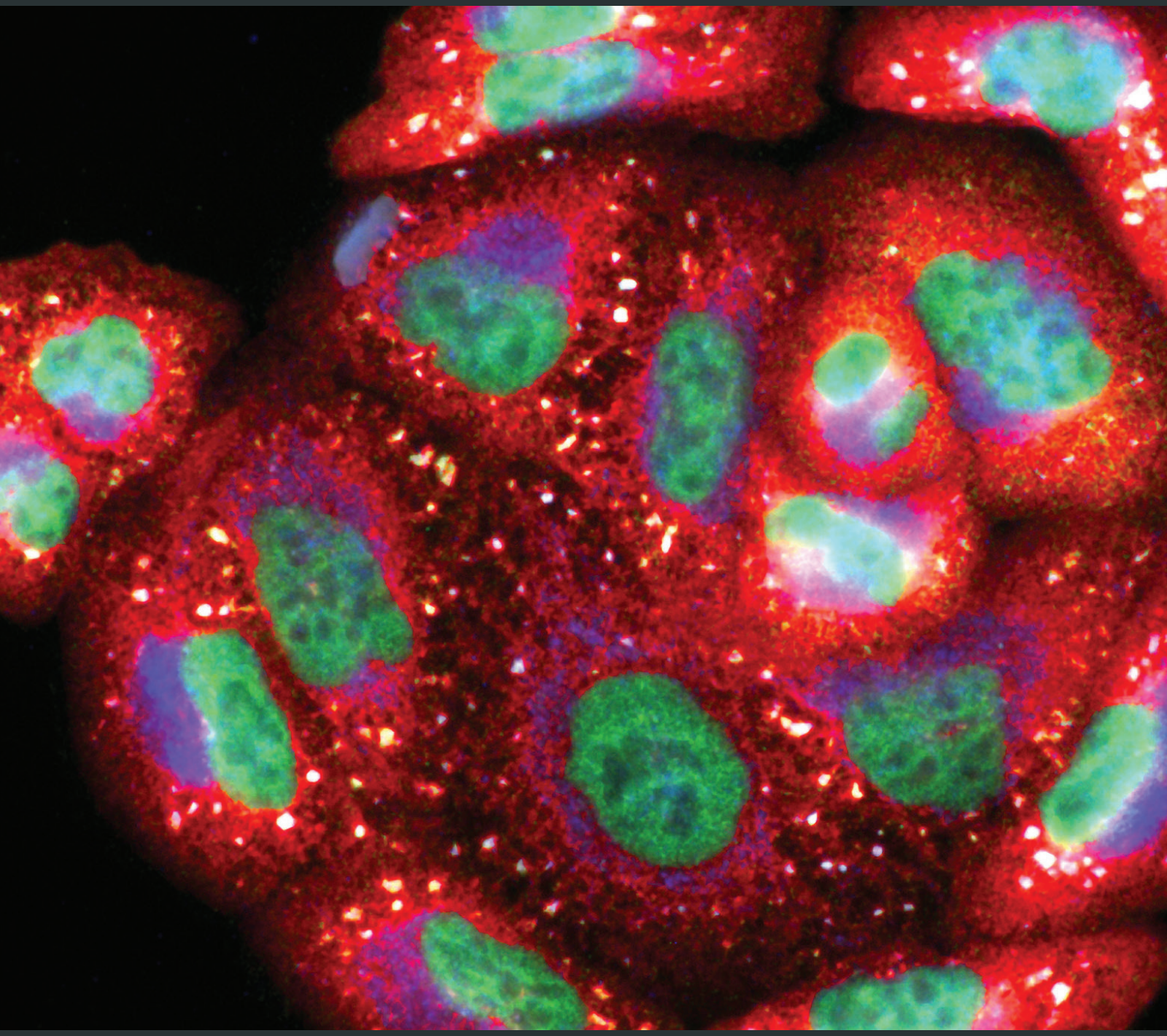
Oxidative Medicine and Cellular Longevity

Neurodegeneration, Neurogenesis, and Oxidative Stress 2015

Guest Editors: Renata Santos, Anne-Laure Bulteau, and Cláudio M. Gomes



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Editorial
Neurodegeneration, Neurogenesis, and Oxidative Stress 2015

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Oxidative stress is implicated in the pathophysiology of a wide variety of neurodegenerative disorders and its role in neurogenesis is becoming increasingly acknowledged. This special issue includes 8 articles that emphasize the implications of oxidative stress in neurodegeneration, neurotoxicity, and neurogenesis.

Several original and review articles discuss the role of oxidative stress in neurodegenerative disorders and upon brain injury. S. K. Singh et al. open the issue by presenting a comprehensive review on the pathology of Alzheimer's disease (AD). Two other review articles focus on specific aspects implicated in AD. L. Zuo et al. discuss how oxidative stress is related to AD progression and to the formation of Aβ plaques and tau neurofibrillary tangles. In addition, the authors examine evidence of epigenetic regulation of Aβ plaque formation in AD neurons and discuss different potential therapeutic approaches. J. S. Cristovão et al. concentrate on the role of metal ions in AD and overview different proteins implicated in AD whose metal binding properties may underlie important biochemical and regulatory processes occurring in the brain during the pathophysiological process. Hyperphosphorylation and aggregation of tau in neurons not only are a central feature in AD but are present in other neurodegenerative diseases, termed tauopathies. S. M. A. Naini and N. Soussi-Yanicostas review the relationship between tau pathology and oxidative stress and present arguments in favor of the hypothesis that these are key components of a pathologic vicious circle in tauopathies. In a different direction, X. Hu et al. summarize recent literature describing the contribution of oxidative stress to brain damage after intracerebral hemorrhage. H. J. Olguín et al. describe dopamine dysfunction as a consequence of oxidative stress and discuss its implication in disease conditions, such as in Parkinson's disease. In an original article, A. Seguin et al. present interesting data on a drug screen performed using two different models of Friedreich's ataxia, yeast and *Drosophila*. In the original article by B. P. Carreira et al., the authors examine the role of nitric oxide in neurogenesis in adult hippocampus following seizures. They show that although nitric oxide is beneficial in the early stages of production of newborn neural cells, it is detrimental to the survival of newly differentiated neurons due to inflammation.

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Review Article

Metals and Neuronal Metal Binding Proteins Implicated in Alzheimer's Disease

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Alzheimer's disease (AD) is the most prevalent age-related dementia affecting millions of people worldwide. Its main pathological hallmark feature is the formation of insoluble protein deposits of amyloid-β and hyperphosphorylated tau protein into extracellular plaques and intracellular neurofibrillary tangles, respectively. Many of the mechanistic details of this process remain unknown, but a well-established consequence of protein aggregation is synapse dysfunction and neuronal loss in the AD brain. Different pathways including mitochondrial dysfunction, oxidative stress, inflammation, and metal metabolism have been suggested to be implicated in this process. In particular, a body of evidence suggests that neuronal metal ions such as copper, zinc, and iron play important roles in brain function in health and disease states and altered homeostasis and distribution as a common feature across different neurodegenerative diseases and aging. In this focused review, we overview neuronal proteins that are involved in AD and whose metal binding properties may underlie important biochemical and regulatory processes occurring in the brain during the AD pathophysiological process.

1. Alzheimer's Disease: Hallmark Amyloid Aggregation and Neuronal Dysfunction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by cognitive decline. The neuropathology hallmarks are gross atrophy of the cortex and hippocampus, and the accumulation of amyloid-beta (Aβ) into senile plaques and of hyperphosphorylated tau into neurofibrillary tangles. The deposition of Aβ and hyperphosphorylated tau aggregates in the human brain occurs in opposite directions with an orderly neuroanatomical pattern. Amyloid plaques first appear in the neocortex and slowly progress through the striatum, the basal cholinergic nuclei, the brain stem, and finally the cerebellum [1]. The deposition of tangles begins in the brain stem and progresses towards the neocortex [2]. Thus, the common presence of amyloid plaques and tau neurofibrillary tangles in the cortex only happens at late stages of the disease.

AD is heterogeneous and multifactorial with sporadic and familial forms [3–6]. The large majority of patients have the sporadic form or late onset dementia (later than 65 years). The few remaining patients have the familial form with early onset dementia (around 30 years to 65 years) and may present different symptoms. These patients have mutations in one of three genes encoding proteins essential for Aβ formation: the amyloid precursor protein (APP) and presenilins 1 and 2 (PSEN1/2) [7–10]. Presenilins are components of catalytic subunit of γ-secretase multicomplex, responsible for the cleavage of APP and formation of Aβ. The origin of the sporadic form is complex involving multiple genetic and environmental risk factors, for example, the presence of apolipoprotein E-ε4 allele, mitochondrial dysfunction, head injury, or a compromised brain blood barrier [3, 11]. Despite the fact that AD is the most common form of dementia of the elderly and affects millions of people worldwide, the exact cause of this disorder is still unknown. The genetic evidence

obtained from the rare familial form of AD supports the hypothesis that the accumulation of Aβ plaques is at the origin of the disease. This is the foundation for the amyloid-β cascade hypothesis [12] which has been the central theory in AD research for the last three decades. According to this hypothesis, the deposition of Aβ is the initial event and it is sufficient to trigger the cascade of pathological and clinical changes in AD, which are the formation of senile plaques and neurofibrillary tangles and subsequent neuronal death, vascular damage, and dementia [12]. Although senile plaque deposition is an early event in the disease, as observed in postmortem human brains [1], plaque accumulation in the brain does not correlate with dementia [13] implying that other mechanisms are associated with neurodegeneration. Notably, therapies designed until now that aimed at targeting amyloid plaques and APP proved to be largely unsuccessful. An increasing amount of data challenges the amyloid-β cascade hypothesis.

Therefore, efforts to integrate the other pathogenic features of AD and multiple etiology pathways into a more global model are now needed. During the course of AD, tau is hyperphosphorylated and accumulates in the somatodendritic compartment as paired helical filaments and straight filaments [14]. In neurons, tau is the major microtubule associated protein and stabilizes its structure. Tau interacts with tubulin promoting its assembly into microtubules. The level of phosphorylation regulates the activity of tau and hyperphosphorylation suppresses its microtubule assembly activity. In addition, hyperphosphorylated tau sequesters normal tau and other microtubule associated proteins that further contribute to microtubule disassembly [15]. Therefore, the abnormal phosphorylation of tau results in loss of normal function and gain of toxic function in the AD brain. The formation of neurofibrillary tangles does correlate with cognitive decline and with neuronal and synapse loss [13, 16].

Senile plaques are extracellular deposits composed mainly of amyloid peptides ranging from 39 to 43 amino acids, which are natural metabolites of APP generated by sequential cleavage by β-secretase and γ-secretase l [17]. The APP is a transmembrane protein necessary for neurogenesis, for neurite outgrowth and guidance, and for synapse formation and repair [18]. APP is processed in different ways through different enzymes leading to the formation of amyloidogenic and nonamyloidogenic precursors. The processing of APP results in the formation of soluble α- and β-secreted APP (sAPP) which is cleaved by α- and β-secretase, respectively. As a product in the nonamyloidogenic pathway, sAPPα promotes neuronal survival and neurite outgrowth, among other beneficial neuronal functions. Contrarily, sAPPβ is not involved in the beneficial functions of sAPPα, participating in synapse pruning. Aβ is secreted through sequential APP cleavage by β- and γ-secretases, resulting in peptides that can range from 39 to 43 amino acids. The Aβ peptides are catabolized by multiple amyloid degrading enzymes, for example, neprilysin and insulin-degrading enzyme [19]. It is the imbalance between the production and clearance of Aβ that triggers its deposition as amyloid plaques. However, several studies suggest that Aβ has a physiological role in the synapses and its complete removal induces neuronal cell death [20–22]. In addition to the aggregates, Aβ is also present in soluble oligomeric forms in APP-transgenic mice and human diseased brains [20]. Compared to Aβ aggregates, the soluble oligomers are highly neurotoxic [23]. Therefore, it is possible that aggregation of Aβ into plaques is a neuroprotective mechanism that eliminates the toxic oligomeric forms [15].

The normal functions of synapses are impaired during the course of AD. Synapse loss correlates with dementia suggesting that it is important for disease progression and for the degeneration process [24]. Dense plaque deposition causes the surrounding neurites to bend and change trajectory, which can lead to changes in synapse signal transmission. Also, gliosis and oxidative stress are observed in the vicinity of plaques. During normal development of the brain, microglia are involved in synaptic pruning after birth and it is possible that in the diseased AD brain the recruitment of activated microglia around the plaques participates in the synapse loss [24]. In addition to aggregates, the oligomeric forms of Aβ obtained from cultured cells or from human AD brain disturb synapses and lead to cognition impairment in injected mice [25–27]. Comparably, evidence also shows that soluble forms of tau are toxic for synapses [28]. The molecular mechanisms that lead to synapse dysfunction and neuronal loss downstream of Aβ and tau are not completely identified but different pathways are implicated such as mitochondrial dysfunction, oxidative stress, inflammation, and dysregulation of metal homeostasis.

2. Metals and Metal Binding Proteins Implicated in AD

Metal ions play essential roles in the brain and there is solid evidence pointing to their homeostatic dysfunction across different neurodegenerative diseases (e.g., [29–31]). This includes the first row transition metals, iron, copper, and zinc and also calcium, whose homeostasis is important for neuronal function and during aging [32–34]. One major hypothesis for this cross talk, which has been put forward since a number of years and which has been elegantly reviewed in [35], proposes that AD is as much as a metallopathy as a proteinopathy. Indeed, age-related metal ion dysfunction altered levels of neuronal metal ions in AD-affected areas including accumulation in protein deposits, and the interplay between metal ions and AD pathological proteins indicates a close relationship between protein misfolding, aggregation, and metal ion homeostasis. In AD patients, it has been shown that Cu^{2+}, Zn^{2+}, and Fe^{2+} are found in the core and rims of senile plaques [36, 37] and colocalize with Aβ [38]. This has led to the suggestion that metal ion sequestration into plaques could lead to deficient distribution of these metals in the neighbouring regions [39]. Moreover, it is described that in AD patients Zn^{2+} is decreased in serum and blood but increased in the cerebrospinal fluid and neocortical tissue [40–42]. In addition, Zn^{2+}, Cu^{2+}, and Fe^{2+} are increased in the neuropil of AD patients [36, 43]. In agreement with a role of metal ions in pathology, molecules designed to chelate Zn^{2+} and Cu^{2+} from amyloid-beta aggregates [44, 45] were

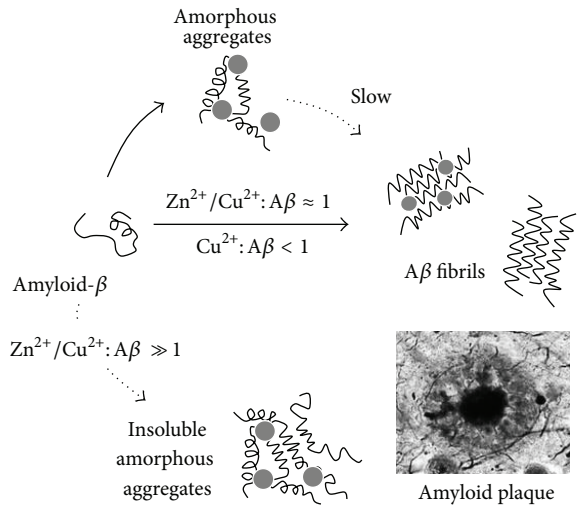

Figure 1: Modulation of amyloid-β aggregation by Cu^{2+} and Zn^{2+} binding. Aβ aggregation into fibrils is a complex pathway that involves multiple intermediate precursor species. The scheme is a simplification depicting direct effects of Cu^{2+} and Zn^{2+} on Aβ aggregation. Superstoichiometric levels of Cu^{2+} and Zn^{2+} (Zn^{2+}/Cu^{2+}: A$\beta \gg 1$) result in insoluble and amorphous aggregates rather than organized fibrils, while equimolar Cu^{2+} and Zn^{2+} (Zn^{2+}/Cu^{2+}: A$\beta \approx 1$) induce amorphous aggregates, which slowly convert to fibrils. At subequimolar Cu^{2+} levels (Cu^{2+}: A$\beta < 1$), the kinetics of fibril formation are accelerated. The AD amyloid plaques, depicted in a representation at the bottom right corner of the figure, contain high levels of Zn (1055 μM), Fe (940 μM), and Cu (390 μM), as reviewed in [35]. See text for details.

found to decrease Aβ deposits in mice models due to Aβ solubilisation [45]. Here, as a contribution for a broader molecular and biochemical analysis of protein-metal cross talks in neurodegeneration, we undertake an overview of proteins with metal binding properties which are implicated in AD (Table 1).

2.1. Amyloid-β. Metal ions have been acknowledged as important players of the pathological effects of Aβ aggregation in AD and have been considered as possible modulators of Aβ misfolding and aggregation due to their binding to the Aβ peptide [46–49] and to amyloid fibrils [50, 51]. Cu^{2+}, Zn^{2+}, and Fe^{2+} bind to Aβ influencing its aggregation pathway and are found in and nearby extracellular senile plaques [29, 36]. The binding of metal ions to Aβ invariably results in aggregation which may either be into amyloid fibers or into amorphous aggregates, depending on the metal ion, stoichiometry, and environmental conditions [49]. In spite of contradictory findings, there seems to be a consensus that (a) superstoichiometric levels of Cu^{2+} and Zn^{2+} result in insoluble and amorphous aggregates rather than organized fibrils [49, 52–55]; (b) equimolar Zn^{2+} and Cu^{2+} induce amorphous aggregates, which slowly convert to fibrils [56, 57]; and (c) at subequimolar Cu^{2+} levels, the kinetics of fibril formation are accelerated [52, 58, 59] (Figure 1). The observation that high levels of Zn^{2+} and Cu^{2+} seem to shift aggregation into oligomeric precursors rather than organized fibrils has important consequences in brain function, as these Aβ precursors are now known to be the neurotoxic self-propagating species causing neurodegeneration. Furthermore, Cu^{2+} and Fe^{2+} participate in ROS production causing oxidative stress and neuronal damage, thus being one of the causes that potentiate Aβ toxicity [60–62]. Indeed, the formation of H_2O_2 as a product of the interaction between Aβ and Cu^{2+} can generate hydroxyl radicals, which are related to AD pathology [63]. Superoxide has also been recently shown to be an intermediate of the reaction leading to the production of H_2O_2 by Cu^{+}-Aβ and O_2 [64]. Zinc and copper chelators inhibit Aβ plaque deposition in AD patients [44, 65, 66], further suggesting that amyloid pathology may arise from the dysregulation of these metal ions. Excess of iron increases Aβ production [67] and leads to the formation of annular protofibrils [68] and slows down the formation of ordered cross-β fibrils [69] towards the formation of shorter and less ordered aggregates [53, 69] which are potentially more toxic.

2.2. Tau. Tau is a disordered cytosolic protein involved in microtubule assembly and stability whose aggregation and toxic deposition are triggered by hyperphosphorylation. This results in the formation of intracellular tau paired helical filaments (PHF), which ultimately gather to form the characteristic neurofibrillary tangles (NFT) [70, 71], a process which is modulated by metal ions [30] (Figure 2). Zn^{2+} binds tau and promotes its hyperphosphorylation [72]; however, low concentrations of zinc induce fibril formation whereas high concentrations induce granular aggregates [73]. Fe^{3+} also binds to hyperphosphorylated tau and induces its aggregation [74, 75], mostly into PHF [75]; however, reduction to Fe^{2+} can reverse aggregation of tau [75]. Excess of iron is accumulated in NTF [76, 77] generating oxidative stress due to the Fenton reaction and perpetuating tau hyperphosphorylation [78]. The role of Cu^{2+} in tauopathies is controversial. Some studies suggest that tau binds Cu^{2+} [79], inhibiting its aggregation

Table 1: Effect of metal ions on selected metal-binding proteins implicated in AD.

Protein	Metal	Effect	Model	Reference
Aβ	Cu^{2+}	Modulates aggregation. Presence of Cu^{2+} in Aβ aggregates decreases toxicity; however, presence of Cu^{2+} in soluble Aβ accelerates cell death. Substoichiometric levels of Cu^{2+} render Aβ aggregates more toxic.	Synthetic Aβ, HEK cells, primary hippocampal cells, and PC12 cells	[49, 52–55, 57–59]
		Increases oxidative stress and neurotoxicity.	Synthetic Aβ, primary neuronal cells	[60, 63, 126, 127]
	Zn^{2+}	Modulates aggregation. Zn^{2+} leads to less toxic Aβ aggregates.	Synthetic Aβ, HEK cells, and primary cortical cells	[49, 53, 54, 57]
	Fe^{2+}	Modulates aggregation promoting the formation of annular protofibrils.	Synthetic Aβ	[49, 68, 69]
		Increases protein levels by disruption of APP processing.	Primary cortical neurons, APP/PS1 mice model, and HEK cells	[67]
		Increases oxidative stress.	M17 neuroblastoma cells, *Drosophila* model	[61, 62]
Tau	Cu^{2+}	Modulates phosphorylation.	Tg-AD mice model, SH-SY5Y cells, and AD mice model	[81, 82]
		Modulates aggregation.	Peptide from tau first microtubule-binding repeat	[80]
	Zn^{2+}	Induces phosphorylation through Zn^{2+} PP2A inhibition.	Rat brain slice cultures, primary neuronal cells	[72]
		Induces fibril formation via disulfide cross-linking.	Recombinant tau protein	[73]
	Fe^{2+}	Modulates aggregation.	Recombinant tau protein, isolated hyperphosphorylated tau from human AD brain tissue	[74, 75]
		Induces imbalance in Cdk5/p25 function that causes a decrease in tau phosphorylation and an increase in oxidative stress.	Primary hippocampal cells	[78, 128]
APP	Cu^{2+}	Increases APP expression levels and Aβ secretion. Promotes APP trafficking and its redistribution.	SH-SY5Y cells, polarized epithelial cells, MDCK-APP-cherry cells, primary cortical neurons, N2a cells, and APP/PS1 mouse model	[81, 83, 85, 86]
		Increases oxidative stress. Cu^{2+}-metalated APP ectodomain promotes neuronal cell death.	Recombinant APP protein and mutants, primary neuronal cells	[90, 91]
	Zn^{2+}	Inhibits ferroxidase activity.	Human brain tissue	[92]
		Increases APP expression levels and amyloidogenic cleavage that leads to accumulation of Aβ.	SH-SY5Y cells, APP/PS1 mice model	[84]
	Fe^{2+}	APP interacts with ferroportin and promotes iron export.	Human brain tissue, HEK293 cells	[92, 93]
Presenilin	Ca^{2+}	Overexpression of PS1 decreases Ca^{2+} release from ER and downregulates Ca^{2+}-dependent mitochondrial transport proteins. Expression of PPS1 M146V causes inhibition of Ca^{2+} channels.	HEK293 cells, human brain tissue, SH-SY5Y cells, SK-N-SH cells, and APPswe/PS1dE9 mice model	[95–97]
MT3	Cu^{2+}	Decreases protein levels.	Human brain tissue, Tg2576 mouse model	[100, 101]
		MT3 interacts with Aβ inhibiting/modulating Aβ aggregation and cytotoxicity.	Recombinant MT3 protein and synthetic Aβ, SH-SY5Y cells, primary cortical cells, and Tg2576 mouse model	[104–107]
	Zn^{2+}	Metal swapping between MT3 and Aβ lowers ROS production and decreases neurotoxicity.	Recombinant MT3 protein and synthetic Aβ, SH-SY5Y cells	[109]
		MT3 increases sAPPα levels and reduces Aβ production.	N2a Swedish APP cells	[102]
ZnTs	Zn^{2+}	Increases expression levels and colocalization with amyloid plaques.	APP/PS1 mouse model, human brain tissue	[110–112, 129]

Table 1: Continued.

Protein	Metal	Effect	Model	Reference
ProSAP/Shank scaffold proteins	Zn^{2+}	Zn^{2+} sequestering by Aβ decreases Shank1 and ProSAP27Shank3 protein levels and promotes synapse loss by disruption of Homer1b and Shank1 scaffold.	Primary hippocampal cells, human brain tissue, and Cos7 cells	[115–117]
	Ca^{2+}	Homers 2 and 3 interact with APP inhibiting APP processing and consequently reducing Aβ secretion.	HEK293 cells, C57/Black6 mouse model	[130, 131]
Ferritin	Fe^{2+}	Increases protein levels. Present within and around amyloid plaques and neurofibrillary tangles.	Human brain tissue	[119–121]
S100B	Ca^{2+}	Increased expression of S100B contributes to overexpressing βAPP in diffuse amyloid deposits.	Primary neuron cells	[132]
	Zn^{2+} Ca^{2+}	S100B interacts with tau resulting in the inhibition of tau phosphorylation via Ca^{2+}/calmodulin-dependent kinase II.	Bovine S100B, SH-SY5Y cells	[133, 134]
S100A9	Ca^{2+}	Increases protein levels. Present near amyloid plaques. Interacts with Aβ *In vitro* and forms linear and annular aggregates. Knockout of the S100A9 gene reduces neuropathology due to reduced Aβ and APP C-terminal levels.	Human brain tissues, Tg2576 mice model, SH-SY5Y cells, and S100A9 recombinant protein	[135–139]
S100A7	Ca^{2+}	Expression of exogenous S100A7 inhibits Aβ production and promotes α-secretase activity.	Primary corticohippocampal cells	[140]

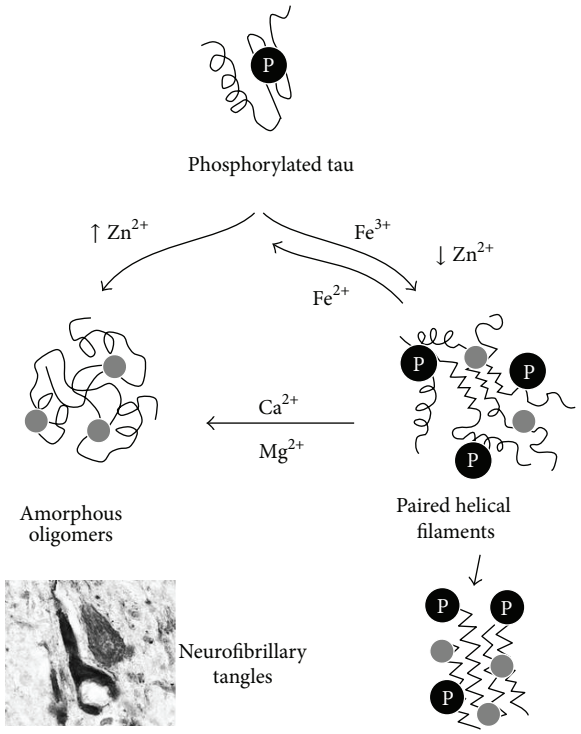

Figure 2: Modulation of tau aggregation by metal ions. Hyperphosphorylated (P) tau undergoes aggregation, which is influenced by metal ion binding. Tau phosphorylation facilitates Fe^{3+} binding that promotes the formation of paired helical filaments (PHF) and further tau fibrillation. The reduction of Fe^{3+} to Fe^{2+} reverts PHF formation. Zn^{2+} binding at high ratios promotes the formation of amorphous tau oligomers, whereas, at low ratios, PHF are formed. Both Ca^{2+} and Mg^{2+} binding to PHF favour the conversion into amorphous off-pathway aggregates. A neurofibrillary tangle is depicted in a representation at the bottom left corner of the figure. See text for details. Adapted from [30].

in vitro [80] while promoting tau hyperphosphorylation in hippocampal neurons [81]. Other studies however suggest that addition of copper-bis(thiosemicarbazone) complexes that increase intracellular copper in AD mice brains inhibits tau phosphorylation [82].

2.3. Amyloid-Beta Precursor Protein. Abnormal processing of the amyloid precursor protein leads to neurotoxic Aβ production. The proteolytic processing of APP is influenced by metal ions, by protein ligands, and by the APP oligomerization state. Cu^{2+} and Zn^{2+} promote APP expression [83–85] and possibly interfere with Aβ metabolism. Cu^{2+} enhances APP dimerization and increases extracellular release of Aβ [86]; yet, other studies suggest that high copper concentrations modulate APP processing leading to reduced Aβ production [87]. Interestingly, APP contains a copper binding domain and a site that favours Cu^{+} coordination, which has led to the suggestion that it could act as a neuronal metallotransporter [87]. Recent structural and biochemical studies have uncovered a high-affinity binding site within the E2 domain that binds competitively Cu^{2+} and Zn^{2+} at physiological concentrations [88]. Metal binding results in large conformational changes and in different structural states that regulate the function of APP and Aβ metabolism [89]. Indeed, APP can be a mediator of Cu neurotoxicity since it was shown that in primary neuronal cultures APP loaded with Cu^{2+} induces cell death [90]. This may possibly involve catalytic reduction of Cu^{2+} to Cu^{+} leading to an increase in oxidative stress in neurons [91]. The links between APP and metal metabolism are further emphasized by the interaction of APP with ferroportin, to promote iron export and its ferroxidase activity [92, 93]. APP ferroxidase activity is inhibited by Zn^{2+} binding contributing to Fe^{2+} accumulation in AD brains [92].

2.4. Presenilin-1. Presenilin-1 (PS-1) is a component of the γ-secretase multicomplex, responsible for the cleavage of APP. Presenilins have an activity as low-conductance passive ER Ca^{2+} leak channels which is independent of γ-secretase activity [94]. Overexpression of presenilin results in increased Ca^{2+} release whose levels are restored by γ-secretase inhibitors [95]. Mutations in presenilins as in familial AD forms result in downregulation of Ca^{2+} channels and Ca^{2+}-dependent mitochondrial transport proteins, strengthening the relationship between Ca^{2+} homeostasis and presenilin [94, 96, 97]. A recent study based on the effects of metal chelators on γ-secretase suggests that Ca^{2+} and Mg^{2+} stabilize γ-secretase and enhance its activity [98].

2.5. Metallothionein 3. Metallothioneins are a family of ubiquitous proteins with metal binding properties and antioxidant

activity [99]. Neuronal metallothionein 3 (MT3), which is involved in the transport and homeostasis of Zn^{2+} and Cu^{2+}, plays an important role in several AD related pathways. MT3 is decreased in AD patients [100] and in Tg2576 mice [101], which can lead to aberrant neuritic sprouting [100]. Additionally, MT3 increases sAPPα (soluble amyloid precursor protein α) levels and reduces Aβ production [102], through an increase in ADAM10 (a disintegrin and metallopeptidase 10). ADAM10 is a protein responsible for the cleavage of APP-derived peptides and activation of the nonamyloidogenic pathway [103]. Mechanistically, it has been reported that the β-domain of MT3 interacts with Aβ, abolishing Cu^{2+} mediated aggregation [104, 105] and ROS production [104]. It has also been suggested that rapid metal exchange between Zn^{2+}-MT3 and Cu^{2+}-Aβ [106] or Zn^{2+} release by MT3 [107] promotes structural changes in Aβ aggregates. In agreement with this, in primary neuron cultures, MT3 inhibits the formation of toxic Aβ aggregates alleviating their neurotoxic effects [105, 108]. One possible mechanism for this effect may be related to the observed metal swapping between MT3 and soluble and aggregated Aβ, which abolishes the production of Cu-induced ROS [104, 109].

2.6. Zinc Transporter 3. Zinc transporter 3 (ZnT3) is a synaptic Zn^{2+} transporter responsible for loading zinc into presynaptic vesicles. This protein is highly expressed in the brains of AD transgenic mice, in which it colocalizes with amyloid plaques [110–112], where zinc is also found at high concentrations. Zinc sequestering within amyloid plaques has been suggested to provoke an imbalance in the cellular environment with concurrent effects on overall metal metabolism and protein homeostasis [35]. ZnT3 has been shown to decrease with aging and AD, contributing to the aggravation of zinc-mediated cognitive decline [113]. In the AD Tg2576 transgenic mouse model with a ZnT3 knockout, cerebral Aβ deposition was nearly abolished by the lack of synaptic Zn^{2+} [58, 59]. ZnT3 and other zinc transporters, such as ZnTs 1, 4, 5, 6, and 7, are also found upregulated in amyloid plaques of human AD brains near Zn^{2+} enriched terminals [60], revealing a cross talk between zinc induced amyloid plaques and zinc transporters. In ZnT3 knockout mice, the addition of metal chaperones results in restoration of expression of the synaptic proteins PSD-95, AMPAR, and NMDAR2b, due to the restitution of hippocampal zinc content [113].

2.7. ProSAP/Shank Scaffold Proteins. ProSAPs/Shanks are zinc-regulated multidomain proteins that are important scaffolding molecules of the postsynaptic density (PSD), a protein dense structure composed of both membranous and cytoplasmic proteins localized at the postsynaptic plasma membrane of excitatory synapses [114]. Deregulation of ProSAP/Shank has been reported in AD: in patients brains and in transgenic mice models, the accumulation of Aβ oligomers is accompanied by reduction of synaptic scaffold protein levels, such as Shank1 and ProSAP2/Shank3 [115], and disruption of the Homer1b and Shank1 scaffolds [116]. Interestingly, sequestration of Zn^{2+} by Aβ leads to less mature synapses by decreasing Shank1 protein levels at the postsynaptic density in hippocampal neurons [117]. Future studies will further elucidate the mechanistic cross-links between the presence of Aβ, zinc levels, and the scaffolding PSD proteins in the context of AD [118].

2.8. Ferritin. Ferritin is the major intracellular iron storage protein in the body. It has elevated levels in AD brain tissue [119–121] and is found in the vicinity of AD plaques [120], suggesting that ferritin trapped within the plaque inclusions may block the transport of iron between cells. The loss of integrity of hippocampus tissue of AD patients is linked with the increase of ferritin [122] and with a reduction of ferroportin protein levels [123]. Effectively, the impact of iron on AD outcomes is not fully explored but a recent longitudinal study has shown that ferritin is strongly associated with cerebrospinal fluid apolipoprotein E levels; in turn, ferritin is elevated by the Alzheimer's risk allele, APOE-ε4 [124]. This study speculates that the APOE-ε4 genotype raises the baseline iron load in the AD brain, lowering the threshold for iron-mediated neuronal loss, a hypothesis that remains to be experimentally addressed.

2.9. S100 Proteins. S100 proteins are a family of at least 21 different vertebrate-specific proteins with two Ca^{2+}-binding EF-hand type sites and in some cases additional sites for Zn^{2+} and Cu^{2+} [125]. S100 proteins are part of the inflammatory response and a number of these proinflammatory cytokines (S100B, S100A6, S100A7, S100A1, S100A9, and S100A12) have been implicated in neurodegenerative disorders, such as AD.

S100B is a proinflammatory cytokine that triggers glial cell proliferation in a RAGE-dependent manner [141]. RAGE is an immunoglobulin-like cell surface receptor that is upregulated in AD and triggers the expression of proinflammatory cytokines and mediates Aβ transport across the blood-brain barrier [142–144]. At high micromolar concentrations, S100B promotes neuroinflammatory processes and neuronal apoptosis [145]. Increased expression of S100B by plaque-associated astrocytes in AD contributes to the appearance of dystrophic neurites overexpressing βAPP in diffuse amyloid deposits [132]. Astrocytic overexpression of S100B is correlated with the degree of neurite pathology in Aβ aggregates and is induced by interleukin-1 (IL-1), which is secreted by activated microglia present in the plaques [146]. TNFα, a cytokine with high levels in AD, decreases S100B expression in astrocytes but increases its extracellular levels which can lead to RAGE activation [147]. Furthermore, studies demonstrated increased susceptibility to neuroinflammation and neuronal dysfunction after infusion of Aβ in transgenic mice overexpressing S100B [148]. Interestingly, S100B interacts with tau in a Zn^{2+} dependent fashion that could be responsible for neurite outgrowth [133]. Other studies, however, suggest that the S100B:tau interaction is mediated by Ca^{2+}/calmodulin-dependent kinase II and results in the inhibition of tau phosphorylation [134].

S100A6, S100A9, and S100A12 also have consistently high levels in samples of AD patients [135, 149]. In particular, S100A9 is found near neuritic plaques [136, 137] and was

found to coaggregate with Aβ *in vitro* and form toxic aggregates [136, 138]. Knockout of S100A9 in a transgenic mouse resulted in reduced Aβ levels in the brain and the animals presented an improved spatial reference memory [139]. In agreement with these observations, knockdown of S100A9 in the AD Tg2576 mice model reduced Aβ and APP C-terminal levels and decreased BACE activity [137]. Induction of S100A9 levels increased intracellular Ca^{2+} levels, which in turn upregulated secretion of the inflammatory cytokines IL-1β and TNFα [150]. On the opposite, expression of exogenous S100A7 in primary corticohippocampal neuron cultures derived from Tg2576 transgenic embryos inhibits the generation of Aβ and promotes the activity of α-secretase [140]. Interestingly, S100 proteins have been found to have amyloidogenic properties [151–155]. This feature, along with the high abundance of S100 proteins in protein deposits, their metal binding properties, dysregulation of Ca^{2+} signalling, and the high levels of Cu^{2+} and Zn^{2+} in the plaques, will certainly translate into the elucidation of new functions of S100 proteins in AD pathomechanisms.

3. Conclusion

Metal homeostasis and balance depend on a number of biochemical processes and proteins, many of which operate in the neuronal environment and in the extracellular synaptic space or at its interface. The biochemistry of this particular cellular moiety is deeply altered upon aging and under neurodegeneration, with wide changes in protein levels, signalling molecules, and metal ion concentrations. Changes in protein and metal ion homeostasis are hallmark features across amyloid-forming neurodegenerative diseases and as we have here overviewed, a number of proteins implicated in AD are directly regulated by metal-protein interactions; in some cases, metal ions are even directly involved as modulators of aggregation pathways. Uncovering the mechanistic details of this cross talk at the biochemical levels in respect to effects on synaptic protein networks, Aβ metabolism and intra- and extracellular protein aggregation in the context of concurrent affected processes such as oxidative stress and neuroinflammation are thus among the major challenges in modern molecular neurosciences.

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

Oxidative Stress in Intracerebral Hemorrhage: Sources, Mechanisms, and Therapeutic Targets

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Intracerebral hemorrhage (ICH) is associated with the highest mortality and morbidity despite only constituting approximately 10–15% of all strokes. Complex underlying mechanisms consisting of cytotoxic, excitotoxic, and inflammatory effects of intraparenchymal blood are responsible for its highly damaging effects. Oxidative stress (OS) also plays an important role in brain injury after ICH but attracts less attention than other factors. Increasing evidence has demonstrated that the metabolite axis of hemoglobin-heme-iron is the key contributor to oxidative brain damage after ICH, although other factors, such as neuroinflammation and prooxidases, are involved. This review will discuss the sources, possible molecular mechanisms, and potential therapeutic targets of OS in ICH.

1. Introduction

Intracerebral hemorrhage (ICH) remains a significant cause of morbidity and mortality throughout the world, although studies of ICH intervention have increased dramatically in the past decades [1]. Currently, there is no effective surgical or medical treatment available to improve the functional outcomes in patients with ICH because of its multiple injury mechanisms [1, 2]. Numerous preclinical studies show that secondary brain injury after ICH is caused by the interaction of cytotoxicity, excitotoxicity, oxidative stress (OS), and inflammation from the products of red blood cell lysis and plasma components [3, 4]. However, the precise pathophysiological mechanisms underlying ICH remain to be completely elucidated.

OS is a condition in which the overproduction of free radicals, mainly reactive oxygen species (ROS), exceeds the antioxidant capacity and subsequently leads to cell injury via directly oxidizing cellular protein, lipid, and DNA or participating in cell death signaling pathways [5]. OS has been implicated in neurodegenerative diseases of the central nervous system and stroke [6, 7]. There are three major types of ROS: the superoxide radical ($O_2^{\bullet-}$), the hydroxyl radical ($^{\bullet}OH$), and hydrogen peroxide (H_2O_2) [8]. Reactive nitrogen species (RNS) are another major type of free radicals, which mainly consist of nitric oxide (NO) and its derivatives. NO is produced in neurons, endothelial cells, and activated astrocytes by nitric oxide synthase (NOS). Under physiological conditions, NO mediates neurotransmission and regulates neuronal survival, proliferation, and differentiation. Under pathological conditions, however, excessive NO can lead to OS via various mechanisms [9]. Moreover, NO reacts with $O_2^{\bullet-}$ to form the more toxic compound peroxynitrite ($ONOO^-$), which can cause oxidation and nitration of tyrosine residues in proteins [10].

Experimental studies have confirmed that OS plays a pivotal role in cerebral injury following ICH. The oxidative products of macromolecules significantly increased, whereas antioxidant enzymes, such as superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase, correspondingly decreased as a result of ICH [11, 12]. Free radical scavengers proved to be effective in neuroprotection in animal ICH models [13, 14]. Moreover, oxidative markers, leukocyte 8-hydroxy-2′-deoxyguanosine and lipid hydroperoxides, are detected in association with long-term and short-term clinical outcomes, respectively, after spontaneous ICH [15, 16]. However, no antioxidant has been applied in patients with ICH because of the unclear mechanism of OS-related injury.

The current review attempted to illustrate the knowledge regarding ICH-related OS and its possible molecular mechanism and to discuss the potential targets of intervention for future research.

2. Primary and Secondary Brain Injury after ICH

Primary brain injury is caused by immediate physical disruption to the neurovascular architecture surrounding the hemorrhagic site due to sheering force and the mass effect of an ICH. Surgical clot evacuation targeting the primary injury has failed due to the extra adverse effects of the surgical procedure [17, 18]. Many clinical trials on minimally invasive surgery for ICH evacuation have thus been performed with potentially improved functional outcomes [19, 20]. However, there are several drawbacks, such as the long time required for an adequate blood evacuation, a relatively high risk of infection and rehemorrhage, and intensive labor and resource consumption [21]. After the onset of bleeding, hematoma enlargement further exacerbates brain damage in 20–40% patients within 24 hours [22]. Hypertension may be a modified factor affecting hematoma growth [23].

When initial bleeding stops and a stable hematoma is formed, a cascade of events occurs to induce secondary brain injury. Thrombin is instantly produced after ICH to stop bleeding, but it also contributes to early neural and endothelial injury [24]. Inflammatory cells infiltrate and damage perihematoma viable brain tissue by excreting a variety of cytokines and chemokines [25]. Another contributor to brain injury after ICH is hemoglobin and its metabolite released via erythrocyte lysis in hematoma [26]. As the major component of hemoglobin, heme can be degraded into iron, carbon monoxide, and biliverdin by heme oxygenase (HO). Iron overload in the brain after hemorrhage subsequently generates abundant ROS, resulting in neurotoxicity [27]. These overlapped mechanisms interact and result in blood brain-barrier (BBB) disruption, neuronal loss, and gliosis with permanent neurological deficits.

3. OS in ICH

ROS are byproducts of cellular metabolism and are mainly generated by mitochondria in living cells [28]. They are highly active with a short half-life, making them very difficult to analyze directly in the laboratory [29]. OS is usually assessed by indirectly measuring the oxidized products of macromolecules. 8-Hydroxy-2-deoxyguanosine (8-OHdG) is a widely used biomarker of in vivo oxidative DNA damage. Both malondialdehyde (MDA) and 4-hydroxy-trans-2-nonenal (HNE) are lipid peroxides produced by free radical attack. Dinitrophenyl (DNP) and protein carbonyl can be measured to quantify protein oxidative damage. The detection of oxidized hydroethidine (HEt) is specifically used to assess $O_2^{\bullet-}$ production in vivo because HEt can pass through the BBB and be selectively taken up by neuron and glia cells and oxidized by $O_2^{\bullet-}$ to ethidine (Et), which provides a red fluorescence signal [30].

In a rodent ICH model, 8-OHdG and DNP increased along the same time course, with peak production at 3 days after ICH, suggesting the presence of OS in ICH [12]. Additionally, the level of MDA increases and is correlated with apoptosis following ICH, indicating that OS contributes to ICH-induced brain injury [31]. Moreover, brain white matter is also damaged as a result of protein oxidation in a porcine ICH model [32].

Recently, OS was reported to exert a prognostic effect in ICH patients. A prospective study analyzing blood samples from 64 ICH patients revealed that elevated level of leukocyte 8-OHdG was associated with lower 30-day Barthel Index independent of traditional prognostic factors [15]. Another prognostic study reported that the serum lipid hydroperoxide (ROOH) concentration was a predictor of poor clinical outcome in ICH survivors and was positively correlated with short-term mortality [16]. In contrast, Mantle et al. observed similar levels of protein carbonyl and antioxidants in ICH and control cases, suggesting that there may be no increased oxidative damage in ICH [33]. This unexpected result is questionable because the tissue (peritumor or aneurysm tissue) used as a control for the oxidative measurement may be pathologically compromised with potentially elevated levels of OS and therefore is not a qualified control [34].

4. Free Radical Sources after ICH

4.1. Mitochondria Dysfunction. Physiologically, 1–3% of all electrons in the electron transport chain in mitochondria leak, generating superoxide radicals, that can be neutralized by normal antioxidant systems [28]. During ICH, mitochondria dysfunction occurs, and substantial ROS production follows [35, 36]. Kim-Han et al. detected an obvious reduction in the oxygen consumption rates of mitochondria in ICH patients, indicating that mitochondria dysfunction, and not ischemia, is responsible for the decreased oxygen metabolites after ICH [35]. Direct evidence of ROS from malfunctioning mitochondria was reported in a recent study, which found that a mitochondrial ROS-specific scavenger can significantly alleviate the increased ROS following ICH [37]. The mechanism of excessive ROS formation by mitochondria after ICH remains unclear but may be partially attributable to mitochondrial permeability transition pore (MPTP) because the inhibition of MPTP can attenuate ROS production [37].

4.2. Hb-Heme-Iron. As the most abundant erythrocyte protein, hemoglobin (Hb) is released into the extracellular space via complement-mediated cell lysis in the hours after ICH and is a potent mediator of OS-induced injury [38, 39]. Both in vitro and in vivo investigations have shown that ROS is highly produced after exposing Hb to cell culture or injecting Hb into mouse striatum [39–41]. Katsu et al. studied the temporal change of ROS in a Hb-injection rat model and observed remarkable ROS production as early as 1 h, which increased at 24 h [42]. Recently, NO, a form of RNS, has also been found to be overproduced because of NOS activation and leads to BBB disruption after infusing Hb into rat brain [43]. Regarding the prooxidant mechanism of Hb, it is commonly believed that iron released from its

degradation is responsible for oxidative damage because an iron chelator may block Hb-induced neurotoxicity [44]. In fact, Hb itself can release a large amount of superoxide during spontaneous, nonenzymatic oxidation to oxyhemoglobin and methemoglobin [45, 46].

Heme, released from methemoglobin, quickly oxidizes to form hemin, which also triggers oxidative damage in brain tissue around the hematoma. An in vitro experiment demonstrated that hemin exposure leads to cell death, preceded by a significant, iron-dependent increase in ROS [47]. Nevertheless, another in vitro study showed that hemin could stimulate lipid peroxidation, irrespective of iron mediation, because the reaction could not be inhibited by deferoxamine or transferrin [48]. Hence, the mechanism of hemin-related oxidative damage partly involves its breakdown to iron by HO, similar to that of Hb [49]. Indeed, hemin is redox-active and can react with peroxides to produce cytotoxic free radicals [48, 50]. Moreover, hemin can intercalate into the cell plasma membrane, facilitating lipid peroxidation [51]. Given the effect of hemin in preclinical studies, biphasic functions are observed. Hemin-induced brain injury is evidenced by increased brain water content at 24 hours after intracerebral hemin infusion [46]. In contrast, systemic hemin treatment is neuroprotective after ICH [52]. Although the mechanisms underlying the protection provided by systemic hemin administration are poorly understood, it is clear that most hemin is in circulation rather than in the brain.

Iron overload is involved in secondary brain injury, leading to neuronal death, brain edema, and neurodeficits after ICH [53, 54]. Intracerebral iron overload begins within 24 h, peaks at 7 days, and continues for at least a month after hemorrhage [55]. Excessive iron in the extracellular space induces oxidative damage via the Fenton reaction, which yields ROS, especially toxic hydroxyl radicals [56]. Direct evidence of iron-mediated oxidative injury has shown that injecting $FeCl_2$ into rat brain causes oxidative DNA damage [11, 12]. The strongest finding supporting the hypothesis of iron-mediated oxidative brain injury is that iron chelators decrease iron accumulation, attenuate ROS generation, exert anti-inflammatory effects, and improve neurological function [57, 58].

4.3. Inflammatory Cells. Neuroinflammation is recognized as a vital factor in the pathophysiology of ICH-induced brain injury and is characterized by microglia activation, leukocyte infiltration, and cytokine and chemokine production [3, 4, 25]. In addition to the release of inflammatory factors, the activation of inflammatory cells following ICH, initially to remove oxidative toxins, also participates in ROS production.

As one type of innate immune cell within the brain, microglia are rapidly activated within 1 h after ICH, peaking at 3–7 days and persisting for several weeks [59]. Hb is a powerful activator of microglia via toll-like receptors [60]. The imbalance of the phenotypic shift between the M1 and M2 phenotypes of microglia contributes to a large release of ROS in addition to proinflammatory factors [61]. Cell experiments have shown that microglia can induce ROS production in vitro [58, 59]. Furthermore, the inhibition of microglia was reported to decrease the ROS production and brain damage volume in an ICH animal model [62].

