considerably along the lifespan [32]. Finally, DR measures diffusion perpendicular to the WM fibers while diffusion parallel to the fibers is estimated by DA. MD is considered a nonspecific marker of degeneration which reflects a decrease in membrane or other barriers to free water diffusion.

2.3. The Interpretation of DTI Diffusion Indices. The predictive value of conversion to dementia was investigated by a few studies [48]. van Bruggen and colleagues reported higher parameters of Receiver Operator Characteristic curve (ROC) for DR (0.94) and FA (0.94) in both the corpus callosum and left cingulum, while DR and DA in the fornix showed only fair (0.78) indices [48].

Only a few reports investigated regions of overlap between indices [7, 23, 49–52]. Overall, there is still considerable variation among studies in the interpretation of multiple indices [52]. Animal models have proposed that increased

MD would be more suggestive of myelin breakdown [53], while an increase in DR or DA is more associated with axonal damage [54]. Most authors describe an increase in DA not accompanied by FA changes as gross tissue loss, widespread tissue damage, and increase of extracellular space [23] which in turn may be a consequence of axonal atrophy secondary to Wallerian degeneration [8, 23]. Conversely, significantly reduced DR without differences in DA has been interpreted as a disruption of myelin integrity in the absence of axonal structural irregularities [8, 15]. These changes would indicate specific damage of the myelin sheaths that restrict DR [8].

Another caveat that restricts the interpretation of diffusion indices is the discrepancy of anatomical findings among DTI investigations. Such constraint may be associated with the different levels of AD severity between participants, which may be responsible for diverse patterns of distribution of DTI changes. While most studies investigated mild to severe individuals [18, 55–57] and mild to moderate participants [7, 50, 58], mild AD patients were investigated by others [4].

2.4. Corpus Callosum and Diffusion Alterations. The atrophy of the corpus callosum (CC) has been considered the anatomical correlate of Wallerian degeneration of commissural nerve fibers [4]. The impact of CC atrophy, as predictor of cognitive decline, has been demonstrated in a three-year followup of elders with age-related WM leukoaraiosis [8].

The pattern of neuronal disruption in CC has been discussed by a few studies, but unclear results may rely on the different methods of anatomical parcellation designed for investigation. Based on the Wallerian degeneration hypothesis and on the AD neuronal degeneration pattern [4], earlier stages of WM degeneration should be associated with the involvement of posterior CC subregion, while on later stages the anterior segment of the CC would exhibit atrophic changes [4].

Recent DTI studies have addressed the progression of WM disruption in the CC based on the retrogenesis hypothesis. The CC comprises late-myelinating fibers in the genu [4, 24] and early-myelinating fiber in the splenium. The posterior CC subregion receives axons directly from the temporoparietal lobe, which are the same brain regions primarily affected by AD pathology [24]. Conversely, late-myelinating fibers connect the frontal lobes to the limbic system [52]. One study [59] reported lower FA and higher RD in the body of the CC.

An increasing evidence body has described early DTI changes in the genu of the CC on early (i.e., preclinical) stages of AD. One investigation showed a similar FA profile in the CC between AD and MCI participants who later converted to clinical AD [48]. The CC also exhibited large clusters of voxels with significant differences between MCI converters and nonconverters, especially a decreased FA and an accompanying increase in DR [48]. Taken together, it seems plausible to suppose that in the CC both mechanisms (i.e., myelin breakdown and Wallerian degeneration) may be associated with WM disconnection. Furthermore, these mechanisms may be involved in region-specific illness effects.

3. Results

Our review of DTI studies included 11 studies (Table 1). Results are discussed in the following topics.

4. Discussion

4.1. Regional Differences among DTI Indices. When analysing brain structural and biomolecular changes of AD, some critical points should be taken into account: the characterization of a region specificity, seen as the variability of DTI changes among tracts; the time dependence, defined as the biological processes across different stages of the disease (preclinical and clinical dementia stages); and the hypothetical mechanisms responsible for these modifications in the axonal fibre [8].

According to Brickman and colleagues [60], DTI differences were found in both early- and late-myelinating fibers. In this study, decreased FA and DA and increased DR indices in late-myelinating fibers were proportionally observed, when compared to early-myelinating fibers, in amnestic MCI individuals. These findings provide strong support for the retrogenesis hypothesis. Huang and colleagues also found evidence favouring that WM pathology may be heterogeneous and vary from one tract to another [59]. Hence, the pattern of WM disruption in amnestic MCI takes place initially in limbic and commissural tracts and later on clinically established dementia may progress to the two remaining tracts—projection and association fibers. Similar results for these fibers have been previously reported [11, 59, 61]. These preliminary findings also suggest that cortical atrophy and progression of WM disruption from amnestic MCI to AD may follow a cortical thinning pattern, spreading over time from temporal and limbic cortices to frontal and occipital cortices [59].

The presence of macroscopic WM lesions, often described in the clinical setting as WM burden of vascular origin, may be distinguished from microscopic lesions in terms of brain pathology. Following this assumption, one population-based DTI study [62] reported a few overlapping areas between macro- and micro-WM lesions; instead, distinct areas of macro- and microchanges were found to be predominant [62]. Findings of FA decreases and increases were exhibited in widespread regions, with the fornix being associated with microscopic WM lesions, while periventricular areas were more linked to WM burden [62]. The lack of notable effects of WM burden on DTI findings was also reported by another study [52]. When controlling for the WM burden-effect between AD participants and controls, roughly all areas of anisotropic changes, including MR increases and FA decreases, remained statistically significant [52].

Taken together, the majority of TBSS-based investigations have attempted to establish a pattern of DTI changes that would characterize AD evolution. The sum of evidence

Table 1: Main DTI-TBSS studies carried out with multiple indices: AD and control comparisons.

Authors	Sample	Voxelwise contrast	Corpus callosum subregion			Fornix	Temporal lobe	Uncinate fasciculus	Occipital lobe
			Anterior	Middle	Posterior				
Acosta-Cabronero et al., 2010 [50]	AD[1] ($n = 25$)	AD-controls[†]	—	↑DA/RD/MD	↑DA/RD/MD	↑DA/RD, ↓FA	↑DA/RD/MD, ↓FA	—	—
Agosta et al., 2011 [76]	AD[3] ($n = 23$) Controls ($n = 15$) a-MCI ($n = 15$)	AD-controls[†] a-MCI-controls	↑DA/RD/MD ↑DA	↑DA/RD/MD ↑DA	↑DA/MD ↑DA	↑DA/MD, ↓FA —	↑DA/RD/MD, ↓FA ↑DA	↑DA/RD/MD, ↓FA ↑DA	— ↑DA
Alves et al., 2012 [52]	AD[2] ($n = 23$) a-MCI ($n = 18$) Controls ($n = 17$)	AD-controls[†] a-MCI-controls[†]	↑DA/RD/MD, ↓FA ↓FA	↑DA/RD/MD, ↓FA ↓FA	— —	— —	↑DA/RD/MD, ↓FA ↓FA	↑DA/RD/MD, ↓FA ↓FA	— —
Bosch et al., 2010 [7]	AD[2] ($n = 15$) a-MCI ($n = 16$) Controls ($n = 15$)	AD-controls[†] a-MCI-controls[†]	↑DA/DR/MD, ↓FA —	↑DA/MD ↑MD	↑DA/DR/MD —	— ↑MD	↑DR/MD, ↓FA ↑DR	↑DA/DR/MD, ↓FA ↑DR/MD	↑DR/MD, ↓FA ↑DR/MD
di Paola et al., 2010 [8]	AD[1] ($n = 38$) MCI ($n = 38$) Controls ($n = 40$)	AD-controls[†]	↑DR, ↓FA	↑DR/DA	↑DR/DA	—	—	—	—
Gold et al., 2010[**] [70]	High risk ApoE4 ($n = 37$) Low risk ApoE4 ($n = 20$)	High-low risk APOE4[†]	—	—	—	↓FA	↓FA, ↑DR	↓FA	↓FA
O'Dwyer et al., 2011 [23]	AD[2] ($n = 9$) a-MCI ($n = 14$) na-MCI ($n = 19$) Controls ($n = 40$)	AD-controls[†] MCI-controls[†]	↑DA/RD/MD, ↓FA ↑MD/DA	↑DA/RD/MD, ↓FA ↓FA	↑DA/RD/MD, ↓FA —	↑DA/RD/MD, ↓FA —	↑DA/RD/MD, ↓FA —	↑DA/RD/MD, ↓FA —	↑DA/RD/MD, ↓FA —
Stricker et al., 2009 [58]	AD[1] ($n = 16$) Controls ($n = 14$)	AD-controls[λ]	—	—	↓FA	↓FA	↓FA	↓FA	↓FA
Salat et al., 2010 [49]	AD[2] ($n = 20$) Controls ($n = 54$)	AD-controls NA[†]	↓FA, ↑DA/DR	—	↑DA/DR	↑DA	↓FA, ↑DA/DR	—	↓FA, ↑DA/DR
Shu et al., 2011 [51]	AD[3] ($n = 16$) a-MCI ($n = 17$) Controls ($n = 19$)	AD-controls[†] MCI-controls[†] MCI versus AD[†]	↑MD/DA/DR, ↓FA ↓FA ↑MD	↑MD/DA/DR, ↓FA — —	↑MD/DA/DR, ↓FA ↓FA ↑MD,	↑MD/DA/DR — —	↑MD/DA/DR, ↓FA ↑MD	↑MD/DA/DR, ↓FA ↓FA —	↑MD/DA/DR, ↓FA ↓FA ↑MD
Vernooij et al., 2008 [63]	832 patients from the community	MCI-controls[*]	↓FA, ↑DA/DR	↓FA	↓FA, ↑DA/DR	↓FA, ↑DA/DR	↓FA, ↑DA/DR	—	↓FA, ↑DA/DR

Note: DA: axial diffusivity; MD: mean diffusivity; DR: radial diffusivity; a-MCI: amnestic mild cognitive impairment; na-MCI: nonamnestic mild cognitive impairment; AD: Alzheimer's disease. Method of voxelwise contrast: [(†)]familywise error rate; [(λ)]permutation based approach; [(*)]not informed. Alzheimer's clinical severity: [(1)]mild; [(2)]mild to moderate; [(3)]severe; [(**)]only female subjects included.

regarding a gradient pattern for AD so far remains inconclusive and no firm conclusions can be drawn. On the other hand, whether a gradient of posterior-anterior changes or anterior-posterior changes predominates, or even a combination of these patterns occurring simultaneously, is still under intense debate [7, 8, 15, 23, 49, 52, 58].

4.2. Risk Factors for Dementia and Retrogenesis. Cognitive alterations associated with DTI changes might be related with early age alterations during the neurodevelopment stages. For instance, a larger proportion of DTI changes, around 85%, might be related to lower intelligence coefficient (IQ), as pointed by previous investigations [14].

Overall, age has been pointed as one of the most important risk factors for DTI changes [21, 25]. An increasing number of DTI studies indicate that age effects may follow an anterior to posterior gradient on WM changes [49, 60, 63–65]. In one recent investigation, a dissociation pattern of DTI changes was associated with age, with larger effect sizes reported in neocortical late-myelinating fibers in comparison with early-myelinating fibers [60]. These findings are also supported by histological studies, which suggest the development of early-myelinating fibers already at prenatal and perinatal periods, in contrast with the later maturation of neocortical fibers [66, 67]. Moreover, the ageing process may be related to FA alterations, regardless of WM atrophy. Indeed, the decrease of WM volume may fail to show an association with FA changes, as pointed by previous studies [65]. Possibly, only some diffusion properties are prone to affect volume such as the degree of myelination and axon degeneration [25]. In general the cellular microstructure of tissue influences the overall mobility of diffusing molecules and works as intracellular barriers. In summary, the inner properties of FA in the axonal cytoskeleton and microtubules are not fully elucidated and deserve further investigation [35].

Regarding vascular disease, one study showed statistically significant DTI differences between WM hyperintensities (noted by visual scan) and apparent normal WM areas [68]. On the other hand, FA-MD and MD-DR between overlapping areas of macro- and microlesions in WM were interpreted as reflecting demyelination and axonal loss within the fibre and early vascular disease [68].

The presence of a ApoE4 allele was investigated in relation to WM disruption and temporal atrophy in nondemented subjects at risk for AD by Bui and colleagues [69]. FA decreases and DR increases were found in the cingulum, inferior longitudinal fasciculus and inferior frontooccipital fasciculus. Interestingly, no significant MD increases were found. Accordingly, there were no significant correlations between diffusion indices and medial temporal volume. In another investigation, FA decreases and DR increases in the AD group remained after controlling for GM volume [58].

Taken together, these findings strongly suggest that retrogenesis hypothesis may be the driving force behind age-mediated changes for some tracts. However, retrogenesis hypothesis may not fully capture the anatomical changes that occur throughout aging [60]. In fact, well-established late-myelinating fibers, such as the fornix [23] and the superior longitudinal fasciculus [60], may not present overt age-related effects. Finally, taking into account that the interpretation of multiple indices is not clearly established, further studies should comprehensively analyse the application of DTI indices to the understanding of complex interactions between vascular disease and degeneration.

4.3. Alternative Hypothesis of Brain Atrophy Progression. In addition to the Wallerian degeneration and retrogenesis hypotheses, a third mechanism of cortical atrophy, in which GM neuronal atrophy may follow axonal damage, has been debated [68]. Previous studies reported higher hippocampal atrophy associated with DTI changes in the fornix and hippocampal tracts (seen as FA decreases combined with DA and DR increases). Conversely, other WM connexions were less associated with hippocampal volume, such as those of periventricular territories [68]. Interestingly, volumetric decreases and DTI changes of WM tract located in the CC (genu and body portions) showed independent effects of age [62]. Additionally, one study involving amnestic MCI participants reported areas less likely to develop overlapping changes due to micro- and macro-WM lesions, among them the fornix and the temporal lobe [12].

The summary of current evidence, although still scarce and preliminary, suggests that particular tracts that are located in temporal and parietal areas may show higher sensitivity to induce cortical atrophy in the surrounding areas and that distinguished and interacting processes, that is, WM atrophy and WM diffusion changes, may be potentially pathologically different.

4.4. Limitations of DTI Studies Carried out through TBSS. DTI may be a useful tool for anatomical quantification of microscopic lesions and shed light on the mechanisms of AD pathology, particularly in terms of gradient of progression. Notwithstanding the increasing evidence based on multiple indices studies and the possibility of hypothesising different underlying mechanisms, DTI proxies are not suitable for directly determining the histological background of brain pathology [70, 71]. Hence, multimodal studies incorporating VBM, PET techniques, and conventional neuropathological studies may be necessary to clearly validate DTI parameters. Another awaited achievement is the use of high resolution techniques to assess difficult areas such as the fornix and hippocampus. High resolution DTI is based on optimized sequences for the medial temporal lobe and enables a detailed investigation of each individual fibre bundle to image voxel. In spite of that, DTI alterations in multiple indices may help to elucidate early pathological changes in preclinical stages of AD. The accurate prediction of cognitively healthy individual to convert to clinical AD still remains a significant research challenge. Thus, the idea of a biomarker profile, rather than the single use of one of these techniques, may offer more robust predictive power to determine who is going to convert to AD with acceptable reliability [72]. Another promising use of DTI is the support vector machine approaches, which consists in the statistical analysis of sensitivity and specificity of DTI indices in the differential diagnosis between groups. One study reported a sensitivity of 93% and a specificity of

92.8 in the discrimination between controls and MCI individuals [73] while in other investigations this discrimination yielded a sensitivity of 90.32% and a specificity of 90.41% [74]. One question raised by support vector investigations is the search for the most accurate DTI indices in voxel-wise analysis. Statistical significant FA differences between controls and MCI were described by some [23, 51, 52] but not all investigations [7, 8, 75]. Nevertheless, non-FA indices (DA, RD, and MD) failed to show significant results for MCI-control discrimination [8, 51, 52]. Discrepant results may partially be explained on the fact that some studies [7, 23, 52] employed the threshold-free cluster enhancement, which is the most conservative statistical method [76].

Most studies found DA and DR to be more accurate in indicating WM disruption in comparison with FA [23]. However, whether DA or DR increases, but not FA decreases, should be highlighted in the interpretation of DTI findings is still under debate. One has to take into account that DA and DR change in the same direction [51]. As a result, FA changes along fibers may be modified by increases of DA or DR, which may potentially suppress the effect of the changed diffusivity on FA. Such characteristic may also explain major widespread changes in MD and DR, which are absolute diffusion metrics, in comparison to FA [52]. Accordingly, the relation between FA and WM changes presents considerable variations over the disease course, apparently becoming less pronounced on later stages for some WM tracts. A few tracts, like the internal capsule, may exhibit DA increase with no significant change in DR [51].

Another important constraint of DTI studies is the interpretation of multiple indices, based mostly on animal model studies, which lack a consistent pathological validity [23]. For instance, the proper interpretation of DA may be a controversial issue, since both increases and decreases have been reported in the literature [7, 48, 50, 77]. Possibly, the lack of such association might be associated with axonal fibre organization or, alternatively, with DTI calculation in crossing fibre zones, as reported by some studies [23, 78].

Finally, one aspect that remains relevant is the discrepancy between studies concerning techniques of DTI acquisition and processing: the anatomical segmentation for the extraction of DTI values of regional tracts. For instance, the segmentation of CC has generated some controversy regarding the assumed topography of callosal fibers [15]. The other concern is related to partial volume effects, which may underestimate thinner anatomical regions such as the fornix and some limbic structures [48]. Future studies shall incorporate higher resolution MRI and apply automated voxel-based technique (VBM) to overcome these limitations.

Notwithstanding these limitations, multiple diffusion indices approach may be employed as one useful tool for the preclinical diagnosis of dementia. Other biomarkers of risk to dementia, such as the presence of ApoE4 and inflammatory markers (IL6, CRP), may be associated with a steeper decline on cognitive status or greater neuronal loss [79]. Neuroplasticity refers to compensatory and neuroprotective mechanisms which maintain brain structure and activity [80]. The increase in neuronal activity, one of the variables that induce myelination, has been shown to be modulated by plasticity mechanisms which may be extended into old age [79]. Future research on DTI will need to explore how DTI changes would be related to mechanisms of brain atrophy, neuronal compensation, and plasticity.

5. Conclusions

The susceptibility of neuronal fibers to the interactions of myelin breakdown, axonal damage, and swelling and other microstructural events may be more deeply appreciated through DTI studies. Moreover, DTI may help mapping the progression of circuit disruption along AD evolution, enabling the establishment of patterns of subclinical features associated with disrupted neuronal pathways. Future neuroimaging studies of dementia will need to transpose with greater accuracy and reliability the complex interpretation of DTI indices, especially on early- and late-myelinating fibers, from animal models to clinical studies. Finally, evidence from DTI provides also a useful surrogate marker of neuronal loss and synaptic disruption and, in addition to cerebrospinal fluid and PET techniques, may be incorporated in the multimodal staging of dementia.

In summary, the use of DTI multiple indices in addition to other standard multimodal methods in dementia research may help to determine the contribution of retrogenesis hypothesis to the understanding of neuropathological hallmarks that lead to AD.

Abbreviations

AD: Alzheimer's disease
CC: Corpus callosum
DA: Axial diffusivity
FA: Fractional anisotropy
GM: Gray matter
MD: Mean diffusivity
MCI: Mild cognitive impairment
DR: Radial diffusivity
DTI: Diffusion tensor imaging
WM: White matter
TBSS: Tract-based spatial statistics.

Conflict of Interests

The authors report no conflict of interests.

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Review Article

Seizure-Induced Oxidative Stress in Temporal Lobe Epilepsy

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An insult to the brain (such as the first seizure) causes excitotoxicity, neuroinflammation, and production of reactive oxygen/nitrogen species (ROS/RNS). ROS and RNS produced during status epilepticus (SE) overwhelm the mitochondrial natural antioxidant defense mechanism. This leads to mitochondrial dysfunction and damage to the mitochondrial DNA. This in turn affects synthesis of various enzyme complexes that are involved in electron transport chain. Resultant effects that occur during epileptogenesis include lipid peroxidation, reactive gliosis, hippocampal neurodegeneration, reorganization of neural networks, and hypersynchronicity. These factors predispose the brain to spontaneous recurrent seizures (SRS), which ultimately establish into temporal lobe epilepsy (TLE). This review discusses some of these issues. Though antiepileptic drugs (AEDs) are beneficial to control/suppress seizures, their long term usage has been shown to increase ROS/RNS in animal models and human patients. In established TLE, ROS/RNS are shown to be harmful as they can increase the susceptibility to SRS. Further, in this paper, we review briefly the data from animal models and human TLE patients on the adverse effects of antiepileptic medications and the plausible ameliorating effects of antioxidants as an adjunct therapy.

1. Introduction

Epilepsy is a serious neurological disorder manifested by recurrence of unprovoked seizures resulting in devastating effects on patients and the caregivers. The seizures are generated due to abnormal hypersynchronous paroxysmal cerebral discharges from the neurons which eventually results in irreversible damage to them and their surroundings. About 50% of reported cases of epilepsy are acquired [1]. The acquired causes such as head injury or infection or exposure to toxic chemicals can initiate one or more seizures or status epilepticus (SE) [2, 3]. Depending on the severity of the first insult, a varying period of latent period was reported during which a cascade of neurobiological changes takes place. These neurobiological changes culminate in the development of spontaneous recurrent seizures (SRS) resulting from synaptic reorganization into hyperexcitable and hypersynchronous neural networks [4]. According to International League Against Epilepsy (ILAE) multiple seizure episodes that occur within 24 hr are considered as a single event and hence SE is regarded as a single event. Established epilepsy refers to occurrence of two or more unprovoked recurrent seizures [2, 3]. A seizure occurring for a short duration is usually benign and self-limiting. Generalized convulsive SE is regarded as a clinical emergency due to significant morbidity and mortality [5]. Generalized convulsive SE in humans is attributed to continuous seizure lasting for 30 min or more consisting of two or more seizure episodes where the patient remains unconscious between the episodes [6, 7]. Considering the severe brain pathology associated with generalized convulsive SE, any seizure lasting for more than 5 min is treated as an emergency in clinics [6, 7]. It has been reported that some patients show nonconvulsive SE where EEG abnormalities are associated with impairment of consciousness that lasts at least 30 min without any obvious convulsive seizures [8]. The clinical signs of nonconvulsive SE are multifaceted exhibiting behavioral/cognitive changes such as confusion, agitation, hallucinations, facial automatisms with jerks, aphasia, nausea, pupillary abnormalities, and cardiorespiratory and thermal alterations [9]. Nonconvulsive SE is often underrecognized when compared to generalized convulsive SE [10]. The current antiepileptic drugs (AEDs) are merely symptomatic and do not prevent the progression of the disease. The greatest disadvantage with AED therapy

is that its discontinuation makes the brain more vulnerable to the recurrent seizures and may get worse with time [11, 12].

In general, epilepsy afflicts more than 65 million people worldwide and over 100,000 new cases are added every year [13]. Among the epileptic patients, about 30% of them are refractory to the current AEDs [14]. Temporal lobe epilepsy (TLE) is one of the most common forms of partial or focal epilepsy which is associated with head traumas, brain malformations, infections, and febrile seizures [15]. In the United States alone over 3 million people suffer from epilepsy. In developing countries, the incidence is even higher due to a likelihood of cerebral infection in children during primitive obstetric services, head traumas in adults resulting from impacts, and a general susceptibility of elderly population to seizures. Severity of epilepsy depends on factors such as age, race, genetics, and socioeconomic and other environmental factors [13, 16]. The exact etiology of epilepsy is not well understood, but any kind of insult to the brain depending on its severity has a potential to induce seizures which can later develop into epilepsy. An alarming rise of epilepsy among different age groups, inconsistent cause and prognosis, morbidity, mortality, and above all its medically intractable nature in some of the patients make it of a top priority for research. Animal models have been instrumental in understanding the pathophysiology of epilepsy and for the preclinical studies for new drug discovery [17, 18]. In this review, we provide the information from animal models and human patients on the harmful role of ROS/RNS (reactive oxygen species/reactive nitrogen species) that are generated as a consequence to seizure and also discuss the role of gliosis, adverse effects of AEDs, and potential benefits of antioxidant supplements in TLE.

2. Oxidative Stress and Temporal Lobe Epilepsy

Studies have indicated that the loss of inhibitory neurons in the hippocampus during SE can alter the steady state of excitation and inhibition between neuronal populations towards hyperexcitability [19, 20]. This hyperexcitability initiates reactive gliosis and also results in mitochondrial dysfunction in neurons due to the generation of free radicals of oxygen and nitrogen species within the hippocampus and dentate gyrus. These changes will lead to neurodegeneration.

2.1. Free Radicals of Oxygen and Nitrogen Species. In normal physiological conditions, ROS and/or RNS levels are fairly well regulated to perform important functions such as autophagy, chemical signaling, cell division, and mitogen activated protein kinase signaling and apoptosis [21]. Due to the highly reactive nature of these molecules, the ROS and/or RNS are tightly regulated. Mitochondrial dysfunction due to ROS and RNS is frequently observed after seizures during epileptogenesis and is normally associated with neurodegeneration [22].

Free radicals contain one or more unpaired electrons in the outermost shell which confers them for being chemically reactive. Free radicals are generated by a loss of an electron or a gain of an electron during a homolytic cleavage [23, 24]. The resultant effect of homolytic cleavage is formation of two free radicals which may or may not carry an electric charge. Due to the presence of an excess electron or a lack of electron in their outermost orbits, these radicals behave as strong oxidants or reductants. Free radicals are highly unstable and reactive species, which initiate a chain reaction by pulling electrons from the nearby molecular fragments to form stable bonds, as a result the proteins and lipids will change their morphology and function. Such effects on DNA result in cross-linking of base pairs leading to mutation of a gene. Important free radicals of oxygen species include hydroxyl radical ($OH^{\bullet}$), superoxide anion (O_2^{-}), hydrogen peroxide (H_2O_2), singlet oxygen (O), alkoxy radical (RO), peroxy radical (ROO), and hypochlorite (HOCl). Widely known free radicals of nitrogen species include nitric oxide radical ($NO^{\bullet}$), peroxynitrite radical ($ONOO^{-}$), nitroxyl anion HNO^{-}, nitrosonium cation (NO^{+}), higher oxides of nitrogen (N_2O_3, $NO_2^{\bullet}$), and S-nitrosothiols (RSNO) [25–28]. The production of these radicals within the cell in excessive amount can lead to oxidative stress.

2.2. Free Radical Production and Oxidative Stress. An oxidative stress generally refers to a biochemical state where ROS or RNS production is unregulated resulting in damage to the cell membrane, proteins, enzymes, and DNA components within the nucleus and the mitochondria [24]. A majority of RNS are generated from the interactions of nitric oxide (NO) and oxygen. NO is an important second messenger, which can also behave like a free radical due to the presence of an unpaired electron in the outermost orbit (6 valence electrons from oxygen and 5 from nitrogen) [28]. NO is produced from the substrate, L-arginine via the enzyme NO synthase (NOS) involving nicotinamide adenine dinucleotide phosphate (NADPH) and oxygen. There are three major isoforms of NOS: (a) neuronal NOS (nNOS) produced by neurons, (b) endothelial NOS (eNOS) expressed mainly endothelial cells, and (c) inducible NOS (iNOS) induced in immune cells, astrocytes, microglia, and also neurons. The roles performed by NO vary based on its synthesis from the NOS isoforms and the tissues in which it is produced [29, 30]. The physiological concentrations of NO produced by nNOS mediate calcium dependent protein modification (S-nitrosylation), energy metabolism (through cytochrome C oxidase), synaptic plasticity, and neuroprotection. The NO produced by eNOS results in calcium dependent cyclic guanosine monophosphate (cGMP) mediated vasodilation to maintain vascular tone of cerebral blood vessels. The NO by iNOS is important for immune response or killing pathogens by generating free radicals [31–37]. However, excessive amount of NO produced by iNOS-mediated mechanism is harmful to the host cells.

Generation of free radicals under normal conditions within a cell is depicted in Figure 1. In the cytoplasmic membrane, NADPH oxidase (NOX) reduces O_2 to superoxide anion (O_2^{-}). Superoxides can also be generated from O_2 from xanthine oxidase during the production of uric acid. These superoxides are converted into H_2O_2 in the presence of

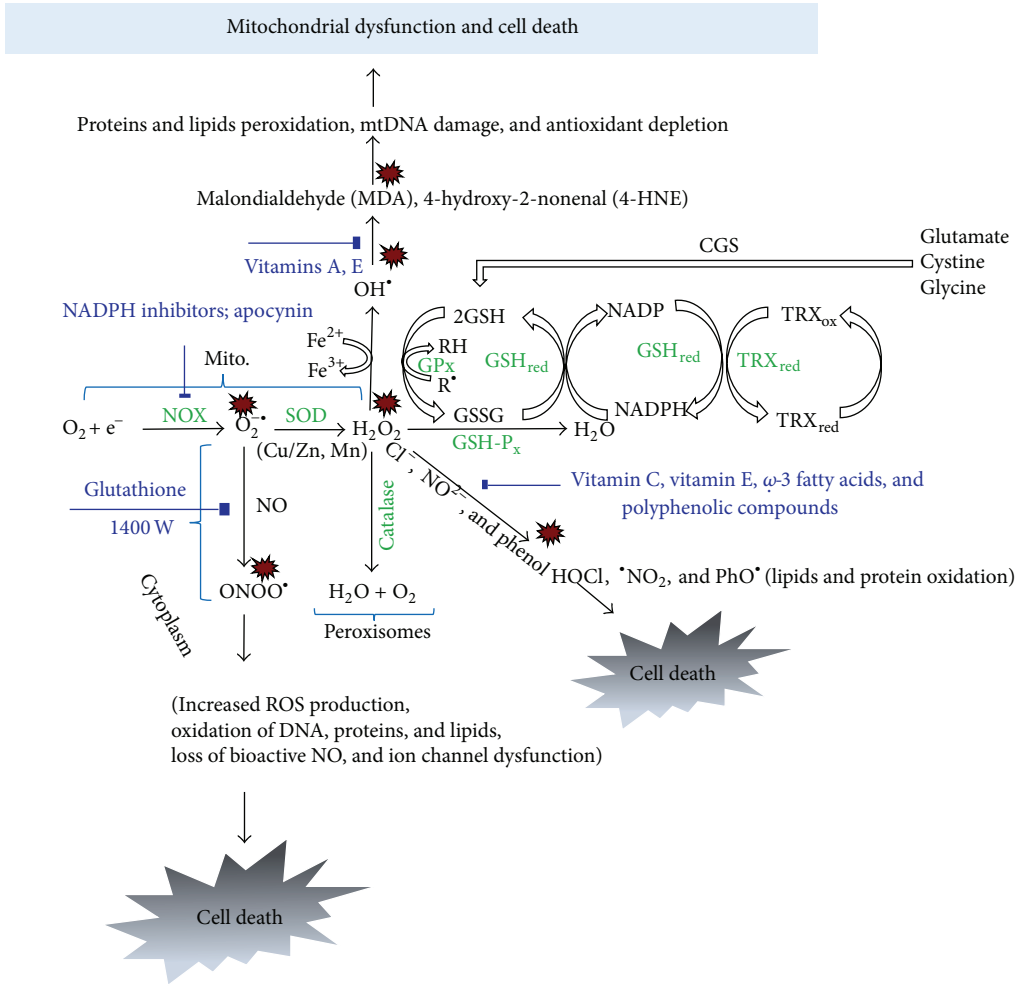

Figure 1: Biochemical reactions of ROS/RNS and their elimination by cellular endogenous antioxidants. Components in blue represent nonenzymatic antioxidants; green represents oxidative and antioxidant enzymes; and small red explosion sign represents generation of free radicals. NOX is the key enzymatic source of ROS. It reduces oxygen to superoxide anion and hydrogen peroxide. $O_2^{\bullet}$ forms H_2O_2 which is the most reactive radical among its group that is produced via Fenton reaction. $OH^{\bullet}$ leads to lipid peroxidation by producing harmful metabolites such as MDA and 4-HNE leading to mitochondrial dysfunction and cell death. It also produces $HOCl^{\bullet}$ and $PhO^{\bullet}$ which are extremely toxic oxidants that disrupt tight junctions and increase paracellular permeability. H_2O_2 is eliminated by CAT, in peroxisomes, and GPx (location varies). At rapid rates, superoxide anions compete with NO which results in the formation of highly reactive molecule called peroxynitrite ($ONOO^{\bullet}$), in cytoplasm, leading to increased ROS production, oxidation of DNA, RNA, and proteins, ion channel dysfunction, and loss of bioactive $NO^{\bullet}$. Peroxynitrite inactivates Mn-SOD, thereby increasing the flux of superoxide anions available to react with NO. SOD catalyzes the reduction of superoxide anions into H_2O_2, in mitochondria in the presence of enzymes GPx and CAT; H_2O_2 gets converted into water and oxygen. Antioxidant enzymes such as GPx oxidize GSH to GSSG and GSH_{red} recycles GSH back from GSSG. NADPH gets reduced to NADP by GSH_{red}. GSH/GSSG is a commonly used biomarker of oxidative stress in biological systems. However, GPx also catalyzes H_2O_2 into H_2O by using reduced TRX_{red}. Antioxidant defense against toxic oxygen intermediates comprises an intricate network which is heavily influenced by nutrition (vitamins A, E, and C and fatty acids). CGS plays an important role in glutathione metabolism and acts as an antioxidant in glial cells such as astrocytes. Extracellular oxidized cysteine is reduced to cysteine by thioredoxin reductase or glutathione that helps to maintain the steady state balance between antioxidants and ROS [24, 41, 80]. ROS, reactive oxygen species; NADPH, nicotinamide adenine dinucleotide phosphate; NOX, NADPH oxidase; SOD, superoxide dismutase (Cu/Zn—copper/zinc, Mn—manganese); CAT, catalase; $O_2^{-\bullet}$, superoxide anion; H_2O_2, hydrogen peroxide; NO, nitric oxide; $ONOO^{-}$, peroxynitrite; HOCl, hypochlorous acid; $PhO^{\bullet}$, phenoxy radical; $OH^{\bullet}$, hydroxyl radical; GSH, glutathione; GSSG, oxidized glutathione; TRXox/red, thioredoxin reduced and oxidized; TRX_{red}, thioredoxin reductase; GSH_{red}, glutathione reductase; GPx, glutathione peroxidase; CGS, cystine/glutamate antiporter system.

superoxide dismutase (SOD). H_2O_2 is a lipophilic molecule, which crosses lipid membranes into peroxisomes where it is finally eliminated by catalase (CAT) releasing H_2O and O_2 [38]. However, if the antioxidant action of SODs or CATs is impaired then, the reaction of superoxide with H_2O_2 yields toxic OH radicals in the presence of Fe^{2+} (called Fenton and Haber-Weiss reaction). These OH radicals can also be generated by superoxides when they react with hypochlorite (HOCl) [24, 38]. The hypochlorite (HOCl) arises when chloride (Cl^{-}) reacts with H_2O_2 catalyzed by peroxidases.

The OH radical is a harmful free radical of oxygen which has a short half-life but remains highly reactive. Further, the hydroxyl radical can also react with NO to form peroxinitrate ($ONOO^-$), a powerful oxidizing agent that can cause lipid peroxidation, tyrosine nitration, and cytotoxicity [24, 27, 39].

Besides the major pathways of free radical production, other enzymes and pathways can contribute to excessive accumulation of ROS/RNS in cells. While they are not the primary sources of ROS/RNS, these enzymes and pathways are capable of accelerating the process of neurodegeneration. NOX also mediates the production of superoxide radicals in the hippocampus. At basal level, these play a role in learning and memory consolidation [40, 41]. However, under pathological conditions such as TLE, NOX overproduces superoxide ions to initiate neurodegeneration. Hence, the compounds that inhibit NOX enzymes could be beneficial in the treatment of epilepsy. A review by Sorce and colleagues describes the advantages of inhibiting of NOX during reactive gliosis and neuronal injury in rat models [42, 43]. In addition to NOX, the cyclooxygenase-2 (COX-2) enzymes have been found to upregulate ROS levels via the production of prostaglandins (specifically, F_2 and H) [44]. In an *in vitro* model of rat cortex, it has been shown that the prostaglandins stimulate astrocytes to produce proinflammatory cytokines, which initiated neuronal death [45]. COX-2 is also responsible for a number of inflammatory responses in tissues involving neutrophils of the immune system [46]. COX-2 inhibition prevented lipid peroxidation within the mice brain and hence COX-2 could be another potential drug target for epilepsy [44, 47].

2.3. Free Radical Neutralization by Endogenous Antioxidant System. Cells possess native antioxidant systems to neutralize free radicals when produced in excess [68]. In the cytoplasm, SOD enzyme is coupled to copper and zinc ions (Cu-Zn SOD, also known as SOD-1), and, in the mitochondria, it is coupled to manganese (Mn-SOD, also known as SOD-2). SOD is an important antioxidant enzyme that scavenges superoxide radicals by catalyzing them into water and molecular oxygen. SOD-1 levels were low in cerebrospinal fluids of human patients with refractory epilepsy [69] suggesting that low levels of SOD-1 increase ROS. Intravenous administration of SOD-1 increased the seizure threshold in amygdala kindling rat models of epilepsy [70]. Experiments with SOD-2 knockout mice have been shown to be susceptible to kainate induced neurodegeneration and neuronal cell death [71].

The fate of H_2O_2 for conversion into H_2O and O_2 is determined by CAT enzymes (in mitochondria and peroxisomes), glutathione peroxidase (GSH-Px, in cytosol and also found extracellularly combined to selenium), and glutathione-S-transferase (GST, in cytosol and microsomes) [72]. The reactions include utilization of reduced glutathione (GSH) to combine with H_2O_2 to form H_2O to release oxidized glutathione (GSSG) [73, 74]. Thus availability of reduced GSH becomes an important antioxidant reserve of the cell. The reduced GSH is resynthesized from GSSG by glutathione reductase (GSH_{red}) utilizing NADPH. The NADPH for this process is generated by thioredoxin reductase (TRX_{red}) found in endoplasmic reticulum by utilizing oxidized thioredoxin (TRX_{ox}) [75–77]. In addition to the antioxidant enzymes, peroxiredoxins, a ubiquitous family of antioxidant enzymes, degrade H_2O_2 and peroxynitrites to H_2O and nitrites [78, 79].

2.4. Susceptibility of Brain to Oxidative Stress. While brain accounts for about 2% of body weight, it consumes 20% of the total inspired oxygen at rest [89]. This is due to a high metabolic rate of the neurons and the need for large amounts of ATP to maintain ionic gradient to sustain normal neurotransmission. Hence, mitochondria are found abundant in neurons' synaptic terminals to supply ATP, which is generated through oxidative phosphorylation [90]. In mitochondria, during normal oxidative phosphorylation, free radicals are also generated in small quantities from electron transport chain (ETC) complexes 1 and 3 [91]. In addition, brain contains large amounts of readily oxidizable polyunsaturated fatty acids which are necessary for the lipid membrane's structure and function. During oxidative stress, polyunsaturated fatty acids become susceptible to lipid peroxidation. This affects the permeability of the membrane to ions and signal transduction [92, 93]. Further, neurons are also rich source of iron, an important element in many cellular processes and physiological functions. During oxidative stress, high amounts of iron can prove harmful as iron participates in the redox reactions to generate ROS via Fenton and Haber-Weiss reaction [94]. Furthermore, CAT enzyme levels (essential for the cleavage of H_2O_2) are low in the brain compared to other organs, for example, 1/10th of liver CAT activity, making it susceptible to oxidative damage [38, 39, 95, 96]. However, under normal conditions, the innate antioxidant systems provide antioxidant protection against the ROS/RNS damage during metabolic processes [97, 98].

3. Seizure Insult Increases Oxidative Stress

Oxidative stress and mitochondrial dysfunction have been long recognized as key mechanisms in several neurological disorders. Emerging evidence confirms that oxidative stress manifests as a consequence of the first seizure insult, which turns out later to become the cause of epileptogenesis [99]. During brain injury that results from seizures in rodent models, a significant increase in neuronal glucose uptake and metabolism was observed [81, 100]. Cerebral blood flow is found to increase in order to cope with hypermetabolism of glucose, thus resulting in buildup of lactate, thus overwhelming the normal glycolysis and tricarboxylic acid (TCA) cycle. The recurrent seizures can also result in overproduction of mitochondrial superoxide radicals in rodent models [48] that can be converted to hydroxyl radical via Fenton and Haber-Weiss reaction. The hydroxyl radical in the presence of Cu^{2+} and Fe^{2+} ions readily oxidizes proteins, lipids, and DNA resulting in altered protein function, membrane permeability, and gene expression, respectively. These events increase neuronal excitability and also decrease seizure threshold.

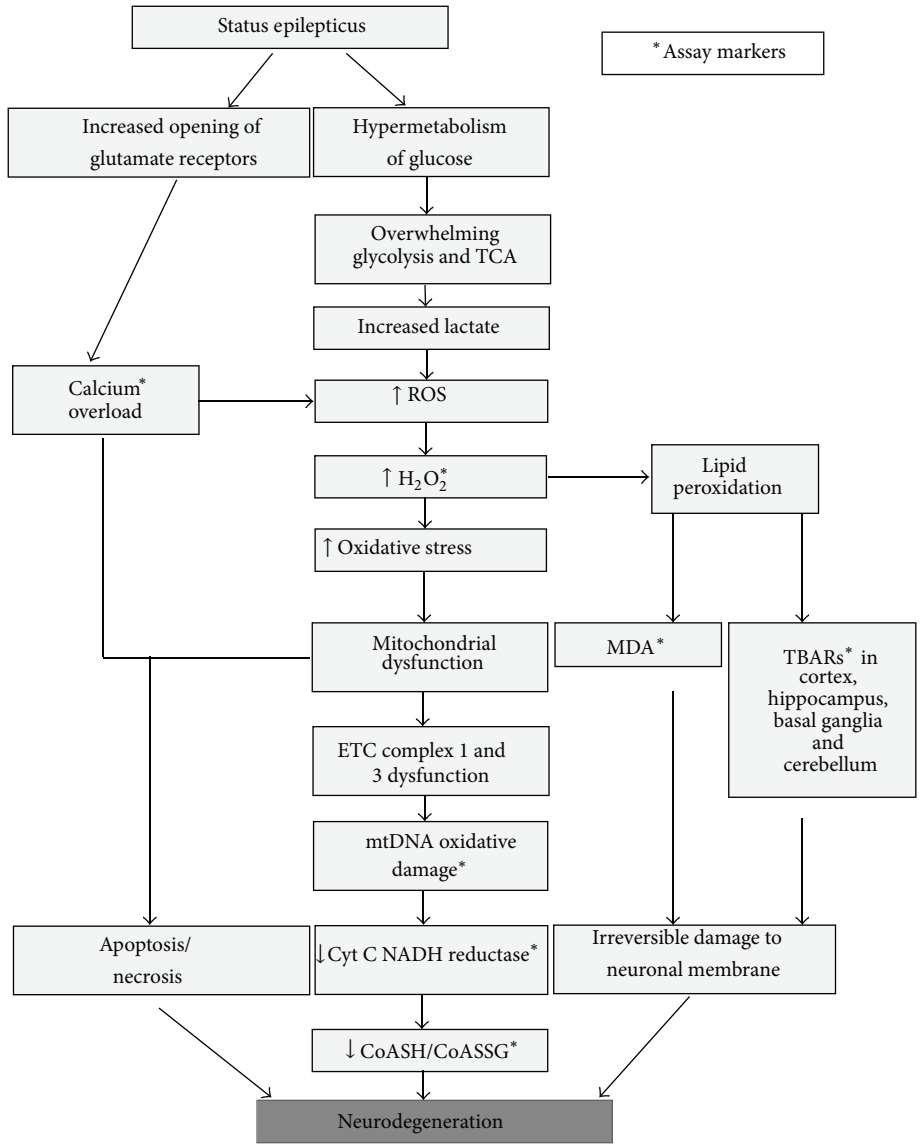

Figure 2: Post-SE pathways in neurodegeneration. SE increases glutamate receptors subunits interactions (NMDA, AMPA, and metabotropic), receptor turn-over, and their trafficking to the postsynaptic membrane. This leads to rapid calcium influx and calcium overload. As a result of this, several calcium dependent enzymes get activated in uncontrolled manner. This results in the activation of several signaling pathways that causes mitochondrial swelling, decrease in ATP, and increase in ROS, which results in oxidization of protein, lipid, and DNA, causing neuronal death. In addition, hypermetabolism, overwhelming glycolysis, and TCA cycle during SE further increase ROS/RNS. High production of lactate can cause cerebral lactic acidosis thereby increasing the production of ROS causing further damage due to mitochondrial dysfunction. Excessive calcium and ROS leads to the collapse of mitochondrial membrane potential, activation of mitochondrial matrix enzymes, and opening of mitochondrial permeability transition pores, decreasing ATP production. ROS are produced in mitochondria through the activity of ETC as a by-product of oxidative phosphorylation. CoASH/CoASSG and GSH/GSSG (described in Figure 3) ratio also decrease in brain tissues during this process and following SE, due to increased oxidative stress [44, 81–84]. TCA: tricarboxylic acid cycle; ETC: electron transport chain; mtDNA: mitochondrial DNA; Cyt C NAD: cytochrome NADH reductase; CoASH: coenzyme A; CoASSG: coenzyme A glutathione disulfide; SE: status epilepticus.

Several lines of evidence showing the link between oxidative stress and the mitochondrial dysfunction due to seizures have been observed in human patients and rodent models of TLE (Table 1; Figures 2 and 4). Briefly, it is summarized here.

(i) An increase in calcium overload due to excitotoxicity and increased ROS production during seizures predisposing neurons to degeneration [101–103]. There is an increased oxidation of macromolecules of the neurons after SE prior to the neuronal loss [104, 105].

(ii) Presence of neuronal death predominantly in CA3 and/CA1 regions of hippocampus following the first seizure [106], thus the TLE. Another example for CA1 hippocampal neurodegeneration is shown in Figure 4 (7 days after seizure).

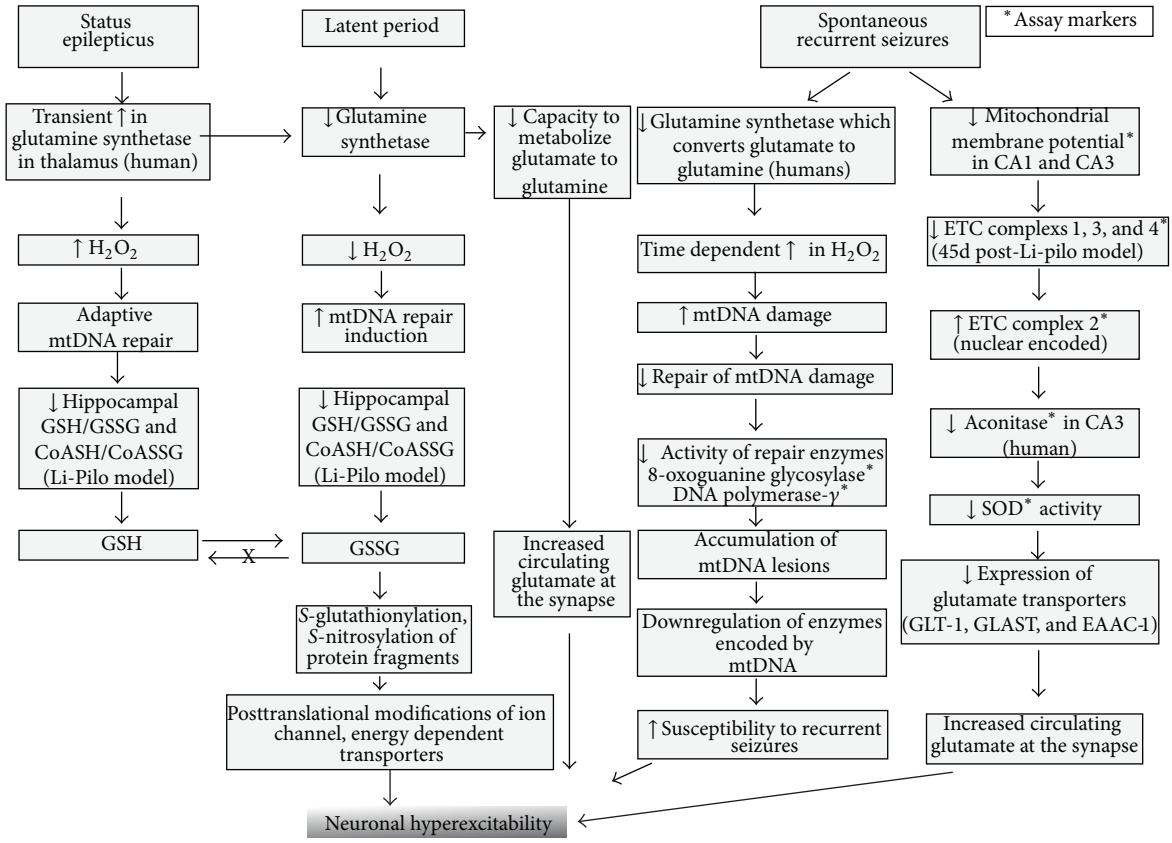

Figure 3: Sequence of events during status epilepticus, latent period, and SRS. The damage to the specific areas of the brain during SE can initiate varying period of neurobiological changes that can lead to the development of SRS. The enzymes free radicals and the pathways involved in these disorders are common in all types of insult (SE, latent period, and SRS), as described in Figures 1 and 2, but with subtle differences. The concentration of antioxidant enzymes rises after an initial insult (imitating their protective role) such as glutamine synthetase in SE; later it reduces which may or may not recover after few days/weeks depending upon the severity of the insult. Latent period is generally characterized by a series of slow neurodegenerative changes in the brain leading to epileptogenesis. The concentration of GST falls during latency that affects glutamate metabolism. High levels of the glutamate in the extracellular and intracellular space can lead to neuronal excitability through activated calcium signaling, as described in Figure 2. Levels of H_2O_2 return to the basal levels with mtDNA repair and low GSH/GSSG and CoASH/CoASSG ratio. During latent period, nitrosylation of protein fragments and posttranslational modifications of ion channels and transporters will further lead to hyperexcitability of neurons [24, 48, 82, 83, 85, 86]. SRS: spontaneous recurrent seizures; GST: glutamine synthetase; mtDNA: mitochondrial DNA; X: reverse reaction does not occur; Li-Pilo: lithium- pilocarpine model.

(iii) Changes in the mitochondrial membrane potential and increased NADPH levels as a consequence to seizures in rodent models and human patients [107].

(iv) A significant increase in neuronal glucose uptake and enhanced metabolism in brain following the first seizure [81, 100].

(v) Inactivation of mitochondrial aconitase levels after SE [48].

(vi) A reduction of mitochondrial N-acetyl aspartate (a metabolite synthesized from aspartate and acetyl-coenzyme A) in hippocampus from human epileptic patients [108–110].

(vii) Dysfunctional electron transport chain complexes (1, 3, and 4) after SE [82, 111–113].

(viii) A rise in mitochondrial H_2O_2 production, lipid peroxidation (increased malondialdehyde, MDA, and thiobarbituric acid, TBA), and mitochondrial DNA (mtDNA) damage following a seizure [58, 85, 114–116].

(ix) An increase in seizure susceptibility in aging mice and/or SOD mice due to compromised innate antioxidant mechanisms [117, 118].

(x) NMDA receptor antagonists [119] and antioxidant supplements (SOD mimetics, vitamin C, vitamin E, and melatonin) administration preventing seizure-induced neuronal death [120–124].

4. Oxidative Stress Increases Hyperexcitability during Epileptogenesis

The period of epileptogenesis (latent period) follows immediately after an initial insult from the seizures. There is a transient increase in glutamine synthetase enzyme during this

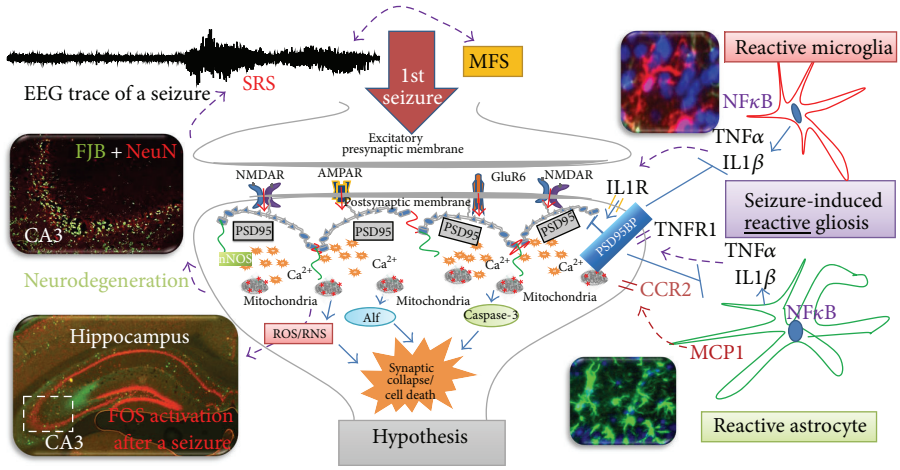

Figure 4: Schematic representation of a synapse, with postsynaptic ionotropic glutamate receptors (NMDA, AMPA, and KA/GLUR6), its associated glial cells, and extrasynaptic effects of a seizure. First seizure due to hyperexcitability of neurons (as evident from increased Fos expression in the hippocampus) induces reactive gliosis at a later stage, which produces inflammatory cytokines and iNOS that are mediated by NFκB transcription. These in turn sensitize postsynaptic neurons and decrease their seizure threshold. Reactive astrocytes also downregulate glutamate uptake, thus increasing the concentration of glutamate at the synapse. These events contribute to further hyperexcitability of neurons as evident from increased spiking activity on EEG. These changes in turn lead to neurodegenerative changes after 3 days following the first seizure (Fluoro-Jade-B (FJB)+, neuronal nuclei protein (NeuN), the markers used to detect neurodegeneration) [30, 35, 87, 88].

Table 1: Time dependent changes in the biomarkers of oxidative stress in rodent models of epilepsy and in human epileptic patients.

(a)

Rat kainate model	4 hr	8 hr	16 hr	24 hr	48 hr	3–7 d	3 week	Human patients	
GSH/GSSG ratio	↑			↑				GSH/GSSG ratio	↓
Lipid peroxidation (TBA assay)	↑	↑	↑	↑	↓	↓	↓	Lipid peroxidation (TBA assay)	↑
Protein oxidation	↑			↑				Protein oxidation	↑
SOD					↑		↑	SOD	↑
NADPH oxidase						↑	↑	catalase	↑
catalase					↑			aconitase	
aconitase		↓	↓		↓			DNA damage (OdG assay)	↑
DNA damage (OHdG assay)			↑	↑	↑				

(b)

Rat kindling model	4 hr	8 hr	16 hr	24 hr	48 hr	3–7 d	3 week
GSH/GSSG ratio			↓	↓	↓		
Lipid peroxidation (TBA assay)				↑			↑
SOD				↓			
aconitase			↓		↓		

(c)

Rat Pilocarpine model	4 hr	8 hr	16 hr	24 hr	48 hr	3–7 d	3 week
GSH/GSSG ratio				↓			
Lipid peroxidation (TBA assay)	↑	↑		↑	↑	↑	
SOD	↑	↑		↑	↑	↑	
catalase				↑			

(d)

Mice kainic acid model	4 hr	8 hr	16 hr	24 hr	48 hr	3–7 d	3 week
GSH/GSSG ratio					↓		
Lipid peroxidation (TBA assay)				↑	↑		↓

Rat kainate model [48–52], Rat kindling model [53–57], Rat pilocarpine model [58–60], Mouse kainate model [61–64], Human patients [65–67].

period in nonreactive astrocytes, which converts excess glutamate to inactive glutamine in the thalamus of human epileptic patients [108]. During the later course, glutamine synthetase activity gets downregulated in reactive astrocytes. As a result, excessive amount of nonmetabolized glutamate is released from astrocytes which accumulate in the extracellular space [125–129]. This results in hyperexcitability of neurons and later an onset of SRS. Astrocyte mediated glutamate release is discussed in later paragraphs. Although H_2O_2 increases during SE, it later decreases during the latent period due to activation of antioxidant systems [85, 130]. As the neuronal excitability increases, the SRS develops, which results in time dependent increase in H_2O_2 due to gradual depletion of antioxidant systems (GSH, coenzyme-A-SH) [131, 132]. As a consequence, accumulation of oxidized form of antioxidant enzymes, namely, GSSG and coenzyme-A-SSG, increases in the brain. A steady increase in ROS causes mitochondrial DNA damage resulting in downregulation of mitochondrial enzyme synthesis that is required for oxidative phosphorylation. Further electron transport chain complexes (1, 3, and 4) are affected [82, 111, 112]. The ROS also modifies the proteins subunits of excitatory ion channels and inactivates the energy-dependent glutamate transporters contributing to a further increase in neuronal hyperexcitability [71, 83]. An increase in ROS production is also contributed to a decrease in SOD and aconitase activity [48] (Figures 2 and 3). The neuronal hyperexcitability is further compounded by the loss of inhibitory GABAergic neuron populations of hippocampus and dentate gyrus leading to increased seizure susceptibility (Figures 4 and 5) [106, 133].

5. Mitochondrial Dysfunction and Lipid Peroxidation in TLE

The brain, being an organ with a low tolerance for hypoxic conditions due to neuronal need for oxygen, is particularly susceptible to ROS/RNS changes in mitochondria. Mitochondrial degeneration affects the stability of nuclear DNA (leading to chromosomal alterations), RNA, proteins, and lipids of the cell and also leads to defective calcium and glutamate homeostasis [41]. This increases the modulation of neuronal excitability and the synaptic transmission, an underlying mechanism in seizure production [84]. Waldbaum and colleagues investigated the changes that occur in the brain during the latency period that leads to the development of epilepsy [130]. Mitochondrial DNA gets repaired soon after the acute brain insult as a defensive mechanism; however this could be prolonged if the production of ROS/RNS during the insult is high. The concentration of H_2O_2 returns back to the basal levels during latency period but the production of ROS and RNS continues leading to the development of SRS [130]. It has also been suggested that certain protective enzymes, antioxidants, and coenzymes may be permanently damaged during this process [83, 113, 134, 135]. Furthermore, changes in DNA/RNA structure, compromised glutamate and calcium homeostasis, and depletion of antioxidant defense mechanism could lead to epileptogenesis [81, 85, 100]. According to Waldbaum and Patel, these changes affect all age groups. These disorders are most prevalent in the older people due to a reduced activity of antioxidant system which leads to the accumulation of free radicals resulting in neurodegeneration [117]. Waldbaum and Patel further proposed that ROS-induced mitochondrial DNA damage and decreased function of the electron transport chain are the major detrimental factors of neuronal death [136]. Oxidative stress leading to mitochondrial DNA alterations is also documented in patients with myoclonic epilepsy [137, 138].

Several hours after SE, the aconitase enzyme levels were found to reduce in mitochondria. Aconitase [an iron-sulphur protein] converts citrate into isocitrate in the TCA cycle. As TCA gets affected, the production of NADPH, flavin adenine dinucleotide ($FADH_2$), and ATP reduces, which contributes to the development of SRS [48].

Lipid peroxidation, in general, is the conversion of fatty acids in the lipid bilayer to reactive species, resulting in neurodegeneration. As described earlier, polyunsaturated fatty acids are also present in large amounts within the inner membrane matrix of the mitochondria and are especially susceptible to lipid peroxidation by generating ROS [4]. Lipid peroxidation affects the permeability of the membrane, calcium pump activity, and most of the membrane bound enzymes [26, 92]—this is repeated. Studies revealed increased malondialdehyde (MDA) (measured as thiobarbituric acid reactive substances, TBARS) and F_2-isoprostane levels that are derived from arachidonic acid cycle demonstrating that the lipid peroxidation indeed occurs during seizures [49, 139, 140]. Hydroxyl radicals that produce lipid peroxidation have also been found in the brains of rodent models of epilepsy [26, 93, 114, 116].

6. Role of Glia during Inflammation and Epileptogenesis

Gliosis (astrogliosis and microgliosis) occurs as a response to brain injury, which is characterized by proliferation and hypertrophy of the glial cells. Representative brain sections from 7 days after SE that were immunostained with glial markers are shown in Figures 4 and 5. Gliosis leads to formation of glial scar around the neurons that are under oxidative stress. Gliosis has both beneficial and detrimental consequences, which depends on their reactive state [141, 142].

6.1. Role of Astrogliosis. Astrocytes are the important source of antioxidants (neurotrophins) in the central nervous system (CNS) and play key role in cellular defense mechanism. Their protective role is regulated by nuclear factor erythroid related factor 2 (Nrf2), a transcription factor that mediates the production of antioxidants [143]. The Nrf2 activation is responsible for the regulation of antioxidant enzymes such as SOD, CAT, glutathione peroxidase (GSH-Px), and reduced form of GSH (GSHred). Astrocytes also play important roles in maintaining potassium homeostasis; glutamate uptake and release; lining of the blood brain barrier (BBB); providing nutritional, structural, trophic, and metabolic support to

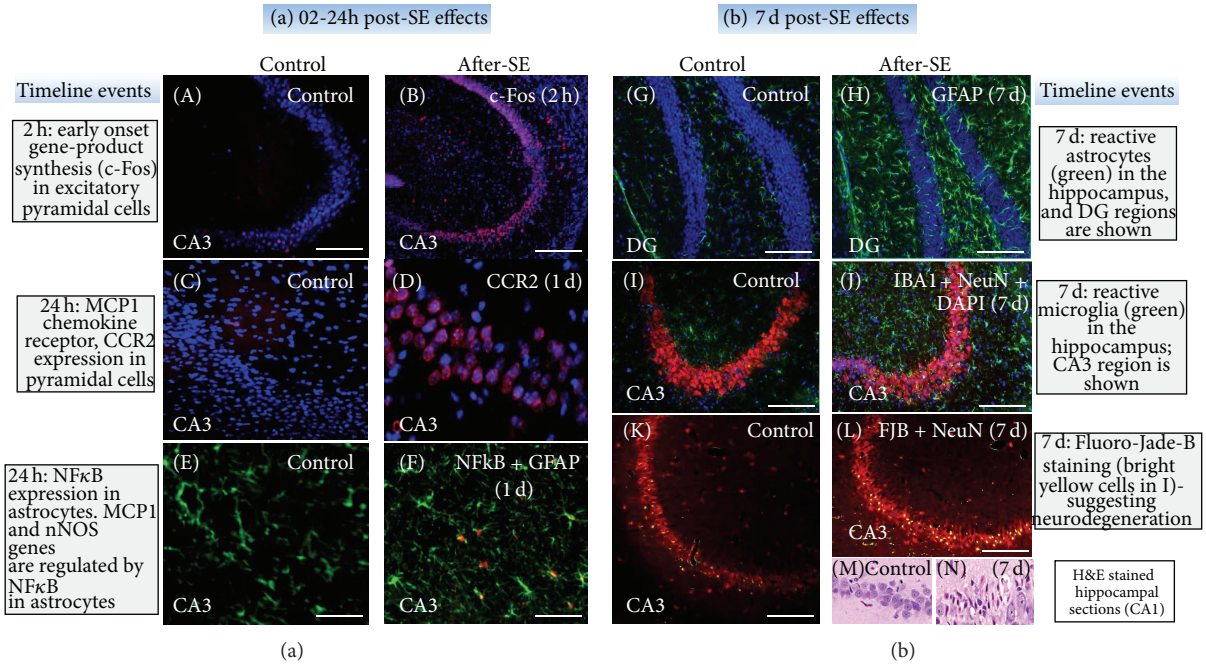

Figure 5: Immunohistochemistry (IHC) of the brain sections from kainate mouse and rat models of epilepsy at 2 h, 24 h, and 7 days after SE. (a) c-Fos ((A), (B)) expression was more widespread in the hippocampal formation at 2 h after SE (B). More than 3-4-fold increased expression (quantified data not shown) of c-Fos in CA3 pyramidal cell layer was observed (B). CCR2 ((C), (D)) and astrocytic NFκB expression ((F), orange) at 24 hours after SE. (b) By 7 days after SE, there was increased astrogliosis ((H), GFAP, green) and microgliosis ((J), IB1A is marker for microglia, green) compared to controls ((G), (I)). SE induced neurodegeneration (FJB +ve neurons) was observed in CA3 of hippocampus (L). There were increased FJB +ve cells in CA3 of hippocampus (green label in (L), all scale bars 100 μm). The same area was invaded by reactive astrocytes and microglia (green cells in (H) and (J)). Hematoxylin and Eosin stained hippocampal sections ((M), (N)) with pyknotic nucleus and shrunken cytoplasm are evident due to SE-induced changes at 7 days post-SE.

neurons; modulating synaptic activity; free radical scavenging; water transport and production of cytokines and NO [144]. Nonreactive astrocytes have also been found to play a neuroprotective role in recovering the neurons from brain injury by releasing trophic factors. The trophic factors include nerve growth factors, fibroblast growth factors, transforming growth factor-β, platelet-derived growth factor, brain-derived growth factor, and ciliary neurotrophic factor [141, 144]. All these factors play a part in stimulating neurite growth [144–147] and also promote angiogenesis, in case of cerebral ischemia, by expressing neuropilin-1 [144, 148].

Astrocytic glutamate transporters and neuronal glutamate receptors are known to play an important role in the pathogenesis of epilepsy. In normal brain, glutamate is taken up via astrocyte glutamate transporters from the extracellular space and metabolized to inactive glutamine to prevent excessive excitatory effects on neurons. Eid and coworkers have also shown a defect in the glutamine-glutamate cycle in hippocampal sclerosis patients that contributes to epileptogenesis [127, 129, 149]. Any alteration in this cycle is deleterious and can contribute to the hyperexcitability of neurons [150]. During the seizure insult, these astrocytic glutamate transporters become dysfunctional and lead to massive accumulation glutamate in astrocytes. This results in a release astrocytic glutamate (due to impaired astrocyte glutamate metabolism) into the extracellular spaces through a calcium dependent mechanism [150, 151]. This astrocytic glutamate release is also thought to be involved in amplifying the excitotoxicity of neurons [142, 152, 153]. The role of astrocytic glutamate in epileptogenesis has been debated for some time. However, it is largely agreed that the synaptic modulation by reactive astrocytes is one of the many causes of SRS [154]. Decreased expression and/or dysfunctional glutamate transporters in astrocytes, GLT-1 and GLAST, have been shown to be one of the key factors of human epilepsy [155].

The astrocytes become reactive, after the first seizure, due to changes at genetic, molecular, and cellular levels [141, 156]. A majority of these changes are observed at the transporter level in TLE during hippocampal sclerosis [157, 158]. In a normal astrocyte, the amount of glial fibrillary acidic protein (GFAP) was low as revealed by immunohistochemistry (Figure 5). Seven days after SE, GFAP was overexpressed, a hallmark of reactive astrocytes [159–161] (Figure 5). These reactive astrocytes secrete cytokines and chemokines such as IL-1B, tumor necrosis factor (TNF-alpha), interleukins (IL-1, IL-6, IL-10), and interferons (IFN-α, IFN-β) [162, 163], and chemoattractant protein-1 (MCP1). In addition to these factors, MCP1 may increase the calcium mediated glutamate release to worsen the epileptic state by producing hyperexcitability and a further production

of ROS/RNS [164–167]. However, it is difficult to predict the effects of individual cytokine in reactive astrogliosis, as we can observe only net combined effects of all the cytokines in *in vivo* models. The cytokines are also known to produce pleiotropic effects. For example, excessive production of IL-6 and TNF-α promotes demyelination, thrombosis, leukocyte infiltration, and BBB disruption [162, 163], while under normal conditions IL-6 and TNF-alpha have neuroprotective effects in ischemic injury and excitotoxic injury models [168, 169]. Hence, the specific contribution of astrocyte cytokine release to the processes involved in the development of epilepsy remains to be established. Perhaps, the role of astrocytes changes at different stages of epileptogenesis.

6.2. Role of Microgliosis. Under normal conditions, microglia cells play a beneficial role to engulf the cellular debris and prevent cellular toxicity from spreading to the bystander neurons and also to recruit distant microglia to the site of injury. The inactive or ramified microglia has a small cell body with thin and slender branches. Activated microglia shows different morphology at different stages of activation [170]. Generally activated microglia, by 7 days after SE, has large cell body with/without thick projections/branches (Figure 4). At the time of injury or during excitotoxicity, these ramified microglia become active/reactive and undergo morphological changes [171–174]. However, during the early stages of insult, microglia are involved in neuroprotection and neurogenesis by releasing neurotropic and anti-inflammatory molecules [175]. Nonreactive microglia secrete neuroprotective factors such as brain derived neurotropic factor (BDNF) and NGF [176–180] and thrombospondin [174]. Microglia are mobile; they move to the site of injury and secrete proinflammatory cytokines and upregulate the expression of cell-surface molecules and membrane proteins [181, 182]. On the other hand when microglial cells becomes reactive, they can activate several inflammatory pathways/cyclooxygenase-2 (COX-2), interleukin (IL)-3, IL-6, Il-1B, tumor necrosis factor alpha (TNF-α), prostaglandins, tissue plasminogen activator (tPA), MCP-1, vascular endothelial growth factors, lymphotoxin, matrix metalloproteinases, and macrophage inflammatory protein-1α [172–178]. The amount of secretion of such factors depends upon the severity of the insult. For example, activation of tPA, along with other factors, has been shown to play a role in the mossy fiber sprouting (MFS) which is observed in chronic epilepsy [183–186]. Further, an increased number of activated microglia near the damaged tissues [36, 187], especially at CA1 and CA3 regions of the hippocampus, prove their harmful role during epilepsy (Figure 5). It has also been proposed that microglial activation can sustain the development of SRS by initiating aberrant neurogenesis and also the migration of neuroblasts in the dentate gyrus [188]. Our ongoing work demonstrates increased expression of chemokine receptor 2 (CCR2), the receptor for MCP-1 (Figure 5). Incidentally, MCP-1 production by astrocytes is mediated through nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) and was also found upregulated following 24 h after SE (Figure 5).

6.3. Crosstalk between Neuron and Glia during Epileptogenesis. Seizures during the SE subsequent spiking activity and repeated SRS activate the resident glial cells (astrocytes and microglia) to become hypertrophic and reactive (Figures 4 and 5). As discussed above, the reactive glial cells release proinflammatory mediators which in turn act on the neurons to decrease their seizure threshold. There is also increased expression of redox-sensitive transcription factors activator protein-1 (AP-1) and NFκB leading to an activation of NADPH oxidase in microglia cells. The activation of NADPH oxidase on microglia cells results in the formation of cytochrome b_{558} in the electron transport chain, which leads to an increase in extracellular superoxide production through iNOS. These factors may affect neuroblasts and/or those neurons that were recovering during post-SE phase of epileptogenesis (Figure 4).

Overall, the chain of events that occur following seizures is summarized below; (a) increase in intracellular calcium due to activation of NMDAR during and soon after SE or seizure (illustrated in Figure 4); (b) activation of phospholipase A_2 (arachidonate release) and phospholipase C (not shown); (c) immediate early gene expression such as c-Fos; (d) altered kinase activity, altered phosphorylation of enzymes, receptors, and ion channels (not shown); (e) altered ion channel function as evident from increase spiking activity; (f) change in subunit expression of excitatory and inhibitory receptors; (g) altered synaptic morphology, remodelled dendritic spines; (h) enhanced neurogenesis in dentate gyrus; (i) MFS leading to altered connectivity; (j) oxidative damage to proteins, lipids, and DNA; and (k) neurodegeneration through apoptosis inducing factor (AIF) or caspase-3 mediated pathway (illustrated in Figure 4). The emerging hypothesis in our laboratory is that targeting the postsynaptic membrane proteins could be protected against the recurrence of seizures. The postsynaptic density protein-95 (PSD-95), a scaffolding protein that links the nNOS with glutamate receptors, is depicted in Figure 4. Modulating protein-protein interactions involved in disease pathways is an attractive strategy for developing drugs but remains a challenge though. One approach is to target certain domains within proteins that mediate these interactions. One example of such a domain is the PDZ domain of PSD-95, which is involved in interactions between many different proteins in a variety of cellular contexts. Because PDZ domains have well-defined binding sites, they are promising targets for drug discovery in epilepsy research.

7. Treatment Options for TLE

7.1. Antiepileptic Drugs: Beneficial and Adverse Effects. Several AEDs have been tried for TLE. AEDs used to suppress seizures in epileptic patients have multiple mechanisms of action [189]. For example, phenytoin reduces the amplitude of sodium channels by inactivating them; ethosuximide blocks Ca^{2+} channels; phenobarbital blocks $GABA_A$ receptors and possibly sodium channels; and carbamazepine (CBZ) prevents convulsions by potentiating certain GABA receptors subtype containing $\alpha 1$, $\beta 2$, and $\gamma 2$ subunits [190]. A long

term use of AEDs leading to impairment of the endogenous antioxidant system has been investigated. AEDs, namely, valproic acid, phenytoin, CBZ, and levetiracetam, are shown to increase lipid peroxidation and decrease GSH/GSH-Px [4]. CBZ is implicated in reduction of CAT enzyme activity while phenobarbital valproic acid (VPA) and CBZ are shown to reduce SOD enzyme activity [4]. In rat cortical astrocyte cell culture assays CBZ, oxcarbazepine, and topiramate are demonstrated to cause oxidative stress leading to reduced activity of astrocyte glutamine synthetase [191, 192]. Phenobarbital, CBZ, and valproic acid after their hepatic metabolism result in reactive intermediates that can lead to covalent binding to macromolecules [193, 194]. From our ongoing proteomics studies from 7 days post-SE mouse model (kainate), we have identified downregulation of VPA transporter protein. VPA is a broad spectrum AED and one of the most widely prescribed drugs for epilepsy worldwide. Its effects are mediated by an action on the inhibitory system, γ-aminobutyric acid (GABA), through enhancement of GABA synthesis and release [195]. VPA is also histone deacetylase (HDAC) inhibitor and has a neuroprotective role [196]. A review by Cárdenas-Rodríguez et al. summarizes the effects of AEDs on the markers of oxidative stress in human epileptic patients [197]. Although AEDs control seizures, their role to elicit systemic toxicity and to contribute to oxidative stress needs to be carefully considered during therapy. Moreover, since AEDs only control symptomatic seizures, an adjunct therapy such as dietary supplements and neuroprotectants would be beneficial. In this review, the role of dietary supplements in epilepsy is briefly discussed below.

7.2. Role of Enzymatic and Nonenzymatic Antioxidants. The cells possess endogenous antioxidant system to neutralize and scavenge free radicals when produced in excessive amounts. As explained earlier in this paper, these scavengers are enzymes such as SODs, catalases, glutathione/glutathione peroxidase system, and thioredoxin reductases. The other nonenzymatic antioxidant systems include cysteine/glutamate antiporter and dietary supplements such as vitamins E and C, polyphenols, melatonin, and ketogenic diet.

7.3. Glutathione and Cysteine/Glutamate Antiporter. GSH has been found to be low in epileptic patients by about 150% when compared to nonepileptic patients [131]. Reduced glutathione, a tripeptide with a free sulfhydryl group, is required to combat oxidative stress and to maintain homeostasis in the cell. Selenium (Se) acts as a catalyst for GSH-Px activity and has similarly been studied in children with epilepsy. It has been found that blood serum Se concentrations are lower in epileptic children than healthy children [198, 199]. But low levels of selenium detected in epileptic patients did not exhibit typical signs of selenium deficiency such as generalized fatigue, light sensitivity, and heart palpitations [193].

Cysteine/glutamate antiporter system (CGS) is a protective antioxidant mechanism. The neurons exchange intracellular excitotoxic glutamate for oxidized cysteine from the extracellular space. GCS is found in both neurons and glial cells (astrocytes, microglia) in the brain [200–204]. Glutamate exported by CGS is responsible for the extracellular glutamate concentration in the brain which is later taken up astrocytes to be converted into inactive glutamine. The oxidized cysteine imported into the cell is essential for the synthesis GSH by enzyme thioredoxin reductase 1. Thus CGS acts like a bridge that connects the antioxidant defense with neuronal excitability. The CGS system gets impaired during an increased extracellular glutamate (during astrocytic glutamate release) and/low intracellular cysteine. Thus an increase in extracellular glutamate apart from inhibiting CGS is also responsible for hyperexcitability of neurons. Thus an inhibition of CGS can lead to depletion of endogenous glutathione reserves succumbing to oxidative stress and cell death termed as "oxidative glutamate toxicity." Impaired CGS system has also been implicated in other neurological disorders apart from epilepsy [205–209]. Therefore, the drugs that enhance CGS can be beneficial.

7.4. Antioxidant Diet Supplements. (i) *Vitamin C.* Vitamin C, due to its water soluble nature, was found to be effective in eliminating free radicals within the brain circulation. The recommended dietary allowance (RDA) for vitamin C is 75–90 mg/day for adults. Red peppers, oranges, grape fruits, and kiwi fruits are the rich sources of vitamin C [210]. In rat models of epilepsy, pilocarpine increased lipid peroxidation during SE. Vitamin C caused a decrease in lipid peroxidation and increase in CAT enzyme activity. Further, vitamin C also increased the latency to the onset of seizures after SE while reducing the mortality rates in rat models [211].

(ii) *Vitamin E.* Vitamin E was found to exert its anticonvulsive effects by upregulating catalase activity in pilocarpine rodent models of epilepsy [123, 124, 211]. The RDA for vitamin E is 15 mg/day (22.4 IU) for adults. Wheat germ oil, sunflower seeds, almonds, and hazelnuts are the rich sources of vitamin E [210]. During pilocarpine induced seizures, vitamin E concentrations were found to decrease in brain cortex [212]. Frantseva and colleagues in kindling rat models of epilepsy showed that antioxidant treatment (vitamin E and glutathione) reduced neuronal death and lipid peroxidation; however, it did not prevent development of recurrent seizures [53].

(iii) *Polyphenols.* Cloves, peppermint, cocoa, oregano, flaxseeds, and chestnuts are the rich sources of polyphenols [213]. Food groups such as polyphenols derived from commercial and organic grape juice and yerba mate have been demonstrated to prevent neurodegeneration and seizures [214, 215]. Branco and colleagues have found that organic yerba mate is found to reduce seizures by increasing SOD and CAT activity in rodent models [215].

(iv) *Melatonin.* Melatonin has been found to act as scavenger of hydroxyl radicals to prevent lipid peroxidation in the CNS [216]. Melatonin rich plant sources include St. John's wort, fennel seed, sunflower seed, fenugreek seed, and black mustard seed [217, 218]. Although not approved by FDA,

0.3–5 mg/day for an adult was found to be beneficial in sleep disorders [219]. Since melatonin has both lipophilic and hydrophilic properties, it is speculated that it could be an effective antioxidant. In a mice study, when melatonin was given concurrently or 30 min prior to induction of seizure with kainate, it attenuated the lipid peroxidation [122]. Interestingly, in the same study melatonin when given 15 minutes after SE had no effect on the seizure suggesting that a high level of melatonin prior to seizure induction has beneficial effects.

(v) *Ketogenic Diet*. A typical ketogenic diet is a high-fat, low carbohydrate diet containing long chain fatty acids providing 3-4 grams of fat for every gram of carbohydrate and protein [220]. The ketogenic diet has been demonstrated to reduce mitochondrial ROS/RNS due to a change in source of energy using fewer carbohydrates and more fat-derived ketone bodies [221–224]. This protective effect can be traced to high acetone concentrations present in the brains of human patients on ketogenic diet. Ketogenic diet demonstrated anticonvulsive effects in epileptic children with congenital abnormalities such as mutations glutamate transporter, GLUT-1 [225], and a deficiency of pyruvate dehydrogenase [223, 226]. High-fat diet was found to initiate epilepsy in infant mice that lack mitochondrial uncoupling protein (UCP) isoforms and this effect was neutralized by a low-fat diet [227, 228]. These data infer the protective effects of a high-fat diet during epileptic seizures, however, the age of the individual being an important criterion.

7.5. NOS Inhibitors to Prevent Epileptogenesis. NOS inhibitors such as N-propyl-L-arginine (L-NPA) and nitro-L-arginine methylester (L-NAME) have been tested in experimental rodent models of epilepsy [31, 36, 229–233]. L-NPA, a selective nNOS inhibitor, reduced the frequency of epileptiform spikes, severity, and duration of seizures during 7 days after SE in kainate mouse (C57BL/6J) model of epilepsy [36]. Studies showed that a broad spectrum NOS inhibitor, L-NAME, had a controversial role on hippocampal damage or protection in rat models (quote our papers from Siobhan and 200), while aminoguanidine selective iNOS inhibitor significantly reduced seizures in a kainate mouse model of epilepsy [229]. Another potent and highly selective inhibitor of iNOS, 1400 W, has been studied for its effects on inhibiting iNOS in both *in vivo* and *in vitro* models [87, 234]. 1400 W is a slow, tight binding, and a highly selective pharmacological inhibitor of human iNOS with a dissociation constant (Kd) value of 7 nM and a selectivity of 5,000-fold for iNOS [30, 234]. Due to its selective action, 1400 W is found to have little or no cardiovascular side effects and does not interfere with the physiological activities mediated by nNOS [234]. 1400 W was found to be most effective during pathological increase in iNOS levels in various organs [30, 234–236]. 1400 W is BBB permeable and biologically active *in vivo* and effective in ameliorating the neuropathological changes in traumatic brain injury and stroke models by decreasing glutamate release [237–239]. Additional advantage of iNOS inhibitors is that they attenuate BBB leakage [240]. Serum albumin (SA) is considered as a biomarker for BBB leakage [241, 242]. Recent studies in the hippocampus suggest that increased SA levels are responsible for hyperexcitability of neurons and SRS due induction of reactive astrogliosis as validated by increased GFAP levels [240, 242]. Our recent proteomics studies of hippocampus from 7 days post-SE mice provide evidence for concomitant increased levels of both SA and GFAP. In those studies, 1400 W reduced SA and GFAP to their basal levels (63-64). From our ongoing work, immunohistochemistry of brain sections from 7 days after SE in an organophosphate rat model revealed an important polarizing effect of 1400 W on gliosis. It decreased reactive microglia, which could be due to decreased levels of glutamate and SA, but increased the number of nonreactive glial cells (data not shown). A recent article highlights the therapeutic importance of drugs that polarize glial cells from reactive to nonreactive state [243]. Nonreactive gliosis is neuroprotective [244–247]. Our EEG analyses from 1400 W treated rats at 7 days after SE confirmed a decrease in spike rate when compared to vehicle treated in a diisopropyl fluorophosphate (DFP) model suggesting that 1400 W decreases neuronal hyperexcitability by reducing proinflammatory cytokines and by promoting neurotropic activity. Although 1400 W is a highly specific iNOS inhibitor and is emerging as a promising disease modifying drug for epilepsy, its mechanism of action is not yet clear. It has been found that 1400 W was able to reduce phosphorylation of c-Jun N-terminal kinase (JNK), but it was unable to prevent seizures from occurring [229, 234], possibly due to inappropriate dosing regimen. JNK acts as a signaling molecule during stress, such as UV radiation and oxidative stress and phosphorylation. JNK is also responsible for neurodegeneration and apoptosis [38]. Hence, an inhibition of JNK prevents neurodegeneration [248, 249] and may offer antiepileptic therapeutic option by iNOS inhibitors.

8. Conclusion

In summary, oxidative stress plays a key role in epileptogenesis after the first seizure. Through progressive neurobiological changes, the first seizure later becomes a cause for recurrent seizures in TLE. The acute effect of oxidative stress is neurodegeneration, which is mediated by seizure-induced reactive gliosis. Oxidative stress targets mitochondrial DNA and lipid peroxidation which affect ATP depletion and further contributes to excessive production of ROS/RNS. These changes override endogenous antioxidant protective mechanisms. These changes will induce rearrangement neural circuits, neuronal loss and neurogenesis, and aberrant migration of neuroblasts thus contributing to hyperexcitability and SRS onset. Breaking this vicious cycle is critical by developing new and effective drugs which can prevent epileptogenesis. The current AEDs in combination with neuroprotectants and/or antioxidants could be effective in disrupting the vicious cycle. Role of antioxidant supplements, ketogenic diet, COX-2 inhibitors, NOS inhibitors, and PSD-95 blocking peptide are some of the options currently being explored to complement existing AEDs to control epilepsy. Initial success of these treatment options in different animal

models and some human patients is encouraging. However, intense investigation is required to fully evaluate the potential of a combination of drugs to cure established epilepsy and refractory epilepsy.

Conflict of Interests

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

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Review Article

Dietary Factors in the Etiology of Parkinson's Disease

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Parkinson's disease (PD) is the second most common neurodegenerative disorder. The majority of cases do not arise from purely genetic factors, implicating an important role of environmental factors in disease pathogenesis. Well-established environmental toxins important in PD include pesticides, herbicides, and heavy metals. However, many toxicants linked to PD and used in animal models are rarely encountered. In this context, other factors such as dietary components may represent daily exposures and have gained attention as disease modifiers. Several *in vitro, in vivo*, and human epidemiological studies have found a variety of dietary factors that modify PD risk. Here, we critically review findings on association between dietary factors, including vitamins, flavonoids, calorie intake, caffeine, alcohol, and metals consumed via food and fatty acids and PD. We have also discussed key data on heterocyclic amines that are produced in high-temperature cooked meat, which is a new emerging field in the assessment of dietary factors in neurological diseases. While more research is clearly needed, significant evidence exists that specific dietary factors can modify PD risk.

1. Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disease with a prevalence of ~1% among those over 60 years of age. PD is characterized by dopaminergic neuron loss in the substantia nigra followed by striatal dopamine depletion, which results in cardinal motor symptoms such as bradykinesia, postural instability, resting tremor, and rigidity. ~10% of PD cases are caused by genetic factors: mutations in the alpha-synuclein, Parkin, *PINK*, *LRRK2*, and other genes [1]. However the remaining ~ 90% of patients are sporadic, arising from unknown causes. Environmental factors are thought to play a crucial role in progression of the disease. Pesticide exposure has been repeatedly linked to PD [2]. Rotenone and paraquat have been shown to induce dopaminergic neuron loss in the substantia nigra and striatum in animals, resulting in development of PD-like symptoms [3]. Further, these pesticides have been linked as PD risk factors in humans. Another very well-known PD-causing toxin is 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). MPTP's dopaminergic toxicity was discovered after hospitalization of several people who were synthesizing homemade opioid drugs in early 1980's. MPTP is metabolized into MPP+ after crossing blood-brain-barrier. MPP+ is transported into dopaminergic neurons via dopamine active transporter and interferes with complex I activity in mitochondria, eventually increasing reactive oxygen species production and oxidative stress in the cell [3].

Although rotenone, paraquat, MPTP, and other toxicants have been repeatedly used to model PD, exposure to these specific compounds does not likely account for a significant number of PD cases due to relatively rare exposures in most environments. The search for compounds that are encountered frequently and exposed throughout the life is still ongoing. Traditionally, examination of dietary factors in PD has received less attention compared to other environmental exposures. However, several dietary habits have been shown to modify the risk of developing PD. Here, we critically review findings on the association of dietary groups, vitamins/antioxidants, metals, and fat, with modifying PD risk. The role of industrial and agricultural contaminants in PD has been reviewed numerous times [2, 4, 5], the focus of this review is on natural components of the diet, or compounds formed during preparation.

2. Vitamins

2.1. Vitamin A and Carotenoid. Carotenoids (alpha- and beta-carotene) are precursors of vitamin A in human. Egg yolks, organ meats, and milk are rich sources of vitamin A, while carotenoid rich diet includes carrots, sweet potatoes, and peaches, as well as other fruits and vegetables. Previously, vitamin A and beta-carotene were shown to inhibit alpha-synuclein fibril formation and destabilize formed fibrils in dose-dependent manner *in vitro* [6]. While several human studies did not identify a link with vitamin A and PD [7–10], Miyake et al. found a protective effect of beta-carotene in PD in a Japanese population [11]. In this study, dietary habits of 249 PD patients were compared with 368 controls. Beta-carotene at highest quartile (>4080.9 μg/day) was inversely associated with PD [odd's ratio (OR) 0.56, 95% confidence interval (CI) 0.33–0.97]. Although there is no recommended daily allowance for beta-carotene due to lack of evidence, one study reported that daily intake range is 2–7 mg for women in US [12]. When data was stratified by sex, beta-carotene consumption remained significant only in women (P value = 0.001). Thus, current data on a role for vitamin A in PD development is extremely limited, with quantifiable effects only at the highest doses.

2.2. Vitamin B. Vitamin B complexes are found in meat, fish, cereal, dairy products, and some vegetables (i.e., potato) and fruits (i.e., banana). Although there are several types of vitamin B, the focus of this discussion is on vitamin B2 (riboflavin), B6 (pyridoxine), B9 (folate), and B12 (cobalamin).

Homocysteine is a metabolite of methionine that is essential for the DNA synthesis and has been shown to exert adverse effects of mitochondrial alterations. Vitamins B6, B9, and B12 indirectly regulate level of homocysteine [13, 14]. Folate-deficient diets result in increases in homocysteine [15]. High homocysteine level damages DNA and depletes energy reserves, subsequently inducing neuron apoptosis [16, 17].

In one study on the effects of folate deficiency, two-month-old C57B1/6 mice were subjected to a diet lacking folate or control diet containing 2 mg folate/kg of food for two months followed by intraperitoneal (ip) MPTP injection at subtoxic doses or saline [15]. Mice fed with control diet did not exhibit differences in motor activity between MPTP or saline groups. Similarly, motor activity in folate-deficient mice was not significantly different from mice with control diet. However, MPTP-induced motor activity impairment and loss of nigral dopaminergic neurons were exacerbated in folate-deficient mice. Further, vitamin B2 deficiency in rodents was shown to decrease circulating iron levels and increase iron turnover, resulting in disturbance of iron metabolism, which is one of the well-established hypotheses in PD [18, 19]. Therefore, in animal models, vitamin B deficiency appears to exacerbate neurotoxicant-induced motor deficits and pathology.

Epidemiological studies presented variable findings. Higher intake of vitamins B6, B9, and B12, but not B2, was associated with lower risk of PD in a German population [20]. A Rotterdam study that examined 7,983 individuals found that while vitamin B6 was protective against PD in dose-dependent manner (only in smokers), vitamins B9 and B12 were not significant [21]. It is not clear whether higher vitamin B6 intake prevents or simply delays PD. Given that significance was only achieved when combined with another known PD protective factor (smoking), there is currently no evidence that B6 alone would modify PD etiology. Lower intake of folate was detected in 249 PD patients, compared to 368 healthy controls, but the association was not significant after adjustment for potential dietary confounding factors [22]. The loss of significance after adjusting for multiple factors illustrates the difficulty in identifying single disease modifying dietary factors in human studies. In another study, Coimbra and Junqueira reported low levels of riboflavin in 31 PD patients [23]. Riboflavin supplementation (30 mg) with 8-hour intervals for 6 months gave rise to promising improvements in motor activity of patients. The protective effect remained intact, even when supplementation was stopped. In contrast, data from the Honolulu Heart Study (HHS), with 30 years of followup of approximately 8,000 men from Japanese-Okinawan ancestry reported that total vitamin B intake was not significantly associated with PD (using 137 patients) [9]. This study has utilized extensive data collection of patients' dietary habits and any environmental toxicant exposures over decades, such as pesticides, resulting in significantly more power compared to retrospective case-control studies. In two large cohort studies entitled "Nurse Health Study (NHS)" and "Health Professionals Follow-up Study (HPFS)" that contain 121,700 females and 51,529 males, respectively, average folate intake was determined as 482 μg/day in men and 366 μg/day in women, where folate levels were not significantly different between PD patients and healthy controls [24]. The questionnaire was used to assess daily consumption of particular food for last 12 months. No association was detected between vitamins B6, B9, and B12 and PD in US population [25]. One possibility is that folate might be protective only in neurotoxin-induced PD models, when administered at high doses, often prior to the neurotoxic insult.

2.3. Vitamin C. Numerous studies have suggested that there is no clear association between vitamin C and human PD [9–11]. Vitamin C was found to increase dopamine synthesis in human neuroblastoma cell line SK-N-SH [26]. Although vitamin C is the most potent antioxidant among other vitamins, exogenous administration may not affect disease development due to limited access to the brain. Access to the brain is restricted by high water solubility and the requirement of active transport at the choroid plexus to enter the brain [27].

2.4. Vitamin D. Major vitamin D rich foods are fortified milk, liver, and saltwater fish [28]. Vitamin D is metabolized into its active form, 1,25-dihydroxyvitamin D (1,25-(OH)2 Vit D or calcitriol) in the cytoplasm of neurons and glial cells [29, 30]. The vitamin D receptor (VDR) is activated by binding to calcitriol which increases calcium uptake in bones.

Although a protective role of vitamin D against PD is not well established, there are number of laboratory studies

suggesting that exogenous administration may be protective: MPP+ toxicity in primary mesencephalic dopaminergic neurons was decreased by low doses of vitamin D (1–100 nM) *in vitro* [31]. Pretreatment of rats with calcitriol prior to 6-hydroxydopamine (6-OHDA) administration attenuated neuronal toxicity *in vivo* [32]. It is worth noting that significantly lower bone mass index and vitamin D deficiency were detected in PD patients [33]. Further, risk factors for hip fracture and falling in PD patients were associated with lower vitamin D plasma levels [34]. Treatment of a 47-year-old male PD patient with very high dose of vitamin D (4000 IU daily) with ongoing conventional therapy delayed tremor and rigidity, while other Parkinsonism symptoms did not show any alterations [35]. This report represents a single case and more additional robust studies have either not been conducted or have failed to show a direct protective effect of vitamin D in PD. A recent systemic review and meta-analysis included seven studies with 1,008 PD cases and 4,536 controls [36]. Statistical analysis indicated a ~twofold increase in risk of PD in individuals with vitamin D-deficient diet [OR 2.2, 95% CI 1.5–3.4]. Further research on association of vitamin D with PD needs to be performed. In particular, more robust epidemiological studies need to be conducted. Distance from the equator has been linked to prevalence of other disorders such as multiple sclerosis. While there have been many hypotheses to account for this relationship, sunlight exposure and vitamin D levels are plausible factors [2]. Given the studies mentioned above, PD prevalence and progressions rates along with distance from the equator and vitamin D levels should be assessed [37].

Examinations of genetic polymorphisms in the VDR as a factor in modulating PD risk or disease development have produced variable results [38]. The rs4334089 polymorphism in the receptor gene has been found significantly associated with PD in a US population study including a discovery phase of 770 Caucasian families with PD history and a validation case-control study (267 cases, 267 controls) [39]. However, the same polymorphism was not found associated with PD in Chinese Han [40] and Taiwanese populations [41].

2.5. Vitamin E. Vitamin E is found at high levels in vegetable oils, nuts, and whole-grain products. It has strong antioxidant capacity. Pretreatment of neurons with vitamin E alleviated MPTP-induced dopaminergic neuron toxicity *in vitro* [42]. Further, vitamin E-deficient mice exhibit heightened sensitivity to MPTP [43–45].

Association of vitamin E with PD has been identified in a number of studies in the last two decades. Serum levels of vitamin E in PD patients were significantly lower than controls [46]. One of the first studies compared frequency of early-life consumption of 31 foods in 106 PD patients and their spouses [47]. This study found that PD patients are less likely to eat vitamin E-containing foods (peanut and salad dressing) than those without PD. A few years later, a relatively large-sampled study was conducted using HHS data [48]. Here, all subjects were followed for 30 years and 24-hour recall for vitamin E-containing food was evaluated. Overall, this study showed an insignificant trend towards an association of high total vitamin E intake with decreased risk of PD. However, consumption of legumes was strongly protective. Unfortunately, the strength of these types of studies is limited by recall accuracy because 24-hour recalls potentially fail to reflect true dietary habits due to underreporting by some groups [9]. The primary reason why legumes are protective, but other foods containing high levels of vitamin E are not remains unclear. One possible explanation might be due to the presence of another unidentified compound that is present in legumes, but is absent or at low levels in other vitamin E rich foods.

Data from NHS and HPFS were used to assess dietary components over one year [8]. Female and male smoker PD patients were found to eat fewer nuts, which are rich in vitamin E content, while no difference was reported for mayonnaise and creamy salad dressings, contradicting the results of Golbe et al. [47]. However, it is difficult to conclude whether having the disease changed the food preferences or if nuts are really protective against PD. Differences in the lipid composition between vitamin E rich foods could also be contributing factors. Miyake et al. found that higher vitamin E intake (>9.759 mg/day) is significantly associated with decreased risk of PD in women (OR 0.33, 95% CI 0.15–0.71; $P = 0.006$) [11]. Interestingly, meta-analysis of eight epidemiological studies reported that moderate intake of vitamin E is protective with a relative risk of 0.81 [10]. Moreover, vitamin E supplementation has been tested as a therapeutic against PD in the DATATOP study [49]. In this study, 800 patients were supplemented with α-tocopherol, the biologically active component of vitamin E, for more than a year. No beneficial effect of α-tocopherol was observed during follow-up evaluation of PD symptoms. Vitamin E supplements contain racemic-α-tocopherol that has lower activity than RRR-α-tocopherol in foods [8, 50]. Thus, higher intake of supplementation might be needed to detect a protective effect. Also, penetration rates through blood-brain-barrier of these two vitamin E forms might be different. In addition, the use of vitamin E supplementation as a therapeutic is difficult to achieve due to the fact that more than half of dopaminergic neurons in substantia nigra of PD patients are already lost in the stage where symptoms are apparent [51].

In conclusion, the data for a protective or preventative role of vitamin E appears to be stronger than other vitamins. Further, low dietary levels could potentially increase risk. However, data on supplementation in patients already diagnosed with PD has failed to show a disease modifying effect.

3. Flavonoids

Flavonoids are the most common groups of polyphenols in human diet [52]. Many plant-based foods and beverages are rich in flavonoids, such as berry fruits and citrus fruits [53]. Flavonoids have high antioxidant capacity [54]; they have been shown to modulate oxidative-related enzymes and regulate mitochondrial function in neurons [52, 55]. These findings point to a potential protective role of flavonoids in PD.

Nobiletin, a flavonoid that is found in citrus fruit peel, was found to improve MPTP-induced motor and cognitive deficits in mice [56]. Although nobiletin administration (50 mg/kg) via ip injections for 2 weeks did not prevent loss of dopaminergic neurons in the midbrain of MPTP-induced PD model mice, motor deficits were alleviated significantly compared to mice that did not receive the injections.

A potential neuroprotective role of flavonoids in PD was recently examined using anthocyanins and proanthocyanidins. Strathearn et al. reported that the treatment of primary midbrain cultures with blueberry, grape seed, hibiscus, blackburrant, or Chinese mulberry extracts rescued rotenone-induced loss of dopaminergic neurons [57]. Here, blueberry and grape seed extracts were shown to rescue disruption of mitochondrial respiration, suggesting that the protective effect might be mediated via enhancement of mitochondrial function.

Epidemiological findings have also suggested that consumption of flavonoids in the human diet lowers PD risk. One study used NHS and HPFS datasets, which consist of 22.7 and 20 years of follow-up data, respectively, from 805 PD patients (438 men and 367 women) [58]. The major sources of flavonoids in this study were apples, tea, blueberry, strawberry, red wine, and orange/orange juice. Although the flavonoid intake was not significant in pooled PD incidents, men showed significant inverse association of flavonoid intake at highest quartile with PD [Hazard ratio (HR) 0.60, 95% CI 0.43–0.83]. The use of estrogen as a possible mechanism underlying gender difference was tested, but no association was found. Consumption of anthocyanin-rich fruits, strawberries, and blueberries reduced PD risk in pooled sample (HR 0.77, 95% CI 0.62–0.97). Another study, including 41 years of follow-up data from 2388 men and 2136 women in Finland, reported the association of berry consumption with increased risk of PD in men [relative ratio (RR) 1.80, 95% CI 0.85–3.82] [59].

One of the unavoidable controversies in epidemiological studies involving berries, as well as other fruits and vegetables, is the presence of pesticide exposure. Although, now many countries have strict regulations on use of pesticides and herbicides in agricultural areas, any exposure of fruits to pesticides could potentially reduce neuroprotective capacity. Another careful consideration will be to test subclasses of flavonoids separately. The specific content of flavonoids, such as flavanones and anthocyanins, varies between fruits. These subclasses exhibit differences in chemical properties and their ability to cross the blood-brain-barrier [60]. It is therefore possible that evaluation of fruit as one group might lead to misleading or weak associations.

4. Calorie Intake

Dietary restriction has been repeatedly shown to prolong lifespan and decrease age-related diseases [61–63]. Low calorie intake attenuated age-related decline in dopamine signaling and increased resistance of nigral neurons to excitotoxic or oxidative stress [64, 65]. Dietary restriction in mice enhanced expression of neurotrophic factor, especially brain-derived neurotrophic factor (BDNF), in hippocampus, resulting in an excitoprotective effect, preventing excitotoxicity which is caused by neurotransmitters such as glutamate [66–68]. Protein chaperones, such as hsp70, that help cells to resist various stressors, were also induced *in vivo* [69, 70].

In *C. elegans* treated with 6-OHDA, dietary restriction prevented dopamine depletion and dopamine cell loss, suggesting protective effect in anterior deirid and posterior deirid neurons [71]. Duan and Mattson (1999) have shown that vulnerability of dopaminergic neurons to MPTP was significantly decreased by lower calorie intake in adult male mice [69]. In another study, male Sprague-Dawley rats were subjected to low calorie diet for two or eight weeks and then injected intrastriatally with 6-OHDA [72]. Here, no differences on striatal dopamine terminal density or apomorphine-induced rotational behavior were observed in normal and restricted diet groups. It is possible that two months of low calorie diet might not be long enough to be protective against 6-OHDA toxicity in rats. A longer exposure to calorie-restricted diet was tested in rhesus monkeys [73]. Here, 13 adult rhesus monkeys were subjected to normal or calorie-restricted diet for 6 months followed by 2.4 mg MPTP injection through carotid artery. Behavioral assessment six weeks after MPTP injection revealed improved motor activity in calorie-restricted monkeys. Imaging and postmortem brain examination confirmed low calorie intake prevented MPTP toxicity, with decreased presynaptic dopamine loss and greater presynaptic dopamine activity in left and right basal ganglia measured using PET scan.

Epidemiological studies have suggested contradicting results on the protective effect of low calorie intake in PD. Low calorie intake is associated with reduced risk of PD [25]. In contrast, although individuals with lowest body-mass index experienced the lowest incidence of PD, the difference failed to reach significance level in Honolulu Heart Study [9]. In US, average calorie intake is 2,700 kcal for women and >3,000 kcal for men. It has been concluded that low calorie intake (1,600–2,000 kcal) beginning at approximately 20 years of age would have protective effect against PD [74].

5. Caffeine

Decreased risk of PD resulting from caffeine intake has been repeatedly identified in US, European, and Asian populations [75, 76]. Common sources of caffeine are coffee, tea (especially black tea), soft and energy drinks, and chocolate. Caffeine is known to be a central nervous system stimulant and an adenosine receptor antagonist [77, 78]. While adenosine receptor antagonists were shown to improve motor activity in MPTP administered primates [79, 80] and caffeine intake reduced MPTP-induced dopamine depletion in mice [81], agonist of the receptor disrupted dopamine transmission, resulting in exacerbated motor deficits in rodents [82].

There are number of case-control, cohort studies, and meta-analyses regarding caffeine intake and PD risk in different populations. Data from HPFS reported an inverse association between coffee (RR 0.5, 95% CI 0.1–2.1) and tea consumption (RR 0.6; 95% CI 0.3–1.2) with PD in very large population of males [83]. Interestingly, the Nurse Health

Study that includes more than 120,000 female nurses did not find a significant inverse association, although a U-shaped dose-response curve between coffee intake and PD was observed, suggesting that individuals with moderate consumption of coffee (1–3 cups per day) had the lower risk of PD [84]. Another larger cohort study, HHS, gave different conclusions due to consideration of period of consumption. Grandinetti et al. reported that coffee intake decreased risk of PD, but it was no longer significant after adjustment for smoking [85]. A few years later, another study used the same HHS data, including more PD patients (102) and longer follow-up, and observed significant inverse association of coffee with PD, independent from alcohol and smoking habits [86]. Liu et al. also reported similar findings in a very large cohort study of US population [87]. Total caffeine intake was protective in dose-dependent manner also in a Chinese population (including 157 PD cases) and remained as a significant association after being stratified by smoking [88]. A strong protective effect was also observed in black tea consumption, as reported in other epidemiological studies in other Chinese and US studies [89–91], although no significant association was observed in green tea consumption [88].

It is noteworthy to mention that the inverse association of caffeine intake in women is not as straightforward as in men, a potentially major factor in the variable findings on the relation between the amount of coffee consumption and PD risk. In addition to the U-shaped relation between coffee intake and PD, implicating that the moderate consumption of coffee is neuroprotective against the disease [84], another large case-control study with 392 cases reported that only high coffee intake was significantly associated with lower risk of PD (OR 0.58, 95% CI 0.38–0.89) [92]. Rugbjerg et al. also reported the inverse association of highest coffee consumption, but not tea, with lower PD incidence [93]. In contrast, the relation was not significant in Finnish female population [94]. The contradicting results may be due to estrogen levels in women. Ascherio et al. proposed the association between coffee intake and estrogen use in two different studies. Higher caffeine intake in women who did not use hormone therapy was associated with a lower PD risk and mortality, while among estrogen users higher intake increased PD risk and mortality significantly [84, 95]. It is surprising that Liu et al. reported the opposite. The higher intake of coffee intake was associated with lower risk of PD among hormone users, but the association could not reach significance threshold [87]. Reconciling differences is a difficult task, especially given that the role of hormone use on neuroprotective mechanisms of caffeine is not known. It has been emphasized that the type of hormone, duration of use, and recipient's age affect the health outcome [96]. The relation between caffeine intake, estrogen use, and PD is difficult to explain and requires more careful consideration of case-control study design with discrepancies among hormone usage when dealing with female populations. The discrepancies described above illustrate the importance of considering influence of other potentially neuroprotective factors in PD such as smoking and estrogen.

6. Fatty Acid Intake

Total dietary fat intake is supplied in three categories: Saturated fatty acids, unsaturated fatty acids, and cholesterol. Cholesterol typically provides only 1% of fat intake, while the remaining 99% comes from fatty acids [21]. Cholesterol is found in animal products, such as meat, eggs, milk, and butter. Fatty acids are divided into two groups: saturated and unsaturated fatty acids. Dairy products and meat are rich in saturated acids. Monounsaturated fatty acids (MUFAs) are found in sunflower oil, peanut oil, and olive oil, which are commonly used in Mediterranean diet. There are different types of polyunsaturated fatty acid (PUFA): vegetable oils that contain omega-6 and omega-3 is abundant in fish and marine products.

Approximately 60% of structural material of the brain is composed of lipid. Synthesis of brain lipid requires essential fatty acids, suggesting that balance in dietary fatty acid intake is crucial for brain function [97–99]. PUFAs are involved in neural function and cerebral structure [100, 101]. Two types of omega-3 fatty acid, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are important for lipid bilayer composition [102, 103]. In omega-3 deficiency, growth factors in brain, especially BDNF, fail to be produced [104]. Here, we have summarized findings on the effect of fatty acid intake in brain function and PD.

MUFAs and PUFAs have been shown to have anti-inflammatory and neuroprotective properties, by reducing the oxidative stress and inhibiting neuronal apoptosis [105–110]. PUFAs help regulation of dopamine activity in basal ganglia, controlling movement [111, 112]. Omega-3 fatty acid is crucial for neurogenesis in olfactory bulb and myelination by oligodendrocytes, which has been shown to be significantly affected in PD [113]. Moreover, omega-3 deficiency causes alteration of the dopamine mesocorticolimbic pathway, which is anatomically relevant to PD [114, 115]. Zimmer et al. reported that dopamine levels in cerebral areas and D2 receptor mRNA expression in frontal cortex were lower in rat fed with omega-3 deficient rats [115].

Supplementation or higher intake of unsaturated fatty acids was shown to alleviate neurotoxin-induced PD-like syndrome. Samadi et al. have shown that DHA reduced and delayed levodopa-induced dyskinesia in monkey [116]. A protective role of omega-3 fatty acid (120 mg EPA and 180 mg DHA) supplementation for three months has also been reported in 6-OHDA-lesion model of PD [117]. In another study, mice were supplemented with DHA (424 mg/kg) before seven MPTP ip injections at 20 mg/kg free base, which produces moderate dopamine denervation [118]. High DHA supplementation also prevented MPTP-induced decrease in dopamine in striatum and loss of tyrosine hydroxylase (TH)-positive nigral neurons. In contrast, in biophysical studies, PUFAs cause enhanced oligomerization of alpha-synuclein and insoluble alpha-synuclein aggregate formation and increased Lewy-like inclusions *in vitro* [119–121]. Studies with rodent models also reported that diet with high concentration of PUFA upregulated alpha-synuclein expression [122, 123]. Moreover, DHA-specific diet (0.69% DHA) increased alpha-synuclein accumulation in brainstem,

followed by increased astrocyte activity in A53T (a disease-causing mutation) alpha-synuclein mice [124]. Given the key role that alpha-synuclein plays in sporadic and genetic PD cases, elevated PUFA diet studies need to carefully evaluate behavioral, biochemical, and especially pathological endpoints.

As the neuroprotective effect of PUFA has been repeatedly shown, both *in vitro* and *in vitro*, high fat diet (HFD) has been shown to be associated with increased risk of PD. HFD increases nigral dopaminergic neurons susceptibility to environmental insults [125]. For example, HFD fed rats (60% and 20% of calories from fat and carbohydrates, resp., compared to 10% calories from fat in regular diet) for 5 weeks were unilaterally injected with 6-OHDA into the medial forebrain bundle [126]. HFD rats showed enhanced striatal dopamine depletion and increased dopamine turnover in substantia nigra. A later study from the same group reported an increase in iron deposition, impaired dopamine function in dorsal striatum, and affected iron transport proteins in substantia nigra in HFD rats, suggesting the role of interplay between high fat intake and iron metabolism in PD [127].

Epidemiological studies have also suggested a protective role of unsaturated fatty acids in PD, while saturated fatty acids were associated with increased risk of developing the disease. Three large cohort studies using NHS, HPFS, and HHS data reported lower PUFA intake in PD patients compared to healthy controls [9, 20, 128–130]. Fish oil supplementation (180 mg EPA and 120 mg DHA for 3 months) had an antidepressant effect in PD patients with major depression symptoms [131]. Comparison of dietary habits of 89 PD cases and 336 healthy controls showed that high level of PUFA intake was protective in those exposed to paraquat [132]. The results on other types of fat intake are controversial. Total and animal fat intake was associated with increased risk of PD [7, 25, 133]; however, some studies reported no association with the disease progression [11, 20, 21, 128–130]. A very recent study analyzed fat intake of 1,087 PD patients and almost 300,000 controls from National Institutes of Health-American Association of Retired Persons Diet and Health Study [134]. Subclasses of fat (PUFA, MUFA, cholesterol, etc.), as well as overall fat intake, did not show any significant association with PD. Currently, it is the largest prospective analysis of dietary intake, considering types of fat intake separately. However, using an older population and having dietary assessment only at the baseline, rather than a follow-up report, has considerable limitations. Overall, it appears that PUFA intake may modulate PD risk. Diet and toxicant interaction studies are beginning to identify important interactions. Understanding the mechanistic bases of such interactions could potentially lead to new therapeutic approaches.

7. Metals

Exposure to metals can be due to different sources: occupational, dietary, or as contaminant in water or air. Occupations such as welders, smelters, and miners are at high risk of exposure to metals such as manganese and iron [135, 136]. Overall, occupational exposures to metals is typically higher in terms of dose, but rarer, compared to dietary consumption at high levels from supplementation or other sources. The route of exposure also needs to be considered, which is often inhalation in occupational settings.

7.1. Iron. Iron accumulation in PD has been studied for decades in imaging, *in vitro,* and *in vivo* studies. Iron accumulates more in substantia nigra of PD cases than it does in other brain regions [137]. The iron hypothesis in PD suggests that Fenton's reaction induces production of hydroxyl radical and higher oxidation states of iron [138–140]. Hydroxyl radicals are toxic to neurons by inducing lipid peroxidation and subsequent cell death. Lewy bodies in PD brains are iron-positive and iron has been shown to induce alpha-synuclein accumulation [141, 142]. Reactive microglia, a common pathological finding in PD brains, contain high levels of iron [143, 144].

Mice that received high iron diet were shown to be more vulnerable to environmental insults. Lan and Jiang have shown that mice fed with high iron diet for a month and received MPTP injection had lower levels of glutathione, higher levels of oxidized glutathione, enhanced formation of hydroxyl radicals and oxidized lipids, accompanied by loss of striatal dopamine and DOPAC in brainstem, compared to mice with control diet [145]. Abnormal iron intake exacerbates MPTP-induced toxicity *in vivo* [146] and enhances alpha-synuclein fibrillation in human BE-M17 neuroblastoma cells overexpressing A53T alpha-synuclein [147]. In contrast, iron-deficient rodents have shown impaired dopamine transport [148], decreased expression of dopamine receptor 1 and 2 in dose- and time-dependent manner [149–151], suggesting that balance in iron is needed for proper dopaminergic activity and TH activity. Therefore, although chelation of iron as a therapeutic approach was suggested with additional support that chelation of iron *in vitro* with desferoxamine prevents MPTP-induced cell death [152], *in vivo* efficacy was likely limited because of two major reasons: many chelators cannot cross blood-brain-barrier and they can deplete all iron and TH synthesis will also be inhibited [153].

