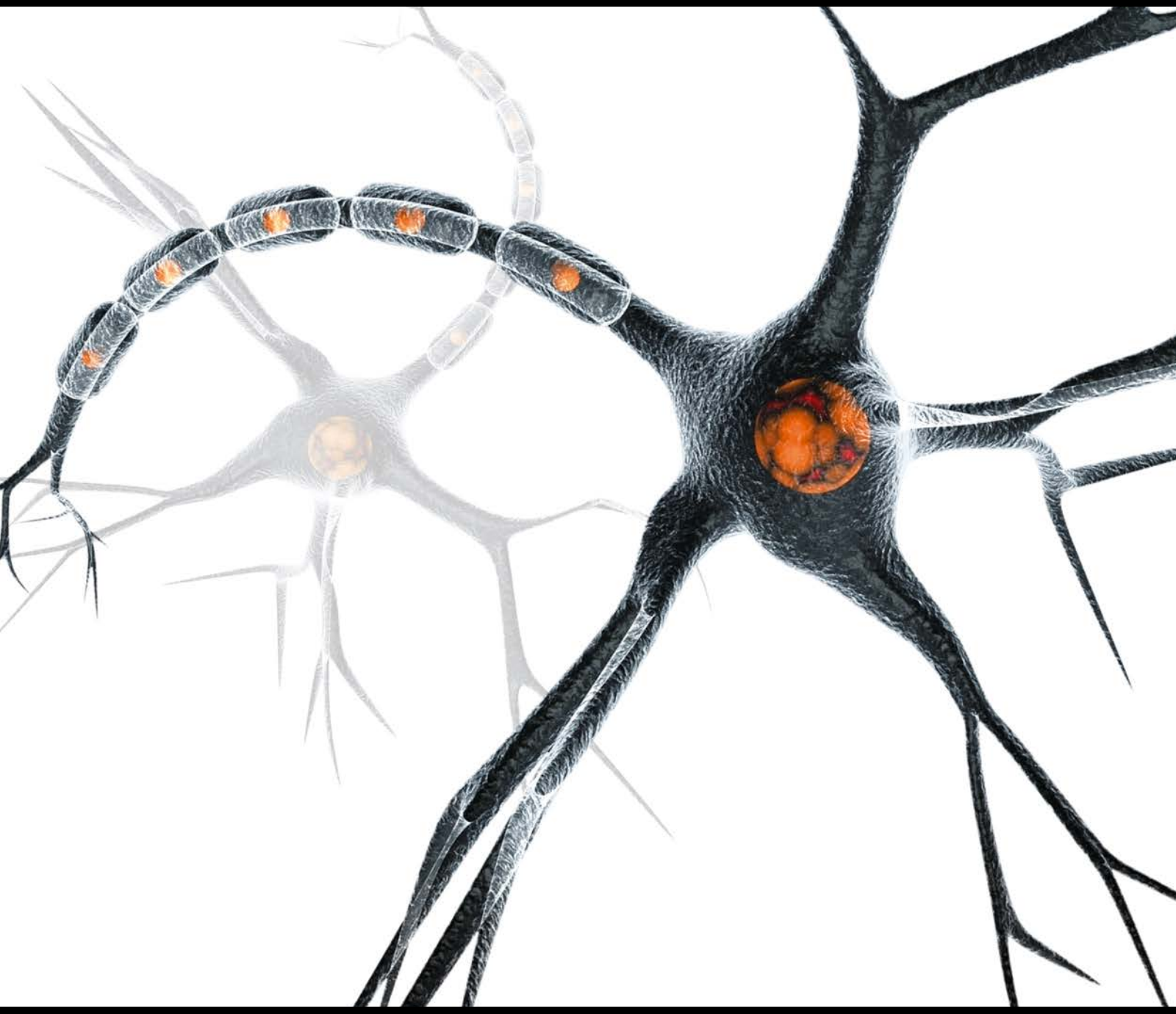
Neural Plasticity

DNA Damage, Neurodegeneration, and Synaptic Plasticity

Guest Editors: Daniela Merlo, Inmaculada C. Ibañez, Rosanna Parlato, and Gerhard Rammes



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Editorial Board

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Editorial

DNA Damage, Neurodegeneration, and Synaptic Plasticity

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This special issue includes four reviews and four original research articles focusing on the physiological and pathological role of DNA damage and repair mechanisms, synaptic plasticity, and neurodegeneration. Moreover, the emerging roles played in memory and neurodegeneration by different factors involved in the stress response, chromatin remodeling, and nucleolar function are specifically addressed. Finally, the present collection includes articles outlining current treatments for neurodegenerative diseases including Alzheimer's disease (AD) and Niemann-Pick type C disease (NPC).

Unrepaired DNA lesions and deficiencies in DNA repair systems are implicated in the progressive neuronal loss observed in many neurodegenerative pathologies although neuronal DNA damage and repair may also play a role in cognitive function and dysfunction. The formation of double strand breaks (DSBs), the most lethal form of DNA damage, may be a physiological process that modifies chromatin organization and gene expression involved in information processing, learning, and memory and may be caused by normal brain activity. Conversely, pathologically elevated β-amyloid peptide (Aβ), a major culprit for the pathogenesis of AD, worsens DNA damage by eliciting aberrant synaptic activity.

We still have limited understanding of the precise molecular mechanisms underlying failures in memory formation and consolidation and the synaptic dysfunction associated with the early onset of neurodegenerative diseases. Therefore, this special issue aims to contribute to the knowledge on these mechanisms involved in both cognitive impairment and neurodegeneration to indicate novel therapeutic targets.

In the review article by L. Narciso et al., "The Response to Oxidative DNA Damage in Neurons: Mechanisms and Disease," the authors outline the notion that the mechanisms controlling genome stability are of key importance in the development and function of the nervous system. They discuss and present different neurological diseases characterized by impairment of DNA repair pathways focusing on base excision repair (BER). Moreover, they stress the evidence that the mechanisms controlling DNA damage in neurons may vary depending on the brain region and suggest the need for further investigation to determine whether differences in the response to DNA damage underlie the brain-region selectivity observed in neurodegenerative diseases.

Factors known to function in DNA recombination/repair machineries have been shown to play a role in long-term memory (LTM). The research article by E. Castro-Pérez et al., "Identification and Characterization of the V(D)J Recombination Activating Gene 1 in Long-Term Memory of Context Fear Conditioning," reports that the *V(D)J* recombination activating gene 1 (RAG1), which encodes a factor that introduces DSBs in immunoglobulin and T-cell receptor genes, is induced in the amygdala, but not in the hippocampus, after context fear conditioning. In functional studies, ablation of RAG1 expression causes a significant impairment in LTM, thus suggesting that RAG1 may play a role in LTM consolidation. This work further supports the

notion that DNA recombination/repair machineries might be involved in learning and memory processes. Interestingly, the authors suggest that an integrated control of the introduction of DSBs, DNA repair, DNA rearrangement, epigenetics, and transcriptional and translational mechanisms may orchestrate gene regulation in memory formation.

Aβ is the key player in the amyloid-cascade hypothesis of AD which appears to be associated with DSB induction in aging and AD. The article by E. Gruz-Gibelli et al., "The Vitamin A Derivative All-Trans Retinoic Acid Repairs Amyloid-β-Induced Double-Strand Breaks in Neural Cells and in the Murine Neocortex," indicates a role of all-trans retinoic acid (RA), a derivative of vitamin A, in the repair of Aβ-induced DBSs. The authors test the DNA and the cellular protection activity of RA both on neuronal SH-SY5Y cells and on astrocytic DI TNC1 cells and, in addition, on extracts from cortex of young and old mice. The authors suggest that RA, besides increasing cell viability in the cortex of young and aged mice, might also target DNA repair of genes involved in synaptic maintenance. Therefore, exploring mechanisms involved in Aβ-induced DSBs might provide additional means to target pathological Aβ-induced changes.

Epigenetic silencing of rDNA by DNA methylation is a common feature of mild cognitive impairment (MCI) and AD and it is considered an emerging disease marker. Interestingly, rDNA silencing in the nucleolus perturbs diverse nucleolar functions including global chromatin regulation and biogenesis of ribosomes. Among the chromatin-remodeling enzymes, poly(ADP-ribose) polymerase-1 (PARP-1) may control synaptic plasticity and memory consolidation by regulating the expression of immediate early genes and rDNA transcription. Recently, it has been shown that the maintenance of the late phase of long-term potentiation (L-LTP), a model for long-term memory, requires nucleolar integrity and the expression of new rRNAs. The research article by J. Zeng et al., "Nucleolar PARP-1 Expression Is Decreased in Alzheimer's Disease: Consequences for Epigenetic Regulation of rDNA and Cognition," shows that the nucleolar PARP-1 may be a sensitive marker of synaptic deficits in AD. The authors suggest a novel role for PARP-1 dysregulation in AD pathology by showing that PARP-1 positive nucleolar staining in CA1 and CA4 hippocampal pyramidal neurons of AD patients is significantly reduced compared to controls. They also propose an intriguing model in which the loss of nucleolar PARP-1, resulting in DNA methyltransferase activation, might account for the rDNA silencing observed in AD.

AD is a complex multifactorial disorder which may require complex approaches to treatment. Early disease detection, combination therapies, and lifestyle choices are all likely contributors to the successful eradication of the pathology. In the review article "Current Research Therapeutic Strategies for Alzheimer's Disease Treatment," J. Folch et al. consider the effectiveness of treatments aimed at reducing Aβ production through both the inhibition of β and γ secretase enzymes and the dissolution of existing cerebral Aβ plaques to be modest. Interestingly, they present alternative strategies centered on the inhibition of the downstream Aβ signaling, particularly acting at synaptic level. The interaction of Aβ and prion protein (PrPC) activates Fyn kinase which then modifies synaptic signaling through NMDA glutamate receptors. This mechanism underlies excitotoxicity and dendritic spine loss. Thus, the authors propose Fyn kinase blockers Masitinib and Saracatinib as effective molecules in treating AD symptoms in experimental mouse models of the disease. In fact, Saracatinib is currently in Phase II and Masitinib is in Phase III clinical trials for mild-to-moderate AD.

In the research paper by G. D'Arcangelo et al., "Miglustat Reverts the Impairment of Synaptic Plasticity in a Mouse Model of NPC Disease," the authors investigate in NPC, a rare disease with progressive neurological deterioration and cognitive decline until severe dementia, the mechanism of action of Miglustat, a recent approved drug for the treatment of the disease. In particular, they study synaptic plasticity phenomena and evaluate ERKs activation in the hippocampus of NPC1−/− mice, a well described animal model of the disease. The authors show an impairment of LTP in NPC1−/− mouse slices which is associated with lack of ERKs phosphorylation. They also find that *in vivo* Miglustat administration in NPC1−/− mice can rescue synaptic plasticity deficits, restore ERKs activation, and counteract hyperexcitability. Overall, these data indicate that Miglustat may be effective for treating the neurological deficits associated with NPC, such as seizures and dementia.

Parkinson's disease (PD) is the second most common neurodegenerative disorder. This is characterized by the progressive loss of midbrain dopaminergic (mDA) neurons in the substantia nigra pars compacta (SNpc) and the presence of α-synuclein-containing protein aggregates termed Lewy bodies (and/or Lewy neurites) in affected neurons. Several transcription factors, playing a role in development and survival of mDA neurons, might be involved in the progressive loss of these neurons. The review article "Neuroprotective Transcription Factors in Animal Models of Parkinson Disease" by F.-X. B. de Thé et al. reports mechanisms by which these transcription factors control neuronal survival and activity, including genomic stability and synaptic maintenance. The authors suggest that a better understanding of these modes of action could help to identify novel neuroprotective approaches, for example, based on direct protein delivery strategies.

In the review article "Chronic Stress and Glucocorticoids: From Neuronal Plasticity to Neurodegeneration," S. Vyas and collaborators discuss the cause-effect relationships between prolonged stress, elevated levels of glucocorticoids (GCs), and cognitive/mood related disorders including AD and PD. Particularly, the authors present a comprehensive view on the cellular mechanisms through which stress and GCs may influence the pathogenesis of AD and PD.

We believe that this special issue, by focusing on DNA damage/repair mechanisms involved in learning and memory and neurodegeneration, will be instrumental to identify new potential approaches to design effective therapeutic strategies.

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Review Article

Chronic Stress and Glucocorticoids: From Neuronal Plasticity to Neurodegeneration

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Stress and stress hormones, glucocorticoids (GCs), exert widespread actions in central nervous system, ranging from the regulation of gene transcription, cellular signaling, modulation of synaptic structure, and transmission and glial function to behavior. Their actions are mediated by glucocorticoid and mineralocorticoid receptors which are nuclear receptors/transcription factors. While GCs primarily act to maintain homeostasis by inducing physiological and behavioral adaptation, prolonged exposure to stress and elevated GC levels may result in neuro- and psychopathology. There is now ample evidence for cause-effect relationships between prolonged stress, elevated GC levels, and cognitive and mood disorders while the evidence for a link between chronic stress/GC and neurodegenerative disorders such as Alzheimer's (AD) and Parkinson's (PD) diseases is growing. This brief review considers some of the cellular mechanisms through which stress and GC may contribute to the pathogenesis of AD and PD.

1. Introduction

Stress is broadly defined as an actual or anticipated threat of well-being or disruption of organism homeostasis [1]. Although the sensing and reaction to stress evolved to promote adaptation, modern workstyles and lifestyles represent challenges that render individuals susceptible to physical and mental disorders [2–5]. Multiple factors influence an individual's ability to cope with stress, for example, early life experiences, gender, or personality traits. Both vulnerability and resilience may be determined by genetic and epigenetic (gene environmental interactions) background [5–9].

Since the discovery of the communication between hypothalamus and pituitary in early 70s that opens a new window in our understanding of the brain-body communication, there are plethora of studies describing the high biological significance of stress and its responses which enables various adaptive processes to changing conditions. The most easily measureable and critical physiological response to stress involves the release of glucocorticoids (glucocorticoids, GCs). These hormones are synthesized and secreted into systemic circulation from the adrenal glands following stimulation by the anterior pituitary hormone adrenocorticotropic hormone (ACTH) [1]. The release of ACTH itself is increased in response to the secretion of corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) from neurons in the hypothalamic paraventricular nucleus (PVN). Together, the hypothalamus, pituitary, and adrenal glands constitute the so-called hypothalamo-pituitary-adrenal (HPA) axis, which plays an essential role in the adaptive response to psychogenic (e.g., fear) and physical (e.g., cellular lesion or pathogen invasion) stressors. The adaptive responses that are initiated by GCs occur in multiple tissues and involve alterations in numerous

physiological (e.g., metabolic, cardiovascular, and immune) as well as behavioral (e.g., emotion, cognition, and motor) processes [1, 10–12]. Normally, GC-driven negative feedback mechanisms at the different levels of the HPA axis serve to normalize GC secretion and restore homeostasis; however, and depending on the type, duration, and intensity of the stressful stimulus, GC hypersecretion may persist and become a potential threat for health [1].

There is now abundant evidence that GCs can exert profound modulatory effects on a variety of brain functions from early development through to late life [12]. Their actions are mediated by two receptors: the mineralocorticoid receptor (MR) and glucocorticoid receptor (GR), which belong to the superfamily of nuclear receptors that act as transcription modulators [13, 14]. In the brain, GR is ubiquitously expressed, whereas MR expression is more restricted to just a few structures (hippocampus, locus coeruleus, amygdala, prefrontal cortex, and nucleus of the solitary tract, as well as PVN neurons). MR is also present in nonneuronal cells, namely, in glia and epithelial cells of the choroid plexus and ependyma [15].

Binding assays using ^{3}[H] corticosterone have shown the MR has a 10-fold higher affinity (K_d = 0.5 nM) for GC compared to GR (K_d = 5 nM), which means that, at basal GC levels, MR is occupied and activated [16] whereas GR is only activated when GC levels reach a certain level, for example, during the circadian peak of GC secretion and during stress [17]. Importantly, brain MR and GR both respond to the same endogenous ligand (cortisol in humans and larger mammals, corticosterone in rodents); further, MR and GR were reported to colocalize in the same pyramidal and granular neurons of the hippocampus [17]. Given the GR and MR colocalization and relatively small difference in their affinity for endogenous GCs, the question arises as to whether they regulate distinct genes and/or coregulate transcription by heterodimerization. Heterodimerization of GR and MR was shown with high concentration of GC (stress level) in the nuclei of cultured hippocampal neurons. Moreover, evidence suggests that their cellular responses through regulation of distinct gene expression (as homodimers) depend strongly upon specific recruitment of coregulators [18, 19].

Synthetic GCs (e.g., dexamethasone, methylprednisolone) are routinely used in clinical situations due to their powerful anti-inflammatory and immunosuppressive actions. However, a growing body of evidence suggests that high GC exposure in early life can adversely program the HPA axis and increase the susceptibility to develop metabolic, neuropsychiatric, and neurodegenerative disorders [5, 20, 21]. In addition, there is now ample experimental evidence where elevated GC levels and prolonged exposure to stressful conditions induce structural remodeling of neurons with synaptic loss as well as alterations in glial functions, which are frequently maladaptive [22]; see also Figure 1. In this brief review we discuss some of current knowledge about cellular targets and mechanisms through which stress and altered GC levels trigger changes in the brain that may lead towards the development and progression of neurodegenerative pathologies such as Alzheimer's (AD) and Parkinson (PD) disease.

2. From Stress-Driven Brain Programming to Neurodegenerative Pathologies

In addition to nongenomic mechanisms that are still incompletely identified [23], chronic stress and GC levels most likely influence neuronal function and connectivity by activating GR-mediated transcription. GRs are normally located in the cytoplasm in association with chaperone proteins such as the heat shock proteins Hsp90 and 70 and the immunophilins FKBP51 and FKBP52. Upon GC binding, conformational change of the GR-chaperone complex results in nuclear translocation of the GR [24, 25]. In the nucleus, GR binds to specific regions of DNA, which possess glucocorticoid response elements (GRE) within the promoters of target genes, leading to cell-type and context-dependent gene expression [26–28]. Transcriptional regulation by GR may occur by (a) direct binding of GR homodimers to GRE within DNA sequences to stimulate transcription, for example, *mitogen-activated protein kinase phosphatase-1* gene; (b) direct binding to negative GRE elements to repress transcription; the gene encoding the prohormone from which ACTH is derived (proopiomelanocortin, *POMC*), *CRH,* and the *CRH receptor* genes are examples of negatively regulated genes; and (c) trans-repression or "tethering," that is, association with other transcriptional factors that inhibit the transcriptional activity of GR. In the brain, identification of GR-modulated genes is difficult due to the anatomical complexity and cellular heterogeneity. Nevertheless, transcriptomic studies in the hippocampus have identified functional classes of GR target genes which include genes coding for neurotransmitter catabolism, neurotrophic factors and their receptors, signal transduction, energy metabolism, and cell adhesion [29].