Neutrophils are the earliest leucocytes to enter the brain after ICH. The role of neutrophils in radical production during ischemic brain stroke has been confirmed by reduced radical formation after neutrophil depletion [63]. OS-related brain injury is part of the pathogenesis mechanism of neutrophil infiltration after ICH [64]. The inflammation linked to OS following ICH indicates that neuroinflammation and OS are intercalated in ICH-induced secondary brain injury.

5. Prooxidase in ICH

The process of OS is related to the activation of many prooxidases in many diseases. The prooxidases that are reported in ischemia stroke include NADPH oxidase (NOX), cyclooxygenase (COX), xanthine oxidase, and nitric oxide synthase [NOS] [65]. In ICH, NOX and NOS are most commonly studied [10, 66–68].

5.1. NADPH Oxidase. NOX is a major source of ROS and is mainly composed of five subunits: a large $gp91^{phox}$ and a smaller $gp22^{phox}$ subunit in the plasma membrane and $p47^{phox}$, $p67^{phox}$, and $p40^{phox}$ subunits in cytoplasm [65]. Once cytosolic $p47^{phox}$ is phosphorylated upon stimulation, it binds to the components of the plasma membrane and activates NOX, which can transfer electrons from NADPH to oxygen, forming superoxide [66]. Seven NOX isoforms, NOX1 to NOX5 and Dual Oxidases 1 and 2, have been identified among which NOX2 ($gp91^{phox}$) is abundant in the brain [67–69]. Tang et al. found that the OS resulting from activation of NOX2 contributes to the severity of ICH and promotes brain injury by comparing wild-type and $gp91^{phox}$ knockout mice [67]. The $gp91^{phox}$ knockout hemorrhagic mice showed lower levels of oxidative product, ICH volume, brain water content, neurological deficit, and mortality rate [67].

Another study by Zia and colleagues showed that the induction of NOX2 could cause OS and worsen brain injury, whereas the inhibition of NOX2 by apocynin suppresses ROS production and confers neuroprotection in rabbit pups with germinal matrix hemorrhage-intraventricular hemorrhage (GMH-IVH) [68]. Moreover, OS resulting from NOX2 activation not only deteriorated ICH-related injury but was associated with the occurrence of ICH in hypertensive mice [70]. However, the same NOX2 inhibitor that exerts a protective property in the GMH-ICH model by preventing $p47^{phox}$ subunit translocation exhibits no effects on enhanced NOX2 activity, lipid peroxidation, brain edema, or neurological dysfunction in a rat ICH model [71]. It is possible that different species (rabbit versus rat), hemorrhagic locations (GMH-IVH versus basal ganglia hemorrhage), and bleeding (autologous artery blood versus collagenase) are responsible for these opposing conclusions.

5.2. Nitric Oxide Synthase. There are three isoforms of NOS accounting for NO production: neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS). The first two are constitutively expressed, and their activities are

calcium dependent, whereas the last one is synthesized by the induction of proinflammatory cytokines, independent of calcium regulation [72].

The activation of NOS after ICH has been demonstrated in many studies. Using the autologous blood model, Zhao et al. reported the temporal profile of iNOS and nuclear factor-κB (NF-κB) and found that the maximal detection of iNOS paralleled the peak concentration of NF-κB at 3 days after ICH, suggesting that iNOS may be mediated by NF-κB because the downstream gene products of NF-κB include iNOS [73]. Other investigators detected NOS overexpression and suggested the role of the NOS/NO/$ONOO^-$ pathway in BBB disruption using the Hb-injection rat model [10, 43]. In contrast, administering nNOS inhibitor after ICH was found to protect BBB integrity and decrease both neuronal death and neurological deficits [74]. Moreover, iNOS knockout mice present significantly less brain edema after collagenase-induced ICH [75]. Therefore, NOS might be a therapeutic target.

The molecular mechanisms for NOS activation after ICH are primarily NF-κB dependent [9, 73]. Thrombin and proinflammatory cytokines, such as TNF-α and IL-1, can induce iNOS expression in microglia via the PKC/p38MAFP/NF-κB pathway [76]. Hemin also activates the NF-κB transcription factor via an undefined mechanism [77, 78]. In addition, high levels of glutamate activate NOS through the NMDA receptor with subsequent Ca^{2+} influx by phosphorylating IKB and NF-κB translocation [74, 79].

6. Antioxidative System in ICH

6.1. Heme Oxygenase. Extracellular heme binds to hemopexin to enter neuronal cells through the hemopexin receptor or heme carrier protein 1 [80]. Intracellular heme is then degraded into iron, carbon monoxide, and biliverdin. HO is the rate-limiting enzyme for this catabolic process with two active isoenzymes: the inducible HO-1 and the constitutively active HO-2. HO-1 is barely detected in the brain under normal conditions but is induced in microglia/macrophages after ICH, whereas HO-2 is normally expressed in neurons, accounting for the vast majority of HO activity in the brain [81].

The antioxidant effects of these enzymes on ICH-induced secondary brain injury are debatable and have been thoroughly reviewed by Chen-Roetling et al. [82]. Their roles are variable, depending on the different ICH models used and various cellular types affected [82]. Compared to wild-type mice, HO-2 knockout was found to attenuate brain injury, remarkably reducing cell loss, striatal protein, and lipid oxidation in a blood-injection model, but worsened the outcome by increasing perihematomal lesion volume, neuroinflammation, and edema in a collagenase-injection model [83, 84]. Conversely, HO-1 knockout exerted a beneficial effect on outcome in a collagenase-induced ICH model [85]. These disparate conclusions are partly explained by the diverse injury mechanisms between the blood- and collagenase-injection ICH models and the different distributions and expression timing of HO-1 and HO-2 [82].

6.2. Superoxide Dismutase. SOD is a key antioxidant enzyme that can detoxify $O_2^{\bullet -}$ to H_2O_2, which is further converted to H_2O by catalase or GPx. According to the specific cellular distribution and metal cofactors, SOD can be categorized into copper/zinc SOD (SOD1) in the cytosol and manganese SOD (SOD2) in the mitochondria and extracellular SOD (SOD3) [86].

Experimental animal studies have shown that free radical scavenging systems are destroyed after ICH. More specifically, evidence suggests that the levels of SOD1 and SOD2 decrease as the ROS level increases 1 day after lysed erythrocyte infusion in rats [87]. Chen et al. confirmed the damaged antioxidant system with elevated lipid oxidation and decreased SOD activity 1 day after ICH in the ventricle [88]. Clinically, decreased plasma SOD and reduced total superoxide scavenger activities have been observed in ICH patients within 1 day after onset [89]. However, SOD1 was found to increase from 1 day after ICH induced by whole blood infusion and peak at 7 days in one study [90]. These contrasting results require further investigation.

Given the protective effect of SOD1, exogenous or endogenous enhancement of SOD1 has been attempted to alleviate the oxidative damage in ICH. SOD1 overexpression in transgenic rats was linked to reduced OS, BBB disruption, and neuronal apoptosis in a Hb-injection model [42]. A recent study on cell replacement therapy in ICH found that neural stem cells (NSCs) overexpressing SOD1 3 days after ICH could increase neuronal survival, indicating that SOD1 enhancement alone or combined with other treatments may be effective in ICH [91]. Moreover, SOD1 hyperexpression is also protective against the spontaneous occurrence of ICH in hypertensive mice by decreasing superoxide. Fewer occurrences, smaller size, and a lower number of ICH are observed in SOD1 transgenic mice than those in SOD1-deficient mice [92]. However, chemically synthesized SOD with extended half-life and improved BBB permeability was reported to have no effect in a collagenase-induced ICH model when intravenously administered [93]. This failure may be partially ascribed to the insufficient dosage used [93].

6.3. Nuclear Factor Erythroid-2 Related Factor 2 (Nrf2). Nrf2 is a basic region-leucine zipper protein that controls the genomic regulator of the cellular antioxidant defense system, including the HO and SOD mentioned above [94]. ROS can activate the Keap1/Nrf2/ARE pathway to counteract oxidative damage after ICH as an adaptive response [90, 95, 96]. Keap1 is an OS sensor and negatively regulates Nrf2. Once exposed to ROS, Nrf2 dissociates from Keap1, translocates to the nucleus, and activates antioxidant response element (ARE) dependent cytoprotective genes that mediate cell survival [97]. Nrf2 increases significantly from 22 h and peaks at 8 h, whereas Keap1 shows a corresponding decrease in the perihematoma region in ICH rats [90]. These opposing expression changes suggest that Nrf2 is activated by Keap1 suppression after ICH. Moreover, the neuroprotection of Nrf2 indicates that Nrf2 knockout mice suffer more brain damage associated with the increased production of ROS and apoptosis [95, 96] and that Nrf2 activation could reduce

peroxide formation by augmenting the antioxidative capacity and hematoma clearance after ICH [98]. Hence, Nrf2 activation by pharmaceutical drugs is a promising target to attenuate OS-induced brain injury following ICH.

Recently, dimethyl fumarate (DMF), a fumaric acid ester that has been approved by the FDA as a treatment for patients with relapsing-remitting multiple sclerosis (MS) [99], demonstrated a beneficial effect by activating Nrf2 in rodent ICH models [100, 101]. In the study by Zhao et al., rats and mice, including Nrf2 knockouts, were initially subjected to intracerebral injection of blood and were then treated with DMF at a clinically relevant dose [100]. The results showed that treatment with DMF activated Nrf2, induced antioxidative enzymes, reduced brain edema, and ultimately enhanced neurological function. Additionally, enhanced hematoma resolution was observed in in vitro experiments by evaluating the phagocytic functions of primary microglia in culture. Iniaghe and colleagues found that upstream casein kinase 2 promoted Nrf2 translocation to exert a neuroprotective effect after DMF treatment [101]. These findings are important. Because DMF is currently approved for clinical use for MS, clinical translation will be relatively easy once the efficacy of DMF on ICH is confirmed in a clinical trial.

7. OS and Death Signaling Pathways in ICH

Numerous brain stroke studies have revealed that ROS/RNS not only directly oxidize cellular macromolecules, such as lipids, proteins, and nucleic acids, associated with oxidative damage, but also are involved in the death signaling pathways. The molecular mechanisms of ROS-mediated cell death in brain ischemia have been thoroughly studied and reviewed elsewhere [29, 65]. Briefly, there are three major OS-mediated pathway activations, including the PI3K/Akt, MAPK/P38, and NK-κB pathways [29]. Cytochrome c-mediated apoptosis is another critical pathway that is mitochondria dependent [102]. These OS-induced death signaling pathways have also been discussed in subarachnoid hemorrhage [103].

Free radicals can induce apoptosis, and antioxidant therapy can reduce neuronal apoptosis after ICH [104, 105]. Few studies have focused on the precise mechanism of ROS/RNS-induced apoptosis or necrosis in the setting of ICH. In vitro Hb oxidative neurotoxicity was attenuated by inhibitors of protein kinase C (PKC) and protein kinase CK2, suggesting that the PKC/CK2 pathway might participate in Hb-induced apoptosis, independent of HO activity [106]. However, the ERK pathway is involved in heme-mediated neuronal death by affecting HO-1 activity [107, 108]. The NF-κB pathway has also been detected in mediating Hb-induced apoptosis [108]. Moreover, the JNK pathway was reported to be activated following iron infusion, and the inhibition of JNK activation reduces apoptotic neuronal cell death and improves functional outcome [109, 110]. Other studies have shown that caspase cascades are activated by OS after hemoglobin explosion in primary neuronal cultures [36] and that ROS-induced apoptosis is related to cytochrome c release in the ICH model [95].

Although the ROS-mediated apoptotic signal pathway after ICH remains unclear, recent findings have shown that MMP-9 is an important mediator linking ROS/RNS with cell death following ICH [42, 95, 111, 112]. MMP-9 has been reported to elevate early, with a peak at 2-3 days, and is associated with apoptosis in the acute phase of ICH [113, 114]. The MMP inhibitor, GM6001, ameliorated neuronal death when administered within 72 h in a mouse ICH model [113]. Both in vitro and in vivo experiments have shown that Hb-induced ROS contributes to MMP-9 activation [42, 111]. NO derived from iNOS has also been reported to directly activate MMP-9 [112]. Moreover, a recent study by Ding et al. demonstrated that superoxide, NO, and their potent toxic metabolite peroxynitrite ($ONOO^-$) participate in the activation of MMP-9 via the following two mechanisms [115]. First, $ONOO^-$ directly modifies pro-MMP through S-nitrosylation and then activates MMP-9. Second, NF-κB is indirectly upregulating and mediates the transcription of MMP-9 [116]. The strong evidence supporting MMP-9-mediated OS-induced cell death is based on the fact that scavenging or decomposing ROS/RNS significantly decreases MMP-9 activity and subsequent neuronal death. SOD1 overexpression or free radical scavenger U83836E successfully reduced OS, MMP-9 levels, and subsequent apoptosis after intrastriatal Hb injection [42, 111]. iNOS inhibition by osteopontin to prevent NO production also suppressed MMP-9 activation and rescued neuronal cells in the perihematoma region in a mouse collagenase-induced mouse ICH model [112]. Additionally, FeTPPS, a type of $ONOO^-$ decomposition catalyst, decreased the levels of $ONOO^-$ and MMP-9 activity, followed by reduced apoptosis, in a Hb-injection rat model [115]. Therefore, ROS/RNS and MMP-9 may constitute a crucial cell death pathway in ICH (Figure 1).

8. Therapeutic Targets and Clinical Trial

Given the abovementioned multiple sources of ROS generation and injured oxidant scavenger systems during OS-induced damage in ICH, several potentially therapeutic targets are discussed.

8.1. Blocking the Sources of ROS Production. Because intraparenchymal blood is the origin of many prooxidant toxins, including Hb, heme, and iron, it is reasonable to suppose that blood evacuation may reduce oxidative damage if the surgery results in no or minimal additional new injury. Hence, minimally invasive surgery (MIS) for clot evacuation may represent a therapeutic strategy for the prevention of secondary oxidative damage. Animal studies have demonstrated that MIS alone or combined with other therapy can improve neurofunction with decreased oxidative injury and reduced apoptosis [117, 118]. More recently, clinical trials with small sample sizes investigating newly applied mechanical devices have reported promising outcomes [119, 120]. A multicenter, randomized, controlled study by our center comparing MIS with routine craniotomy is ongoing and involves 2448 ICH patients [121]. The clinical results will provide valuable information regarding the effect of MIS on the prognosis of patients with ICH.

Strategies targeting chelating individual prooxidants have been investigated. Haptoglobin is a blood protein primarily synthesized by hepatocytes that is also produced locally by

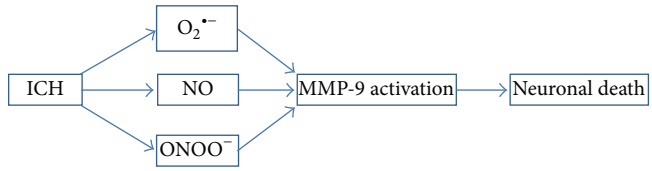

Figure 1: The OS-induced death pathway mediated by MMP-9. Hb released into extracellular space via complement-mediated cell lysis after ICH is a potent oxidant which can produce a plenty of free radicals such as superoxide ($O_2^{\bullet-}$), NO, and their conjunctive metabolite, peroxynitrite ($ONOO^-$). These ROS/RNS activate MMP-9 possibly through NF-κB activation and finally lead to neuronal death. ICH: intracerebral hemorrhage; MMP-9: matrix metalloproteinases-9.

oligodendrocytes in the brain. Haptoglobin binds extracellular Hb, preventing Hb-mediated oxidative damage [122]. Animals that are hypohaptoglobinemic exhibit more brain damage after ICH, whereas those overexpressing haptoglobin are relatively protected. Therefore, haptoglobin is a potential therapeutic target for the prevention of brain injury following ICH [123]. Sulforaphane, a Nrf2 activator, has been shown to elevate haptoglobin and reduce brain injury in an ICH animal model [96]. Hemopexin is another blood protein known to bind heme with high affinity [124]. Hemopexin-deficient mice show increased protein oxidation, tissue heme, and augmented ICH damage [125]. This protein may also be a target to alleviate brain injury after ICH. Additional work must be performed to further establish its efficacy.

Deferoxamine mesylate (DFO), an iron chelator, is a promising agent for ICH treatment that has been confirmed to be effective in many preclinical studies [126–128]. The preliminary results in clinical trials are also encouraging. The phase I clinical trial has determined the tolerability, safety, and maximum tolerated dose of DFO in patients with ICH [129]. The phase II trial (High Dose Deferoxamine [HI-DEF] in Intracerebral Hemorrhage) is now underway, with the initial results indicating that DFO can reduce perihematoma edema, a major predictor of clinical outcome [130].

Other possible interventional targets include prooxidant enzymes, which are activated during ICH. Theoretically, inhibiting or deactivating these enzymes would be beneficial. However, controversy remains regarding the use of prooxidase inhibitors. For example, the beneficial effect of apocynin given 2 h after ICH is not achieved by its acting as an intracellular inhibitor of NADPH oxidase [71]. Tetrahydrobiopterin, which has been reported to limit the superoxide generation from NOS and chemically reduce superoxide, fails to reduce neurological deficits 24 h after ICH in mice [131]. One possible reason for this inefficacy is that many prooxidases consist of several isoforms, and their functions usually differ or can even be opposing [72, 132]. Completely, and not selectively, suppressing their activation would negate the benefits gained from some protective isoenzymes. It would be useful to identify the agent specifically acting on the detrimental isoform for a certain prooxidase.

As mentioned above, the various effects of heme oxygenases (HO-1 and HO-2) relevant to different ICH models indicate that they are challenging targets in the treatment of ICH [84, 133]. HO inhibitors may attenuate the neurotoxicity of the iron release from heme/hemin decomposition, but the toxicity of heme can enhance oxidative damage. A combination approach using two or more agents to increase HO activity while detoxifying iron with chelators has been proposed [82].

8.2. Scavenging Excessive ROS/RNS. Because of the impaired defense system after ICH, an alternative treatment is to neutralize the overproduced ROS and restore the normal function of endogenous antioxidant enzymes and scavengers. There is substantial support for the use of free radical scavengers in the management of brain injury secondary to ICH. Many free radical scavenging drugs have been evaluated in clinical trials to improve the outcome of ICH.

NXY-059 (disufenton sodium) is a free radical-trapping agent that significantly reduced disability and hemorrhagic transformation in acute ischemic stroke patients in the SAINT-I clinical trial [134]. Accordingly, the efficacy of NXY-059 treatment was also explored in ICH patients in the Cerebral Hematoma and NXY Treatment trial (CHANT) [135]. However, the result was disappointing, with no treatment effect observed on functional outcome, despite tolerability and safety. Edaravone is another free radical scavenger that has been marketed for clinical use in acute ischemia stroke treatment since 2001, with preclinical success in ICH [136–138]. Although good neurological function has been observed in preclinical studies, the clinical effect of edaravone in ICH remains unclear because of a lack of multicenter, randomized, double-blind clinical trials [139].

PPARγ agonists have been reported to play antioxidative roles by upregulating catalase and SOD directly or activating the Nrf2 pathway, and the Safety of Pioglitazone for Hematoma Resolution in ICH (SHRINC) clinical trial has been launched [140–142]. The SHRINC study will provide important information regarding the safety and clinical outcome of PPARγ agonists in ICH.

9. Conclusion

OS has been established as an important pathogenesis of brain injury in ICH. Upon bleeding into the parenchyma, elevated glutamate, infiltrating inflammatory cells, and the metabolic products of erythrocyte lysis are the sources of active free radical generation. Free radical overproduction is accompanied by prooxidase activation and antioxidase inhibition, causing OS in ICH. The direct biomolecule oxygenation and indirect cell death signaling pathway activations by

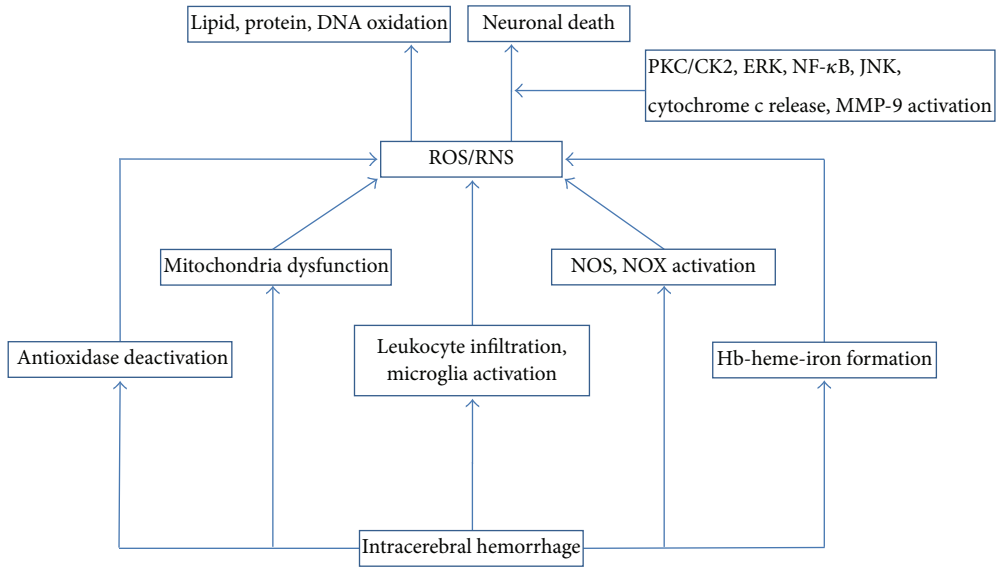

Figure 2: The sources of oxidative stress and the cell death pathways induced by oxidative stress following intracerebral hemorrhage. Oxidative stress after ICH is a consequence of prooxidant overproduction as well as deactivation of antioxidases such as SOD. The Hb-heme-iron metabolic axis due to erythrocyte lysis represents the major sources of ROS. Neuroinflammation evoked by ICH involves the activation of microglia and the infiltration of leukocyte which is another important contributor to the production of ROS. Activation of prooxidases including NOS and NOX during ICH also releases plenty of free radicals. Other factors which can generate ROS include mitochondria dysfunction. Oxidative stress causes cell death by direct oxidation of lipid, protein, and DNA or via induction of neuronal death mediated by PKC/CK2, ERK, NF-κB, JNK signaling pathways as well as cytochrome c release, and MMP-9 activation. PKC: protein kinase C; ERK: extracellular signal-regulated kinase; NF-κB: nuclear factor kappa B; JNK: c-Jun N-terminal kinase; ROS: reactive oxygen species; RNS: reactive nitrogen species; NOS: nitric oxide synthase; NOX: nicotinamide adenine dinucleotide phosphate oxidase; MMP-9: matrix metalloproteinases-9.

ROS/RNS are responsible for the OS-induced brain damage after ICH (Figure 2).

Future research should focus on developing new antioxidant compounds that can both block the sources of oxidative stress in ICH and neutralize the existing overproduction of free radicals. More importantly, efforts should be made to identify the molecular mechanism underlying the effect of OS on cell death in ICH. Additionally, because ICH-induced brain damage is ascribed to a complex pathogenic mechanism, focusing on one specific pathway, such as single antioxidant treatment, is not sufficient to achieve significant clinical improvement. Therefore, one drug with multifaceted function or combined surgical and pharmaceutical treatment or two or more drug interventions with distinctive mechanisms may be promising future treatments. For these reasons, the ultimate results of the clinical trials of DFO and pioglitazone in ICH are high anticipated because both drugs have multiple beneficial effects and reduce oxidative damage [140, 143].

Conflict of Interests

The authors declare that there is no conflict of interests regarding this work.

Authors' Contribution

Xin Hu and Chuanyuan Tao contributed equally to this work.

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Review Article

Overview of Alzheimer's Disease and Some Therapeutic Approaches Targeting Aβ by Using Several Synthetic and Herbal Compounds

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Alzheimer's disease (AD) is a complex age-related neurodegenerative disease. In this review, we carefully detail amyloid-β metabolism and its role in AD. We also consider the various genetic animal models used to evaluate therapeutics. Finally, we consider the role of synthetic and plant-based compounds in therapeutics.

1. Alzheimer's Disease (Overview)

Alzheimer's disease (AD) is the most common neurodegenerative disorder characterized by progressive memory loss. In 1907, Alois Alzheimer was the first to report a case of intellectual deterioration with the histological findings of senile plaques and neurofibrillary tangles [1]. An estimated 4.5 million Americans have AD and, as the elderly population continues to grow, the prevalence could increase by threefold to 13.2 million by 2050 [2]. The scenario seems more alarming, as it is estimated that by the year 2020, approximately 70% of the world's population aged 60 and above will be living in developing countries, with 14.2% in India. Previous reports suggest that age-adjusted prevalence of AD to be 1.91% in a community residing population in a southern Indian province of Kerala, as a part of the cognition in older adults in Trivandrum (COAT) study [3]. The reported incidence rates for AD have been lower in Asian countries than in the industrialized world [4, 5]. The impact of AD on health care costs, including direct and indirect medical and social services, is currently estimated to be greater than $100 billion per year [6]. In addition, there is currently no cure for AD; therefore, the major challenges for the near future will be the development of new therapies and therapeutic targets for disease modification and prevention.

To date, there are two major neuropathological features for the diagnosis of AD, namely, the extracellular plaque formation and neurofibrillary tangles (NFTs) formation intracellularly. The former comprises amyloid-β protein (Aβ) while the latter involves neurofibrillary tangles (NFTs) consisting of paired helical filaments of hyperphosphorylated tau protein. These histopathological lesions are mainly confined in the hippocampus region of the brain and in the cerebral cortex, the two large forebrain domains related to memory and other higher cognitive functions. The characteristic pathology in due course leads to the typical clinical symptoms, for example memory impairment, general cognitive decline, and personality changes associated with AD. The causes of AD are still rather poorly known, with different etiologies (e.g., Aβ overproduction, genetics, Aβ impaired clearance, and NFT formation) leading to senile plaques, neurofibrillary tangle (NFT) formation, and extensive neuronal death. However, several studies and evidence point to Aβ as critical in the pathogenesis of AD. According to the amyloid cascade hypothesis, Aβ peptides form aggregates and toxic assemblies which initiate several processes leading

to neuronal dysfunction and ultimately large-scale cell death [7]. The prevalence of AD varies among several different factors, including age, genetics, comorbidities, and education level. There is no way to absolutely diagnose AD without performing an autopsy. There is no cure for AD; however promising research and development for early detection and treatment is underway.

1.1. History. Alzheimer's disease was discovered by a German Neurobiologist and Psychiatrist named Alois Alzheimer in 1906 [9]. Before Alzheimer's 1906 discovery, both scientists and the nonscience community viewed dementia as a "natural" progression of age, and "senility was accepted as a part of aging" according to Natalie Whaley in her honours thesis on the social history of Alzheimer's disease. First time, AD was observed in Auguste D., a 51-year-old woman. Her family observed some unusual behavioural changes in her personality and then they brought her to Dr. Alois Alzheimer in 1901. The family reported problems with memory loss, speaking difficulty, loss of good judgement, disorientation to time and place, and problem with abstract thinking. Later on, Dr. Alzheimer described that she is having an aggressive form of dementia, memory impairment, problem using language, and behavioural changes [10]. Dr. Alzheimer also noted many other abnormal symptoms, including rapid mood swing, personality changes, loss of initiative, sleeping longer than usual, and loss of interest in usual activity [11]. Dr. Alois followed her for about five years, until her death, in 1906. After death, he performed an autopsy and found dramatic shrinkage of the cerebral cortex, deposition of fat bodies in blood vessels, and atrophied brain cells [9]. He discovered neurofibrillary tangles and senile plaques, which have become indicative of AD [11]. The condition was first discussed in the medical literature in 1907 and named after Alzheimer in 1910.

1.2. Pathology and Pathophysiology of Alzheimer's Disease. The brain of AD patient often shows marked atrophy, with broadened sulci and shrinkage of the gyri. In the majority of cases, every part of the cerebral cortex is involved. However, the occipital lobe is often relatively spared. The cortical ribbon may be thinned and ventricular dilatation is apparent, especially in the temporal horn, due to atrophy of the amygdala and hippocampus. During the last couple of decades, the pathology of AD has been extensively studied whereby animal models have provided valuable information in understanding of the pathogenic mechanisms of AD. Furthermore, AD pathology can be divided into three broad sections: (a) positive lesions (lesions related to accumulation), (b) negative lesions (those that are due to losses), and (c) inflammation and plasticity (those that are due to the reactive processes). The first category involving positive lesions is very common and easy to detect and constitutes the basis of the diagnosis. Both the neuronal and synapse loss are difficult to evaluate, as they do not belong to the diagnostic criteria but could be the alterations that are more directly related to the cognitive deficit. Furthermore, microscopic studies on AD brain revealed significant neuronal loss, in addition to shrinkage of large cortical neurons. Many neuropathologists believe that loss of synapses neurons in association with shrinkage of the dendritic arbor of large neurons is the critical pathological substrate. The main neuropathological hallmarks of AD are senile plaques and neurofibrillary tangles, although these two are not unique to AD and can be found in other human neurodegenerative disorders and in clinically normal individuals as well. Apart from senile plaques, two other types of amyloid-related plaques are found in the brains of AD patients: burnt-out plaques, which consist of an isolated dense amyloid core and diffuse plaques, which contain poorly defined amyloid but no well-circumscribed amyloid core. It is believed that the abnormal processing of the amyloid-β protein precursor through amyloidogenic pathway results in different fragments, the most toxic of which is the Aβ_{42} peptide [12]. Aβ_{42} readily self-aggregates and forms clumps of insoluble fibrils in the brain, thereby triggering the formation of senile plaques. It has been postulated that Aβ_{42} is mainly responsible for initiating a cascade of events leading to neuronal dysfunction, later followed by death. Although increasing evidence supports the hypothesis that the accumulation of Aβ is very decisive to the pathogenesis of AD [13], some investigators believe that Aβ is not exclusively responsible for the neuronal alterations that underlie its symptoms [14].

Neurofibrillary tangles (NFTs) are the other important characteristic histopathological features of AD. Neurofibrillary tangles are found inside neurons and are composed of paired helical filaments of hyperphosphorylated microtubule-associated protein tau (MAPT). Accumulation of NFTs intracellularly may cause dysfunction of the normal cytoskeletal architecture of neurons with subsequent death. Senile plaques (Aβ) and neurofibrillary tangles (NFTs) are not distributed evenly across the brain in AD but are confined to vulnerable neural systems.

Other pathological modifications commonly discovered in the brains of AD patients include granulovacuolar degeneration, neuropil threads, and amyloid angiopathy. The latter one is a distinct vascular lesion and found in many AD brains, consisting of amyloid deposition in the walls of small- to medium-sized cortical and leptomeningeal arteries due to which the involved vessels may become compromised with resultant hemorrhage.

After microscopic examination, observation of sufficient amount of senile plaques and neurofibrillary tangles suggests important pathological criteria for the diagnosis of AD. Because of the presence of amyloid-β in senile plaques and to a variable degree in cerebral blood vessels in the AD brain, the roles of this important protein and its precursor peptide, amyloid-β protein precursor, have been widely investigated [15], although the exact nature of their roles in the pathogenesis of AD remains unclear. Increasingly, the importance of differential neuronal vulnerability and the relationship of this to the morphological and biochemical characteristics of AD are being recognized. The most consistent neurochemical change associated with AD has been the well-documented decline in cholinergic activity that has inspired many attempts to treat AD with cholinergic drugs. However, additional deficiencies

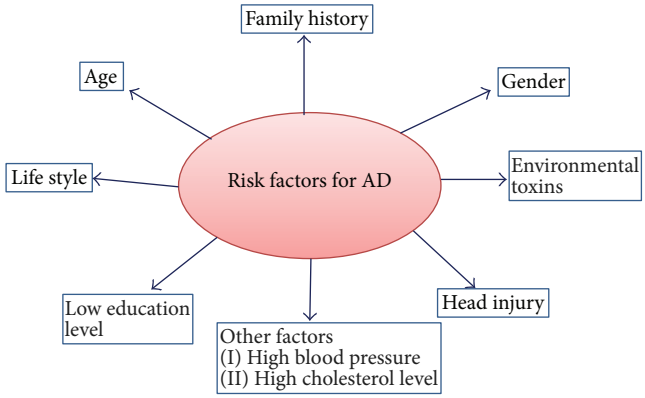

Figure 1: Showing different causal and risk factors for Alzheimer's disease.

in glutamate, norepinephrine, serotonin, somatostatin, and corticotrophin-releasing factors have also been described.

1.3. Risk Factors for AD. While scientists know that distortion of nerve cells in case of Alzheimer's disease occurs, why this happens is still unknown. However, they have discovered certain risk factors that increase the likelihood of developing Alzheimer's (Figure 1).

1.3.1. Age. With increasing age, the risk of developing AD becomes higher. Most patients develop AD after the age of 65 years. The risk of developing AD reaches 50% for individuals above the age of 85 years. Statistically speaking, about 5% of men and women between the ages of 65 and 74 have Alzheimer's disease, and nearly half of those aged 85 and older may have the disease. Despite its prevalence, Alzheimer's disease is not a normal part of aging. The age-specific incidence rates for Alzheimer's disease demonstrate a doubling of incidence for about every six years of added life, which indicates an exponential increasing risk with increasing age of individuals. This exponential risk is somewhat similar across studies, regardless of geographic region, even if the underlying absolute incidence rate differs.

1.3.2. Familial History and Genetics. Another risk factor involves family history. Researchers have shown that those who have a parent, brother, or sister with AD are more likely to develop the disease than individuals who do not have a first-degree relative with AD. The vast majority of AD cases are not genetically inherited, although some genes may act as risk factors [16]. The risk increases if more than one family member have the illness. Genetically identified forms of AD, which usually have an onset before the age of 65, have been identified and account for 0.1% of disease cases [17]. Scientists have identified three genes that described people who will develop Alzheimer's, but only a very small percentage of individuals with AD (about 1%) carry these genes. The apolipoprotein E (APOE-ε4) is carried by about 25% of individuals and increases the risk of developing AD, but it is not sure that individuals with APOE-E4 will develop the disease. Scientists believe that the vast majority of AD cases are caused by a complex combination of genetic and nongenetic determinants.

1.3.3. Other Risk Factors. Besides age, family history, and genetics, other important risk factors exist that may contribute to AD risk. In this context, some promising research suggests that strategies for keeping and living overall healthy aging may help maintain brain health and may even provide some protection against AD. These factors include eating habits, healthy lifestyle, staying socially and physically active, and avoiding excess alcohol and tobacco.

Some of the strongest evidence links brain health to heart health. The risk of developing Alzheimer's disease or vascular dementia appears to be increased by many conditions that damage the heart and blood vessels. These include heart disease, diabetes, stroke, high blood pressure, and high cholesterol. It is a common saying in medical practice that "work with your doctor to monitor your heart health and treat any problems that arise." Further, studies of donated brain tissue provide additional evidence for the heart-head connection. These studies suggest that plaques and tangles are more likely to cause Alzheimer's symptoms if strokes or damage to the brain's blood vessels is also present.

2. Different Hypotheses Postulated Related to Alzheimer's Disease

2.1. The Tau Hypothesis of Alzheimer's Disease. Tau protein plays a critical role in pathophysiology of AD. The tau hypothesis focuses primarily on the role of the microtubule binding tau protein, which is the main component of NFTs in AD. Hyperphosphorylation of tau protein results in NFT formation. This hypothesis proposes a mechanism for neurotoxicity based on the loss of microtubule-stabilizing tau protein that leads to degradation of the cytoskeleton [18]. However, it is not clear whether tau hyperphosphorylation is responsible or is caused by the formation of abnormal helical filaments [19]. Tauopathy-like diseases also support the tau hypothesis in which the same protein is significantly misfolded [20]. However, a majority of research groups support the alternative hypothesis that amyloid-β is the primary causative agent for AD [19].

2.2. The Cholinergic Hypothesis of Alzheimer's Disease. The cholinergic hypothesis is the oldest AD hypothesis [21]. This hypothesis proposed that AD is caused by the reduced synthesis of a neurotransmitter called acetylcholine in neurons. The cholinergic hypothesis was formulated over 30 years ago and suggests that a dysfunction of acetylcholine-containing neurons in the basal forebrain contributes substantially to the cognitive decline observed in AD patients [22]. In addition to the dysfunction and neuronal loss in basal forebrain regions, confirmation of cholinergic losses comes from studies that report deterioration in the activity of acetylcholine esterase (AChE) and choline acetyltransferase (ChAT), reduced acetylcholine (ACh) release, and decreased level of nicotinic and muscarinic receptors in the AD affected

brain [23]. This observation led to the formation of the cholinergic hypothesis, considered the oldest hypothesis of AD [24, 25]. The cholinergic hypothesis has not had widespread support, largely because medications intended to treat acetylcholine deficiency have not been very effective, although 4 of the 5 approved drugs work on just this mechanism. Other cholinergic effects have also been proposed, for example, initiation of large-scale aggregation of amyloid leading to generalized neuroinflammation [26].

2.3. The Mitochondrial Cascade Hypothesis. The mitochondrial cascade hypothesis was first proposed by Swerdlow and Khan, 2004, which postulates that mitochondrial dysfunction is the primary cause of Aβ deposition, neurofibrillary tangle (NFT) formation, and synaptic degeneration in AD [27]. The mitochondrial cascade hypothesis takes several conceptual liberties. It assumes that similar physiologic mechanisms underlie AD and brain aging. It postulates that because AD mitochondrial dysfunction is systemic, it cannot simply represent a consequence of neurodegeneration. The mitochondrial cascade hypothesis argues that non-Mendelian genetic factors contribute to nonautosomal dominant AD. Finally, it posits that AD brain mitochondrial dysfunction drives to amyloidosis, tau phosphorylation, and cell cycle reentry. Mitochondrial dysfunction is observed in several AD tissues [28], including platelets, fibroblast, mitochondria, and brain. There are basically three mitochondrial enzymes that are found to be defective. This includes reduced activities of α-ketoglutarate dehydrogenase complex, cytochrome oxidase, and pyruvate dehydrogenase complex [29]. Through special analysis of AD brains, level of cytochrome oxidase is found to be normal, but the enzyme itself is structurally altered [30]. In AD, oxidative stress and proteasome dysfunction have been postulated to facilitate mitochondrial dysfunction [31]. Also, studies on cytoplasmic hybrid (cybrid) indicate that mtDNA at least, in part, accounts for reduced cytochrome oxidase activity in AD [32].

2.4. Amyloid Cascade Hypothesis. Although the exact cause of AD is still a matter of debate, the amyloid cascade hypothesis is the best accepted and most studied hypothesis among those mentioned above. The presence of amyloid plaques is considered to be the main characteristic of AD pathology. The primary constituent of senile plaques identified so far is Aβ peptide, which is produced on account of proteolytic processing of the amyloid-β protein precursor (AβPP) by β- and γ-secretases [33, 34]. Furthermore, cloning of the AβPP gene [35] has allowed the disease to be examined at molecular and biochemical levels. Subsequently, mapping of several familial forms of AD (fAD) mutations in the AβPP gene [36], the association of AD with Down's syndrome, and higher prevalence of AD with increased numbers of AβPP all established the critical role of AβPP in AD pathogenesis [33, 37, 38]. The central role of AβPP in AD etiology is further supported by the identification of fAD mutations in presenilin 1 (PS1), which involves AβPP cleavage and generates Aβ and AICD fragments.

In the early 1990s, it was proposed that the main essence of the amyloid cascade hypothesis is increased production or decreased clearance of Aβ peptide, the culprit behind AD [39, 40]. Accumulation of the hydrophobic Aβ peptide (Aβ_{40} and Aβ_{42}) results in its self-aggregation and formation of insoluble plaques, triggering a cascade of events resulting in death of the neuronal cells and thus causing AD (Figure 2).

A large portion of fAD cases is accounted for by mutations in the presenilin 1 (PS1) gene [41]. PS1 is one of the four main membrane proteins in presenilin complex which associates with three other membrane proteins to form the γ-secretase complex. Unlike in AβPP, fAD mutations in PS1 are scattered throughout the length of molecules. Many of these mutations result in modified cleavage of AβPP, causing enhanced production of the longer Aβ_{42} peptide, which is more prone to self-aggregate as compared to the smaller Aβ_{40} [42] and is shown to be more toxic *in vitro*. Since the level of Aβ_{42} is found to be much higher in AD patients, it was postulated that the rise in levels of Aβ_{42} triggered the cascade of the distorting events resulting in AD [43]. Although there is increase in Aβ_{42} due to many fAD mutations, some mutations in PS1 do not elevate Aβ_{42} levels but rather decrease Aβ_{40} levels. This has led to yet another possibility that an enhancement in the A$\beta_{42/40}$ ratio, instead of the absolute levels of Aβ_{42}, is pathogenic and triggers the deleterious events leading to the disease. This view is supported by the observation that increased A$\beta_{42/40}$ ratio is generally inversely related to the age of onset of AD [44].

3. Amyloid-β Protein Precursor (AβPP) and Its Function

The amyloid-β protein precursor (AβPP) gene is located on chromosome 21 in humans. Alternative splicing of the AβPP transcript generates 8 isoforms, of which 3 are most common: AβPP695, AβPP751, and AβPP770. The 695 amino acid form predominantly expresses in the CNS, and 751 and 770 amino acid forms express ubiquitously [45]. AβPP belongs to type I transmembrane proteins that include the amyloid precursor-like proteins (APLP1 and APLP2) in mammals and the amyloid precursor protein-like (APPL) in *Drosophila*.

The exact physiological function of AβPP is not well known and remains an important issue in AD research. In many studies, overexpression of AβPP shows a positive effect on cell health and growth. This effect is epitomized in transgenic mice that overexpress wild-type AβPP and have enlarged neurons [46]. AβPP knockout mice are viable and fertile, showing a comparatively subtle abnormal phenotype [47, 48]. APLP1 and APLP2 knockout mice also survive and are fertile. However, double null mice AβPP/APLP2 and APLP1/APLP2 and triple null mice AβPP/APLP1/APLP2 show early postnatal lethality [49, 50]. Interestingly, AβPP/APLP1 mice are viable [50], suggesting that APLP2 is critical when either AβPP or APLP1 is absent.

Further, the similarity in proteolytic processing and topology between Notch and AβPP suggest that AβPP may function as a membrane receptor like Notch. Indeed, different AβPP ligands have been identified, such as Aβ [51],

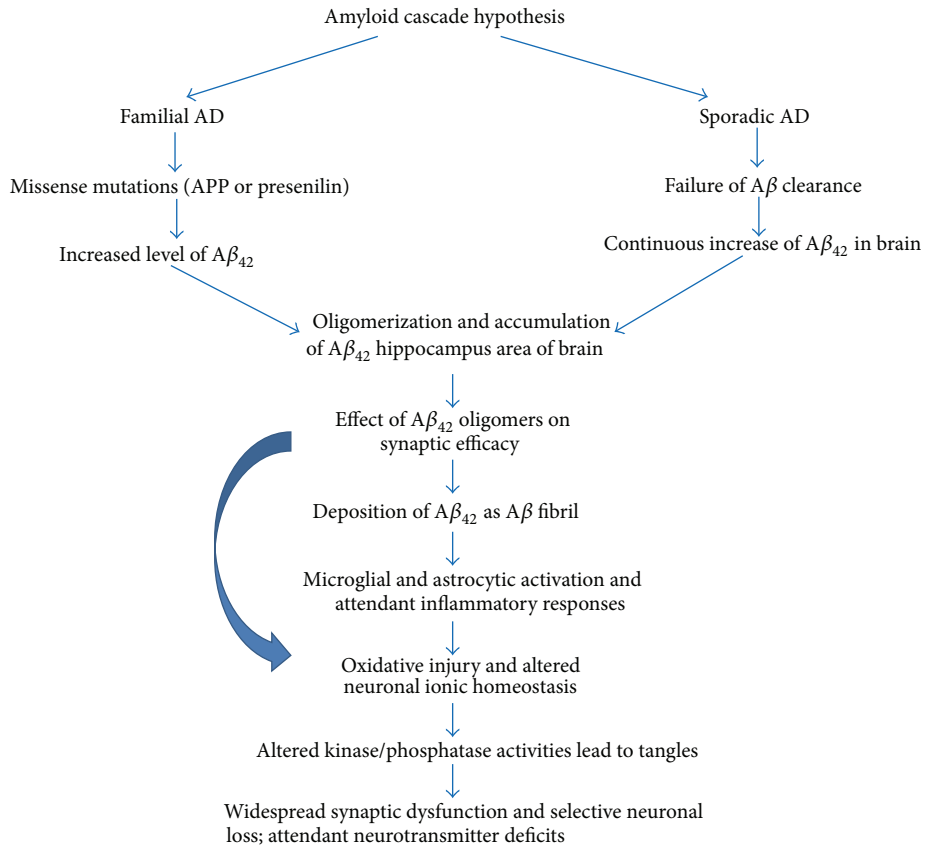

Figure 2: The amyloid cascade hypothesis of Alzheimer's disease. This hypothesis represents the classic theory of the origins of AD. Both familial forms of Alzheimer's (fAD) and later-onset forms with no known etiology (sporadic AD) lead to the production of excess $A\beta_{42}$. Once this toxic peptide begins to aggregate, a cascade of events is triggered that produces the biological and neurological symptoms of Alzheimer's disease.

netrin-1 [52], and F-spondin [53]. However, while binding of AβPP by these ligands can affect AβPP processing, the exact downstream signalling events triggered by such binding remain to be clarified and an authentic membrane receptor function for APP remains speculative.

Although AβPP has been the subject of much study since its identification, its physiological function remains unclear. AβPP has important roles in neurites' outgrowth and synaptogenesis, cell adhesion, calcium metabolism, neuronal protein trafficking along the axon, and transmembrane signal transduction, among others, all requiring additional *in vivo* experimental evidence [54]. AβPP generates various fragments during proteolytic processing and these AβPP metabolites serve various functions. Therefore, the net effect of full-length AβPP on biological activity may be a combination of its metabolites' functions, depending on the proportion of levels of each AβPP metabolite. In adult animals, intracerebral injections of the AβPP ectodomain can improve cognitive function and synaptic density [55, 56]. The sites most responsible for the bioactivity of the AβPP ectodomain appear to be its two heparin-binding domains [57]. Overall, studies from various research groups suggest that AβPP plays an important role in protein trafficking regulation.

4. Proteolytic Processing of AβPP and Generation of Aβ Peptide

As described above, AβPP is a type I transmembrane protein. It is synthesized in the endoplasmic reticulum (ER) and transported to the trans-Golgi-network (TGN) through the Golgi apparatus where the highest concentration of AβPP is found in neurons at steady state [58–60]. One of the most prominent areas of AD research is the study of the generation of Aβ after AβPP processing. Aβ generation takes place in ER and Golgi/TGN [60]. Further, AβPP can be transported from the TGN to TGN-derived secretory vesicles to the cell surface where it is either reinternalized via an endosomal/lysosomal degradation pathway [61, 62] or cleaved by α-secretase to produce a soluble molecule, $A\beta PPs_{\alpha}$ [63]. Some reports also suggest involvement of endosomal/lysosomal system in Aβ generation [64]. Unlike Aβ which is neurotoxic, studies suggest that $A\beta PPs_{\alpha}$ is neuroprotective, making the subcellular distribution of AβPP an important factor in neurodegeneration [65]. Therefore the characterization of the mechanisms involved in APP transport and trafficking are crucial to understanding the pathogenesis of AD.