A case-control study, which includes 126 PD cases and 432 controls from the Detroit area reported that higher dietary iron intake was significantly associated with increased risk of PD (OR 1.88, 95% CI 1.05–3.38) [25]. An interesting relation between iron, animal fat, and PD has been reported in a case-control study of New Yorkers [154]. Although dietary iron itself was not significantly associated with PD, the highest quartile of animal fat intake accompanied by low transferrin saturation level was very strongly associated with PD (OR 9.0, 95% CI 2.7–29.9). Transferrin level is an indicator of iron stores, suggesting that, in case of low transferrin levels, there are more free iron atoms to induce oxidative stress [155]. It is noteworthy that intake of particular type of iron through diet matters. Dietary iron can be presented in three types: (1) Heme iron (found in red meat and absorbed well by the human body), (2) nonheme iron (found in vegetables, such as spinach and in grain/cereal and not well absorbed by body), and (3) supplementation. Logroscino et al. (2008) reported that dietary iron intake is moderately associated

with increased PD risk (RR 1.30, 95% CI 0.94–1.80; $P = 0.02$) in NHS and HPFS data [156]. While nonheme iron intake was related with increased risk of PD (RR 1.27), heme iron intake had no effect in disease progression. Iron supplementation was found related to PD only in men, suggesting a potential gender-specific metabolism of iron. In contrast, in a Japanese population, after being adjusted for several confounders (vitamin E, vitamin B6, caffeine, alcohol, cholesterol, smoking, sex, and age), higher dietary intake was protective against PD with a P value lower than 0.0001 [11]. This result suggests the interplay of iron with other dietary factors and importance of confounder effects. Fukushima et al. compared blood iron levels of PD patients and controls, as well as dietary habits [157]. It has been reported that dietary iron intake was not associated, but instead iron exposure via contamination of drinking water and airborne metals was more prominent in China. Finally, it should be noted that while many retrospective studies have identified links between dietary iron and PD, large highly powered prospective studies have failed to identify a convincing link [156].

Extra caution is needed to evaluate the cause of iron accumulation in the brain of PD patients. Iron uptake in the gut is highly regulated. Thus, genetic predisposition might also play an important role in iron accumulation in the brain. In two different genetic studies, PD patients were shown more likely to have polymorphism in transferrin [158] and hemochromatosis gene [159]. Neurotoxic models of PD, such as rotenone, disrupt iron homeostasis [160], suggesting that dietary iron intake, genetic factors, and environmental exposures, might play a combinatorial role in accumulation of iron in PD brains.

7.2. Manganese. Occupational manganese exposure at chronic and high levels in welders, miners, and smelters was associated with increased incidence of Parkinsonian-like symptoms [161]. A population-based case control study in Detroit also reported increased risk of PD with >20 years of manganese occupational exposure [162]. Besides occupational settings, manganese is a natural product of most of the foods: legumes, nuts, grains, some fruits, and vegetables. Different types of cereals and mixed nuts have high manganese levels, ranging from 20 mg/kg to 40 mg/kg [163]. However, the relation between dietary intake of manganese and PD is not very clear. Miyake et al. conducted a case-control study using Japanese PD patients and healthy controls and evaluated their dietary habits over a month [11]. In this study, dietary manganese intake did not affect PD risk after adjustments for confounders. In China, manganese exposure from nutrients was not associated with PD [157]. In another study, 250 PD patients and 388 controls from western Washington State participated in a case-control study to determine relation between dietary manganese with PD risk [130]. Although manganese intake alone was not significant, diets with low manganese and high iron or high manganese and high iron were significantly associated with increased risk of PD incidence (OR 1.2 and 1.9). These results are in agreement with a previous rat study [164], suggesting a potential synergetic effect of two heavy metals in the disease pathogenesis. Many foods are rich in both manganese and iron, including spinach, peas, nuts and seeds, which is a fact that further epidemiological studies should consider carefully.

7.3. Magnesium and Calcium. Magnesium is a cofactor for crucial processes in cell, such as protein and nucleotide synthesis, cell cycle activities, and mitochondrial integrity. It also modulates calcium and potassium transport via pumps, carriers, and channels [165].

Magnesium supplementation has been found to be protective in neurotoxicant-induced PD models [166, 167]. It also inhibits spontaneous and Fe-induced aggregation of alpha-synuclein [142]. Oyanagi et al. investigated the effects of calcium and/or magnesium deficiency over two generations in Wistar rats [168]. Rats that have received low magnesium diet (14 or 40 mg/100 g of food), compared to rats with control diet (70 mg magnesium/100 g of food), showed lower dopaminergic neuron count, more active microglia, and decreased fiber size of myelin fibers in substantia nigra. Dopaminergic neurodegeneration in the substantia nigra was more evident in magnesium-deficient rats than calcium and magnesium deficient rats. The same study also suggested that magnesium deficiency is toxic to the dopaminergic system only if occurring in fetal and newborn periods of life, suggesting the importance of magnesium in early development of dopaminergic neurons. The synergistic effect of calcium and magnesium was confirmed in a mouse model as well. Mice fed with low calcium/magnesium-deficient diet displayed cataleptic behavior, which was inhibited by treatment with L-DOPA in a dose-dependent manner, and these mice had significantly lower TH activity in substantia nigra [169]. Since magnesium was found to decrease NMDA activity [170], hypofunction of dopamine might be due to supersensitivity of NMDA receptors enhanced by lack of magnesium.

In humans, magnesium showed a protective effect against PD in a Japanese population (OR 0.33, 95% CI 0.17–0.73 for highest quartile >312.9 mg/day; $P = 0.002$) [11]. A relatively small-sized case control study in Sweden reported that lower dietary magnesium intake was associated with lower brief smell identification test score ($P = 0.012$), which is an olfactory function test in which PD patients generally perform less efficiently than healthy controls [171].

8. Alcohol

The association between alcohol consumption and PD risk has been investigated in several epidemiological studies. Most studies are adjusted for sex and age while some considered smoking, which is often concomitant with alcohol.

A small twin study consisting of 31 monozygotic twin pairs reported that the unaffected twin drank more alcohol than the affected twin (RR 0.5) [172]. A Spanish study (128 PD patients and 256 controls) showed that drinking more than 50 g/day of alcohol ($P < 0.001$) was inversely associated with PD in males, while the association was absent in female [173]. A crude inverse association of alcohol (beer, wine, and liquor) with PD was observed in a Swedish population, although the significance disappeared after adjustment for

smoking [174]. Similarly, in Leisure World cohort study (395 cases and 2,320 controls from Southern California), PD risk was lower for drinkers of 2+ alcoholic drinks/day (OR 0.77, 95% CI 0.58–1.03) [175]. In contrast, an Italian case-control study reported that individuals with light to moderate alcohol drinking exhibited reduced PD risk compared to ones with heavy drinking or nondrinkers [176]. In another cohort study, in Swedish population, PD was associated with increased risk of alcohol use disorder (HR 1.3, 95% CI 1.25–1.53) with the highest risk in lowest age group, <44 years old (HR 2.39, 95% CI 0.96–5.93) [177]. To date, few studies have convincingly identified alcohol use as a significant risk factor for PD. A major strength of this study is that it analyzes 13–17 years of follow-up data of 1,083 PD patients hospitalized with an alcohol use disorder and 658 PD patients with appendicitis among 602,930 individuals in total. Although the datasets are rather large, the alcohol data was not adjusted for potential confounding factors, such as smoking. Another possible explanation why the findings of this study were inconsistent with previous findings might be due to their study design. Here, the alcohol consumption of the control was not known. The fact that PD patients admitted to hospital with appendicitis were not questioned for alcohol consumption becomes a significant limitation.

A meta-analysis was performed recently to investigate association of alcohol consumption with PD risk [178]. Meta-analysis of 32 studies including 9,994 cases among 677,550 subjects found a significant association of beer with decreased risk of PD (RR 0.59, 95% CI 0.39–0.90), but not with wine and liquor. In conclusion, the consumption of alcohol was associated with lower risk of PD in most of case-control and cohort studies, while the association generally disappeared after adjustment with smoking. Careful adjustment for age, smoking habit, caffeine intake, amount of alcohol, and types of alcohol, including compounds associated with specific alcoholic beverages (ex. tannins in wine) that have been consumed, needs to be performed in future studies.

9. Byproducts of Preparation

The discussion above focuses on several natural components of the diet. However, few human foods are consumed in the "raw" state. The preparation process itself may result in the formation of neurotoxicants.

Heterocyclic amines (HCA) were isolated and characterized in fried and broiled meat more than 30 years ago [179]. At that time, only five compounds were identified: 3-amino-l,4-dimethyl-5H-pyrido[4,3-b]-indole (Trp-P-1), 2-amino-9H-pyrido[2,3-b]indole (AaC), 2-amino-3-methyl-9H-pyrido[2,3-b]indole (MeAaQ), 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MelQx), and 2-amino-3-methylimidazo[4,5-f]quinoline (IQ). Since then, more than 20 HCAs were identified in cooked meat [180]. The amount of HCAs formed during the cooking process depends on time, temperature, and type of meat [181]. Several HCAs were identified as mutagens *in vitro* and *in vivo* studies, including in nonhuman primates [182–184].

Association of some HCAs with neurological disorders and neurotoxic mechanisms has been investigated. Two of those are Trp-P-1 and 3-amino-1-methyl-5H-pyrido[4,3-b]indole (Trp-P-2). Unilateral infusion of Trp-P-1 and Trp-P-2 in the rat striatum caused increase in dopamine and decrease in its metabolites in striatum, suggesting inhibition of monoamine oxidase [185]. Impairment in dopamine catabolism by Trp-P-1 and Trp-P-2 has been reported in the following studies [186, 187]. Moreover, Trp-P-2 was found to inhibit L-amino acid decarboxylase in human brainstem [188]. In the same study, the most potent inhibitors of dopamine synthesis after Trp-P-2 were identified as 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) and Trp-P-1.

PhIP is a heterocyclic amine that may account for up to ~75% of the genotoxic material from the crust of cooked meat [189, 190], implicating that it can be consumed repeatedly and in high doses throughout the life. PhIP is highly mutagenic and is associated with several cancer types including breast, prostate, and colon [184]. Although PhIP and its N-hydroxylated metabolite, N-OH-PhIP were shown to cross blood-brain-barrier [191], their effects on nervous system were unknown. High dose PhIP administration caused tremor and freezing in female mice (personal communication with Drs. K. Turteltaub and A. Director-Myska). Recent neurotoxicity studies on PhIP *in vitro* using primary midbrain cultures have been performed [192]. PhIP and N-OH-PhIP treatments at 1 μM were selectively toxic to primary dopaminergic neurons isolated from embryonic day 17 rat embryos. Here, dopaminergic neurotoxicity of PhIP and its metabolite were mediated by oxidative stress, which can be prevented by pretreatments with the antioxidant, N-acetylcysteine and compounds with antioxidant capacity, such as blueberry extract.

Harmane (1-methyl-9H-pyrido[3,4-b]indole) is another HCA, that is produced endogenously and is exposed exogenously from meat and other foods [193]. Blood levels of harmane were associated with essential tremor, which is one of the most common neurological diseases, characterized by action tremor of the hands [194, 195]. Case-control study including 150 essential tremor and 135 controls reported that blood harmane concentration was ~50% higher in cases than controls (OR 1.56, 95% CI 1.01–2.42) [196]. The same group was able to replicate the association in a separate case-control group from New York [197]. Recently, blood harmane concentrations were compared in 113 PD cases and 101 controls [198]. Blood harmane was significantly elevated in PD patients compared to healthy controls (OR 2.31, 95% CI 1.46–3.67).

The data from a limited number of studies on HCAs and PD suggests that these mutagenic compounds should be evaluated as dopaminergic neurotoxicants and etiological factors in PD. There are several gaps in the literature that need to be addressed. Future studies with HCA administration in animals will help to understand whether HCA exposure in animals can reproduce the clinical PD phenotypes. Moreover, epidemiological studies that investigate meat cooking time and doneness preferences of PD cases and controls will be very useful to reveal the association between HCA and PD.

10. Conclusion and Future Research

Parkinson's disease is a common neurodegenerative disorder, affecting individuals especially over 60 years of age. There is no known prevention or certain cure for the disease. Although rare mutations have been identified that cause familial PD, the majority of incidence remains as a mystery. For a long time, environmental factors have been a major concern related to disease pathogenesis. A multitude of environmental and occupational exposures have been implicated as PD risk factors. However, both increasing disease prevalence and the legislation reducing the use of many pesticides have renewed the search for the frequently encountered environmental factors that modify disease risk.

Here, in this review, we summarized key findings regarding the modulating effects of dietary factors in PD. The presence of contradicting findings on a single nutrient in the literature is a common problem. Especially, as discussed throughout the review, epidemiological studies might not have confirmed the findings from *in vitro* and *in vivo* studies. To solve this controversy, there are several points that need to be considered related to study design.

(1) Epidemiological studies in the literature usually reported opposite findings on a single nutrient. Careful consideration is needed in the design of the study. A homogenous cohort or case-control population and large number of data will increase the chance of validation of results in further studies. In addition, while multivariate analysis including sex and age is essential, the adjustment for other dietary factors and habits (such as smoking) is highly recommended to increase the power of the study. Three such studies are HHS, NHS and HPFS, including approximately 8,000, 120,000 and 50,000 individuals, respectively. Patients' dietary habits, amount of food intake, and life styles have been followed and recorded for 20–30 years, creating very valuable datasets. Establishing more studies with large sample size and long follow-ups will prevent many crucial limitations in most epidemiological studies. Further improvement will be achieved by recruiting samples in early ages in life, instead of 30–50 years of age as in these three studies. By the time PD symptoms appear, significant nigral dopamine neuron loss has occurred. Testing dietary habits and intake of specific food groups in different stages of life, including early development and puberty, will also increase the strength of studies. *In vivo* studies utilizing chronic or even multigenerational studies on consumption levels of dietary factors will likely provide significant mechanistic insight.

(2) The common limitation of epidemiological studies on dietary habits comes from the self-administered food questionnaires. PD patients may recall specific food consumption at a higher rate than controls in an effort to implicate risk factors. This is also known as *recall bias*. There are additional findings that having the disease might change food preferences [199, 200], suggesting that food surveys can lead the false results.

(3) Investigation of dietary factors has inherent difficulties. A single food does not contain a single micronutrient. For example, dairy products are rich in vitamins A, B, and D, and also fat. A very recent meta-analysis included seven studies with more than a thousand PD patients among more than 300,000 individuals and identified dairy product as a risk factor for PD [201]. However, determination of which natural component or contaminant in these contributes to the association of the disease is important for early prevention and to identify mechanisms of pathogenesis.

(4) The effect of combinations of food molecules needs to be considered. This type of approach is usually seen in combination of molecules from the same group: total vitamin intake and overall fat intake. However, evaluating composition of different groups of macronutrients is also crucial. For example, PUFAs are known to have a protective effect against neuroinflammation and oxidative stress, which are two common mechanisms in neurodegenerative diseases. Assessment of PUFAs along with vitamins and flavonoids, two large groups of antioxidants, might increase significance of their association with the disease. The traditional Mediterranean diet is defined with the high intake of olive oil, legumes, vegetables, and fruit, along with lower consumption of meat, poultry, and animal fats [202]. In a small case-control study, higher Mediterranean diet score was significantly associated with lower risk of PD [203]. Prospective and cohort studies with larger sample sets evaluating a particular diet or nutritional pattern are needed to understand dietary risk factors for PD.

(5) Although genetic and environmental factors (pesticides, occupational exposures, and dietary habits) in PD have been investigated extensively, limited data on interactions are available. Genetic predisposition might greatly modulate the association of environmental factors with PD. A new advanced approach to genome-wide association studies investigates the effect of environmental factors in relation with polymorphisms or mutations in genomic level. A recent study followed this approach and showed combinatorial association of a polymorphism in glutamate receptor gene, *GRIN2A*, and caffeine intake in PD risk [204]. This study also independently validated findings of previous genome-interaction studies [205]. Future studies that consider more than one genetic and environmental factor in the risk of PD will generate more consistent findings and pave the way to reveal underlying mechanisms in the disease pathogenesis.

Conflict of Interests

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

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Research Article

Alleviation of Kainic Acid-Induced Brain Barrier Dysfunction by 4-O-Methylhonokiol in *In Vitro* and *In Vivo* Models

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This experiment was designed to investigate whether 4-O-methylhonokiol (MH), a principal ingredient of *Magnolia (M.) officinalis* bark, alleviated acute intraperitoneal (i.p.) kainic acid- (KA-) induced brain blood barrier dysfunction (BBBD) *via* pathological examination and cytological analyses of the brain tissues of mice. KA (10–30 mg/kg) time- and dose-dependently increased the water content of brain tissues and induced edema and encephalopathy. However, pretreatment with MH (5 and 20 mg/kg, i.p.) significantly reduced the water content of the brain compared to that observed in the KA control group. Furthermore, MH significantly and dose-dependently reversed the remarkable variations in evan's blue dye (EBD) staining and malondialdehyde (MDA) levels that were induced by KA (10 mg/kg, i.p.). MH also decreased the elevated seizure scores that were induced by KA (10 mg/kg, i.p.) in mice in a manner similar to scavengers such as DMTU and trolox. Additionally, MH significantly scavenged intracellular ROS and Ca^{2+} within hippocampal cells. The tight junction seals mediated by claudin (Cld-5) were also found to be modulated by MH. MH efficiently reduced 1,1-diphenyl-2-picrylhydrazyl (DPPH) (IC_{50}, 52.4 mM) and $^{\bullet}OH$ with an electron spin resonance (ESR) signal rate constant of $4 \times 10^9\ M^{-1} \cdot S^{-1}$, which is close to the reactivity of the vitamin E analog trolox. Taken together, these results suggest that MH may enhance radical scavenging in lipid and hydrophobic environments, which may be important for the physiological activity of the barrier.

1. Introduction

2-[4-Methoxy-3-(2-propenyl)phenyl]-4-(2-propenyl)phenol(MH) is a principal ingredient of the bark of *Magnolia (M.) officinalis*. *Magnolia* bark has been used in traditional medicine to treat various disorders [1, 2]. MH has potent antifungal and antibacterial activities and apparently also has anti-inflammatory and neurotrophic activities [3–5]. MH has a biphenyl (ortho- and parapositions) structure containing two allyl groups, which are beneficial for increasing its affinity for endothelial cells [6]. The ortho-allyl group may potentially form a six-member ring after absorption of the hydroxyl group, but a single para-allyl group cannot form a six-member ring with hydroxyls. Honokiol has been found to be a potent scavenger of hydroxyl radicals due to its allyl groups [6] or phenolic constituents [7, 8]. Phenolic compounds have strong free radical scavenging activities [9, 10]. Additionally, the methylation in the structure of phenolic compounds reduces their overall hydrogen bonding potential [11]. This reduction in hydrogen bonding potential increases the lipophilicity or membrane permeability of these compounds [11]. Lin et al., reported that honokiol possesses the abilities to block excitatory amino acid- (EAAs-) evoked cation signals [12] and to inhibit glutamate-induced cell damage [13]. Moreover, honokiol has antinociceptive actions in glutamatergic pain [14] and antioxidants that suppress the oxidation of low-density lipoproteins due to its strong radical scavenging activities [15].

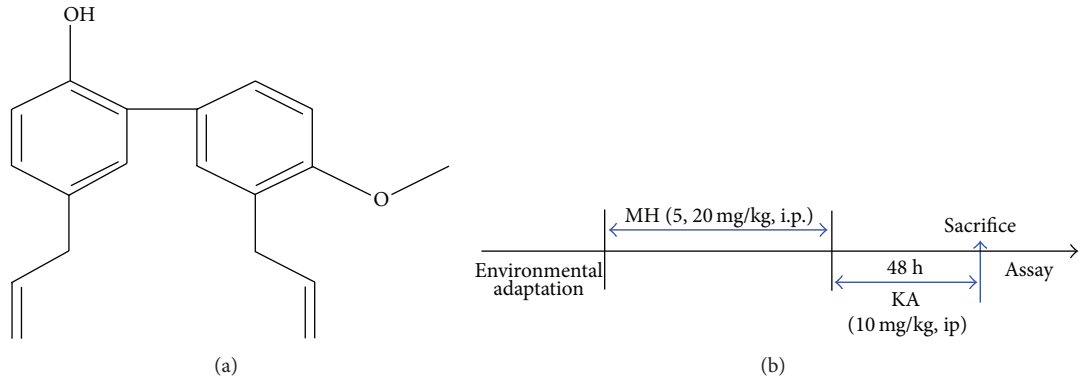

Figure 1: Chemical structure of MH (a) and experimental scheme (b).

Electron spin resonance (ESR) is a sophisticated spectroscopic technique that detects free radicals or inorganic complexes in chemical and biological systems [16–18]. ESR spectroscopy of spin-trapped radicals has become the method of choice for the detection and identification of the free radicals that are formed in biological systems [19–21]. The spin-trapping technique utilizing nitrones has been applied to the detection of free radicals for over thirty years. Nitrone spin traps are used in ESR studies because they specifically react with free radicals to form a radical adduct with a longer lifetime than the initial free radicals. For biological applications, nitrone spin traps, such as 5,5-dimethyl-l-pyrroline-*N* oxide (DMPO), have been used most frequently.

EAAs, such as glutamate, are acknowledged as the primary neurotransmitters that mediate synaptic excitation in the vertebrate central nervous system (CNS) [22]. Glutamate has dual actions on CNS neurons; glutamate acts as an excitatory neurotransmitter at physiologic concentrations and acts as a neurotoxic substance when present in excessive amounts. Glutamate has also been implicated in the initiation of nerve cell death in stroke, epilepsy, and other forms of CNS insult. Moreover, glutamate kills neuronal cells through either the receptor-mediated pathway or *via* the inhibition of cysteine uptake and the oxidative pathway [23]. It is well known that KA, a glutamate analogue, induces elevations in intracellular Ca^{2+} and extracellular glutamate levels *via* the coactivation of NMDA receptors. Glutamate-evoked Na^{+} influx has also been proposed to contribute to acute forms of neurotoxicity [24, 25]. Brain blood barrier dysfunction (BBBD) has been described after KA administration [26, 27].

The brain blood barrier (BBB) is a vascular system that regulates the passage of materials between the peripheral circulation and the CNS. The BBB is a unique membranous barrier that tightly segregates the circulating blood [28, 29]. Moreover, the BBB is extremely important "guardian" that regulates the access of drugs to the CNS in both physiological and pathological circumstances [30]. Many CNS diseases and injuries, such as epilepsy, stroke, multiple sclerosis (MS), encephalomyelitis (EAE), and others, are accompanied by BBBD [31–34]. Moreover, barrier dysfunction related to faulty out-transport of metabolites may be responsible for epileptogenesis in the presence (or absence) of other parenchymal abnormalities [10, 35]. The permeability of the endothelial barrier is regulated by two different routes: the paracellular pathway, which controls the permeability through interendothelial junctions, and the transcellular pathway, which involves caveolae-mediated vesicular transport [36]. BBBD and subsequent edema are major contributors to the pathogenesis of epilepsy. The formation of brain edemas has been linked to the inability of the barrier to maintain the necessary ion gradients. Furthermore, alterations in barrier ion homeostasis have been linked to epilepsy [37, 38]. In this study, we examined the therapeutic effects of MH in KA-induced brain barrier dysfunction that are mediated through redox repair.

2. Materials and Methods

2.1. Chemicals. MH (molecular weight = 280.4, molecular formula = $C_{19}H_{20}O_2$), the chemical structure of which is shown in Figure 1(a), was isolated from the bark of *M. officinalis* as described elsewhere [39]. The bark of *M. officinalis* was dried in the shade at room temperature and stored in a dark, cold place until use. The air-dried bark of *M. officinalis* (3.0 kg) was cut into pieces and extracted twice with 95% (v/v) ethanol (by weight, four times as much ethanol as dried plant was used) for 3 days at room temperature. After filtration through 400-mesh filter cloth, the filtrate was refiltered through filter paper (Whatman, number 5) and concentrated under reduced pressure. The extract (450 g) was then suspended in distilled water, and the aqueous suspension was extracted with n-hexane, ethyl acetate, and n-butanol. The n-hexane layer was evaporated, and the residue (70 g) was measured chromatographically on silica gel with an n-hexane : ethyl acetate (9 : 1) solution to extract a crude fraction that included MH. This fraction was repeatedly purified by silica gel chromatography using n-hexane : ethyl acetate as the eluent to obtain pure MH (Figure 1). The purity exceeded more than 99.5%. MH was identified by 1H NMR (400 MHz, $CdCl_3$) 1 : 3.36 (2H, d, J = 7 Hz, H-7), 3.44 (2H, d, J = 7 Hz, 7-H), 3.89 (3H, s, OMe), 5.05–5.14 (5H, m, H-9, H-9, OH), 5.93–6.07 (2H, m, H-8, H-8), 6.92 (1H, d, J = 7 Hz, Ar-H), 6.97 (1H, d,

J = 8 Hz, Ar-H), 7.04–7.08 (2H, m, Ar-H), 7.24–7.31 (2H, m, Ar-H). 13C NMR (100 MHz, $CDCl_3$) 1 : 34.5 (C-7), 39.6 (C-7), 55.8 (OMe), 111.2 (C-3), 115.7 (C-4), 115.8 (C-9), 116.1 (C-9), 128.0 (C-1), 128.1 (C-6), 129.0 (C-3), 129.2 (C-1), 130.0 (C-5), 130.4 (C-6), 130.7 (C-2), 132.4 (C-5), 136.7 (C-8), 138.0 (C-8), 151.0 (C-2), and 157.2 (C-4). The ethanol extract of *M. officinalis* was composed of 16.6% MH, 16.5% honokiol, 12.9% magnolol, and 42–45% other components. These results agree with previously published data [40], and this compound may possibly be the same compound demonstrated by Zhou et al. [41]. MH was kindly provided by the Bioland Cooperation (Daejeon, South Korea). KA ((2*S*,3*S*,4*S*)-carboxy-4-(1-methylethenyl)-3-pyrrolidineacetic acid) was purchased from Tocris (Ellisville, MO, USA). Cld-5 was purchased from Abcam Inc. (Cambridge, MA, USA). The OXYTEK thiobarbituric acid reacting substances (TBARS) assay kit was purchased from Alexis (Farmingdale, NY, USA), and 6-carboxy-2′,7′-dichlorofluorescein diacetate (DCFH-DA) and fura-4/AM were purchased from Molecular Probes Inc. (Eugene, OR, USA). DMPO was purchased from Enzo (Plymouth Meeting, PA, USA). Evan's blue dye (EBD), Na_2SO_4, acetone, ferrous sulfate ($Fe_2SO_4{\cdot}7H_2O$), hydrogen peroxide (H_2O_2, 30%), diethylenetriamene pentaacetate (DTPA), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,7-tetramethylchroman-2-carboxylic acid (trolox), *N*,*N*′-dimethylthiourea (DMTU), 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT), and all other chemicals were of high quality and were obtained from Sigma (St. Louis, MO, USA).

2.2. Animals. Male ICR mice (Samtako, Osan, Korea) weighing 30–35 g were used for the *in vivo* experiments (n = 7-8). The animals were housed in acrylic cages (45 cm × 60 cm × 25 cm) with water and food available *ad libitum* under an artificial 12 h light/dark cycle (lights on at 7:00) and constant temperature (22 ± 2°C). The mice were housed in a departmental room for 1 week prior to testing to ensure that they had adapted to the new environment. All experiments involving animals were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publication number 85-23, revised 1985). The institutional animal care and use committee of Chungbuk National University approved the protocol.

2.3. Primary Hippocampal Neuronal Cell Culture and KA Exposure. Primary cultures of rat hippocampal neurons were prepared from the hippocampi of E18-19 Sprague Dawley (SD) rat embryos and cultured according to a previously described method [42]. The hippocampi were dissected and incubated with 0.25% papain in Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution at 37°C for 20 min. The cells were then mechanically dissociated with fire-polished Pasteur pipettes by trituration and plated on poly-L-lysine-coated cover slips in a 35 mm culture dish. The cells were maintained in neurobasal/B27 medium containing 0.5 mM L-glutamine, 25 μM glutamate, 25 μM 2-mercaptoethanol, 100 U/mL penicillin, and 100 μg/mL streptomycin under a humidified atmosphere of 95% air and 5% CO_2 at 37°C, half of the medium was changed every 2 days, and the hippocampal neurons were cultured for 12–14 days before KA (100 μM) exposure. MH (30–100 μM) was added 0.5–1 h prior to KA treatments.

2.4. Cell Viability Assays. Cell viability assays were performed as previously described [43]. After exposure for the indicated times, the neurons were assayed for viability using MTT (Sigma, St. Louis, MO, USA), which was added at a final concentration of 5.0 mg/mL for 4 h. MTT was then removed, and the neurons were lysed in 200 μL of dimethyl sulfoxide (DMSO). Absorbance was measured at 570 nm on a SpectraMax M2 multimode microplate reader (Sunnyvale, CA, USA). The data are expressed as the percentages of unexposed neurons that remained in the presence of KA.

2.5. Intracellular ROS Measurement. The production of ROS in the neurons was determined as previously described using DCFH-DA (Molecular Probes, Eugene, OR, USA) [44]. The cells were incubated with 10 μM DCFH-DA at 37°C for 30 min. After the DCFH-DA was removed, the cells were recorded. The DCFH-DA-loaded cells were placed in a SpectraMax M2 multiwell fluorescence microplate reader (Sunnyvale, CA, USA) with excitation and emission wavelengths of 515 nm and 552 nm, respectively. The protein concentrations were determined with Bradford assays.

2.6. Intracellular Calcium Measurement. The acetoxymethylester form of fura-4 (Molecular probes, Eugene, OR) was used as the fluorescent Ca^{2+} indicator. The hippocampal cells were incubated for 60 min at room temperature with 5 μM fura-4/AM and 0.001% Pluronic F-127 in a HEPES-buffered solution composed of the following (in mM): 150 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, and 10 glucose with the pH adjusted to 7.4 with NaOH. The cells were then washed with HEPES-buffered solution and placed on a SpectraMax M2 multiwell fluorescence microplate reader (Sunnyvale, CA, USA). The emitted fluorescence was calculated using a fluorescence analyzer and converted to intracellular free Ca^{2+} concentration $[Ca^{2+}]_i$.

2.7. Determination of Brain Edema. Water content measurements of the brains of mice that were sacrificed 48 h after the administration of KA (i.p.) were performed. A control group of mice received saline. The water contents of the brain tissues were detected by measuring the ratios of the body weights and brain weights. Briefly, the brains (n = 5-6) were quickly removed and weighed (brain weight), and the percentages of water content were calculated as [brain weight/bodyweight] × 100%.

2.8. Evaluation of Brain Barrier Integrity. A variety of studies have used the peripheral injection of Evan's blue dye (EBD) as a barrier tracer marker [45]. To estimate barrier integrity, the mice were intravenously injected with EBD (1%, Sigma, St. Louis, MO, USA) in phosphate-buffered saline (PBS, pH 7.4) that had been sterilized by passage through a Millex-GP0 22 μm filter (Millipore, Bedford, MA, USA). Five minutes

later, KA (10–30 mg/kg, i.p.) was administered to the mouse. MH was injected into the abdominal site 50 min prior to EBD. Two days later, the mouse was killed by cervical dislocation and the brain was excised. Approximately 1.0 g of brain was minced and dispersed in 6.0 mL 0.5% Na_2SO_4 solution, and the dye was extracted by the addition of 14 mL acetone. After 3.5 h of extraction, the dye concentration was determined using a spectrophotometer at OD_{590}. MH (5–20 mg/kg, i.p.), DMTU (50 mg/kg, i.p.), and trolox (50 mg/kg, i.p.) were administered 50 min prior to EBD injection.

2.9. Measurement of Seizure Activity. Male mice were grouped ($n = 5$ or 6 mice/group) and pretreated (i.p. injection) with MH (5–20 mg/kg) or NaCl (0.9%). Seizures were induced in mice in the KA and MH + KA groups *via* the injection of KA (10–30 mg/kg, i.p.). The mice in the control group received an equal volume of 0.9% NaCl at the same time points. The mice were pretreated (i.p.) with MH and scavengers such as trolox (50 mg/kg, i.p.) and DMTU (50 mg/kg, i.p.) 50 min prior to the KA injections. Male ICR mice weighing 30–35 g were injected with saline or KA dissolved in saline (10–30 mg/kg, i.p.). Seizure activity was rated during a 3 h period after the KA challenge according to the following scale devised by Racine et al. [46]: stage 1 (facial clonus), stage 2 (nodding), stage 3 (forelimb clonus), stage 4 (forelimb clonus with rearing), and stage 5 (rearing, jumping, and falling). Two days after the KA treatment, the hippocampi of the other seizing mice were dissected to evaluate the dysfunctions.

2.10. Lipid Peroxidation Assay. Lipid peroxide formation was analyzed by measuring the TBARS in the homogenates as described by Suematsu et al. [47]. The OXYTEK TBARS assay kit was used for these measurements. Lipid peroxidation was determined using the protocol of these authors *via* the measurement of the absorbance at 532 nm and is expressed as nmol of malondialdehyde (MDA)/mg of protein. The protein concentrations of the hippocampi were determined using the Bradford assay.

2.11. Western Blotting Assay. Tissues were lysed in the lysis buffer for 30 min on ice with vortexing every 5 min. The lysates were then centrifuged at 14,000 rpm for 5 min to remove the insoluble material. Protein concentrations were determined by the Bradford method (Bio-Rad) using BSA as the standard. For Cld-5, the protein was separated on 16% SDS-PAGE gels. The gels were subsequently transferred onto PVDF membranes (Amersham Hybond TM-P, GE Healthcare, Buckinghamshire, UK) by electroblotting for 2 h at 60–75 V. The membranes were then blocked with 5% nonfat milk solution in Tris-NaCl buffer (TNT) containing 0.5% Tween-20 and incubated with primary antibodies as indicated. Monoclonal donkey anti-rabbit IgG horseradish peroxidase-conjugated secondary antibodies were used at 1 : 3,000. Proteins were detected by enhanced chemiluminescence using a commercial kit (Amersham Hybond TM-P, GE Healthcare, Buckinghamshire, UK).

2.12. DPPH Assay. The scavenging of the stable free radical DPPH by MH was assayed spectrophotometrically [48]. DPPH in ethanol (0.1 mM, control) was mixed thoroughly with various concentrations of MH (1–100 mM), and the absorbance was read at 517 nm. The degree of DPPH radical scavenging activity of MH was calculated as a percentage of inhibition (% inhibition) where

$$\%\ \text{inhibition} = \left[\frac{\left(A_{\text{control}} - A_{\text{sample}}\right)}{A_{\text{control}}}\right] \times 100. \quad (1)$$

2.13. $^{\bullet}OH$ Scavenging Activity by ESR. $^{\bullet}OH$ was generated by the Fenton Reaction System, and the generated $^{\bullet}OH$ rapidly reacted with the nitrone spin trap DMPO [21]. The resultant DMPO/$^{\bullet}OH$ adduct was detected with an ESR spectrometer. MH (0.2 mL) at various concentrations was mixed with DMPO (0.2 M, 0.2 mL), Fe_2SO_4 (2.0 mM, 0.2 mL), and H_2O_2 (2.0 mM, 0.2 mL) in a phosphate buffer solution (100 mM, pH 7.2), and the mixture was transferred to a quartz flat cell for ESR measurement. The measurements were performed in an ESR cavity at room temperature (24-25°C). After the reaction, the ESR spectrum was recorded at room temperature using an ESR (JEOL JESTE-300) spectrometer (JEOL, Inc., Tokyo, Japan) equipped with a TE102 cavity. The experimental conditions were as follows: magnetic field, 339.3 ± 10 mT; power, 2.2 mW; modulation frequency, 9.44 GHz; amplitude, 10×10; and sweep time, 0.5 min. The results are indicated as the time required to produce a 50% inhibition or as the decrease in signal peak height (IH_{50}) by ESR.

2.14. Statistical Analyses. The data are presented as the means ± the SEMs. The statistical comparisons were made using one-way analyses of variance (ANOVAs). P values < 0.05 were considered statistically significant. When significant variations were found, the individual values were compared with Holm-Sidak tests.

3. Results

3.1. Effects of MH on KA-Induced Brain Edema. To measure brain edema, we evaluated the brain water content at 48 h after i.p. treatment. As shown in Figure 2, there was a significant increase in brain water content in the KA group compared to the control group that was dependent on administration dose and time. The amount of dye extravasation in brain was markedly increased compared to the control brain at 48 h after KA treatment ($17.77 \pm 0.34 \times 10^{-2}$ versus $16.02 \pm 0.31 \times 10^{-2}$). In contrast, the instillation of the control solution had no effect on the increase in water contents. Moreover, pretreatment with MH and KA resulted in a decrease in brain water content compared to the administration of KA alone ($P < 0.05$). The highest dose of the MH used in this study (20 mg/kg) inhibited the increase in water content to a degree similar to that achieved by scavengers. Scavenger treatment was associated with a significant decrease in brain weight/body weight ratios.

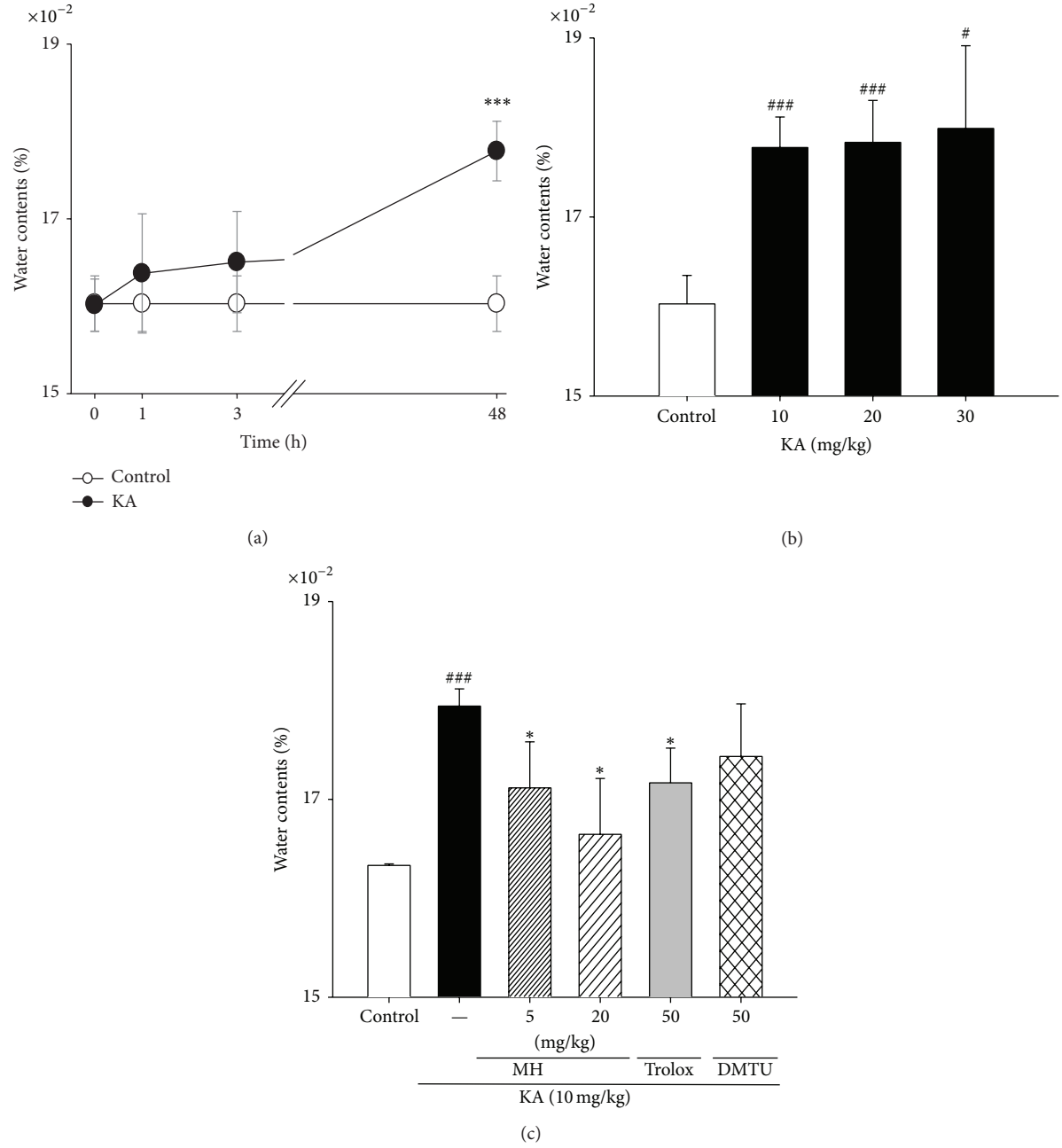

Figure 2: Changes and protective effects of MH, scavengers on the brain water content in KA-induced mice. (a) Male mice were grouped (n = 5 or 6/group). Seizures were induced by KA injection (10 mg/kg, i.p.); the mice in the saline group received an equal volume of 0.9% NaCl. Water contents were measured 0, 1, 3, and 48 h after KA or control injection. (b) Male mice were grouped (n = 5 or 6/group). Seizures were induced by KA injection (10–30 mg/kg, i.p.); the mice in the control group received an equal volume of 0.9% NaCl. (c) Male mice were grouped (n = 5 or 6/group) and pretreated with MH (5–20 mg/mouse, i.p.), trolox (50 mg/kg, i.p.), DMTU (50 mg/kg, i.p.), or NaCl (0.9%). Fifty minutes after MH or saline pretreatment, seizures in the KA and MH + KA groups were induced by KA injection (10 mg/kg, i.p.); the mice in the control group received an equal volume of 0.9% NaCl. The water contents of brain tissue was detected by measurement of the ratio of brain weight and body weight. The percentage of water content was calculated as [brain weight/bodyweight] × 100%. All weight data are presented as means ± SE. $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ versus control group. $^{*}P < 0.05$, $^{***}P < 0.001$ versus KA group.

3.2. Changes in KA-Induced Brain Barrier Permeability due to MH. EBD uptake after the i.p. administration of KA was measured to evaluate the loss of barrier integrity due to KA. The results of the EBD uptake studies are shown in Figure 3. The dye uptake in the mice that were given only saline prior to KA injection was 170.28 ± 18.63 mg/g wet tissue. The increase in barrier permeability was largely reversed within 3 hours. KA significantly inhibited the uptake of EB to 73.02 ±

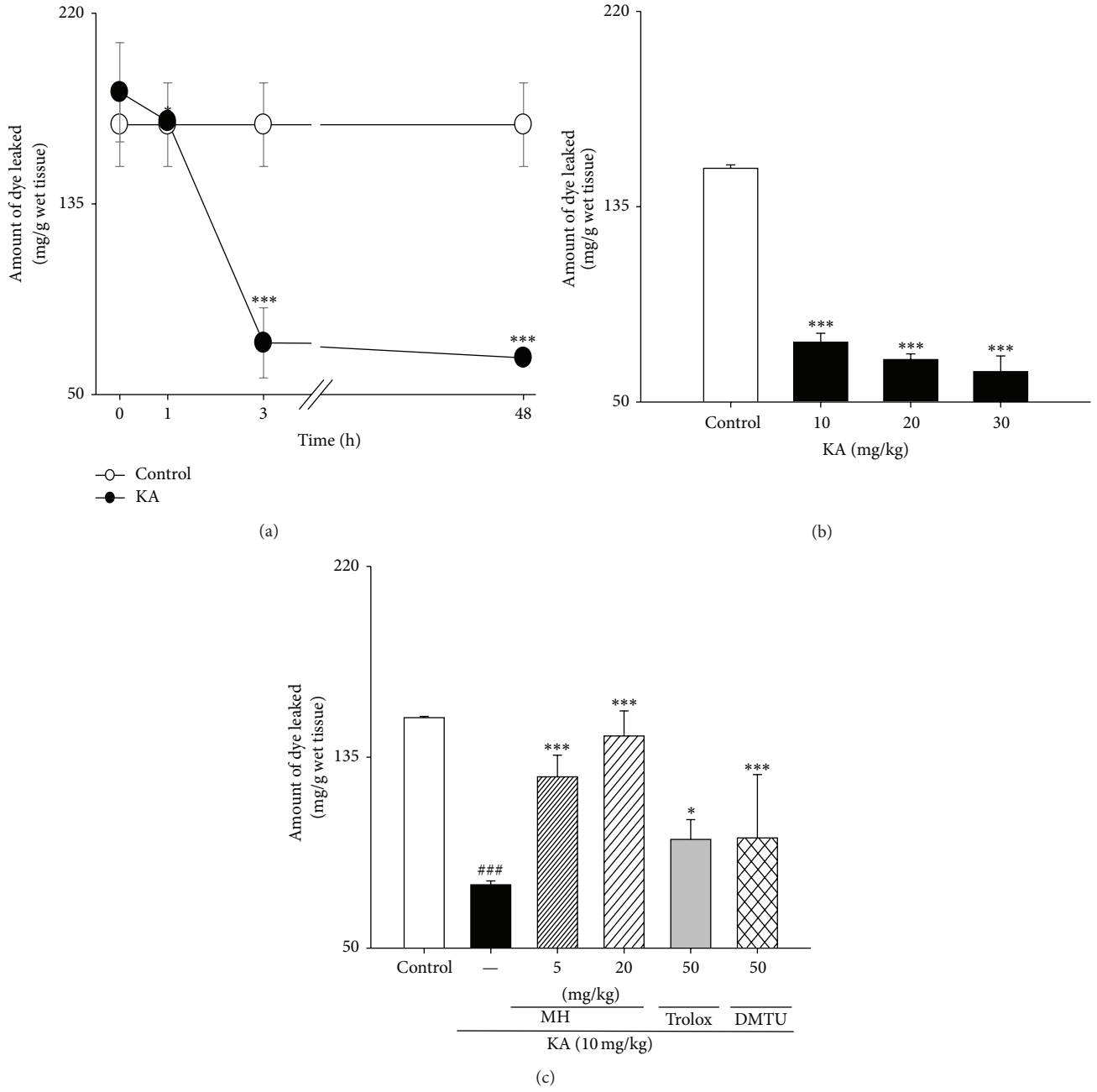

Figure 3: Changes and ameliorative effect of MH, scavengers on the BBB permeability in KA-induced mice. (a) Male mice were grouped (n = 5 or 6/group). Seizures were induced by KA injection (10 mg/kg, i.p.); the mice in the control group received an equal volume of 0.9% NaCl. Permeability was measured colorimetrically 0, 1, 3, and 48 h after KA or control injection. (b) Male mice were grouped (n = 5 or 6/group). Seizures were induced by KA injection (10–30 mg/kg, i.p.); the mice in the control group received an equal volume of 0.9% NaCl. (c) Male mice were grouped (n = 5 or 6/group) and pretreated (i.p. injection) with MH (5–20 mg/kg, i.p.), trolox (50 mg/kg, i.p.), DMTU (50 mg/kg, i.p.), or NaCl (0.9%). Fifty minutes after MH or control pretreatment, seizures in the KA and MH + KA groups were induced by KA injection (10 mg/kg, i.p.); the mice in the control group received an equal volume of 0.9% NaCl. EBD (100 mg/kg, i.v) was injected 5 minutes prior to KA administration. Two days after the KA administration, dye in the brain tissue was extracted and determined colorimetrically. All data are presented as means ± SE. $^{###}P < 0.001$ versus control group. $^{*}P < 0.05$, $^{***}P < 0.001$ versus KA group.

15.65 mg/g wet tissue compared to the control, while EB uptake remained unaltered until 48 hours (66.32 ± 3.15 mg/g wet tissue). KA produced a dose-related inhibition of dye extravasation. In contrast, MH pretreatment significantly reversed the KA-elicited EBD permeability in the brain; the levels reached 126.28 ± 17.07 and 144.45 ± 27.75 mg/g wet tissue in the KA + MH 5 group and the KA + MH 20 group, respectively (Figures 3(a) and 3(c)). Similar results

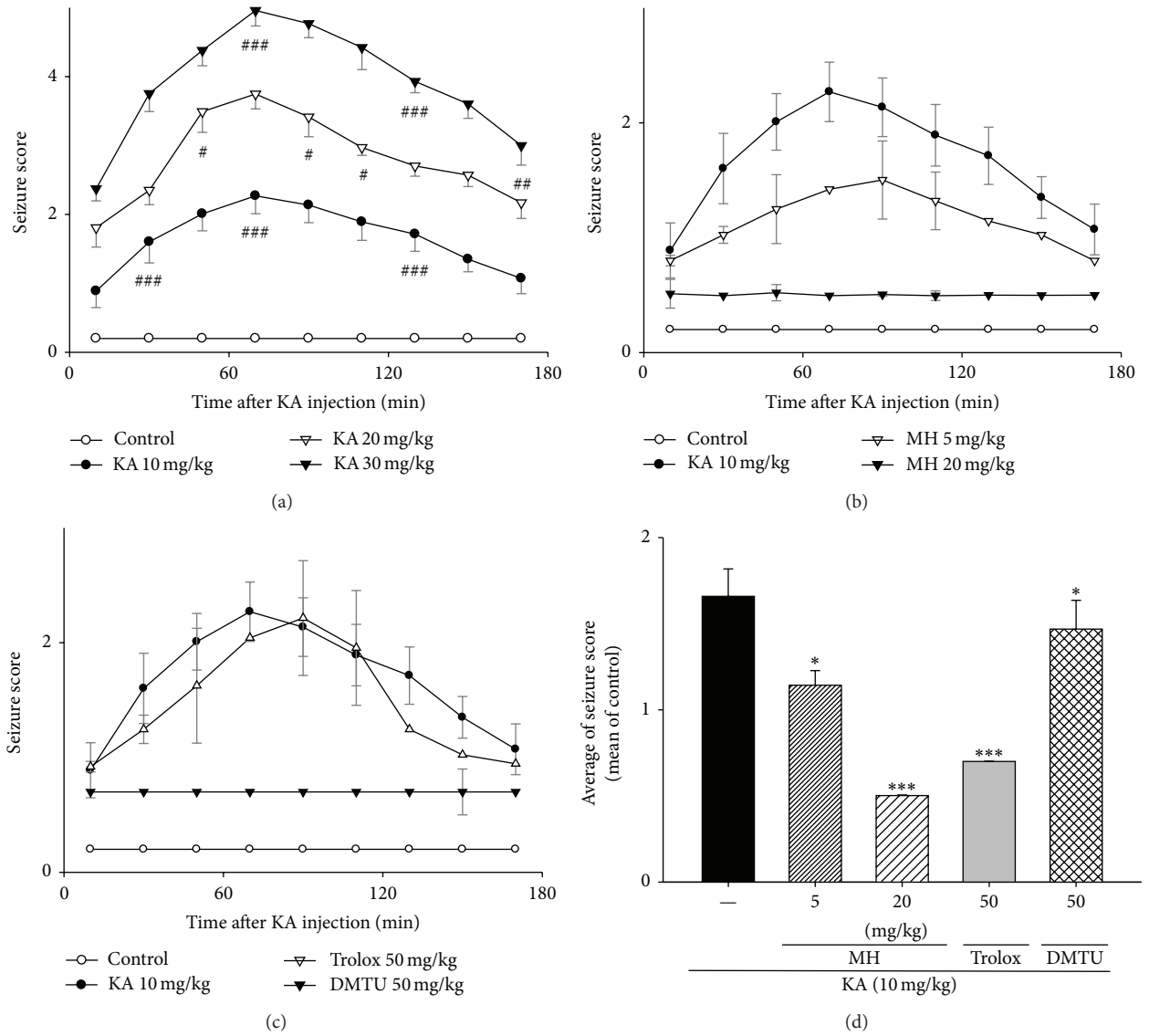

Figure 4: Effect of MH, scavengers on KA-induced convulsion in mice. Seizure scores were rated according to the scale devised by Racine. Seizures were induced by KA injection (10–30 mg/kg, i.p.); the mice in the control group received an equal volume of 0.9% NaCl. Male mice were grouped (n = 5 or 6/group) and pretreated (i.p. injection) with MH (5–20 mg/kg), scavengers such as trolox (50 mg/kg, i.p.) and DMTU (50 mg/kg, i.p.), or NaCl (0.9%). Fifty minutes after the MH or control pretreatment, seizures in mice in the KA, scavengers + KA, and MH + KA groups were induced by KA injection (10 mg/kg, i.p.); the mice in the control group received an equal volume of 0.9% NaCl. The preconvulsive behavior was scored within 3 h after KA injection. Scores at various time points after seizure induction were analyzed by two independent observers, after which they were averaged and statistically compared by the Holm-Sidak test. All data are presented as means ± SE. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus control group. $^{*}P < 0.05$, $^{***}P < 0.001$ versus KA group.

were obtained after the administration of DMTU or trolox, which are scavengers, rather than MH (Figure 3(c)). Taken together, these findings demonstrate that pretreatment with MH increased the KA-elicited decrease in the permeability.

3.3. MH Protected against KA-Induced Excitotoxicity in Mice. Mice were pretreated with MH (5–20 mg/kg, i.p.) to evaluate the protection of MH against KA-induced excitotoxicity. Equal volumes of 0.9% NaCl solution were administered to control mice in the same manner. Fifty minutes after the MH or saline pretreatment, KA (10–30 mg/kg) or 0.9% saline was injected. The convulsive behaviors were scored for 3 h after KA injection. Compared with the saline group in which no neurologic anomalies were observed following saline induction, the convulsive behaviors of the KA-treated mice initially occurred within 30 min; the seizure scores reached 3 after 1 h and substantially increased to 4-5 within 3 h following KA injection (Figure 4(a)). Delayed seizure onsets and decreased seizure scores were observed in the mice that were administered MH and the mice that received the scavengers trolox (50 mg/kg, i.p.) or DMTU (50 mg/kg, i.p.) (Figures 4(b) and 4(c)). The maximal seizure values were

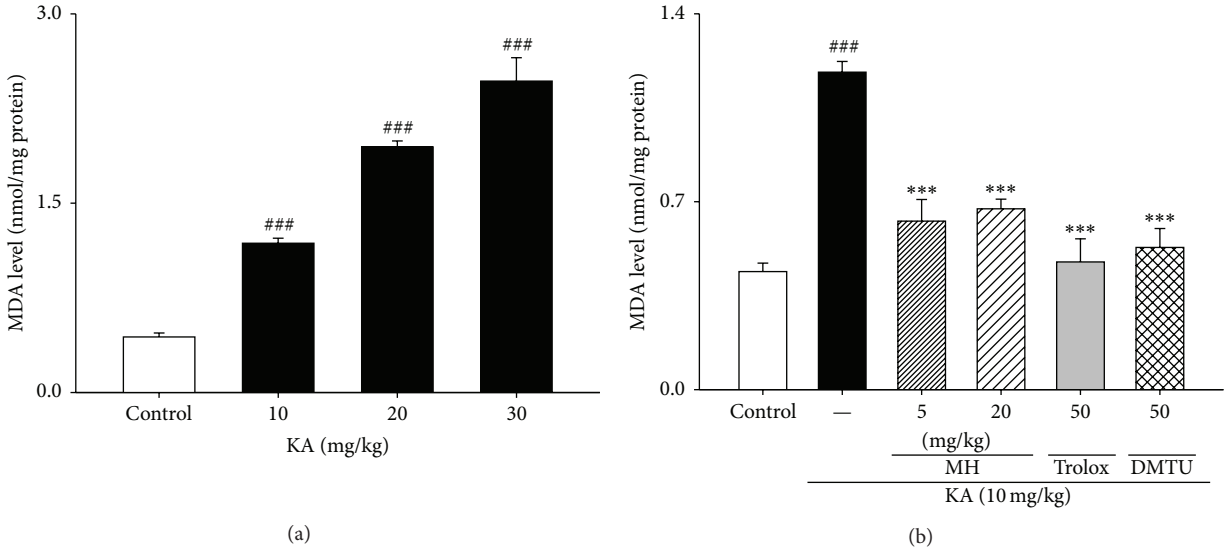

Figure 5: Effect of MH, scavengers on the MDA levels in KA-treated hippocampal tissue homogenates. Male mice were grouped (n = 5 or 6/group) and pretreated (i.p. injection) with MH (5–20 mg/kg), scavengers such as trolox (50 mg/kg, i.p.) or DMTU (50 mg/kg, i.p.), or NaCl (0.9%). Fifty minutes after the MH or saline pretreatment, seizures in the KA, scavengers + KA, and MH + KA groups were induced by KA injection (10 mg/kg, i.p.); the mice in the control group received an equal volume of 0.9% NaCl. All data are presented as means ± SE. $^{###}P < 0.001$ versus control group. $^{***}P < 0.001$ versus KA group.

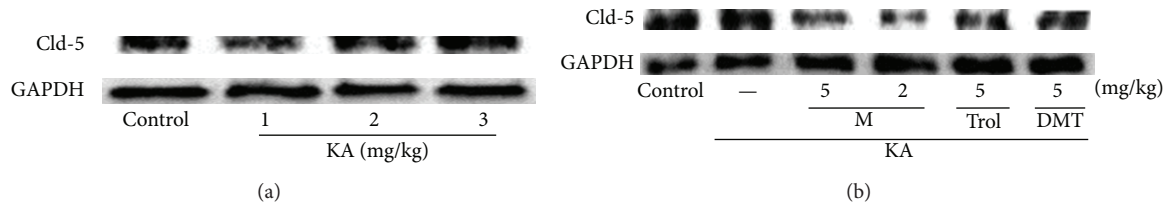

Figure 6: Effect of MH and scavengers on cld-5 levels in KA-treated hippocampal neuron. Immunoblots of lysed mice hippocampus 2 days following administration of MH or KA are shown. Neurons were exposed to KA at concentrations of 0, 10, 20, and 30 mg/kg for 48 h. Neurons were exposed to 10 mg/kg of KA at 0.5–1 h after 5–20 mg/kg of MH, trolox (50 mg/kg, i.p.), and DMTU (50 mg/kg, i.p.) pretreatment. GAPDH levels were measured to confirm equal protein loading.

reached after 1.30–2 h; thereafter, the seizure scores gradually decreased. As shown in Figure 4(d), we arranged the average seizure scores at each time point. Significant decreases in the average of seizure scores were observed in the mice that were administered MH or the scavengers trolox (50 mg/kg, i.p.) or DMTU (50 mg/kg, i.p). Taken together, these results indicate that MH may alleviate KA-induced status epilepticus.

3.4. Inhibition of Hippocampal MDA Levels by MH. The mechanism underlying these findings may be mediated by reactive oxygen scavenging. Free radicals may be one of the major causes of excitotoxic lesions. Therefore, we estimated free radical generation using a TBARS assay. TBARS levels are an indicator of lipid peroxidation and were significantly and dose-dependently increased in the hippocampi of the mice that were treated with KA compared to the control group that did not receive the stressor agent (Figure 5(a)). However, pretreatment with the 5 and 20 mg/kg doses of MH significantly prevented the KA-induced increase in TBARS levels. The animals that were treated only with either dose of MH exhibited no alterations in TBARS levels (data not shown).

3.5. Alterations in Hippocampal Cld-5 Protein Levels due to MH. Cld-5 can be the critical transmembrane protein that determines the barrier properties. Cld-5 tightens the paracellular cleft. As a further test, we investigated the alterations in the hippocampal tissues that were induced by KA (Figure 6). The expression of Cld-5 was dose-dependently induced by KA. Moreover, slight changes in Cld-5 expression levels were observed following the trolox and DMTU treatment. These data suggest that MH may potentially inhibit KA-induced barrier dysfunction *via* redox sensitive repair, which may be important for the physiological activity of the barrier.

3.6. Effect of MH and the Scavengers on KA-Induced Neuronal Loss, Oxidative Stress, and $[Ca^{2+}]_i$ Influx in Primary Cultured Hippocampal Cells. The cell death of primary hippocampal neurons was assessed with MTT assays to evaluate the protective capabilities of MH. Neurons were exposed to KA

at concentrations of 0, 30, 50, 70, and 100 μM for 48 h (Figure 7(a)). Figure 7 shows that cell death was rapid; growth was inhibited to 55.5 ± 2.2% of the control levels 48 h after KA exposure. When the cells were exposed to 100 μM KA, significant cell death occurred after 48 h. Exposure of the hippocampal neurons to KA (100 μM) for 48 h elicited a significant decrease in cell survival, whereas KA-induced neuronal loss was inhibited by 65.8 ± 2.0% and 77.1 ± 4.0% following the addition of 30 and 100 μM MH, respectively (Figure 7(a)). Moreover, inhibition of cell death to 75.0 ± 3.3% and 75.2 ± 0.1% occurred following exposure to 100 μM of trolox and DMTU, respectively (Figure 7). These results indicate that MH protected the neurons against KA-induced cytotoxicity. Because KA induces oxidative damage in cultured murine neurons, we examined whether MH affected the 100 μM KA-induced changes in ROS levels in primary hippocampal neurons using the DCFH-DA assay. Low levels of ROS were found in the controls (3186 ± 230 fluorescence intensity), and these values were considered physiological. In contrast, a significant increase in ROS concentration was observed (4209 ± 187 fluorescence intensity) following treatment with 100 μM KA for 48 h. Furthermore, as shown in Figure 7(b), the ROS levels were 4163 ± 96 and 3598 ± 204 intensity following the 30 and 100 μM doses of MH, respectively. Trolox and DMTU also induced inhibitions of ROS production at the high doses of 100 μM (Figure 7(b)). Taken together, these results indicate that treatment with 100 μM KA elevated ROS production and that pretreatment with 30 to 100 μM MH significantly decreased ROS production. Regarding oxidative glutamate toxicity, a 100-fold increase in intracellular ROS results in an elevation of cytosolic Ca^{2+} that precedes cell death. To investigate the mechanism by which MH protected against KA-induced neurotoxicity, we examined whether MH could inhibit the KA-induced elevation in intracellular $[Ca^{2+}]_i$ in cultured hippocampal neurons. We measured $[Ca^{2+}]_i$ levels using the Ca^{2+} indicator fura-4. As shown in Figure 7, the 100 μM KA treatment led to a significant elevation of $[Ca^{2+}]_i$ (data not shown). The percentages of the inhibitions of the elevation of $[Ca^{2+}]_i$ due to MH in the hippocampal neurons were 8.9%, 11%, and 20% at the MH doses of 30, 50, and 100 μM, respectively, the levels of the normal controls were taken as 100% (Figure 7(c)). Thus, the MH treatments in the range of 30 to 100 μM dose-dependently inhibited the KA-induced elevations in $[Ca^{2+}]_i$. However, DMTU was strongly suppressed following treatment with trolox. These results indicate that the treatment of the cultured hippocampal neurons with 100 μM KA elevated $[Ca^{2+}]_i$ and that this effect was attenuated by MH.

3.7. Reduction of DPPH Radical Activity by MH. The activity of MH is generally attributed to its antioxidative efficacy. To identify the redox potential of MH, the reduction of the DPPH radicals was analyzed using a spectrophotometric method. Figure 6 shows that MH scavenged the DPPH radicals in a dose-dependent manner and that the IC_{50} of MH was approximately 52.4 mM (Figure 6). Trolox was used as a positive control and found to scavenge 100% of the DPPH radicals at 0.25 mg/mL.

3.8. $^{\bullet}$OH Reduction Activity of MH. The $^{\bullet}$OH generated by the Fenton reaction system was trapped by DMPO, and this trapped $^{\bullet}$OH was detected with an ESR spectrometer. As shown in Figure 9(a), the typical 1 : 2 : 2 : 1 ESR signal of the DMPO/$^{\bullet}$OH adduct ($AN = AH = 14.4$ G) was observed. Each spectrum was obtained 15 min after the initiation of the Fenton reaction. MH inhibited the Fenton reaction by reacting with $^{\bullet}$OH. Additionally, the DMPO/$^{\bullet}$OH adduct signal gradually decreased over time. The decay rate exhibited approximately pseudo-first-order kinetics over the period of measurement, and the half-life of the DMPO/$^{\bullet}$OH signal was estimated to be 10.24 min. The activities of DMTU, trolox, and MH were 5.4, 7.65, and 9.65 min, respectively. The rate constant of the reaction of MH with $^{\bullet}$OH (3.4×10^9 $M^{-1}\cdot S^{-1}$) was calculated from the competition with the spin trap DMPO and found to be close to the reactivity of trolox (3.5×10^9 $M^{-1}\cdot S^{-1}$) (Figure 9(b)). The initial velocity of the signal decay rate of MH seemed to be slightly faster than that of trolox.

4. Discussion

Our results clearly demonstrated that MH, a principal ingredient of the bark of *M. officinalis,* had significant antioxidant/neuroprotective effects against KA-induced excitotoxicity in mice. The cytological, biochemical, and behavioral results agreed with the fact that MH inhibited seizures *in vivo* and inhibited edema and permeability variations. We found that MH reduced the MDA and Cld-5 levels in the hippocampal tissue. Moreover, MH was found to possess DPPH radical and $^{\bullet}$OH reduction activities.

KA is a specific agonist of the KA receptor and a selective ionotropic glutamate agonist. Glutamate toxicity is the major contributor to pathological cell death within the nervous system and appears to be mediated by ROS [49]. Excitotoxicity is thought to play an important role in the neural damage that occurs in pathological conditions such as trauma, stroke, epilepsy, and hypoglycemia. Under such pathological conditions, the excess release of L-glutamic acid and other EAAs can lead to excitotoxic lesions in the brain that result from the overexcitation of nerve cells [50]. Behavioral activity can provide strong evidence that antioxidants or free radical scavengers are capable of counteracting the neuronal damage induced by KA (Figure 4). Schulz et al. provided direct *in vivo* evidence that KA-induced neuronal damage is mediated by free radicals. Our DPPH and ESR data indicate that MH has radical reduction activity (Figures 8 and 9).

The BBB is intimately interconnected with the causes and effects of and treatments for seizures. The endothelium is a semipermeable barrier that lines the vasculature and regulates fluid and solute exchange between the blood and the interstitial space. There are two pathways that allow solutes to traverse the endothelium, the transcellular and paracellular pathways. The transcellular pathway, also known as the transcytotic pathway, is defined by the caveolae-dependent vesicle-mediated transport of macromolecules, across the endothelial barrier. The transcellular route crosses the apical and basal cell membranes and is primarily mediated by

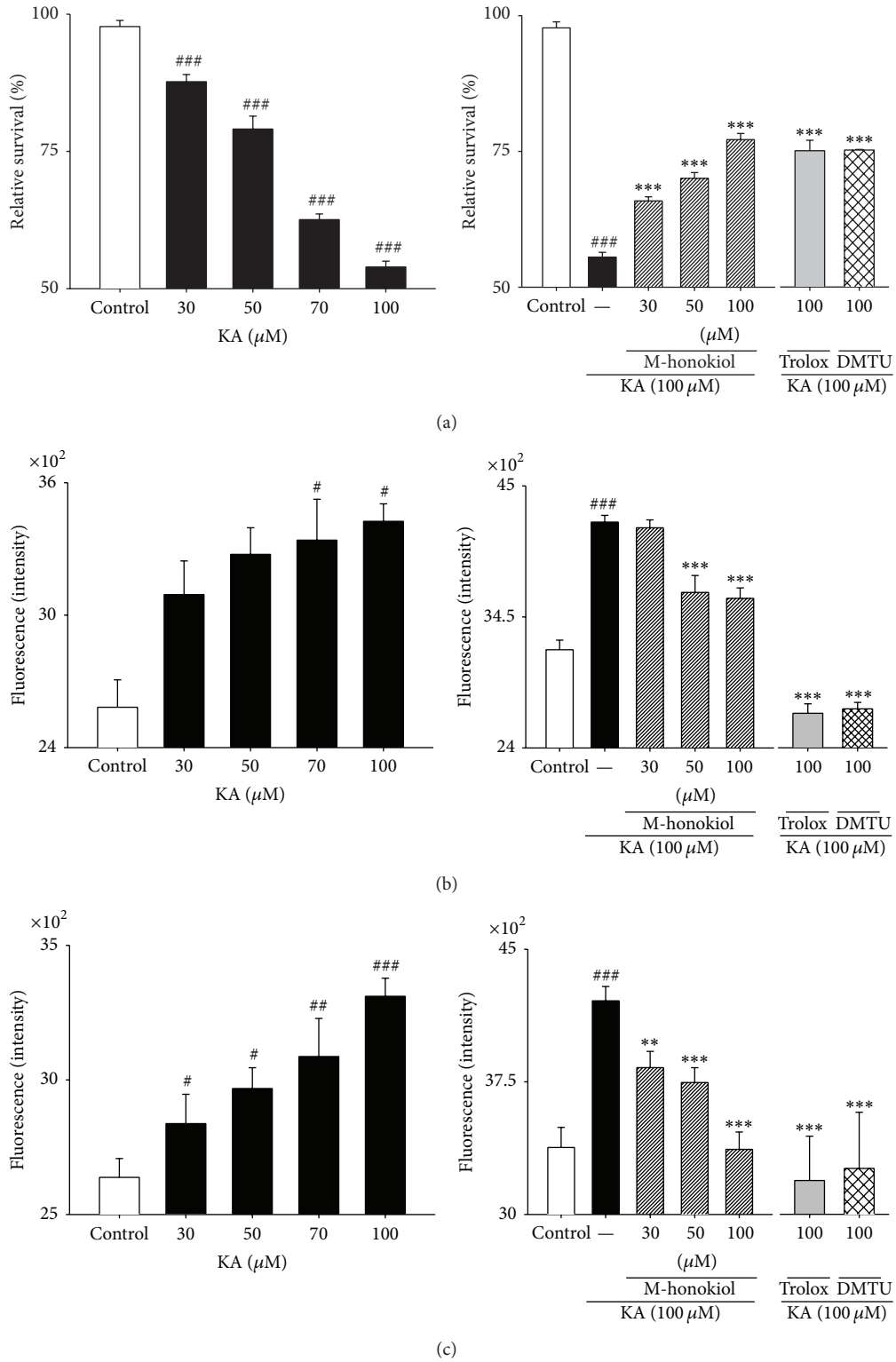

Figure 7: Effect of MH, scavengers on KA-induced neuronal loss and Oxidative stress and $[Ca^{2+}]_i$ influx in primary cultured hippocampal cells. Concentration data for KA-induced toxicity in primary cultured hippocampal neurons. Examination of the dose effect of KA on neuronal viability by the MTT assay. Neurons were exposed to KA at concentrations of 30, 50, and 100 μM for 48 h. Cell viability at 48 h after KA exposure was measured by the MTT assay. Examination of the dose effect of KA on neuronal ROS level and $[Ca^{2+}]_i$ the DCFH-DA and Ca^{2+} indicator with fura-4 assay. All data are presented as means ± SE. $^{###}P < 0.001$ versus control group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus KA group.

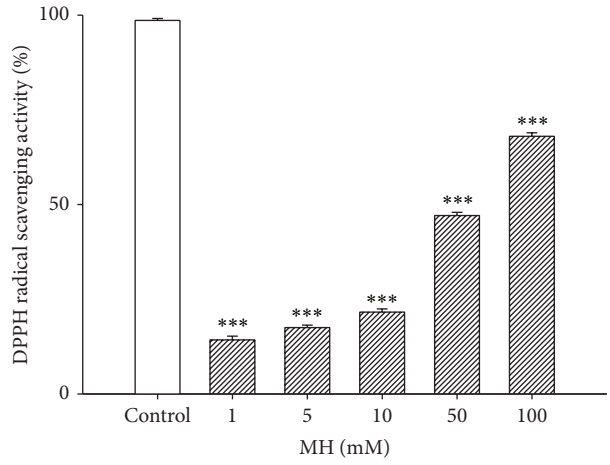

Figure 8: DPPH radical-scavenging activity of MH. 1–100 mM of MH was used. Trolox, as a positive control, scavenged 100% of the DPPH radical at 0.25 mg/mL. All data are presented as means ± SE. $^{***}P < 0.001$ versus control group.

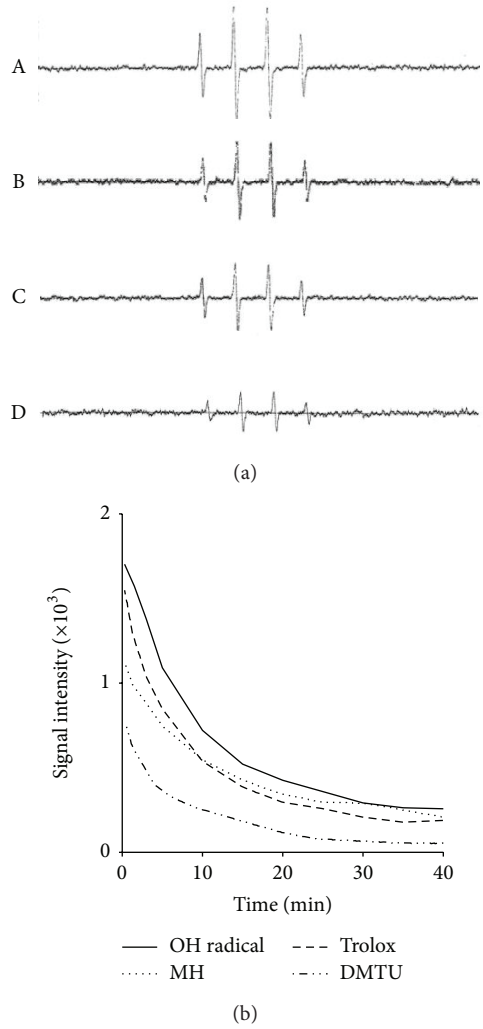

Figure 9: Reduction activity of MH on •OH. (a) ESR spectra of DMPO/•OH adduct were generated in a Fenton Reaction System. The solutions with a final volume of 0.1 mL contained 2 mM ferrous sulfate, 2 mM H_2O_2, and 100 mM phosphate buffer (pH 7.2). Reactions were started by addition of ferrous ammonium sulfate (2 mM final concentration) and the steady-state ESR were recorded at 15 min after the Fenton Reaction. Line A; DMPO/•OH adduct, B; MH (1 mg/mL), C; trolox (1 mM), and D; DMTU (1 mM). (b) Time course of •OH degradation induced by Fenton Reaction System. DMPO/•OH adduct, MH (1 mg/mL), trolox (1 mM), and DMTU (1 mM).

channels, carriers, pumps, and vesicles. In contrast, the paracellular route passes through the intercellular and lateral spaces between cells that are in contact and is mediated by the tight junctions (TJs). As shown in Figure 3, MH increased the permeability of the BBB following KA-induced neurotoxicity in mice. This increase in permeability may account for the superior antioxidant activity. It is well known that KA induces free radicals [51, 52]. Free radicals actually inhibit the sodium pump [53]. Potassium ions are crucial for normal action potential generation in all excitable tissue. Another possibility is related to the facts that MH is a small molecule and a dimer of allyl-phenol. The ortho-allyl group may potentially form a six-member ring after the absorption of the hydroxyl group. Additionally, phenolic compounds are commonly found in plants and have been reported to have multiple biological effects that include antioxidant activity [54–56]. Finally, lipid solubility has long been recognized as an important factor in diffusion across biological membranes; this is partially due to hydrogen bonding affinity, which is key factor that determines the rate at which a drug passively crosses the BBB. The presence of hydroxyl groups on peptides tends to promote hydrogen bonding with solvating water, which leads to a concomitant decrease in the partition coefficient (i.e., the lipophilicity) and a subsequent decrease in membrane permeability [57]. Accordingly, MH may enhance radical scavenging in lipid and hydrophobic environments, which may be important for the physiological activity of the barrier. To assess the impaired barrier permeability for EAAs, we investigated the alterations in endothelial tight junctional protein expression. The barrier properties are primarily determined by endothelial junctional complexes that consist of TJs and adherens junctions (AJ). In general, TJs seal the interendothelial cleft to form a continuous blood vessel, while the AJs are important for initiating and maintaining endothelial cell-cell contact [58, 59]. The molecular architecture of the TJ has been recently reviewed in detail [60, 61]. Clds are the principal barrier-forming proteins. Clds have two functional subcategories: (1) Clds that specifically increase paracellular permeability through the formation of paracellular channels (pore forming Clds), and (2) Clds that are generally reduce paracellular permeability (sealing Clds) [62]. Brain endothelial cells possess Cld-5, Cld-12, and possibly other Clds. Cld-5 is a critical component of the maintenance of the barrier [63]. Cld-5 is responsible for limiting paracellular ion movement selectively, which produces the high ER of the barrier [64, 65]. Some Clds can also regulate charge selectivity by acting as electrostatic barriers to either anions or cations. Alternatively, the high ER or low conductance of the potential paracellular

pathway emphasizes the extreme effectiveness of the tight junctions in occluding this pathway by effectively reducing the movement of ions.

Brain edema is commonly observed in conditions of impaired BBB function. The early process of vasogenic edema formation may also initiate seizures. Based on our edema results (Figure 2), we speculate that MH may reduce edema formation early in epilepsy by controlling the rate of ion transport across the barrier. However, many questions remain concerning the mechanisms that are responsible for barrier ion transport and how they are altered by epilepsy.

Additionally, we quantitatively estimated $^{\bullet}$OH levels to examine the rate constants of the interaction of MH with $^{\bullet}$OH. In this work, we studied the reactions of MH with radicals using the ESR technique. As shown in Figure 9, the DMPO/$^{\bullet}$OH adduct was suppressed by the presence of MH or scavengers. MH reduced $^{\bullet}$OH at relatively low concentrations (1 mg/mL). The DMPO/$^{\bullet}$OH adduct occurred at approximately 10.24 min, whereas those for DMTU, MH, and trolox occurred at approximately 5.4, 7.65, and 9.65 min, respectively. Generally, the second-order rate constant for DMPO/$^{\bullet}$OH ($3.4 \times 10^9\ M^{-1}\cdot S^{-1}$) is estimated with mannitol as a competitive standard [k ($^{\bullet}$OH + mannitol) = $2.7 \times 10^9\ M^{-1}\cdot S^{-1}$]. The rate constant of the reaction of MH with $^{\bullet}$OH ($4 \times 10^9\ M^{-1}\cdot S^{-1}$) was calculated from the competition with the spin trap DMPO and was found to be close to the reactivity of trolox (Figure 9(b)). The initial velocity of the signal decay rate for MH seemed to be slightly faster than that of trolox. In summary, MH efficiently reduced DPPH (IC_{50}, 52.4 mM) and $^{\bullet}$OH with a rate constant of $4 \times 10^9\ M^{-1}\cdot S^{-1}$, which is close to that for the reactivity of trolox, which is a vitamin E analog.

5. Conclusion

These results suggest that MH may enhance redox scavenging mechanisms in lipid and hydrophobic environments, which may be important for the physiological activity of the barrier. Thus, MH can be a useful agent against redox-associated development or the progression of epilepsy.

Conflict of Interests

The authors declare that they have no conflict of interests with anyone at their institute or with any other party for publishing the data of the present study.

Acknowledgments

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Review Article

From Pathways to Targets: Understanding the Mechanisms behind Polyglutamine Disease

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The history of polyglutamine diseases dates back approximately 20 years to the discovery of a polyglutamine repeat in the androgen receptor of SBMA followed by the identification of similar expansion mutations in Huntington's disease, SCA1, DRPLA, and the other spinocerebellar ataxias. This common molecular feature of polyglutamine diseases suggests shared mechanisms in disease pathology and neurodegeneration of disease specific brain regions. In this review, we discuss the main pathogenic pathways including proteolytic processing, nuclear shuttling and aggregation, mitochondrial dysfunction, and clearance of misfolded polyglutamine proteins and point out possible targets for treatment.

1. Introduction

Polyglutamine (polyQ) diseases are inherited, fatal neurodegenerative disorders caused by an expansion of a coding trinucleotide (CAG) repeat, which is translated to an abnormally elongated glutamine (Q) tract in the respective mutant proteins. There are nine known polyQ diseases: dentatorubral-pallidoluysian atrophy (DRPLA), Huntington's disease (HD), spinal-bulbar muscular atrophy (SBMA), and six spinocerebellar ataxias (SCA 1, 2, 3, 6, 7, and 17). Except for SBMA, which is X-linked, members of this disease group are inherited in an autosomal dominant manner [1]. It also appears that the shared expansion of polyQ tract confers some shared neurodegenerative pathways on the diseases. Although the region of the brain that is affected differs according to each disease, the observed cell death is aggravated by the trafficking of the protein to specific cellular compartments where it can increase the rate of aggregation. Both nuclear and cytoplasmic aggregates are present in polyQ diseases and contain parts of the respective disease proteins, ubiquitin, and several important homeostatic proteins [2]. The recruitment of ubiquitin, heat shock proteins, and proteasomal subunits into these aggregates implies that protein quality control mechanisms such as the ubiquitin-proteasome system (UPS) are involved in polyQ pathogenesis [3]. It has also been discussed that the cleaved protein is more toxic than the full-length variant. An initial proteolytic cleavage of the respective disease proteins may generate a fragment containing the elongated polyQ stretch which is more aggregate prone and hence more toxic for the cell [4, 5]. What is also interesting about this group of proteins is that although they are all ubiquitously expressed in embryonic stages and adulthood, the pathology of the disease only occurs in neuronal cells [6]. One possible explanation for this phenomenon is the high energy demand of neurons and hence their dependency on oxidative energy metabolism. This points dysfunctional mitochondria as a shared mechanism of neurodegeneration [7]. In this review we focus on what we consider to be the most important pathways in pathology of Huntington's disease and spinocerebellar ataxias: proteolytic processing, nuclear

shuttling and aggregation, mitochondrial dysfunction, and intracellular protein degradation systems (Figure 1).

2. Proteolytic Processing

Early studies of the common characteristics of polyQ diseases revealed that small fragments of mutant proteins containing the expanded polyQ stretch harbored cytotoxic characteristics [8, 9]. Proteolytic cleavage, the proposed source of these breakdown products, was suggested as an early or initial step in the molecular disease development. This mechanistic concept is commonly known as the *toxic fragment hypothesis* [10]. The presence of proteolytically derived fragments of mutant proteins was reported for all polyQ diseases introduced in this review, namely, SCA 1 [11], SCA 2 [12], SCA 3 [13, 14], SCA 6 [15], SCA 7 [16, 17] SCA 17 [18], and HD [19, 20]. Currently, several classes of endogenous proteases have been linked to the proteolysis of polyQ proteins including the groups of caspases [21–24] and calpains [20, 25–29].

For SCA 1 and 2, neither an inherent cytotoxicity and aggregation propensity nor a clear impact on pathology is evident for mutant protein fragments, demanding further characterization [11, 12]. For ataxin-2, the disease protein in SCA 2, mutant fragment constructs were shown to exhibit an aggregate formation potential *in vitro* [30], but further studies revealed a decreased cytotoxicity of N-terminally truncated mutant ataxin-2 compared to the full-length protein [31]. Even so, for the majority of polyQ diseases a correlation between proteolytic processing of mutant proteins and disease progression is generally accepted.

In a SCA 3 cell model, the expression of a fragment of ataxin-3 containing an elongated polyQ stretch induced apoptosis and cell death as well as a severe ataxia in a mouse model, showing a more rapid manifestation of a SCA 3-reminiscent phenotype when compared to mice expressing full-length mutant ataxin-3 [8]. In addition, polyQ-containing ataxin-3 fragments were shown to form aggregates on their own and were also able to recruit full-length protein into the insoluble inclusions [32, 33]. In HD, *in vitro* data showed that the progressive truncation of mutant huntingtin (mHtt) protein and the length of the polyQ expansion correlate with the aggregation propensity and an increase in apoptotic stress [34, 35]. Mouse studies revealed a similar result when animals expressing the polyQ expanded exon 1 of huntingtin (Htt) showed a progressive neurological phenotype recapitulating characteristics of HD. This suggests that the N-terminal polyQ-containing portion of Htt was sufficient to induce neurodegeneration *in vivo* [9]. An important observation is that these disease fragments were detectable in human HD and SCA 3 brain and lymphoblasts [13, 20, 36] and were found to be an important component of neuronal intranuclear inclusions [37–39]. Similar results were retrieved from two mouse models of SCA 7 expressing polyQ expanded ataxin-7. In brain tissue of these animals N-terminal ataxin-7 fragments were observed which appeared in nuclear aggregates in correlation with onset of the disease phenotype [16, 17]. As with much of the current research on polyQ diseases, not all observations are in agreement. An HD mouse model expressing a polyQ expanded fragment of Htt encompassing exons 1 and 2 exhibited neither neurotoxic effects nor an HD phenotype, despite the presence of nuclear inclusions [40]. This illustrates that not all fragment species feature neuropathological characteristics. Another noteworthy investigation made on a SCA 3 gene trap mouse model showed that expression of a fusion protein comprising β-galactosidase and the N-terminal portion of ataxin-3 without the polyglutamine tract led to the formation of cytoplasmic inclusion bodies and to a phenotype reminiscent of the neurological symptoms observed in SCA 3 mice and patients [41]. Furthermore, C-terminal polyQ fragments of the α1A calcium channel, disease protein in SCA 6, showed a polyQ independent cytotoxic nature. However, the expansion of the polyQ stretch within the fragment resulted in its increased resistance to proteolysis entailing an accumulation of this toxic species [15].

The first proteases which were shown to cleave polyQ expanded proteins were caspases. This family of cysteine proteases is associated with apoptotic pathways and inflammation but is also known to be involved in a variety of other cellular functions like cell proliferation, differentiation, and migration [42, 43].

Caspases are involved in cell death mechanisms and an increase in activation of caspases has been detected in the course of polyQ diseases. Presence of apoptotic cell death and caspase activation was shown in human HD brains as well as in mouse and cell models of HD [44–51], although this goes against previous studies that did not find apoptotic nuclei in the R6/2 mouse model of HD [52]. Cell death pathways and caspases were also reported to be switched on in other polyQ diseases like SCA 3 [8, 53] and SCA 7 [54, 55]. In the case of SCA 7, activated caspase-3 was recruited into inclusions in cell culture and human SCA 7 brain, and its expression was upregulated in cortical neurons [54]. In general, inhibition of caspases has been shown to ameliorate disease progression and phenotype in HD mice [44, 49].

Within the polyQ diseases reviewed, the first discovery of caspase-mediated cleavage of a disease-causing protein was made for HD [21]. This *in vitro* study indicated a specificity of caspase-3 for huntingtin and a polyQ expansion dependent cleavage. Further studies identified caspase-1 dependent cleavage of huntingtin and confirmed caspase-3-mediated fragmentation, whereas caspases-7 and -8 appeared not to cleave full-length huntingtin [22]. Moreover, caspase-3 selectively processed expanded huntingtin and resulting N-terminal fragments formed cytoplasmic and nuclear inclusions [48]. Direct evidence for caspase-mediated huntingtin cleavage was gained from early stage HD postmortem human tissue and transgenic mice. In these brain tissues, not only mutant but also wild type huntingtin are substrates for caspase cleavage. The early disease stage of these samples suggests that caspase-mediated proteolysis of mHtt may precede neurodegeneration [23].

Multiple studies have begun to elucidate the specific caspases responsible for cleavage of huntingtin. A broad inhibition of caspases with Z-VAD-FMK in clonal striatal cells led to a reduction of specific huntingtin fragments and an increased viability without changing levels of inclusions,

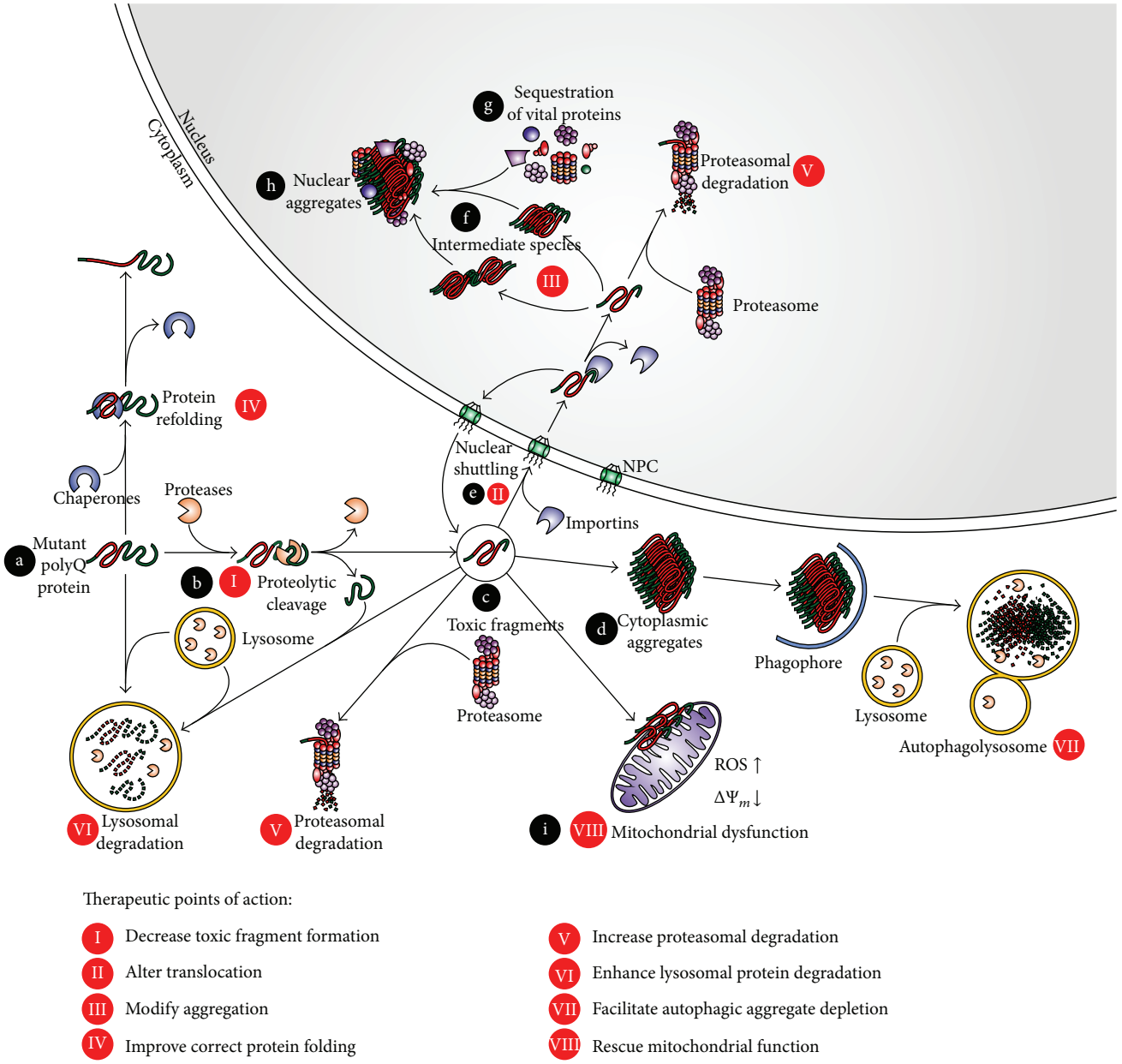

Figure 1: A model of the common molecular mechanisms behind polyglutamine pathology. Schematic illustration of the intracellular fate of the polyglutamine (polyQ) expanded protein, from the unprocessed mutant protein to a protein aggregate. The mutant protein (a) is proteolytically processed by endogenous enzymes (b) forming toxic fragments (c). These fragments form aggregates in the cytoplasm (d). Alternatively, toxic breakdown products can translocate into the nucleus (e) and generate nuclear aggregates (h) by forming intermediate species (f) and sequestering further vital proteins (g). Accumulation of polyQ species can damage important cellular components and lead, for example, to mitochondrial dysfunction (i). The visualized pathways point possible sites for therapeutic engagement: prevention of proteolytic events (I) can decrease levels of toxic fragments. Alteration of nuclear shuttling (II) and modulation of aggregation (III) can ameliorate the detrimental effects of toxic species. As polyQ expansions lead to misfolded proteins, structural refolding assisted by enhanced chaperone activity (IV) might be beneficial. An increased degradation of polyQ proteins and aggregates via proteasomal (V), lysosomal (VI), and autophagosomal (VII) pathways can reduce the amounts of toxic species inside the cell. Finally, attenuating the consequences of polyQ toxicity (VIII), like impaired mitochondrial function, can improve the cellular viability.

whereas treatment with the caspase-3 specific inhibitor Z-DEVD-FMK reduced aggregates without changing cleavage or increasing cell viability [46]. The generation of mouse lines expressing caspase-3 and caspase-6 resistant polyQ expanded huntingtin by eliminating specific cleavage sites unveiled a strong relevance of cleavage at the 586 amino acid caspase-6 site of huntingtin. Removing the caspase-6, but not caspase-3, recognition sites in mHtt appeared to be sufficient to protect from neuronal dysfunction and neurodegeneration *in vivo* [56]. A further study showed that caspase-6, but not

caspase-3, is activated before the onset of motor abnormalities in murine and human HD brain. Caspase-6 activation correlated directly with the size of the polyQ expansion and inversely with the age at onset [56]. Moreover, medium spiny neurons (MSNs) expressing caspase-6 resistant mHtt showed a decreased susceptibility for NMDAR-induced excitotoxicity and no caspase-6 activation compared to MSNs expressing unmodified mHtt [56–58]. By contrast, two caspase-6 knockout HD mouse models showed that production of a 586 amino acid derived proteolytic fragment was not prevented in the brain, disagreeing with a direct involvement of caspase-6 in mHtt cleavage [59, 60].

Correlating with the results for huntingtin, caspases-1 and -3, but not caspases-7 and -8, were reported to cleave ataxin-3 *in vitro* producing specific fragments [22]. The impact of caspase cleavage was confirmed in a cell based model, showing that predominantly caspase-1-mediated fragmentation of expanded ataxin-3 resulted in increased aggregation and treatment with caspase inhibitors prevented inclusion formation *in vitro* [61]. Interestingly, a different *in vitro* study showed that mutant ataxin-3 was cleaved to a lesser extent than wild-type ataxin-3 after a common initial proteolytic step, suggesting that generated mutant fragments cannot be further degraded. This may result in an accumulation of aggregation-prone expanded ataxin-3 fragment species [62]. In a *Drosophila* model cleavage of ataxin-3 appeared to be conserved and also caspase-mediated, featuring neuronal loss which was mitigated by a sextuplet caspase site mutation in ataxin-3 [63]. A recent publication reported involvement of CDK5 in caspase-mediated ataxin-3 cleavage, showing that RNAi of CDK5 in a *Drosophila* model for SCA 3 resulted in an enhanced SCA 3 toxicity [64]. Contrary to results pointing to an involvement of caspases in the molecular pathology of SCA 3, an *in vitro* study based on patient-derived iPSCs demonstrated that upon excitotoxic stress ataxin-3 cleavage and aggregation were prevented neither by pharmacological inhibition of caspases-1 and -3 nor by treatment with a pan-caspase inhibitor but was abolished by inhibiting calpain activity [65].

In the case of SCA 7, *in vitro* assays identified caspase-7 as the responsible proteolytic enzyme for ataxin-7 fragmentation. The mutation of two specific caspase-cleavage sites in ataxin-7 not only resulted in a resistance of polyQ expanded ataxin-7 to caspase cleavage but also attenuated cell death, aggregate formation, and transcriptional interference in cells. Fragments of ataxin-7 corresponding to products of caspase-7 cleavage were also found in SCA 7 mice, which furthermore exhibited an increased caspase-7 activation and recruitment into the nucleus by expanded ataxin-7 [24]. Nonetheless, full-length expanded ataxin-7 can form inclusions without evidence for cleavage [54].

TBP (TATA-binding protein), the disease protein in SCA 17, was reported to show fragmentation and fragment-dependent formation of aggregates in SCA 17 mice [18], but *in vitro* assays did not show a TBP substrate-specificity for caspases [22], suggesting different proteolytic enzymes to be involved in truncation of TBP.

A second group of proteolytic enzymes that were associated with cleavage of polyQ expanded proteins are calpains, a class of calcium-dependent cysteine proteases. These ubiquitously expressed enzymes exhibit a multitude of regulatory cellular functions and are specialized in modulating structure, localization, and activity of their substrates [66, 67].

In human HD tissue and in brains of HD mouse models an increased expression level of calpains, namely, of calpains-1, -5, -7, and -10, and elevated enzyme activity have been reported [25, 26, 68, 69]. Interestingly, an age-dependent attenuation of calpain activity was observed in an HD mouse model, suggesting alterations in calcium signaling mechanism with disease progression [70]. Furthermore, wild-type and mutant huntingtin were identified as calpain substrates and calpain-dependent proteolytic cleavage products of huntingtin were detected in murine and human HD tissue [25, 27, 46, 71]. Caspase-3 cleavage derived huntingtin fragments undergo further proteolysis by calpains, generating smaller products and suggesting a proteolytic pathway of serial processing events [20]. Additionally, calpain-derived mHtt fragments were shown to accumulate in the nucleus [26], which correlates with cytotoxicity and aggregation in HD [34, 35]. In cell models, the inhibition of calpain cleavage of mutant huntingtin by mutating putative cleavage sites within the huntingtin protein resulted in a decreased proteolysis, aggregation, and toxicity [26]. The mutation of Ser-536 to aspartic acid in order to mimic phosphorylation abolished huntingtin proteolysis at this cleavage site and reduced mutant huntingtin toxicity, pointing to an involvement of phosphorylation events as modulators of calpain cleavage [72]. Concurrent with their activation after ischemic injury, calpains were also shown to cleave full-length huntingtin in infarcted rat cortex and striatum producing N-terminal fragments [73].

Although initial studies stating that an involvement of calpains in SCA 3 was not detectable [61, 63], calpain-dependent proteolysis of ataxin-3 has been reported corresponding to observations in HD [28, 29, 65]. Several putative calpain cleavage sites within the ataxin-3 protein were identified [28, 29, 65, 74], accounting for the generation of a C-terminal polyQ-containing and aggregation-prone fragment [33]. After activation of calpains *in vitro,* fragments of respective sizes were generated. This effect was suppressed when the endogenous calpain inhibitor calpastatin (CAST) was coexpressed in treated cells and aggregation of mutant ataxin-3 was induced or decreased [28]. In a double mutant CAST KO/SCA 3 mouse model, the knockout of the endogenous calpain inhibitor led to higher ataxin-3 fragmentation, amplified aggregate load, increased neurodegeneration, and, in conclusion, to a more severe behavioral phenotype [29]. Reciprocally, overexpression of CAST using adenoassociated viral vectors in a lentiviral mouse model of SCA 3 resulted in reduced ataxin-3 proteolysis and in decreased size and number of intranuclear inclusions of ataxin-3 and neuroprotection via calpain inhibition [74]. In line with these observations, CAST was shown to be depleted in murine and human SCA 3 brain tissue [74]. The neuronal specificity of the molecular mechanisms underlying SCA 3 pathology has been demonstrated by an approach using SCA 3 patient-derived IPSCs. After neuronal differentiation and glutamate-induced calcium influx, excitation-induced ataxin-3 cleavage

and aggregation were triggered. This was observed only in neurons, not in glial cells or fibroblasts, and was abolished by calpain inhibition [65].

Although caspases and calpains are reported to account for the majority of cleavage effects on polyQ expanded disease proteins, several fragmentation events could not be explained by their proteolytic activity. An important group of enzymes to consider is the lysosomal cathepsins, which has been shown to process mutant huntingtin. An involvement of cathepsins-D, -B, -L, and -Z [75–77] has been indicated to produce fragments termed cp-A and cp-B [78]. For the cp-A fragment it was illustrated that the protease responsible for its formation has cathepsin-D-like properties in immortalized neurons and gamma-secretase-like properties in primary neurons, pointing to a cell type specific involvement of different proteolytic enzymes [79]. A further screen for identification of novel proteases using 514 protease-specific siRNAs detected 11 enzymes including three members of the matrix metalloproteinase (MMP) family cleaving huntingtin. When knocking down the most promising candidate MMP-10 in a striatal cell line cleavage of mutant huntingtin was prevented. In line with this work, MMPs were shown to be upregulated in HD mouse models and loss of function of *Drosophila* MMP homologs also ameliorated mutant huntingtin-induced neuronal dysfunction [80]. A very interesting novel explanation for the appearance of toxic fragments of huntingtin is that observed missplicing of huntingtin transcripts accounts for shortened N-terminal huntingtin variants [81]. A likewise fascinating attempt to explain ataxin-3 cleavage was done by showing that the intrinsic proteolytic property of ataxin-3's Josephin domain may lead to an autolytic processing of the disease protein [82]. However, C14A ataxin-3 mutants lacking proteolytic activity exhibited no differences neither in subcellular localization nor in proteolysis [62].

As a multitude of publications show that proteolytic processing of polyQ expanded proteins by a variety of enzymes represents a pivotal step in the molecular pathomechanism of polyQ diseases, modulating the activity of cleavage-responsible proteases or decreasing the levels of toxic fragments could be reasonable approaches for therapeutic treatment.

There are various ways to approach treatment. One method is to inhibit the proteolytic activity of caspases, calpains, cathepsins, or MMPs directly. Using such methods beneficial effects were achieved for HD [26, 46, 80, 83, 84] and SCA 3 [28, 61, 63, 65]. But attention should be paid to potential adverse effects as well [84]. A similar approach is to target the expression of endogenous inhibitors, such as calpastatin, as was done in SCA 3 [28, 29, 74].

A second approach is to modulate alternate pathways and achieve off-target benefits. Treating R6/2 mice with a tetracycline derivative delayed disease progression and death by reducing the levels of caspases-1 and -3 [42] through upstream regulation of Apaf-1 [85]. Reducing elevated calpain activity in HD mice also had beneficial off-target benefits [68, 69]. In addition, CDK5 was reported to act against caspase cleavage of huntingtin by phosphorylation at S434 [86]. In SCA 3, decreasing CDK5 levels via RNAi in *Drosophila* enhanced mutant ataxin-3 toxicity [64]. Another option is to use a genetic approach to modulate cleavage such as induction of exon 12 skipping in huntingtin pre-mRNA using oligonucleotides. This modification prevented the translation of the caspase-targeted region around amino acid 586 and thereby inhibited the formation of an N-terminal fragment implicated in HD toxicity [87].

3. Aggregation

As the pathological hallmark of polyQ diseases [1], aggregation has been widely discussed as therapeutic target. Although it serves as an easy readout for screens, cell models, and neuropathology, the exact role of aggregates in the neurodegeneration observed in polyQ diseases is still under debate. In the field of polyQ diseases, aggregates were identified as intranuclear inclusions in mouse models of Huntington's disease [52] and subsequently confirmed in HD patients [37, 88]. This was quickly followed by an identification of aggregates containing the disease protein in SCA 3 [32, 38] and SCA 1 [89] and cytoplasmic aggregates in SCA 6 [90], SCA 7 [54, 91], and SCA 17 [92]. In SCA 2, the initial reports of the absence of aggregation [93, 94] have since been challenged [95, 96].

What was initially observed as large fibrillar inclusions is most likely the end stage of protein aggregation and nucleation. The beginning steps feature monomeric species which transform into oligomeric structures and protofibrils/fibrils, although the correlation between these intermediates may not be linear. Some of these species may be direct pathway intermediates while others may not be directly relevant to the inclusion formation seen in patients [97]. Recent advances are being made and assays developed which will help in studying this pathway of aggregation, monomer addition, and isolating specific aggregate species [98]. Work in the field of HD on oligomer formation is bringing the field closer to understanding the mechanisms behind nucleation. The conversion of monomers to oligomers in HD is described as a packing of the N-terminal Htt segment into the oligomer core [98], elongation of fibrils follows, and a third step involves the ability of oligomers to seed monomer elongation. The work suggests that oligomer dissociation rates are similar to association rates and that oligomers serve as both on-pathway and off-pathway intermediates in fibril formation. It seems important to thus consider the aggregation pathway as an ebb and flow of intermediates which feed into multiple pathways. Ataxin-3 was also shown to have a multistep aggregation process where the first step involves the aggregation of the protein independent of the polyQ domain and a second step which is unique to the polyQ expansion and produces highly stable amyloid-like aggregates [99]. In a discussion about aggregation pathways, it is also important to note that kinetic differences between nucleation and protein folding in the nucleus and in the cytoplasm probably play a large role in the observed differences we see in inclusions between nuclear protein aggregates such as in SCA 1 and SCA 3 and cytoplasmic proteins such as SCA 2 and SCA 6 [100]. For HD, the study of the aggregation pathway pointed to

"at least three" aggregation pathways which can be influenced by various inhibitors, molecules, and interactions [101]. Inhibiting each pathway has different effects on neurotoxicity. The same was shown for ataxin-3 where different amyloid aggregates affect Ca^{2+} regulation by different mechanisms [102]. Altering the specific pathways of aggregation is a potential therapeutic strategy which may not decrease the total amount of aggregation but could decrease neurotoxicity.

A widely discussed topic is the exact cytotoxic nature of aggregates. By looking at the specific location of inclusions in patient brains, a discrepancy arose between the neurons which have the inclusions and the neurons which are known to degenerate [103]. In HD, the medium spiny neurons which are selectively lost present with much less aggregation than the large interneurons [103]. This and similar findings suggest that the large aggregates are protective. But the other work, such as in SCA 1, reiterates the relationship between aggregates and cytotoxicity. Patients who have a specific histidine interruption in the expanded polyQ tract of ataxin-1 have a decreased amount of aggregation and absence of disease [104]. The issue with such findings is that it does not provide insight into what is happening with intermediate oligomeric species which are more correlated to the onset of symptoms than to the formation of large protein aggregates [105, 106].

Just to highlight how complex it is to tease out the exact role of aggregates, in SCA 7 and SCA 6, different types of nuclear inclusions were identified. In SCA 7, they differ in their size, composition, and distribution of key proteins [54, 91] and detection with a p62 antibody found different subsets of cytoplasmic aggregates in SCA 6 [107]. Furthering our understanding of the interplay between neuronal types can tell us more about the effect of aggregation in specific populations and how that affects the health of surrounding cells. It is difficult to come to a conclusive decision on aggregation and to pull apart the protective properties from the cytotoxic ones without further information.

The discussion on toxicity of aggregates is also relevant for the screening of large libraries of therapeutic compounds or genetic modifiers. Using aggregation as readout is intuitive since if any part of the pathways of aggregation is toxic, then reducing the eventual product of large readout aggregates could also be considered reducing the intermediate toxic species. However, the field should be cautious about blocking the conversion of toxic oligomeric species to possible beneficial aggregates or shifting the balance of different conformation in an unfavorable direction [108]. It is also possible to look at increasing the overall rate of aggregation which could decrease the amount of time in which toxic intermediates can do damage but would cause an overall increase in total large aggregates. Targeting the depletion of specific species with antibodies or upregulated clearance is also a therapeutic possibility. Also, although extracellular aggregate transmission has not been proven for polyglutamine diseases, it could be possible to target the prion-like spread of smaller fibrils and oligomers [109, 110].

In general, a focus on aggregation has allowed the field to gain knowledge about various biological pathways involved in polyQ induced neurodegeneration. As previously described, cleavage plays a large role in the kinetics of aggregation and the mechanisms of toxicity. In the search for intermediate steps between proteolysis and aggregation, it was demonstrated in various cell models that polyQ-containing fragments or polyQ stretches themselves are generally able to form soluble oligomeric structures, mediating cytotoxicity and representing a starting point for subsequent aggregation [111–113]. These oligomeric species could also be identified in brain tissue of HD mouse models and patients [113, 114].

Looking at aggregates has also allowed us to see the recruitment of proteasomal subunits and look into the dysregulation of the ubiquitin system in neurodegeneration. But aggregation is slowly becoming an avoided topic in polyQ research. Hopefully recent advances in understanding aggregate intermediates will open a door to a better analysis of aggregation in neurodegeneration. This can lead to a renewed interest in understanding the complexity behind protein folding and nucleation in polyQ diseases.

4. Nuclear Transport

One aspect relevant to both of the aggregation of these proteins and to their general function is their ability to shuttle between the nucleus and cytoplasm. This transport modulates how they both perform their regular function and cause neurodegeneration. Nuclear transport encompasses many features of protein function such as transcription, avoidance of protein clearance machinery, import of a toxic fragment, and many other cellular processes. The current evidence suggests that the nucleus is a large site of toxicity in cells and blocking nuclear transport in animal models has shown that this pathway is a possible therapeutic target [109, 115, 116]. In general, nuclear entry is a highly controlled process and at the heart of that regulation is the nuclear pore complex which serves as a selective gatekeeper of entry [117]. The nuclear pore complex recognizes a group of proteins known as karyopherins which are carrying protein cargo for entry and exit out of the nucleus. Karyopherins recognize their cargo by the presence of specific nuclear localization signal (NLS) and nuclear export signal (NES) on proteins (reviewed by [118]). The most direct way for a protein to be transported by a karyopherin is to have an identifiable NLS or NES (or combination), but secondary features such as the visibility of this signal and posttranslational modifications such as phosphorylation and cleavage which alter the signal also play a large role.

Within the polyQ diseases discussed here, NES and/or NLS have been found for the disease proteins of SCA 1, SCA 3, SCA 7, and HD [11, 119–123].

In SCA 1, specifically, it has been shown that regulation of nuclear localization is relevant to disease progression and ataxin-1 stability. It was shown early on that blocking the NLS on ataxin-1 prevents the protein from causing neurodegeneration *in vivo* [11]. It was later explained that phosphorylation at S776 and the subsequent binding and release from 14-3-3 can mask the NLS, stabilize ataxin-1, and

modulate its localization [124] which is important for the nuclear interaction of ataxin-1 with splicing factors RBM17 and U2AF65 [125]. 14-3-3 is a protein that is involved in regulating many cellular processes by binding phosphorylation sites and the example of ataxin-1 demonstrates how factors outside of direct nuclear shuttling influence localization and affect the direct pathomechanisms of disease.

SCA 3 and HD have been the two most widely studied in the possible therapeutic regulation of transport to modify disease. The focus on nuclear transport has been a consequence of studies where fusing mutant fragments of Htt to exogenous NES prevented nuclear transport and inhibited the toxicity of the fragment [126, 127] and the reverse happened when it was fused to an NLS [126]. This work was reproduced in a mouse model which had a shorter lifespan correlated to an added NLS [128]. It has been difficult to tie together the cellular events that cause transport with the known pathways of nuclear entry. It is known, for example, that ataxin-3 and huntingtin enter the nucleus in response to cellular stress and heat shock, but the exact mechanism of transport is not elucidated [129–131]. Also, phosphorylation of both proteins has been linked to nuclear transport. Phosphorylation of huntingtin on N17 releases it from the endoplasmic reticulum to allow nuclear entry but also prevents export from the nucleus during stress response [132] and modulates its neurotoxicity [133]. In the case of ataxin-3, CK-2 dependent phosphorylation of S340 and S352 within the third UIM (ubiquitin interacting motif) has been suggested to control nuclear entry [134]. The current research is also focused on understanding the karyopherins involved in the recognition of the NLS and NES sites of these proteins with the aim of modulating disease. CRM1, or exportin-1, has been shown to interact with both ataxin-3 and huntingtin NES sites [132, 135] and suggested to be an exporter of ataxin-7 [122]. Karyopherins B1 and B2 have also been published as possible mediators of huntingtin localization which act on a putative huntingtin NLS [136]. Cellular and oxidative stress were shown to alter the activity of CRM1 and to affect the localization of polyQ proteins by posttranslational modifications of karyopherins or subsets of the nuclear pore complex [137].

Also of note is the importance of the NLS site in SBMA. The androgen receptor (AR) is kept in the cytoplasm by heat shock proteins which mask this nuclear localization site but, upon binding to the androgen ligand, the NLS is exposed and the androgen receptor translocates to the nucleus where it activates androgen-responsive genes (reviewed in [138]). The presence of the androgen receptor in the nucleus in the presence of the ligand is considered necessary for disease development as mice with an NLS deletion showed delayed onset of phenotype and reduced motor deficit [139]. It is important to note this nuclear function of the AR as the proteins in SCA 1, SCA 3, and HD may also have similar important roles in the nucleus, although aggravating their nuclear presence may overwhelm those beneficial roles and cause neurotoxicity.

In those polyQ diseases where an NLS or NES has not been identified, localization of the protein has still proved to be important to pathogenesis. Recent work using a polyQ antibody has demonstrated that the localization of ataxin-2 within the cell corresponds to disease stages of SCA 2. Cytoplasmic presence corresponded to early stage and nuclear presence and aggregation to final stages of the disease [140]. The mislocalization of ataxin-2 has also been shown to be a potent modifier of ALS/TDP43 toxicity [141] and it has also been suggested that ataxin-2 is important for SCA 3 neurodegeneration. This points to the possibility that the localization of ataxin-2 is important in modulating other neurodegenerative diseases [142]. In SCA 6, the C-terminal peptide of the alpha 1A subunit of the P/Q-type voltage-gated calcium channel with the expanded polyQ tract is also toxic to cells depending on its nuclear localization [143]. Although the exact mechanism behind this translocation is not known, the current hypothesis is that it is important for disease progression.

One way to affect localization is to target the polyQ expansion of the protein. It was shown that the expansion of the CAG repeat in Htt reduces its interaction with Tpr, a nuclear pore protein, which is involved in nuclear export [144] and the expansion of ataxins-3 and -7 has also been linked to nuclear retention [122, 145].

Overall, nuclear trafficking and localization are a summation of many processes that happen within the cell starting from cleavage of the protein, aggregation, modulation of mitochondrial response, and involving all functions of the protein such as transcriptional regulation. The list of proteins with altered subcellular localization in neurodegeneration includes NFkB, ERK1/2, TDP43, Smad, E2F1, CREB, and many others [146]. Because of this wide breadth of cellular mechanisms involved in nuclear localization, it should always be considered an aspect of therapeutic intervention.

5. Clearance Mechanisms

It is known that polyQ proteins are associated with the formation of intracellular aggregates, possibly through the formation of toxic fragments, but the important question of what clearance mechanisms are involved remains. The two main clearance routes of organelles and proteins in eukaryotic cells are the ubiquitin-proteasome system (UPS) together with heat shock response and the autophagy-lysosomal pathway. While proteasomes predominantly degrade short-lived nuclear and cytoplasmic proteins as well as misfolded and unfolded proteins from the endoplasmic reticulum, the autophagic system can degrade organelles and cytoplasmic protein complexes [147, 148].

The interplay of heat shock proteins, chaperones, and the UPS is important for protein clearance [149]. During oxidative or cellular stress heat shock proteins are dramatically upregulated. They bind to misfolded proteins and remodel them back to their native formation. If refolding is not possible, degradation by the proteasome is initiated. Failure in one of the systems can be compensated partially by the upregulation of the other, but prolonged failure results in protein aggregation and dysfunctional homeostasis of cells [150]. Many wild-type ataxins as well as huntingtin have been shown to interact with components of the UPS under

normal conditions. Yeast two hybrid assays demonstrated an interaction of ataxin-3 and the ubiquitin and proteasome binding factors HHR23A and HHR23B [151, 152]. Ataxin-1 was shown to interact with the ubiquitin-like protein A1Up [153], the ubiquitin-specific protease USP7 [154], and the E2 ubiquitin-conjugation enzyme UbcH6 [155, 156]. Moreover, ataxin-7 was indicated to interact with the S4 subunit of the 19S proteasome [157].

In line with the fact that normal function of polyQ proteins involves interaction with the quality control system is the knowledge that molecular heat shock proteins, ubiquitin, and proteasomal subunits are found in neuronal aggregates in postmortem brains of patients. In HD patients and animal models, aside from the N-terminal part of mHtt, ubiquitin, molecular chaperones including GRP78/BiP, HSP70, and HSP40, and the 20S, 19S, and 11S subunits of the 26S proteasome were also found ([37, 158], reviewed in [159]). Similar results were described for SCA 1 [160], for SCA 3 [161], and for SCA 7 [54, 157]. Together, these findings indicate that ubiquitin, heat shock proteins, and subcomplexes of the 26S proteasome are redistributed to the site of polyQ protein degradation.