In addition to altering gene expression, growing evidence suggests that epigenetic mechanisms represent a means through which stress and GCs can leave long-lasting "memories" of past experiences which, in turn, contributes to shaping the organism's physical and mental health trajectory [21, 30, 31]; see Figure 1. Broadly, epigenetics refers to stable changes in the regulation and/or function of DNA, RNA, and/or proteins that do not involve alterations of their primary sequences. Two well-known examples of epigenetic marks induced by environmental stimuli (e.g., stress) are DNA methylation and histone modification. The first evidence of epigenetic programing in the brain by early life adversity showed that poor maternal care in rats leads to methylation of exon 1_7 in the *GR* promoter, being accompanied by aberrant behaviors and altered HPA axis responses during adulthood [32, 33]. Subsequently, similar mechanisms were reported in humans who had experienced childhood adversity [34] and in infants born to depressed mothers [35]. The earlier studies in rats were replicated in mice in paradigms of prenatal GC exposure and early postnatal stress; we showed that these pre- and postnatal manipulations resulted in epigenetic modifications of the promoters of neurotransmitter (*dopamine receptor 2*) [36], *GR*, and various GR target genes [37, 38] with long-lasting maladaptive behavioral consequences.

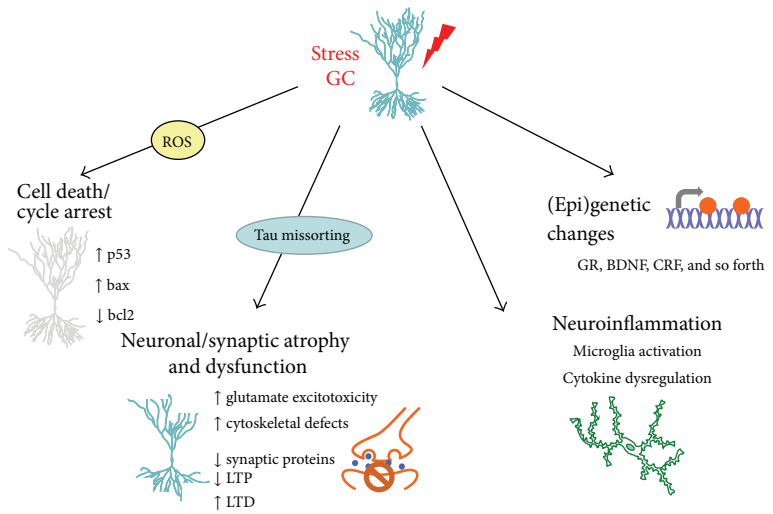

Figure 1: Cellular targets and actions of chronic stress mediated by glucocorticoid receptors. This schema depicts some cellular targets and mechanisms that are targeted by glucocorticoids (GCs), whose actions are mediated by glucocorticoid receptors (GR). GCs are secreted under conditions of stress; neuronal damage and brain pathologies are a common consequence of persistently elevated GC secretion. GC can trigger mitochondrial dysfunction and the apoptotic machinery, as well as cell cycle arrest and cell death. In addition, stress/GC may induce neuronal atrophy and synaptic dysfunction/loss by stimulating hyperphosphorylation of the cytoskeletal protein Tau, thus disturbing the integrity of the cytoskeleton and missorting Tau at synapses. Together, these latter events may eventually result in the degradation of synaptic proteins and receptors and consequently, synaptic plasticity. Stress and GC are also established as modulators of microglial activation and neuroinflammatory processes. Lastly, accumulating evidence indicates that stress and GC can influence neuronal structure and function through epigenetic mechanisms.

Recent studies also suggest that early life events (e.g., intrauterine infections, maternal stress, and poor maternal and perinatal nutrition) may play a role in the onset of Alzheimer's disease (AD), an age-related neurodegenerative disorder characterized progressive memory and cognitive deficits [39]. From this perspective, AD is probably not determined by a single etiologic factor but results from the interplay between genetic and environmental factors throughout life, possibly explaining why monozygous twins can be discordant for AD. Albeit this is still controversial and the literature is sparse, it has been suggested that adverse events in early life, for example, maternal stress and poor maternal and perinatal nutrition, can potentially predispose eventually to AD through epigenetic programing of specific genes/pathways related to AD neurodegeneration. For example, maternal separation for the first 3 weeks of rodent life is shown to result in increase of AD cellular pathways (e.g., APP misprocessing and Tau hyperphosphorylation; see below) followed by synaptic and neuronal damage as well as cognitive deficits in adulthood [40] suggesting the potential impact of early-life stress exposure to the precipitation of AD neurodegeneration later in life. While most current research on epigenetic mechanisms focuses on DNA methylation, one recent study demonstrated that GC, acting via GR, increase the levels of histone deacetylase 2 (HDAC2), an enzyme regulating DNA expression, in the CK-p25 mouse [41]. In general, how early life stressors reprogram the fetal brain and contribute to late-life development of neurodegenerative disorders (e.g., AD) is emerging as an exciting, new research field [42].

Experimental evidence in animal studies indicates that stressful events in early life can impact the etiopathogenesis of another neurodegenerative disorder, Parkinson's disease (PD), which is characterized by both motor and nonmotor symptoms. Depression, anxiety, apathy and interestingly fatigue are common nonmotor features occurring in around 30 to 58% of patients before the onset of motor symptoms in PD patients. In addition, the prevalence of cognitive impairment in PD ranges from 19 to 36% [43]. The cellular mechanisms underlying these nonmotor symptoms in PD may share similarities to AD, particularly with respect to the molecular pathways activated by stress.

Maternal separation was reported to exacerbate motor deficits and nigrostriatal lesion in an experimental model of PD [44]. In an interesting study, pups of female animals, exposed to the bacterial endotoxin lipopolysaccharide (LPS) during pregnancy, showed loss of dopaminergic (DA) neurons. Since loss of dopaminergic neurons as well as related motor deficits is a characteristic feature of PD pathology, the above findings suggest that high LPS levels in mothers might interfere with the development of DA neurons in the fetus, thus enhancing susceptibility to PD [45]. Accordingly, developmental stress may represent the first imprint in the brain and accumulatively with later stressful stimuli to affect nigrostriatal neurochemical reserve and precipitate the PD phenotype [46].

3. Chronic Stress and GC as a Risk Factor for AD

AD is a multifactorial neurodegenerative disorder with complex etiopathology. Besides early life stress (see above), accumulating clinical evidence strongly suggests that chronic

stress in adulthood as well as elevated GC levels may have a role in the development of AD pathology and related dementia [47, 48]. In fact, high levels of cortisol are commonly found in AD patients' plasma, saliva, and/or CSF [49–53]; AD patients also show higher total daily secretion of cortisol [54]. The potential link between stress/GC and AD described above is strengthened by emerging evidence that stress may advance the age of onset of the familial form of AD [47, 48, 55] and that cortisol levels in AD patients correlate with their memory deficits [56, 57] suggesting a role for GC on AD. Nevertheless, in the absence of longitudinal studies it is not clear from the available evidence as to whether elevated GC secretion is a cause or a consequence of AD disease.

An important brain area in unraveling the interrelationship between stress, elevated GC, and AD pathology is the hippocampus, which is among the first areas affected in AD patients. Hippocampal lesions in AD brain are not only associated with the deficits in declarative, spatial, and contextual memory but could also be responsible for the suggested HPA axis dysregulation and the subsequent overproduction of GC found in AD patients due to the inhibitory role that hippocampus exhibits on HPA axis. Indeed, previous studies from our laboratories (and others) have shown that hippocampal neurons are particularly vulnerable to the adverse effects of stress and GC, their effects being manifested as dendritic atrophy and apoptotic cell death [22, 58]. Moreover, a large number of studies have shown that stress and elevated GC levels affect neurogenesis in adult brain with subsequent impairments of mood and cognitive behavior [59, 60]. More specifically, both acute and chronic exposure stress reduces adult neurogenesis, affecting hippocampal cell proliferation and, in certain studies, survival of newborns [61, 62]. In addition, administration of corticosterone showed the ability of glucocorticoids to damage neurogenesis in adult brain by inhibiting cell proliferation, differentiation and survival [63] while the deleterious effect of stress and/or corticosterone on neurogenesis is GC-dependent [64]. In a vicious cycle, alteration in neurogenesis of adult brain is recently shown to impact on GC negative feedback on the central elements regulating HPA axis activity [65, 66]. Moreover, perturbations in adult neurogenesis may also be related to the cognitive deficits associated with AD whereas contradictory findings support both increases and decreases of neurogenesis in brain of AD patients and Tg animal models [67]. Here, it is also worthwhile noting that stress and GC interfere with hippocampal-prefrontal cortex (PFC) connectivity [68] and dendritic and synaptic plasticity in the PFC, thus disrupting executive functions [58]. These PFC structural deficits are also likely to have consequences for central regulation of the HPA axis providing another neuroanatomical link between HPA axis dysregulation and subsequent GC hypersecretion and AD pathology.

4. Impact of Stress and GC on Neurodegenerative Mechanisms of AD

At the molecular level, AD pathology is characterized by amyloid beta (Aβ) that forms deposits (senile plaques) and hyperphosphorylated forms of the cytoskeletal protein Tau that aggregate into neurofibrillary tangles (NFT) [69–71]. Aβ is the proteolytic product of a large transmembrane protein called amyloid precursor protein (APP), which is sequentially cleaved by β-secretase (BACE-1) and γ-secretase (a complex of enzymes) to generate the production of Aβ; this cellular pathway is often called APP misprocessing. Many studies have demonstrated that the products of APP misprocessing trigger neuropathological processes associated with AD such as synaptic malfunction (including impairment of long-term potentiation), neuronal atrophy and synaptic disintegration and loss [72] as well as mitochondrial dysfunction, oxidative stress, and glial activation [73].

Although still a subject of debate, several studies suggest that Aβ also triggers the abnormal hyperphosphorylation of Tau, NFT formation, and neuronal loss. Moreover, cumulative evidence suggests that the detrimental effects of Aβ are abolished in Tau-KO mice, highlighting the essential mediatory role of Tau protein in the neuro- and synaptotoxic effects of Aβ [73–77]. Further support for an essential role of Tau in the establishment of AD pathology derives from clinical findings that have consistently shown that the cognitive deficits in AD patients correlate better with NFT rather with Aβ deposition *per se*. Moreover, Gómez-Isla et al. [78] demonstrated a strong correlation between neuronal loss in the cerebral cortex and increased NFT burden with disease progression; no such correlation was found with Aβ. In addition, the reduction of hippocampal volume in AD patients correlates better with CSF levels of phosphorylated Tau than with those of Aβ [79].

The evidence of a causal relationship between stress/GC and AD includes that from studies showing that either high GC levels and/or stress increase the production of Aβ and exacerbate memory deficits in transgenic mouse models of AD [80, 81]. Specifically, chronic immobilization stress in transgenic mice expressing the amyloid precursor protein (APP) V717ICT-100 (a mutation which results in aggressive early onset AD) accelerates the appearance of extracellular Aβ deposits and worsens memory deficits. Similar findings were obtained *in vivo* when young (prodromal) 3XTg-AD mice were treated with the synthetic GC, dexamethasone [80]; the same authors also reported dexamethasone-induced APP misprocessing in the N2A cell line, a finding matched by our own observations in PC12 cells [82]. Further, Green et al. demonstrated that GCs upregulate the transcription of *APP* and *β-secretase*, whose promoters contain a glucocorticoid response element (GRE) [80]. Consistent with the above, our studies in middle aged rats showed that stress and chronic GC drive APP processing towards the generation of Aβ and its precursor molecule (C99), both of which have neurotoxic and cognition-impairing properties [83] (see also Figure 1). The latter changes were accompanied by increases in the levels of β-secretase (BACE-1) and Nicastrin, a protein found in the γ-secretase complex. Further experiments that attempted to mimic intermittent stressful events that may exert cumulative effects over the lifetime indicated that GC potentiate the APP misprocessing pathway in previously stressed rats receiving Aβ-infusions [83] (see Figure 2).

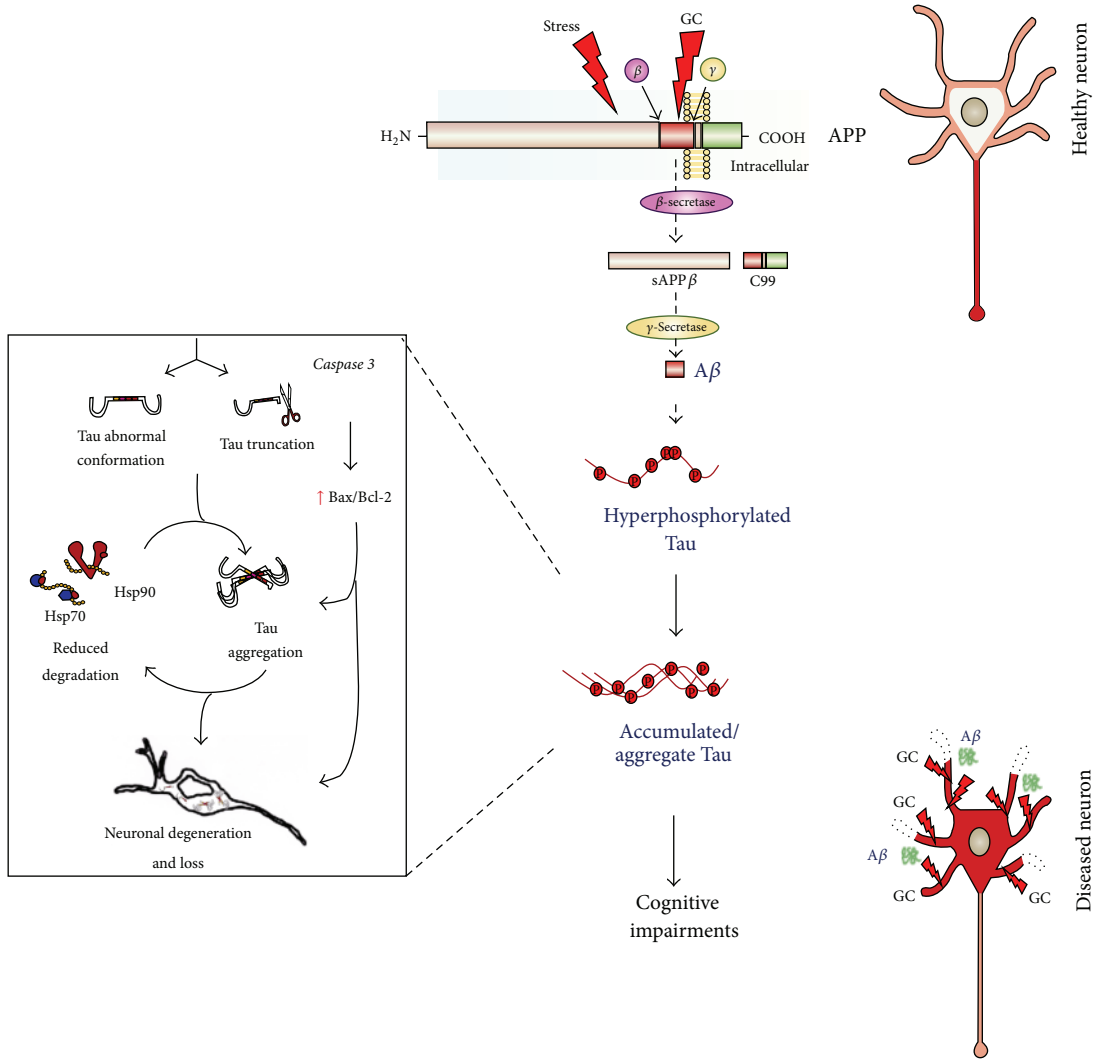

FIGURE 2: Proposed model through which chronic stress and glucocorticoids (GCs) may contribute to Alzheimer disease (AD) pathology. The model illustrates how chronic stress and high GC levels can trigger AD pathology; the figure is based on experimental evidence obtained in cellular and animal models of AD. Extended exposure to stress/high GC levels activates the amyloidogenic pathway of amyloid precursor protein (APP). This so-called misprocessing of APP involves the sequential cleavage of APP by β- and γ-secretases, resulting in the generation of toxic amyloid β (Aβ). Subsequently, the cytoskeletal protein Tau, which is mainly localized in axons (red in the representation of a healthy neuron), becomes aberrantly hyperphosphorylated, catalyzed by glycogen synthase kinase (GSK3β) and/or cyclin-dependent kinase 5 (CDK5). Hyperphosphorylated Tau is trafficked to, and accumulates in, the somatodendritic compartment, where it oligomerizes and forms insoluble aggregates (red in the diseased neuron). In addition, the abnormal conformation adopted by Tau and caspase 3-mediated truncation of Tau is accompanied by dysregulation of the molecular chaperones Hsp90 and Hsp70, which normally serve to promote Tau degradation (left panel). This cascade of events causes neuronal atrophy and loss, followed by cognitive impairments.

In addition to triggering the amyloidogenic pathway, high levels of GC and stress can also instigate the aberrant hyperphosphorylation of Tau protein that also characterized AD brain. Among the first reports suggesting a potential connection between GCs and Tau was that from Stein-Behrens et al. [84] who demonstrated that GC exacerbate kainic acid-induced hippocampal neuronal loss with a contemporaneous increase in Tau immunoreactivity. A later study showed that chronic treatment of 3xTg AD mice with dexamethasone leads to the somatodendritic accumulation of Tau in the hippocampus, amygdala and cortex [80].

Supporting those earlier studies, we showed that chronic stress or GC increase the levels of aberrantly hyperphosphorylated Tau in the rat hippocampus and PFC [85] (see Figure 2). Importantly, the hyperphosphorylation occurred at certain Tau epitopes that are strongly implicated in cytoskeletal dysfunction and synaptic loss (e.g., pSer262) [86, 87] and hippocampal atrophy (e.g., pThr231) [88] in AD patients. Here, it is pertinent to note that the extent of phosphorylation at Thr231- and Ser262-Tau correlates strongly with severity of memory impairment, speed of mental processing, and executive functioning in AD patients [89–91].

Although chronic stress and GC treatment exert similar, but not identical, effects on individual Tau phosphoepitopes *in vivo* and *in vitro* [82], the overall evidence points to GC as the key mediator of the AD-like pathology induced by stress. On the other hand, some studies have suggested a role for at least one other stress-related molecule, namely, corticotrophin-releasing hormone (CRH), as deletion of the *CRH receptor 1* gene in mice was found to block the detrimental effects of stress on Tau phosphorylation [92, 93].

As shown at Figure 2, information on the mechanisms underlying stress/GC-induced hyperphosphorylation of Tau is only just beginning to emerge. For example, *in vitro* experiments indicate that the effects of stress/GC are mediated by glycogen synthase kinase 3 (GSK3) and cyclin-dependent kinase 5 (CDK5), both of which have well-established roles in Tau hyperphosphorylation and the subsequent disruption of microtubules, features seen in the AD brain [82]. We now also know that GC exposure increases Tau accumulation by affecting turnover of the protein by reducing its degradation [82]; the latter appears to result from dysregulation of molecular chaperones (e.g., Hsp90 and Hsp70) that are responsible for Tau proteostasis [94] (see Figure 2). Interestingly, both these heat shock proteins also serve to maintain GR in a high affinity state, suggesting that these proteins may be the point at which GC/GR signaling intersects with the cellular machinery that regulates Tau degradation. Using a transgenic mouse that expresses human P301L-Tau (the most common Tau mutation), we recently showed that chronic stress triggers different aspects of Tau pathology in addition to inducing, its aberrant hyperphosphorylation and aggregation of Tau into insoluble forms [94]. Adding to the mechanistic understanding of stress-driven aggregation of Tau, we also showed that chronic stress enhances caspase 3-mediated truncation of Tau at its C-terminal, leading to an abnormal conformation of Tau in the hippocampus (Figure 2). This truncation-dependent misfolding of Tau into an abnormal conformation is known to facilitate nucleation and recruitment of other Tau molecules into neurotoxic aggregates [95, 96] before NFT are formed [95, 97, 98].