Processing of AβPP takes place by two different pathways: amyloidogenic pathway, which results in the generation of

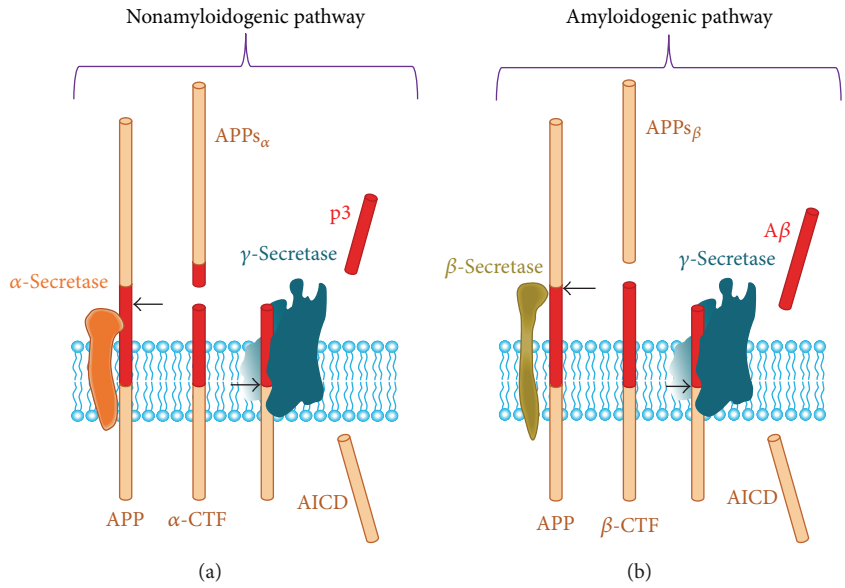

Figure 3: Processing of the amyloid-β protein precursor (AβPP) occurs by two pathways. (a) Nonamyloidogenic processing of AβPP involving action of α-secretase followed by γ-secretase as shown in the figure. (b) Amyloidogenic processing of AβPP involving β-secretase followed by the action of γ-secretase. Both processes generate soluble ectodomains (AβPPs$_\alpha$ and AβPPs$_\beta$) and a similar intracellular C-terminal fragment (AICD). The Aβ peptide starts within the ectodomain and continues into the transmembrane region (red). Adapted from Thinakaran and Koo [8].

toxic Aβ fragment of 42 amino acid, and nonamyloidogenic pathway, which is required for normal functioning of neurons. There is involvement of three different types of serine proteases in the AβPP processing which are α-, β-, and γ-secretases. The quantitatively and functionally most important proteolytic processing of AβPP is mediated through nonamyloidogenic pathway, that is, cleavage of AβPP by the action of α- and γ-secretases. Action of α-secretase releases the AβPPs$_\alpha$ ectodomain and a carboxy terminal fragment (AβPP-CTF$_\alpha$). Action of γ-secretase later generates a small p3 and AICD fragment (Figure 3). AβPPs$_\alpha$ has been suggested to show neuroprotective and synapse-promoting activities [66], but the mechanism behind this and identification of the receptor mediating these effects has not yet been identified.

Cleavage of AβPP by the action of β-secretase and γ-secretase results in Aβ_{42} generation, called amyloidogenic pathway (Figure 3). Action of β-secretase releases the ectodomain AβPPs$_\beta$ and rests AβPP carboxy-terminal fragment (AβPP-CTF$_\beta$) which is further cleaved by the γ-secretase producing the Aβ peptide(s) and the AβPP intracellular domain (AICD) fragment. The biological function of all the above fragments (AβPPs$_\beta$, Aβ, and AICD) generated through amyloidogenic pathways is still to be explored, although Aβ release is associated with synaptic activity and synaptic transmission onto neurons [67]. The AICD fragment is thought to be a nuclear signalling molecule [68], but this is also not fully explored [69].

5. Definition of Amyloid-β Peptide

Definition of amyloid-β follows the guidelines of nomenclature established in the November 2006 meeting of The Nomenclature Committee of the International Society of Amyloidosis. Amyloid-β is defined as peptides of 36–43 amino acids that are primarily involved in AD as the main component of the amyloid plaques found in the brains of AD patients. Amyloid-β, also defined as protein deposits found *in vivo*, can be distinguished from nonamyloid protein deposits by observing under an electron microscope. Amyloid-β has a characteristic fibril appearance, a unique pattern of X-ray diffraction, and an affinity for the dye Congo red of histological samples, which results in an apple green birefringence under plane-polarized light [70]. The term amyloid-β was initially reported to restrict as extracellular deposits only. However, many types of amyloid-β have since been reported to begin intracellularly, resulting in the characteristic extracellular amyloid deposits found upon cell death. Therefore, amyloid is no longer restricted to extracellular inclusion but also includes those intracellular inclusions having typical amyloid appearance [70].

6. Physiological Function of Aβ Peptide

Multiple lines of evidence reveal that overproduction of Aβ through AβPP processing results in a neurodegenerative cascade leading to self-aggregation, synaptic dysfunction, formation of intraneuronal fibrillary tangles, and gradual neuron loss in the hippocampus [71]. There are two main toxic species, Aβ_{40} and Aβ_{42}, with Aβ_{42} being more hydrophobic in nature and more prone to self-aggregation which results in Aβ fibril formation [72]. Previous studies on familial form of AD (fAD) mutations consistently show rise in the ratio of A$\beta_{42/40}$ [73], thereby indicating that elevated levels of Aβ_{42} relative to Aβ_{40} are crucial for AD pathogenesis,

likely achieved by providing the core for Aβ assembly into oligomers, fibrils, and amyloidogenic plaques [74]. Although the majority of Aβ peptides are secreted from the cell, Aβ can be generated in several subcellular compartments within the cell, such as the ER, Golgi/TGN, and endosome/lysosome. In addition, internalization of extracellular Aβ can be done by a cell for its degradation. The presence of intracellular Aβ implies that Aβ may accumulate within the neuronal cell and contribute to AD pathogenesis. Confirming the presence of intracellular Aβ, intraneuronal Aβ immunoreactivity has been found in the hippocampal and entorhinal cortical regions which are more prone to early AD pathology with mild cognitive impairment (MCI) in AD patients [75]. The accumulation of intracellular Aβ paves the way for extracellular plaque formation in Down's syndrome (DS) patients [76] and the level of intraneuronal Aβ reduced as the extracellular Aβ plaques accumulate [77]. Studies of transgenic mouse models consistently confirm evidence for intracellular accumulation of Aβ as an early event in the neuropathological phenotype, with decreasing intraneuronal levels of Aβ as extracellular plaques build up [78–80]. Intraneuronal Aβ can also disrupt amygdala-dependent emotional responses by modulating the ERK/MAPK signalling pathway [81]. Previous reports also suggest reduction in Aβ neurotoxicity due to inhibition of dynamin-mediated but not clathrin-mediated Aβ internalization [82]. One recent study by Friedrich et al. suggests that intracellular Aβ can self-aggregate within the cell and disrupt the vesicular membrane, contributing to its pathological effect [83].

Aβ was originally considered a neurotoxic species confined to the brain of aged or demented persons. Later findings suggest that the presence of soluble Aβ species in the bodily fluids of many species [84] and in the conditional cell culture media [85] has disproved this concept and inferred a physiological function for Aβ. Low levels of Aβ enhance hippocampal long-term potentiation and improve memory, indicating its novel positive, modulatory role in neurotransmission and memory [86], while excessive Aβ causes neuronal loss as well as synaptic dysfunction. One study using a transgenic *Caenorhabditis elegans* model found that intracellular Aβ aggregation in muscle cells may trap excess free copper to reduce copper-mediated cytotoxic effects [87]. However, whether Aβ can form intracellular aggregates in human peripheral cells to exert a physiologically protective function remains to be determined.

7. Mechanism of Formation of Aβ Fibril

The mechanism of formation of Aβ oligomer *in vivo* remains unclear. In this context, Glabe suggests that the complexity of the oligomer formation can be assumed by the fact that multiple Aβ oligomer conformations are produced via different pathways [88]. The mechanisms of fibril formation of extracellular and intracellular oligomers may also vary. The fibrillization of Aβ into senile plaques is a complex process involving several steps [89–91]. After Aβ is released from cells, it can bind to several proteins: for example, albumin, α1-antichymotrypsin, apolipoprotein E, and complement proteins [92]. Presence of Aβ as stable soluble dimers is detected in both cell culture media and brain homogenates [93]. Total Aβ concentration may be the critical determinant of fibril formation. In normal brain, breakdown of Aβ takes place immediately after its production from the cells before fibrillization or deposition, while, in the aging brain, increased production of Aβ and its reduced rate of clearance may lead to Aβ fibrillization, further leading to disease condition and AD pathogenesis. Recent studies reveal three different types of Aβ oligomers: (a) very short oligomers ranging from dimer to hexamer size [94, 95]; (b) small oligomers ranging from 17 to 42 kDa which are Aβ-derived diffusible ligands (ADDLs) [96]; and (c) protofibrils that can be seen in electron microscopy as short fibril intermediates of less than 8 nm in diameter and less than 150 nm in length. Protofibrils are short-lived structures detected during *in vitro* formation of mature amyloid fibrils [97–99]. However, relationships between the aforementioned oligomers remain unclear. Moreover, all oligomeric forms of Aβ derived intermediates, that is, oligomers, ADDLs, protofibrils, and mature Aβ fibrils, are potentially neurotoxic and may be a key cause of neurotoxicity in AD. Aβ exists mainly in two alloforms: Aβ_{40} and Aβ_{42}, which follow distinct oligomerization pathways [94, 100]. Each peptide showed different behaviour at the earliest stage of assembly and monomer oligomerization. Kinetic studies of Aβ fibril formation have shown that formation of Aβ_{42} self-aggregates is faster than Aβ_{40} and forms fibril [98, 101]. It is also well reported that the fibrillogenic and neurotoxic property of Aβ_{42} is higher than that of Aβ_{40}. The initial phase of fibrillization of Aβ_{42} monomers involves formation of pentamer/hexamer units called paranuclei (Figure 4). These paranuclei are initial structures that can further oligomerize to larger units and form large oligomers, protofibrils, and fibrils. Monomers, paranuclei, and large oligomers are predominately unstructured with only short β-sheet/β-turn and helical elements. During formation of protofibrils, essential conformational changes take place when the unstructured, α-helix, and β-strand elements convert into β-sheet/β-turn structures. Paranuclei could not be detected for Aβ_{40} at similar concentrations of the peptide. Aggregate-free Aβ_{40}, when carefully prepared, existed as monomers, dimers, trimers, and tetramers, in rapid equilibrium [94]. The important residue promoting the pentamer/hexamer formation is Ile41. Addition of later residue to Aβ_{40} is sufficient to induce paranuclei formation [94]. A natural propensity to form paranuclei is the only feature of Aβ_{42}. This important finding may explain the predominantly strong association of Aβ_{42} with AD. Paranuclei formation in Aβ_{42} is blocked by oxidation of Met35 and produces oligomers indistinguishable in morphology and size and from those produced by Aβ_{40} [95]. Preventing the fibrillization of toxic Aβ_{42} paranuclei through selective Met35 oxidation thus represents a potential therapeutic target for AD treatment. The most important feature of controlling early oligomerization of Aβ is the length of the C-terminal as compared to 34 physiological relevant alloforms of Aβ [95]. The primary amino acid residue in Aβ_{42} is a side chain of residue 41 which is crucial for effective paranuclei formation and self-aggregation into oligomer formation. Aβ_{40} self-aggregation is particularly

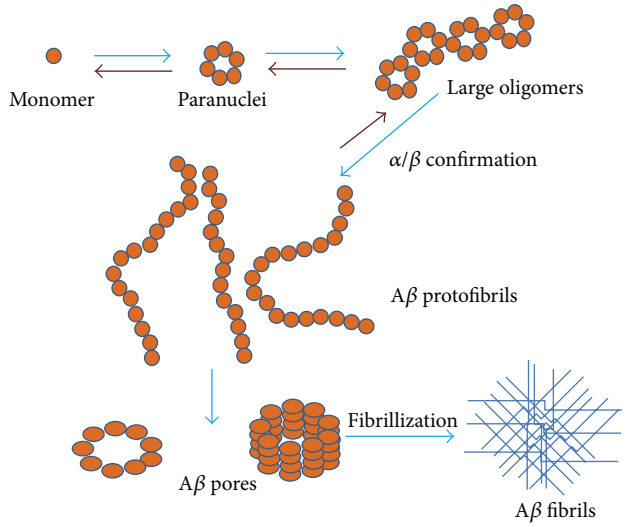

FIGURE 4: A model showing $A\beta_{42}$ oligomerization and fibrillization: the equilibrium between monomer to paranuclei and from paranuclei to large oligomers is rapid and reversible. The conversion of oligomers to protofibrils is slower but also reversible. Conversion of protofibrils into fibrils is an irreversible step. Basically, the monomers, paranuclei, and large oligomers do not have any definite structure instead of some β-turn/β-sheet and helical (α) elements. Essential conformational changes occur during protofibril formation where the unstructured, α-helix, and β-strand elements transform into β-sheet/β-turn structures.

critical to substitutions of Glu22 or Asp23 and to truncation of the N terminus [94]. Whereas $A\beta_{42}$ oligomerization is largely unaffected by substitutions at positions 22 or 23 or by N-terminal truncations, it is significantly affected by Phe19 or Ala21 substitutions. The above statement reveals that Aβ oligomerization differs between $A\beta_{40}$ and $A\beta_{42}$ which are controlled by specific regions and residues.

8. Aβ Toxicity

Alzheimer's disease is considered by some researchers to be a disease of the synapses and has been termed "synaptic failure" [102]. While Aβ can destroy neurons, synaptotoxicity may be more appropriate for earlier stages of AD that are best categorized by synaptic loss rather than neuronal death. Loss of dendritic spines or synaptic terminals may cause the associated deterioration in cognitive functions that characterizes AD. However, it is still unclear whether the synaptotoxic and neurotoxic actions of Aβ are a separate mechanistic process or if the actions follow a common mechanism [103]. As discussed above, the pentameric and hexameric oligomers may be the building blocks of the more toxic decameric and dodecameric complexes. Both cross-linked oligomeric forms of Aβ and Aβ fibril were significantly more toxic than disaggregated Aβ (dimers threefold and tetramers 13-fold more toxic than monomers). One of the main results is the fact that monomers have very low toxicity, while toxicity rises substantially only when Aβ self-associates, although it is still challenging to establish a degree of increasing toxicity with the number of monomers in the given oligomer because of the decrease in occurrence frequency of higher order oligomers. Monomeric Aβ has the propensity to adopt different conformations in water solutions, including momentarily extended β-sheet conformations in the central and C-terminal regions, connected by turn between them, or β-hairpin [104].

Several other lines of study support a role for oligomeric form of Aβ as the toxic entity in AD patients. Human brain shows soluble oligomers with similar structural properties as observed *in vitro* by antioligomeric antibodies staining; the same oligomers were also observed *in vivo* [105]. Aβ oligomer toxicity *in vitro* has been attributed to several distinct mechanisms, including but not limited to membrane disruption and direct formation of ion channels. There have been numerous reports of increased membrane conductance or leakage in the presence of Aβ oligomers ranging from small globulomers to large prefibrillar assemblies [106, 107], with some evidence presented to support formation of discrete ion channels of pores [108, 109].

Fibrillar Aβ, on the other hand, has been shown to bind to a wide array of cell surface proteins, including the receptor for advanced glycation end products (RAGE) complex and AβPP [110], leading in some cases to increased free radical formation and oxidative stress. Similarly, binding to the α-7 nicotinic receptor can mediate N-methyl-D-aspartate (NMDA) receptor activity with broad effects on cellular metabolism [111]. Any or all of these effects may play a role in loss of synaptic function, leading to symptomatic AD. Other proposed interactions, such as dysregulation of calcium channels, may be confounded by membrane disruption effects, making them harder to confirm.

It is important to note that since Aβ exists *in vitro* and *in vivo* as a continuum of different oligomeric states, none of which are particularly stable, it is difficult to distinguish biological effects induced by one specific type of nonfibrillar oligomer. Therefore, it is entirely feasible that Aβ has significantly different physiological effects when in different oligomeric forms. Thus, it is difficult to exclude any of

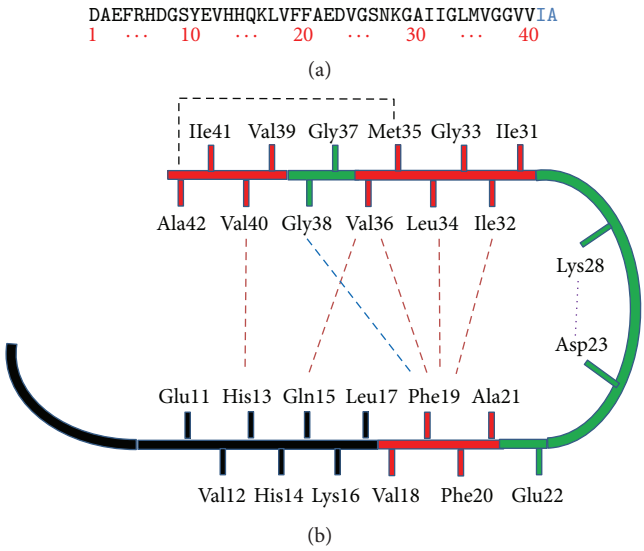

FIGURE 5: (a) Sequence of $A\beta_{42}$ that is derived from human $A\beta PP$. (b) Structural constraints in $A\beta_{40}$ and $A\beta_{42}$ fibrils. NMR measurements of $A\beta_{40}$ fibrils have shown that residues 1–10 are unstructured and residues 11–40 adopt a β-turn-β fold. Side chain packing is observed between Phe19 and Ile32, Leu34 and Val36, Gln15 and Val36, and His13 and Val40 (orange dashed line). In $A\beta_{42}$ fibrils, residues 1–17 may be unstructured (in black), with residues 18–42 forming a β-turn-β fold. Molecular contacts have been reported within the monomer unit of $A\beta_{42}$ fibrils between Phe19 and Gly38 (blue dashed line) and between Met35 and Ala42 (black dashed line). In both $A\beta_{40}$ and $A\beta_{42}$, the turn conformation is stabilized by hydrophobic interactions (red residues) and by a salt bridge between Asp23 and Lys28 (purple dashed line).

the putative mechanisms for involvement of $A\beta$ oligomers in progression of AD without further study.

$A\beta_{40}$ is the common, more soluble form of $A\beta$. $A\beta_{42}$ has two extra amino acids on the end of the peptide (Figure 5(a)). One of these, 42nd amino acid, is an alanine, which can loop back to form a salt bridge with 35th amino acid, methionine (Met35) (Figure 5(b)). This extra hairpin turn of $A\beta_{42}$ makes it less soluble and more toxic. The toxicity of $A\beta_{42}$ is much greater than $A\beta_{40}$.

9. Role of $A\beta$ in AD Pathogenesis

As discussed so far, it is clear that $A\beta$ is one of the hallmarks for Alzheimer's disease. $A\beta$ is generated from $A\beta PP$ processing by β- and γ-secretases through the amyloid cascade pathway. $A\beta$ is one of the main toxic peptides which has a critical role in AD pathogenesis. $A\beta$ normally has a propensity for self-aggregation, resulting in $A\beta$ fibril formation which ultimately form senile plaques extracellularly, causing neuronal damage and synaptic dysfunction. Although $A\beta$ aggregates are mainly found in the hippocampus area of postmortem brain of AD patients, they are also distributed to some extent in the cortex area of brain. As we know, the hippocampus is the prime memory storage part of the brain, so these aggregates affect the surrounding neurons in hippocampus area and are responsible for AD pathology. The temporal profile of pathological features, together with genetic risk factors for AD, has led to the hypothesis that accumulation of $A\beta$ oligomers during early, preclinical stages of the disease initiates a cascade of events resulting in synaptic dysfunction, neuronal loss, and atrophy within the temporoparietal and hippocampal regions. This neurodegeneration, in turn, causes neuronal dysfunction, cognitive decline, and ultimately complete loss of memory [112].

10. Therapeutic Approaches for AD

There is no cure for AD; however, drug treatments are available to help relieve symptoms in several aspects of the disease, and researchers around the world are focusing on finding better treatments, preventive strategies, and ultimately a cure. A variety of cellular mechanisms can lead to the generation of Alzheimer's disease. Along with $A\beta$, microtubule-associated protein tau is another hallmark of AD [113]. In the case of AD, tau becomes hyperphosphorylated, aggregated, and finally accumulated as NFT [114]. Tau plays an important role, not only as axonal protein but also as regulator of dendritic function, particularly mediating early $A\beta$ toxicity during AD [115]. Therefore, $A\beta$ and tau became targets in drug development for AD. Many clinical trials targeting these two proteins have been implemented; several lines of research are still under investigations. There are several therapeutic approaches being investigated for the treatment of AD (Figure 6). Therapeutic strategies are basically categorized in the following three ways: (i) treatments that prevent the onset of the disease by sequestering the primary progenitors; (ii) disease-modifying therapies termination or the reversal of disease progression; and (iii) symptomatic treatments that treat the cognitive symptoms of the disease and protect from further cognitive decline. Among the therapeutic strategies mentioned in Figure 6, amyloid-based therapies using synthetic- as well as herbal-based antiamyloid approach are highlighted here.

10.1. Amyloid-Focused Therapies. Along with tau-focused therapeutic approaches, amyloid-focused treatment strategies are also in development in order to prevent the aggregation and accumulation of insoluble $A\beta$ and/or clear $A\beta$ plaques postformation. Still, studies have reported that soluble $A\beta$ peptides may similarly be protective *in vivo* as an ameliorative response to free radical toxicity [116, 117].

10.1.1. Inhibition of $A\beta$ Aggregation. Since $A\beta$ aggregation is hypothesized to be the most crucial step of the pathogenic process of AD, the strategy to inhibit $A\beta$ aggregation has emerged as a promising approach to treat AD. Numerous synthetic as well herbal compounds have been identified as inhibitors of $A\beta$ aggregation; however, the mechanistic interaction between $A\beta$ and these compounds is still not clear [118]. To gain insight into the mechanism of inhibition, it is necessary to understand the structure of $A\beta$. While the structure of $A\beta$ has not been resolved by crystallography,

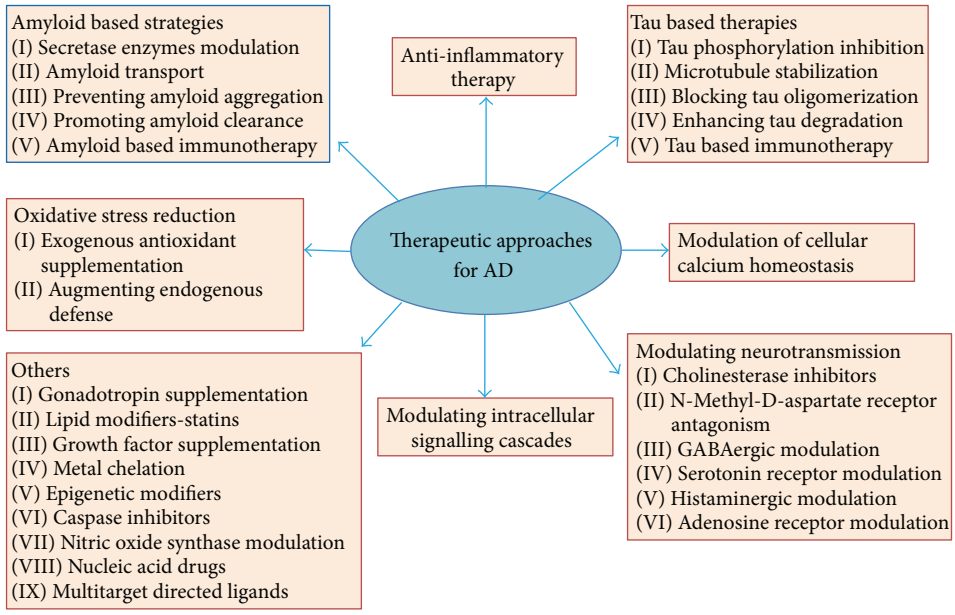

Figure 6: Different therapeutic approaches for the treatment of AD.

several structures have been predicted by different techniques, including nuclear magnetic resonance (NMR) spectroscopy and computer simulation [119–121]. Petkova et al. proposed a broadly used structural model for Aβ fibrils using solid state NMR (SS-NMR), as shown in Figure 5(b) [122]. In this model, residues 1–10 are structurally disordered, while residues 12–24 and 30–40 adopt β-strand conformations and form parallel β-sheets by internal hydrogen bonding. Figure 5(b) shows the secondary structure for a single Aβ_{42} monomer within the fibril. Residues 25–29 contain a 180° bend of the protein backbone that brings the two β-sheets in contact through side chain-side chain interactions. A single cross-β unit is a double-layered β-sheet structure with a hydrophobic core and a hydrophobic face. The only charged residues in the core are Asp23 and Lys28, which form a salt bridge to stabilize the β-sheet structure.

This model contains information relevant to the design of inhibitors for Aβ aggregation. For example, compounds that have interaction propensity with the hydrophobic core of Aβ peptide would disrupt monomer-monomer interaction, thereby destabilizing the formation of small oligomeric aggregates or nuclei. Compounds that recognize residues 12–24, which are included in the interaction between fibril units, would interfere with lateral association, while compounds interacting through hydrogen bonds formation with amino or carboxyl groups of residues 12–24 or 30–40 are expected to inhibit soluble aggregate elongation.

Most compounds showing inhibitory capability toward Aβ aggregation are aromatic in nature, such as resveratrol, coumarin, and nicotine [123–125]. It is hypothesized that the aromaticity plays an important role by breaking the hydrophobic interaction between Aβ monomers. Aromatic compounds can interact with residues Phe19 and Phe20 of Aβ peptide via π-π stacking interactions.

Another finding that the derivatives of penta peptide, KLVFF (residues 16–20 of Aβ), can inhibit Aβ aggregation supports this speculation [126, 127]. Therefore, to achieve better inhibitory capability toward Aβ aggregation, aromatic compounds have been modified and functionalized on their aromatic centre.

11. Different Model Organisms to Study Alzheimer's Disease

As the rate of occurrences of AD is growing continuously, pressure is mounting on the research community to develop a suitable and effective drug treatment. While other age-related diseases like heart disease and cancer can now be successfully studied, treated, and to a certain extent cured, AD, and other age-related human neurodegenerative diseases such as Parkinson's disease, is still not curable. This is not only due to a poorer understanding of AD, the complexity of the brain, and its relative inaccessibility, but it is also due to a lack of "natural" disease models. For example, the dog naturally mimics some AD features including Aβ cortical pathology, loss of neuronal cells, and learning and memory deficits, but it does not develop neuritic plaques and NFTs [128]. Primates do develop forms of both but are not well studied. Also, though rodents will readily develop cancer, the senile plaques and NFTs formation have never been reported [129].

Thus, transgenic AD mouse and rat models have enabled the scientific community to overcome the lack of a suitable natural model for the study of AD. The major limitation is that rodents do not naturally have anything close to AD. The AD in these models is imposed. Since the study of AD in humans is methodologically and ethically complex and critical, AD transgenic models provide an approach to understanding

AD pathogenesis, so as to recognize new biomarkers and to design new therapeutics, although they do not utilize normal physiology. In addition, transgenic AD models allow investigation of the early stages of the disease, something that is problematic with human postmortem tissue [130]. In contrast numerous drug targets have "cured" these mouse models while having no benefit in clinical trials of AD patients [131]. The problem is that AD pathology is a response whose removal has limited, if any, benefit for humans but is an imposed abnormality for the transgenic rodents.

11.1. Transgenic Mice Model of AD. The mouse model of AD was established in the mid-1990s with the development of the PDAPP model [132], followed by the Tg2576 in subsequent years [133] and APP23 [134] models, being currently the most extensively used amyloidosis models in AD research. The PDAPP model expresses human AβPP carrying the Indiana familial AD mutation (V717F) driven by the platelet-derived growth factor-β promoter, whereas both Tg2576 and AβPP23 models express human AβPP with the Swedish mutation (K670N/M671L) driven by the hamster prion protein and murine Thy-1 promoters, respectively. All the above-mentioned models support the amyloid cascade hypothesis; they show progressive Aβ deposition in both diffuse and neuritic plaques, cerebral amyloid angiopathy, microgliosis, (limited) hippocampal atrophy, astrocytosis, synaptic and neurotransmitter alterations, and cognitive and behavioural deficits, relevant to human AD neuropathological profile [135–138]. AβPP-based models confirm the principal role of AβPP and Aβ in the Alzheimer disease process and allow target identification and subsequent preclinical evaluation of various symptomatic and disease-modifying drugs, primarily targeting the amyloid cascade. The major drawback of these models, however, is the lack of NFT formation, although hyperphosphorylated tau may be present.

The discovery of early-onset mutations in the PSEN genes aids in development of PSEN1 and PSEN2 transgenic mouse models. Even though an increased $A\beta_{42}/A\beta_{40}$ ratio in some of these models has been observed, they are void of plaque pathology. Further, few behavioural and cognitive discrepancies are present in these models; they lack NFT development like AβPP-based models as well. They are mainly useful for the development of double transgenic AβPP/PSEN mice, which display an elevated $A\beta_{42}/A\beta_{40}$ ratio and accelerated Aβ pathology compared to the single AβPP model they are based on, thereby supporting the modifying role of PSEN. In addition, these AβPP/PSEN mice display amyloid-associated inflammation, neuronal loss, cognitive decline, and BPSD-like behavioural alterations [139, 140]. The major loophole of all the above-mentioned models—lack of NFT formation—was moderately overcome by the development of transgenic mice having human tau insertion and the subsequent crossing of tau and AβPP models, the latter including enhanced amyloid deposition accompanied by tau hyperphosphorylation, NFT-like formation, and obvious death of neurons, thereby supporting the amyloid cascade hypothesis affirming that Aβ pathology mediates tau pathology. However, there is no colocalization of plaques and NFT in AD brain of AβPP/tau mice. This limitation was compensated with the development of the triple transgenic (3xTg) mouse [78]. Instead of crossing independent mutant mouse lines, two transgenic constructs (mutant AβPP and tau) were microinjected into single-cell embryos of homozygous mutant PSEN1 mice, thereby preventing segregation of AβPP and tau genes in succeeding generations. In accordance with the amyloid cascade theory, these 3xTg mice develop Aβ plaques prior to NFT pathology with a temporal and spatial profile equivalent to AD, in addition to inflammation, synaptic dysfunction, and cognitive decline [141].

The generation of transgenic rodent research models that develop some of the pathological hallmarks of AD has given a substantial boost to drug discovery efforts and has also raised many intriguing questions about the underlying disease process. However, one should never neglect the potential danger of uncritical extrapolating from mouse/rat to humans. The fact that at the moment no animal model recapitulates all aspects of human AD reflects the limitations of using a rodent system to model a human condition that takes decades to develop and primarily involves higher cognitive functions.

11.2. Caenorhabditis elegans Transgenic AD Model. *Caenorhabditis elegans*, a free-living small nematode of approximately 1.2 mm in length, has several characteristics that make it useful as a model organism. The nematodes are transparent, which allows the study of embryonic development and gene expression in living animals under the microscope. It was first used to study molecular and developmental biology by Syndey Brenner in the 1970s [142]. This invertebrate was the first animal for which an entire genome was sequenced and has become one of the most popular model organisms to study neurodegenerative disease, as demonstrated by the development of numerous transgenic disease models, including for Alzheimer's disease (discussed below).

Several AD-related genes and pathways found in humans have orthologues in *C. elegans*. The nematode genome encodes three orthologues for PSEN1: (i) sel-12, (ii) hop-1, and (iii) spe-4. The first has been found in a screen for suppressors of the egg-laying defective phenotype in lin-12 gain-of-function worms [143]; it facilitates Notch/lin-12 signalling, functional mostly during embryonic development. The second, that is hop-1, homolog of PSEN1 [144], in fact shares more homology to human PSEN2; and the third, spe-4, has no clear human counterpart [144]. Three genes, aph-1, pen-2, and aph-2, combine together to form a functional complex of γ-secretase. In addition, an orthologue of Aβ (apl-1) has been described in *C. elegans* [145]. Similar to *Drosophila*, the APL-1 protein does not contain the Aβ sequence; neither does *C. elegans* display BACE1-like activity.

There are basically three Aβ-expressing nematode models which have been developed. When expressed in muscle cells, $A\beta_{1-42}$ induced the formation of amyloid-immunoreactive inclusions. A subset of these deposits also binds the Aβ-specific dye thioflavin S, showing that amyloid fibrils are formed similar to human AD. In addition, paralysis of the nematodes occurred, thereby indicating a muscle cell specific toxicity of Aβ [146]. Nematodes expressing $A\beta_{1-42}$ in neuronal cells also develop Aβ deposits but display only a very

subtle phenotype [147]. Interestingly, oligomeric Aβ were detected in these strains that might be similar to the neurotoxic Aβ-derived diffusible ligands [148]. These transgenic models provide important insight into the toxicity of specific Aβ species but do not allow screening of chemical or genetic modifiers of AβPP processing.

To create tauopathy models of nematode, both wild-type and mutated human tau proteins were expressed in neurons of *C. elegans*, inducing a progressive phenotype of defective motility (uncoordinated phenotype), which was more deceptive in the mutants. Interestingly, these transgenic lines also display hyperphosphorylation of tau [149], which is linked to GSK-3β activation. Future genome-wide screens will display which modifier genes are linked to the complex disease process and characterize diagnostic or therapeutic drug targets.

11.3. Drosophila melanogaster as a Transgenic Model of AD. *Drosophila melanogaster* are the most commonly used species of *Drosophila* in the laboratory worldwide for research purposes. Their use in the modelling of human neurodegenerative disease is based on the inherent assumption that the fundamental aspects of cell biology are conserved throughout evolution in higher organisms. This is supported by the fact that ~75% of human disease-related genes have homologs in *Drosophila*, suggesting that molecular mechanisms of any disease in humans may be conserved in the fly.

There are many compelling reasons to study AD in the *Drosophila* model. The *Drosophila* brain has approximate 300,000 neurons and is organized into the area with separate, specialized functions such as memory, learning, olfaction, and vision, similar to human. *Drosophila*, on account of its very short generation time (10–12 days) and easy maintenance, is a popular model in genetic research. Although one could argue that the *Drosophila*'s maximum lifespan of 55–80 days is significantly greater than that of the worm (~18 days), it is still much shorter than that of the mouse (2-3 years), making it ideal for studying a progressive age-related disease such as AD.

In addition, the *Drosophila* has an unrivalled battery of genetic tools, including a fully sequenced genome; an extensive library of mutant stocks including RNA interference (RNAi) and knockout (KO) lines; sophisticated transposon-based methods for gene manipulation; systems for spatial and temporal specific ectopic gene expression; and balancer chromosomes. Balancer chromosomes are unique: composed of multiple inversions that prevent recombination, together with dominant, lethal, and visible markers. They allow the maintenance in long-term culture of lethal or deleterious mutations in heterozygotes, without the necessity to set up specific crosses.

The combination of such extensive genetic tools and practicality makes the *Drosophila* ideal for genetic screening. A variety of screening methods are available in the *Drosophila*, involving chemical mutagenesis (EMS), genetic deletion kits, or mobile genetic elements (P, EP, and GS elements). Genetic screens are powerful experiments providing an unbiased forward genetic approach, which allows the discovery of genes or metabolic pathways not immediately apparent in the pathogenesis of AD.

11.4. Drug Screens Using Drosophila AD Models. Another potential use for a characterized *Drosophila* disease model is its use for novel drug screening. The secreted Aβ peptide fly model was verified as a platform for drug discovery by testing the efficacy of a drug used to treat human AD patients and was shown to slow progression of AD [150]. The drug memantine, a noncompetitive glutamate antagonist, is effective in slowing progression of human AD [151]. In addition, the life span of flies expressing two copies of $A\beta_{42}$ or one copy of $A\beta_{42}$-arctic was increased when flies were treated with MK-801, an inhibitor of the excitatory action of glutamate on the NMDA receptor [150]. A therapeutic intervention that is effective in human AD patients is, therefore, also effective in the fly; thus the fly AD model is useful for testing novel human drugs. Congo red, which binds to Aβ and has been shown to reduce neurodegeneration in a fly model of polyQ disease [152] and a mouse model of Huntington's disease [153], has also been shown to reverse the reduced life span of flies expressing two copies of $A\beta_{42}$ or one copy of $A\beta_{42}$-arctic [150].

The AβPP processing model in *Drosophila* has also been used in drug validation studies. Ubiquitous expression of AβPP, BACE, and DPsn resulted in reduced longevity and a visible wing phenotype, which were used for screening β- and γ-secretase inhibitors [154]. Feeding flies with either β- or γ-secretase inhibitors resulted in an increased survival of AβPP/BACE/DPSn expressing transgenic flies, making this fly model useful for investigating drugs that modulate AβPP processing and have the potential to decrease Aβ-induced cellular degeneration [154]. Singh in his doctoral thesis showed neuroprotective effect of some herbal compounds targeting anti-Aβ therapeutic approach using *Drosophila* model of AD [155].

12. Aβ and Metallosis

12.1. Metal Ions and Aβ Toxicity. In case of AD, elevated metal ions concentrations have been demonstrated in several studies [156–159], in particular copper and zinc which are associated with both the aggregation and the neurotoxicity of Aβ peptides, and proposed as an important factor in neuropathology of AD [156, 159–162].

An extensive number of reports have provided empirical data showing metal mediated toxicity of amyloid-β, but a detailed NMR or X-ray diffraction atomic structure is yet to be described [163, 164]. However, Azimi and Rauk [165] were able to use MD simulations to demonstrate that Aβ-copper coordinated structures can form both parallel and antiparallel conformations. Zinc ions have been shown to form intermolecular complexes while copper ions tend to form intramolecular complexes cross-linking multiple peptides [166–168]. The schematic in Figure 7(a) shows the details of metal ion mediated Aβ toxicity which results in fibril formation and leads to AD. Figure 7(b) depicts Aβ-copper interactions [162]. Interaction of Aβ amino acid

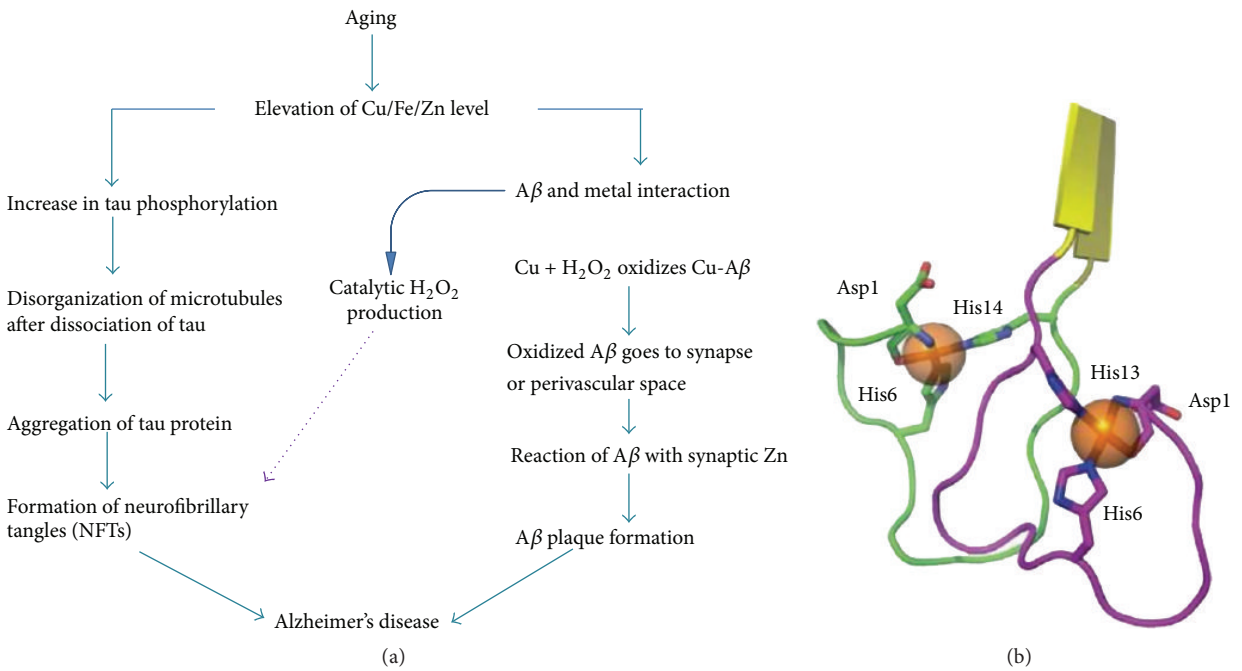

Figure 7: (a) Proposed model for AD pathology based on abnormal metal interaction. Cu and Fe levels increase during aging in the CNS and result in increase in metal and Aβ interaction. Cu binding to Aβ results in ROS production and autooxidation of Aβ peptide. Oxidized Aβ contributes to synaptic pathology and plaque formation. Metals may also promote phosphorylation of tau and hence enhance formation of NFT which further contribute to AD pathology. (b) Model showing the amyloid-copper interaction. Notice the coordination sites at His 6 from one peptide together with His13 and His14 from the second peptide.

residues with copper is shown in figure which leads to Cu mediated Aβ toxicity. Copper has been shown to interact with amyloid-β at the His13 and His14 residues on one peptide with the His6 residue on the other peptide.

Histidine is well known as Zn^{2+} and Cu^{2+} ligand for many other proteins and peptides [169, 170]. Coordination of Zn^{2+} to His13 and His14 but not His6 has been found to be critical to induce $A\beta_{1-40}$ aggregation [171, 172]. Cu^{2+} is able to compete with Zn^{2+} for binding to histidine residues of Aβ and at low concentration it inhibits the ability of Zn^{2+} to induce aggregation, but at higher Cu^{2+} concentrations aggregation does occur [173].

It has been reported that the levels of copper (0.4 ± 0.1 mg/g of wet weight of plaque) and zinc (1.2 ± 0.2 mg/g) are found to be high in the senile plaques found within AD brains [157]. Use of a Cu-Zn chelator, such as clioquinol, inhibits Aβ accumulation in AD transgenic mice [174, 175], which highlights the importance of studying copper and zinc binding to Aβ.

12.2. ROS Generation by Metal Mediated Aβ Toxicity. The coordination of metal ions such as copper, iron, and zinc to Aβ also results in the chemical reduction of these metals and the subsequent generation of hydrogen peroxide from molecular oxygen together with other available biological reducing agents such as cholesterol, in a catalytic manner [176–178]. In the case of $A\beta_{42}$, the reduction of copper is independent of the aggregation state of the peptide, as both soluble and fibrillar forms show copper-reducing ability.

The generation of hydrogen peroxide in the presence of reduced metals, and in the absence of sufficient detoxifying enzymes such as catalase and glutathione peroxidase, gives rise to the toxic hydroxyl radical via Fenton chemistry [176]. The generation of hydrogen peroxide contributes to Aβ toxicity. In support of this, cellular toxicity can be rescued by the addition of catalase [178, 179]. Further, resistance to Aβ toxicity is associated with an enhanced ability to degrade hydrogen peroxide [180], and catalase inhibitors can enhance Aβ toxicity. The potentiation of Aβ toxicity by copper is the greatest for $A\beta_{42} > A\beta_{40} >$ rodent $A\beta_{40}$, which corresponds to the peptide relative activities in reducing copper(II) to copper(I) [176]. These data support a role for Aβ in the generation of hydrogen peroxide via metal ion reduction and for oxidative processes in the augmentation of Aβ to potentiate the AD cascade. A summary of the proposed role of metal ions in AD has already been shown in Figure 7(a).

12.3. Neuroprotective Role of Metal Chelator against Aβ Induced Toxicity. To prevent metal mediated neurotoxicity of Aβ, researchers are focusing on chelation therapy. Chelation therapy is the use of metal specific chelators which are able to chelate extra metal ions present in brain; hence reduced possibility of interaction of these metals with Aβ, and ultimately a slowdown of metal, mediated Aβ-toxicity.

Traditional metal chelators have been used to sequester or redistribute metal ions from metal-bound Aβ species in order to suppress metal mediated Aβ neurotoxicity *in vitro* and *in vivo* [181–184]. Cherny et al. [174] initially reported that

Cu/Zn chelators solubilize Aβ from tissues of postmortem AD brain. They choose the clioquinol (CQ, 5-chloro-7-iodo-8-hydroxyquinoline) based on its ability to cross the blood-brain barrier as tested in AD transgenic mice (Tg2576) and found significantly reduced levels (~65%) of Aβ-aggregates as well as ROS generation in CQ treated mice as compared to control.

Ongoing research in this area focuses on the prevention of metal mediated Aβ neurotoxicity and ROS production by metal chelating therapy, which is an emerging trend in current research. There is immense need to develop such a suitable metal chelator that can prevent Aβ aggregation by effectively sequestering extra metal ions. Several groups focused on developing such type of new molecules [182, 185, 186]. More particularly, in a pioneering work, Lakatos et al. developed two carbohydrate-containing compounds, N,N′-bis[(5-β-D-glucopyranosyloxy-2-hydroxy) benzyl]-N,N′-dimethyl-ethane-1,2-diamine (H2GL1) and N,N′-bis[(5-β-D-glucopyranosyloxy-3-tert-butyl-2-hydroxy) benzyl]-N,N′-dimethyl-ethane-1,2-diamine (H2GL2), that are shown to be promising therapeutic tools against AD, based on *in vitro* studies [185]. In this context, we designed and synthesized novel compound L, 2,6-pyridinedicarboxylic acid, 2,6-bis[2-[(4-carboxyphenyl)methylene]hydrazide], to test the *in vivo* neuroprotective efficacy in a well-established *Drosophila* transgenic model system. Recently Singh et al. reported the neuroprotective role of a novel copper chelator against copper mediated Aβ toxicity [162].

13. Neuroprotective Role of Flavonoid against AD

Naturally occurring as well as synthetically synthesized dietary flavonoids have been extensively used as alternative candidates for Alzheimer's treatment, taking into account their antioxidative, antiamyloidogenic, and anti-inflammatory properties. Experimental evidence from different studies supports the hypothesis that certain flavonoids may protect against AD, in part by interfering with the generation and assembly of amyloid-β peptides into neurotoxic oligomeric aggregates and also by reducing tau aggregation. Dietary supplementation studies using flavonoid-rich plant or food extracts have shown their ability to influence cognition and learning in humans and also in animal models of diseases [187–192]. Presently, there is no direct association between flavonoid consumption and improvement in neurological health. Nevertheless, the potential beneficial effect of flavonoids in the brain seems to be related to their ability to interact with intracellular neuronal and glial signalling pathways, thus influencing the peripheral and cerebral vascular system, protecting vulnerable neurons, enhancing existing neuronal function, or stimulating neuronal regeneration.

Flavonoids are naturally occurring polyphenolic compounds widely spread in plants. They are present in foods and beverages of plant origin such as a variety of fruits, vegetables, cocoa, cereals, tea, and wine [193]. The six main subclasses of flavonoids include (1) flavonols (e.g., kaempferol, quercetin), present in onions, leeks, and broccoli; (2) isoflavones (e.g., daidzein, genistein), found mainly in soy and soy products; (3) flavones (e.g., apigenin, luteolin), present in parsley and celery; (4) flavanols (e.g., catechin, epicatechin, epigallocatechin, and epigallocatechin gallate (EGCG)), abundant in green tea, red wine, and chocolate; (5) flavanones (e.g., hesperetin, naringenin), primarily found in citrus fruit and tomatoes; and finally (6) anthocyanidins (e.g., pelargonidin, cyanidin, and malvidin), sources of which include berry fruits and red wine.

It was thought that the ability of flavonoids to promote memory, learning, and cognitive function was mediated by their antioxidant capacity [194]. Nevertheless, due to their limited absorption and their low bioavailability in the brain, increasing evidence demonstrates that they are able to interact with the cellular and molecular components of the brain responsible for memory, having the potential to protect vulnerable neurons, enhance existing neuronal function, stimulate neuronal regeneration, and induce neurogenesis [194, 195]. Recent study from our lab showed the neuroprotective property of a novel synthetic flavonoid derivative against Aβ-induce neurotoxicity in *Drosophila* model of AD [196].

14. Neuroprotective Role of Natural Polyphenols in AD

Nature has gifted mankind with a plethora of vegetables, flora-bearing fruits, and nuts. Natural polyphenols are the most commonly found chemical compounds in consumable herbal beverages and food worldwide [197, 198]. They constitute a large group of phytochemicals with more than 8000 identified compounds. The variety of bioactive nutrients present in these natural products play a central role in prevention and cure of various human neurodegenerative diseases, such as Alzheimer's disease (AD), Parkinson's disease, and other kinds of neuronal damage. Plants have a long history as a rich source of new bioactive compounds for drug discovery and may have advantages in relation to efficacy. Several reports documented the effectiveness of herbal extracts over isolated material, in protection against lipid peroxidation [199], and anticancer effects [200]. For example, a mixture of carotenoids has been found to be more effective than any one single carotenoid in protecting liposomes against lipid peroxidation [199].

Polyphenolic compounds from medicinal plants are key sources of neuroprotective agents against AD. Using the structure of these bioactive ingredients as templates for synthetic drugs offers a wide range of potential neuroprotective compounds [201]. In the past few decades, several studies attempted to measure the effect of total plant extract on AD and to isolate the active component responsible for the neuroprotective effects [202, 203].

Natural polyphenols reveal their antioxidant effect by reducing free radical species and/or encouraging endogenous antioxidant capacity. Thus, the antioxidant properties positively contribute to their neuroprotective effects. Furthermore, some of them influence synthesis of endogenous antioxidant molecules in cells via activating Nrf/ARE pathway [204]. Apart from antioxidant property, most of them appear to have a number of different molecular targets, affecting several signalling pathways and showing pleiotropic

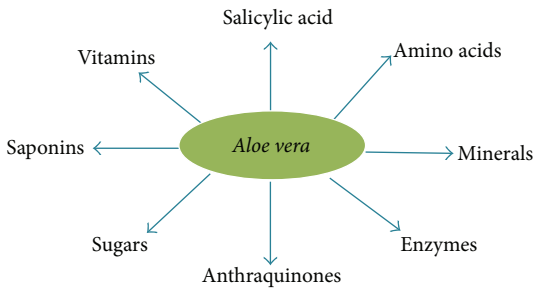

Figure 8: Illustration of the different components present in *Aloe vera*.