The carboxyl terminus of the HSC70-interacting protein (CHIP) is a HSP70 cochaperone as well as an E3 ubiquitin ligase that protects cells from proteotoxic stress. The ability of CHIP to interact with HSP70 and function as a ubiquitin ligase places CHIP in a pivotal position in protein quality control [162] and makes CHIP a frequently analyzed protein in polyQ refolding and degradation. It was shown that CHIP directly interacts and colocalizes to ataxin-1, ataxin-3, and huntingtin aggregates [163, 164]. Additionally, CHIP promotes ubiquitination of wild-type and mutant ataxins-1 and -3 and huntingtin as well as decreasing steady state levels of mutant ataxins-1 and -3 and huntingtin by inducing degradation. Therefore, CHIP suppresses aggregation and toxicity in cell culture and *Drosophila* [163, 164]. Suppression of CHIP resulted in an increased formation of microaggregates and toxicity in a SCA 3 transgenic mouse model [165]. Moreover, overexpression of CHIP together with ataxin-1 led to reduction of ataxin-1 solubility and thus increased formation of aggregates [166]. Another HSP70-dependent E3 ligase that is shown to act redundantly to CHIP on some substrates is parkin [167]. Parkin (*PARK2*, mutated in an autosomal recessive form of PD), which mediates the targeting of proteins for proteasomal degradation, is known to interact and modulate ataxin-2 and ataxin-3 but not ataxin-1 [166, 168–171]. Wild-type and polyQ expanded ataxin-3 deubiquitinate parkin directly and parkin ubiquitinates and facilitates the clearance of wild-type and mutant ataxin-2 and ataxin-3 by proteasomal degradation [168–170]. Additionally, it was demonstrated that parkin forms a complex with the expanded polyQ protein, HSP70, and the proteasome. This decreases cytotoxicity in SCA 2 and SCA 3 by reducing proteasomal impairment. No direct interaction of huntingtin and parkin has been described to date although studies confirmed the colocalization of parkin and huntingtin in mouse brain as well as in patient samples [168]. Additionally, a partial suppression of parkin in an HD mouse model slightly aggravates the neurological phenotype [172]. The interaction or modulation of polyQ disease proteins by parkin can offer an explanation of the parkinsonian phenotype in SCA 2 and SCA 3. Also it is noteworthy that ataxin-1 interacts and is modulated by an E2 ubiquitin-conjugation enzyme, called UbcH6, which regulates the transcriptional repression of expanded ataxin-1 and the rate of ataxin-1 degradation [155, 156]. The binding and ubiquitination of huntingtin by the E2 ubiquitin-conjugation enzyme E2-25K is not influenced by the length of the polyQ stretch [173]. But it is shown that the expression of E2-25K modulates the aggregation and toxicity of mutant huntingtin and that E2-25k is recruited to aggregates in HD and SCA 3 patients [174]. Together these findings indicate a clear influence and impairment of the UPS in all polyQ diseases discussed with the exception of SCA 6. Here, the proteasome has not been implicated in disease progression and there is no evidence for the ubiquitination of aggregates.

Unfolding and remodeling of proteins is necessary for them to pass through the narrow pore of the proteasome barrel, which thus precludes clearance of oligomers and aggregated proteins [175]. A number of polyQ diseases have been associated with decreased chaperone and proteasome activity in patients, cell, and animal models of SCA 1, SCA 3, SCA 7, SCA 17, and HD [176–182]. Nonetheless, there was work demonstrating that, in a SCA 7 knock-in mouse model, no significant impairment of the UPS was found [183]. Also, in recent studies on HD degradation, rapid and complete clearance of polyQ expanded huntingtin in neuronal cells and *in vitro* was shown [184] and dynamic and reversible recruitment of proteasomal subunits into inclusion bodies was observed in living cells [185]. In addition, several groups demonstrated that inhibition of the proteasome in cell culture and mammalian cells results in increased aggregation and cytotoxicity in SCA 3 and HD [181, 186], whereas an overexpression of p45 (ATPase of 19S subunit of proteasome) stimulates degradation of ataxin-3 [187]. Whether the proteasomal enzymatic machinery is able to cleave between successive glutamine residues remains unclear [184, 185, 188–190].

One widely accepted theory is that degradation of misfolded polyQ proteins is a team effort between autophagy and the UPS. Besides the above mentioned involvement of the UPS it is known that the aggregation-prone polyQ proteins and fragments strongly depend on autophagy for their clearance [191]. In SCA 7, the unmodified truncated protein was shown to be degraded via macroautophagy *in vitro* [192] and it was shown that macroautophagy and proteasomal degradation play a role in degrading mHtt [76, 184]. In these studies they demonstrated that blocking autophagy resulted in reduced cell viability and increased number of aggregates and stimulating autophagy promoted clearance of wild-type and mutant huntingtin as well as its caspase derived N-terminal fragment of huntingtin [76]. Specifically targeting the N-terminal huntingtin for the UPS decreased its levels and thus decreased aggregation [184]. Furthermore, it was shown that a polymorphism in an autophagy related gene (ATG7) modulates the age at onset of HD patients [193, 194].

For SCA 1, SCA 3, SCA 6, and SCA 7 an increased susceptibility of cytoplasmic aggregates to autophagic degradation

was shown compared to nuclear polyQ inclusions [195–200]. Impairment of the autophagic system is demonstrated by an increased number of autophagosomes, endosomal-lysosomal-like organelles, and multiple vesicular bodies. This was shown in brain and lymphoblasts of HD patients and in primary neurons and brain of HD transgenic mice [52, 201–203]. Characterization of a SCA 1 transgenic mouse model also indicated changes in the autophagic flux by vacuolar formation with autophagic origin and significant altered LC3-II/-I ratio [204]. Similar results were found in ataxin-7 transgenic mice where LC3 levels were significantly altered and wild-type ataxin-7 levels were stabilized by autophagy whereas no stabilizing effects were described for mutant ataxin-7 [196]. Additionally, it was shown that full-length and cleaved fragments of ataxin-7 are differentially degraded. While full-length wild-type and mutant ataxin-7 was primarily found in the nucleus and therefore degraded by the UPS, fragments of ataxin-7 which were located in both the cytoplasm and nucleus were found to be degraded similarly by autophagy and the UPS [197]. Pharmacological activation of autophagy by treatment with a p53 inhibitor led to increased autophagic activity together with reduced ataxin-7 toxicity and therefore represents a possible therapeutic approach in the treatment of SCA 7 [205].

p62 acts as a cargo receptor for degradation of ubiquitinated targets by autophagy [206]. Studies in human post-mortem brain samples from SCA 3, SCA 6, and HD patients revealed p62 positive cytoplasmic, axonal, and nuclear aggregates. This again indicates an involvement of the autophagic system in the clearance of aggregated polyQ proteins [107, 207, 208]. p62 also contributes to recruitment of proteasomes to nuclear aggregates of ataxin-1 and to the degradation of ataxin-1 [209]. As discussed earlier, mammalian proteasomes may not be able to cleave (polyQ) sequences and seem to release polyQ-rich peptides. An initial study about a cytosolic enzyme called puromycin-sensitive aminopeptidase (PSA) showed that it is able to digest polyQ sequences [210]. However, in cultured cells, *Drosophila*, and mouse muscles, PSA overexpression decreased aggregate content and toxicity of mutant huntingtin and mutant ataxin-3 by enhancing autophagy [211].

As discussed earlier in this review, aggregates including polyQ protein fragments are believed to cause neuronal death. Therefore, reducing the amount of aggregates is an important therapeutic strategy. This reduction can be achieved by enhancing the above described mechanisms: chaperone mediated refolding of polyQ proteins or degradation of misfolded proteins by autophagy or the UPS. Heat shock proteins were shown to accumulate in aggregates of HD, SCA 1, SCA 3, and SCA 7 and this led to an interest in modulating the molecular chaperone machinery as a possible therapeutic strategy for polyQ diseases. An overexpression of HSP40/HDJ-2 suppressed ataxin-3 and ataxin-1 aggregation *in vitro* [3, 160], but not in huntingtin exon 1 overexpressing cell lines [185]. Moreover, modulation of the chaperone system in HD, SCA 1, SCA 3, and SCA 17 studied *in vitro* [212, 213], yeast [214], *C. elegans* [215], *Drosophila* [216], mammalian cells [186, 217–219], and animal models [220–226] demonstrated controversial results. As the overexpression of single members or the combination of different members of the molecular chaperone system gave controversial and transient effects, the development of combinatorial therapies was proposed. Combining treatment with histone deacetylase (HDAC) inhibitors was promoted in recent years. It was shown that the oral administration of 17-(allylamino)-17-demethoxygeldanamycin (17-AAG) markedly suppressed eye degeneration, inclusion formation, and lethality in a SCA 3 *Drosophila* model and also neurodegeneration in an HD *Drosophila* model by induction of HSP70, HSP40, and HSP90 expression [227]. Valproic acid (VPA) an antiepileptic drug which also acts as an HDAC inhibitor and promotes expression of small molecules including HSP70 was shown to alleviate the phenotype of SCA 3 in *Drosophila* [228] and in HD transgenic mice [229]. Furthermore, a combined treatment of lithium (induces autophagy and downregulates HDAC1) and VPA produced several beneficial effects and prolonged median survival in HD transgenic mice [230]. In HD patients, valproic acid is discussed to have beneficial effects on psychiatric symptoms [231] but was also shown to have side effects like developing Parkinson's syndrome with an axial dystonia [232]. The HDAC inhibitor sodium butyrate was shown to delay the onset, ameliorate the neurological phenotype, improve the survival in SCA 3 transgenic mice, and improve the survival of neurons in an ataxin-7 cell model [55, 233]. An analog of this compound, sodium phenylbutyrate, was successfully tested in HD mice [234] and was shown to be safe and well tolerated by HD patients [235], but a phase II clinical trial (started 2006) was abandoned with no cited results.

Although attempts at modulating the proteasome system have been made, upregulation of this pathway is challenging and thus attention has shifted to enhancing autophagy [236]. In polyQ diseases, it has been demonstrated that modulation of one system has direct effects on the other. An HSP90 inhibitor (17-DMAG) resulted in a reduction of neuropathology in a SCA 3 transgenic mouse model although the biggest induction was of LC3-II and beclin and not in heat shock proteins as expected [237]. Beclin modulation has been previously shown to rescue motor symptoms and ataxin-3 clearance in a lentiviral-based rat model [199, 200] and in HD cell culture and primary neurons [237, 238].

Autophagy can also be upregulated by mTOR-(mammalian target of rapamycin-) dependent and mTOR-independent pathways. Autophagy can be induced in all mammalian cell types by rapamycin, an inhibitor of mTOR. Rapamycin treatment of cells expressing aggregation-prone polyQ disease proteins enhanced the degradation of polyQ proteins, reduced the number of aggregates, and protected cells, flies, and mice from mutant protein-associated degradation in SCA 3 and HD [239–241]. Lithium, which is normally used to treat bipolar disorders, was shown to have beneficial effects in polyQ diseases by an mTOR-independent pathway. It targets various intracellular enzymes, including glycogen synthase kinase 3β and inositol monophosphatase by lowering inositol and IP3 levels [242]. Induction of autophagy by lithium led to enhanced clearance of autophagy substrates, like mutant huntingtin fragments as well as mutant ataxin-1 and ataxin-3 *in vitro*, in

Drosophila and mouse models [240, 243–246]. Additionally, a combinatory treatment of lithium and rapamycin protected an HD *Drosophila* model against neurodegeneration by enhancing macroautophagy [247]. Other substances having a beneficial effect on mutant huntingtin toxicity and clearance by activating an mTOR-independent pathway are rilmenidine and trehalose [248]. Trehalose together with rapamycin again showed an additive effect on the clearance of mutant huntingtin [249]. Very recently, the first nanomedical approach in treating HD was presented. It was demonstrated that europium hydroxide nanorods reduced huntingtin aggregation by inducing autophagic flux [250].

6. Mitochondrial Dysfunction

As the field of research in polyQ diseases is progressing, more is understood about the common mechanisms behind neurodegeneration. Over the last decade an emerging role in the pathogenesis of several neurodegenerative disorders such as Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS) [251, 252] has been assigned to mitochondrial dysfunction and impaired energy metabolism. This can be explained by the high energy demands of neuronal cells and their inability to produce ATP by glycolysis and hence dependence on functional mitochondria for oxidative phosphorylation. Recent findings also support the involvement of dysfunctional mitochondria in polyglutamine diseases. Most insights were gained in the field of Huntington's disease but several studies also highlight the role of mitochondria in the pathology of spinocerebellar ataxias.

Metabolic defects and loss of body weight at early stages of the disease are well described symptoms of polyQ disease patients in HD [253, 254], SCA 1 [255], and SCA 3 [256] as well as in the respective disease mouse models [9, 41, 257]. For HD and SCA 3 patients, an inverse correlation between body mass index and CAG repeat number was reported [256, 258]. In SCA 1 patients this weight loss appears despite a balance between energy intake and expenditure and patients show an increase of energy expenditure and fat oxidation at a resting state which might be a cause of altered autonomic nervous system activity and gait ataxia [255].

Another common feature of polyQ diseases is metabolic alterations. Advanced magnetic resonance imaging techniques are used to study alterations in metabolite concentrations in distinct brain regions of patients and mouse models. Increased lactate production was found in cortex and basal ganglia of HD patients [259] while cerebellum and brain stem of SCA 1 patients showed decreased total NAA (N-acetylaspartate + N-acetylglutamate, tNAA) concentrations and elevated glutamine, total creatine, and myoinositol concentrations compared to controls [260, 261]. The levels of tNAA and myoinositol correlated with patients' ataxia scores. Similar changes in metabolite concentrations were seen in conditional SCA 1 and a SCA 1 knock-in mouse models. Interestingly, the metabolite levels almost went back to baseline when expression of the transgene was suppressed at early stages of the disease in the conditional mouse model and alterations in metabolite levels were observed in knock-in mice months before any pathology was detected [261, 262].

Apart from alterations in metabolite concentrations, oxidative stress and changes in ATP production caused by deranged respiratory chain complex activities indicate mitochondrial dysfunction in polyQ disease. As previously reviewed, HD patients show reduced complexes II, III, and IV activities in putamen and caudate, while alterations in complex I activity were found in muscles only [263]. Several studies also point to dysfunctional respiratory chain complex and increased oxidative stress in SCA 2, 3, and 12 [264–270]. Decreased complex II activity was found in lymphoblasts from SCA 3 patients, in cells from transgenic mice and in SCA 3 cell models [269]. In cells expressing human, polyQ expanded ataxin-3, decreased activities of the antioxidant enzymes catalase, glutathione reductase and superoxide dismutase, and consequently mitochondrial DNA damage were detected [266]. Similar findings of increased catalase levels and DNA damage were gained from SCA 3 patient samples compared to healthy controls [270]. A recent study also suggests that the disease characteristic aggregates can be reduced in a neuronal SCA 3 cell model by treatment with an extract of *Gardenia jasminoides* which was shown to reduce the production of reactive oxygen species [271].

While the precise pathways which lead to the observed problems in mitochondrial bioenergetics remain elusive, localization of polyQ disease causing proteins to the mitochondria and their actions at the mitochondria have been subjects of intensive research. For SCA 3, it is known that both normal and polyQ expanded ataxin-3 localize to mitochondria [62] and that degradation of polyQ expanded ataxin-3 via the UPS is promoted by an ubiquitin ligase in the outer mitochondrial membrane called MITOL [272]. Localization to the mitochondria was also shown for mutant huntingtin. Also, mitochondria from HD patient lymphoblasts and from brain of transgenic mice expressing full-length mHtt had decreased membrane potential and defects in mitochondrial calcium handling [273].

An important role in regulating mitochondria mediated cell death in polyQ disease has been ascribed to the B-cell lymphoma 2 (Bcl-2) family of proteins. These proteins regulate the permeability of the outer mitochondrial membrane and thereby control cell survival, morphology, dynamics, and membrane potential of mitochondria. Bcl-2 family members can be both prosurvival and proapoptotic. The main family members inhibiting cell death are Bcl-2 and B-cell lymphoma-extra large (Bcl-xL) while the BH3-only proteins Bax and Bcl-2 antagonist (Bak) form pores in the mitochondrial membrane and thus initiate apoptosis. For SCA 3 and SCA 7 it was shown that the mRNA and protein levels of Bcl-xL were downregulated in cerebellar neurons when polyQ expanded ataxin-3 and ataxin-7, respectively, were overexpressed leading to activation of caspase-3 and caspase-9, two main caspases involved in mitochondrial induced apoptosis [53, 274]. Recently, it was shown that a direct interaction between ataxin-3 and Bcl-xL exists and suggested that ataxin-3 promotes the interaction between Bcl-xL and Bax [274]. SCA 3 and SCA 7 *in vivo* models also showed increased levels of Bax mRNA and protein which

can be explained by increased levels of active phospho-p53, a transcription factor known to enhance the transcription of Bax [53, 274–276]. Similarly, Bax levels were found to be increased in HD cell and mouse models [51, 268, 277] as well as in the caudate nucleus of HD patients compared to healthy individuals [278]. Moreover, polyQ expanded ataxin-3 was found to decrease mRNA and protein levels of the prosurvival Bcl-2 by affecting Bcl-2 mRNA stability [279, 280]. For HD, the alterations of Bcl-2 levels remain controversial. While expression of mHtt decreased Bcl-2 protein levels in different cell lines and in brain of HD mouse models [281–283], other studies did not find alterations in well studied models like R6/1 [284].

PolyQ proteins are also known to influence the transcription of multiple genes coding for important mitochondrial proteins. One example is the impairment of peroxisome proliferator-activated receptor-γ (PPAR-γ) coactivator-1α (PGC-1α) expression and function. PGC-1α is a transcriptional master coactivator controlling mitochondrial biogenesis, metabolism, and antioxidant defense [285–287]. Alterations in levels and activity of PGC-1α have been found in HD patients and mouse models [288, 289] and polymorphisms of PGC-1α have been described to modify the age at onset in HD patients [290]. PGC-1α has also been considered a potential therapeutic target by showing that PGC-1α levels were restored and phenotype and survival of HD mice were improved by treatment with bezafibrate, a pan-PPAR agonist [291]. While PGC-1α emerges as an important player in HD pathogenesis, little is known about the involvement of this master coactivator in other polyQ disorders. The question also remains: whether this mechanism is exclusive to HD or is a common feature of many polyQ diseases.

Apart from changes in mitochondrial bioenergetics and transcription of important proteins associated with mitochondrial function and cell death, alterations in shape and motility of mitochondria have been observed in HD. Both retrograde and anterograde mitochondrial transport along axons were shown to be impaired by mHtt in cultured neurons of mouse and rat models [292, 293]. While fragmented mitochondria have been reported for many HD cell models and patients over the last decades, recent studies link this observation to GTPase dynamin related protein-1 (DRP-1). DRP-1 is one of the shaping proteins which regulate mitochondrial fission and fusion. Costa et al. [294] described a higher basal activity of calcineurin which phosphorylates DRP-1 and thereby increases its activity and translocation to mitochondria thus leading to mitochondrial fragmentation in HD models. A direct interaction between mHtt and Drp-1 and an increased enzymatic activity were also shown in brain tissue of HD patients and an HD mouse model [295]. Since the balance between fission and fusion is known to be crucial for mitochondrial function and since neuronal death caused by increased mitochondrial fragmentation has been reported for other neurodegenerative disorders like AD and PD [251], it seems that a better understanding of this pathway would be insightful into understanding the mechanisms and possible therapeutic opportunities in polyglutamine diseases.

7. Concluding Remarks

The neurodegenerative disorders belonging to the group of polyglutamine diseases reviewed here share features such as an inverse correlation of the CAG length with age at onset, neurological features as main presentations of the disease, and an autosomal dominant mode of inheritance. The polyglutamine expansion in these unrelated proteins converges them into common pathogenic mechanisms which can result in corresponding therapeutic interventions. In this review we describe these pathways and possible points of therapeutic entry. First, it is possible to target the stability and conversion of the expanded protein by enhancing protein refolding and degradation or preventing proteolytic cleavage and creation of the toxic fragment. Another option is to decrease the ability of the protein to reach the site of toxicity by altering its ability to translocate between the nucleus and cytoplasm. Enhancing the lysosomal and proteasomal degradation and facilitating autophagic aggregate clearance are exciting current prospects for therapy. Also, modifying the pathways of aggregation remains a viable therapeutic approach as does facilitating mitochondrial health and function. Overall, the field of polyglutamine disease offers many possibilities for disease intervention (Figure 1), although no current therapy is available.

Abbreviations

AD: Alzheimer's disease
ALS: Amyotrophic lateral sclerosis
AR: Androgen receptor
ATP: Adenosine triphosphate
Bak: Bcl-2 homologous antagonist/killer
Bax: Bcl-2-associated X protein
Bcl-2: B-cell lymphoma 2
Bcl-xL: B-cell lymphoma-extra large
CAST: Calpastatin
CDK: Cyclin-dependent kinase
CHIP: C-terminus of the HSC70-interacting protein
DRP-1: GTPase dynamin related protein-1
DRPLA: Dentatorubral-pallidoluysian atrophy
HD: Huntington's disease
HDAC: Histone deacetylase
HSP: Heat shock protein
Htt: Huntingtin
iPSC: Induced pluripotent stem cell
mHtt: Mutant huntingtin
MITOL: Mitochondrial ubiquitin ligase
MMP: Matrix metalloproteinase
MSNs: Medium spiny neurons
mTOR: Mammalian target of rapamycin
NES: Nuclear export signal
NLS: Nuclear localization signal
NMDAR: N-Methyl-D-aspartate (NDMA) receptor
PSA: Puromycin-sensitive aminopeptidase
PD: Parkinson's disease

polyQ: Polyglutamine
PGC-1α: Peroxisome proliferator-activated receptor-γ (PPAR-γ) coactivator-1α
SBMA: Spinal-bulbar muscular atrophy
SCA: Spinocerebellar ataxia
TBP: TATA-binding protein
tNAA: N-Acetylaspartate + N-acetylglutamate
UIM: Ubiquitin interacting motif
UPS: Ubiquitin-proteasome system
VPA: Valproic acid.

Conflict of Interests

Any financial conflict of interests is disclosed by all sides.

Authors' Contribution

All authors contributed equally to this study.

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Review Article

Magnetic Resonance Spectroscopy: An In Vivo Molecular Imaging Biomarker for Parkinson's Disease?

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Parkinson's disease (PD) is a neurodegenerative disorder caused by selective loss of dopaminergic neurons in the substantia nigra pars compacta which leads to dysfunction of cerebral pathways critical for the control of movements. The diagnosis of PD is based on motor symptoms, such as bradykinesia, akinesia, muscular rigidity, postural instability, and resting tremor, which are evident only after the degeneration of a significant number of dopaminergic neurons. Currently, a marker for early diagnosis of PD is still not available. Consequently, also the development of disease-modifying therapies is a challenge. Magnetic resonance spectroscopy is a quantitative imaging technique that allows in vivo measurement of certain neurometabolites and may produce biomarkers that reflect metabolic dysfunctions and irreversible neuronal damage. This review summarizes the abnormalities of cerebral metabolites found in MRS studies performed in patients with PD and other forms of parkinsonism. In addition, we discuss the potential role of MRS as in vivo molecular imaging biomarker for early diagnosis of PD and for monitoring the efficacy of therapeutic interventions.

1. Introduction

Parkinson's disease (PD) is the most common neurodegenerative movement disorder. The disease affects approximately 7 million people globally and has a very high socioeconomic impact. Clinically, PD is characterized by bradykinesia, akinesia, muscular rigidity, postural instability, and resting tremor, including also nonmotor symptoms such as cognitive and psychiatric impairment [1]. Neuropathological hallmarks of PD are degeneration of dopaminergic neurons in substantia nigra pars compacta (SNc) and formation of inclusions called Lewy bodies, mainly composed of α-synuclein, within the central and peripheral nervous system [2, 3]. The loss of dopaminergic neurons in the SNc results in decreased levels of dopamine in the putamen of the dorsolateral striatum, leading to dysfunction of direct and indirect pathways of movement control that involve corticobasal ganglia-thalamocortical loops [2].

In PD, the patients fulfill the clinical criteria when approximately 60–70% of nigrostriatal neurons are degenerated and 80% of content of striatal dopamine is reduced [4]. In the "preclinical" PD phase nonmotor symptoms, such as olfactory dysfunction, constipation, rapid eye movement behavior disorder, mood disorders, and depression, precede motor symptoms reflecting the dysfunction of dopaminergic or nondopaminergic neurons. This clinical condition describes a stage of disease called "prodromal" [5]. Detection of prodromal PD phase is becoming an important goal for determining a definite diagnosis and for choosing a suitable treatment strategy. Currently, PD treatment is symptomatic. One of the major challenges in PD is the development of disease-modifying therapies, such as neuroprotective or cell-base restorative agents. The clinical symptoms appear after the degeneration of a significant number of dopaminergic neurons and, in this advanced stage, the disease-modifying therapies may be ineffective to attenuate progression of the neurodegeneration. Thus, the identification of specific and

sensitive biomarkers is extremely important to facilitate early and differential diagnosis, monitor disease progression, and assess efficacy of current and future treatments.

Magnetic resonance spectroscopy (MRS) is a noninvasive imaging technique, for exploration in vivo of intracellular metabolic status, and may provide a neuroimaging biomarker of normal biological and pathological processes or response to a therapeutic intervention. This paper presents an overview of MRS and its implications for detection in vivo of neurodegeneration processes in PD. In addition, the potential role of MRS as in vivo molecular imaging biomarker to confirm early and differential PD diagnosis and to assess response to therapy is discussed.

We performed a review of peer-reviewed literature using Pubmed/Medline. The search was limited to studies reported in English language and published from January 1990 to February 2014 (Tables 1, 2, and 3). A combined search was performed using the following terms: magnetic resonance spectroscopy, proton magnetic resonance spectroscopy, phosphorus magnetic resonance spectroscopy, Parkinson's disease, atypical parkinsonian disorders, progressive supranuclear palsy, multiple-system atrophy, and corticobasal degeneration, N-acetylaspartate, differential diagnosis, and dopaminergic therapy. We selected the articles according to the following criteria: (1) MRS studies that involved humans; (2) studies that compared spectra of PD patients to those of healthy controls; and (3) single and multivoxel MRS studies.

2. Proton Magnetic Resonance Spectroscopy

MRS is a noninvasive imaging technique that can be used to measure the concentrations of different low-molecular weight chemicals. The technique is based on the same physical principles of magnetic resonance imaging (MRI), that is, the detection of energy exchanges between external magnetic fields and specific nuclei within atoms. MRS is used in vivo for the study of different nuclei, including ^{1}H, ^{31}P, ^{13}C, ^{15}N, ^{19}F, and ^{23}Na. The main nucleus studied today in neurospectroscopy is ^{1}H, which provides information on markers of neurons, myelin, energy metabolism, and other metabolically active compounds.

The metabolites detectable with proton MRS (^{1}HMRS) include the prominent resonances of N-acetylaspartate (NAA), choline-containing compounds (Cho), creatine + phosphocreatine (Cr), myoinositol (mI), lactate (Lac), and a variety of other resonances that might not be evident depending on type and quality of spectra as well as on the pathological condition [6].

NAA, which resonates at 2.02 parts per million (ppm), represents the largest proton metabolic concentration in the human brain after water. Indeed, the concentration of NAA reaches the order of 10 μmol/g. NAA is widely interpreted as a neuronal marker and implicated in several neuronal processes, including lipid and protein synthesis, mitochondrial functioning, and osmoregulation. NAA synthesis occurs in mitochondria and requires acetyl-CoA and L-aspartic acid as substrates. NAA has been proposed to serve as a mitochondrial shuttle of acetyl-CoA used for fatty acid synthesis. NAA is reduced in many brain disorders in presence of neuronal or axonal loss. The Cho peak (3.2 ppm) represents a combination of several choline-containing compounds, including free Cho, phosphorylcholine, and glycerophosphorylcholine, and to a small extent acetylcholine. Free Cho acts as a precursor to acetylcholine, while glycerophosphorylcholine is a product of breakdown of membrane phosphatidylcholine and acts as an osmoregulator. The concentration of Cho is relatively low on the order of 0.5 to 1.5 μmol/g. The Cho peak is often viewed as a marker of membrane turnover or inflammation in ^{1}HMRS studies. The Cr peak (3.03 ppm) is composed of creatine and phosphocreatine. These metabolites buffer the energy use and energy storage of cells. The concentration of total Cr is estimated on the order of 8 to 9 μmol/g. Cr concentration is often used as an internal standard because it is considered to be relatively stable, showing slow increase with age. Thus, total Cr is often used as an internal reference (i.e., a denominator in metabolite signal ratio). The mI peak (3.56 ppm) represents the presence of myoinositol and myoinositol phosphate. MI is suggested as a glial marker, osmoregulator, intracellular messenger, and detoxifying agent. The Lac (1.3 ppm) is an end product of anaerobic glycolysis; thus the increase in Lac concentrations often serves as an index of altered oxidative metabolism, that is, in ischemia, hypoxia, and cancer [6]. The amino acids glutamine (Glu), glutamate (Gln), and γ-aminobutyric acid (GABA) (2.1–2.4 ppm) are involved in excitatory and inhibitory neurotransmission. MRS at high field strengths improves the quantitation of these compounds [7]. MRS is implemented as single-voxel and multivoxel method. Single-voxel spectroscopy detects the signal from a single region during one measurement, whereas multivoxel or MR spectroscopic imaging (MRSI) or chemical shift imaging (CSI), using additional phase-encoding pulses, obtains the signal from multiple regions at the same time and provides the information of spatial distribution of major cerebral metabolites [8]. The metabolite concentrations are expressed in terms of semiquantitative ratios such as NAA/Cr, NAA/Cho, Cho/Cr, and mI/Cr. In relative quantification, one of the metabolite peaks measured is used as the concentration standard and serves as the denominator of peak ratios. As a result, the total number of quantifiable metabolites is decreased by one. Furthermore, alterations in the peak ratio do not necessarily reflect a change in the concentration of the numerator. The alteration may be caused by change in the concentration of the numerator, the denominator, or both or may be due to changes in relaxation behavior. The assumption that the concentration of certain reference metabolites (e.g., total creatine and choline) remains constant may be incorrect under normal conditions, as well as in many pathologic states. It is therefore advisable to obtain concentration expressed in standard units (such as millimoles per kilogram wet weight) by applying absolute quantification.

MRS is applied to help researchers in the understanding of pathophysiological mechanisms and clinicians in the diagnosis and follow up of neurological disorders. Currently, in care of PD patients, MRS coupled with a careful clinical

assessment is assuming a certain importance for the differential diagnosis of PD with initial motor symptom from atypical parkinsonian disorders (APDs), supporting the diagnostic accuracy of the neurologist's assessment, and, consequently, improving the disease management.

3. Metabolic Changes Detected by ^{1}HMRS in Parkinson's Disease

The first ^{1}HMRS studies were designed to identify possible alterations of metabolic status of cortical-basal ganglia structures involved in motor dysfunctions in PD patients versus healthy control subjects (Table 1). Abnormal ^{1}HMRS spectra were reported in basal ganglia. In particular, a significant reduction of NAA/Cho ratios was found in the lentiform nucleus of PD patients compared with control subjects [9]. Choe et al. [10] showed asymmetric decrease of NAA/Cr ratios in the contralateral SN to the symptomatic side in PD with unilateral symptoms. However, a study reported a significant increase of total Cr levels in prefrontal cortex, but no change in NAA and Cho in SN of PD patients [11]. Other studies reported metabolic alterations also in cortical structures. Reduced NAA and Cho levels in temporoparietal cortex [12, 13] and reduced NAA levels in motor cortex [14], posterior cingulated cortex [15], and presupplementary motor area [16] were observed. Some studies reported no metabolite differences between the PD patients and the control subjects in either metabolite ratios or absolute concentration of NAA, Cho, and Cr in cortical-basal ganglia loop [17–20]. A ^{1}H-MRSI study of Tedeschi et al. [19] found that there were no significant differences between PD patients and control subjects of NAA, Cho, and Cr ratios in brainstem, caudate, thalamus, lentiform nucleus, and association cortices. These findings were in agreement with previous single-voxel ^{1}H-MRS studies that showed no significantly reduced NAA in lentiform nucleus [17] and putamen and thalamus [18].

The recent development of ^{1}HMRS at high magnetic field strengths led ^{1}HMRS to play a more important role as imaging tool in the identification of metabolite changes in PD. Indeed, ^{1}HMRS of the brain with high magnetic field strengths has many advantages which include better signal-noise ratio and increased spectral, spatial, and temporal resolution, allowing detection of a greater number of metabolites (such as Glu/Gln and GABA), more reliable estimation of peak area, and hence more precise quantification compared with ^{1}HMRS at 1.5 Tesla [26].

A 3D ^{1}HMRSI at 3 Tesla study reported in PD patients changes not only of NAA/Cr ratios but also of mI/Cr ratios, with significant differences of two metabolites between rostral and caudal SN regions [21]. In particular, in the rostral SN regions PD patients showed a trend towards decreased NAA/Cr and mI/Cr ratios compared with control subjects whereas in the caudal SN regions the metabolite ratios were increased in PD patients compared with control subjects. Another 3 Tesla ^{1}HMRSI study investigated metabolite distribution throughout the whole brain. In particular, reduced NAA/Cr and Cho/Cr ratios in bilateral temporal gray matter and increased Cr in right temporal gray matter in PD patients versus control subjects were found [22].

^{1}HMRS at high magnetic field strengths was used also to detect and quantify Glu and GABA, which did not show abnormalities in ^{1}HMRS at 1.5 Tesla studies [9, 20]. ^{1}HMRS at 3 Tesla studies found reduced Glu levels in the posterior cingulated gyrus [23], but not in the lentiform nucleus [27] in PD patients compared with control subjects. The cortical Glu reduction is confirmed by a study on animal model of PD. In this study, performed on 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-lesioned nonhuman primates, Fan et al. [28] found that Glu levels were reduced in the primary motor cortex on the side ipsilateral to the lesion. A ^{1}HMRS at 4 Tesla study, comparing the metabolic profile of 10 PD patients with that of matched controls, reported decreased Glu, NAA, and glutathione levels and increased Cho levels in SN of PD patients [24]. In addition, the authors reported a fourfold higher GABA/Glu ratio in SN versus cerebral cortex. A ^{1}HMRS at 7 Tesla study reported that GABA concentrations in the pons and putamen were significantly higher in mild to moderate PD patients than healthy controls [25]. These findings on GABA are consistent with animal model studies that found elevated GABA levels in the striatum [29–31].

Significant alterations in neurochemical levels may provide new evidences able to elucidate the pathophysiological mechanisms underlying of PD. Reduced NAA levels, observed in all cerebral structures of patients with PD by ^{1}HMRS, reflect not only wide neuronal degenerations, which involves the corticobasal ganglia-thalamocortical networks, but also metabolic dysfunctions. Indeed, since NAA is synthesized in neuronal mitochondria in an energy-dependent manner, its decrease in PD would confirm the hypothesis that the dysfunction of the mitochondrial electron transport chain, as a result of a defect in complex I, is a primary or secondary event in PD pathogenesis [32]. Although the reduction of NAA levels is a condition that may occur also in other neurodegenerative diseases, this finding in PD patients may be indicative of impairments in mitochondrial metabolic system that hypothetically contribute to neuronal degeneration. The mitochondrial dysfunction in PD is also supported by a study of Bowen et al. [33], who reported in occipital lobe of PD patients high levels of Lac, metabolite which accumulates in most disease associated with a deficiency in mitochondrial oxidative metabolism.

^{1}HMRS of the NAA levels might represent a useful in vivo imaging biomarker also for the prediction of cognitive decline in PD. Interestingly, decreased NAA/Cr ratios were found in anterior cingulated cortex of PD patients compared with controls. These low NAA levels significantly correlated with poorer executive function and more severe psychotic symptoms in PD patients [34]. In addition, changes of NAA and Cho levels in early cognitive impairment phase of PD patients were observed. In particular, a recent ^{1}HMRS at 3 Tesla reported that NAA/Cr ratios in the occipital lobe of PD patients with mild cognitive impairment were lower than in control subjects and Cho/Cr ratios in the posterior cingulated of PD patients with mild cognitive impairment were higher

Table 1: Main results of MRS studies in Parkinson's disease versus controls.

MRS technique	Number of PD patients	Main results versus control subjects	Mean (SD) of metabolite ratios in controls	Mean (SD) of metabolite ratios in PD patients	Significant differences were indicated by the following:	Reference
^{1}HMRS at 1.5 T	6	Reduction of NAA/Cho ratios in the lentiform nucleus	NAA/Cho: 2.11 (0.39)	NAA/Cho: 1.29 (0.28)	$P \leq 0.02$.	[9]
^{1}HMRS at 1.5 T	15 (in 7 the symptomatic side was on the left and in 8 it was on the right)	Reduction of NAA/Cr ratios in the left symptomatic side of SN	NAA/Cr: 1.37 (0.22)	NAA/Cr: 1.20 (0.36)	$P = 0.001$	[10]
^{1}HMRS at 1.5 T	10	Increase of total Cr in the prefrontal cortex	Cr: 0.61 (0.16) IU	Cr: 0.76 (0.08) IU	$P < 0.05$	[11]
^{1}HMRS at 1.5 T	20	Reduction of NAA/Cr and Cho/Cr ratios in the temporoparietal cortex	NAA/Cr: 2.06 (∗) Cho/Cr: 0.75 (∗)	NAA/Cr: 1.78 (∗) Cho/Cr: 0.47 (∗)	For NAA/Cr $P < 0.05$ For Cho/Cr $P < 0.01$	[12]
^{1}HMRS at 1.5 T	17	Reduction of NAA/Cr ratios in the temporoparietal cortex	NAA/Cr: 2.20 (0.38)	NAA/Cr: 1.78 (0.30)	$P < 0.05$	[13]
^{1}HMRS at 1.5 T	10	Reduction of NAA/Cr ratios in the motor cortex	NAA/Cr: 1.34 (0.11)	NAA/Cr: 1.21 (0.12)	$P < 0.05$	[14]
^{1}HMRS at 1.5 T	12	Reduction of NAA/Cr ratios in the posterior cingulated cortex	NAA/Cr: 1.78 (0.39)	NAA/Cr: 1.53 (0.20)	$P = 0.03$	[15]
^{1}HMRS at 1.5 T	44	Reduction of NAA/Cr ratios in the presupplementary motor area	NAA/Cr: 1.47 (0.16)	NAA/Cr: 1.39 (0.17)	$P = 0.045$	[16]
3D ^{1}HMRSI at 3 T	9	Reduction of NAA/Cr and mI/Cr in the rostral SN regions and increase of NAA/Cr and mI/Cr in the caudal SN regions	NAA/Cr_{ros}: 3.34 (1.23) mI/Cr_{ros}: 0.82 (0.43) NAA/Cr_{cau}: 2.03 (0.67) mI/Cr_{cau}: 0.64 (0.34)	NAA/Cr_{ros}: 2.45 (1.55) mI/Cr_{ros}: 0.59 (0.52) NAA/Cr_{cau}: 4.92 (2.96) mI/Cr_{cau}: 1.95 (1.43)	$P = 0.054$ for NAA/Cr_{ros} $P = 0.248$ for mI/Cr_{ros} $P = 0.002$ for NAA/Cr_{cau} $P = 0.021$ for mI/Cr_{cau}	[21]
^{1}HMRSI at 3 T	20	Reduction of NAA/Cr and Cho/Cr ratios in bilateral temporal gray matter and increase of total Cr in the right temporal gray matter	NAA/Cr_{left}: 1.27 (0.09) Cho/Cr_{left}: 0.18 (0.032) NAA/Cr_{rigth}: 1.32 (0.121) Cho/Cr_{left}: 0.18 (0.034) Cr_{rigth}: 1995 (225) IU	NAA/Cr_{left}: 1.13 (0.131) Cho/Cr_{left}: 0.14 (0.028) NAA/Cr_{rigth}: 1.17 (0.213) Cho/Cr_{left}: 0.13 (0.034) Cr_{rigth}: 2243 (361) IU	$P \leq 0.01$ for NAA/Cr_{left} and Cho/Cr_{left} $P \leq 0.05$ for NAA/Cr_{right} $P \leq 0.01$ for Cho/Cr_{right} $P \leq 0.05$ for Cr_{right}	[22]
^{1}HMRS at 3 T	12	Reduction of Glu/Cr ratios in the posterior cingulated gyrus	Glu/Cr: 0.555 (0.07)	Glu/Cr: 0.474 (0.092)	$P = 0.019$	[23]
^{1}HMRS at 4 T	10	Reduction of Glu, NAA, and glutathione and increase of Cho in the SN. Increase of GABA/Glu ratio in SN versus cerebral cortex	NA	NA	NA	[24]
^{1}HMRS at 7 T	13	Increase of GABA in the pons and putamen	$GABA_{pons}$: 1.0 (0.2) μmol/g $GABA_{putamen}$: 1.6 (0.2) μmol/g	$GABA_{pons}$: 1.6 (0.4) μmol/g $GABA_{putamen}$: 2.1 (0.4) μmol/g	$P < 0.001$ for $GABA_{pons}$ $P < 0.05$ for $GABA_{putamen}$	[25]

Cho: choline-containing compounds; Cr: creatine + phosphocreatine; GABA: γ-aminobutyric acid; Glu: glutamate; ^{1}HMRS: proton magnetic resonance spectroscopy; ^{1}HMRSI: proton magnetic resonance spectroscopy imaging; IU: institutional units; mI: myoinositol; MRS: magnetic resonance spectroscopy; NA: not applicable; NAA: N-acetylaspartate; PD: Parkinson's disease; SD: standard deviation; SN: substantia nigra; T: Tesla. ∗: DS not done.

than in healthy control subjects and cognitively normal PD patients [35].

The cerebral regional variability of Cho levels observed in some studies [12, 22, 24, 35] makes it difficult to understand what role the Cho can have in the pathophysiology of PD. The reduction of Cho levels observed in some studies [12, 22] might be related to damage of membrane structure of neuronal cells within the corticostriatal system. On the other hand, the trend to increase of Cho and Cr [11, 22, 35] could be a minimal sign for neuroinflammation. Indeed, since higher concentrations of Cho and Cr are present in glial cells than neurons, they may be elevated in neuroinflammation condition [36], although an increase of glial marker mI has been not reported in PD. Therefore, the available evidence is not sufficient to ascribe to Cho and Cr a role as biomarkers of neuroinflammation in PD. Finally, ^{1}HMRS changes in Glu and GABA levels reported in PD may reflect alterations of the balance between excitatory and inhibitory processes in the corticobasal ganglia-thalamocortical networks involved in motor control.

4. ^{1}HMRS in Parkinson's Disease and Atypical Parkinsonian Disorders

In early stage of PD the motor symptoms can easily be mistaken for any number of disorders. Indeed, it is very likely that the PD may be confused with various APDs, such as progressive supranuclear palsy (PSP), multiple-system atrophy (MSA), especially the Parkinson's variant of multiple-system atrophy (MSA-P), and corticobasal degeneration (CBD). A differentiation of these clinical entities may be challenging, particularly in the early stages of motor symptoms of the disease, where overlapping clinical signs lead to a high rate of misclassification. However, a differentiation between APDs and PD is important for making easier early diagnosis and for choosing a specific treatment strategy.

MRS plays an important role in the differentiation of PD from APDs, especially in early stage of disease where a differentiation of these conditions is not easy (Table 2).

^{1}HMRS of striatal structures might differentiate PD from APDs by virtue of reduced NAA/Cr ratios in MSA but not PD. In particular, ^{1}HMRS showed reduced NAA/Cr ratios in the lentiform nucleus in six of seven MSA-P cases, whereas normal levels of putaminal NAA were found in eight of nine PD subjects [17]. A study by Abe et al. [37] showed that, as compared to normal controls, patients with PSP, CBD, MSA, and PD had significant reduction of NAA/Cr ratios in the putamen, whereas patients with PSP, CBD, and MSA, but not PD, had significant reduction of the NAA/Cr ratios also in the frontal cortex.

Moreover, patients with CBD showed significant reduction of NAA/Cr ratios in the frontal cortex and putamen as compared to patients with PD and MSA. Patients with PSP showed a significant reduction of NAA/Cr ratios in the putamen as compared with patients with PD and MSA. Guevara et al. [38], using MRSI on a 1.5 Tesla scanner, found that patients with PSP and MSA-P had lower NAA concentrations in the pallidum, putamen, and lentiform nucleus compared to healthy controls and patients with PD. In another ^{1}HMRS study, in which the single voxel was localized to the lentiform nucleus, Federico et al. [39] showed that NAA/Cho ratios were significantly reduced in MSA and in PSP patients compared to PD patients and to controls. Moreover the NAA/Cr ratios were significantly reduced in MSA, PSP, and PD patients compared to controls, but only in MSA compared to PD patients. However, other MRS studies showed reduced NAA/Cr and NAA/Cho ratios in the lentiform nucleus not only in APD but also in PD [42]. A study investigated 24 patients with MSA compared to 11 PD patients and 18 controls by applying multiple regional single voxel ^{1}HMRS including putamen, pontine basis, and cerebral white matter (WM) at 3 Tesla [40]. Significant NAA/Cr reductions have been shown in the pontine basis of patients with both MSA-C (cerebellar ataxia variant of MSA) and MSA-P, while putaminal NAA/Cr was only reduced in the patients with MSA-P. Eight of the 11 MSA-P patients compared to none of the PD and control group were classified correctly by combining individual NAA/Cr reductions in the pontine basis and in the putamen. These results suggest that combined assessment of NAA/Cr in the pontine basis and putamen by higher magnetic field may be effective in differentiating MSA-P from PD in terms of the high specificity of reduced NAA/Cr in the pontine basis or in the putamen in patients with MSA-P. A recent 3D ^{1}H-MRSI study at 3 Tesla demonstrated a clear differentiation between PD and APDs, by comparing NAA/Cr ratios in SN regions [41]. In particular, for PD patients, NAA/Cr ratios in the caudal voxels were greater than those in the rostral voxels, whereas for healthy controls and APDs patients these ratios were reversed.

Overall, the striatal NAA differences found in APDs but not in PD patients compared with healthy controls contrast with the findings of the study of Gröger et al [41]. These authors, using ^{1}H-MRSI at higher magnetic field strengths, showed NAA differences only in PD patients and attributed these findings to neuronal loss in the SNc as primary pathological mechanism of PD. It is likely that the high-field MRSI, measuring metabolite changes from several areas of the brain simultaneously, provides more reliable and accurate results, making ^{1}HMRSI of the NAA, as expression of neuronal integrity and function, an in vivo imaging biomarker for differential diagnosis.

5. ^{1}H- and ^{31}P-MRSI in Early Diagnosis of Parkinson's Disease

Phosphorus (^{31}P)-MRSI is an imaging technique that measures the levels of compounds related to the energy metabolism of the brain including low-energy metabolites, such as free phosphate (Pi) and adenosine mono- and diphosphate (AMP and ADP), and the high-energy phosphates such as adenosine triphosphate (ATP) and phosphocreatine (PCr). Since mitochondrial dysfunction seems to be an early event inducing PD, imaging techniques such as ^{31}P-MRSI, able to detect a possible alteration of the brain energy metabolism, could be useful tool for early diagnosis of PD.

Table 2: Main results of MRS studies in Parkinson's disease and atypical parkinsonian disorders.

MRS technique	Disease	Main results	Mean (SD) of metabolite ratios in controls	Mean (SD) of metabolite ratios in patients	Significant differences were indicated by the following:	Reference
^{1}HMRS at 1.5 T	MSA-P and PD versus controls	Reduction of NAA/Cr ratios in the lentiform nucleus	NAA/Cr: 1.76 (0.96)	$NAA/Cr_{MSA\text{-}P}$: 1.35 (0.29) NAA/Cr_{PD}: 1.82 (0.28)	$P < 0.02$ MSA-P versus controls $P > 0.05$ PD versus controls	[17]
^{1}HMRS at 1.5 T	PSP, CBD, MSA, and VP versus controls	Reduction of NAA/Cr ratios in the more affected frontal cortex	NAA/Cr: 2.1 (0.2)	NAA/Cr_{PSP}: 1.4 (0.5) NAA/Cr_{CBD}: 0.9 (0.4) NAA/Cr_{MSA}: 1.2 (0.2) NAA/Cr_{VP}: 1.7 (0.1)	$P < 0.01$ PSP, MSA and VP versus controls; $P < 0.001$ CBD versus controls	[37]
	PSP, MSA, CBD, and PD versus controls	Reduction of NAA/Cr ratios in the more affected putamen	NAA/Cr: 2.2 (0.2)	NAA/Cr_{PSP}: 1.4 (0.2) NAA/Cr_{CBD}: 1.0 (0.4) NAA/Cr_{MSA}: 1.8 (0.3) NAA/Cr_{PD}: 1.5 (0.2)	$P < 0.01$ PSP and PD versus controls; $P < 0.001$ CBD versus controls; $P < 0.05$ MSA versus controls	
^{1}HMRS at 1.5 T	MSA-P and PSP versus controls and PD	Reduction of NAA levels in the putamen	NAA: 7.1 (1.6) mM	NAA_{PSP}: 5.26 (0.9) mM $NAA_{MSA\text{-}P}$: 5.27 (0.6) mM NAA_{PD}: 6.88 (1.2) mM	$P = 0.003$ MSA-P versus controls $P = 0.023$ MSA-P versus PD $P = 0.002$ PSP versus controls $P = 0.016$ PSP versus PD	[38]
		Reduction of NAA levels in the pallidum	NAA: 6.52 (1.5) mM	NAA_{PSP}: 4.07 (1.0) mM $NAA_{MSA\text{-}P}$: 5.54 (1.1) mM NAA_{PD}: 6.36 (0.8) mM	$P < 0.001$ PSP versus controls $P < 0.001$ PSP versus PD	
		Reduction of NAA levels in the lentiform nucleus	NAA: 6.77 (1.2) mM	NAA_{PSP}: 4.6 (0.6) mM $NAA_{MSA\text{-}P}$: 5.4 (0.7) mM NAA_{PD}: 6.62 (0.8) mM	$P = 0.003$ MSA-P versus controls $P = 0.027$ MSA-P versus versus PD $P < 0.001$ PSP versus controls $P < 0.001$ PSP versus PD	
^{1}HMRS at 1.5 T	MSA and PSP versus controls and PD	Reduction of NAA/Cho ratios in the lentiform nucleus	NAA/Cho: 2.02 (0.43)	NAA/Cho_{MSA}: 1.39 (0.31) NAA/Cho_{PSP}: 1.45 (0.28) NAA/Cho_{PD}: 1.82 (0.28)	$P < 0.001$ MSA versus controls $P < 0.001$ PSP versus controls $P < 0.001$ MSA versus PD $P < 0.05$ PSP versus PD	[39]
^{1}HMRS at 1.5 T	MSA, PSP, and PD versus controls and MSA versus PD	Reduction of NAA/Cr ratios in the lentiform nucleus	NAA/Cr: 1.86 (0.29)	NAA/Cr_{MSA}: 1.32 (0.30) NAA/Cr_{PSP}: 1.40 (0.17) NAA/Cr_{PD}: 1.65 (0.41)	$P < 0.001$ MSA versus controls $P < 0.001$ PSP versus controls $P < 0.05$ PD versus controls $P < 0.01$ MSA versus PD	
^{1}HMRS at 3 T	MSA-C and MSA-P versus controls; MSA-P versus PD	Reduction of NAA/Cr ratios in the pontine basis	NA	NA	$P < 0.0001$ MSA-C versus controls $P < 0.0001$ MSA-P versus controls $P = 0.001$ MSA-P versus PD	[40]
	MSA-P versus controls and PD	Reduction of NAA/Cr ratios in the putamen	NA	NA	$P = 0.009$ MSA-P versus controls $P = 0.002$ MSA-P versus PD	

Table 2: Continued.

MRS technique	Disease	Main results	Mean (SD) of metabolite ratios in controls	Mean (SD) of metabolite ratios in patients	Significant differences were indicated by the following:	Reference
3D ^{1}HMRSI at 3 T	PD versus controls and ADPs	Lower NAA/Cr ratios in the rostral SN regions than those in the caudal SN regions	$(NAA/Cr)_{ros}$: 2.56 (0.73) and $(NAA/Cr)_{cau}$: 1.85 (0.51)	In PD $(NAA/Cr)_{ros}$: 1.97 (1.24) and $(NAA/Cr)_{cau}$: 3.47 (2.37). In ADPs $(NAA/Cr)_{ros}$: 2.29 (1.37) and $(NAA/Cr)_{cau}$: 2.09 (0.92)	$P < 0.0001$ PD versus controls; $P < 0.0001$ PD versus APDs	[41]
	APDs versus controls	Lower NAA/Cr ratios in the caudal SN regions than those in the rostral SN regions	$(NAA/Cr)_{ros}$: 2.56 (0.73) and $(NAA/Cr)_{cau}$: 1.85 (0.51)	$(NAA/Cr)_{ros}$: 2.29 (1.37) and $(NAA/Cr)_{cau}$: 2.09 (0.92)	$P = 0.977$ APDs versus controls	

APDs: atypical parkinsonian disorders; CBD: corticobasal degeneration; Cho: choline-containing compounds; Cr: creatine + phosphocreatine; ^{1}HMRS: proton magnetic resonance spectroscopy; ^{1}HMRSI: proton magnetic resonance spectroscopy imaging; MRS: magnetic resonance spectroscopy; MSA: multiple-system atrophy; MSA-C: cerebellar ataxia variant of multiple-system atrophy; MSA-P: Parkinson's variant of multiple-system atrophy; NA: not applicable; NAA: N-acetylaspartate; PD: Parkinson's disease; PSP: progressive supranuclear palsy; SD: standard deviation; SN: substantia nigra; T: Tesla; VP: vascular parkinsonism.

Combined ^{31}P- and ^{1}H-MRSI at 3 Tesla measuring absolute ADP, ATP, Cr, and PCr concentrations in two well-defined cohorts of patients with early and advanced PD have been performed to evaluate brain energy metabolism [43]. In the putamen and midbrain of both PD groups compared to control a bilateral reduction of high-energy phosphates such as ATP and PCr as final acceptors of energy from mitochondrial oxidative phosphorylation was found. In contrast, low-energy metabolites such as ADP and Pi were within normal ranges. Patients with early PD, with clearly lateralized motor symptoms, exhibited a significant reduction of putamen high-energy phosphates in the less affected hemisphere with a less pronounced dopaminergic cell loss. Therefore, mitochondrial dysfunction would seem to be a rather early occurring event in the pathophysiology of dopaminergic degeneration in PD, although a recent ^{31}P- and ^{1}H-MRSI study at 3 Tesla did not detect metabolic abnormalities in early PD compared with controls [44]. Since few ^{31}P-MRSI studies explored brain energy metabolism in PD, it is not still clear if this technique may be an imaging biomarker for detection of mitochondrial dysfunction and then for early diagnosis of PD.

6. ^{1}H-MRSI in Detection of Metabolic Changes in Parkinson's Disease after Treatment

Currently, the available drugs for treating motor symptoms of PD include the replacement therapy with dopamine precursor Levodopa, MAO-B inhibitors, and more recently direct-acting dopamine agonists. When pharmacological treatments are not adequate to control symptoms, surgical techniques such as deep brain stimulation can ameliorate the motor symptoms of PD.

Some ^{1}HMRS studies investigated the effects of PD therapy on neurochemical and metabolic profile in cortical-basal ganglia structures (Table 3). In a study evaluating the metabolism of striatum of PD patients versus control subjects, Holshouser et al. [45] reported that NAA/Cho ratios were significantly low in PD patients who did not use levodopa/carbidopa, whereas the ratios were normal in levodopa-treated patients compared with controls. Similarly, Ellis et al. [46] found a significant reduction in putaminal NAA/Cho ratios contralateral to the most affected side in 9 drug-naïve patients with idiopathic PD, but not the 7 levodopa-treated patients compared with controls.

These data suggest that dopaminergic treatment may affect NAA levels in the striatum of PD patients, despite the fact that only prospective studies, in which cerebral metabolic levels are assessed by MRS before and after therapy, could confirm this hypothesis. In a study of Clarke et al. [20] the lentiform nucleus of five PD patients was studied by ^{1}HMRS before and 10 minutes after administration of apomorphine. No metabolic differences in NAA, Cho, Cr, and Glu + Gln levels between PD patients and control subjects and between spectra obtained from patients before and after apomorphine therapy were detected. However, a ^{1}H-MRS study reported that dopaminergic therapy effected the neurochemical status of the motor cortex in de novo PD patients [47]. Indeed, this study showed an increase in Cho/Cr and in NAA/Cr ratios in the motor cortex and an improvement in motor performance in PD patients 6 months after pergolide treatment. In the same way, a ^{1}H-MRS study evaluating the changes in the concentrations of some brain metabolites, in PD patients before and after deep brain stimulation of bilateral subthalamic nucleus, found that after the treatment the cortical NAA/Cho and NAA/Cr ratios were increased significantly and correlated highly with clinical improvement of motor performances [48]. These findings suggest that the clinical treatment-induced improvement might be the result of partial restoration of neuronal functions which in turn may increase the cortical metabolite levels. Overall, NAA recovery could be used as a biomarker of neuronal function for monitoring the response to pharmacological and nonpharmacological therapy of PD.

Table 3: Main results of MRS studies in PD patients treated with dopaminergic drugs and DBS.

MRS technique	Drug	Main results	Mean (SD) of metabolite ratios in treated patients	Mean (SD) of metabolite ratios in nontreated patients	Mean (SD) of metabolite ratios in controls	Significant differences were indicated by the following:	Reference
^{1}HMRS at 1.5 T	Levodopa/carbidopa	Low NAA/Cho ratios in striatum of nontreated PD patients	NAA/Cho: 1.80 (0.48)	NAA/Cho: 1.60 (0.33)	NAA/Cho: 1.83 (0.62)	$P = 0.012$ for nontreated patients versus controls	[45]
^{1}HMRS at 1.5 T	Levodopa	Reduction in putaminal NAA/Cho ratios contralateral to the most affected side in drug-naïve patients	NAA/Cho: 1.15 (0.19)	NAA/Cho: 0.97 (0.14)	NAA/Cho: 1.26 (0.28)	$P = 0.009$ for nontreated patients versus controls	[46]
MRS technique	Treatment	Main results	Mean (SD) of metabolite ratios before treatment	Mean (SD) of metabolite ratios after treatment	Mean (SD) of metabolite ratios in controls	Significant differences were indicated by the following:	Reference
^{1}HMRS at 1.5 T	Pergolide	Significant increase of Cho/Cr ratios in the motor cortex after therapy	Cho/Cr: 0.71 (0.13)	Cho/Cr: 0.82 (0.13)	NA	$P < 0.05$	[47]
^{1}HMRS at 1.0 T	DBS of the STN	Cortical increase of NAA/Cho and NAA/Cr ratios	NAA/Cho: 1.3238 (0.3196) NAA/Cr: 1.6303 (0.6361)	NAA/Cho: 2.5583 (1.2993) NAA/Cr: 1.7057 (0.4167)	NA	$P = 0.04699$ for NAA/Cho; $P = 0.0326$ for NAA/Cr	[48]

Cho: choline-containing compounds; Cr: creatine + phosphocreatine; DBS: deep brain stimulation; ^{1}HMRS: proton magnetic resonance spectroscopy; MRS: magnetic resonance spectroscopy; NA: not applicable; NAA: N-acetylaspartate; PD: Parkinson's disease; SD: standard deviation; STN: subthalamic nucleus; T: Tesla.

7. Conclusions and Future Directions

PD is a neurodegenerative disease whose insidious onset makes its early diagnosis difficult, which is important to slow down the disease progression and optimise the therapy. During the past two decades, significant progresses have been made in the discovery and assessment of potential biomarkers for the early differential diagnosis and monitoring treatment efficacy.

In vivo MRS can provide a useful and objective tool for detection of cerebral metabolic changes in patients with PD and has been shown to meet many of criteria of an ideal imaging biomarker. Indeed, MRS has good test-retest reliability and, compared with other in vivo imaging biomarkers, such as positron emission tomography (PET) and single photon emission tomography (SPECT), is noninvasive and cheap, and it does not require contrast agents for the molecular imaging involving exposure to radioactive substances. In addition, compared with some in vitro molecular biomarkers, such as mRNA and protein expression levels, that require a complex analysis, MRS is not restricted to specialized centres to perform the analysis, making its extension to general public health centres possible.

The recent technical advances of MRS, including the availability of higher magnetic fields and the development of reliable methods for absolute metabolite quantification and for a better identification of metabolite signals, allowed achieving in vivo detailed information on pathophysiology of PD. In particular, the reduction of NAA levels in cortical-basal ganglia networks reflects neuronal loss and mitochondrial metabolic dysfunction in PD. On the same time, changes of Glu and GABA concentrations detected in vivo in basal ganglia of PD patients could be suggestive of dysfunction of neuronal excitatory and inhibitory activities which are involved in the control of movements. Several studies demonstrated the usefulness of MRS to achieve a differential diagnosis of PD versus other forms of parkinsonism, especially in early stages of disease in which signs and symptoms of different forms of parkinsonism have greater overlap. In addition, there is evidence that MRS may be an in vivo imaging biomarker not only for early and differential diagnosis but also for treatment of PD. However, the studies performed so far are extremely heterogeneous in terms of number of enrolled PD patients, MRS techniques used to identify and to process the metabolite signals, and methods used to calculate the metabolite concentrations. Moreover, technical MRS factors, including different echo- and relaxation times, voxel sizes, field strength, and pulse sequence, may be responsible for result variations observed in some studies. Therefore, multicenter studies on larger samples of PD patients, MRS at high magnetic fields, standardized methods for acquisition and processing of spectroscopic metabolite signals, and use of the absolute quantification of tissue metabolite concentrations are required to definitively

ascribe to MRS the role of in vivo molecular imaging biomarker for PD.

Conflict of Interests

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

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Research Article

Glatiramer Acetate and Nanny Proteins Restrict Access of the Multiple Sclerosis Autoantigen Myelin Basic Protein to the 26S Proteasome

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We recently showed that myelin basic protein (MBP) is hydrolyzed by 26S proteasome without ubiquitination. The previously suggested concept of charge-mediated interaction between MBP and the proteasome led us to attempt to compensate or mimic its positive charge to inhibit proteasomal degradation. We demonstrated that negatively charged actin and calmodulin (CaM), as well as basic histone H1.3, inhibit MBP hydrolysis by competing with the proteasome and MBP, respectively, for binding their counterpart. Interestingly, glatiramer acetate (GA), which is used to treat multiple sclerosis (MS) and is structurally similar to MBP, inhibits intracellular and *in vitro* proteasome-mediated MBP degradation. Therefore, the data reported in this study may be important for myelin biogenesis in both the normal state and pathophysiological conditions.

1. Introduction

Myelin basic protein (MBP) is one of the major autoantigens in the pathogenesis of multiple sclerosis (MS) [1] and experimental autoimmune encephalomyelitis [2]—animal model of MS. MBP and its peptides have been extensively studied as important components of the autoimmune pathology of the central nervous system (CNS). A number of MBP peptides were found to be strongly associated with MHC class II [3, 4] in MS patients. Although MS is thought to be mainly a CD4+ T cell-mediated disease, myelin-specific cytotoxic lymphocytes, which recognize MHC I-restricted MBP peptides, can lyse human oligodendrocytes in cell culture [5] and cause severe EAE in mice [6]. The fragments of intracellular proteins that are presented on the MHC class I molecules are generated mainly by the multicatalytic proteinase complex—a 26S proteasome [7]. The majority of cellular proteins are degraded by the 26S proteasome in a ubiquitin-dependent manner [8]. The polyubiquitin chains interact with the 19S regulatory particle, which catalyzes the deubiquitination and denaturation of the substrate and its translocation into the 20S catalytic chamber [9, 10]. Interestingly, recent data indicate that proteasome substrates may be polymonoubiquitinated [11] or even modified by single ubiquitin moieties [12]. Moreover, the number of proteins, such as ornithine decarboxylase [13] and p21 [14], can be degraded by the 26S proteasome without ubiquitination in an ATP-dependent manner [15]. Uncapped 20S proteasome particles are also active in the degradation of either completely or regionally disordered nonubiquitinated proteins, such as α-synuclein

[16] and p53 [17]. Recently we have shown that the 26S proteasome can hydrolyze MBP at physiologically relevant concentrations without ubiquitination *in vitro* and in mature oligodendrocytes [18]. Therefore, proteasome-mediated MBP degradation, which generates myelin antigenic peptides [19, 20], is of a critical importance for the pathogenesis of CNS-related autoimmune diseases.

MBP is known to be highly flexible and intrinsically disordered [21], suggesting that electrostatics forces may primarily determine its interactions with other proteins. We have previously found that the ubiquitin-independent proteolysis of MBP seems to be charge-mediated, as 26S proteasome less efficiently degrades deiminated MBP bearing a decreased positive charge [18]. Therefore, the intracellular counterparts of MBP may restrict its accessibility to the 26S proteasome. Alternatively, proteins that mimic MBP may compete with proteasome for MBP binding. Intracellular MBP may bind Ca^{2+}-activated calmodulin (CaM), actin, tubulin, and proteins containing SH3 domains. MBP is believed to be associated with the cytoskeleton and interacts with actin in oligodendrocytes *in vivo* [22]. The ability of MBP to polymerize actin depends on the net positive charge of the MBP molecule [23]. Full-length MBP is known to bind CaM, a highly acidic calcium sensor, under near-physiological conditions [24]. MBP is a major calcium-dependent CaM-binding protein in human brain white matter MBP, and CaM is colocalized in cultured myelin [25]. An 18.5-kDa MBP has been shown to bind to several SH3 domains, including that of Fyn, a member of the Src family of tyrosine kinases that is involved in a number of signaling pathways during CNS development [26]. The surface charge density of the Fyn-SH3 domain is negative, and the rate of its binding to MBP depends on the MBP net positive charge [27]. In the present study, we investigated whether the interaction of MBP or 26S proteasome with a number of charged proteins could interfere with ubiquitin-independent MBP degradation.

2. Materials and Methods

2.1. Proteins. MBP was prepared from bovine brains according to [28]. The obtained protein was purified by reverse phase HPLC on a C_4 10/250 column (Macherey-Nagel). Actin from porcine muscle, lysozyme from chicken egg, calmodulin from bovine brain, and BSA were obtained from Sigma. Recombinant histone H1.3 was obtained from *E. coli*, and recombinant human ubiquitin and recombinant human K48-tetraubiquitin were obtained from Boston Biochem. GA (Copaxone) is a commercially available drug from Teva; for the experiments, it was desalted into 20 mM Tris-HCl pH 7.5 using a HiTrap Desalt column (GE Healthcare Life Sciences).

2.2. Cultured Cells and Transfection Procedures. HEK293 cells were grown at 37°C and 5% CO_2 in DMEM supplemented with 10% fetal calf serum and antibiotics (penicillin-streptomycin). The cells were transfected with the pBudCE4.1/EF-FLAG plasmid carrying human MBP or the human histone H1.3 sequence. The cDNA transfections were accomplished using Lipofectamine LTX with Plus reagent (Life Technologies). All of the procedures were performed according to the manufacturer's instructions.

2.3. Cycloheximide Chase Experiments. To study the proteasomal degradation of MBP and histone H1 in HEK293 cells, cycloheximide (100 μg/mL) was added to transfected cells for the indicated times, and the cells were lysed using RIPA buffer (150 mM NaCl, 0.5% sodium deoxycholate, 50 mM Tris-HCl pH 8, 0.1% SDS, 1% NP-40, and protease inhibitors mixture). Protein lysates prepared from an equal number of cells were resolved via SDS-PAGE and blotted onto nitrocellulose membranes. MBP and histone H1 were visualized using an anti-FLAG antibody (A8592, Sigma-Aldrich). β-Actin was used as a loading control and detected using a specific antibody (sc-81178, Santa Cruz Biotechnology).

2.4. Purification of the Proteasome from Mouse Liver. Briefly, a BALB/c brain was homogenized using a Dounce homogenizer into three parts w/w lysis buffer containing 30 mM Tris-HCl (pH 7.5), 2 mM ATP, 1 mM EDTA, 5 mM $MgCl_2$, 1 mM DTT, 10% glycerol, 150 mM NaCl, and a protease inhibitor cocktail. The prepared brain homogenate was subjected to three repeated freeze-thaw cycles, and further cell debris was removed via two consecutive centrifugations at 4°C (1,500 g for 20 min and 13,000 g for 30 min). Ammonium sulfate was added to the supernatant to 40% saturation, and the mixture was agitated for 40 min at 4°C. The precipitate was collected by centrifugation (13,000 g 10 min at 4°C), dissolved in buffer containing 20 mM Tris (pH 7.5), 10% glycerol, 150 mM NaCl, 1 mM ATP, 1 mM DTT, 1 mM EDTA, and 5 mM $MgCl_2$, and loaded on a Superose 6 column (GE Healthcare Life Sciences). The fractions (1 mL each) were collected, and the proteasome activity was quantified using Suc-LLVY-MCA as a substrate. To distinguish between the activity related to the 20S proteasome and that related to the 26S proteasome, the assay was performed with or without 0.02% SDS. The buffer used to measure the activity of the proteasomes contained 20 mM Tris (pH 7.5), 1 mM ATP, 1 mM DTT, and 5 mM $MgCl_2$. The fractions containing the 26S proteasome were subjected to ion-exchange chromatography on a MonoQ column using a NaCl gradient (275–1000 mM in 20 column volumes) in buffer containing 20 mM Tris (pH 7.5), 10% glycerol, 0.1 mM ATP, 1 mM DTT, and 0.1 mM EDTA. The fractions containing the 26S proteasome were dialyzed into storage buffer (25 mM Tris-HCl [pH 7.5], 1 mM DTT, 1 mM ATP, 5 mM $MgCl_2$, and 10% glycerol). The concentration of proteasome was determined with a Bradford assay. For a long-term storage, up to 40% glycerol was added to the proteasome, and the purified proteasome was stored at −20°C for two months.

2.5. Native PAGE. Proteasome samples (200 ng) were loaded on a 4% gel (acrylamide: N,N′-methylenebisacrylamide 37.5 : 1, 180 mM Tris-borate buffer [pH 8.3], 5 mM $MgCl_2$, 1 mM DTT, and 1 mM ATP). Electrophoresis was conducted for 1.5 h at 4°C and 180 V. The gels were soaked in buffer containing 20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM

DTT, and 1 mM ATP supplemented with 100 mM Suc-LLVY-MCA for 10 min at 37°C and visualized on a Versa Doc Imaging system (Bio-Rad) using the trans-UV excitation and the 530BP emission filter.

2.6. Proteasome Ultracentrifugation. The 20S and 26S proteasomes from BALB/c mouse brains were separated by ultracentrifugation. The tissue was homogenized (Dounce homogenizer, Thomas Scientific) in 3 V buffer containing 20 mM Tris-HCl (pH 7.5), 10% glycerol, 150 mM NaCl, 1 mM EDTA, 5 mM $MgCl_2$, 1 mM DTT, and protease inhibitor cocktail (Roche), and the homogenates were centrifuged (16,000 g, 4°C 30 min) to remove cell debris. To study MBP binding to the proteasome, the homogenates were incubated with purified bovine MBP in the presence of 1 μM PS-341 for 30 min at 4°C. Further homogenates were separated by ultracentrifugation in 10% to 55% glycerol gradient in buffer containing 20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM DTT, and 1 mM ATP at 125,000 g for 18 h at 4°C. The proteasome activity in the resulting fractions was measured using Suc-LLVY-MCA as a substrate in the presence and absence of 1 μM PS-341 and 0.02% SDS.

2.7. Association of MBP with 26S. Bovine MBP (1 μg) and PS-341-pretreated 26S (3 μg) were incubated for 1 h in 100 μL of buffer containing 20 mM Tris-HCl (pH 7.5), 20% glycerol, 1 mM DTT, 1 mM ATP, 200 μg/mL BSA, 0.1% NP-40, and 100 mM NaCl at 4°C. MBP-26S complexes were precipitated with the addition of rat monoclonal anti-MBP (ab7349, Abcam) or mouse polyclonal anti-hRpn10 antibodies (H00005710-B01P, Abnova), followed by incubation with the protein G-sepharose. The resulting immunoprecipitates were subjected to Western blotting analysis and further stained for MBP (ab77895, Abcam) and hRpn2 (ab21638, Abcam).

2.8. In Vitro Protein Degradation by Proteasome. The proteasome samples were mixed with bovine MBP and one of tested proteins (actin, CaM, histone H1.3, GA, lysozyme, BSA, GST, Ub, and K48-Ub_4) in buffer containing 20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM DTT, and 1 mM ATP and incubated for 2 h at 37°C. The MBP concentration in the reaction mixture was 90 ng/μL, the proteasome concentration was 50 ng/μL (proteasome to substrate 1 : 250), and the concentrations of tested proteins were 60, 180, 360, and 600 ng/μL.

2.9. Chymotrypsin-Like Proteasome Activity Assay. 26S proteasome (0.1 μg/μL) was mixed with Suc-LLVY-MCA substrate (25 μM) in buffer containing 20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM DTT, and 1 mM ATP with or without the tested protein (1 μg/μL). The rate of hydrolysis was measured on a Varioskan Flash plate fluorimeter (Thermo Scientific) at an excitation of 360 nM and emission of 460 nM.

2.10. Surface Plasmon Resonance. The SPR measurements were performed on a Biacore T200 apparatus. The ligands were immobilized on CM4 chips (~1,500 RU) using an amino-coupling kit according to the manufacturer's instructions. All of the analyte binding measurements were performed with HBS-EP+ as the continuous running buffer at 25°C. Actin, GA, and histone H1.3 were injected at a concentration of 5.0 μM and a flow rate of 25 μL/min for 200 s. The binding sensorgrams were analyzed using the BIAevaluation Software.

3. Results

To test for a possible direct interaction of MBP with 26S proteasome, we incubated PS-341-treated 26S proteasome with MBP and further immunoprecipitated proteins using either anti-MBP or anti-hRpn10 antibodies. In both cases, the eluates contained MBP and 26S proteasome, whereas no cross-reactivity of anti-hRpn10 and anti-MBP antibodies with MBP and the proteasome, respectively, was observed, suggesting that the proteasome binds MBP *in vitro* (Figure 1(a)). The 26S proteasome consists of two subparticles, namely, a hollow barrel-shaped 20S particle that contains multiple proteolytic sites and a regulatory 19S subunit that is required to recognize the polyubiquitination signal [9]. The fractionation of proteasomes mixed with MBP by glycerol gradient centrifugation demonstrated that MBP was coeluted with 26S but not with 20S proteasomes (Figure 1(b)). This finding agrees with our previous observations suggesting that MBP-proteasome interaction is charge-mediated, as the acidic isoelectric point of the majority of 19S regulator subunits is below 6 (pI values of 19S subunits of eukaryotic proteasome are listed in [29]).

To obtain further details on the mechanism of proteasomal MBP degradation, we further made an attempt to intercept MBP before it could reach the proteasome or, alternatively, mimic MBP to compete with it for proteasome binding. To this end, we selected a number of proteins (Table 1) that could potentially interfere with the hydrolysis of MBP by proteasome: (i) actin [30] and CaM [31], which are known to bind MBP *in vitro* and *in vivo*; (ii) the anti-MS drug glatiramer acetate (GA), which is structurally similar to MBP [32]; (iii) positively charged and intrinsically disordered histone H1.3 [33]; (iv) mono- and tetra-ubiquitin (Ub, Ub_4), which can bind the ubiquitin receptors of the 19S regulator [34, 35]; (v) the slightly acidic globular proteins GST and BSA and basic lysozyme with compact globular structure which were used as controls. The hydrolysis of MBP was monitored in a ubiquitin-free *in vitro* system containing purified 26S proteasome, ATP, test proteins, and none of the components of the ubiquitination system. The rate of MBP hydrolysis was analyzed by SDS-PAGE (Figure 2(a)). Neither Ub_4 nor mono-Ub, which, respectively, binds to the ubiquitin interaction motifs of Rpn10 [34] and N-terminal segment of Rpn13 [35], significantly changes the rate of MBP hydrolysis by the proteasome. This result suggests that the Ub-binding domains are not involved in MBP recognition by the 19S regulator. GST, BSA, and lysozyme did not change the rate of MBP hydrolysis, while actin, histone H1.3, CaM, and GA obviously inhibited MBP degradation (Figures 2(a) and 2(b)). We further tested the ability of actin, GA, and histone H1.3 to bind to MBP and the 26S proteasome.

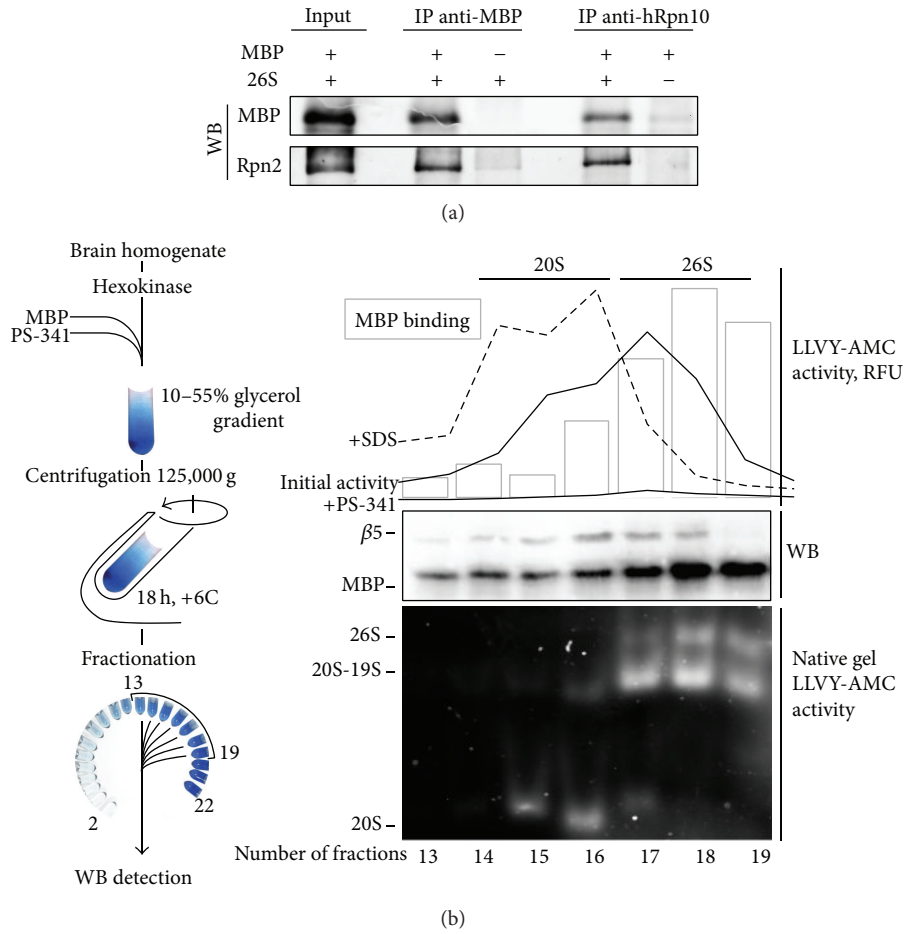

Figure 1: (a) Western blotting analysis of the immunoprecipitation of the 26S proteasome complex with bovine MBP using monoclonal anti-MBP or anti-hRpn10 antibodies. (b) PS-341-treated 20S and 26S proteasomes from BALB/c brain homogenate were preincubated with MBP, separated by ultracentrifugation, and analyzed for bound MBP by Western blotting, as indicated. The presence or absence of the 19S regulatory particle verified by Western blotting for β5 proteasome subunit, native PAGE, and LLVY-AMC activity profiles in the presence or absence of SDS and PS-341.

According to the SPR measurements, MBP interacted with negatively charged actin but not with GA and histone H1.3 (Figure 2(c), left panel). In contrast, the 26S proteasome bound GA and histone H1.3, but not actin (Figure 2(c), right panel). Therefore, proteins with detected inhibitory activity were evidently divided into two subgroups, particularly those that bind MBP and those that bind the 26S proteasome. Interestingly, among proteasome binders, GA itself was resistant to proteasomal hydrolysis, whereas histone H1.3 was degraded by 26S proteasome to some extent (Figure 2(d), left panel). According to the precise densitometry analysis, the observed migration of the GA molecular weight distribution to the less heavy masses is explained by the dynamic processes of aggregation/disaggregation rather than by 26S-mediated hydrolysis (Figure 2(d), right panel).

The extent of MBP hydrolysis in the presence of actin, CaM, histone H1.3, and GA (Figures 2(a) and 2(b)), as estimated by densitometry analysis, was plotted as a function of the concentration of inhibitory proteins (Figure 3). Furthermore, the experimentally observed extent of MBP hydrolysis in the presence of different concentrations of actin and CaM was compared with the theoretical amount

Table 1: Panel of tested proteins.

Protein	MW	[1]pI
MBP from bovine brain	18.3	11.3
Actin from porcine muscle	41.8	5.2
Ubiquitin human (Ub)	8.6	7.4
BSA	66.4	5.6
Glatiramer acetate (GA)	~7 (5–9)	~10.3
Histone H1.3 human	22.2	11.0
Ub_4	34.4	6.6
GST human	48.8	~6.5
Apo-calmodulin human (CaM)	16.7	4.1

[1]Isoelectric points of proteins listed in the Table 1 were calculated using ExPASy Compute pI/Mw tool http://web.expasy.org/compute_pi/.

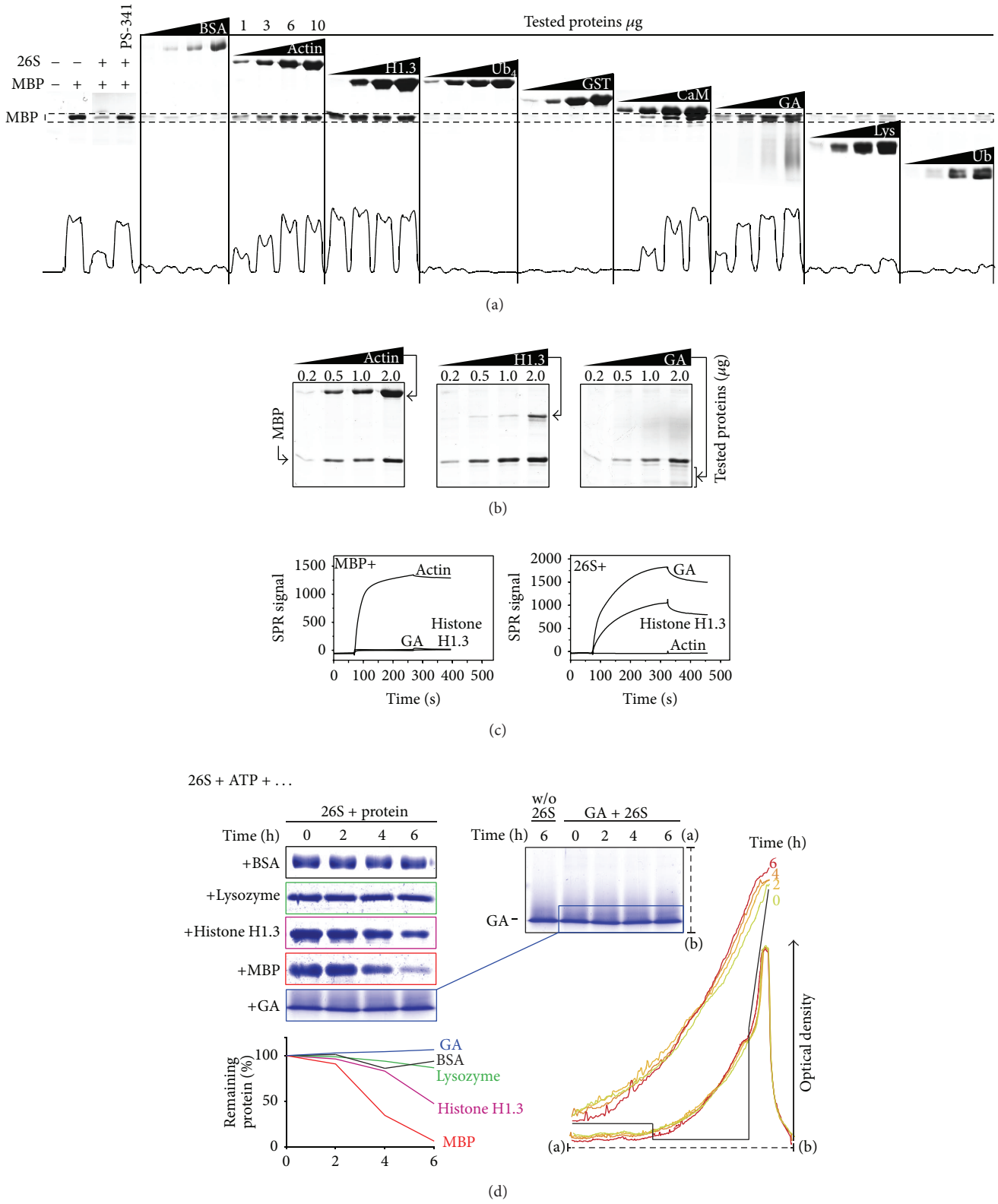

FIGURE 2: (a and b) Degradation of bovine MBP by 26S proteasome in the presence of tested proteins (1–10 μg) as indicated. The bottom curve on panel (a) represents the densitometry analysis of the remaining MBP (dashed area on top). (c) Sensorgrams from SPR measurements for the interaction between GA, histone H1.3, CaM, and immobilized MBP (left panel); 26S proteasome and immobilized actin, GA, and histone H1.3 (right panel). (d) Degradation of BSA, lysozyme, histone H1, GA, and MBP by 26S proteasome in presence of ATP as monitored by PAGE. The percentage of protein remaining was calculated as the ratio of protein at the indicated time-points relative to the initial protein. The insertion shows the overlaid densitometry profiles of GA samples incubated with the 26S proteasome.

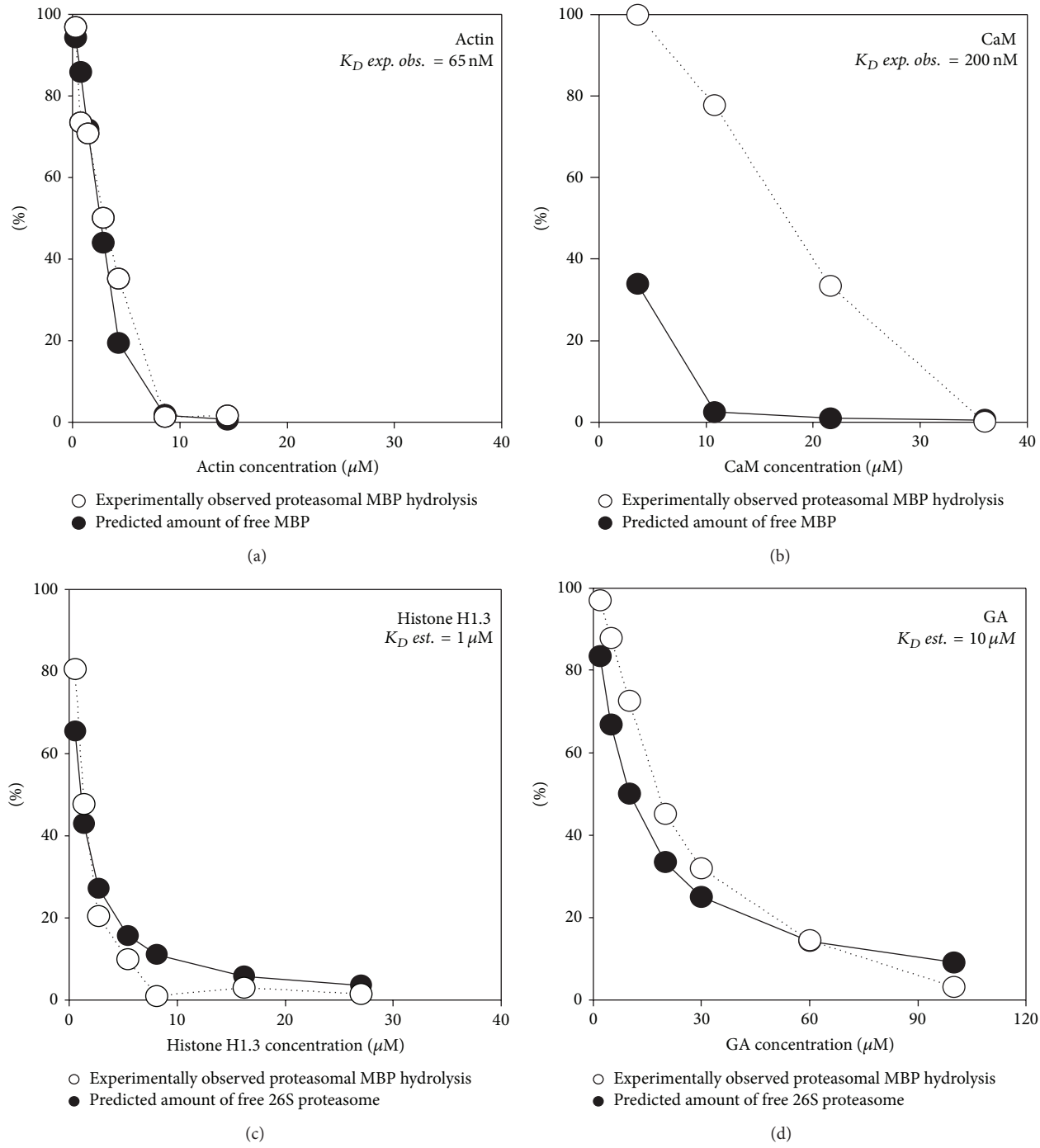

Figure 3: Plots represent the percentage of MBP hydrolysis (opened circles) by 26S proteasome in the presence of the indicated concentrations of actin (a), CaM (b), histone H1.3 (c), and GA (d). The theoretical percentage of uncomplexed MBP (filled circles on panels (a) and (b)) was calculated using previously reported K_D (*exp.obs.*) values for the MBP-actin and MBP-CaM interactions, respectively. The theoretical percentage of histone H1.3- or GA-bound 26S proteasome (filled circles on panels (c) and (d)) was calculated using an asymptotic approximation of the percentage of MBP hydrolysis, assuming that the bound proteasome cannot degrade MBP (theoretical K_D *est.*).

of uncomplexed MBP calculated using previously reported dissociation constants (K_D) for the MBP-actin (Figure 3(a)) and MBP-CaM (Figure 3(b)) interactions. The reversed task was accomplished using experimental curves that represent the inhibition of proteasome-mediated MBP proteolysis by GA and histone H1.3. Using the asymptotic approximation of the percentage of MBP hydrolysis, we estimated the theoretical K_D of 26S-histone H1.3 (Figure 3(c)) and 26S-GA (Figure 3(d)) interaction by assuming that bound 26S proteasome cannot degrade MBP.

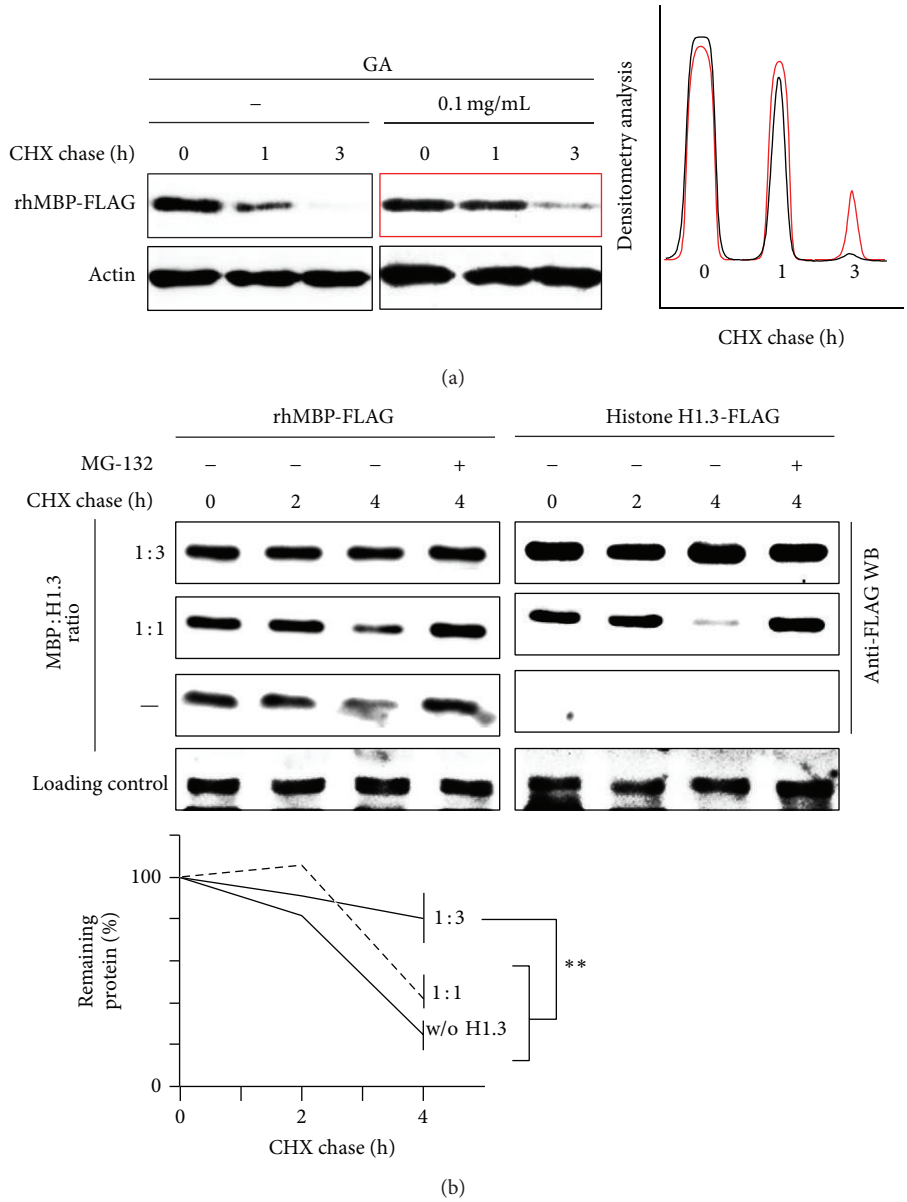

Figure 4: (a) HEK293 cells were transfected with cDNA coding for human MBP with C-terminal FLAG epitope (hMBP-FLAG). After 4 h, the cells were incubated for an additional 20 h with or without GA, as indicated. The cells were then subjected to a cycloheximide chase followed by Western blotting. (b) The HEK293 cells were transfected with cDNAs coding for rhMBP-FLAG along with cDNA coding for the human histone H1.3-FLAG, as indicated. After 24 h, the cells were subjected to a cycloheximide chase followed by western blotting analysis. The percentage of protein remaining was calculated as the ratio of protein at the indicated time-points relative to the initial protein. The data are represented as the mean ± SEM from three separate experiments. ∗∗ denotes $P < 0.01$.

We monitored degradation of MBP in HEK293 cells to determine if the discovered inhibitory effects may be observed *in vivo*. To this end, we transiently transfected HEK293 cells with cDNA coding for human MBP (hMBP) and either cotransfected cDNA coding for human histone H1.3 or added GA into the culture medium. The cells were treated with cycloheximide (CHX), harvested at the indicated time-points, and further subjected to western blotting analysis. Both GA (Figure 4(a)) and histone H1.3 (Figure 4(b)) decreased the intracellular proteasome-mediated MBP hydrolysis. Similar to the *in vitro* assays, transiently transfected histone H1.3 was partially degraded by the proteasome in HEK293 cells.

4. Discussion

Here, we showed that CaM and actin protect MBP from proteasomal hydrolysis. Previous findings indicate that the dissociation constant (K_D) of MBP-CaM interaction is approximately 200 nM [36] for recombinant murine MBP

or 148 nM for MBP from porcine brain [25], as determined by SPR. The interaction of MBP with actin is characterized by a K_D of 66.6 nM for G-actin or 65.3 nM for F-actin [30]. Importantly, the rate of *in vitro* MBP hydrolysis in the presence of actin agrees with the predictions of concentration of "free" MBP based on previously reported dissociation constants (Figure 3(a)). We failed to correlate the amount of unbound MBP with the rate of CaM-mediated inhibition of proteasomal MBP degradation. We further reasoned that this observation is due to different mechanisms of MBP binding. MBP-actin interaction is believed to be charge-mediated, as the ability of MBP to polymerize actin depends on the net positive charge of the MBP molecule—the rate and extent of actin polymerization induced by 18.5 kDa MBP charge isomers correlate with the charge reduction caused by posttranslational modifications [23]. The MBP in MBP-actin assemblies is structurally heterogeneous but gains ordered secondary structure elements (both α-helices and β-sheets), particularly in the terminal fragments and in a central immunodominant epitope [37]. In summary, the interaction of MBP with actin involves the majority of the protein sequences, which effectively masks MBP from the proteasome. Unlike MBP-actin complexes, the interaction of MBP with CaM is less related to its charge: the binding properties of the two MBP charge isoforms—C1 and C8—are very similar [36]. More importantly, MBP contains a distinct CaM-binding segment, which is located near the C-terminus and corresponds to residues 138–156 of human 18.5 kDa MBP. $MBP_{138–156}$ interacts mainly with the C-terminal lobe of CaM, and a conformational change accompanies binding [31]. Thus, the limited surface of protein-protein contact may reduce the ability of CaM to protect MBP from proteasomal hydrolysis.

Relevant data regarding the intracellular proteasome concentration in mammalian cells are lacking; however, the concentration of 26S proteasome in the cytoplasm of yeast is estimated to be 140–200 nM [38]. This concentration is similar to that observed in our *in vitro* assays. CaM is known to interact with a number of target proteins, including myosin light chain kinase, calcineurin, neuronal nitric oxide synthase, and phosphodiesterase. The maximum free Ca^{2+}-CaM concentration in HEK 293 cells is only 50–60 nM at resting conditions, while the total available calmodulin concentration (apo-CaM and Ca^{2+}-CaM) is 6–10 μM [39, 40]. In turn, the total concentration of actin in nonmuscular cells is typically 2-3 mg/mL (46–70 μM) [41]. Approximately 60% of cellular actin is polymerized, and the rest of the protein is mostly bound to profilin and thymosin-β4; the concentration of free monomeric actin is estimated to be 2 μM [42]. Because MBP may bind actin filaments, the intracellular concentration of actin accessible for interaction with MBP may be estimated to be 20–40 μM. Proteasomal MBP degradation was significantly inhibited at actin and CaM concentrations of 10–20 μM. Thus, we suggest that CaM is unlikely to protect MBP from the 26S proteasome *in vivo*, whereas actin is a potential "nanny protein" [43] for MBP.

Proteins and polypeptides that mimic MBP were shown to inhibit proteasomal MBP degradation by competing with it for 26S proteasome binding. Both histone H1.3 and the MS therapeutic agent GA, which mimics MBP in both charge and structure, could inhibit the 26S-mediated MBP proteolysis *in vitro* and *ex vivo*. The deconvolution of the K_D of histone H1.3- and GA-26S proteasome interaction based on the inhibition curves results in values of 1 and 10 μM, respectively (Figures 3(c) and 3(d)). Histone H1.3 protects MBP from proteasomal hydrolysis in a "suicidal" manner, whereas GA seems to be resistant to proteasome-mediated hydrolysis. According to the mechanism of action proposed for GA in MS, this agent acts mainly on the periphery and not in the CNS. Thus, the direct competition of GA with MBP for 26S proteasome binding inside oligodendrocytes seems to be questionable. We further suggest that GA may affect intracellular MBP processing in antigen-presenting cells outside the CNS, especially in the context of the recently reported proteasome-dependent presentation of MHC II-restricted antigens [44].

5. Concluding Remarks

In this study, we showed two possibilities to protect MBP from proteasome-mediated hydrolysis in order to compensate lack of control via ubiquitination system. First, 26S proteasome failed to recognize MBP when it is associated with naturally occurring MBP-binding proteins, including but probably not restricted to actin and CaM. Importantly, this interaction, which is characterized by large surface contact and accompanied by neutralization, is evidently more effective than "key-lock" binding. These results suggest that a number of negatively charged proteins that are known to be engaged in protein-protein interactions with MBP may potentially serve as "nanny proteins" that partially defend MBP from intracellular degradation. Second, polypeptides that mimic MBP restrict its access to the 26S proteasome. Further studies should identify possible physiologically relevant basic and intrinsically disordered "gatekeepers" that can protect MBP from proteasome-mediated degradation.

Conflict of Interests

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

Acknowledgments

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Review Article

Derailed Intraneuronal Signalling Drives Pathogenesis in Sporadic and Familial Alzheimer's Disease

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Although a wide variety of genetic and nongenetic Alzheimer's disease (AD) risk factors have been identified, their role in onset and/or progression of neuronal degeneration remains elusive. Systematic analysis of AD risk factors revealed that perturbations of intraneuronal signalling pathways comprise a common mechanistic denominator in both familial and sporadic AD and that such alterations lead to increases in Aβ oligomers (Aβo) formation and phosphorylation of TAU. Conversely, Aβo and TAU impact intracellular signalling directly. This feature entails binding of Aβo to membrane receptors, whereas TAU functionally interacts with downstream transducers. Accordingly, we postulate a positive feedback mechanism in which AD risk factors or genes trigger perturbations of intraneuronal signalling leading to enhanced Aβo formation and TAU phosphorylation which in turn further derange signalling. Ultimately intraneuronal signalling becomes deregulated to the extent that neuronal function and survival cannot be sustained, whereas the resulting elevated levels of amyloidogenic Aβo and phosphorylated TAU species self-polymerizes into the AD plaques and tangles, respectively.

1. Introduction

Alzheimer's disease involves a gradual decline of synaptic function which is clinically presented as dementia [1, 2]. AD brains are defined by the presence of two different protein aggregates: plaques and tangles. Plaques are assemblies of extracellularly deposited Aβ peptides predominantly comprising the Aβ40 and the highly amyloidogenic Aβ42 peptides. These peptides are the products of sequential processing of APP (amyloid precursor protein) by BACE1 and-γ-secretase [3]. It is generally assumed that in AD homeostasis of Aβ40 and 42 species is altered resulting in increased formation of oligomeric Aβ (Aβo) and subsequent aggregation into plaques. Tangles comprise intracellular assemblies of hyperphosphorylated TAU, a protein which as monomer—among other functions—binds to and stabilizes microtubules [4, 5]. There is high degree of consensus that in AD kinase and/or phosphatase activities are deregulated, resulting in hyperphosphorylation of TAU. TAU then loses its ability to bind to microtubules and consequently acquires a high propensity to oligomerise and further aggregate in tangles [6].

Decades of AD research have culminated in a wealth of data on virtually every aspect of AD etiology and pathogenesis. This has led to detailed insights into the mechanisms of AD, such as APP processing or TAU-phosphorylation, but a coherent picture encompassing AD pathology (i.e., cause/etiology, mechanisms of development, structural changes of neurons, and clinical manifestations) is still in its infancy. This review attempts to contribute to this discussion by proposing mechanisms that may help to design a conceptual framework of AD pathology.

2. Intraneuronal Signaling and Endocytosis Are Dysregulated in AD Leading to Increased Aβo Formation and TAU-Phosphorylation, which in Turn Further Derange Signalling

2.1. Aβ Oligomers Impact Intraneuronal Signalling in Familial AD. Sporadic AD is often phrased as idiopathic to emphasise that the cause of the neuronal degeneration and symptoms is unknown. Although undoubtedly true for

Table 1: Sporadic and familial AD risk genes and nongenetic positive risk factors and possible pathogenic mechanisms [8, 132].

Risk factor	Possible mechanism(s)*		References**
	Aβ homeostasis	Cellular signaling	
Genetic			
APP	APP processing	Erk1/2	[61]
PS1	Change in Aβ40/Aβ42 ratio	Wnt-signalling, Erk1/2, Akt, and Ca^{2+} signaling	[61, 64–66, 133]
PS2	APP processing	Erk1/2	[65, 134, 135]
BACE	APP processing	cAMP-PKA-CREB signaling	[68]
ApoE4	Aβ clearance	Erk1/2, JNK	[136–140]
SORLA	APP processing	Neurotrophin signaling	[137, 141, 142]
EPHA1	?	Ephrin signalling (Erk1/2)	[143, 144]
MS4A6A/MS4A4A	?	Signalling	[132]
CD2AP	?	PI3K-Akt-GSK3 (podocytes)	[145]
CLU	Aβ sequestering	Leptin/clusterin signalling; p53-Dkk1-JNK pathway	[146–148]
β2-AR	?	PKA, Erk1/2, and JNK	[149, 150]
CD33	Aβ clearance		[151]
PICALM	APP processing	Regulation of receptor-mediated endocytosis?	[152]
BIN1	APP processing	Ca^{2+} dyshomeostasis	[153]
ABCA7	Aβ clearance	?	[154]
Nongenetic			
Smoking	?	Erk1/2 activation by oxidative stress	[155, 156]
Obesity	?	Cytokine-induced activation of MAPKs (p38, JNK); leptin signalling	[157–160]
Traumatic brain injury (TBI)	APP processing	Activation of MAPKs (Erk1/2, p38, and JNK), Akt, GSK3β	[8, 161]
Type II diabetes	?	Insulin signalling, cytokine-induced activation of MAPK's (p38, JNK)	[158–160, 162]
Stress (hormones)	?	Glucocorticoid-induced activation of Erk1/2, JNK; oxidative stress-induced JNK-dependent APP processing	[163–166]
Anaesthetics		Activation of MAPKs (Erk1/2, JNK)	[167–170]
Ageing	APP processing	Impaired Ca^{2+} dyshomeostasis and signalling, elevated cytokine signalling ("inflammaging"), impaired mitochondrial function with altered redox signalling (MAPKs, PI3K/Akt)	[171–174]

*Not exhaustive. **Including reviews with original research papers cited.

individual patients, epidemiological studies have revealed several positive and negative AD risk factors which may hold clues as to the mechanism of AD pathogenesis (Table 1). Remarkably, these risk factors are highly diverse, consisting of genetic, lifestyle, and environmental cues with various degrees of disease penetrance. For instance, ApoE variants and zygosity are either protective against or strongly increase the risk of AD [7]. Ageing, smoking, traumatic brain injury, or metabolic diseases such as diabetes are examples of nongenetic modifiers [8]. In rare familial cases mutations in APP or its processing machinery comprise highly penetrant risk factors which in itself suffices to trigger AD.

Irrespective of their nature and origin, at a certain point these risk factors converge to a common mechanism involving synaptic failure, Aβ and TAU pathology, and subsequent neuronal loss. Thus, a key question of understanding AD is not what causes AD, as these are multifactorial and heterogeneous among patients, but how these may converge mechanistically to trigger AD pathology. Once understood, principally every condition impacting this mechanism could be considered as contributing to AD and effective therapeutic options targeting this mechanism could be rationalised for treating AD.

The discovery of genetic risk factors causing early onset AD has been extremely instructive to reveal such common mechanism since in these exceptional cases only one defined cause, namely, altered APP processing, triggers AD providing an relatively "simple" paradigm to investigate pathogenesis. From numerous studies on the mechanism of APP-dependent neurotoxicity, a picture emerges in which Aβo, but not plaques or monomers, comprises prime candidates

Table 2: Neuronal receptors impacted by Aβo [19, 175] and possible effects on downstream signalling pathways.

Receptor	Signal transduction pathway	References*
NMDAR (NR2B subtype)	Erk1/2, CamKIV	[95, 176–182]
mGluR5 (with PrP^C)	PKC, MAPKs (Erk1/2, p38, and JNK)	[79]
nAchR (α7 subtype)	Erk1/2, Akt, and JAK-STAT	[183, 184]
Wnt receptor	Wnt signalling (GSK3)	[185, 186]
IR/IGF	PI3K-Akt	[176, 187]
Amylin receptor	Erk1/2, PKA	[177]
RAGE	p38	[188]
Neurotrophin receptors	Erk1/2, Akt	[45, 189]
β2AR	PKA, Erk1/2, and JNK	[149, 190, 191]

*Including reviews with original research papers cited.

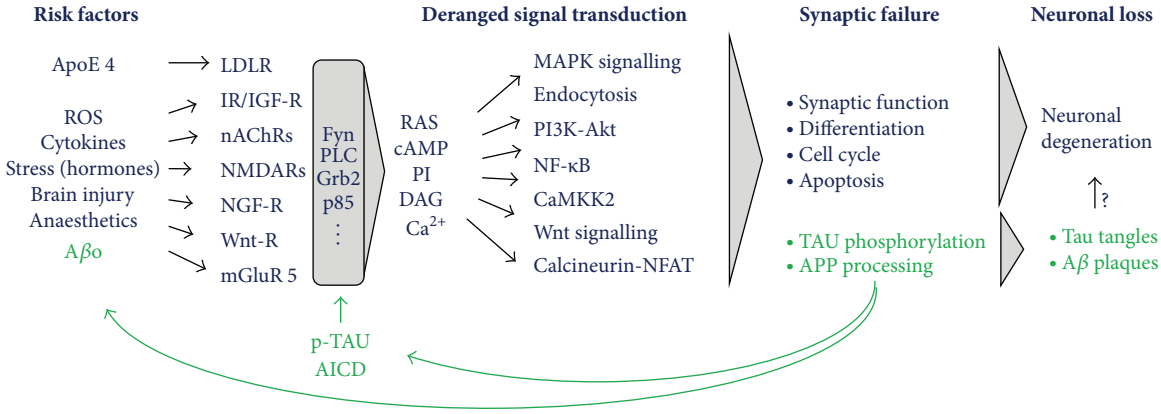

Figure 1: APP, its processing products and TAU are part of an intraneuronal signalling network required for neurogenesis, neuronal function, and survival which go awry in AD. Aβo and AD risk factors modulate receptor mediated intraneuronal signalling and endocytosis which impacts Aβ homeostasis and TAU-phosphorylation. TAU-hyperphosphorylation leads to decreased microtubule binding, somatodendritic redistribution, and altered signalling. Apart from a modulatory role of Aβo, AICD, and phosphorylated TAU on signalling, their formation is also controlled by signalling implying a positive feedback loop which could overtime lead to a dysfunction of signalling cascades underlying synaptic integrity and neuronal survival. High levels of Aβo and hyperphosphorylated-TAU species will, due to their intrinsic amyloidogenic propensity, ultimately aggregate into plaques and tangles. Risk factors which impact these signalling processes, either directly or indirectly (i.e., through impacting Aβo levels), will set off this cascade of events culminating in synaptotoxicity and pathology. Note that the schematic is highly simplified and intended to depict general principles. For a more exhaustive insight into the signalling pathways impacted in AD, see [30]. Abbreviations are as follows: LDLR: low density lipoprotein receptor; IR: insulin receptor; IGF-R: insulin-like growth factor receptor; nACHR: nicotinic acetylcholine receptor; NMDAR: N-methyl-D-aspartate receptor; NGF-R: nerve growth factor receptor; Wnt: Wingless Int; PrPc: cellular prion protein; RAS: rat sarcoma; cAMP: cyclic adenosine monophosphate; PI: phosphoinositides; DAG: 1,2-diacylglycerol; mGluR5: metabotropic glutamate receptor; MAPK: mitogen-activated protein kinase; PI3K: phosphoinositide 3-kinase; NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells; CamKK2: calcium/calmodulin-dependent protein kinase 2; and NFAT: nuclear factor of activated T-cells.

responsible for synaptic failure, TAU-phosphorylation, and neuronal loss [9–15]. Also the AICD, another APP processing product, may play a role here [16–18]. Aβo has been shown to bind directly to, or modulate indirectly, numerous neuronal receptors [19] implying that these impact synaptic signalling cascades including MAPK, Akt, Wnt, and Rho pathways (summarized in Table 2 with references and Figure 1). It appears that Aβo acts as a nonspecific pathological receptor ligand/agonist, both at the pre- and postsynaptic membrane. In addition, Aβo binds to membranes directly which appear to involve GM1 ganglioside and as such are thought to induce structural and functional changes which may impact Ca^{2+} signalling and synaptic plasticity [20, 21]. A global impact of Aβo on different signalling pathways and their respective signalling components is consistent with the widely held view that kinase and phosphatase activities are imbalanced early on in the pathogenesis in diseased neurons [22], resulting in improper hyperphosphorylation of downstream substrates including TAU [6, 23].

Also APP processing itself is controlled by signal transduction pathways. GPCR's, like GPR3 and β2-adrenergic, receptors mediate their effects on APP processing through interaction with β-arrestin and γ-secretase [24, 25]. Activation of JNK3 MAPK by Aβo phosphorylates APP at T668, thereby increasing its endocytosis and subsequent processing [26, 27]. Also, Ras-Erk1/2 and PI3K-Akt signalling pathways activate APP-expression [28] or PS1, a subunit of the APP processing machinery [29]. These results suggest that Aβo

increases its own formation by modulating APP processing through these signalling pathways (Figure 1).

2.2. Signalling and Endocytosis: Intimate Partners in Crime. Cell signalling and endocytosis are increasingly recognized as intertwined and bidirectionally controlled processes [31–33]. Receptor internalisation by endocytosis is a common response upon ligand binding to desensitize cells. Internalised receptors are shuttled to early endosomes, which act as a sorting station for recycling to the plasma membrane or to the lysosome for degradation. Signal propagation is not restricted to the plasma membrane but (may) continue(s) after internalisation. Endosomes marked with active signalling pathways, referred to as signalling endosomes [34], prolong and even intensify signalling while transported through the cell. To illustrate this, activation of the NGF receptor at the presynaptic membrane transiently activates RAS-Erk1/2 signalling [31, 35]. Upon internalization, signalling is sustained as NGF-receptors remain actively coupled with Erk1/2, however, in this context via RAP1, while being transported to the nucleus in order to phosphorylate substrates such as CREB and Erk5. Another example of a close link between signalling and endocytosis entails Wnt signalling. Here, internalization of activated receptors is required to control GSK3 activity and β-catenin stability [36, 37]. Conversely, signalling controls the endocytic pathway itself by impacting the phospholipid turnover. Increases of PIP3 by activation of PI3K allows (apart from Akt) membrane recruitment of Rho and Arf GEFs and GAPs in turn modulating their respective GTPases involved in vesicular trafficking (among other functions such as cytoskeletal rearrangement) [38].