It is interesting to note that chronically elevated GC secretion, usually in response to stress, is a major cause of major depressive illness [99]. In light of the increasing volume of data implicating high GC levels in AD, it is important to consider that epidemiological studies implicate depression as a risk factor for the development of AD; this is supported by the observation that previously depressed subjects have increased amyloid plaque and NFT loads [100]. Different studies have in fact sought to discriminate between subjects undergoing normal aging from those suffering from depression or AD through the measurement of the various APP cleavage products [101–104]. While much remains to be discovered about the potentially important role of depression in AD pathology, it is interesting to note that antidepressant drugs, whose actions often involve reductions in GC secretion, inhibit the proteolytic cleavage of APP into amyloidogenic products [104, 105].

Lastly, it deserves mentioning that a recent epidemiological study found that the prevalence and incidence of dementia in war veterans suffering from posttraumatic depression (PTSD) is twice as high as that in age-matched PTSD-free subjects [106]. While PTSD is a condition quite distinct from major depression, these findings hint at the important influence lifetime stressful experiences can have on mental health, possibly through epigenetic mechanisms. The findings are also interesting since PTSD patients usually show hypoactivity of the HPA axis (versus hyperactivity in depression), suggesting that just a single—but major stressful—event involving transient GC hypersecretion can have long-lasting neuropathological consequences.

4.1. Inflammation and AD: Role of GCs? Chronic inflammation is one of the central pathological features of AD with reactive microglia and astrocytes surrounding senile β-amyloid plaques observed in both postmortem AD brain and animal models [107, 108]. Evidence from human studies suggests that glial activation is an early event; thus inflammatory markers are present in mild cognitive impairment cases that eventually progress to AD [109]. Thus proinflammatory cytokines produced by activated glia in response to amyloid fibrils would be expected to activate HPA axis and increase GC levels. *In vitro* studies clearly show that Aβ can be taken up through phagocytosis in microglia and thereafter degraded [110, 111]; thus, in AD setting, microglial likely have a beneficial role early in pathology. However, elevation of proinflammatory cytokines such as IL-1β may also participate in mood disorders such as depression [112] in AD.

The importance of immune-related responses in the emergence of Aβ burden, tau pathology, and dementia is gaining momentum as molecular comprehension of their actions is increasingly unraveled by human genetic and animal studies. Recent genome-wide association studies have identified variants in at least 16 genes involved in microglia/macrophage functions as risks for developing AD [113]. Among them, *ε4* allele of *APOE* gene is a known strong risk factor, accelerating the age of onset of AD. APOE is produced by both microglia and astrocytes; it regulates not only lipid and Aβ metabolism but also microglial chemotaxis and proinflammatory cytokine expression [114]. Recently, another strong link was found between variants in *TREM2* gene and AD. TREM2 is specifically expressed in myeloid cells where it promotes phagocytosis whilst inhibiting cytokine production [115]. These and most other GWAS genes identified [113] are involved in aberrant microglial/macrophage responses with regard to Aβ clearance and spread of Tau pathology.

In addition to genetic susceptibility, prolonged exposure of Aβ affects microglial functions. Thus, crucial microglial functions such as motility and phagocytosis were impaired in APP/PS1 mice [116]; also in these mice the levels of Aβ receptors (SRA, CD36, RAGE) and Aβ degrading enzymes (neprilysin, MMP9) were decreased with concomitant increase in proinflammatory cytokines TNF-α and IL-1β [117]. Age, a primary risk factor for AD, is also an important contributor to dysfunction of innate immune responses. Microglial dystrophy and fragmentation observed in aging brain [118] occur before the appearance of abnormal Tau suggesting dysfunctional microglia could contribute to appearance of Tau pathology.

Chronic stress through GCs is known to prime and augment neuroinflammatory processes in the cortex and hippocampus upon subsequent proinflammatory challenges such as LPS [119, 120]. Peripheral infections and stress are both known to affect the activation state of microglia and in AD pathology both could have detrimental effects on the functions of microglia. There is little known on how glucocorticoids influence glial functions during prodromal to emergence and progression of AD pathology. It would be important to understand whether GC through GR has any role in Aβ degradation in astrocytes or myeloid cells.

5. Role of Glucocorticoids in Onset and Progression of Parkinson's Disease

Parkinson's disease (PD) is a complex systemic and progressive neurodegenerative disease associated with both motor and nonmotor symptoms. The cardinal motor symptoms such as akinesia, resting tremor and rigidity mostly arise from preferential and substantial loss of dopaminergic neurons (50–60%) in the substantia nigra pars compacta (SNpc) with significant dopamine depletion in the sensorimotor striatum. The nonmotor symptoms include olfactory dysfunction and sleep behavior disorder as well as mood changes and cognitive impairment as discussed above. One principle histopathological feature is the presence of Lewy bodies (LBs), which are proteinaceous inclusions containing mainly structurally altered presynaptic protein, alpha-synuclein, which, as recent evidence shows, plays a central role in PD pathology. Alpha-synuclein LB deposition was used by Braak et al. [121] as a principle pathological marker to monitor the progression and severity of PD. PD is believed to originate from olfactory nucleus and autonomic nervous system progressing in an ascending manner to many brain regions such as substantia nigra, striatum, raphe, locus coeruleus, hypothalamic nuclei, hippocampus, amygdala, and cerebral cortex accounting for both motor and nonmotor symptoms [121–123]. Thus, for example, PD patients with cortical LBs also suffer from dementia and visual hallucinations [124].

While several gene mutations have been identified in familial forms of PD, the majority of PD cases are sporadic and of unknown etiology. Nevertheless, significant advances in the last decade on PD genetics, particularly genome-wide association, as well as pathophysiological mechanisms in various PD model systems, have contributed much to our comprehension of PD. Cellular processes such as oxidative and nitrative stress, mitochondrial dysfunction, and deregulated intracellular calcium levels as well as damaged proteostasis related to alpha-synuclein aggregation are the most studied and relate to dopamine neurodegeneration [125].

As in AD patients, the HPA axis is likely dysregulated in PD patients. Specifically, previous studies [54, 126–128] including our own work [129] show that plasma cortisol levels are significantly higher in idiopathic PD patients compared to control subjects; however, these high levels do not correlate to disease duration or to L-3,4-dihydroxyphenylalanine (L-DOPA) treatment. Interestingly, the diurnal pattern of cortisol secretion in PD patients, in particular the normally quiescent nocturnal cortisol secretory pattern, is affected [54].

6. The Neurodegenerative Potential of Altered GC Levels in PD Pathology

Chronically elevated GC levels in PD patients suggest that HPA regulated-stress responses may impact PD pathology. Indeed, the role of stress was proposed as one of the underlying causes of PD as clinical reports show that stress triggers the appearance of PD symptoms or exacerbates the motor symptoms [130–132]. The role of stress in PD is supported by few experimental studies such as food deprivation and tail-shock and maternal separation aggravate motor deficits in the 6-hydroxydopamine (6-OHDA) PD model (6-hydroxydopamine local injections lesions the nigrostriatal pathway) [133]. In combined chronic stress exposure with 6-OHDA lesion, stress was shown to worsen the 6-OHDA-driven motor deficits, aggravate the neurodegeneration of nigrostriatal system, and completely block compensatory recovery of motor tasks [131, 134]. The precise actions of high GC levels in motor control following nigrostriatal lesions are yet not known. Analysis of GR expression in PD brains revealed that GR levels were reduced in the SNpc and augmented in the putamen, compared to age-matched control subjects; similar results were found in MPTP- (1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine-) treated mice [129]. GCs are known to profoundly modulate dopaminergic neurotransmission. The role of GC on the limbic arm of the dopaminergic circuitry related to reward and motivation as well as neuropsychiatric diseases has been extensively investigated (see below). Thus, from its known roles in mesolimbic circuitry, it has been postulated that GR also likely affect motor automated or habitual skills of the sensorimotor circuitry in the striatum by influencing NMDA/AMPA receptor functions in D1 and D2 receptor-medium spiny neurons (Figure 3). Indeed, it has been shown that chronic stress leads to opposing structural changes in the limbic/associative and sensorimotor striatal circuitry with atrophy in the former and hypertrophy of sensorimotor striatum, leading to habit behavior [135]. In addition, the roles of both glucocorticoids and noradrenaline were recently reported in habit memory [136]. It is possible that GR-mediated changes in the putamen during the prodromal stage of PD play a role in preventing the appearance of motor symptoms, culminating in dopamine depletion and death of dopaminergic neurons in the substantia nigra.

Altered stress responses most likely play an important role in nonmotor PD symptoms, particularly anxiety, depression, and mild cognitive impairment, which often precede motor symptoms. Interestingly, there is also evidence in PD for lower novelty-seeking and high harm avoidance personality traits with anxiety-associated symptoms [43, 137]. These observations suggest that, in the initial disease stage, stress-related alterations in GC-GR activity could impact both the motivation/cognitive-associated dopaminergic as well as nondopaminergic (serotonergic and noradrenergic)

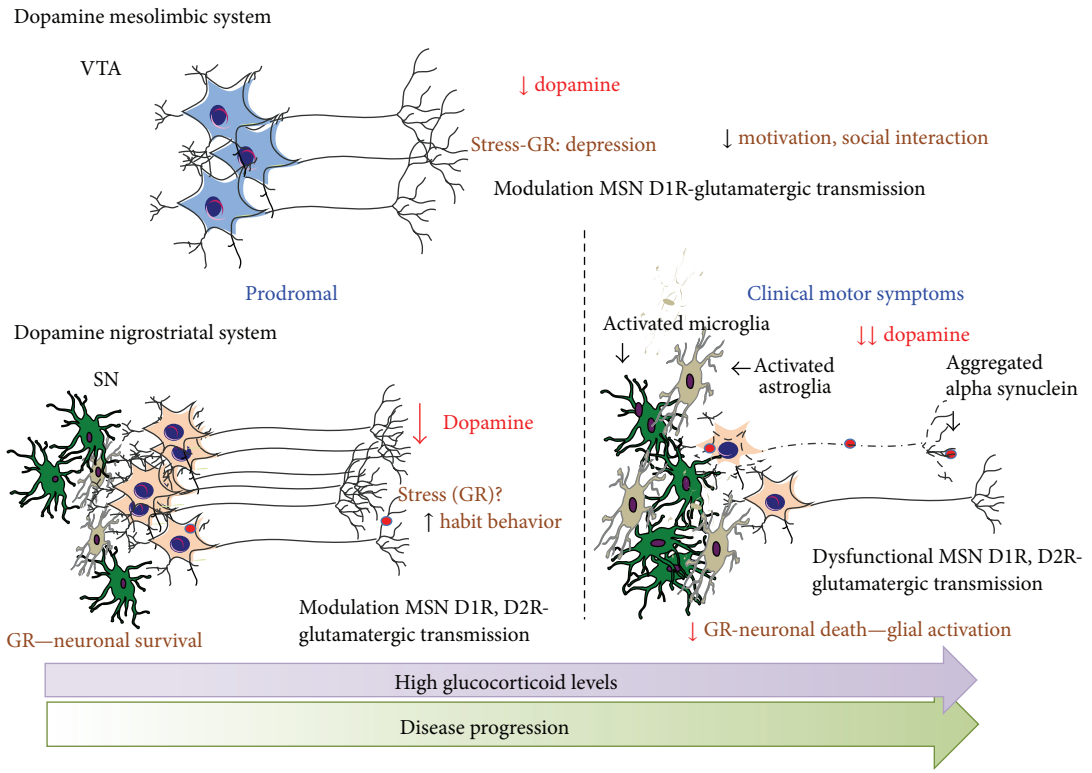

Figure 3: Putative impact of elevated GC levels on GR function in nigrostriatal and mesolimbic dopaminergic systems in PD. Stress-level elevation of GCs may be an early feature of PD, potentially impacting both motor and nonmotor dopaminergic systems. Mesolimbic dopaminergic circuitry is likely affected through structural and functional changes occurring in D1R MSNs. These changes lead to depression and reduced motivation and social interaction which are key prodromal features of PD. Dopaminergic neurons in VTA are relatively spared in PD. In the nigrostriatal system, high levels of GCs initially protect dopaminergic neurons of substantia nigra through dampening the immune responses, namely, mediated by activated microglia and astrocytes. In the putamen, high stress levels of GCs through GR augment habit learning and may act to prevent the appearance of motor symptoms. With disease progression, GR function is affected, leading to chronic glial and immune activation, which exacerbates dopamine neurodegeneration with significant dopamine depletion in the striatum. Changes in GR activity may also affect striatal D1 and D2R MSNs further participating in the appearance of clinical motor symptoms.

neuronal circuitry. This would also implicate dopaminergic neurons in the ventral tegmentum area (VTA), which although relatively spared in PD are well-known to regulate reward and aversion by stress and have been implicated not only in addiction but also depression involving the transcriptional factor CREB and BDNF [138–141]. On the other hand, dorsolateral dopamine neurons in the SN (vulnerable in PD) were shown to respond to tasks involving working memory [142]; thus, their demise could explain, in part, the cognitive deficits observed in PD. Studies on the dopaminergic transmission during stress have revealed the complexity of the system. In fact, firing patterns of dopamine neurons in VTA correlated with depressive-like behaviors in mice, although the effect appears to depend on the stress paradigm used to induce the depressive-like behavior [139, 143]. Electrophysiological evidence implicates changes in both D1R and D2R-medium spiny neurons (MSNs) in the ventral striatum [144], but the depressive-behaviors seems to preferentially affect D1R MSNs [145] (Figure 3). Glutamatergic receptors, NMDA and AMPA receptor functions were shown to be also altered in the D1R MSNs, notably NMDAR-dependent LTD, reduced AMPA/NMDA receptor ratio and increased endocytosis of AMPA receptors [146].

7. Role of Glucocorticoid Receptors in Inflammation-Induced Neurodegenerative Processes and Nonmotor Symptoms in Parkinson's Disease

Accumulating evidence points to inflammation resulting from chronic activation of innate and adaptive immune cells as playing an important role in both neurodegenerative processes and in nonmotor symptoms of PD. Using radiolabeled ligand ^{11}C-PK-11195 for translocator protein, Positron Emission Tomography (PET) studies in PD patients revealed an early activation of microglia in many brain regions including basal ganglia and midbrain [147, 148]. Furthermore, postmortem studies as well as analyses of serum and cerebrospinal fluid from PD showed high levels of proinflammatory mediators such as TNF-α, IL-1β, iNOS, IFN-γ, and COX-2 [149]. In line with observations in PD patients, presence of inflammatory mediators and glial reactivity in striatum and substantia nigra is a key feature in many of the experimental animal models of PD [150]. Evidence from recent genome-wide studies points to involvement of the immune system in the etiology of idiopathic PD. A number of susceptibility loci identified relate to genes

expressed in immune cells such as HLA-DQB1, LRRK2 or BST-1 [151, 152]. In addition, identified PD risk factors [such as age, environmental toxins (e.g., heavy metals or pesticides,) traumatic brain injury, and bacterial or viral infections] activate immune responses in periphery and brain.

7.1. GR Regulation of Inflammation Important for Dopamine Neuronal Survival. Activated microglia functioning as innate-immune competent cells are likely involved in releasing the above inflammatory molecules, thereby inducing dopamine neurodegeneration. Indeed, the important role of these proinflammatory mediators in promoting degeneration of dopaminergic neurons of substantia nigra was demonstrated using mice with specific knockout of these genes [153–156]. Many of the proinflammatory mediators found in PD patients are transcriptional targets of GR. The synthetic analogue of GCs, dexamethasone, was shown to attenuate dopamine neuronal loss by precluding activated microglia from releasing toxic inflammatory molecules [157, 158]. In adrenalectomized mice (lacking endogenous production of GCs), dopamine neuronal loss was augmented following MPTP intoxication indicating that endogenous GCs do play a role in protecting dopamine neurons [159]. Examination of GR in microglia revealed an increase in nuclear localization of GR following MPTP treatment in mice, which coincided with a rise in systemic corticosterone levels, indicating that GR is activated in microglia during the degeneration of dopamine neurons [129]. The unequivocal evidence that GR in microglia normally protects dopamine neurons was provided by experiments with mice in which the GR gene was selectively deleted in microglia/macrophages. MPTP treatment in these mice resulted in increased dopamine neuronal loss as well as increased microglial activation and expression of proinflammatory mediators [129]. Indeed, the absence of GR in microglia resulted in sustained activation of NF-κB as was shown in these microglial GR mutants. The above findings have a significant relevance for PD pathogenesis as nuclear expression of p65 subunit of NF-κB, indicative of transcriptional activity, was found in the substantia nigra microglia of PD postmortem [160].

Inflammatory reaction mediated by immune-competent cells such as microglia is normally a very tightly regulated process of limited duration. It is very likely that the processes involved in the regulation of glial immune responses including the expression and secretion of inflammatory mediators are compromised in PD and also AD resulting in a chronic inflammatory state with sustained activation of glia spanning many years. One likely factor contributing to dysfunction of glial immune responses is aging. Immune-regulatory processes are compromised in aging (immunosenescence) and also during chronic stress [161] where there is an increased susceptibility to infections as well as proinflammatory cytokine production [162]. In aging, microglia show enhanced sensitivity to inflammatory stimuli, a process called "priming" which could be also induced by chronic stress and a dysregulated HPA axis. In this regard, there are several studies showing that chronically elevated GCs levels in response to different stressors cause proinflammatory cytokine production and sensitization or "priming" of microglia. Importantly, subsequent inflammatory or toxic stimuli result in aggravation of neuronal injury [119, 120, 163]. Moreover high and sustained GCs can exacerbate inflammation because of GC resistance whereby GR activity is affected. Thus it is plausible that GR transcriptional activity regulating inflammatory response of microglia is compromised in AD and PD patients who display persistently high GC levels.

7.2. GR, Inflammation and Nonmotor PD Symptoms. Recent experimental evidence shows that glia and peripheral immune cells are activated upon chronic psychogenic stress and that their actions are important in mood and behavior [164–167]. Glial production of potent proinflammatory cytokines such as TNF-α, IL-6, and INF-γ are implicated in depression through stimulation of the kynurenine pathway (shift of serotonin synthesis from tryptophan to kyneurin) in activated astroglia, microglia, and infiltrating peripheral immune cells. Kynurenine, produced from tryptophan by activation of indoleamine 2,3-dioxygenase (IDO), can be further converted to kynurenic acid or quinolinic acid, the latter affecting the function of both monoaminergic and glutamatergic neurons. Quinolinic acid toxicity with increased glutamate release results in lipid peroxidation and nitrative stress [168, 169] Evidence shows that the kynurenic acid/tryptophan ratio is altered in CSF and serum in PD patients [170].