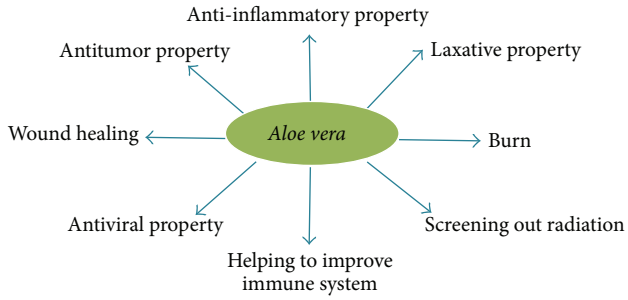

Figure 9: Figure showing the medicinal properties of *Aloe vera*.

activity on cells [205]. For instance, polyphenolic compounds can modulate activity of NF-κB or SIRT1 exerting neuroprotective effects. Recent studies have shown anti-Aβ activity of compounds from natural sources *in vitro* and *in vivo* [206–208]. Still, evidence for the capability of common edible elements to inhibit Aβ oligomerization *in vivo* remains a challenge.

Aloe vera has been used as medicinal agent since Roman times [209]. *A. vera* contains different bioactive components (Figure 8) harbouring over 75 biologically active compounds [210] known to have a wide range of pharmacological activities (Figure 9), including anti-inflammatory, wound healing, antioxidative, antiarthritic, antidiabetic, and antitumorigenic effects [211].

Aloe vera has always been preferred as a herbal remedy and is one of the most popular herbal plants. Major value added products from *Aloe* are gel and juice.

Recently, it has been reported that *Aloe vera*, supplemented orally to mice, is effective on wound healing. *Aloe vera* acts as a free radical scavenger and has other antioxidant properties on diabetic patients by controlling elevated anions in an alloxan- or STZ-induced diabetic animal models [212, 213].

15. Conclusion

Currently the accumulated experimental evidence leans toward strongly supporting the toxic role of Aβ within the pathophysiology of AD. However, the existence of some data regarding the role of Aβ in the normal physiology of the brain suggests that this peptide may act in different modes at different times, according to diverse conditions. So far, it appears that at the initial stages of development and in the young brain, when in physiological doses (i.e., picomolar to nanomolar range) and in soluble, oligomeric forms, Aβ can show neuroprotective, antioxidant, and trophic properties, even facilitating synaptic plasticity. On the contrary, in many potentially adverse conditions, Aβ may deploy multiple toxic effects, contributing significantly to neuronal damage, as seen in AD. Some of these conditions appear to be associated with Aβ itself, such as high concentrations and fibrillar or aggregated states, presence of free metals, brain tissue previously injured or aged, and decreased antioxidative mechanisms. Moreover, it is necessary to remark that both trophic and toxic effects may not be mutually exclusive. In other words, they might coexist and cross-modulate each other, even throughout advanced stages of AD, complicating an approach based upon antiamyloidogenic therapy, at least theoretically. This functional duality may also underlie the modest success and the high rate of collateral consequences of such therapies. In summary, blockade, inhibition, or modulation of those sites, effects, and negative processes in which Aβ is involved, but simultaneously respecting those sites and physiologic processes in which Aβ is also taking part, remain a major challenge for therapeutic research in the future.

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

The Role of Dopamine and Its Dysfunction as a Consequence of Oxidative Stress

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Dopamine is a neurotransmitter that is produced in the substantia nigra, ventral tegmental area, and hypothalamus of the brain. Dysfunction of the dopamine system has been implicated in different nervous system diseases. The level of dopamine transmission increases in response to any type of reward and by a large number of strongly additive drugs. The role of dopamine dysfunction as a consequence of oxidative stress is involved in health and disease. Introduce new potential targets for the development of therapeutic interventions based on antioxidant compounds. The present review focuses on the therapeutic potential of antioxidant compounds as a coadjuvant treatment to conventional neurological disorders is discussed.

1. Introduction to Dopamine

Dopamine (DA) plays a vital role in reward and movement regulation in the brain. In the reward pathway, the production of DA takes place in the ventral tegmental area (VTA), in nerve cell bodies. From there, it is released into the nucleus accumbens and prefrontal cortex. In vivo, the concentration of DA in the VTA is 4.8 ± 1.5 nM, while in red nucleus, it is 0.5 ± 1.5 nM [1]. The pathway for motor functions is different. In this pathway, the substantia nigra cell bodies are responsible for the production and discharge of DA into the striatum. DA plays multiple functions in the brain. Calabresi et al. reported the role of DA in the modulation of behavior and cognition; voluntary movement; motivation; punishment and reward; inhibition of prolactin production; sleep; dreaming; mood; attention; working memory; and learning [2].

DA can be a precursor in the biosynthesis of other related catecholamines such as norepinephrine and epinephrine (Figure 1). Norepinephrine is synthesized from DA by the catalytic action of DA β-hydroxylase in the presence of L-ascorbic acid and molecular oxygen (O_2). Norepinephrine then acted upon by the enzyme phenylethanolamine *N*-methyltransferase with *S*-adenosyl-L-methionine (SAMe) as a cofactor to produce epinephrine.

The biosynthesis of DA and other catecholamines can be limited by the action of enzyme tyrosine hydroxylase (TH) [3]; therefore, regulatory mechanisms of TH could be promising for improving gene therapy approaches and other treatment modalities [4]. After the synthesis of DA, it is incorporated into synaptic vesicles by the action of vesicular monoamine transporter 2 ($VMAT_2$), where it is stored. DA is discharged by exocytosis into the cell membrane and dumped into the synapse.

2. Dopamine Receptors

In the synapse, DA binds to either postsynaptic or presynaptic DA receptors or both. This bond, regardless of the receptor, generates an electric potential in the presynaptic cell [5].

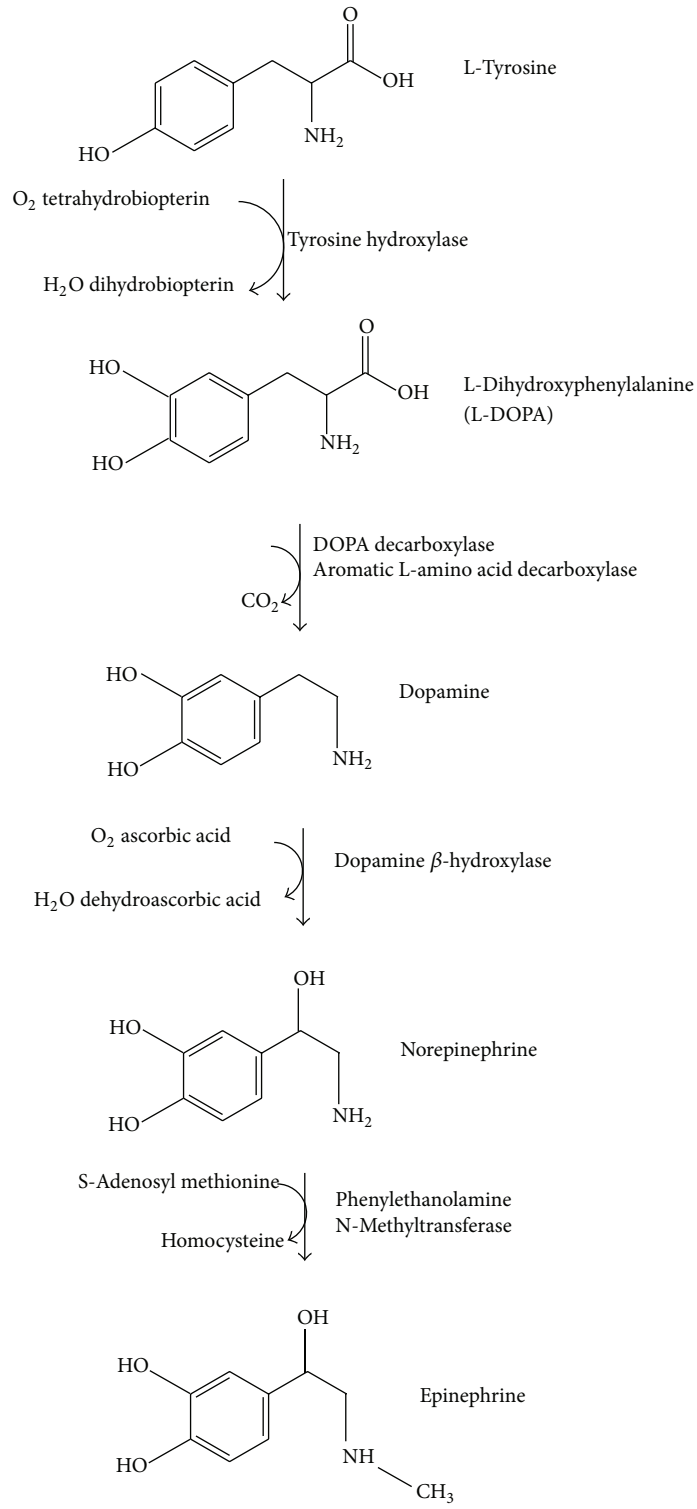

Figure 1: Catecholamine biosynthesis.

In the case of postsynaptic DA receptors, the signal is propagated to the postsynaptic neuron, while, in the case of presynaptic DA receptors, the signal can either excite the presynaptic cell or inhibit it. Presynaptic receptors with an inhibitory potential, also known as autoreceptors, inhibit the synthesis and release of neurotransmitters and thus function to maintain normal levels of DA. After carrying out its synaptic function, DA is taken up again into the cytosol by presynaptic cells through the actions of either high-affinity DA transporters (DAT) or low-affinity plasma

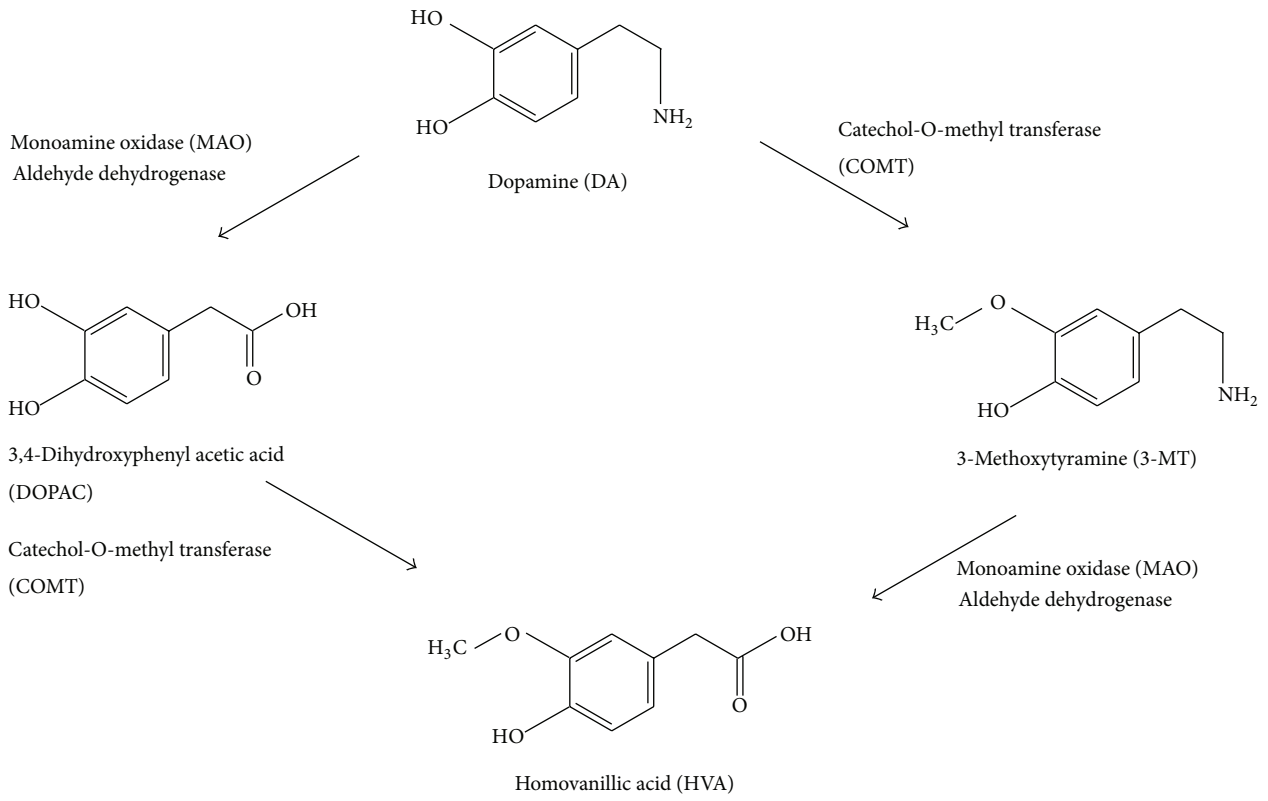

Figure 2: Dopamine metabolism.

membrane monoamine transporters. Once in the synaptic neuron, amphetamine exercises a reverse influence on the action of DA transporters (DAT) and forces DA molecules out of storage vesicles and into the synaptic gap [6]. The DA transporter is a sodium-coupled symporter protein responsible for modulating the concentration of extraneuronal DA in the brain [7]. The DA now in the cytosol is then repackaged into vesicles by the action of vesicular monoamine transport, $VMAT_2$ [8].

3. Metabolism of DA

The enzymatic breakdown of DA to its inactive metabolites is carried out by catechol-*O*-methyl transferase (COMT) and monoamine oxidase (MAO) (Figure 2). This degradative action can be performed by the MAO isoforms MAO-A and MAO-B. It should be noted that COMT is predominantly expressed by glial cells. In neurons, this enzyme is either missing or found at very low levels. MAO-B is mainly found in astrocytes, whereas MAO-A predominates in catecholaminergic neurons like the cells of the SN. MAO breaks down dopamine to 3,4-dihydroxyphenylacetaldehyde (DOPAL), which in turn is degraded to form 3,4-dihydroxyphenylacetic acid (DOPAC) by the action of the enzyme aldehyde dehydrogenase (Figure 3) [9].

Another pathway for the metabolism of DA involves the enzyme COMT, which converts it to 3-methoxytyramine (3-MT). Then, 3-MT is reduced by MAO to HVA and eliminated in the urine. As a result, the inhibition of monoamine oxidase has been considered as an adjunctive therapy in neurodegenerative disorders such as Alzheimer's and Parkinson's disease (PD) [10]. However, MAO inhibitors are used to increase DA levels and not to decrease hydrogen peroxide production. Actually, neurons have different antioxidant systems, for example, catalase and glutathione, to cope with H_2O_2 production. Furthermore, the MAO-derived DOPAC metabolite is probably much more toxic than H_2O_2. The inactivation of DA in the brain, striatum, and basal ganglia is mediated by reuptake via DAT followed by enzymatic action of MAO, which breaks it down to DOPAC. Nevertheless, there are few DATs in the frontal cortex, and this leads to the breakdown of DA via another pathway that involves the norepinephrine transporter (NET) on neighboring norepinephrine neurons, proceeded by the enzymatic action of COMT that breaks DA down to 3-MT [11], which may be a way to design therapies against neurological disorders. The velocity of DA degradation is usually faster in the DAT pathway than in NET. In mice, DA is degraded in the caudate nucleus via the DAT pathway within 200 milliseconds, in comparison with 2,000 milliseconds in the frontal cortex [11]. Nondegraded DA is repackaged by $VMAT_2$ in the vesicles for reuse.

Dopaminergic neurons are found principally in the VTA of the midbrain, the substantia nigra pars compact, and the arcuate nucleus of hypothalamus. The axons of these neurons project to different areas of the brain through major pathways known as mesocortical, mesolimbic, and nigrostriatal pathways [12]. The mesolimbic pathway connects the VTA to the nucleus accumbens. The somata of the neurons

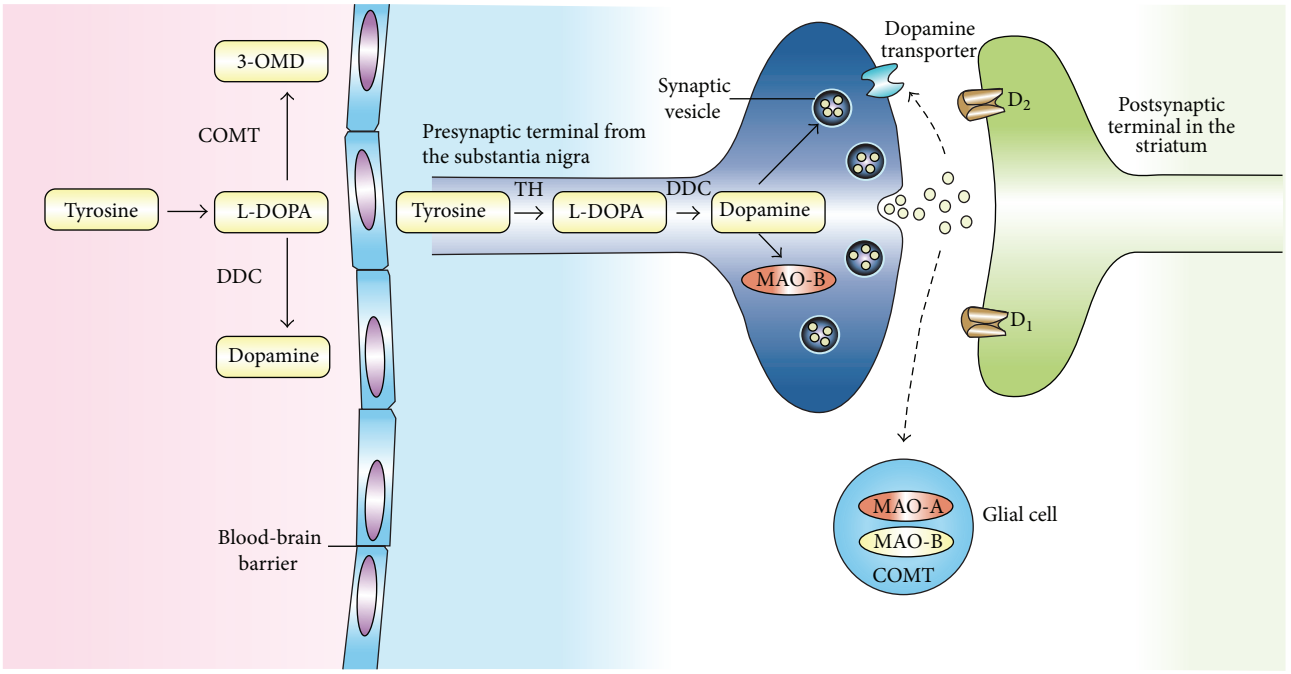

Figure 3: Dopamine metabolism pathways.

originate in the VTA, and, from there, DA is transported to the nucleus accumbens through the amygdala and the hippocampus. The nigrostriatal pathway joins the substantia nigra with the neostriatum. The neuronal somata are located in the substantia nigra, and the axons of these neurons are ramified into the caudate nucleus and putamen. This pathway is also connected to the basal ganglia motor loop. All of the innervations originating from these pathways explain many of the effects produced when the DA system is activated [13]. For instance, the VTA and the nucleus accumbens connected through the mesolimbic pathway are central to the brain reward system [14].

The modulation of extracellular DA levels occurs by two mechanisms, designated as tonic and phasic DA transmission. The former takes place when a small amount of DA is discharged independent of neuronal activity. This type of discharge is usually regulated by the activity of neurons and neurotransmitter reuptake [15]. The latter occurs when DA is released by the activity of DA-containing cells. Schultz et al. in a study carried out in monkeys reported that this activity is characterized by the irregular pacemaking activity of single spikes and rapid bursts of typically 2–6 spikes in quick succession [16], while Brozoski et al. affirmed that concentrated bursts of activity result in a greater increase of extracellular DA levels than would be expected from the same number of spikes distributed over a longer period of time [17], as a consequence of dopamine metabolism.

4. The Reuptake

DA reuptake can be inhibited by cocaine and amphetamines, but each has a different mechanism of action [18]. Cocaine is a DA transporter and norepinephrine transporter blocker. It inhibits the uptake of DA, which results in an increase in DA lifetime, thereby producing an overabundance. Disruptions in these mechanisms following chronic cocaine use contribute to addiction, due, in part, to the unique architecture of the mesocortical pathway. By blocking dopamine reuptake in the cortex, cocaine elevates dopamine signaling at extrasynaptic receptors, prolonging D_1-receptor activation and the subsequent activation of intracellular signaling cascades, and thus induces long-lasting maladaptive plasticity [19]. Although Barr et al. have identified a novel mechanism by which cocaine promotes activation of D_1-expressing nAcc neurons, the enhancement of inositol 1,4,5-trisphosphate receptors (IP_3R) mediated responses via σ_1R activation at the endoplasmic reticulum, resulting in augmented Ca^{2+} release and amplified depolarization due to subsequent stimulation of transient receptor potential canonical channels (TRPC) [20].

5. Role of Dopamine in Oxidative Stress

It is well known that mitochondrial dysfunction and oxidative stress contribute in a significant way to the development of PD [21].

A loss of 5–10% of dopaminergic neurons has been found in every decade of aging and an increase in brain oxidative damage is associated with age, and aging is considered a risk factor for PD. The expansive nature of oxidative damage includes mitochondrial dysfunction, DA autooxidation, α-synuclein aggregation, glial cell activation, alterations in calcium signaling, and excess-free iron. An increased incidence of PD may be correlated with alterations in the transcriptional activity of various pathways, including nuclear factor erythroid 2-related factor 2, glycogen synthase kinase 3β,

mitogen activated protein kinase, nuclear factor kappa B, and the reduced activity of superoxide dismutase, catalase, and glutathione with aging [22]. PD is a neurodegenerative disease that usually affects people older than 65 years [23].

Blast-wave-induced traumatic brain injury results in increased hypothalamic expression of oxidative stress markers and activation of the sympathoadrenal medullary axis, due to increased sympathetic excitation. This mechanism may involve elevated AT1 receptor expression and NADPH oxidase levels in the hypothalamus, which is related to DA [24].

The pathway to mitochondrial dysfunction begins with oxidative phosphorylation, which produces superoxide radicals, formed by one superoxide anion, one hydroxyl radical, and free radicals (FR) that come from organic compounds. Alcoxyl, peroxyl, hydrogen peroxide, and singlet oxygen [25], are byproducts that are deposited in the mitochondria, thereby making this organelle the main site for the generation of reactive oxygen species (ROS) within the cell and the first line of defense against oxidative stress [26]. However, superoxide also functions as a signaling molecule, different from signals mediated by hydrogen peroxide, hydroxyl radicals, or peroxynitrite. Although, a well-known role of superoxide is a precursor of reactive hydroxyl radicals by the superoxide-dependent Fenton reaction, the formation of peroxynitrite results in damage to target molecules and leads to pathological disorders, as was reported by Afanas'ev [27]. This author suggested that superoxide signaling depends on nucleophilic reactions. It is necessary to clarify that an oxidant is an element or compound in an oxidation-reduction (redox) reaction that accepts an electron from another species. Due to the fact that it gains electrons, a superoxidant is often a molecule that contains many oxygen atoms and offers a high oxidant capacity.

Studies have suggested that mitochondrial c-Jun N-terminal kinase (JNK) plays a role in the etiology of 6-hydroxydopamine- (6-OHDA-) induced oxidative stress [28]. These authors suggest that 6-OHDA induced cell death through activating PI3K/Akt pathway and inhibiting JNK pathway. On this basis, it was suggested that inhibitors that block the association of JNKs within the mitochondria might be useful neuroprotective agents for the treatment of PD [27], and probably dysfunction in the projections of dopaminergic neurons of the nigrostriatal DA pathway from the substantia nigra to the dorsal striatum would slowly lead to PD [29].

Oxidative stress and hydrogen peroxide (H_2O_2) have been implicated as the underlying factors in the initiation and progression of PD. Increases in endogenous H_2O_2 in the dorsal striatum attenuated electrically evoked DA release and also decreased basal DA levels [30]. The degeneration of the nigrostriatal pathway in PD is associated with oxidative stress and oxidized DA [31]. On the other hand, selenium transport protein (selenoprotein P) and Sepp1 expressed by neurons of the substantia nigra of the midbrain indicate a role for Sepp1 in the nigrostriatal pathway, which suggests that local release of Sepp1 in the striatum may be important for signaling and/or synthesis of other selenoproteins with neuroprotective activity [32]. Selenoprotein P (Sepp1) and its receptor, apolipoprotein E receptor 2 (apoER2), account for brain retaining selenium better than other tissues, Sepp1-apoER2 interactions supply selenium for maintenance of brain neurons, to protect the severe neurodegeneration and death in mild selenium deficiency [33].

Pharmacological inhibition of brain inflammation and endoplasmic reticulum stress prevented glucose intolerance due to Aβ oligomers (AβOs), which act via a central route to affect peripheral glucose homeostasis [34]. Aβ oligomers affect the hypothalamus and reveal a link between hypothalamic dysfunctions in metabolic disorders [35]. The consumption of β-phenethylamine- (β-PEA-) containing food for a long time is a neurological risk with many pathological consequences [36]. β-PEA toxicity is associated with hydroxyl radical (HO) production and oxidative stress generation in dopaminergic areas of the brain. β-PEA toxicity may be blocked by inhibition of mitochondrial complex-I [37].

PD has a multifactorial mechanism. Oxidative stress and neuroinflammation, including activation of NADPH-dependent oxidases, play a major role in the progression of dopaminergic cell death [38]. A possible role for DNA repair systems in ageing and neurodegenerative diseases after DNA damage was observed in the brain of individuals affected by neurodegenerative diseases. A study of DNA repair gene polymorphisms (XRCC1 Arg399Gln, XRCC3 Thr241Met XPD Lys751Gln, XPG Asp1104His, APE1 Asp148Glu, and HOGG1 Ser326Cys) suggested that APE1, XRCC1, and XRCC3 genetic variants might be a risk factor for PD by increasing oxidative stress, which might cause the loss of dopaminergic cells in the substantia nigra and locus coeruleus, which could in turn lead to abnormal signal transmission and the development of PD [39].

NADPH oxidase (NOX) was originally identified in immune cells, playing an important microbicidal role. In neurodegenerative and cerebrovascular diseases, inflammation is increasingly being recognized as contributing negatively to neurological outcome, with NADPH oxidase as an important source of superoxide. The activated enzyme complex transports electrons to oxygen, thus producing the superoxide anion ($O_2^{\bullet -}$), a precursor of reactive oxygen species, and is the advantage of a targeted NADPH oxidase inhibitor that would inhibit the production of superoxide [40]. Indeed, Nox1/Rac1 could serve as a potential therapeutic target for PD because dopaminergic neurons are equipped with a Nox1/Rac1 superoxide-generating system; however, stress-induced Nox1/Rac1 activation causes oxidative DNA damage and neurodegeneration [41].

Another possible etiology of PD could be due to the loss of serum response factor (SRF), which leads to a decrease in the levels of antiapoptotic proteins, brain-derived neurotrophic factor (BDNF), and Bcl-2, all of which are considered to be a key cause of increased sensitivity to oxidative stress and dysfunction of the SRF-activating mitogen-associated kinase pathway [42]. Organs with a reduced capacity for regeneration like the brain are highly affected by inflammation, and neuroinflammation is recognized as a major contributor to epileptogenesis [43].

Peripheral inflammation provokes brain immune response involving microglial activation, elaboration of proinflammatory

Table 1: Studies of drugs that alter levels of dopamine or its metabolites in clinical disorders. ↑ up, ↓ down.

Drug	Clinical disorder	Dopamine or metabolites	Ref.
Rasagiline	Antidepressant	MAO-A and MAO-B in the brain ↓	[86]
Methamphetamine (METH)	Addiction	Expression of fosb, fra1, and fra2 in the nucleus accumbens (NAc) ↓	[87]
Ladostigil	Antidepressant	MAO-A and MAO-B in the brain ↓	[86]
Risperidone/donepezil	Parkinsonian features	Dopamine transporter activity ↑	[88]
Cocaine, heroin, or methamphetamine	Addiction	Extracellular dopamine in CNS ↑	[89]
1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)	Parkinsonian features	Dopamine and TH ↓	[90]
PAOPA	Schizophrenia	Active site of the dopamine D(2) receptor ↓	[91]
Methylphenidate	Cocaine addiction	Dopamine transporter ↓	[92]
Phenelzine	Depression and anxiety disorders	Dopamine levels in brain ↑	[93]
Amphetamine	Attention deficit hyperactivity disorder	Extracellular dopamine ↑	[94]
L-DOPA	Parkinson disease	Brain dopamine levels ↑	[95]
3,4-Methylenedioxymethamphetamine	Addiction	Brain dopamine levels ↑	[96]
Flupenthixol, perphenazine, and zotepine	Tauopathies	Dopamine D(2) receptor ↓	[97]
Asenapine	Acute schizophrenia, manic episodes, bipolar I disorder	Brain dopamine levels ↑	[98]
Pramipexole	Depression	Dopamine receptor D(3) ↑	[99]

cytokines, and reactive oxygen species. Thus, inflammation produces a secondary injury to neurons. A significant part of this response in the brain is mediated by cyclooxygenase (COX) and COX-2 through downstream proinflammatory prostaglandin (PG) signaling [44]. The anti-inflammatory effect of COX in the brain is mediated by PGE_2 EP_4 signaling, and the findings of Shi et al. [45] identify the PGE(2) EP3 receptor as a novel proinflammatory, proamyloidogenic, and synaptotoxic signaling pathway. Furthermore, the authors suggest a role of COX-PGE(2) EP3 signaling in the development of AD. These data suggest that LPS induced proinflammatory gene expression in the hippocampus and isolated adult microglia is decreased by a EP_4 selective agonist. EP_4 agonists significantly reduced levels of proinflammatory cytokines and chemokines in plasma, indicating that the activation of peripheral EP_4 gives protection to the brain against systemic inflammation. This suggests that an attractive strategy to prevent the onset and/or delay the progression of neurodegenerative diseases should address the mechanism that is directly implicated in controlling oxidative stress and the inflammatory response. This hypothesis is supported by the work of Kato et al., who proposed that microglial modulation may be a key target in the treatment of various psychiatric disorders [46].

The central nervous system and dopaminergic neurotransmission are associated with the development of addiction. This assertion is supported by the argument that drugs such as nicotine, cocaine, and amphetamine directly or indirectly increase the mesolimbic DA reward pathway and by the neurobiological theory that the DA pathway is pathologically altered in addicted persons [47]. Cocaine, nicotine, and amphetamine have both direct and downstream effects on dopaminergic systems. Cocaine affects the HPA axis and brain nuclei responsible for movements. Cocaine's rewarding effects are through its action on dopaminergic signaling pathways. Therefore, any therapeutic strategy for the abuse of these drugs should target the improvement of the efficacy and tolerability of DA transporters and other molecular targets (Table 1) in clinical disorders.

6. The Endocrine System and Dopamine

The depletion of DA may lead to upregulation of the renin-angiotensin system (RAS) to compensate for DA depletion [48]. Nevertheless, hyperactivation of the RAS has many consequences, among which are the aggravation of NADPH oxidase activity and exacerbation of oxidative stress and the microglial inflammatory response and dopaminergic neuron loss [49].

DA is the primary neuroendocrine inhibitor of prolactin secretion by the anterior pituitary gland [50]. The pathway to this inhibitory action begins in the hypothalamic arcuate nucleus, whose neurons produce DA, which is emptied into hypothalamohypophyseal blood vessels of the median eminence, responsible for supplying blood to the anterior pituitary gland, the location of lactotrope cells. These cells

Table 2: Neuroprotector and antioxidant effect of compounds that alter the dopaminergic metabolism.

Substance	Effects	Tissue or animal models	Ref.
Carnosic acid (CA)	Protection against lipid peroxidation and GSH reduction levels and antiapoptotic and antioxidative action	Human neuroblastoma SH-SY5Y cells	[100]
Hesperidin	Reduction in glutathione peroxidase and catalase activity, total reactive antioxidant potential	Striatum mice	[101]
Carnosic acid	Prevent apoptosis through an increase in glutathione S-transferase P (GSTP) expression via activation of the PI3K/Akt/NF-κB pathway	Human neuroblastoma SH-SY5Y cells	[102]
Alkaloids from *Piper longum* (PLA)	Upregulate the activities of SOD, GSH-Px, CAT, the content of GSH, and the total antioxidant capacity and decrease the content of NOS and the content of MDA, NO	Sustantia nigra and striatum of rats	[103]
Novel (E)-3,4-dihydroxystyryl aralkyl sulfones and sulfoxides	Neuroprotective, antioxidative, and antineuroinflammatory properties	Neuronal cells	[104]
Fenofibrate	Protected against decreased level of DA and excessive production of reactive oxygen species (ROS)	Rats	[105]
2-[[(1,1-Dimethylethyl)oxidoimino]-methyl]-3,5,6-trimethylpyrazine (TBN)	Remarkable neurorescue effects to increase the number of dopaminergic neurons and reduce ROS	Mice and rats	[106]
D-440 is a novel highly selective D_3 agonist	Neuroprotection in cell survival and apoptosis	Dopaminergic MN9D cells	[107]
Gallic acid	Significantly increased passive avoidance memory and total thiol and GPx contents and also decreased MDA levels	Nigral tissue	[108]
Garcinia indica extract	Acted as an effective neuroprotective agent for striatal dopaminergic neurons	Rat	[109]
(±)-α-Lipoic acid	Induced reversal of oxidative stress	Human neuroblastoma SH-SY5Y cells	[110]

secrete prolactin continuously in the absence of DA. Thus, DA is sometimes referred to as the prolactin-inhibiting factor (PIF), prolactin-inhibiting hormone (PIH), or prolactostatin [51].

Wang et al. discovered that D_1 and D_4 receptors are responsible for the cognitive-enhancing effects of DA, whereas D_2 receptors are more specific for motor actions [52]. In humans, antipsychotic drugs that have been found to reduce the activities of DA lead to impairments in concentration and reductions in motivation and inability to experience pleasure (anhedonia) [53]. The prolonged use of DA has been associated with tardive dyskinesia, an irreversible movement disorder [54]. Gonadal hormones are greatly affected by antipsychotic drugs. In women, these drugs are associated with low levels of estradiol and progesterone, while, in men, they significantly reduce the levels of testosterone and dehydroepiandrosterone (DHEA) [55].

The gynecological effects of antipsychotic drugs in women center on hyperprolactinemia, whose main consequences are amenorrhea, cessation of the normal ovarian cycle, loss of libido, occasional hirsutism, false positive pregnancy tests, and the long-term risk of osteoporosis [56]. In men, hyperprolactinemia produced by antipsychotics causes gynecomastia, lactation, impotence, loss of libido, and hypospermatogenesis [57]. Other effects of these drugs include weight gain, drooling, diabetes, sexual dysfunction, dysphoria (abnormal depression and discontent), fatigue, heart rhythm problems, stroke, and heart attack [56].

7. Neuroprotective Substances That Alter Dopamine Metabolism

Several studies have reported that antioxidants play an important role in Parkinson's disease [58], and the administration of antioxidant drugs might be used to prevent neuronal death produced by oxidative mechanisms in dopamine metabolism (Table 2).

8. Dopamine Metabolism and Antidepressants

Many drugs with antidepressant and antipsychotic properties, including drugs of abuse and endogenous chemicals such as DA, are primarily metabolized in the liver by cytochrome P450 (CYPs) enzymes. Moreover, this degradation can also occur in extrahepatic organs and the brain. Knowledge of brain CYP-mediated metabolism may help in understanding why patients respond differently to drugs used in psychiatry and may predict the risk for psychiatric disorders, including neurodegenerative diseases and substance abuse [59].

Wood reported the role of opioid and cannabinoid transmission in the modulation of food palatability and pleasure of food consumption and noted that this pathway is independent of brain DA [60]. This may explain why food motivation in animals is independent of brain DA concentration. Nevertheless, other consummatory pleasures as feeling or motivating to a person may be more associated with DA.

The brain reward system is strongly associated with DA, which functions to provoke feelings of enjoyment and reinforcement, both of which motivate a person to perform certain works. The release of DA in areas such as the nucleus accumbens and the prefrontal cortex is principally due to rewarding experiences such as food, sex, drugs, and neutral stimuli that are associated with them [61]. Behavioral activation and effort-related processes are regulated by DA of the mesolimbic area, a critical component of brain circuitry.

The principal source of DA in the brain is the dopaminergic neurons of the midbrain. DA is involved in the control of movement and in error signals for reward prediction, motivation, and cognition [61].

Schizophrenia, autism, attention deficit hyperactivity disorders, and drug abuse are other pathological disorders that have been associated with DA dysfunction.

The firing of dopaminergic neurons has been hypothesized to be motivational as a consequence of reward anticipation. The basis of this hypothesis hinges on the fact that a greater reward than expected leads to an increase in the firing of dopaminergic neurons, which consequently increases desire or motivation towards the reward [61]. Nevertheless, recent findings have revealed that some dopaminergic neurons react in consonance with the expectations of reward neurons, while others seem to respond to unpredictability. Moreover, the same findings showed a predominance of reward neurons in the ventromedial region of the substantia nigra pars compact and in the ventral tegmental area. Neurons in these areas project mainly to the ventral striatum and thus might transmit value-related information in regard to reward values [62]. Nonreward neurons are predominant in the dorsolateral area of the substantia nigra pars compacta, which projects to the dorsal striatum and may relate to orienting behavior. Ideas on the role of DA in desire, motivation, and pleasure emanated from studies carried out in animals. In one such study, rats were subjected to depletion of the neostriatum by 99% using 6-hydroxydopamine and nucleus accumbens DA. Foraging behavior is modulated by DA through the activation of brain systems that register reward upon finding a food source [63]. Highly palatable food raises DA levels in monkey, but a prolonged presence of this palatable food makes DA levels decline [64].

DA in the mesolimbic pathway increases general arousal and goal directed behaviors and decreases latent inhibition. These effects augment the creative drive to generate ideas. Thus, creativity is a three-factor model in which the frontal lobes, the temporal lobes, and the mesolimbic DA system [65] play a part. Some authors suggest that the frontal cortex and striatum are more sensitive to oxidative burden, which could be related to the parallel monoamine perturbations [66].

9. Consideration for Treatments

Individuals suffering from schizophrenia display an increase in the activity of the dopaminergic system in the mesolimbic pathway. There is decreased activity in the mesocortical pathway. Therefore, these two pathways are blamed for the different sets of symptoms in schizophrenia.

Antipsychotic drugs act as DA antagonists [67]. Psychosis and schizophrenia produce highly abnormal dopaminergic transmission. Nevertheless, clinical studies associating schizophrenia with brain DA metabolism have produced controversial or negative results [68]. The levels of HVA in the cerebrospinal fluid are the same in schizophrenics and controls [69]. Antipsychotic drugs have an inhibitory effect on DA at the level of the receptors and block the neurochemical effects in a dose-dependent manner. Typical antipsychotics commonly act on D_2 receptors while they atypically act on D_2 and D_1, D_3 and D_4 receptors, with a low affinity for DA receptors in general [70].

Levodopa is a DA precursor used in various forms to treat PD and dopa-responsive dystonia. Other inhibitors that can be coadministered with levodopa use an alternative metabolic route for producing DA involving catechol-O-methyl transferase. However, oxidative stress and mitochondrial dysfunction can be produced by an increase in endogenous 6-OHDA [71].

As a theoretical possibility, an increase in endogenous 6-OHDA would trigger the formation of Lewy bodies in dopaminergic neurons and eventually lead to their degeneration. Such neurodegeneration could be attenuated using potent antioxidants together with L-DOPA. This would ultimately delay the progression of PD [72]. L-DOPA binds to GPE (Gly-Pro-Glu) by the N-terminal tripeptide of insulin-like growth factor-I. This bond is naturally cleaved in the plasma and brain. GPE has neuroprotective effects since it crosses the blood-CSF and the functional CSF-brain barriers and binds to glial cells, and this tripeptide might represent a promising strategy to supply L-DOPA to Parkinson's patients [73].

The effects of DA on immune cells depend on their physiological state. DA can activate resting T cells, but it can also inhibit them on being activated [74].

This chapter could provide a novel insight into our understanding of the biological mechanisms of neurological disorders and a potential explanation that showed perspectives associated with DA deficits in common clinical disorders that have remained in humans through evolution.

9.1. Amphetamines to Treat DA Disorders. Amphetamine acts to increase DA concentration in the synaptic gap through a mechanism that is different from that of cocaine. The structures of amphetamine and methamphetamine are similar to those of DA [75].

Both have two pathways of entrance into the presynaptic terminal bouton, direct diffusion through the neuronal membrane or uptake via DA transporters [76]. The main target of many drugs, such as psychostimulants, nootropics, antidepressants, and some recreational drugs including cocaine, is the DAT. Some stimulants increase the concentration of DA in the presynaptic cleft, an increase that gives rise to an excitatory effect when these drugs are consumed [77].

By increasing the action of the direct pathway in the basal ganglia, DA reduces the effect of the indirect pathway. Macchi et al. found that insufficient DA biosynthesis in dopaminergic neurons causes PD, a condition in which one loses the ability to execute smooth, controlled movements [78].

In addition to the above functions, DA also plays an important role in the neurocognitive function of the frontal lobe by controlling the flow of information from the brain. Hence, DA disorders in this region of the brain can cause a decline in neurocognitive functions, especially in memory, attention, and problem-solving. Moreover, decreased concentrations of DA in the prefrontal cortex are thought to contribute to attention deficit disorder [79].

10. Expert Commentary

Disorders such as schizophrenia and PD are associated with altered immune function and changes in brain DA receptors and DA signaling pathways. L-DOPA, DA agonists, inhibitors of DA metabolism, or brain grafts with cells expressing a high level of TH are possible treatment methods for PD because of their ability to correct or bypass an enzymatic deficiency that is the key characteristic of this disease. Another promising target in PD treatment is PPAR-γ, which is a key regulator of the immune response. Treatment can also be achieved using agonists with the potential to impact pro- and anti-inflammatory cytokine expression in immune cells at the transcriptional level [80]. Intrastriatal expression of DA synthesizing enzymes could be a promising approach to gene therapy. Expression could be achieved using adenoassociated virus vectors/marrow stromal cells (MSCs) or nonviral intravenous agents involving rat transferrin receptor monoclonal antibodies (TfRmAb) targeted to PE glycated immunoliposomes. The detention or removal of nitrating agents may protect against protein inactivation and limit neuronal injury in PD, thus suggesting the necessity of developing therapeutic agents capable of doing this without interfering with normal neuronal function [81].

The emergence of a highly interesting new area of nonpharmacological treatment of TH dysfunction has occurred in the past few years. TH normalization could provide neuroprotection in PD patients. These new approaches focus on the use of dietetic therapy or the active constituents of plants and phytomedicines, which are believed to provide protection for people suffering from PD [82].

Zhang et al. found that the activation of Akt, a serine/threonine kinase that promotes cell survival and growth, increases the ability of neurons to survive after injury and regenerates lost neuronal connections [83]. These authors suggest that Akt-signaling pathway disinhibition could provide a valuable strategy to enhance survival, function, and integration of grafted DA neurons within the host striatum and improve survival and integration of different forms of neural grafts.

11. Five-Year View

In the last few years, the identification of the relationship between immune and neurodegenerative diseases has been demonstrated based on the effect of monoclonal antibodies. Several antibodies that recognize linear Aβ segments also react with fibrils formed from unrelated amyloid sequences. This suggests that reactivity with linear segments of Aβ does not mean that the antibody is sequence specific [84].

In fact, clinical trials on PD have shown that transplants of embryonic mesencephalic DA neurons form new functional connections within the host striatum, but the therapeutic benefits have been highly variable. One obstacle has been poor survival and integration of grafted DA neurons [85].

This mini review indicates that novel therapies may offer significant improvements and target new mechanisms of neurological disorders. These novel therapeutic strategies involve drugs that act not only on the targets of the dopamine transporter but also on other molecular targets to improve drug efficacy and tolerability and obtain the needed improvements in protein homeostasis to alter the metabolism of DA. We recommend that further studies be carried out in different animal and human models.

Key Points

(i) Dysfunction of dopamine pathways has been implicated in development of Parkinsonism.

(ii) Common biochemical markers of dopamine are used to monitor its effect and its role in disorders.

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

Nitric Oxide Regulates Neurogenesis in the Hippocampus following Seizures

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Hippocampal neurogenesis is changed by brain injury. When neuroinflammation accompanies injury, activation of resident microglial cells promotes the release of inflammatory cytokines and reactive oxygen/nitrogen species like nitric oxide (NO). In these conditions, NO promotes proliferation of neural stem cells (NSC) in the hippocampus. However, little is known about the role of NO in the survival and differentiation of newborn cells in the injured dentate gyrus. Here we investigated the role of NO following seizures in the regulation of proliferation, migration, differentiation, and survival of NSC in the hippocampus using the kainic acid (KA) induced seizure mouse model. We show that NO increased the proliferation of NSC and the number of neuroblasts following seizures but was detrimental to the survival of newborn neurons. NO was also required for the maintenance of long-term neuroinflammation. Taken together, our data show that NO positively contributes to the initial stages of neurogenesis following seizures but compromises survival of newborn neurons.

1. Introduction

Neurogenesis, a multistep process that gives rise to functional and integrated new nerve cells from self-renewal and multipotent neural stem cells (NSC) [1, 2], occurs throughout adulthood in many animal species, including humans [3, 4]. Adult neurogenesis involves proliferation, migration, differentiation and fate determination, survival, maturation, and integration of newborn cells into the preexisting neuronal network. Two main areas are recognized as neurogenic niches in the adult mammalian brain: the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus of hippocampus. Particularly in dentate gyrus, new nerve cells are formed locally at the border between the granular layer and the hilus (SGZ), migrate short distances along the inner granular zone (IGZ), and extend long axonal projections to the CA3 pyramidal cell layer of the hippocampus [5, 6]. It has been shown that neurogenesis can be modulated by different physiological and environmental factors. Hormones, some growth factors, learning, exercise, and antidepressants seem to activate and stimulate the proliferation of NSC [7–9], while aging or inflammation has the opposite effect [10–12]. The neurogenic response to lesion involves neuroinflammation that activates resident microglia cells. In these conditions, microglia cells release inflammatory cytokines and reactive oxygen and nitrogen species like NO [13].

NO is a free radical gaseous molecule that results from the conversion of L-arginine into L-citrulline, catalyzed by the nitric oxide synthase family of enzymes. Particularly in inflammatory conditions, the expression of the inducible nitric oxide synthase (iNOS) is involved in the production of high levels of NO. NO is an important cellular messenger with different cell targets, being involved in many physiological mechanisms in cardiovascular, immunological, and nervous systems [14]. During neurogenesis, particularly in the early

stages, such as proliferation, the neurogenic response mediated by NO depends on the pathophysiological state of the tissue, source of NO, and time of exposure [15]. Despite the intensive investigation on the effect of NO on the proliferation of NSC, there is a lack of information about the role of NO in migration, differentiation, and survival on newborn cells following brain injury.

In this work we investigated the role of NO from inflammatory origin in the regulation of hippocampal neurogenesis after a brain insult. We analyzed the proliferation of NSC, migration, differentiation, and survival of newborn cells using a KA-induced seizure mouse model [16, 17]. We show that NO increased the proliferation of early-born cells, particularly in the SGZ, and the number of neuroblasts, following *status epilepticus* (SE). Furthermore, NO was important for the maintenance of long-term neuroinflammation, which may be the leading cause to its detrimental effect on the survival of newborn cells in the dentate gyrus. Taken together, our data show that NO is a promising target for promoting the proliferation and migration of NSC following seizures, although its presence may compromises long-term survival of newborn cells.

2. Materials and Methods

2.1. Materials. 5-Bromo-2′-deoxyuridine (BrdU), normal goat serum (NGS), paraformaldehyde (PFA), and Triton X-100 were purchased from Sigma Chemical (St. Louis, MO, USA). Mouse anti-neuronal nuclear (NeuN) and mouse anti-glial fibrillary acid protein (GFAP) were purchased from Millipore (Billerica, MA). DAKO fluorescent mounting medium was obtained from DakoCytomation (Glostrup, Denmark). Rat anti-BrdU was obtained from Oxford Biotechnology and doublecortin (C-18) (DCX) from Santa Cruz Biotechnology (Dallas, Texas, USA). Rabbit anti-cleaved caspase-3 was obtained from Cell Signaling (Danvers, MA, USA). Anti-rat and anti-rabbit IgG conjugated with Alexa Fluor 488 and anti-rat and anti-mouse IgG conjugated with Alexa Fluor 594 secondary antibodies were purchased from Molecular Probes (Invitrogen, Paisley, UK). Kainic acid was obtained from Ocean Produce (Canada) and sodium thiopental from B. Braun Melsungen (Germany).