As discussed above, intracellular signalling is deregulated in AD by risk factors and Aβo and thus inevitably will impact endocytosis. Indeed, sporadic AD is characterised by an abnormal activation of the endocytic pathway, with associated increases in PI3K and RAS signalling and Rab5 levels, and comprises early neuropathological alteration even before Aβ pathology ensues [39–41]. Consistent with its role as a pathological receptor ligand, Aβo also increases internalisation of receptors by endocytosis [42–44]. An even more general effect on the endocytic pathway is expected by Aβo triggered receptor-mediated activation of phospholipid signalling through neurotrophin or insulin signalling pathways [45–48]. Likewise, stress-activated p38 MAPK, a kinase activated in AD, stimulates Rab5 which leads to acceleration of endocytosis [49].

Note that AICD, another APP processing product, activates signalling through interaction with Shc and Grb2, adaptor proteins that link with Ras/Erk1/2 and PI3K/Akt pathways (reviewed in [18]) and (perhaps as a consequence) trigger endocytic dysfunction [16]. In addition, GSK3 is also increased by AICD through inhibition of Wnt signalling [50]. Thus, apart from Aβo, other APP processing products also impact signalling and endocytosis in AD and may therefore, at least in part, contribute to the development of AD pathology independently of Aβ [17].

As BACE1 and γ-secretase are localised at endosomes, amyloidogenic, neurotoxic processing of APP requires endocytosis [51–54] and is controlled by vesicular trafficking [55]. Conditions that alter the residence time and/or levels of APP or its processing enzymes at the endocytic compartment impact Aβ production or clearance accordingly [55–58]. For instance, Arf6, a small GTPase controlled by phospholipid signalling, mediates endosomal sorting of BACE1 and thereby APP processing [59]. Or the already abovementioned JNK-driven phosphorylation of APP at T668 facilitates its endocytosis and processing [27]. Similarly, ApoE receptors facilitate the internalization of APP to the endosomal compartment [60].

Taken together, the processing of APP is controlled by signalling pathways which impact expression and endocytic localisation of APP and its processing machinery. Thus, aberrant activation of pathways that increase endocytic APP levels also allows more processing and hence elevates Aβo and AICD formation. This model implies a positive feedback loop as APP processing itself is activated by its own products through signalling (Figure 1). In this way, subtle genetic or nongenetic AD risk factors which lead to relatively small perturbations of signal transduction pathways could, if unchecked, trigger over time a large buildup of Aβo/AICD and thus amplify these subtle alterations into large derangements of signalling and associated endocytosis.

2.3. Derailed Intraneuronal Signalling Is a Common Denominator in Sporadic and Familial AD. As discussed above, in familial AD increased formation of Aβo impacts receptor-mediated signalling. In addition, APP, its processing machinery, and the AICD impact signalling independent of Aβ formation (reviewed in [61]). For instance, PS1, a subunit of the γ-secretase complex, cleaves numerous transmembrane signalling receptors and transducers other than APP CTFs, including Notch, cadherins, ErbB4, LDL receptor related proteins, and so forth [62, 63]. In addition, PS1 and PS2 impact signalling pathways directly. Deletion of PS1 and/or PS2 activates Erk1/2 activity in cell line models, whereas an early onset FAD mutation in PS1 results in constitute activation of CREB-phosphorylation which is associated with neurodegeneration [64–67] and BACE1 regulates the cAMP/PKA/CREB pathway independent of Aβ [68]. Thus, APP and components of its processing machinery impact neuronal signalling pathways independent of APP processing. Hence, FAD mutations can modulate signalling in potentially two ways: through elevated Aβo formation via abnormal APP processing and/or independently of Aβ through altered interactions with signalling pathways. Perhaps through these combined effects on signalling such mutations represent particularly aggressive and penetrant forms of AD.

Considering that signalling and associated endocytosis is abnormal in FAD, it begs the question how this relates to sporadic AD. As amyloidogenic processing of APP is controlled by signalling and endocytosis, it is highly relevant to observe that AD risk factors, although very heterogeneous, have common mechanistic underpinnings by impacting intracellular signal transduction pathways (summarized in Table 1). For example, ApoE4 and traumatic brain injury, two entirely unrelated AD risk factors, both directly

activate common signalling pathways (such as Erk1/2). In fact, for most nongenetic AD risk factors no direct impact on Aβ homeostasis can be hypothesized but involve altered signalling. Metabolic disorders like obesity or diabetes are associated with high levels of cytokines which activate AD relevant pathways in neurons. Likewise, glucocorticoids produced under conditions of chronic stress impact AD relevant signalling cascades in their own right. AD risk factors, such as stroke or head injury involve glutamate receptor-mediated excitotoxicity and impact Ca^{2+} signalling in a way which mechanistically resembles Aβo-instigated activation of Erk1/2 by NMDA receptors. Ageing, the most prominent risk factor for AD, involves, apart from the abovementioned risk factors, altered redox signalling as a result of age-related decline of mitochondrial activity with concomitant increases in ROS production.

In summary, a common denominator in both FAD and sporadic AD comprises perturbation of intraneuronal signalling with associated changes in the endocytic pathway. As outlined above this may result in a vicious, self-enforcing cycle of deranged signalling and Aβ production driving the pathogenesis (Figure 1). In early onset FAD, this autocatalytic mechanism is directly and potently impacted by mutations in APP or its processing machinery. In late onset and sporadic AD initial, probably relatively minor, alterations of signalling by one or more AD risk factors may overtime set off this mechanism which once in motion drives AD pathogenesis.

This scenario resembles a domino system where tumbling of the stones (deranged signalling) is both cause and effect (autocatalytic effect), yet in order to let it happen a "risk factor" such as a sufficiently strong push, windfall, or vibration, is required to set off the cascade. To extent the metaphor further, FAD mutations could be seen as alterations of the core autocatalytic mechanism itself, for instance, as thinner domino stones, which make the system more unstable and thus more sensitive to risk factors. The opposite may be true for "protective" APP mutations (thicker stones, more resilient to risk factors) like the recently discovered Icelandic mutation which decreases APP processing [69].

From this perspective it can be envisaged that AD risk factors comprise a patient-specific constellation which determine the onset and progression of altered signalling and consequently AD pathogenesis. By extension any genetic, environmental, pharmacological, or lifestyle factor impacting this mechanism can, depending on the direction of the effect, be considered as a positive or negative AD risk factor.

2.4. A Signalling Function of Phosphorylated TAU Contributes to AD Pathogenesis. Besides Aβ polymerization and deposition into plaques, hyperphosphorylation and aggregation of TAU into intracellular tangles are other pathological features of AD. The identification of clinical mutations in TAU leading to FTLD strongly suggests that TAU in AD has an important role in pathogenesis [70–72]. Consistent with this notion, in many experimental paradigms a TAU-dependent neuronal degeneration was observed [23]. However, a key question remains as to the mechanism involved especially in relation to changes in signalling and Aβ homeostasis.

A study in transgenic APP mice, a model of early onset AD without TAU-tangle formation, revealed that deletion of the endogenous TAU mouse gene rescues cognitive decline without impacting plaque formation [73]. These findings position TAU as a downstream mediator required for APP-instigated neuronal toxicity, a feature not involving a loss-of-function (i.e., decreased microtubule stabilization), but a gain-of-toxic function which, however, does not involve TAU tangles [74]. Instead it was shown that TAU regulates postsynaptic NMDAR signalling directly by a mechanism involving recruitment of Src kinase Fyn to the PSD95-NMDA receptor complex [75, 76]. Combined with the observation that, like deletion of TAU, lowering of NMDAR-Erk1/2 signalling rescues APP-driven toxicity [75, 77] it appears that in AD such TAU function potentiates NMDA receptor signalling [76, 78]. Likewise, Aβo activation of the mGluR5 receptor through PrP^C may also involve Fyn-TAU interaction [26, 79]. In other words TAU has, besides its well-known function in binding and stabilizing microtubules, a role in intracellular signalling. This raises the distinct possibility that when TAU's signalling activity goes awry it may contribute to AD pathogenesis.

Albeit TAU's signalling function is a somewhat neglected feature, a far more general role of TAU in signalling (apart from impacting NMDA receptors) can be considered. Table 3 shows numerous TAU interactors which are transducers of receptor-mediated signalling implying that TAU can modify their activity through these interactions. These interactors function in a variety of pathways both pre- and postsynaptically. Indeed, apart from impacting postsynaptic NMDA receptor activity, TAU activates presynaptic growth factor signalling through interaction with Src family kinases [80–82] or phospholipid signalling by activation of PLCγ [83] and would provide a mechanistic explanation as to the role of TAU in neurite outgrowth [84, 85] and cell cycle reentry [86, 87] in cell line models.

From Table 3 it can also be appreciated that many interactors through their SH3 domains bind to the proline-rich domain (PRD) of TAU. Notably, TAU PRD is hyperphosphorylated in AD suggesting that these interactions are controlled by TAU-phosphorylation (and indirectly the relevant signalling cascades). Quantifying the TAU-SH3 interaction by surface plasmon resonance and sedimentation assays indicated this may indeed be the case [88, 89]. Phosphorylation-mimicking mutations of TAU were shown to increase or decrease (depending on the TAU-isoform) the affinity to Fyn or Src SH3 domains, consistent with the requirement of TAU-phosphorylation for regulation of NGF-RAS-Erk1/2 signalling [80]. Moreover, clinical FTLD-causing TAU-mutations were found to strongly increase the affinity to SH3, that is, phenocopying the effects of hyperphosphorylation [88]. Thus, these mutations could directly impact signalling, similar as in AD, which may contribute to neuronal degeneration. In fact, it may provide an explanation as to the mechanism of FTLD mutations in TAU which do not impact its aggregation propensity [90] such as R406W [91–94], which possesses an increased affinity to Fyn-SH3 of about 45 times [88]. Collectively, it seems possible that

Table 3: Binding partners of TAU (modified from [4, 192]).

Binding partner	Region of TAU involved	Function/identity of binding partner	References
β-tubulin	Repeat domains	Cytoskeleton	[193]
F-actin		Cytoskeleton	[194]
ApoE3	Repeat domains	Lipid carrier	[195, 196]
Fgr	Proline-rich domain	Src kinase family	[89]
Fyn	Proline-rich domain	Src kinase family	[82, 89]
Lck	Proline-rich domain	Src kinase family	[82, 89]
cSrc	Proline-rich domain	Src kinase family	[82, 89]
Grb2	Proline-rich domain	Growth factor signalling	[89]
c-Abl		Src kinase family	[197]
p85α	Proline-rich domain	Regulator PI3K, phospholipid signalling	[89]
PLCγ		Phospholipid signalling	[89, 198]
GSK3β	N-terminal	Kinase	[199]
Calmodulin	Repeat domain	Ca^{2+} signalling	[200, 201]
14-3-3	Proline-rich domain and repeat domain	Signalling scaffold	[202–204]
Annexin A2		Ca^{2+} signalling, membrane trafficking	[205]
Pin1	Proline-rich domain	Peptidyl-prolyl cis/trans isomerase regulates phosphorylation of TAU	[206, 207]

deregulation of signalling by hyperphosphorylated TAU constitutes a toxic gain-of-function of TAU driving pathogenesis in AD (Figure 1).

2.5. Deregulated Signalling by Aβo or Other AD Risk Factors Triggers TAU-Hyperphosphorylation. An important question however remains as to how TAU becomes hyperphosphorylated in the first place. The facts that TAU is a substrate of many of the kinases operating in the pathways modulated by Aβo (Table 2) or by AD risk factors or genes (Table 1) and that TAU is phosphorylated by neurons challenged with Aβo or other stresses/conditions [95–98], provide a mechanistic explanation as to TAU hyperphosphorylation in AD [5, 23, 99, 100]. Once phosphorylated, TAU may impact intracellular signalling further implying a positive feedback mechanism such as that proposed for TAU-potentiated NGF-Erk1/2 activation [80] and/or by recruitment of Fyn to NMDA receptors [75].

Another salient feature entails the somatodendritic redistribution of TAU in diseased neurons, a prerequisite for impacting postsynaptic signalling. This feature of TAU is controlled by phosphorylation of microtubule binding repeat domains which strongly reduces its affinity to microtubules [101]. Hyperphosphorylation of TAU (and presumably detachment from microtubules) is a prerequisite—by an as yet unclear mechanism—to cross an axonal diffusion barrier allowing TAU to invade the somatodendritic space [102]. Phosphorylation of TAU at the repeat domains, in particular Ser262, is required to elicit Aβ-instigated neurotoxicity [95, 103, 104], indicating that detachment from microtubules entails an important feature of AD pathogenesis. Moreover, Ser262 is one of the earliest sites phosphorylated in the course of pathogenesis [22] and its phosphorylation acts as a priming site for further, more extensive phosphorylation at sites that may control its signalling function [105]. These results indicate a sequential mechanism of TAU-phosphorylation by Aβo and other AD risk factors affecting its subcellular distribution and signalling.

Collectively TAU-phosphorylation comprises a gain-of-toxic function driving AD pathogenesis. Activation of signalling pathways by Aβo and/or by other triggers (see Table 1) leads to hyperphosphorylation of TAU which subsequently decreases its microtubule binding and alters its somatodendritic redistribution and signalling function. These effects may be amplified by a feedback mechanism as formation of Aβo and phosphorylated TAU not only control but are also controlled by signalling (Figure 1). The resulting deregulation of intraneuronal signalling contribute to neurodegeneration (see below), whereas the elevated levels of Aβo and phosphorylated TAU, which have a high propensity to aggregate, lead to Aβ plaques and TAU tangles.

3. Considerations on the Mechanism of Altered Signalling in Neurodegeneration

3.1. Intraneuronal Signalling Defines Fate and Function of a Neuron for Better and for Worse. As discussed above deregulation of signalling may drive the neuronal degeneration in AD. The question, however, remains as to how mechanistically "deregulation" of pathways lead to neuronal degeneration. Neuronal function and survival depend on a balance between neurotrophic and neurotoxic cues setting off signalling cascades which define the outcome ranging from proliferation, differentiation, synaptic plasticity to apoptosis. A classical example entails growth factor signalling which sustains neuronal survival and can trigger differentiation or even antagonize the effects of toxic insults, whereas neurons without sufficient trophic support are prone to undergo apoptosis, as it occurs in a developing nervous system [106, 107].

Thus, properly regulated and balanced signalling, in function of its developmental state, defines fate and function of a neuron [108]. Accordingly, pathological conditions, such as in AD, which off-balance signalling are expected to decrease neuronal integrity.

The underlying mechanisms of how signalling leads to altered neuronal function are poorly understood but extensive work on Erk1/2 signalling revealed insights which may be applicable to other neuronal signalling pathways as well [109]. Erk1/2 signalling is particularly relevant for AD since its aberrant activation is an important driver of neurodegeneration [109, 110]. Erk1/2 kinases are responsive to a wide variety of functional (learning and memory), trophic, and pathogenic stimuli leading to different, even opposing, outcomes including survival, proliferation, differentiation, and neuronal cell death [110–112]. Thus, the signal as such is not predictive of the outcome and additional layers of control exist to determine specificity of Erk1/2 activation. Compartmentalization is a prominent mechanism to ensure specificity as it directs and concentrates the kinase (or sometimes the whole signalling pathway) to appropriate substrates within the cell [113]. This remarkable feature involves several scaffold, anchor, and retention factors which bind to Erk1/2 and often also other signalling molecules determining its subcellular action and allowing crosstalk with other pathways [113].

Localisation of Erk1/2 to specify its output is in part controlled by the kinetics of the signal (reviewed in [114, 115]). During transient activation, Erk1/2 remains predominantly cytoplasmic promoting proliferation, whereas its sustained activation is needed for nuclear concentration and results in differentiation. Chronic stress causes prolonged Erk1/2 activation in the nucleus which contributes to cell death [109, 116]. Thus, the widely different outcomes of Erk1/2 signalling depends, at least in part, on its kinetics as it dictates its subcellular localisation and as such specifies accessibility of substrates (reviewed in [113, 115]). In several model systems, sustained Erk1/2 activation involves a nuclear accumulation which is associated with detrimental outcomes [116–120]. For instance, neurons challenged with stress trigger a persistent nuclear retention of activated Erk1/2 and elicit proapoptotic effects and cell death [109, 116]. Accordingly, it seems likely that the chronic activation of Erk1/2 in AD, presumably by Aβo and possibly other risk factors (see Tables 1 and 2), leads to an aberrant, prolonged nuclear accumulation contributing to neuronal demise.

3.2. Diseased Neurons in AD Display Signalling Configured for Immature Neurons. The insights obtained from the studies on Erk1/2 revealed that spatiotemporal control of Erk1/2 signalling determines its impact on neuronal function and survival [109] and as such provide a conceptual framework of the underlying mechanisms as to how derailed Erk1/2 signalling contributes to neuronal degeneration in AD. We anticipate that this concept is likely applicable to other signalling pathways as well.

In fact hyperphosphorylation of TAU in AD can be considered a reflection of such global deregulation of signalling in adult neurons [6] and illustrates how this may lead to inappropriate outcomes in function of the developmental state. As outlined above, hyperphosphorylation of TAU may lead to increased microtubule dynamics and the potentiating of pathways (such as Erk1/2) resulting in aberrant cell cycle entry and apoptosis. Such functional outcomes are expected to be detrimental in mature, postmitotic neurons of the adult brain. However, in a developing brain hyperphosphorylated TAU is fully appropriate as, in this context, neurons require dynamic microtubules to mediate sufficient synaptic plasticity, proliferation, and differentiation but also susceptibility to undergo apoptosis when trophic support by target cells is insufficient [108]. In other words, neuronal signalling in AD involving TAU-hyperphosphorylation appears to be geared to a situation resembling an immature brain.

Perhaps, a similar situation may apply for APP and its processing as well, given the neurotrophic properties of APP and its cleavage products [121]. Addition of APP to PC12 cells stimulates neurite outgrowth [122], whereas in transgenic mice expression of human APP results in increased neurogenesis [123, 124]. Moreover, the AICD promotes signalling associated with neurite outgrowth [18, 50], and secreted sAPPα impacts proliferation of embryonic stem cells [125]. Remarkably, at low concentration, Aβ has neurotrophic activity but only in undifferentiated neurons but is toxic to mature neurons [126–129]. Thus, APP and its processing products may have a role in proliferation and differentiation, functions that are particularly relevant in a developing brain, but, when unchecked, toxic to mature neurons.

Collectively it can be envisaged that APP, its processing products, and TAU are part of an intraneuronal signalling network required for neurogenesis, neuronal function, and survival which needs to be appropriately tuned to the developmental status. Accordingly, pathological conditions or risk factors which off-balance such signalling network to a state resembling immature neurons will be detrimental for mature neurons.

3.3. Considerations on Drug Discovery for Alzheimer's Disease. As discussed above aberrant activation of signalling cascades underlies mechanistically neurodegeneration in AD. As such it may provide a conceptual framework for successful drug discovery as it assumes that interventions aimed at normalizing signalling are expected to be neuroprotective, to reduce Aβ levels and TAU-phosphorylation and consequently plaque and tangle formation. In this way a fundamental mechanism driving pathogenesis in AD will be targeted and thus anticipates the minimum to preserve the function of still healthy neurons in the diseased brain and possibly may even restore dysfunctional synaptic activity of affected, but still living, neurons in symptomatic patients. However, given the multitude of pathways involved and considering their important neuronal functions, pharmacological modulation of one, specific target safely to achieve that goal will be a major challenge. Another confounding factor comprises the heterogeneity of sporadic patients, presumably reflected by the heterogeneity of risk factors each with their specific effects on the nature and effect size of the signalling pathways.

Aβ-directed therapeutic approaches to reduce Aβ levels have been and are still heavily explored and are expected to

normalize signalling, at least to some extent, and thus have therapeutic potential. However, a possible downside may be that in symptomatic patients TAU-hyperphosphorylation has kicked in already to a level able to derange signalling and neuronal function in a feed forward fashion independent of Aβo (from that point on perhaps mechanistically similar to how clinical TAU mutations in FTLD lead to neurodegeneration). Thus, such approach would be most successful in a preventive setup very early in the development of AD. Another consideration is that the therapeutic intervention itself should not inadvertently impact neuronal signalling for the worse. For instance, inhibiting γ-secretase will, on one hand, lead to lowered Aβo levels and most likely to cognitive improvement in transgenic APP mouse models of familial AD but on the other hand may also impact signalling pathways (such as increased Erk1/2 activity [65]), independent of APP processing, which may impair a therapeutic response in sporadic AD patients. Likewise inhibition of CDK5, a prominent TAU-kinase and considered an attractive drug target for AD [130], may lead to sustained Erk1/2 activity and consequently neuronal apoptosis [131].

Nevertheless, promising drug targets to be considered for therapeutic intervention comprise components of signalling pathways impacted in AD [6, 130] although there is a risk—given the overall deregulation of signalling—that downregulation of only one kinase (or pathway) might be too limited to result in a satisfying therapeutic response. From this perspective, an interesting point of intervention may comprise the convergence where receptors relay their environmental cues to second messengers such as Ca^{2+} and/or small GTPases modules (Figure 1). Downregulating, but not fully inhibiting, the activity of such relay systems may lead to a more global normalization of signalling in AD and thus may constitute a promising therapeutic avenue.

Conflict of Interests

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

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Research Article

Wavelet Analysis Increases Sensitivity and Specificity of Spirography for Ambulatory Tremor Discrimination

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The most frequently seen types of tremor are essential (ET) and parkinsonian tremor (PT) and in some patients clinical characteristics of these tremor types overlap. It is vital to distinguish between these two types of tremor in order to reach the right diagnosis and select the appropriate treatment. One of the widely used methods for tremor detection and discrimination, appropriate for a quick ambulatory assessment of the patient's tremor, is spirography. With spirography, the tremor can be observed through several parameters, for example, tremor spectrum and spiral image, which give useful information for its identification. Standard spirography parameters of ET and PT can overlap; therefore, these parameters are often not enough for identification of the observed tremor. To increase the specificity and sensitivity of spirography for PT, ET and normal, tremor free controls, we used the wavelet analysis with Morlet wavelet transform. To facilitate analysis, comparison, storage, and retrieval of spirography tremor records we also developed an integrated computer assisted spirography system that increases the convenience of outpatient tremor identification and follow-up. We conclude that wavelet analysis of spirography records increases the sensitivity and specificity of the method, thus, facilitating the distinction between ET and PT.

1. Introduction

Tremor is the most frequent movement disorder. There are several types of tremor, but the most common types are Parkinsonian (PT) and essential tremor (ET) [1]. In clinical practice, tremor is mostly diagnosed by using clinical examination only. However, by using only clinical examination, ET is accurately diagnosed in 50–63% of cases, whereas the PT in 76% of the pathologically confirmed cases [2]. The occasional overlap between different types of tremor makes diagnosing even more difficult. For example, more than 50% of patients with PT that have pathognomonic resting tremor also have postural tremor [2].

Patients with ET or PT have an increased activity in the cerebellothalamocortical circuit. In Parkinson's disease, the increased activity in the cerebellothalamocortical circuit is caused by the dopaminergic dysfunction of the pallidum triggers. In ET, the GABAergic dysfunction of the cerebellar dentate nucleus and brain stem, possibly caused by neurodegeneration in these regions, lead to tremulous activity within the cerebellothalamocortical circuit [3].

To reach the right diagnosis and select the appropriate medical treatment, it is necessary to discriminate between these two types of tremors. Additional methods for more precise tremor assessment have been used. For example: subjective scales for clinical assessment of tremor [4], functional tremor evaluation tests, tests for evaluation of tremor's impact on activities of daily living [5], physiological measurement methods—such as surface EMG [6], accelerometry [7], tremor tracking in electromagnetic field [8], video method [9] and also computer assisted spirography method (CAS) [10, 11].

Computer assisted spirography method (CAS) has already been used as an additional diagnostic tool for tremor discrimination for the last 20 years [10]. It is an appropriate method for a quick ambulatory assessment of

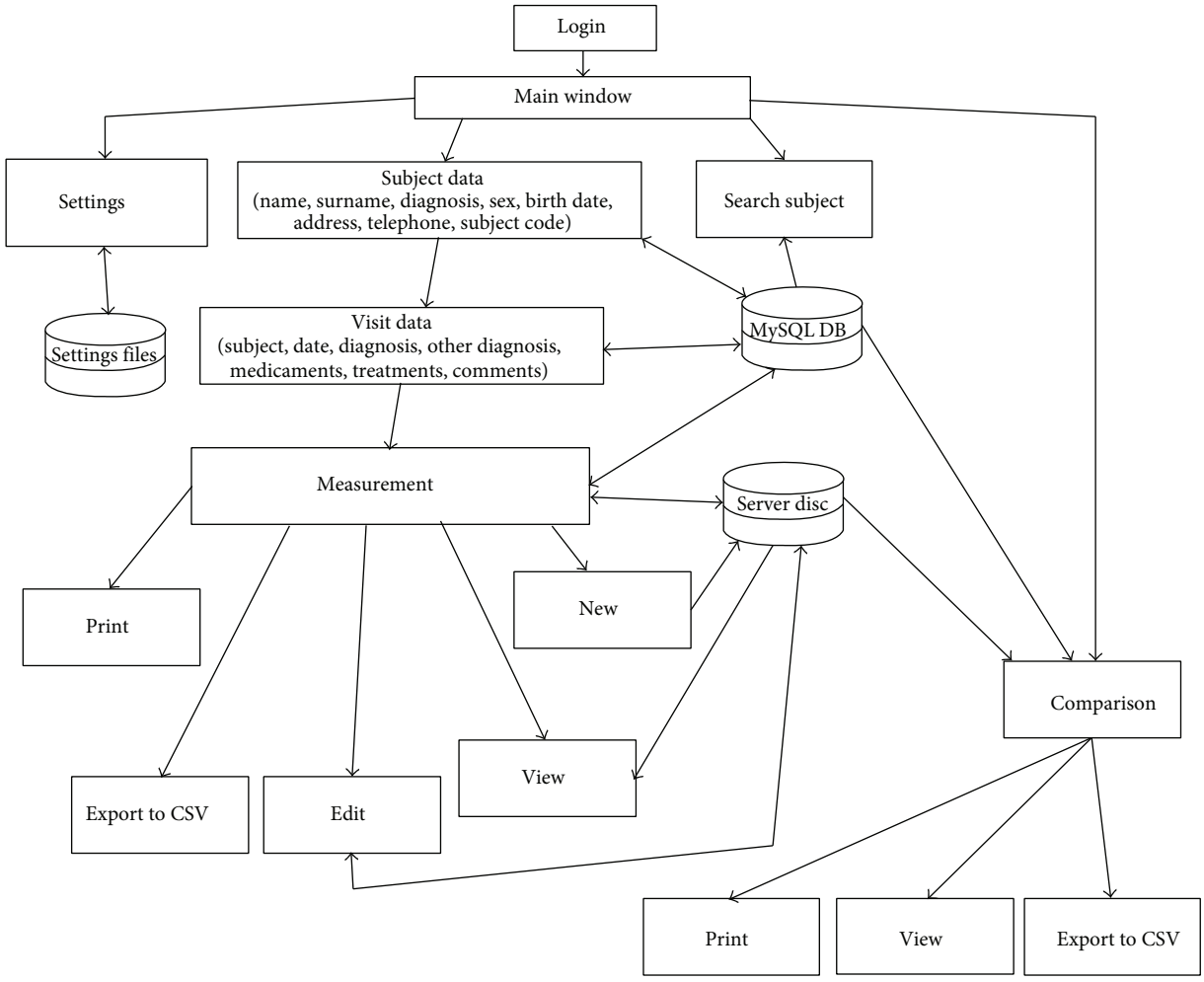

Figure 1: The structure presentation of the CAS application.

the tremor, because it allows precise measurement of tremor amplitude and frequency, and also accurately quantifies the observed tremor in comparison with traditional clinical examination and other subjective methods. The tremor can be viewed through different parameters such as tremor spectrum, pressure spectrum, radius-angle transform, and velocity-time transform [10].

Although the standard parameters of CAS have been useful for intra- and interpatient tremor evaluation, the differentiation of tremors is still not perfect in some cases, especially because of overlapping different tremor types (e.g., ET and PT) and a variable expression of tremor amplitude over time [2].

At the Department of Neurology, University Medical Centre Ljubljana we included a new parameter for spirography analysis, the wavelet transform scalogram calculated with the wavelet analysis method using the Morlet wavelet transform in time-frequency representation, to increase the specificity and sensitivity of the CAS [12]. The Morlet wavelet transform is a type of continuous wavelet transform and the most popular complex wavelet used in practice, which mother wavelet is defined as

$$\psi(t) = \frac{1}{\sqrt[4]{\pi}} \left(e^{jw_0 t} - e^{w_0^2/2} \right) e^{t^2/2}, \quad (1)$$

where w_0 is the central frequency of the mother wavelet.

To facilitate analysis, comparison, storage, and retrieval of spirography tremor records, we also developed an integrated computer assisted spirography system for patient record keeping that increases the convenience of ambulatory tremor identification, differentiation and follow-up.

2. Materials and Methods

2.1. Participants. Spirography records from fifty participants (20 with PT, 15 with ET, and 15 controls; 24 males and 26 females; average age was 61 years) were selected from the Department of Neurology, University Medical Centre Ljubljana database. The patients' and controls' hospital

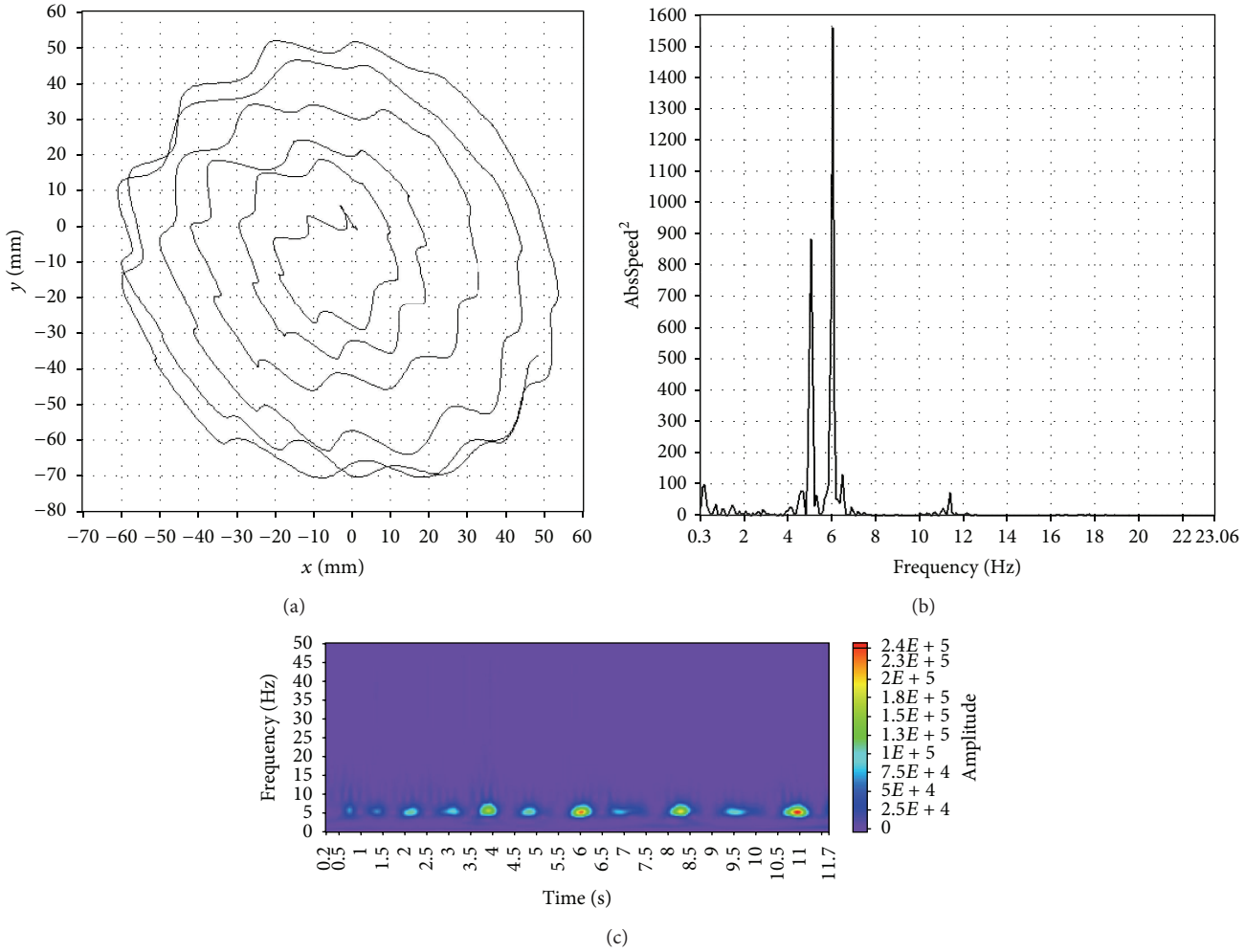

Figure 2: A typical example of spiral image (a), tremor spectrum (b), and wavelet transform scalogram (c) for an ET patient.

records were examined and selected by a senior neurologists [13, 14], so that patients with other parkinsonian syndromes (parkinson-plus syndromes, multiple-system atrophy, cortico-basal degeneration, iatrogenic parkinsonism), hepatic diseases, and alcoholism were excluded from this retrospective study. The study was approved by the Medical Ethical Committee of the University in Ljubljana and was in accordance with the Helsinki Declaration of 1975.

2.2. Patient Evaluation Protocol. Previously, spirography data of patients with neurological diseases were stored on several computers using different recording, storage, data retrieval and analysis protocols. We developed an integrated computer assisted spirography system that facilitates analysis, comparison, storage and retrieval of patients' spirography tremor records (Figure 1) and has been routinely used at the Department of Neurology for several years. Hospital staff can access via the intranet the common patients' database using a single user interface for measurements, data analysis and retrieval of patient records. Thus, the system increases the convenience of ambulatory tremor identification, discrimination and patient follow-up. For proper administration of patient data, the user is first required to fill in subject and visit data input fields. Then the user can initiate a new measurement, view the recorded measurements, and edit or delete previously recorded measurements. The comparison between previously chosen measurements is another important part of CAS application. It allows comparing between two or more selected measurements through all observed parameters. All observed measurements can be exported to a comma separated value (CSV) file format. Tremor measurements can be made at a stationary computer, connected to the database via intranet, or on a laptop and later merged with the patient's database on the stationary unit.

The three spirography parameters, retrospectively evaluated in our study, were the drawn spiral image for qualitative evaluation [15], tremor spectrum [16], and a newly introduced parameter the wavelet transform scalogram [12].

During the measurement the participants sat on a chair and freehandedly drew a spiral on a programmed graphical tablet with the tablet's pen [17]. During the drawing process the participant was required to keep the pen in contact with the tablet's surface for the whole time. Patients with severe tremor were not always able to do that. However, the

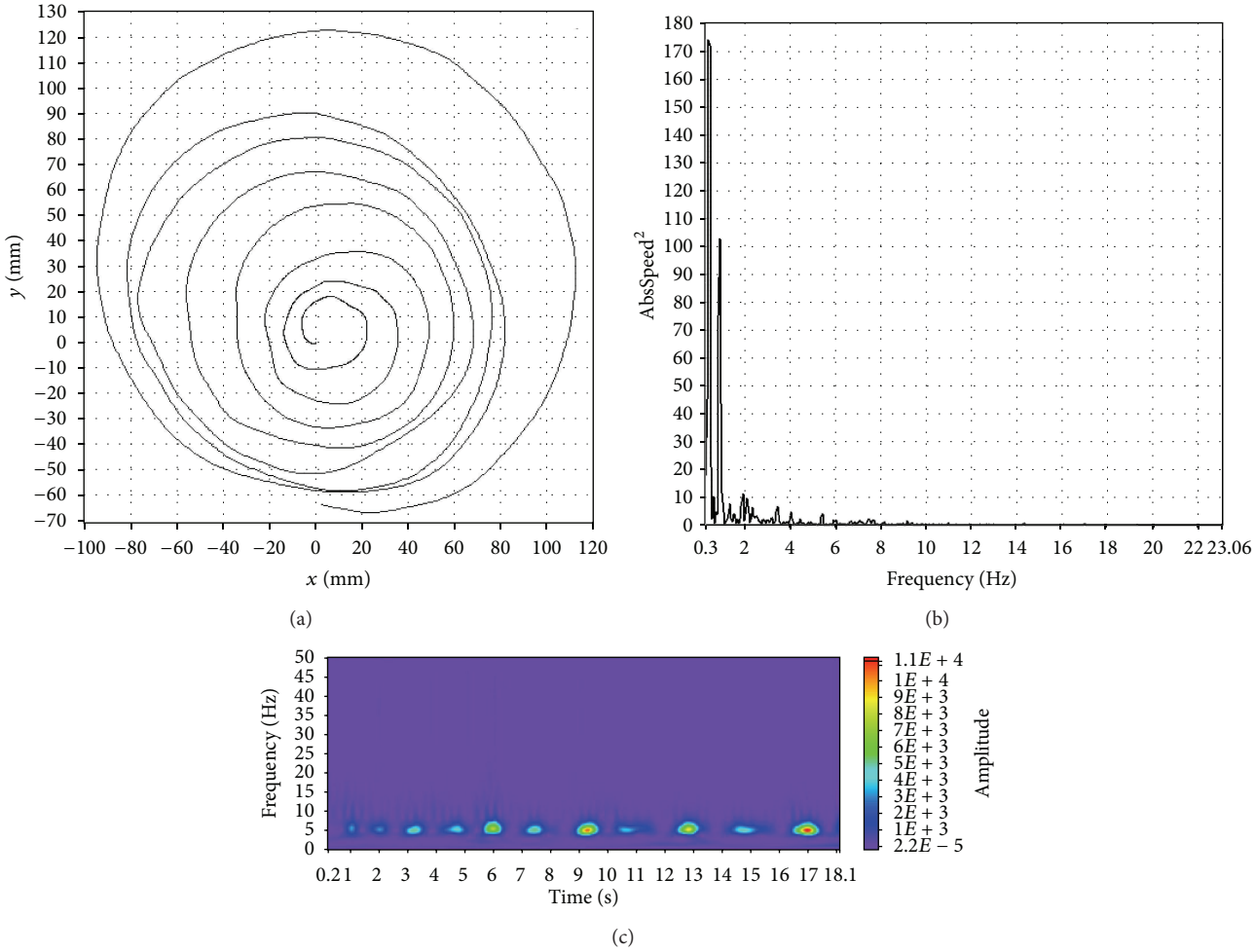

Figure 3: A typical example of spiral image (a), tremor spectrum (b), and wavelet transform scalogram (c) for a PT patient.

digital tablet can still track the pen's position even if it is raised for a few millimeters above the surface. This made the measurements on patients with severe tremor easier and also without the need for custom adjustments.

The spirography parameters (spiral images, tremor spectra, and wavelet transform scalograms) were evaluated visually by three experts, trained in spirography. Based on the results, the specificity and sensitivity of the three parameters for detection of PT, ET and healthy controls were calculated as follows [18]. For example, the sensitivity of spiral images for ET is the proportion of people with ET who will have a positive result ($a/(a+c)$) and its specificity is the proportion of people without ET who will have a negative result ($d/(b+d)$). Therefore, a is the number of patients with ET that test positive, c is the number of patients with ET that test negative, b is the number of persons without ET that test positive and d is the number of persons without ET that test negative.

3. Results

Visual characteristics of spiral images, tremor spectra, and wavelet transform scalograms enable the distinction among patients with ET or PT and tremor free, healthy controls. A representative example of a spiral image, a tremor spectrum and a wavelet transform scalogram for an ET patient is shown in Figure 2. Spiral images, tremor spectra, and wavelet transform scalograms, for a PD patient and for a healthy control, are shown in Figures 3 and 4, respectively.

A patient with ET usually has a symmetrical and wavy spiral, a tremor spectrum with one or two dominant frequency peaks between 4 and 12 Hz and a wavelet transform scalogram filled with many oval shaped islands of activity (Figure 2). Patients with PD tremor (Figure 3) typically draw an asymmetrical spiral that is usually compressed at the base; their tremor spectrum has multiple peaks of activity up to 8 Hz and a wavelet transform scalogram with a continuously present activity during the time of drawing. Healthy, tremor free controls tend to draw a symmetrical spiral, have a tremor spectrum with peaks below 2 Hz and a wavelet transform scalogram with very little activity that is usually concentrated in the second half of the record (Figure 4).

We calculated the specificity and sensitivity of the three spirography parameters for diagnosing ET, PT and differentiating healthy controls. There was no difference among the patients' spirography parameters with regard to gender. The

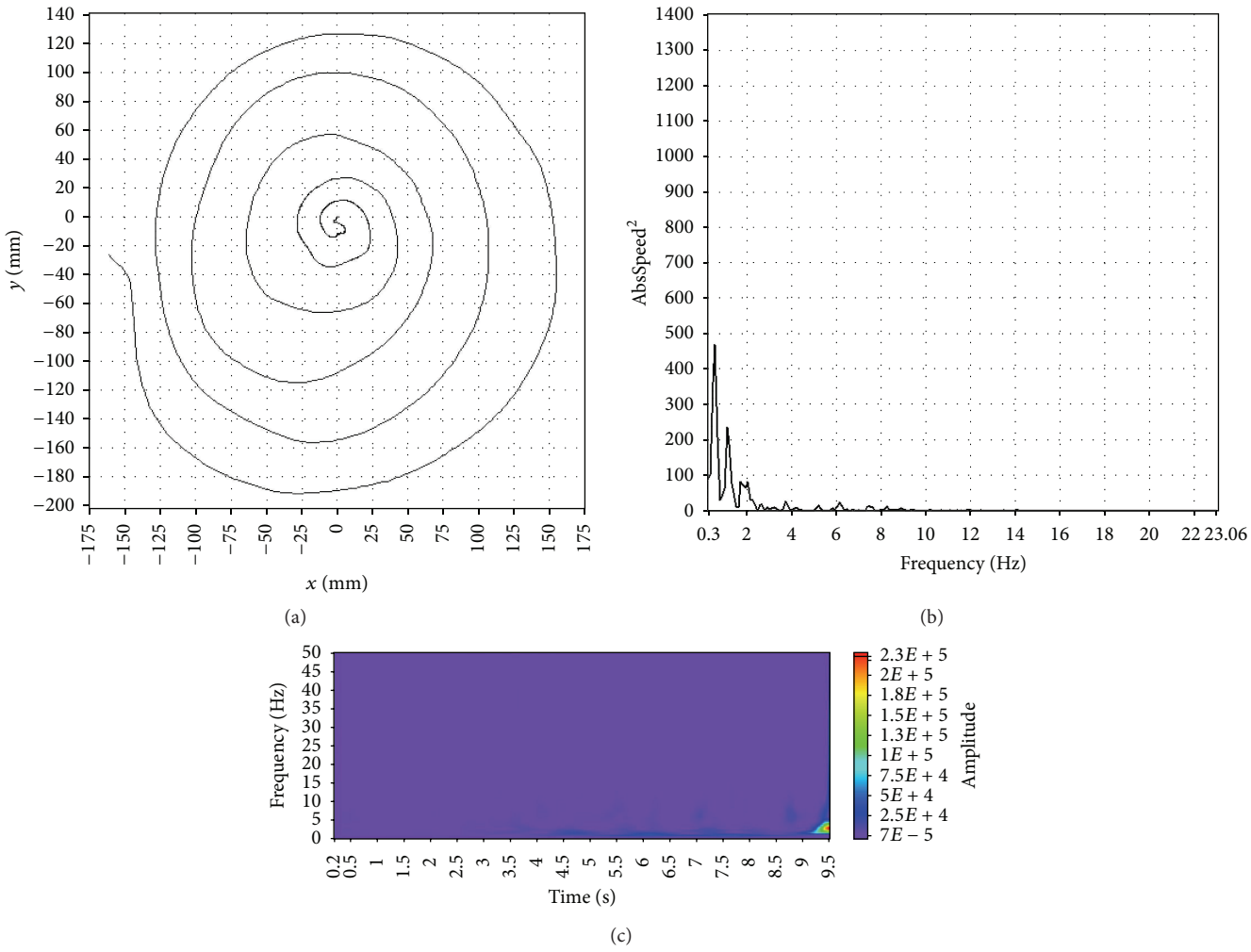

Figure 4: A typical example of spiral image (a), tremor spectrum (b), and wavelet transform scalogram (c) for a healthy, tremor free control.

Table 1: Specificity and sensitivity for diagnosing ET (the number of patients is 15).

Spirography parameter(s)	Specificity ET	Sensitivity ET
Spiral image (S)	0.96	0.84
Wavelet transform scalogram (W)	0.99	0.87
Tremor spectrum (T)	0.89	0.73
S & T	0.97	0.82
S & T & W	0.97	0.91

Table 2: Specificity and sensitivity for diagnosing PT (the number of patients is 20).

Spirography parameter(s)	Specificity PT	Sensitivity PT
Spiral image (S)	0.75	0.65
Wavelet transform scalogram (W)	0.91	0.92
Tremor spectrum (T)	0.71	0.53
S & T	0.82	0.67
S & T & W	0.92	0.88

results are shown in Tables 1, 2 and 3 for ET, PT and controls, respectively.

3.1. Specificity. Diagnosing ET by visual examination of wavelet transform scalograms from spirography records had the highest specificity (0.99) and was similar to the specificity for differentiating controls by wavelet transform from ET and PT (0.97). Diagnosing PT only on the basis of the patient's frequency spectrum showed the lowest specificity (0.71). The lowest specificity in all three groups was noted for the tremor spectrum parameter (0.89, 0.71 and 0.80 for ET, PT, and controls resp.). Combining spiral image and tremor spectrum analysis improved specificity in all three groups compared to analysis with tremor spectrum or spiral image only (0.97, 0.82 and 0.86 for ET, PT and controls resp.) but did not reached the specificity for wavelet transform analysis only. Diagnosing tremor by combined visual analysis of all three parameters (i.e., spiral drawing, tremor spectrum, and wavelet transform scalogram) further increased the sensitivity and specificity of spirography for ET,

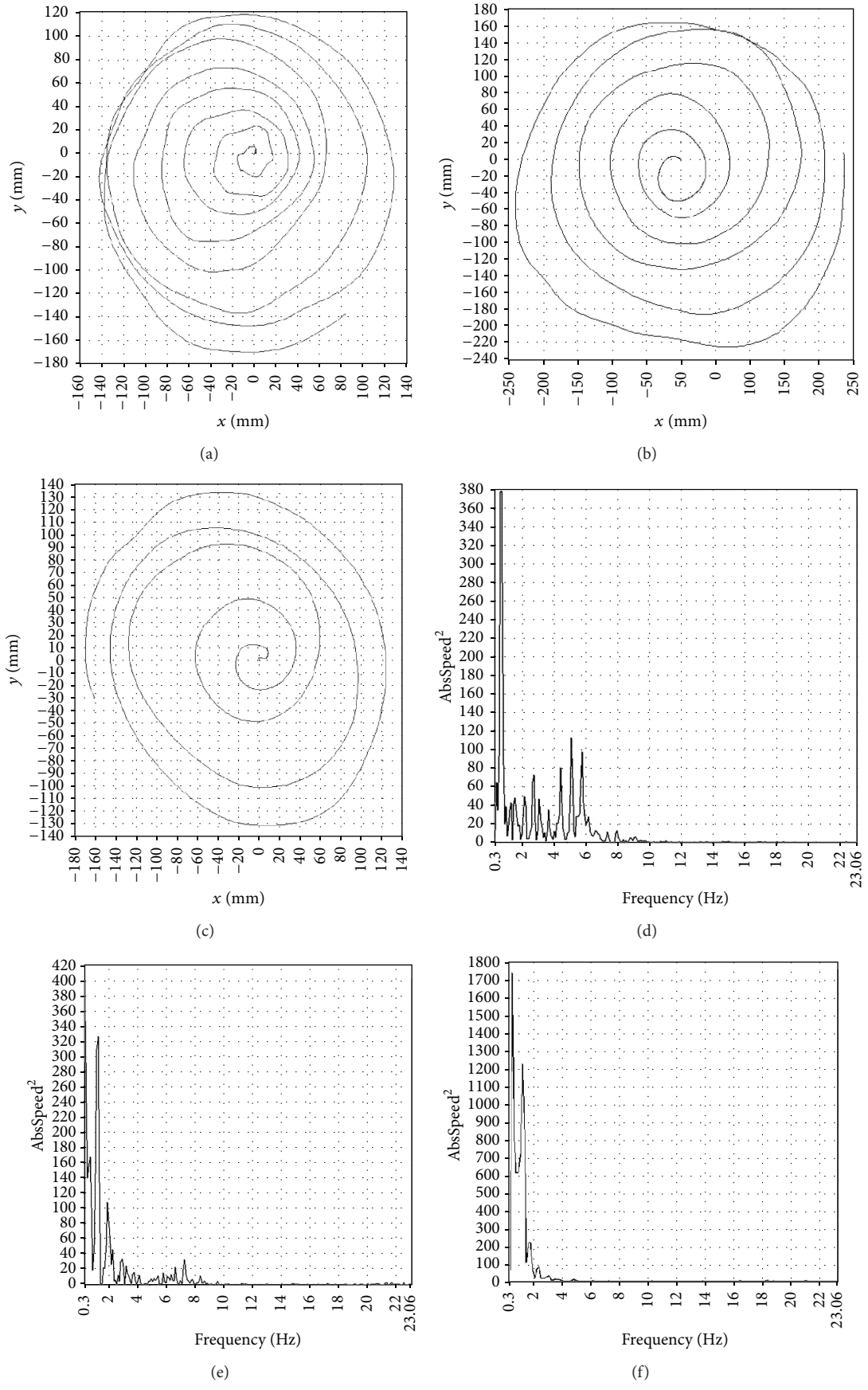

Figure 5: Continued.

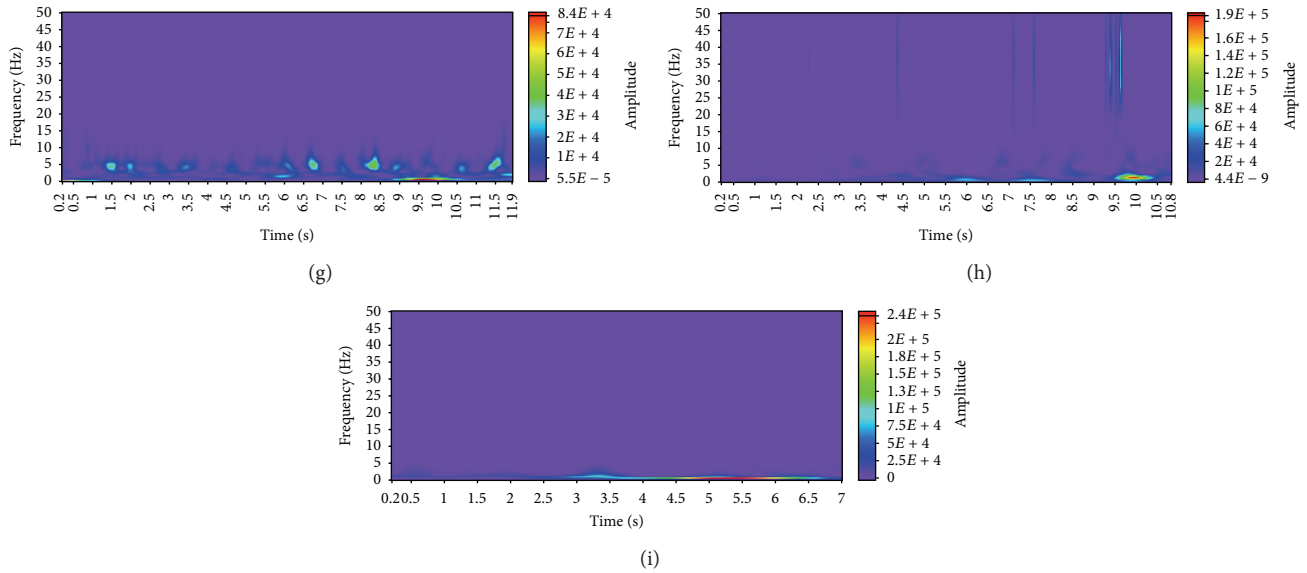

Figure 5: An example similar spirography parameters results for an ET ((a), (d), (g)), a PT ((b), (e), and (h)) and a healthy control ((c), (f), and (i)).

Table 3: Specificity and sensitivity for differentiating controls (the number of patients is 15).

Spirography parameter(s)	Specificity controls	Sensitivity controls
Spiral image (S)	0.79	0.59
Wavelet transform scalogram (W)	0.97	0.89
Tremor spectrum (T)	0.80	0.58
S & T	0.86	0.73
S & T & W	0.93	0.87

PT, and controls. However, diagnosing by wavelet transform scalogram only was better than after combined analysis with all three parameters for ET and controls (0.99 versus 0.97 for ET and 0.97 versus 0.93 for controls). The combination of spiral image, tremor spectrum, and wavelet transform scalogram analysis slightly improved the specificity for PT diagnosis compared to wavelet transform scalogram only (0.92 versus 0.91).

3.2. Sensitivity. Combining spiral image, tremor spectrum, and wavelet analysis showed the highest sensitivity for diagnosing ET (0.91) compared to diagnosing PT or differentiating controls (0.88 and 0.87 resp.). The lowest sensitivity was for diagnosing PT by tremor spectrum only (0.53). Analysing spirography records by wavelet transform scalograms only showed the highest sensitivity for differentiating PT and controls (0.92 and 0.89 resp.). The highest sensitivity for ET was achieved with the combined visual analysis of spiral image; tremor spectrum and wavelet transform scalogram (0.91). However, combining spiral image, tremor spectrum, and wavelet transform scalogram analysis reduced the sensitivity for PT and for controls compared to diagnosing with the wavelet transform scalogram only (0.88 versus 0.92 for PT; 0.87 versus 0.89 for tremor free controls).

4. Discussion

The results of spirography tremor analysis show that, compared to spiral image and tremor spectrum evaluation, the wavelet transform scalogram has the highest sensitivity and specificity for detection of healthy controls and patients with ET and PT. Therefore, the wavelet transform scalogram is the most useful single spirography parameter to distinguish between these two common types of tremor. The main advantage of wavelet transform scalogram, compared to tremor spectrum, is that wavelets are localized in both time and frequency; it can detect amplitude change of a specific frequency with respect to time, whereas tremor spectrum is only localized in frequency and is useful for amplitude analysis of frequency component over the whole time span of a signal.

The sensitivity and specificity of all spirography parameters is lower for PT than for ET since spirography is more sensitive to action tremor (i.e., postural or kinetic tremors) than to resting tremors. PT is usually a resting tremor, most pronounced when the affected body part is completely supported against gravity. In ET, tremor of the hands is typically an action tremor. However, some patients with Parkinson's disease also have an associated action tremor [1]. Differentiation of PT from ET is also difficult because of an overlapping tremor frequency range (4–12 Hz for ET and 3–7 Hz for PT). The frequency of ET may decrease with age, thus, further complicating tremor diagnosis.

When the distinction between PT and ET with spirography is in doubt, a reliable diagnosis can be achieved by evaluating the wavelet transform scalogram or combining the visual analysis of tremor spectrum and spiral image.

Evaluating tremor with all three spirography parameters simultaneously (i.e., spiral image, tremor spectrum, and wavelet transform scalogram) does not lead to a consistent, further increase in sensitivity, and specificity of spirography for PT, ET, and healthy controls.

In Figure 5 we can see an example of similar spiral image and tremor spectrum parameters for PT and ET patients and a healthy, tremor free, and control participant. Therefore, the differentiation among PT and ET patients and the healthy control is possible only by the wavelet transform scalogram.

Previous spirography studies have evaluated tremor through different parameters [10, 19, 20], but none of them have used the wavelet transform scalogram nor compared the sensitivity and specificity of spiral image, tremor spectrum and wavelet transform scalogram for ET, PT, and tremor free controls. To our knowledge, this is the first spirography study that includes the wavelet transform scalogram as an additional parameter for tremor identification. It is also the first study that compares the sensitivity and specificity of spiral image, tremor spectrum, and wavelet transform scalogram in patients with ET or PT and in tremor free controls.

5. Conclusions

Because of the overlapping characteristics of different tremor types and variable expression of tremor amplitude over time, tremor differentiation is often difficult and conventional spirography parameters are often not enough for identification of the observed tremor. To improve tremor type differentiation with spirography, we introduced the wavelet transform scalogram. Compared to the spiral image and the tremor spectrum, the wavelet transform scalogram has the highest specificity and sensitivity for ET, PT, and tremor free controls. Thus, wavelet analysis increases sensitivity and specificity of spirography for ambulatory tremor discrimination.

Conflict of Interests

The authors report no conflict of interests.

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Research Article

Transgenic Rat Model of Huntington's Disease: A Histopathological Study and Correlations with Neurodegenerative Process in the Brain of HD Patients

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Rats transgenic for Huntington's disease (tgHD51 CAG rats), surviving up to two years, represent an animal model of HD similar to the late-onset form of human disease. This enables us to follow histopathological changes in course of neurodegenerative process (NDP) within the striatum and compare them with postmortem samples of human HD brains. A basic difference between HD pathology in human and tgHD51 rats is in the rate of NDP progression that originates primarily from slow neuronal degeneration consequently resulting in lesser extent of concomitant reactive gliosis in the brain of tgHD51 rats. Although larger amount of striatal neurons displays only gradual decrease in their size, their number is significantly reduced in the oldest tgHD51 rats. Our quantitative analysis proved that the end of the first year represents the turn in the development of morphological changes related to the progression of NDP in tgHD51 rats. Our data also support the view that all types of CNS glial cells play an important, irreplaceable role in NDP. To the best of our knowledge, our findings are the first to document that tgHD51 CAG rats can be used as a valid animal model for detailed histopathological studies related to HD in human.

1. Introduction

Huntington's disease (HD) is an autosomal dominant inherited disorder belonging to the group of systemic brain atrophies. General clinical symptoms are defined by early changes in personality and cognitive functions, followed by typical involuntary choreatic movements; advanced stages of the disease include bradykinesia, rigidity, and dementia. The first symptoms appear frequently between 35 and 50 years of age. The disease is always fatal with an average survival of 10–15 years after the onset of the first symptoms. The disease may develop at any time, even during childhood and adolescence. Of note, juvenile form of HD represents 6% of HD patients [1].

Histopathologically, HD is characterised by premature death particularly of medium-sized (mainly GABA-ergic) striatal neurons but large interneurons are mostly spared (e.g., [2]). Regardless of the development of reactive gliosis within the striatum, loss of the grey matter is extensive and results in the compensatory enlargement of lateral brain ventricles. Due to the general atrophy, the brain weight can decrease by 40% (e.g., [3]).

Severity of the degeneration is evaluated using grading system proposed by Vonsattel and coauthors [2], which classifies HD into five grades (0–4). In grade 0, patients have already clinical symptoms; however, neuronal loss in the head of the caudate nucleus (CN) can be detected only on microscopic level. In grade 1, neuronal loss is accompanied by reactive gliosis, which is evident primarily in the head and tail, less in the body of CN. Grade 2 is characterised by mild to moderated striatal atrophy. In grade 3, due to the progression of atrophy, the medial outline of the CN becomes straight. In grade 4, neuronal loss is a keystone (reaches 95%) and therefore the medial part of the CN is concave. Moreover, brains from patients with late onset of clinical symptoms might show changes occurring in usual aging in addition to those observed in HD (e.g., [2]).

Genetic mutation on short arm of chromosome 4, which causes HD, was discovered in 1983 [4]. Lately, in 1993, gene IT15 (interesting transcript 15), which codes unstable protein huntingtin (htt) comprising variable number of CAG repeats, was identified [5]. Such molecular defect is based on the expansion of this triplet that codes amino acid glutamine. Therefore, HD can be included into the group of polyglutaminopathies.

Mutant form of huntingtin (mhtt) comprises up to 40 repeats and individuals with 36–39 CAG repeats are in risk of developing adult (late-onset) form of HD. Juvenile form of HD develops in patients with 55 and more repeats (e.g., [6]). Although wild-type huntingtin is expressed in all cell types, with the highest concentration in the brain [7], its functions are not yet fully understood [8]. Expanded polyglutamine forms intracellular deposits, particularly in a form of intranuclear and neuropil aggregates (e.g., [8, 9]). It is evident that mhtt displays specific toxicity to striatal (mainly GABA-ergic), less cortical neurons. However, the role of mhtt in the pathogenesis of HD appears highly controversial, ranging from being essential in pathogenesis of the disease (e.g., [6, 8]) to being neuroprotective [10, 11]. Moreover, mhtt also accumulates in nuclei of astrocytes causing their dysfunction, particularly in relation to glutamate uptake. This may further promote vulnerability, especially of striatal medium-sized spiny neurons, to the excitotoxic damage [12]. Therefore, turning our attention to glutamate uptake in astrocytes might be of particular importance for preventing glutamate excitotoxicity in HD [12, 13]. Nevertheless, the presence of mhtt in neural cells is not the only mechanism, which is involved in HD pathogenesis. Metabolic and mitochondrial dysfunctions, oxidative and nitrative stress, and also apoptosis play an important role as well (e.g., [14]).

Generation of transgenic mice as well as transgenic rats advanced significantly the understanding of HD pathology. Indeed, over 20 different rodent models of this disease have been generated to date (for the review see [15]). The firstly introduced transgenic model of HD termed R6 [16] and other generated lines, such as R6/1 and R6/2 (with 115 and 145 CAG repeats, resp.), belong to the most frequently used models of HD (e.g., [17, 18]). These models of HD are characterized by early onset and fast progression of behavioural deficits (later also by motor dysfunction) and presence of intranuclear polyglutamine (polyQ) inclusions (4 weeks postnatally), but without the evident reduction of neurons even in aged mice [16, 19]. Therefore, they simulate rather juvenile than adult form of HD.

In 2003, von Hörsten and coworkers generated the first transgenic rat model of HD (tgHD51 rats; Sprague-Dawley background), which carries a truncated htt cDNA fragment with 51 CAG repeats under the control of the native rat htt promoter [20]. Due to relatively smaller number of CAG repeats, this model exhibits a high degree of similarity to the late-onset form of HD. These rats survived up to 24 months and exhibited slow cognitive decline and not as much of motor deficit (e.g., [9, 20–24]). Neuropil aggregates of polyglutamine and typical intranuclear inclusions in neurons appeared in the brain approximately at 6–9 months of age [9]. Despite the fact that tgHD51 rats displayed enlarged lateral brain ventricles, stereological analysis revealed only a subtle decrease in the number of neurons in the striatum, while no changes were observed in number of neurons in the frontal cortical layer V of 12-month-old tgHD51 rats when compared with age-matched wild-type controls [24].

Astrocytes are the most numerous type of glial cells in mammalian CNS. Currently, they are considered as highly active cellular component of the CNS parenchyma with functional pleiotropy essential for neuronal survival and function (e.g., [25–27]). Besides others, they are closely associated with neuronal synapses ("tripartite synapse"—[28]), secrete and degradate proteins of extracellular matrix, and are responsible for uptake and release of different neurotransmitters, primarily of the glutamate. It has been suggested that enhanced release of glutamate and other substances may represent an early event in a number of, if not all, neurodegenerative diseases (e.g., [29]).

The communication between neurons and local blood flow mediated by astrocytes is elementary for the maintenance of functional microenvironment in the grey matter of the entire CNS parenchyma; therefore the term neuronal-glial-vascular unit is used [30]. On the other hand, under pathological conditions, the perivascular end-feet can restrict transport or diffusion across the blood-brain interface [31].

Glial fibrillary acidic protein (GFAP [32]) is essential for immunohistochemical identification of astrocytes. However, GFAP is densely expressed only within the cell body and larger processes of astrocytes, unlike the numerous fine processes representing the majority of the total volume of the astrocytes, which are GFAP-negative [30]. Any type of the CNS injury (primarily the acute damage) initiates morphological changes of some astrocytes, which become reactive (e.g., [33]). They are hypertrophic with longer and thicker main processes and increased expression of GFAP due to the formation of bundles of gliofilaments (e.g., [34]). In the acute phase of reaction, astrocytes also reexpress intermediate filaments significant for glial precursors—nestin and vimentin (e.g., [35, 36]).

Beta-subunit of Ca^{2+} binding protein (S100β) is another putative astrocytic marker (e.g., [37]). S100β is produced, stored, and released primarily by astrocytes; however, it is also expressed by many other cells in the CNS and other body regions. S100β is localized in the cytoplasm and nucleus and

involved in the regulation of a number of cellular processes (e.g., [38, 39]). Regardless of large body of studies, only few of them are dealing with morphology of S100β^+ cells (e.g., [39, 40]). Most of experimental or clinical studies are related to detection of concentrations of S100β within the tissue, or its plasma (or CSF) levels, whose changes are significant for various diseases, probably due to release of S100β from damaged astrocytes (e.g., [38]).

NG2 glia (polydendrocytes or synantocytes) represent a fourth type of glia in the CNS (e.g., [41]). They exist abundantly in both grey and white matter of the mature CNS in rodents as well as human. They constitute the major group of cells undergoing mitosis in the adult rodent brain and are almost as numerous as astrocytes [42]. NG2 cells are primarily described as the precursors of myelinating oligodendrocytes (OLPs). However, many of the NG2 cells remain in the NG2-positive state for a significant time and have a unique capacity to communicate with nearby cells, forming multiple contacts with astrocytes, microglia, oligodendrocytes, and even neurons [43]. In human brain, significant morphological changes related to the progression of pathology were studied particularly in multiple sclerosis and gliomas [44].

Microglia, the immunocompetent highly motile cells of the CNS, are extremely plastic and undergo a variety of structural changes based on their location and current role [45]. In the grey matter, the most frequent is ramified form, which express protein Iba1 (ionized calcium-binding adapter molecule 1) also known as AIF-1 (allograft inflammatory factor 1). The density of this marker significantly increases with activation of cells. It is obvious that the activation of microglia is a basic mechanism in the defence of the CNS, also in relation to neurodegenerative processes (e.g., [45, 46]). Although the role of microglia in neurodegeneration is still controversial, it is evident that in human brain they are activated in early stages of NDP of different phenotype, primarily in HD (e.g., [46, 47]), Parkinson's disease (e.g., [48]), and Alzheimer's disease (e.g., [49, 50]). It is possible that microglia transform to phagocytes and target neurons as the disease progresses but appear to be dysfunctional with increasing amounts of ingested debris [48].

It is commonly known that the neurodegenerative process of HD phenotype is a chronic process, morphologically characterized by the progressive degeneration of neurons, principally in the striatum, but gradually affecting almost all parts of the brain. This results in a reduction of grey matter and brain atrophy with compensatory enlargement of the lateral brain ventricles. Nevertheless, also as the second component of the brain parenchyma, the glial cells play an irreplaceable role in this process. The reaction of astrocytes to any damage of the CNS parenchyma in a sense of their conversion into the reactive intensely GFAP-positive subset is well known already for long time. Although the participation of other types of glial cells, particularly of microglia and NG2 glia, in neurodegenerative process has been studied in last two decades, the histopathological interrelations among all above-mentioned cell types have not been well described yet. Moreover, the validation of existing transgenic rat model of HD51 from this point of view is still lacking.

2. Materials and Methods

All animal procedures were performed in accordance with the directive of the EEC (86/609/EEC) and the use of animals in our experiments was reviewed and approved by the Animal Ethical Committee of Charles University in Prague, Faculty of Medicine in Hradec Králové.

2.1. Animals. Male homozygous tgHD51 CAG rats (+/+; $n = 18$) and their wild-type (wt) littermates (−/−; $n = 18$) were obtained from H. P. Nguyen and O. Riess, the Department of Medical Genetics, University of Tübingen, Germany. Rats were sacrificed at the age of 2 or 3 months (tgHD $n = 3$; wt $n = 2$), 6 months (tgHD $n = 2$; wt $n = 3$), 12 months (tgHD $n = 4$; wt $n = 5$), 18 months (tgHD $n = 5$; wt $n = 4$), and 22–24 months (tgHD $n = 4$; wt $n = 4$).

2.2. Postmortem Specimens. The brains of three patients with approximately 2-, 8-, and 20-year clinical manifestation of HD (sex/age: ♀/52, ♂/38, and ♀/52) were studied. Three control brains of patients (sex/age/weight: ♂/33/1440 g, ♀/43/1510 g, and ♂/56/1400 g) with no history of neurologic disorder or brain lesion were also taken for the study. The clinical features of HD (described in autopsy records) were characteristic for the given stages in all three patients. However, detailed neurological records or results of genetic testing were not available because old archival material was used. Surprisingly, the total brain weight was markedly reduced in all HD cases independent of the sex and duration of the illness (duration/sex/weight: 2 y/♀/1160 g, 8 y/♂/1150 g, and 20 y/♀/1120 g) in comparison with control human brains. The severity of striatal histopathological changes was graded (grades 0–4) according to Vonsattel and coauthors [2].

Paraffin blocks of brain tissue from autopsies were taken from the neostriatum (the caudate nucleus and putamen) at the level of the globus pallidus and at the level of the nucleus accumbens. The blocks were donated by Fingerland's Department of Pathology, Faculty Hospital in Hradec Králové.

2.3. Histology and Immunohistochemistry. Animal brains were processed either with formalin fixation (4% neutral formaldehyde) and embedded in paraffin or with 4% paraformaldehyde (in 0.1 M phosphate buffer) fixation and frozen sections preparation. The transcardial perfusion with fixative solution under deep anaesthesia followed by postfixation (for 3 days or 2 hours, resp.) was made in all animals. Brains were transversely cut using the Brain Blocker (Better Hospital Equipment Corp., USA) to obtain the identical blocks of brain tissue. After postfixation, the brain hemispheres were separated and processed separately.

2.3.1. Paraffin Sections. Histological processing was the same for both the experimental material and autopsies. Serial coronal sections (7 μm thick; 120 sections per each rat brain hemisphere), prepared by conventional histological processing, allowed us to study the same region with different antibodies. Findings obtained by immunofluorescent detections (double-labelling) were mostly confirmed by a single

Table 1: Antibodies used.

Antibody	Host	Dilution	Source	Report
Nestin	Rat monoclonal anti-mouse	1 : 4	DSHB	Progenitor cells marker
Nestin	Mouse monoclonal anti-human	1 : 200	Millipore	Progenitor cells marker
β-III tubulin	Mouse monoclonal	1 : 20	Exbio	Neuronal marker
MAP2	Mouse monoclonal	1 : 500	Sigma-Aldrich	Neuronal marker
MAP2	Rabbit polyclonal	1 : 700	Millipore	Neuronal marker
NeuN	Mouse monoclonal	1 : 100	Millipore	Marker of mature neurons
Synaptophysin	Mouse monoclonal	1 : 20	Dako	Marker of neuronal synapses
Vimentin (Cy3 conjugated)	Mouse monoclonal	1 : 100	Sigma-Aldrich	Astrocyte and radial glia marker
NG2	Rabbit polyclonal	1 : 400	Millipore	Oligodendrocyte precursor and pericyte marker
APC	Mouse monoclonal	1 : 200	Calbiochem	Oligodendrocyte and astrocyte marker
GFAP	Mouse monoclonal	1 : 400	Sigma-Aldrich	Astrocyte marker
GFAP	Rabbit polyclonal	1 : 400	Dako	Astrocyte marker
S100β	Mouse monoclonal	1 : 1000	Sigma-Aldrich	Astrocyte marker
S100β	Rabbit polyclonal	1 : 300	Dako	Astrocyte marker
Iba1	Mouse monoclonal	1 : 300	Millipore	Microglia and macrophage marker
PolyQ-huntingtin	Mouse monoclonal	1 : 20000	Millipore	Polyglutamine inclusions marker

antibody detection using peroxidase-antiperoxidase (PAP) immunohistochemistry on parallel paraffin sections.

2.3.2. PAP Immunohistochemical Detection. For immunohistochemical detection, deparaffinized and rehydrated sections were used. Pretreatment in microwave 3 × 5 min at 800 W in the sodium citrate buffer (pH 6.0) and washing in 0.01 M PBS was mostly performed. For detection of polyglutamine deposits the pretreatment with 98% formic acid (5 min at room temperature) was required. Incubation in blocking solution (water solution of H_2O_2) for 20 min was followed by incubation with primary antibodies (Table 1) performed overnight at 4°C. Sections were then washed and incubated with the appropriate biotinylated secondary antibody (Jackson ImmunoResearch Lab., USA, 1 : 500) for 45 min at room temperature and subsequently with a streptavidin conjugate of peroxidase (Dako, CR) also for 45 min. Visualization of bound antibody was performed using DAB (Sigma-Aldrich, CR) and H_2O_2.

2.3.3. Cryostat Sections. Postfixed blocks of rat brains were placed stepwise in solutions with gradually increasing sucrose concentrations (10%, 20%, and 30%) for cryoprotection at 4°C. Serial coronal cryostat free-floating sections (30 μm thick) were cut. The slices were incubated with 5% Chemiblocker (Millipore, MA, USA) and 0.2% Triton in PBS at 4°C.

2.3.4. Immunofluorescent Double-Labelling Technique. The sequential technique for immunofluorescent double-labelling of antibodies (Ab) was same for both types of sections. Avidin or appropriate secondary Ab was labelled with Cy-3 or Alexa Fluor 660 (red) and with Alexa Fluor 488 or 594 (green) and nuclei were counterstained with DAPI (blue). The negative control, omitting the primary antibody, was made in each labelling.

Photomicrographs were made with Lucia G/F software version 4.82 (Laboratory Imaging, Prague, Czech Republic) or Quick Photo Camera 2.3 software (Promicra, Prague, Czech Republic).

2.4. Quantitative Analysis. In order to characterize the progression of NDP in the striatum of tgHD51 rats, we used the quantitative analysis of the median diameter of neuronal nuclei as a marker of proposed significant process in a course of neurodegeneration in tgHD rats; it means the shrinkage of striatal neurons. We would like also to determine the onset of significant neuronal degeneration in the striatum of tgHD51 rats and the possible participation of age-related changes.

The region of rat brain, used for analysis, was determined according to the brain atlas [51] from the level 14 (+0.95 from bregma) dorsally in a sequence of 3 slices, each 7 μm thick, in the distance of 15 sections (i.e., approximately 105 μm); it means that analysed part of rat brain was of about 220 μm

thick. Selected sections were labelled with NeuN antibody, which marks selectively the nuclei of mature neurons, using the PAP immunohistochemical detection.

The groups of tgHD and wt rats were divided into two basic subgroups of "young" 3- and 6-month-old (0–6 months) and "old" 12-, 18-, and 24-month-old (>6 months) rats. The number of analysed sections was the following: 6 sections in the "young_wt" group, 6 sections in the "young_tgHD" group, 18 sections in the "old_wt" group, and 24 sections in the "old_tgHD" group. Neuronal nucleus median diameter was obtained from 50 independent measurements in the central area of the striatum on each analysed section. Due to possible distortions of the shape of neuronal nucleus in the section, the largest size of the nucleus was considered the nucleus diameter. Each group of rats of the same age was represented by the set of all medians in given group.

2.5. Statistical Analysis. Statistical analyses of the differences between groups were performed using MS Excel 2007 (Microsoft Corp., Redmond, WA, USA) and NCSS 2007 (NCSS LLC, Kaysville, Utah, USA). The median diameters were compared using Kruskal-Wallis Multiple-Comparison Z-Value Test (Dunn's Test). The test with Bonferroni correction was used to adjust multiple comparisons with the family-wise α at 0.05.

3. Results

In tgHD51 rats, the NDP in the striatum starts to develop only after 6 months of age. Surprisingly, the most distinct changes in striatal grey matter develop by the end of the first year of age (probably between 9 and 12 months). The end of the first year represents the turn in the development of morphological changes related to the progression of NDP within the striatum of tgHD51 rats. These findings correspond to the course of HD in human brain, where the motor and behavioural changes precede the loss of striatal neurons [20]. We demonstrate possible parallels between the HD progression in humans and the above-described transgenic rat model and prove the validity of our findings for human HD pathology. The cases demonstrated here represent the sequence of 3 stages (grades 1-2, grade 3, and grade 4) of the progression of HD in human brain.

It is almost impossible to dissociate the alterations referring to neurons and glia in a course of NDP because of very close relationship and mutual influence of both main components of striatal parenchyma. However, we would like to stress some features specific for each of them in a course of the development of NDP within the striatum of both rat and human brains. For that reason, we described their involvement in progression of NDP separately.

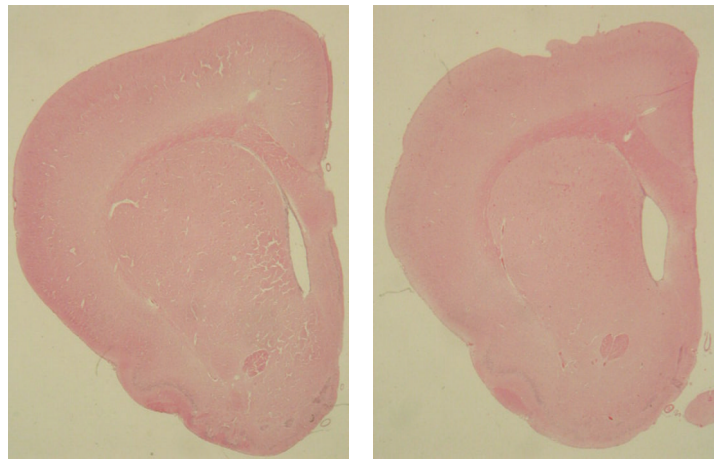
3.1. Neuronal Degeneration during the Progression of HD. When we compare the brains of 2- and 3-month-old, (young adult) wild-type and tgHD rats, there is no difference in morphology of the striatum. Also lateral brain ventricles are narrow, of the same shape in both mentioned groups (Figure 1(a)). Only in 12-month-old tgHD51 rats appears the identifiable enlargement of lateral ventricles, which documents developing striatal atrophy. The process gradually progresses resulting in prominent widening of lateral ventricles (Figure 1(b)), with concave medial outline of the striatum in the oldest (22–24-month-old) tgHD rats, which is fully comparable with the progression of HD in human brain.

3.1.1. Degeneration of Striatal Neurons. Striatal neurons (β-III-tubulin$^+$/MAP2$^+$) or their nuclei (NeuN$^+$ or DAPI$^+$) form typical clusters in all tested groups of wt (e.g., Figures 2(a), 2(b), and 3(a)) and tgHD rats (e.g., Figures 2(c)–2(f) and 3(b)) and in human postmortem samples of intact striatum (Figure 4(a)). However, with the progression of HD in humans, gradual decrease in number of neurons is significant, particularly in advanced stages of HD (grade 3—Figure 4(b) and grade 4—Figure 4(c)).

Nuclei of striatal neurons are very characteristic, especially due to their large size and fine loosely arranged chromatin in comparison with significantly smaller nuclei with more densely arranged chromatin of glial cell. Despite the fact that striatal neurons become gradually smaller in course of HD progression (compare Figures 3(a) and 3(b)), such specific features of neuronal and glia nuclei always enable their distinguishing. Our immunohistochemical analysis of neuronal nuclei by NeuN shows slow but already significant progression of neuronal degeneration in the striatum from 12 to 24 months of age of tgHD rats (Figure 3(b)) when compared with age-matched controls (Figure 3(a)) and younger tgHD rats. The most typical for NDP in tgHD51 rats is a gradual decrease in size of neuronal bodies and nuclei (with maintenance of nucleo-cytoplasmic rate), which results in the disintegration and disappearance of affected neurons (Figures 2(d)–2(f)), ultimately scavenged by microglia (Figures 12(b) and 12(c)).

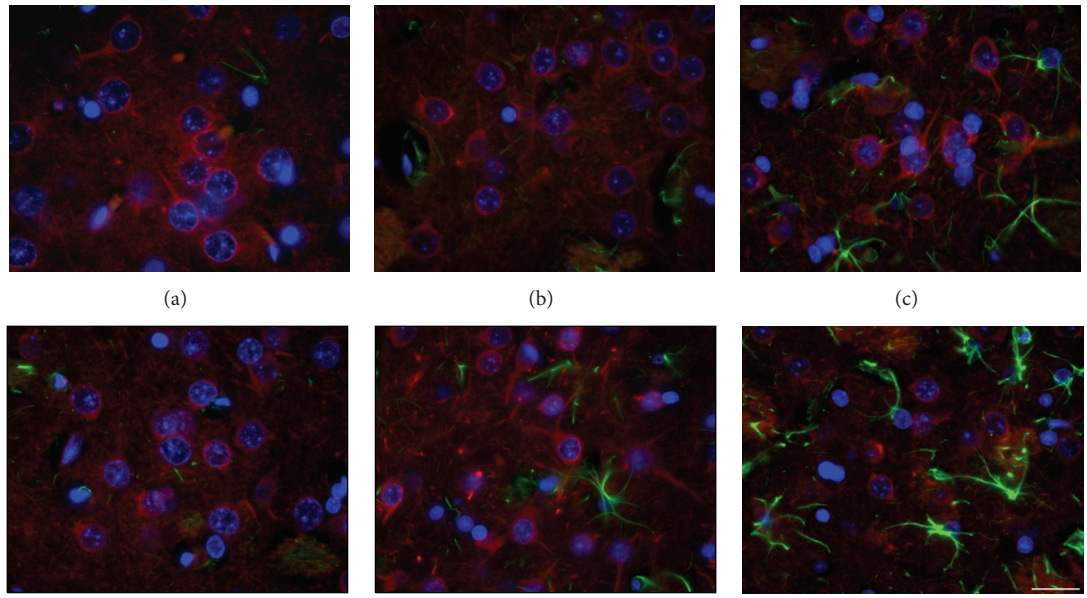
In the human HD brain, grades 1-2 (with approximately 2-year clinical manifestation), the degeneration and loss of neurons were only random; therefore, the loosening of the neuropil has not been apparent yet. On the other hand, in grade 3 (approximately 8-year clinical history), neuronal degeneration was already obvious (Figure 4(b)). Depletion of neurons (particularly in the CN and putamen (Pu)) accompanied with rarefaction of the neuropil resulted in a reduction of striatal volume and noticeable enlargement of the lateral ventricles. In grade 4 (with approximately 20-year clinical diagnosis) the entire corpus striatum (CN, Pu, and globus pallidus) was affected by degeneration of neurons and neuropil (resulting in severe striatal atrophy) and therefore the concomitant astrogliosis here prevailed (Figures 4(c), 10(b), and 10(c)). The remaining striatal neurons (marked by their prominent nuclei) gradually became smaller with the progression of NDP, like in the brain of tgHD rats.

Additionally, we confirmed that, alike in human HD brain, neuronal degeneration is selective, that is, affecting primarily certain groups of neurons in the striatum of investigated senescent tgHD rats and moreover that age-related changes contribute to final extent of NDP.



(a) (b)

Figure 1: (a) Lateral brain ventricles are quite narrow in young (3-month-old) control wt rats, unlike (b) notably enlarged ventricles in 18-month-old tgHD51 rats, owing to the progression of striatal atrophy which confirms the development of NDP. *Haematoxylin and eosin*. *Direct magnification* 1.5x.



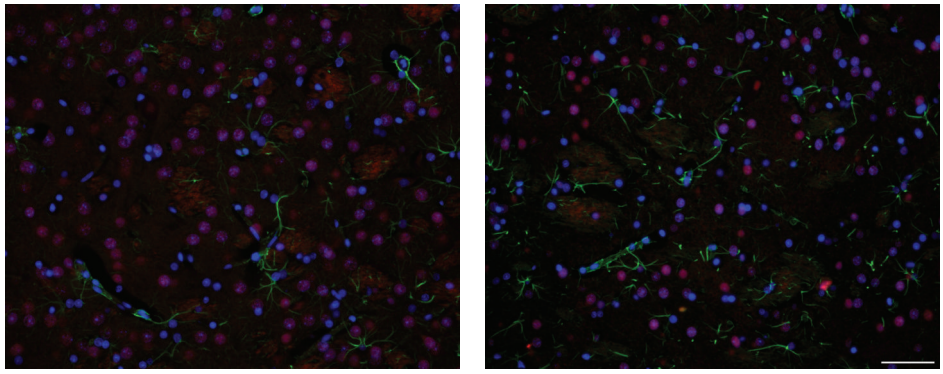
(a) (b) (c)

(d) (e) (f)

Figure 2: Significant decrease in the size (diameter) of the striatal neurons in a course of the development of NDP is marked by accompanied changes in the diameter of their nuclei, due to the maintenance of the nucleo-cytoplasmic rate. Detection of the β-III-tubulin$^+$ neurofilaments, which fill in the entire cytoplasm of neuronal body and processes, enables to document the shrinkage not only of neuronal nuclei but also of the bodies of neurons, due to either physiological ageing process (a–c) or that caused by the progression of neuronal degeneration in tgHD rats (d–f). The number of GFAP$^+$ astrocyte grows up only slightly in course of ageing (a–c), but significant increase (astrogliosis) is evident in oldest tgHD rats (f). (a) wt—2-month-old, (b) wt—12-month-old, (c) wt—22-month-old, (d) tgHD—6-month-old, (e) tgHD—12-month-old, (f) tgHD—24-month-old rats. *Anti-β-III-tubulin (red) + anti-GFAP (green) + DAPI (blue)*. *Bar* 20 μm.

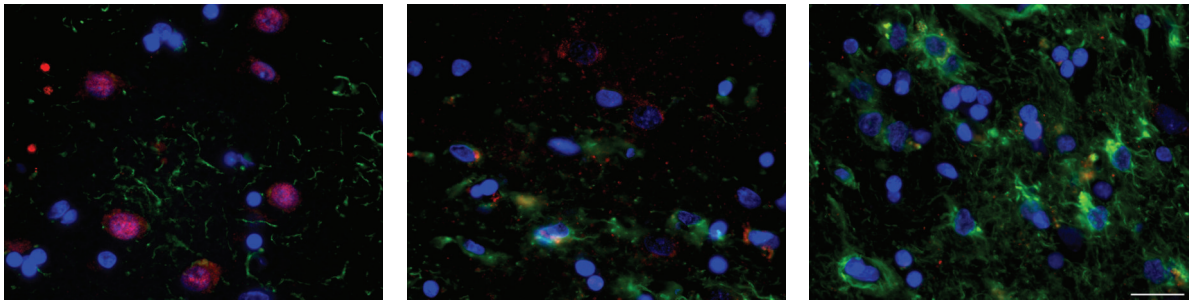
3.1.2. Quantitative Analysis of Neuronal Degeneration in the Striatum of tgHD Rats. We supposed that neuronal degeneration in the striatum of tgHD rats manifests primarily by the decrease in a volume/size of neuronal bodies including their nuclei. In order to precisely characterize the progression of NDP within the striatum of tgHD51 rats, our morphological findings were supplemented by quantitative analysis of the diameter of neuronal nuclei labelled with NeuN. Also the proportion of age-related changes in this process was assessed.

The progression in decrease of the median diameter of neuronal nuclei with age of rats in both wt and tgHD groups of rats is documented by Progress Chart (Figure 5(a)).



(a) (b)

Figure 3: Significant difference in the number and size of neurons/neuronal nuclei ($NeuN^+$) is evident if we compare (a) 2-month-old wt rats and (b) 18-month-old tgHD rats; (b) concomitant reactive astrogliosis is already developed in 18-month-old tgHD rats; it is also apparent that the degeneration of neurons in tgHD rats is typically selective (alike in human HD brain). *Anti-NeuN (red) + anti-GFAP (green) + DAPI (blue)*. *Bar* 50 μm.



(a) (b) (c)

Figure 4: In postmortem samples of human HD brain, (b, c) gradual progression of chronic striatal NDP marked by massive neuronal degeneration and severe concomitant astrogliosis is evident in comparison with (a) intact control brain. (a) Control (♂/56), (b) HD duration 8 years, grade 3 (♂/38), and (c) HD duration 20 years, grade 4 (♀/52). *Anti-NeuN (red) + anti-GFAP (green) + DAPI (blue)*. *Bar* 20 μm.

In the first two groups of rats, that is, 2-3-, and 6-month survivors, no differences in the median diameter of NeuN-positive nuclei were detected when compared within the individual group or among the groups. Striking decrease in the median diameter of neuronal nuclei by 26% was detected in 12-month-old tgHD rats unlike the parallel wt group, in which the decrease was only 7.6%, when compared with the youngest animals in both mentioned groups. Surprisingly, further progression in decrease of the median diameter of neuronal nuclei was not so rapid; however, finally the reduction reached 28.7% in 24-month-old tgHD rats, when compared with age-related degeneration in 24-month-old wt rats (only 8.3%).

Statistical characteristic of the groups of rats using Box Plot (Figure 5(b)) enables the multiple comparison of the median diameter of neuronal nuclei of the following groups of rats: "young _wt" and "young_tgHD" are groups of rats 3 and 6 months (0–6 months) old; "old_wt" and "old_tgHD" are groups of rats 12, 18, and 24 months old (>6 months). Results of Kruskal-Wallis Multiple-Comparison Z-Value Test (Dunn's Test) of the mentioned groups of rats (Table 2) indicate significantly different pairs of groups (Bonferroni test: medians are significantly different if Z-value is > 2.6383). It is evident that the median diameter of neuronal nuclei in "old_tgHD" group is significantly statistically different from all other evaluated groups. Differences among three remaining groups are statistically insignificant.

Table 2: Results of Kruskal-Wallis Multiple-Comparison Z-Value Test (Dunn's Test) of the groups of rats: "young_wt" and "young_tgHD" are groups of rats 3 and 6 months (0–6 months) old; "old_wt" and "old_tgHD" are groups of rats 12, 18, and 24 months old (>6 months). Asterisk indicates significantly different pairs of groups (Bonferroni test: medians are significantly different if Z-value is > 2.6383). Statistically significant is a difference in the median diameter of neuronal nuclei in "old_tgHD" group in comparison with all other evaluated groups.

Group	young_tgHD	young_wt	old_tgHD	old_wt
young_tgHD				
young_wt	0.2844			
old_tgHD	4.9557*	4.5959*		
old_wt	1.813	1.4646	4.5135*	

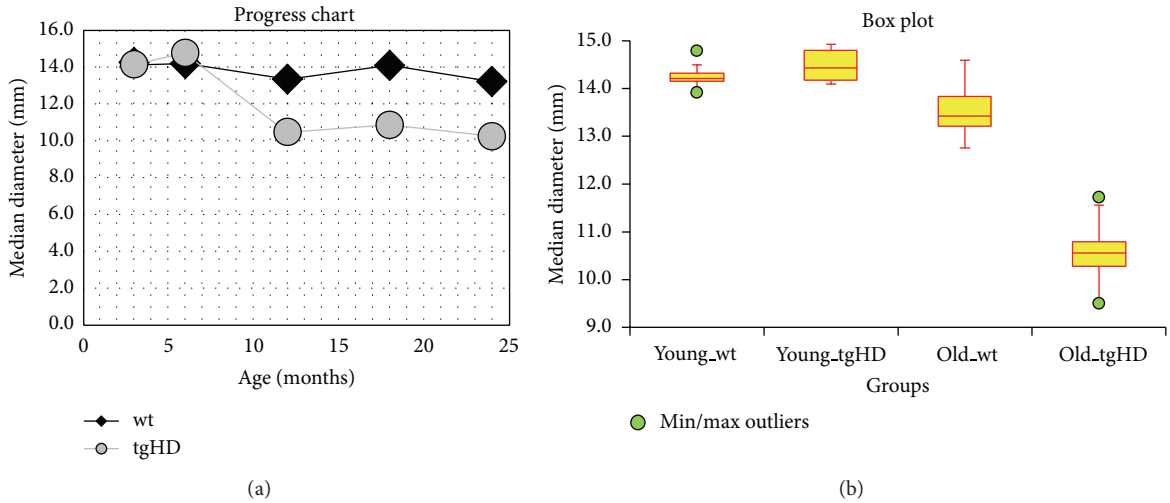
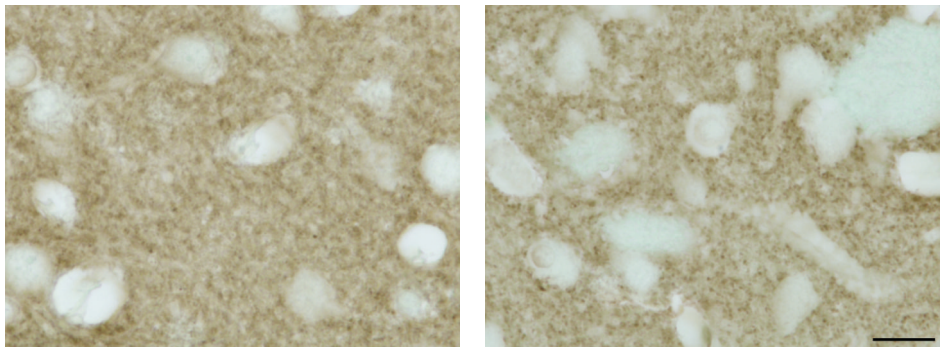


FIGURE 5: (a) Progress Chart: the progression in decrease of the median diameter of neuronal nuclei with age of rats in wt groups (black line) and tgHD51 rats (grey line). (b) Box Plot: statistical characteristic of the groups of rats. The multiple comparison of the median diameter of neuronal nuclei of the groups of rats: "young_wt" and "young_tgHD" are groups of rats 3 and 6 months (0–6 months) old; "old_wt" and "old_tgHD" are groups of rats 12, 18, and 24 months old (>6 months).

We can conclude that the rate of neuronal degeneration reaches maximum at the end of the first year of animal's age and then, in the following 12 months, it proceeds rather slowly. Moreover, it is potentiated with age-related changes particularly in the oldest animals. Unexpectedly, the transitional amelioration of the process up to slight improvement appeared in both groups (wt and tgHD) of 18-month survivors. Neuronal degeneration in wt rats can be attributed only to the debit of the aging process; the decrease in size of nuclei was slow and the difference between 2-3-month-old rats and 24-month-old ones was only 8.3% (Figure 5(a)).

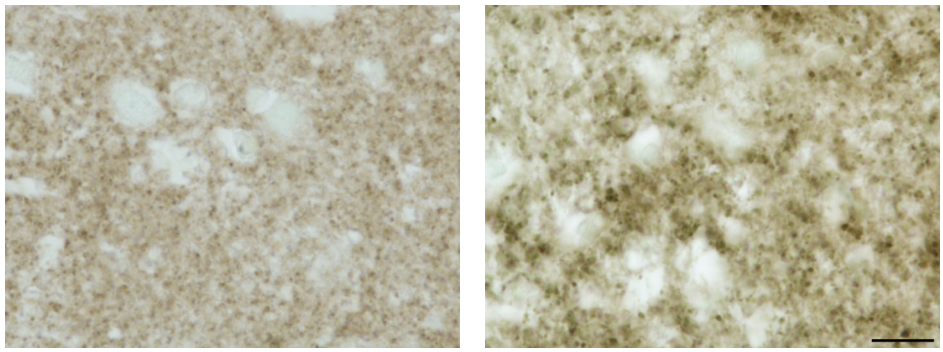
3.1.3. Rarefaction of Neuropil and Alterations in Morphology of Synapses. Striatal atrophy, in the case of HD, is primarily caused by the degeneration of striatal neurons. Of course, the most prominent feature, seen on histological preparations, is a gradual reduction of neuronal bodies marked by the nuclei. Indeed, the reduction in a volume of neuropil is at least of the same importance. Large amounts of dendrites (with dendritic spines) extending from neuronal bodies (labelled with anti-MAP2—Figure 12, less with anti-β-III-tubulin—Figure 2) with myriads of synapses represent the essential component of the neuropil. Although the rarefaction of neuropil is not based only on the degeneration of this network of neuronal processes and synapses, it demonstrates the progression of such process in both human and rat brains (Figures 8(a)–8(d)). In addition, we also proved the alterations in a character of synapses. In control brains of both rats and humans, synaptophysin-positive synapses are very fine, of uniform size and shape, and plentiful (Figures 6(a) and 7(a)). With the progression of NDP, most of synapses become coarser, more prominent, but of variable size, and some of them are intensely labelled for synaptophysin; consequently, their number gradually decreases (Figures 6(b) and 7(b)). Despite different size of synapses in rat (Figures 6(a) and 6(b)) and human (Figures 7(a) and 7(b)), the mentioned alterations are of the same character. Since the severity of the striatal damage is also influenced by duration of NDP, the changes in morphology of synapses, and particularly the loosening of neuropil, are certainly more prominent in advanced stages of HD in human brain (Figure 7(b)) than in terminal stage of NDP in tgHD rats (Figures 6(b) and 8(d)). Additionally, the alterations in glial component and the ageing-associated changes (see Section 3.2.) also markedly influence loosening of the neuropil. Indeed, the pattern of such process in this basic aspect is the same for both tgHD rats and HD patients.

3.1.4. Polyglutamine Deposits. Detection of polyglutamine deposits using polyQ-huntingtin provides interesting findings, which give a complete histopathological picture of HD progression. In wt rats, polyQ detects a normal polyglutamine domain huntingtin encoded by lower number (about 35 or less) of consecutive glutamine repeats; therefore, only fine polyQ deposits are spread in the nuclei of striatal neurons (Figure 8(a)). In contrast, the pathogenic alleles usually contain 39 or more glutamine repeats, which results in production of mhtt and increased density of intranuclear polyQ expression in relation to the progression of NDP of HD phenotype (Figures 8(b)–8(d)). It is necessary to emphasize that increased density/concentration of intranuclear polyQ deposits is also potentiated by the shrinkage (decreased volume) of nuclei in degenerated neurons in course of the progression of NDP (compare Figures 8(a) and 8(b)–8(d)). Similarly, the number of affected polyQ$^+$ glial cells significantly increases with advancing NDP (Figures 8(c) and 8(d)), most likely in relation to the development of reactive astrogliosis; that is, mhtt is probably preferentially



(a) (b)

Figure 6: (a) In young control animals (2 months old), synapses within the neuropil are very numerous, fine and of uniform size; (b) the most conspicuous alteration in old (here 18 months) tgHD rats is variable size and enlargement (coarsening) of most of synapses; however, also their decreased number participating in loosening of neuropil is evident. *Anti-synaptophysin counterstained with* 0.1% *methyl green*. *Bar* 10 μm.



(a) (b)

Figure 7: (a) Synapses in intact (control, ♂/56) human brain are (like in rats) uniform and densely accumulated within the neuropil; (b) they also become coarser and of variable size with the progression of NDP in HD patients (here ♀/52, grade 4, duration 20 years); continuous decrease in their number significantly participates in rarefaction of the neuropil, most prominent in terminal stage of NDP. *Anti-synaptophysin counterstained with* 0.1% *methyl green*. *Bar* 10 μm.

expressed by reactive astrocytes. Surprisingly, using polyQ-huntingtin antibody, neither typical large intranuclear nor neuropil aggregates were seen. Moreover, neurons in adjacent cortex also exhibit intranuclear but more cytoplasmic polyQ deposits; therefore, they are more densely stained in comparison with striatal neurons, particularly in tgHD rats (Figures 8(e)–8(g)). On the contrary, only few cortical glial cells express polyQ, which corresponds to the absence of typical reactive gliosis in this region. The presence of very densely labelled (hyperchromic) degenerated/shrunk cortical (primarily pyramidal) neurons—demonstrated also by other histological staining methods—unequivocally confirms that, in tgHD51 rats, NDP in the striatum is accompanied with the degeneration of cortical neurons (Figures 8(f) and 8(g)) alike in human HD brain.

Moreover, the highest density of deposits in shrunk/hyperchromic terminally degenerated neurons (less prominent in glial cells) corresponds to the hypothesis that accumulation of mhtt results in the cell death.

3.2. Glial Cells during Progression of HD. It is evident that the developments of changes in glial cell morphology, and certainly also in their function, are conditioned by the intensity and rate of neuronal degeneration in the context of the neuron-glia relationship.

3.2.1. Astrocytes. Protoplasmic astrocytes are the most numerous component of the striatal parenchyma. Despite their standard visualisation by detection of GFAP, most of them are GFAP-negative.

The shape of astrocytes changes during the progression of NDP; however specific prominent alterations occur only in human HD brains, where the astrogliosis gradually develops (Figures 10(b) and 10(c)). In tgHD rats, the main $GFAP^+$ processes get coarser, albeit less branched. Indeed, the most distinct part of the processes (enlarged and densely $GFAP^+$) becomes their vascular end-feet (Figures 9(b) and 9(c)). Hence, the main astrocytic processes and the perivascular

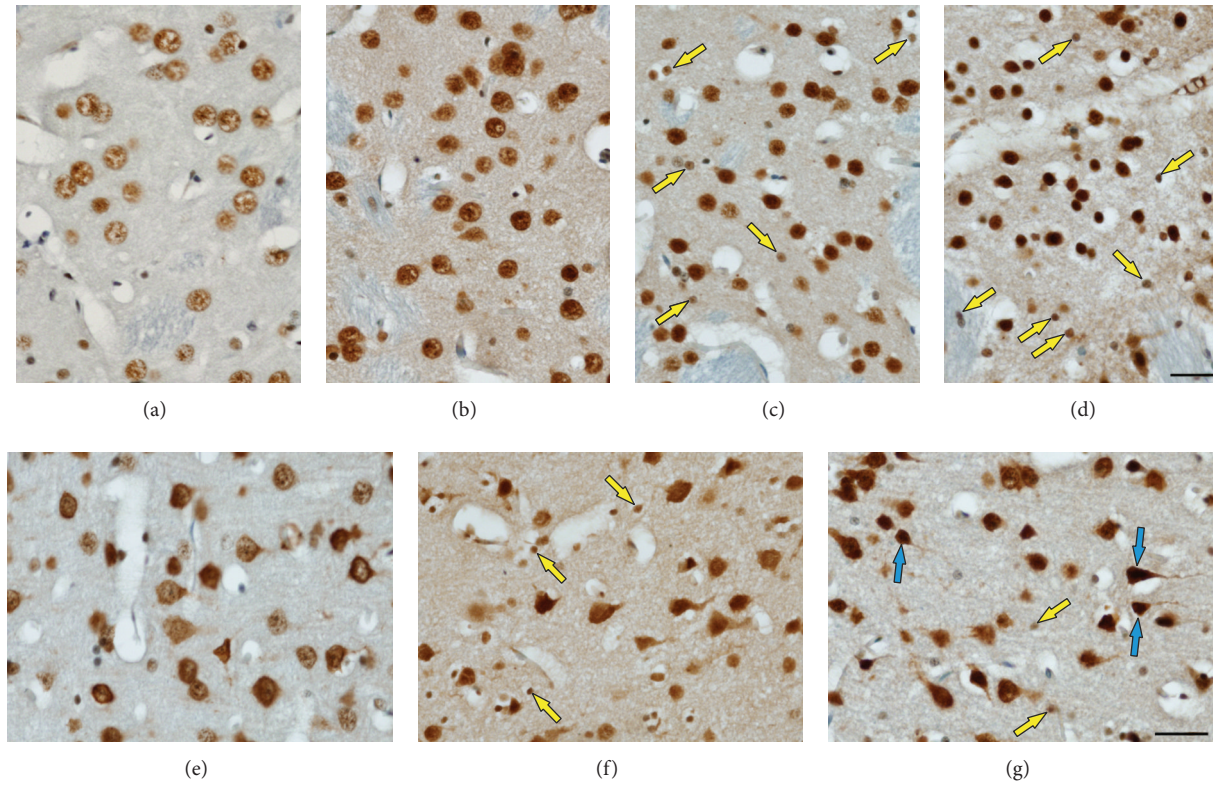


Figure 8: (a) Only fine polyQ deposits are spread in the nuclei of striatal neurons in control 6-month-old rats unlike (b) a higher density of polyQ deposits in age-matched tgHD rats; (c) significantly increased density of polyQ inclusions, in both neurons and some glial cells *(yellow arrows)*, is already in 12-month-old tgHD rats and (d) particularly in aged (here 18-month-old) tgHD rats; increased density of polyQ expression is probably also influenced by the shrinkage of nuclei during the degeneration of neurons; the progression of rarefaction of the neuropil is also apparent. (e) In the cortex of 2-month-old wt rats, perinuclear polyQ positivity is only in some neurons, unlike increased concentration of polyQ (mhtt) deposits particularly within the cytoplasm, primarily of pyramidal neurons, in (f) 12-month- and (g) 18-month-old tgHD rats; in aged tgHD rats, the significant number of degenerated (hyperchromic) neurons is present *(blue arrows)*; only few polyQ$^+$ and subsequently degenerated glial cells *(yellow arrows)* and particularly loosening of the neuropil are also characteristic for the progression of NDP in the cortex of tgHD51 rats. *Anti-polyQ counterstained with* 0.1% *methyl green*. *Bar* 20 μm.

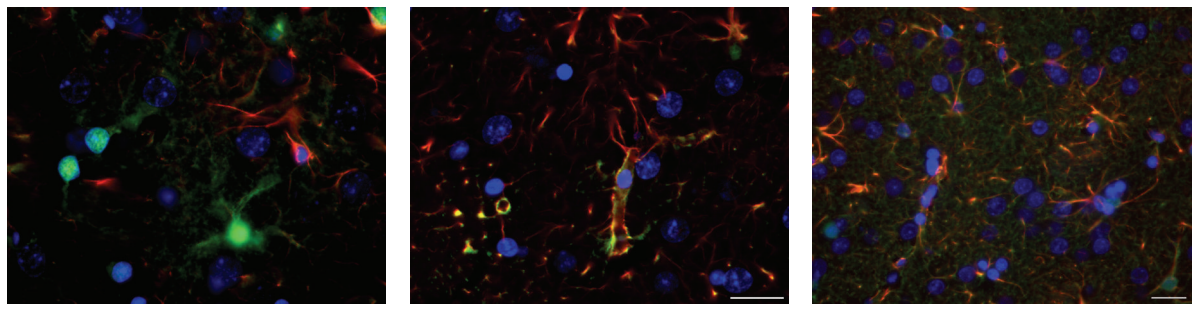
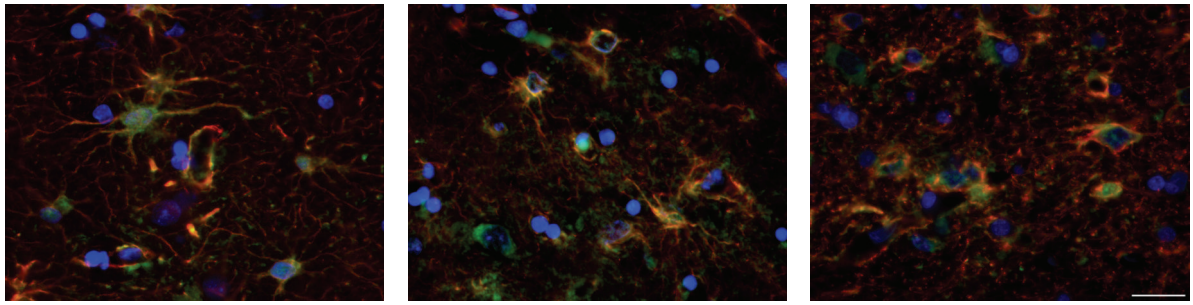


Figure 9: (a) In young (2-month-old) wt rats S100β^+ astrocytes are predominantly GFAP-negative; with the progression of NDP in (b) 18-month-old tgHD rats and (c) 24-month-old tgHD rats the main GFAP$^+$ processes become coarser, albeit less branched, and the most distinct are their vascular end-feet (thickening of the perivascular membrane in form of "rings"); the number of not only S100β^+/GFAP$^-$ but also S100β^+/GFAP$^+$ astrocytes increases primarily around the vessels; the coexpression is seen mainly within the thick astrocytic processes terminating by the end-feet on the vessel wall. *Anti-GFAP (red) + anti-S100β (green) + DAPI (blue)*. *Bar* 20 μm.



(a) (b) (c)

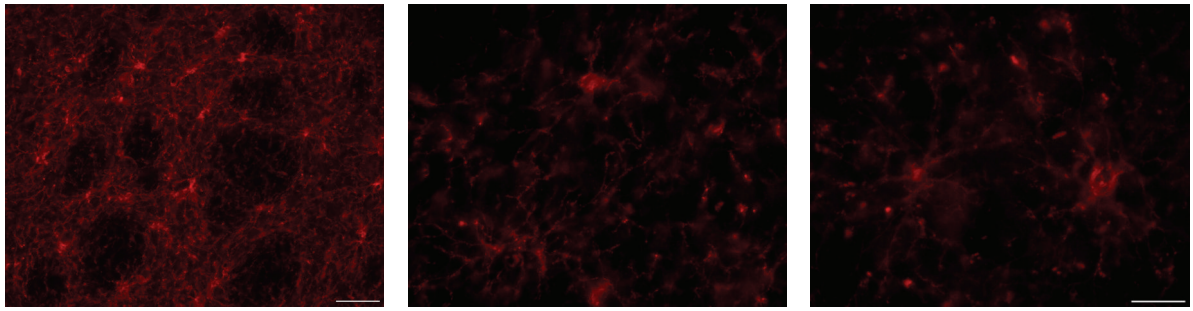
Figure 10: In HD human brain, significant "reactive" astrogliosis develops with the progression of NDP (b, c) in comparison with control brain (a); also the fine GFAP$^+$ processes become numerous and of characteristic arborization; specific terminal swellings are in most of the densely GFAP$^+$ processes; although the amount of S100β^+/GFAP$^-$ is not significantly influenced by NDP, the regressive changes (shrinkage) affect also S100β^+ cells. (a) Control (♂/56), (b) HD duration 8 years, grade 3 (♂/38), and (c) HD duration 20 years, grade 4 (♀/52). *Anti-GFAP (red) + anti-S100β (green) + DAPI (blue). Bar* 20 μm.

limiting membrane are the most prominent GFAP$^+$ structures in striatal parenchyma, not only in brains of tgHD rats surviving for 12–24 months (Figures 9(b) and 9(c)), but also in old wt rats, as well as in postmortem specimens of HD patients (Figures 10(b) and 10(c)). Due to only slow development of neuronal degeneration in the striatum of tgHD rats, the subsequent astrogliosis progresses also slowly—with insignificant onset after 6 months of age of tgHD rats—and becomes more distinct just in 18–24-month-old animals (Figure 9(c)). Age-related changes not only are seen in old wt rats, but also participate in progression of reactive gliosis in tgHD rats. Less (in wt rats) or more (in tgHD rats) developed striatal atrophy is manifested in senescent rats by denser accumulation of (smaller) nuclei of neurons and glia (compare Figures 2(a)–2(c)).

In HD human brains, besides the gradually increased number of reactive astrocytes, their fine GFAP$^+$ processes become numerous although shorter in comparison with control brains and of characteristic arborization, forming a fine loosely arranged network, whose density increases with the progression of the disease. Moreover, we identified the specific terminal swellings in most of densely GFAP$^+$ processes, whose number and also size slowly but gradually increase with the progression of HD (Figures 10(b) and 10(c)).

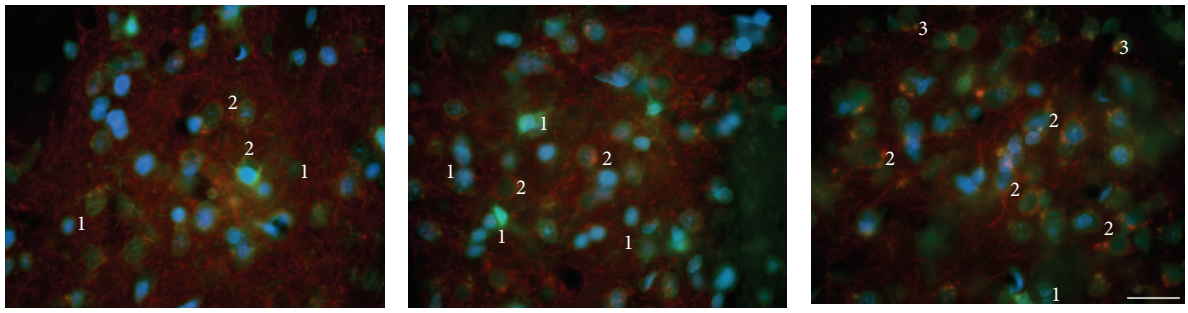
Despite the fact that the astrocytes, engaged in NDP, are described as "reactive," it is necessary to point out that they are of quite different structure in comparison with typical reactive astrocytes appearing after the acute brain injury. First of all, in both HD patients and tgHD rats, generation of reactive astrocytes proceeds gradually and slowly, unlike the almost immediate appearance of reactive astrocytes after the acute brain damage. Indeed, their bodies are not significantly enlarged (hypertrophic); contrariwise, a part of them also undergoes the degeneration and they are scavenged by microglia (Figures 12(c), 13(c), and 13(d)). Additionally, enlarged GFAP$^+$ vascular end-feet, which typically highlight the wall of vessels in developing NDP (Figures 9(b), 10(b), and 10(c)), and increased amount of GFAP$^+$ gliofilaments forming thick bundles within the cytoplasm of reactive astrocytes belong to significant features of concomitant astrogliosis in brain of both tgHD rats and HD patients. On the contrary, we never found the reexpression of intermediate filaments nestin and vimentin, which is considerable feature of hypertrophic reactive astrocytes after the acute brain damage.

3.2.2. S100β Protein-Positive Glia. S100β protein is glial-specific and it is expressed primarily by astrocytes, although not by all of them. Since this protein participates in many processes related to glial-glial and neuronal-glial crosstalk, we were interested in relevance of S100β expression by astrocytes in relation to developing NDP of HD phenotype. Surprisingly, the number of S100β^+ astrocytes is not markedly influenced by the progression of chronic NDP either in tgHD rats or in human HD brain. The majority of S100β-positive astrocytes are GFAP-negative, primarily in healthy brain. Intensely S100β labelled are cell bodies (incl. nuclei) and large processes terminated by end-feet. Although less prominent, expression is also in fine astrocytic processes. Since S100β^+ cells in the striatum are mainly the astrocytes, they may reflect the changes caused by the progression of NDP (Figures 9(b), 9(c), 10(b), and 10(c)), as well as by ageing process in controls. Such alterations in astrocyte immunoreactivity/morphology were primarily described on GFAP$^+$ astrocytes but naturally affect all subtypes of astrocytes. In both NDP and ageing, bodies of numerous either S100β^+ or GFAP$^+$ astrocytes slightly decrease in size and their processes become coarser (Figures 9 and 10). Moreover, the coexpression of S100β and GFAP increases, although rather differently in tgHD rats and human HD brains. In tgHD rats, the coexpression is slightly enhanced in end-feet (Figures 10(b) and 10(c)), unlike significant coexpression in both the cytoplasm of cell bodies and end-feet in human HD brains (Figures 10(b) and 10(c)). It is likely that different intensity of S100β/GFAP coexpression depends on a severity of the damage of striatal parenchyma which resulted, except others, in thickening of perivascular limiting membrane (i.e., vascular end-feet). Contrariwise, we could not confirm the expression of S100β by NG2 glia either in controls or in relation to striatal NDP.