Another means by which glial activation and proinflammatory cytokines promote mood anomalies in PD is through reducing neurogenesis in hippocampal subgranular zone, thus affecting hippocampus-mediated regulation of mood and cognition [171].

8. Conclusion

Clinical and preclinical studies suggest that chronic stress/elevated GC levels may be an etiological factor in the development and progression of both AD and PD pathologies. Growing evidence indicates that the pathological manifestations of chronic stress include neuronal and synaptic atrophy/malfunction as well as immunosuppression, but our understanding of the underpinning mechanisms is still poor and calls for more research not only to identify therapeutic inroads but, also, preventative measures or ways to delay onset of disease.

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

Nucleolar PARP-1 Expression Is Decreased in Alzheimer's Disease: Consequences for Epigenetic Regulation of rDNA and Cognition

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Synaptic dysfunction is thought to play a major role in memory impairment in Alzheimer's disease (AD). PARP-1 has been identified as an epigenetic regulator of plasticity and memory. Thus, we hypothesize that PARP-1 may be altered in postmortem hippocampus of individuals with AD compared to age-matched controls without neurologic disease. We found a reduced level of PARP-1 nucleolar immunohistochemical staining in hippocampal pyramidal cells in AD. Nucleolar PARP-1 staining ranged from dispersed and less intense to entirely absent in AD compared to the distinct nucleolar localization in hippocampal pyramidal neurons in controls. In cases of AD, the percentage of hippocampal pyramidal cells with nucleoli that were positive for both PARP-1 and the nucleolar marker fibrillarin was significantly lower than in controls. PARP-1 nucleolar expression emerges as a sensitive marker of functional changes in AD and suggests a novel role for PARP-1 dysregulation in AD pathology.

1. Introduction

Alzheimer's disease (AD), the most common cause of dementia in the elderly, is an irreversible progressive neurodegenerative disorder clinically characterized by memory loss and cognitive decline [1]. AD is characterized pathologically by synaptic loss and by the accumulation of extracellular beta-amyloid (Aβ), neuritic plaques, and hyperphosphorylated tau in intracellular neurofibrillary tangles (NFT) [2–4]. Of these, synaptic loss most closely correlates with cognitive decline [5], whereas beta-amyloid accumulation, the presence of neuritic plaques, and NFT are the pathological markers required to make a definitive diagnosis of AD [6].

Failure of synaptic plasticity has been proposed as the mechanism underlying memory impairment in AD [7, 8]. The chromatin-remodeling enzyme poly(ADP-ribose) polymerase-1 (PARP-1) plays important roles in synaptic plasticity and memory consolidation in both *Aplysia* and rodents [9–11]. This enzyme engages in poly(ADP)-ribosylation (PAR), using nicotinamide adenine dinucleotide (NAD+) to form branched ADP-ribose polymers on nuclear acceptor proteins, such as DNA polymerases, ligases, and histones. This epigenetic modification results in the loosening of chromatin structure allowing repair proteins and transcription factors to access the DNA [12, 13]. PARP-1 activation leads to the expression of genes required for memory consolidation such as immediate early genes [14] and ribosomal RNA genes (rDNAs) in the nucleolus [15]. In addition, PARP-1 has also been shown to regulate multiple areas of nucleolar function, including the inheritance of rDNA chromatin structure, editing of precursor rRNA, and biogenesis of ribosomes in the nucleolus [16, 17]. Since synaptic plasticity has been shown to be impaired in AD, we hypothesized that this impairment may be due to a loss of PARP-1 and a disruption of PARP's role in the nucleolus in maintaining nucleolar integrity. To begin addressing this hypothesis, we compared PARP-1

Table 1: Autopsy case material.

Case number	Age-sex	Diagnosis	Braak	PARP1 DAB	PARP1 confocal	PARP1/Fib confocal
1	78 F	AD	VI	Y	Y	
2	75 M	AD	VI	Y	Y	
3	77 M	AD	V	Y	Y	
4	65 F	AD	VI	Y		
5	89 F	AD	V	Y		
6	75 M	AD	V	Y		
8	87 F	AD	V	Y		
10	85 M	AD	V	Y		
11	86 M	AD	VI		Y	Y
12	76 M	AD	V		Y	Y
13	88 F	AD	V		Y	Y
14	90 M	AD	V		Y	Y
15	78 M	AD	V		Y	Y
16	65 F	AD	VI		Y	Y
17	78 F	AD	VI		Y	Y
19	81 F	Control	II	Y		
20	76 F	Control	II	Y	Y	
21	76 F	Control	II	Y		
23	69 M	Control	I	Y		
24	71 M	Control	II	Y	Y	
26	71 M	Control	II	Y		
27	71 M	Control	II	Y		
29	97 M	Control	II		Y	Y
30	93 M	Control	I		Y	Y
31	71 M	Control	I		Y	Y
32	86 M	Control	I		Y	Y
33	71 M	Control	0		Y	Y
34	44 M	Control	0		Y	Y

expression in postmortem hippocampal brain tissue derived from patients with neuropathologically confirmed AD to control hippocampal brain tissue from patients without significant neuropathology. We show that PARP-1 positive staining of nucleoli in CA1 and CA4 hippocampal pyramidal cell neurons in AD is significantly reduced compared to controls. We suggest that memory impairment in AD may be due, in part, to this novel finding. This loss of nucleolar PARP-1 in AD appears due in part to a mislocalization of the protein from the nucleolus. Here, we present a model in which the loss of nucleolar PARP-1 precedes changes in nucleolar function and integrity seen in early stages of AD.

2. Materials and Methods

2.1. Case Material. Paraffin-embedded tissue blocks from the hippocampus were collected from deidentified archived material from the Alzheimer's Disease Research Center (ADRC) at Emory University School of Medicine, Sun Health Research Institute Brain and Body Donation Program of Sun City, Arizona [18, 19], Kings County Hospital Center, and State University of New York Downstate Medical Center.

Postmortem brain tissue was acquired from two groups of individuals (Table 1): (1) the AD group consisted of tissue from male and female patients with neuropathologically confirmed AD that meet the criteria for the diagnosis of "definite" Alzheimer's disease according to the Consortium to Establish a Registry for Alzheimer's Disease [20] and a high likelihood that dementia was due to AD by NIA Reagan criteria [21] and (2) the control group consisted of individuals, both male and female, of similar age to the AD group with no known history of dementia or neurologic disorder and without significant neuropathology. The AD cases had Braak scores of V-VI and the controls had Braak scores of 0, I, or II (Table 1).

2.2. Tissue Preparation. The samples were deparaffinized, hydrated, and submerged in 10 mM citrate buffer (pH 6.0) and microwave irradiated (15 min) for antigen retrieval. Then the samples were used for light or confocal microscopy as indicated by "Y" in Table 1.

2.3. Immunohistochemistry for PAR and PARP-1 by Light Microscopy. After antigen retrieval, slides were rinsed for 5 min with 0.1% triton X-100 in phosphate-buffered saline (PBS-Triton), treated with 3% H_2O_2 for 20 min, rinsed with PBS-Triton for 5 min, blocked in 2% normal horse serum in

PBS-Triton for 30 min, and incubated with primary antibody (anti-PAR polyclonal, 1 : 200; Cat # 4336-BCP-100, Trevigen; and PARP-1 monoclonal antibody, 1 : 200; Cat # 1522G, AbD Serotec) overnight in a humidity chamber. The sections were then rinsed in PBS-Triton and incubated for 1 h in biotinylated secondary antibody horse anti-mouse (1 : 200) diluted in blocker (VECTASTAIN ABC systems, Vector Laboratories), rinsed again, and developed using the ABC system (Vector Laboratories, Burlingame, CA), using standard histologic procedures. For controls, sections were treated as mentioned above with omission of primary antisera (1 : 200).

2.4. Immunohistochemistry for PARP-1 by Confocal Microscopy

2.4.1. Single Immunohistochemistry for PARP-1. For single immunofluorescent visualization, the samples were blocked for 1 h with 2% normal goat serum (NGS) in PBS-Triton and then incubated overnight with PARP-1 monoclonal antibody (1 : 200) diluted in blocker. After rinsing 3 times for 10 min each in PBS-Triton, the samples were incubated 4 h with goat anti-mouse-biotin F(ab) fragment (1 : 200) in blocker buffer, rinsed 3 times for 10 min each in PBS-Triton, and incubated for two hours with Strep Alexa 647 (1 : 200) and DAPI (1 : 500) in blocker buffer. The sections were then rinsed in PBS-Triton and in distilled water, immersed for 5 minutes in 70% ethanol containing 0.3% Sudan Black, rinsed in distilled water, and mounted on glass slides with Prolong Gold (Molecular Probes, Eugene, OR). For controls, sections were treated as mentioned above with omission of primary antisera.

2.4.2. Double Immunohistochemistry for PARP-1 and Fibrillarin. The double immunohistochemistry was similar to the single immunohistochemistry except for (a) a second primary antibody (rabbit anti-fibrillarin antibody, 1 : 100; Cat # ab5821, Abcam) which was used during the incubation overnight and (b) a second secondary antibody (fluorescein goat anti-rabbit; 1 : 200 Invitrogen, Thermo Fisher Scientific, Glen Island, NY) which was used during the incubation with secondary antibodies.

2.5. Quantification. Qualitative assessment of the immunohistochemistry using light and confocal microscopy was performed and staining was determined to be either strong (for light microscopy) or high intensity (for confocal microscopy), weak or absent. Images were taken of each slide at a magnification of 400x and all the cells in three randomly chosen fields within the designated region were counted for presence or absence of nucleolar staining. For confocal microscopy, all images were taken at the same parameters preset on sections stained with no primary antibodies. Statistical studies using paired t-tests were performed.

3. Results

3.1. Loss of PARP-1 from the Nucleolus of Neurons in AD. Using light microscopy we compared PAR and PARP-1 levels in AD and controls. We found no significant differences in the nuclear staining of PAR in neurons in hippocampal regions CA1–4, entorhinal and temporal cortices, or subiculum (data not shown). In contrast, PARP-1 immunohistochemistry showed positive staining in the nucleus with strong staining of the nucleolus in controls and weak nuclear staining with little to no staining in the nucleoli within neurons in AD (Figure 1 compare (a) and (b)). Interestingly, the only exception was dentate gyrus where no differences between AD and controls were observed. In controls, the percentage of pyramidal neurons with PARP-1 positive nucleoli was 63.9% in CA1 and 51.1% in CA4. In contrast, the percentage of PARP-1 positive nucleoli in pyramidal neurons in AD was 28.7% in CA1 and 30.4% in CA4 (Figures 1(c) and 1(d)).

We used confocal microscopy to confirm our results showing loss of PARP-1 nucleolar staining in AD. Consistent with the light microscopy data, we found that 66.1% and 62.2% of CA1 and CA4 hippocampal pyramidal cell nucleoli stained positive for PARP-1 in controls, whereas, in AD, nucleolar PARP-1 staining was present in only 29.3% and 32.0% of CA1 and CA4 pyramidal cells, respectively (Figure 2).

3.2. Nucleolar Marker Fibrillarin Is Not Significantly Downregulated in Nucleoli of Hippocampal Pyramidal Cells. To test whether other nucleolar proteins are affected in AD, we performed double immunohistochemistry with PARP-1 and fibrillarin, a nucleolar protein involved in pre-rRNA processing. If the loss of PARP-1 nucleolar staining was due to general damage and structural loss of nucleoli from cells, then we would also expect to see a comparable loss of fibrillarin and other nucleolar proteins. However, a loss of PARP-1 with preserved fibrillarin staining in AD would indicate that loss of PARP-1 is selective. Control cases exhibited high intensity nucleolar staining and a higher percentage of PARP-1 and fibrillarin colocalization (Figures 3(a)–3(d)) compared to AD (Figures 3(e)–3(h)). There is a significant loss ($p = 0.017$) of PARP-1 nucleolar staining in CA1 pyramidal cells in AD compared to controls. In contrast, fibrillarin staining in CA1 is not significantly different between AD and controls (Tables 2 and 3). The loss of PARP-1 from the nucleolus, therefore, appears to be a selective departure and may reflect a departure from the nucleolus due to mislocalization of the protein (Tables 2 and 3).

4. Discussion

In this study, we demonstrated that there is a loss of PARP-1 from hippocampal pyramidal cell nucleoli in AD, suggesting that PARP-1 nucleolar function may be compromised in AD. Recently, our group demonstrated that the maintenance of late-phase long-term potentiation (L-LTP), a model for long-term memory, requires nucleolar integrity and the expression of new rRNAs—the latter being regulated by PARP-1 [22]. Therefore, we hypothesize that PARP-1 and nucleolar integrity are required for long-term memory. Recently, in a study complementary to ours, it was demonstrated that chronic deficits in nucleolar function alter synaptic plasticity

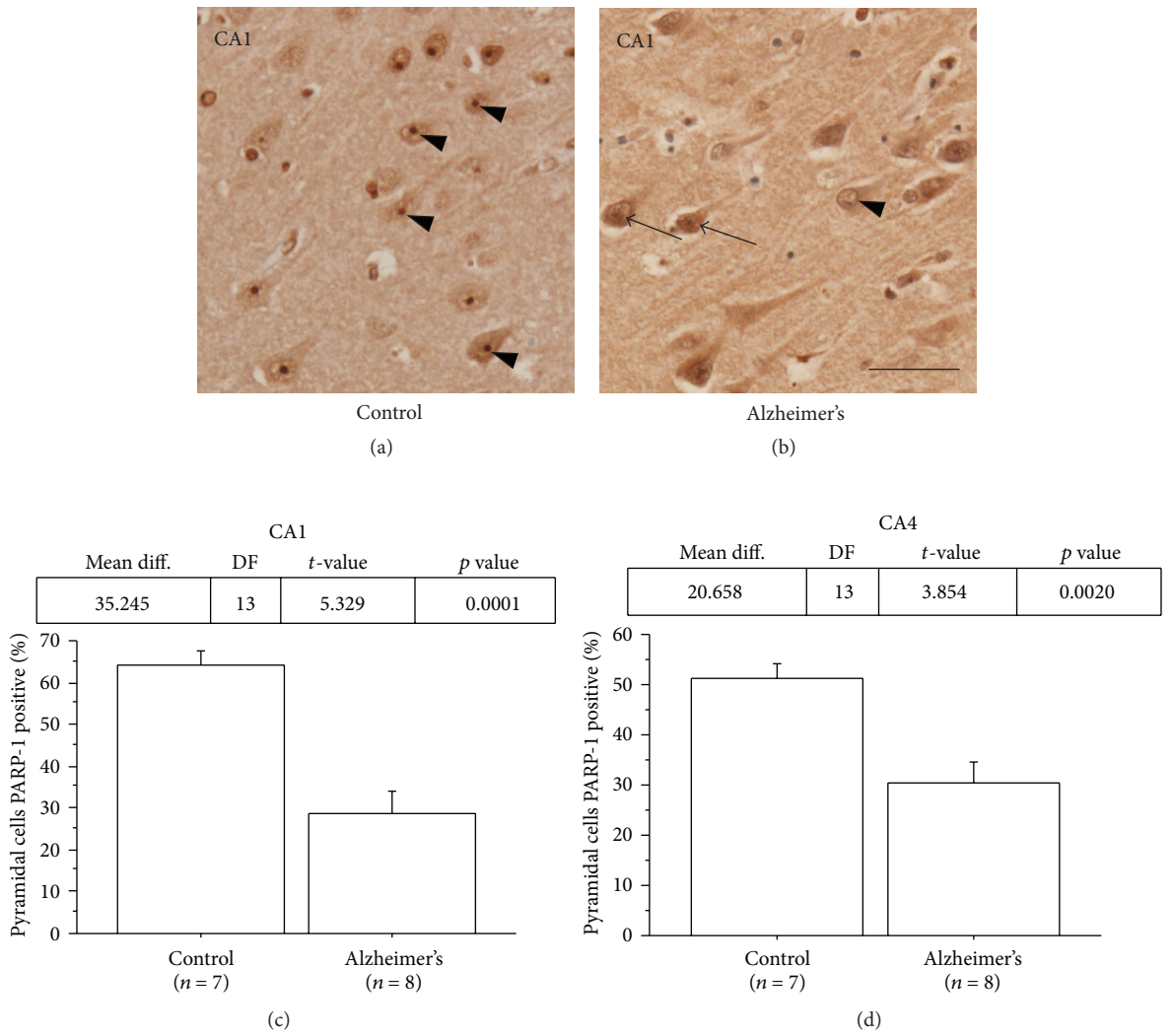

Figure 1: Nucleolar PARP-1 immunoreactivity in AD ranged from absent to dispersed and less intense compared to that of controls. ((a) and (b)) Representative immunostaining with diaminobenzidine (DAB) of human hippocampal pyramidal neurons in CA1 region. (a) Prominent nucleolar staining of PARP-1 (arrows) was seen in most of pyramidal neurons of a control case. (b) The nucleolar staining of PARP-1 ranged from absent (arrowheads) to a more dispersed pattern with less intensity of label (arrows) in pyramidal neurons of an AD case. ((c) and (d)) Percentages of CA1 and CA4 hippocampal pyramidal neurons with PARP-1 positive nucleoli were significantly lower in AD cases compared to controls. (Control, $n = 8$; AD, $n = 8$; $^{*}p < 0.05$.) Scale bar = 50 μm.

Table 2: CA1.

	% of pyramidal cells nucleoli	Mean	t-test
Control Alzheimer's	PARP1(+)/Fib(+)	58.80 29.74	0.039 (∗)
Control Alzheimer's	PARP1(+)/Fib(−)	7.17 2.53	0.329
Control Alzheimer's	PARP1(−)/Fib(+)	6.05 21.52	0.024 (∗)
Control Alzheimer's	Total PARP1(+)	68.60 32.27	0.017 (∗)
Control Alzheimer's	Total Fib(+)	64.85 51.69	0.242

$^{*}p < 0.05$.

Table 3: CA4.

	% of pyramidal cells nucleoli	Mean	t-test
Control Alzheimer's	PARP1(+)/Fib(+)	55.50 26.15	0.033 (∗)
Control Alzheimer's	PARP1(+)/Fib(−)	5.68 4.58	0.830
Control Alzheimer's	PARP1(−)/Fib(+)	1.74 24.78	0.031 (∗)
Control Alzheimer's	Total PARP1(+)	61.18 30.73	0.051
Control Alzheimer's	Total Fib(+)	57.24 50.56	0.450

$^{*}p < 0.05$.