2.2. Animals. Two-month-old C57BL/6J (iNOS$^{+/+}$) mice or B6.129P2-*Nos2*^{tm1Lau}/J (iNOS$^{-/-}$) male mice were obtained from Charles River (Barcelona, Spain) or The Jackson Laboratory (Bar Harbor, ME, USA), respectively. The animals were kept in animal facilities with food and water *ad libitum* in a 12 : 12 light-dark cycle. The weight of the animals varied between 18 and 26 g. All experiments were performed in accordance with NIH and European guidelines (86/609/EEC) for the care and use of laboratory animals. Furthermore, the animals were housed in our licensed animal facility (International Animal Welfare Assurance number 520.000.000.2006). In addition, all the people working with animals have received appropriate education (FELASA course) as required by the Portuguese authorities. This study is included in two projects approved and financed by the Foundation for Science and Technology (FCT, Portugal, PTDC/SAU-NEU/102612/2008 and PTDC/NEU-OSD/0473/2012) that approved the animal experimentation described. The animal experimentation board at the Center for Neurosciences and Cell Biology also approved the use of the animals in this study.

2.3. Administration of Kainic Acid in Mice. KA was dissolved in a sterile saline solution (0.9% NaCl in water) and injected subcutaneously (25 mg/kg), as previously described by our group [15]. All animals that received KA developed grade five seizures or higher according to 1972s Racine's six-point scale modified for mice [18]. In animals injected with saline solution alone, no seizures were observed and were used as controls. At least three animals survived in each experimental group.

2.4. Assessment of NSC Proliferation by 5-Bromo-2′-deoxyuridine Incorporation. To assess proliferation of NSC, all animals treated with KA or saline solution were treated with BrdU (intraperitoneal (i.p.) injections, 4 doses, 50 mg/kg) 2 hours apart, in a total of 200 mg/kg, up to 12 hours before sacrificing the animals at different time points (Figure 1(a)). In order to analyze distribution of NSC along dentate gyrus, all animals were treated with BrdU (i.p. injections, 4 doses, 50 mg/kg) every 12 hours, three and seven days after KA or saline administration. Three weeks later, mice were sacrificed (Figures 1(b) and 1(c)). In both experiments mice were transcardially perfused with 0.9% NaCl followed by 4% PFA in 0.01 M phosphate buffer saline (PBS, 7.8 mM $Na_2HPO_4{\cdot}2H_2O$, 2.7 mM $NaH_2PO_4{\cdot}H_2O$, 154 mM NaCl, and pH 7.2), following deep anesthesia with Eutasil (20% sodium pentobarbital). Brains were removed and kept overnight in 4% PFA for further fixation and then dehydrated in 20% sucrose/0.2 M phosphate buffer (PB, 48 mM $NaH_2PO_4{\cdot}H_2O$, 152 mM $Na_2HPO_4{\cdot}2H_2O$, and pH 7.2), at 4°C. Coronal sections from the hippocampal region were cryosectioned (30 μm thick, in 8 series) and stored in an antifreeze solution (0.05 M PB, 30% ethylene glycol, and 30% glycerol), at 4°C.

2.5. Immunohistochemistry. Free-floating coronal hippocampal sections were processed for immunohistochemistry against DCX or BrdU and NeuN, BrdU, and GFAP or BrdU and cleaved caspase-3. Brain sections were treated with 1 M HCl for 20 min at 65°C, for DNA denaturation and then blocked for 1 hour with 5% NGS in 0.25% Triton X-100 in 0.01 PBS. Slices were then incubated with the primary antibodies, goat anti-DCX (1 : 400) or rat anti-BrdU (1 : 50) and mouse anti-NeuN (1 : 200) or mouse anti-GFAP (1 : 7000) or rabbit anti-cleaved caspase-3 (1 : 600), 48 h at 4°C. After rinsing with 0.25% Triton X-100 in PBS and with 2% block solution (NGS), the sections were incubated with the correspondent secondary antibodies (1 : 200), in 2% block solution (NGS), for 2 h in the dark, at room temperature. After rinsing with 0.25% Triton X-100 in PBS, the sections were kept in PBS 0.1 M solution, at 4°C, until setting in 2% gelatin-coated slides with DAKO fluorescence mounting medium.

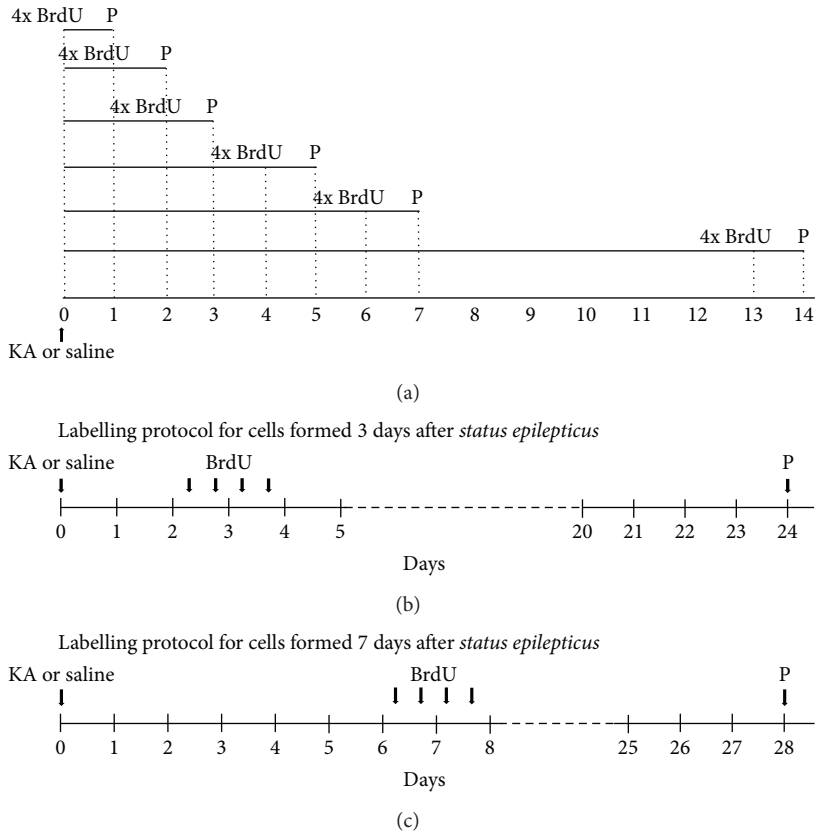

Figure 1: Experimental protocol for assessment of NSC proliferation (a) and differentiation (b and c). (a) KA or saline solutions were subcutaneously injected (25 mg/kg). Intraperitoneal injections (i.p.) of BrdU (4 doses, 50 mg/kg) were administrated every 2 hours, up to 12 hours before transcardiac perfusion (P). (b) Administration protocol of BrdU 3 days after SE. (c) Administration protocol of BrdU 7 days after SE. Intraperitoneal injections (i.p.) of BrdU (4 doses, 50 mg/kg) were administrated every 12 hours. Perfusions (P) were performed 3 weeks after BrdU treatment, following anesthesia.

2.6. Analysis of BrdU Incorporation. The distribution of the newborn cells in the dentate gyrus was analyzed in SGZ, IGZ and outer granular zone (OGZ) (Figure 2) and the numbers of BrdU+ cells were counted in each zone using an epifluorescence microscope (20x objective, Axiovert 200, Zeiss, Jena, Germany).

Images (0.73 μm z-stacks) from 50 BrdU+ cells of each brain were acquired in a laser scanning microscope LSM 510 META or LSM 710 (Zeiss, Jena, Germany) with Argon/2 (488 nm) and DPSS 561-10 (561 nm) lasers (63x oil-immersion objective). Orthogonal projections in y-axis were performed and counted the number of BrdU+/NeuN+ or BrdU+/GFAP+ or BrdU+/Casp3+ cells were counted. The percentage of cells which show colocalization of markers was obtained by dividing the total number of BrdU+/NeuN+ or BrdU+/GFAP+ or BrdU+/Casp3+ cells by 50 BrdU+ cells.

2.7. DCX and GFAP Immunoreactivity. DCX and GFAP immunoreactive areas were analyzed using ImageJ software. Snap images were acquired in a Zeiss Axioimager (Zeiss, Jena, Germany) with a 20x objective. The threshold value was set for each staining and the percentage of dark background area was measured, excluding more anterior and posterior dentate gyri.

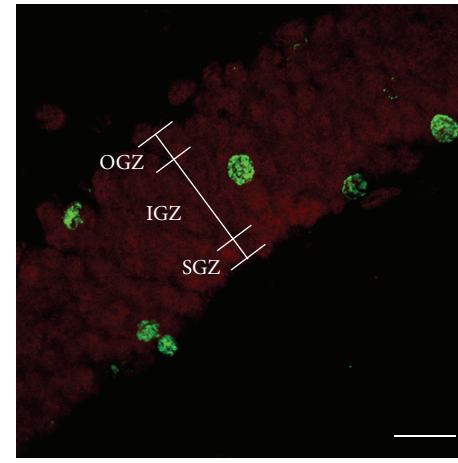

Figure 2: Schematic representation of the three zones of the dentate gyrus: the subgranular zone (SGZ), inner granular zone (IGZ), and outer granular zone (OGZ). The number of BrdU+ cells (green) was counted in each zone.

2.8. Statistical Analysis. The data are expressed as means ± SEM. Statistical significance was determined by using two-factor analysis of variance (ANOVA), followed by *post hoc*

Table 1: NO increased NSC proliferation following treatment with KA.

Days	$iNOS^{+/+}$ saline		$iNOS^{+/+}$ KA		$iNOS^{-/-}$ saline		$iNOS^{-/-}$ KA	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
1	16,82	0,91	9,39	1,04	11,31	2,86	7,72	1,43
2	23,13	1,31	33,23	1,86	12,10	1,33	13,35	3,87
3	24,45	1,77	$63,36^{***}$	1,36	16,71	4,05	25,64	0,53
5	26,32	1,12	$82,69^{***}$	5,87	15,58	0,96	18,36	1,99
7	21,60	0,49	$82,45^{***}$	3,44	20,08	0,61	$87,08^{\#\#\#}$	8,39
14	21,71	1,77	$37,48^{**}$	1,99	17,15	1,38	27,33	2,16

Evaluation of cell proliferation in the subgranular zone (SGZ) of $iNOS^{+/+}$ versus $iNOS^{-/-}$ mice, at several time points after SE, assessed by BrdU incorporation. Following seizure induction, there is a time-dependent increase in cell proliferation in $iNOS^{+/+}$ animals, peaking at day 5. In $iNOS^{-/-}$ mice, only 7 days after SE the number of BrdU+ cells significantly increases in the SGZ. At least 3 surviving animals were used for each experimental group. Data are expressed as means ± SEM. Two-factor ANOVA: $N = 3$ to 5, $^{**}p < 0.01$ and $^{***}p < 0.001$, significantly different from $iNOS^{+/+}$ saline; $^{\#\#\#}p < 0.001$, significantly different from $iNOS^{-/-}$ saline.

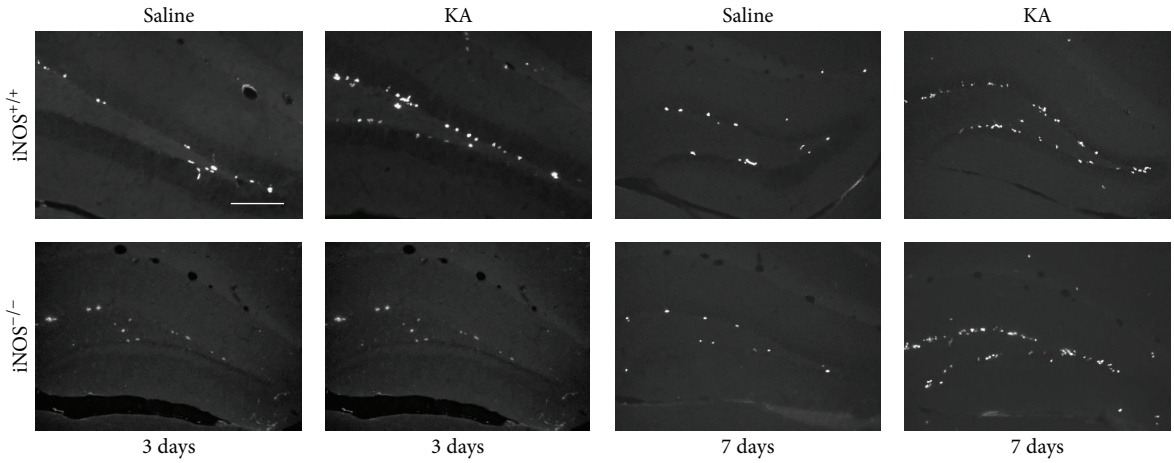

Figure 3: Number of BrdU+ cells in the dentate gyrus increased with KA treatment. Representative images of BrdU+ cells (white), 3 and 7 days after KA or saline treatment in $iNOS^{+/+}$ or $iNOS^{-/-}$ mice. Scale bar: 50 μm.

Bonferroni's test in GraphPad Prism 5 software. Differences were considered significant when $p < 0.05$.

3. Results and Discussion

3.1. NO Is Involved in the Proliferation of NSC in the Dentate Gyrus following SE

3.1.1. Proliferation of Neural Stem Cells in the Hippocampus following Seizures Comprises a NO-Dependent and NO-Independent Phase. In order to investigate the role of NO in cell proliferation, we used an *in vivo* KA-induced seizure mouse model, as described in Section 2. Proliferation of newborn cells was evaluated by the incorporation of BrdU, a thymidine analogue. The number of BrdU+ cells in the dentate gyrus was assessed by immunohistochemistry (Figure 3).

In $iNOS^{+/+}$ mice, treatment with KA increased significantly the incorporation of BrdU in the SGZ from 3 days after treatments up to 14 days, when compared to saline-treated mice (Table 1, two-factor ANOVA; treatment: 31.95, $F = 151.5$, $df = 3$; time: 29.71, $F = 84.57$, $df = 5$; treatment × time (interaction): 33.70, $F = 31.97$, $df = 15$), with a peak at 5 days after treatment with KA (82.70 ± 5.87 cells/section, $p < 0.001$). In $iNOS^{+/+}$ mice treated with saline solution, the number of BrdU+ cells did not change significantly during the analyzed period of time ($p > 0.05$ for all time points). These results are in line with previous findings that seizures in mice trigger neuroinflammation and stimulate cell proliferation in the SGZ of the dentate gyrus [11, 17, 19, 20]. Cell proliferation in the hippocampus is also increased in different acute injured-animal models, such as stroke [21, 22] and traumatic brain injury [23, 24].

In $iNOS^{-/-}$ mice, BrdU incorporation was unchanged with KA treatment up to 5 days after seizures (7.73 ± 1.43 cells/section at 1 day after SE ($p > 0.05$), 13.35 ± 3.87 cells/section for 2 days after seizures ($p > 0.05$), 25.64 ± 0.53 cells/section for 3 days after SE ($p > 0.05$), and 18.36 ± 1.99 cells/section for 5 days after SE ($p > 0.05$)). Interestingly, the number of BrdU+ cells in the dentate gyrus of $iNOS^{-/-}$ mice was significantly increased 7 days after seizures (87.08 ± 8.40 cells/section, $p < 0.001$), compared with saline-treated $iNOS^{-/-}$ mice (20.08 ± 0.61 cells/section). Finally, the incorporation of BrdU returned to basal levels 14 days after seizures with KA treatment (27.33 ± 2.17 cells/section, $p > 0.05$). In

saline-treated iNOS$^{-/-}$ mice the incorporation of BrdU was similar for all time points ($p > 0.05$). In these animals, proliferation seems to be regulated by two different mechanisms: one that is NO-dependent up to 5 days after seizures and another that is regulated by a NO-independent mechanism, at 7 days after seizures.

Production of inflammatory factors from microglia, such as NO, has already been reported as essential for proliferation of NSC in the hippocampus [25]. Our group previously described the mechanism by which NO triggers the initial proliferation in SVZ cells [15, 26]. In those studies, we reported that NO is able to bypass the epidermal growth factor receptor and directly activate upstream components of ERK 1/2/MAPK signaling pathway, resulting in increased cell proliferation of NSC in early stages [15].

Moreover, late proliferation depends on the activation of cGMP and PKG, suggesting a biphasic mechanism of proliferation trigged by NO [9]. Interestingly, we observed an increase in proliferation of NSC 7 days after seizures in iNOS$^{-/-}$ mice, which suggests that proliferation at this time is independent of NO. There are some other potential signaling pathways that may play a role in NSC proliferation at this time point. For instance, the NO-cGMP pathway is an important mediator of the proliferative effects of neuropeptide Y in the hippocampus [27–29]. Also in a model of SE, activated microglia induce the expression of insulin-like growth factor-1 (IGF-1) and stimulates the proliferation of progenitor cells in SGZ by a MAPK-dependent mechanism [30]. There are several factors expressed by microglia cells that can regulate neurogenesis and NSC can also regulate the activation of microglia cells, so it is possible that this interaction microglia-NSC may function as some kind of compensatory mechanism to regulate proliferation of NSC, independently of NO.

3.2. Involvement of NO on Migration and Distribution of Newborn Cells in the Dentate Gyrus following Seizures

3.2.1. Distribution of Newborn Cells Formed 3 Days after Seizures in the Dentate Gyrus Is Independent of NO. We next investigated the role of NO in the distribution of newborn cells in the dentate gyrus after 21 days following proliferation dependent on NO (that occurs at 3 days post-seizure), to evaluate whether the cells remained in the subgranular zone or are redistributed to the outer layers of the dentate gyrus. iNOS$^{+/+}$ or iNOS$^{-/-}$ mice were treated with either saline or KA and BrdU was injected in all animals 3 days later. The distribution of the new cells formed at this time point was assessed in the SGZ, IGZ, and OGZ of the dentate gyrus, 21 days after BrdU administration. BrdU+ cells increased with KA treatment in iNOS$^{+/+}$ and iNOS$^{-/-}$ mice (Figure 4(a)). The total number of BrdU+ cells significantly increased with KA treatment in both iNOS$^{+/+}$ and iNOS$^{-/-}$ mice (Figure 4(b), two-factor ANOVA; genotype: 5.68, $F = 2.334$, $df = 1$, $p > 0.05$; treatment: 49.79, $F = 20.47$, $df = 1$, $p < 0.001$; genotype × treatment (interaction): 0.76, $F = 0.3113$, $df = 1$, $p > 0.05$). For iNOS$^{+/+}$ mice, treatment with KA duplicated BrdU+ cells (29.24 ± 2.91 BrdU+ cells/section, $p < 0.05$) compared to saline-treated mice (12.59 ± 1.66 BrdU+ cells/section). For iNOS$^{-/-}$ mice, KA treatment also doubled the number of BrdU+ cells (37.99 ± 7.75 BrdU+ cells/section, $p < 0.01$) compared to saline-treatment (16.66 ± 2.89 BrdU+ cells/section).

In iNOS$^{+/+}$ mice, KA treatment significantly increased BrdU+ cells in SGZ (16.68 ± 1.56 BrdU+ cells/section, $p < 0.01$) comparatively to saline-treated mice (9.49 ± 1.18 BrdU+ cells/section) (Figure 4(c), two-factor ANOVA; treatment: 25.52, $F = 14.81$, $df = 3$, and $p < 0.001$; regions: 41.85, $F = 36.43$, $df = 2$, and $p < 0.001$; treatment × regions (interaction): 1.62, $F = 0.4690$, $df = 6$, and $p > 0.05$). For these mice, BrdU+ cells also increased with KA treatment in IGZ (9.35 ± 1.97 BrdU+ cells/section, $p < 0.01$) when compared with saline-treated mice (2.48 ± 0.47 BrdU+ cells/section). BrdU+ cells did not change significantly in OGZ with KA treatment. Similarly, in iNOS$^{-/-}$ mice (Figure 4(c)), BrdU+ cells significantly increased after seizures in SGZ (17.98 ± 2.82 BrdU+ cells/section, $p < 0.05$) and IGZ (12.37 ± 3.78 BrdU+ cells/section, $p < 0.05$), compared with saline-treated iNOS$^{-/-}$ mice. These results suggest that the distribution of NSC born 3 days after the insult is regulated by a NO-independent mechanism.

3.2.2. Abolishment of NO Does Not Affect Distribution of Newborn Cells Formed 21 Days following Seizures in the Dentate Gyrus. We next investigated the role of NO in the distribution of newborn cells in the dentate gyrus after 21 days following proliferation during a phase that is not dependent on NO (that occurs at 7 days post-seizure). iNOS$^{+/+}$ or iNOS$^{-/-}$ mice were treated with either saline or KA, BrdU was injected in all animals 7 days later, and perfusions performed 28 days after BrdU injection (7 days after seizures followed by 21 days).

Treatment with KA in cells formed 7 days after seizures did not change significantly the number of BrdU+ cells along the dentate gyrus (21 days after the cells were labeled with BrdU around day 7) for none of the genotypes (Figures 5(a) and 5(b), two-factor ANOVA, treatment: 10.61, $F = 2.180$, $df = 1$, and $p > 0.05$; genotype: 15.95, $F = 3.278$, $df = 1$, and $p > 0.05$; treatment × genotype (interaction): 0.45, $F = 0.09155$, $df = 1$, and $p > 0.05$). In iNOS$^{+/+}$ and iNOS$^{-/-}$ mice, the number of BrdU+ cells in KA-treated mice was similar in all zones of the dentate gyrus, compared to the respective saline controls (Figure 5(c), two-factor ANOVA; treatment: 11.25, $F = 4.592$, $df = 3$, and $p < 0.01$; regions: 48.07, $F = 29.44$, $df = 2$, and $p < 0.001$; treatment × regions (interaction): 3.95, $F = 0.8072$, $df = 6$, and $p > 0.05$).

According to our study, at 7 days after KA treatment the proliferation of newborn cell is regulated by a NO-independent mechanism. As described before, there are some other factors that can lead to activation of signaling pathways possible involved in regulation of proliferation of NSC in the hippocampus at this stage of the neurogenesis. For instance, IGF-1 produced by activated microglia cells can increase the proliferation of NSC in the SGZ by a mechanism dependent of the MAPK signaling [30]. Also there is the possibility of a compensatory mechanism regulated by NSC, independently

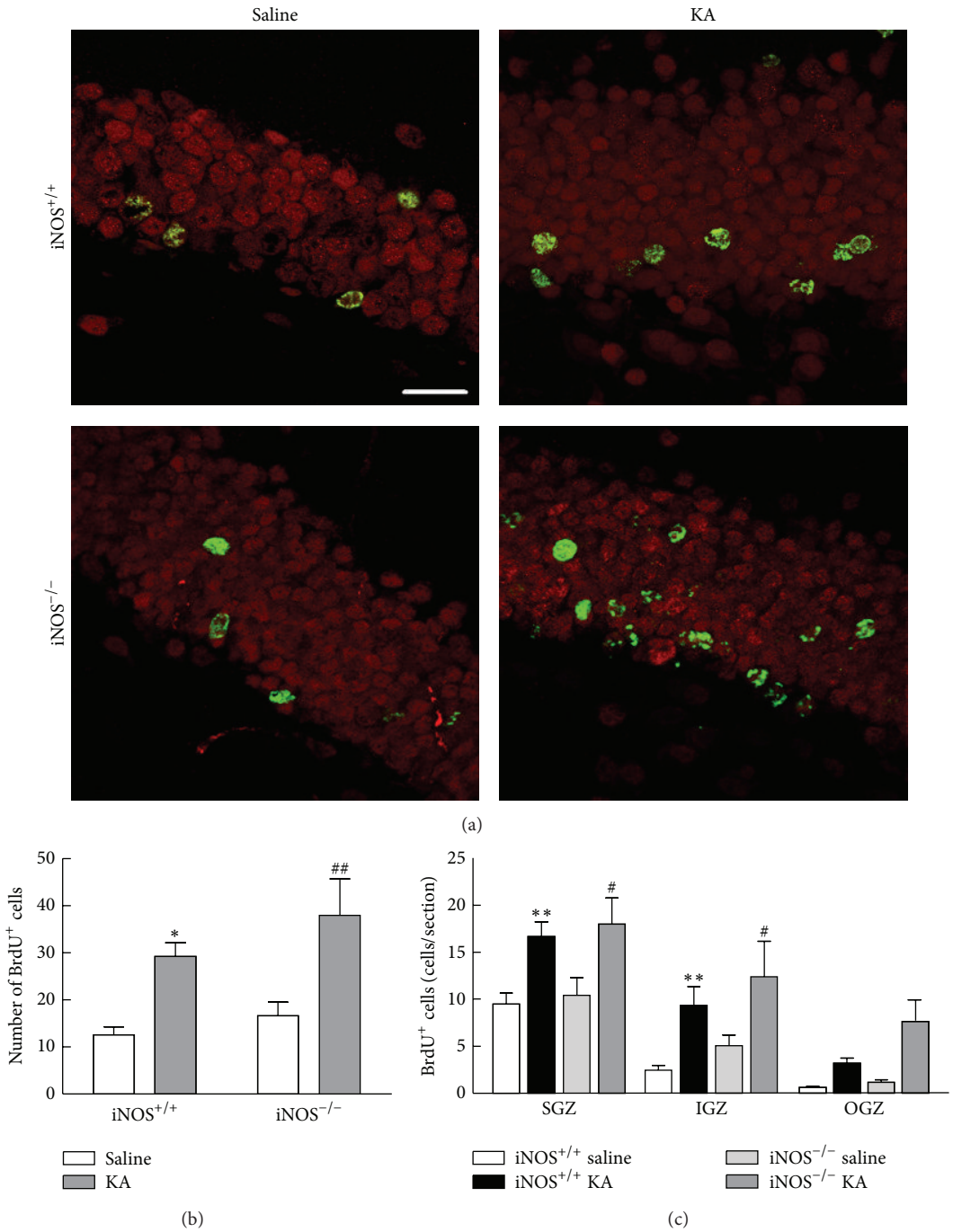

FIGURE 4: The number of BrdU+ cells in the dentate gyrus increased following seizures, 21 days after BrdU treatment, by a NO-independent mechanism. (a) Representative images of the distribution of BrdU (green) and NeuN (red) positive cells in different layers of the dentate gyrus of the hippocampus, 3 days after induction of seizures with KA or saline treatment, in iNOS$^{+/+}$ and iNOS$^{-/-}$ mice. Scale bar: 20 μm. (b) Number of BrdU+ cells in iNOS$^{+/+}$ and iNOS$^{-/-}$ mice. Data are expressed as means ± SEM. Two-factor ANOVA (Bonferroni's posttest): $N = 5$ to 7; $^{*}p < 0.05$, significantly different from iNOS$^{+/+}$ saline; $^{\#\#}p < 0.01$, significantly different from iNOS$^{-/-}$ saline. (c) BrdU+ cells in different layers of the dentate gyrus, namely, in the SGZ, IGZ and OGZ of iNOS$^{+/+}$ vs iNOS$^{-/-}$ mice, 3 days following SE. Data are expressed as means ± SEM. Two-factor ANOVA (Bonferroni's posttest): $N = 5$ to 7; $^{**}p < 0.01$, significantly different from iNOS$^{+/+}$ saline; $^{\#}p < 0.05$, significantly different from iNOS$^{-/-}$ saline.

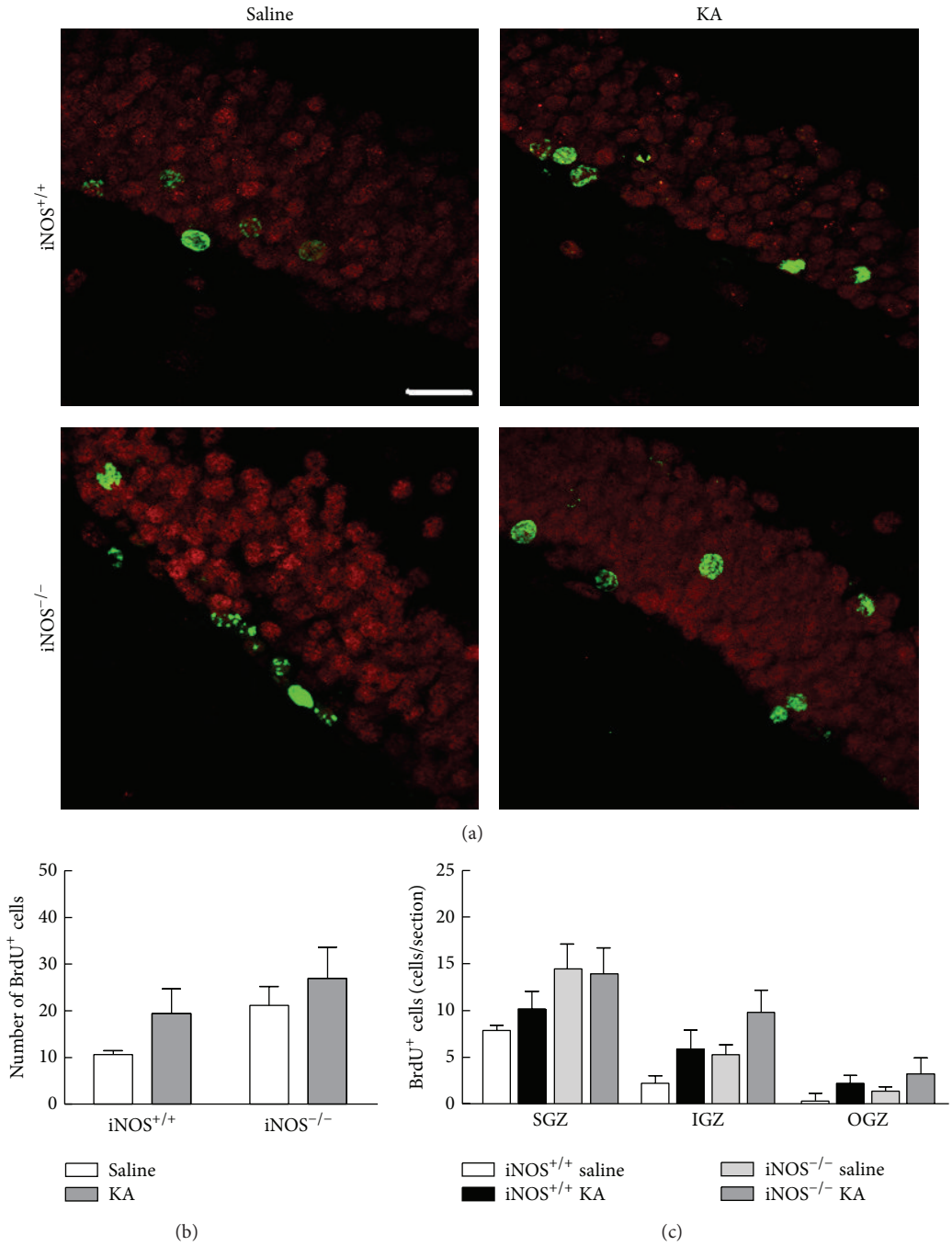

Figure 5: The number of BrdU+ cells in the dentate gyrus born 7 days after SE is not affected by NO, 21 days after treatment with BrdU. (a) Representative images of BrdU (green) and NeuN (red) positive cells in the dentate gyrus, 7 days after treatment with KA or saline in iNOS$^{+/+}$ and iNOS$^{-/-}$ mice. Scale bar: 20 μm. (b) Number of BrdU+ cells in iNOS$^{+/+}$ and iNOS$^{-/-}$ mice. Data are expressed as means ± SEM. Two-factor ANOVA (Bonferroni's posttest), $N = 4$ to 6, $p > 0.05$. (c) BrdU+ cells in the three regions of the dentate gyrus. Data are expressed as means ± SEM. Two-factor ANOVA (Bonferroni's posttest), $N = 4$ to 6, $p > 0.05$.

of any other factor produced by microglia cells. Thus, the distribution of newborn cells in the dentate gyrus was similar in iNOS$^{+/+}$ and iNOS$^{-/-}$ mice after seizures, suggesting that NO is not involved in how cells are distributed along the dentate gyrus 7 days after seizures, but other factors may be involved.

3.3. NO Has Different Effects in Neuronal and Astrocytic Differentiation

3.3.1. Early Neuronal Differentiation following Seizures Is Dependent on NO. Here, we identified immature neurons in iNOS$^{+/+}$ and iNOS$^{-/-}$ mice treated with either saline or

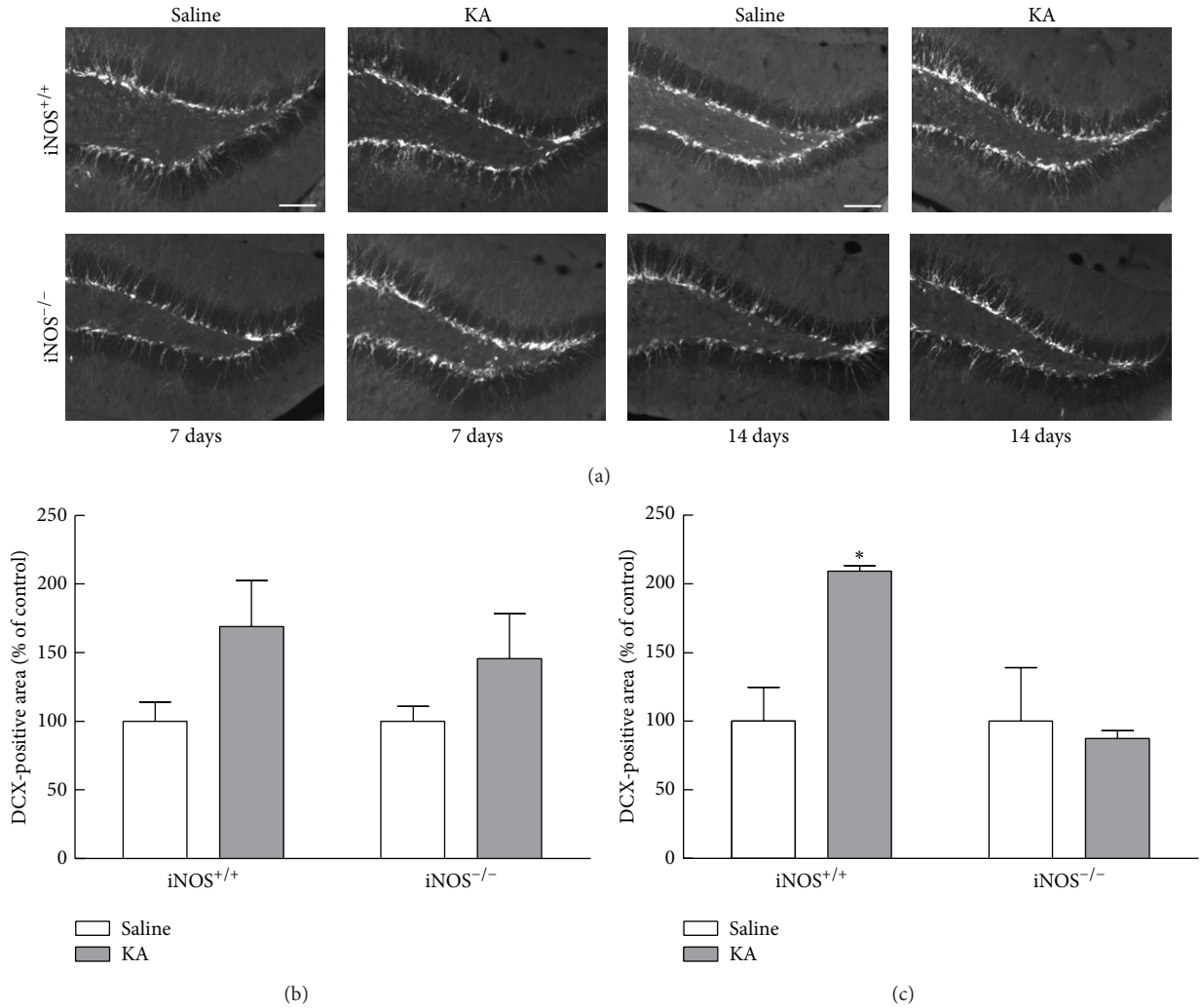

Figure 6: DCX immunoreactivity is dependent on NO, 14 days after seizures. (a) Representative images of DCX (white) immunoreactivity in the dentate gyrus, 7 and 14 days after KA or saline treatment in iNOS$^{+/+}$ and iNOS$^{-/-}$ mice. Scale bar: 100 μm. (b) DCX-immunoreactive area 7 days after SE. (c) DCX-immunoreactive area 14 days after SE. Data are expressed as means ± SEM. Two-factor ANOVA (Bonferroni's posttest): $N = 3$ to 6, $^{*}p < 0.05$, significantly different from iNOS$^{+/+}$ saline.

KA, as described in Section 2. DCX is a specific marker for neuroblasts and immature neurons [31] born in the first two weeks of the neurogenic process. Accordingly, we chose to analyze DCX-immunoreactive area at 7 days and 14 days after KA treatment.

The DCX-immunoreactive area was increased in iNOS$^{+/+}$ mice, 14 days after seizures compared to saline-treated mice, but not in KA-treated iNOS$^{-/-}$ compared to saline-treated mice (Figure 6(a)). At 7 days after seizures, the percentage of DCX-immunoreactive area tends to increase with KA treatment in both iNOS$^{+/+}$ (169.01 ± 33.50% of control, $p > 0.05$) and iNOS$^{-/-}$ (145.64 ± 32.75% of control, $p > 0.05$), although this increase is not significant compared to saline-treated mice (Figure 6(b), two-factor ANOVA; genotype: 0.79, $F = 0.1382$, $df = 1$, and $p > 0.05$; treatment: 18.90, $F = 3.326$, $df = 1$, and $p > 0.05$; genotype × treatment (interaction): 0.78, $F = 0.1381$, $df = 1$, and $p > 0.05$). Later, at 14 days after seizures, the DCX+ area doubled in iNOS$^{+/+}$ mice (209.32 ± 4.07% of control, $p < 0.05$), when compared to saline-treated mice of the same genotype (100.00 ± 24.75% of control). In iNOS$^{-/-}$ mice, treatment with KA did not change the DCX-immunoreactive area (87.57 ± 5.48% of control, $p > 0.05$), at 14 days after seizures, when compared with the saline-treated mice (100.00 ± 39.13). (Figure 6(c), two-factor ANOVA; genotype: 23.01, $F = 6.420$, $df = 1$, and $p < 0.05$; treatment: 14.57, $F = 4.065$, $df = 1$, and $p > 0.05$; genotype × treatment (interaction): 23.01, $F = 6.420$, $df = 1$, and $p < 0.05$).

Our results showed that NO from an inflammatory origin increases the number of neuroblasts/immature neurons in the dentate gyrus, at least at 2 weeks after seizures. It has been shown that the number of DCX+ neuroblasts significantly increased following treatment with L-NAME, a NOS inhibitor, and KA together [32]. Moreover, in the same study, inhibition of NOS alone increased the number of BrdU+ newborn cells in the hilus, which suggests a role for NO in

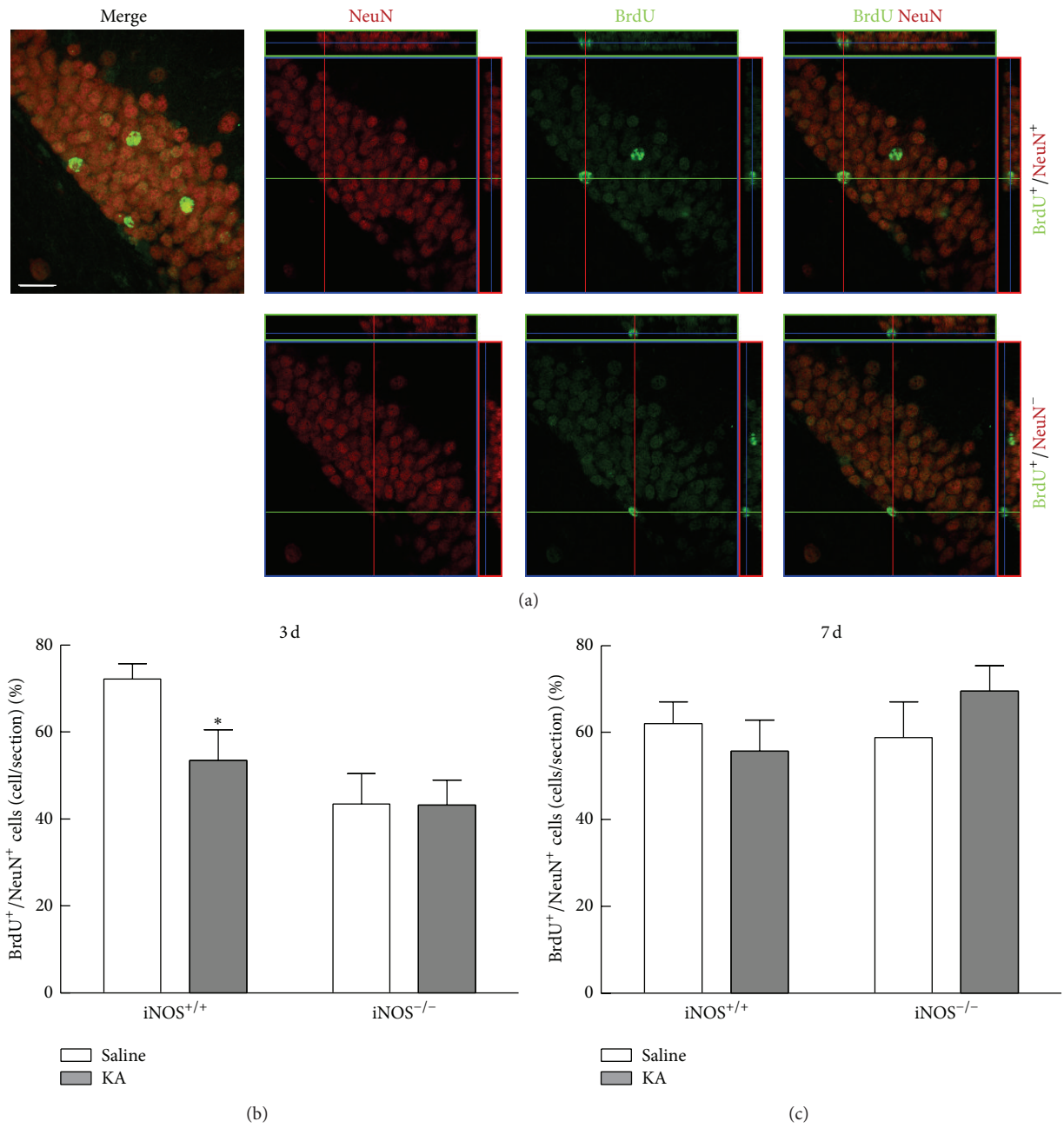

FIGURE 7: NO decreases the number of newborn neurons born 3 days after seizures in iNOS$^{+/+}$ mice. (a) Orthogonal projections of representative images of BrdU-positive cells (BrdU+) shown green and NeuN-positive (NeuN+) cells, shown red. Scale bar: 20 μm. Assessment of the percentage of BrdU-NeuN colocalizing cells in the dentate gyrus of iNOS$^{+/+}$ or iNOS$^{-/-}$ mice, 3 days (b) or 7 days (c) following SE. At least 3 surviving animals were used for each experimental group. Data are expressed as means ± SEM. Two-factor ANOVA (Bonferroni's posttest): $N = 4$ to 7; $p < 0.05$, significantly different from iNOS$^{+/+}$ saline.

their correct migration into the granular zone of the dentate gyrus.

3.3.2. NO Limits Survival of the Cells That Proliferate Earlier (3 Days) but Not Later (7 Days) after Seizures. To investigate the survival at 21 days of cells formed 3 and 7 days after seizures, colocalization of BrdU+/NeuN+-cells was assessed by immunohistochemistry. NeuN is a neuronal marker for mature neurons and colocalization with BrdU allows the investigation of new neurons formed at the time point of treatment with BrdU. Images of 50 BrdU+ cells of each animal were acquired and orthogonal projections in y-axis were performed for each image (Figure 7(a)).

At 21 days after treatment with BrdU, the percentage of new neurons born 3 days after seizures decreased in iNOS$^{+/+}$ mice treated with KA (53.50 ± 7.04% of BrdU+/NeuN+-cells,

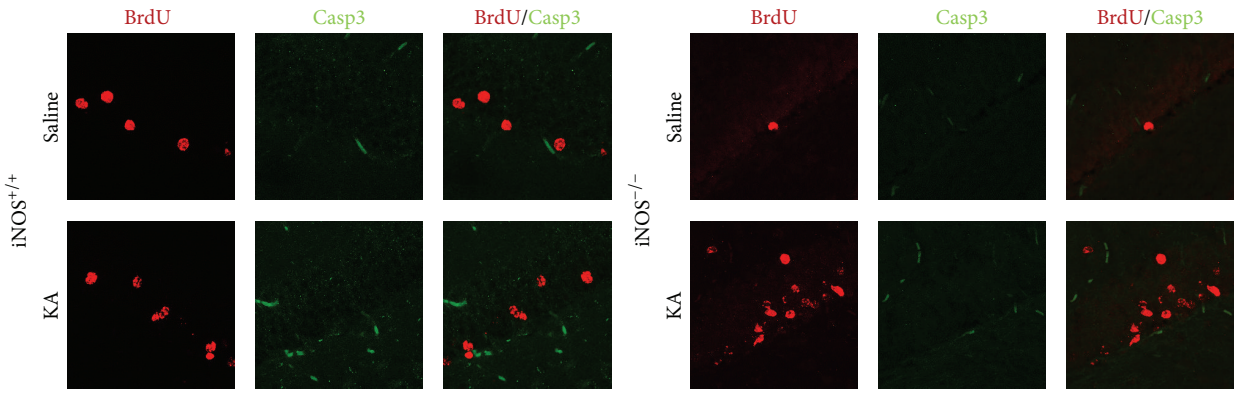

FIGURE 8: Absence of BrdU/Casp3 colocalization in cells formed 3 days after treatment with saline or KA in iNOS$^{+/+}$ and iNOS$^{-/-}$ animals. Representative images of BrdU-positive cells (red) and cleaved caspase-3 (Casp3, green). At least 3 surviving animals were used for each experimental group. Scale bar: 20 μm.

$p < 0.05$), compared to saline-treated mice (72.29 ± 3.48% of BrdU+/NeuN+-cells), but not in iNOS$^{-/-}$ mice (43.50 ± 6.95% of BrdU+/NeuN+-cells for saline and 43.20 ± 5.68% of BrdU+/NeuN+-cells for KA-treated mice, $p > 0.05$) (Figure 7(b), two-factor ANOVA: genotype: 7.79, $F = 2.682$, $df = 1$, and $p > 0.05$; treatment: 32.65, $F = 11.25$, $df = 1$, and $p < 0.01$; genotype × treatment (interaction): 7.30, $F = 2.516$, $df = 1$, and $p > 0.05$). These results suggest that survival of newborn cells after seizures is regulated by a NO-dependent mechanism, similar to proliferation of NSC in these conditions. However, NO seems to result in the formation of less new neurons after seizures.

NO may be toxic to neurons and neuronal apoptosis was evident after administration of a NO donor in a febrile seizure rat model [33]. NO has also been proposed as an inhibitor of cell-cycle progression in many cell types, through activation of p53 or Rb signaling pathways [34, 35]. This relationship of NO and programmed cell death might influence the survival rate of the newborn cells. Moreover, NO does not necessarily need to be directly toxic to newborn neurons but may be involved in maintenance of inflammation that will condition the survival of the new cells.

For neurons born 7 days after seizures, the number of new neurons in iNOS$^{+/+}$ mice was very similar between treatments, with 55.6 ± 7.22% of BrdU+/NeuN+-cells ($p > 0.05$) in KA-treated mice and 62.00 ± 5.03% of BrdU+/NeuN+-cells for saline-treated mice. In iNOS$^{-/-}$ mice, treatment with KA (69.50 ± 5.85% of BrdU+/NeuN+-cells) also did not change the number of new neurons, compared to saline-treated mice (58.80 ± 8.31% of BrdU+/NeuN+-cells) (Figure 7(c), two-factor ANOVA; genotype: 3.15, $F = 0.5348$, $df = 1$, and $p > 0.05$; treatment: 0.53, $F = 0.09010$, $df = 1$, and $p > 0.05$; genotype × treatment (interaction): 8.07, $F = 1.372$, $df = 1$, and $p < 0.05$). These results suggest that cells that proliferate in a NO-independent phase become neurons that survive better than cells that proliferate earlier (3 days) after the onset of seizures.

In order to confirm the effect of NO in survival of the cells that proliferated 3 days after seizures, we assessed the colocalization of BrdU with a cell death marker, cleaved caspase-3, by immunohistochemistry. Representative images of each animal were acquired (Figure 8). Colocalization of BrdU with cleaved caspase-3 was not observed, which suggests that the decrease in the survival of the new neurons formed 3 days after seizures in iNOS$^{+/+}$ animals does not result from apoptosis of the proliferating cells at this time point (21 days after treatment with BrdU). It is possible that cell death occurs earlier or by other mechanisms.

3.3.3. Astrogliogenesis Is Not Affected by Abolishment of NO after Seizures. We were interested in understanding whether the proliferating cells could be differentiating into astrocytes. In order to analyze this, we assessed GFAP+ cells formed 3 and 7 days after seizures by immunohistochemistry 21 days after BrdU injection. GFAP is a protein expressed by astrocytes, and colocalization with BrdU allows the identification of newborn astrocytes at the time point of treatment with BrdU (Figure 9(a)).