(a) (b) (c)

Figure 11: NG2 glia forms a dense 3D network, particularly prominent on thick (30 μm) slices of the rat brain; there is no significant alteration in density and arrangement of this network if we compare control wt rats with age-matched tgHD rats. (a, b) 18-month-old wt rat; (c) 18-month-old tgHD rat. *Anti-NG2. Bar (a)* 50 μm *and (b, c)* 20 μm.



(a) (b) (c)

Figure 12: (a) Microglia *(green)* in the striatum of young (6-month-old) wt rats were mostly of small size, that is, nonactivated, although some neurons are here also scavenged (physiological degeneration); (b) the number of microglia increases quite slowly with the progression of NDP in tgHD rats; however (c) significant increase is only in the oldest animals (from 18 months of age). The double-staining with MAP2 antibody enables to document different stages of the degeneration of striatal neurons *(red)* up to the removal of their remnants; moreover, the accumulation of debris *(red)* in the cytoplasm of microglia *(green)*; (b, c) 1—inactive microglia, 2—scavenged neuron (early stage of phagocytic process), and 3—remnants of the ingested material (phagosomes) in the cytoplasm of microglia. Additionally, significant gradual reduction in the number of dendrites (i.e., rarefaction of the neuropil) with the progression of NDP is evident in aged tgHD rats. (b) tgHD—12-month-old rat; (c) tgHD—18-month-old rat. *Anti- MAP2 (red) + anti- Iba1 (green) + DAPI (blue). Bar* 20 μm.

3.2.3. NG2 Glia (Polydendrocytes). They are closely related to the development of myelinating oligodendrocytes, whose precursors are considered. We were interested in their possible alterations related to the progression of NDP in tgHD rats and also in senescence. Due to technical reasons (particularly the length of formalin fixation and the use of paraffin sections only), we were not able to detect NG2 glia cells in postmortem samples of human brains. In the rat brain, we did not prove any significant changes either in their number or in morphology in a course of the development of NDP, even in ageing process. In all examined samples, they were numerous (e.g., they outnumbered the $GFAP^+$ astrocytes) and large number of their fine branched processes forms a very dense three-dimensional network throughout the entire striatal parenchyma, particularly in the grey matter (Figures 11(a)–11(c)). We also did not prove colabelling of NG2 with astrocytic markers, such as GFAP or S100β, which might suggest that astrocytes in response to chronic slowly developing NDP are generated in prolonged time window.

3.2.4. Microglia. They represent a special type of professional phagocytes occurring only in CNS, which are spread out primarily within grey matter (Figures 12(a) and 13(a)), but in lower number they are present also in white matter. In agreement with previous studies we document their upregulation with the progression of NDP, particularly in advanced stages, in both tgHD51 rats and HD brains (Figures 12 and 13), unlike their only slowly growing number in ageing control animals. In the oldest (18–24-month-old) wt control rats, their number is evidently higher, which confirms physiological increase of neuronal degeneration in aged animals. Interesting morphological findings appeared in relation of microglia ($Iba1^+$) to degenerated neurons ($MAP2^+$). This double-staining enables

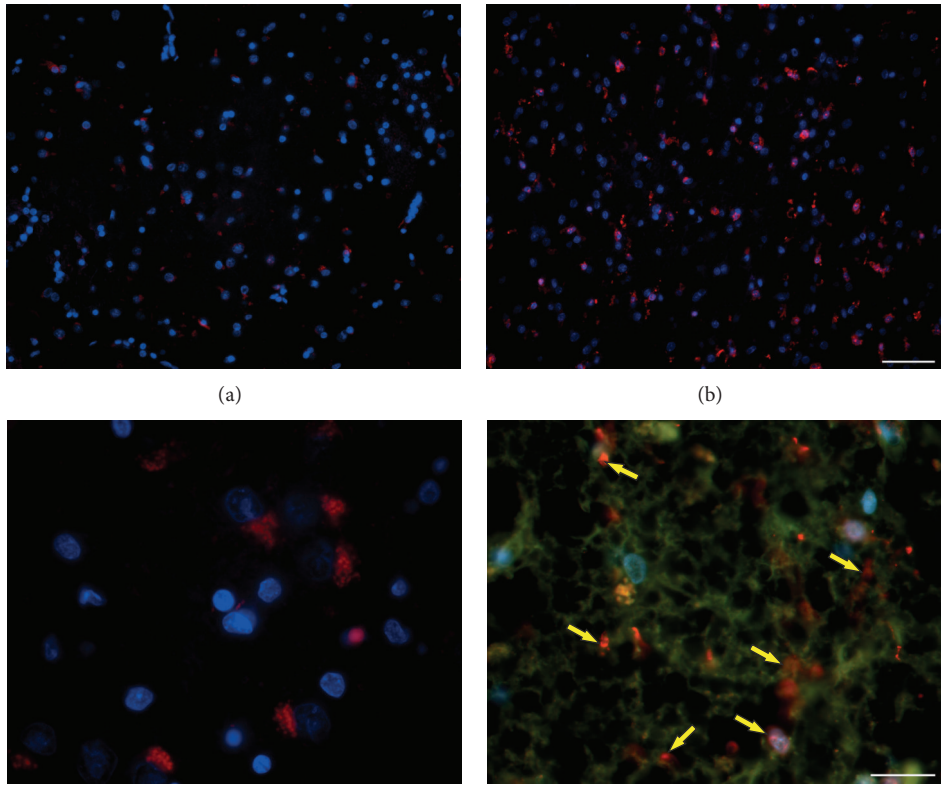


FIGURE 13: (a) In control human brain (♂/33), microglia *(red)* are mostly inactive/small (here in CN), but (b) their number and size significantly increase in advanced stage of NDP in HD brain (CN) (♀/52, HD duration 20 years, grade 4); (c) degenerated neurons within the CN are scavenged by microglia labelled with densely red stained lysosomes inside their cytoplasm (♂/38, HD duration 8 years, grade 3); (d) in advanced stage of NDP (♀/52, HD duration 20 years, grade 4), almost all neurons in CN already degenerated and they were replaced by reactive astrocytes; S100β^+ astrocytes also form a typical network; however, the neuropil is only sparse; microglia are numerous, particularly related to vessels *(yellow arrows)*. *(a, b, and c) Anti-Iba1 (red) + DAPI (blue)*; *(d) anti-Iba1 (red) + anti-S100β (green) + DAPI (blue)*. *Bar: (a, b)* 50 μm; *(c, d)* 20 μm.

to document the consequence of stages of neuronal degeneration and removal of neurons, including the accumulation of ingested debris inside the microglial cells (Figures 12(b) and 12(c)). Indeed, glial cells (mainly astrocytes) degenerate as well and are scavenged in both rat and human brain under the pathological (e.g., Figure 13(d)) as well as physiological conditions.

In human brain, in relation to advancing NDP, the growing number of microglia is also observed (Figure 13(b)), especially in comparison with normal control brains (Figure 13(a)). However, the large number of microglia is present only in relation to degenerated and scavenged neurons; when most of striatal neurons are destroyed (i.e., in seriously damaged CN - grade 4, 20-year ongoing clinical manifestation of HD), a subsequent decrease in number of microglia is detected (Figure 13(c)). Concurrently, the majority of remaining microglia (intensely Iba1$^+$) are settled close to vessels (Figure 13(d)). By contrast, in the Pu of the same sample, degenerated scavenged neurons and therefore also the microglia were still present in a large amount.

In summary, the hallmark of NDP in tgHD51 rats is a slow degeneration of striatal neurons, manifested primarily by gradual decrease in size of neuronal bodies/nuclei (with maintenance of nucleo-cytoplasmic rate) accompanied with the rarefaction of neuropil. Using the quantitative analysis, we clearly demonstrated for the first time that the turn point in the progression of neurodegenerative process in tgHD51 rats is before the end of the first year of animal age. Then, between 12 and 24 months of age, the further progression is gradual but at a slower rate, resulting in death of many neurons. Moreover, we confirmed that the development of NDP within the striatum is accompanied with gradual degeneration of cortical, particularly pyramidal neurons. We also documented significant participation of the glia, of which function in the development of NDP is irreplaceable. Most prominent is the involvement of GFAP$^+$ astrocytes, particularly their transformation into the specific type of reactive astrocytes. This transformation is responsible for alterations in a structure (and therefore also in a function) of the perivascular glial limiting membrane, loosening of neuropil, and other changes. Surprisingly, we cannot confirm noticeable changes in morphology or number of NG2 glia in tgHD rats, unlike significant participation of microglia and, although less prominent, involvement of S100β^+ cells in NDP

in both tgHD and human HD brains. Certainly, the entire process is potentiated by ageing changes. Our results confirm the complexity of the entire process, in which the neuron-glia crosstalk is crucial.

4. Discussion

The aim of our study was primarily motivated by the absence of histopathological characteristics of chronic neurodegenerative process of HD phenotype in transgenic HD51 CAG rats, which, unlike the other transgenic animal models of HD, survive up to 2 years. Moreover, this transgenic model comprises relatively smaller number (51) of CAG repeats. Both mentioned hallmarks create conditions for similarity to the late-onset form of HD. For this reason, we tried to define to which extent is possible to make a parallel between rat tgHD model and real NDP in human HD brain from histopathological point of view. On the other hand, behavioural symptoms were already widely studied on these animals *(see below)*.

Despite the fact that HD takes place exclusively within human brain and each type of existing animal models is not able to replicate completely mechanisms participating in NDP of the HD phenotype, transgenic models represent a crucial part in the field of the research on HD pathogenesis.

4.1. Mutant Huntingtin and Polyglutamine Deposits. It is generally known that HD is a neurodegenerative movement disorder caused by genetic mutation and morphologically characterized by progressive but selective loss of neurons, primarily within the striatum, followed by the development of reactive gliosis (e.g., [52]). Regardless of some *in vitro* studies which indicated formation of polyQ inclusions that reduce levels of mhtt and the risk of neuronal death [10, 11], it was suggested that the aberrant protein huntingtin (mhtt) with an expansion of N-terminal polyglutamine tract causes preferentially degeneration of striatal neurons in patients with HD (e.g., [6]). Moreover, a gain of a new toxic function of the mhtt results in the loss of former protective functions of wild-type htt, which ultimately leads to the death of neurons [8]. Accumulation of polyQ within the neurons and inside the neuropil is primarily described in a form of aggregates. Our findings showed fine polyQ-huntingtin-positive deposits within the nuclei of striatal neurons in control wt rats and their increase up to very dense accumulation in course of the progression of NDP in tgHD51 rats. On the other hand in cortical (particularly pyramidal) neurons, polyQ$^+$ deposits were not only intranuclear but also particularly cytoplasmic. PolyQ-huntingtin antibody labels not only expanded polyglutamine (mhtt) but also wild-type (normal) huntingtin; therefore the deposits are also present in the brain of control rats. Hence, increased density of polyQ expression in tgHD51 rats is related to increased number of glutamine repeats, that is, to the accumulation of mhtt. Htt is particularly not only spread within the cytoplasm of neuronal bodies and dendrites [7] but also present in the nucleus [53]. Mhtt is present in both nuclear and cytoplasmic compartments, extended polyQ aggregated in the cytoplasm and then it is transported to the nucleus [54]. Increasing concentration of the mhtt in the nucleus accelerates the onset and progression of NDP; however, also extranuclear polyQ might contribute to the initiation of NDP [55]. The same but almost exclusively intranuclear polyQ deposits were also found in glial cells in both brain regions. Accumulated mhtt causes the dysfunction of astrocytes, particularly in relation to glutamate uptake, since it decreases the expression of glutamate transporters. This impairment may promote vulnerability, especially of striatal medium-sized spiny neurons, to the excitotoxic damage and their degeneration (e.g., [12, 13]). Astrocytes expressing mhtt transform into reactive phenotype, which is characterized by markedly decreased expression of glutamate transporters (GLAST and GLT-1) and glutamate uptake. These alterations appear already in early stages of HD (grade 0) and progress in a course of the disease. Therefore, it was suggested that the presence of mhtt in astrocytes and their conversion into reactive glia may contribute to HD pathogenesis [13].

4.2. Reactive Astrogliosis. Reactive astrogliosis is a gradual continuous process of progressive alterations in gene expression and cellular changes. The intensity and extent of the reactive gliosis are determined primarily by signals from damaged cells (e.g., [56, 57]). Up to now, the origin and the way by which the increased number of astrocytes appears in damaged CNS are not fully elucidated and still remain a matter of intensive debate. Considering various types of CNS disorders, marked differences in development of astrogliosis should be taken into the account. During acute CNS injury, reactive gliosis develops within two days and reaches its maximum 1-2 weeks following the insult, while chronic injuries, such as neurodegenerative diseases, are characterised by slow development of astrogliosis, which may last even several years in humans [58].

Owing to the fact that only very low (if any) response of neural progenitors to the CNS damage is found in all nonneurogenic regions, the source for generation of GFAP$^+$ reactive astrocytes remains under discussion—two plausible possibilities are proposed: (1) transformation or dedifferentiation from resident astrocytes which has been confirmed after different neurotoxic lesions in rodent brain (e.g., [59, 60]) or the activation of "quiescent" protoplasmic astrocytes (a subset of GFAP$^+$ astrocytes) acquiring stem cell properties in adult mouse cerebral cortex after acute injury [61]; (2) differentiation of glial progenitors expressing Olig2 and NG2 [62, 63]. For that reason we were surprised with the absence of any obvious involvement of NG2 glia and principally also S100β-positive astrocytes in reactive gliosis. On the contrary, both mentioned possibilities were described in relation to the acute lesion of rodent brain. How the generation of new reactive astrocytes occurs in human brain suffering from chronic neurodegenerative process or in brain of transgenic animals modelling neurodegenerative diseases, as well as the participation of NG2 in the progression of NDP, particularly in HD patients, remains unclear.

It is evident that the development of reactive gliosis and the alterations in astrocyte morphology are conditioned by the intensity and rate of neuronal degeneration in

the context of the neuron-glia relationship [64]. Our findings also document significant difference between typical reactive astrocytes, which change their phenotype in reaction to the acute brain injury or excitotoxic lesion, used formerly as an animal model of HD (e.g., [58, 65]) and "reactive" astrocytes developing during slow progression of chronic neurodegenerative process of HD phenotype. The most conspicuous are changes in expression of GFAP, that is, in the number and arrangement of gliofilaments (the principal astrocytic intermediate filaments) within the cytoplasm. Reactive astrocytes become more stellate with only few but coarse main processes, especially those terminating as the end-feet on the vessel wall. They are intensely labelled for GFAP, due to not only increased amount of $GFAP^+$ gliofilaments but particularly their accumulation to thick bundles (e.g., [34, 64]). This alteration is evident in both tgHD and human HD brains and progresses with the development of NDP. Furthermore, this process occurs not only in HD, but also in other neurodegenerative diseases, such as Parkinson's (e.g., [48, 56]) and Alzheimer's disease (e.g., [66]). Indeed, similar changes, although to a lesser extent, are typical for astrocytes in a course of ageing in both human and rodents (e.g., [66]). Significant for those "reactive" astrocytes involved in NDP is also the absence of reexpression of other intermediate filaments, nestin and vimentin (abundantly expressed in immature astrocytes), whose adaptation is characteristic for immediate activation of astrocytes not only in case of the acute brain injury (e.g., the formation of the glial scar) but also in neurotoxic models of HD [64]. Moreover, these astrocytes do not become markedly hypertrophic (e.g., [34]).

4.3. Neuronal-Glial-Vascular Unit. Except for others, very important is also the relationship of astrocytes to the vessels. Our previous findings documented the thickening of end-feet (and this way of the perivascular limiting membrane), which markedly outlines vessel walls forming typical "rings" with the progression of NDP in excitotoxic rat model of HD [64]. Our findings also demonstrate that those coarser end-feet not only are intensely GFAP-positive but also frequently coexpress S100β protein in both tgHD rat and HD human brains. These gradual alterations in the architecture of the vascular end-feet might contribute to the restriction of transport or diffusion across the blood-brain interface (e.g., [31, 56]). Additionally, the specific enlargement at the tips of astrocytic processes in a form of intensely GFAP-positive "terminal swellings" was found in advancing NDP in human HD brains (grades 3 and 4). However, there is no evidence, which structure in neuropil is in contact with these modified endings. Hypothetically, they might belong to those extensions that lost contact with any opposite structure. In agreement with the other authors, we have indicated participation of ageing changes [67] in the above-mentioned alterations of astrocytic morphology in the oldest groups of control animals.

4.4. CNS Phagocytes: Microglia and Astrocytes. It is also well known that astrocytes are involved in degradation of different metabolites and toxic products under the normal conditions. Their ability to function as phagocytes is then enhanced under the pathological conditions (e.g., [68]). It was proposed that chronically activated astrocytes and microglia can damage neurons via release of highly toxic products (e.g., [66]) and also that microglia play an important role in pathogenesis of Parkinson's disease. This latter suggestion is based on the fact that, with progressive neuronal loss, increasing amounts of α-synuclein and neuromelanin accumulate in the extracellular space due to dysfunction in microglia phagocytic ability [49]. In general, debris accumulation is supposed to be one of key features of the progression (if not direct initiation) of neurodegenerative diseases.

4.5. NG2 Glia. To our surprise, we did not find any significant alterations in number and morphology of NG2 polydendrocytes in the presence of pathology. They formed a typical dense network in all tested groups of tgHD and wt rats. Due to technical reasons, we were unfortunately not able to follow them in postmortem specimens of human brains, either with HD or intact. It is probably the main reason why the involvement of NG2 glia in NDP has not been reported yet in brains of HD patients. The elegant study employing postmortem specimens of human brain tissue with multiple sclerosis (MS) lesions or glioma (using both frozen and paraffin sections) was published by Staugaitis and Trapp [44]. They also discussed difficulties with detection of NG2 glia in routinely processed surgical and autopsy tissue. However, no obvious decrease in density of NG2 glia was found in the grey matter of cerebral cortex with MS lesions [44, 69]. Indeed, most of other studies which reported the relationship of NG2 glia to human CNS pathology are based only on *in vitro* experiments (e.g., [70]).

4.6. Participation of Neurons and Glial Cells in Neurodegenerative Process. It is obvious that glia, particularly GFAP-positive astrocytes, play a critical role in the progression of NDP. Nevertheless, their involvement is always in a context of changes affecting simultaneously all components—cellular and noncellular—of the nervous tissue microenvironment. Also microglia is suggested to be an important player in complex response of nervous tissue to the chronic damage, principally by the activation of cascade of the immune response.

The evaluation of the contribution of $GFAP^+$ astrocytes to neurodegenerative process remains still unclear, albeit their dual role—neuroprotective and neurodegenerative—was already mentioned by some authors (e.g., [27, 56, 71]). On the one hand, astrocytic degeneration accompanying degeneration of the neurons obviously participates in the processes resulting in disintegration of the nervous tissue (loosening of the neuropil, thickening of perivascular glial limiting membrane, and others). On the other hand, there are some indications that changes in astrocyte character and function belong to neuroprotective mechanisms in damaged nervous tissue.

Regardless of the interesting and challenging findings related to the different types of glial cells, the degeneration and loss of neurons, primarily within the striatum,

accompanied with loosening of the neuropil still remain the hallmark of HD. However, the disintegration of the neuropil not only results from gradual reduction of neuronal processes, but also includes the alterations of the glial cells. Indeed, regression of the neuropil represents the progressive disruption of the striatal microenvironment, including the vascular niche, essential for the viability and functioning of all the components of striatal tissue, which even more contributes to worsening of the entire process. The reduction of the grey matter results in a severe brain (primarily striatal) atrophy in advanced NDP.

4.7. Rats Transgenic for HD. Transgenic HD51 CAG rats were used also by other research groups, mainly to validate changes in their behaviour, which indicates impaired striatal function (e.g., [9, 21–24]). The tests proved an early learning and memory impairment (starting between 9 and 12 months) and delayed motor deficits (with the onset around 12 months); moreover, these deficits develop prior to any diagnosed striatal dysfunction. Additionally, age- and genotype-dependent changes in psychomotor performance and in the frequency of choreiform movements were proved in homozygous, less heterozygous tgHD rats in comparison with controls at the age of 15 months and particularly at 20 months [22].

Concerning morphological evaluation, it was shown that polyQ aggregates appear within the striatum of tgHD rats at 12 months of age [9, 23] and the density of their accumulation increases with age, which is consistent with our observations. Furthermore, the quantitative analysis of total striatal volume was carried out in animals from 3 to 15 months of age (using stereological method), which confirmed significant gradual decrease in striatal volume from 12 to 15 months in tgHD rats compared with wt littermates [9] or in 12-month-old rats (compared with 6-month-old and wt rats), respectively [24]. The only reference of Kántor and coauthors [24] provides quantitative analysis of total numbers of neurons and neuronal density in the striatum and a part of frontal cortical layer V in groups of 6- and 12-month-old tgHD51 and wt rats. The results related to the striatum are comparable with our observations. However, the hallmark of neurodegenerative process in the brain of tgHD rats is gradual decrease in size of striatal neurons, unlike the significant decrease in their number in postmortem samples of HD brains. Therefore, we used median diameter of neuronal nuclei to document the onset and progression of NDP in 5 groups of tgHD rats and age-matched wt rats. Nevertheless, to analyse the density of striatal neurons (i.e., their number per defined square unit) becomes rather problematic in the oldest rats (1.5–2 years old) due to significant shrinkage of the striatum. Kántor and coauthors also concluded that, unlike striatal neurons, the cortical neurons (in layer V) did not reveal significant degeneration in 12-month-old tgHD rats. We documented the presence of significant number of hyperchromic degenerated neurons in this age in comparison with age-matched control rats, which is consistent with changes within the cortex of human HD brain [2]. Moreover, already in 6 months old tgHD rats, degenerated cortical neurons appeared. On the other hand, in both tgHD51 rats and HD patients, the extent of neuronal degeneration in the cortex never reaches extensive degeneration within the striatum; therefore, it cannot be accompanied with reactive gliosis.

5. Conclusions

In this study, histopathological features, significant for alterations in human brains suffered from HD and brains of tgHD51 CAG rats, were compared. The significant difference is primarily in the intensity of neuronal degeneration which, even in the oldest rats, does not reach the severity of the damage characteristic for advanced stages of HD in humans. In tgHD51 rats, quite large amounts of striatal neurons display only gradual decrease in their size and do not fully degenerate and disappear until the death of animal (at about 2 years of age). Therefore, also concomitant reactive astrogliosis is not extensive in old tgHD rats, unlike the advanced stages of HD in humans. On the other hand, striatal atrophy (the reduction of grey matter) develops gradually, and it is distinguishable from 12 months of age in tgHD rats, manifested primarily by compensatory enlargement of lateral brain ventricles. The reason for striatal shrinkage is the same in rats and humans; it originates from the gradual degeneration of neurons resulting in rarefaction of the neuropil including the alterations in the density and character of synapses. Our data also support the view that all types of CNS glial cells play an important, irreplaceable role in neurodegenerative process. There are different indications that changes in their character and function try to balance the development of serious irreversible damage of the striatum as long as possible. However, when this battle exceeds all their capacities, the system collapses and their involvement becomes detrimental, finally resulting in death of an individual. All the available results in this field directly or indirectly confirm that the response of nervous tissue to any damage is always complex (moreover in relation to the entire organism) and therefore has to be considered in this context.

To the best of our knowledge, our findings are the first to document that tgHD51 CAG rats can be used as a valid animal model for detailed histopathological studies related to HD in human. On the other hand, since HD occurs only in humans and we are not able to reproduce fully this process in animals till now, the interpretation of all findings has to be always carefully weighted and sufficiently critical.

Conflict of Interests

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

Authors' Contribution

All authors have read and approved the final paper.

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Review Article

The Emerging Use of *In Vivo* Optical Imaging in the Study of Neurodegenerative Diseases

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The detection and subsequent quantification of photons emitted from living tissues, using highly sensitive charged-couple device (CCD) cameras, have enabled investigators to noninvasively examine the intricate dynamics of molecular reactions in wide assortment of experimental animals under basal and pathophysiological conditions. Nevertheless, extrapolation of this *in vivo* optical imaging technology to the study of the mammalian brain and related neurodegenerative conditions is still in its infancy. In this review, we introduce the reader to the emerging use of *in vivo* optical imaging in the study of neurodegenerative diseases. We highlight the current instrumentation that is available and reporter molecules (fluorescent and bioluminescent) that are commonly used. Moreover, we examine how *in vivo* optical imaging using transgenic reporter mice has provided new insights into Alzheimer's disease, amyotrophic lateral sclerosis (ALS), Prion disease, and neuronal damage arising from excitotoxicity and inflammation. Furthermore, we also touch upon studies that have utilized these technologies for the development of therapeutic strategies for neurodegenerative conditions that afflict humans.

1. Introduction

The ability to image cells, tissues, and whole animals has been at the forefront of medical technological advance since the advent of the first microscope and has resulted in the evolution of various imaging modalities, including X-ray, magnetic resonance imaging (MRI), ultrasound (US), positron emission tomography (PET), computed tomography (CT), and optical imaging. Optical imaging, in particular, employs light in the visible and near-infrared spectrum to visualize various cellular processes and has evolved from observing anatomical differences between tissue slices from a single time point to imaging multiple biological features longitudinally in a noninvasive manner in the same animal [1]. Additionally, the use of visible light photons for imaging is an attractive option as it is less harmful than repeated use of ionizing radiation utilized in most other medical imaging modalities.

Noninvasive or *in vivo* optical imaging is particularly advantageous for the study of neurodegenerative diseases. In contrast to conventional techniques that show an absolute reliance on access to brain tissue, which for the most part is only available postmortem, *in vivo* optical imaging permits the study of the tissue within the contextual influences of the intact animal. Moreover, *in vivo* optical imaging contributes towards the reduction in the number of animals used in basic research and drug development. For instance, the same animal can be imaged multiple times in order to monitor visually, often in real time, the progression or regression of infection or disease. In effect, an animal used in an experiment serves as its own control. This, in turn, avoids the need to sequentially sacrifice animals at different time points, allowing significant reductions in the number of animals used per study. With *in vivo* methods, fewer animals can deliver data with greater statistical significance. Additionally,

more accurate animal models can be created that can bear the characteristics of a longitudinal study design, internal experimental control, and quantitative data. In short, *in vivo* optical imaging methods not only guide appropriate endpoint tissue sampling for histology or biochemical analysis but also benefit scientific inquiry and obey the principles of humane experimental techniques in medicine.

To date, most of the work that has been performed so far has utilized rodent models, most likely due to the availability of transgenic mice and the extensive knowledge of mice genetics and biology that exists. Therefore, in this review, we discuss how the emerging use of *in vivo* optical imaging in combination with reporter gene technology, particularly in mouse models, is contributing towards a better understanding of the intricate molecular underpinnings of neurodegenerative diseases and also how this technology is leading to the development of potential therapeutic options.

2. *In Vivo* Optical Imaging Capabilities

Several instruments are currently available to perform *in vivo* optical imaging, each with varying capabilities. Fluorescent and bioluminescent reporters are most commonly used, and most instruments are able to read data from both, including the NightOwl (Berthold Technologies), *In Vivo* imaging systems (Bruker), iBox Scientia Small Animal Imaging System (UVP), and the PhotonIMAGER (Biospace Lab). As well, the Mousepod is an accessory for the Odyssey CLx Infrared Imaging System (Li-Cor Biosciences). Several optical imaging systems are also able to be used in conjunction with other medical imaging modalities (MRI, PET, and CT) such as the IVIS series and FMT series of imaging systems (Perkin Elmer). In fact, systems are now being produced with integrated medical imaging, such as the IVIS Spectrum CT (Perkin Elmer), which has a built-in microCT. While these systems provide invaluable information, the mode or method of sedation of experimental animals used can exclude some research studies, such as those involving the sleep-wake cycle and the examination of the physiology of the immune system [2]. Recently, optical imaging of nonsedated animals by way of the In Actio Module for the PhotonIMAGER (Biospace Lab) through rapid acquisition of photons has been developed as a means of addressing this limitation. When choosing an instrument for *in vivo* optical imaging, it is important to consider the method of light detection and the software used to analyze images. Due to their high sensitivity, cooled CCD cameras are most often used. In fact, all of the above-mentioned instruments employ the use of a CCD camera except the Odyssey CLx Infrared Imaging System, which uses the nearly equivalent avalanche photodiode. As well, the software capabilities should be considered depending on the experiment. When two or more reporters are used with different emission wavelengths or tissue autofluorescence is an issue, spectral unmixing can be used to tease apart the different wavelengths. Imaging of several animals simultaneously can be performed on instruments that come equipped with a multiple mouse manifold to deliver anesthetic gas, such as the IVIS series from Perkin Elmer, which decreases the technician hands on time required. Alternatively, instruments without a multiple manifold can still be used to image several mice under injectable anesthetic, providing they fit in the CCD field of view; however, signal can only be measured for each mouse if the software is able to define multiple regions of interest (ROIs) for photon measurement. Multiple ROI capabilities are also of importance when the reporter used differentially localizes to multiple regions of the animal or multiple probes are used.

3. Optical Imaging Reporters

Reporting the location and expression of molecular signals for optical imaging requires reporters that emit light; two of the most commonly used are fluorescent and bioluminescent reporters. Fluorescence relies on a variety of excitation and emission wavelength filter pairs for varying fluorescent reporters, whereas bioluminescence requires a substrate to complete the biochemical reaction to produce light [3]. Both methods of light generation possess inherent advantages and disadvantages during experimental setup, and, moreover, data analysis and reporter choice must be determined based on the requirements of the experiment(s) to be performed (Figure 1). A general limitation of visualizing fluorescent light in optical imaging is endogenous light absorption, which can be easily illustrated by holding different colour laser pointers to one's fingers and examining the degree of light transmission through the tissue. Green light results in little to no light transmission through tissue, whereas red light is more easily transmitted. This is due to endogenous absorption of light by hemoglobin and melanin in the lower part of the visible spectrum limiting the depth of light penetration (Figure 2) [4]. Therefore, animal positioning during imaging is of the utmost importance and must be adjusted so that the light signal is placed closest to the camera detector. In addition, multimodal imaging that combines photon information with structural information generated by MRI or CT, for example, plus the application of algorithms is providing improved ways to enhance spatial resolution and to reconstruct 3D models of light production within tissues.

4. Fluorescent Reporters

Fluorescent proteins absorb light photons at a wavelength specific to the protein, which then excites electrons to a higher energy state. As the electrons return to ground state, energy is released as light at a different wavelength generating a colour on the visible spectrum [5]. Imaging with fluorescent protein reporters has several advantages. Firstly, experimental setup is relatively easy, as once a reporter with certain fluorescence is chosen, it is integrated into the animal and imaged with the corresponding excitation/emission wavelengths for that fluorophore. Secondly, there are many fluorescent reporters available that emit light at varying wavelengths throughout the visible and near infrared spectrum. This enables multiple reporters to be used simultaneously by choosing fluorescent proteins with little spectral overlap. Although the setup is relatively easy, it can be difficult to interpret fluorescent

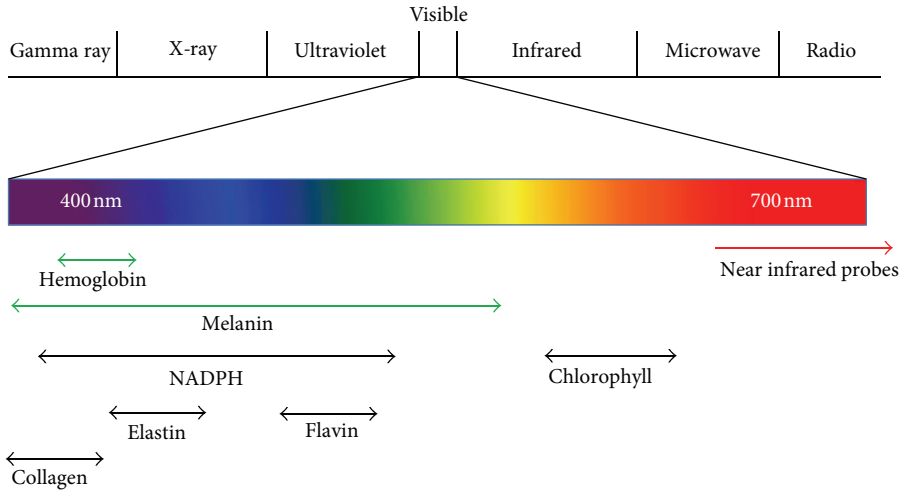
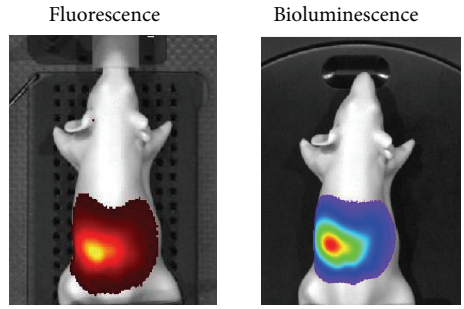


Figure 1: General location of absorption, tissue autofluorescence, and near infrared probes on the visible light spectrum. Factors contributing to tissue absorption (green arrows) and autofluorescence (black arrows) are indicated below.



Source	Protein	Chemical reaction
Auto-fluorescence	Yes	No
Substrate	None	Luciferin
Requirements	None	ATP + O_2
Probes	Many	A couple
Mice per image	Several	Several
Imaging time	Milliseconds	Minutes

Figure 2: Comparison between fluorescent and bioluminescent reporters for use in *in vivo* optical imaging. The luciferase used for comparison in this figure is firefly luciferase.

data. Autofluorescence of skin, fur, and tissue, due to several cellular components, including NADPH, flavin coenzymes, elastin, and collagen, can interfere significantly with signal from fluorescent reporters if emission wavelengths overlap (Figure 2) [6]. Additionally, chlorophyll present in standard mouse food autofluoresces thus interfering with many common reporters [7]. To compensate for autofluorescence, software has been developed with advanced mathematical modeling to separate the sources of different wavelengths. This particular feature is referred to as spectral unmixing; nevertheless, many optical imaging instruments and software lack this capability [8, 9]. While fluorescent proteins have been traditionally used, nonprotein based fluorophores commonly used in cellular imaging, such as fluorescein and CyDyes, are alternative fluorescent probes for use in *in vivo* optical imaging, and recently, quantum dots have been developed for optical imaging. Quantum dots are small, inorganic nanoparticles that emit a specific wavelength of light depending on their size, from ultraviolet to near-infrared, and can be conjugated to molecules that localize fluorescence to an area of interest [10]. They offer increased brightness and stability over fluorescent proteins and providing a means to manipulate the wavelength emitted by simply altering the size of the nanoparticle. However, since nonprotein based

Table 1: Commonly used luciferase reporter systems. Information on some of the available luciferases for use in *in vivo* optical imaging experiments, including sources, emission wavelengths, substrates, and selective advantages for each.

Source	Emission wavelength (nm)	Substrate	Advantages
Bacteria (*Vibrio* and *Photobacterium* species)	478–545 (dependent on species)	FMNH2 + O_2 + long chain fatty aldehyde	Exogenous substrate not required
Firefly (*Photinus pyralis*)	560	D-luciferin + ATP + O_2	Most commonly used and modified for red-shifted emission
Sea pansy (*Renilla reniformis*)	480	Coelenterazine + O_2	Different substrate allows multiplexing with firefly luciferase
Copepods (*Gaussia princeps* and others)	470	Coelenterazine + O_2	Small size and secreted
Deep-sea shrimp (*Oplophorus gracilirostris*)	460	Furimazine + O_2	Small size and secreted

reporters cannot replicate *in vivo*, they cannot be made into fusion proteins to monitor promoter activity.

5. Bioluminescent Reporters

Bioluminescence is most commonly used for *in vivo* optical imaging and refers to the light that is generated by a chemical reaction between the substrate, luciferin, and oxygen, in which luciferase acts as the enzyme to accelerate the reaction [11]. When the electron of this reaction product returns to ground state, energy is emitted in the form of light, similar to fluorescence. There are several different bioluminescent reporter systems, each isolated form a different species and generating light at varying wavelengths (summarized in Table 1). Whereas fluorescence data analysis can be difficult to interpret due to tissue autofluorescence, there is no endogenous tissue bioluminescence; therefore, all detected light directly results from the luminescent reporter. Nevertheless, the experimental setup is slightly more challenging compared to fluorescence. As a luciferin substrate is required for most of the bioluminescent chemical reactions, and is not endogenous to animal models, it must be supplied exogenously. Therefore, experimental consistency is important to produce comparable results. To establish the optimal dosage of luciferin and the optimal time to image the animal after injection of the substrate, a kinetic curve is initially generated. While the need for a kinetic curve is only required once, it can become challenging when studying an experimental animal from birth to adulthood due to alterations in metabolism that can alter the kinetics of luciferin processing. Nevertheless, the fact that luciferin is able to cross the blood brain barrier (BBB) is especially pertinent for neuroimaging [12]. A recent advancement that may eliminate the use of exogenous luciferin in some models is the use of the bacterial luciferase (*lux*) gene cassette that contains both the luciferase and luciferin genes [13]. Light is generated in the location of the reporter without relying on the bioavailability and/or kinetics of the luciferin substrate. Besides the obvious increase in gene size required for engineering the cassette, there are several drawbacks that accompany this technique, including the lower degree of gene expression than traditional firefly luciferase and also the shorter emission wavelength (490 nm and 560 nm, resp.). The latter drawback may be a problem for deep tissue imaging due to the absorption of the signal [13]. In addition, the use of a secreted luciferase may increase the resulting signal, as the substrate no longer requires entry into the cell expressing the luciferase; however, this may also lead to diffusion throughout the body, thus preventing accurate localization of the signal.

6. *In Vivo* Optical Imaging in the Study of Neurodegeneration

The use of *in vivo* optical imaging technology is emerging as an important addition to the array of tools currently available for the study of neurodegenerative conditions. These diseases and disorders of the central nervous system (CNS) are characterized by the progressive loss of neuronal function and structure that eventually culminates in cell death. There are several different types of neurodegenerative diseases classified largely by the identity of the neuronal cell population that is afflicted. These include Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis (ALS), and Prion diseases. Their complex etiology is common amongst the majority of neurodegenerative diseases; they are not monogenic or polygenic diseases and pathogenesis is multifaceted by events that are, most often, independent of genetic mutations. At the molecular level, the events responsible for neurodegeneration include oxidative stress, axonal transport deficits, protein oligomerization and aggregation, calcium deregulation, mitochondrial dysfunction, neuron-glial interactions, neuroinflammation, DNA damage, and aberrant RNA-processing. One of the greatest risk factors for neurodegeneration is advanced chronological age, in combination with mitochondrial DNA mutation and oxidative stress damage. Due to the extended life expectancy in the developed world, the prevalence of many of these diseases is expected to rise. Therefore, identification of tools that can assist in the rapid detection and quantitative assessment of the neuropathological status of diseased individuals is of the utmost importance, not only for diagnostic purposes but also

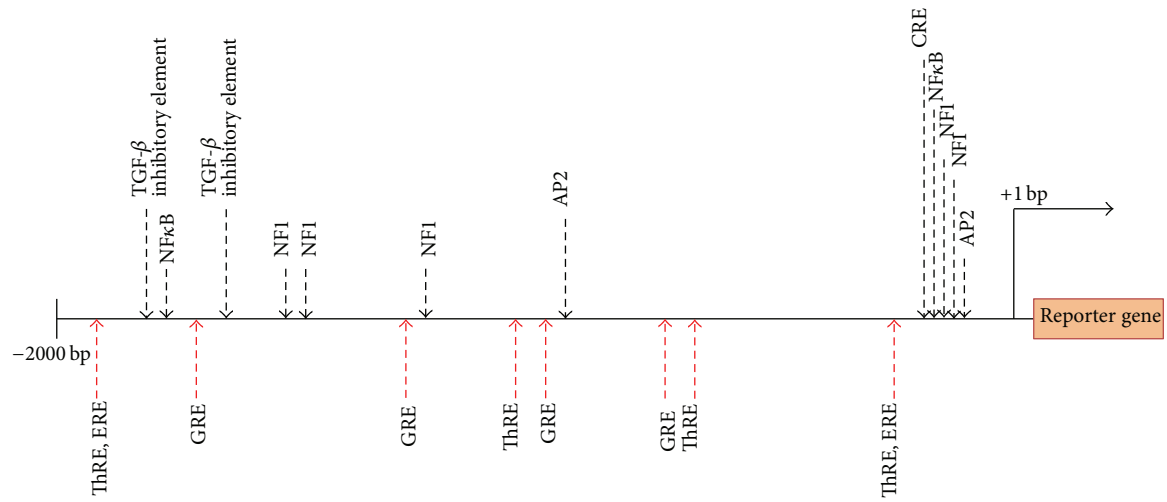


FIGURE 3: Putative response element and transcription factor binding sites within the mouse GFAP 5′-upstream region. The binding sites of transcription factors and some elements are shown above the line. Hormone response elements binding sites are shown below the line. For a detailed description please refer to Laping et al. [14]. ThRE: thyroid hormone response factor element; ERE: estrogen response element; GRE: glucocorticoid response element, NF1: nuclear factor 1; AP2: activator protein 2; TIE: TGF-β inhibitory element; CRE: cAMP response element; NFκB: nuclear factor κB. Elements and features are not depicted to scale.

for the development and evaluation of effective therapeutic options.

One promising approach by which *in vivo* optical imaging is contributing to the study of neurodegenerative diseases is through the use of transgenic mice in which a reporter gene (i.e., green fluorescent protein (GFP) or the enzyme *luciferase*) is under the control of an "activatable" promoter that acts as a disease biomarker. To this end, the glial fibrillary acidic protein (GFAP) promoter has been harnessed most often (Figure 3). GFAP is a major intermediate filament protein of astrocytes whose expression is highly regulated and is induced during astrocyte activation in response to multiple factors, notably from brain injury and disease including degenerative conditions [15–17]. The regulation of GFAP is most likely due to multiple sites within the promoter region of the gene. Although some promising sites have been identified, their significance and contribution to the overall regulatory control is still under investigation. Nevertheless, there are a plethora of sites for hormones, growth factors, inflammatory cytokines, and transcription factors (Figure 3). Additionally, epigenetic mechanisms such as phosphorylation and methylation are also likely to exert significant influence over GFAP transcription. Moreover, GFAP has also been shown to fluctuate under the circadian light-dark cycle [18]. In addition to GFAP, other similarly utilized promoters include heme oxygenase-1 promoter (HO-1), a marker for oxidative stress [19]; toll-like receptor 2 (TLR2) promoter, involved in the regulation of the inflammatory response of microglial cells [20]; microtubule associated protein 1 light chain 3 (LC3) promoter, a marker for autophagy [21]; and the growth-associated protein-43 (GAP-43) promoter, strongly upregulated in adult injured neurons as a part of the regenerative process [22] (Figure 4).

7. Alzheimer's Disease

Alzheimer's disease (AD) is the most common cause of dementia in adults and is characterized by the extracellular accumulation of amyloid plaques composed of aggregated amyloid β (Aβ) peptide, as well as intracellular neurofibrillary tangles composed of hyperphosphorylated and aggregated Tau protein. This, in turn, is highly neurotoxic. Research into the neuropathology of AD has been aided tremendously by generation of transgenic mice that accurately recapitulate the deposition of Aβ, often by overexpressing Aβ containing specific familial mutations in the amyloid precursor protein (APP) gene. Nevertheless, these models are also hindered by the fact that they do not show any overt clinical neurological symptoms of the disease and do not succumb to the deposition of Aβ in the brain. Therefore, *in vivo* diagnosis of AD pathology in the brains of these mice has proved to be challenging and most often can only be accomplished postmortem or through laborious learning and memory tests that are not only challenging but also quite often subjective. To delineate whether *in vivo* optical imaging would be a successful application for the study of AD, two widely used transgenic mouse models, transgenic lines APP23 and CRND8, were crossbred with reporter mice that express *luciferase* under the GFAP promoter to generate bigenic mice whose *luciferase* expression can be visualized [23]. In these bigenic mice, age and transgene dependent increases in *luciferase* signal were readily observed which correlated with the onset of robust Aβ deposition in the brain. In general, the CRND8:GFAP-*luciferase* mice showed a much earlier inflection in the bioluminescence emitting from the GAFP promoter than the APP23:GFAP-*luciferase* mice. Nevertheless, the signal emitted from these bigenic mice was

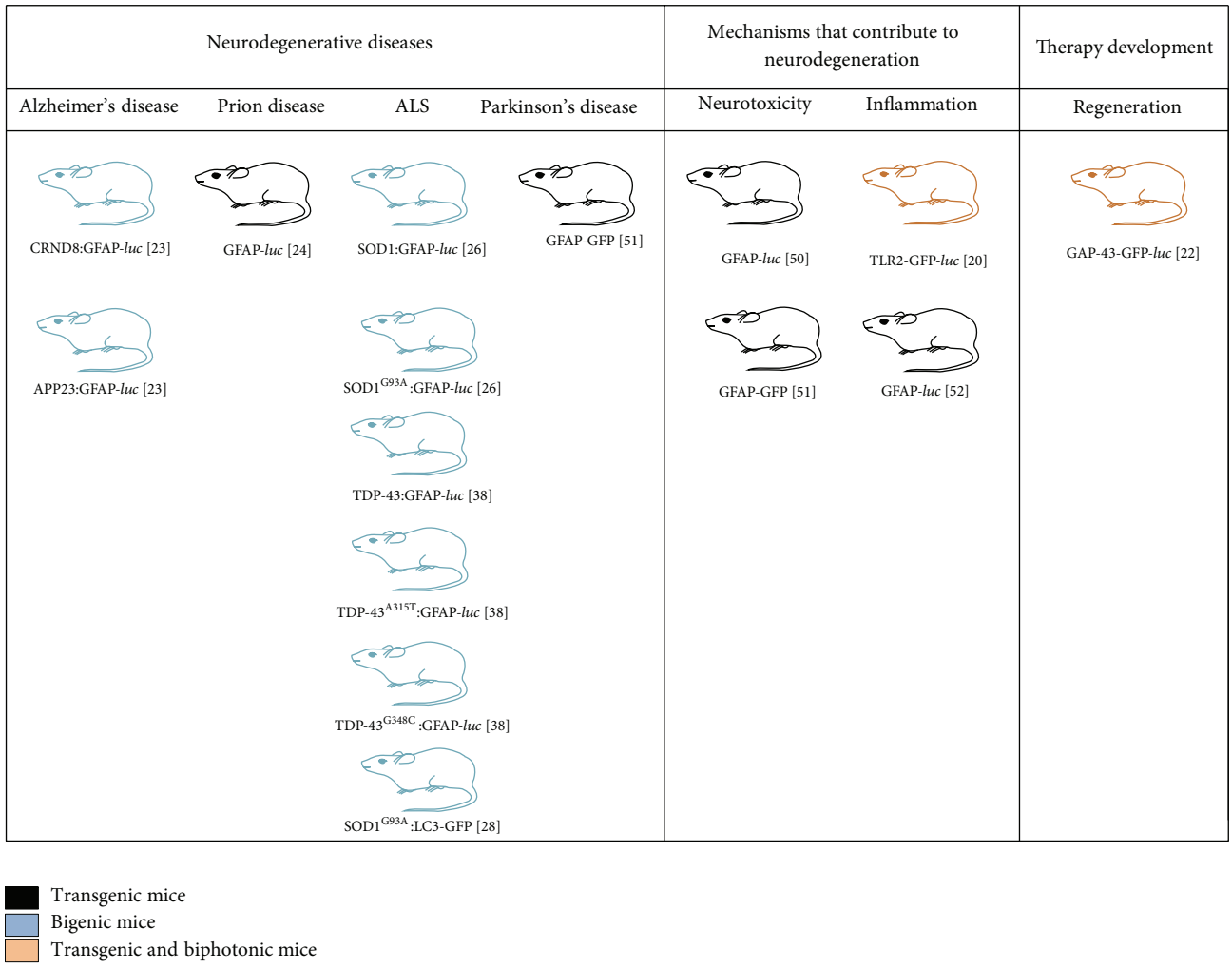


Figure 4: Bigenic and transgenic reporter mouse models that have been used for the study of neurodegeneration by *in vivo* fluorescent and bioluminescent optical imaging technology.

far above the signal emitted from the control mice that were only GFAP-*luciferase*. *In vivo* optical imaging, therefore, permitted the diagnosis of a neurological disease in these mice in the absence of any overt signs of neurological dysfunction. Additionally, utilizing *in vivo* optical imaging technology, the visualization of accelerated deposition of Aβ in live APP23:GFAP-*luciferase* mice upon inoculation with brain homogenate derived from aged APP23 mice was possible [23]. Conceivably, the bioluminescence paradigm utilized in the study could be adapted to the study of any AD transgenic mouse lines to draw general conclusion about the molecular mechanisms contributing to the disease and permitting the early diagnosis of the disease in experimental animal models.

8. Prion Diseases

Prion diseases are rare, fatal neurodegenerative diseases caused by the misfolding and the subsequent replication of the infectious PrP^{Sc} molecule. The molecular mechanism(s) involved in the conversion of the cellular prion protein (PrP^{c}) to the pathological isomer (PrP^{Sc}), and the subsequent cascade of molecular events that contribute to the neurodegenerative process, remain elusive. Unlike other neurodegenerative diseases, wild-type mice can be inoculated with an infectious dose of prion inoculum and the course of disease progression can be monitored. Disease progression is highly reproducible when inoculating with a mouse-adapted prion strain and unlike many other neurodegenerative disorders, it can recapitulate neurological symptoms along with the pathology that is characteristic of the disease in humans. For this reason, prion models are potentially very useful for evaluating biomarkers of neuronal health and testing neuroprotective therapeutics. Astrocytic gliosis occurs simultaneously with prion replication thus permitting the use of transgenic GFAP-*luciferase* to monitor the progression of prion disease. To date, the application of *in vivo* optical imaging technology to the study of prion diseases has shown that the Rocky Mountain Lab (RML) scrapie strain in mice can be diagnosed at ~55 days after intracranial inoculation, which represents half

the time required for the emergence of clinical symptoms, thus providing an early diagnostic criterion [24]. Alternate routes of prion infection that involve prion neuroinvasion from peripheral tissues, such as intraperitoneal inoculation and oral gavage, also resulted in detectable bioluminescent signals. Moreover, an inverse relationship was observed between the dose of prion inoculum administered and the point of bioluminescence inflection that was observed, relative to mock treated mice, over a wide range of prion dilutions. This study shows that alterations to bioluminescence signals between infected and control transgenic mice can indeed serve as a semiquantitative surrogate biomarker of prion replication. Undoubtedly, *in vivo* optical imaging technologies provide a new window of opportunity to test therapeutic interventions and visualize their effect on the onset of disease or progression. Moreover, this also affords the opportunity to optimize and refine classical bioassays by requiring fewer mice and shorter experimental time-courses. It is tempting to speculate whether genetically engineered mice with higher levels of *luciferase* expression would provide greater sensitivity and be conducive for earlier detection of astrocytic gliosis in parallel with the earliest replication of prions following inoculation [24].

9. Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS) is an adult-onset neurological disorder characterized by the progressive degeneration of motor neurons leading to muscle weakness, atrophy, paralysis, and subsequently death. The lifespan of individuals diagnosed with the clinical onset of ALS is often just five years. The pathological events contributing to the loss of motor neurons and the exact pattern of ALS spread are not fully understood. Novel findings utilizing *in vivo* optical imaging and bigenic reporter mice that possess both GFAP-*luciferase* and the $SOD1^{G93A}$ mutation have contributed new insights into the pathobiology of ALS. In general, experimental animals possessing the human SOD1 mutation G93A develop features that resemble human familial and sporadic ALS [25]. In bigenic mice ($SOD1^{G93A}$:GFAP-*luciferase*), *in vivo* optical imaging revealed that there are several successive stages of repeated increases in the expression of GFAP [26]. The first round of GFAP-*luciferase* increase corresponded with the asymptomatic stage at 25–30 days with prominent signal emanating from the lumbar spinal cords projections and peripheral neurons (projection areas of sciatic nerves). The second round corresponded with the clinical onset of the disease (85–90 days) which is characterized by distinct behavioral deficits and hind-limb paralysis. The peak signal at 113 days corresponded precisely with the induction of hind-limb paralysis. In the second round, prominent GFAP-*luciferase* signals emanated once again from peripheral sciatic neurons and Schwann cells. The authors suspect that the first round of GFAP promoter activation was most likely due to the expression of GFAP in astrocytes and glial progenitor cells, whereas the second round of promoter activation was most likely due to the activation of astrocytes in response to the ensuing pathology. In general, these studies revealed that toxicity to motor neurons in ALS was not noncell autonomous and that populations of nonneuronal cells, perhaps glial cells, can also affect the viability of motor neurons.

In vivo optical imaging of the $SOD1^{G93A}$:GFAP-*luciferase* mice also showed an increased signal contribution from the corticospinal tract and upper motor neurons near the end stages of the disease [26]. Further *ex vivo* imaging of the affected brains to delineate the specific region(s) of signal occurrence confirmed that the signals mainly arose from the cortex and brainstem areas. These particular regions are implicated in the control of respiratory functions and the swallowing reflex, which suggests that damage within this region may contribute to the dramatic weight loss and breathing difficulties that is often associated with ALS [26]. Imaging also provided some evidence for "dying-back" neuropathy, or denervation in ALS, which may be initiated by the loss of neuromuscular junctions [27]. Recapitulation of the $SOD1^{G93A}$:GFAP-*luciferase* neuropathy with SOD1:GFAP-*luciferase* mice that have undergone precise mechanical denervation using the cut-and-crush method of sciatic nerve injury provided some credible evidence for this hypothesis [26].

Analogous to the cellular role played by the ubiquitin-proteasome pathway, autophagy is considered to prevent the accumulation of abnormal proteins that may be toxic to the cell. Nevertheless, in ALS pathology, autophagy could also be involved in the process of motor neuron death. Microtubule associated protein 1 light chain 3 (LC3) is a marker for autophagy and bigenic mice possessing the fusion of the promoter region of LC3 to GFP and also the G93A mutant of human SOD1 has been generated in order to monitor *in vivo* autophagy in a mouse model of ALS [28]. *In vivo* optical imaging of $SOD1^{G93A}$:LC3-GFP at presymptomatic (10 weeks), early symptomatic (17 weeks), and late symptomatic (19 weeks) stages of the disease revealed a strong fluorescent signal *in vivo* over the T_3–S_1 level at 17 and 19 weeks of age in the double transgenic mice. *Ex vivo* autophagy imaging of spinal cord sections also showed a progressive increase of the fluorescence signal from 17 to 19 weeks in these mice in the anterior horn at the $L_{4\text{-}5}$ level, and the fluorescence signals were clearly observed in the gray matter of the spinal cord with a progressive increase of the signal and decreases in large motor neurons. Taken together, these results suggest that although the activation of autophagy may be induced during the onset of ALS, the fusion of the autophagosome to the lysosome may become insufficient at the end stages of the disease, possibly contributing to motor neuron cell death [28].

The occurrence of ALS and frontotemporal lobar degeneration with ubiquitin inclusions (FTLD-U) in some families, and the discovery that the transactive response DNA-binding protein 43 (TDP-43) is present in the cytoplasmic aggregates of both diseases, provided the first set of clues that the two diseases may share a common underlying mechanism [29]. TDP-43 is a DNA/RNA binding protein that contains an N-terminal domain, two RNA-recognition motifs, and a glycine-rich C-terminal domain thought to be important in the mediation of protein-protein interactions [30, 31]. It serves a plethora of cellular functions but its implication in

neurodegenerative diseases was primarily substantiated by the discovery of dominantly inherited missense mutations in TDP-43, which are present in patients with familial form ALS [21, 32–36]. Additionally, in neurodegenerative diseases, TDP-43 can be found in cytoplasmic ubiquitinated inclusions, where it shows poor solubility, hyperphosphorylation, and cleavage into smaller fragments [29]. Early mouse models expressing wild-type TDP-43 or mutant TDP-43 (A315T and M337V) exhibited early paralysis followed by death [37]. Moreover, many of these transgenic animals also exhibited increased ubiquitination of TDP-43 without the accumulation in inclusion bodies. Altogether, these observations raised questions about the validity and the usage of these animals as appropriate experimental models for the study of human forms of ALS. Many of these characteristics were primarily attributed to the high-level of neuronal expression of the transgene. Therefore, to better recapitulate the human version of the disease, alternate rodent models have been generated that show not only ubiquitous expression of the transgene, but also, more importantly, moderate levels, mainly due to the transgenes being under the control of their own promoters [38]. *In vivo* optical imaging of bigenic versions of these alternate rodents (i.e., TDP-43:GFAP-*luciferase*, TDP-43^{A315T}:GFAP-*luciferase*, and TDP-43^{G348C}:GFAP-*luciferase*) showed that astrocytes are activated as early as 20 weeks in the brain, during a 52-week examination period, in TDP-43^{G348C}:GFAP-*luciferase* mice. Moreover, the induction of astrogliosis in the brain and the spinal cord of all three bigenic models preceded the appearance of cognitive and motor abnormalities by up to 6–8 weeks.

10. Neuronal Damage Arising from Trauma

One of the primary causes of CNS neuronal damage is trauma to the brain which can initiate chronic molecular events that may be important epigenetic factors that predispose an individual to neurodegenerative conditions such as Alzheimer's disease [39, 40], Parkinson's disease [41], and ALS [42, 43], at a later time in life [44–46]. Emerging evidence also suggests that mild traumatic brain injury (TBI), which consists of concussive and mild concussive trauma, such as those encountered during sporting activities, can provoke a distinctive neurodegenerative state known as chronic traumatic encephalopathy (CTE) [47–49]. Trauma to the brain consists of the primary injury that disrupts brain tissue, followed by a cascade of secondary events that may spread by multiple molecular mechanisms. Secondary injuries consist of molecular events such as blood-brain-barrier (BBB) disruption, edema, oxidative stress, excitotoxicity, inflammation, and cell death. Clinical presentation of secondary injuries is usually delayed and, therefore, can be sensitive to therapeutic intervention. As such, secondary injury processes may serve as viable option(s) for imaging and therapeutic targets for the diagnosis and treatment of CNS neuronal damage caused by trauma. Some of the specific mechanisms of CNS neuronal injury that have been examined using *in vivo* optical imaging technology include neuronal excitotoxicity and inflammation. Insights gained from these studies can contribute to a better understanding of the molecular mechanisms associated with the secondary injury caused by trauma to the brain and, also, how best to curb these pathological features in an effort to circumvent the probability of developing a neurodegenerative pathology at a later time.

Trauma to the CNS may lead to excitotoxic events in the brain. Excitotoxicity is defined as cell death resulting from the toxic actions of excitatory amino acids (EAA). Since glutamate is the major excitatory neurotransmitter in the mammalian CNS, neuronal excitotoxicity usually refers to the injury and/or death of neurons arising from prolonged exposure to glutamate and the associated excessive influx of ions into the cell through glutamate-mediated receptors. The resulting ion overload (i.e., Ca^{2+}) is particularly neurotoxic, leading to the activation of enzymes that degrade proteins, membranes, and nucleic acids. The overactivation of glutamate receptors can also impair cellular ion homeostasis, activate nitric oxide synthesis, generate free radicals, and induce programmed cell death. In experimental animal models, excitotoxicity can be induced through treatment with kainic acid (KA), a potent agonist for a subtype of glutamate receptors. *In vivo* optical imaging of excitotoxicity has been delineated through both GFAP-GFP and GFAP-*luciferase* mice. In these mice, significant elevation of GFAP signal was detected in the brain after subcutaneous treatment with KA [50, 51]. Additionally, in the GFAP-GFP mice, symptoms of Parkinson's disease were induced by the neurotoxicant 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). The effect of MPTP was also visualized after subcutaneous injection of the agent [51]. These reporter mouse models can therefore serve as useful tools to study the neuropathological consequences of excitotoxicity and neurotoxicants. Specifically, these models may permit the identification of key upstream molecular events that instigate or contribute to neuronal damage, which in turn will provide not only novel insights into the molecular basis of how neuronal cells die but also potential approaches for therapeutic intervention by uniquely targeting mechanisms involved in excitotoxic/neurotoxic signaling cascade.

Paradoxically, the inflammatory response can either aggravate or ameliorate the ensuing neuropathology associated with trauma to the brain. However, since the inflammatory response parallels that of secondary tissue injury, much interest has focused on the possibility of minimizing, or altogether arresting, certain components of inflammation in order to reduce secondary damage. Research from such a venture has broad applicability and is pertinent to the study of various neurodegenerative diseases. Several CNS resident cells, such as astrocytes and microglia, have innate inflammatory capacity and the live imaging of the activation of these cells has contributed some novel findings to the inflammatory mechanism operating under neurodegenerative conditions. The live imaging of inflammation in bigenic reporter mice (GFAP-*luciferase*) revealed that sex and estrogen levels are strong determinants of astrocyte activation/response caused by cerebral ischemia [52]. Following cerebral ischemia, GFAP-signals were markedly stronger in female transgenic mice than in males. However, these signals were diminished upon the entry of female mice into

estrus or upon the pharmacological application of estrogen. Additional findings from this work suggest that the extent of the ischemia, based on the degree of signal intensity, is dictated by the size of the injury only in the male mice. No such correlation was observed in any of the experimental groups of female GFAP-*luciferase* mice used in the study.

The inflammatory response mediated by microglial cells can be regulated by Toll-like receptor 2 (TLR2) activation. Within the mouse brain, TLR2 expression is very low but is dramatically upregulated in response to infection and/or injury to the brain [53, 54]. Nevertheless, the mechanisms behind TLR2 activation, the long term consequence of activation, and brain region specific expression patterns of TLR2 are unknown. For these purposes, the TLR2-GFP-*luciferase* transgenic mice have provided some much needed understanding to the microglial activation process [20]. In a model of ischemia, the TLR2-GFP-*luciferase* mice showed TLR2 activation as early as 6 hours after the ischemic event. Interestingly, the activation was initially observed in the olfactory bulb (OB), even preceding its expression in the area of ischemic lesion. Moreover, longitudinal monitoring of TLR2 activation showed that the signal was detectable over the period of several months after the initial ischemic attack, implying that postischemic inflammatory process is much longer than previously understood. The biphasic nature of microglia activation (acute activation in OB followed by chronic activation at the site of ischemic lesion) was suggested to be a result of the distinct neuroanatomical location maintained by the OB. Specifically, the OB is located at a region that is considered to be at the interphase between the external environment and the brain. Perhaps this distinct location permits the expression of a unique subclass of microglia that may exist in a perpetually primed or alert state. This hypothesis was further supported by the parallel activation of TLR2 signal in the OB and at the site of intracranial inoculation of LPS. Furthermore, the OB was also able to translate TLR2 response and microglial activation signals, caused by inhalation of LPS, from the external environment into the brain.

11. *In Vivo* Optical Imaging for Diagnostic and Therapeutic Purposes

Drug discovery and evaluating the effectiveness of newly developed therapies are a priority that can be promptly addressed through the use of *in vivo* optical imaging. For example, novel protective monoclonal antibodies were discovered in mice infected with a bioluminescently tagged influenza A virus through binding of the hemagglutinin H1 and H5 subtypes [55]. Additionally, to assess drug efficacy, tumour response to Gefitinib, an epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor, was evaluated in mice injected with fluorescently labeled tumourigenic A549 cells and found to reduce tumour size over time [56]. Similarly, a mouse model with stainless steel implants in the knee was inoculated with methicillin-sensitive *Staphylococcus aureus* (MSSA) to determine the optimal antibiotic use at different doses [57]. Therefore, it is reasonable to assume that the use of *in vivo* optical imaging and reporter mice technology for the study of neurodegenerative diseases will undoubtedly provide a reliable avenue for the development of novel diagnostic assays that show both improved sensitivity and specificity over current options. Moreover, these applications can also contribute towards the development of novel, disease-modifying therapies whose delivery and efficacy can be monitored in a longitudinal manner, permitting the use of less experimental animals and minimizing the variability that would emerge from using large sample sizes.

Nevertheless, the major hurdle for *in vivo* optical imaging, with respect to diagnostic and therapeutic molecule development for neurodegenerative diseases and disorders, remains the delivery of molecular agents across the restrictive BBB. The BBB ensures restrictive passage of molecules to the CNS in order to maintain proper functioning environment for the brain. Free passage of molecules would, therefore, disrupt intricate brain homeostasis. Passage of potential molecules across the BBB is also further hindered by the addition of fluorescent moieties or contrast agents that would be required for direct visualization. Not surprisingly, many of the molecules that have been developed are used to target receptors on the endovascular region which are upregulated during many pathological events of the brain. Nevertheless, one particular ligand that has been successfully evaluated, particularly using *in vivo* optical imaging technology, as a diagnostic tool for AD is the oxazine dye AOI987 [58, 59]. AOI987 has a low molecular weight, readily traverses the BBB, and shows high affinity towards Aβ plaque. It is well demonstrated that Aβ deposition precedes and most likely is involved in the induction of neuronal atrophy. Therefore, the deposition and subsequent quantification of Aβ load in the brain of affected individuals are imperative as detection of amyloid deposition may be the first step(s) towards diagnosis and subsequent optimization of treatment strategies for the ensuing neuropathology. Apart from the aforementioned properties, AOI987 absorbs and emits in the near infrared (NIR) fluorescence spectrum thus minimizing the impact of tissue autofluorescence and light-scattering that would be otherwise observed from dyes with a shorter absorption and emission spectrum. Another ligand that has been studied using *in vivo* optical imaging is the curcumin-derived NIR fluorescent probe CRANAD-2 which also shows a high affinity for Aβ [60]. Uniquely, upon intercalation with amyloid plaques, the probe not only increases in fluorescence and quantum yield but also undergoes a shift in the emission spectra by 90 nm. This particular spectral feature of CRANAD-2 is particularly intriguing as it may offer the ability to discriminate amyloid-bound probe from unbound probe, thereby enhancing the target-to-background signal. The aberrant aggregation of proteins/peptides is a common theme among most age related neurodegenerative diseases, including Parkinson's disease, Huntington's disease, ALS, and FTLD. Although the specific protein aggregates and the downstream cellular factors that are vulnerable differ, shared disease mechanisms are increasingly apparent among these diseases. It is, therefore, tempting to speculate whether the aforementioned ligands, their unique properties, and/or the technology used in their synthesis would be applicable for the detection and study of other CNS protein aggregation

diseases using *in vivo* optical imaging technology. Versatile amyloid-specific fluorescent probes can have a very positive impact in the drug delivery and diagnostics fields for a wide range of neurodegenerative conditions and their delivery, function, and efficacy will undoubtedly be aided by *in vivo* optical imaging capabilities. Several other recent advances have been made that readily permit and/or assist in the transfer of molecules across the BBB, including potent viral vectors and nanoparticle technology, and it is foreseeable that these could be harnessed for *in vivo* optical imaging applications.

Apart from providing insights into the disease process reporter mice harboring transgenes can also provide highly specific mechanistic information on the biological specificity and efficacy of therapeutic agents. The most commonly used GFAP-*luciferase* transgene can provide novel insight into the degree of CNS injury recovery (or lack thereof) in response to a therapeutic. Another pertinent addition to the diversity of reporter mice currently available for *in vivo* optical imaging (Figure 4) is the GAP-43:*luciferase*-GFP mouse [22]. A unique property of this reporter mouse is that GAP-43 promoter is neuron specific and can, therefore, be utilized to sense neuronal response(s) to CNS injury. GAP-43 is a neuron specific phosphoprotein that is involved in neurite outgrowth and plasticity [61]. The induction of GAP-43 coincides with early neuronal development and is often considered to be mostly silent in the adult CNS. Nevertheless, it is strongly upregulated in the adult injured neuron and deregulation of the protein has also been observed in several neurodegenerative diseases [62–67]. Taken together, the upregulation of GAP-43 may represent a biomarker of regeneration within the adult CNS whose expression is induced in response to neuronal injury. Thus, the GAP-43:*luciferase*-GFP mouse may represent not only a suitable *in vivo* model to assess the innate regenerative process of the mature CNS but also a qualitative marker of the efficacy of therapeutic agents to promote this repair.

The therapeutic use of stem cells for regenerative and/or neuroprotective purposes has benefitted enormously by the application of *in vivo* optical imaging, specifically in tracking their survival. In one instance, neural stem cells (NSC) genetically engineered to overexpress glial-cell derived neurotrophic factor (GDNF) and also to express the *luciferase* gene have been tracked, quantified, and characterized *in vivo* upon grafting to the mouse brain in a Huntington's disease model (HD) [68]. Using *in vivo* optical imaging of *luciferase* gene expression, grafted GDNF-*luciferse* NSCs were shown to not only survive after the transplantation process but also migrate via the rostral migratory stream, the natural pathway for NSCs of the subventricular zone. The overexpression of GDNF by the NSCs, in turn, was shown to protect striatal projection neurons from an excitotoxic model of HD and to minimize the behavioral abnormalities associated with the disease [68].

12. Conclusion

The continuous refinement of currently available reporter gene mouse models for various neurodegenerative diseases, together with technical improvements in small animal *in vivo* optical imaging technology, has led to rapid progress in monitoring neurodegenerative disease pathobiology noninvasively in living animals. Insights from the pathophysiological processes related to disease initiation and progression can result in the identification of new molecular targets or treatment strategies. Novel functional imaging probes or contrasting agents directed towards disease-specific alterations have also been developed for improved diagnosis. Some of the most significant developments for *in vivo* optical imaging include the use and refinement of the bacterial *lux* gene cassette to eliminate luciferin use and the shift of fluorescent reporter emission wavelengths to the near-infrared spectrum to avoid tissue autofluorescence issues. Activatable probes that remain optically inactive until they are enzymatically cleaved by specific enzymes that are activated during disease are also revolutionizing our understanding of disease processes within living animals [1].

Although *in vivo* optical imaging is able to provide information on specific reporter location, it is not without a number of practical hurdles. One of the most challenging hurdles is the limited spatial resolution in tissue due to absorption and the difficulty in ascribing depth to the reporter signal as images are commonly acquired as planar and two-dimensional (2D). To circumvent this, other medical imaging modalities, such as microCT, can be used in tandem with optical imaging to generate three-dimensional (3D) images. Instrumentation and computer software are currently available to reconstruct images from a second modality (CT, MRI, or PET) with the 2D optical scans, using intricate algorithms based on the shape of the animal, how light passes through various thicknesses of tissue, and the scattering pattern, to precisely pinpoint where the signal of interest is originating from. Another technical advancement that has intriguing applications for *in vivo* optical imaging is improvements to the speed at which CCD cameras can process images that enables freely moving animals to be imaged. The In Actio Module, provided by Biospace Lab, records video through two CCD cameras at 45 frames per second; one is dedicated to imaging the subject, and the other records the light emitting reporter location and intensity. While still requiring much optimization, this technology opens the door to more exciting developments in free moving, *in vivo* animal imaging.

The ability of *in vivo* optical imaging technology to assess neurological disease states is gaining tremendous traction. The burgeoning choice of probes and animal models now require careful validation to confirm the specificity of imaging readouts. Undoubtedly, however, the establishment of effective biomarkers and end-points, capable of defining critical parameters such as genetic, metabolic, or behavioral signatures of specific neurodegenerative disorders, will provide faster, more effective, and less expensive ways to diagnose disorders (Figure 5). Moreover, this can also lead to better evaluation of drug efficacy and to the identification of potential subgroups of patients who are more likely to elicit enhanced responses from therapeutic intervention. Furthermore, the currently applied imaging protocols for disease diagnosis and therapy guidance need to be relentlessly replicated and subsequently standardized in order to compare

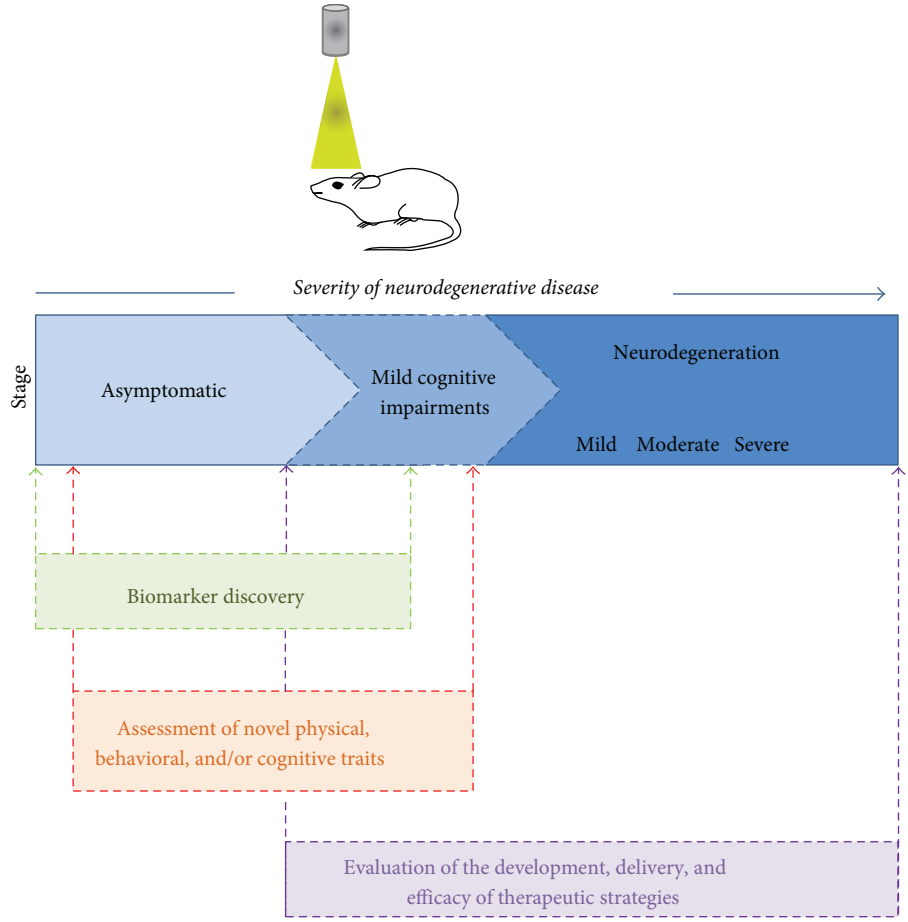


Figure 5: The selective advantage of utilizing *in vivo* optical imaging and small transgenic reporter animals in the study of neurodegeneration for the discovery of biomarkers and novel traits (physical, behavioral, and cognitive) and for visualizing the delivery and efficacy of therapeutic agents and strategies.

and delineate experimental results between various research groups in order to draw definite conclusions.

In vivo optical imaging holds great promise not only in animal models but also for clinical imaging of the human brain. Advantages include the avoidance of radiation and radio-labeled tracers/agents in detection, the relative ease by which it can be performed without the need for complex surgical techniques, minor discomfort to the patient, and the relatively low cost for clinical/bedside implementation. To achieve success, major efforts in probe development and instrumentation is still required to overcome several technical challenges such as the potential toxicity of imaging probes or contrast agents given the larger quantities that must be administered to human patients, the ability to discriminate true cerebral signal from extracerebral contamination, and the degree of tissue penetrance. In the case of the latter, the dimensions of the imaging object (i.e., the human brain) also require powerful and large excitation source and an extremely sensitive detection camera. Additionally, the camera integration time must be optimized to sufficiently sample changes in fluorescence over time and thus measure fluorescence dynamics, which is imperative for longitudinal studies. A considerable challenge in the efforts to translate *in vivo* optical imaging findings from laboratory animals (i.e., rodents) to humans will be the need to perform similar set(s) of studies in nonhuman primates (NHP). NHP models offer sizeable advantages over those that use rodents and other small species because of their neurobiological similarity to humans and their longer life span, which makes it possible to study individual subjects over several years, an imperative requirement for neurological diseases. However, at the present time, this field is virtually unexplored. Ultimately, application of *in vivo* optical imaging of neurodegenerative diseases has tremendous potential to provide improved patient care and lead to the development of personalized precision medicine with greater efficacies and potentially fewer side effects.

Conflict of Interests

The authors declare that they have no conflict of interests regarding the publication of this paper.

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Review Article

Apocynin, a Low Molecular Oral Treatment for Neurodegenerative Disease

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Accumulating evidence suggests that inflammatory mediators secreted by activated resident or infiltrated innate immune cells have a significant impact on the pathogenesis of neurodegenerative diseases. This may imply that patients affected by a neurodegenerative disease may benefit from treatment with selective inhibitors of innate immune activity. Here we review the therapeutic potential of apocynin, an essentially nontoxic phenolic compound isolated from the medicinal plant *Jatropha multifida*. Apocynin is a selective inhibitor of the phagocyte NADPH oxidase Nox2 that can be applied orally and is remarkably effective at low dose.

1. Introduction

Ageing societies are facing an increasing prevalence of neurodegenerative diseases. Some relatively prevalent examples are Alzheimer's and Parkinson's disease and less prevalent are Huntington's and Lou Gehrig's disease (amyotrophic lateral sclerosis; ALS). All neurodegenerative diseases have in common that no effective treatment exists that can stop the progressive deterioration of neurological functions. Of note, none of the neuroprotective agents that have been tested in the clinic have an efficacy that goes beyond symptoms control.

In recent years the insight has been growing that inflammatory reactions from resident or infiltrated innate immune cells may have a significant impact on the pathogenesis of neurodegenerative disorders [1]. A recognized central player is the microglia, a glial cell that belongs to the myeloid lineage and is often indicated as the macrophage of the CNS. This intriguing new insight may imply that drugs with proven efficacy in the protection of peripheral organs against the cytotoxic function of innate immune cells, such as mononuclear or polymorphonuclear phagocytes (resp., MNCs and PMNs), might also be useful for the treatment of the neuroinflammatory component of neurodegenerative diseases. A major hurdle that drugs need to take is to cross the blood brain barrier and penetrate the CNS parenchyma where the neurodegenerative process takes place. In this review we will discuss preclinical studies highlighting the potential of apocynin, a small phenolic antioxidant, as treatment of neurodegenerative diseases.

2. Apocynin, a Pharmacologically Active Plant Phenol

Apocyin (4′-hydroxy-3′-methoxyacetophenone or acetovanillone) was identified as the biologically active substance in the roots of *Picrorhiza kurroa* Royle ex Benth, a perennial plant growing in the alpine Himalaya. Extracts from the roots are used in the Ayurvedic medical tradition of India and Sri Lanka for the preparation of ethnic medicines for the treatment of ailments of liver, heart, joints, and lungs. We have prepared a 95% ethanolic root extract under controlled conditions in the laboratory and subjected the preparation to an activity-guided purification using the oxidative burst of human polymorphonuclear/neutrophilic granulocytes (PMN) as an experimental test for acute inflammation [2].

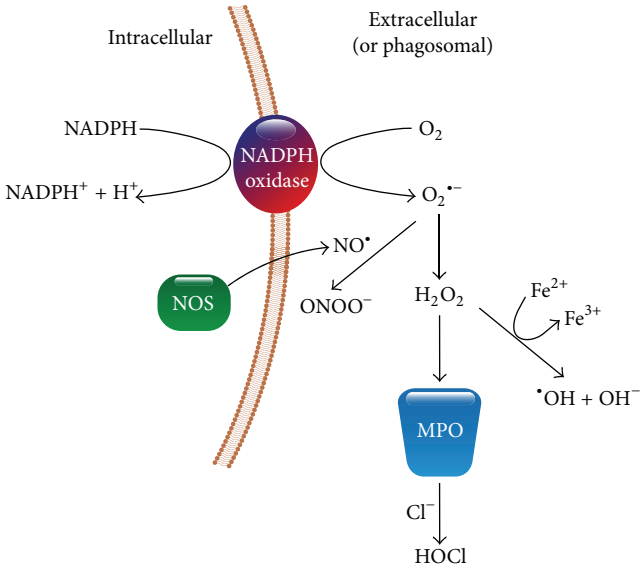

Figure 1: Reactive oxygen species produced in the phagocyte oxidative burst. MPO: myeloperoxidase; NOS: nitric oxide (NO) synthase.

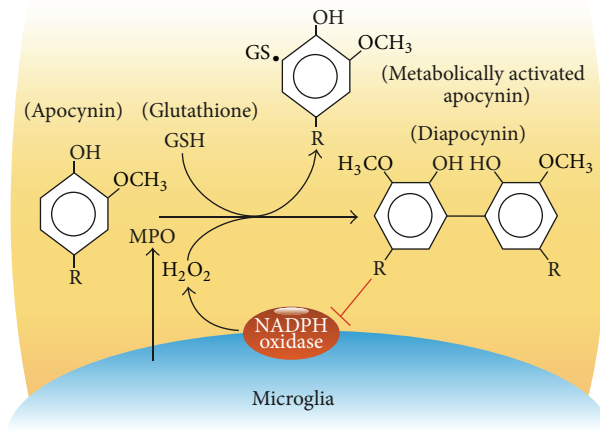

Figure 2: *Inhibition of microglia Nox2 by metabolically activated apocynin*. Receptor-mediated activation of microglia cells induces production of reactive oxygen species and release of myeloperoxidase (MPO). The MPO-catalyzed reaction of apocynin with H_2O_2 leads to production of a reactive intermediate that stabilizes by binding to free thiol groups, for example, GSH, or by dimerization. Dimeric apocynin (diapocynin) inhibits Nox2 activity.

The read-out assay we used was based on the generation of luminol-enhanced chemiluminescence by human PMN stimulated with zymosan opsonized in human serum. The essence of the assay is that the serum-opsonized yeast particles stimulate the PMN via surface-exposed receptors of immunoglobulins or complement factors. The activation signals relayed via these receptors lead to the emptying of cytoplasmic granules (degranulation) and the assembly of the phagocyte NADPH oxidase Nox2. The Nox2 enzyme complex is assembled from membrane-bound ($gp91^{phox}$, $p22^{phox}$) and cytoplasmic ($p40^{phox}$, $p47^{phox}$, $p67^{phox}$, and Rac2) subunits [3]. The assembly process involves phosphorylation of subunits by specific kinases and formation of thiol-bridges. The assembled complex takes up electrons from NADPH and transfers these onto free molecular oxygen leading to formation of superoxide anion (O_2^-; one electron reduction) and hydrogen peroxide (H_2O_2; two electron reduction). Both oxidants have cytotoxic activity as could be shown using red blood cells from different species [4]. The oxidative burst of PMN comprises a cascade of strongly reactive oxygen species, collectively indicated as ROS (Figure 1). By reaction of O_2^- with nitric oxide the strongly cytotoxic peroxynitrite is formed. In the presence of Fe^{2+} ions H_2O_2 is converted into highly reactive hydroxyl radicals ($OH^{\bullet}$), which via peroxidation of membrane lipids affect the fluidity of cell membranes. Myeloperoxidase released by degranulation of the PMN catalyzes the reaction of H_2O_2 with halide molecules (Cl_2, Br_2, and J_2) forming highly toxic hypohalides (OCl^-, OBr^-, and OJ^-). ROS are essential components of the intracellular killing of phagocytosed microbes, but when released into the extracellular milieu they are important mediators of the tissue destructive activity of activated PMNs [5, 6].

It can be envisaged that components of chemically complex plant extracts can interfere with the read-out assay at multiple levels and may also exert nonspecific effects such as killing of the PMN or scavenging of the oxyradicals. This implies that successful activity-guided purification needs to be well focused and carefully controlled for nonspecific effects to avoid false positive results. Notwithstanding these hurdles, we were able to demonstrate a highly specific activity of apocynin in the assay. Apocynin was found to be metabolically activated in an MPO-catalyzed reaction with H_2O_2 [7] forming a symmetrical dimer, diapocynin [8] (Figure 2). The observation that the reaction intermediate could be trapped with GSH led us to hypothesize that metabolically activated apocynin might block the formation of thiol bridges between the membrane-bound and cytosolic components that assemble functional Nox2. It was later found, however, that diapocynin directly inhibits Nox2 superoxide production and that this activity is independent of MPO [8]. An important finding with apocynin has been that it inhibits the oxidative burst of PMNs, without impeding the intracellular killing of bacteria. This implies that treatment with apocynin may prevent collateral damage to tissues infiltrated by activated PMN without impeding their bactericidal function.

3. Efficacy of Apocynin in AIMID Animal Models

The initial target disease in which we tested the clinical effect of apocynin was the WAG/Rij ($RT\text{-}1^u$) rat model of collagen-induced arthritis (CIA), which is an accepted preclinical model of the autoimmune inflammatory disease (AIMID) rheumatoid arthritis (RA). In this model, PMNs have a clear pathogenic role [9], reflecting the situation in RA patients [6]. In the rat study we chose to administer apocynin at

a dose range of 0,3 to 200 μg/mL drinking water, which was provided ad libitum. It was observed that already at the lowest dose of 0,3 μg/mL, corresponding to a daily oral dose of 6 μg, the arthritis was almost completely suppressed [10]. No effect of apocynin on serum levels of anti-collagen autoantibody or of IL-6, an important pathogenic cytokine in CIA and RA, was observed, suggesting high selectivity for the inflammatory component of the disease. Independent from us, Hougee et al. demonstrated in a mouse CIA model that orally administered apocynin restores the blocked production of cartilage proteoglycan in the arthritic joint [11]. An intriguing side effect of the treatment, illustrating the powerful anti-inflammatory effect of apocynin, was the dramatic suppression of the necrotizing skin lesions at the sites where the immunizing antigen/CFA formulation was injected [12].

Since its initial identification as potent anti-inflammatory agent in 1990, apocynin has become an established inhibitor of the oxidative burst in neutrophils as demonstrated in a wide range of *in vivo* models for immune-mediated inflammatory disorders affecting peripheral and central organs. Of particular importance for this review are the promising clinical effects observed in models of neurodegenerative disease, including ALS, Alzheimer, and Parkinson's disease. In these models the antioxidant activity of apocynin is not targeted to the neutrophil but to the "macrophage of the brain," that is, microglia.

4. Microglia

The brain contains various cell types with the capacity to exert immune functions including astrocytes, microglia cells, and macrophages located in the meninges and perivascular spaces of brain arteries and capillaries [13]. For their immune tasks these cells are equipped with conserved receptors for pathogen-associated or damage-associated molecular patterns, which relay activation signals to the cells for inducing inflammatory effector mechanisms [14].

Microglia are innate immune cells ubiquitously distributed within the central nervous system where they are engaged in tight interactions with neurons, oligodendrocytes, and astrocytes. However, only microglia release MPO after stimulation, being a requisite for the metabolic activation of apocynin.

Microglia in the healthy CNS have a ramified resting phenotype. Opposite to earlier concepts, microglia in the healthy brain are not resting but are highly dynamic cells that carry out homeostatic surveillance of the extracellular environment by the extension and retraction of their protrusions and phagocytosis of tissue debris, which could otherwise cause inflammation [15]. Activated microglia are found in diseased CNS tissue, such as within demyelinated cortical grey matter lesions in the MS brain, surrounding amyloid plaques in Alzheimer brain and in the degenerating substantia nigra in Parkinson's disease [16, 17]. Although the diverse expression profiles of microglia appear to reflect a broad, continuous spectrum of activation states, two activation states at both ends of the spectrum can be recognized corresponding to the M1 and M2 state designated for macrophages [18]. "Classically activated" M1 microglia, for example, induced by LPS or IFNγ, have proinflammatory functions which are exerted by the secretion of proinflammatory cytokines such as TNF-α, IL-1β, and IL-12 and toxic substances such as reactive oxygen and nitrogen species. "Alternatively activated" M2 microglia, such as induced in a milieu containing high IL-4 or IL-13 levels, have anti-inflammatory and tissue regenerative activities, which are mediated by cytokines such as IL-4, IL-10, or TGF-β and repair factors such as insulin-like growth factor, arginase-1, or chitinase-like-1. Not only the cytokine milieu, but also the redox state of the microenvironment, which is directly related to NADPH oxidase activity, determines the functional differentiation of microglia towards an M1 or M2 phenotype [18].

5. Apocynin as a Potential Treatment of Neurodegenerative Disease

M1 microglia cells are the main resource of Nox2 in the brain. The expression by M1 microglia of activated Nox2 producing ROS is an essential component of microglia-mediated neurotoxicity. The broadly accepted notion that microglia-derived ROS are important mediators of neurodegenerative brain injury raises the question whether the favorable pharmacological profile and low toxicity of apocynin can be used for neuroprotective treatment. Microglia cells not only express Nox2 but also secrete MPO after activation and could thus potentially exert metabolic activation of apocynin. Several authors have reported on the beneficial effect of apocynin on (models of) acute neurological disorders, such as ischemia, intracerebral hemorrhage, and stroke (reviewed in [19]). What are the perspectives for apocynin in chronic neurodegenerative disorders?

In vitro studies using cultured microglia have implicated Nox2-derived ROS in the proliferation and functional polarization of microglia [20, 21]. A crucial finding has been that inhibition of Nox2 promotes alternative and anti-inflammatory microglia activation during neuroinflammation [22]. This implies that suppression of Nox2 with apocynin might restore a healthy balance between a proinflammatory M1 and an anti-inflammatory/proregenerative M2 phenotype of microglia. Others have shown that apocynin lowers the production of IL-1β, TNF-α, and nitric oxide by microglia, thus interrupting a self-perpetuating cycle of detrimental activity. While the exact neurotoxic mechanism of activated microglia in neurodegenerative disease is still uncertain, it is also of considerable interest that the release of the excitotoxin glutamate requires Nox2 activity and that this can be inhibited by apocynin [23]. Taken together, these data suggest a potentially beneficial role of apocynin in neurodegenerative disease. Indeed, promising effects of apocynin have been observed in mouse models of some major neurodegenerative diseases.

Amyotrophic Lateral Sclerosis (*ALS, Lou Gehrig Disease*). Amyotrophic lateral sclerosis is a common adult-onset neurodegenerative disease affecting motor neurons. The disease

leads to rapidly progressing motoric impairment and death usually within 5 years. While in the majority of patients the cause of the disease is unknown, in a subset of cases the disease has a genetic cause, namely, mutations in the superoxide dismutase-1 (SOD1) gene [24, 25]. The mutation enhances oxidative stress by dysregulated production of superoxide anion due to reduced dismutation to H_2O_2. Mutant SOD1 expressing astrocytes are linked to ALS pathology because of their reduced capacity to absorb glutamate [26] and/or by their release of neurotoxic factors [27]. Selective silencing of mutant SOD1 [28] or replacement of mutant by wild-type microglia [29] in the mutant SOD1 mouse model strongly point to a central pathogenic role of microglia. Harraz et al. have used oral apocynin to control progression of neurodegeneration in a SOD1 mutant mouse model and observed promising effects [30]; it was observed that administration of apocynin in the drinking water significantly prolonged survival and delayed the onset of motoric defects. This study shows that orally administered apocynin can build up a sufficiently high concentration within the CNS parenchyma for mitigating neurotoxic levels of ROS production. However, these promising data could not be reproduced in another study using the same mutant mouse strain [31].

Alzheimer Disease (*AD*). Alzheimer disease is an ageing-associated progressive neurological disorder leading to irreversible dementia. Neuropathological hallmarks of AD are senile plaques of misfolded and fibrillar amyloid-β aggregates and intraneuronal tangles of tau protein within the cerebral cortex [32]. Activated microglia cells were found clustered around senile plaques producing neurotoxic agents like ROS, NO, and TNF-α. Activation of microglia Nox2 by oligomeric and/or fibrillar amyloid-β [33, 34] and expression of activated Nox2 in Alzheimer brain [35] have been reported.

Lull et al. have tested apocynin at a daily oral dose of 10 mg/kg via the drinking water in a $hAPP(751)_{SL}$ transgenic mouse model of AD [36]. They observed in apocynin-treated mice a significant reduction of plaque size within cortex and hippocampus and a reduction of microglia numbers in the cortex, but not in the hippocampus. However, a behavioral feature of AD observed in this mouse model, that is, performance in the Morris water maze swim test, which tests spatial memory organized in the hippocampus, was not markedly improved by the treatment. The limited clinical effect of apocynin in the model might be due to the absence of clear neuroinflammation, while this is more prominent in AD patients, and because of the fact that plaque formation does not necessarily predict cognitive decline.

Parkinson's Disease (*PD*). The pathological hallmark of PD is a progressive degeneration of dopamine producing neurons in the substantia nigra (SN), a pigmented structure located in the bottom of the midbrain. Via the release of dopamine, the SN has a central role in the coordination of various neurological functions, including reward, addiction, and movement. The latter function is particularly disturbed in PD. To compensate for dopamine loss, a metabolically stable precursor of dopamine (L-DOPA) is given, which in a substantial number of patients causes typical involuntary movements known as hyperkinetic syndrome. While in the vast majority of (sporadic) PD patients the cause of the disease is not known, in a small fraction a genetic cause has been found, namely, mutations in several genes, including alpha-synuclein, parkin, leucine-rich repeat kinase 2, PTEN-induced putative kinase 1, and ATP13A2 [25]. The observation that users of heroin contaminated with MPTP developed PD symptoms [37] enabled generation of a clinically relevant animal PD model. After conversion of MPTP into MPP+ by monoamine oxidase B in astrocytes, MPP+ is concentrated in dopaminergic cells via uptake through the specific dopamine transporter, where it blocks complex I of the mitochondrial respiratory chain. The ensuing redox stress causes amongst others dysregulation of cellular Ca^{2+} leading to cell death. Just like in ALS and AD, neurodegeneration in PD is found to be associated with microglia Nox2 activation, which is thought to contribute significantly to the pathogenic process [38].

Using an *in vitro* system, Gao et al. demonstrated that ROS generated by microglia Nox2 enhances the sensitivity of dopaminergic neurons to MPP+ [39]. A beneficial effect of apocynin on neurotoxic effects mediated by microglia has been shown in a mouse model of PD [40].

6. The Effect of Apocynin in a Nonhuman Primate Parkinson's Disease Model

Repetitive injection of a low dose of MPTP in common marmosets, a small-bodied neotropical primate, elicits a neurological disease that at the level of clinical and neuropathological presentation closely approximates PD [41]. We have used this MPTP model in 5 marmoset twins to test whether oral apocynin is also effective in a higher species [35]. For oral administration, apocynin was dissolved in Arabic gum; one sibling of each twin was given apocynin containing gum and the other was given only the gum. Treatment with apocynin (100 mg/kg, TID) started one week before PD induction with MPTP (1 mg/kg, via subcutaneous injection for 8 days). Apocynin limited the typical body weight loss associated with the parkinsonian syndrome. Also the motor function in the apocynin treated monkeys was improved, indicating an anti-Parkinson efficacy of apocynin. Moreover, the number of surviving dopamine neurons was increased by apocynin, indicating a neuroprotective efficacy. Remarkably, apocynin has a similar molecular structure as homovanillic acid (HVA), a metabolite of dopamine. An explanation of the protective efficacy of apocynin in PD might also be related to the compensation of the reduced level of the natural available o-methoxycatechol HVA.

7. Perspective for Treatment of Human Patients

Apocynin is a potentially attractive oral prodrug because of its low general toxicity and the fact that its specific antioxidant action is elicited after metabolic activation by MPO releasing phagocytic cells. Safety data of apocynin are scarce, but those available show low toxicity and high stability (partly reviewed in [19]). The LD50 after oral dosing in mice has been

estimated at 9 g/kg. In rats about 80% of intraperitoneally injected apocynin at 120 mg/kg was recovered in unchanged form in a urine sample collected 20 hours later. An intravenous dose of 420 mg/kg apocynin in mice caused minimal signs of toxicity [12].

To our knowledge, apocynin has not been tested in human neurodegenerative disease patients. However, Peters et al. have evaluated the therapeutic potential of inhaled apocynin on ozone-induced bronchial hyperresponsiveness to methacholine in asthmatic patients as a model of inflammatory lung disease [42]. The authors could exclude scavenging of ozone by apocynin and concluded that the effect was mitigating ROS production by PMNs and eosinophils that had infiltrated the lung upon ozone exposure.

The mouse studies discussed in this review show that low doses of apocynin administered via the oral route reach the CNS parenchyma in a sufficient concentration to inhibit the microglia oxidative burst and inhibit neurodegeneration. Taking the very low systemic toxicity and the highly specific mode of action of apocynin into account it would be an attractive perspective to test the therapeutic value in human neurodegenerative disease.

Conflict of Interests

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

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Review Article

Potential Therapeutic Strategies for Alzheimer's Disease Targeting or Beyond β-Amyloid: Insights from Clinical Trials

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Alzheimer's disease (AD) is a progressive neurodegenerative disorder with two hallmarks: β-amyloid plagues and neurofibrillary tangles. It is one of the most alarming illnesses to elderly people. No effective drugs and therapies have been developed, while mechanism-based explorations of therapeutic approaches have been intensively investigated. Outcomes of clinical trials suggested several pitfalls in the choice of biomarkers, development of drug candidates, and interaction of drug-targeted molecules; however, they also aroused concerns on the potential deficiency in our understanding of pathogenesis of AD, and ultimately stimulated the advent of novel drug targets tests. The anticipated increase of AD patients in next few decades makes development of better therapy an urgent issue. Here we attempt to summarize and compare putative therapeutic strategies that have completed clinical trials or are currently being tested from various perspectives to provide insights for treatments of Alzheimer's disease.

1. Introduction

Alzheimer's disease (AD) is named after the German physiologist who first presented neuropathological characteristics of the dementia at the 37th meeting of Society of Southwest German Psychiatrists in 1906 [1]. Alzheimer's studied a patient with progressive memory loss for five years and analyzed the brain post mortem using silver staining [2]. This contributed to the identification of neuritic plagues and neurofibrillary tangles (NFT) [1], two characteristics employed to identify the dementia to date. It took another 20 years to determine β-amyloid (Aβ) and tau which are major components of neuritic plagues and NFTs, respectively [3], marking the modern era of study of AD research.

AD, as a progressive neurodegenerative disorder, deprives patients of their memory and even lives. Memory loss is the most notable symptom [4, 5] at the early stage but as the disorder advances, difficulties with language, perception, and execution of movement become prominent [6], followed by neuropsychiatric and behavioral abnormality, muscle mass loss, and mobility deterioration [6]. Loss of normal daily living in those with dementia is inevitable.

In addition to the affliction and sufferings to patients, Alzheimer's disease can cost society substantially, especially in developed countries. The expenditure of AD was around \$100 billion per year [7]; the bill was about €177 billion in Europe solely in 2008 [8]. Due to deteriorating abilities to live on their own, caregivers are necessary for progressed AD patients. Burdens on these caregivers' life including physical, psychological, and economic aspects [9–11] can be a major concern.

The global prevalence of dementia for people over the age of 60 is estimated as high as 40 million in 2001, and the figure is forecasted to double every 20 years [12, 13], indicating that Alzheimer's disease has become a modern epidemic. In the near future, surging number of AD patients will become an overbearing social issue. Therefore, the need for therapeutic strategies for this devastating disease is urgent.

Currently, Food and Drug Administration (FDA) approved AD drugs are still limited within two categories: cholinesterase inhibitors and memantine [14–16] (a NMDA receptor antagonist). Unfortunately, the effects and benefits of these drugs are marginal and work only to alleviate the symptoms [17–19]. However, in recent years, fundamental

researches focusing on the pathogenesis of AD paved the way for development of new treatments targeting the radical source of Alzheimer's disease [20]. Numerous trials have been or are currently being conducted to determine effects of various compounds on AD in different stages.

Alzheimer's disease causes major impairment of individual health and social economy due to the limited effective therapeutic approaches. With the explosive explorations based on two hallmarks of AD, numerous clinical trials targeting on or off Aβ have been or are being conducted. In this paper, we will briefly summarize successes and failures in clinical trials in Alzheimer's disease and try to give a systematic review in an attempt to derive insights from previous experience.

2. Therapeutic Targets Focusing on Aβ Cascade Hypothesis (Table 1)

2.1. Inhibition of Aβ Production. Studies of familial Alzheimer's disease (FAD) motivate the discovery of responsible genetic factors, establishing Aβ-centered theory for AD. Amyloid precursor protein (APP) experiences sequential cleavages by β-secretase and γ-secretase and gives rise to the dementia culprit β amyloid (Aβ) that is thought to initiate soluble oligomers, insoluble fibrils, and accumulated plagues (Figure 1). APP can be alternatively processed by α-secretase within the Aβ region and generate a longer C-terminal fragment under the first cleavage. In terms of curbing production of Aβ, the three crucial enzymes processing APP have been therapeutic targets in drug development. The rationale is to inhibit β-/γ-secretase while promoting the α-secretase activity to become the priority strategy.

2.1.1. β-Secretase (BACE1) Inhibitor. Beta-site APP-cleaving enzyme 1 (BACE1) is the protease responsible for the initial cleavage of APP, giving rise to the production of neurotoxic suspect Aβ [21, 22]. Mounting evidence corroborate the availability of BACE1 inhibition. BACE1 knock-out mice indicated a close correlation between the BACE1 inhibition and the Aβ decline [23, 24]. It is reported that BACE1 inhibition improved memory deficits [25] and rescued Aβ-driven cholinergic dysfunction [26] in APP transgenic mice. Although the BACE1-deficient animal model presented a relatively benign phenotype with high viability, suggesting that the possibility of targeting β-secretase would be a safe therapeutic approach, further testing indicated that the drastic inhibition would result in hypomyelination and behavioral abnormalities such as seizures [27–30]. This is because, except from APP, BACE1 has a series of substrates, like neuregulin-1, related to myelination [29, 31]. AD pathology onset was postponed in the APP × BACE1+/− mice; however, it hinted at a partial inhibition that might mitigate the potential safety problems [32, 33]. It has been noted that the discrepancy between potency-required molecular weight and CNS penetration-required size [34, 35] poses another challenge.

Many BACE1 inhibitors are derived from approved drugs for type 2 diabetes with properties regulating insulin metabolism. Nuclear peroxisome proliferator activated receptor gamma (PPARγ) functions as a transcription factor regulating gene expression [36], modulating inflammation response, promoting microglia-mediated Aβ endocytosis, and declining cytokine secretion [37]. Thiazolidinediones can activate PPARγ to inhibit β-secretase and promote ubiquitination to degrade amyloid load [38]. PPARγ agonists like thiazolidinediones derivatives rosiglitazone and pioglitazone soften the peripheral insulin resistance [39], which aggravates AD neuropathology, and this decline of insulin sensitivity helps in Aβ proteolysis. The study of rosiglitazone has been developed to a large phase 3 trial; however, it has been discontinued due to cardiac risk concerns [40]. Pioglitazone has recently progressed into a phase 3 clinical trial after precluding a previously reported bladder risk. But due to the involvement of substrate complexity and some adverse effects, other phase 3 clinical trials for BACE1 inhibitors are still lacking.

However, several novel drugs are currently under investigation. Based on conjugation to a penetrant carrier peptide [41, 42], the potent CNS impermeable compound, CTS-21166, has completed the phase 1 trial. It showed a good tolerance and a reduction of plasma Aβ level in healthy volunteers [43]. A phase 1b dose-escalating study for MK-8931 demonstrated a positive effect in reducing the level of toxic proteins in addition to safety and good tolerance. A phase 2 trial recruiting 200 mild-to-moderate patients was expanded to a larger 1960-participant phase 3 trial, including conventional cognitive and functional primary outcomes, and it recently passed an interim safety evaluation.

Another BACE1 inhibitor, LY2886721, though it appeared to be safe and lowered Aβ42 in cerebrospinal fluid by more than two-thirds in phase 1 trial [44], was terminated due to the fact that 4 out of 45 patients showed liver abnormalities during the phase 2 trial. Besides, RG-7129 was also terminated in its phase 3 trials in 2013. These terminations again signaled that significant challenges are remaining: whether BACE1 inhibitors will be safe in the long run and if lowering BACE1 activity will slow cognitive decline.

2.1.2. γ-Secretase Inhibitors (GSI) and Modulators (GSM). γ-secretase is a transmembrane protease responsible for the eventual cleavage of amyloid precursor protein (APP) to generate Aβ (Figure 1), thus it is considered a principal therapeutic target in Alzheimer's disease [45, 46]. This enzyme complex consists of four components: Aph1, Pen2, glycosylated nicastrin, and endoproteolyzed presenilin as the catalytic core [47], and it is involved in myriads of physiological process. The versatility places hurdles in the way of γ-secretase targeted drug development. In the human body, aside from APP, there are more than 50 different substrates that γ-secretase is capable of reacting with, many of which are neuronal substrates [48]. Importantly, γ-secretase is also responsible for cleavage of Notch 1, which leads to the release of the Notch intracellular domain (NICD), subsequently translocated to the nucleus to regulate genes involved in cell development, cell survival, and cell fate determination [49]. Thus, inhibition of γ-secretase needs to be cautiously designed to particularly circumvent the drawbacks caused

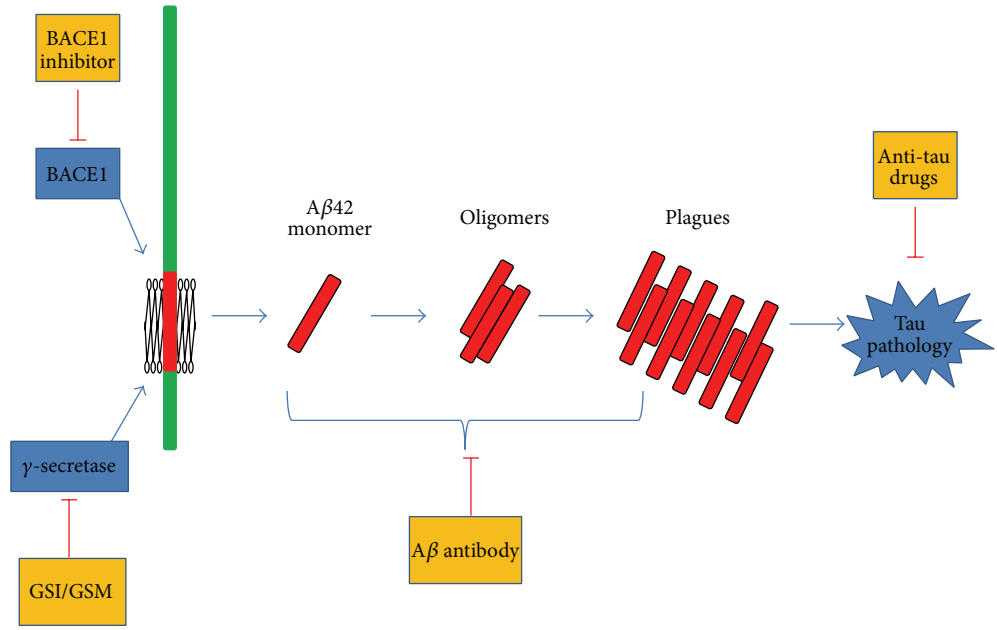

Figure 1: β-amyloid hypothesis based therapeutic targets. APP, after sequentially being cleaved by BACE1 and γ-secretase, gives rise to a neuron toxic molecule Aβ42. This peptide can exist as monomers or aggregates into oligomers and plagues. The assembly of Aβ42 triggers downstream effects and induces tau phosphorylation. BACE1 inhibitors and GSI/GSM aim to prohibit the production of pathological Aβ, and vaccines or Aβ antibodies promote clearance mechanism. As for tau, GSK-3β inhibitors and other antiaggregates are potential therapeutics targeting on blocking tau hyperphosphorylation or aggregation.

by Notch signaling abnormality. Haematological [50] and gastrointestinal [51] toxicity, skin reactions [52, 53], and changes to hair color [54] are the most commonly reported adverse effects of γ-secretase inhibitor.

Several γ-secretase inhibitors (GSIs) have been launched in clinical trials. Many reduced the Aβ production in plasma or CSF (cerebrospinal fluid), but few successfully avoided the Notch-induced side-effects. Semagacestat decreases Aβ level in plasma and downregulates its generation in the central nervous system (CNS) [55]. Semagacestat is the first γ-secretase inhibitor that have been taken into Phase 3 clinical trials. While phase 1 trial suggested a dose-dependent decline of Aβ synthesis in CSF [55], phase 2 trial began exhibiting skin-related side effects. Although Aβ level in plasma has significantly decreased, it was not duplicated in CSF and no effects on cognition and function were found. Two pivotal phase 3 trials were reluctantly started; however they were discontinued due to increased risk of skin cancer and infection and lack of efficacy [56]. Fall of semagacestat, a potentially promising drug candidate, repeated disappointing results of other GSIs, which deemed that a deeper understanding of interaction between 4 subunits and their substrates is necessary.

Different GSIs present favor to interact with subunits of γ-secretase, exhibiting target specificity. DAPT and L685458 indicated the smallest selectivity, while MRK-560 and sulfonamide based GSIs strongly prefer to inhibit PS1 instead of PS2 [57, 58]. Aph1 heterogeneity is critical for individual survival, suggesting that targeting of Aph1b γ-secretase specifically would be more tolerated [59], although the feasibility of drug design still remains difficult to determine.

Accordingly, the second generation Notch-sparing γ-secretase inhibitors aimed at selective inhibition of specific sites took the spotlight. Avagacestat (BMS-708163), begacestat, and NIC5-15 are such Notch-sparing GSIs under clinical trials. It was reported that avagacestat (BMS-708163) has 137-fold selectivity for APP over Notch in cell culture and robustly reduces CSF Aβ levels without causing Notch-related toxicity in rats and dogs, although this is still being researched [60]. Phase 2 trials have to be terminated due to the adverse effects of gastrointestinal and dermatological system in addition to the lack of cognitive improvement compared to placebo counterparts. Begacestat decreased the Aβ concentration in the plasma but not in CSF [49, 61], and a phase 1 clinical trial in combination with cholinesterase inhibitor donepezil has been completed, further data was unavailable. Another Notch sparing GSI candidate, NIC5-15, a natural monosaccharide [62], is currently under a phase 2 trial and demonstrated good tolerance and safety [63].

Given that the unresolved adverse effects brought on by GSIs are tricky to address, the concept of γ-secretase modulators (GSMs) was established with the expectation of nonsteroidal anti-inflammatory drugs (NSAIDs). A subset of NSAIDs, like ibuprofen, indomethacin, and sulindac sulfide, disconnected from their cyclooxygenase (COX) properties were discovered to be able to selectively reduce the production of Aβ42 at the cost of elevated shorter peptide Aβ38 [64, 65]. Surprisingly, this downregulation of toxic Aβ level

Table 1: RCTs based on β-amyloid in recent years.

Mechanism	RCT	Status	Estimated end	Dementia stage	Enrollment	Duration	Reported outcomes	Details of drugs/RCTs
↓Aβ production								
BACE1 inhibitor	Pioglitazone	Phase 2; completed	2005.1	Mild-to-moderate	25	18 months	Dose-dependent changes in plasma Aβ levels	Insulin sensitizer, class of PPARγ agonists
	CTS-21166	Phase 1; completed	2008.2	Healthy	56			
	MK8931	Phase 3; ongoing	2018.3	Mild-to-moderate	1960	≈6.5 years		With enhanced BBB permeability
	E2609	Phase 1; completed	2013.9	MCI/mild AD	65			
	NIC5-15	Phase 2; ongoing	2013.12		40			Notch-sparing, insulin-sensitizer
GSI/GSM	Begacestat	Phase 1; completed	2009.10	Elder healthy	49			Selectively inhibits cleavage of APP over Notch [208]
	CHF 5074	Phase 2; completed	2012.4	MCI	96	12 weeks		NSAID
	EVP-0962	Phase 2; completed	2013.10	MCI/early stage	52	14 days		
α-secretase activator	Atorvastatin	Phase 3; completed*	2007.7	Mild-to-moderate	600	80 weeks		Tested with AchEI
	Simvastatin	Phase 3; completed	2007.10	Mild-to-moderate	400	18 months		
	Etazolate	Phase 2a; completed	2009.8	Mild-to-moderate	159		Safe and well tolerated	↑ α-secretase activity, acting as a GABA-A receptor modulator and a PDE-4 inhibitor [209]
↓Aβ aggregation/oligomers	Epigallocatechin-3-gallate (EGCg)	Phase 2/3; ongoing	2015.6	Early stage	50	18 months		Prevents the Aβ aggregation via binding to the unfolded peptide
	Scyllo-inositol (ELND005/AZD103)	Phase 2; completed	2010.5	Mild-to-moderate	350	18 months	Insufficient to support/refute benefits [210]	
	Tramiprosate (3APS)	Phase 3	unknown	Mild-to-moderate	950		Suggesting disease-modifying effects [211]	
	PBT2	Phase 2; completed	2007.12	Mild AD	80	12 weeks	Well-tolerated, ↓CSF Aβ42, and improved executive function [212]	

Table 1: Continued.

Mechanism	RCT	Status	Estimated end	Dementia stage	Enrollment	Duration	Reported outcomes	Details of drugs/RCTs
↑Aβ clearance								
Active immunotherapy	Affitope AD02	Phase 2; completed	2013.12	Early stage	335	>1 year		N-terminal Aβ1-6, a synthetic peptide
	Affitope AD03	Phase 1; completed	2011.11	Mild-to-moderate	28			i.h. with or without adjuvant aluminum
	UB 311	Phase 1; completed	2011.4	Mild-to-moderate	19			N-terminal Aβ1-14
	V 950	Phase 1; completed	2012.1		86			formulated on Aluminum-containing adjuvant
	CAD 106	Phase 2; completed	2012.12	Mild AD	177		A favourable safety profile [213]	N-terminal Aβ1-6; i.m. of adjuvanted CAD106;
	BAN2401	Phase 2; ongoing	2016.12	MCI/mild AD	800	18 months		mAb against Aβ oligomers
	BIIB037	Phase 1; ongoing	2014.11	Prodromal to mild	160			Administered via intravenous (IV) infusions in subjects
Passive immunotherapy	Ponezumab	Phase 2; completed	2011.8	Mild-to-moderate	198	24 months		
	Crenezumab	Phase 2/3; ongoing	2016.5	Mild-to-moderate	361	24 months		
	Gammagard (IVIg)	Phase 2, completed	2010.4	Mild-to-moderate	24	6 months	Improved cognition	
		Phase 3; completed	2012.12	Mild-to-moderate	390	70 weeks	Showed no significant effect	
		Phase 2; ongoing	2014.10	MCI	50	24 months		
	AMBAR	Phase 2/3; ongoing	2016.12	Mild-to-moderate	350			
	Gantenerumab	Phase 3; ongoing	2019.3	Mild	1000	>5 months	Phase 1 RCT↓brain Aβ; high doses, AE	Mainly targets Aβ plagues
	Solanezumab	Phase 3; ongoing	2016.12	Mild	2100		No benefits in primary outcomes	Mainly targets soluble oligomeric Aβ
	AAB-003	Phase 1; ongoing	2014.8	Mild-to-moderate	104	52 weeks		Previously treated with AAB-003
	GSK933776	Phase 1; completed	2011.5		50			
	SAR228810	Phase 1; ongoing	2015.1	Mild-to-moderate	48	14.5–22 months		

Table 1: Continued.