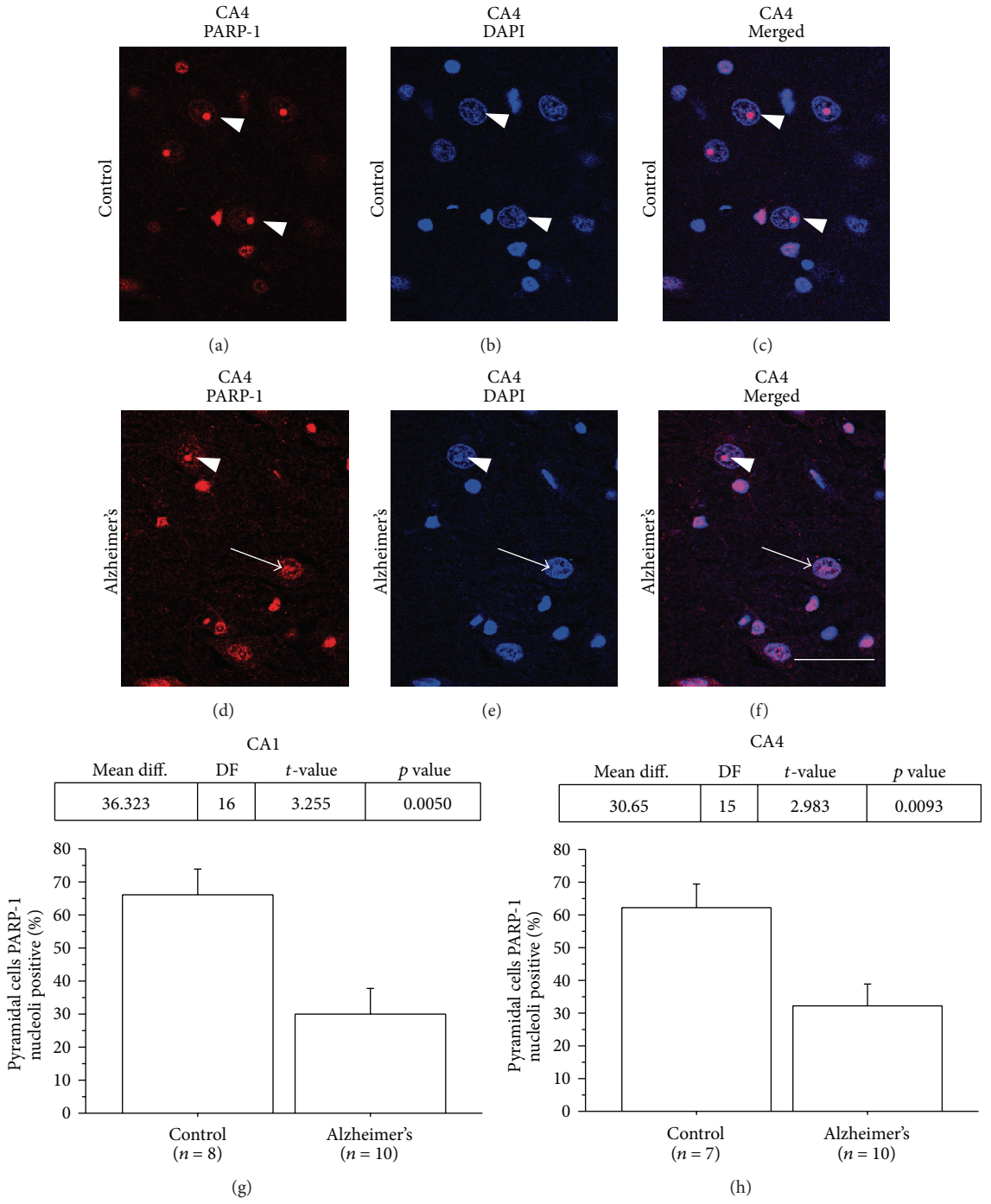

Figure 2: PARP-1 nucleolar immunoreactivity is altered in hippocampal pyramidal cells in AD brains. Representative confocal microscopy of PARP-1 immunostaining (red) with DAPI nuclear counterstaining (blue) of CA4 hippocampal pyramidal neurons. In controls brains (a–c) a high percentage of pyramidal cell nucleoli have intense and well- delineated PARP-1 staining (arrowheads). In contrast, in AD brains (d–f), the percentage of intensely stained and well-delineated nucleoli is less than in the controls and there is a more dispersed pattern with weak label intensity ((d) and (f), arrow). ((g) and (h)) The percentage of CA1 (g) and CA4 (h) hippocampal pyramidal neurons with PARP-1 positive nucleoli staining was less in AD cases compared to controls. (Control, $n = 8$ and $n = 7$ for CA1 and CA4, resp.; AD, $n = 10$ for both CA1 and CA4; $^{*}p < 0.05$.) Scale bar = 25 μM.

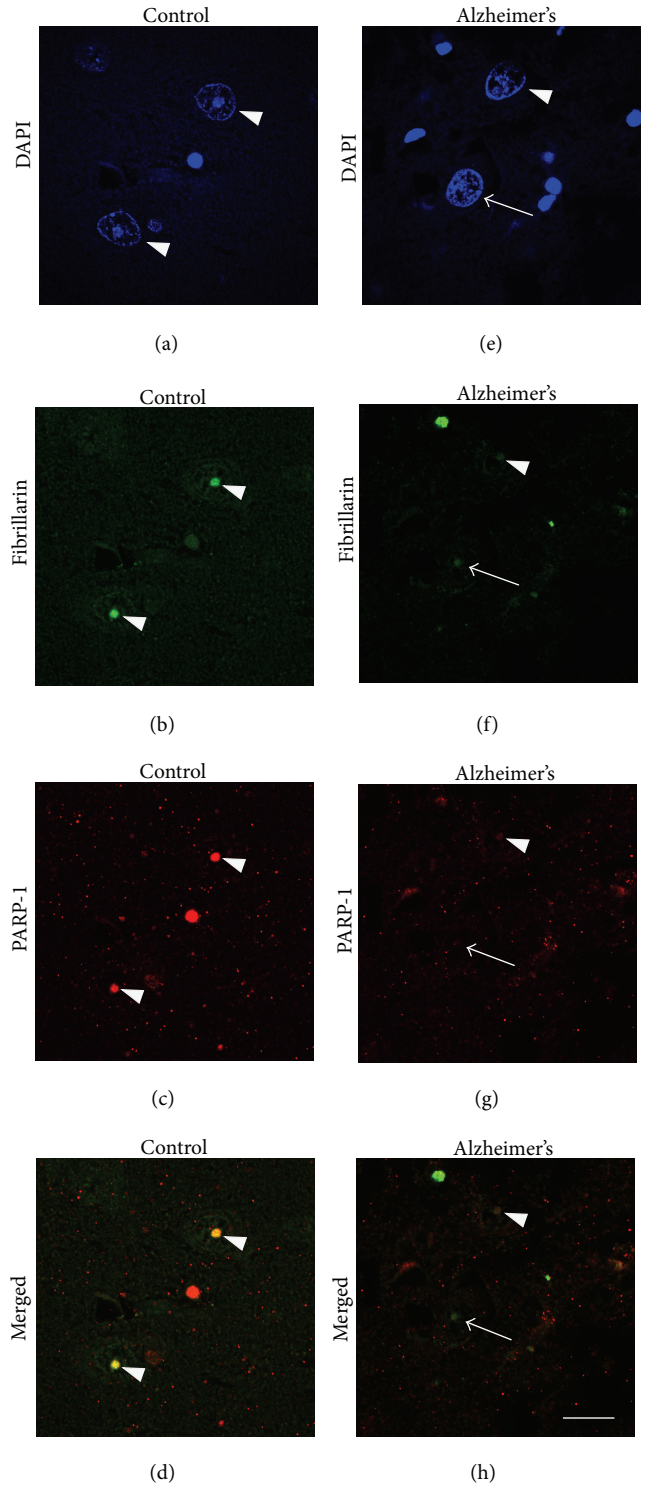

Figure 3: Nucleolar proteins in hippocampal pyramidal cells are altered in AD. ((a)–(h)) Representative figures show colocalization ((d) and (h), yellow) of fibrillarin ((b) and (f), green) and PARP-1 ((c) and (g), red) in the nucleoli of pyramidal neurons. Control cases exhibit high intensity staining (a–d) compared to AD (e–h) (arrowheads). In AD compared to controls, there is a lower percentage of nucleoli that are both PARP-1(+) and fibrillarin(+) ((f)-(g), arrowhead) in CA1 (see Table 2) and CA4 (see Table 3) pyramidal cells and a higher percentage of nucleoli PARP-1(−)/fibrillarin(+) ((f) and (g), arrow) in CA1 (see Table 2) and CA4 (see Table 3), suggesting that different nucleolar proteins are affected in different ways in AD. Scale bar = 20 μm.

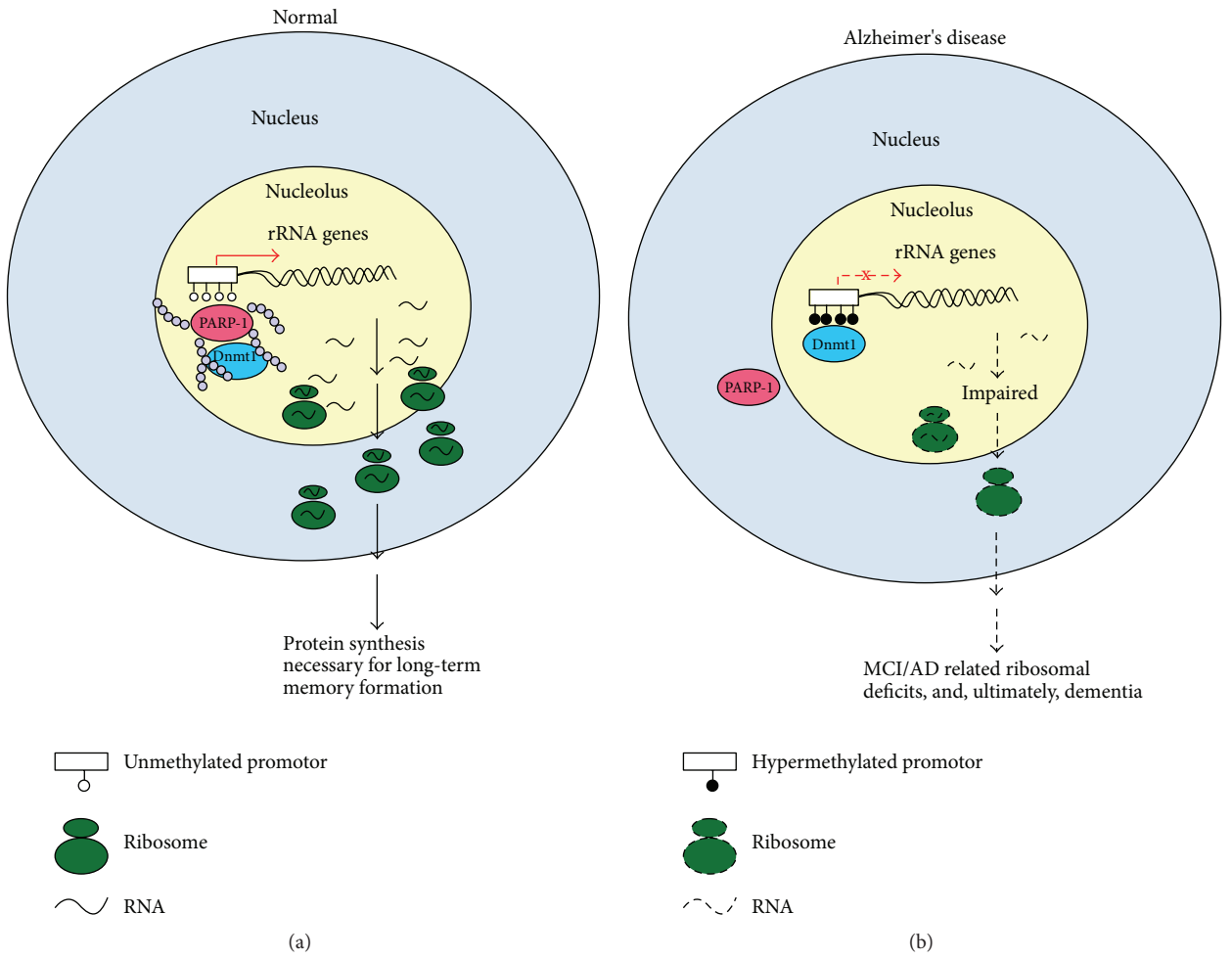

Figure 4: Model. Ribosome biogenesis in Alzheimer's disease. (a) Normal neuron. PARP-1 activity (PAR) prevents rDNA methylation. PAR = poly(ADPribosyl)ation. (b) AD neuron. Absence of PARP-1 in nucleoli allows DNA methyltransferase (Dnmt1) to methylate rDNA promoters silencing rRNA transcription resulting in nucleolar disruption.

and learning and memory [23]. In addition, PARP-1 has also been shown to regulate multiple areas of nucleolar function, including the inheritance of rDNA chromatin structure, editing of precursor rRNA, and biogenesis of ribosomes in the nucleolus [16, 17].

There is a previous immunohistochemical study of PARP-1 and PAR staining in AD, which found an increase in nuclear PARP-1 and PAR in frontal and temporal lobe tissues [24]. Hippocampus was not examined and the nucleolar compartment was not assessed. It is possible that the PARP-1 nucleolar loss has a differential degree of sensitivity in different areas of the brain in AD and may be a finding specific to the hippocampus. We found that the CA1 and CA4 subregions of the hippocampus exhibit vulnerability to the nucleolar PARP-1 loss in AD, which mirrors the vulnerability to AD neuropathological change and to ischemic damage. Interestingly, chronic deficits in nucleolar function have been shown to lead to neurodegeneration with differential cellular vulnerability in the hippocampus [25].

PARP-1 has shown to be activated secondary to oxidative stress and DNA damage [24, 26–28] and, in mild to moderate stress, is thought to be part of the repair mechanism but may lead to cell death via consumption of NAD+ when overactivated. We suggest that PARP-1 may act via two distinctly different mechanisms in AD. We hypothesize that the loss of PARP-1 from nucleoli of hippocampal pyramidal cells may be an early and persistent finding in AD. This loss of nucleolar PARP-1 from hippocampal pyramidal neurons may lead to deficits in synaptic plasticity and, thus, to cognitive impairment. In contrast, late in AD, it is possible that PARP-1 is overactivated and contributes to cell death in frontal and temporal cortices as shown by Love et al. [24]. We suggest that both pathways may contribute to cognitive impairment in AD. Furthermore, we speculate that the loss of PARP-1 from hippocampal pyramidal cells in AD may help to explain some of the selective vulnerability of the CA1 and CA4 regions of the hippocampus. That is, there is a loss of the physiologic PARP-1 activation required for long-term synaptic plasticity

and memory consolidation [9–11, 14, 15] and also a region specific loss of the reparative activation of PARP-1 associated with mild to moderate stress.

The nucleolus has emerged as an important structure to study in relation to AD neuropathology. In a study of postmortem brains from the Nun Study of Aging and Alzheimer's Disease, a longitudinal study examining the onset of AD, it was found that asymptomatic AD cases, in which autopsied brain samples revealed common AD lesions in spite of the subjects having had normal cognition, exhibited significant hypertrophy of nucleoli (+80.2%) in CA1 neurons compared to MCI or controls [29]. There was also hypertrophy of cell bodies and nuclei but it was the nucleoli which had the largest change. This suggests a compensatory mechanism preventing the impairment of cognition despite the presence of typical AD pathology [29]. Based on these findings, we hypothesize that it was the maintenance of nucleolar function (and, therefore, rRNA synthesis) which prevented the cognitive deficits in these individuals with AD neuropathology.

Aberrations in the epigenetic code of acetylation, methylation, and PARylation are a common denominator of neurodegenerative diseases [30–32]. Nucleolar impairment may also be a common denominator in several neurodegenerative disorders such as Huntington's, Parkinson's, and Alzheimer's' disease [33]. Epigenetic silencing of rDNA by DNA methylation has been found to be a common feature of mild cognitive impairment (MCI) and AD and may represent a new marker of the disease [34]. The rDNA silencing occurs in the nucleolus, perturbing nucleolar functions such as global chromatin regulation [35] and biogenesis of ribosomes [17]. This gene silencing is consistent with previous reports of a decrease in ribosomes in the inferior parietal lobe of MCI and AD patients [36]. Impairing the expression of rRNAs (essential components of ribosomes) or any of the steps of ribosome biogenesis can produce nucleolar stress, leading to changes in gene expression and a reduction in ribosomes and protein synthesis resulting in cellular dysfunction.

To date, the factors leading to increased rDNA methylation in MCI and AD are unknown. Since PARP-1 has been shown to regulate genomic methylation patterns by inhibiting the activity of DNA methyl-transferase [37], we propose that PARP-1 displacement from the nucleolus in AD leads to hypermethylation of rDNA. There is then downregulation of rRNA expression and of ribosomal biogenesis (see Figures 4(a) and 4(b)). Without new ribosomes, the synthesis of new proteins is impaired and the formation of new memories disrupted.

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 Response to Oxidative DNA Damage in Neurons: Mechanisms and Disease

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There is a growing body of evidence indicating that the mechanisms that control genome stability are of key importance in the development and function of the nervous system. The major threat for neurons is oxidative DNA damage, which is repaired by the base excision repair (BER) pathway. Functional mutations of enzymes that are involved in the processing of single-strand breaks (SSB) that are generated during BER have been causally associated with syndromes that present important neurological alterations and cognitive decline. In this review, the plasticity of BER during neurogenesis and the importance of an efficient BER for correct brain function will be specifically addressed paying particular attention to the brain region and neuron-selectivity in SSB repair-associated neurological syndromes and age-related neurodegenerative diseases.

1. Introduction

Each cell in the human body receives tens of thousands of DNA lesions per day by a variety of sources. Therefore, cells have evolved a multifaceted response to counteract the potentially deleterious effects of DNA damage. The cellular response to DNA damage involves execution of DNA repair and activation of a repertoire of DNA damage signalling molecules (DNA damage response, DDR). The main DNA repair pathways, nucleotide excision repair (NER), base excision repair (BER), mismatch repair (MMR), homologous recombination (HR), and nonhomologous end-joining (NHEJ), are devoted to the repair of specific DNA alterations and complementary in some respects. NER is a multistep process that deals with damage causing significant distortion of DNA structure, such as UV-induced damage and bulky adducts (reviewed in [1]). BER corrects DNA from oxidation, deamination, and alkylation including single-strand breaks (SSB) which are all lesions that cause little distortion to the DNA helix structure (reviewed in [2]). MMR is an evolutionarily highly conserved repair pathway that corrects mismatches generated during DNA replication and escape proofreading (reviewed in [3]). Recombinational repair deals with the most lethal form of DNA damage, double strand breaks (DSB), by using an homologous DNA sequence as in the case of HR or requiring little or no sequence homology for efficient repair as in the case of NHEJ (reviewed in [4]).

The appropriate repair of DNA damage and resolution of replication problems is orchestrated by the DDR, through the action of sensors, transducers, and effectors that coordinate DNA repair with ongoing cell physiology. Signal transducers include ATM and ATM-Rad3-related (ATR) that are DNA damage-activated kinases that respond to different types of DNA lesions. Downstream of these proteins is two families of checkpoint kinases (Chk), the Chk1 and Chk2 kinases, that are targets of regulation by ATR and ATM kinases, respectively (reviewed in [5]).