In iNOS$^{+/+}$ or in iNOS$^{-/-}$ mice, seizures did not change the number of new astrocytes (BrdU-GFAP colocalizing cells) 3 days following SE (3.50 ± 0.34%, $p > 0.05$), compared with saline-treated mice (2.71 ± 0.61%) (Figure 9(b), two-factor ANOVA, genotype: 36.46, $F = 10.30$, $df = 1$, and $p < 0.01$; treatment: 3.39, $F = 0.9584$, $df = 1$, and $p > 0.05$; genotype × treatment (interaction): 0.00, $F = 0.0005183$, $df = 1$, and $p > 0.05$).

Furthermore, seizures did not change the number of BrdU-GFAP colocalizing cells in both genotypes, 7 days after SE (3.60 ± 0.51% or 2.75 ± 1.11% of BrdU-GFAP colocalizing cells in iNOS$^{+/+}$ mice or iNOS$^{-/-}$ mice, resp., $p > 0.05$). The percentage of BrdU-GFAP co-localizing cells in saline-treated mice was very similar between iNOS$^{+/+}$ and iNOS$^{-/-}$ mice (2.00 ± 0.71% versus 1.20 ± 0.37%, resp.) (Figure 9(c), two-factor ANOVA; genotype: 7.07, $F = 1.473$, $df = 1$, and $p > 0.05$; treatment: 25.76, $F = 5.369$, $df = 1$, and $p < 0.05$; genotype × treatment (interaction): 0.01, $F = 0.001353$, $df = 1$, and $p > 0.05$).

In vitro studies reported that exposure to pathological levels of NO (0.1 mM for 24 hours) promotes astroglial fate determination in NSC over neuronal commitment or selectively depletes early neuronal progenitor cells [36]. In this particular model, astrogliogenesis seems to be positively

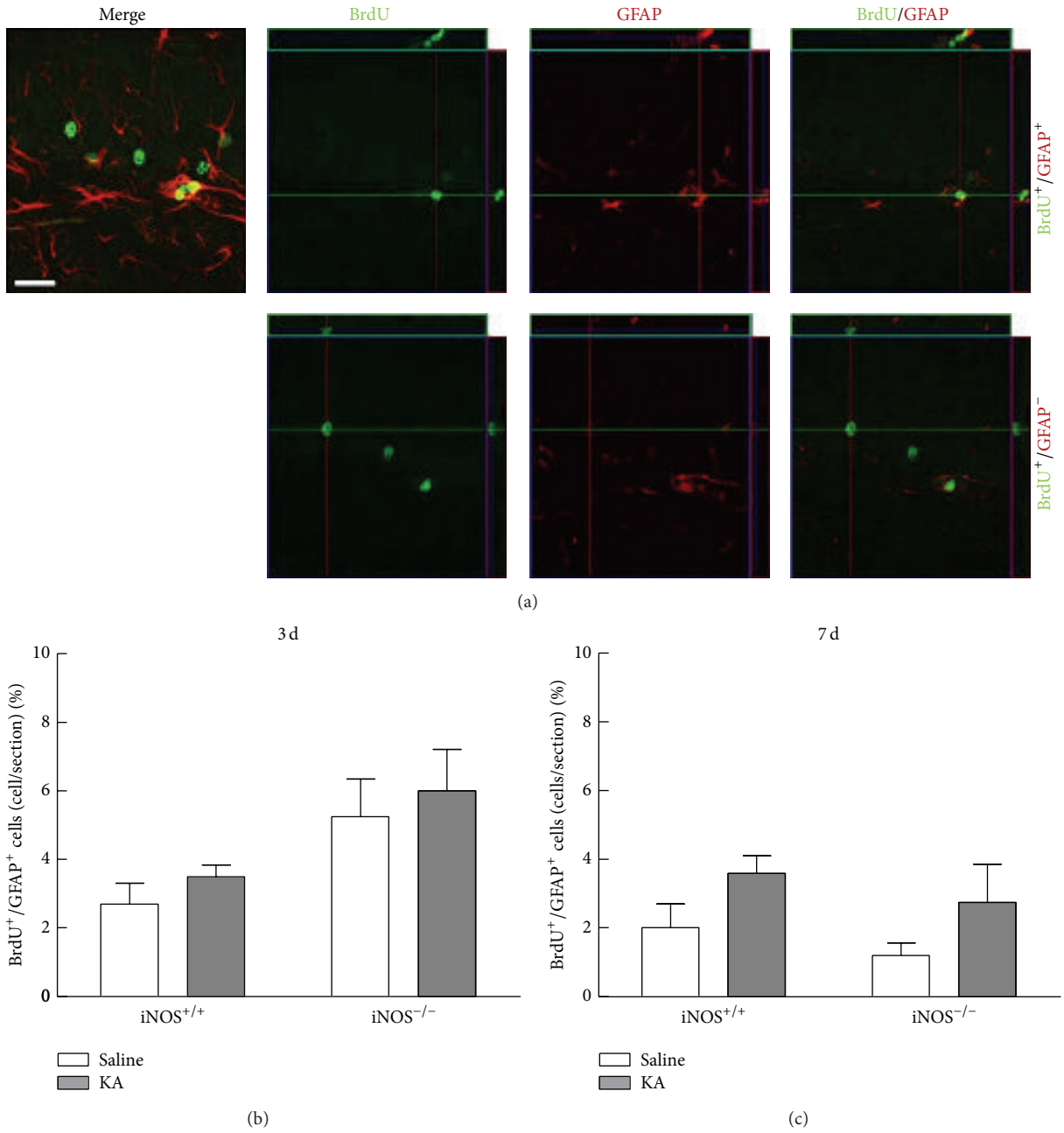

FIGURE 9: Differentiation of newborn cells formed 3 and 7 days after SE into astrocytes is not affected by NO. (a) Orthogonal projections of representative images of BrdU-GFAP colocalizing cells. Scale bar: 20 μm. Percentage of BrdU-GFAP colocalizing cells, 3 days (b) or 7 days (c) following SE. Data are expressed as means ± SEM. Two-factor ANOVA (Bonferroni's posttest), $N = 4$ to 7, $p > 0.05$.

regulated by exposure to NO. Here we show that exposure to NO originated from iNOS is not involved in astroglial differentiation from neural stem cells after a brain injury, which did not change *per se* the number of newborn GFAP+ cells.

3.4. NO Is Important for Astrogliosis in iNOS$^{+/+}$ Mice 28 Days after Treatment. Next, we evaluated the possible involvement of NO in neuroinflammation, 28 days after seizures. For this purpose, GFAP immunoreactivity was assessed by immunohistochemistry and the intensity of GFAP staining used as a measure for astrogliosis (Figure 10(a)). In iNOS$^{+/+}$ mice, KA treatment increased GFAP immunoreactivity (170.45 ± 15.74% of control, $p < 0.05$) when compared to saline-treated mice (100.00 ± 23.87% of control), 28 days after treatment (Figure 10(b), two-factor ANOVA, genotype: 28.27, $F = 6.721$, $df = 1$, and $p < 0.05$; treatment: 6.41, $F = 1.527$, $df = 1$, and $p > 0.05$; genotype × treatment (interaction): 6.42, $F = 1.527$, $df = 1$, and $p < 0.05$). Here we show that GFAP-immunoreactive area was increased 28 days after seizures, in a NO-dependent manner, suggesting that neuroinflammation is still present at this time.

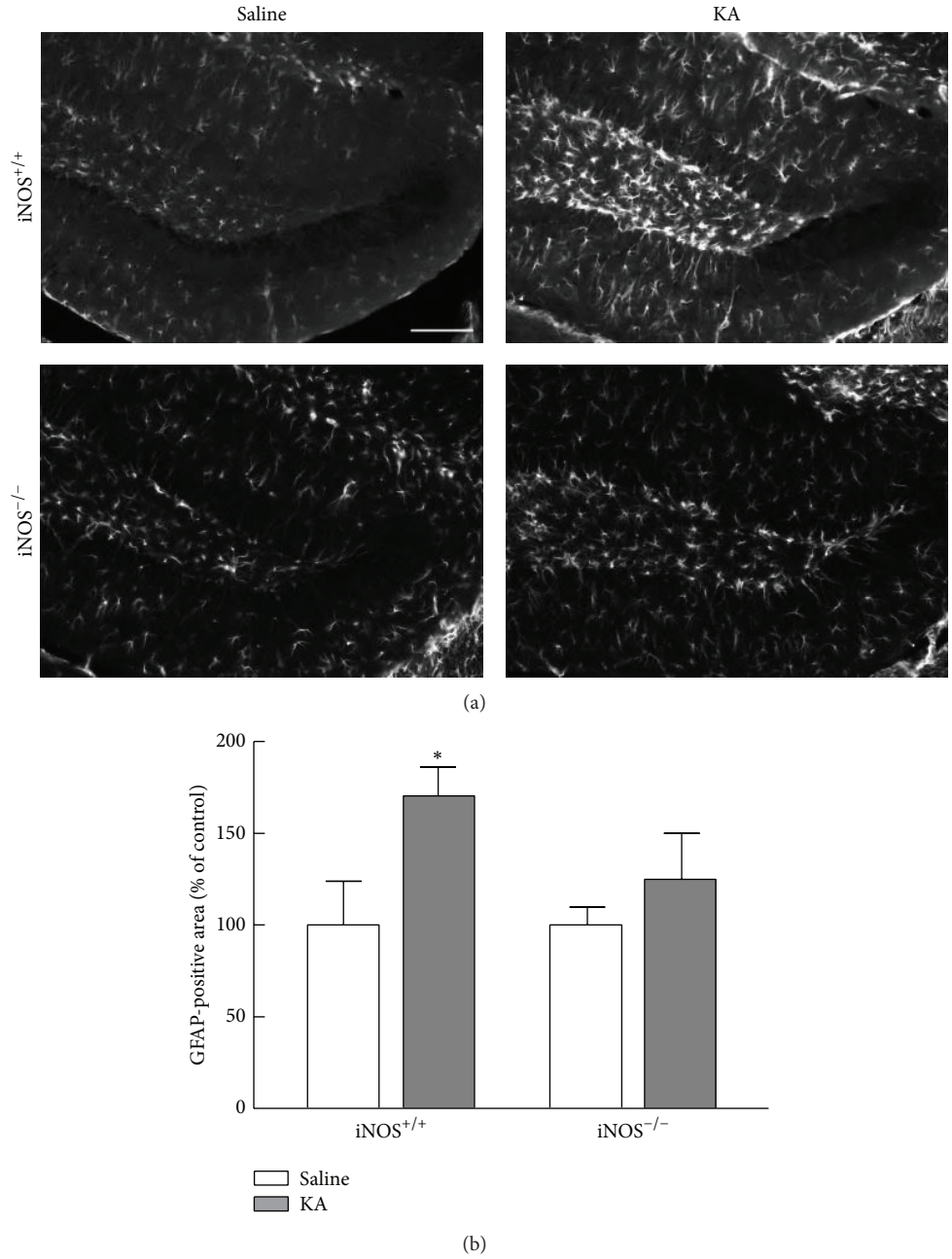

Figure 10: Astrogliosis is affected by abolishment of NO, 28 days after seizures. (a) Representative images of GFAP (white) immunoreactivity 28 days after KA or saline treatment in $iNOS^{+/+}$ or $iNOS^{-/-}$ mice. Scale bar: 100 μm. (b) GFAP immunoreactivity 28 days following SE. Data are expressed as means ± SEM. Two-factor ANOVA (Bonferroni's posttest): $N = 4$ to 5; $^{*}p < 0.05$, significantly different from $iNOS^{+/+}$ saline.

Previously, our group studied neuroinflammation 5 days after seizures and showed an increase in the number of reactive astrocytes in either $iNOS^{+/+}$ or $iNOS^{-/-}$ treated with KA; thus the process is independent of NO production [15]. Here we show that activation of astrocytes is maintained up to 28 days after seizures. However, the astrogliosis is not observed at this time point in the mice lacking iNOS, suggesting that late astrogliosis, but not early astrogliosis, is NO-dependent. This prolonged neuroinflammation may condition survival of the newborn neurons in $iNOS^{+/+}$ KA-treated mice, as observed.

3.5. Regulation of Physiological versus Pathophysiological Neurogenesis by NO. The role of NO in regulation of neurogenesis is still unclear. Overall, NO seems to negatively regulate neurogenesis in physiological conditions, while in pathophysiological situations it shows proneurogenic action. Several studies reported a decrease in proliferation of NSC [37–39] and survival of the newborn cells [36]. NO can also modulate differentiation of new precursors by increasing neuronal [38, 39] or astrocytic differentiation [36].

After a brain insult, NO has been reported as proneurogenic factor, since an increase in proliferation of NSC is

reported in most of the injury-induced models, including stroke and seizures [22, 40]. Although differentiation is positively regulated by NO following brain insults [38], the survival of the newborn cells seems to be decreased by NO [41].

Our results, together with previous findings, suggest that not only is proliferation of NSC regulated by NO-dependent mechanisms following a lesion, but also differentiation and survival of the newborn neurons are regulated by the presence of NO following seizures. The fact that NO is important to maintain neuroinflammation up to 28 days after seizures may have influence on survival of newborn cells and may contribute to the failure of new neurons to efficiently survive in such conditions.

4. Conclusions

With this work we aimed to understand the involvement of NO produced from iNOS in hippocampal neurogenesis in a *status epilepticus* mouse model. Our results showed that production of NO in an inflammatory context increased proliferation of the early-born NSC following a brain insult. Early differentiation of neuroblasts and immature neurons increased following seizures by a NO-dependent mechanism. We also showed that the distribution of newborn cells along the dentate gyrus was modified by seizures, but NO was not involved in this phenomenon. Furthermore, survival of new neurons formed at an early stage (3 days after seizures) is decreased by NO. In fact, NO is showed to be important in maintenance of neuroinflammation up to 28 days after seizures, which may provide an aggressive environment for the newborn cells, which fail to survive.

Altogether, these findings help to better understand the involvement of NO produced by iNOS in different stages of adult neurogenesis following injury and open the possibility to explore new NO-based therapeutic approaches to brain repair after an insult, knowing when NO is proneurogenic and when it impairs survival of newborn neurons.

Abbreviations

BrdU: 5-Bromo-2′-deoxyuridine
GFAP: Glial fibrillary acid protein
IGZ: Inner granular zone
iNOS: Inducible nitric oxide synthase
KA: Kainic acid
NeuN: Neuronal nuclear
NGS: Normal goat serum
NO: Nitric oxide
NSC: Neural stem cells
OGZ: Outer granular zone
PFA: Paraformaldehyde
PBS: Phosphate-buffered saline
SGZ: Subgranular zone
SE: *Status epilepticus*
SVZ: Subventricular zone
DCX: Doublecortin.

Conflict of Interests

The authors declare that they have no conflict of interests.

Authors' Contribution

Bruno P. Carreira and Daniela F. Santos contributed equally to this work.

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Review Article

Tau Hyperphosphorylation and Oxidative Stress, a Critical Vicious Circle in Neurodegenerative Tauopathies?

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Hyperphosphorylation and aggregation of the microtubule-associated protein tau in brain, are pathological hallmarks of a large family of neurodegenerative disorders, named tauopathies, which include Alzheimer's disease. It has been shown that increased phosphorylation of tau destabilizes tau-microtubule interactions, leading to microtubule instability, transport defects along microtubules, and ultimately neuronal death. However, although mutations of the *MAPT* gene have been detected in familial early-onset tauopathies, causative events in the more frequent sporadic late-onset forms and relationships between tau hyperphosphorylation and neurodegeneration remain largely elusive. Oxidative stress is a further pathological hallmark of tauopathies, but its precise role in the disease process is poorly understood. Another open question is the source of reactive oxygen species, which induce oxidative stress in brain neurons. Mitochondria have been classically viewed as a major source for oxidative stress, but microglial cells were recently identified as reactive oxygen species producers in tauopathies. Here we review the complex relationships between tau pathology and oxidative stress, placing emphasis on (i) tau protein function, (ii) origin and consequences of reactive oxygen species production, and (iii) links between tau phosphorylation and oxidative stress. Further, we go on to discuss the hypothesis that tau hyperphosphorylation and oxidative stress are two key components of a vicious circle, crucial in neurodegenerative tauopathies.

1. Introduction

The tauopathies are a class of neurodegenerative disorders characterized by hyperphosphorylation and aggregation of the microtubule-associated protein tau (MAPT) into paired helical filaments (PHFs) or straight filaments (SFs), forming neurofibrillary tangles (NFTs) in brain. Unlike amyloid-beta (Aβ) aggregation, which is associated with Alzheimer's disease (AD), tau tangles are found in multiple neurodegenerative disorders such as progressive supranuclear palsy (PSP), corticobasal degeneration (CBD), Pick's disease, dementia pugilistica, frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17), and many other disorders including AD [1]. Mutations in the *MAPT* gene have been linked with several familial early-onset tauopathies [1]. More than 50 pathogenic mutations have been identified in the *MAPT* gene [1], providing evidence that tau alterations alone can cause neurodegeneration. It has been shown that abnormal tau hyperphosphorylation impairs its binding to microtubules and its capacity to promote microtubule assembly, resulting in its self-aggregation into NFTs, microtubule disorganization, and impaired transport along axonal microtubules [2, 3].

In addition to tau hyperphosphorylation, a growing body of evidence suggests that oxidative stress (OS) is another component of the pathophysiology of tauopathies. According to the OS theory of aging, which was deduced from genetic studies showing that manipulation of antioxidant defenses affects longevity in several animal models, brain neurons are seen as a crucial target of oxidative attacks. Moreover, OS has been implicated in the disease process in several neurodegenerative disorders, including AD [4]. In AD, the link between the production of toxic Aβ peptide and OS is well documented and has been the subject of several recent

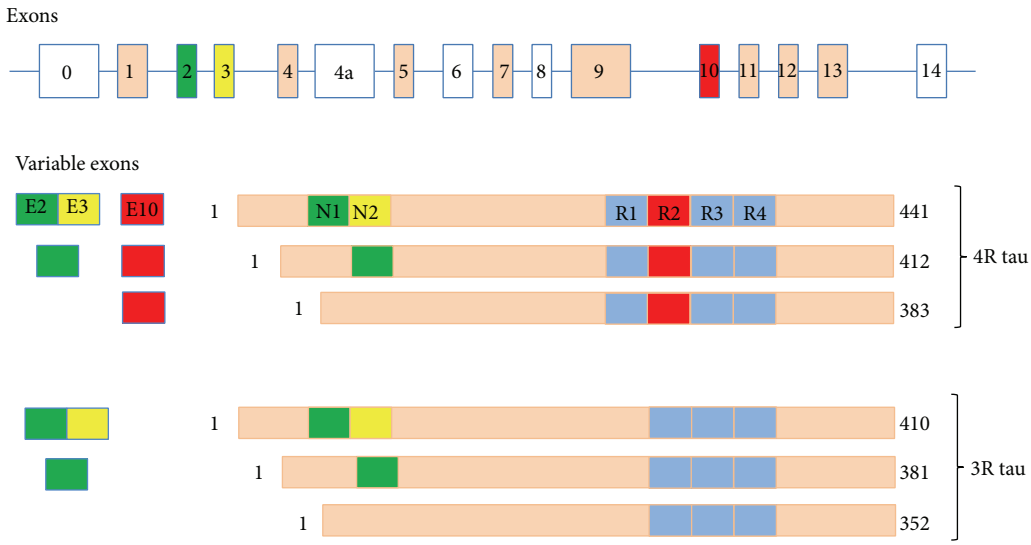

Figure 1: The *MAPT* gene, the variable exons, and the six tau isoforms in the adult human brain generated by alternative splicing. The constitutively spliced exons are shown in beige. E0, E4a, E6, E8, and E14 are not transcribed in human brain. Alternative mRNA splicing of E2 (green), E3 (yellow), and E10 (red) generates six tau isoforms ranging from 352 to 441 aminoacids. Three isoforms have four repeats each (4 repeat) and three isoforms have three repeats each (3 repeat). The repeats are shown with R (R1 to R4). The exons and introns are not shown to scale.

reviews [5, 6]. However, other data support an essential role for OS in tau hyperphosphorylation, tau polymerization, and tau toxicity. The accumulation of OS markers is now seen as a further hallmark of tau pathology in both patients and animal models. However, the precise role of OS in tauopathies remains far from clear.

Here, after a brief summary of our knowledge of tau structure and function, we review evidence suggesting that OS is both a late consequence of tau pathology paralleling the course of the disease, and an early cellular response to injuries linked to tau toxicity. Lastly we discuss the hypothesis that tau hyperphosphorylation and OS are the two key elements of a vicious circle, crucial in tau pathology.

2. Structure and Posttranslational Modifications of the Tau Protein

Tau is a highly soluble, natively unfolded, and phosphorylated protein predominantly located in axons of mature neurons [7–9]. Tau is also found in the neuronal somatodendritic compartment [10] and nucleus [11], and to a lesser extent in astrocytes and oligodendrocytes [12]. Six tau isoforms are expressed in the CNS in human adults [13]. The six isoforms are generated *via* alternative splicing of a single *MAPT* gene located at 17q21.31 and comprising 16 exons [14]. Tau isoforms range in length from 352 to 441 amino acids, and from 45 to 65 kDa, with exons 1, 4, 5, 7, 9, 11, 12, and 13, translated in all tau isoforms. Tau isoforms differ by the presence of three (3R) or four (4R) carboxy-terminal tandem repeat sequences of 31 amino acids, corresponding to microtubule-binding domains (MBDs). Tau isoforms also differ by the absence (0N) or presence of one (1N) or two (2N) N-terminal repeated sequences encoded by alternative exons 2 and 3. The 3R- and 4R-tau isoforms are found in equal amounts in the adult human brain; while the 0N, 1N, and 2N tau isoforms comprise about 37%, 54%, and 9% of total tau, respectively [15, 16] (Figure 1).

The tau protein found in the PNS is named "big tau," in reference to its high molecular weight compared with isoforms expressed in CNS neurons. This higher weight stems from the inclusion of exon 4a in the amino-terminal half of the protein [17]. Expression of different tau isoforms is tissue-specific [13] and dynamic during development, probably owing to its key role in cytoskeletal plasticity during embryogenesis and early development. In humans, only the shortest tau isoform (3R/0N) is expressed in fetal brain, whereas all six isoforms are found in adult CNS [18].

Posttranslational modifications are a major factor behind the diversity of tau. Besides phosphorylation, the crucial posttranslational modification in tau, this protein also undergoes an array of other posttranslational changes, such as *O*-glycosylation [19], ubiquitination [20], SUMOylation [21], nitration [22], glycation [23], acetylation [24], cross-linking by transglutaminase [25], isomerization [26], conformational alteration, and proteolytic cleavage [27], all of which are involved in tau regulation and pathology. *O*-GlcNAcylation (a form of tau *O*-glycosylation) reduced tau phosphorylation in cerebral cortex and hippocampus in rat [28], and in Alzheimer's disease, *O*-GlcNAcylation levels negatively correlate with tau phosphorylation [29]. Tau acetylation at lysine residue 280 increased tau fibrillization and decreased tau-dependent microtubule assembly *in vitro*. Tau acetylation was also found to be strongly associated with tau hyperphosphorylation and tau inclusions in Alzheimer's disease, corticobasal degeneration, progressive supranuclear palsy, and in tau transgenic mouse models of tauopathies [24, 30].

However, the precise functions of tau modifications remain enigmatic.

3. Functions of the Tau Protein

The main function recognized for tau is promoting microtubule polymerization and stabilization. Microtubules form part of the cytoskeletal framework in all eukaryotes and are composed mainly of heterodimers of α- and β-tubulin forming tubular polymers. Microtubules play a major role in cytoskeletal maintenance and act as highways for intracellular transport of organelles, vesicles, proteins, and signaling molecules [31].

The 4R-tau isoform displays the highest affinity for microtubules and efficiently promotes microtubule assembly, likely due to the presence of the interrepeat sequence located between the first and second MBDs [16]. Binding of tau to microtubules has been visualized by NMR spectroscopy [32] and cryo-EM [33]. However, some studies have challenged the primary function of tau as main regulator of microtubule stability and assembly. In cell culture, tau colocalizes with the most dynamic microtubules [9], those showing the highest basal turnover rate among microtubule populations [34]. Moreover, siRNA-mediated knockdown of tau does not impair microtubule polymerization [35]. In addition, tau, *via* its MBD, interacts with other proteins such as actin [36], presenilin-1 (PS-1) [37], tau phosphatases [38], RNA [39], and DNA [40], strongly suggesting that tau has multiple functions apart from its putative role as modulator of microtubule dynamics.

The N-terminal domain of tau interacts with Src homology 3 (SH3) domains in a number of signaling proteins [41], such as Fyn tyrosine kinase. Tau also promotes process extension in oligodendrocytes by connecting Fyn to microtubules [12]. Tau also stimulates Src-mediated actin rearrangements after growth factor stimulation [42]. Interestingly, the binding of tau to neuronal plasma membrane components and SH3 domains is affected by its phosphorylation [43, 44]. Other functions of tau include binding and activation of phospholipase C (PLC) γ [45], inhibition of the enzyme histone deacetylase-6 (HDAC-6) [46], modulation of the cellular response to heat shock [47], and adult neurogenesis [48]. Lastly, it was recently shown that the 2N/4R tau isoform plays a role in microglial activation in humans [49]. These data suggest that tau is a scaffolding protein able to (i) bind to at least two signaling proteins, (ii) restrict signaling molecules and transduction pathways to defined cellular compartments, and (iii) regulate several signaling pathways [50].

Notwithstanding these multiple hypothetical functions, the relationship between tau and microtubules is well established. In particular, tau appears to modulate axonal transport *via* interaction with motor proteins, such as detachment of kinesin from microtubules and directional reversal of dynein, without affecting the speed of transport [51–53]. High levels of tau accumulation are detected in the axonal region near the synapse [54], which may facilitate cargo delivery to presynaptic terminals [51]. Moreover, while several observations suggest that removal or overexpression of tau *in vitro* or *in vivo* does not impair axonal transport [55, 56], other studies suggest that overexpression of wild-type or mutant tau in either cell or mice models of tauopathy impairs axonal transport [57, 58]. These discrepancies may be due to differences in study design or model systems used.

Four tau knockout (KO) lines have been independently generated in mice [59–62]. All four lines are viable and fertile, with no apparent phenotypes. Harada et al. [59] generated the first mouse tau KO strain. Apart from the absence of an overt phenotype, these mice showed reduced microtubule stability and mild disorganization of small-caliber axons. In another tau KO line, Dawson et al. [60] reported a delay in neuronal maturation in *in vitro* culture from the tau KO line. However, in both KO lines, microtubule-associated protein (MAP) 1A was found to be elevated during early developmental stages, suggesting that overexpression of MAP1A is able to compensate for the loss of tau. Interestingly, an increased accumulation of MAP1A was also reported in the third tau KO line, which was generated by Fujio et al. [62]. In addition, despite normal levels of MAP1B in tau KO mice generated by Harada et al. [59], cross-breeding of this line with MAP1B-deficient mice resulted in cerebral developmental defects and early lethality [63], suggesting a further functional overlap between tau and this MAP.

Precise investigations of tau KO mice generated by Harada et al. [59] revealed progressive behavioral impairments and motor deficits in older individuals. These phenotypes comprised muscle weakness, impaired balance, hyperactivity, and learning deficits from 12 months of age onward [64]. More recently, complex motor deficits linked to dopaminergic neuronal loss and increased iron accumulation in neurons in substantia nigra were reported in another tau KO line [65]. Tau KO mice also showed deficits in neuronal circuit formation as revealed by electrophysiology [66]; alongside these negative consequences, tau KO mice are more resistant to epileptogenesis, excitotoxicity, and beta-amyloid toxicity [67, 68].

Tau overexpression in neuronal cell cultures results in an increased number of neurites per cell [69]. Moreover, while insertion of human *MAPT* gene in mice leading to expression of all six tau isoforms does not induce severe neuropathology [70], expression of this transgene in a mouse tau KO context resulted in tau hyperphosphorylation and accumulation of insoluble 3R tau [71]. These mice also develop neurodegeneration and age-dependent memory loss [72, 73]. By contrast, hind-limb clasping and spinal cord abnormalities accompanied with somatodendritic distribution of the human tau protein were detected in human 2N4R tau cDNA mouse, while endogenous mouse tau was present [70]. A transgenic line with KI insertion of human 2N4R tau cDNA in the first exon of the mouse *MAPT* gene displayed increased neuronal survival accompanied by better performance in a novel object recognition task [74]. Apart from different types of transgenes and the presence or absence of endogenous mouse tau yielding different phenotypes, the promoter type also profoundly affected the observed phenotypes. Severalfold overexpression of the shortest human tau isoform to endogenous mouse tau resulted in axonal degeneration and progressive motor weakness [75].

Table 1: Major pathogenic tau mutations.

Mutation	Type	Effect	Reference
P301L	Substitution	Neuronal impairment, NFT formation	Vogelsberg-Ragaglia, et al., 2000 [94]
P301S	Substitution	Tau hyperphosphorylation and aggregation	Bugiani et al., 1999 [95]
ΔK280	Deletion	Tau aggregation and decreased microtubule affinity, neural loss	Vogelsberg-Ragaglia, et al., 2000 [94]
VPR triple mutant (R406W, P301L, and V337M)	Substitution	Tau hyperphosphorylation, aggregation, and decreased microtubule affinity	Tatebayashi, et al., 2002 [96] Tanemura, et al., 2002 [97]
N296N	Silent	Greater 4R to 3R ratio (splicing)	Spillantini, et al., 2000 [98]

The precise mechanism of hyperphosphorylated tau-mediated neurotoxicity remains to be elucidated, but two major mechanisms have emerged to date: (i) toxic loss of function, with loss of physiological tau function held responsible for tau pathology [76–78] and (ii) toxic gain of function with highly phosphorylated tau displaying ill-defined toxic effects in neuronal cells [72, 79, 80]. Interestingly, it was shown that hyperphosphorylated tau relocates to dendritic spines, where it may exert its toxic effect by impairing trafficking and/or synaptic anchoring of glutamate receptors [81].

More than 50 mutations of the *MAPT* gene have been identified to date that are associated with neurodegenerative tauopathies [1]. Table 1 lists the most important pathogenic tau mutations. Many of these mutations are missense mutations or small deletions, which modify tau sequence. In particular, several missense mutations such as G272V, P301L, P301S, V337M, G389R, and R406W decrease the *in vitro* affinity of tau to microtubules, resulting in deficits in microtubule assembly and stability [15, 82, 83]. By contrast, missense mutations S305N and Q336R slightly increase the ability of tau to promote microtubule assembly [84, 85]. In addition, most of these missense mutations enhance tau aggregation [86, 87]. Lastly, several mutations impair binding of tau to protein phosphatase 2A, one of the major tau phosphatases in brain neurons [88]. Other mutations are located in either intronic sequences close to the 5′ splice site of exon 10 (positions +3, +11, +12, +13, +14, +16, +19, and +29) or exonic sequences, also impairing exon 10 splicing (N279K, ΔK280, L284L, N296H, ΔN296, P301L, P301S, G303V, and S305N). All these mutations disrupt the physiological 1 : 1 ratio of 4R to 3R tau isoform in the adult human brain. In turn, the increased production of 4R tau leads to its assembly onto NFTs and ultimately neurodegeneration [89]. Other mutations in intronic sequences adjacent to the stem-loop structure in exon 10 increase accumulation of soluble 3R tau isoform through alteration of *MAPT* gene splicing. Interestingly, these mutations lead to neuronal apoptosis and increased accumulation of tau degradation products, but not the formation of NFTs [90]. Finally, several pathologic tau mutations are located in exon 10, such as ΔK280, ΔN296, and N296H, and induce tau protein accumulation and affect *MAPT* RNA levels [83, 91–93].

Although these results point to tau as a key factor essential for proper microtubule assembly and dynamics, they also highlight the complex and still poorly understood function of this protein.

4. Phosphorylation, Oligomerization, Aggregation, and Propagation of the Tau Protein

The activity of the phosphoprotein tau is mainly regulated by phosphorylation. Similar to the expression of different tau isoforms, tau phosphorylation is also developmentally regulated, which is important for cytoskeletal plasticity during early development. Tau phosphorylation also influences the structure, distribution, and function of the protein in neurons [99].

Tau phosphorylation is increased in both physiological and pathological contexts, but it is still unclear whether the mechanisms involved in physiological and pathological tau hyperphosphorylation overlap. Tau phosphorylation is markedly increased during embryonic development [100], likely due to the increased need for neuronal plasticity. Several cellular stress conditions such as OS [101–103], heat stress or hypothermia [103, 104], and even starvation [105] modulate tau phosphorylation. Hyperphosphorylated tau is also identified as the main component of NFTs [106]. High levels of hyperphosphorylated tau have been detected in the cerebrospinal fluid (CSF) of patients suffering from tauopathies. Levels of hyperphosphorylated tau in CSF also correlate with hippocampal atrophy in prodromal AD, also called "mild cognitive impairment" (MCI) [107, 108]. Tau is hyperphosphorylated in all tauopathies, but hyperphosphorylation states differ among and within disorders [109]. Importantly, no single phosphorylation site is specific for tauopathies, and hyperphosphorylation is characterized by an overall increase in tau phosphorylation at multiple residues. Also, tau hyperphosphorylation is defined as an increase in either the number of phosphorylated sites per tau molecule or the fraction of tau molecules phosphorylated at a given site. Thus 1.9 moles of phosphate per mole of tau is found in a healthy human brain, against 6 to 8 moles of phosphate per mole of tau in an AD brain [110]. However, the precise phosphorylation state of tau is difficult to define in *postmortem* biopsy material, due to the labile nature of phosphorylated tau, which quickly becomes dephosphorylated after excision [111].

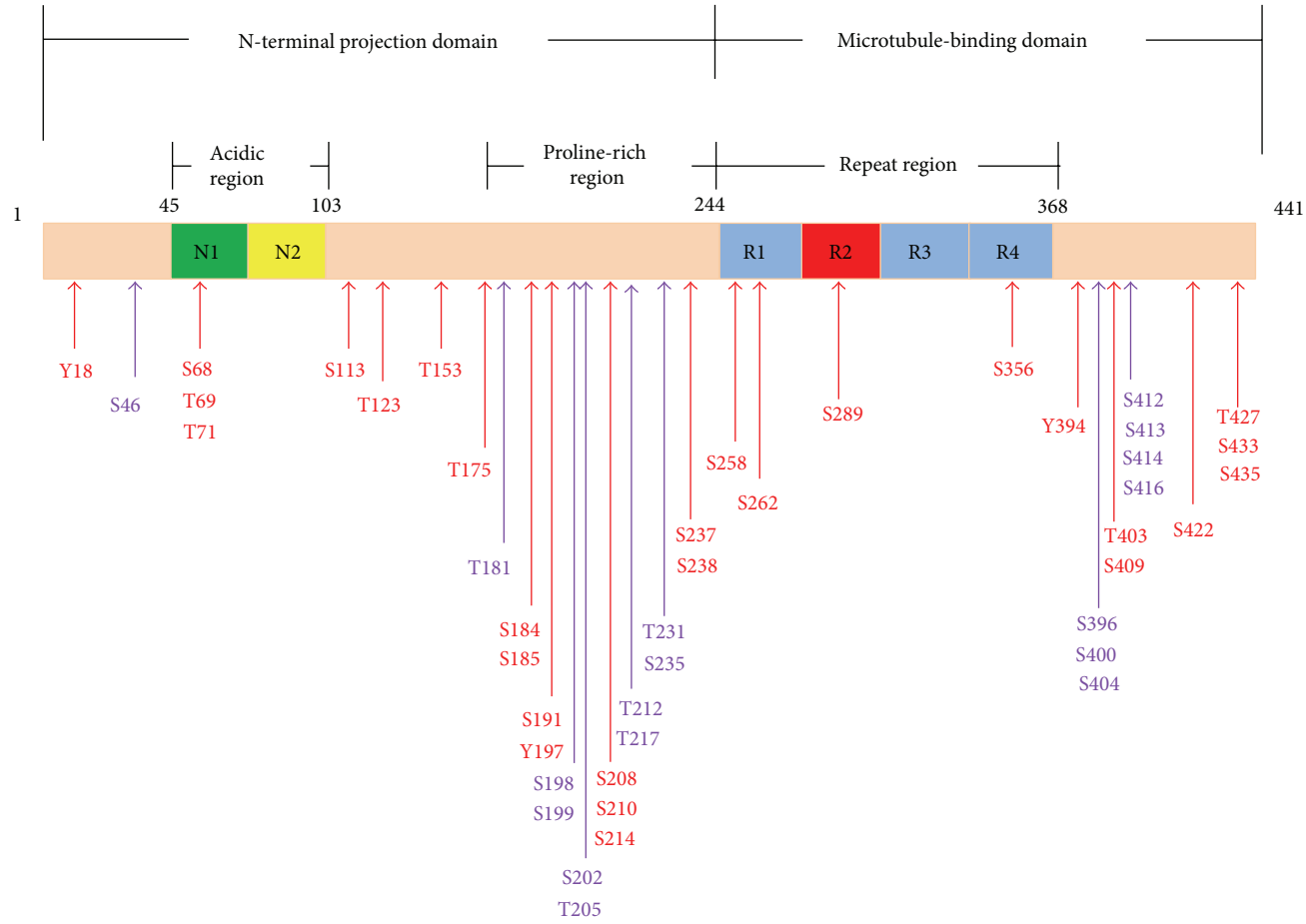

FIGURE 2: Schematic representation of the distribution of tau phosphorylation sites on the longest tau isoform (441 amino acids). The two amino-terminal inserts are demonstrated by E2 and E3. The microtubule-binding domains are represented with R(1–4). Physiologic tau phosphorylation comprises approximately 10 phosphorylated residues. The physiologically phosphorylated residues are shown in purple and cluster in the proline-rich domain (PRD) and in the C-terminal region. The number of phosphorylated residues rises to 45 on the longest tau isoform from Alzheimer brain with the appearance of phosphorylated residues shown in red. The two amino-terminal inserts and repeat regions are not physiologically phosphorylated in adult human brain. A number of phosphorylation sites are detected in both regions on the longest tau isoform from Alzheimer brain.

Nearly 45 phosphorylated sites have been identified on tau extracted from AD brain, that is, more than half of all the 85 theoretical phosphorylable residues on the longest tau isoform (2N4R) (Figure 2). This situation contrasts with the approximately 10 and 18 phosphorylated residues found on soluble tau extracted from adult human brain and fetal rat brain, respectively. Most hyperphosphorylated residues in AD are clustered in the C-terminal and the proline-rich domains of tau, and very few sites are located within the N-terminal region and MBD. Most phosphorylated sites have been identified by mass spectrometry or Edman degradation, and a few sites located mostly at the N-terminal region have been identified only by phosphospecific antibodies [112].

Tau hyperphosphorylation is thought to result from an imbalance in the function of several protein kinases and phosphatases [109]. While tau is phosphorylated by a large number of kinases *in vitro* [112], the identity of the kinases, which are responsible for physiological or pathological phosphorylation of tau *in vivo*, has so far remained elusive. In particular, as no single kinase is able to phosphorylate all pathological tau residues, several kinases may be involved in tau hyperphosphorylation. Tau kinases fall into the two main groups of proline-directed and non-proline-directed kinases. Proline-directed tau kinases GSK3β and CDK5 are important tau kinases that phosphorylate tau at a large number of serine and threonine residues and play an important role in tau pathology in AD [113, 114]. Furthermore, GSK3β activation and CDK5 overexpression were shown to induce tauopathy-related phenotypes in mouse models [115, 116]. Activity of nonreceptor tyrosine kinases (such as Fyn and c-Abl) have also been linked to AD pathology [117]. It has been proposed that tau phosphorylation by different kinases likely follows a sequential pattern, in which phosphorylation of given residues facilitates that of other phosphorylation sites. Such a sequential pattern of kinase activities has been suggested by *in vitro* observations of the effect of DYRK1A and CDK5 kinases on GSK3β-mediated tau phosphorylation [118, 119]. In addition, the activation of one kinase may activate a second

one through a "kinase cascade," as what was observed by the stimulatory effect of CK1 and c-Abl kinases on CDK5 [120, 121].

Phosphatases dephosphorylating tau *in vitro* include PP1, PP2A, PP2B, and PP5 [122]. PP2A displays the strongest dephosphorylating effect on tau [123] and may be an important regulator of tau phosphorylation *in vivo* [124]. A decrease in PP2A activity mediates hyperphosphorylation of tau in hypothermia [104]. Studies also suggest that PP2A expression and activity are markedly decreased in AD [125, 126]. Furthermore, an AD-like pathology has also been observed secondary to treatment with okadaic acid, a potent inhibitor of phosphatases 1 and 2A in rats [127, 128].

Increased tau phosphorylation decreased its affinity for microtubules: pathologic phosphorylation of tau at Ser396 and Ser404 decreases its binding affinity for microtubules [129]. Similarly, phosphorylation at S214 and T241 in the proline-rich domain reduces the binding of tau to microtubules [2].

Aggregation-prone tau species display toxicity in cell culture [130] and in a transgenic mouse model [131]. However, whereas it was previously thought that aggregated tau was toxic, more recent studies suggest that soluble and prefibrillar tau species are more likely to be implicated in neurodegenerative processes [132]. In particular, several observations suggest that tau oligomers, but not tau aggregates, are the toxic species. In AD patients, neuronal loss exceeds NFT number by at least one order of magnitude [133], and some familial tau mutations associated with frontotemporal dementia cause very few aggregated tau inclusions [134]. Tau oligomers and truncated tau species display toxicity *in vitro* [135, 136]. Following expression of the aggregation-prone $\text{tau}^{\Delta K280}$ construct in N2a neuroblastoma cells, the toxic tau species were identified as prefibrillar forms of tau before the β-sheet containing aggregates were detected [130]. Moreover, tau overexpression in several *Drosophila* models induces neurodegeneration without NFT formation [137, 138]. However, it must be borne in mind that the lifespan of *Drosophila* neurons is one order of magnitude lower than that of mammalian neuronal cells. Lastly, whereas accumulation of soluble forms of the tau protein correlates with neuronal and synaptic dysfunction and toxicity in several mouse models [80, 81, 139], in two different conditional mouse models of tauopathies, transgene silencing resulted in memory recovery while NFTs were still present [80, 139].

In vitro, recombinant full-length and nonphosphorylated tau is able to form filaments following interaction with negatively charged compounds such as sulfated glycosaminoglycans, RNA, or fatty acids [39, 140–142]. Moreover, both wild-type and mutant tau bearing G272V, ΔK280, P301L, P301S, S305N, V337M, or R406W mutations do not form filaments *in vitro* in the absence of heparin or other negatively charged compounds. However, the exact mechanism by which heparin induces formation of tau fibrils remains a matter of debate.

Interestingly, in a given tauopathy, tau aggregation propagates in a sequential and predictable fashion from one brain region to another, in a manner similar to that described for prion proteins. This stereotypical spatiotemporal spreading of tau aggregation has been described in AD [143] and argyrophilic grain disease (AGD) [144], suggesting that tau pathology spreads along defined neuronal pathways. *In vitro* studies have demonstrated intercellular transfer of tau inclusions in cultured cells [145]. Sulfated glycosaminoglycans at the cell surface are required for internalization of aggregated tau [146]. This phenomenon has also been observed *in vivo* in mice expressing wild-type human 4R tau isoform, following injection of brainstem extracts from mice expressing human tau^{P301S} [147]. Also, formation of tau inclusions is accelerated when filaments assembled from recombinant human tau^{P301S} were injected into the brains of young tau^{P301S} transgenic mice before the formation of tau aggregates [148]. In addition, transgenic mice overexpressing human tau^{P301L} in restricted areas of the entorhinal cortex and subiculum showed a propagation of the pathology in other synaptically connected brain regions [149, 150]. The data suggest that filamentous tau can convert soluble tau to fibrillar tau, forming tau inclusions.

5. Neurodegenerative Tauopathies

Tauopathies are heterogeneous disorders with partially overlapping clinical, neuropathological, and genetic characteristics, forming a spectrum of disorders. These diseases are characterized by microtubule-associated tau protein abnormalities [1]. Tau inclusions occur in neurons, astrocytes, and oligodendrocytes [151, 152].

Intraneuronal tau inclusions display different morphologic features in tauopathies, ranging from NFTs and neuropil threads to dystrophic neurites and Pick bodies. Tau aggregates are also differentiated by the phosphorylation and isoform content of tau, allowing a molecular classification of tauopathies. A biochemical classification based on the electrophoretic profile of tau discriminates four distinct classes [153].

Class I tauopathies are characterized by three major electrophoretic tau isoforms at 60, 64, and 69 kDa and a minor isoform at 72/74 kDa. This profile corresponds to the aggregation of the six tau isoforms in the human brain and characterizes AD, Down syndrome, and several other tauopathies. Class II forms are characterized by two major electrophoretic isoforms at 64 and 69 kDa and a minor band at 74 kDa. This profile characterizes several tauopathies such as CBD, AGD, and PSP. The profile of Class III tauopathies displays two major bands at 60 and 64 kDa that correspond to tau isoforms lacking exon 10 encoded sequences (3R tau). This profile is found in Pick's disease and FDTP-17. Lastly, in Class IV tauopathies, a major band at 60 kDa and two minor bands at 64 and 69 kDa are found that correspond to tau isoforms devoid of sequences encoded by exons 2, 3, and 10. This class comprises type I and type II myotonic dystrophy.

While many familial tauopathies are caused by mutations in the *MAPT* gene [154], other forms are caused by specific environmental factors. In particular, tau aggregations are found in chronic traumatic encephalopathy (CTE), a neurodegenerative disease first described in boxers and termed dementia pugilistica. In this case, the etiology of the disease

is linked to repetitive blast and/or injury to the brain. CTE is characterized by progressive neurodegeneration with widespread deposition of hyperphosphorylated tau as NFTs.

Brain trauma is thought to induce the dissociation of tau from microtubules *via* mechanisms such as intracellular calcium influx, glutamate receptor-mediated excitotoxicity, and kinase activation, leading to hyperphosphorylation of intracellular tau [155–157]. In addition, a sporadic tauopathy, endemic to the island of Guadeloupe, has been linked to the consumption of annonacin, a naturally occurring toxin with mitochondrial complex I inhibitor properties [158]. Low nanomolar concentrations of the toxin have been shown to cause neurodegeneration and induce redistribution of tau from axons to the somatodendritic compartment in neuronal culture [159]. *In vivo*, the toxin causes an increase in both tau levels and tau phosphorylation. This toxin also increases neuronal somatodendritic accumulation of hyperphosphorylated tau in transgenic tau^{R406W} mice following a brief 3-day exposure [160].

Initial causative events and precise pathological processes remain largely elusive in tauopathies. Mutations in the *MAPT* gene have been identified in a large number of rare familial tauopathies, but environmental factors such as injuries and toxins, most of which remain to be identified, are also suspected to play a role in the far more frequent sporadic forms of the disease. In particular, multiple lines of evidence point to OS as an important agent in the pathophysiology of neurodegenerative tauopathies.

6. Oxidative Stress Is a Common Feature of the Pathophysiology of Neurodegenerative Diseases

Reactive oxygen species (ROS) are chemically reactive molecules containing oxygen, such as superoxide anion (O_2^-), hydroperoxyl radical (HO_2), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH^-). Under normal conditions, cells permanently produce limited amounts of ROS by multiple biochemical processes. The "professional" ROS producers, such as mitochondria, peroxisomes, and endoplasmic reticulum, are the major sources. In mitochondria, ROS are produced permanently as a byproduct of ATP production by the electron transport chain. It is well documented that elevated levels of ROS are highly toxic to cells, in which they damage essential macromolecules, such as DNA, RNA, proteins, and membrane lipids. Importantly, it has been proposed that accumulation of ROS and oxidative damage on macromolecules contribute not only to the physiology of ageing, but also to the pathophysiology of many diseases, including various types of neurodegenerative disorders.

Cells have developed several strategies to manage ROS. In particular, they synthesize several enzymes that display antioxidant properties. Among this large family of antioxidant proteins are catalase, glutathione peroxidase (GPx), and superoxide dismutase (SOD), which protect against the damaging effects of reactive oxygen species (ROS). In particular, the chief defense against superoxide anion produced in the course of respiration is mitochondrial SOD2. However, ROS detoxification is not 100% efficient, and residual superoxides and peroxides persist in cells. In addition, it has been shown that microglial cells produce ROS, either in an attempt to eliminate pathogens or during the first steps of neuroinflammatory processes. Several recent studies have established a direct, albeit complex relationship between neurodegenerative diseases of the AD/tauopathy types and microglial activation [49, 161, 162]. However, this novel and very interesting field of investigation lies outside the scope of this review.

Thus while controlled amounts of ROS are permanently made as a byproduct of cellular metabolism and inflammatory processes under physiological conditions, a pool of antioxidant molecules is permanently produced to detoxify toxic oxygen species. In this context, oxidative stress refers to an imbalance between ROS levels and available antioxidant molecules. In particular, it is well established that sustained oxidative stress can be very damaging to cells, such conditions leading to cell apoptosis as the consequence of activation of the JNK pathway [163, 164].