Mechanism	RCT	Status	Estimated end	Dementia stage	Enrollment	Duration	Reported outcomes	Details of drugs/RCTs
Anti-tau								
↓tau production	Valproate	Phase 3; complete	2009.12	Mild-to-moderate	313	2 years	Did not show cognitive benefits and prevention of behavioral defects; associated with reduced brain volumes	
	Lithium	Phase 2; ongoing	2019.4		80		A pilot study was insufficient to support or refute the efficacy [214]	
↓tau fibrillization/deposition	Nicotinamide	Phase 1/2; ongoing	2014.7	Mild-to-moderate	50	24 weeks		Vitamin B3
	TRx0237	Phase 3; ongoing	2015.12	Mild/mild-to-moderate	700/833	18 months/15 months		
	Methylene blue (Rember)	Phase 2; completed		Mild-to-moderate	321	6 months	Showed uncertain results	
	Davunetide (AL108)	Phase 2; completed	2008.1	MCI	144	12 weeks	Showed benefits on memory	
	BMS-241027	Phase 1; completed	2013.10	Mild	40	9 weeks		

RCT: randomized controlled trial; PPAR: peroxisome proliferators activated receptor; BBB: blood brain barrier; MCI: mild cognitive impairment; GSI: γ secretase inhibitor; GSM: γ secretase modulator; NSAID: nonsteroidal anti-inflammatory drugs; AChEI: acetylcholinesterase inhibitor; GABA: γ-aminobutyric acid; PDE: phosphodiesterase; CSF: cerebrospinal fluid; i.h.: subcutaneous injection; i.m.: intramuscular injection; mAb: monoclonal antibody; AE: adverse event.

*RCTs with a combination of another drug.

Data sources: http://www.clinicaltrials.gov/.

lacks the inhibitory effect on Notch or other substrates [64]. This finding promoted the GSMs as promising therapeutic candidates for Alzheimer's disease, because the Notch-induced drawbacks may be avoided and on the other hand, the overproduction of shorter and more soluble Aβ38 seems less likely to aggregate and is less pathogenic.

Among the compounds described above, tarenflurbil (R-flurbiprofen) relates to ibuprofen structurally and pharmacologically. Phase 1 trials with a broad dose range (400 to 1600 mg/day) revealed a low drug exposure in the brain [66], while phase 2 trials narrowed this spectrum (400–800 mg, twice daily) and showed trivial benefits on function with the lowest dosage (400 mg). Although undesirable side effects like nausea, dizziness, and diarrhea were observed, this compound is still considered tolerable [67]. After modification, phase 3 trial suggested neither functional improvement nor clinical efficacy in the mild AD patients [68] and thus the results were disappointing. The weak potency of tarenflurbil can be attributable to low CNS penetration as shown in phase 1 trial, and on the other hand, NSAID residue activity curbed Aβ clearance mechanism mediated by microglia [69].

Another GSM CHF-5074 based on R-flurbiprofen ameliorated brain Aβ load and improved the animals' performance in behavior tests. The drug's safety and tolerability have been evaluated and are undergoing a phase 2 trial. Published data indicated that this compound may have an additional function of acting independently of Aβ42 [70, 71]. Nevertheless, a balance between lipophilicity and potency of these compounds must be considered. The remarkably increased potency in 2nd and 3rd generations of GSMs relies heavily on the increase of lipophilicity, which has been proved to result in off-targets, like hepatotoxicity [72].

2.1.3. α-Secretase Activator. APP can be cleaved by an alternative α-secretase rather than β-secretase in the first step to circumvent the generation of pathological Aβ peptide. Hence, increasing the chance of α-cleavage could be an effective approach to decrease the Aβ formation and promote soluble APP production to protect neurons [73]. Agonists of muscarinic, glutamate, and serotonin receptors (and the agonists or antagonists of transmitters receptors would be discussed in following section), statins, oestrogens, testosterone, and protein kinase C activators belong to this drug classification that can motivate α-secretase activity, and they have been launched in clinical trials, but data indicating their use in AD is limited [74].

Etazolate (EHT-0202), a selective $GABA_A$ [75] receptor modulator, has completed a phase 2 trial in patients with mild to moderate AD. It presented a good oral bioavailability and an elevation of sAPPα [76]. Bryostatin-1, a macrocyclic lactone, caused a decline of brain Aβ40/42, improved behavior test in AD mouse model [77], and was under a phase 2 trial, but the specific information is inaccessible.

Statin drugs such as atorvastatin and simvastatin lower peripheral cholesterol production to prevent heart attacks and other expressions of cardiovascular disease. Atorvastatin, in combination with cholinesterase inhibitor, has completed a phase 2 clinical trial and achieved a beneficial cognition and function [78], but failed to repeat the outcome in a 641-patient phase 3 clinical trial [79, 80]. Simvastatin can penetrate BBB and long-term statin treatment can decline Aβ level. In a 35-normal participant phase 4 trial of Simvastatin, it was reported to reduce phospho-tau-181 in CSF, while not total tau or Aβ level [81]. A follow-up study evaluating one year simvastatin treatment in 120 cognitively normal and middle-aged adults, effect on CSF levels of Aβ42, t-tau, and p-tau181, is ongoing.

2.2. Anti-β-Amyloid Aggregation. The pathological Aβ peptides, prone to assembly into aggregate as neuro-/synaptic toxic products spur the idea of inhibition of Aβ aggregation or destabilization of the Aβ oligomers species. However, Aβ aggregations are characterized with a high stability resistance to disaggregation [82] and remain insoluble even with heat or SDS [83]. The fact that amyloid fibrils have an extremely low energy state [82] and the lack of thorough understanding of Aβ aggregation process have complicated the issue. Besides, another challenge would be to access the compounds with high CNS bioavailability and low immunogenicity and toxicity. It is generally believed there are three strategies that block Aβ aggregation: antiaggregate compounds, metal complexing agents and immunization. They can disturb the formation of either soluble oligomers or insoluble plagues.

2.2.1. Nonpeptidic Antiaggregates. The first class of mentioned inhibiting aggregation compounds is nonpeptidic antiaggregates, tramiprosate, derived from proprionic acid that is a primitive representative. The promising outcomes of this agent from the safety and tolerance [84] were neutralized by two following phase 3 trials: the European trial precluded methodological problems that might lead to the negative in the North American trial and demonstrated the poor CNS penetration and the weak potency [85] of this drug.

The second generation of nonpeptidic antiaggregates was expected to meet those challenges. Scyllo-inositol is thought to effectively impede Aβ aggregation, promote misfolding modulation, and accelerate aggregates disassociation [86]. Because this compound can cross blood brain barrier (BBB), with the assistance of inositol transporters, it can achieve a high concentration in CNS via peripheral administration. This drug is being tested in the phase 2 trial with mild-to-moderate Alzheimer's disease patients on the basis of good tolerance and safety profile [87]. Although high doses (1000 mg and 2000 mg) resulted in serious adverse effects, the studies continued to test the low dose (250 mg) cohorts [88]. Epigallocatechin-3-gallate (EGCg), a polyphenol from green tea, via disrupting unfolded peptide, stimulated α-secretase activity and inhibited Aβ aggregation in animal models [89]. This agent was also involved in modulation of cell transduction, regulation of cell survival and death [89], and protection of mitochondrial function. The multiple effects of this natural compound make it a promising candidate, and a phase 3 trial with early AD patients with EGCg is being conducted.

2.2.2. Metal Complexing Agents. After Aβ peptides were produced and released into extracellular fluids, metals like Zn and Cu can motivate oligomerization into fibrils. So metal

chelators or metal complexing agents that can interfere with reaction of metal ions with Aβ are likely to be a therapeutic strategy. Clioquinol (PBT2), metal-induced Aβ inhibitors, also has a potent CNS permeability. PBT2 can redistribute metal ions to neurons promoting metalloproteinase expression and thus an increment of Aβ degradation. A phase 2 trial was completed and it proved a decrease of Aβ42 concentration in CSF and an improvement of cognitive and behavioral performance [90].

2.2.3. Active Immunization. It is conventionally thought that clearance of CNS Aβ requires a BBB permeability property, confining the therapeutic targets in a very narrow realm: medicinal chemistry-driven and small molecules. Nonetheless, incredible work done by Schenk et al. revealed that immunization of PDAPP transgenic mice markedly mitigated amyloid plaque burden, improved neuritic dystrophy, and even reduced existed Aβ plagues [91]. This striking breakthrough suggested that Aβ immunotherapy would be a potential strategy to remove both soluble and aggregated β amyloid [92].

AN-1792, the first anti-Aβ vaccine (with full length Aβ 1–42) tested in active immunization clinical trial, was terminated in the phase 2 trial in patients with early AD due to the fact that some participants developed aseptic meningoencephalitis and cerebral microhemorrhage [93]. The complication is attributed to cytotoxic T cell or autoimmune response [94–96]. Therefore, employing only fragments instead of full length of Aβ or other cell epitopes to circumvent toxicity and inflammation is highlighted. Additionally, the security of adjuvant and delivery approaches must be cautiously considered.

The next generation of vaccine is devoid of any T-cell epitopes. CAD-106, consisting Aβ 1–6 peptides coupled to a Qβ virus-like particle, has recently completed the phase 2 trial in patients with mild AD and did not lead to meningoencephalitis [97]. Two other vaccines, UB 311 (Aβ 1–14) and V950 (Aβ N-terminal conjugated to ISCO-MATRIX), both containing B-cell epitopes, have also recently finished phase 1 trial. However, another vaccine AC-001(Aβ 1–7 conjugated to inactivated diphtheria toxin) discontinued its phase 2 trial in August 2013, because the studied drug elicited a strong antibody response. Another active immunization approach is developed on the foundation of Affitope using short six-amino acid peptides that imitate the native Aβ sequence. AD-01 and AD-02, targeting N-terminal fragments of Aβ, were proved to rescue AD-like symptoms in animal models [98]. Recently, AD-02 has progressed into a phase 2 clinical trial.

2.2.4. Passive Immunization. Another strategy to avoid immune response is direct administration of antibodies. This passive immunization has an approximate potency to remove amyloid plaques and rescue neuritic and glial pathology [99], reduce early tau hyperphosphorylation [100] and cytopathology [101], and reverse abnormal hippocampus synaptic plasticity [102].

Bapineuzumab (AAB-001) is a humanized monoclonal antibody, derived from 3D6, published to promote removal of Aβ plagues and rescue synapse loss in APP transgenic mice brain [99]. However, in a 234-patient phase 2A safety and tolerability trial, this agent indicated no significant alteration on primary measures of cognition and daily activity. For apolipoprotein E (ApoE) ε4 carriers, there is a temporary vasogenic oedema, an adverse effect correlated with dose administration [87]. Given that 4,000 mild AD patients across North America and Europe showed no treatment effect on either cognitive or functional outcomes, the phase 3 trial was terminated. Solanezumab (LY2062430) is a humanized monoclonal IgG1 antibody directed against the mid-domain of the Aβ peptide (Aβ 16–24) and designed to specifically bind soluble species of Aβ. Phase 2 study showed dose-dependent increases of various Aβ species in plasma and CSF, an indication that insoluble Aβ is released from plagues and leaches into fluid [103]. Two trials in phase 3 suggested a limited benefit for cognitive performance as compared to cholinesterase-inhibitor drugs. A third trial started from July 2013 to test demonstrated brain amyloid burden, and data is expected to be read out in December 2016. Gantenerumab, a human IgG1 antibody binding to Aβ fibrils, can elicit phagocytosis to remove Aβ plagues in brain and rescue Aβ oligomers that induced impaired long-term potentiation (LTP) in rats model. An expanded phase 2/3 trial of 770 participants is being conducted and is estimated to be completed in 2016. Crenezumab, a novel humanized antibody with IgG4 backbone, is believed to limit microglia mediated inflammatory cytokines release to avoid vasogenic oedema. It can recognize β amyloid oligomers, fibrils, and plagues with a high binding affinity. Phase 1 study confirmed safety and tolerance, followed by an ongoing phase 2 trial in patients with mild to moderate AD using elevated dose as well as a test for prevention of this progressive dementia. There are several antibodies which have completed or have undergone the early clinical trials, as shown in Table 1.

Many monoclonal antibodies are delivered intravenously, whereas passive immunization can be also accomplished via infusion of intravenous of immunoglobulins (IVIg) from healthy donor. A small study in 8 patients showed increase of Aβ antibodies in serum, decrease of Aβ in CSF, and stabilization of MMSE (mini-mental-state exam) scores over 18 months. A phase 2 trial with 24 patients suggested beneficial cytokine concentrations alteration in plasma. However, two critical phase 3 trials showed no difference between study drug and placebo, though a trend toward benefit for the higher dose, and thus were halted. Another published phase 2/3 clinical trial evaluating infusion of albumin in combination of IVIg is currently conducted in patients with mild to moderate AD.

Active immunization maintains the body with a constant high concentration of immunoglobulin, so this strategy calls for fewer follow-up injections with a reasonable expense. But to tackle with the T-cell induced inflammation would be a tricky issue. Passive immunization is a more effective method especially for elderly people considering their weakened responsiveness to vaccines [104]. Selection of safe epitopes can be readily met, as well as a better control of antibody titer; however, antibody delivery could be inconvenient and costly, and the risk of vasogenic oedema and cerebral amyloid angiopathy might increase.

2.3. Tau. According to Aβ hypothesis, intracellular neurofibrillar tangles (NFTs) induced by altered phosphatase/kinase activity is a downstream event of aggregation of β-amyloid (Figure 1), and NFTs as a catalyst will aggravate the oxidation and further result in neuronal dysfunction, cell death, and transmitter deficits. Tau is normally a highly soluble protein in cytoplasm binding to microtubules as a stabilizer. Formation of NFTs as a result of hyperphosphorylated and misfolded tau protein aggregation is toxic to neurons. The pathological tau proteins lose the capability to aid microtubules in transporting neuronal substance, leading to neuronal dysfunction and apoptosis [105, 106].

2.3.1. Kinase Inhibitors. Protein kinase, a group of critical enzymes responsible for tau overphosphorylation, is a prerequisite for the tau-induced toxicity. However, myriads of kinases mutually play a central role in regulating cell function and guaranteeing a normal physiological condition. The development of tau-targeted therapy is therefore challenging due to redundancy of kinase interactions and uncertainty of which enzyme specifically catalyzes the phosphorylation that we are focusing on [107, 108].

The first class of tau inhibitors aims to modulate tau phosphorylation via decreasing the activity of related kinase since imbalanced interaction between glycogen synthase kinase 3 beta (GSK3β) and protein phosphate 2 (PP2A) enhances tau hyperphosphorylation and NFT formation [109]. GSK3β appears to engage in AD pathogenesis given its impact on cellular signaling and gene description [109]. Recently, it has been reported that GSK3β is responsible for 31% of the pathological phosphorylation sites of tau protein [110] and is found colocalized with NFTs in postmortem brain [111, 112]. Toxic Aβ that promotes GSK3β activity bridges a link between the two hallmarks of Alzheimer's disease [110], implicating that GSK3β inhibitor is a potential drug target.

Lithium and valproate reduced tau phosphorylation and prevented reversed aspects of tauopathy in animal models [113] but did not show cognitive improvement in clinical trials with AD patients [114]. NP-031112 (NP-12), a non-ATP competitive inhibitor of GSK3β, counteracts tau phosphorylation, reverses amyloid burden in brain, prevents cell loss, and rescues spatial memory deficits using animal models [115]. But the phase 2b trial was terminated due to the negative results. Development of some paullone, indirubin, and maleimide family-derived GSK3β inhibitors is in the pipeline, yet stuck in the preclinical trials concerning the cytotoxic effects.

Cyclin dependent kinase 5 (cdk5) is another kinase tightly associated with tau pathology. Cdk5 regulating protein was found in AD brain and thus is probably causing a pathophysiological tau phosphorylation [116]. Cdk5-selective inhibitors were demonstrated to penetrate BBB and reduce elevated Aβ level by regulating cdk5 [117] and are at preclinical status. The test of several compounds targeting other protein kinases, like cdk1/2/9, p38, Erk1/2, JNK, casein kinase, and DYRKIA brought disappointing outcomes, and trials were discontinued due to the poor efficacy or severe adverse effects.

2.3.2. Inhibition of Tau Aggregation. Another scenario to interfere with tau-induced NFT is to inhibit tau aggregation or promote tau assembly disassociation. Rember (methylene blue) is such a tau antiaggregant [118]. Preclinical data revealed a learning deficit reversing property and a completed phase 2 trial proved that this agent can slow down AD progression with a good bioavailability [119, 120]. TRx0237, another methylene blue, has an improved drug absorption, bioavailability, and tolerability. Since 2008, intensive investigation of this agent began, and growing evidence indicated that TRx0237 benefits neuroprotection [121] and Aβ clearance in transgenic mice and improves spatial learning in rats [119, 122]. The antiaggregation properties were reported by some papers, and three phase 3 studies are ongoing.

Epothilone D (BMS-241027) is a microtubule stabilizer, via inhibition of tau release from microtubule to maintain the transportation function of axon, and on the other hand, precludes formation of tau aggregation. This agent restored behavioral and cognitive deficits, inhibited neuron loss, and curbed the tauopathy in animal models [123, 124]. Epothilone can penetrate BBB and exert a better efficacy at low concentration and now undergoes a phase 1 clinical trial. Nicotinamide, the precursor of coenzyme NAD+, reduces phosphorylated tau and protects microtubules stabilization in mouse model [125]. Nicotinamide has been launched into clinical studies suggesting that it is safe and well tolerated and a phase 2 clinical trial is ongoing in patients with mild-to-moderate Alzheimer's disease.

3. Putative Therapies Still Derived from Neurotransmitter System

Neurotransmitters depletion (basically referring to acetylcholine, ACh) and synaptic dysfunction are two classical features of AD [126]. Thus, two hypotheses have been established—cholinergic hypothesis [127] and glutamatergic hypothesis [128], based on which FDA approved therapies—AchE inhibitors and NMDA receptor antagonists—to mitigate AD symptoms were developed. Although drugs regulating transmitters' production, release, and recycling cannot prevent the progression of AD, pursuit of searching novel receptor agonists and antagonists has never stopped (Table 2).

Cholinergic neurons impairment accompanies the early progression of dementia. From animal and human studies, cholinesterase inhibitors administration stimulated memory and learning process [129]. Besides, a marked correlation between loss of cholinergic neurons and deterioration of defected memory was proved in animal models later [130, 131]. Therefore, improvement of cholinergic system, including potentiating effects of acetylcholine (Ach) and inhibiting activity of cholinesterase, is a potential therapeutic goal.

Ach is a ligand for nicotine receptors and exerts an excitatory effect on the postsynaptic neuron, an essential event for long-term potentiation (LTP) and memory formation. Several nicotinic receptor agonists to reinforce this event are being tested in clinical trials. EVP-6124, a selective agonist of the α-7 nicotinic acetylcholine receptor, has finished a phase 1/2 trial showing safe and well tolerated results and recently

(Oct 2013) entered two phase 3 trials to test the cognitive benefits. Quite a few other clinical trials testing nicotinic agonists are ongoing (ladostigil hemitartrate, phase 2; ispronicline, phase 1), completed (RO5313534), or terminated (ABT-089).

A transmitter that indirectly modulates neuron degeneration and memory deficits is serotonin (5-HT). Growing evidence indicated that inhibition of 5-HT_6 could facilitate Ach release and via elevated cholinergic transmission, memory and learning defects were likely to be ameliorated. 5-HT_6 antagonists were widely reported in many studies to rescue anticholinergic drugs-induced amnesia [132]. Recently, two agents, PRX-03140(5-HT_4 antagonist) and SB-742457(5-HT_6 antagonist), completed the phase 2 trials. Lu AE58054, an antagonist of the serotonin 6 (5-HT_6) receptor was recently progressed into a phase 3 trial with 930 mild to moderate AD patients in combination with AchE inhibitor donepezil.

4. Potential Findings of Therapeutics for Alzheimer's Disease from Other Perspectives

In addition to the two hallmarks and neurotransmitter system impairment, there are several other features found in Alzheimer's disease, including inflammation, oxidative stress, mitochondrial dysfunction, neurotrophin deficiency, and so forth. These aspects are not systematically and thoroughly summarized and are likely to be neglected though; they do provide new perspectives in developing AD treatments. Many drugs of great therapeutic potential are under clinical trials (Table 3).

4.1. Anti-Inflammation and Antioxidants. Chronic inflammation is an essential feature of AD and contributes to its pathogenesis in numerous ways. Microglia are brain's resident macrophages that monitor brain activity and play a contributing role in removal of redundant and apoptotic neurons [133, 134], remodeling of normal synapse [135], and protection of CNS from pathogens and detritus [136]. However, they can shift to another phenotype to secrete series of inflammatory factors, exerting detrimental effects on bystander neurons and processes they are involved in. Aggregated Aβ appears to be a robust agent driving this alteration, since markers of activated microglia were densely colocalized within the deposits [137, 138]. Microglia seem incapable of degrading Aβ that they intake [139, 140], leading to a frustrated phagocytosis instead. As clinical trials have been a major disappointment, agents that drive microglia to a phenotype that favors attack on pathogens rather than bystander neurons may hold therapeutic potential.

Based on compelling evidence of the involvement of inflammation in AD pathogenesis, anti-inflammatory drugs have been investigated. COX inhibitors, aiming to reverse the elevated Aβ burden and cognitive deficits caused by overexpression of COX2 [141, 142], showed limited efficacy [143]. Glucocorticoid steroids, considered as potent drugs by declining overexpression of proinflammatory mediators [144], showed poor benefits [145] or adverse effects [146]. Flavonoid administration prevented cognitive impairment associated with inflammation in animal studies [147, 148]; however, the beneficial effects cannot be repeated in human [149].

Another anti-inflammatory agent etanercept, an approved arthritis drug, is a TNF-α antagonist to neutralize the activated microglia secreted cytokines. Modulation of immune system may have benefits for Alzheimer's disease patients and a phase1 clinical trial in combination with supplementation of some specific nutrients is ongoing in mild to moderate AD patients. Curcumin, a natural polyphenol, has anti-inflammatory and antioxidant properties and exhibits other neuroprotective functions like promoting metal chelation, curbing tau aggregation, and facilitating neurogenesis. It undergoes a phase 2 study, but details are not available.

Oxidative injury is the following causal event of inflammation and the study of antioxidants in treatment of AD achieved little success. Alpha-tocopherol, a synthetic vitamin E, is thought to prevent brain cell damage by destroying toxic free radicals and slowing down the cognitive decline in the finished phase 3 trial. In addition, a phase 3 trial of DHA (docosahexaenoic acid), an omega-3 fatty acid, was terminated because cognitive decline was not changed compared to placebo group.

4.2. Mitochondrial Dysfunction. Mitochondrial dysfunction taking place in early AD enhances synaptic damages and neuron apoptosis, so it is considered a causal factor of neurodegeneration [150]. APP and Aβ are transported into mitochondrion reacting with mitochondrial components, leading to an impaired ATP processing and increased oxidative stress level [150, 151]. ApoE4, a risk factor for sporadic AD, harms mitochondrial trafficking and function and promotes mitochondrial apoptosis [152, 153]. Replacing mitochondrial DNA (mtDNA) form one cell line with mtDNA from AD patients supported a mitochondrion cascade hypothesis [154], offering new therapeutic targets. Latrepirdine (dimebon), an antihistamine that preserves mitochondrial structure and function and protects against Aβ induced pore apoptosis, has been tested in a clinical trial in Russia and phase 2 data showed improvement of all outcomes [155] while phase 3 trial did not confirm it [156]. However, a combination of therapy with donepezil was demonstrated as well tolerated from preliminary results in phase 1 trial and further information awaits analysis [157]. AC-1204 is designed to improve mitochondrial metabolism [158] by induction of chronic ketosis, thus rescuing regional cerebral hypometabolism presented in early Alzheimer's disease, and this agent is undergoing a phase 3 clinical.

4.3. Diabetes. Diabetes is another risk factor for Alzheimer's disease [159] in which the insulin resistance and disrupted glucose metabolism [160] can be attributed to a tumor necrosis factor (TNF) induced inflammation pathway [161, 162]. Insulin can mediate Aβ degradation by activating insulin-degrading enzyme (IDE) [163]. A CSF insulin decline in prodromal female AD patients [164], the presence of insulin resistance, and the dysfunctional insulin signaling pathway in dementia brain [165] are documented. Incretin

and liraglutide, two drugs for hyperglycemia, implicating beneficial effects on AD mice [166, 167], reinforced the relationship between diabetes and AD, and a phase 2 study of liraglutide, a glycogen like peptide 1 agonist is still ongoing. These evidences brought the advent of concept "type 3 diabetes," [168] and an intranasal insulin delivery with an ameliorating cognitive function effect [169] has completed its phase 2 study.

4.4. ApoE (Apolipoprotein) and Aβ Export. ApoE (apolipoprotein) is a powerful genetic factor [170, 171] for sporadic AD beyond APP, PS1, and PS2 genes. The isoform ApoE4 substantially promotes the risk of AD and decreases the age of onset [172]. ApoE is generally thought to regulate Aβ clearance and thus influence fibrillogenesis. In CNS, ApoE, responsible for transportation of cholesterol to neurons, is primarily produced in astrocytes [173]. Aβ aggregation and clearance are differently affected in an isoform (ε2, ε3, and ε4) dependent manner; frequency of AD and mean age at clinical onset are 91% and 68 years of age in ε4 homozygote, 47% and 76 years of age in ε4 heterozygote, and 20% and 84 years in ε4 noncarriers [172, 174]. ApoE was found colocalized with amyloid plagues [175] and this coexistence is more abundant in ApoE4 carriers [176]. Additionally, ApoE4 is associated with cognition decline before clinically apparent syndromes [177, 178]. ApoE4, as previously described, can work synergically with other risk factors, like insulin resistance and peripheral vascular diseases [179, 180], thus exerts a confounding effect on AD and triggers inflammatory cascade. After being synthesized, ApoE is lipidated by the ABCA1, a process regulated by nuclear receptor liver X receptor (LXR) or retinoid X receptor (RXR), and transported to form lipoprotein particles. The complex particle binds soluble Aβ, promoting transfer via neuron surface receptors such as low-density lipoprotein receptor (LDLR), low-density lipoprotein receptor-related protein 1 (LRP1), and heparin sulphate proteoglycan (HSPG) [181, 182] into neurons where degradation can be finished with proteolysis in lysosome. ApoE ε4 isoform has less affinity of binding Aβ compared to ε3, showing a less efficient clearance phenotype [183, 184]. Stimulation of LXR/RXR enhances removal of Aβ [185, 186] while inhibition of ABCA1 impairs Aβ clearance in ApoE4 rather than ApoE3 mice [187]. Therefore, the molecules and receptors involved in ApoE metabolism can be potential therapeutic targets for drug development.

Recent studies demonstrated that oral administration of bexarotene, a RXR agonist and a FDA approved anticancer drug, reduces Aβ plaques and improves cognitive function in an ApoE-dependent manner in amyloid mouse model [186], and a phase 2 clinical trial is currently ongoing to determine its safety and effect on abnormal proteins in the brain with 300 mg for one month compared to placebo. Other drugs that aim to regulate ApoE expression (LXR agonist TO901317) [185, 188], block ApoE-Aβ interaction, disrupt ApoE4 domain (CB9032258, phthalazinone analogue) [189], mimic the receptor binding region [190] (COG112), and so forth, have shown benefits of reversing Aβ burden in vivo or in vitro, but did not reach the clinical trials yet. ApoE-targeted therapies are still at the early stage of development and relevant approaches and strategies are required to carefully evaluate them though, showing a huge promising battle with Alzheimer's disease.

4.5. Neurotrophin. Nerve growth factor (NGF) as a neurotrophin plays a critical role promoting survival and maintaining the function of cholinergic neurons [191, 192]. In AD patients, transcription and translation levels of NGF were changed [193, 194], suggesting that NGF supplementation probably is a treatment approach for Alzheimer's disease. NGF with unfavorable size and polarity is a peptide that cannot cross BBB [193, 195], so to safely and efficiently deliver it to the brain will be a great challenge [196, 197]. However, efforts have been made to overcome this obstacle. An example of strategy is as follows: CERE-110 uses adeno-associated virus to transfer a gene that makes NGF and is injected into AD patients' brain. This approach undergoes a phase 2 study.

5. Concluding Remarks

Aβ cascade hypothesis was firstly proposed in 1992 [198] assuming that β-amyloid would be the suspect initiating pathogenesis of Alzheimer's disease. So a series of explorations focusing on physiological and pathological processes that participate in the production, aggregation, and clearance of Aβ have been widely studied. The identification of two crucial enzymes (γ-secretase and BACE1), responsible for the cleavage of the presumably pathogenic Aβ from its precursor, suggests that the cure of AD may be around the corner.

However, failures in many large clinical trials using Aβ-targeted drugs (Table 4) and FDA approved compounds with marginal efficacy questioned the validity of Aβ cascade hypothesis. Indeed, Aβ hypothesis, having dominated the AD realm for two decades, has always been controversial. One of the most unfavorable evidences was the finding that amyloid plagues were diffused in AD patients' brain postmortem (and neuroimaging outcomes confirmed the autopsy findings), which is abundant in healthy people [199, 200]. Nevertheless, plenty subsequent investigations put forward the oligomeric form of Aβ, rather than plagues, as the actual culprit for synapse dysfunction [201, 202] and the following amplifying events. This significant finding, at least partially, defended the validity of Aβ cascade hypothesis. But, still, why do therapeutic strategies targeting the secretases only have marginal efficacy? First, the two versatile secretases (BACE1 and γ-secretase) are at the same time responsible for processing other substrates, which unfortunately are either vital to metabolism normality or tricky to avoid targeting. The undesirable side effects are so overwhelming that they prohibit drug's efficacy and approval. Second, the drug permeability through blood brain barrier (BBB) is another considerable problem. Most drugs described above have a poor capability to cross BBB, so it is reasonable to see numerous clinical trials, including those having progressed to phase 3, fail. Instead of questioning the plausible hypothesis, it is more imperative to cautiously design clinical studies and interpret the outcomes.

Table 2: RCTs targeting neurotransmitter systems in recent years.

Mechanism	RCT	Status	Estimated end	Dementia stage	Enrollment	Duration	Reported outcomes	Details of drugs/RCTS
Cholinergic agents								
AchE inhibitor	Huperzine A	Phase 2/3; completed	2012.6	Mild-to-moderate	390	6 months	↑cognitive function, daily living activity, global clinical assessment	A natural AChEI; antioxidant and neuroprotective properties [215]
	Ladostigil hemitartrate	Phase 2; ongoing	2016.9	MCI	200	36 months		Antioxidant properties; modulates APP processing
Nicotinic receptor agonist	EVP-6124	Phase 3; ongoing	2017.7	Mild-to-moderate	790	26 weeks	Positive outcomes in a 24-week phase 2b RCT	
	RO5313534	Phase 2; completed	2010.11	Mild-to-moderate	389	6 months		$\alpha 7$ nicotinic receptor agonist; as add-on therapy to donepezil
	Ispronicline (AZD3480)	Phase 2; ongoing	2014.7	Mild-to-moderate	300	1 year		$\alpha 4\beta 2$ and $\alpha 2\beta 2$ nicotinic receptor agonist
	MT-4666	Phase 2; ongoing	2015.5	Mild-to-moderate	450	24 weeks		
	ABT-089	Phase 2; terminated	2013.10	Mild-to-moderate	434	24 weeks		
	MK-7622	Phase 2b; ongoing	2017.8	Mild-to-moderate	830	12–24 weeks	As adjunctive therapy to donepezil	$\alpha 7$ receptor modulator
Glutamatergic agents	AVP-923	Phase 2; ongoing	2014.9	Mild-to-moderate	200	10 weeks	Behavioral problems	NMDA receptor antagonist
Serotoninergic agents	Lu AE58054	Phase 3; ongoing*	2016.1	Mild-to-moderate	≈2500		Positive results in a phase 2 RCT, 278 participants, 6 months; showed positive results	Several phase3 RCTS with donepezil (AchEI);
	SB-742457	Phase 2; completed	2011.8	Mild-to-moderate	684	6 months		

RCT: randomized controlled trial; AChEI: acetylcholinesterase inhibitor; MCI: mild cognitive impairment; NMDA: N-methyl-D-aspartic acid.
Data sources: http://www.clinicaltrials.gov/.
*RCTs with a combination of another drug.

Table 3: Other novel approaches in AD clinical trials.

Mechanism	RCT	Status	Estimated end	Dementia stage	Enrollment	Duration	Reported outcomes	Details of drugs/RCTS
Anti-inflammation and antioxidation	Curcumin	Phase 2; completed	2007.12	Mild-to-moderate	33			NSAID, cholesterol-lowering properties
	Etanercept	Phase 1; ongoing	2015.6	Mild-to-moderate	12	12 months	↑cognitive function with other nutrients.	Approved drug for arthritis; may modulate immune system; benefit AD patients
	dl-alpha-tocopherol (vitamin E)	Phase 3; completed	2012.10	Mild-to-moderate	613			
	PUFA	Phase 1/2; ongoing*	2015.1		100	18 months		Tested alone or together with lipoic acid
	RO4602522	Phase 1; completed	2013.5		17			
PDE inhibitors	PF-04447943	Phase 2, completed	2010.9	Mild-to-moderate	198			Selective PDE 9A inhibitor
	MK0952	Phase 2; completed	2007.11	Mild-to-moderate				Selective PDE 4 inhibitor
	Cilostazol	Phase 4; completed	2013.7	Mild-to-moderate	46			PDE3 inhibitor, Antiplatelet agent in WMHI; ↑pCREB
Tyrosine kinase inhibitor	Masitinib	Phase 3; ongoing*	2015.12	Mild-to-moderate	396			In combination with AChEI and/or memantine
Insulin and GLP1-R agonists	Intranasal insulin (glulisine)	Phase 2/3; ongoing	2015.2	MCI/mild AD	240	12 months	Showed neuroprotection	Diabetes agent
	Exendin-4 (exenatide)	Phase 2; ongoing	2016.7	MCI/early stage	100	3 years		
	Liraglutide	Phase 2; ongoing	2017.1	early stage	206	12 months		
Modulating mitochondrial function	AC-1204	Phase 2/3; ongoing	2015.1	Mild-to-moderate	480	26 weeks		
	Latrepirdine (Dimebon)	Phase 3; completed	2009.12	Mild-to-moderate	598	6 months		
RXR agonist	Bexarotene	Phase 2; ongoing	2014.3	Mild-to-moderate	20			Approved anticancer drug; linked to key pathways relevant to AD and Aβ
NGF delivery	CERE-110	Phase 2; ongoing	2014.12	Mild-to-moderate	50	24 months	24 months	Designed to help neurons function better; uses a virus to transfer NGF gene
	Encapsulated Cell biodelivery of NGF	Phase 1b	2011.12		6	12 months		

RCT: randomized controlled trial; NSAID: nonsteroidal anti-inflammatory drugs; PDE: phosphodiesterase; WMHI: subcortical vascular disease; pCREB: phosphorylated cAMP-response element binding protein; AChEI: acetylcholinesterase inhibitor; GLP1-R: glucagon-likepeptide1 receptor; MCI: mild cognitive impairment; RXR: retinoid X receptors; NGF: nerve growth factor.

*RCTs with a combination of another drug.

Data sources: http://www.clinicaltrials.gov/.

Table 4: Terminated trials targeting Aβ hypothesis.

Mechanism	RCT	End	Enrollment	Duration	Main reasons
BACE1 inhibitor	Rosiglitazone	2009.2	693	24 weeks	Unimproved cognitive status
	LY2886721	2013.8	128		AE: 4 cases of liver damage in a phase 2 study in June 2013
GSI/GSM	Semagacestat	2011.5	164	>7 months	Unimproved cognitive status, but worsening functional ability; AE: skin cancers and infections
	Avagacestat	2010.6	209	24 weeks	AE: gastrointestinal and dermatological abnormalities like diarrhea, nausea, vomiting, rash, and itching skin; nonmelanoma skin cancers; and worsened cognition
	tarenflurbil	2008.5	1684	18 months	Insufficient pharmacodynamics: poor capability to penetrate the BBB
Active immunology	AN1792	2003.9	375		AE: 6 patients developed aseptic meningoencephalitis due cytotoxic T cell response in phase 2a trial
	ACC-001	2014.2	126	24 months	Showed a serious side effect in phase 2 trial
Passive immunology	Bapineuzumab (AAB-001)	2012.6	1331	18 months	Showed no treatment effect on either cognitive or functional outcomes in two phase 3 trials
GSK3β inhibitor	Tideglusib (NP12)	2012.6	306	45 weeks	Missed its primary endpoint and some secondary endpoints

RCT: randomized controlled trial; AE: adverse event; BBB: blood brain barrier.
Data sources: http://www.clinicaltrials.gov/; http://www.alzforum.org/.

Given limited benefits from inhibition of Aβ production, more focus should be converted to the clearance strategy. Delivery of antibodies may be a good choice due to the safety leverage compared to vaccine. Besides, there are quite a few ongoing clinical trials using passive immunization. From Table 1, antibodies are capable of binding and clearing multiple forms of Aβ. It is important because there is equilibrium between oligomers and plagues of Aβ [203]. For a single-target antiaggregate disrupting formation or enhancing disassembly of Aβ oligomers, plagues as a reservoir will replenish and maintain the balance [204, 205]. So the property of simultaneously interfering different processes during Aβ aggregation suggested that passive immunization might be of a promising value.

In recent years other AD risk factors have been widely studied. Though no groundbreaking outcomes have been shown, it provided quite a few unprecedented opportunities. First, the validated AD specific biomarkers need to be carefully developed and examined. Biomarkers should be able to at least precisely indicate the response to therapeutic intervention to avoid misinterpretation of clinical trial data. Besides, current animal models have serious limitations. Most transgenic mouse models published in AD studies overproduce Aβ solely mimicking familial Alzheimer's disease, might not suffice phenotypes of sporadic AD accounting for the dominant populations.

In addition, AD is a disorder that is too intricate and too factor-driven to be entirely understood from its pathogenesis. As we discussed previously, various factors (Aβ, tau, inflammation, and apoE) complicatedly interact with each other. So the conventional "one protein, one drug, one disease" hypothesis would not work for Alzheimer's disease. From the successful experience in therapeutic development in multifactorial diseases like AIDS, atherosclerosis, cancer, and depression, multitarget drugs or combination therapy can possibly generate more benefits. Since drugs with more than one target could possibly mitigate a redundancy effect in such a complex nerve network, this combination therapy or similar approach multitarget-directed ligands (MTDLs) might bring new hope in search of therapeutics for Alzheimer's diseases [206, 207]. In this novel fashion, some combinations with approved drug are under clinical trials (Tables 1, 2, and 3, RCTs marked with $*$).

Notwithstanding these challenges, with more scientific insights from basic researches and cooperation between laboratories and pharmaceutical companies, it is very likely to find the optimum treatment for Alzheimer's disease in the near future.

Abbreviation

AD: Alzheimer's disease
Aβ: Amyloid β
NFT: Intracellular neurofibrillar tangles
FDA: Food and Drug Administration
APP: Amyloid precursor protein
NMDA: N-Methyl-D-aspartic acid
PS: Presenilin
NICD: Notch intracellular domain
GSI: γ-Secretase inhibitors
CSF: Cerebrospinal fluid
CNS: Central nervous system
CSF: Cerebrospinal fluid
GSM: γ-Secretase modulators
NSAID: Nonsteroidal anti-inflammatory drugs
BACE1: Beta-site APP-cleaving enzyme 1
FAD: Familial Alzheimer's disease
BBB: Blood brain barrier
COX: Cyclooxygenase
GABA: γ-Aminobutyric acid
ApoE: Apolipoprotein E
MMSE: Mini-mental-state exam
GSK3β: Glycogen synthase kinase 3 beta
PP2A: Protein phosphatase 2
cdk5: Cyclin dependent kinase 5
TNF: Tumor necrosis factor
IDE: Insulin-degrading enzyme
LXR: Liver X receptor
RXR: Retinoid X Receptor
LDLR: Low-density lipoprotein receptor
LRP1: Low-density lipoprotein receptor-related protein 1
HSPG: Low-density lipoprotein receptor-related protein 1
ABCA1: ATP-binding cassette transporter 1
Ach: Acetylcholine
LTP: Long-term potentiation
5-HT: Serotonin
NGF: Nerve growth factor.

Conflict of Interests

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

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Research Article

Reduction of Experimental Cerebral Malaria and Its Related Proinflammatory Responses by the Novel Liposome-Based β-Methasone Nanodrug

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Cerebral malaria (CM) is a severe complication of and a leading cause of death due to *Plasmodium falciparum* infection. CM is likely the result of interrelated events, including mechanical obstruction due to parasite sequestration in the microvasculature, and upregulation of Th1 immune responses. In parallel, blood-brain-barrier (BBB) breakdown and damage or death of microglia, astrocytes, and neurons occurs. We found that a novel formulation of a liposome-encapsulated glucocorticosteroid, β-methasone hemisuccinate (nSSL-BMS), prevents experimental cerebral malaria (ECM) in a murine model and creates a survival time-window, enabling administration of an antiplasmodial drug before severe anemia develops. nSSL-BMS treatment leads to lower levels of cerebral inflammation, expressed by altered levels of corresponding cytokines and chemokines. The results indicate the role of integrated immune responses in ECM induction and show that the new steroidal nanodrug nSSL-BMS reverses the balance between the Th1 and Th2 responses in malaria-infected mice so that the proinflammatory processes leading to ECM are prevented. Overall, because of the immunopathological nature of CM, combined immunomodulator/antiplasmodial treatment should be considered for prevention/treatment of human CM and long-term cognitive damage.

1. Introduction

Human cerebral malaria (CM) caused by *Plasmodium falciparum* is a major cause of malaria mortality. Survivors of CM experience developmental and behavioral impairments [1, 2]. Therefore, antiplasmodial treatment may not be sufficient for preventing cognitive CM consequences.

There is common agreement that CM is the result of cerebral vascular obstruction by parasitized erythrocytes as well as additional factors, such as immunopathological components [3, 4]. Experimental cerebral malaria (ECM) depicted in mice is a reliable model for unraveling CM pathogenesis [5] and can be prevented by immunomodulation [6–9]. In general, infection of C57Bl/6 mice with low inoculums of *P. berghei* ANKA- (PbA-) infected erythrocytes (PE) (5×10^4–10^5) leads to experimental cerebral malaria (ECM) [10]. ECM progresses rapidly, with death occurring at relatively low parasitemias approximately one week after inoculation. The mortality rate due to ECM is at least 90%; surviving mice later succumb to severe anemic malaria at high parasitemias [11].

It is well established that, in the context of neuroinflammatory diseases, glucocorticosteroids modulate T cells, macrophages, microglia, and the blood brain barrier (BBB) [12]. In a previous study, we demonstrated that administration of a novel liposomal nanodrug based on the steroid β-methasone hemisuccinate (BMS) as its active pharmaceutical ingredient (API) (also referred to as nanosterically stabilized

liposomes (nSSL-BMS)) prevents murine cerebral symptoms and creates the therapeutic time-window necessary for administration of effective antiplasmodial drugs. Brain histology and RNA expression of cytokine-related genes suggest that nSSL-BMS alleviates the immunopathological processes that induce ECM [6]. In these experiments, murine immune status was examined at a single time point (9 days after inoculation, the peak of cerebral disease); in addition, cytokine and chemokine levels were measured only indirectly, via mRNA levels. As RNA levels do not necessarily correlate with protein levels [13], analysis of cytokine and chemokine protein levels at several time points is essential. Our current study was designed to address these limitations, thereby enabling a better understanding of the mechanism of action of the steroidal nanodrug. The results indicate the role of integrated immune responses in ECM induction and show that the new steroidal nanodrug nSSL-BMS reverses the balance between the Th1 and Th2 responses in malaria-infected mice, preventing the proinflammatory processes leading to ECM.

2. Materials and Methods

2.1. Preparation of nSSL-BMS. nSSL-BMS was prepared and characterized according to our previously published protocols [6, 14]. The nSSL-BMS were composed of HSPC/cholesterol/PEG-DSPE at a mole ratio of 55 : 40 : 5 hydrated with 250 mM calcium acetate, with a mean size of 82.2 ± 0.73 d·nm (polydispersity index of 0.12 ± 0.01) and a drug to lipid mole ratio of 0.17 ± 0.06.

2.2. Ethics Statement. All procedures adhered to the Australian National Health and Medical Research Council guidelines for animal research and were approved by both the University of Sydney Animal Ethics Committee and the Animal Ethical Care Committee of The Hebrew University of Jerusalem.

2.3. Animals. Six- to 8-week-old female C57BL/6 mice were obtained from the Australian Animal Research Centre (Perth, Australia) and housed in the Medical Foundation Building Animal House, University of Sydney (Sydney, Australia), in group cages under a 12 h light-dark cycle with food and water ad libitum.

2.4. Induction of Experimental Cerebral Malaria (ECM). *Plasmodium berghei* ANKA (PbA) was maintained in vivo by serial transfer of parasitized erythrocytes (PE) from infected to naïve mice. Intraperitoneal injection of experimental mice with PbA from peripheral blood of infected donor mice at an inoculum of 5×10^4 PE resulted in >90% incidence of terminal ECM signs on days 7-8 after inoculation (PoI), at parasitemias of up to 25% (mostly below 20%). Parasitemia was monitored by thin blood smears prepared from tail blood. These were stained with a 25% Giemsa solution, examined under a light microscope, and parasitemia determined as the percentage of infected red blood cells per 10,000 erythrocytes. ECM was determined according to neurological symptoms, that is, presentation of one or more signs of neurological deficit including ataxia, convulsions, limb paralysis, poor righting reflex, roll-over, and coma. Brains were selected at random for histology. Brain pathology observed in mice dying of ECM (generally on day 7 PoI) included hemorrhages and mononuclear cell accumulation in small vessels. In the absence of neurological symptoms, on average at 14 days PoI, nontreated mice succumbed to hyperparasitemia (above 25%) and severe anemic malaria (SM), as has been reported in other cases where mice are resistant to *P. berghei* ANKA-induced ECM [15].

2.5. Treatment Protocols and Mouse Euthanasia. Infected mice were administered 20 mg/kg nSSL-BMS or equal amounts of empty liposomes by i.v. injection on days 3, 5, and 7 PoI. Infected control mice were injected with 5% dextrose or equal amounts of empty liposomes. The empty liposomes were injected in a separate experiment. At least five mice from each group were chosen at random and deeply anesthetized, and brains were removed following intracardial perfusion with 20 mL cold PBS, snap frozen in liquid nitrogen, and stored at −80°C until use.

2.6. Quantification of Total Brain Soluble Proteins. Frozen brains were homogenized in 0.5 mL 1% (v/v) protease inhibitor cocktail solution in phosphate buffered saline (PBS) by a bullet blender, using zirconium beads. Homogenates were centrifuged at 12,000 rpm (17,200 ×g) for 10 minutes at 4°C. The supernatant containing soluble proteins (~200 μL) was stored at −80°C until analysis.

Total soluble protein in each sample was measured using the bicinchoninic acid assay (BCA, Pierce Biotechnology, USA); 25 μL sample was added to a 96-well flat bottom plate in duplicate and incubated with 200 μL working reagent at 37°C for 30 min in the dark. An intense purple color developed and the absorbance was recorded by a SpectraMax 190 spectrophotometer (Molecular Devices, USA) at 562 nm. Protein concentration in unknown samples was estimated by comparison to a standard curve of bovine serum albumin (BSA).

2.7. Brain Cytokine Measurement. Murine brain cytokine proteins interferon gamma (IFN-γ), tumor necrosis factor (TNF), monocyte chemoattractant protein-1 (CCL-2/MCP-1), and monokine induced by interferon gamma (CXCL-9/MIG) were measured using the Cytometric Bead Array Flex Set (BD Biosciences). Briefly, antibody-conjugated beads (1 : 50 dilution in 5 μL Capture Bead Diluent) were incubated with 5 μL protein sample for one hour in a 96-well round bottom plate, at room temperature. Phycoerythrin-labelled detection fluorescent antibodies (1 : 50 dilution in 5 μL Detection Reagent Diluent) were added to detect the corresponding bead-protein complexes. The plate was further incubated for one hour at room temperature in the dark and detected by a Beckman Coulter cytomics FC500 MLP flow cytometer. Data analysis was performed using FlowJo software (Tree Star, Inc). The concentration of each individual cytokine was

revealed by the intensity of fluorescence of the relevant bead population compared to the standard curves.

Murine cytokine CXCL-10/IP-10 was measured by the Mouse CXCL-10/IP-10 DuoSet ELISA Development Kit (R&D Systems, USA) according to the manufacturer's protocol. Briefly, a NUNC immunoplate (96-well flat) (Thermoscientific, Australia) was coated with capture antibody in PBS overnight at room temperature. The plate was washed three times with PBS containing 0.05% (v/v) Tween 20 in a blocking buffer, 1% w/v BSA in PBS, was added and the plate incubated at room temperature for 1 h, followed by rinsing three times with washing buffer. Brain protein samples in PBS and CXCL-10/IP-10 standards were plated in duplicate and incubated for 2 h at room temperature, following which unbound proteins were removed by repetitive washes with washing buffer. Biotinylated goat anti-mouse CXCL-10/IP-10 detection antibody was added to detect the bound proteins. Samples were further incubated at room temperature for 2 h and washed three times and the protein-antibody complex was labeled with diluted Streptavidin-horseradish peroxidase (HRP, R&D Systems, USA) for 20 min in the dark at room temperature. After three washes, tetramethylbenzidine (TMB) (Sigma, USA) was added and the plate was incubated for an additional 20 min. The reaction was stopped by addition of sulfuric acid. Absorbance was read at 450 nm by a SpectraMax 190 spectrophotometer (Molecular Devices, USA). The concentration of CXCL-10/IP-10 was estimated from the standard curve using GraphPad Prism 5 software.

Cytokine concentrations estimated were normalized to the mg total protein.

2.8. Statistics. In vivo and cytokine results were evaluated for statistical significance using one-way ANOVA and Tukey test in GraphPad Prism and presented as mean ± S.E.M. Significant differences of $P < 0.05$ are noted by an asterisk.

3. Results

We found that nSSL-BMS consistently prevented ECM; nontreated mice and mice treated with empty liposomes developed irreversible cerebral disease, while nSSL-BMS treated mice did not show ECM symptoms and later developed severe life-threatening malaria (characterized by anemia) (Table 1). The control mice developed irreversible CM within a week PoI, with relatively low parasitemias (less than 25%, mostly below 20%). Mice which survived ECM following nSSL-BMS administration developed severe malaria and were euthanized a week later (two weeks PoI), at parasitemias above 35% (Figure 1).

On day 7 PoI, the brains of moribund control mice contained elevated levels of all tested proinflammatory proteins (Figure 2). In contrast, the brains of nSSL-BMS-treated mice displayed much lower levels of the proinflammatory cytokines and chemokines IFN-γ, TNF, CCL-2, and CXCL-9. These relatively low levels remained stable throughout the experiment. CXCL-10 levels were similar in all groups of infected mice. On day 14 PoI, CXCL-10 levels in nSSL-BMS-treated mice were slightly elevated (all other mice were moribund due to ECM and were euthanized) (Figure 2).

Table 1: ECM rates after treatment of PbA-infected mice with nSSL-BMS.

Group*	[a]ECM	SM	Survival on day 8 after inoculation
Control	38[b] (95%)	2 (5%)	2/40 (5%)
Empty liposomes	12 (100%)	0 (0%)	0/12 (0%)
nSSL-BMS	2 (6.5%)	29 (93.5%)	29/31 (93.5%)

All results depict significant differences relating to experimental cerebral malaria (ECM) versus severe anemic malaria (SM) and nSSL-BMS versus control groups.
*Cumulative results of three experiments.
[a]Mice were euthanized when signs indicated the onset of irreversible disease.
[b]Number of mice in the group.

4. Discussion

In the context of neuroinflammatory diseases, glucocorticosteroids (GC) modulate T cells, macrophages, microglia, and the blood brain barrier [12]. A previous study using the murine model of CM showed that, in contrast to free β-methasone hemisuccinate (BMS), a novel superior steroid formulation, liposomal BMS (nSSL-BMS), prevented ECM and created a therapeutic time-window that enabled antiplasmodial treatment and total cure. Brain histology and mRNA measurements of cytokine and chemokine related genes suggested that nSSL-BMS alleviates the immunopathological process that induces ECM [6]. While cytokines and chemokines may reach the brain through the damaged blood brain barrier that accompanies ECM [16], local production of cytokines and chemokines in CM has been emphasized as the important triggering element [17–20]. The examined samples were taken after thorough blood perfusion, indicating that the results most likely reflect the activity of molecules that either were produced in the brain or were produced elsewhere but were bound to local receptors. In addition, this murine model depicts brain damage without respiratory stress, a remote syndrome, thus excluding possible effects of systemic damage. Therefore, the current study stresses the role of cerebral immune responses mediated by actual Th1-related proteins in the initiation of ECM. The results demonstrate that the new steroidal nanodrug nSSL-BMS reverses the balance between the Th1 and Th2 responses so that the inflammatory process and the corresponding ECM are prevented.

It is common practice to measure gene expression levels via mRNA expression, as in our previous study using this mouse model [6]. However, RNA levels may not reflect the presence and levels of actual cytokines and chemokines due to the many processes which occur between transcription and translation. Protein stability is a significant factor; the half-life of different proteins can vary from minutes to days, whereas the degradation rate of mRNA falls within a

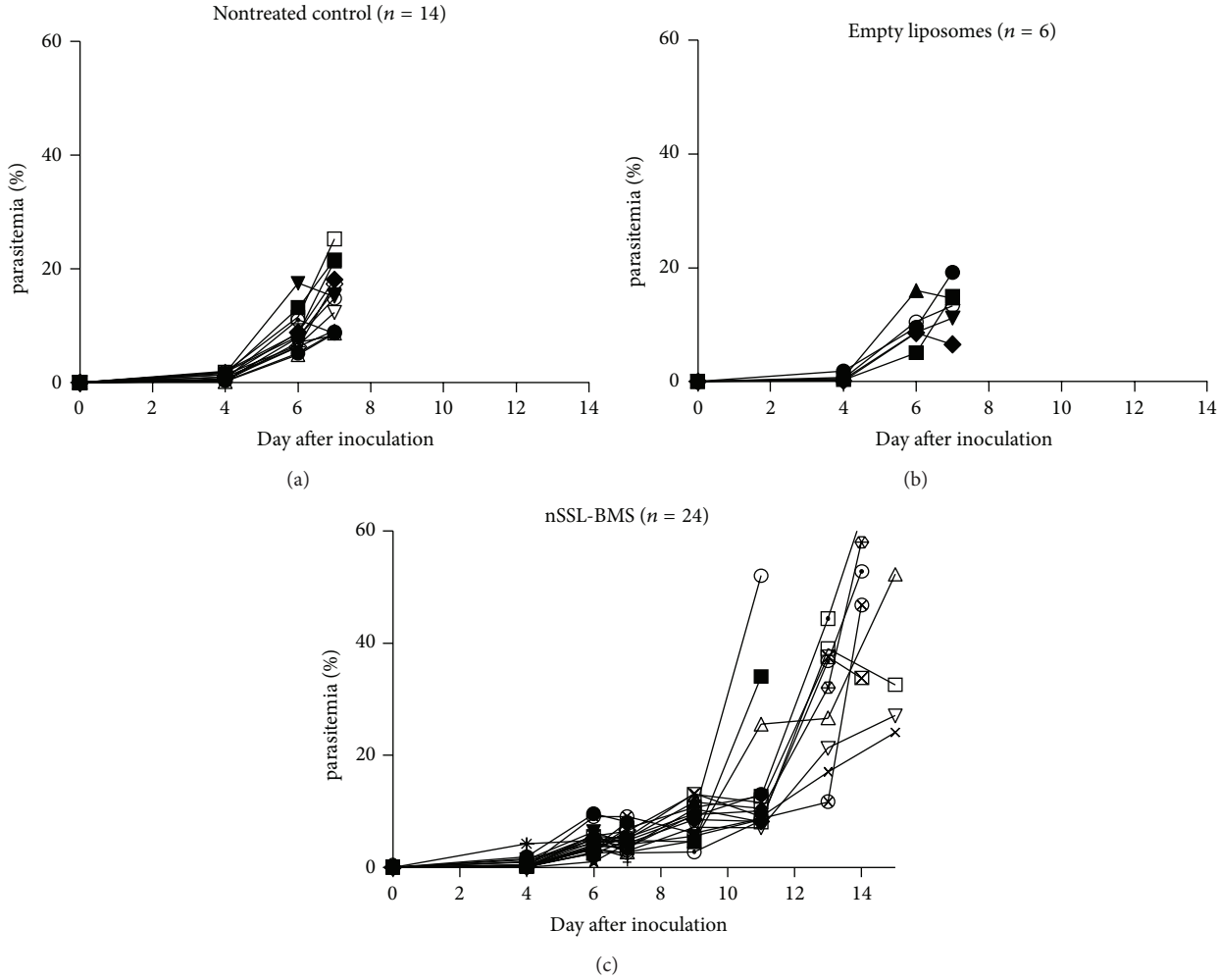

Figure 1: Effect of nSSL-BMS on murine PbA infection. Each line represents a single mouse. The empty liposomes were examined in a separate experiment. The results in the nSSL-BMS injected mice are significantly different compared to the controls (survival and parasitemia on days 8–14 PoI).

much tighter range (2–7 hrs for mRNAs versus 48 hrs for proteins). The biochemical diversity of proteins means that individual correlation levels with associated mRNA are likely to vary widely [13]. Therefore, in order to complete the understanding of the mechanism of action of our steroidal nanodrug, in this study, we measured actual levels of the relevant cytokines and chemokines.

We selected IFNγ and TNF (proinflammatory cytokines) and CCL-2, CXCL-9, and CXCL-10 (proinflammatory chemokines) as representative molecules for examining the relationship between the level of Th1-related immune responses and ECM occurrence in mice treated by nSSL-BMS. IFNγ and TNF are central factors in the cascade of events leading to human CM [21, 22] and ECM [23–25]. IFNγ induces TNF production, which in turn upregulates adhesion molecules on brain endothelial cells, leading to an increase in the adhesion of platelets and parasitized erythrocytes [26]. CCL-2, which recruits monocytes, memory T cells, and dendritic cells to inflammation sites produced by either tissue injury or infection, is implicated in the pathogenesis of several diseases characterized by monocytic infiltrates, including neuroinflammatory processes [27]. An increase in proinflammatory Th1-related chemokines, for example, CCL-2, CXCL-9, and CXCL-10, is associated with ECM [17, 28–30]. CXCL-9, related to CXCL-10, is chemoattractant for T cells and NK cells and affects the growth, movement, and activation state of cells that participate in immune and inflammatory responses [31]. In response to IFNγ, CXCL10 is secreted by several cell types, including monocytes, endothelial cells, and fibroblasts. CXCL10 has been associated with several roles, including chemoattraction of monocytes/macrophages, T cells, NK cells, and dendritic cells; promotion of T cell adhesion to endothelial cells; antitumor activity; and inhibition of bone marrow colony formation and angiogenesis. Several of these activities are related to many Th1-type inflammatory diseases [32]. Elevated plasma levels of CXCL10 have been tightly associated with CM mortality [33, 34].

De Miranda et al. [35] found that on day 5 PoI PbA-infected mice presented anxiety signs, histopathological

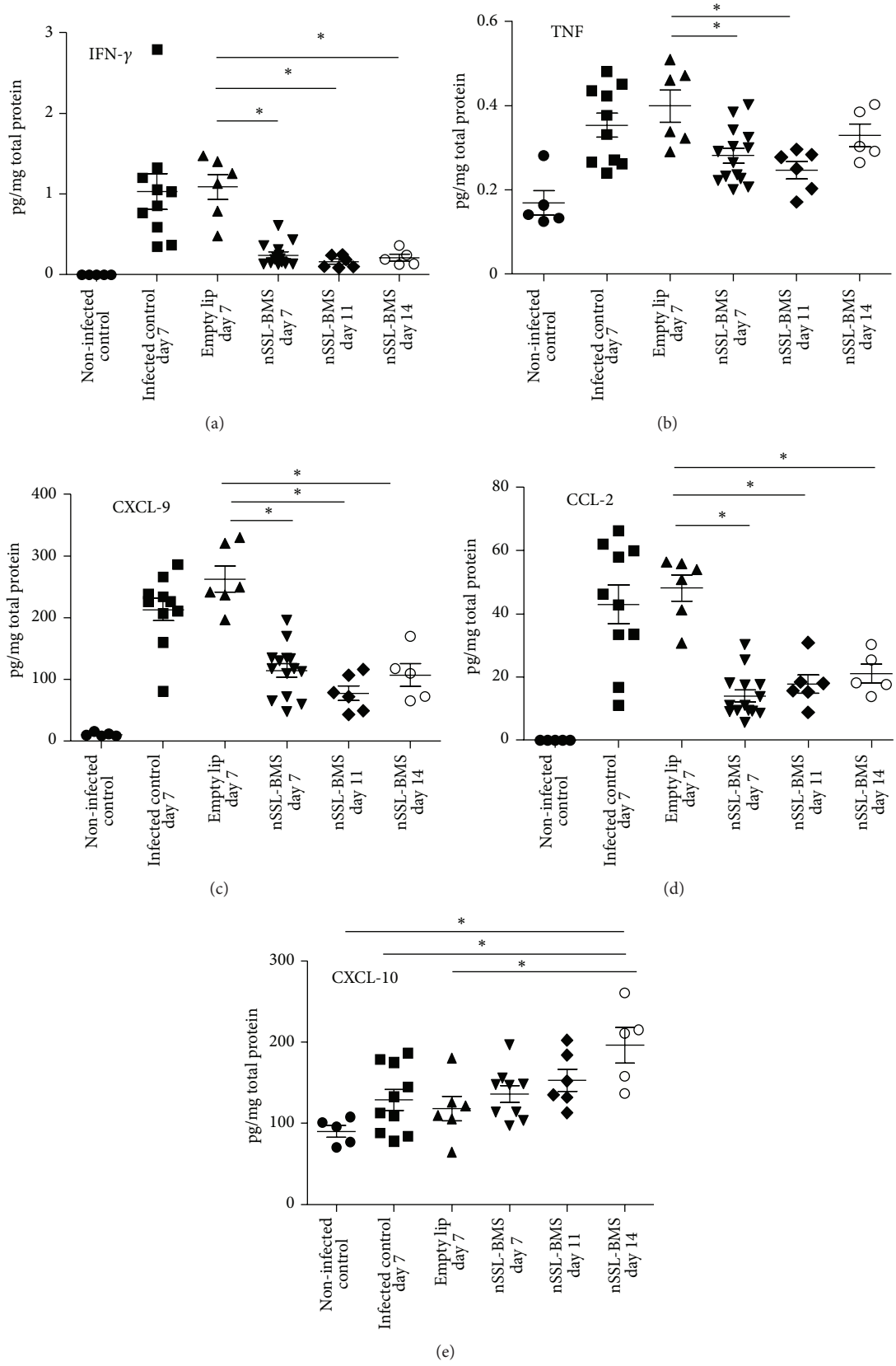

Figure 2: Brain cytokine and chemokine levels in PbA-infected mice treated with nSSL-BMS. Each symbol represents a single mouse. *Significant, P value < 0.05. Noninfected control, $n = 5$. Infected control day 7, $n = 10$. Empty nSSL day 7, $n = 6$. nSSL-BMS day 7, $n = 9$. nSSL-BMS day 11, $n = 6$. nSSL-BMS day 14, $n = 5$.

alterations in the brainstem, cerebrum, and hippocampus, and increased cerebral levels of proinflammatory cytokines. These findings suggest involvement of central nervous system inflammatory mediators in the anxiety symptoms observed in CM. We found that PbA-infected nontreated mice and infected mice injected with empty liposomes presented increased levels of cerebral proinflammatory cytokines and chemokines. This was most significant on day 7 PoI, when control mice develop irreversible CM. In contrast, there was a reduction in IFN-γ, TNF, CCL-2, and CXCL-9 levels in mice treated with nSSL-BMS. Although CXCL-10 levels were similar in all infected groups up to day 11 PoI, an increase was observed in nSSL-BMS-treated mice (the only surviving group) on day 14 PoI. All other immune parameters in the surviving mice were relatively low and similar to previous days. Wilson et al. [33] and Campanella et al. [28] have suggested a central role for CXCL-10 in the development of ECM. Wilson et al. demonstrated decreased levels of CXCL-10 and improved survival in mice 11 days PoI, following injection of artemether and atorvastatin. In treated mice, no difference in CXCL-10 levels was noted on day 5 PoI, compared to control levels. Although these investigators relate the improved survival to reduced deleterious effects of CXCL-10, it should be stressed that the most striking event in CM induction is the impairment of the BBB, which starts early in plasmodial infection. Campanella et al. showed an increase in CXCL-10 levels at a similar, relatively late stage after infection. Moreover, their CXCL-10 deficient mice were only partially protected against CM [28]. In our experiments, treatment with nSSL-BMS did not alter CXCL-10 level until day 11 PoI; increased levels (compared to nontreated infected mice) were observed on day 14 PoI. These later days PoI are much less relevant to the induction and development of the cerebral syndrome (which was fatal in nontreated mice 6 or 7 days PoI). These results indicate that either CXCL-10 is not necessarily a major player in ECM induction or its role is masked by the activity of other immune factors.

In our experiments, a correlation was observed between the immunological effects of nSSL-BMS and ECM prevention. Although individual cytokines and chemokines may contribute to the induction of (E)CM [36, 37], the end result of malarial infection is determined by a repertoire of immune responses. A synergistic effect may increase disease severity; for example, IFNγ and TNF, together with other cytokines and chemokines, synergize in the upregulation of adherence molecules, which stimulate immunopathology [25, 38]. This concept applies to a variety of chemokines [28]. The opposite process—CM avoidance or alleviation—is also dependent on a battery of cytokine responses (and/or chemokines or antioxidants) rather than on a single component [2, 28, 39, 40]. In a similar fashion, the end result of immune (and antiparasitic) intervention is also a balance of synergistic and antagonistic reactions.

The relevance of CM research using mouse models has been discussed [5]. It has been emphasized that drug validation is impossible without in vivo experiments. Moreover, some newly approved therapies are based on drugs previously tested for efficacy in mice (e.g., artemisinin combination therapy). Although the efficacy of glucocorticosteroids for CM treatment has been questioned, the nanosteroidal drug nSSL-BMS is a superior, novel nanodrug which displays a lack of toxicity as well as improved delivery to the inflamed brain [6]. This compound reduced ECM and the corresponding Th1 responses, confirming the hypothesis that the cerebral syndrome is a result of a Th1 shift in the balance of Th1/Th2 responses. Adjunctive therapy coupled to antiplasmodial treatment has been shown to prevent ECM cognitive defects [2]. Because of the immunopathological nature of CM, combined nSSL-BMS/anti-plasmodial-based therapy is suggested for prevention/treatment of CM and long-term cognitive damage.

5. Conclusions

The results confirm the hypothesis that the cerebral syndrome in ECM is a result of a dynamic shift in the balance of Th1/Th2 responses. A novel liposome-based nanosteroidal drug, β-methasone hemisuccinate (nSSL-BMS), reduces both cytokine and chemokine proinflammatory responses that lead to CM in a murine model. Our steroidal nanodrug prevents ECM by inducing multiple changes in the immunopathological process; therefore, combination therapy using antiplasmodial treatment and this novel drug would be of great importance in a clinical setting.

Conflict of Interests

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

Authors' Contribution

Jintao Guo and Judith H. Waknine-Grinberg have equally contributed.

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Clinical Study

Improvement of Oxidative and Metabolic Parameters by Cellfood Administration in Patients Affected by Neurodegenerative Diseases on Chelation Treatment

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Objective. This prospective pilot study aimed at evaluating the effects of therapy with antioxidant compounds (Cellfood, and other antioxidants) on patients affected by neurodegenerative diseases (ND), who displayed toxic metal burden and were subjected to chelation treatment with the chelating agent calcium disodium ethylenediaminetetraacetic acid ($CaNa_2EDTA$ or EDTA). *Methods*. Two groups of subjects were studied: (a) 39 patients affected by ND and (b) 11 subjects unaffected by ND (controls). The following blood parameters were analyzed before and after three months' treatment with chelation + Cellfood or chelation + other antioxidants: oxidative status (reactive oxygen species, ROS; total antioxidant capacity, TAC; oxidized LDL, oxLDL; glutathione), homocysteine, vitamin B12, and folate. *Results*. After 3-months' chelation + Cellfood administration oxLDL decreased, ROS levels were significantly lower, and TAC and glutathione levels were significantly higher than after chelation + other antioxidants treatment, both in ND patients and in controls. Moreover, homocysteine metabolism had also improved in both groups. *Conclusions*. Chelation + Cellfood treatment was more efficient than chelation + other antioxidants improving oxidative status and homocysteine metabolism significantly in ND patients and controls. Although limited to a small number of cases, this study showed how helpful antioxidant treatment with Cellfood was in improving the subjects' metabolic conditions.

1. Introduction

The influence of nutrition and dietary supplements on the course of neurodegenerative diseases (ND) has been recently studied, particularly, the effects of nutritional factors, such as polyunsaturated fatty acids, vitamins, milk proteins, gluten, and probiotics, on the development, relapse rate, and progression of multiple sclerosis (MS) [1]. Recently malnutrition at the time of diagnosis has been associated with a shorter duration of disease in amyotrophic lateral sclerosis (ALS) [2]. Nutritional approaches have been proposed to reduce the risk and improve the management of Alzheimer's disease (AD) [3]. Cell damage due to both oxidative stress and depletion of endogenous antioxidants could be considered mechanisms of injury for ND. Indeed, the use of antioxidants seems to help prevent the formation of reactive species and counteract the damage to DNA, lipids, proteins, and other biomolecules [4]. For example, serum nitric oxide and peroxynitrite levels have been shown to be higher in patients affected by Parkinson's disease (PD) [5] than in controls, establishing a relationship between serum levels of these oxidants and the severity of the disease. In this context, other studies have demonstrated the usefulness of phenolic compounds and N-acetylcysteine as antioxidants in ND and PD, respectively [6, 7].

Mitochondrial dysfunction is considered to be the basis of the development and progression of several neurologic diseases with different aetiologies, including ND [8]. Therefore, mitochondria have become an interesting target of drug therapy [9]. Particularly, as reported by Mao et al.'s study, mitoQ, a mitochondria-targeted antioxidant, can delay

disease progression and alleviate pathogenesis in an experimental autoimmune encephalomyelitis mouse model of MS [10].

We had previously shown how helpful antioxidant compound Cellfood was in improving mitochondrial respiratory metabolism of endothelial cells and inhibiting hypoxia-induced reactive oxygen species (ROS) generation *in vitro* [11]. In this prospective study we evaluated the influence of Cellfood treatment on blood parameters in patients affected by ND and subjected to chelation therapy with the chelating agent calcium disodium ethylenediaminetetraacetic acid ($CaNa_2EDTA$ or EDTA), administered intravenously. Two groups of subjects were studied: 39 patients affected and 11 subjects unaffected by ND (controls). All subjects were affected by chronic body burden of heavy metals and were treated with EDTA to remove metal intoxication [12–14]. The subjects were also daily treated with antioxidant therapy to help the detoxification process: some of them received Cellfood and some other antioxidants [15].

Oxidative status represents the result of the balance between ROS generation and antioxidant capacity of the organism. Blood parameters could possibly highlight oxidative stress conditions. In this study serum concentrations of ROS, total antioxidant capacity (TAC), oxidized LDL (oxLDL), cholesterol profile (total cholesterol (TC); HDL; and LDL), and the blood GSH/GSSG red-ox couple were assessed. The latter is mostly responsible for maintaining homeostasis of cell red-ox state [16]. Homocysteinemia and metabolically related vitamins (vitamin B12, active vitamin B12, serum, and erythrocyte folate) were also determined. In fact, if vitamin levels are inadequate, hyperhomocysteinemia can have a prooxidant effect causing or promoting oxidation.

The aim of this prospective pilot study was to evaluate the influence of Cellfood treatment during chelation therapy and to compare it with that of other antioxidants.

2. Materials and Methods

2.1. Patient Recruitment. Out of 80 consecutive subjects who had undergone a medical checkup in an outpatient medical center, only 50 were selected and enrolled for this study due to their compliance in following the protocol, for example, receiving chelation therapy once a week by personal choice and taking daily antioxidants. Antioxidants were distributed at random.

Twenty patients were affected by MS; fifteen of them had been previously treated with conventional drugs against MS (e.g., immunosuppressant agents, such as mitoxantrone and azathioprine, broad-spectrum immunomodulatory agents, such as glatiramer acetate and interferon β, monoclonal antibodies, such as rituximab and natalizumab, and the recently discovered fingolimod, a sphingosine-1-phosphate-receptor modulator) [17, 18]. However, all these patients had interrupted previous therapies almost 2 months before starting chelation treatment. Five of these MS patients had never been previously treated with drugs.

Nineteen patients affected by ND were also recruited as well as 11 subjects not affected by any known disease but previously exposed to environmental or occupational heavy metals, who decided to start chelation therapy and acted as controls. Subjects' age ranged from 18 to 75.

All subjects provided written informed consent to participate in this study. Declaration of Helsinki and all procedures involving human participants were approved by the Milan University's Ethical Advisory Committee (number 64/14).

2.2. Study Design. All subjects (ND and controls) underwent chelation therapy for 3 months. EDTA is endowed with antioxidant properties; in fact, without any added vitamin C, it can decrease oxidative DNA damage and lipid peroxidation [19]. However, since its administration occurred once a week, the subjects were treated daily with antioxidants. At the beginning (basal values) and at the end of the treatments (after 3 months), blood lipid panel, homocysteine metabolism, and some oxidative stress parameters were evaluated.

2.3. Chelation Test. All ND patients and controls had been subjected to "chelation test" in order to verify their possible burden by toxic metals. Generally, for the "chelation test," EDTA (2 g), diluted in 500 mL physiological saline (Farmax srl, Brescia, Italy), is slowly (in about 2 hours) administered intravenously in subjects who are invited to collect urine samples before and after the first intravenous EDTA treatment. Urine collection following chelation treatment lasted 12 hours. Urine samples are accurately enveloped in sterile vials and sent to the Laboratory of Toxicology (Doctor's Data Inc., St. Charles, IL, USA) to be analysed, as previously reported [12]. Briefly, samples are acid-digested with certified metal-free acids (digestion takes place in a closed-vessel microwave digestion system), diluted with ultrapure water, and carried out via inductively coupled plasma with mass spectrometry (ICP-MS) utilizing collision/reaction cell methods coupled with ion-molecule chemistry, a reliable new method for reducing interference. Urine standards, both certified and in-house, are used for quality control and validation of data. To avoid the potentially great margin of error due to fluid intake and sample volume, results were reported in micrograms (μg) per g of creatinine.

When the first "chelation test" showed intoxication by heavy metals, our subjects started chelation therapy once a week.

2.4. Antioxidant Supplementation

2.4.1. Cellfood Treatment. Cellfood (Eurodream, La Spezia, Italy) is an antioxidant nutritional supplement containing 78 ionic/colloidal trace elements and minerals combined with 34 enzymes and 17 amino acids, all suspended in a solution of deuterium sulphate, efficient in protecting against oxidative damage *in vitro* [20]. A gradually increasing concentration of Cellfood was administered daily to subjects (22 ND patients and 6 controls) according to the following scheme: the first, second, and third day 1 drop in mineral water three times a day, the fourth, fifth, and sixth day 2 drops, the seventh and eighth day 3 drops three times a day, that is, 1 drop more three

times a day, and finally 20 drops altogether were given three times a day. The treatment lasted three months.

2.4.2. Other Antioxidant Treatments. Twenty-two patients (17 ND and 5 controls) took daily other antioxidants, instead of Cellfood. Particularly, 10 of them (6 ND and 4 controls) took α-lipoic acid (400 mg/day), the other 10 glutathione (Ultrathione, 500 mg/day), alone or together with multivitamin complexes, aminoacid and mineral mixtures, or probiotics. Also these treatments lasted three months.

2.5. Evaluation of Blood Parameters

2.5.1. Sample Collection. Biochemical parameters were measured in blood drawn from patients before starting therapy with EDTA and antioxidants (basal values) and after three months.

Peripheral blood samples were collected after overnight fasting into preevacuated and light-protected tubes, with no additive or with EDTA, in order to evaluate oxidative status (ROS; TAC; oxLDL) and glutathione and homocysteine metabolism (homocysteine, Hcy; holotranscobalamin, active B12; serum folate, s-Fol; erythrocyte folate, ery-Fol).

Serum aliquots were used to measure ROS, TAC, oxLDL, active B12, and s-Fol concentrations while EDTA whole blood was used for glutathione and ery-Fol levels determination. The remaining EDTA whole blood sample was centrifuged within 30 minutes to obtain plasma for total Hcy determination.

All the aliquots, except for the one used for blood counting, were immediately frozen and stored at −80°C ready for assay.

A 12-hour urine sample was used for the "chelation test," as previously described.

2.5.2. Oxidative Status. Serum ROS expressed as Carratelli Units (UCarr), oxLDL concentrations, and TAC were measured by using a commercial enzyme-linked immunoabsorbent assay (ELISA, Mercodia, Uppsala, Sweden) on the EASIA reader (Medgenix Diagnostics, Fleurus, Belgium) and by using spectrophotometric commercial kits (dROMs test, Diacron International, Grosseto, Italy; OXY-adsorbent test, Diacron International, Grosseto, Italy) on F.R.E.E. analyzer (Free Radical Elective Evaluator analyzer, Diacron International, Grosseto Italy) (Diacron), respectively.

Total and free glutathione concentrations were assessed by HPLC method followed by fluorescent detection using a commercially available kit (Chromsystems Instruments & Chemicals, Munich, Germany). Total glutathione is the sum of oxidized (GSSG) and free (GSH) glutathione existing in the sample prior to reduction. Briefly, since chromatography can only determine GSH, GSSG present in the sample was converted into GSH by using a reduction reagent which reduced one GSSG molecule to 2 GSH molecules obtaining total glutathione. GSSG concentration was calculated by subtracting the GSH amount from the total glutathione. GSH/GSSG ratio was also calculated and used as an oxidative stress marker.

2.5.3. Homocysteine Metabolism. Plasma Hcy levels were measured using homocysteine liquid enzymatic assay (Sentinel Diagnostics, Milan, Italy) on Modular P analyser (Roche Diagnostics, Indianapolis, IN, USA). Serum active B12, s-Fol, and ery-Fol concentrations were determined using the relevant Abbott Microparticle Enzyme Immunoassay (MEIA) kits (Holotranscobalamin-Active-B12 and Architect Folate, Abbott Laboratories, Abbott Park, IL, USA) on Architect analyser (Abbott).

2.5.4. Lipid Panel. Serum total cholesterol (TC), high-density lipoprotein (HDL) cholesterol, and low-density lipoprotein (LDL) cholesterol concentrations were determined using the routine tests on Modular P analyser. Total/HDL cholesterol and LDL/HDL cholesterol ratios were calculated together with oxLDL/HDL and oxLDL/LDL ratios.

2.6. Statistical Analysis. Data were analyzed by analysis of variance (ANOVA) with the solution type as main factor. Post hoc comparisons were made using Tukey's honestly significant difference test (HSD).

3. Results

3.1. Patients' Characteristics. As shown in Figure 1, eleven of these subjects were classified as controls (C) because they were not affected by ND or other known diseases. Six of them took Cellfood and five took other antioxidants. Thirty-nine patients were classified as ND: 20 MS, 5 ALS, 9 PD, and 5 AD. Twenty-two of them took Cellfood while the other seventeen received other antioxidants. The subjects of these two groups were matched for age, sex, disease duration, and previous drug treatments. Mean age of each group was 43 ± 5. Subjects' basal values were obtained before the beginning of each treatment.

3.2. Chelation Therapy. The first "chelation test" of our subjects showed intoxication by heavy metals with prevalence of lead, cadmium, aluminium, and gadolinium (used as contrast agent in magnetic resonance imaging to diagnose MS) (data not shown). Consequently, the subjects were administered chelation therapy once a week.

3.3. Oxidative Status Parameters. Figure 2 shows the active B12, s-Fol, and Hcy concentrations as basal values and after 3 months' treatment (chelation + Cellfood or chelation + other antioxidants). After chelation + Cellfood treatment, low basal levels of active B12 improved significantly, both in controls and in ND patients; moreover, a significant decrease in Hcy levels but no significant variations in s-Fol concentrations were observed. Basal ROS levels were significantly higher and basal TAC levels were significantly lower in ND patients than in controls, as shown in Figure 3. Of note, in both controls and ND patients, only chelation + Cellfood treatment improved TAC and ROS values significantly compared with chelation + other antioxidants. Figure 4 shows that cholesterol profile improved more in controls than in ND patients after both antioxidant treatments, while chelation + Cellfood

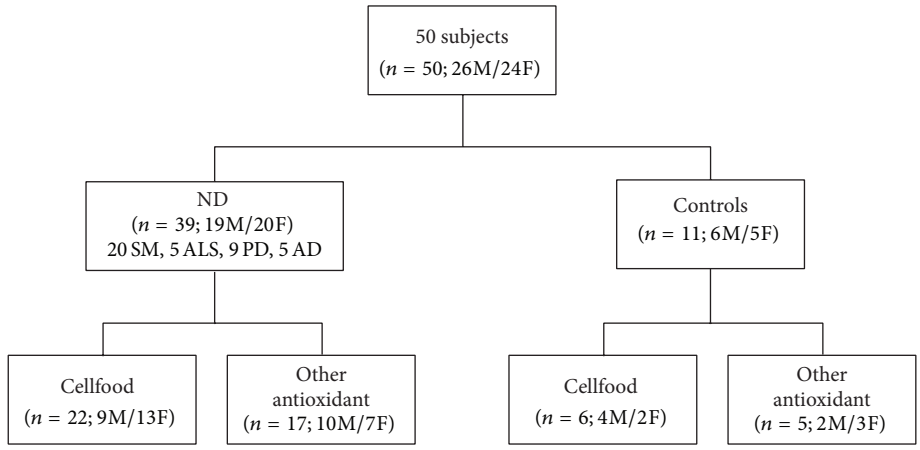

Figure 1: Scheme of enrolled subject's characteristics.

treatment was significantly effective on both groups. Notably, also oxLDL levels decreased, even if not significantly so, in both groups. In addition, as reported in Figure 5, all ratios improved after both treatments in ND patients and in controls [21, 22].

High ROS levels were associated with low GSH values. At baseline, GSH levels were significantly higher in controls than in ND patients. Chelation + Cellfood treatment significantly increased GSH levels in ND patients (Figure 6). On the whole, our findings showed that chelation + Cellfood treatment was much more efficient than other antioxidant treatments.

4. Discussion

Much attention has been recently devoted to red-ox processes involving ROS in neurodegenerative diseases (ND) [23]. In particular, the protective role of antioxidants could possibly have clinical implications in PD, AD, ALS, and MS. Antioxidants contained in natural foods are really considered part of nutraceuticals, that is, compounds with a significant role in modifying and preserving healthy physiological functions [24]. Even if different ND may have unrelated pathophysiology, the role of ROS is recognised in the etiology of all ND. The effectiveness of nutritional antioxidants in some human diseases has been reported in this context [25, 26]. Therapies administrated to patients affected by serious ND cannot be limited to antioxidant integrators. The literature indicates that specific antioxidants can have chelating capacities and that a synergism exists between antioxidants and chelating agents [27].

To the best of our knowledge, ours is the first study showing the improvement in antioxidant capacity due to a treatment with antioxidants in subjects affected by chronic body burden of heavy metals on chelation therapy: some of them were affected by ND while others were unaffected by known diseases. We examined how oxidative and metabolic parameters can improve in ND patients. EDTA chelation therapy was administered to all the subjects recruited as required by urine chelation test results (data not shown). We measured blood levels of some oxidative and metabolic parameters before starting antioxidant treatments and three months after. Our results highlight how helpful chelation + antioxidant treatment was; α-lipoic acid or glutathione, whether or not associated with vitamins, aminoacids, minerals, and probiotics, was taken daily by about 50% of the subjects and Cellfood was taken by the remaining 50%.

Cellfood is a nutraceutical, antioxidant supplement containing natural trace elements and minerals combined with enzymes and amino acids, suspended in a solution of deuterium sulphate, efficient in protecting against oxidative damage [20]. In a previous study we showed that Cellfood treatment *in vitro* increased mitochondrial metabolism in endothelial cells [11]. In this study we showed that chelation + Cellfood was significantly more efficient than chelation + other antioxidants in modifying ND patients and controls oxidative and metabolic parameters. In fact, oxidative status parameters showed that chelation + Cellfood improved TAC and GSH values significantly by reducing ROS levels significantly and lowering oxLDL. Moreover, chelation + Cellfood improved cholesterol profile increasing HDL levels significantly. The effects of Cellfood were more evident in ND patients than in controls. In addition, chelation + Cellfood treatment increased active B12 and serum folate and reduced homocysteine levels significantly, as expected.

Some authors suggested that dietary polyphenols can protect subjects against ND. Indeed, olive polyphenol administration increased the levels of the nerve growth factor and brain-derived neurotrophic factor in mice brains [28]. Similarly, α-lipoic acid prevented damage induced by 6-hydroxydopamine or by chronic use of L-DOPA in dopaminergic neurons in a PD animal model [29]. A new glutathione derivative, endowed with an 8-hydroxyquinoline group as a metal-chelating moiety, displayed neuroprotective activities *in vitro* [30]. Some traditional vitamins seem to act as antioxidants on ND [31]. Moreover, a multivitamin supplement enriched with phytosterol reduced serum cholesterol in hyperlipidemic rats [32]. In addition, treatment with a novel oral nutraceutical formula containing omega-3 and omega-6 fatty acids and vitamins (vitamin A and vitamin E) reduced the risk of sustained disability progression without

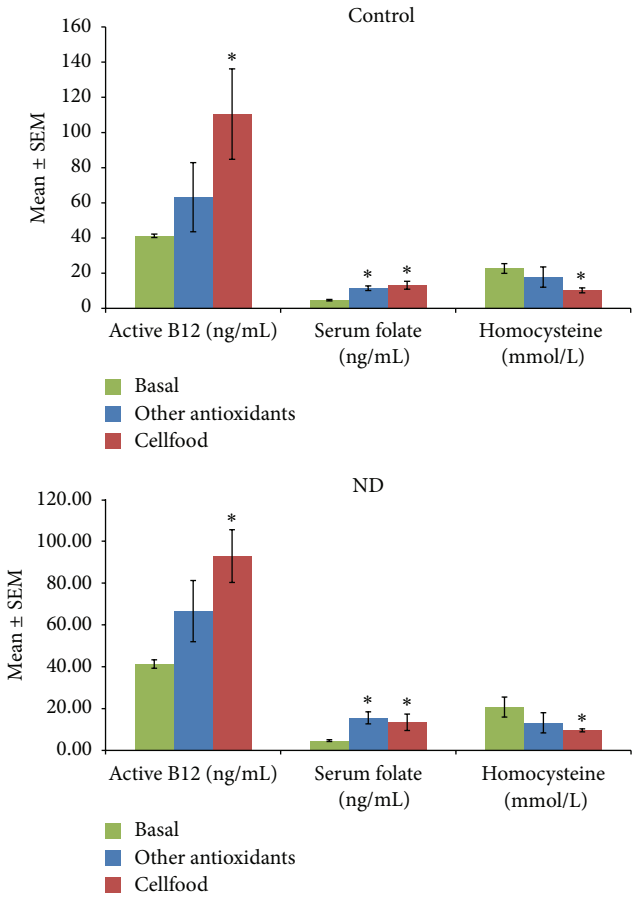

Figure 2: Serum active Vit B12, folate, and homocysteine levels measured in subjects unaffected (controls) or patients affected by neurodegenerative diseases (ND). All subjects' concentrations were determined before the beginning (basal values) and after three months of treatment with Cellfood or other antioxidants (see text). $^{*}P < 0.05$ versus all basal values.

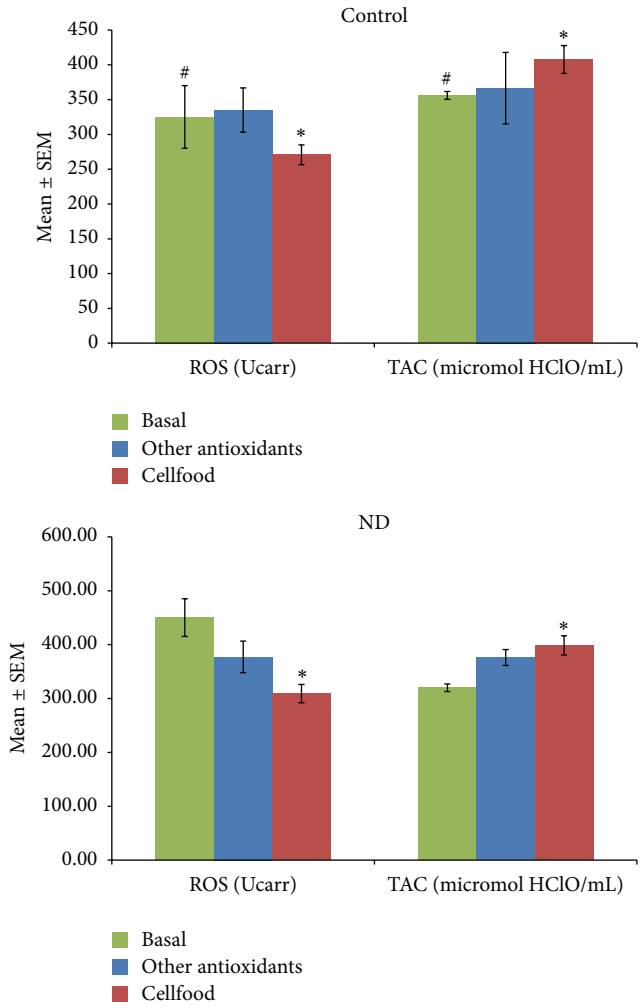

Figure 3: Serum reactive oxygen species (ROS) levels and total antioxidant capacity (TAC) measured in subjects unaffected (control) or patients affected by neurodegenerative diseases (ND). All subjects' concentrations were determined before the beginning (basal values) and after three months of treatment with Cellfood or other antioxidants (see text). $^{*}P < 0.05$ versus all basal values; $^{\#}P < 0.05$ C versus ND (basal values).

any serious adverse events in patients with relapsing remitting MS [33]. As mitochondrial dysfunction has been associated with the aging process and a large variety of human disorders such as ND [9], mitochondria became an interesting target for therapy. For instance, mitochondria-targeted antioxidants have been recently developed to treat PD [34].

Previous results indicated a correlation between elevated plasma cholesterol levels and cognitive impairment in AD patients [35]. In MS patients serum oxidized LDL concentrations have been proposed as a marker of clinical staging, since an increase in their levels was associated with expanded disability status scale [36]. Moreover, according to Kardys et al., high cholesterol affected retinal nerve fibre layer thickness in MS patients with optic neuritis [37]. In addition, as reported by Weinstock-Guttman et al., higher LDL levels associated with an increasing number of contrast agents caused ever increasing brain lesions in MS interferon beta-treated patients [38]. Therefore, the lowering of TC and TC/HDL ratios in our subjects treated with chelation + antioxidants suggests that these molecules could be given to MS patients. At baseline all our subjects' parameters of lipid profile generally were within normal values, and, interestingly, after a 3-month chelation + antioxidant treatment, there was a significant increase in HDL levels and decrease in TC concentrations in both groups on Cellfood. Moreover, according to Pawlak et al.'s report [21], lipoprotein ratios confirmed the beneficial effects of antioxidants in preventing the risk of cardiovascular diseases; this appeared more evident in controls than in ND patients. These ratios can help predict the degree of clinical benefits lowering risk levels well below the target of secondary prevention. These promising results are confirmed by the decreasing levels of oxLDL (an important biomarker of lipoprotein abnormalities and oxidative stress associated with atherosclerosis) and the decreasing oxLDL/LDL ratio (a new and more potent biomarker than standard lipid assessment) [21].

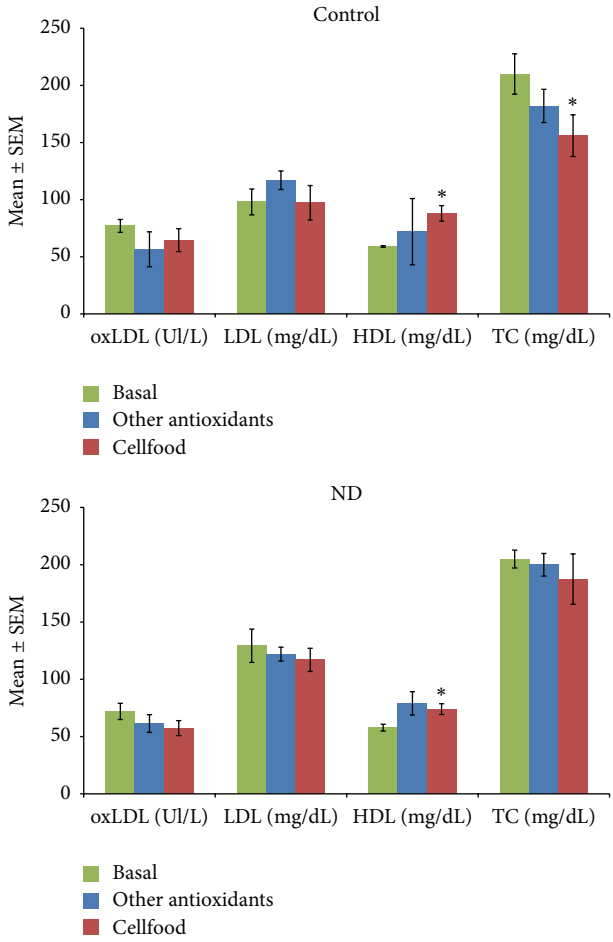

Figure 4: Serum oxidized LDL (oxLDL), LDL, HDL, and total cholesterol (TC) levels measured in subjects unaffected (controls) or patients affected by neurodegenerative diseases (ND). All subjects' concentrations were determined before the beginning (basal values) and after three months of treatment with Cellfood or other antioxidants (see text). $^{*}P < 0.05$ versus all basal values.

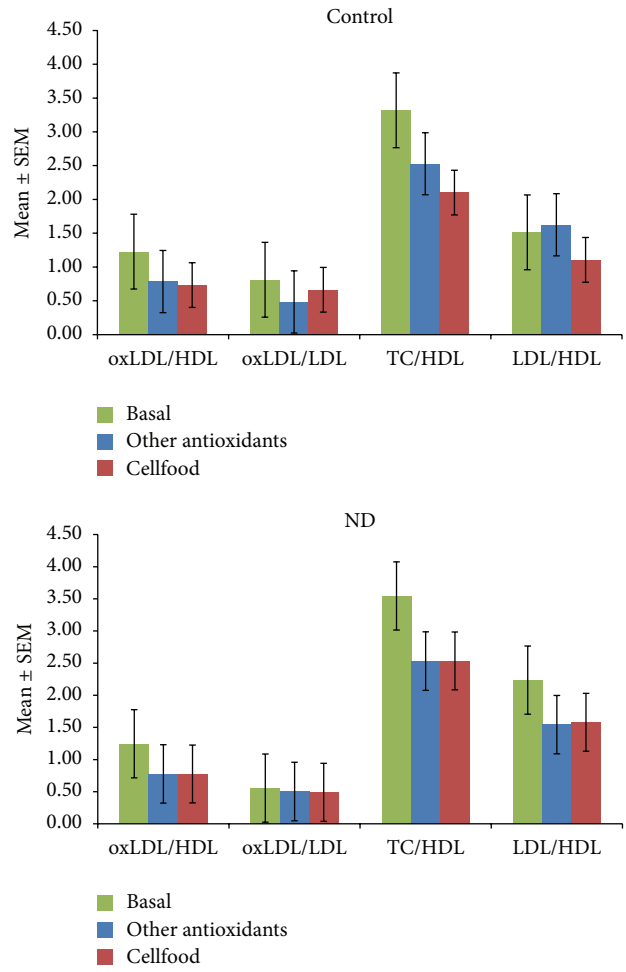

Figure 5: Oxidized LDL (oxLDL)/LDL ratios, oxLDL/LDL ratios, total cholesterol (TC)/HDL ratios, and LDL/HDL ratios calculated from the levels of subjects unaffected (controls) or patients affected by neurodegenerative diseases (ND). All subjects' values were determined before the beginning (basal values) and after three months of treatment with Cellfood or other antioxidants (see text).

According to other authors' findings, homocysteine induces oxidative stress by promoting ROS production, by increasing NADPH oxidase, and by decreasing thioredoxin [39, 40]. Consequently, we decided to evaluate also Hcy metabolism.

Homocysteine, a metabolic intermediate of methionine, is the crucial aminothiol for the biosynthesis of other aminothiols (methionine and cysteine) and for cell red-ox balance (glutathione biosynthesis). Hyperhomocysteinemia has been implicated in the pathogenesis of atherosclerosis and is considered an independent marker of ischaemic stroke. A lack of vitamin B12 and/or folate leads to an accumulation of Hcy in the blood and is responsible for macrocytic anaemia and, often, irreversible neurological damage [41]. Neurological damage due to vitamin deficiency is quite a common condition: up to 75% of B12-deficient patients can have neurological or neuropsychiatric symptoms even if not anaemic. The earliest marker of a negative B12 balance is probably a low active B12 level (the complex vitamin B12-transcobalamin II) which is the critical transporter of cobalamin to peripheral tissues and can reflect vitamin B12 availability in the body [42]. In our study, after chelation + Cellfood treatment all subjects showed a significant increase in active B12 levels and a significant decrease in Hcy concentrations. Additionally, both chelation + antioxidant treatments increased folate concentrations significantly and helped control prooxidant molecule production. Chronic oxidative stress condition, in fact, could cause irreversible damage to cellular homeostasis.

Glutathione (GSH) is produced intracellularly from three amino acids (glutamate, cysteine, and glycine). GSH is oxidized to GSH disulfide (GSSG) by gluthatione peroxidase (GP) and then regenerated as GSH by the reaction with GSH reductase (GR) [43]. GSH plays a key role in cell resistance against oxidative damage by providing enzymes involved in ROS metabolism with reducing equivalents, by eliminating potentially toxic oxidation products, and by reducing oxidized protein thiols [44]. GSH availability under oxidative

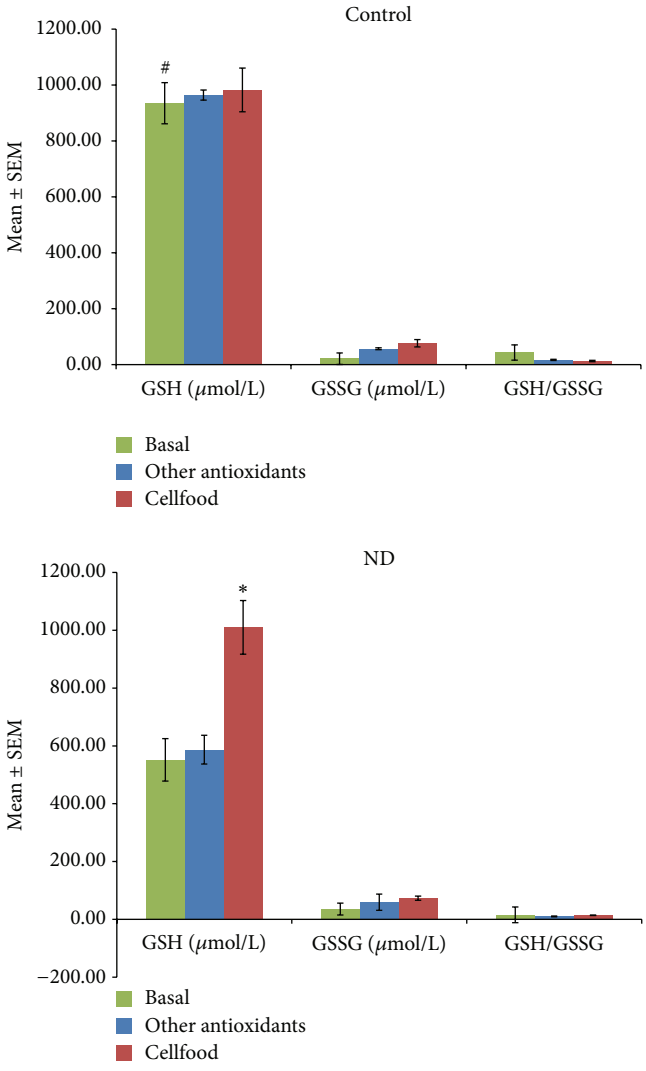

Figure 6: Free glutathione (GSH) and oxidized glutathione (GSSH) levels and GSH/GSSH ratios measured in blood obtained from subjects unaffected (control) or patients affected by neurodegenerative diseases (ND). All subjects' values were determined before the beginning (basal values) and after three months of treatment with Cellfood or other antioxidants (see text). $^{*}P < 0.05$ versus all basal values; $^{\#}P < 0.05$ C versus ND (basal values).

conditions is ensured by GSH recycling and biosynthetic pathways, which can be upregulated when oxidative stress occurs. Measurement of GSH and its disulfide forms (i.e., GSSG) in blood and their ratios is considered an index of the whole-organism oxidative status and a useful indicator of disease risk in humans [45]. Recently, Aoyama and Nakaki demonstrated that GSH function disorder is implicated in the aetiology of some neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, progressive supranuclear palsy (PSP), Huntington's disease (HD), and multiple sclerosis [44]. According to this study, our ND patients showed lower GSH levels at baseline than controls. However, three-month treatment with chelation + Cellfood increased significantly GSH levels in ND patients.

Our subjects showed an oxidative stress condition probably due to accumulation of toxic metals in tissues which weaken the antioxidant system even in apparently healthy subjects (controls). Several studies indicate that exposure to heavy metals could affect the antioxidant potential of blood in people exposed to toxic elements. The mechanisms of metal-induced damage in mammalian systems include the production of ROS by altering cellular oxidative status of membranes and tissues and/or by directly lowering antioxidant reserves [46, 47]. Latency period (sometimes years) between a probable exposure to toxic substances and the onset of clinically evident ND depends on individual reactions and is not to be underestimated.

As concerns the exposure to toxic substances, during a prevention program in some Italian towns, we showed a decrease in TAC only in one population of a southern Italian village due to the high levels of arsenic in local spring water; accumulation of this toxic metal in tissues appeared to weaken the antioxidant defence system in apparently healthy subjects [48].

In conclusion, our study, though limited to a small number of cases, showed that easily detectable blood parameters can be a useful tool during chelation therapy with EDTA and antioxidant treatment.

These treatments can help counteract nutritional/environmental/occupational/pharmacological toxicity, if any.

Finally, chelation + Cellfood treatment was significantly more efficient than chelation + other antioxidant treatments in improving most parameters both in controls and in ND patients. This pilot study suggests that antioxidant treatment with Cellfood helps improve metabolic conditions.

Conflict of Interests

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

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Review Article

Gambling Disorder during Dopamine Replacement Treatment in Parkinson's Disease: A Comprehensive Review

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Gambling Disorder (GD) is characterized by "the failure to resist gambling impulses despite severe personal, family or occupational consequences". In the fifth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-V), GD replaces the DSM-IV diagnosis of Pathological Gambling (PG). GD estimated prevalence ranges between 0.4% and 3.4% within the adult population and it seems to be more common in patients with Parkinson's disease (PD). In this population, GD recently has become more widely recognized as a possible complication of dopamine agonist (DA) therapy. This association has aroused great interest for the dramatic impact GD has on patients' quality of life. Management of PG in patients with PD could be demanding. It is based on patient and caregiver education, modification of dopamine replacement therapy, and in some cases psychoactive drug administration. In this review article, the authors provide an overview of GD pathogenesis during DA therapy as well as a summary of available treatment options.

1. Introduction

Gambling Disorder (GD) is characterized by "the failure to resist gambling impulses despite severe personal, family or occupational consequences." In the fifth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-V), GD replaces the DSM-IV diagnosis of Pathological Gambling (PG) [1]. DSM-IV classified this disorder as an Impulse Control Disorder (ICD) [2]. GD differs from PG in that it requires 4 rather than 5 criteria for diagnosis and excludes the "Illegal Acts" criterion [1]. The DSM-5 work group moved PG to the category "Addiction and Related Disorders" [3]. The rationale for this change is that the growing scientific literature on PG reveals common elements with substance use disorders. Brain imaging studies and neurochemical tests have made a "strong case that [gambling] activates the reward system in much the same way that a drug does" [4].

GD estimated prevalence ranges between 0.4% and 3.4% within the adult population [5–7]. GD, along with compulsive sexual behaviour, compulsive buying, the addiction-like compulsive use of dopamine replacement therapy, or dopamine dysregulation syndrome (DDS) [8], seems to be more common in patients with Parkinson's disease (PD) than in the general population [9]. GD is reported as a side effect of dopamine agonist (DA) therapy used in PD [10, 11], with a dramatic impact on the quality of life of patients and their caregivers. This review describes some aspects of GD pathogenesis during DA therapy and its management.

2. Epidemiology and Risk Factors

GD prevalence in North America is reported to be between 0.4% and 1.9% within the adult population [10, 12–14]. In

PD, some evidence suggests that GD is associated with an early onset disease, longer disease duration and high novelty seeking personality traits [10, 15, 16]. Other independent risk factors include younger age, male sex, cigarette smoking, prior personal or family history of alcohol addiction and impulse traits [17–20]. According to the available data, GD prevalence rates in PD may vary considerably, ranging from 6% in PD patients not receiving DA to 17% among those on DA treatment [21]. In PD patients under DA therapy, concurrent levodopa use increases the risk to develop GD by approximately 50% [17]. GD involves a subset of patients only, suggesting an underlying susceptibility, mediated by PD-specific factors such as a dysregulation of dopaminergic system, which may also modulate underlying temperament traits. The psychological profile of PD patients may have a role as a risk factor, since impulse sensation seeking personality traits and addiction proneness characterized PD patients who develop GD.

Some authors suggest that DA, but not L-dopa treatment, may worsen executive functions in patients affected by early/mild PD [22]. DAs, compared with L-dopa, have significantly greater affinity for D3 receptors (approximately 20 to 100 times more affinity for D3 than D2), and little or no affinity for D1 receptors [23].

Voon et al. observed that GD was associated with DAs but not with agonist subtype or doses: both D1/D2 (pergolide) and D2/D3 (ropinirole and pramipexole) agonists were equally implicated [10, 21]. However, the authors do not rule out D3 mechanisms, given that pergolide may have greater D3 than D1 receptor affinity [24]. Other authors confirmed these data, finding that agonist dose and duration were non-significant.

No differences were observed between pramipexole, ropinirole, and pergolide in their association with GD [25], and DA doses did not predict GD development [26].

Thondam and coworkers reported a case of a young patient that developed severe, socially disruptive impulsivity manifesting with pathological gambling during a long-term bromocriptine therapy [27].

Other retrospective reports suggest a different role of specific dopamine receptor agonists, considering their different dopamine receptor affinity [28, 29]. These authors found an increased prevalence of GD in PD patients treated with pramipexole, compared with other dopamine receptor agonists. In these patients, GD may develop for an excessive stimulation of D3 receptors.

The role of DA dose in increasing GD risk is still not clear [19, 30]. Perez-Lloret et al., documented that PD patients with impulse-control disorder symptoms were exposed to higher dopamine doses than those without them (1.6 ± 0.1 versus 1.0 ± 0.1 daily-defined doses). However, using a dose-response pharmacodynamic model authors disclosed a significant nonlinear dose-response relationship between dopamine agonists and frequency of ICD symptoms [31].

Moreover, recently in a retrospective study performed on 20 patients with PD, Castrioto and coworkers documented that high chronic dopaminergic treatment (mean levodopa equivalent daily doses 1420/mg) induced pathological hyperdopaminergic behaviours in 8/20 patients, which had resolved in 7/8 patients when the dosage was reduced (mean levodopa equivalent daily doses 320/mg) [32].

Mood disorders also represent powerful risk factors, and low performance in cognitive tasks requiring frontal function has been associated with GD [15, 33, 34].

3. Clinical Manifestations

GD is defined as failure to resist gambling impulses despite severe consequences on personal, family or professional life. It is often under-recognized: patients very rarely give spontaneous information about it, do not understanding that GD may be related to PD treatment [35].

Lives of patients affected by GD become dominated by gambling behaviour, leading to overwhelming financial burdens, inability to maintain a career, and eventually the disintegration of family relationships [36]. The most frequent attitudes are slot machines, lottery scratch cards and bingo, and GD occurs more frequently during the "on" phase [37].

4. GD: Neurotransmitters and Pathophysiology

GD shares diagnostic features with substance use disorder (SUD), and its pathophysiology involves specific neurotransmitter systems, brain regions and neural circuitries. The main neural pathways seem to be corticostriato-thalamo-cortical circuitry and mesocorticolimbic pathway for reward and reinforcement processes.

4.1. Neurotransmitters. Dopamine (DA), a neurotransmitter implicated in reward and reinforcement, is probably involved in GD pathogenesis and some authors used positron emission tomography (PET) to test whether GD is associated with abnormalities in D2 and D3 receptor levels, as observed in SUD. They compared D2 and D3 receptor binding between subjects with and without GD, and assessed the relationship between binding profiles, impulsiveness (a known predisposing trait) and gambling severity. Unlike with substance use disorder, there appear to be no marked differences in D2/D3 levels between healthy subjects and pathological gamblers, suggesting that low receptor availability may not be a necessary feature of addiction. However, relationships between [11C]-(+)-PHNO binding and gambling severity/impulsiveness suggests involvement of the D3 receptor in impulsive/compulsive behaviours. These authors suggest that strategies focused on the D3 system may be effective in treating some individuals with GD [38].

Several functional imaging studies provided further evidences about the involvement of specific brain regions in PG behaviours. Areas such as prefrontal cortex (ventromedial and orbitofrontal areas), ventral striatum (nucleus accumbens) and amygdala showed a reduced activation in pathological gamblers during fMRI studies, suggesting a relationship with aberrant reward and response inhibition [39]. In another fMRI study about motivational and emotional states in men with and without PG, subjects with PG reported stronger gambling urges and showed relatively reduced activation of

frontal cortical, basal ganglionic and thalamic brain regions while viewing gambling tapes, during the period prior to the onset of subjective motivational or emotional response [40]. In a [11C] raclopride positron emission tomography (PET) study, Steeves et al. assessed dopaminergic functions during gambling in PD patients. PG patients demonstrated a greater reduction in binding potential in the ventral striatum during gambling compared with control subjects, reflecting greater dopaminergic release. Similar findings are reported in subjects with chemical addictions [41]. In a recent study, the authors found that PD patients with PG have abnormal resting state dysfunction of the mesocortico-limbic network on SPECT imaging, possibly associated with a drug-induced overstimulation of relatively preserved reward-related neuronal systems [42]. All these findings, based on different functional imaging studies, show that PG shares many features with drugs addiction such as the relation with a deficiency of the mesolimbic dopaminergic reward system.

Most recently, neuroimaging has provided new evidence that increased susceptibility for ICD and addiction associated with the impulsive personality trait may be dependent on normal variations in brain function that, in PD, interact with DA agonist exposure to produce pathological behaviours [43].

Similarly to what has been observed in drugs addicts, a reduced activation in prefrontal cortex (ventromedial area) has been observed in patients with GD. These data support the view of GD within the spectrum of behavioural addictive disorder.

The development of GD in patients receiving low doses of dopaminergic drugs suggests that a genetic predisposition may play a role in some cases [44]. Lee et al. described a variant of the serotonin 2A receptor gene (HTR2A) associated with GD in PD patients receiving DA therapy, mainly those taking low doses of dopaminergic drugs [45].

Other neurotransmitters may have a role in GD pathophysiology. Serotonin (5-HT) has been implicated in control over motivated behaviours. Abnormalities in 5-HT function have been described in subjects with PG [46]. Dopamine has been involved in reinforcing and rewarding behaviours, and it has long been associated with these processes in drug addiction [6].

Norepinephrine (NE) system has been involved in drug relapse, reward and sensitization, and high NE levels have been observed in CSF and urine samples of subjects with GD as compared to controls [47, 48]. A decrease of GABAergic neurotransmission system is involved in the expression of behavioural impulsivity [49–51].

4.2. Pathophysiology. In scientific literature there are different hypotheses for the association between GD and dopaminergic stimulation in PD patients.

Even if D1, D2 and D3 receptors are involved in motor responses, the activation of D1 and D2 receptors, located in the dorsal striatum, is associated mainly with the motor effects of the medications. In contrast, the activation of D3 receptors located in mesolimbic pathways and in areas such as nucleus accumbens and olfactory tubercle is involved in motivation and reward behaviours [23]. GD may be due to an excessive stimulation of this D3 receptor subtype [29, 52–55].

PD is characterized by a massive loss of dopaminergic neurons in the substantia nigra, with a pronounced depletion of dopamine in the nigrostriatal pathway and a decreased stimulation of the striatal D1 and D2 receptors [56]. This depletion leads to disturbance in the cognitive, limbic, and associative corticobasal gangliathalamo-cortical loops, and might predispose to the occurrence of GD in PD. PD patients, even in the absence of dementia or depression, are likely to show a range of clinically significant impairment in executive functions, most probably linked to degeneration in the basal ganglia-thalamo-cortical circuits (striatal-frontal tracts), secondary to cell loss within the substantia nigra (SN) (due to decreased dopaminergic transmission in fronto-striatal loops) [57, 58].