DNA breaks arising from oxidative damage are a major threat for the genome stability of mature neurons [6]. This type of damage is mostly repaired by BER/SSBR. In this review, the plasticity of DNA repair during neurogenesis, the key role of BER/SSBR, and its brain region selectivity

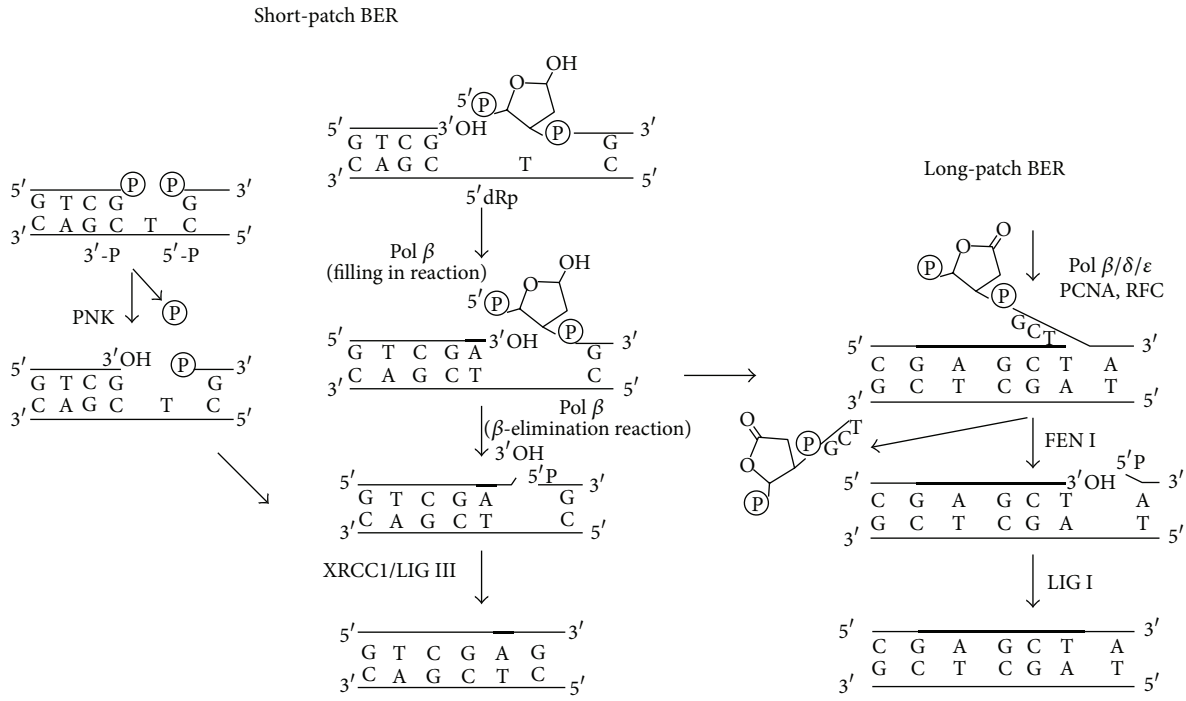

Figure 1: Simplified scheme for the short- and long-patch base excision repair pathways.

in neurological diseases will be specifically addressed by providing an update of recent findings. Moreover, original data on the characterization of the response to oxidative stress in neurons from different brain areas will be presented.

2. Plasticity of DNA Damage Type and Repair during Neurogenesis

The regulatory networks of differentiation programs include genes that are involved in the response to DNA damage and cell death execution. As a consequence of this gene reprogramming, the mechanisms that deal with the maintenance of genome stability can change substantially in the transition from neurogenesis to nervous system maturation. By using *in vitro* cell differentiation systems, several studies have shown that DNA repair is downregulated during differentiation (reviewed in [7]). Indeed, the first evidence of differentiation-associated downregulation of DNA repair was provided by Hanawalt's laboratory in human hNT neurons [8]. In particular, when the repair of UV-induced DNA lesions was compared between terminally differentiated human hNT neurons and their precursor NT2 cells, it was clear that postmitotic neurons display attenuated global DNA repair but efficiently repair expressed genes (a pathway that was later called transcription-domain associated repair) [9]. Also, the mechanisms that control chromosome integrity, namely, telomerase and telomere-associated proteins, function as distinct telomere protection mechanisms during the processes of neurogenesis and neuronal maturation because of differentiation-associated transcriptional control. This impacts the response to DNA damaging agents as shown by the extreme sensitivity to telomere damage of newly generated neurons that are deficient in both the telomerase and the TRF2 telomere-binding protein [10]. The DNA damage response (DDR), the sophisticated cell network that monitors genome integrity, is also affected by differentiation-associated gene reprogramming. Carlessi et al. [11] showed that the differentiation of immortalized human neural stem cells *in vitro* is accompanied by an upregulation of ATM and the DNA-dependent kinase DNA-PK, sharp downregulation of ATR and Chk1, transient induction of p53, and the onset of apoptosis in a fraction of cells. The response to ionizing radiation (IR), including apoptosis, was dependent on ATM as shown by its attenuation following targeted silencing of ATM. Similarly, it was shown that DDR signalling and radiosensitivity were altered in terminally differentiated astrocytes as compared to their progenitors, neural stem cells (NSC) [12]. While NSC activated canonical DDR upon exposure to IR, astrocytes lacked functional DDR signalling with transcriptional repression of ATM leading to radioresistance. Astrocytes retained the expression of NHEJ genes and DNA-PK was shown to be the key player in the response to DNA damage. The efficiency of BER during neural differentiation was addressed by Sykora et al. [13]. They showed that terminally differentiated human SH-SY5Y neuroblastoma cells are more sensitive to oxidative damage than their undifferentiated counterparts. This is at least partially due to attenuated BER in postmitotic neurons that correlates with diminished protein levels of long-patch BER components (that are shared by DNA replication), such as flap endonuclease-1 (FEN1), proliferating cell nuclear antigen (PCNA), and DNA ligase 1 (Lig1) (Figure 1).

The evidence for the plasticity of DNA repair/DDR during cell differentiation, as inferred from these *in vitro*

studies, is strengthened by *in vivo* studies in animal models where the effects of specific defects in DNA repair/DDR on neural development have been specifically investigated. During neural development, neural progenitors undergo symmetric divisions that expand the size of the progenitor pool before switching to an "asymmetric" mode of division wherein each round produces one progenitor cell and one "postmitotic" neuron. Newborn neurons then migrate from the proliferative zones to various CNS regions where they undergo further differentiation and become integrated into functional networks. In the early developmental stages, DNA repair plays a key role in the formation of a functional nervous system and the integrity of specific repair pathways is required along the developmental program [6]. This is well illustrated by mouse models with germline deletions of either Xrcc2 or DNA ligase 4 (Lig4), which are essential for the repair of DSB through HR and NHEJ, respectively. Xrcc2 $^{-/-}$ embryos display massive apoptosis in the brain by E10.5 when neural progenitor proliferation occurs, whereas no apoptotic cells are detectable in the brains of Lig4 $^{-/-}$ embryos until E12.5 when neural progenitors are differentiating into neurons [14]. Indeed in these different stages, cells are susceptible to different types of DNA damage. During proliferation, the most common type of damage is replication stress that is acted upon by HR and NHEJ. HR requires the presence of a sister chromatid and therefore this pathway is not available in neurons that have exited the cell cycle. In this cell type, NHEJ becomes the pathway responsible for DSB repair.

Challenging examples of the effect of the type of damage and its subsequent processing on the pathological outcome are two human syndromes, ataxia telangiectasia (A-T), a childhood neurodegenerative syndrome [15], and ATR-Seckel syndrome that presents severe neurodevelopmental defects [16]. These syndromes involve full or partial inactivation of the kinases ATM and ATR, respectively. These kinases respond to different types of damage that occur frequently during neural development: ATM to DSB while ATR is activated by RPA-coated single-stranded DNA, a lesion that may occur during replication fork collapse. Their lack of function leads to very different clinical outcomes: in the case of ATM to neurodegeneration and in the case of hypomorphic mutations of ATR to neurodevelopmental defects.

Oxidative DNA lesions, including SSB, are expected to be a frequent type of damage encountered by noncycling cells. The pathway of election for removal of oxidative DNA damage is BER. Targeted deletion of DNA polymerase β (Polβ), the main BER polymerase, causes neonatal lethality in mice [17]. Histological examination of the embryos showed extensive cell death in newly generated postmitotic neuronal cells in the developing central and peripheral nervous systems.

In conclusion, the plasticity of DNA repair/DDR during neurogenesis sets the tolerance to different types of DNA damage at different levels depending on the cell stage.

3. ROS: Sources and Role in Neurons

ROS and reactive nitrogen species (RNS) are generated by cellular metabolism and by exogenous agents. Metabolism-generated ROS can cause approximately 10.000 oxidative lesions per genome per day [18]. Neurons carry a high load of mitochondria. Almost 50 years ago, the first evidence was provided showing that the respiratory chain of mitochondria produces ROS [19]. The electrochemical gradient produced by the respiratory chain is used to synthesize ATP; however, some of these electrons inevitably leak out of the pathway leading to the production of $O_2^{-\bullet}$. These radical species can be very dangerous when produced in excess, but they are also important in redox signalling from the organelle to the rest of the cells. Controlled ROS generation is indeed necessary for optimal functioning of the CNS through fine-tuning of redox-sensitive signalling pathways. Brain mitochondria can also absorb large amounts of hydrogen peroxide when they utilize glycolysis as energy source [20]. Under the condition of neuronal cell damage, mitochondria are considered the main source of ROS during glutamate excitotoxicity [21]. ROS accumulation can damage neurons and mitochondria mediate both neuronal apoptosis and necrosis. Mitochondria act as platforms for the activation of caspases during apoptosis and participate in the dysregulation of Ca^{2+} homeostasis during necrosis. Another important source of ROS in damaged neurons are the nonmitochondrial nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOX family) [22]. NOX enzymes are not only restricted to microglia but also expressed in neurons, astrocytes, and neurovascular system. There is now clear evidence for their role in various neurodegenerative diseases [23].

ROS-induced DNA damage has little, if any, specificity along the DNA strand. ROS-induced DNA damage includes base modification, deoxyribose modification, DNA cross-links, abasic sites, SSB, and DSB. SSB can occur directly by the disintegration of the oxidized sugar [24] or indirectly during BER of base damage as repair intermediates. SSB can also arise as a result of incorrect activity of DNA topoisomerase 1 (Top1) in which the enzyme remains covalently attached to the 3′ end of the break (Top1 cleavage complex) (reviewed in [25]). The 3′- and/or 5′-termini of most SSB must be restored to conventional 3′-hydroxyl and 5′-phosphate moieties to allow gap-filling and DNA ligation (Figure 1). Defective SSB repair (SSBR) can result in neurological diseases (see below). Although less frequently than SSB, DSB can also arise following replication past unrepaired SSB or when SSB encounter the transcription machinery or arise in close proximity. If not repaired they may have a dramatic impact on development.

4. BER/SSBR in Neuronal Cells

BER/SSBR is the main DNA repair mechanism in the removal of oxidized DNA bases and oxidized DNA break termini that are formed at high frequency in neuronal cells. BER proceeds through five steps: (i) base removal by a specific DNA glycosylase (DG); (ii) incision at the resulting abasic site by an AP-endonuclease (APE1); (iii) processing of the produced blocked termini of the gap; (iv) gap-filling by a DNA polymerase; and (v) resealing of the damaged DNA strand by a DNA ligase [26–28] (Figure 1). A brief biochemical characterization of the enzymes involved in these steps is provided below with special emphasis on their role in neurogenesis as inferred from *in vitro* and *in vivo* studies.

4.1. DNA Glycosylases: DNA Lesion Recognition and Removal. The first and most specific step of BER is the recognition of damaged DNA bases by distinct DGs. Eleven DGs have been identified in mammals and all of them recognize the specific DNA base by the same mode of action, that is, flipping base out of the DNA helix into an active site pocket [29]. Monofunctional DGs, such as uracil-DNA glycosylases (UDGs) and thymine DNA glycosylase (TDG), present only glycosylase activity and catalyze the base lesion excision mainly by hydrolyzing the N-glycosidic bond to generate an AP site [29]. The bifunctional DGs have an additional lyase activity and process the AP site via β or β/δ elimination reaction. They include the DGs specific for oxidized bases, such as 8-oxoGuanine (8-oxoG) DNA glycosylase (OGG1) and endonuclease VIII-like proteins [27].

UDGs are monomeric protein that recognize and excise uracil base from DNA [27]. Mammalian cells have five distinct UDGs: nuclear UNG2 is devoted to the repair of incorporated uracil (U:A base pair) whereas UNG1, SMUG1, TDG, and probably MBD4 all contribute to the repair of uracil in the U:G base pair (formed by the deamination of cytosine) [30]. Humans and mice have two different UNG isoforms, UNG1 and UNG2 localized in the mitochondria and in the nucleus, respectively [31]. The role of UNG1 in neurodegeneration is shown by the phenotype of conditional transgenic mice expressing a mutated version of UNG1 that present decreased mitochondrial respiration, apoptosis, neurodegeneration, and altered behaviour [32]. Feeding animals with a folate-deficient diet induced the degeneration of CA3 pyramidal neurons in Ung KO mice but not in wild-type animals [33]. Furthermore, folate depletion increased nuclear mutation rates in Ung KO mouse embryo fibroblasts (MEFs) [33] due to high levels of uracil in DNA [34] as a consequence of pool imbalance and/or cytosine deamination due to decreased levels of S-adenosylmethionine [35]. Depletion of UNG1 in cultured rat hippocampal neurons was also sufficient to induce DNA damage, upregulation of p53, and apoptosis [36].

Four different splice forms of OGG1 are present in mammalian cells, but only two of them are involved in the repair of 8-oxoG: OGG1-1a in nuclear DNA and OGG1-2a in mitochondrial DNA [37]. OGG1 initiates a canonical BER pathway that proceeds by the action of APE1, Polβ, and XRCC1/Lig3 to repair the damaged base [38, 39]. OGG1 is widely expressed and active in human and rodent brains [40]. Ogg1 KO mice have been used to examine the role of oxidative DNA damage in neuropathology. Liu et al. [41] reported that OGG1 protects neurons against cell death and its absence determines poorer functional outcome in mice under ischemic conditions. Aged Ogg1 KO mice showed a decreased spontaneous locomotor behaviour and a decrease in striatal dopamine levels [42]. During replication, the bypass of 8-oxoG by Polδ determines the formation of the mismatch 8-oxoG:A that is recognized by MUTYH that mediates the removal of the adenine mispaired with 8-oxoG. Subsequently, Polλ reconstitutes the correct 8-oxoG:C pair, thus allowing the intervention of OGG1 [43]. Both nuclear and mitochondrial isoforms of MUTYH are present in mammalian cells [44]. The analysis of single (Ogg1 KO or Mutyh KO) or double (Ogg1/Mutyh DKO) mutant mice revealed that Ogg1 KO mice exhibited severe striatal neurodegeneration, whereas mice lacking MUTYH or both OGG1 and MUTHY were resistant to neurodegeneration under the condition of oxidative stress [45]. These findings clearly indicate that 8-oxoG accumulation in neurons and microglia leads to neurodegeneration and suggest that the lack of MUTYH may protect the brain from oxidative stress by preventing SSB accumulation.

The mammalian homologs of the *Escherichia coli* endonuclease VIII, encoded by the Nei gene, are termed Nei-like (NEIL) 1, Nei-like 2, and Nei-like 3. NEIL1 and NEIL2 recognize oxidized pyrimidines such as thymine glycol, 5-hydroxycytosine, dihydrothymine, dihydrouracyl, and 5-hydroxyuracyl [46]. NEIL1 interacts with replication proteins and is preferentially involved in replication-coordinated BER [28]. NEIL2 seems to have a crucial role in the repair of oxidized bases in active genes (transcription-coupled BER, TC-BER) as suggested by its interaction with RNA polymerase II, TFIIH, CSB, and LIG3 both *in vitro* and *in vivo* [47, 48]. Neil2 KO mice indeed accumulate oxidative DNA damage mostly in transcribed regions of their genome [48]. TC-BER has been suggested also for the repair of 8-oxoG [49], requiring the involvement not only of OGG1 and RNA Pol II but also of NER factors such as XPA, CSB, and UVSSA, indicating the need for strict control of oxidative damage in active genes. NEIL3 recognizes and excises oxidation-induced hydantoin lesions [50]. Neural stem/progenitor cells from adult Neil3 KO mice are impaired in proliferation and hippocampal neurons present synaptic irregularities. Moreover, Neil3 KO mice are affected by learning and memory deficits, demonstrating that NEIL3 is pivotal for maintaining adult neurogenesis [51, 52].

The alkyladenine DNA glycosylase (AAG) mediates alkylation-induced tissue damage and whole-animal lethality [53]. In transgenic mice overexpressing AAG (Aag-Tg mice), alkylating agents induce extreme cerebellar toxicity and dramatically impaired motor function. Interestingly, these effects are prevented in Aag KO mice [54, 55] suggesting that AAG activity, in the presence of alkylation damage, determines an accumulation of toxic BER intermediates, while loss of AAG prevents their formation and promotes cell survival [54].

4.2. AP-Endonuclease 1: The Processing of Abasic Sites. APE1 is a multifunctional protein that has a central role in BER by processing the AP sites and in transcriptional regulation by redox activation of transcription factors [56]. APE1 cleaves the DNA sugar-phosphate backbone at a position immediately 5′ of AP sites to prime DNA repair synthesis but it can also correct oxidized abasic sites (reviewed in [57]). Ape1 knockdown in cortical neurons induced the accumulation of oxidative DNA damage after glutamate treatment, suggesting that APE1 has a pivotal role in the repair of oxidative DNA damage in neurons [58]. On the other hand, overexpression of APE1 is neuroprotective in neurons exposed to cisplatin [59] or hydrogen peroxide [60]. Moreover, the DNA repair function of APE1 protects differentiated neuroblastoma cells from apoptosis induced by hydrogen peroxide [61]. APE1 interacts with CDK5, which in turn phosphorylates APE1 at

Thr 232, thus reducing its endonuclease activity and resulting in the accumulation of DNA damage in cortical neurons and in neuronal death after treatment with the neurotoxin 1-methyl-4-phenylpyridinium (MPP+) [62].

Besides APE1, the processing of SSB with 3′ and/or 5′ blocked termini generated by ROS involves end-processing factors that are instrumental in completing repair [27]. Human syndromes with varied neuropathology have been causally associated with defects in these factors. Their function and role will be addressed in the section on the role of BER/SSBR in human pathology (see below).

4.3. XRCC1: The Key Player in the Coordination of BER/SSBR. The scaffold protein XRCC1 orchestrates the coordination of the whole BER/SSBR process [26, 63]. XRCC1 interacts with several enzymes involved in BER/SSBR such as Lig3 [64], APE1, polynucleotide kinase/phosphatase PNKP, FEN1 [65], and DNA glycosylase OGG1 [66]. Moreover, XRCC1 interacts with PCNA and this interaction plays a central role in the DNA repair during DNA replication [67]. Xrcc1 KO mice die early in embryogenesis, indicating an essential role of XRCC1 in development [68]. Cerebellar granule cells from Xrcc1 heterozygous mice and Xrcc1 knockdown in human neuroblastoma cells show an accumulation of SSB and reduced survival following oxidative stress [69]. Neural-specific inactivation of Xrcc1 in mice induces loss of cerebellar interneurons, which causes a strong neuropathology. Moreover, loss of XRCC1 leads to the accumulation of SSB in the whole nervous system and abnormal hippocampal function [70].