Several lines of evidence suggest that the balance between ROS and antioxidant defenses is particularly fragile in brain neurons. More importantly, perturbations of this delicate equilibrium are suspected to play a crucial role in many neurodegenerative diseases, first in Alzheimer's disease and more recently in other tauopathies. We know that neuronal cells display unique features and properties: they are permanently postmitotic cells that also show high energy and oxygen consumption, suggesting an elevated rate of ROS production. Brain neurons also contain high levels of transition metals, which can adopt at least two oxidation states, including a reactive one. Iron and copper are tightly linked with the cellular redox state. Fe^{2+} can generate the hydroxyl radical through the fenton reaction [165]. Moreover, the brain has low levels of antioxidants relative to other organs [166].

7. Brain Neurons Undergo Oxidative Stress in Tauopathies

A large body of evidence indicates that Aβ deposition in both AD patients and transgenic animal models is associated with an accumulation of OS markers. Similarly, 20 years ago, the first evidence for OS was detected in Pick's disease and CBD patients. Increased levels of heme oxygenase-1 (HO-1), a putative marker of oxidative injury, were detected in Pick bodies in Pick's disease patients and neuropil threads and glial inclusions in patients with CBD [167]. More recently, OS was also found to be associated with tauopathies of the FTLD spectrum of disorders [168] and PSP types [169]. Several OS markers are notably increased in these tauopathies, such as malondialdehyde (MDA), which is formed following the reaction of ROS on polyunsaturated lipids, or 4-hydroxynonenal (HNE) and thiobarbituric acid reactive substances (TBARS) produced by peroxidation of intracellular lipids [168, 170, 171]. Oxidative damage of the glycolytic enzymes fructose bisphosphate aldolase A (aldolase A) and phosphoglycerate kinase-1 (PGK-1) was also detected in the frontal cortex in PSP cases [168].

In addition to OS markers, several studies have reported an activation of antioxidant defenses in several tauopathies. In particular, in several PSP patients, an increased level of SOD2 was detected in the subthalamic nucleus [172], and high levels of Cu/Zn-SOD were found in cerebrospinal fluid [173]. Interestingly, increased levels of SOD, Glutathione peroxidase (GPx), and HNE-conjugated GPx were found in PSP patients that correlated positively with age and thus disease progression [173].

The overexpression of both OS markers and antioxidant enzymes in both patients with different types of tauopathies and animal models strongly suggests that OS is a key component of the pathophysiology of these diseases. However, these data also raise the important question of the role of OS in tau pathologies, a fundamental issue for the development of efficient therapeutic strategies. In other words, is OS an early causal factor in the pathophysiological process or is it merely a consequence of the different cell injuries induced by tau hyperphosphorylation.

8. Oxidative Stress, an Early Marker of Tauopathies

Several studies performed on various cellular or animal models of tauopathies have established that overexpression of mutant forms of human tau underlying various types of dominantly inherited tauopathies increases both the expression of OS markers and the sensitivity of neurons to oxidant molecules, such as H_2O_2 or paraquat. Following overexpression of wild-type tau in N2a neuroblastoma cells, an increased susceptibility to H_2O_2 was observed, linked to peroxisome depletion in neurites due to inhibition of transportation along microtubules [57]. More recently, a proteomic analysis of transgenic mice carrying human tau^{P301L} protein underlying FTDP-17 identified proteins involved in mitochondrial respiration and metabolism (mainly components of respiratory complex V) and antioxidant enzymes (peroxiredoxins 5 and 6, glutathione S-transferase, and GPx) as the two major classes of downregulated proteins. Furthermore, biochemical analysis of these mice showed an increased ROS production and lipid peroxidation in the brain, elevated activities of antioxidant enzymes, and evidence of mitochondrial dysfunction. In this model, increased ROS levels were already detected in 12-month-old tau^{P301L} mice but were more pronounced and statistically significant in 24-month-old individuals, correlating with the age-specific increase in tau pathology [174]. This progressive increase in ROS that parallels the progression of the disease suggests that ROS production is a mere consequence of the pathophysiological process.

However, other studies suggest that oxidative stress is present at earlier stages of the pathologic process in AD [175, 176], and more recently in other tauopathies [177]. Accumulation of a truncated tau fragment has been described in sporadic AD cases, and cultured cortical neurons from a transgenic rat model expressing this truncated protein displayed high levels of OS markers and an increased susceptibility to ROS, resulting in a dramatic increase in mortality upon exposure [178]. These findings suggest that truncation of tau is an early event that precedes OS in this model.

OS and mitochondrial dysfunction accompanied by behavioral deficits were detected prior to tau hyperphosphorylation and NFT accumulation in transgenic mice expressing human tau^{P301S} protein, which underlies another autosomal dominant form of FTDP-17 [177]. In this model, a decreased expression of MnSOD was detected from 7 months of age onward, while tau hyperphosphorylation and tangle formation were only detected 3 months later and were associated with GSK3β activation. In these mice also, at 7 and 10 months of age, elevated protein carbonyl levels (an indicator of protein oxidation) were detected in the cerebral cortex, suggesting that ROS production occurs early in the disease process. Importantly, while markers of lipid peroxidation and protein oxidation were markedly elevated at 12 and 24 months of age, ROS levels in isolated brain cortex mitochondria were only mildly increased at the same stages. This result indicates that mitochondria are not the major source of ROS in these transgenic tau^{P301S} mice [177].

In a recent study, several lines of evidence suggest that OS is a primary and causal player in the neurotoxicity induced by tau mutations, through induction of both apoptosis and dysregulated cell cycle activation [179]. First, using a *Drosophila* model that overexpresses human mutant tau^{R406W} associated with FTD, Dias-Santagata et al. [179] showed that genetic downregulation of the antioxidant enzymes SOD2 or thioredoxin reductase (Trxr) increases neurodegenerative phenotypes induced by tau^{R406W} expression. In close agreement with these data, treatment of tau^{R406W} flies with 30 mM paraquat leads to an increased mortality rate, underlining the elevated sensitivity of these flies to OS. Next, using a transgenic line expressing another tau construct, designated tau^{E14}, in which 14 S/T phosphorylation sites were mutated to glutamate to mimic phosphorylation, it was shown that flies overexpressing tau^{E14} and heterozygous for either $Sod2^{n283}$ or $Trxr^{\Delta 1}$ mutations displayed an increased number of apoptotic cells. Importantly, in these flies, levels of tau hyperphosphorylation were not modified by the downregulation of the antioxidant enzymes. These results clearly show that heterozygous *Sod* or *Trxr* mutations enhance the toxicity of tau^{E14} independently of tau phosphorylation levels. As the JNK signaling pathway is one of the best characterized responses to oxidative damage [163, 180], the authors investigated JNK activation in tau^{E14} individuals and tau^{E14} flies heterozygous for either $Sod2^{n283}$ or $Trxr^{\Delta 1}$. Interestingly, compared with levels seen in tau^{E14} individuals, a 6- or 9-fold increased activation of the JNK pathway was detected in tau^{E14} flies heterozygous for either $Sod2^{n283}$ or $Trxr^{\Delta 1}$, respectively, suggesting that JNK pathway activity induced by ROS leads to induction of apoptosis. Moreover, in these flies, activation of the JNK pathway was only detected in brain neurons, the sole brain cell type showing significant levels of apoptosis. Finally, while proliferating brain neurons are extremely uncommon in adult wild-type flies, approximately 40 and 100 PCNA and phosphorylated histone 3 (PH3) expressing neurons were detected, in tau^{R406W} individuals

and tau^{R406W} flies heterozygous for either *Sod2*^{n283} or *Trxr*$^{\Delta 1}$, respectively. Thus this study identified both activation of JNK pathway-mediated apoptosis and cell cycle dysregulation as pathogenic processes directly induced by ROS produced as a consequence of the pathophysiological processes induced by tau hyperphosphorylation.

Altogether, these studies identify OS as an early cellular dysfunction in the pathophysiology of tauopathies, which induces both JNK-mediated apoptosis and a dysregulated cell cycle in brain neurons. These data also suggest that either tau hyperphosphorylation *per se,* microtubule disorganization, or some other not yet identified consequences of abnormal tau accumulation promote ROS production and thereby OS in brain neurons. However, the source of ROS in the different models used, that is, mitochondria versus other producers such as microglia, and the precise role of oxidant molecules in the pathologic processes are two key issues that remain unresolved.

9. Oxidative Stress Can Promote Tau Hyperphosphorylation and Aggregation

Although the results described above clearly establish that expression of tau mutants underlying tauopathies in either human patients or animal models induces OS in neurons, another line of evidence also suggests that accumulation of ROS can directly stimulate tau hyperphosphorylation and aggregation.

Several studies showed that OS leads to increased tau phosphorylation in neuronal cultures [181, 182]. Moreover, carbonyl-4HNE facilitates aggregation of phosphorylated tau *in vitro* [183] and induces tau hyperphosphorylation [184, 185]. Oxidative events also produce oxidized fatty acids, which have been shown to stimulate tau polymerization *in vitro* [186]. Moreover, in mice deficient in mitochondrial SOD2, an increased tau hyperphosphorylation parallels mitochondrial dysfunction and OS [187]. More recently, because folate deficiency has been linked to neurological disorders [188], a transgenic zebrafish line deficient in folate was produced through overexpression of γ-glutamylhydrolase (γ-GH), an enzyme that converts active polyglutamyl folates into inactive monoglutamyl folates. Interestingly, this zebrafish transgenic line develops OS associated with tau hyperphosphorylation and aggregation and Aβ plaque formation [189].

However, the mechanism by which OS affects tau phosphorylation remains controversial. In rabbits, clear evidence of mitochondrial injury, OS, increased levels of tau phosphorylation and nuclear translocation of GSK3β was observed following intracisternal injection of aggregated Aβ42. Moreover, in the same model, treatment with lithium chloride (LiCl), an inhibitor of GSK3β, did not prevent mitochondrial DNA damage or tau hyperphosphorylation, suggesting that the translocation of GSK3β may represent an additional event unrelated to tau hyperphosphorylation [190]. GSK3β has been identified as one of the major serine/threonine kinases involved in tau phosphorylation/hyperphosphorylation. It was shown that treatment of primary rat cortical neuron cultures with cuprizone, a copper chelator, in combination with oxidant agents Fe^{2+} and H_2O_2, significantly increased GSK3β activity and pathologic tau hyperphosphorylation. By contrast, concomitant treatment of these cultures with LiCl significantly decreased GSK3β activity and reduced abnormal tau phosphorylation, identifying GSK3β as the kinase involved in tau phosphorylation following OS conditions in this model [191]. More recently, it was shown that, in neuronal PC12 cells cultured with 100 μM H_2O_2, treatment with low doses of GSK3β inhibitors protected the cells against H_2O_2-induced OS and apoptosis. By contrast, higher concentrations of GSK3β inhibitors induce opposite effects relative to apoptosis and tau phosphorylation, demonstrating the key role, albeit ill defined, of this kinase in the disease process. Taken together, these data suggest that GSK3β plays important roles in tau pathologies, and fine modulations of its activity may prevent apoptosis, as well as tau phosphorylation, induced by OS [192].

However, beside GSK3β, OS affects other signaling pathways and/or kinases mediating tau hyperphosphorylation. In particular, several tau kinases belong to the family of stress-activated protein kinases, which are activated in response to OS [193, 194]. In particular, it has also been shown that HNE directly activates two members of the stress-activated kinase family, JNK and p38, in NT_2 neuronal cells [195].

Another possible link between OS and pathologic tau phosphorylation is peptidyl prolyl *cis*-trans isomerase 1 (PPIase1) or Pin1. It has been shown that this enzyme is significantly downregulated and oxidized in AD hippocampus. Because Pin1 has been implicated in dephosphorylation of tau protein, it can be hypothesized that *in vivo* oxidative modifications of Pin1, as found in AD hippocampus, reduce Pin1 activity, leading to increased tau phosphorylation [196].

Insulin may also play a role in OS-induced tau phosphorylation. First, it has been established that OS conditions lead to decreased insulin secretion and sensitivity [197, 198]. Second, while insulin is highly sensitive to OS, it plays an important regulatory role in tau phosphorylation in neuronal cell cultures [199, 200], and abnormal insulin levels in mice lead to tau hyperphosphorylation in brain neurons [201, 202].

Thus, whereas accumulation of toxic hyperphosphorylated tau species has been shown to stimulate the production of ROS and thus OS conditions, these data strongly suggest that, in turn, OS directly promotes tau hyperphosphorylation. In this context, tau hyperphosphorylation and OS appear as two elements of a crucial "vicious circle" leading to a progressive coordinated increase in both ROS and abnormal tau and ultimately to cell death.

10. Antioxidants in Therapeutic Approaches

Following up the OS theories of aging and neurodegeneration, several antioxidant substances have been tested in different models of tauopathies, showing interesting therapeutic properties at least in these models. In mice overexpressing the smallest human tau isoform, which develop age-dependent filamentous tau inclusions accompanied by neuronal loss and behavioral alterations, dietary supplementation of vitamin E reduced mortality, decreased the number of tau-containing inclusions in the spinal cord, and improved behavioral

phenotypes [203]. In another study using a cell model of tauopathy induced by expression of a truncated tau fragment, treatment with the antioxidants vitamin C or vitamin E significantly decreased ROS production [204]. Curcumin, a naturally occurring substance found in turmeric (*Curcuma longa*), is another antioxidant displaying an interesting therapeutic potential. Treatment with curcumin decreased Aβ-induced tau hyperphosphorylation in PC12 cells [205] and okadaic acid-induced ROS production and tau hyperphosphorylation in mice [206]. In addition, in mice lacking superoxide dismutase, antioxidant treatment with catalytic antioxidant EUK189 decreases accumulation of OS markers and tau hyperphosphorylation [187]. Antioxidant therapy has also been shown to inhibit the progression of tau pathology in 3xTg-AD mice, an aggressive mouse model of AD [207]. In these mice, treatment with EUK-207 (a superoxide dismutase/catalase mimetic) from 4 to 9 months of age, that is, before the onset of symptoms, markedly ameliorates disease phenotypes [207]. Similarly, after chronic administration of antioxidant coenzyme Q10 to tau^{P301S} mice, lipid peroxidation was reduced, and survival and behavioral deficits were markedly improved [208]. Also, treatment of these mice with the antioxidant methylene blue diminished oxidative damage, such as oxidized nucleic acids, and tau hyperphosphorylation [209]. Lastly, treatment with vitamin E or overexpression of antioxidant enzyme thioredoxin peroxidase markedly ameliorated the neurodegenerative phenotype of tau^{R406W} transgenic flies [179].

Although much evidence points to antioxidant substances as potential therapeutic agents for the treatment of neurodegenerative diseases of the AD and tauopathy types, translation of the results obtained in animal models into clinical therapeutic strategies has not yet led to significant advances. Hence a better understanding of the role of OS in these diseases is essential, and antioxidant strategies hold promise for slowing down or halting the progression of neurodegenerative tauopathies.

11. Conclusion

Both OS and tau hyperphosphorylation appear as key elements in the pathophysiology of tauopathies. However, the relationship between intracellular ROS and tau hyperphosphorylation remains unclear. Accumulation of hyperphosphorylated tau has been shown to cause OS, but ROS have also been shown to stimulate tau hyperphosphorylation. In this context, a better understanding of the role of OS in these pathologies may serve primarily to define novel markers of early stages of the disease and then to develop therapeutic strategies to attenuate, halt, or reverse disease progression. In addition, close interplay between tau hyperphosphorylation and OS suggests that these events are two key components of a vicious circle that plays a crucial role in the pathologic process in tau pathologies, including AD.

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

The Role of Oxidative Stress-Induced Epigenetic Alterations in Amyloid-β Production in Alzheimer's Disease

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An increasing number of studies have proposed a strong correlation between reactive oxygen species (ROS)-induced oxidative stress (OS) and the pathogenesis of Alzheimer's disease (AD). With over five million people diagnosed in the United States alone, AD is the most common type of dementia worldwide. AD includes progressive neurodegeneration, followed by memory loss and reduced cognitive ability. Characterized by the formation of amyloid-beta (Aβ) plaques as a hallmark, the connection between ROS and AD is compelling. Analyzing the ROS response of essential proteins in the amyloidogenic pathway, such as amyloid-beta precursor protein (APP) and beta-secretase (BACE1), along with influential signaling programs of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and c-Jun N-terminal kinase (JNK), has helped visualize the path between OS and Aβ overproduction. In this review, attention will be paid to significant advances in the area of OS, epigenetics, and their influence on Aβ plaque assembly. Additionally, we aim to discuss available treatment options for AD that include antioxidant supplements, Asian traditional medicines, metal-protein-attenuating compounds, and histone modifying inhibitors.

1. Introduction

Alzheimer's disease (AD) is the most prevalent type of dementia with over five million people affected in the United States and 35 million worldwide [1]. The existence of amyloid-β (Aβ) plaques and tau neurofibrillary tangles (NFTs), leading to synaptic loss, is the major hallmark of AD pathogenesis [2–5]. Aβ, a 36–43 amino acid peptide that has been shown to clump together, forms amyloid aggregates that act in a prion fashion [6]. Zinc (Zn), Copper (Cu), and Iron (Fe) ions have also been implicated in the protein aggregation process, with Cu and Zn spatially correlated with amyloid plaques [7]. These plaques are often found in aging neurons, together with NFTs that are formed from hyperphosphorylated tau proteins. With time, this buildup of plaques and tangles can trigger the neurodegeneration associated with AD, resulting in cognitive deterioration with impaired speech, vision, behavior, and eventually death [8, 9]. Although individual facets of AD pathogenesis are understood, the mechanism of neurodegeneration is complex due to the fact that AD develops differently in each patient [10–12]. One possible vehicle for deposition and accumulation of Aβ in AD is oxidative stress (OS), mediated by the production of reactive oxygen species (ROS) (Figure 1) [3, 13–15].

Particularly in biological systems, ROS are a category of important free radicals such as superoxide and hydroxyl radicals produced as a byproduct of oxidative phosphorylation in the mitochondria's electron transport chain (ETC), with smaller amounts originating from cellular membrane, endoplasmic reticulum (ER), and peroxisomes [16–20]. Interestingly, Aβ can form complexes with Cu and generate hydrogen peroxide via the reduction of Cu^{2+} [21]. The body can be exposed to ROS directly from exogenous sources, such as tobacco smoke and radiation [22–24]. ROS can also act

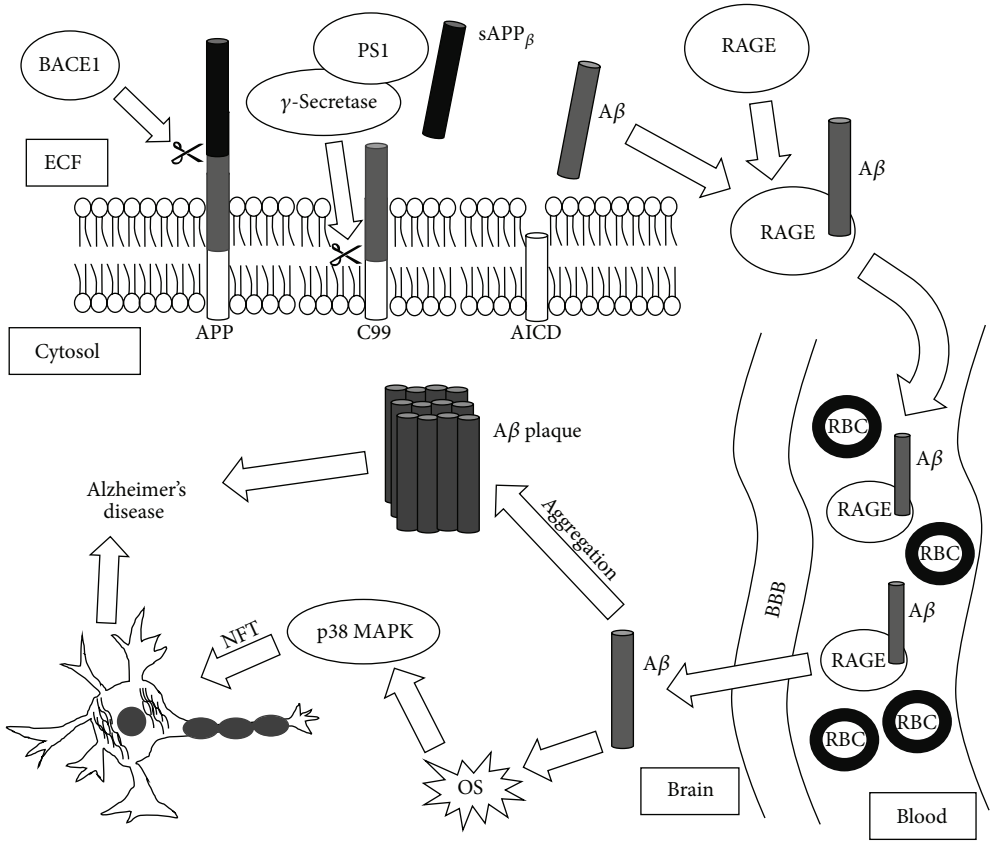

Figure 1: The role of amyloidogenesis in Alzheimer's disease. Amyloid-β originates as amyloid-beta precursor protein in the plasma membrane, which is cleaved sequentially by beta-site APP cleaving enzyme 1 and gamma-secretase. Following interaction between receptors for advanced glycation end products, which leads to its uptake in the brain, amyloid-β can form insoluble plaques and induce neurofibrillary tangles in neurons, the hallmarks of Alzheimer's disease. Aβ, amyloid-β; AICD, amyloid intracellular domain; APP, amyloid-beta precursor protein; BACE1, beta-site APP cleaving enzyme 1; BBB, blood-brain barrier; C99, residual 99 amino acids from C-terminal of APP; ECF, extra cellular fluid; p38 MAPK, p38 mitogen-activated protein kinase; OS, oxidative stress; PS1, presenilin 1; NFT, neurofibrillary tangle; RAGE, receptor for advanced glycation end products; RBC, red blood cell.

as necessary signaling molecules [25–27]. However, if their concentration increases markedly or the body cannot remove the excess species efficiently, these molecules can cause cellular damage. ROS oxidize essential cellular components such as DNA, proteins, and lipids, leading to disruption in cell homeostasis [28, 29].

In the process of aging and neurodegenerative diseases, the decline of normal antioxidant defense mechanisms increases the brain's vulnerability to oxidative damage known as OS [30]. ROS have the ability to modify crucial molecules in the cell, including proteins shown to be involved in neurodegenerative diseases [31]. Misregulation of ROS, due to either mitochondrial dysfunction, age, or both, has been implicated in numerous neurodegenerative diseases. By connecting ROS production to Aβ plaque formation, a more complete map of amyloidogenesis can allow researchers to focus their efforts on viable treatment options for AD.

2. Importance of ROS in Neurology

2.1. Natural Formation and Function of ROS. OS can be indicated by cell damage and impairment of cell signaling as a direct or indirect result of the accumulation of ROS in the cell. In some biological contexts, ROS provide essential molecular services. For example, neutrophils generate superoxide via NADPH oxidase in order to sequester or eliminate pathogens [32]. In many cases, superoxide forms from oxidative phosphorylation that occurs in the respiratory chain of mitochondria, especially in the sites of NADH dehydrogenase (complex I) and cytochrome bc1 (complex III) [13, 17]. The ETC transfers electrons from a series of protein complexes that act as electron donors and acceptors, with diatomic oxygen acting as the ultimate electron acceptor. Leakage in the ETC does occasionally occur, and premature redox reactions between oxygen and complexes I and III produce superoxide radicals [33]. Complexes I and III are also susceptible to malfunction during the process of aging, which can lead to additional OS [34]. ROS can be generated from pathological conditions such as hyperglycemia and hypoxic insults [3, 35, 36]. Aging is associated with increased OS due to long-term exposure of ROS and insufficient defense mechanisms in the brain [31, 34, 37]. The accumulation of such ROS eventually leads to significant cell damage [34, 38–41].

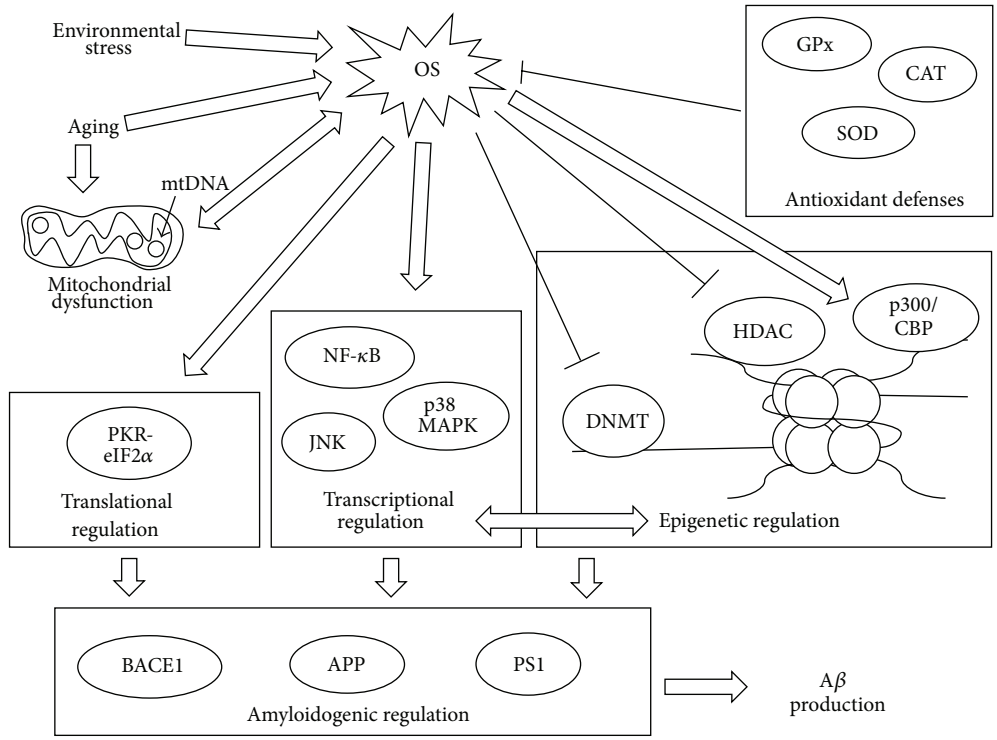

Figure 2: The role of oxidative stress in the multiple domains of amyloid-β production regulation. Oxidative stress, caused from reactive oxygen species production, creates an environment which is epigenetically, transcriptionally, and translationally favorable for amyloid-β production. Aβ, amyloid-β; APP, amyloid-beta precursor protein; BACE1, beta-site APP cleaving enzyme 1; CAT, catalase; DNMT, DNA methyltransferase; eIF2α, eukaryotic translation initiation factor-2alpha; GPx, glutathione peroxidase; HAT, histone acetyltransferase; HDAC, histone deacetylase complex; JNK, c-Jun N-terminal kinase; p38 MAPK, p38 mitogen-activated protein kinase; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; OS, oxidative stress; PKR, double-stranded RNA dependent protein kinase; PS1, presenilin 1; SOD, superoxide dismutase.

2.2. Inorganic-Related Pathoetiology. Exposure to environmental factors such as pollutants, chemicals, and radiation can disrupt the balance between prooxidant and antioxidant levels, thereby inducing OS [5, 42]. Elevated ROS are due to activated phagocytes in chronic inflammation under stress, whereas the declining antioxidant levels are associated with mutated antioxidant enzymes or toxins [42]. Poisoning in herbicides, such as paraquat, can indirectly influence neurotransmitter metabolism by altering redox cycling [42] and has been linked to Parkinson's disease [43]. Nutritional factors also play crucial roles in AD development: excess Fe deposits can increase the formation of ROS while Zn can contribute to Aβ peptide aggregation [5, 44]. Recent studies show a detection of higher Fe concentration in AD patient brains, particularly in the area of the hippocampus and the parietal cortex. Fe-induced ROS can damage the cell membrane via lipid peroxidation and the subsequent neurotoxicity leads to potential cell death [5, 45]. Though present in high concentrations in Aβ aggregates, Fe has not been copurified with Aβ. On the other hand, Cu and Zn copurify with Aβ in human *postmortem* brains [46]. In a study by Chen et al., Zn addition was exclusively found to encourage protofibril formation. This process occurred without nucleation of Aβ oligomers [47]. Additionally, Cu has been found to form enzymatic complexes with Aβ that mirror the antioxidant superoxide dismutase (SOD). These Aβ·Cu^{2+} complexes have long been understood to directly generate hydrogen peroxide via Cu^{2+} reduction [48]. More recently, Mayes et al. demonstrated how Aβ fibrils, when bound to Cu^{2+}, could convert hydrogen peroxide into hydroxyl radicals [49]. Although further investigation on the mechanism is necessary, environmental and nutritional-derived OS proposes a novel approach to therapeutic strategies in neurodegenerative diseases (Figure 2) [5].

2.3. Endogenous Antioxidant Defense Mechanisms. ROS molecules are natural byproducts of normal cellular biochemistry. In order to maintain homeostasis, the body has evolved several endogenous antioxidant molecules and enzymes to mitigate ROS-induced cytotoxicity [41]. Some of the better-studied antioxidant enzymes include SOD, catalase (CAT), and glutathione peroxidase (GPx) (Figure 2). Humans possess three types of SOD. SOD1 and SOD3, which contain Zn and Cu as cofactors, are located in the cytoplasm and extracellular space, respectively. SOD2 is located in the mitochondria and binds to manganese as a cofactor [50]. These metalloproteins can facilitate the dismutation of superoxide into oxygen and hydrogen peroxide [29, 50]. Peroxide, still a ROS molecule, is further processed by the antioxidant CAT. CAT is a nearly universal enzyme for

organisms exposed to oxygen and catalyzes the decomposition of hydrogen peroxide into water and oxygen [51]. The selenium containing GPx restores oxidized membrane lipids [52], while also reducing hydrogen peroxide to water [53]. With the coordination of these antioxidants, a healthy cell can effectively control the potential dangers of ROS.

2.4. Dysfunction due to ROS Production and Neurodegeneration. While these antioxidants can help protect an organism from oxidative damage, they themselves can also be oxidized by ROS. Choi et al. examined the oxidative modifications that SOD1 could undergo in AD brains [54]. SOD1 was found to be oxidized and carbonylated in the brain, and its activity was markedly reduced in patients with AD. The downregulation of intracellular SOD is found to contribute to the acceleration of Aβ oligomerization and initiate early onset of cognitive impairment [55]. SOD1 was also observed in protein aggregates, implicating its role in AD pathogenesis [54]. Casado et al. and Ansari and Scheff both demonstrated reduced levels of SOD, CAT, and GPx in AD patient blood samples and human *postmortem* frontal cortex samples, respectively [56, 57]. Additionally, glutathione (GSH) redox cycling is reported to be essential in the brain's detoxification of ROS [41]. As the most abundant antioxidant, GSH acts in the first response to OS [58]. Reduced antioxidant capacity is a hallmark of AD, and the ensuing OS can lead to neurodegeneration. This oxidative imbalance illuminates the potential brain damage and cellular dysfunctions arising from OS [3, 41, 59]. If the level of ROS exceeds the protection of endogenous antioxidant pathways in persistence, cell death is likely and is almost universally implicated in neurodegeneration [38].

Le Bras et al. examined how increased ROS could activate cell-death machinery [60]. ROS are able to promote mitochondrial membrane permeability, releasing calcium (Ca^{2+}). ROS can also discharge amplification factors of mitochondria-induced apoptosis, activate caspases, and induce DNA damage. By initiating these hallmarks of mitochondria-induced apoptosis, ROS have the capacity to trigger neuronal death. Furthermore, mitochondrial DNA (mtDNA) is another target of oxidation by ROS (Figure 2) [61]. Liu et al. showed how large sections of mtDNA were mutated in patients with neurodegenerative diseases and how mutations could make mitochondria more vulnerable to apoptosis [61]. In addition, Patten et al. determined how ROS affected apoptosis through other means. For example, ROS have been shown to stabilize p53 and activate c-Jun N-terminal kinase (JNK) [62]. Activation of these central elements in mitochondria-induced apoptosis can lead to eventual neurodegeneration.

2.5. Aging and Oxidative Stress. Many neurodegenerative diseases are associated with old age [63], and most symptoms appear in people over age 60 [64]. Recent research has suggested that the elderly are more prone to OS (Figure 2) [65, 66]. Complex I deficiencies are more prevalent in aging patients, suggesting that a less efficient ETC can create higher concentrations of ROS than in younger people [67]. Hamilton and Holscher used an AD mouse model to show that neurogenesis was markedly reduced in aging mice, together with increased Aβ plaque formation as a result of OS [65]. Additionally, mitochondria can be viewed as dynamic organelles, which are constantly undergoing a process of fusion and fission [68]. Conley et al. and Hauptmann et al. have demonstrated how mitochondrial dysfunction is more common with age [69, 70]. If the mitochondrial fission-fusion homeostasis is disrupted, accelerated ROS production will ensue (Figure 2) [71]. The resulting increase in ROS is detrimental for the cell due to superoxide and hydrogen peroxide's reactivity with essential molecules, including DNA and lipids [72]. Mitochondria accumulate membrane damage as they age, which can assist increased ROS production. Hauptmann et al. observed that mitochondrial dysfunction could begin as early as three months in an AD mouse model [70]. This is critical since mitochondrial dysfunction is generally viewed as one of the first steps of AD pathogenesis [37].

3. Neuropathological Characteristics of Alzheimer's Disease

3.1. Amyloidogenic Pathway. One of the major pathological indications of AD is the formation of extracellular plaques loaded with Aβ peptide [4, 73, 74]. Understanding the process in which Aβ is formed is likely of the utmost importance in the search for an effective AD treatment. The amyloid-beta precursor protein (APP) is an integral membrane protein whose normal function is not yet fully understood. By altering APP levels in transgenic mice, it was suggested that APP is important in synapse plasticity [75, 76]. APP is processed in two distinct mechanisms: the amyloidogenic, or plaque forming, and the nonamyloidogenic. In the nonamyloidogenic route, APP is processed by an α-secretase and then γ-secretase to yield an APP intracellular domain and soluble N-terminal fragment called p3 [77]. The majority of APP enters the nonamyloidogenic pathway, and other factors, such as mutations, environmental stimuli, and aging, are likely to influence this pattern; however, the mechanism remains unclear [4].

The sequential enzymatic breakdown of APP by the beta-site APP cleaving enzyme 1 (BACE1) and the γ-secretase complex with presenilin 1 (PS1) highlights the amyloidogenic pathway [2, 4, 5, 37, 78–80]. Other products formed from these actions include a truncated type of sAPP ($sAPP_{\beta}$) and the residual 99 amino acids of APP (C99) from the cleavage of APP by BACE1. The remaining amyloid intracellular domain (AICD) is formed due to the liberation of Aβ cleaved from C99 by γ-secretase, leaving AICD in the plasma membrane (Figure 1) [81]. Though Aβ has observed beneficial characteristics, such as its function as an antimicrobial peptide [82], it is highly associated with formation of bulky plaques that ultimately result in neuronal degradation. Soluble Aβ oligomers are recognized as the most neurotoxic form of Aβ [41, 55, 83]. Its ability to bind to preexisting Aβ aggregates or lipid membranes (e.g., gangliosides) and potential to alter other cytoskeletal proteins can lead to synaptotoxicity within neurons [84]. Additionally, the activity of BACE1 is markedly higher in old age, linking age with Aβ plaque production [85]. The receptor for advanced glycation end products (RAGE)

is an important Aβ receptor and the binding of Aβ to RAGE facilitates transportation across the blood-brain barrier (BBB) [86], thereby aiding in accumulation of Aβ protein within the brain (Figure 1). Cho et al. described how RAGE could stimulate BACE1 expression through RAGE's ability to generate an intracellular Ca^{2+} response that activates nuclear factor of activated T-cells 1 (NFAT1), a BACE1 activator. BACE1 then cleaves APP to form Aβ, which completes the feedback loop by acting as a RAGE activator [87].

3.2. Connection between Aβ-Induced OS and Tau Neurofibrillary Tangles (NFTs). Another pathophysiological characteristic of AD is the formation of intracellular NFTs consisting of an abnormal accumulation of hyperphosphorylated tau protein [4, 73]. Normally, tau serves to assemble and stabilize microtubule structures and is essential for the transportation of vesicles containing neurotransmitters within the neuronal axons. The excess phosphorylated tau aggregates and forms insoluble helical filaments that limit the transportation of neurotransmitters. As a result, NFTs interfere with communication between neurons and eventually lead to cognition impairment. Like Aβ oligomers, tau aggregates are cytotoxic [4].

Aβ-induced OS alters cellular signaling pathways and initiates a phosphorylation response. An increase in activation of JNK and p38 mitogen-activated protein kinase (MAPK) is noticeable in AD *postmortem* brains, suggesting a possible linkage between dysregulation of MAPK signaling pathway and AD pathogenesis [88]. Giraldo et al. demonstrated that p38 MAPK polypeptide is involved in tau hyperphosphorylation. The p38 MAPK and other kinases can be activated in response to Aβ accumulation. Activated p38 MAPK polypeptide phosphorylates tau protein, producing a hyperphosphorylated tau response. This study illustrates a positive correlation between tau aggregation and activated p38. Therefore, the activation of p38 is an indicator for tau hyperphosphorylation, further supporting the pathological association between Aβ and tau in AD (Figure 1) [88].

3.3. Aβ Formation Leads to Apoptosis in Neurons. Aβ has been associated with neurodegeneration and is found at elevated levels in AD brains [89]. The increase in Aβ causes neurodegeneration by activating apoptotic death signals such as caspase pathways in neurons [90–93]. Ferreiro et al. stated that Aβ was involved in depleting Ca^{2+} amounts in the ER [90], resulting in a high level of cytosolic Ca^{2+} that causes the mitochondrial membrane to lose its chemical potential, inducing mitochondrial apoptotic events. They demonstrated that lower levels of GSH, in response to increased Ca^{2+} release, result in ROS accumulation [91]. Aβ has also been shown to increase the activity of calcineurin (CaN), a protein phosphatase that catalyzes dephosphorylation of Bcl-2-associated death promoter (BAD). As a proapoptotic protein, BAD triggers cytochrome c release after translocating to the mitochondria [92]. In addition, Aβ proteins associate with the caspase cascade, leading to proteolysis of caspase targets and eventual apoptosis [93].

3.4. Neurodegeneration Results in Decreased Cognitive Ability, Dementia, and Memory Loss. Aβ has been referred to as an initiator in the mitochondrial, ER, and caspase-responsive apoptotic pathways, which collectively lead to neurodegeneration [90–93]. Neuronal atrophy is an essential characteristic of AD, as well as memory deficits, a loss of cognitive ability, and dementia [8, 94]. A study of 764 participants attempted to map Aβ in the brain. Posterior cortical regions, associated with memory retrieval in younger participants, show Aβ deposits in the elderly with AD [95]. Aging mice expressing an AD-linked APP variant formed Aβ plaques, resulting in memory loss [96]. The isolated Aβ protein induced memory deficits when injected into young rats [96]. Similarly, Aβ dimers extracted from the cerebral cortex of AD patients were found to affect learned behavior memory when administered to normal rats. The Aβ dimers were concluded to be the smallest toxic species for synapse structure [97]. Therefore, Aβ has been reliably shown to induce AD effects in a variety of experimental settings.

4. The Role of ROS in Alzheimer's Disease

4.1. Epigenetic Alteration of Aβ (Methylation and Acetylation). ROS activity has long been understood to affect DNA transcription through its oxidation of DNA and related proteins [98, 99]. Epigenetics refers to the changes in gene expression through chemical processes, such as histone modification and DNA methylation, without the disruption of DNA sequence. Acting as an anchor for DNA, histones contain N-terminal tails that can be methylated, sumoylated, phosphorylated, and ubiquitinated, as well as other posttranslational modifications. Histone acetylation and deacetylation are the most well-studied mechanisms, with histone acetyltransferases (HATs) and histone deacetylases (HDACs) attaching or removing acetyl groups to the histone tails, respectively. Acetylation neutralizes the positive charge associated with the histone tail, limiting the attraction among the negative phosphate groups of DNA. Relaxed DNA offers easier access for gene transcription [100]. DNA methyltransferases (DNMTs) are closely tied to the process of histone acetylation, modifying DNA structure by transferring methyl groups to cytosine-guanine (CpG) dinucleotides. Generally, methylated CpG sequences can induce histone modifications that repress the transcriptional complex's ability to access DNA [100]. Oxidation of the guanine residue in CpG dinucleotides can also affect the epigenetic regulatory complexes in a similar manner, placing emphasis on OS in the regulation of CpG sites [101]. These sites are particularly important in AD, as the promoter regions of APP and BACE1 contain 65 and 36 CpG sites, respectively [3]. The presence of these sites adds significance to the idea that the essential genes of amyloidosis are potentially regulated in an epigenetic manner. Additionally, epigenetics can be influenced by environmental stimuli; however, it can also change naturally during growth and development [102].

Recently, explorations of epigenetic regulation mechanisms present a novel insight into OS and its relation to AD [3, 103]. Several studies have revealed the epigenetic control of Aβ production in the progression of AD [104,

105]. Chromatin remodeling has also been reported to assist in the upregulation of BACE1 and Aβ production [106, 107]. Sung et al. and Chouliaras et al. have shown that not only is there global decrease of DNA methylation in the hippocampus of *postmortem* AD patients, but also APP-related mutations cause an epigenetic shift in an AD model cell line [102, 103]. Clearly, epigenetic mechanisms are meaningful in Aβ plaque formation. Gu et al. studied what possible agents could provoke this epigenetic shift in AD patients [3]. When neuroblastoma cells were treated with hydrogen peroxide, there was a significant increase in histone acetylation together with a decrease in DNA methylation. This histone hyperacetylation and DNA hypomethylation resulted in increased APP and BACE1 transcription, possibly by a gain of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activity [3]. This study shows how OS can cause Aβ plaque formation through means of epigenetic mechanisms and offers promise for treatment approaches directed at this pathway. Cytosine in a CpG site of DNA undergoes frequent methylation and regulates gene expression during development, differentiation, pathogenesis, and aging [3, 102]. Besides DNA methylation, DNA hydroxymethylation describes a different biological role in the epigenetic modification of AD. 5-Hydroxymethylcytidine (5-hmC) and 5-methylcytidine (5-mC) levels are significantly decreased in AD brains [103]. The linkage between epigenetic dysregulation and AD is evidently supported by previous studies with correlation to OS [3, 103]. The modified transcriptional expression of AD-related genes (APP, BACE1, and PS1) enhances Aβ production and contributes to AD pathogenesis and development [3, 108]. Furthermore, epigenetic mechanisms associated with OS, especially altered methylation or CpG oxidation, exacerbate the progression of oxidative DNA damage (Figure 2) [3].

4.2. Activation of Stress-Related Signal Pathway Increases BACE1 and APP Transcription. Due to the neuropathological nature of AD, many studies have investigated how the Aβ formation pathway can be manipulated. Using the potent DNMT inhibitor S-adenosylhomocysteine (SAH), Lin et al. were able to hypomethylate PS1 and APP promoters, accompanied by increased expression of PS1 and APP. As a result, Aβ protein production is increased [109]. Guo et al. showed that JNK and p38 MAPK, stress-related MAPKs, are activated with addition of anisomycin and induce intracellular Aβ production in neuroblastoma cells [110]. APP and BACE1 were found to be upregulated as a result of demethylation of their promoters. Simultaneously, transcription of HAT p300/CREB-binding protein (CBP) was increased, while transcription of DNMTs and HDACs was downregulated [110]. This study confirmed that Aβ overproduction could occur during times of cellular stress through a hypomethylation/hyperacetylation-dependent pathway. Increasingly, evidence suggests the contribution of epigenetic dysregulation to AD pathogenesis [25, 102]. The epigenetic modification in AD-related genes is a result of activation of JNK and p38 MAPK pathways [3, 111]. In the presence of anisomycin, the reduction of DNMT activity induces an overexpression of APP, BACE1, and PS1 [102, 112]. In addition, Gu et al. have identified hydrogen peroxide as an activator for the distinct MAPK cascades. DNA methylation is markedly reduced in APP and BACE1 promoters after treatments with hydrogen peroxide [3]. The finding further suggests the role of OS in modulating DNA methylation and histone acetylation in specific AD-related genes (Figure 2) [3, 25].

4.3. Translational Regulation of Aβ. In a separate investigation, the addition of hydrogen peroxide in human neuroblastoma cells resulted in enhanced expression of BACE1, supporting the observation that OS can heighten BACE1 levels [111]. JNK is believed to be responsible for this increase in BACE1 mRNA, while JNK signaling is correlated with tau-induced toxicity [38]. Moreover, eukaryotic translation initiation factor-2alpha (eIF2α) was found to translationally regulate initiation of BACE1 protein synthesis [111]. The eIF2α undergoes phosphorylation upon its activation, and elevated levels of phosphorylated eIF2α have been reported in AD brains [113]. Phosphorylated eIF2α generally stops protein translation in response to cellular stress; however certain stress response genes, such as BACE1, are activated by eIF2α [111]. Double-stranded RNA dependent protein kinase (PKR) responds to cellular hardship in a proapoptotic manner by activating other stress signaling cascades including eIF2α [114]. Therefore, PKR-eIF2α expands BACE1 protein expression via translational regulation in response to OS (Figure 2) [111]. Suppression of aberrant eIF2α phosphorylation ameliorated AD symptoms in a mouse AD model [113].

5. Treatment

5.1. Antioxidant Supplements. Promising strategies for AD treatment fall on those that can decrease Aβ oligomer and phosphorylated tau levels, promote neuroprotection, and alleviate OS [112, 115]. With the view that ROS are the instigators in Aβ production, it is understandable that much research has focused on the clinical opportunity of antioxidants in alleviating AD symptoms. Gubandru et al. measured the effects of several antioxidant supplements on certain OS markers such as advanced glycation end products (AGEs), protein carbonyls (CRBNLs), and malondialdehyde (MDA) [73]. Although the sample size was small (21 AD patients and 10 controls), results demonstrated how the antioxidant supplement Rivastigmine decreases AGEs in AD patients, while Donepezil restores GSH and total antioxidant capacity (TAC). Therefore, antioxidant supplements offer potential strategies to ameliorate AD in dementia patients [73].

JNK and NF-κB are well-known activators of the amyloidogenic pathway and are responsive to OS [116, 117]. As a free radical scavenger, molecular hydrogen (H_2) can specifically reduce hydroxyl radicals. To that end, Wang et al. demonstrated how the reducing agent hydrogen-rich saline could decrease neural inflammation and OS induced by Aβ [118]. Specifically, mice were injected with Aβ and then treated with hydrogen-rich saline for 10 days. OS markers, including the levels of 8-hydroxydeoxyguanosine (8-OHdG), JNK, and NF-κB, were all reduced in the hydrogen-rich saline administered group. Therefore, hydrogen-rich saline

inversely regulates the activation of JNK [118]. This study suggests that hydrogen-rich saline can be used to relieve the symptoms of neuroinflammation [119] and OS in AD patients by attenuation of JNK and NF-κB-induced OS response [118].

Rutin is a naturally occurring glycoside that acts as both an anti-inflammatory and an antioxidant agent. Wang et al. previously demonstrated rutin's ability to inhibit Aβ plaque formation and relieve OS [120]. In another study directly aimed at rutin's role in protecting against AD, mice treated with rutin displayed favorable levels of antioxidant markers, such as increased SOD and GPx activity, reduced memory deficits, and fewer Aβ oligomers [41]. Inflammatory cytokines interleukin- (IL-) 1β and IL-6 were also found to be at lower levels in treated murine brains. In addition, rutin supplementation enhances the activity of SOD, GPx, and CAT [121]. Due to its demonstrated antioxidant and anti-inflammatory properties, rutin shows great potential as a future treatment for AD patients.

Resveratrol, normally found in grapes and red wine, is a phytoalexin that is produced by plant species as a defense mechanism against fungal attack. Its neuroprotective/antioxidant properties have been shown useful in AD treatment [94]. For example, resveratrol can protect astrocytes, the human brain's most plentiful cell, against ROS damage. Astrocytes are important sources of GSH, a major antioxidant in the body, and a decline in GSH levels occurs in aged brains due to the increased vulnerability against OS [122]. Resveratrol provides a shield for astrocytes, which in turn modulates GSH levels and reinforces its antioxidant activity [122]. In addition, resveratrol hinders cellular apoptosis through influencing intracellular signaling pathways and antioxidant mechanisms, such as reducing NF-κB activation and scavenging ROS intermediates. Particularly, resveratrol activates sirtuin protein, a NAD-dependent HDAC, and ultimately improves mitochondrial bioenergetic efficiency through a pathway mediated by sirtuin-1 (SIRT1) and inhibits the formation of Aβ fibrils [94, 123]. Thus, resveratrol's potential to protect neurons from Aβ- and OS-induced toxicity displays promising therapeutic applications during AD progression.