L-dopa, in this stage of disease, may improve certain cognitive functions that are associated with the severely depleted dorsal striatum, while at the same time impairing other cognitive functions, associated with the relatively intact ventral striatum [59]. Thus, one explanation is that excessive, targeted dopamine stimulation of intact ventral striatal receptors in early or mild PD leads to an "overdose" of ventral striatal-cortical circuitry that can manifest itself in the clinical phenomenon of impulsive-compulsive behaviours, such as GD. These behaviours are maintained by ongoing dopaminergic stimulation of a sensitized ventral striatal system, which is manifested clinically as an increased drive for certain behaviours and maintained by an inability to learn from negative decision outcomes [60].

Therefore, all these data have provided insight into the neurochemical, neuroanatomical and functional basis of GD developing in PD patients showing that DA agonists may induce changes in brain function that impair patients' ability to learn appropriately from both reward and punishment.

5. GD Management in Patients with PD

GD management in patients with PD is challenging and there are limited data to support any particular therapeutic strategy. Its association with DA therapy suggests that dopaminergic treatment modifications may be effective. Compulsive behaviours often resolve after DA tapering, switching to a different agonist or discontinuing DA entirely [28, 29, 61].

Some authors reported that 80% of patients discontinuing or significantly decreasing DA doses, or switching to a different agonist, experienced full or partial remission of GD symptoms [29, 62]. Many PD patients are reluctant to discontinue DA treatment because of the motor benefits associated with their use. Moreover, when reducing DA doses, a withdrawal syndrome (DAWS) may occur in a subset of patients, causing profound disability [63].

The first step in GD management is to try to prevent it. Before starting DA therapy, risk factors such as male sex, young age and a history of drug abuse should be taken into consideration. Another aspect is to identify subjects with GD, also involving caregivers.

Very limited data supporting the use of psychiatric drugs for GD in PD exist. The neurobiological similarities between GD and substance use disorders suggest that specific pharmacotherapies may be helpful in treating GD.

Atypical antipsychotics, antidepressants, mood stabilizers and various psychosocial interventions have been proposed to treat GD in patients with PD [28, 64]. The role of these various agents in the management of GD is not well established, and only few case reports have been published on this topic [8].

5.1. Serotonin Reuptake Inhibitors (SSRIs). Serotonin system has long been associated with impulse control and different studies support its role in GD [39, 65]. Decreased serotonin function within ventral medial prefrontal cortex may cause disinhibition and contribute to GD development. Drugs affecting serotonin neurotransmission may represent potential treatment for GD.

SSRI, though effective in obsessive-compulsive disorders, provide questionable benefit in GD, since they may facilitate dopaminergic transmission and could worsen gambling attitude. In one trial on fluvoxamine versus placebo, this drug was associated with a statistically significant improvement in GD [66], but this data was not confirmed in another study [67]. Paroxetine effect on GD was analyzed in two studies. The first one showed a significant effect of paroxetine on GD, evaluated with the Gambling Symptoms Assessment Scale [68]. The other one failed to demonstrate a significant benefit [69]. Sertraline did not prove to be superior to placebo [70]. Citalopram seems to be effective on GD in the general population [71]. SSRIs could have some short-term efficacy on GD in the general population, so they may be helpful in patients with co-occurring anxiety or mood disorders. Existing reports on the efficacy of SSRI for GD treatment in patients with PD have not been encouraging [72].

5.2. Mood Stabilizers and Antipsychotics. Lithium and valproate, an antiepileptic drugs that increase the GABA levels [73, 74], have been reported to be effective on gambling and manic symptoms compared to placebo in patients with GD and bipolar disorders [64, 75]. Recently patients with a dopamine dysregulation syndrome, a pathological condition that contribute to behavioral disturbances, and who were all refractory to medication adjustments responded by the addition of valproic acid [76].

There are some case reports on atypical antipsychotics usage in GD treatment. Low-dose risperidone may be effective in controlling GD behaviour in PD patients [11, 29, 54]. A positive effect of high-dose quetiapine in controlling gambling behaviour in a patient with PD have been observed [77]. N-desmethylclozapine, the major active plasma metabolite of the atypical antipsychotic clozapine has a potent partial agonist activity on dopamine D2/D3 receptors [78, 79]. Some authorsreport the effectiveness of clozapine on persistent gambling behaviour following discontinuation of DA therapy [72, 80, 81], although its use requires careful monitoring due to potential risk of agranulocytosis [78].

Controlled studies on atypical antipsychotic drug for GD in non-PD subjects showed that olanzapine is not effective in gambling behaviour treatment [65, 82, 83]. Some authors found that aripiprazole, a partial dopamine D2/D3 receptor agonists, may be effective in treating impulsive/compulsive and addictive behaviours via regulation of reward pathway circuitries [84, 85], although Cohen et al. reported in 3 non-PD patients that aripiprazole may induced PG [86]. Recently, Gaboriau and coworkers in a retrospective study performed in 166 non-PD patients with history of PG reported that in 8 of these patients, aripiprazole induced the development of PG that improved after its dismission [87]. In PD patients, aripiprazole-treatment may be related to exacerbation of motor symptoms [88]. A randomized trial on bupropion, a drug with monoamine reuptake inhibition and nicotinic receptor antagonism properties, failed to demonstrate its efficacy in GD [89].

Topiramate may play a role in GD treatment. It has been known to have a positive effect on binge eating disorder associated with obesity, and compulsive impulsive sexual behaviours in patients with psychiatric disorders [90, 91]. Topiramate has multiple mechanisms of action, in particular it is able to bind the GABAA receptors increasing the GABA levels with a concentration-dependent effect [73, 74, 92] and recently it has been shown to inhibit levodopa-induced dyskinesia in animal models, suggesting a possible inhibitory effect on dopaminergic drugs [93]. In a recent case report, the authors suggest that topiramate may be an effective therapy in PD patients with PG [94].

An open non-randomized trial on zonisamide in fifteen PD patients with GD demonstrated a marked reduction in impulsive behaviour severity, without clinically significant side effects [95].

5.3. Opioid Antagonists. Dopaminergic systems that influence rewarding and reinforcing behaviours have been implicated in GD. Gambling triggers dopamine release, which in turn may reinforce the pathological behaviour [96].

Opioid receptor antagonists are thought to decrease dopamine neurotransmission in the nucleus accumbens and in the motivational neurocircuitry. Their efficacy have been studied in PG treatment for their indirect modulation of mesolimbic dopamine circuitry and their role in alcohol and opiate dependence treatment [96].

In non-PD patients, a positive effect of naltrexone in PG treatment was demonstrated in a double-blind, placebo-controlled trial, with a statistically and clinically significant difference. Naltrexone was more effective in gamblers with more severe urges than in those who describe their urges to gamble as moderate [97]. An open-label study suggested naltrexone efficacy in reducing urge intensity to gamble when given in high doses (50 to 250 mg/day) [98]. These data were confirmed in another study in which naltrexone was administered at doses typically used in alcohol or opiate dependence, with a good safety profile [99]. However, clinical use of naltrexone is limited by its side effects. In a case report series, the authorsobserved that naltrexone could be an

effective option for GD treatment in PD patients who develop GD after DA therapy [100].

Nalmefene, another opioid antagonist, has been found to be effective in non-PD subjects with GD, but its efficacy is connected with dosage [101]. Another multicentre randomized controlled trial demonstrated that low dose nalmefene (25 mg/day) is effective on GD symptoms in the short-term, with few adverse events and without the dose-dependent hepatotoxicity of naltrexone [102].

5.4. Behavioural Therapies. In addition to pharmacological treatments, psychosocial interventions may be considered in GD management. Involving caregivers in the management of GD may be useful. Counselling and limiting access to money and medications in conjunction with tapering DA treatment have been effective in some patients [35].

Several non-pharmacological treatments have been studied in GD, such as behavioural, cognitive, and psychoanalytic therapy. Cognitive behavioural therapy or attendance at Gamblers Anonymous meetings may play a role in selected groups of patients with GD, having been associated with better outcome in non-PD subjects [103]. They were seen effective on gambling severity and frequency, and these effects were maintained over time [104, 105]. However, their effectiveness have not been examined in subjects with PD.

5.5. Deep Brain Stimulation. Deep brain stimulation (DBS) surgery of the subthalamic nucleus (STN) or globus pallidus internus may markedly improve "off"-medication motor symptoms, and STN DBS has the potential to allow significant reduction in drug dose [106]. Therefore, STN DBS could be seen as a treatment option for patient with dopaminergic drug related behaviours.

However, the relationship between DBS and GD seems to be complex. Few case reports and small case series have reported contrasting effects of STN DBS on dopamine misuse and GD, while a recent prospective study found clear beneficial effects of STN DBS on these disorders.

The efficacy of STN stimulation for GD in PD patients has not been fully clarified. Existing data are contradictory. Some case series suggest that bilateral DBS of STN may improve GD, allowing a decrease in levodopa dose or DA discontinuation [107]. Other evidence shows that GD may begin in the early postoperative period, and 71% of patients with pre-operative GD remained unimproved or worsened post-operatively [108–111]. Up to date, ICDs should not be considered an indication for DBS.

In an observational study on 110 consecutive parkinsonian patients scheduled for STN DBS surgery, Eusebio et al. suggest that STN DBS may reduce compulsive use of dopaminergic medication and its behavioural consequences. Whether this improvement is the result of STN DBS or the consequence of better treatment management remains to be established. Most of the addictive behaviours improve after STN DBS partly as a result of the lower dosage of dopaminergic medication but also possibly through a specific effect of STN DBS in the limbic circuit of motivation and reward [112].

6. Conclusions

In conclusion, there are very limited evidence on the efficacy of the treatment of GD symptoms in PD patients.

Management of GD in patients with PD under DA therapy is based on both patient and caregiver education (i.e., psychosocial interventions, such as counselling and cognitive behavioural therapy), modification of dopamine replacement therapy dosage to the lowest effective daily dose, or increase in L-dopa treatment and in some cases administration of topiramate or zonisamide, that up to date represent the only therapeutic option available in PD patients. Quetiapine and olanzapine represent a secondary line of therapy, although there are very few data supporting their role in PD.

Conflict of Interests

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

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Research Article

Improvements in Memory after Medial Septum Stimulation Are Associated with Changes in Hippocampal Cholinergic Activity and Neurogenesis

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Deep brain stimulation (DBS) has been found to have therapeutic effects in patients with dementia, but DBS mechanisms remain elusive. To provide evidence for the effectiveness of DBS as a treatment for dementia, we performed DBS in a rat model of dementia with intracerebroventricular administration of 192 IgG-saporins. We utilized four groups of rats, group 1, unlesioned control; group 2, cholinergic lesion; group 3, cholinergic lesion plus medial septum (MS) electrode implantation (sham stimulation); group 4, cholinergic lesions plus MS electrode implantation and stimulation. During the probe test in the water maze, performance of the lesion group decreased for measures of time spent and the number of swim crossings over the previous platform location. Interestingly, the stimulation group showed an equivalent performance to the normal group on all measures. And these are partially reversed by the electrode implantation. Acetylcholinesterase activity in the hippocampus was decreased in lesion and implantation groups, whereas activity in the stimulation group was not different from the normal group. Hippocampal neurogenesis was increased in the stimulation group. Our results revealed that DBS of MS restores spatial memory after damage to cholinergic neurons. This effect is associated with an increase in hippocampal cholinergic activity and neurogenesis.

1. Introduction

It is widely accepted that brain function can be modulated by electrical stimulation of focal brain structures; furthermore, that electrical stimulation may possibly be used to treat patients with brain dysfunction. In particular, deep brain stimulation (DBS) has been used to treat various types of movement disorders and psychiatric disorders [1–3]. Recently, DBS of memory-associated brain structures were tested as a possible treatment for Alzheimer's-type dementia, with some studies providing promising results. For example, some authors report that hypothalamic stimulation modulates limbic activity and improves certain memory functions [4], and others report that DBS of the nucleus basalis magnocellularis (NBM) improves cognitive functioning in patients with Parkinson's disease-related dementia [5]. Furthermore, stimulation of the entorhinal region was found to enhance memory for spatial information when applied during learning [6]. In animal studies, high frequency stimulation activates specific amino acids in the hippocampus

that may be involved in the enhancement of short-term memory formation [7]. Also, formation of water maze memory is facilitated after bilateral stimulation of the entorhinal cortex [8].

Clinical evidence obtained from patients with dementia or other neurological disorders shows that DBS could be used as a tool to enhance memory function. However, this evidence is not strong, as the clinical trials were not randomized and case-controlled or did not employ large sample sizes. Therefore, disease-specific animal experiments are necessary to advance the clinical application of DBS. There are varied results due to the administration of 192 IgG-saporin [9–14]. We made the experiment with a rat model of dementia by intracerebroventricular (ICV) 192 IgG-saporin injections. And we found that spatial memory is profoundly impaired in this model [15]. Here, we used the 192 IgG-saporin rat model of dementia to confirm the effects of DBS on spatial memory function.

Degeneration of cholinergic basal forebrain neurons, including the medial septum (MS), is a common feature of Alzheimer's disease and vascular dementia and has been correlated with cognitive decline [16–19]. Projections from the MS to the hippocampus consist of more than three types of fibers, including cholinergic, GABAergic, and glutamatergic fibers [20–22] and are the primary source of cholinergic input to the hippocampus [23, 24]. Furthermore, the MS is reported to regulate hippocampal activity through acetylcholine, GABA, and glutamate [25–28].

DBS therapy for dementia has not yet established the most effective stimulation site and parameter. In general, high frequency stimulation leads to long term potentiation, and low frequency stimulation induces long term depression [29]. However, recent reports showed that long term potentiation could be induced by low frequency stimulation [30, 31]. Some studies evaluating the hippocampal effect by the MS stimulation are mostly divided into low frequency (<10 Hz) or high frequency (>100 Hz) stimulation [32–34]. Therefore, we stimulated the MS with midfrequency (60 Hz) stimulation trains to evaluate the therapeutic potential of this frequency range for reversing spatial memory impairments induced by the loss of cholinergic neurons.

2. Materials and Methods

2.1. Animals. The present study was conducted according to the guidelines for the care and use of laboratory animals of the NATIONAL RESEARCH COUNCIL, USA. Rats were housed one to three per cage in a temperature- and humidity-controlled room, and all rats had free access to food and water with a 12-hour light/12-hour dark cycle.

Fifty male Sprague Dawley rats (200–250 g) were randomly assigned to one of the following groups. Rats in the normal ($n = 11$) group had no surgical procedure. Rats in the lesion ($n = 15$) group had ICV administration of 192 IgG-saporin. Rats in the implantation ($n = 14$) group had ICV administration of 192 IgG-saporin and implantation of an electrode in the MS. Rats in the stimulation ($n = 10$) group had ICV administration of 192 IgG-saporin and electrical stimulation of the MS.

2.2. Surgical Procedure. Thirty-nine rats were anesthetized with a mixture of ketamine (75 mg/kg), acepromazine (0.75 mg/kg), and rompun (4 mg/kg) and secured in a stereotaxic frame. After scalp incision, rats were injected bilaterally with 8 μL of 192 IgG-saporin (0.63 μg/μL, Chemion, Temecula, CA) into ICV following coordinates relating to Bregma: AP: −0.8 mm, ML: ±1.2 mm, and DV: −3.4 mm. The solutions were delivered at a rate of 1 μL/min. The syringe was left in place for 5 min after injection.

After the administration of 192 IgG-saporin, 24 rats had an additional procedure for electrode implantation. A hole was drilled in the skull at the level of the MS, and a tungsten electrode was implanted in the MS (AP + 0.6 mm, ML 0.1 mm, and DV − 6 mm). The stimulation electrode was fixed with dental cement (Long Dental Manufacturing, Wheeling, IL). This group was further divided into two groups, with 12 rats originally in the implantation group and 12 rats originally in the simulation group. However, two rats in the stimulation group were not stimulated due to a problem with the wire. Therefore, the implantation group consisted of 14 rats, and the stimulation group consisted of 10 rats.

2.3. Stimulation Electrode and Parameters. A tungsten stimulation electrode (200 μm diameter, 5 μm parylene coating) was inserted into the MS. The tip of electrode was tapered by electrochemical etching to reduce damage to the target area electrical stimulation consisted of pulses (120 μs duration, 50 μA) delivered at 60 Hz. Stimulation parameters were monitored in real time at the beginning and end of stimulation with an oscilloscope (HDS 1022M, Owon, Korea). Rats were stimulated daily beginning a week after surgery until the end of behavioral testing (5 consecutive hours per day, every day for 2 weeks). Stimulation was delivered after the daily water maze training session.

2.4. Behavioral Test: Morris Water Maze. Two weeks after surgery, rats were trained in the Morris water maze as previously described [15]. Training consisted of four trials per day for 5 days with the platform in a fixed position. For each training trial, the rat was placed into the pool at one of four semirandomly chosen starting points and given 60 s to reach the platform. Any rat that did not reach the platform within 60 s was led to the platform by the experimenter and allowed to remain on the platform for 10 s. After 48 hours from the final training trial, the rats were given a probe trial lasting 60 s, during which the platform was removed from the pool. Swim paths were recorded using a video tracking system. During training trials, swim distance, latency to reach the platform, and swim speed were measured. During the probe trial, swim distance, swim speed, swim time in each quadrant, the time spent in the platform zone, and the number of platform crossings were also measured.

2.5. Histological Evaluation. Immediately after behavioral testing (probe test), 8 out of 11 rats from the normal group, 8

out of 15 rats from the lesion group, 8 out of 14 rats from the implantation group, and 6 out of 10 rats from the stimulation group were anesthetized with a mixture of ketamine, acepromazine, and rompun and perfused with normal saline and cold 4% paraformaldehyde. Brains were removed, postfixed, and transferred to 30% sucrose for 4 days. The brains were sectioned into 30 μm sections using a freezing microtome and stored in a cryoprotectant solution (0.1 M phosphate buffer (pH 7.2), 30% sucrose, 1% polyvinylpyrrolidone, and 30% ethylene glycol) at −20°C. Anatomical landmarks from a stereotaxic atlas [35] were used to localize the MS and hippocampus.

Floating sections were used to detect location of electrode, cholinergic cells, and neurogenesis. Cresyl violet staining was performed to confirm the location of electrode. To perform immunohistochemistry, sections were incubated in 0.3% H_2O_2 for 30 min to inactivate endogenous peroxidase activity. They were blocked with 5% normal serum and incubated with polyclonal antibodies against choline acetyltransferase (1 : 200, ChAT, Chemicon, Temecula, CA) or doublecortin (1 : 200, Santa Cruz Biotechnology Inc., Santa Cruz, CA) overnight at 4°C. The sections were incubated with biotinylated secondary antibodies, followed by the avidin-biotin complex method (ABC Elite, Vector Labs, Burlingame, CA). They were visualized with diaminobenzidine (DAB) using a DAB substrate kit (Thermo, Fremont, CA).

2.6. Acetylcholinesterase (AChE) Assay. The remaining rats (3 out of 11 rats from the normal group, 7 out of 15 rats from the lesion group, 6 out of 14 rats from the implantation group, and 4 out of 10 rats from the stimulation group) were anesthetized with a mixture of ketamine, acepromazine, and rompun and decapitated with a guillotine. The brains were quickly removed to acquire protein for AChE assay. The MS and hippocampus were dissected with fine forceps from 1 mm coronal brain slices. The samples were homogenized in lysis buffer (Intron, Seongnam, Korea) and placed in ice for 30 min. The samples were centrifuged for 20 min at 12,000 rpm, and the protein in supernatant was measured using the bicinchoninic acid protein assay reagent kit (Pierce, Rockford, IL). The protein samples were stored at −70°C. The enzymatic activity of AChE was determined using the method of Ellman et al. [36] with some modifications as previously described [15]. Briefly, 20 μL triplicate samples were mixed with the reaction mixture (0.2 mM dithiobisnitrobenzoic acid (Sigma, St. Louis, MO), 0.56 mM acetylthiocholine iodide (Sigma, St. Louis, MO), 10 μM tetraisopropyl pyrophosphoramide (Sigma, St. Louis, MO), and 39 mM phosphate buffer, pH 7.2) at 37°C. After 30 min, the optical density was measured at 405 nm.

2.7. Data Analysis. Indices of water maze probe test were expressed as a percentage of the values of the normal group. Doublecortin immunopositive cells were counted in 10 coronal sections per group, located 3.0 to 3.6 mm posterior to bregma. The number of doublecortin immunopositive cells was presented as mean ± standard error of the mean (SEM). One-way analysis of variance was used for overall analysis of experiments except training trials, followed by a least significant difference test or Tukey honestly significant difference test as post hoc tests at each time point. P values less than 0.05 were considered statistically significant. Water maze training trials were analysed with Linear Mixed Model. Statistical analyses were performed using PASW (version 18; SPSS Inc., Chicago, IL) and SAS version 9.2 (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. Spatial Memory Testing. The results of water maze training are shown in Figure 1(a). The latencies of the first day were decreased under the 20 seconds on the final day of the training trails. From the first day to the fifth day, the differences among the days were statistically significant ($P < 0.0001$) regardless of group. However, there was no significant difference among the groups depending on the time passage ($P = 0.3897$). Taken together, these data show that latency to reach the platform declined progressively across training days for all groups, indicating progressive learning of the hidden platform location. Water maze probe test indices were expressed as a percentage of values for the normal group (Figure 1(b)). The lesion, implantation, and stimulation groups showed no differences from the normal group in motor-related behavior, evidenced by similar swim distances and speeds. These findings suggest no effect of cholinergic lesion, electrode implantation, or electrical stimulation on motor function. However, the amount of time that the lesion group spent in the target quadrant was decreased to 70% of normal group values. Also, the amount of time that the lesion group spent in the platform zone was significantly decreased to 26% of normal group values ($P = 0.006$), whereas it was only decreased to 70% ($P = 0.472$) and 98% ($P = 0.965$) for the implantation and stimulation groups, respectively. Moreover, the number of platform crossings was significantly reduced to 27% ($P < 0.001$) and 61% ($P = 0.039$) for the lesion and implantation groups, respectively, whereas it was only decreased to 95% for the stimulation group ($P = 0.805$).

3.2. Histological Evaluations. The location of stimulating electrodes in the MS was confirmed with cresyl violet staining (Figure 2).

Intraventricular 192 IgG-saproin injections produced denervation of ChAT immunopositive neurons in the MS, which is thought to be part of the basal forebrain complex (Figure 3). The ChAT immunopositive neurons in normal rats were evenly distributed in the MS, and the structure of the cell bodies and dendrites were wholly intact as shown in Figure 3(a). In contrast, lesion, implantation, and stimulation groups, which were injected with 192 IgG-saporin, showed noticeable damage of cell body and dendrite structures.

We quantified the number of hippocampal cells containing doublecortin, which is expressed in various neurogenesis stages from the differentiation phase to the axonal and dendrite targeting phase, using immunohistochemistry (Figure 4). The lesion group showed a significant decline in numbers of doublecortin immunopositive cells to 77.6% of

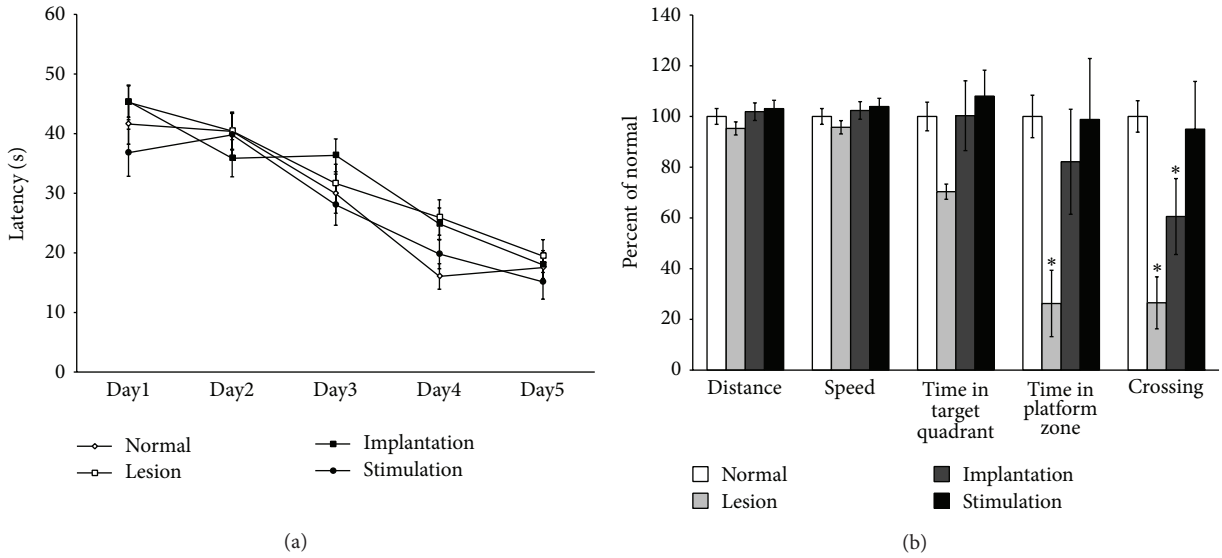

Figure 1: Effect of MS-DBS on spatial memory. Latency indicates the time required for the rat to find the escape platform during training trails. During training trails, all groups gradually acquired the location of the platform (a). After a delay of two days, spatial memory was improved by MS-DBS (b). Time spent in the platform zone ($P < 0.05$) and number of crossings ($P < 0.005$) was significantly different between lesion and normal groups. However, the stimulation group did not differ from the normal group. There was no disruption of motor function in any group. Data are shown as mean ± SEM (a). Indices are expressed as the percentage of normal group values (b). MS: medial septum; DBS: deep brain stimulation: Dist: distance.

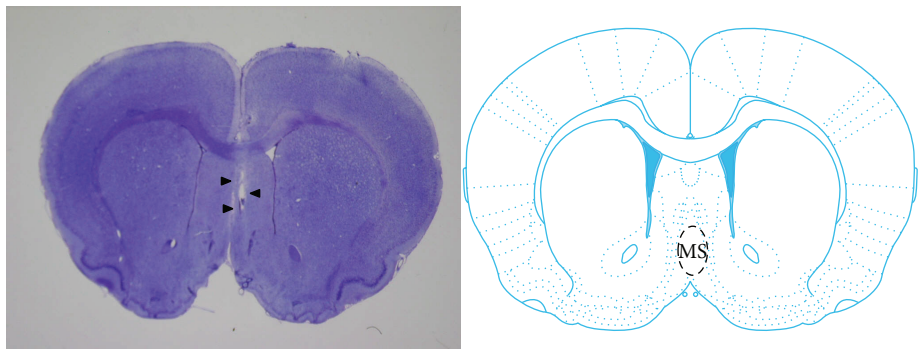

Figure 2: Cresyl violet stained coronal section and slide of atlas [35] at MS level. Arrow heads show the tract of the electrode in the MS.

normal group values ($P = 0.002$), which confirms the effect of damaged basal forebrain cholinergic neuron on hippocampal neurogenesis. The implantation group did not show a significant difference from the lesion group ($P = 0.142$). However, the stimulation group showed a significant increase in the number of doublecortin immunopositive cells compared with the lesion group ($P = 0.002$).

3.3. AChE Assay. In the medial prefrontal cortex, AChE activity was declined in the lesion and implantation groups (Figure 5), although there were no statistically significant differences from the normal group. AChE activity in the stimulation group was higher than that in the normal group, and it was more significantly increased than that in the implantation group ($P = 0.028$). There was a statistically significant decline in hippocampal AChE activity in the lesion group ($P = 0.045$) and implantation group ($P = 0.001$) compared with the normal group. Interestingly, hippocampal AChE activity in the stimulation group was similar to that in the normal group and it is more significantly increased than that in the lesion ($P = 0.038$) and implantation groups ($P = 0.001$).

4. Discussion

Because medications for dementia have limitations, such as side effects or temporary efficacy, the study of alternative therapies is needed. DBS has been approved by the US Food and Drug Administration and can safely be used for movement disorders like Parkinson's disease or tremor. Recently,

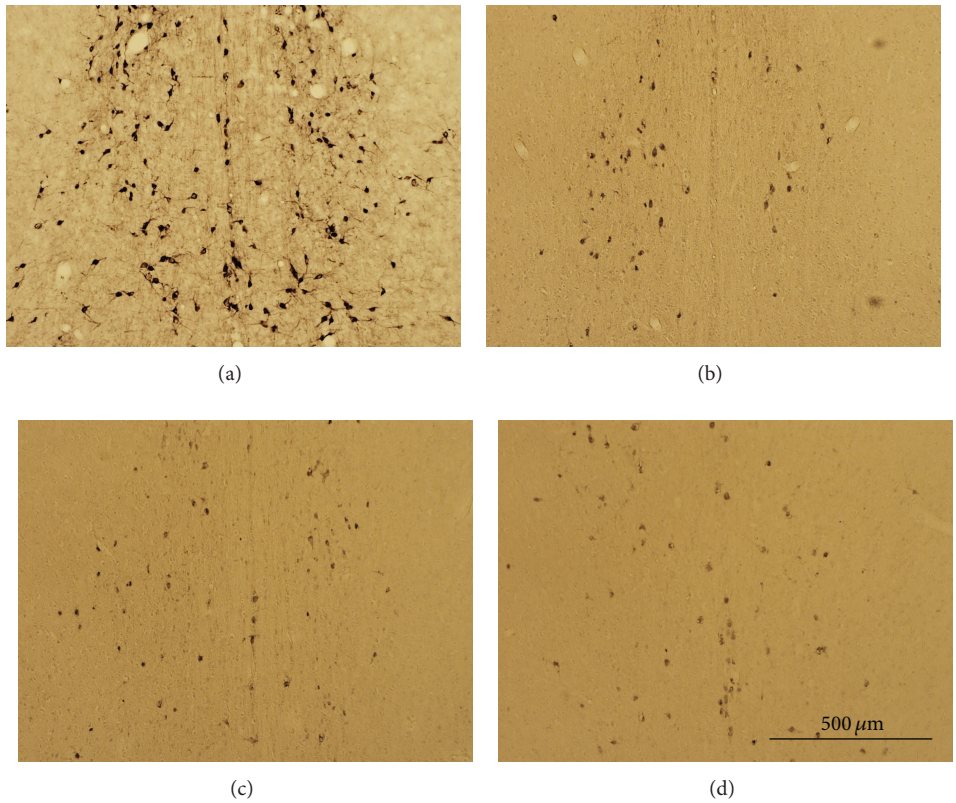

FIGURE 3: Representative pictures showing the effects of the cholinergic lesion on the basal forebrain. The normal group had numerous ChAT immunopositive neurons in the MS (a). The lesion (b), implantation (c), and stimulation (d) groups displayed a loss of cholinergic neurons in the MS. Scale bar represents 500 μm. ChAT: choline acetyltransferase; MS: medial septum.

the possibility that DBS can enhance memory function has been reported in some clinical cases and experimental studies [4–8]. However, the mechanisms of DBS are unclear, and effective stimulation parameters, such as duration and location, are still not well defined. Therefore, experimental studies are needed.

Longer durations of MS-DBS might provide greater therapeutic effects than shorter durations of stimulation. Therefore, we stimulated the MS starting 1 week before the behavioral test and continued stimulating until the end of the test. Laxton et al. [37] reported possible improvements in cognition and slowing of the rate of cognitive decline by continuous fornix/hypothalamus DBS for 12 months. Compared to baseline, an increase in brain metabolism in the temporal and parietal cortical regions was observed 1 month after DBS and was sustained for 12 months. We confirmed that motor function was unaltered in the stimulation group, which received electrical stimulation for 2 weeks.

Although we do not know which stage of memory processing was affected by MS-DBS, long term spatial memory was improved. An interesting finding in this study was that the impairment in spatial memory by 192 IgG-saporin was rescued by MS-DBS. Therefore, we propose that 60 Hz MS-DBS has positive effects on spatial memory.

It has been reported that ICV injection of 192 IgG-saporin damaged both cholinergic basal forebrain neurons and Purkinje cell in the cerebellum [38]. Also, Cerebellar Purkinje cell loss in AD patients has been reported. Because of the similarity in the loss of Purkinje cell [39, 40], using of 192 IgG-saporin is not a big problem for making a dementia model. When 192 IgG-saporin is injected into the ICV rather than direct administration in MS, cholinergic cells have higher survival rate. In this study, we administrated 192 IgG-saporin into the ICV. And averagely 35% of the cholinergic cells were intact in the MS. Our finding of residual ChAT immunopositive cells and AChE activity indicates that basal forebrain cholinergic neurons were not completely damaged by administration of intraventricular 192 IgG-saporin. We believe that MS-DBS influenced the activity of the hippocampus via projections from the MS through remaining cholinergic neurons despite injection of 192 IgG-saporin.

A single shock or brief tetanic stimulation of the MS was found to sharply enhance population spikes evoked in the hippocampal CA1 pyramidal cell layer, and a comparable facilitation of population spikes was produced by microapplication of acetylcholine at the same site [41]. Both cholinergic and GABAergic mechanisms have been proposed to explain this effect. MS facilitation of hippocampal activity is mediated by inhibition of inhibitory interneurons [42]. Cholinergic- and GABAergic-mediated septal drive plays a role in the tuning of signal conversion within the hippocampus [43]. Low-frequency stimulation of the MS and commissural

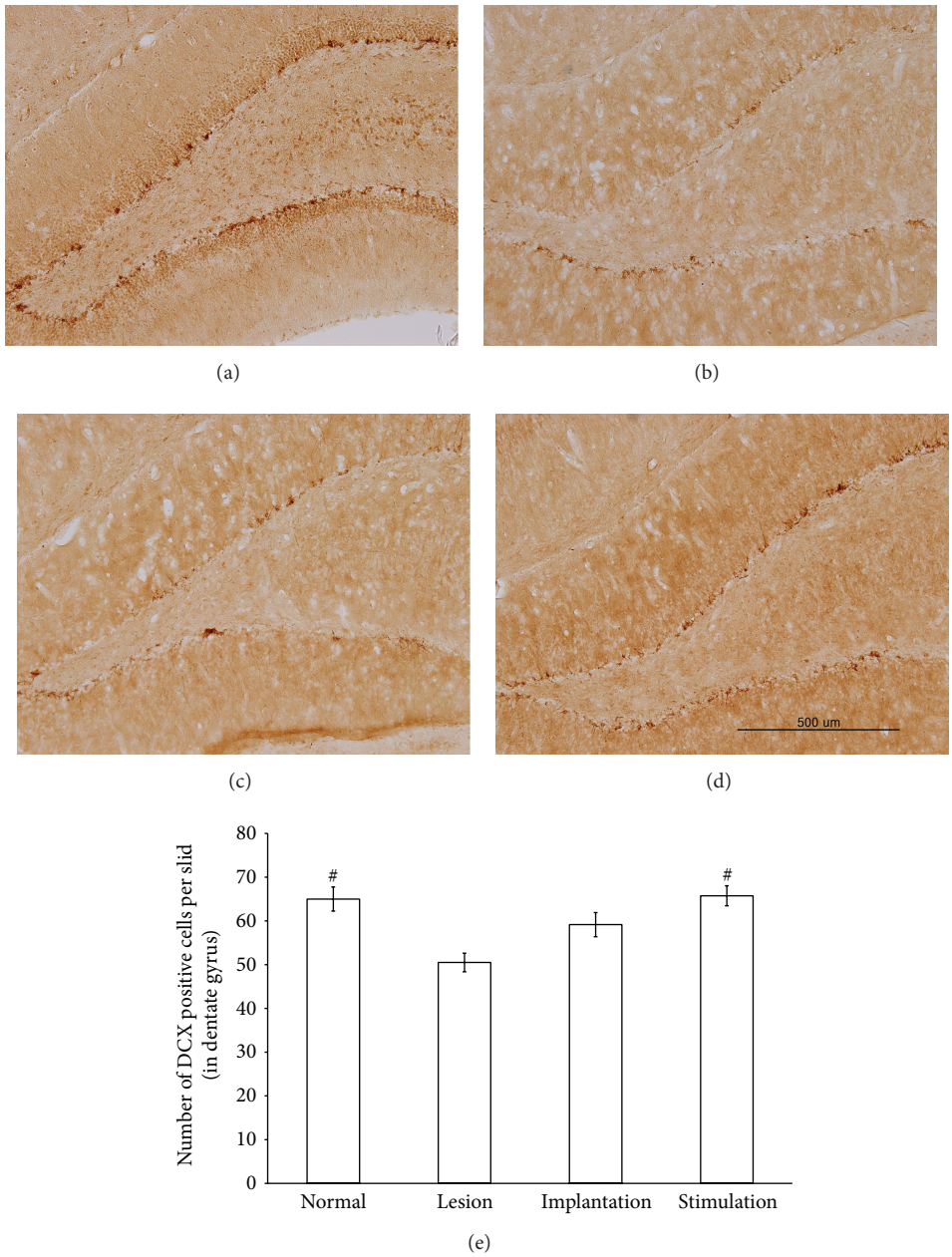

Figure 4: Effects of MS-DBS on adult hippocampal neurogenesis revealed by doublecortin immunohistochemistry. Representative pictures show the effects of basal forebrain cholinergic deficits and MS-DBS on hippocampal neurogenesis (a–d). Many doublecortin immunopositive cells were observed in the normal group (a). However, the number of these cells was decreased in the lesion (b) and implantation (c) groups, in which basal forebrain cholinergic neurons were damaged, but not in the stimulation group (d). After counting the immunopositive cells (e), we found that the number of cells in the normal group was significantly different from that in the lesion group ($P < 0.05$). However, there was no difference between the lesion group and the implantation group, which was only implanted with an electrode in the MS. The number of doublecortin immunopositive cells was significantly increased in the stimulation group ($P < 0.05$), which had damaged basal forebrain cholinergic neurons and received electrical stimulation of the MS.

fibers induces NMDA-dependent, long-lasting potentiation of hippocampal synapses [30]. Electrical stimulation of the MS could possibly affect these systems, although we did not investigate GABAergic or glutamatergic changes in this study. Moreover, the increase in hippocampal acetylcholine by MS-DBS could affect hippocampal theta rhythm and spatial memory. Considerable research has demonstrated correlations between hippocampal theta rhythm and learning and memory [44–46], and positive correlations between hippocampal acetylcholine and theta rhythm have also been reported [47–49].

The other potential mechanism through which MS-DBS could enhance spatial memory is via hippocampal neurogenesis. Adult hippocampal neurogenesis is restricted

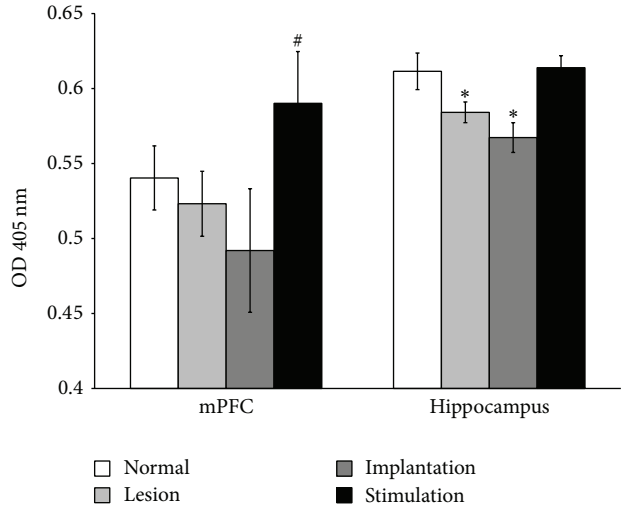

Figure 5: Effect of MS-DBS on AChE activity. In the prefrontal cortex, AChE activity of the stimulation group was significantly increased more than that in implantation group ($P < 0.05$). Hippocampal AChE activity in the lesion ($P < 0.05$) and implantation ($P < 0.005$) groups was significantly less than that in the normal group. However, AChE activity in the stimulation group was equivalent to that in the normal group. The AChE activity was expressed as the optical density of the colorimetric reading at 405 nm. Values are mean ± SEM. OD: optical density.

to the subgranular zone of the dentate gyrus (DG), and new neurons continue to be generated throughout the lifespan. Hippocampal neurogenesis is thought to be associated with hippocampus-dependent memory. Knockdown of adult hippocampal neurogenesis impairs spatial memory [50], and treatments that disrupt hippocampal neurogenesis impair hippocampus-dependent memory [51, 52]. Adult hippocampal neurogenesis is thought to consist of several developmental processes [53]. Doublecortin (DCX) is a protein that promotes microtubule polymerization and can serve as a marker of adult neurogenesis during the stages of late mitotic neuronal precursors and early postmitotic neurons [54, 55]. DCX is expressed specifically in newly generated neurons. DCX is not expressed in GFPA-expressing astrocytes. Also the study shows absence of DCX expression during neuronal regeneration or lesion induced gliogenesis [56]. We confirmed that a deficiency of basal forebrain cholinergic neurons reduces the number of DCX immunopositive cells in the subgranular zone of the DG. This result supports previous findings that damage of basal forebrain cholinergic neurons decreases neurogenesis in the granule cell layer of the DG and increases the number of apoptotic cells [57–59]. Interestingly, Stone et al. reported that electrical stimulation of the entorhinal cortex promotes proliferation in the DG and the integration of these neurons into hippocampal circuits supporting spatial memory [8]. In this study, stimulation of the MS after cholinergic damage recovers the number of DCX immunopositive cells, suggesting that MS-DBS promotes neurogenesis in the DG, which, in turn, may promote hippocampus-dependent learning and memory. We speculate that MS-DBS may increase hippocampal acetylcholine and thereby promote hippocampal neurogenesis. Cholinergic activation increases proliferation of hippocampal neural stem cells and enhances the survival of newborn neurons [60, 61].

Unexpectedly, the behavioral test deficits of lesioned rats seen during the probe trials appear to be partially reversed by the electrode implantation, even in the absence of electrical stimulation. Insertion effect is common phenomenon after deep brain stimulation surgery for Parkinson disease or neuropathic pain patients [62, 63]. After the insertion of electrode into the subthalamic nucleus for PD patients, glucose metabolism has been changed in the absence of stimulation [64]. In the unstimulated hippocampus, enzyme activity is changed in the narrow area surrounding the electrode [65]. In animal study, it has been reported that the different thickness of electrode induced a various regional neuroinflammation and recognition deficits [66]. This different effect on memory from our results could be caused by the thickness of used electrode (280, 150 μm versus 200 μm) and the site of implantation (subthalamic nucleus versus MS). Because the insertional effects are still unclear, the relevant studies are needed in the future.

In conclusion, we found that MS-DBS increases hippocampal acetylcholine and neurogenesis and restore spatial memory from cholinergic malfunction due to basal forebrain cholinergic deficiency. Although we do not know which stage of memory processing is affected by MS-DBS, our study is important in that it confirms the therapeutic effect of MS-DBS.

Conflict of Interests

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

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Review Article

ATP Release through Lysosomal Exocytosis from Peripheral Nerves: The Effect of Lysosomal Exocytosis on Peripheral Nerve Degeneration and Regeneration after Nerve Injury

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Studies have shown that lysosomal activation increases in Schwann cells after nerve injury. Lysosomal activation is thought to promote the engulfment of myelin debris or fragments of injured axons in Schwann cells during Wallerian degeneration. However, a recent interpretation of lysosomal activation proposes a different view of the phenomenon. During Wallerian degeneration, lysosomes become secretory vesicles and are activated for lysosomal exocytosis. The lysosomal exocytosis triggers adenosine 5′-triphosphate (ATP) release from peripheral neurons and Schwann cells during Wallerian degeneration. Exocytosis is involved in demyelination and axonal degradation, which facilitate nerve regeneration following nerve degeneration. At this time, released ATP may affect the communication between cells in peripheral nerves. In this review, our description of the relationship between lysosomal exocytosis and Wallerian degeneration has implications for the understanding of peripheral nerve degenerative diseases and peripheral neuropathies, such as Charcot-Marie-Tooth disease or Guillain-Barré syndrome.

1. Introduction

Lysosomes are acidified, enzyme-containing intracellular organelles that break down phagocytosed materials, cell debris, and waste materials [1]. Therefore, lysosomes (conventional lysosomes) are considered to be the end-point of a final degradative pathway, the final destination of internalized macromolecules [2, 3]. However, it was recently demonstrated that lysosomes play an additional role in regulating exocytosis (secretory lysosomes) in addition to degrading old materials [4]: regulated secretion. This mature lysosome exocytic process can be triggered following an increase in the free Ca^{2+} concentration above $1\,\mu M$. A microtubule-dependent step then provides the movement of exocytic lysosomes towards the plasma membrane [5]. Lysosomal vesicles are usually acidified by its H^+-ATPase [4]. Chemicals that cause alkalinization of lysosomes can trigger lysosomal exocytosis [6].

Lysosomal exocytosis is required for plasma membrane repair via extracellular Ca^{2+} influx [7]. Plasma membrane resealing by lysosomal exocytosis is triggered within seconds after cell injury [7, 8]. Synaptotagmin VII, a plasma membrane Ca^{2+} sensor in lysosomal exocytosis, provides a mechanism by which a rise in intracellular Ca^{2+} upregulates the fusion of lysosomal vesicles with the plasma membrane [9, 10]. However, our understanding of the role of the lysosomal contents in the exocytic process of the peripheral nervous system (PNS) remains limited.

ATP is well established as a free energy source involved in biochemical pathways. However, ATP is now recognized as both an intracellular energy source and an extracellular messenger. Thus, ATP is a transmitter of relevant purinergic

signaling in all nerves [11, 12]. In central synapses, there may be a corelease of ATP with other neurotransmitters or a separate release of ATP [13, 14]. ATP is a functionally important extracellular signaling molecule in the central nervous system (CNS) because activation of P2X and P2Y receptors in postsynaptic neurons, microglia, and astrocytes can trigger significant Ca^{2+} entry into the cytoplasm [15–17]. A recent study revealed that both resting microglia and activated microglia after nerve injury express P2X4, P2X7, and P2Y12 ATP receptors [18] and that released ATP contributes to the activation of the resting microglia near the activated microglia [19]. A previous report indicated that nonadrenergic, noncholinergic autonomic nerves contain ATP concentrated in lysosomal vesicles in vivo [20]. A considerable amount of ATP is stored and released by astrocytes and microglia through lysosomal exocytosis [21–24]. Contrary to a previous study [24], recently, it was reported that ATP release from microglia is dependent on the exocytosis via a vesicular nucleotide transporter (VNUT) but not lysosomal vesicles [25]. However, compared with glial cells in the CNS, the mechanism of ATP release via vesicular exocytosis in Schwann cells and peripheral nerve axons and their behaviors to Wallerian degeneration by released ATP in the PNS are not well known. Therefore, in this review, we discuss the dynamics of ATP related to lysosomal exocytosis in the PNS and the role of lysosomal exocytosis during Wallerian degeneration (Figure 1).

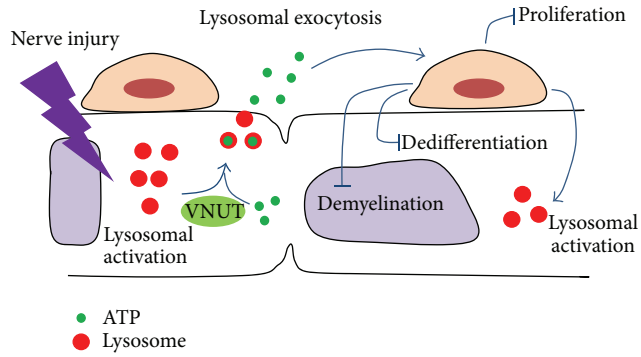

Figure 1: Model of lysosomal exocytosis events in Schwann cells during Wallerian degeneration. After peripheral nerve injury, secretory lysosomal activation is increased, which triggers lysosomal exocytosis during Wallerian degeneration. Through lysosomal exocytosis, Schwann cells release ATP into the extracellular space. The released ATP transmits to neighboring Schwann cells and promotes lysosomal activation and subsequent lysosomal exocytosis.

2. ATP Release through Lysosomal Exocytosis in the PNS

ATP is a significant signaling molecule in the PNS, as it plays an important role in chemical communication between several cell types [26, 27]. During Schwann cell development, extracellular ATP inhibits Schwann cell proliferation and differentiation [28]. In primary Schwann cells, extracellular ATP also triggers the release of ATP or amino acids [29, 30]. How can Schwann cells and peripheral neurons then release ATP into the extracellular space? One ATP-releasing mechanism in the PNS is secretory lysosomal exocytosis.

2.1. ATP Release from Schwann Cells through Lysosomal Exocytosis. In Schwann cells, uridine triphosphate or glutamate induces ATP release through vesicular exocytosis [31, 32]. Inhibitors of exocytosis that inhibit the formation of vesicles from the Golgi complex or prevent the delivery of vesicles disrupt ATP release from Schwann cells [31]. Recently, our group found that lysosomal vesicles are an exocytic ATP-releasing vesicle in Schwann cells [33]. Lysosomal-associated membrane protein 1 (LAMP1), a lysosomal vesicle marker, colocalizes with quinacrine, a specific ATP-combining chemical, in primary Schwann cell granules in culture, thus indicating that ATP is stored in lysosomal vesicles [33].

Fusion between exocytic vesicles and cell membranes is necessary to release vesicular contents. Vesicle-associated membrane protein 7 (VAMP7), a member of the vesicular soluble NSF attachment protein receptor (SNARE) family, is involved in Ca^{2+}-dependent lysosomal exocytosis, and its interaction with synaptotagmin VII (SytVII), a member of the synaptotagmin family of Ca^{2+}-binding proteins, is required for lysosomal exocytosis [34, 35]. In in vivo and in vitro Schwann cells, SytVII/VAMP7-positive vesicles are also observed in lysosomal vesicles [33]. The existence of SytVII and VAMP7 indicates that lysosomal exocytosis in Schwann cells is a Ca^{2+}-dependent process.

VNUT has the capacity to transport cytosolic ATP into vesicles [36]. Intracellular vesicles that contain ATP through the interaction of VNUT are fused with the plasma membrane and, then, ATP through vesicular exocytosis is released into the extracellular space [36]. In Schwann cells, VNUT also induces the entry of ATP into lysosomal vesicles [33]. Thus, during Wallerian degeneration, VNUT induces ATP to enter lysosomal vesicles, and ATP is subsequently released through Ca^{2+}-triggered lysosomal exocytosis in Schwann cells. These studies demonstrate that ATP is stored in lysosomal vesicles via VNUT following stimulation, and ATP secretion from Schwann cells occurs through Ca^{2+}-dependent lysosomal exocytosis during Wallerian degeneration.

2.2. ATP Release from Peripheral Neurons via Lysosomal Exocytosis. ATP is liberated from stimulated peripheral nerves [37] and is important for signaling injurious nociceptive information [38]. ATP, as a neurotransmitter, is released from exocytic vesicles at presynaptic terminals and is the medium of the communication between the cells [28, 39–44]. In peripheral neurons, lysosomal vesicles contain a considerable amount of ATP in vivo [20]. However, the characteristics of ATP release via lysosomes in neurons remain to be elucidated. In dorsal root ganglion (DRG) neurons, the existence of lysosomal exocytosis and vesicular ATP release was reported separately [28, 43, 44]. Recently, our group reported that ATP is stored in lysosomes and is released from lysosomal exocytosis in cultured DRG neurons [45]. In primary DRG cultures, staining for quinacrine, an ATP-binding chemical, is visualized in lysosomal vesicles [45]. Quinacrine-positive

vesicles were observed in neuronal soma and in the tip of elongating processes of cultured DRG neurons [45]. VNUT-positive vesicles containing quinacrine staining are also observed in neuronal soma and the tips of the elongating processes of cultured DRG neurons which are similar to the distribution of quinacrine-stained lysosomal vesicles [45]. Thus, in peripheral neurons, ATP was thought to enter into lysosomal vesicles through VNUT.

DRG neurons are pseudopolar neurons that contain both central and peripheral processes. Thus, ATP released from DRG neurons could affect DRG-glia interactions in the PNS and CNS. The existence of ATP-containing lysosomal vesicles in the tips of neuronal processes suggests the possibility that ATP released from DRG neuronal axon terminals through lysosomal exocytosis may induce microglial activation and neuropathic pain in the spinal cord dorsal horn after nerve injury [46], as well as Schwann cell proliferation or differentiation during Schwann cell development in peripheral nerves [28]. For example, lysosomal exocytosis is involved in axonal degradation during Wallerian degeneration. The high concentration of extracellular ATP, which is released from Schwann cells, inhibits axonal degradation in peripheral nerves during Wallerian degeneration [45]. Thus, neuronal mechanisms of ATP release through lysosomal exocytosis may increase our understanding of physiological or pathophysiological neuron-glia interactions in both PNS and CNS.

3. Lysosomal Exocytosis and Schwann Cell Demyelination

After nerve injury, during Wallerian degeneration, demyelination of Schwann cells occurs via fragmentation of the myelin sheath into ovoid-like structures near Schmidt-Lanterman incisures (SLI) [47–49]. Lysosomal activation is increased in Schwann cells during Wallerian degeneration [50, 51]. Increased lysosomal activation (conventional lysosomes), which indicates an increased number of acidified lysosomal vesicles, affects myelin fragmentation in Schwann cells during Wallerian degeneration [51]. It seems likely that the activated lysosomal vesicles engulf and remove myelin fragment debris in Schwann cells during Wallerian degeneration. However, the mechanisms by which the reduced lysosomal activation inhibits demyelination have not been studied previously. On the other hand, lysosomal exocytosis also occurs in Schwann cells during Wallerian degeneration. Recently, our group presented evidence that increased lysosomal exocytosis inhibits myelin fragmentation in Schwann cells during Wallerian degeneration [52]. Several lysosomal exocytosis activators (highly concentrated extracellular ATP and NH_4Cl) inhibit myelin fragmentation in sciatic explant cultures during Wallerian degeneration [52]. In contrast, sciatic nerve explant incubation with both a lysosomal exocytosis activator and inhibitor (metformin and chlorpromazine) for 3 days restores myelin fragmentation [52]. Thus, we believe that one mechanism by which lysosomal exocytosis inhibits demyelination is through enhanced release of ATP from Schwann cells into the extracellular space in the PNS. The increased extracellular ATP level may induce Ca^{2+}-dependent alkalization of existing acidified lysosomal vesicles in Schwann cells during Wallerian degeneration [24, 30] and may reduce the amount of activated conventional lysosomes. Decreased lysosomal vesicles may affect the inhibition of myelin fragmentation during Wallerian degeneration. In addition, the increased ATP concentration in the extracellular space may induce the alkalization of lysosomal vesicles and subsequently enhance lysosomal exocytosis in neighboring Schwann cells. In addition to the increased extracellular ATP, it is possible that unidentified secretory proteins induced by lysosomal exocytosis in Schwann cells prevent myelin fragmentation and degradation. Thus, further evaluation is needed to reveal the underlying proteins released by lysosomal exocytosis in demyelination during Wallerian degeneration.

During Wallerian degeneration, recruited macrophages into the peripheral nerves engulf the debris of myelin sheaths [53, 54]. Because macrophages express several ATP receptors [55, 56], the extracellular ATP may activate the recruited macrophages and, subsequently, inhibit the removal of myelin debris by the macrophage. However, because ex vivo Wallerian degeneration system is closed, the recruitment of macrophage into the sciatic nerve explants could be excluded. Thus, we think that the inhibition of demyelination in ex vivo sciatic nerves through the increased ATP concentration is not involved in the effect of macrophages [52].

Are there effects of lysosomal exocytosis during Wallerian degeneration other than ATP secretion in denervated Schwann cells? Lysosomal exocytosis is also involved in Schwann cell remyelination. Lysosomal vesicles in Schwann cells contain a compact myelin-consisting protein [57]. This secretory vesicle fuses with the plasma membrane through lysosomal exocytosis in Schwann cells and promotes remyelination by the addition of myelin protein to the plasma membrane [57]. Thus, several studies showed that lysosomal exocytosis in Schwann cells closely affects myelin sheath dynamics in response to stimuli.

4. Lysosomal Exocytosis and Schwann Cell Dedifferentiation and Proliferation

During Wallerian degeneration, Schwann cells detached from axons undergo dedifferentiation and reenter the cell cycle to promote axonal regeneration. These dedifferentiated Schwann cells are similar to their immature state during Schwann cell development. The transition from myelinating Schwann cells to their dedifferentiated state involves several regulatory proteins. Extracellular signal-regulated kinase (ERK), c-jun, and p38 mitogen-activated protein kinase (p38 MAPK), members of the MAPK family, induce the initiation of Schwann cell dedifferentiation and act as negative regulators of myelin differentiation in Schwann cells [58–61]. The p75 neurotrophin receptor (NGFR), which is a low affinity nerve growth factor receptor, is activated in demyelinating myelinated Schwann cells after nerve injury and is involved in the Schwann cell dedifferentiation process during Wallerian degeneration [50, 51, 62]. p75NGFR induction also mediates lysosomal activation in demyelinating Schwann cells during

Wallerian degeneration [51]. Thus, understanding the relationship between lysosomal vesicles and members of the MAPK family or neurotrophin receptors during Wallerian degeneration may help to identify the molecular mechanism of Schwann cell dedifferentiation.

Using a sciatic nerve explant system, our group found that p38 MAPK and ERK1/2 are involved in lysosomal exocytosis in Schwann cell dedifferentiation during Wallerian degeneration [63]. A lysosomal exocytosis activator (i.e., highly concentrated ATP) induces the downregulation of p-p38MAPK and p-ERK1/2 in Schwann cells during Wallerian degeneration [63]. Lysosomal exocytosis is involved in p75NGFR expression and lysosomal activation during Wallerian degeneration. Highly concentrated ATP (2 mM) inhibits conventional lysosomal activation and the expression of p75NGFR in the denervated state of Schwann cells during Wallerian degeneration [63]. At this time, a decrease in conventional lysosomal activation is likely to induce the reduction in acidified vesicles for scavenging myelin fragments and the transfiguration into secretory vesicles (secretory lysosomal activation). Thus, these studies indicate that lysosomal exocytosis affects Schwann cell dedifferentiation during Wallerian degeneration. In addition, increased lysosomal exocytosis blocks Schwann cell proliferation, which is involved in axonal regeneration during the process of peripheral nerve regeneration [63].

Which molecules released through lysosomal exocytosis affect Schwann cell dedifferentiation during Wallerian degeneration? During Schwann cell development, increased extracellular ATP (300 μM) inhibits the proliferation and differentiation of Schwann cells cocultured with DRG neurons [28, 64]. Because Schwann cells return to an immature developmental stage during Wallerian degeneration, it is possible that ATP released through lysosomal exocytosis could affect dedifferentiating Schwann cells during Wallerian degeneration. ATP released from dedifferentiated Schwann cells during Wallerian degeneration may function as a neurotransmitter in the peripheral nervous system and communicate with neighboring Schwann cells to inhibit their dedifferentiation. Thus, these studies have confirmed that ATP and lysosomal exocytosis in the PNS are closely related to Wallerian degeneration.

5. Concluding Remarks

The role of lysosomal exocytosis in the PNS has been studied recently. The current belief is that lysosomal exocytosis is involved in Schwann cell demyelination, remyelination, dedifferentiation, and proliferation during Wallerian degeneration. In addition, secretory vesicles affect axonal degeneration during Wallerian degeneration. In the PNS, an important role for lysosomal exocytosis is that it releases ATP from peripheral neurons and Schwann cells. ATP may function as a neurotransmitter and affect nerve degeneration during Wallerian degeneration. According to our previous studies [52, 63], peripheral nerve injury should increase ATP release through lysosomal exocytosis into the extracellular space of the sciatic nerves and the increased ATP should have inhibited Wallerian degeneration in the injured sciatic nerves without any treatments of lysosomal exocytosis activators. However, Wallerian degeneration in the injured sciatic nerves without any treatments is ongoing [52]. Then why does not the inhibition of Schwann cell dedifferentiation and proliferation through secretory lysosomal ATP release occur in vivo during Wallerian degeneration? We believe that extracellular ATP released from Schwann cells or peripheral axon terminals is easily degraded in the extracellular environment in vivo [65]. The efficient prevention of ATP degradation in the extracellular space is likely to regulate the processes of Schwann cell dedifferentiation and proliferation during Wallerian degeneration. Consequently, these recent results have opened up a new research area to understand the mechanisms of peripheral nerve degeneration and regeneration. Furthermore, the regulation of ATP release in peripheral nerves may make lysosomal exocytosis a potentially valuable therapeutic target for peripheral nerve degenerative diseases and peripheral neuropathies, such as Charcot-Marie-Tooth disease or Guillain-Barré syndrome.

Conflict of Interests

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

Authors' Contribution

Na Young Jeong and Junyang Jung contributed equally to this paper.

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Review Article

Therapeutically Targeting Neuroinflammation and Microglia after Acute Ischemic Stroke

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Inflammation has a pivotal role in the pathogenesis of ischemic stroke, and recent studies posit that inflammation acts as a double-edged sword, not only detrimentally augmenting secondary injury, but also potentially promoting recovery. An initial event of inflammation in ischemic stroke is the activation of microglia, leading to production of both pro- and anti-inflammatory mediators acting through multiple receptor signaling pathways. In this review, we discuss the role of microglial mediators in acute ischemic stroke and elaborate on preclinical and clinical studies focused on microglia in stroke models. Understanding how microglia can lead to both pro- and anti-inflammatory responses may be essential to implement therapeutic strategies using immunomodulatory interventions in ischemic stroke.

1. Introduction

Stroke is the second leading cause of death worldwide and most victims suffer from disabilities such as paresis and speech defects [1]. One of the major causes of stroke is an interruption of cerebral blood flow resulting in ischemia [2]. The incidence and mortality of stroke increase with age, and as the elderly population is rapidly growing in most developed countries ischemic stroke is a common societal burden with substantial economic costs [3]. Although great advances have been made in understanding the diverse mechanisms of neuronal cell death induced by ischemic stroke, clinically effective neuroprotective therapies are limited [1]. Recent studies suggest that cells other than neurons may be involved in the pathogenesis of ischemia and that a functional "neurovascular unit" comprises neuronal, glial, and vascular elements [4, 5].

After ischemic stroke, an inflammatory response is initiated within a few hours, with the activation of microglia and astrocytes and the production of chemoattractants, cytokines, and chemokines [6–8], with the subsequent infiltration of blood-derived cells such as leukocytes [9, 10]. These cells interact with one another via intricate signaling pathways. Recent studies show that systemic inflammatory status prior to and at the time of stroke is a key determinant of acute outcome and long-term prognosis [11, 12]. Inhibiting inflammatory responses after stroke can prevent brain injury and, therefore, improve neurological outcome [13]. Conversely, it has been suggested that suppressing inflammation could be detrimental and long-term functional recovery could be worse when inflammation after stroke is inhibited [14, 15]. Taken together, inflammatory responses after ischemic insult could be beneficial or detrimental, probably depending on the stage of stroke and environments; nevertheless, more work is needed to elucidate the role of inflammation during stroke.

Microglia are activated after ischemic stroke, changing shape and phenotype, similar to macrophages in systemic inflammation. Activated microglia have the potential to phagocytose, present antigens, and produce cytokines and matrix metalloproteinases (MMPs) that disrupt the blood brain barrier (BBB) [10]. Peripheral leukocytes can then infiltrate into the brain and further exacerbate inflammation and brain damage [15].

Interestingly, microglial activation causes the release of a number of inflammatory mediators that are either cytotoxic or cytoprotective [16]. Microglial phagocytosis contributes to restoration of tissue homeostasis by clearing pathogens and necrotic cells, suppressing inflammation, and facilitating brain repair [9, 17]. Recent studies, thus, suggest therapeutically targeting microglia in stroke. Here, we focus on the roles of microglia in neuroinflammation after ischemic stroke.

2. Microglia under Normal Physiological Conditions

In the resting state, microglia survey the CNS microenvironment by continuously extending and retracting ramified processes [18, 19]. They control synapse number and remodeling in the developing brain and clear debris in the healthy adult CNS [20]. Depending on the brain area, microglia can express different proteins and display various morphologies [21] and respond differently according to injuries [22]. Microglia are mostly located in the gray matter, where they ramify radial processes [21]. The activation and cell fate of microglia are influenced by their location. After focal ischemia for an hour followed by 24 h of reperfusion, the number and length of microglial processes decrease and the expression of CD11b increases in the ischemic core, indicating that microglia in this region are activated [23]. On the other hand, microglia remained inactivated with more ramified processes in the penumbra-salvageable region after reperfusion around the peri-infarct area [24]. Taken together, quiescent microglia are not simply "resting," but rather they continuously survey and prepare to change phenotype and function in response to a variety of stimuli in their surroundings.

3. Microglia during Acute Ischemic Stroke

Inflammation of acute ischemic stroke is a dynamic process induced by brain-resident microglia and blood-derived leukocytes [25, 26]. Activation of microglia is the first step of the inflammatory process even within minutes [7, 27]. Two to three days following ischemia, the activation and amplification of microglia peak and continue for several weeks [28, 29]. Meanwhile, infiltration of neutrophils begins after 1 day of stroke, followed by infiltration of macrophages after 2 days of stroke [25]. Although the precise roles of microglia in ischemic stroke have not yet been fully understood, recent studies strongly suggest multiple functions. The population of microglia increased in the ipsilateral hemisphere of stroke, while it remained at basal levels in the contralateral hemisphere [30]. In the ischemic environment, microglia can phagocytose tissue debris as well as secrete proinflammatory cytokines, resulting in further damage [31]. In contrast, microglia also can secrete anti-inflammatory mediators [32, 33] to alleviate inflammation. Defective microglial activation/proliferation significantly increased the size of infarction and the number of apoptotic neurons after stroke [34], which supports the pivotal role of microglia after ischemic stroke.

3.1. Different Phenotypes of Activated Microglia: M1 and M2. During microglial activation after ischemic stroke, cell morphology is changed either to M1, the typically activated phenotype, or to M2, an alternatively activated phenotype; this phenotypic switch depends on the type of stimulation (Figure 1). M2 microglia are regarded as "healing cells" that contribute to recovery after damage and secrete anti-inflammatory mediators such as interleukin- (IL-) 10, transforming growth factor- (TGF-) β, IL-4, IL-13, and insulin growth factor- (IGF-) 1, as well as various neurotrophic factors [32, 35–39]. On the other hand, M1 microglia are considered as proinflammatory, producing proinflammatory meditators such as tumor necrosis factor- (TNF-) α, IL-1β, and interferon- (IFN-) γ. M1 microglia express CD80, CD86, and MHC class II on the cell membrane and present antigens to T cells [40]. In addition, M1 microglia tend to induce neuronal cell death more readily than M2 microglia [41]. For this reason, inhibiting the M1 phenotype has been suggested as a plausible therapeutic strategy in cerebral ischemia models. In ischemic stroke, the M2 phenotype is dominant in both local microglia and newly recruited macrophages at earlier stages, but the M1 phenotype population increases progressively in peri-infarct regions, suggesting that neurons under ischemic condition trigger changes toward the M2 phenotype in microglia and macrophages [41]. Considering these opposing roles of microglia, stroke therapies should not be focused on simply suppressing microglia but instead on balancing the beneficial and detrimental reactions of microglia.

As there is no single specific marker for microglia and activated microglia changes to amoeboid morphology with an enlarged cell body and stout processes, it is difficult to distinguish them from macrophages and myeloid-derived cells that infiltrate the injured brain tissue [42]. Since microglia and macrophages originate from primitive myeloid cells, a number of markers such as CD11b, F4/80, and Iba-1 are the same [35, 43]. Although different phenotypes of activated microglia express unique cell surface proteins, markers for each phenotype have not been determined specifically because of the similarities to other cell types. To date, several markers have been identified for activated M1 and M2 microglia (Table 1). As a marker for M1, MHC class II is used as M1 microglia participate in antigen presentation in immune reactions [44]. On the other hand, M2 microglia express high levels of antigen-presenting molecules such as Ym-1 and CD206 [24].

3.2. Differential Microglial Expression in Ischemic Stroke (Ischemic Core versus Penumbra). As the number of microglia increases after ischemic stroke, the pattern of microglial response is different depending on the location of the lesion. In the ischemic core, globular $Iba1^+ED1^+$ cells appear 7 days after transient ischemia [45]. In another study, when measured 24 h after cerebral ischemia followed by reperfusion, microglia/macrophages in the ischemic core expressed high levels of CD11b, indicating activation and formation of an amoeboid phenotype [23]. Twenty-four hours after focal ischemia without reperfusion, few $CD11b^+CD68^+$ cells were

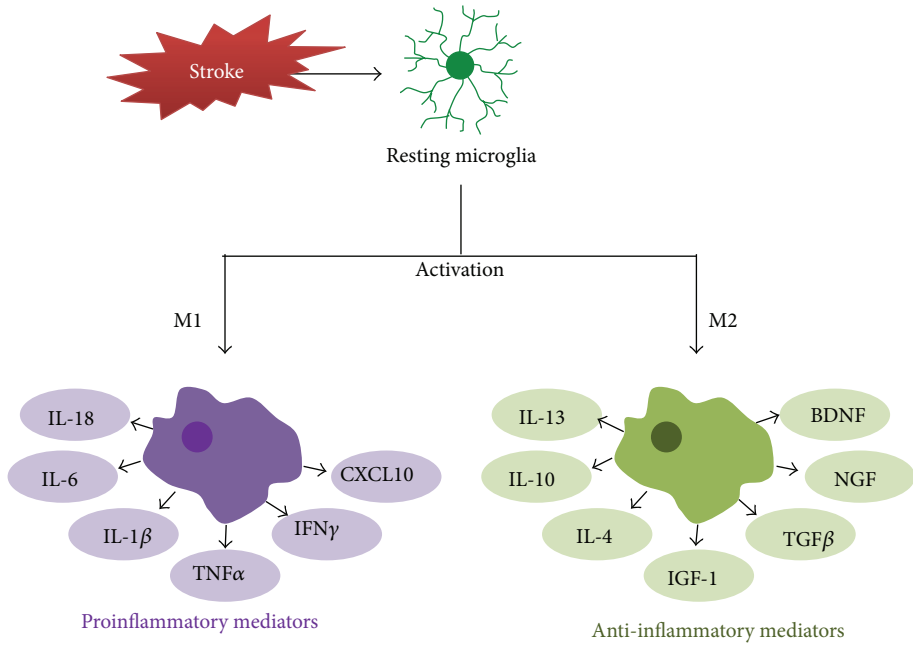

Figure 1: Phenotypes and activation of microglia after ischemic stroke. Under ischemic conditions, microglia change their morphology and become activated. Activated microglia are characterized as either an M1 classically activated phenotype or an M2 alternatively activated phenotype. Microglial activation induces transcription associated with the inflammatory mediators. According to their phenotypes, microglia can promote proinflammatory (by M1) or anti-inflammatory (by M2) machinery. IGF-1: insulin-like growth factor 1; IL-1β: interleukin-1 beta; IL-6: interleukin-6; IL-10: interleukin-10; TGF-β: transforming growth factor-beta; TNF-α: tumor necrosis factor-alpha.

Table 1: Markers for distinguishing activated and resting microglia.

Name	Markers	References
CD11b	Both resting and activated M/M	[24, 75]
CD45	Nucleated hematopoietic cell surface	[30, 76]
CD68 (ED1)*	Active phagocytosis M/M	[24, 45, 77]
Iba-1	Both resting and activated M/M	[78, 79]
F4/80	Both resting and activated M/M	[35, 43]
IB4	Both resting and activated M/M	[15]
Ym-1	Activated M/M (M2)	[24]
Iba-1$^+$, CD206$^+$	Activated M/M (M2)	[24]
Iba-1$^+$, CD16/32$^+$	Activated M/M (M1)	[41]

*CD68 and ED1 are virtually the same molecule (CD68 is used more in the human context, while ED1 is the name of that protein in rodents). M/M: microglia and macrophages.

identified in the ischemic core, and CD68 (same as ED1 in rodents), marker for phagocytosis, was highly expressed by day 7. At 24 h, Ym-1 and CD206, markers for the M2 phenotype, were exclusively found in the ischemic core, suggesting that the microglia/macrophages participate in tissue repair in the ischemic core [24]. Another study also confirmed M2 phenotype dominance of microglia/macrophages by finding Iba1$^+$/CD206$^+$ expression at 24 h after stroke in the ischemic core; the expression was highest at 5 days after insult, decreasing after 14 days [41]. As disease severity increases, however, microglia decrease in number and disintegrate; numerous dead CD11b$^+$ cells were found in the ischemic core at 72 h after stroke [46], and, similarly, CD11b$^+$ cells showed disintegration in the ischemic core at 7 days after permanent focal cerebral ischemia induced by photochemically induced thrombosis (PIT) method or middle cerebral artery occlusion (MCAO), whereas there are an increased number of microglia and macrophages in the penumbra [47, 48]. Altogether, it is assumed that ischemia induces injury to microglia in the infarct core in the early phase, and, subsequently, M2 microglia/macrophages migrate into the area during the first week, followed by a decrease in microglial number afterwards. In contrast, the number of M1 microglia/macrophages increases over the first 2 weeks (Table 2). Microglia respond dynamically to ischemic stroke, as an early "anti-inflammatory" M2 phenotype, followed by a transition to a "proinflammatory" M1 phenotype. Severe ischemic state of core environment including ischemic neurons could prime microglial M1 phenotype or death. These dual roles of microglia suggest that stroke therapies should be shifted from simply suppressing microglia toward adjusting the balance between beneficial and detrimental microglial responses.

Unlike the ischemic core, microglia in the penumbra seem to be highly activated [22]. In a permanent ischemic stroke model, CD68 is expressed on ramified CD11b$^+$ cells in the penumbra at 6 h and, continuously, increases in the hypertrophic amoeboid cells of the ischemic core [24]. However, it

Table 2: Microglial marker expressions in the ischemic core and peri-infarct zone.

Location	Marker	Model	Expression (after reperfusion)	Reference
Ischemic core	Iba1	1.5 h tMCAO	24 h, 4–7 d (peak)	[45]
	Iba1$^+$, ED1$^+$	1.5 h tMCAO	7 d	[45]
	CD11b	1 h tMCAO	24 h	[23]
	CD68	pMCAO	7 d	[24]
	Ym-1	pMCAO	24 h	
	CD206	pMCAO	24 h	
	Iba1$^+$, CD206$^+$	1 h tMCAO	1 d, 5 d (peak)	[41]
	Iba1$^+$, CD16/32$^+$	1 h tMCAO	3 d, 14 d (peak)	
Peri-infarct zone	Iba1$^+$, ED1$^-$	1.5 h tMCAO	3.5 h, 7 d (peak)	[45]
	CD11b$^+$, CD68$^+$	pMCAO	6 h	[24]
	F4/80	pMCAO	24 h, 3 d (peak)	[49]
	CD11b	pMCAO	1 h	[23]
	CD68	pMCAO	24 h	[24]
	CD68$^+$, MHC II$^+$	pMCAO	3–7 d	[51]

pMCAO: permanent middle cerebral artery occlusion and tMCAO: transient middle cerebral artery occlusion.

should be noted that CD11b can be expressed on both resident and infiltrating phagocytes. Within 90 min of transient focal ischemia after reperfusion, the number of Iba1$^+$ED1$^-$ cells increases from 3.5 to 7 days and then decreases by day 14 [45]. After 8 and 24 h of focal cerebral ischemia, the length and the number of processes of microglia/macrophages in the penumbra decrease, demonstrating their activation [23]. In addition, CD11b and F4/80 are prominently expressed in the penumbra [23, 49]. Ym-1$^+$ and CD206$^+$ cells were not detected in the penumbra from 1 to 7 days after permanent MCAO [24], while the IBA-1$^+$ and CD206$^+$ cells were highest at day 5 in the penumbra after 60 min of transient MCAO [41]. The difference in animal models may account for the discrepancies between these two studies. Given that the infiltrating cells are recruited much more in permanent MCAO [50], this might be coming from a higher number of infiltrating cells or a lower survival of resident cells. Most CD68$^+$ microglia/macrophages were located in the penumbra [24, 46]. Markedly proliferating resident microglia with few infiltrating blood-derived macrophages after focal cerebral ischemia were detected at 2 and 3 days of transient MCAO over 3 days after 30 or 60 min of occlusion but significantly reduced in 60 min of occlusion compared to 30 min of occlusion [7]. Moreover, in the penumbra of permanent focal ischemia, CD68$^+$ microglia were accompanied by increased expression of MHC class II on the cell surface from days 3 to 7 [51]. Taken together, proliferating and activated microglia predominate in the penumbra, and their number increases over the first week after stroke. Although these studies are limited because they did not differentiate resident microglia from infiltrating macrophages, one implication is that the change of microglial phenotype occurs dynamically and consistently and the location of the microglia is crucial. This suggests that therapeutic approaches to regulate microglia need to be targeted specifically by brain region (Table 2).

In clinical studies, abundant activated microglia have been found histologically in the ischemic core as well as penumbra within 1-2 days after onset [52, 53]. Similar to the rodent stroke model, these cells remained for several weeks and were predominantly placed in the peri-infarct zone. Positron emission tomography (PET) with ^{11}C-labled PK11195 enables *in vivo* imaging the presence of activated microglia [54–56]. ^{11}C-labled PK11195 has significant binding potential in the core and penumbra at 2 days and remained until 30 days, however, with less specificity to differentiate inflammatory cells due to binding mitochondrial peripheral benzodiazepine receptors which are expressed in activated microglia, macrophages, astrocytes, granulocytes, and lymphocytes [57, 58]. Recently, increased [^{18}F]-fluoro-2-deoxy-D-glucose (^{18}F-FDG) PET imaging in the peri-infarct area shows association with activated microglia and infiltrated cells [47]. Further studies are needed to clarify the glucose metabolism and microglial response after ischemic stroke.

4. Therapeutic Approaches Modulating Microglial Response

4.1. Genes and Cells. The cytokine IL-1 has been strongly implicated in the pathogenesis of ischemic brain damage. Although ischemic damage compared with wild-type mice was not significantly altered in mice lacking either IL-1a or IL-1b alone, mice lacking both forms of IL-1 exhibited dramatically reduced ischemic infarct volumes compared with wild type (total volume: 70%; cortex: 87% reduction) [59].

Toll-like receptors (TLRs) are signaling receptors in the innate immune system that trigger specific immunological responses to systemic bacterial infection. Microglia express TLRs which lead to gene expression of proinflammatory cytokines [35]. TLR4 and TLR2 are the most marked TLRs

in microglia, and they are increased after ischemia [15]. In addition, TLR4 was localized with CD11b-positive microglia in the ischemic striatum [60]. In this respect, TLR4 but not TLR3 or TLR9 knockout (KO) mice had significantly smaller infarct areas and volumes at 24 h after ischemia-reperfusion compared with wild-type mice [60]. Some previous studies focused on an important role of TLR2 signaling in brain ischemia. Temporal analysis with flow cytometry of the microglial/macrophage activation profiles in TLR2-KO mice and age-matched controls revealed reduced microglial/macrophage activation after stroke and reduced capacity of resident microglia to proliferate, as well as decreased levels of monocyte chemotactic protein-1 (MCP-1) and consequently lower levels of CD45 high/CD11b$^+$ expressing cells [61].

Recently, MMPs have been regarded as important molecules in neuroinflammation as well as neuronal apoptosis. Several reports have shown that activated microglial cells are crucial in white matter lesion (WML) pathology. A transplanted microglial cell line (HMO6) and mesenchymal stem cell line (B10) migrated to sites of WMLs, including the corpus callosum (CC) and caudoputamen (CP), reduced the severity of WMLs, and inhibited the accumulation and activation of microglia and astrocytes. Transplantation of both cell types reduced the level of MMP-2 mRNA in microglia of the CC. MMP-2 protein level and activity were also both greatly reduced in the same region. These results indicate that transplantation of either microglial cells or mesenchymal stem cells could inhibit chronic cerebral ischemia-induced WML formation by decreasing MMP-2 expression in microglia and decreasing MMP-2 activity in the CC region [62].

Expressions of MMP-1, -3, -8, and -9 were significantly induced by single or combined treatment with the immunostimulants lipopolysaccharide (LPS) or phorbol myristate acetate (PMA) in primary cultured microglia and BV2 microglial cells. Inhibition of MMP-3 or -9 significantly suppressed the expression of inducible nitric oxide synthase (iNOS) and proinflammatory cytokines and the activities of nuclear factor-kappa B (NF-κB), AP-1, and p38 mitogen-activated protein kinase (MAPK) in LPS-stimulated microglia [63]. Taken together, various microglia-derived cytokines, signal receptors, and neuroinflammatory proteins reported that their knockout models may play a neuroprotective role in ischemic brain injury sufficiently.

4.2. Chemicals. Propofol confers neuroprotection against focal ischemia by inhibiting microglia-mediated inflammatory response in a rat model of ischemic stroke. Propofol treatment reduced infarct volume and improved the neurological functions. Moreover, molecular studies showed that mRNA expression of microglial markers CD68 and Emr1 significantly increased, and mRNA and protein expressions of proinflammatory cytokines TNF-α, IL-1β, and IL-6 were augmented in the peri-infarct cortical regions of vehicle-treated rats 24 h after MCAO [64].

2,3,5,4′-Tetrahydroxystilbene-2-O-β-D-glucoside (TSG), an active component, has been reported to be beneficial for human health and used as an antiaging agent. Recent studies have shown that TSG presents numerous pharmacological properties including antioxidant, free radical-scavenging, anti-inflammation, and cardioprotective effects. Microglia BV2 cell lines were used to investigate the antineuroinflammatory effects of TSG. TSG reduced LPS-induced microglia-derived release of proinflammatory factors such as TNF-α, IL-1β, and nitric oxide (NO). Further, TSG attenuated LPS-induced NADPH oxidase activation and subsequent reactive oxygen species (ROS) production [65].

Cryptolepine significantly inhibited LPS-induced production of TNF-α, IL-6, IL-1β, NO, and prostaglandin E2 (PGE2). Protein and mRNA levels of cyclooxygenase-2 (COX-2) and iNOS were also attenuated by cryptolepine [66]. Kalopanaxsaponin A, a triterpenoid saponin isolated from *Kalopanax pictus*, inhibited iNOS, COX-2, and TNF-α expressions in LPS-stimulated microglia, while kalopanaxsaponin A increased anti-inflammatory cytokine IL-10 expression [67]. Fucoidan treatment significantly inhibited excessive production of NO and PGE2 in LPS-stimulated BV2 microglia. It also attenuated expression of iNOS, COX-2, MCP-1, and proinflammatory cytokines, including IL-1β and TNF-α. Moreover, fucoidan exhibited anti-inflammatory properties by suppressing NF-κB activation and downregulating extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), MAPK, and AKT pathways [68]. Geniposide decreased the secretion of TNF-α, IL-1β, IL-6, IL-8, and IL-10 from cultured microglial cells. It also downregulated TLR4 mRNA expression in the microglia [69]. Treatment with LXA(4) ME suppressed neutrophil infiltration and lipid peroxidation levels, inhibited the activation of microglia and astrocytes, reduced the expression of TNF-α and IL-1β, and upregulated the expression of anti-inflammatory cytokines IL-10 and TGF-β 1 in the ischemic brain [70]. Compared with the vehicle group, rosuvastatin prevented the impairment of neurological function and decreased the infarct volume. The increases in activated microglia, macrophages, and superoxide levels usually caused by ischemia/reperfusion were significantly ameliorated by rosuvastatin. Rosuvastatin also inhibited the upregulation of gp91^{phox} and p22phox, phosphorylation of NF-κB, and induction of COX-2 and iNOS [71]. These studies suggest that chemicals such as propofol, TSG, and cryptolepine have experimentally neuroprotective effects and they may be therapeutic target for clinical application.

4.3. Augmentation of Anti-Inflammatory Response. Early studies showed that the administration of the anti-inflammatory cytokine IL-10 protects against permanent MCAO in mice. IL-10 was overexpressed in astrocytes, microglia, and endothelial brain cells in IL10T compared with wild-type mice. Four days following MCAO, IL-10T mice showed a 40% reduction in infarct size that was associated with significantly reduced levels of active caspase 3 compared with wild-type mice [72]. Subcutaneous administration of IGF-1 also resulted in significantly reduced infarct volumes and an increase in motor-sensory functions in normotensive rats [73].

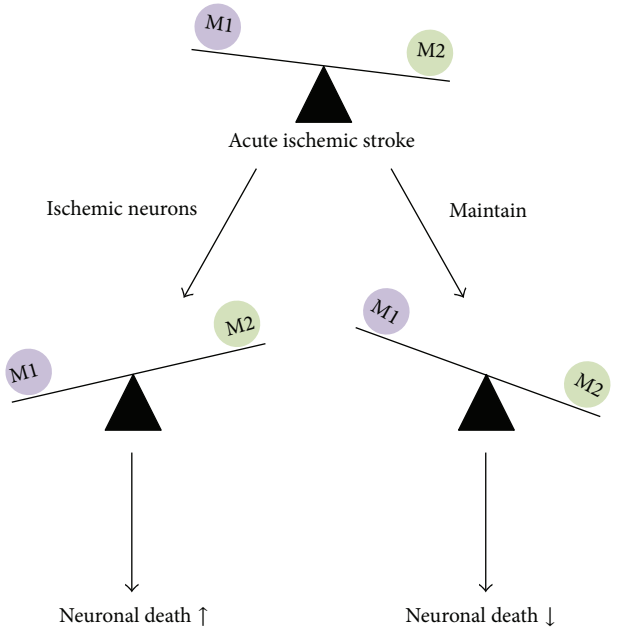

Figure 2: The roles of microglia in ischemic stroke. The balance between proinflammatory and anti-inflammatory responses is important for determining outcomes after stroke.

TIPE2 (TNF-α-inducible protein 8-like 2 or TNFAIP8L2) is essential for maintaining immune homeostasis. Some genetic studies suggested the role of TIPE2 in the regulation of TLR function and the link between TLRs and ischemic cerebral injury. The genetic ablation of the TIPE2 gene significantly increased the cerebral volume of infarction and neurological dysfunction in mice subjected to MCAO [74].

Therefore, exogenous administration or overexpression of pivotal factors may be strong candidates for the treatment of ischemic stroke.

5. Conclusion

We have summarized recent evidence suggesting that microglia have critical functions during ischemic stroke. Inflammation associated with microglia plays an important role in the pathogenesis of ischemic stroke. Although several trials for anti-inflammatory treatment have proven to be effective for treating acute stroke in animal models, they have unfortunately been ineffective in clinical trials. Increasing evidence suggests that inflammatory response is a double-edged sword, as it not only exacerbates secondary brain injury in the acute stage of stroke but also contributes beneficially to brain recovery after stroke (Figure 2). Microglia could serve as powerful cellular targets in ischemic stroke. Successful microglial replacement therapy is encouraging since manipulation of microglia may be effective for treating other neurological conditions. However, there is still much to be done in order to translate promising preclinical findings into clinical practice. Further studies should consider the pro- and anti-inflammatory responses by microglia, not separately but as a whole. Improving our understanding of the dynamic balance between pro- and anti-inflammatory responses and identifying the discrepancies between preclinical studies and clinical trials may lead to the design of more effective therapies.

Conflict of Interests

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

Authors' Contribution

Youngjeon Lee, Sang-Rae Lee, and Sung S. Choi contributed equally to this paper.

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Review Article

Evidence of Inflammatory System Involvement in Parkinson's Disease

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Parkinson's disease (PD) is a chronic neurodegenerative disease underpinned by both genetic and environmental etiologic factors. Recent findings suggest that inflammation may be a pathogenic factor in the onset and progression of both familial and sporadic PD. Understanding the precise role of inflammatory factors in PD will likely lead to understanding of how the disease arises. *In vivo* evidence for inflammation in PD includes dysregulated molecular mediators such as cytokines, complement system and its receptors, resident microglial activation, peripheral immune cells invasion, and altered composition and phenotype of peripheral immune cells. The growing awareness of these factors has prompted novel approaches to modulate the immune system, although it remains whether these approaches can be used in humans. Influences of ageing and differential exposure to environmental agents suggest potential host-pathogen specific pathophysiologic factors. There is a clear need for research to further unravel the pathophysiologic role of immunity in PD, with the potential of developing new therapeutic targets for this debilitating condition.

1. Introduction

Parkinson's disease (PD), characterized by a loss of dopaminergic neuron, is a common movement disorder, affecting over 4 million individuals worldwide. Although a subject of intense research, the mechanisms underlying PD pathogenesis remain incompletely understood. However, a broad range of studies conducted over the past few decades, including epidemiological, genetic, and postmortem analysis, as well as *in vitro* and *in vivo* modeling, have contributed significantly to our understanding of the pathogenesis of the disease. It is generally accepted that both genetic and environmental factors contribute to the development of PD. Several genes have been identified including *SNCA, PARKIN, DJ-1, PINK1,* and *LRRK2*, whose mutations are responsible for rare familial forms of PD. Despite such progress, the functions of the products of most susceptibility genes have not been fully elucidated. The large number of susceptibility genes probably reflects the complexity of the pathogenesis that contributes to the development of PD. While genetic and chemical models of the disease have established oxidative stress and mitochondrial and proteasomal dysfunction as disease-perpetrating events, the mechanism mediating dopaminergic deficit in sporadic PD remains unknown [1]. The search for a PD pathoetiologic mechanism has uncovered dysfunction of the immune system, particularly innate neuroinflammatory response, as a potential etiologic factor [2, 3]. In addition, recent reports have also implicated the adaptive immune system in PD pathogenesis. Research on susceptibility genes identified by GWAS indicates that some autoimmune diseases such as Crohn's disease may share mutations on the same gene *LRRK2*, which has exemplified the significance of immune system in the pathogenesis of PD. Interestingly, other PD genes have also been reported to have a functional role in immune system. DJ-1 has been reported to regulate mast cell activation and IgE-mediated allergic responses [4].

Manzanillo and colleagues have shown that parkin has a role in ubiquitin-mediated autophagy of *M. tuberculosis*. They reported that both parkin-deficient mice and flies are sensitive to various intracellular bacterial infections [5]. We propose that the likelihood of a common mechanism fundamental to the etiology of all genetic backgrounds of PD is the dysregulation of the immune system, which makes the patients vulnerable to environmental challenge, such as infections or chemical exposure. Although it is still far away to conclude that PD is an autoimmune disease, a better understanding of the cross-talk between the immune system and the CNS will be crucial to harness natural beneficial responses for therapeutic strategies. Here, we review the involvement of immune system (Figure 1) and inflammatory factors (Table 1) in the pathogenesis of PD and discuss potential therapeutic targets of immune regulation in PD.

2. Molecular Mediators

2.1. Cytokines and Other Soluble Signaling Proteins. Although the etiology of PD is unknown, it is generally believed that the genetic and environmental factors cause the damage of dopaminergic neurons. Necrosis of neurons is observed following acute brain injury and neurotropic infections or as a result of the release of damaging chemicals such as glutamate, nitric oxide (NO), and reactive oxygen species (ROS). Damaged neurons themselves then induce the local inflammation and may exacerbate immune-mediated disease. Alternative explanation indicates that neuroinflammation may serve as an initiation factor of DA neuron degeneration. This has been supported by researchers using ploy(I:C) (TLR3 agonist) and LPS (TLR4 agonist) to induce the loss of DA neuron at the SNc of murine [6, 7]. The expression and function of PD genes such as LRRK2 and SNCA in immune cells are also in support with this possibility [4, 5]. Mutations of these genes may increase the individual's vulnerability to infections and activate the immune system and lead to dopaminergic neuron demise [8, 9].

Abundant evidence in humans demonstrates a role for chronic inflammation and innate immune activation in PD. Cytokines such as IL-2, IL-4, IL-6, IL-10, TNF-α, and IFN-γ increased in the serum of PD patients [10, 11] and association between systemic markers of inflammation and idiopathic PD risk has been reported [12]. Postmortem studies found upregulated levels of cytokines (including IL-1, TGF-α, IFN-γ, and IL-6) in the CSF and nigrostriatal regions of individuals with PD relative to age-matched healthy controls [13–16]. Of particular interest, matrix metalloproteinase-3 (MMP-3) can stimulate microglia to produce proinflammatory and cytotoxic molecules such as TNF-α, IL-6, and IL-1β as well as MMP-3, which in turn contribute to neuronal damage [17]. MMP-3 has also been reported to damage blood-brain barrier (BBB) and amplify neuroinflammation in an MPTP mouse model of Parkinson's disease [18]. In addition, MMP-3 can be induced in astrocyte by polyI:C and impair neurodevelopment [19]. Furthermore, proteins of the complement system, a serum-mediated mechanism designed to clear antibody and various immune targets, are found in extra neuronal Lewy body postmortem. IL-18 synergizes with IL-12 to produce IFN-γ in NK cells [20, 21] and IL-12, IFN-γ, and TNF-α in monocyte [22]. IL-18 and its receptor in multiple sclerosis (MS) have been intensively studied [23–25] while its role in PD is under investigation. In animal models, IL-18 null mice showed reduced dopaminergic neuron loss upon 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) treatment [26]. Interestingly, IL-18 gene promoter polymorphism [27] and IL-17 and IL-10 gene polymorphism [28] have been reported to be associated with the risk for developing sporadic PD in the Han Chinese populations. This finding suggests that innate immune activation occurred in association with or in response to Lewy body formation [29]. Serum levels of TNF are elevated in PD patients and the serum levels of IL-6 correlate with Hoehn and Yahr staging [30]. Taken together, these observations indicate that an active inflammatory process with definite innate immune involvement is ongoing in the CNS of PD patients.

2.2. Toll-Like and Other Pattern Recognition Receptors. Innate immune responses are generally initiated following recognition of pathogen-associated molecular patterns (PAMPS), conserved structures expressed by infectious agents. Equally important are endogenous signals for innate responses, known as damage-associated molecular patterns (DAMPS), which are nuclear and cytosolic proteins including DNA, heat shock proteins (HSP), ATP, oxidized membrane lipids, and aggregated and modified or misfolded proteins. It has been recently shown that extracellular α-synuclein may act as a DAMP for microglia, increasing the expression of TLR-1, -2, -3, and -7, MyD88, MMP-9, TNF-α, and IL-1β [31].

The innate immunity sensors include both cell-associated pattern recognition receptors (PRRs), which include toll-like receptors (TLRs), NOD-like receptors (NLR), RIG-like receptors (RLRs), C-type lectin receptors, scavenger receptors, and N-Formyl met-leu-phe (fMLP) receptors, and soluble PRRs, such as complements, collectins, pentraxins, and natural antibodies. For many of these PPRs, their expression pattern and role in neurodegenerative disorders are still under investigation. TLRs play a significant role in noninfectious diseases such as atherosclerosis, asthma, and inflammatory bowel disease (IBD) [32–34]. TLR activation induces several signalling pathways via the intracellular adaptor protein Myd88 and TIR-domain-containing adapter-inducing interferon-β (TRIF) and production of a wide range of immune-regulatory mediators such as cytokines and chemokines. The involvement of TLRs in neurodegenerative diseases is evidenced by pathology studies of human disorders, as well as by data from experimental animal models [35–37]. As is so often the case in the context of immune regulation, TLR-mediated responses can both aggravate inflammation or contribute to its control.

Another group of PRRs, namely, NOD-like receptors (NLRs), initiate the assembly of inflammasomes which activate caspases and, in this way, promote production and secretion of mature IL-1β and IL-18. Aβ and prion proteins are potent activators of the (NLR family, pyrin domain-containing) NLRP3 inflammasome [38–40]. NLRP1 and

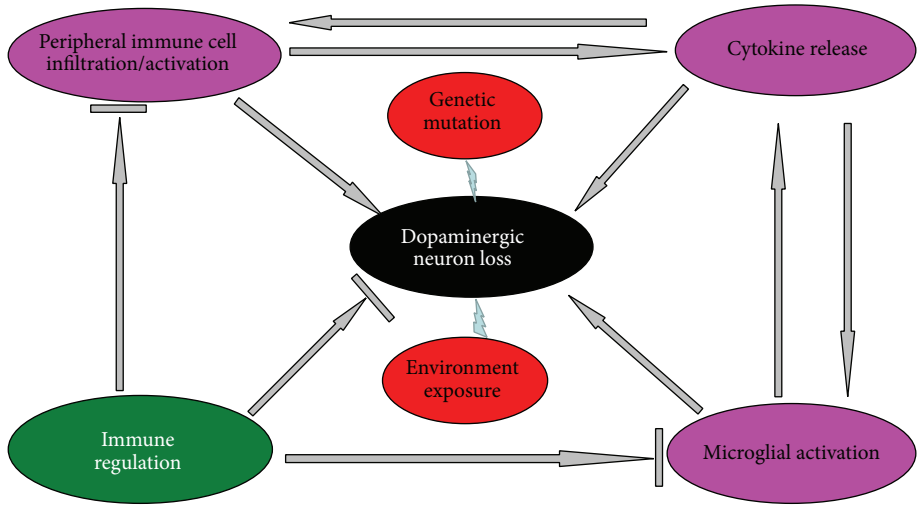

Figure 1: The paper is summarized in the schematic drawing. Genetic mutations and environmental exposures can selectively induce dopaminergic neuron loss in the SNc. The mechanism may involve both regional (microglia) and systematic (peripheral) immune dysregulation. The dysregulated immune system can either elicit or exacerbate dopaminergic neuron loss at SNc by direct contact or by overrelease of cytokines and other immune mediators and thus immune regulation may have great therapeutic potential in PD treatment.

Table 1: Inflammatory factors involved in Parkinson's disease.

Cytokines and other soluble molecules	IL-1, IL-2, IL-4, IL-6, IL-10, TNF-α, IFN-γ, TGF-α, IL-6, MMP-3, IL-17, and IL-18
Pattern recognition receptors (PRRs)	TLRs (TLR-1, -2, -3, and -7), NLRs, and complements
Immune cells	Microglia, monocyte, NK cell, T-cell, and B cell

NLRP5 are known to contribute to neuronal death [41]. Aβ induced IL-1β release in microglia through NLRP3 [42]. Although data mining indicates an upregulation of NLRP4 and a downregulation of NR2C2, which is associated with NLRP10, in the fibroblast cells from LRRK2 mutated patients [43], the direct contribution of NLRs to the pathogenesis of PD has not been reported. Yet other receptors important in neuronal-glia interactions are the purinergic receptors P1 (adenosine) and P2 (ATP) receptors. Experimental evidence indicates that A2A adenosine receptors (ARs) play a pivotal role in the inhibition of inflammatory processes. Ohta and Sitkovsky reported that the stimulation of A2A receptor signaling suppressed the inflammation and inhibited the tissue damage in mouse models of liver injury and endotoxin-induced septic shock [44]. The inhibitory effect was through regulating IL-18 activity [45–48] and thus may be candidate of therapeutic target for IL-18-initiated diseases, including MS, RA, and asthma. Adenosine receptors can mediate both potentially neuroprotective and neurotoxic effects. Their roles in different neurodegenerative diseases are not elucidated yet. In PD, the expression of both P1 and P2 receptors is altered [49, 50]. In attempts to clarify the functional significance of these changes, much attention has been devoted to the role of the adenosine A2A receptor in PD since antagonists for this receptor improve clinical symptoms and protect against toxin-induced neuronal degeneration [51, 52]. Thus, PRRs play an ambivalent role in neurodegenerative diseases, depending on the mode of activation.

2.3. The Complement System. Complements, which serve as soluble PRRs in the activation of innate immune system, were found in extra neuronal Lewy bodies postmortem. This has been proposed to be important for elimination of aggregated proteins in PD by phagocytosis by microglial cells expressing complement receptors [29]. However, the broad and often profoundly unregulated expression of complement components indicates that complement activation may equally contribute to the demise of neurons and axons. In the MPTP model, mannose binding lectin was found to be increased in SNc area earlier than the neuronal loss, which indicates that the complement system activation may contribute to the dopaminergic neuron damage [53]. The fact that neurons and oligodendrocytes express low levels of complement regulatory proteins renders these cells particularly vulnerable to complement-associated death [54].

3. Microglia, the Resident Immune Cells

Inflammation via activated glial cells has been reported as an important factor responsible for pathogenesis of PD [55–57]. The presence of activated microglia has long been reported in PD patients, but the mechanism and role of this activation remain controversial [58].

The classic activation protects neurons from injury and maintains homeostasis in the brain. After the resolution of the activation, microglia return to the resting state. In PD patients, persistent activation of microglia has been observed [59]. Activated microglia have also been found in the SN

and/or striatum in animal models of PD [53, 60–62]. An accumulation of activated microglia around dopaminergic neurons has been found in three postmortem human brains with MPTP-induced Parkinsonism [63]. Taken together, these data indicate a high association between the microglia activation and the dopaminergic neuron degeneration. The recently discovered genetic association between the HLA regions with late-onset sporadic Parkinson's disease [64] strengthens the possible relevance of antigen presenting cells (APC) in PD. Although the exact causal link between microglia activation and dopaminergic neuron injury in PD remains controversial, several lines of evidence have suggested that persistent microglia activation exerts deleterious effects that result in the loss of dopaminergic neurons.

The first harmful effect of microglia was through the activation of NFκB pathway [65, 66] and consequently increased release of proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) [67–69], while it decreased release of anti-inflammatory cytokines such as IL-4, IL-13, IL-10, and tumour growth factor-β (TGF-β). However, both proinflammatory and anti-inflammatory cytokines were observed to be elevated in other studies [10, 70], indicating the complexity of microglia activation. Persistent exposure to high concentrations of the proinflammatory cytokines threatened the viability of dopaminergic neurons. For example, chronic expression of TNF-α or IL-1β in the SN of rats can induce the dopaminergic neuron death [69, 71, 72]. Moreover, the TNF receptor, also a death-signaling receptor, has been found to be widely expressed on dopaminergic neurons in human SN pars compacta (SNpc) [73] and contributes to phenotype that likely contributes to the selective vulnerability of dopaminergic neurons in PD patients. Correspondingly, mice carrying homozygous mutant alleles for both TNF receptors 1 and 2 were protected against MPTP-induced dopaminergic neurotoxicity [74]. Activated microglia can also release chemokines and recruit peripheral immune cells to brain parenchyma and this will be reviewed later in this paper.

The second noxious effect of activated microglia in PD was an increased production of reactive oxygen species, such as NO and superoxide. These reactive species can directly cross the membrane of dopaminergic neurons, overwhelm the endogenous antioxidant systems, and ultimately cause oxidative stress and degeneration of dopaminergic neurons [75].

Thirdly, microglia may also present endocytosed or lysosomal peptides to CD4 T lymphocyte through the expression of MHC class II [76, 77], which can propagate the inflammatory process. In light of these findings, activated microglia certainly appear to be toxic and inexorably exacerbate the death of dopaminergic neurons. However, modest activation of microglia is necessary and beneficial for brain health. With respect to their role in promoting neuronal injury, the focus should also be on plasticity of the microglial response rather than identifying them as a solely negative factor.

However, the pinpoint for the microglia transit from protective into neurotoxic ones is not clear. Microglia were activated acutely when dopaminergic neuron death occurred in the SN of many MPTP- or 6-hydroxydopamine- (6-OHDA-) induced animal models, which usually resemble the end stage of clinical PD. However, PD develops gradually and becomes a chronically acquired state. Thus, a complete profile of microglial activation during the entire course of PD may help us reach a better understanding of progression of the disease. In fact, the plasticity of microglial states is dependent on not only the type of stimulus but also the duration and magnitude of stimulation [78]. A delicate equilibrium of microglial-derived factors might determine the neurotrophic or neurotoxic effect of activated microglia. The modulation of microglial functional states may be a useful tool to intervene in the progression of PD.

4. Peripheral Leukocyte Infiltration: Recruited Immune Cells from Blood

Primarily taken as an immune privileged organ, the brain parenchyma infiltration of peripheral leukocyte is tightly regulated at the level of the blood-brain barrier (BBB) [79, 80]. However, the peripheral leukocyte migration and infiltration in the brain do occur in PD.

Since the invasion of T cells in the brain was first reported [58, 81], numerous studies have confirmed the infiltration of immune cells in the brain in both postmortem PD patients and animal models [82, 83]. These brain-invading lymphocytes consisted of a heterogeneous population of both CD8+ and CD4+ cells. A similar profile of peripheral leukocyte infiltration was also present in MPTP mouse models of PD, which further validated the relevance of the model to the human syndrome [82]. While still under investigation, the infiltration of regulatory T cells (Treg) was generally accepted to have protective effect on dopaminergic neurons through suppressing microgliosis either by direct contact or secreting cytokines that attenuate inflammatory responses [84, 85]. Interestingly, both the experimental model and the clinical syndrome exhibit BBB dysfunction [53, 86]. Rentzos and colleagues also report that circulating RANTES (Regulated on Activation, Normal T Cell Expressed and Secreted), which is a C-C beta-chemokine with strong chemoattractant activity for T lymphocytes and monocytes [87], was increased in PD patients, which indicates that the recruitment of T lymphocytes to sites of inflammation in the central nervous system of PD patients may be through the interaction of RANTES and its receptor CCR5.

Besides direct function at the inflammatory site, the peripheral immune cells may also get involved in the pathogenesis of PD systemically. CD4+ T and CD19+ B cells have been reported to be reduced in the peripheral blood of PD patients due to a reduced development/proliferation or because the recruitment to the brain is unclear [88]. Another study by Niwa and colleagues showed an increase of NK cells and a decrease of Th1 cells in the peripheral blood and they were associated with disease severity in patients with PD [89]. A strong upregulation of peripheral monocyte percentage and the CCR2 on its surface has also been reported in PD patients [90]. Interestingly, many of the genes identified to be the cause of familial PD have also been found to be expressed

in peripheral immune cells and play important roles. The first identified mutation to cause PD was the SNCA gene, which is expressed in a wide variety of immune cells including T cells, B cells, NK cells, microglia, and monocytes [91–94]. LRRK2 and DJ-1 were also found to be expressed in different populations of human peripheral blood mononuclear cells (PBMCs) and may contribute to autoimmune diseases such as Crohn's disease, leprosy [95, 96], and multiple sclerosis [97]. Parkin was recently found to mediate resistance to intracellular pathogens. The authors managed to show that the parkin-deficient mice and flies are sensitive to many intracellular bacterial infections, which include *M. tuberculosis*, indicating that this gene has a conserved role in metazoan innate defence [5]. Taken together, these results suggest the existence of highly regulated immunopathogenic mechanisms at work in PD that may ultimately be harnessed as therapeutic(s).

5. PD and Infectious Disease

Since the Poskanzer and Schwab hypothesis (PSH) that Parkinson's disease was due to influenza infection in the 1950s, the relationship between PD and viral infection has received much attention. Although the original hypothesis has been proven to be fault, the association between pathogen exposure and Parkinson's disease is still being actively pursued. Although infection has not been shown to be associated with PD by some researchers [98], more recently, it was reported that viral infections such as mumps, scarlet fever, influenza, whooping cough, and herpes simplex infections were significantly related to PD development [99]. It was also suggested, but never proven, that intrauterine influenza infection may be related to PD. Alpha-synuclein has also recently been proposed as a prion-like protein that can migrate from affected to unaffected neurons [100, 101]. Further evidence is needed to establish the relationship between PD and pathogen infections.

6. Immune Regulation as Therapeutic Targets

Evidence for the involvement of the immune system (both peripheral immune cells and brain resident microglia) in the development and progression of PD has inspired immunotherapeutic approaches to prevent neuronal loss and to aid neuronal growth. In general, researchers have tried to block the effects of microglia-derived inflammatory mediators [102] or modulate the peripheral immune system [76, 103]. Strategies aimed at harnessing the inflammatory response in preclinical models of PD have included use of anti-inflammatory gene therapy approaches: overexpression of a dominant negative TNF molecule to block native TNF signalling has been shown to effectively protect neurons from 6-OHDA-induced cell death even after delayed administration [104, 105]. Another approach has been the use of NSAIDs (nonsteroid anti-inflammatory drugs) as their use seems to reduce the risk of PD development [106–109]. Anti-inflammatory compounds, such as naloxone, minocycline, and dexamethasone, can reduce microglia activation and neuronal damage in different models of nigral degeneration [110–113]. Alternatively, the potential neuroprotective compounds may be hydrogen sulphide-releasing l-DOPA derivatives that reach the brain and reduce the level of IL-6/TNF and NO from microglia [114]. More specific blockade of inflammation has been achieved successfully with inhibitors of COX-2 [115], which has been shown to be increased in PD SN [116].

Peripheral immune system modulation has been mainly designed to prime T cells *in vivo* with different agents and then transfer them to the periphery of animal models with induced dopaminergic neuron death. Another common strategy is to induce Treg (regulatory T cells)/tolerance. Indeed, Treg transfer into MPTP-treated animals attenuated loss of nigral DA neurons [84, 85]. Of particular interest is the stem cell transplant therapy. These neural stem cells (NSCs) and mesenchymal stem cells (MSCs) are no longer believed to primarily replace damaged cells but rather to modulate immune responses and rescue the dopaminergic neurons by secreting a variety of soluble factors [53, 117].

7. Conclusions

There is increasing evidence that dysregulated inflammatory responses are implicated in PD. While a direct causal relationship between an infectious agent and PD has yet been unequivocally proven, studies of specific host-pathogen responses that are relevant to select individuals will provide further pathophysiologic clues. Based on current evidence, interventions aimed at either blocking microglia-derived inflammatory mediators or modulating the peripheral immune cells may be potentially useful therapies that are worth exploring. The role of PD genes in modulating the immune system will hopefully unravel pathophysiologic clues that could lead to development of new therapeutic targets.

Conflict of Interests

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

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Research Article

An *In Vivo* Microdialysis Study of FLZ Penetration through the Blood-Brain Barrier in Normal and 6-Hydroxydopamine Induced Parkinson's Disease Model Rats

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FLZ (N-[2-(4-hydroxy-phenyl)-ethyl]-2-(2,5-dimethoxy-phenyl)-3-(3-methoxy-4-hydroxy-phenyl)-acrylamide) is a novel synthetic squamosamide derivative and a potential anti-Parkinson's disease (PD) agent. The objective of the present study was to investigate the penetration of free FLZ across the BBB and the effects of P-gp inhibition on FLZ transport in normal and 6-hydroxydopamine (6-OHDA) induced PD model rats. *In vivo* microdialysis was used to collect FLZ containing brain and blood dialysates following intravenous (i.v.) drug administration either with or without pretreatment with the specific P-gp inhibitor, zosuquidar trihydrochloride (zosuquidar·3HCl). A sensitive, rapid, and reliable ultraperformance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) technique was developed and validated to quantitate free FLZ levels in the dialysates. No significant differences were observed in the brain/blood FLZ area under the concentration-time curve (AUC) ratio between normal and PD model rats. However, pretreatment with zosuquidar·3HCl markedly increased the AUC ratio in both rat models. In addition, FLZ penetration was similar in zosuquidar·3HCl-pretreated normal and PD rats. These results suggest that P-gp inhibition increases BBB permeability to FLZ, thereby supporting the hypothesis that P-gp normally restricts FLZ transfer to the brain. These findings could provide reference data for future clinical trials and may aid investigation of the BBB permeability of other CNS-active substances.

1. Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder after Alzheimer's disease (AD). The prevalence of PD increases with age and is approximately 0.3% in the general population, which rises rapidly to 3% for individuals over the age of 65 years [1].

Since the early 1960s, the standard treatment for PD has involved the pharmacologic replacement of dopamine with the dopamine precursor, 3,4-dihydroxy-L-phenylalanine (L-DOPA). Supplementation of L-DOPA with carbidopa, an inhibitor of aromatic L-amino acid decarboxylase (and hence the peripheral metabolism of L-DOPA), represented a marked improvement in therapy and is still a mainstay of PD treatment [2]. Many other pharmacotherapies, such as dopamine agonists, catechol-O-methyltransferase (COMT) inhibitors, monoamine oxidase-B (MAO-B) inhibitors, anticholinergic agents, amantadine, and glutamate receptor antagonists, have also been used [3]. However, there is still no cure for PD and no solid evidence for efficacious disease-modifying strategies. In addition, motor complications in the advanced stages of the disease, adverse effects of dopaminergic therapy, and nonmotor symptoms (e.g., loss of sense of smell, sleep disturbances, mood disorders, orthostatic hypotension, and constipation) remain enormous challenges during long-term therapy. Thus, new neuroprotective therapeutic agents are urgently needed [3].

FLZ (N-[2-(4-hydroxy-phenyl)-ethyl]-2-(2,5-dimethoxy-phenyl)-3-(3-methoxy-4-hydroxy-phenyl)-acrylamide; Figure 1) is a novel synthetic derivative of squamosamide, which was first isolated from *Annona glabra* (Pond Apple) [4]. FLZ

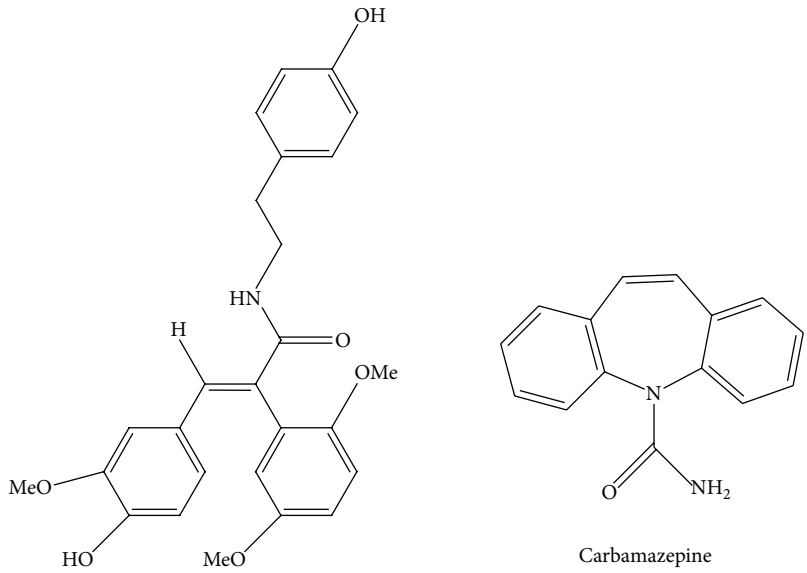


Figure 1: Structure of FLZ and carbamazepine (IS).

inhibits the lipopolysaccharide-induced production of certain inflammatory mediators [5], has potent neuroprotective effects [6–12], and may be a potential treatment for PD [6, 7, 13] and AD [8, 14–16].