4.4. The Resynthesis and Ligation Steps. Once damaged termini at SSB are restored to their conventional hydroxyl configuration, gap-filling and ligation will continue either via short-patch (SP) or long-patch (LP) BER, with distinct repair patches: one nucleotide in SP-BER and two or more nucleotides in LP-BER [65] (Figure 1). Polβ is involved in the resynthesis step in SP-BER while in LP-BER it incorporates the first nucleotide and Polδ and Polε [71, 72] are possibly involved in the elongation step. PCNA participates in LP-BER, but a PCNA-independent and Polβ-dependent LP-BER has also been reported [73, 74]. In the SP-BER, after the dNMP insertion, the deoxyribose-phosphate (dRP) is removed by the dRP-lyase activity of Polβ [75] and the repair is completed by ligation by the Lig3/XRCC1 complex. In LP-BER, the dRP is displaced and Polβ/Polδ polymerize tracts of DNA longer than one base [71, 76]. The strand displacement produces a flapped substrate that is refractory to ligation; this structure is recognized and excised by FEN1 [77, 78], followed by ligation by DNA ligase 1 (Lig1) [79]. Disruption of the coordination between Polβ and FEN1 in the processing of the flap structure leads to CAG repeat expansion that results in mutant Huntingtin protein expression in Huntington's disease (HD) [80, 81].

The choice of the BER subpathway is determined by multiple factors such as the type of lesion and the DNA glycosylase involved in its removal [38, 72, 82], protein-protein interaction, cell-cycle phase [65, 83], and/or differentiation status [13, 84]. As mentioned above, Sykora et al. [13] showed that differentiated human neuroblastoma cells are more sensitive to oxidative damage and present lower levels of proteins involved in LP-BER, such as FEN1, PCNA, and Lig1, thus relying mostly on Polβ-dependent BER for protection from endogenous damage. More recently, it has been shown that 50% Polβ reduction in a mouse model of Alzheimer accelerates synaptic and cognitive deficits determined by impaired autophagy and neurodegeneration [85].

5. Defects in SSBR in Neurological Diseases

PNKP is a DNA repair factor exhibiting both 5′-DNA kinase and 3′-phosphatase activities. Its capacity to remove 3′-phosphate generates 3′-OH termini, thus rendering DNA termini accessible for polymerases after base excision by the bifunctional glycosylases NEIL1 or NEIL2 [27]. PNKP interacts not only with the scaffold protein XRCC1 [86, 87] but also with XRCC4, a factor involved in NHEJ for the repair of DSB [88–91]. Mutations in PNKP have been recently identified as the cause of microcephaly with seizures (MCSZ), a syndrome characterized by profound neurodevelopmental microcephaly and early-onset seizures [92–94]. PNKP inactivation in murine neural progenitors induced neurodevelopmental abnormalities and postnatal death. In mice, in which a tamoxifen-inducible promoter was used to inactivate Pnkp after neurogenesis in different neural compartments, specific neural populations, including oligodendrocytes, were affected. These findings indicate that PNKP is required not only for neurogenesis but also for genome maintenance in mature neuronal cells, involving both BER and NHEJ [95].

Aprataxin (APTX) removes AMP from the 5′-termini of DNA breaks resulting from abortive DNA ligation events [96–98]. Loss of APTX in neuronal cells induces a defect in SSBR [98] and sensitivity to genotoxic agents [99]. Patients with loss of functional APTX are affected by ataxia with oculomotor apraxia-1 (AOA1), with progressive cerebellar ataxia [100, 101].

Tyrosyl-DNA phosphodiesterase 1 (TDP1) removes trapped topoisomerase peptides from 3′-termini of DNA breaks resulting from abortive topoisomerase 1 activity [102] and is involved also in the cleaning of others 3′-modified termini [103, 104]. Tdp1 KO mice show late onset progressive atrophy in the cerebellum and patients with loss of TDP1 are affected by spinocerebellar ataxia with axonal neuropathy (SCAN1) [105].

6. BER in Age-Related Neurodegenerative Diseases

Defective DNA repair has also been associated with age-related neurodegenerative disorders [106–109] such as Alzheimer's disease (AD) and Parkinson's disease (PD); however, the specific contribution of DNA damage to the etiology of these disorders has yet to be determined.

Parkinson's disease neurons present oxidative stress and genomic instability [110]. Dopaminergic neurons in the *substantia nigra* (SN) *pars compacta* of PD brains have high levels of mitochondrial DNA deletions, possibly related to

respiratory chain deficiency [111]. In addition, an upregulation of the mitochondrial isoforms of the DNA glycosylases MUTYH and OGG1 was found in SN of PD patients [112, 113]. A significant proportion of dopaminergic neurons from PD patients was positive for phosphorylated APE1, while the proportion of dopaminergic neurons positive for total APE1 is similar in PD patients and normal individuals [62].

High levels of DNA breaks have been reported in neurons from AD patients [110]. Moreover, neurons from AD patients accumulate oxidized DNA bases both in nuclear and mitochondrial DNA [110]. Wang et al. have shown high levels of the oxidized DNA bases 8-oxoG in both nuclear and mitochondrial DNA from brains of Mild Cognitive Impairment (MCI) patients, the phase between normal aging and early dementia [114]. This finding suggests that oxidation of DNA bases is an early event in AD pathology and may play a role in neurodegeneration. Analysis of peripheral leukocytes derived from AD and MCI patients revealed increased levels of ROS-induced DNA damage [115]. Furthermore, treatment of human primary fibroblasts with oxidizing agents induces a gene expression pattern typical of fibroblasts from AD patients [116].

An alteration in gene expression of DNA repair genes has been observed in AD by several authors. A decrease of OGG1 activity has been described in brains from AD patients compared to healthy individuals [117]. Lower levels of UDG are also present in brains from AD patients compared to healthy controls [118]. MutT Homolog 1 (MTH1), which is critical to avoid the incorporation of oxidized DNA bases in nuclear DNA during replication, is downregulated in the hippocampus from AD patients compared to controls [119]. In addition, AD tissues have decreased levels of Polβ [120]. Huang et al. have shown that APE1 levels are similar in AD and healthy individuals, but AD brains have higher levels of phosphorylated APE1 [62].

An impairment of BER function has been described in sporadic AD patients: both UDG activity and Polβ activity are decreased in cell extracts from AD brain tissues. BER impairment is also present in MCI brains, where it correlates with the severity of the disease [118]. Further, both affected and nonaffected brain regions have a diminished BER activity, suggesting that BER dysfunction is a general feature of AD brains that could occur at the earliest stages of the disease and be pivotal in the progression of AD [118]. A study of BER capacity in mitochondrial extracts from AD brains shows that 5-hydroxyuracyl incision and DNA ligase activity are lower in AD brains [121]. A recent study [122] has evaluated markers of oxidative DNA damage, DNA repair, and cell cycle in hippocampus from three groups: (i) clinical-pathological AD, with AD neuropathology and clinical dementia (CP-AD), (ii) pathological AD, with neuropathology without clinical dementia (P-AD), and (iii) normal aging. Oxidative DNA damage was high in all groups, but subjects with CP-AD present reductions of DNA repair and cell-cycle inhibition markers and increases in cell-cycle progression and cell death markers when compared to both P-AD and normal subjects. No differences in all the markers were present between P-AD patients and normal subjects. These results indicate that cognitive decline may be associated with DNA repair impairment and cell-cycle deregulation.

7. Brain Region Selectivity and Neuronal Vulnerability in Neurological Diseases

The brain is the most complex organ of the human body and is characterized by different regions having distinct and specific functions. This functional multiplicity results from the presence of several different cell populations. Investigation of DNA repair activities in the brain showed differential activity patterns related both to the specific DNA repair system and the brain region analysed. An additional element, which distinguishes the DNA repair systems operating in the human brain, is the subcellular compartment where they act. Analysis of BER in five mouse brain regions [123], namely, caudate nucleus, frontal cortex, hippocampus, cerebellum, and brain stem showed that the activities of three major DGs, OGG1, UDG, and NTH1 are higher in the nucleus with respect to mitochondria and that the cerebellum is the region having the highest levels of nuclear DG activity. In contrast, mitochondrial glycosylase activities showed a pronounced variation among the brain regions analysed, which manifested a general decline associated with age. Instead, nuclear glycosylase activities decline appears to be limited to the cerebellum [123]. Analysis of mRNA expression pattern of NEIL1, NEIL2, OGG1, and NTH1 glycosylases confirmed a wide distribution of BER enzymes both in human and rodent brain regions, except for NEIL3 whose expression was revealed in a few cell populations and at early stages of postnatal development [40]. Again, the cerebellum is one of the brain areas showing the highest levels of DG transcripts. The preferential activity/expression of BER in the cerebellum could be related to the marked vulnerability and susceptibility of this brain region to neurodegenerative events observed in different human neurodegenerative syndromes associated with BER/SSBR defects. For instance, AOA1 and SCAN1 are characterized by a marked cerebellar atrophy leading to progressive ataxia. Aptx, the gene mutated in AOA1, is widely expressed in the nervous system and it has been detected in cerebellum, basal ganglia, cerebral cortex, and spinal cord [100, 101]. For many neurological aspects, AOA1 resembles A-T but lacks typical extraneurological features such as immunodeficiency and cancer susceptibility [124]. As revealed in two autopsied cases, the pronounced atrophy of AOA1 patients cerebellum is caused by a severe loss of Purkinje cells [125, 126]. Degeneration of posterior columns, spinocerebellar tracts, and anterior horn cells of the spinal cord was also observed [125]. Apart from cerebellar ataxia, other prominent clinical phenotypes of AOA1 patients are axonal sensorimotor neuropathy, cognitive defects, and chorea. The presence of aprataxin in the caudate nucleus [100], the *in vivo* detection of caudate nucleus hypoperfusion [127], and a reduction of dopamine transporter density in caudate and putamen of AOA1 brains [128] suggested that aprataxin mutations in basal ganglia could affect the function of this brain region, thus leading to choreoathetosis. However, it should be noted that morphological alterations of basal ganglia have not been observed in AOA1 patients. Cognitive

disturbances have also been found in AOA1 patients which are consistent with a possible disruption of the frontocerebellar pathways [127].

SCAN1 has a later onset compared to AOA1. In the human brain, Tdp1, the gene mutated in SCAN1, is highly expressed in different regions including the cerebellum (granule and Purkinje cells), dentate nucleus, spinal cord, and dorsal root ganglia [129], similar to mouse brain. Although there are not reported autopsy studies from SCAN1 patients, the brain expression profiles of TDP1 in nondiseased brains together with progressive ataxia and axonal sensorimotor neuropathy typical of SCAN1 individuals are consistent with the involvement of the TDP1 expressing regions in the pathogenesis of SCAN1. It is noteworthy that SCAN1 patients do not manifest cognitive defects as compared to AOA1 individuals [102].

Another gene involved both in nuclear and mitochondrial BER and associated with cerebellar dysfunction is Pnkp. Recently, the finding of severe cerebellar atrophy [93] in two Dutch siblings affected by MSCZ with a homozygous mutation in Pnkp and in 11 individuals of nine Portuguese families affected by early-onset recessive AOA [130] confirmed the cerebellum as one of the most vulnerable brain regions in DNA repair syndromes. As reported in AOA1 patients, beside ataxia, cognitive impairments were also observed [93, 130] in several individuals, leading even to severe dementia in some cases [130]. Although PNKP has been identified in normal and pathological human cerebellum [131], a detailed analysis of expression levels and/or activity of PNKP in human brain is not yet available.

A clear picture emerging from the analysis of these diseases is that the cerebellum appears to be the brain region with the highest vulnerability to defects in BER/SSBR activities. The high expression/activity levels of BER core proteins (OGG1, UDG, NTH1, NEIL1, and NEIL2) and end-processing DNA repair factors such as APTX and TDP1 in the cerebellum could be suggestive of a high susceptibility of this brain region to DNA lesions and especially to oxidative DNA damages. Consistently, it has been shown that cerebellar granule neurons and CA1 neurons are particularly vulnerable to oxidative stress stimuli compared to other neurons such as cortical and CA3 neurons [132, 133]. This feature correlates with the marked loss of cerebellar granule cells in aged individuals [134, 135] and the decline of BER glycosylase activities specifically detected in the cerebellum during aging [123]. Importantly, data derived from transcriptomic analyses on cerebellar granules and cortical neurons showed that some genes related to the DNA damage response and repair (such as Hmgb2 and Pold1) are markedly more active in the cerebellar neurons [136]. A parallel result was obtained on CA1 neurons as compared to CA3 neurons [136]. The finding that, under basal conditions, cerebellar granule neurons have 25% lower ATP levels with respect to cortical neurons could also account for the selective vulnerability of cerebellum to oxidative stress, a condition requiring a high energy demand to cope with DNA damages.

As described for SCAN1 and AOA1, clinical phenotypes characterizing age-related neurodegenerative diseases are associated with the specific brain regions and population of neurons targeted, even though important causality issues

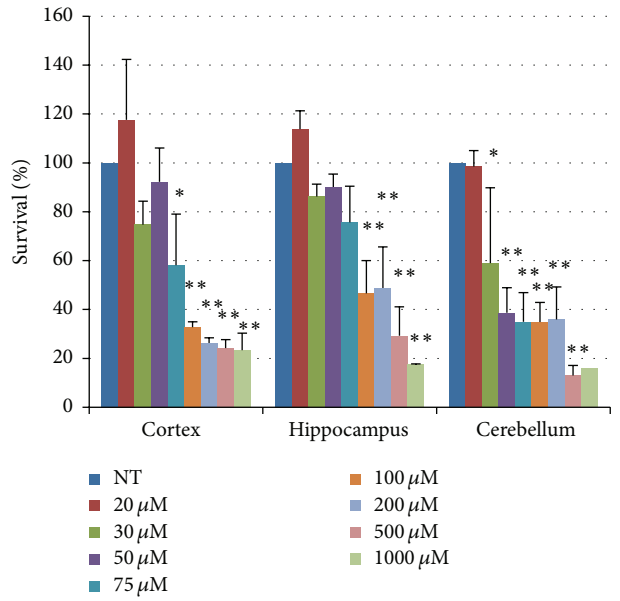

Figure 2: Cell survival as detected by the MTT assay in neurons from different brain regions treated with increasing H_2O_2 doses for 30 min. Error bars indicate the standard error. $^{*}p < 0.05$ and $^{**}p < 0.01$ versus untreated (NT) by Least Significant Difference (LSD) test. Two-way ANOVA did not show any significant difference in the response to treatment in the neurons derived from the three different brain regions (cell cultures were prepared as previously described) [137–139]. In more detail, C57BL wild-type mice were sacrificed at postnatal day 5 (P5) for cerebellar granule cell cultures, whereas at day 17 embryos were collected from pregnant mice for preparation of cortical and hippocampal neurons. Brain regions were dissected and dissociated and cultures were maintained in appropriate media.

remain to be addressed [140]. In AD, early memory deficits are caused by the selective degeneration of pyramidal neurons in the entorhinal cortex, subiculum and CA1 of the hippocampus, and accumulation of β-amyloid in frontal, temporal, and parietal cortex. In particular, cholinergic neurons are the most affected neuronal type [141]. In PD, dopaminergic neurons of the SN are targeted [142] and their degeneration accounts for the major clinical manifestations of PD. Another neuronal type specifically targeted in neurodegenerative diseases is GABAergic neuron, whose loss characterizes both spinocerebellar ataxia-1 (SCA1) and Huntington's disease (HD), two polyglutamine diseases. It is noteworthy that the cell population affected in SCA1 is represented by giant Purkinje cells of the cerebellum [143], whereas spiny neurons of the striatum degenerate in HD [144]. Interestingly, the genes causing SCA1 and HD are expressed in both cerebellum and striatum; however, the reason why both regions are not affected in both diseases is not known.

Although there is no evidence for a region- and neuron-specific DNA damage response in neurodegenerative diseases, it is tempting to speculate that neurons that are selectively affected in these diseases could be both selectively vulnerable to specific DNA damage inducers (e.g., ROS) and/or less responsive to DNA repair.

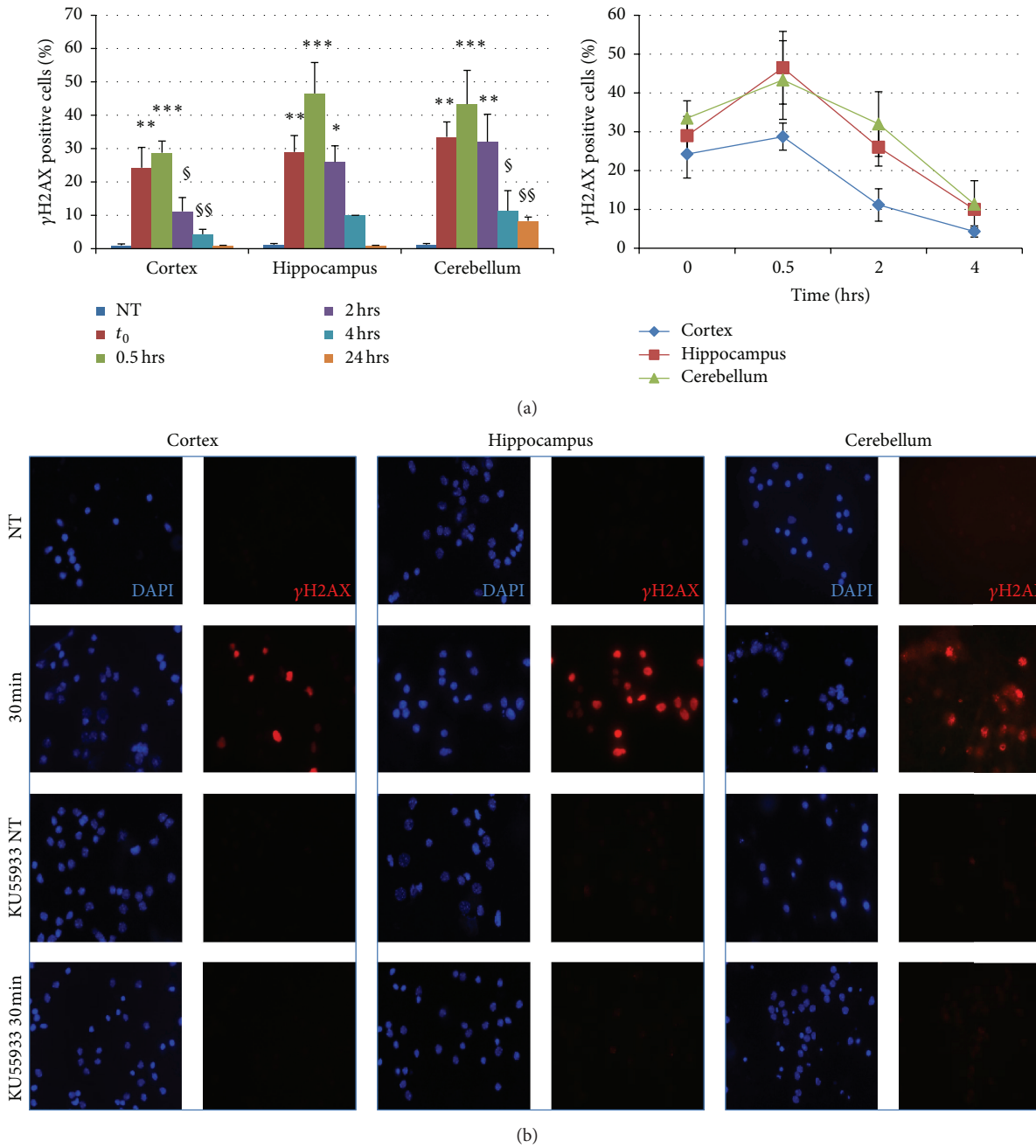

FIGURE 3: H_2O_2-induced DNA damage and repair as detected by γH2AX foci formation in neurons derived from different brain regions. (a) Left: percentage of cells bearing 10 or more γH2AX foci at different times after treatment with 20 μM H_2O_2 for 30 min. One-way ANOVA and *post hoc* analysis Least Significant Differences (LSD) indicated a significant increase of γH2AX positive cells immediately and 30 minutes after the treatment in all brain regions. Two-way ANOVA did not show any interaction between the treatment and the neuronal cell type. Right: kinetics of γH2AX dephosphorylation following posttreatment times up to 24 hrs. ANOVA test shows any interaction between treatment and the three types of neurons on slopes of focus kinetics. For each time point, at least 100 nuclei were examined. Error bars indicate standard error. $^{*}p < 0.05$ versus untreated (NT) by LSD test; $^{\S}p < 0.05$ versus t_0 by LSD test; $^{**}p < 0.01$ versus untreated (NT) by LSD test; $^{\S\S}p < 0.01$ versus t_0 by LSD test; $^{***}p < 0.05$ versus untreated (NT) by LSD test. (b) Immunofluorescence of γH2AX (red, Ser 139; Millipore, Billerica, MA, USA) in neurons exposed to H_2O_2 (20 μM, 30 min) with or without 1 hr pretreatment with a specific ATM kinase inhibitor KU55933 (10 μM). Nuclei are stained with DAPI in blue. One representative experiment is shown.