Vitamin E includes a group of antioxidant molecules called tocopherols and tocotrienols [124]. Given its free radical scavenging activity and biological significance in treating other diseases, Vitamin E as a therapy for AD has attracted much attention. Interestingly, a study examining the effect of Vitamins A, C, and E for OS concluded that not only could Vitamin E restore antioxidant activity, but it was found to be more effective than Vitamin A and C, in the rat brain [125]. However, in a more recent study there was no significant difference recorded between patients taking Vitamin E (800 IU/day) over those taking a placebo [126]. A review of the effect of Vitamin E in AD treatment highlights the conflicting results of many studies [127]. Mecocci and Polidori concluded that various obstacles, such as the permeability of the BBB, the delicate antioxidant/free radical equilibrium, and vascular factors of AD pathogenesis, could be responsible for reduced Vitamin E efficacy [128]. Focusing on improving Vitamin E uptake in the brain and optimizing a treatment plan should lead to more realistic results for Vitamin E supplementation. These challenges make Vitamin E and other antioxidants difficult but worthy potential AD treatment candidates [41, 120, 122, 128].

5.2. Traditional Medicine. Traditional Chinese and Ayurvedic medicine has led to various potential candidates for AD treatment. Su He Xiang Wan (SHXW) is a combination of herbs used in traditional Chinese medicine for clinical problems including stroke, infantile convulsions, and seizures [129, 130]. Jeon et al. studied the effect of SHXW when inhaled by mice injected with Aβ into the hippocampus [130]. SHXW mice showed reduced memory impairment and suppressed Aβ-induced JNK and p38 activations. In SH-SY5Y cells, repression of Aβ-induced apoptosis was observed, and upregulation of Heme oxygenase (HO-1) and nuclear factor-like 2 (Nrf2) indicated mitigated ROS formation [130]. Together, these findings suggest the promise of SHXW to treat AD patients. Clinical studies remain to be conducted to determine the potential efficacy of SHXW.

The Chinese native *Ginkgo biloba* tree has a long history of practice in Chinese traditional medicine [131]. Investigation of its potential role in western medicine has yielded mixed results. A study was performed on 395 subjects with dementia who were treated with 240 mg/day of EGb 761, an extract from *Ginkgo biloba* leaves. Patients that were treated with EGb 761 scored higher on the Short Syndrome Test (SKT), a measure of cognitive ability [132]. A second study showed similar results on the SKT, as well as improved secondary efficacy variables such as caregiver distress scores, Alzheimer's Disease Activities of Daily Living International Scale, and Verbal Fluency Test [133]. However, a separate long-term study testing the effectiveness of EGb 761 in the prevention of AD showed no significant difference between EGb 761 and the matched placebo in terms of AD diagnosis [134], which requires further investigation.

In India, the spice turmeric is a major constituent in curry powders and has been used for digestive ailments [135]. Curcumin, a molecule found in turmeric, has antioxidant activity [136]. Lim et al. observed the effect of low (160 ppm) and high (5000 ppm) dose curcumin on Alzheimer-like pathology in mice. Both high and low concentrations led to a reduced amount of oxidized proteins, as indicated by western blot analysis of carbonylation levels in murine brains. In addition, low dose curcumin lowered soluble and insoluble Aβ by up to 50%. However, APP expression remained unchanged [136]. Yang et al. demonstrated that curcumin can cross the BBB to bind to Aβ and hinder aggregation of Aβ, reducing Aβ levels in mice with previously established Aβ deposits [137].

5.3. Metal Ion Chelators. As mentioned earlier, Fe, Cu, and Zn have been implicated in various aspects of AD, including Fe-induced cognitive damage [45], Cu- and Zn-mediated amyloid aggregation [138], and Cu-mediated ROS generation [48, 49]. Investigations into the possibility of metal-protein-attenuating compounds (MPACs) that abate proteins from interacting with ions have yielded promising results. Iodochlorhydroxyquin, commonly known as clioquinol, is an MPAC that was the focus of a pilot phase 2 clinical trial carried out by Ritchie et al. Over 9 months, 36 patients

participated in a double-blinded, placebo-controlled study that showed a reduction in plasma Aβ42 levels, with no changes in Cu levels. Although the myelo-optic neuropathy associated with chronic use of clioquinol caused its withdrawal in 1970, clioquinol appears to be safe to use with no adverse effects that were reported in this study [139]. Separately, Lannfelt et al. examined the efficacy of PBT2. A successor to clioquinol, PBT2 is a second-generation 8-OH quinolone MPAC that also demonstrated beneficial effects in targeting Aβ oligomers. Aβ42 levels were lowered dose-dependently of PBT2, and no severe adverse effects were reported [140]. Further research into the efficacy and safety of MPACs could hold much potential in the search for effective AD treatment.

5.4. HDAC Inhibitors. Epigenetically, AD genomes have been found to be globally DNA-hypomethylated and histone-hyperacetylated [141]. This epigenetic profile is beneficial for BACE1 expression and Aβ production, thus leading to AD formation. If these epigenetic changes could be reversed, possibly Aβ aggregation could be suppressed. To test this idea, Sung et al. developed two novel HDAC inhibitors (HDACIs) to determine Aβ levels in response to histone deacetylation [108]. Overall, Aβ40 and Aβ42 levels, two common sizes of the Aβ protein, were lower with exposure to HDACIs *in vitro*. β- and γ-Secretase component transcription was suppressed and transcription of Aβ degrading enzymes, such as matrix metalloproteinase-2 (Mmp2), was increased. Additionally, aged AD mice showed improved learning capabilities and reduced memory deficits when exposed to HDACIs [142]. Addition of curcumin, a p300 inhibitor, reduced the expression of BACE1 via histone H3 acetylation inhibition in an Alzheimer cell line [143], further promoting the idea of epigenetics as an initial step in Aβ production. Thus, these inhibitors can be potentially used as alternative treatment options for AD in clinical settings.

6. Conclusions

The features of AD pathogenesis are interrelated with OS. Although it remains unclear whether OS is a direct cause or a result of AD pathology, evidence demonstrates that Aβ plaques, NFTs, and mitochondrial dysfunction all contribute to and are influenced by the imbalance of the oxidative state in the brain. Overproduction of Aβ protein is increased through upregulation of both APP and BACE1, as well as involvement of transcriptional and translational coordinators. The activation of stress-induced MAPK (e.g., p38 MAPK) signaling pathways further contributes to the hallmarks of AD. Therapies that include a diet with high levels of antioxidants could both guard against deleterious epigenetic changes and alleviate the devastating clinical manifestations of AD. Additionally, compounds derived from traditional Chinese and Ayurvedic medicine could potentially be candidates for clinical trials given their success in the laboratory. MPACs that target the impact of metal ions in OS and protein aggregation, as well as inhibitors of the HAT/HDAC enzymes, restore global epigenetic expression that is altered by OS. Inhibition of this event reduces apoptosis and neurodegeneration observed in histone-altered cells. Studies that continue to elucidate the exact mechanism of OS-induced Aβ production and the effectiveness of antioxidants and small molecule inhibitors will be paramount to the treatment of AD. With increasing understanding of AD pathogenesis, the findings provide promising prospects guiding future clinical investigations and discovery of novel treatment approaches.

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

A Yeast/*Drosophila* Screen to Identify New Compounds Overcoming Frataxin Deficiency

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Friedreich's ataxia (FA) is a rare neurodegenerative disease which is very debilitating for the patients who progressively lose their autonomy. The lack of efficient therapeutic treatment of the disease strongly argues for urgent need to search for new active compounds that may stop the progression of the disease or prevent the appearance of the symptoms when the genetic defect is diagnosed early enough. In the present study, we used a yeast strain with a deletion of the frataxin homologue gene as a model of FA cells in a primary screen of two chemical libraries, a fraction of the French National Chemical Library (5500 compounds) and the Prestwick collection (880 compounds). We ran a secondary screen on *Drosophila melanogaster* flies expressing reduced levels of frataxin during larval development. Half of the compounds selected in yeast appeared to be active in flies in this developmental paradigm, and one of the two compounds with highest activities in this assay partially rescued the heart dilatation phenotype resulting from heart specific depletion of frataxin. The unique complementarity of these two frataxin-deficient models, unicellular and multicellular, appears to be very efficient to select new compounds with improved selectivity, bringing significant perspectives towards improvements in FA therapy.

1. Introduction

Friedreich's ataxia (OMIM #229300, FA) is the most prevalent form of autosomal recessive spinocerebellar ataxia in Caucasians. It is a rather heterogeneous disorder characterized by progressive ataxia and dysarthria [1] usually appearing around puberty, but sometimes much later in life (>60 years). Neurological features include sensory neuropathy, deep sensory impairment, and signs of pyramidal-tract involvement. Nonneurological manifestations include hypertrophic cardiomyopathy (in ~60% of patients) and diabetes (in ~30% of patients). Friedreich ataxia is caused by mutations in the FXN gene, most frequently (96%) arising from an unstable hyperexpansion of GAA triplet repeat in the first intron of the gene [2] which results in decreased transcription of the FXN locus (and to some extent the adjacent PIP5K1B locus [3]) and reduced level of frataxin.

Frataxin is a highly conserved protein with homologues found in bacteria, yeast, invertebrates, plants, and mammals. In eukaryotic cells, the protein is synthesized with a presequence that targets the protein to the mitochondrial matrix. Most of our knowledge about the role of frataxin comes from studies of mutant yeast cells and cells from FA patients (reviewed in [4, 5]). The precise role of frataxin is still a matter

of debate. It is generally recognized to participate in iron-sulfur cluster (ISC) assembly [6–9], but its function as an iron-chaperone in ISC synthesis remains fragile as illustrated by contradictory reports [10–14]. It was rather suggested that frataxin activates the transsulfuration reactions required for ISC biosynthesis [15], and a point mutation in the Fe-S scaffold protein Isu1p bypasses frataxin deletion in yeast [16]. Key features of frataxin-deficient yeast cells, FA patients fibroblasts, and virtually all models generated so far are a hypersensitivity to oxidative insult and the inability to induce proper antioxidant defenses [17–19]. In fibroblasts, this hypersensitivity to oxidative insult has been ascribed to impair signaling of antioxidant defenses resulting from actin stress fibers disorganization [19–21].

The therapeutic arsenal to treat FA is limited and several attempts aim at developing new strategies to better handle this fatal disease. The current trials focus on lowering oxidative insults (e.g., Idebenone, Phase III trial), reducing iron-mediated toxicity (e.g., deferiprone, Phase II trial), increasing antioxidant defense levels (e.g., pioglitazone, Phase III trial), or increasing frataxin expression (e.g., polyamides or erythropoietin or gene therapy) (for general reviews on therapeutic approach to FA, see [22, 23]).

Despite many efforts to overcome any of the abnormalities related to frataxin deficiency, there is currently no efficient treatment to cure or even stop the progression of the disease, mostly because many aspects of the pathological consequences of frataxin depletion are still not fully understood. As a result there is still a need to use new approaches and to identify new molecules to successfully fight FA. Unfortunately, the important genetic instability of frataxin knockdown cell lines, such as murine fibroblast models for Friedreich's ataxia, is a severe limitation in a high-throughput drug screen [24]. Cotticelli et al. [25] recently reported a high-throughput screening of several chemical libraries using a yeast strain with the frataxin gene (*YFH1*) under the control of a galactose inducible/glucose-repressible promoter to mimic frataxin deficiency. Based on a test to evaluate mitochondrial energetics, the screen allowed identifying a number of compounds that were further evaluated on the murine myoblast cell line C2C12 treated with ferric ammonium citrate and buthionine sulfoximine to recapitulate some of the phenotypes of FA cells. In the present study, we used a genuine *yfh1*-deleted yeast strain (Δ*yfh1*) as a model of FA cells in a primary screen of two chemical libraries, a fraction of the French National Chemical Library (5500 compounds, about 12% of the full chemical library) (http://chimiotheque-nationale.enscm.fr/) and the Prestwick collection (880 compounds) (http://www.prestwickchemical.com/). We used as a secondary screen genetically engineered *Drosophila melanogaster* flies expressing reduced levels of dfh that exhibit a strong developmental phenotype [26]. The complementarity of these two frataxin-deficient models, unicellular and multicellular, allowed the identification with an improved selectivity of 6 new compounds with high specific activity in both paradigms, one of them also active in improving heart functions in *Drosophila* with reduced frataxin expression in cardiomyocytes, bringing significant progress towards perspectives in FA therapy.

2. Materials and Methods

2.1. Yeast Strains and Growth Conditions. The *S. cerevisiae* strains used in this study were the cycloheximide resistant wild-type (WT) strain derived from YPH499 (MATa *ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1 cyh2*) and its derivative YPH499Δ*yfh1* (Δ*yfh1*::TRP1). To prevent the accumulation of extragenic suppressor mutations, the Δ*yfh1* mutant was constructed using the YPH499 *yfh1* shuffle strain [27]. In the shuffle strain, the Δ*yfh1* deletion is covered by pRS318-*YFH1*, a plasmid containing the CEN, *CYH2,* and the *YFH1* HindIII genomic fragment. The plasmid was removed by counter selection in anaerobiosis on YPD-TE medium (1% yeast extract, 2% Bacto peptone, 2% glucose, 0.2% Tween 80, 20 mg·L^{-1} ergosterol) containing 10 μg·mL^{-1} cycloheximide, which is toxic in the presence of the *CYH2* allele. To monitor the loss of mitochondrial DNA leading to a rho$^{\circ}$ status, the YPH499Δ*yfh1* strain was regularly crossed with an appropriate Rho tester strain and analyzed using standard yeast genetics procedures [28]. Only isolates with more than 90% Rho^{+} cells were used in the screening procedure. Control strains were the wild-type strain BY4741 (Mat a *his3-Δ1 leu2-Δ0 met15-Δ0 ura3-Δ0*) and its Δ*ggc1* derivative (Mat a *his3-Δ1 leu2-Δ0 met15-Δ0 ura3-Δ0 ggc1*::KANMX4).

2.2. Screening Procedure on Yeast. The chemical libraries were available as a series of 96-well microtiter plates containing the compounds as 0.01 M stock solutions in DMSO. Fresh isolates of YPH499Δ*yfh1*, obtained after plasmid shuffling, were maintained on YPD medium. To screen the chemical libraries, the cells were grown on minimum medium in which frataxin-deficient cells can only grow very poorly. This medium (YNB-Raf) consisted of Yeast Nitrogen Base (Difco) 6.7 g·L^{-1}, supplemented with the required amino acids and 200 mg·L^{-1} adenine and 2% raffinose plus 0.1% glucose as carbon sources. 96-well microtiter plates containing 120 μL YNB-Raf per well were inoculated at an initial $OD_{600\,nm}$ of 0.01. The chemicals were added at a final concentration of 10 μM. The plates were incubated at 30°C for 3 days, and the cell density was measured by reading the optical density ($OD_{600\,nm}$) using a SpectraMax i3 microtiter plate reader (Molecular Device).

Dose-dependence of the compounds was tested (1) by monitoring the Δ*yfh1* cell growth on liquid cultures in YNB-Raf medium and also (2) by agar disc diffusion assays, as described in [29]. Two hundred forty microliters of exponentially growing cell cultures, adjusted to an $OD_{600\,nm}$ of 0.01, was mixed with 10 mL Ultrapure low melting point agarose (0.8% weight/vol in water, Invitrogen) maintained at 30°C after melting and poured onto a square Petri dish (12 cm × 12 cm) containing YNB-Raf solid medium. Sterile paper discs distributed on the agar plates with the lawn of Δ*yfh1* cells were impregnated with 7 μL of each compound (10 mM in DMSO), and growth of the cells around the discs was monitored by scanning the plates at different time points.

2.3. Drosophila Stocks, Culture Methods, and Treatment with Compounds. UAS-fhRNAi (w[1]; Pw[+mC]=UAS-fh.IR2), UAS-mitoGFP (w[1118]; Pw[+mC]=UAS-mitoGFP.AP2/

CyO), and da-GAL4 (P{GAL4-da.G32}2) were obtained from the Bloomington Stock Center. Hand-GS is described in [30]. Stock solutions of the tested compounds (10 mM in DMSO), or similar volumes of DMSO for control conditions, were incorporated in food medium (60 g·L^{-1} yeast, 34 g·L^{-1} corn meal, 50 g·L^{-1} sucrose, 8.6 g·L^{-1} agar, and 25 mL·L^{-1} methyl 4-hydroxybenzoate (200 g·L^{-1} in ethanol)) to a final concentration of 10 μM or 50 μM. To test the compounds on the defective pupariation, female da-GAL4 female flies were crossed with UAS-fhRNAi or w[1118] males and allowed to lay eggs for 3 hours on standard food medium at 26°C. 24 hours after egg laying, first instar larvae (L1) were collected and transferred at 23°C on food medium containing the tested compounds. Three to four samples of 50 L1 were transferred for each tested compound, and the timing of pupariation of these larvae at 23°C was followed up. This 23°C breeding temperature was chosen because, using our standard rearing medium, it led to a final percentage of pupariation of 50% for untreated frataxin-deficient larvae, a percentage well suited to identify both enhancement or suppression of the deleterious phenotype. Statistical significance of differences between treated and untreated larvae was assessed with one tailed t-test analysis. To test the compounds on the heart phenotype, expression of fhRNAi was driven by the heart specific RU486-inducible Geneswitch driver Hand-GS in UAS-mitoGFP;HandGS>UAS-fhRNAi flies as described in [31]. The activity of the Hand-GS driver (and hence the level of frataxin depletion) was controlled by RU486 added to the fly food (40 ng·mL^{-1} of food during development and 100 μg·mL^{-1} during adulthood). The driver was simultaneously used to express a mitochondrial GFP, providing sufficient fluorescence in cardiomyocytes for high-speed video recording through the cuticle of anaesthetized flies.

2.4. In Vivo Imaging of Fly Hearts. UAS-mitoGFP;HandGS >UAS-fhRNAi and UAS-mitoGFP;HandGS>+ 4-day-old adult flies were anesthetized with Triethylamine (FlyNAP). Video movies were acquired on a Zeiss SteREO Lumar.V12 Stereomicroscope, with a NeoLumar S 1.5x objective as described in [30]. For every video, the 501 frames were flattened into one by using the ImageJ function Zproject (Max Intensity). The picture generated was thresholded for light intensity by using the set AutoThreshold function. The anterior part of the heart (abdominal segments A1/A2) was then detected with the Analyze Particles tool from ImageJ (minimum size = 6,000; maximum size = 100,000; circularity = 0–0.99). The vertical row used to measure the diastolic diameter was automatically positioned using the XM variable as the abscissa origin. Statistical significance was assessed by nonparametric Wilcoxon analysis.

3. Results

3.1. Screening of the Chemical Libraries on the Basis of Growth Rescue of the Δyfh1 Yeast Strain with Raffinose as the Main Carbon Source. Frataxin-deficient yeast cells (and more generally yeast mutants affected in oxidative phosphorylation)

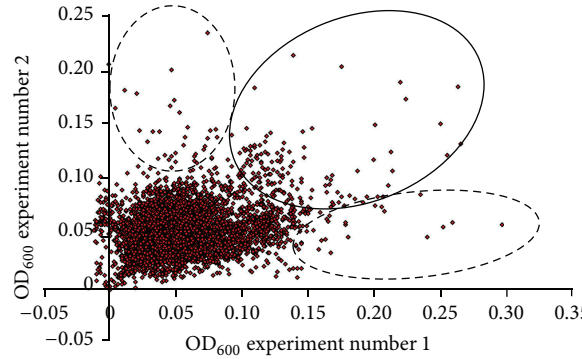


Figure 1: Drugs ability to improve the fitness of frataxin-deficient yeast cells. Pairwise analysis of the growth of the Δ*yfh1* cells in two independent experiments, using the full set of compounds from the French National Chemical Library (5500 compounds) and the Prestwick collection (880 compounds). The regions of the graph circled with dotted lines representing the results of growth improvement in only one condition are maybe therefore attributed to growth of extragenic suppressors. Compounds within the area circled with a solid line were typical of drugs entering the secondary screening in the yeast assay.

show a slow growth phenotype when raffinose is provided as the carbon source [32]. This is because raffinose, unlike glucose, prevents catabolic repression: full utilization of this carbon source thus requires the functioning of both the glycolytic and the oxidative phosphorylation pathways. We used this carbon source in our screen, rather than glycerol (which can only be metabolized by respiration) because we looked for drugs that would improve the mitochondrial functions without necessarily fully restoring them. A primary screening was run in triplicate on all the compounds, and results were compared pairwise. Typical results from one pairwise analysis out of three are presented in Figure 1. The regions of the graph circled with a dotted line represent conditions where the growth rescue is not consistent in the replicates and is most likely due to the appearance of genetic suppressors. The compounds from the region of the graph circled with a solid line were good candidates as active drugs. We selected the 60 compounds that were the most efficient at reproducibly improving the growth of Δ*yfh1* cells in our selection medium (YNB-Raf). In a validation screen, these compounds were tested on the growth of 3 yeast strains under the same experimental conditions: cells of the wild-type strain, Δ*yfh1* cells, and cells of a Δ*ggc1* strain, a strain which is also defective in iron-sulfur cluster biogenesis due to a lack of the mitochondrial GDP/GTP exchanger but has normal frataxin content [33]. This screen allowed us to select 18 molecules which improved the growth of Δ*yfh1* cells, but which had lower (or no) effect on the growth of Δ*ggc1* cells (data not shown). These molecules were studied in a wide range of concentrations (0.1–100 μM) for their effect on the growth kinetics of Δ*yfh1* cells in liquid YNB-Raf medium. Dose-dependence of the compounds was also tested by agar disc diffusion assays [29]. The effects of the 18 selected compounds were very different according to their concentration and to the growth phase of yeast cells.

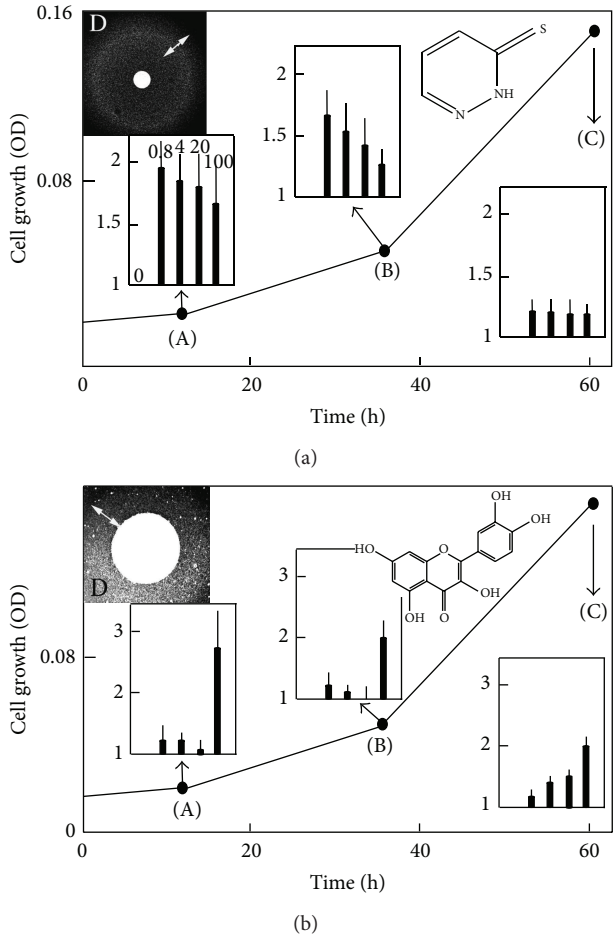


Figure 2: Effect of drugs on the kinetics of Δ*yfh1* cell growth. Panels A (effect of LPS-01-04-L-G10) and B (effect of LPGS-02-C06) show a typical growth curve of Δ*yfh1* cells in liquid YNB-Raf medium with no addition as a reference curve and in inserts, the effect of the drugs on the kinetics of growth evaluated by measuring the cell density ($OD_{600\,nm}$) at 3 different stages of the growth: 12 h (A), 36 h (B), and 60 h (C) after addition of the drug at different concentrations (0.8, 4, 20, and 100 μM). The values in insert graphs represent the increase of growth due to the drug at these various concentrations, as *n*-fold increase of the cell density compared to the DMSO control (1 = no change, 2 = 2-fold increase, etc.). Dose-dependent effect is also illustrated by agar disc diffusion assays (YNB-Raf/agar medium) in panels D: paper discs (diameter of 0.3 cm) were impregnated with 7 μL of the concentrated drugs (10 mM in DMSO), and the pattern of growth of Δ*yfh1* colonies around the discs was photographed after 2–4 days. The zones showing the highest density of colonies are indicated by a double arrow. All of the experiments were performed in quadruplicate.

Some examples are illustrated in Figures 2 and 3. Figure 2 shows a typical growth curve of Δ*yfh1* cells in liquid YNB-Raf medium: there is a very long lag period (about 24 h) before the cells enter a short exponential phase of growth, and then the cells stop growing (stationary phase) to reach a maximum $OD_{600\,nm}$ value of about 0.2 (2 million cells·mL^{-1}) after more than 3 days. We tested the effect of the selected compounds (added at different concentrations in the medium) on these different phases of growth: lag period, beginning of exponential phase, and end of exponential phase (A, B, and C, respectively, in Figure 2). We observed very different dose-dependent effects of the molecules on the growth of frataxin-deficient cells, allowing define categories of compounds acting at different concentrations on different phases of the growth. Examples are shown in Figure 2: some molecules strongly improved the early phases of growth of frataxin-deficient cells at low concentration (0.8 μM) (LPS 01-04-L-G10, Figure 2(a)); some molecules improved all phases of growth at high concentration (100 μM) (LPGS-02-C06, Figure 2(b)), while other molecules had different effects on the growth phases according to their concentration (data not shown). These differences in the effects of the selected compounds were also evidenced in the agar disc diffusion assays: maximum efficiency of a drug at low concentration resulted in a concentric zone of colonies growing better at some distance of the paper disc (Figure 2(a)), while colonies grew better in the immediate vicinity of the paper disc when the drug was more active at the highest concentration (Figure 2(b)). Variants of these patterns (zones of growth improvement/inhibition around the paper discs) were observed with different drugs (Figure 3). These results suggest that the different compounds selected in our screen improved Δ*yfh1* cells viability through distinct molecular mechanisms. One of these compounds was desferrioxamine B (DFOB) provided as deferoxamine mesylate (Figure 3). DFOB is the metal-free form of ferrioxamine B (FOB), the ferric iron complex of this siderophore for which *S. cerevisiae* has a specific transporter, Sit1p [34]. The beneficial effect of this strong iron chelator on Δ*yfh1* cell growth was proportional to its concentration (Figure 3). We also tested the effect of DFOB versus FOB: the iron-containing molecule was more efficient at rescuing cell growth than the iron-free one (data not shown).

Only the compounds presenting a strict specificity toward the Δ*yfh1* phenotypes and exhibiting no cytotoxicity (i.e., no growth inhibition at high concentration) were selected for evaluation of their efficiency *in vivo* in a *Drosophila* model of FA. The 12 selected compounds (8 from the "French National Chemical Library" and 4 from the Prestwick collection) are presented in Figure 4.

3.2. Evaluation of Drug Efficiency In Vivo on a Drosophila Model of FA. In *Drosophila*, several models have been developed to downregulate dfh (the ortholog of fxn) in various tissues by an UAS-GAL4 transgene based RNAi method [26, 31, 35–37]. Ubiquitous inactivation of dfh throughout development, under control of the ubiquitously expressed da-GAL4 driver, leads to a developmental blockage at the third larval stage. Frataxin-deficient larvae do not formed pupae at the expected time, continue to grow, and become giants. Only a fraction of these larvae undergo pupariation much later than controls. The frataxin-deficient larvae also present reduced activities of ISC-containing mitochondrial aconitase and of respiratory complexes II, III, and IV along with hypersensitivity to iron [26].

Therefore, we tested here the ability of the 12 compounds selected in yeast to rescue this developmental phenotype. To this purpose, we followed the timing of pupariation of da-GAL4>UAS-fhRNAi larvae treated with compounds at

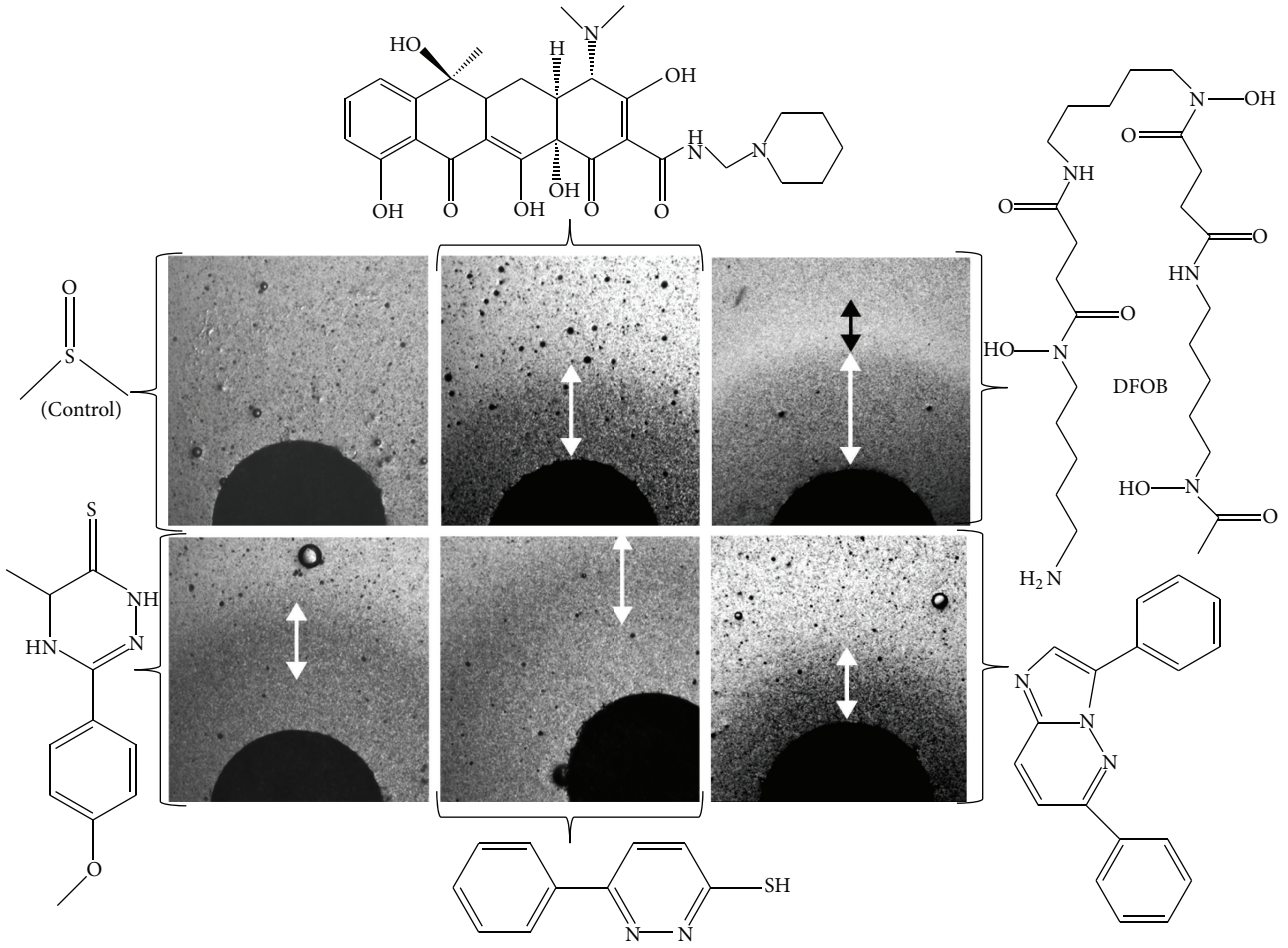
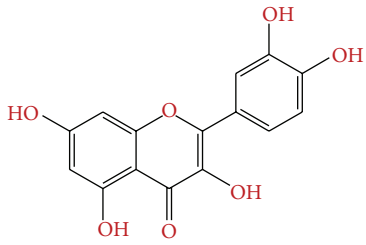


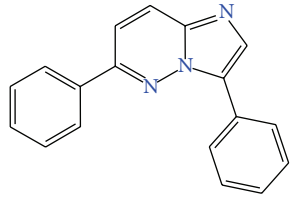
Figure 3: Dose-dependent effect of some drugs evaluated by agar diffusion assays. The agar diffusion disk assays were performed as described in Figure 2. White double arrows represent zones of growth improvement whereas black double arrows represent zones of growth inhibition. Pictures are shown in negative to highlight the contrast. Chemical used were from left to right upper panel DMSO (as a control), rolitetracycline (not evaluated further), and desferrioxamine B (DFOB) and lower panel LPS02-25-L-E10, LPS01-03-L-F03, and LPS02-30-L-H10.

10 or 50 μM. In our breeding conditions, more than 80% of da-GAL4/+ control larvae formed pupae between 140 hours and 190 hours after egg laying (AEL) (Figure 5(b)). As expected, da-GAL4>UAS-fhRNAi larvae presented delayed pupariation: at 263 hours AEL, only 6–8% of these larvae have formed pupae (Figure 5(a)), and 50% never reached pupariation (Figure 5(b)). Two compounds, LPS 01-04-L-G10 and DFOB, improved both the timing of pupariation and the final percentage of larvae reaching pupariation when larvae were treated at 10 μM (Figure 5(b)). Interestingly, the effect of LPS 01-04-L-G10 was more pronounced at the lower dose (10 μM), as was observed in yeast (Figure 2(a)). The dose-dependent effect of DFOB was also similar to that found in yeast: 28.5% of the frataxin depleted larvae reached pupariation at 263 hours AEL with 10 μM treatment and 58.5% with 50 μM treatment (Figure 5(a)). FOB was not tested at this stage. At 50 μM, we detected 4 additional compounds, LPS01-03-L-F03, LPS 02-14-L-B11, LPS02-13-L-E04, and LPS02-25-L E10, with significant improvement of pupariation (Figure 5(a)). Consequently, 50% of the compounds selected in yeast appeared to be active in flies.

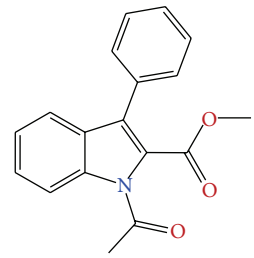
Finally, we investigated whether the two most promising compounds (LPS 01-04-L-G10 and deferoxamine mesylate), active at the lower concentration (10 μM), could have also beneficial effects on the heart dilatation phenotype induced by frataxin depletion in cardiomyocytes. Using the strategy recently described in [31], we measured diastolic diameters of 4-day-old UAS-mitoGFP;HandGS>UAS-fhRNAi adult male flies untreated or treated during development with 10 μM of these compounds. As expected, untreated flies presented heart dilatations compared to age-matched UAS-mitoGFP;HandGS>+ control flies (Figure 5(c)). While treatment with deferoxamine mesylate significantly enhanced the pathological phenotype, we observed a significant rescue with LPS 01-04-L-G10 treatment (Figure 5(c)). Thus, this last compound rescues at least two pathological hallmarks of FA in *Drosophila* models and deserves attention as a leading compound for further improvements.



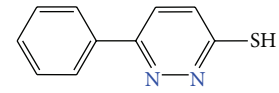
LPGS-02-C06



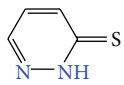
LPS 02-30-L-H10



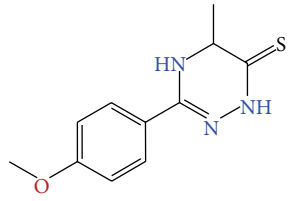
LPS 02-23-L-C05



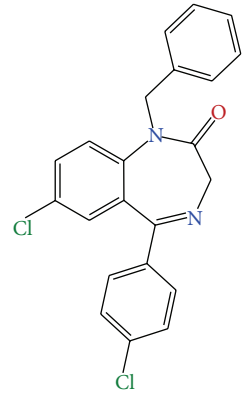
LPS 01-03-L-F03



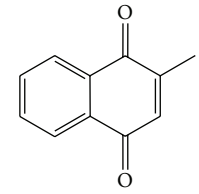
LPS 01-04-L-G10



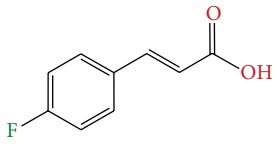
LPS 02-25-L-E10



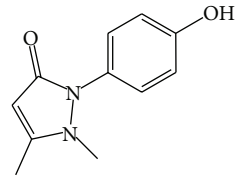
LPS 02-13-L-E04



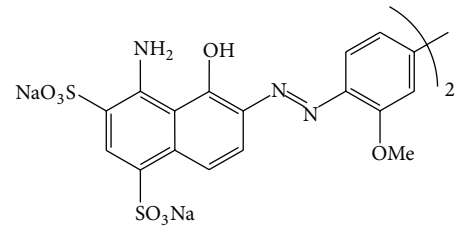
Menadione



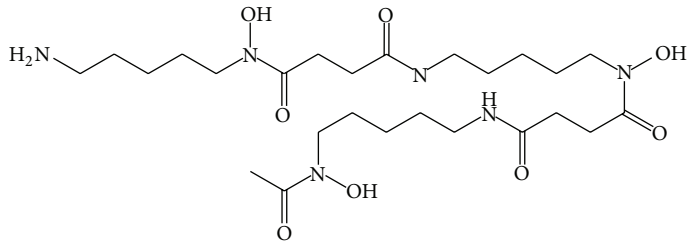
LPS 02-14-L-B11



4-Hydroxy antipyrine



Chicago sky blue



Deferoxamine

Figure 4: Chemical structure of the compounds selected from the yeast-based screen and used in the *Drosophila* based developmental assay.

4. Discussion

Two chemical libraries were screened on frataxin-deficient yeast (*S. cerevisiae*). The first chemical library is the Prestwick collection (880 compounds) and is composed of approved drugs (FDA, EMA, and other agencies). This collection is particularly valuable as the compounds were selected for their known bioavailability and safety in humans. The second chemical library is a subset of the French National Chemical Library, composed of 5,500 compounds. This collection includes a large diversity of functionalized and drug-like compounds, mainly based on heterocyclic scaffolds. Indeed, this library is composed of chemical compounds synthesized by French medicinal chemists over the last decades. The

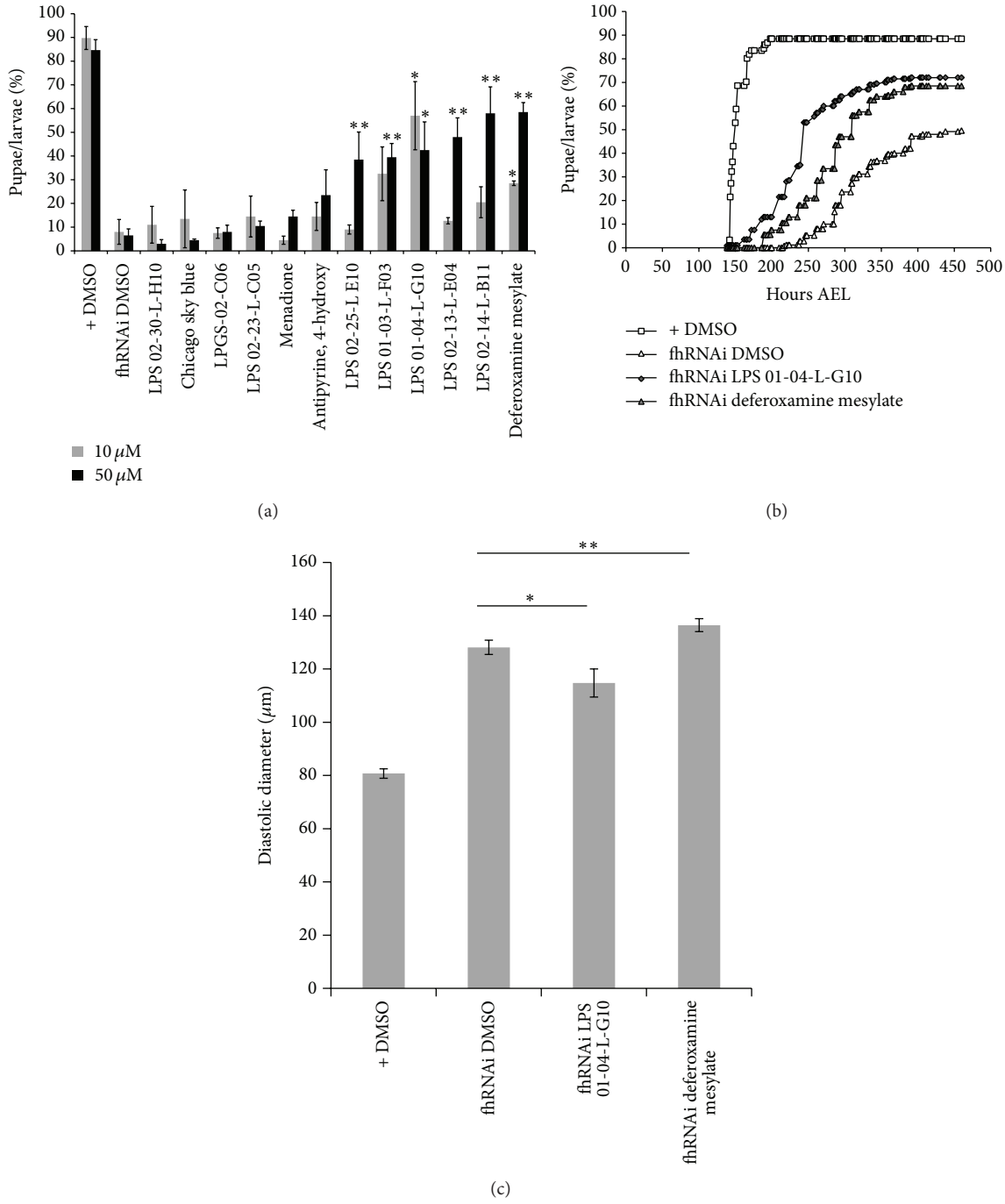


Figure 5: Drugs ability to rescue *in vivo* phenotypes induced by frataxin inactivation in *Drosophila*. (a) The timing of pupariation of da-GAL4>+ (+) and da-GAL4>UAS-fhRNAi (fhRNAi) larvae untreated (DMSO controls) or treated with compounds was followed. Percentages of larvae reaching pupariation 263 hours after egg laying (AEL) are shown. The 12 compounds selected in yeast were tested at 10 μM (grey bars) and 50 μM (black bars). Each treatment condition was tested on 3 to 4 samples of 50 larvae. All values are means (±SEM). Significant differences of da-GAL4>UAS-fhRNAi treated with a compound compared to untreated larvae of the same genotype are indicated: $^{*}P < 5.10^{-2}$ and $^{**}P < 5.10^{-3}$. (b) Percentages of larvae reaching pupariation as a function of time after egg laying for control larvae (+ DMSO) and frataxin depleted larvae and untreated (fhRNAi DMSO) and treated with 10 μM deferoxamine mesylate or LPS 01-04-L-G10 are shown. (c) Diastolic diameters of 4-day-old UAS-mitoGFP;HandGS>+ control ($n = 24$) and UAS-mitoGFP;HandGS>UAS-fhRNAi adult male flies untreated ($n = 26$) or treated during development with 10 μM LPS 01-04-L-G10 ($n = 8$) or deferoxamine mesylate ($n = 17$). All values are means (±SEM). Significant differences between treated and untreated UAS-mitoGFP;HandGS>UAS-fhRNAi flies are indicated: $^{*}P < 5.10^{-2}$ and $^{**}P < 5.10^{-3}$.

screening was performed in triplicate at a single concentration (10 μM) for each tested compound and led to identify 60 compounds that significantly improved the growth of frataxin-deficient yeast cells in liquid medium with raffinose as the main source of carbon. This set of compounds was clustered on the basis of structural features, dose-dependent and specific action on Δ*yfh1* cells, availability, and purity, in order to afford 12 molecules representative of the different chemical families. In order to assess the relevancy of each chemical family towards Friedreich's ataxia, the 12 selected compounds were tested on an animal model of FA, that is, the *Drosophila* model where frataxin-deficient larvae show impairments in the larval to pupal transition, a phenotype previously shown to be associated to decreased activities of several ISC containing enzymes [26]. The 12 compounds were tested for their ability to improve the defective pupariation due to frataxin ubiquitous inactivation. Four approved drugs were identified in yeast from the Prestwick collection (Menadione, Chicago Sky Blue, antipyrine, and desferrioxamine B), but only DFOB was confirmed to be active *in vivo* on the *Drosophila* pupariation assay. Known as an iron chelator, this drug is clinically used under its mesylate salt form to remove excess iron from the body. After 263 hours in presence of 50 μM of DFOB, about 60% of frataxin-mutated larvae had undergone the pupariation step. Yeast cells have a specific transporter for ferric chelate FOB (Sit1p). Owing to the very high binding constant of ferric iron to DFOB (K_D 10^{-31} M, [38]), it is most likely that the DFOB provided to the cells will chelate the ferric iron present in the growth media and therefore be used by the cells as an iron source. Therefore, the rescuing effect of DFOB in the yeast model could be interpreted in different ways: either the drug acts as a classical iron chelator, by decreasing the total cell iron pool available to the cells, or it makes iron more available to the cells by preventing iron precipitation in the mitochondria. This latter hypothesis seems the more likely, since FOB also improved growth of frataxin-deficient cells. Moreover, iron chelators that cannot be used as iron sources by *S. cerevisiae* (bathophenanthroline disulfonic acid, ferrozine, 2, 2′-bipyridyl, salicyl-hydroxamic acid) and that are known to generate iron depletion in yeast did not rescue growth of Δ*yfh1* cells and had even toxic effects ([33] and data not shown). The rescuing effect of DFOB in the *Drosophila* model could be based on a different molecular mechanism than in yeast, although nothing is known about the possible use of FOB as an iron source by *Drosophila*. We are currently testing the effect of FOB versus DFOB in this model.

4-hydroxyantipyrine, one of the main metabolites of the nonsteroidal anti-inflammatory and antipyretic drug antipyrine, showed a slight effect, but this effect was not statistically significant. Surprisingly, menadione was found inactive in the developmental assay in flies. Also named vitamin K3, menadione shows both pro- and antioxidant activities [39] and was previously identified as a hit by Cotticelli et al. [25] in yeast depleted of Yfh1p. The mode of action of menadione is complex. Although the toxic effects of high doses (mM range) of menadione involve reactive oxygen species production [40] and depletion of intracellular glutathione pools [41, 42], pretreatment of yeast cells with low doses of menadione induces a protection against further oxidative insult [40]. The low dose used in our screen (10 μM) may induce such a protective effect in the context of the Δ*yfh1* cells where exposure to oxygen induces both the production of reactive oxygen species and a depletion in intracellular glutathione pools (reviewed in [5]). Eight compounds from the French National Library were tested on the *Drosophila* pupariation assay, and 5 of them showed significant effects. The best compound is 4-fluorocinnamic acid (LPS 02-14-L-B11) which showed similar beneficial effect than deferoxamine in the pupariation assay. Interestingly, in another assay (rescue of the heart dilatation phenotype induced by heart specific frataxin depletion) deferoxamine mesylate increases the pathological phenotype, when LPS 01-04-L-G10 treatment significantly improves it. This points out that frataxin depletion likely impact several pathways whose relative importance may vary between tissues. Therefore one compound targeting one of these pathways may be active in one paradigm but inefficient to rescue another tissue-specific phenotype where a different pathway may be critical. LPS 01-04-L-G10, which is active in two assays in *Drosophila*, is a cinnamic derivative presenting a simple structure and is a good starting point for a hit-to-lead optimization process. In particular, the carboxylic acid function is expected to prevent the crossing of biological barriers and should be modified. Next, the 1,4-benzodiazepin-2-one (LPS 02-13-L-E04) was identified as an efficient compound at 50 μM (about 50% of pupae). This result is promising as many benzodiazepines have been developed as anxiolytic drugs (ex: diazepam). Interestingly, this compound is a derivative of a TSPO ligand named Ro5-4864, which is known to modulate several mitochondrial signaling pathways. The last three active compounds (LPS 02–25-L-E10, LPS 01–03-L-F03, and LPS 02–13-L-E04) exhibit the same thioamide function. The presence of thioamide function may suggest a mode of action through the chelation of iron. However the pyridazine-3-thiol scaffold was never described for this activity and iron chelators, such as desferrioxamine B, Triapine, or Tachpyridine, are generally much more functionalized in order to efficiently trap atoms of iron. Interestingly, LPS 01-04-L-G10 showed the best activity at 10 μM, with about 60% of pupae after 263 hours and up to 72% after 320 hours. As a drug-like scaffold, the pyridazine-3-thiol is a good candidate for a hit-to-lead program.

5. Conclusions

Altogether, the present results open new and promising ways to decipher the molecular basis of frataxin deficiency and to develop original compounds with some efficiency to treat FA. The *Drosophila* based developmental assay, although quite tedious, is robust and should be extremely powerful to further evaluate derivatives of the hits described in this study. In addition it can be completed with other phenotypic assays in flies such as the heart defect rescue assay recently described [31]. These additional developments should help discriminating general or tissue-specific action of the compounds towards development of new drugs for FA.

Disclosure

Alexandra Seguin and Véronique Monnier are co-first authors.

Conflict of Interests

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

Authors' Contribution

Hervé Tricoire and Emmanuel Lesuisse supervised equally this work.

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