A preclinical pharmacokinetic study showed that FLZ can cross the blood-brain barrier (BBB) with no target effects [17]. Our previous work also showed that the binding rate of FLZ to plasma proteins is very high (about 90%). Therefore, for therapeutic purposes, it is critical to examine the ability of free FLZ to cross the BBB. The BBB permeability of a drug can usually be established by sampling the cerebrospinal fluid (CSF) [18] or extracellular fluid (ECF) using a technique called microdialysis [19]. Microdialysis has a number of advantages over traditional methods [20–22]. For example, sampling can be performed dynamically and continuously with no fluid loss, and high-resolution concentration profiles for drugs and metabolites can be obtained from individual subjects. In addition, only drugs that penetrate the BBB are obtained using this technique, whereas samples obtained from brain homogenates also contain drugs residing within the blood trapped in the network of capillaries running throughout brain. Microdialysis can also be used to sample multiple sites within a single animal/person [23, 24]. However, most microdialysis experiments were performed in normal animals rather than in actual animal models of the target disease; this is important because disease status may influence drug disposition in the brain [25–27]. In particular, the BBB is thought to be leaky in individuals with neurodegenerative diseases such as AD or PD [28–31], which has a marked effect on drug passage into the brain.

There are several animal models of PD, such as 6-hydroxydopamine (6-OHDA), rotenone, drosophila α-synuclein overexpression, mouse α-synuclein overexpression, and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model [32]. 6-OHDA and MPTP are the two classic toxin-induced animal models of PD [33]. MPTP is mainly used in nonhuman primates and in mice [33, 34]. In contrast to primates, rodents are less sensitive to MPTP toxicity [35]. Rats are resistant to MPTP toxicity and mouse strains vary widely in the sensitivity to the toxin [36]. However, 6-OHDA is a highly effective toxin for dopaminergic neurons in mice, rats, cats, and primates [32]. 6-OHDA induced lesion is commonly used in rats, which is established by stereotactic techniques and with relatively low maintenance costs.

A previous investigation in normal animals also indicated that FLZ may be a substrate for the multidrug resistance transporter, P-glycoprotein (P-gp) [17], which is encoded by the *ABCB1* gene in humans. However, this study only investigated brain-to-plasma ratios at four independent time points after the administration of FLZ; therefore, the time-dependent effect of P-gp inhibition on the distribution of unbound FLZ in the brain was not determined. Furthermore, P-gp function [31, 37] and mRNA expression levels [38] are reportedly reduced in PD brains versus normal brains.

Zosuquidar·3HCl is an extremely potent P-gp modulator, does not modulate multidrug resistance protein (MRP1) or breast cancer resistance protein (BCRP) mediated resistance [39, 40], and has a significantly lower affinity for CYP3A than for P-gp [41]. As a third generation P-gp inhibitor, it displays characteristics that make it an "ideal modulator" of P-gp mediated multidrug resistance [42]: it binds P-gp with high affinity; it shows highly potent *in vitro* reversal of drug resistance; it has a high therapeutic index (active at doses ranging from 1 to 30 mg/kg in *in vivo* antitumor efficacy experiments); and it has little effect on the pharmacokinetics of coadministered agents [43–45].

The present study used an innovative *in vivo* microdialysis technique to investigate the penetration of unbound FLZ

through the BBB. We also used normal and 6-OHDA induced PD model rats to assess the effects of zosuquidar·3HCl (a specific P-gp inhibitor) mediated P-gp inhibition on free FLZ concentrations in the brain ECF and blood over time. *In vivo* microdialysis is a useful tool for evaluating drug passage across the BBB, particularly when used to study drug transporters in the CNS [20]. As such, the present study addresses a knowledge gap regarding the permeability of the BBB to drugs and the effects of P-gp in normal and PD model animals.

2. Materials and Methods

2.1. Chemicals and Reagents. FLZ (purity = 99.6%; pKa = 13.75 ± 0.46; lipophilic and poorly water-soluble) was supplied by Professor Ping Xie (Institute of Materia Medica, Chinese Academy of Medical Sciences, Beijing, China). Carbamazepine (used as an internal standard (IS)) was obtained from the National Institutes for Food and Drug Control (Beijing, China). Zosuquidar·3HCl was purchased from Shanghai Haoyuan Chemexpress Co., Ltd. (Shanghai, China). Bovine serum albumin (BSA, Australian origin; purity > 98%) was purchased from Beijing SeaskyBio Technology Co., Ltd. (Beijing, China). Heparin sodium (purity ≥ 99%, 150 U/mg) was obtained from Beijing Biodee Biotechnology Co., Ltd. (Beijing, China). Acetonitrile (mass spectrometry (MS) grade) was obtained from Honeywell Burdick & Jackson Inc. (Muskegon, MI, USA). Ringer's solution (145 mmol NaCl, 2.97 mmol $CaCl_2$, and 4.03 mmol KCl) was purchased from Beijing Double-Crane Pharmaceutical Co., Ltd. (Beijing, China). All other chemicals were high-performance liquid chromatography (HPLC) grade. Deionized water was purified using a Millipore water purification system (Millipore, Billerica, MA, USA). The perfusion fluid was prepared by dissolving BSA in Ringer's solution (4%, w/v).

2.2. Experimental Animals. Male Wistar rats (250–350 g; Vital River Laboratories, China) were used in this study. Animals were maintained under temperature-controlled conditions with a 12 h light/dark cycle and allowed food and water *ad libitum*. All rats were allowed to acclimatize for a minimum of 3 days upon arrival before experimentation. All protocols and procedures involving animals were approved by the Animal Care and Welfare Committee of Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College (Beijing, China).

2.3. 6-OHDA Induced Rat Model of PD. PD was induced in rats using 6-OHDA, as previously described [46]. Briefly, rats were anaesthetized with pentobarbital sodium (30 mg/kg, i.p.) and mounted on a stereotaxic apparatus. The skull was cleaned and a burr hole was drilled to allow a needle to be passed into the right substantia nigra pars compacta (anterior-posterior: −5.2; lateral: +2.2; dorsal-ventral: −7.8 relative to bregma) according to Paxinos and Watson [47]. Unilateral infusion of 6-OHDA (10 μg dissolved in 0.1% ascorbic acid (final concentration: 2 μg/μL); purity > 98%; Sigma Aldrich, China) was performed for 5 min at a rate of 1 μL/min. After the infusion, the needle was kept in place for another 10 min to prevent leakage along the needle track. To confirm the onset of PD, the rotational behaviour of the rats, which was induced by i.p. injection of apomorphine (0.5 mg/kg, dissolved in water), was tested 14 days after lesion formation. The number of contralateral rotational turns was recorded over 30 min. Immunohistochemical staining for tyrosine hydroxylase (TH) was also performed to confirm PD [6, 48].

2.4. Experimental Design. Rats were divided into four groups (n = 3 animals per group): three normal rats treated with FLZ only (FLZ only-N); three PD rats treated with FLZ only (FLZ only-PD); three normal rats treated with FLZ plus zosuquidar·3HCl (FLZ + ZOSUQ-N); and three PD rats treated with FLZ plus zosuquidar·3HCl (FLZ + ZOSUQ-PD). The system was equilibrated for 1 h after surgery. FLZ was dissolved in a mixture of dimethyl sulfoxide, polyethylene glycol 400, and sodium chloride injection (1 : 4 : 5 (v/v/v)). All rats received FLZ (35 mg/kg) via intravenous injection into the tail vein (with or without pretreatment with zosuquidar·3HCl). Rats in the FLZ + ZOSUQ-N and FLZ + ZOSUQ-PD groups received an i.v. injection of zosuquidar·3HCl (20 mg/kg, dissolved in 20% ethanol-saline) 10 min before FLZ administration. The dose, route of administration, and timing of P-gp inhibition were based on those described in earlier studies [17, 49–51]. Microdialysis samples were collected from the striatum and the jugular vein at 15 min intervals for 1 h before (blanks) and for 4 h after FLZ administration. The samples were stored at −80°C until they were analysed by ultraperformance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). At the end of the experiment, the rats were decapitated and the brains were removed for histological verification of probe placement. Data were discarded if the probe placement was outside the target area.

2.5. In Vitro Experiments. *In vitro* experiments were performed to predict nonspecific binding and described approaches to reduce the degree of adsorption based on a previous study [52]. Firstly, the nonspecific binding to the tubing was assessed by comparing the FLZ concentrations before and after passing through the tubing. Then different concentrations of BSA (0.5%, 2%, or 4%, w/v) were added to the perfusion fluid to evaluate the nonspecific binding. The most frequently used calibration method, retrodialysis, was used to assess the level of probe recovery. The general requirement of this method is that the extraction fraction should be the same whether solute exchange across the membrane occurs by either gain (sampling) or loss (delivery) [22]. When the gain value was determined, the probe was inserted into the FLZ standard solution and perfused with the blank perfusion fluid, while the loss value was tested and the probe was positioned in blank perfusion fluid and perfused with FLZ standard solution (gain = C_{out}/C_s and loss = $1 - C_{out}/C_{in}$, where C_{in} is the perfusate concentration (inflow to probe), C_{out} is the dialysate concentration (outflow from probe), and C_s is the standard FLZ concentration surrounding the membrane). Firstly, BSA was added to the perfusion fluid inside the membrane but not to the solution

outside the membrane. Next, BSA (0.5% or 4%) was added to both inside and outside the membrane, respectively. The microdialysate samples were collected every 15 min after an hour of equilibration period.

2.6. In Vivo Microdialysis Experiments

2.6.1. Microdialysis Surgery. The microdialysis system comprised a CMA 470 refrigerated fraction collector, a CMA 402 syringe pump, and fluorinated ethylene propylene tubing (CMA, Solna, Sweden). CMA microdialysis probes for the blood (CMA 20, 10 mm in length) and brain (CMA 12, 4 mm in length) were also used. Animals were anaesthetized with urethane (1.4 g/kg, i.p.) before surgery and remained anesthetized throughout the experimental period. The body temperature of each rat was maintained at 37°C until the end of the experiment using a heating pad. The blood microdialysis probe was positioned within the jugular vein toward the right atrium and sutured to the surrounding muscle to prevent it from slipping out. The probe was then perfused with heparinized ringer's solution (1500 U/mL) for 15 min. The microdialysis guide cannula was implanted into the right striatum zone (anterior-posterior: 0.2 mm; lateral: 3.0 mm; dorsal-ventral: 3.5 mm from bregma) and fixed to the skull with stainless steel screws and dental acrylic. The brain microdialysis probe was inserted through the guide cannula.

In the pilot microdialysis experiment, ringer's solution was perfused through the probe at a flow rate of 1.5 μL/min and the dialysate samples were collected every 15 min from 90 min before until 6 h after dosing with FLZ. In all the other groups, the probes were perfused with ringer's solution containing 4% BSA, with the flow rate set at 1.0 μL/min.

2.6.2. In Vivo Recovery. The relative recovery of FLZ using the microdialysis probe was estimated using a retrodialysis method after sample collection and clearing overnight until there is no drug in the tissue and blood. Perfusates containing FLZ (20 ng/mL for the brain and 200 ng/mL for the blood) were passed separately into the brain and blood through the microdialysis probes at a constant flow rate of 1 μL/min. After a 1-hour stabilization period, the dialysates were collected at 15 min intervals for 1 hour. The concentrations of FLZ in the dialysate (C_{out}) and perfusate (C_{in}) were analysed by UPLC-MS/MS as described in Section 2.7. Recovery (R) was expressed by the following equation: $R = 1 - (C_{out}/C_{in})$.

2.7. UPLC-MS/MS Quantification

2.7.1. Preparation of Blood and Brain Samples. The blood and brain samples were prepared by spiking 75 μL of the corresponding IS working solutions (carbamazepine, 10 ng/mL) into 15 μL of dialysate followed by vortex mixing. After centrifuging at 16654 ×g for 10 min, 5 μL of supernatant was injected to the UPLC-MS/MS system.

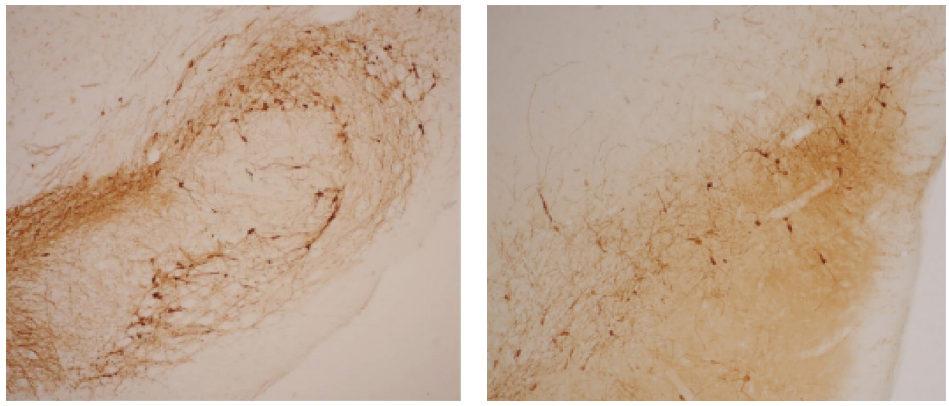
2.7.2. Instrumentation and Chromatographic Conditions. Sample analysis was performed using a Waters ACQUITY UPLC system (Waters Corp., Milford, MA, USA), and a Waters Xevo triple quadrupole tandem mass spectrometer (Waters Corp., Manchester, UK) was used to detect the analytes. The analytes were separated on a BEH (bridged ethyl hybrid) C_{18} analytical column (50 mm × 2.1 mm, 1.7 μm, Waters Co.) using isocratic elution with a mobile phase consisting of acetonitrile and water containing 0.3% acetic acid (28 : 72, v/v) at a flow rate of 0.45 mL/min. The separation was completed within 3.2 min. After each injection, the needle was washed with acetonitrile for 10 seconds to reduce carryover (a strong wash with 90% acetonitrile followed by a weak wash with 10% acetonitrile). The column was maintained at 30°C and samples were kept at 10°C in the autosampler.

The ESI instrument settings were optimized for the analysis, and the appropriate MRM transitions and MS/MS parameters were determined for individual compounds by direct infusion into the mass spectrometer. The optimum operating parameters of the ESI interface in positive mode were as follows: nebulizer, 7.0 bar; gas flow, 900 L/h; desolvation temperature, 500°C; capillary voltage, 3.5 kV; cone voltage, 40 V; and the LC eluent flow during the period from 0.0 to 1.0 min was switched to waste before introduction to the mass spectrometer for data acquisition. The following precursor-to-product ion transitions were subjected to multiple reaction monitoring: m/z 450.17 → 137.03 (cone voltage, 46 V; collision energy, 30 V) and m/z 450.17 → 313.08 (cone voltage, 46 V; collision energy, 16 V) as the quantitative ion pair and qualitative ion pair, respectively, for FLZ and 237.07 → 178.97 (cone voltage, 38 V; collision energy, 32 V) as the quantitative ion pair for carbamazepine (IS). Data acquisition and processing were performed using a Masslynx 4.1 workstation (Waters Corp).

2.7.3. Method Validation. Stock solutions of FLZ and IS were prepared separately in methanol at a target concentration of 1 mg/mL and then diluted with methanol to create working solutions of FLZ at concentrations of 1, 3, 10, 30, 100, 300, 1000, 1500, 2500, 5000, 10000, and 15000 ng/mL and IS at concentrations of 10 ng/mL. All solutions were stored in glass tubes at 4°C for less than 2 months until analysis and were protected from light. FLZ calibration standards (0.1, 0.3, 1, 3, 10, 30, 100, 150, 250, 500, 1000, and 1500 ng/mL) were prepared by spiking the blank perfusate with the appropriate working standard solution of FLZ. Quality control samples were prepared using the working solutions from the same stock solution (0.1, 0.3, 1, 10, 100, and 1000 ng/mL), thereby representing the entire range of concentrations.

Assay validation to meet the acceptance criteria was performed according to the bioanalytical method validation guideline (European Medicines Agency Guideline on Bioanalytical Method Validation, July 21, 2011). The assay was validated in terms of specificity, matrix effect, recovery, calibration curve, precision, accuracy (intra- and interday), lower limit of quantification (LLOQ), and stability (autosampler, freeze-thaw, and long-term).

2.8. Data Analysis and Statistical Procedures. Pharmacokinetic parameters were calculated from the observed data by noncompartmental analysis using the Drug and Statistics for Windows software package (DAS, version 2.0, Chinese



(a) (b)

Figure 2: Immunohistochemistry of tyrosine hydroxyls staining. (a) Dopaminergic neurons in normal rat. (b) Dopaminergic neurons in rat with 6-OHDA lesions.

Pharmacological Association, China). The penetration ratio of FLZ through the BBB was represented by the partition coefficient (Ri), which was calculated as the FLZ area under the concentration-time curve (AUC) in the brain divided by the FLZ AUC in the blood ($Ri = AUC_{brain}/AUC_{blood}$) [53]. A two-sided Student's t-test was used to examine differences between the FLZ only-N and FLZ only-PD groups, the FLZ + ZOSUQ-N and the FLZ + ZOSUQ-PD groups, the FLZ only-N and FLZ + ZOSUQ-N groups, and the FLZ only-PD and FLZ + ZOSUQ-PD groups. All data were expressed as the mean ± the standard deviation (SD). The criterion for statistical significance was set at $P < 0.05$.

3. Results

3.1. 6-OHDA Induced Rat Model of PD. The number of contralateral rotational turns was recorded over 30 min. The rats that rotate more than 210 rotational turns were chosen as the PD rats. Immunohistochemical staining for tyrosine hydroxylase (TH) results showed that the dopaminergic neurons were decreased substantially in the PD rats more than the normal rats (Figure 2).

3.2. Method Validation. An accurate, fast, and sensitive UPLC-MS/MS method was developed and validated for the quantification of FLZ in rat brain and blood dialysates. Sample preparation involved "protein precipitation with methanol." The separation was achieved in 3.2 min, and FLZ and IS were eluted at 2.63 min and 1.55 min, respectively. The LLOQ was 0.1 ng/mL, with no interference by the blank matrix. Two calibration curves (0.1–150 ng/mL and 150–1500 ng/mL; $r > 0.99$) were established to allow the quantification of a wide range of FLZ concentrations. The intraday and interday accuracy were both between 91.7% and 106%, with a precision (represented by the relative standard deviation (RSD)) < 11%. The mean extract recovery was >73.2%. The matrix effect was between 1.04 and 1.10, with an RSD < 13%. The stability of FLZ during preparation, after three freeze/thaw cycles and after storing at −80°C for 45 days, was also monitored. The results indicated that FLZ remained stable under all three conditions.

3.3. In Vitro Experiments. More than 90% of FLZ was bound to the tubing when perfused with ringer's solution containing FLZ. This value was less than 15% after the addition of BSA (0.5%, 2%, or 4%, w/v) to the perfusion fluid. The gain value increased substantially as the BSA concentration in the perfusion fluids increased; however, the loss value changed in the opposite direction. The two values were significantly different. The gain and loss values were very close when the BSA content was added up to 4% to both sides of the membrane. Application of these new perfusion conditions resulted in a marked increase in the relative *in vivo* recovery of FLZ (13.2% and 29.0%, resp., for the brain and blood microdialysis probes). Furthermore, these new conditions were similar to those encountered in actual *in vivo* systems, in which albumin is present in both the plasma and ECF.

3.4. Free FLZ Pharmacokinetics. Because the FLZ levels in the brain dialysate were below the LLOQ after administration of FLZ for 3.5 hours, samples were collected from the FLZ only-N and FLZ only-PD groups during this period only; the mean *in vivo* recovery by the blood and brain probes was 29.0 ± 2.1% and 13.2 ± 3.5%, respectively. The FLZ concentrations in the dialysate were calibrated by the recovery values as the following equation: $C = C_d/R$ (where C is the actual free FLZ concentration in the ECF or blood, C_d is the determined free FLZ concentration in the dialysate, and R is the FLZ recovery by the probe).

Statistical analysis indicated that there was no significant difference between the FLZ only-N and FLZ only-PD groups in terms of free FLZ levels in the blood and brain dialysates over time ($P > 0.05$, Figures 3(a) and 4(a)) (the only exception was the second blood dialysate sample; $P < 0.05$). In addition, there were no significant differences between the two groups in terms of pharmacokinetic parameters (AUC; half-life of drug ($t_{1/2}$); drug clearance from plasma (Cl); Table 1). Similarly, there were no significant differences in FLZ levels or pharmacokinetic parameters between the FLZ + ZOSUQ-N and FLZ + ZOSUQ-PD groups ($P > 0.05$, Figures 3(d) and 4(d) and Table 1).

Table 1: Key pharmacokinetic parameters and FLZ BBB penetration values ($n = 3$ per group).

		FLZ only-N	FLZ only-PD	FLZ + ZOSUQ-N	FLZ + ZOSUQ-PD
AUC (μg/L × h)	Blood	2349 ± 39	3072 ± 621	1562 ± 881	1334 ± 259*
	Brain	145 ± 25	145 ± 18	324 ± 46**	333 ± 103*
$t_{1/2}$ (h)	Blood	1.21 ± 0.22	1.34 ± 0.03	1.11 ± 0.13	1.43 ± 0.25
	Brain	1.15 ± 0.57	1.31 ± 0.29	1.04 ± 0.43	1.14 ± 0.27
CL_z (L/h/kg)	Blood	14.9 ± 0.2	11.7 ± 2.5	28.4 ± 16.8	26.8 ± 4.7**
Ri (AUC_{brain}/AUC_{blood})		0.0617 ± 0.0105	0.0492 ± 0.0158	0.2485 ± 0.1142*	0.2459 ± 0.0270***

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Nonetheless, FLZ levels in the FLZ only-N and FLZ + ZOSUQ-N groups showed a significant group effect over time. Blood dialysate FLZ concentrations were significantly higher in the FLZ only-N group than in the FLZ + ZOSUQ-N ($P < 0.05$, Figure 3(b)) group between the sixth and fourteenth samples (inclusive), and FLZ concentrations in the brain dialysates from the FLZ + ZOSUQ-N group were significantly higher than in those from the FLZ only-N group between the third to the twelfth samples (the only exception was the eleventh sample) ($P < 0.05$, Figure 4(b)). Correspondingly, the mean brain dialysate FLZ AUC was significantly higher for the FLZ + ZOSUQ-N group than for the FLZ only-N group (123% increase; $P < 0.01$, Table 1); however, the mean difference in the blood dialysate FLZ AUC between the two groups was not significant ($P > 0.05$, Table 1).

Statistically significant differences were also observed between the FLZ only-PD group and FLZ + ZOSUQ-PD group. The FLZ concentrations in the blood dialysates (from the first to the tenth samples) from the FLZ + ZOSUQ-PD group were significantly lower than those from the FLZ only-PD group ($P < 0.05$, Figure 3(c)), whereas the FLZ concentrations in the brain dialysate were significantly higher between the fourth and the fourteenth samples ($P < 0.05$, Figure 4(c)). The decrease in the FLZ blood dialysate AUC (56% decrease; $P < 0.05$, Table 1) and the increase in the FLZ brain dialysate AUC (130% increase; $P < 0.05$, Table 1) observed in the FLZ + ZOSUQ-PD group relative to the FLZ only-PD group were statistically significant.

3.5. Comparison of Brain to Blood Free FLZ AUC Ratios. The dialysate brain : blood AUC ratios give an indication of BBB transport as they account for variations in FLZ levels, which might explain any observed differences in dialysate concentrations. Statistical analysis revealed that the 3-fold increase in the brain : blood free FLZ AUC ratio observed in the FLZ + ZOSUQ-N group relative to the FLZ only-N group was statistically significant ($P < 0.05$, Figure 5) and that the 4-fold increase in the brain : blood free FLZ AUC ratio observed in the FLZ + ZOSUQ-PD group relative to the FLZ only-PD group was also statistically significant ($P < 0.001$, Figure 5). The 20% decrease in the blood : brain free FLZ AUC ratio observed in the FLZ only-PD group relative to the FLZ only-N group was not statistically significant ($P > 0.05$, Figure 5). Also, the ratios in the FLZ + ZOSUQ-N and FLZ + ZOSUQ-PD groups were not significantly different ($P > 0.05$, Figure 5).

4. Discussion

A pilot *in vivo* experiment was performed to obtain basic information about the feasibility of using microdialysis to assess unbound FLZ levels in the brain and blood of normal rats. The levels of FLZ in brain and blood dialysate samples were very low in most of the postdose samples with no defined time course for FLZ (data not shown); also, the *in vitro* recovery was less than 1%. This may be explained by the very low recovery of FLZ by the microdialysis probes and/or by the adsorption of the compound to the outflow tubing or to the microdialysis probes themselves [54].

Subsequent *in vivo* microdialysis experiments were performed in rats dosed with FLZ (35 mg/kg, i.v.) under the modified perfusion conditions. There was no significant difference in FLZ BBB penetration or pharmacokinetic parameters in the blood or brain between the FLZ only-N and FLZ only-PD groups, which differs from the previous studies reporting alterations/damage in blood brain barrier after 6-OHDA administration [48, 55] or in PD patients [30, 31]. It may be because of the fact that the 6-OHDA induced rat model was an acute brain injury model which may lead to BBB dysfunction on one side, while increasing P-gp expression on the other side [55]. FLZ was a substrate of P-gp at the same time, which would affect the FLZ penetration to BBB. The brain dialysate FLZ AUC for animals in the FLZ + ZOSUQ-N group was 123% higher than that for animals in the FLZ only-N group, and that in the FLZ + ZOSUQ-PD group was 130% higher than that in the FLZ only-PD group. These observed differences were strongly suggestive of increased FLZ transport across the BBB in zosuquidar·3HCl-treated animals. The mean free FLZ AUC in the blood dialysates from the FLZ + ZOSUQ-PD group was 56% lower than that for animals in the FLZ only-PD group significantly. There was also a 34% reduction in the FLZ AUC for the FLZ + ZOSUQ-N group compared with the FLZ only-N group, although the difference was not statistically significant, possibly due to the increased variability in FLZ + ZOSUQ-N group. Pretreatment with zosuquidar·3HCl caused a reduction in the FLZ AUC for the blood dialysate; this may be because more FLZ penetrated the BBB and became distributed throughout the brain (or other tissues that express P-gp at high levels). The brain to blood free FLZ AUC ratio for animals in the FLZ + ZOSUQ-N group was 3 times higher than that for animals in the FLZ only-N group, and the ratio for animals in the FLZ + ZOSUQ-PD group was 4 times higher than that for animals

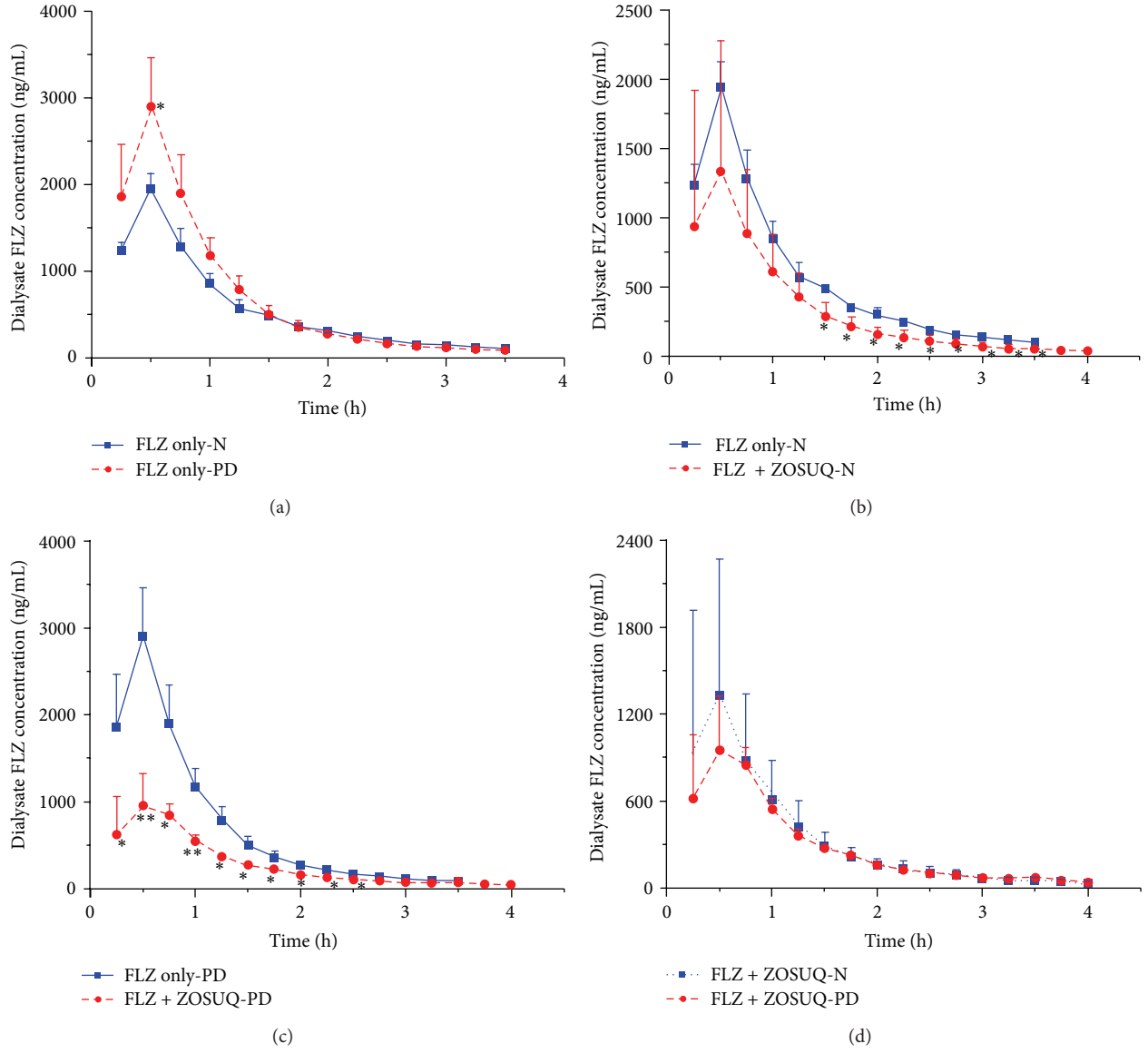


FIGURE 3: Blood dialysate FLZ profiles for the different treatment groups ($n = 3$ per group). (a) FLZ concentrations measured in blood dialysates at different time points in the FLZ only-N and FLZ only-PD groups. A significant difference was observed at the second time point only (30 min). (b) FLZ concentrations measured at different time points in blood dialysates from the FLZ only-N and FLZ + ZOSUQ-N groups. FLZ levels decreased significantly between the sixth and fourteenth samples in the FLZ + ZOSUQ-N group relative to those in the FLZ only-N group. (c) FLZ concentrations measured at different time points in blood dialysates from the FLZ only-PD and FLZ + ZOSUQ-PD groups. There was a significant reduction in FLZ levels in the FLZ + ZOSUQ-PD compared with the FLZ only-PD group (the first to the tenth sample). (d) FLZ concentrations in blood dialysates measured at different time points in the FLZ + ZOSUQ-N and FLZ + ZOSUQ-PD groups. There was no statistical difference in FLZ levels between the two groups. $^{*}P < 0.05$; $^{**}P < 0.01$. Data are expressed as the mean ± SD. Note: FLZ dialysate samples from the FLZ only-N and FLZ only-PD groups were collected for 3.5 hours (brain dialysate concentrations after this period were below the limit of quantification).

in the FLZ only-PD group. Our previous study [17] showed that, compared with that in the control group, the FLZ brain-to-plasma ratio in animals pretreated with zosuquidar·3HCl increased by about 3.1-fold at 0.25 min postdose and by about 14.5-fold to 20.8-fold at the other three time points tested (4, 10, and 30 min postdose). The increases noted in the present study were lower than that. This may be due to the different sampling methods used (the previous study used trunk blood and brain homogenate collected following decapitation) or different data analysis methods (the previous study used the brain homogenate-to-plasma concentration ratio rather than the brain dialysate AUC-to-blood dialysate AUC ratio). There was no significant difference in FLZ BBB penetration between the FLZ + ZOSUQ-N and the FLZ + ZOSUQ-PD groups or between the FLZ only-N and FLZ only-PD groups, further supporting the hypothesis that P-gp limits the ability

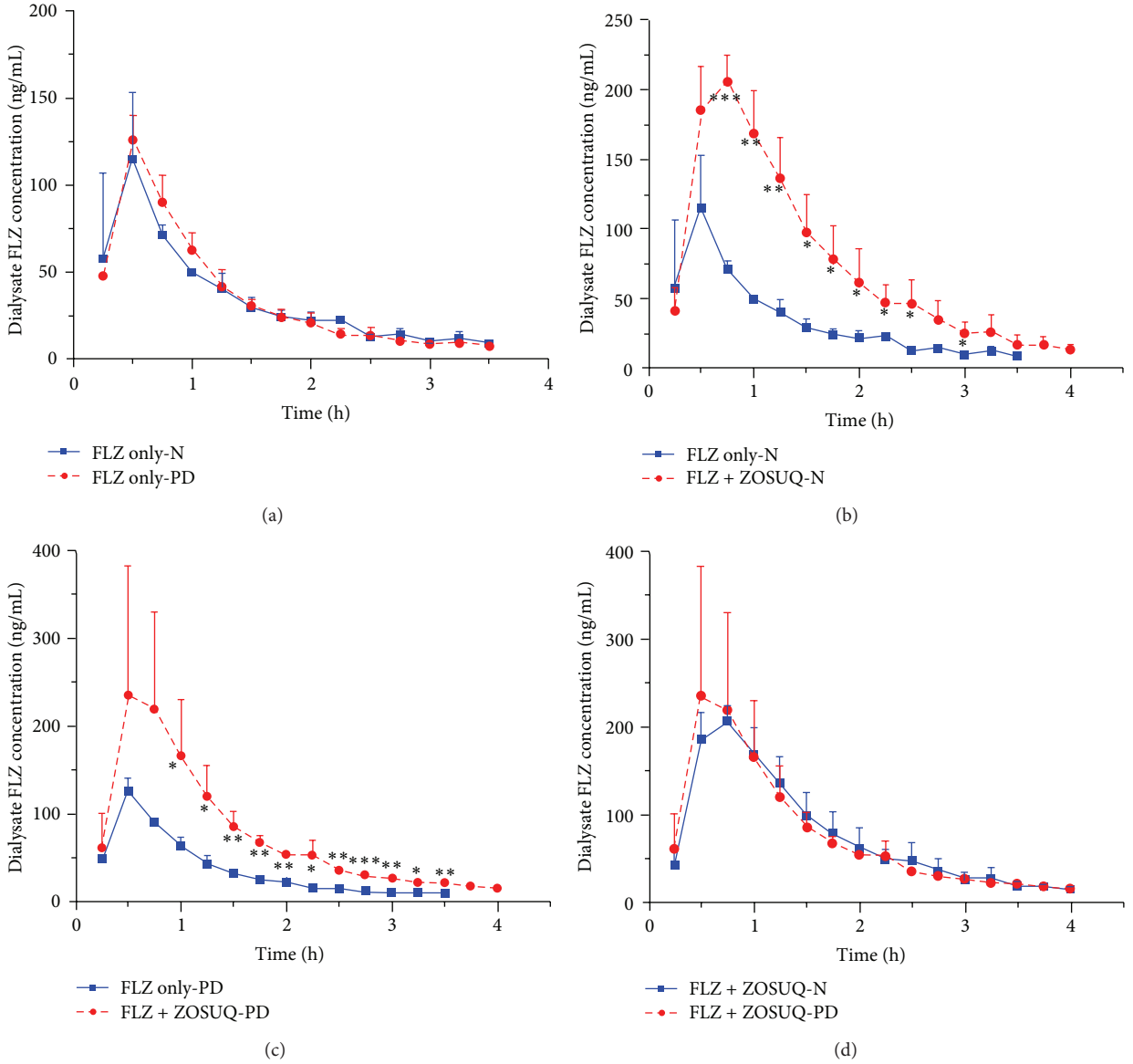


Figure 4: Brain dialysate FLZ profiles in the different groups ($n = 3$ per group). (a) FLZ concentrations in brain dialysate samples collected at different times from the FLZ only-N and FLZ only-PD groups. The differences in FLZ levels between these two groups were not significant. (b) FLZ concentrations in brain dialysate samples taken at different times from the FLZ only-N and FLZ + ZOSUQ-N groups. FLZ concentrations were significantly higher in the FLZ + ZOSUQ-N group than in the FLZ only-N group (between the third and twelfth samples; the eleventh sample is the exception). (c) FLZ concentrations in brain dialysate samples taken at different times from the FLZ only-PD and FLZ + ZOSUQ-PD groups. FLZ concentrations were significantly higher in the FLZ + ZOSUQ-PD group than in the FLZ only-PD group (between the fourth and fourteenth samples). (d) FLZ concentrations in brain dialysate samples taken at different times from the FLZ + ZOSUQ-N and FLZ + ZOSUQ-PD groups. There was no statistically significant difference in FLZ levels between these two groups. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Data are expressed as the mean ± SD. Note: FLZ dialysate samples were collected from the FLZ only-N and FLZ only-PD groups for 3.5 hours (brain dialysate concentrations after this period were below the limit of quantification).

of FLZ to penetrate the BBB [17]. In addition, the $t_{1/2}$ of FLZ in the present study was about 1.1 h (both in the blood and brain), which is longer than that reported previously (about 0.5 h) [17]. This may be due to the high level of FLZ binding to plasma proteins. This result also indicates that it is important to ascertain free drug levels when undertaking pharmacokinetic studies of CNS drugs that bind tightly to plasma proteins.

To the best of our knowledge, this is the first study to examine the effects of a P-gp modulator (zosuquidar·3HCl) on the transport of free FLZ across the BBB. The present study was also the first to use the *in vivo* microdialysis technique to investigate the pharmacokinetic characteristics of FLZ in the rat brain. However, the present study still has limitations. Firstly, the 6-OHDA model, as an acute animal model, differs from the slowly progressive pathology of human PD and

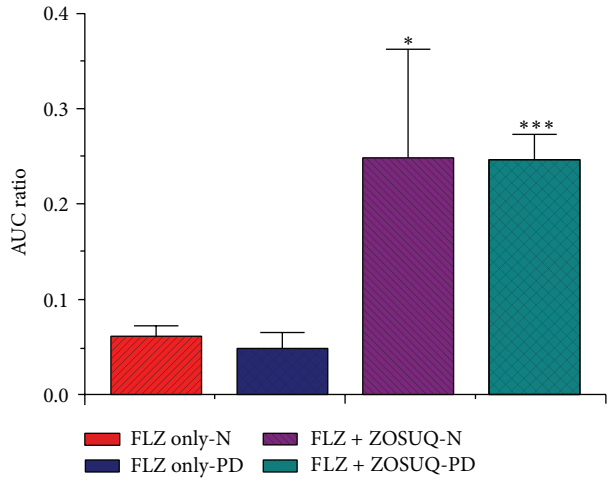


Figure 5: Brain dialysate : blood dialysate FLZ AUC ratios for each treatment group. There was a 3-fold increase in the brain dialysate : blood dialysate FLZ AUC ratio in the FLZ + ZOSUQ-N group relative to that in the FLZ only-N group ($P < 0.05$) and a 4-fold increase in the FLZ + ZOSUQ-PD group relative to that in the FLZ only-PD group ($P < 0.001$). The 20% reduction observed in the FLZ only-PD group relative to the FLZ only-N group was not statistically significant ($P > 0.05$). The mean ratios in the FLZ + ZOSUQ-N and FLZ + ZOSUQ-PD groups were not significantly different ($P > 0.05$). Data are expressed as the means ± SD ($n = 3$ per group).

does not mimic all pathological and clinical features of human Parkinsonism, although it was frequently used in the preclinical studies. We should note the differences between PD model animals and PD patients in future clinical trial of FLZ. Besides, the animals were anesthetized during sampling, which may have altered the pharmacokinetics of the drug. Future studies should use animals that are conscious and able to move freely, and the levels of FLZ and the relative neurotransmitters (such as dopamine and its metabolites) should be determined at the same time to establish a relationship between pharmacokinetics and pharmacodynamics under the normal and disease states. Such studies may help us to identify the pharmacological mechanism of action.

In conclusion, the present study suggests that BBB penetration by FLZ is similar in normal and PD rats. The bioavailability of FLZ in the brains of rats pretreated with zosuquidar·3HCl increased in both normal and PD rats, suggesting that P-gp efflux limits the ability of FLZ to cross the BBB. These findings suggest that P-gp prevents FLZ from reaching its target site in the brain. The approach described herein might be useful for investigating BBB penetration by other drugs used to treat CNS disease.

Abbreviations

AD:	Alzheimer's disease
AUC:	Area under the concentration-time curve
BBB:	Blood-brain barrier
BEH:	Bridged ethyl hybrid
BSA:	Bovine serum albumin
C_{in}:	Concentration of FLZ in the perfusate
Cl:	Drug clearance from plasma
C_{out}:	Concentration of FLZ in the dialysate
CNS:	Central nervous system
COMT:	Catechol-O-methyltransferase
CSF:	Cerebrospinal fluid
ECF:	Extracellular fluid
FLZ:	N-[2-(4-Hydroxy-phenyl)-ethyl]-2-(2,5-dimethoxy-phenyl)-3-(3-methoxy-4-hydroxy-phenyl)-acrylamide
FLZ only-N group:	FLZ only in normal rats
FLZ only-PD group:	FLZ only in PD rats
FLZ + ZOSUQ-N group:	FLZ plus zosuquidar·3HCl in normal rats
FLZ + ZOSUQ-PD group:	FLZ plus zosuquidar·3HCl in PD rats
HPLC:	High-performance liquid chromatography
IS:	Internal standard
L-DOPA:	3,4-Dihydroxy-L-phenylalanine
LLOQ:	Lower limit of quantification
MAO-B:	Monoamine oxidase-B
MPTP:	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MRP:	Multidrug resistance protein
MS:	Mass spectrometry
NSB:	Nonspecific binding
P-gp:	P-Glycoprotein
$t_{1/2}$:	Half-life of drug
PD:	Parkinson's disease
RSD:	Relative standard deviation
SD:	Standard deviation
TH:	Tyrosine hydroxylase
UPLC-MS/MS:	Ultraperformance liquid chromatography-tandem mass spectrometry
Zosuquidar·3HCl:	Zosuquidar trihydrochloride
6-OHDA:	6-Hydroxydopamine.

Conflict of Interests

The authors have no conflict of interests to declare regarding the publication of this paper.

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Research Article

Chronic Dietary Supplementation of 4% Figs on the Modification of Oxidative Stress in Alzheimer's Disease Transgenic Mouse Model

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We assessed the changes in the plasma Aβ, oxidative stress/antioxidants, and membrane bound enzymes in the cerebral cortex and hippocampus of Alzheimer's disease (AD) transgenic mice (Tg2576) after dietary supplementation of Omani figs fruits for 15 months along with spatial memory and learning test. AD Tg mice on control diet without figs showed significant impairment in spatial learning ability compared to the wild-type mice on same diet and figs fed Tg mice as well. Significant increase in oxidative stress and reduced antioxidant status were observed in AD Tg mice. 4% figs treated AD Tg mice significantly attenuated oxidative damage, as evident by decreased lipid peroxidation and protein carbonyls and restoration of antioxidant status. Altered activities of membrane bound enzymes (Na^+ K^+ ATPase and acetylcholinesterase (AChE)) in AD Tg mice brain regions and was restored by figs treatment. Further, figs supplementation might be able to decrease the plasma levels of Aβ (1–40, 1–42) significantly in Tg mice suggesting a putative delay in the formation of plaques, which might be due to the presence of high natural antioxidants in figs. But this study warrants further extensive investigation to find a novel lead for a therapeutic target for AD from figs.

1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder with complex multifactorial pathological changes in the brain. It is most prevalent form of dementia characterized by a progressive impairment of memory, cognitive functions, and behavior in the elderly [1]. It affects millions of people and has become major medical and social burden in developed and developing countries [2]. This disease has been reported to be the sixth leading cause of death. The neuropathology of AD is characterized initially by the deposition of senile plaques mainly composed of amyloid beta protein (Aβ) and neurofibrillary tangles containing hyperphosphorylated tau protein in the brain and later by the loss of neurons and their processes [3, 4]. Cognitive impairment appears to be most closely correlated in time with the loss of neurons and neuronal processes [5]. Accumulation of Aβ peptide might cause an increase in intracellular reactive oxygen species (ROS) and free radicals. The generation of ROS and relative oxidative damage is believed to be involved in the pathogenesis of AD. The ROS can induce functional and structural damage to cell membranes through lipid peroxidation and carbonyl modification of protein which may be involved in the pathogenesis of AD [6]. Other characteristic transforms that occur in AD are the increment of acetylcholinesterase (AChE) [7] around the amyloid plaques.

The relationship between plaque/tangle deposition and the neuronal degeneration is not clearly understood. However, most of the AD cases occur sporadically, resulting from the influence of various nongenetic environmental factors. The mechanisms underlying AD appear to be diverse and so are the potential therapeutic methods for treating AD.

Currently, the interest in the role of dietary antioxidants in human health has been prompted in the area of neurodegenerative disease research. Fruits are good sources of bioactives, and there are a number of commercial polyphenol-rich beverages, which base their marketing strategies on antioxidant potency. Since the last decade, antioxidant has received a special attention as dietary supplements and several studies have shown inhibition of Aβ plaque formation *in vitro* and *in vivo* by natural compounds [8–13]. Curcumin and ginkgo biloba extract have been reported to have protective effect against the progression of AD pathology in AD murine models [14, 15].

The fig (*Ficus carica L.*) is a classical fruit tree associated with the beginnings of horticulture in the Mediterranean basin [16, 17]. Since ancient time, the Mediterranean region and the Middle East countries have been the most important cultivating centres of figs [18]. Compared with other common fruits and beverages, figs are an excellent source of minerals, vitamins, and dietary fiber; they are fat and cholesterol free and contain abundant amino acids [19–22]; it contains the highest concentrations of polyphenols [23]. The fig fruit is well known for its attractive taste and nutritive value due to its antioxidant properties, and it is consumed fresh or dried worldwide [21, 24–26]. The leaves are being used traditionally in the treatment of jaundice [27]. Figs are an excellent source of phenolic compounds, such as proanthocyanidins [23]. Actually, red wine and tea, two well-publicized sources of phenolic compounds, contain lower amounts of phenols than figs [28]. Figs have been reported to have excellent radical scavenging and antioxidant [21] activities. The effect of figs fruits on experimental AD is not yet well studied. To address this, we performed a set of experiments in a transgenic mouse model of AD supplemented with figs for 15 months with a focus on Aβ and oxidative stress.

2. Materials and Methods

2.1. Collection and Preparation. Fresh figs fruits were collected from Al-Jabal Al-Akhdar farms, Oman. The flesh was isolated manually, rinsed with water, dried for 18 h in a drying cabinet at 40°C, and stored at room temperature. The dried fruits will be crushed and extracted with acetone (1 : 1 ratio, weight to volume) under agitation at room temperature. After 48 h, the extract was then filtered and the filtrate will be evaporated to dryness in a drying cabinet at 40°C and stored. After that, the samples were ground into fine powder using a coffee grinder.

2.2. Diet Preparation for the Animals. The ground figs were sent to USA to prepare the diet for the mice. The diet was prepared by mixing the figs (4%) with regular diet as per National Institutes of Health, USA, protocol by Research Diet Inc., NJ, USA.

2.3. Animals and Treatment. Twelve transgenic female (APPsw/Tg 2576) and 6 wild control (nontransgenic) mice (Taconic form, NY, USA) were used. Animals were quarantined for 7 days after shipping and individually housed in plastic cages in an animal room, which was maintained at a temperature of 22 ± 2°C, a relative humidity of 50 ± 10%, and a 12 h light/dark automatic light cycle (light: 0800–2000 h). All these animals are free from pathogens and viruses. Experimental period commenced at the age of 4 months. The animals were divided into three groups: Group 1: wild-type (nontransgenic) control of the APPsw mice fed with regular diet, Group 2: AD transgenic mice also fed with regular diet, and Group 3: AD mice fed with 4% figs fruit diet. Influence of fig supplemented diet on cognitive behavior after 15 months was assessed by using the Morris water maze test (for spatial memory and learning ability). Following the behavioral assessments, oxidative stress, antioxidants, and membrane bound enzymes were investigated in experimental and control mice. All animal experiments in the present study were complied with the Animal Care and Use Committee of the Sultan Qaboos University, Oman (SQU/AEC/2010-11/3).

2.4. Blood and Tissue Sample Collection. The day after completion of the behavioral tests, blood samples were collected from all groups for plasma separation and all the samples were stored at −80°C until being used. Then the animals were decapitated with the head transferred onto the dry ice, followed by rapid dissection of the hippocampus and the cerebral cortex, homogenization in 9 volumes (1 : 9 w/v) of cold saline for preparation of a 10% cerebral homogenate in an ice bath, and centrifugation for supernatant collection. Whole brains were rapidly removed simultaneously and chilled in an ice-cold saline solution. The tissue samples were stored at −80°C until assay.

2.4.1. Morris Water Maze Test. The water maze consisted of a metal pool (170 cm in diameter × 58 cm tall) filled with tap water (25°C, 40 cm deep) divided into four quadrants. In the centre of one quadrant was a removable escape platform below the water level covered with a nontoxic milk powder. The pool was divided into four quadrants (NE, NW, SE, and SW) by two imaginary lines crossing the centre of the pool. For each animal, the location of invisible platform was placed at the centre of one quadrant and remained there throughout training. The mice must memorize the platform location in relation to various environmental cues, and there was nothing directly indicative of the location of the escape platform in and outside of the pool. Therefore, the placement of the water tank and platform was the same in all acquisition trials. Each mouse was gently placed in the water facing the wall of the pool from one of the four starting points (N, E, S, or W) along the perimeter of the pool, and the animal was allowed to swim until it found and climbed onto the platform. During the training session, the mice subject was gently placed on the platform by an experienced investigator when it could

not reach the platform in 60 s. In either case, the subject was left on the platform for 15 s and removed from the pool. The time for animals to climb onto the hidden platform was recorded as escape latency or acquisition time. In order to determine the capability of the animals to retrieve and retain information, the platform was removed 24 h later and the mice were released into the quadrant diagonally opposite to that which contained the platform. Time spent in the region that previously contained the platform was recorded as retention time. In each trial, the animal was quickly dried with a towel before being returned to the cage [29]. All tests were carried out at the end of the experimental period following 4% fig fruit dietary supplementation. ANY-maze software from Ugo Basile, Italy, was used.

2.4.2. Determination of Plasma Aβ (1–40) and Aβ (1–42). Aβ1–40 and Aβ1–42 plasma were measured by commercially available ELISA kits (Araclon Biotech Ltd., Zaragoza, Spain).

2.5. Biochemical Assays in the Brain. Oxidative stress markers such as malondialdehyde (MDA) [30] and total protein carbonyl content [31–33] were assayed in hippocampus and the cerebral cortex. The activities of enzymatic antioxidants, superoxide dismutase (SOD) [34], CAT [35], glutathione peroxidase (GPx) [36, 37], glutathione reductase (GR) activity [38], and the levels of reduced glutathione (GSH) [39] were also analyzed in hippocampus and the cerebral cortex. Furthermore, the activities of membrane bound enzymes such as acetylcholinesterase (AChE) activity [40] and Na^+ K^+ ATPase [41, 42] were also assayed in hippocampus and the cerebral cortex. Protein estimation was conducted according to Lowry et al. [43].

2.6. Statistical Analysis. The statistical analysis was performed using SPSS software version 16.0. The results were expressed as mean ± SEM. All data were statistically analyzed by one-way analysis of variance (ANOVA), followed by Dunnett's *t*-test. A significant difference was determined when $P < 0.05$.

3. Results

3.1. A 4% Fig Rich Diet Improved Spatial Memory in AD Tg Mice. The cognitive ability of the Tg mice was assessed by the Morris water maze test. Wild-type control mice after 15 months were given the task of learning how to find the hidden platform in the Morris water maze, and their performance was found to improve in an experience-dependent manner. In contrast, the Tg mice after 15 months showed a significantly delayed latency to finding the hidden platform compared with the wild control mice (Figures 1(a) and 1(b)). Figs supplementation to Tg mice for 15 months significantly improved the escape latency to find the platform than Tg mice on control diet (Figures 1(a) and 1(b)), which indicates that figs might be able to improve spatial memory in Tg mice.

3.2. Effect of 4% Figs on Aβ (1–40) and Aβ (1–42) Content in Plasma. Plasma levels of both Aβ1–40 and Aβ1–42 were significantly higher in Tg mice on normal diet than wild-type mice on the same diet and figs supplementation ameliorated these levels significantly (Table 1) than Tg mice on normal diet.

Table 1: Figs diet effect on plasma Aβ (1–40, 1–42) levels in Tg mice.

Groups	Plasma Aβ levels (pg/mL)	
	Aβ1–40	Aβ1–42
Control wild	82.01 ± 6.25^a	98.02 ± 7.46^a
Control Tg	1354.68 ± 103.69^b	282.14 ± 21.60^b
4% Figs Tg	840.28 ± 64.17^c	164.05 ± 12.53^c

Data are presented as mean ± SD, and $n = 6$/group.
Values not sharing common superscripts (a, b, and c) differ significantly at $P < 0.05$ (DMRT).

3.3. Effect of 4% Figs on LPO and Protein Carbonyls in AD Transgenic Mice. APPsw (Tg2576) AD mice showed significant increase in LPO levels in both brain regions studied (cortex and hippocampus) compared to wild type (Table 2). However, 4% figs dietary supplemented AD mice for 15 months attenuated the increase in LPO comparable to wild control values. Table 1 depicts significantly elevated levels of protein carbonyls in disease control APPsw (Tg2576) mice compared to wild type (cortex and hippocampus) and 4% figs dietary supplementation significantly brings down protein carbonyl levels in AD mice.

3.4. Effect of 4% Figs on the Antioxidant Enzymes in APPsw (Tg2576) AD Transgenic Mice. Significantly decreased activities of SOD, GPX, GR, and CAT in cerebral cortex and hippocampus were found in AD mice when compared to wild mice (Tables 3 and 4). However, the entire antioxidant enzyme activities were significantly enhanced by 4% figs dietary supplementation in cerebral cortex and hippocampus of APPsw (Tg2576) AD mice.

GSH activity in brain regions of disease control APPsw (Tg2576) mice was significantly decreased in cortex and hippocampus compared to wild-type mice (Table 3). However, 4% figs dietary supplemented mice restored GSH activity to near normal levels in cortex and hippocampus.

3.5. Effect of 4% Figs on Membrane Bound Enzymes in AD Transgenic Mice. AChE activity significantly increased in the cortex and hippocampus of control APPsw (Tg2576) mice. Dietary supplementation of 4% figs for 15 months attenuated AChE activity in the cerebral cortex and hippocampus of AD mice. Disease control APPsw (Tg2576) mice showed significant inhibition in Na^+ K^+ ATPase activity in the cortex and hippocampus and figs dietary supplementation could be able to offer a significant improvement in the activity of membrane bound enzymes (Table 5).

4. Discussion

To our knowledge, this study is the first to investigate the ability of figs to attenuate oxidative stress in AD transgenic

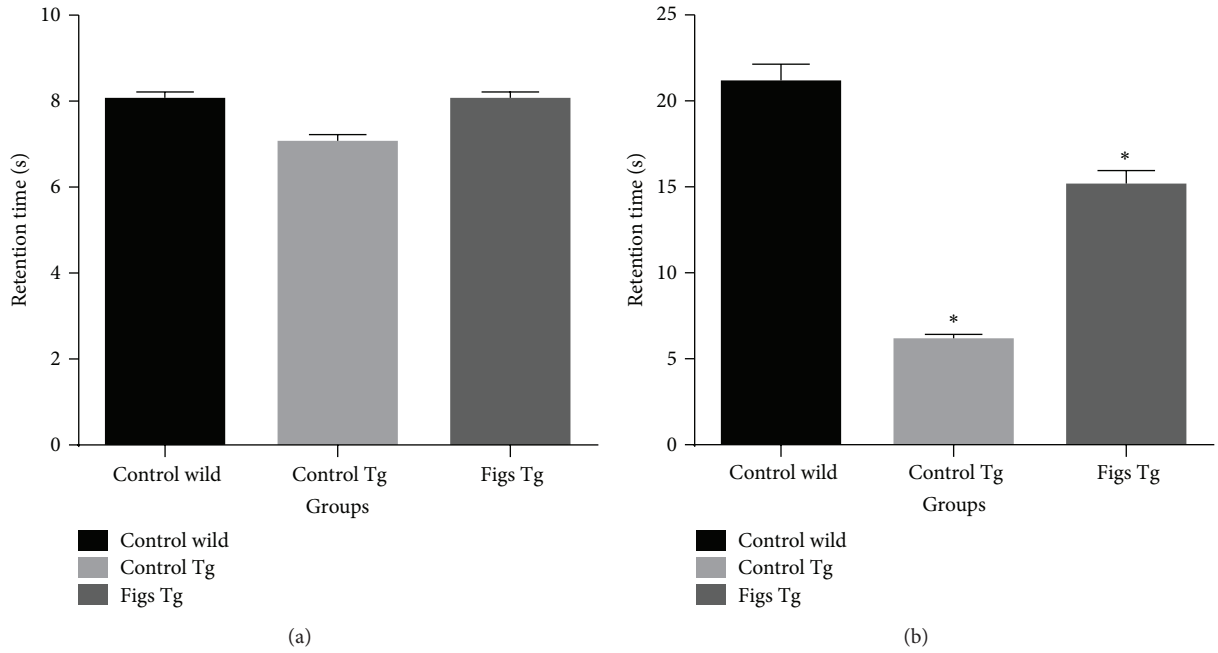

Figure 1: Supplementation with 4% figs ameliorated the decline in spatial memory and learning ability of Tg mice. (a) Retention time in Morris water maze test at the age of 4 months. (b) Retention time in Morris water maze test after treatment of figs diet for 15 months. Data are presented as mean ± SD and $n = 6$/group. $^{*}P < 0.05$ compared to wild-type mice.

Table 2: Effect of figs on lipid peroxidation and protein carbonyls content in brain of Tg mice.

Groups	MDA levels (nmol/mg protein)		Protein carbonyl (nmol/mg protein)	
	Cortex	Hippocampus	Cortex	Hippocampus
Control wild	1.60 ± 0.12^{a}	0.90 ± 0.07^{a}	30.01 ± 0.06^{a}	51.01 ± 0.06^{a}
Control Tg	3.60 ± 0.28^{b}	2.10 ± 0.16^{b}	81.26 ± 0.05^{b}	132.14 ± 0.05^{b}
Figs Tg	2.70 ± 0.21^{c}	1.40 ± 0.11^{c}	49.86 ± 4.61^{c}	101.23 ± 4.61^{c}

Data are presented as mean ± SD, and $n = 6$/group.
Values not sharing common superscripts (a, b, and c) differ significantly at $P < 0.05$ (DMRT).

Table 3: Effect of figs on superoxide dismutase and catalase activity in cortex and hippocampus of Tg mice.

Groups	SOD (U/mg protein)		Catalase (U/mg protein)	
	Cortex	Hippocampus	Cortex	Hippocampus
Control wild	196.03 ± 14.93^{a}	215.04 ± 16.37^{a}	3.80 ± 0.29^{a}	4.30 ± 0.33^{a}
Control Tg	114.06 ± 8.73^{b}	128.06 ± 9.80^{b}	1.70 ± 0.13^{b}	2.20 ± 0.17^{b}
Figs Tg	165.06 ± 12.61^{c}	179.06 ± 13.67^{c}	2.90 ± 0.22^{c}	3.40 ± 0.26^{c}

Data are presented as mean ± SD, and $n = 6$/group.
Values not sharing common superscripts (a, b, and c) differ significantly at $P < 0.05$ (DMRT).

Table 4: Effect of figs dietary supplementation on glutathione dependent antioxidant enzymes in the brain of Tg mice.

Groups	Glutathione peroxidase (nmol NADPH oxidized/min/mg protein)		GSH (mg/g protein)		Glutathione reductase (nmol NADPH oxidized/min/mg protein)	
	Cortex	Hippocampus	Cortex	Hippocampus	Cortex	Hippocampus
Control wild	12.00 ± 0.91^{a}	24.00 ± 1.83^{a}	4.20 ± 0.32^{a}	5.80 ± 0.44^{a}	11.00 ± 0.84^{a}	11.70 ± 0.89^{a}
Control Tg	5.00 ± 0.38^{b}	11.01 ± 0.84^{b}	2.10 ± 0.16^{b}	3.20 ± 0.25^{b}	4.00 ± 0.31^{b}	4.60 ± 0.35^{b}
Figs Tg	9.10 ± 0.70^{c}	17.01 ± 1.30^{c}	3.20 ± 0.24^{c}	4.70 ± 0.36^{c}	8.20 ± 0.63^{c}	9.00 ± 0.69^{c}

Data are presented as mean ± SD, $n = 6$/group.
Values not sharing a common superscripts (a, b and c) differ significantly at $P < 0.05$ (DMRT).

Table 5: Influence of figs dietary supplementation on AChE and Na^+ K^+ ATPase activity in cortex and hippocampus of Tg mice.

Groups	AChE (U/mg Protein)		Na^+ K^+ ATPase (% control)	
	Cortex	Hippocampus	Cortex	Hippocampus
Control wild	2.40 ± 0.06^a	2.80 ± 0.06^a	98.02 ± 7.46^a	97.02 ± 7.39^a
Control Tg	4.40 ± 0.05^b	4.80 ± 0.05^b	36.01 ± 2.74^b	32.01 ± 2.44^b
Figs Tg	3.20 ± 0.06^c	3.60 ± 0.06^c	70.01 ± 5.33^c	69.01 ± 5.25^c

Data are presented as mean ± SD, and $n = 6$/group.
Values not sharing common superscripts (a, b, and c) differ significantly at $P < 0.05$ (DMRT).

mice. Our current results clearly demonstrated that dietary supplementation of figs could significantly improve the learning and memory deficits in AD transgenic mice. Figs diet fed mice spend more time in the target quadrant and made more annulus crossings than the animals fed with the control diet during the probe test [44].

Previous studies proposed a model for neurodegeneration in AD brains based on free radicals/oxidative stress associated with Aβ (1–40 and 1–42) [45, 46]. The increased levels of plasma Aβ in AD were also previously documented [47, 48]. It has been observed that AD transgenic mice could secrete more Aβ1–42 and Aβ1–40 than their wild control littermates throughout their life [49, 50], which coincides with our results. But the effect of figs diet on reducing the plasma Aβ (1–40 and 1–42) levels in Tg mice shows that figs may offer beneficial effect before Aβ plaque formation.

ROS can damage essential cellular constituents such as lipids and proteins, which can be measured by identification of their by-products MDA and protein carbonyl, respectively [51]. We observed increase of MDA and production and protein carbonylation in cerebral cortex and hippocampus of AD Tg mice, indicating that oxidative stress occurs as a consequence of AD, thereby contributing to brain damage. Dietary supplementation of figs notably inhibited the accumulation of MDA and protein carbonyl levels in cortex and hippocampus of Tg mice, which is an oxidized by-product of lipid peroxidation. This context was supported by the previous studies that the figs and figs leaves could reduce MDA level, an index of lipid peroxidation [27] on carbon tetrachloride induced rats [52].

GSH offers primary defense in neurons against oxidative stress and maintains cellular redox homeostasis [53]. In our experiment we observed a significant decrease in the GSH levels in the brain of AD Tg mice compared to wild controls. It is known that GSH depletion is the first indicator of oxidative stress during neurodegenerative diseases [54]. Figs supplementation to AD Tg mice was able to reverse the decrease in GSH levels. Aziz [55] has reported that figs could be able to reverse the GSH levels in lead acetate and carbon tetrachloride induced hepatotoxicity [52] in rats, suggesting the efficacy of figs in preventing the oxidative damage and associated changes.

SOD is responsible for catalyzing the conversion of superoxide anions into hydrogen peroxide [56, 57] which is further decomposed to water and oxygen by CAT [58]. The activities of SOD and CAT were found to be significantly diminished in cortex and hippocampus of AD Tg mice. Figs supplementation in diet to AD Tg mice prevented decrease in the activities of SOD and CAT. Studies have shown that figs could directly inhibit the superoxide anion formation which could enable the restoring of SOD and CAT and our result suggests that the neuroprotective effects of the figs might be due to their antioxidant activity [52, 55, 59].

GPx and GR represent a crucial defensive system to protect cells against ROS [60]. The activity of GPx and GR was significantly decreased in brain regions. Isharat et al. [61] have reported significant decrease in these enzymes in experimental dementia. On other hand, Fan et al. [62] have reported the decreased GPx and GR activity as result of oxidative stress in scopolamine induced amnesia in the hippocampus and cerebral cortex. Figs supplementation significantly attenuated the elevated levels of GPx and GR in brain regions. Figs have shown to be successful in increasing the activity of GPx and GR enzyme in rats with oxidative damage induced by methanol [59].

AChE is an acetylcholine hydrolyzing enzyme that is responsible for the termination of cholinergic response [63]. The AChE activity was found to be markedly elevated in Tg mice brain regions. This observation coincides with previous reports whereby I.C.V. administration of streptozotocin at subdiabetogenic dose has been shown to induce memory deficits along with increase in oxidative stress and AChE activity [64, 65]. AChE activity was significantly increased in hippocampus in L-methionine induced model of vascular dementia [66] and the activity of AChE depends largely on the membrane characteristics, since the enzyme is membrane bound. Barbosa et al. [67] suggested that amyloid beta peptides induce Ca^{2+} influx that leads to increased activity of AChE which is attributed to Ca^{2+} mediated oxidative stress. In our study, dietary supplementation of figs could be able to inhibit AChE activity which was supported by the previous study suggesting that leaf extracts of fig could offer AChE inhibitory activity and antioxidant effects [68]. But the mechanism of anticholinesterase activity reduction by figs appears to be complicated and needs further extensive investigation.

Modification of Na^+ K^+ ATPase activity may induce neuronal death with features of both apoptosis and necrosis [69]. In the current study, the activity of Na^+ K^+ ATPase was found to be decreased in AD Tg mice, which is in line with other studies reporting decrease in the enzyme activity during aging [70, 71]. Na^+ K^+ ATPase is known to be highly

susceptible to changes in the membrane lipids, which may be further attributed to the progressive increase in the lipid peroxidation [72, 73]. ROS overproduction inhibits the activity of ATPase via thiol- and lipid-dependent mechanisms [74]. It has been demonstrated that the reduced activity of Na^+ K^+ ATPase caused by oxidative stress cannot drive the ion pumps to maintain depolarization of neurons and thus may become lethal to neurons [74]. The mechanism of the action of enhancing Na^+ K^+ ATPase effects of figs is uncertain, as its multiple active compounds such as anthocyanins, coumarins, caffeoylquinic acids, ferulic acid, quercetin, fumaric acids, alkaloids, and flavonoids have multifunctional action, making it complex in pharmacological action. Recently we have reported that the date fruits could offer protection to oxidative damage in AD mice [75]. Further inhibition in lipid peroxidation and enhancement of antioxidant enzymes in various disease conditions by figs have already been reported also support our findings [21, 22, 76].

5. Conclusion

In conclusion, figs could improve memory related behavioral deficits, reducing the Aβ and oxidative damage and enhancing the antioxidant system in AD transgenic mice. Protection from Aβ mediated oxidative damage in brain could be potentially considered as a promising strategy for therapeutic intervention in AD. Our results allow us to conclude that figs diet seems to be an effective modifying therapeutic strategy for AD. Further extensive experiments must be done in order to elucidate the molecular mechanisms through which figs diet may mediate its beneficial effect on AD like neurodegenerative disease condition.

Conflict of Interests

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

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Research Article

Poststroke Neuropsychiatric Symptoms: Relationships with IL-17 and Oxidative Stress

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Stroke variably activates interleukin- (IL-) 17 expression, reduces regulatory T cells, and induces oxidative stress, which may support neurodegeneration. Ischemic stroke patients were screened for depressive symptoms (Center for Epidemiological Studies Depression (CES-D)) and cognitive status (Mini Mental State Examination). Proinflammatory cytokines (IL-17, IL-23, and interferon- [IFN-] γ), anti-inflammatory cytokine IL-10, and lipid hydroperoxide (LPH), a measure of oxidative stress, were assayed from fasting serum. Of 47 subjects (age 71.8 ± 14.4 years, 36% female), 19 had depressive symptoms (CES-D ≥ 16), which was associated with poorer cognitive status ($F_{1,46} = 8.44$, $P = 0.006$). IL-17 concentrations did not differ between subjects with and without depressive symptoms ($F_{1,46} = 8.44$, $P = 0.572$); however, IL-17 was associated with poorer cognitive status in subjects with depressive symptoms ($F_{1,46} = 9.29$, $P = 0.004$). In those subjects with depressive symptoms, IL-17 was associated with higher LPH ($\rho = 0.518$, $P = 0.023$) and lower IL-10 ($\rho = -0.484$, $P = 0.036$), but not in those without. In conclusion, poststroke depressive symptoms may be associated with cognitive vulnerability to IL-17 related pathways, involving an imbalance between proinflammatory and anti-inflammatory activity and increased oxidative stress.

1. Introduction

Stroke is a leading cause of disability, and common sequelae such as depression and cognitive impairment contribute significantly to disease burden among survivors. Depression after stroke has been associated with cognitive impairment, as assessed using the Mini Mental State Examination (MMSE) [1, 2]; however, biological mechanisms that may mediate this relationship remain elusive. Depression in medically healthy patients has been associated with increased concentrations of cytokines in peripheral blood [3], which may be relevant to depression after stroke [4]. Previous studies have identified relationships between MMSE scores and peripheral blood inflammatory markers, including C-reactive protein and kynurenine [5, 6], suggesting inflammation as a possible link between depression and cognitive impairment after stroke.

In animal models, the infiltration of T cells that express IL-17 exacerbates neurodegenerative damage in the delayed phase of postischemic injury [7]. In the peri-infarct cortex, apoptosis is the predominant mode of neuronal death, which is heavily influenced by inflammatory and anti-inflammatory cytokine signals released from infiltrating peripheral T lymphocytes and other cell types; however, only a few clinical studies have investigated IL-17 after stroke [8, 9]. In one study, IL-17 expression by peripheral mononuclear cells was associated with poorer neurological outcomes, although relationships with depression and cognitive status were not assessed [10]. IL-17 can induce blood brain barrier disruption

through a mechanism that depends on the production of reactive oxygen species [11], suggesting that IL-17 could exacerbate neurodegeneration through oxidative damage to lipids, protein, and DNA. Recently, behavioral effects of IL-17 were demonstrated in an animal study, which reported that IL-17 expressing T cells exacerbated behavioral deficits during experimental induction of depression-like behaviors [12]. Based on those findings, it was hypothesized that serum IL-17 concentrations would be associated with depressive symptoms and cognitive impairment following acute ischemic stroke. This study explored relationships between IL-17, cognition, depression, and lipid peroxidation.

2. Materials and Methods

This cross-sectional observational study recruited consecutive English-speaking participants admitted to an acute care regional stroke centre with verified acute ischemic infarctions on CT or MR imaging. Patients with a medical history of prestroke dementia, hemorrhagic stroke, decreased consciousness, severe aphasia or dysarthria, significant acute medical or neurological illness other than stroke, and presence of a premorbid diagnosis of an axis I psychiatric disorder other than unipolar depression or chronic medical conditions known to have an inflammatory component were excluded. The protocol was approved by the Research Ethics Board at Sunnybrook Health Sciences Centre. All participants provided written informed consent prior to participation.

Depression was screened using the Center for Epidemiological Studies Depression Scale (CES-D) on which a score ≥16 is a reliable and sensitive indicator of poststroke depression [13]. Medical comorbidity has been found not to interfere with the accuracy of the CES-D to screen depressive episodes [14]. A trained researcher administered the CES-D scale (a self-report instrument assessing the presence and severity of symptoms over the past week) under the supervision of the study psychiatrist. Cognitive status was assessed using the MMSE, which has been validated in stroke [15], and administered by experienced personnel trained by the study psychiatrist. Stroke severity was assessed using the National Institutes of Health Stroke Scale (NIHSS) [16]. The CES-D, MMSE, and NIHSS were administered either on the same day as the blood draw or on the afternoon before. For patients with an available clinical CT scan, lesion location was recorded, stroke lesions were traced, and lesion volumes were recorded.

Within 24 hours of assessment, fasting blood was collected via venipuncture in BD SST Vacutainer (New Jersey, USA) tubes at 7:30 am ± 30 minutes. Serum was separated and stored at −80°C until analyzed. The proinflammatory Th17 cytokines IL-17 and IL-23 were assayed by standard enzyme linked immunosorbent assays according to manufacturers' instructions (Abcam, Toronto, ON, Canada). The Th1 effector cytokine interferon- [IFN-] γ and the anti-inflammatory cytokine IL-10 were measured using a multiplex suspension bead array immunoassay (Luminex Corporation, Austin, TX, USA). The intra-assay variabilities for the ELISA and Luminex kits were less than 15% (for the IL-17 assay, the coefficient of variability was 3.3%). All analyses were performed in a single batch to avoid variability between assays. Assay sensitivities were 0.2 pg/mL for IL-10, 1.0 pg/mL for IFN-γ, 20 pg/mL for IL-23, and 3 pg/mL for IL-17. Undetectable serum concentrations were imputed at their lower limits of detectability for subsequent analyses. As a stable measure of oxidative stress, lipid hydroperoxides (LPH) were assayed spectrophotometrically as described previously [17].

The Kolmogorov-Smirnov test was used to assess normality of distribution. Serum analyte concentrations and lesion volumes were log transformed to obtain normal distributions for use in analyses of covariance (ANCOVA). Patient characteristics were compared between those with and without depressive symptoms using t-tests for continuous measures and Chi-square tests for categorical variables. MMSE scores were compared to patient characteristics using t-tests for categorical variables or Pearson correlations for continuous measures. Variables related to IL-17 concentrations, depressive symptoms, or MMSE scores at trend level were controlled for systematically in models post hoc. Because differential associations between inflammatory and oxidative stress markers have been observed between depressed and nondepressed subjects [18, 19], relationships between serum markers in subgroups of patients with and without depressive symptoms were explored in Spearman correlations, due to smaller sample sizes.

Sample size was chosen based on effect sizes observed previously relating MMSE scores and serum inflammatory markers [5, 6]. Statistical analyses were performed using SPSS statistical software (version 20; SPSS Inc., Chicago, Illinois) or in R (http://www.R-project.org/).

3. Results

382 patients following ischemic stroke were screened for the study and 138 patients were carefully selected who met inclusion criteria and did not meet any criterion for exclusion. A total of 47 patients (aged 71.8 ± 14.4, 36% female) with mild to moderate stroke severity (NIHSS scores 4.9 ± 4.5) who agreed to participate and who had available serum samples were included in this analysis. Nineteen patients screened positive for depressive symptoms (CES-D ≥ 16), and subjects with and without depressive symptoms were similar in demographics and clinical characteristics (Table 1) although trends were noted for age, hypertension, dyslipidemia, and lesion location. Subjects with and without depressive symptoms did not differ in serum markers (Table 2).

The mean MMSE score was 27.1 ± 2.8. MMSE scores were associated with IL-17 concentrations in patients with depressive symptoms ($r = -0.493$, $P = 0.032$). MMSE scores were not associated with other patient characteristics (Table 1) or serum markers (Table 2).

Serum cytokine and LPH concentrations are presented in Table 2. No relationships were observed between the serum analytes and patient characteristics from Table 1, including ASA or NSAID use, NIHSS scores, and time since stroke

Table 1: Clinical demographic characteristics.

	CES-D < 16 $n = 28$	CES-D ≥ 16 $n = 19$	X^2 or t	P
Demographics				
Age (mean ± SD)	68.8 ± 14.1	76.2 ± 14.2	1.77	0.08
Sex (% male)	60.7	68.4	0.29	0.59
Living alone (%)	42.9	31.6	0.61	0.43
Level of education > high school (%)	92.3	94.4	0.08	0.78
History of depression (%)	3.6	18.8	2.17	0.14
Vascular risk factors				
Hypertension (%)	71.4	94.7	3.97	0.05
Diabetes (%)	21.4	36.8	1.34	0.25
Dyslipidemia (%)	60.7	84.2	2.99	0.08
Obesity (BMI ≥ 30) (%)	14.3	21.0	0.37	0.55
Smoking (%)	17.8	21.1	0.08	0.79
Concomitant medications				
Antidepressant use (%)	7.1	15.8	0.89	0.35
ASA use (%)	62.9	73.7	0.58	0.45
NSAID use (other than ASA) %	7.4	21.1	1.83	0.18
Stroke characteristics				
Weeks since stroke (mean ± SD)	3.4 ± 5.3	3.4 ± 4.8	0.04	0.97
NIHSS scores (mean ± SD)	4.6 ± 4.5	5.4 ± 4.7	0.63	0.53
Lesion location				
Anterior (%)	10.7	15.8	0.26	0.61
Posterior (%)	50.0	26.3	2.64	0.10
Intermediate (%)	10.7	26.3	1.95	0.16
Extending (%)	25.0	31.6	0.25	0.62
Lesion side				
Left (%)	46.4	42.1	0.09	0.77
Right (%)	50.0	57.9	0.28	0.60
Bilateral (%)	3.6	0.0	0.69	0.41
Lesion volume (cm^3) (mean ± SD)*	28.2 ± 63.0	20.6 ± 47.5	0.65	0.52

X^2 or t values and corresponding P values reflect results of Pearson's Chi-squared tests for categorical variables and independent t-tests for continuous variables.

*$n = 21$ nondepressed and $n = 15$ depressed.

BMI: body mass index; CES-D: Center for Epidemiological Studies Depression; NIHSS: National Institutes of Health Stroke Scale; NSAID: nonsteroidal anti-inflammatory; ASA: acetylsalicylic acid.

($P > 0.05$). Most patients included in the study had a large-artery atherosclerosis. No correlations were found between serum levels of biomarkers and etiologic origin of ischemic stroke. Lesion volume was correlated with IFN-γ ($\rho = 0.363$, $P = 0.03$) in this cohort, but not with any other serum analyte.

To test the hypothesis that IL-17 concentrations are associated with depressive symptoms, an ANCOVA model to assess differences in IL-17 concentrations between those with and without depressive symptoms, controlling for age and gender, was used. Serum IL-17 concentrations did not differ between patients with and without depressive symptoms ($F_{1,46} = 0.342$, $P = 0.572$).

To test the hypothesis that IL-17 concentrations were associated with MMSE scores, an ANCOVA model predicting MMSE scores controlling for age, gender, and depression was used. Depression was associated with poorer MMSE scores ($F_{1,46} = 8.44$, $P = 0.006$) and there was a significant depression × IL-17 interaction ($F_{1,46} = 9.29$, $P = 0.004$) whereby IL-17 concentrations were associated with poorer cognitive status in patients with depressive symptoms (see Figure 1). The model explained 15.9% of the variance in MMSE scores. The interaction between depressive symptoms and IL-17 in predicting MMSE scores persisted in post hoc models controlling for hypertension, dyslipidemia, history of depression, antidepressant use, NSAID use, lesion location, NIHSS scores, and time between phlebotomy and assay.

In Spearman correlations, serum IL-17 concentrations were associated with higher LPH concentrations in patients with depressive symptoms ($\rho = 0.518$, $P = 0.023$), but not in those without ($\rho = 0.107$, $P = 0.587$). IL-17 was also

Table 2: Serum assay results.

Serum analytes	Median (IQR) CES-D < 16 $n = 28$	CES-D ≥ 16 $n = 19$	t^*	P
IL-17 (pg/mL)	40.4 (32.8–52.2)	38.0 (33.2–50.7)	0.66	0.52
IL-23 (pg/mL)	300 (273–438)	262 (192–382)	1.81	0.08
IL-10 (pg/mL)	0.20 (0.20–2.41)	0.20 (0.20–2.07)	0.10	0.92
IFN-γ (pg/mL)	0.85 (0.18–1.00)	1.00 (0.23–1.00)	0.20	0.84
LPH (nmol/mL)	11.2 (8.25–13.0)	9.06 (7.63–10.5)	0.84	0.41

$^*t$ values and corresponding P values reflect results of Student's t-test using log transformed values.
Percentages of analyte concentrations returned below the limit of detectability were 0% for IL-17, IL-23, and LPH, 66% for IL-10, and 30% for IFN-γ.
CES-D: Center for Epidemiological Studies Depression; IQR: interquartile range; LPH: lipid hydroperoxides.

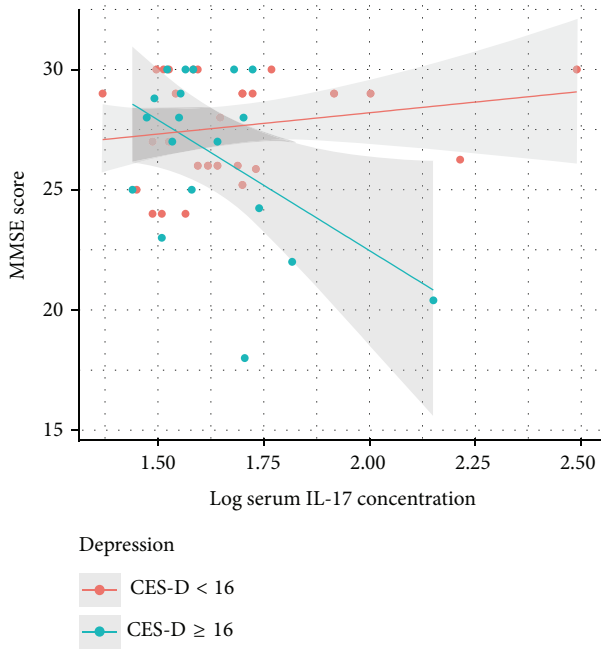

Figure 1: Differential association between peripheral IL-17 concentrations and cognitive status between patients with and without depressive symptoms. MMSE: Mini Mental State Examination, IL-17: interleukin-17, and CES-D: Center for Epidemiological Studies Depression Scale.

associated with lower serum IL-10 concentrations in patients with depressive symptoms ($\rho = -0.484$, $P = 0.036$) but not in those without ($\rho = -0.100$, $P = 0.611$). Similar relationships were not observed with IL-23 or IFN-γ.

4. Discussion

In this study, poststroke depression was not associated with a bias towards peripheral production of IL-17. Relationships between poststroke depression and peripheral concentrations of other cytokines have been inconsistent, with increases noted in some [20–22] but not all previous studies [23–25]. However, in the present study poststroke depression was associated with cognitive impairment, replicating findings from previous studies [1, 2].

Among depressed patients, serum concentrations of IL-17 were associated with poorer cognitive status, consistent with neurodegenerative roles of IL-17 in animal cerebral ischemia models [7]. The significant interaction between depression and IL-17 concentrations predicting MMSE scores suggests that depression may confer neural vulnerability to IL-17 mediated inflammatory pathways. Possible bases for this interaction remain speculative; poststroke depression may be associated with central nervous system (CNS) inflammation that could exacerbate IL-17 expression by T cells when they infiltrate the brain and/or with neurotrophic/neuroprotective deficits that might impair neural resilience to IL-17 mediated neurodegenerative pathways [26].

Additional findings from the present study suggest that IL-17 may be related to the depletion of regulatory T cells (Tregs) and to augmented oxidative stress among subjects with depressive symptoms. Some IL-17 secreting cells (Th17 cells) share a common lineage with regulatory T cells and the expansion of Th17 cells may occur at the expense of Tregs [9]. Among subjects with depressive symptoms, higher IL-17 concentrations were associated with lower IL-10 concentrations, suggesting that poststroke depression may be associated with susceptibility to Treg depletion due to a Th17 response. This may exacerbate neurodegenerative damage, to the detriment of cognitive function, since Tregs are thought to be beneficial after stroke due to their secretion of the anti-inflammatory and neuroprotective cytokine IL-10 into the postinfarct brain [9]. Previously, a polymorphism in the promoter region of the IL-10 gene has been associated with poststroke depression [24], which would be consistent with vulnerability to low IL-10 production among depressed patients.

Given the ability of IL-17 to disrupt the blood brain barrier and contribute to neurodegeneration through increased production of reactive oxygen species [11], LPH associated with IL-17 could reflect peroxidation of blood brain barrier or CNS lipids. In a previous study, serum LPH concentrations were associated with subtle damage to cerebral white matter in patient with bipolar disorder but not in nondepressed controls [17]. The basis for the observation of a relationship between concentrations of IL-17 and LPH specifically among subjects with depressive symptoms after stroke requires further investigation. Findings from a recent study suggested that Th17 cells may be more reactive to oxidized lipids in stroke patients, but depressive or cognitive symptomatology was not assessed in that study [8]. The present results might also reflect greater activation of the NLRP3 inflammasome in peripheral blood mononuclear cells from subjects with depression, as suggested in a recent study of depressed and nondepressed medically healthy subjects [27]. NLRP3 inflammasome activity results in maturation and secretion of IL-1β or IL-18, and NLRP3 inflammasome assembly can be promoted by reactive oxygen species generated by IL-17. In turn, IL-1β or IL-18 resulting from NLRP3 activity can stimulate IL-17 secretion from Th17 or $\gamma\delta$T cells, which may result in a feedforward loop that sustains inflammatory cytokine secretion [28–30]. This would also be consistent

with the findings of one previous study, in which elevated IL-18 concentrations were associated with poststroke depression [25]. More clinical data will be required in order to replicate these findings and to establish roles of these inflammatory and oxidative stress markers in neurodegenerative pathways and their relationships with depressive and cognitive symptoms.

Although these data suggest adequate power to detect the effects observed for relationships between IL-17, cognitive status, and oxidative stress among patients with depressive symptoms, the results are limited by a small sample size. Potential bias may have been introduced at the level of recruitment although the demographics of the included subjects do not differ substantially from those generally seen at our site. Replication in larger cohorts including larger numbers of patients with elevated IL-17 concentrations will thus be informative. Although protein degradation over the course of storage at −80°C may have affected assayed concentrations, the time between phlebotomy and assay did not affect the main results. The present study measured cytokines at a single time point with variable poststroke sampling times, potentially contributing to heterogeneity in the findings; however, cytokine concentrations were not associated with time since stroke in this sample. Nevertheless, future studies should delineate the time course of IL-17 elevations and the relative significance of elevated IL-17 concentrations specifically in acute, subacute, and chronic stages of stroke. Serum cytokine measurements are limited by variable systemic release and half-lives in circulation, and therefore they may not reflect CNS concentrations; however, peripheral T cells are known to enter the brain after stroke and the present results support previous findings to suggest that a peripheral IL-17 bias may be clinically relevant [10]. While the MMSE is largely used as a screening instrument, it has been validated and used extensively in stroke [5, 6, 15]. The MMSE is sensitive to clinically meaningful cognitive impairment, and it is relatively stable over time after stroke [31]. Finally, while the CES-D has been shown to have excellent concurrent validity with diagnostic criteria for depressive episodes and high accuracy in screening, future studies might confirm the present findings using a structured clinical interview for major depressive disorder criteria.

5. Conclusion

These preliminary clinical data would be consistent with vulnerability to IL-17 mediated neurodegenerative pathways in patients with depressive symptoms. The related mechanisms may involve an imbalance between pro- and anti-inflammatory activity and augmented oxidative stress, which may help to characterize poststroke depression and associated cognitive susceptibility.

Conflict of Interests

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

Authors' Contribution

W. Swardfager, N. Herrmann, A. C. Andreazza, R. H. Swartz, S. E. Black, and K. L. Lanctôt conceived of the study, participated in its design and coordination, interpreted results, and helped to draft the paper. W. Swardfager, K. L. Lanctôt and M. M. Khan performed statistical analyses, interpreted results, and drafted the paper. A. C. Andreazza carried out serum assays and participated in analyses and interpretation of results. All authors read and approved the final paper.

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Research Article

Protective Effects of *Borago officinalis* Extract on Amyloid β-Peptide(25–35)-Induced Memory Impairment in Male Rats: A Behavioral Study

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Alzheimer's disease (AD) is a neurodegenerative disorder and most common form of dementia that leads to memory impairment. In the present study we have examined the protective effects of *Borago officinalis* (borage) extract on Amyloid β (Aβ)-Induced memory impairment. Wistar male rats received intrahippocampal (IHP) injection of the Aβ(25–35) and borage extract throughout gestation (100 mg/kg). Learning and memory functions in the rats were examined by the passive avoidance and the Morris water maze (MWM) tasks. Finally, the antioxidant capacity of hippocampus was measured using ferric ion reducing antioxidant power (FRAP) assay. The results showed that Aβ(25–35) impaired step-through latency and time in dark compartment in passive avoidance task. In the MWM, Aβ(25–35) significantly increased escape latency and traveled distance. Borage administration attenuated the Aβ-induced memory impairment in both the passive avoidance and the MWM tasks. Aβ induced a remarkable decrease in antioxidant power (FRAP value) of hippocampus and borage prevented the decrease of the hippocampal antioxidant status. This data suggests that borage could improve the learning impairment and oxidative damage in the hippocampal tissue following Aβ treatment and that borage consumption may lead to an improvement of AD-induced cognitive dysfunction.

1. Introduction

Alzheimer's disease (AD) is the most common cause of dementia that is estimated to affect approximately 36 million people worldwide [1]. AD is a disease that is commonly characterized by a gradual decline of memory, language, and cognitive ability. The nerve cells in the brains of Alzheimer's patients progressively shrink and die. Such neuronal cell death occurs first in the brain regions that are responsible for learning and memory, but it ultimately spreads to the entire brain [2]. It has been reported that the cholinergic system of Alzheimer's patients damages, resulting in decreased acetylcholine-producing choline acetyltransferase activity, decreased choline absorption, and decreased acetylcholine release, as well as decreased cortical acetylcholinesterase activity [3]. Cholinergic neurons of basal forebrain nuclei enter the hippocampus and cerebral cortex, and these neurons are crucial for memory, concentration, and other cognitive procedures [4].

Senile plaques and neurofibrillary tangles are the hallmark pathological features that are observed in the cortex, hippocampus, basal forebrain, and amygdala of an Alzheimer's patient [5]. Neurofibrillary tangles are intracellular fibrillar aggregation of the microtubule-associated

protein tau which is hyperphosphorylated and oxidized. Senile plaques consist of insoluble fibrillar amyloid β (Aβ). Aβ destabilizes cellular Ca^{2+} homeostasis, consequently, inhibits hippocampal long-term potentiation, and disrupts synaptic plasticity [6, 7]. In addition, Aβ induced elevation of reactive oxygen species (ROS) levels in neurons resulting in protein oxidation, lipid peroxidation, ROS formation, and cellular dysfunction, leading to calcium ion accumulation and subsequent neuronal death [8–10]. Furthermore, Aβ causes damage to mitochondrial membranes and hence increases the amount of intracellular H_2O_2, thus affecting the genes downstream by interacting with numerous receptors and damaging neurons, ultimately accelerating cell death and hippocampus alteration [11–13].

The hippocampus is a relevant structure that is highly involved in cognition and psychological function and there is evidence that this structure is rapidly and extremely affected by an injection of the Aβ fragment (Aβ25–35) in rat [14]. As mentioned above, oxidative stress and inflammation following Aβ involve development and progression of AD. Antioxidants that prevent the detrimental consequences of Aβ are consequently considered to be a promoting approach to neuroprotection in AD brain [15]. *Borago officinalis*, also known as borage, is a plant with nutritional value that is also used in traditional medicine in Iran. It has been known for its mood elevating properties as early as the first century A.D. [16].

Dietary use of borage exhibited immune-modulator [17] and blood pressure lowering effects in normal and hypertensive rats [18]. Borage oil has been promoted as an effective treatment for different pathologies, such as diabetic neuropathy and rheumatoid arthritis [19, 20].

Several experimental studies have shown that borage has antioxidant properties by decreasing the level of oxidative stress and free radical scavenging activity [21, 22].

Phytochemical studies reveal that borage contains tannins, resine, ascorbic acid, beta carotene, niacin, riboflavin, rosmarinic acid, and flavonoids [22, 23]. Borage is considered one of the best sources of gamma-linolenic acid (GLA) [24] which is known to have beneficial effects on brain ageing. It has been reported that treatment of aged rats with GLA restores the hippocampal LTP [25] and improves both memory and N-methyl-D-aspartic acid receptor function [26].

As the use of traditional medicine is widespread and plants still represent a large source of natural antioxidants, we seek to determine if borage extract as the best source of both GLA and antioxidant capacity can improve the cognitive and memory ability on Aβ-induced learning and memory deficits in rat.

2. Materials and Methods

The Aβ(25–35) was purchased from sigma-Aldrich company (St Louis, MO, USA). *Borago officinalis* leaves were obtained in dried condition from Research Institute for Islamic and Complementary Medicine (Tehran, Iran). Aβ25–35 was solubilized in sterile water at 1 μg/μL concentration and stored at −20°C.

2.1. Animals. We included 28 male Wistar rats (Pasteur-Iran), weighing 250–300 g in this experimental study. All animals were group-housed and given ad libitum access to food and water. Housing conditions were maintained at a temperature of 21 ± 2°C and the relative humidity of 50 ± 5% on a 12 h light/12 h dark cycle.

The rats randomly were assigned to the following groups:

the control or intact group ($n = 7$) that was left undisrupted;

the sham-operated group;

the Aβ25–35 model group which received single bilateral intrahippocampal (IHP) injections of Aβ25–35 [27];

the borage-treated group that received borage extract (orally, 100 mg/kg) following IHP injection of Aβ25–35 for 14 days [28].

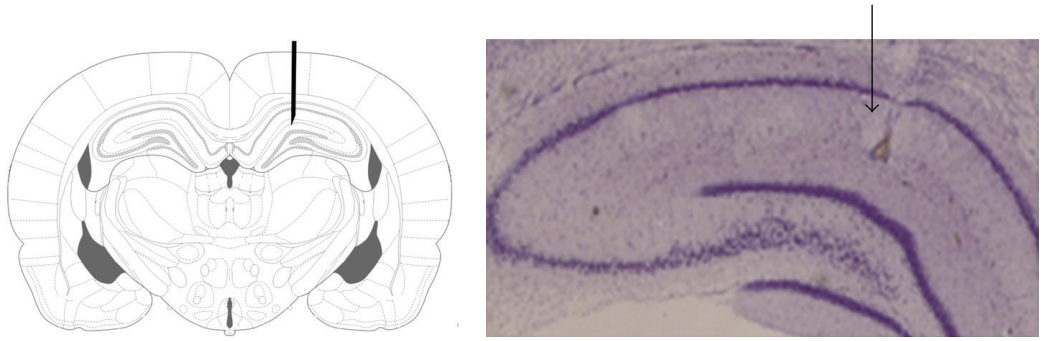
2.2. Preparation of Borage Extract. Dried borage leaves were cleaned and ground into coarse powder by electrically driven device. The powdered material was soaked into aqueous ethanol (80%) for one week with occasional shaking [29]. The extract was filtered through a Whatman filter paper and evaporated to dryness under reduced pressure at a maximum of 40°C using a rotary evaporator. *Borago officinalis* yielded 10.9% dried extract. The extract was completely dissolved in distilled water and kept at 4°C.

2.3. Intrahippocampal Injection of Aβ25–35. The animals were anesthetized with the ketamine (100 mg/kg) and xylazine (10 mg/kg) and transferred to a stereotaxic apparatus (Stoelting, Wood Dale, IL, USA). Injection was made using a 10 μL microsyringe (Hamilton-Reno, NV, USA). Relative to the bregma and with the stereotaxic arm at 0°, the coordinates for the dentate gyrus were posterior −3.6; lateral ±2.3; and dorsal 3 mm [30].

Aβ solution (6 μL) was bilaterally injected into the region over 1 μL/2 min. the cannula was left in place for 2 min after each injection to allow for diffusion. Sham operated rats received vehicle solution. The skin was then sutured and the animals were left to recover in a warm box before returning to their home cages. The injection site was checked by injection trypan blue instead of peptide in preliminary experiments (Figure 1).

2.4. Passive Avoidance Learning. The passive avoidance test was started two weeks after the Aβ injection. The apparatus consisted of two chambers of the same size (20 × 20 × 30 cm). The chambers were separated by a guillotine door. The walls and floor of one compartment consisted of white opaque resin and the walls of the other one were dark. An intermittent electric shock (100 V, 0.3 mA, and 0.5 s) was delivered to the grid floor of the dark compartment by an isolate stimulator [14].

Each rat was gently placed in the white compartment and after 5 s the guillotine door was opened and the animal was allowed to enter the dark module. Immediately after entering



(a) (b)

Figure 1: Schematic (a) and cresyl violet stained (b) photograph representing the microinjection site of Amyloid β into the hippocampus (black arrow).

the dark chamber, the door was closed and an electric shock was delivered through the floor grid (acquisition trial). Then the rats were returned to their home cages. Twenty-four hours later, each rat was again placed in light chamber (retention trial). The interval between the placement in the light chamber and the entry into the dark chamber (STL) and the total time spent in dark compartment (TDS) were recorded in the absence of electric foot shocks, as indicator of inhibitory avoidance behavior.

2.5. Assessment of Spatial Memory. As described previously [31], spatial memory testing was carried out using Morris water maze (MWM) apparatus. The MWM consisted of a circular pool (180 cm in diameter, 60 cm in depth) painted black and filled to a depth of 35 cm with water at a temperature of 22±1°C. Numerous visual cues were present around the room and remained constant during the length of the experiment. The pool was divided into four quadrants with four starting locations, which were referred to as north (N), east (E), south (S), and west (W), each located at equal distances along the pool rim. An invisible platform (10 cm diameter) was located 1 cm below the water in the center of the northern quadrant that remained consistent for all animals across the training trials. The rats were trained between 10:00 a.m. and 12:00 noon for four days. Training consisted of two blocks with four trials. During training, each animal was allowed to swim until they located the hidden platform or until 90 s has elapsed. All groups were trained from each of the starting positions (N, E, S, and W). There was a 30 sec period between the two trials, which was spent on the platform. Rats were allowed to rest for 5 min between two consecutive blocks. Installed above the pool was a video camera (Nikon, Melville, NY, USA) linked to a tracking system to record a number of parameters including the time taken to reach the hidden platform (escape latency) and the length of the swim path (traveled distance). On day 5, a probe trail was performed in which the platform was removed from the pool and each rat was allowed to swim for 60 s. For these probe trails, percentage of time spent in the target quadrant was recorded.

2.6. Histological Verification. For verification of injection position using a light microscope (Olympus, Japan), the trypan blue injected rats were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (pH = 7.3) and the hippocampi were serially sectioned into 10 μm coronal sections by a microtome (Leica Instruments, Germany). After deparaffinization and rehydration, sections were stained in 0.1% cresyl violet for 3 minutes. Finally, the sections were photographed with a digital camera (Olympus, DP 11, Japan) attached to a microscope (Olympus Provis, Ax70, Japan) and the stained slices were qualitatively analyzed for the injection site.

2.7. Ferric Reducing/Antioxidant Power (FRAP) Assay. After memory assessment, the animals were decapitated, brains were removed, and the extracted hippocampi were immediately frozen in liquid nitrogen and maintained at −80°C until processing.

The hippocampus portion was gently homogenized in ice-cold phosphate buffered saline (0.1 M, pH 7.4) to give a 10% homogeny suspension and used for FRAP assay.

Briefly, 50 μL of homogenate was added to 1.5 mL freshly prepared and prewarmed (37°C) FRAP reagent (300 mM acetate buffer (pH = 3.6), 10 mM TPTZ in 40 mM HCl, and 20 mM $FeCl_3 \cdot 6H_2O$ in the ratio of 10 : 1 : 1) in a test tube and incubated at 37°C for 10 min. The absorbance of the blue colored complex was read against reagent blank (1.5 mL FRAP reagent + 50 μL distilled water) at 593 nm. Standard solutions of FeII in the range of 100 to 1000 mM were prepared from ferrous sulphate ($FeSO_4 \cdot 7H_2O$) in distilled water. FRAP values were expressed as nmol ferric ions reduced to ferrous form/mg tissue.

2.8. Statistical Analysis. Data were presented as mean ± S.E.M and analyzed by SPSS version 16 software. The data of the escape latency and traveled distance during the training days were analyzed using two-way analysis of variance (ANOVA), treatment as one factor and training days as the second factor. Statistical analyses of the FRAP value, passive avoidance test, and percent of time spent in target quarter of probe

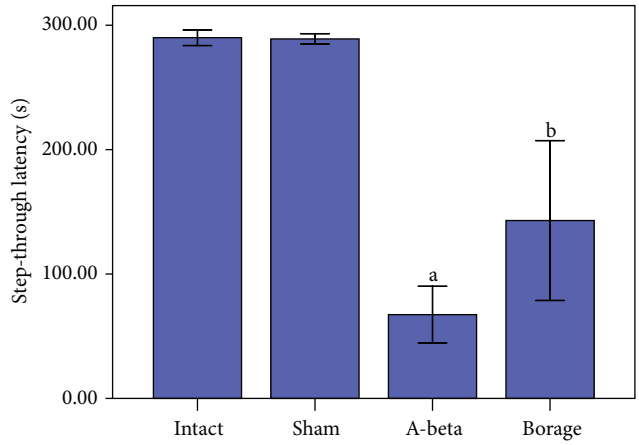


Figure 2: The mean of the step-through latency in the passive avoidance task. Vertical bars show S.E.M. (a $P < 0.001$ versus intact and sham groups; b $P < 0.01$ versus A-beta group).

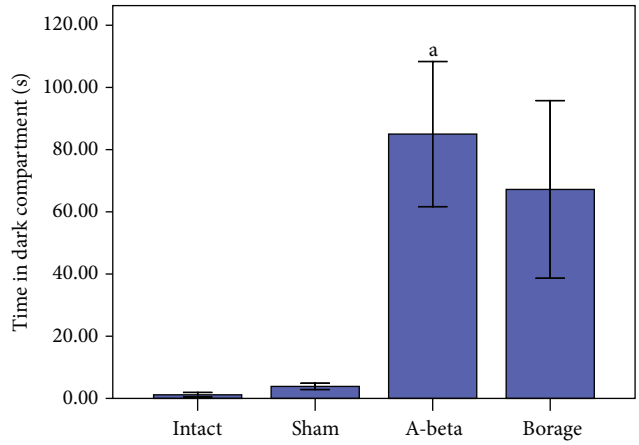


Figure 3: The mean of the time spent in dark compartment in the passive avoidance task. Vertical bars show S.E.M. (a $P < 0.001$ versus intact and sham groups).

trial were performed using one-way ANOVA. Tukey multiple comparison tests were used to analyze the significance of the differences between the groups, when appropriate. Value of $P < 0.05$ was considered significant.

3. Results

Histological analysis showed that injection of Aβ was in their desired location, according to the atlas of Paxinos and Watson (Figure 1) [30].

3.1. Passive Avoidance Task. In the acquisition trial (14 days after the operation), we found no difference between the intact and other groups in the step-through latency (STL, data not shown). However, the IHP injection of Aβ(25–35) reduced STL in the retention trial compared to intact and sham groups ($P < 0.01$, Figure 2).

Furthermore Aβ-treated rats spent more time in dark compartment (TDC) in respect to intact and sham-operated rats ($P < 0.001$, Figure 3). Administration of borage for 14 days caused a significant increase in STL compared with Aβ-treated rats ($P < 0.01$, Figure 2). Borage treatment insignificantly attenuated the TDC when compared to Aβ group (Figure 3).

3.2. MWM Performance. A two-way analysis of variance revealed significant effects of treatment [F (3, 28) = 26.85, $P < 0.001$] and training days [F (3, 24) = 7.8, $P < 0.001$]. In addition, there was a significant interaction between treatment and training days [F (9, 72) = 2.35, $P < 0.05$]. Analysis of the four training days showed that intact group spent less time to find the hidden platform (escape latency) than the other groups (Figure 4) and this time was more in the first day compared to other days. Longer escape latency indicates more sever spatial memory deficits. The post hoc analysis indicated a significant difference between the intact and sham-operated groups and the rats which received Aβ ($P < 0.001$). According to the results, borage administration

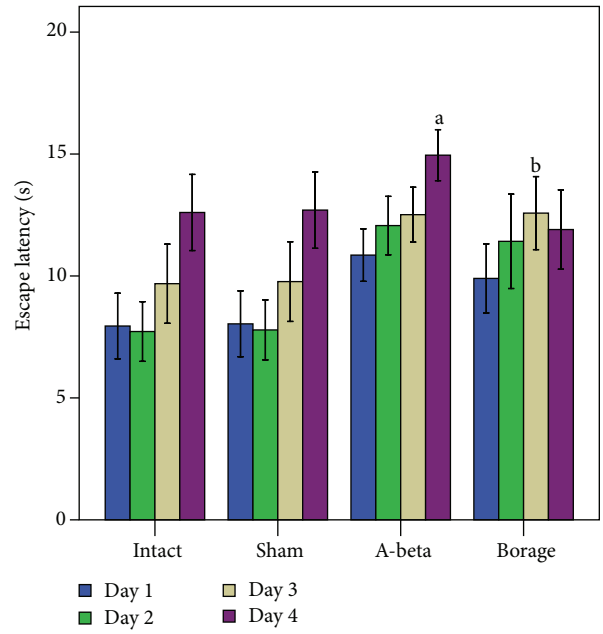


Figure 4: The mean of latencies to find hidden platform in the MWM. Each block represents the average latency of four consecutive trial days. Data present as mean ± S.E.M. (a $P < 0.001$ versus intact and sham groups; b $P < 0.01$ versus A-beta group).

caused significant reduction in escape latency compared with the Aβ-treated group ($P < 0.01$).

In accordance with the latency data, there was a significant effect of treatment [F (3, 28) = 11.23, $P < 0.001$] and training days [F (3, 55) = 21.78, $P < 0.001$] on the traveled distance. There was no significant interaction between training days and treatment. As shown in Figure 5, a significant difference in traveled distance was seen between Aβ-treated rats and intact group ($P < 0.001$). Aβ-treated rats that received *Borago* for 14 days showed less traveled distance compared with Aβ group ($P < 0.001$).

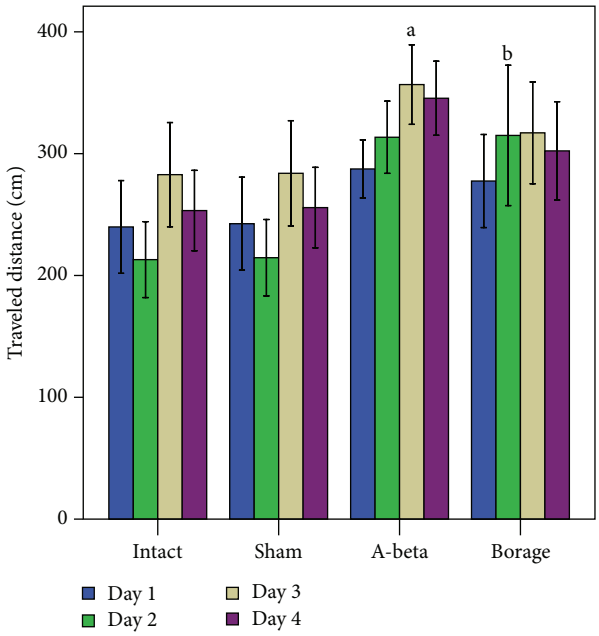


Figure 5: The mean of traveled distance in the MWM. Each block represents the average of traveled distance of four consecutive trial days. Data present as mean ± S.E.M. (a $P < 0.001$ versus intact and sham groups, b $P < 0.001$ versus A-beta group).

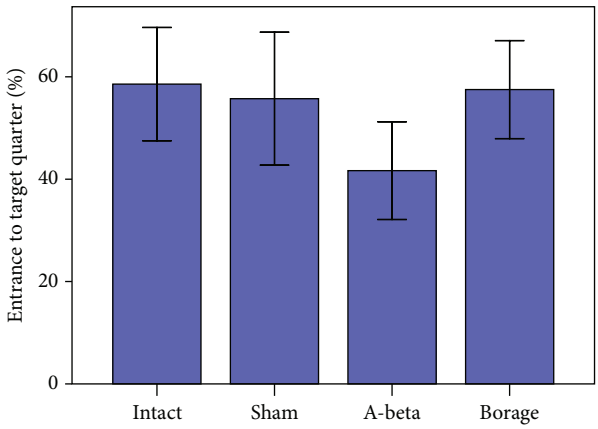


Figure 6: The mean of the percent of entrance to target quarter in the probe trial in the MWM. Data present as mean ± S.E.M.

Percentage of the entrance to target quadrant in the probe trial session was investigated. Results showed that the intact group spent more time in target quadrant (5.87 ± 553) than other groups (sham: 5.57 ± 649; Aβ: 4.67 ± 333; *Borago*: 5.57 ± 0.479, Figure 6).

3.3. FRAP Assay. Aβ caused a significant reduction in FRAP value of homogenate samples as compared with intact and sham-operated animals ($P < 0.001$, Figure 7). Borage treatment increased antioxidant power (FRAP value) of brain homogenate samples ($P < 0.05$, Figure 7).

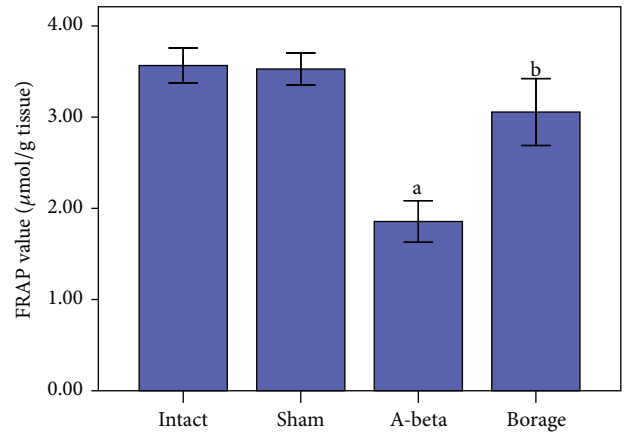


Figure 7: Effect of borage on antioxidant power (FRAP value) of hippocampus homogenate samples following microinjection of Aβ into rat hippocampus. Data present as mean ± S.E.M. (a $P < 0.001$ versus intact and sham groups; b $P < 0.05$ versus A-beta group).

4. Discussion

The major finding of this study was the attenuation of learning and memory impairment by borage following IHP injection of Aβ. Treatment with borage was protective against Aβ-induced oxidative stress in the hippocampus. Aβ plays an important role in the pathophysiology of Alzheimer disease and a close correlation exits between Aβ procedure and the neurodegeneration process of AD [32]. Nitta et al. showed that the performance of the water maze task in β-amyloid-treated rats was impaired and choline acetyltransferase activity significantly decreased in the frontal cortex and hippocampus [33]. The deposition of β-amyloid protein in the brain is related to the impairment of learning and cholinergic neuronal degeneration and the β-amyloid protein-treated rats could be used as animal model for AD [14]. The key brain regions involved in navigation in the MWM task include the striatum, the frontal, and spatially the hippocampus [34, 35]. In the present study, the bilateral IHP injection of Aβ(25–35) induced a significant learning disturbance in the passive avoidance and MWM tasks in the rat. Consistent with our results several experimental studies have shown that the infusion of Aβ(25–35) into the brain induced learning impairment in the passive avoidance and radial-arm maze tasks [14] and memory disturbance in the Y-maze, passive avoidance, and water maze tasks [36]. Similarly, Harkany et al. reported that bilateral injection of Aβ(25–35) in rat induced learning deficits in passive avoidance tasks [37]. Our results of FRAP assay showed that Aβ(25–35) could decrease antioxidant power of the hippocampus. The reaction of FRAP assay is linearly related to molar concentration of the antioxidant(s) present. Consistent with our study several lines of evidence suggest that the Aβ is inserted into the neuronal membrane bilayer and generates oxygen-dependent free radicals and then causes the lipid peroxidation and protein oxidation [38, 39]. Furthermore, the Aβ deposition activates the acute immune response of microglial cells and astrocytes and leads to production and

activation of inflammation-related proteins such as complement factors, cytokines, such as interleukin 1, interleukin 6, and tumor necrosis factor α, thus leading to synaptic damage, neuronal loss, and the activation of other inflammatory participants [40–42]. The brain is sensitive to oxidative stress because of low antioxidant and cell membrane lipid [43]. Therefore, the use of different spices and aromatic herbs as external antioxidant is one of the most common therapeutic strategies for treatment of neurological disease [44, 45] and improvement of brain damage and cognitive function [31, 46, 47]. The present study demonstrated that administration of borage was protective against Aβ-induced memory and antioxidant deficit. As expected following Aβ injection, a significant reduction in antioxidant power, as indicated by FRAP value, was observed. Borage increased the antioxidant power of homogenate samples of hippocampus which was consistent with results from the study by others that extracted antioxidants from borage [48, 49]. Our results showed that borage oil could improve Aβ-induced memory impairment. The protective effect of borage on memory can be related to its function of scavenging free radicals and to its high content of GLA [24].

Indeed, borage's hydroalcoholic extract inhibits linolenic acid oxidation, liposome peroxidation, and/or scavenges 2,2-diphenylpicrylhydrazyl (DPPH) radical in vitro [21]. Several studies evaluated the relationships between antioxidant activity of borage extract and its GLA content [50, 51].

Tasset-Cuevas et al. have shown that both borage seed oil and GLA exert a role in the genomic stability, acting as desmutagenic agents against hydrogen peroxide by scavenging the ROS originated by the model genotoxicant used [52].

Similarly, Duffy et al. observed an increase in learning ability after administration of evening primrose oil which is rich in GLA following uteroethanol exposure in rats [53].

Treatment with GLA rich natural oils was previously shown to partially prevent nerve ischemia and associated conduction anomalies and improved long-term potentiation in the hippocampus in rats with experimental diabetes mellitus [26, 54].

Kavanagh et al. have shown that GLA treatment increased anti-inflammatory cytokines in hippocampus of lipopolysaccharide-treated rats and suggested that these effects may be coupled with fatty acid-induced upregulation of peroxisome proliferator-activated receptor gamma which processes known anti-inflammatory effects [55].

Taken together, it is possible that borage oil inhibits ROS generation, scavenges free radicals, inhibits the effects of inflammatory proteins, suppresses the inhibitory effects of Aβ on learning, and might be implicated in the protection of Aβ-induced neurotoxicity.

It is not clear whether the borage extract or its active ingredient crosses the blood brain barrier and that needs to be investigated further.

5. Conclusion

In summary, we demonstrated that the IHP injection of Aβ(25–35) induced a significant reduction in antioxidant power and learning deficits in passive avoidance and Morris water maze tasks and borage administration significantly attenuated traveled distance and escape latency reduction following injection of Aβ(25–35) and improved Aβ-induced time spent deficiencies. Borage treatment improved step-through latency and time spent in dark compartment in passive avoidance tasks. Also, borage increased the antioxidant power of homogenate samples of hippocampus. Therefore, it is likely that borage may be useful to treat patients with impaired memory function.

Conflict of Interests

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

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