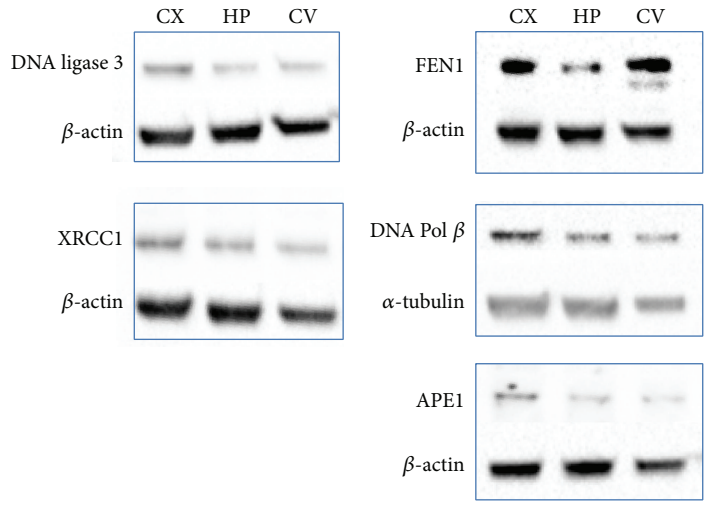

Figure 4: Levels of BER enzymes in neurons derived from cortex (CX), hippocampus (HP), and cerebellum (CV). Immunoblotting was carried out by using antibodies specific for DNA ligase 3 (103 kDa, BD Biosciences Pharmingen, San Diego, CA), FEN1 (43 kDa, Abcam, Atlanta, USA), XRCC1 (85 kDa, Bethyl Laboratories, Inc.), APE1 (35.5 kDa, Santa Cruz Biotechnology, Inc.), and DNA Polβ (38 kDa, Trevigen Inc., Gaithersburg, MD). β-actin (40 kDa, Sigma) and α-tubulin (55 kDa) were used as loading controls. Western blot analysis was conducted on 15 μg of whole cell extracts. One representative experiment is shown.

To address this issue, we have compared the response to oxidative DNA damage of different types of neurons derived from mouse cortex, cerebellum, and hippocampus.

All three types of neurons showed a significant H_2O_2 dose-related decrease in survival (Figure 2). Neurons from cerebellum seem to be more sensitive (LC_{50} = 29.8 μM) to oxidative stress when compared to neurons from cortical and hippocampal regions (LC_{50} = 41.5 and 55.2 μM, resp.). The efficiency of DDR was monitored by the formation and repair of γH2AX foci. The number of foci-positive nuclei immediately after treatment was lower in cortical neurons as compared with neurons from cerebellum and hippocampus, suggesting a less effective DDR in cortex (Figure 3(a), left). During the posttreatment repair time, the percentage of γH2AX foci-positive cells declined in all three types of neurons with the same rate (Figure 3(a), right) and at 24 hr posttreatment the repair was almost completed in all neuronal cell types. The presence of the specific ATM kinase inhibitor KU55933 fully abolished the appearance of γH2AX foci (Figure 3(b)), indicating that this kinase is activated following H_2O_2-induced DNA damage in neurons from the three brain regions analysed. An ATM-dependent DDR has also been described following H_2O_2 treatment in postmitotic myotubes [145] strengthening the importance of this kinase in the signalling of DNA breaks [146]. Finally, when the protein levels of key BER enzymes, such as APE1, XRCC1, Polβ, Lig3, and FEN1, were measured (Figure 4), no significant differences were found by comparing neurons derived from the three different brain regions. A notable exception is FEN1 that was higher in neurons from the cerebellum and the cortex as compared with neurons from hippocampus. It has been shown that the expression levels of DNA replication/repair proteins, including FEN1, predict regional somatic repeat instability in the brain [147]. We confirm elevated expression of FEN1 in the cerebellum, thus supporting the hypothesis that in this brain region it may account for the reduced somatic instability as compared with other regions (e.g., striatum) [81]. Therefore, our data support the hypothesis that brain region differences in BER/SSBR activities and/or DDR efficiency may contribute to the brain region selectivity and neuronal vulnerability in neurological diseases.

8. Conclusions

There is emerging evidence for an important role of BER/SSBR in the control of genome stability in the nervous system. Indeed, a malfunction of almost all the key players of this pathway has been associated with neurological alterations. This finding confirms that neuronal cells need to be protected from oxidative damage that is mostly repaired by BER. Oxidative damage is produced at high levels in the brain due to high level of tissue oxygen consumption. SSB that are produced during BER are a major threat to mature brain as shown by the neurological consequences of defective SSBR. There is some evidence that the mechanisms that control DNA damage in neurons may vary depending on the brain region.

In SSBR-associated neurological syndromes, specific brain areas are affected, with the cerebellum being the most vulnerable one. In contrast, further investigation is required to determine if differences in response to DNA damage underlie the brain region selectivity observed in neurodegenerative diseases.

A better understanding of DNA repair/DDR mechanisms in the nervous system may open new avenues in the design of innovative therapies.

Conflict of Interests

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

Authors' Contribution

Laura Narciso, Eleonora Parlanti, and Mauro Racaniello contributed equally to this work.

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Research Article

Miglustat Reverts the Impairment of Synaptic Plasticity in a Mouse Model of NPC Disease

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Niemann-Pick type C disease is an autosomal recessive storage disorder, characterized by abnormal sequestration of unesterified cholesterol within the late endolysosomal compartment of cells and accumulation of gangliosides and other sphingolipids. Progressive neurological deterioration and insurgence of symptoms like ataxia, seizure, and cognitive decline until severe dementia are pathognomonic features of the disease. Here, we studied synaptic plasticity phenomena and evaluated ERKs activation in the hippocampus of BALB/c NPC1−/− mice, a well described animal model of the disease. Our results demonstrated an impairment of both induction and maintenance of long term synaptic potentiation in NPC1−/− mouse slices, associated with the lack of ERKs phosphorylation. We then investigated the effects of Miglustat, a recent approved drug for the treatment of NPCD. We found that *in vivo* Miglustat administration in NPC1−/− mice was able to rescue synaptic plasticity deficits, to restore ERKs activation and to counteract hyperexcitability. Overall, these data indicate that Miglustat may be effective for treating the neurological deficits associated with NPCD, such as seizures and dementia.

1. Introduction

Niemann-Pick type C disease (NPCD) is a panethnic, fatal, autosomal recessive, neurovisceral lipid storage disorder with infantile and juvenile onset in 95% of cases and adult onset in 5% of cases [1]. The NPC genes, *NPC1* mutated in about 95% of the disease and *NPC2* mutated in the remaining 5%, encode proteins that are involved in intracellular lipid transport. Mutations in *NPC1* and *NPC2* give rise to severe abnormalities in the functioning of this transport system with excess storage of lipids as cholesterol, glycosphingolipids, and sphingosine in the late endosomal and lysosomal intracellular compartments, associated with peripheral and central organ dysfunction [2].

The disease is characterized by progressive neurological deterioration and the insurgence of symptoms like ataxia, seizure, and cognitive decline until severe dementia leading to premature death [2, 3]. Although the genetic defects causing NPCD are well known, very little information is available on the causes of neurological deficits and neuropathology. Current therapeutic approaches for NPCD are limited. N-butyldeoxynojirimycin (Miglustat; Zavesca, Actelion Pharmaceuticals), a drug initially used for the treatment of several dyslipidosis including GM1 gangliosidosis, Gaucher type I, and Tay-Sachs disease, has been approved in 2009 for the treatment of progressive neurological manifestations in adult and pediatric patients affected by NPCD [4–7]. The active principle of the drug is an iminosugar able to specifically inhibit the enzyme glucosylceramide synthase (GCS) that converts the ceramide into the glycosphingolipid glucosylceramide (i.e., the first product in the synthesis of complex glycosphingolipid, including gangliosides). Although undeniable advantages concerning some neurological symptoms have been noticed in clinic, the reason why cells presenting

a bias in the transport system of both cholesterol and all classes of sphingolipids should benefit from the block of glycolipids synthesis is not clear [8].

Changes in the plasma membrane cholesterol content and in the glycosphingolipids/cholesterol ratio are particularly important in affecting lipid rafts, platforms where many transductive signaling processes are generated, fundamental for a variety of cellular functions and important regulators of glutamate receptor activity [9, 10]. We demonstrated in a previous paper that cholesterol depleted hippocampal neurons exhibit an impaired NMDA receptors-mediated synaptic plasticity [11]. Therefore, alteration of a correct dynamic of cholesterol-sphingolipids content in the neuronal plasma membrane might have a key role in neuronal dysfunction associated with NPC disease, such as memory impairment and dementia.

Aim of this study is to evaluate whether synaptic plasticity phenomena, involved in learning and memory processes, are affected in NPC1−/− mice, a well-established mouse model for the NPC disease, and if *in vivo* Miglustat treatment is able to counteract synaptic deficits. To this aim, we employed a multidisciplinary approach both in Wild Type (WT) and in NPC1−/− mice, consisting in (i) electrophysiological recording in acute hippocampal slices and (ii) molecular analysis of intracellular pathway.

2. Methods

2.1. Animals. We first established a colony of NPC1−/− mice that represent a well-known experimental model of NPCD since they display most of the clinical features of the disease including cognitive deficits. Breeding pairs of BALB/cNctr-Npc1m1N/J (Stock number: 003092) mice were purchased from Jackson Laboratories (Bar Harbor, MA, USA). This strain contains a spontaneous mutation in the NPC1 locus [12]. Animals were bred and maintained according to Italian Animal Care Committee rules. Heterozygous (male × female) mice were bred and the genotypes of offspring animals were determined as indicated by Jackson Laboratories in genotyping protocols database by polymerase chain reaction (PCR) [12]. Briefly, PCR was performed using the following primers: WT sense: CTG TAG CTC ATC TGC CAT CG, WT antisense: TCT CAC AGC CAC AAG CTT CC; mutant sense: GGT GCT GGA CAG CCA AGT A, mutant antisense: TGA GCC CAA GCA TAA CTT CC. The expected products were as follows: mutant = 475 bp, WT = 173 bp, heterozygote = 173 bp, and 475 bp. Age-matched WT served as controls.

2.2. Miglustat Administration. The experiments have been performed to study the effects of *in vivo* Miglustat (N-butyldeoxynojirimycin, Sigma) treatment on NPC1−/− mice synaptic activity. In addition to WT mice ($n = 12$) and NPC1−/− untreated mice ($n = 11$), we distributed the treated animals in four groups: WT mice treated by oral administration (gavage) with either saline solution ($n = 5$) or Miglustat (0.2 mg/kg) ($n = 5$) and NPC1−/− mice treated by oral administration (gavage) with either saline solution ($n = 5$) or Miglustat (0.2 mg/kg) ($n = 11$).

NPC1−/− mice showed well-defined symptoms consisting in ataxia and intentional tremor since 50–52 days of age. Before the insurgence of the characteristic symptoms of the pathology (the 38th day of life), NPC1−/− and WT mice have been treated for 20 days. During this temporal window, no gastroenteric side effects linked to immino sugar administration were evident and they were able to feed and did not show insurgence of neurological symptoms presenting a normal function motor system (see Video in Supplementary Material available online at http://dx.doi.org/10.1155/2016/3830424). Because we aimed at evaluating the effects of Miglustat on synaptic plasticity deficits rather than its general effects on clinical symptoms and lifespan, animals were sacrificed at the end of treatment, at 58–60 days of life.

2.3. Extracellular Recordings in Mouse Hippocampus. BALB/cNctr-npc1N mice, genotypically characterized littermates (5–7 weeks old), were used according to the procedures established by the European Union Councils of Animal Care European Communities Council Directive of 24 November 1986 (86/609/EEC). All efforts were made to minimize the number of animals used and their suffering. Under anesthesia with enflurane, they were decapitated and brains were quickly removed and placed in cold, oxygenated artificial cerebral spinal fluid (ACSF) containing the following (in mM): NaCl 124, KCl 2, KH_2PO_4 1.25, $MgSO_4$ 2, $CaCl_2$ 2, $NaHCO_3$ 26, and glucose 10 [13]. The hippocampus was rapidly dissected and slices (450 μm thick) were cut transversely with a McIlwain tissue chopper (Mickle Laboratory Engineering Co., Gomshall, UK) and transferred into a tissue chamber, where they were laid in an interface between oxygenated ACSF and humidified gas (95% O_2/5% CO_2) at 32–34°C (pH = 7.4), constantly superfused at flow rate of 1.2 mL/min. Extracellular recordings of the population spikes (PSs) were made in the stratum pyramidale of the CA1 subfield with glass microelectrodes filled with 2 M NaCl (resistance 5–10 MΩ). Orthodromic stimuli (10–500 mA, 20–90 ms, 0.1 Hz) were delivered through a platinum electrode placed in the stratum radiatum in the Schaffer collateral/commissural CA1 pathways. The test stimulus intensity of 50 ms square pulses was adjusted to elicit a PS of 2-3 mV at 0.03 Hz. PS amplitude was calculated every minute as the average of six recordings performed every 10 s. To exploit basal synaptic transmission, the PS was recorded for 1 hour. After recording stable signals (20–30 min), a tetanic stimulation (100 Hz, 1 s) was delivered to induce long term potentiation (LTP) at the same stimulus intensity used for the baseline responses. Posttetanic potentiation (PTP) and LTP were measured by calculating the PS amplitude prior to and after the tetanus.

Field potentials were fed to an Axoclamp 2A amplifier, acquired through a digital/analogic system (Digidata 1440A, Axon Instruments) and analyzed with the software pCLAMP10 (Axon, Foster City, CA, USA).

Changes in the amplitude of PS after tetanization were expressed as percentages of the basal PS amplitude ($PS/PS_{basal} * 100$, where PS_{basal} is the mean PS amplitude before tetanization). The overall effects on PTP and LTP were measured by calculating the average, for each slice, of the PS amplitudes recorded during the 5 min period before

the tetanus (BST), during the first minute after the tetanus (PTP), and during the 60 min period after the tetanus (LTP).

2.4. Immunoblotting Assays. Hippocampal slices used for electrophysiological experiments were rapidly dissected to isolate CA1 regions and then homogenized in ice-cold RIPA buffer containing 1x complete protease and phosphatase inhibitors (Sigma). Protein concentration was assessed by the Micro BCA Protein Assay Kit (Pierce). Proteins (30 μg) were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on polyacrylamide gels and electrophoretically transferred to nitrocellulose membranes as previously described [14]. Loading of protein samples was verified by Coomassie and Ponceau staining. Primary antibodies directed against phospho-p44/p42 MAPK (Thr202/Tyr204), total p44/p42 MAPK were obtained from Cell Signaling Technology. Membranes were first processed to visualize the phosphorylated forms of proteins, dehybridized (Restore Western Blot Stripping Buffer, Pierce, Rockford, IL, USA), and then reprobed with antibodies directed against total proteins for normalization. Quantitation was carried out by densitometric film analysis.

2.5. Statistics. For electrophysiological experiments, data are expressed as mean measurements ± SEM and n represents the number of slices studied. Data were compared with Student's t-test or the ANOVA test and were considered significantly different if $p < 0.05$. Excel 5.0 software was used for generation of graphs. For ERKs, activation studies data, expressed as mean ± SEM, were statistically analyzed using Student's t-test.

3. Results

3.1. Effect of Miglustat on Basal Synaptic Transmission. In a previous paper, we demonstrated that basal synaptic transmission (BST) recorded in the CA1 region of hippocampal slices from NPC1−/− mice was enhanced with respect to slices from WT mice [15]. In the present study, we analyzed whether *in vivo* Miglustat administration could be able to reduce synaptic transmission in NPC1−/− mice.

In NPC1−/− and WT slices from untreated animals, PS % values at 20 min of the recording time period of BST were 127.1 ± 6 and 104.1 ± 6.1 whereas at 60 min they were 144.7 ± 10.8 and 111 ± 9.3, respectively ($n = 8$ from 8 different animals for each group, $p < 0.05$, Figure 1). In slices from WT animals treated with saline solution ($n = 5$ from 5 different animals) and with Miglustat ($n = 5$ from 5 different mice), the BST values did not significantly change compared to the untreated mice (105.1 ± 5.9 and 103.2 ± 6.3, resp., versus 104 ± 6.1 at 20 min; 109.1 ± 7.1 and 109.7 ± 8.7 versus 111 ± 9.3 at 60 min, Figure 1), thus excluding any event due to gavage technique or Miglustat unspecific effects. Indeed, in slices from NPC1−/− mice treated with saline solution ($n = 5$ from 5 different animals), the PS values did not vary with respect to the untreated (129.4 ± 7.2 versus 127.1 ± 6 at 20 min; 147.2 ± 11.2 versus 144 ± 10.8 at 60 min). After 20 days of oral Miglustat administration, PS recorded in the CA1 region of hippocampal slices from NPC1−/− mice ($n = 11$ from 11 different animals) remained stable for all the recording time compared to NPC1−/− saline treated mice: value at 20 min 95.2 ± 5.6 versus 129.4 ± 7.2 ($p < 0.01$) and value at 60 min 107.4 ± 7.3 versus 147.2 ± 11.2 ($p < 0.01$), indicating a counteracting effect of the drug on the hyperexcitability observed in the NPC1−/− mice (Figure 1).

3.2. Impairment of Synaptic Plasticity in CA1 Hippocampal Region of NPC1−/− Mice Was Rescued by Miglustat Treatment. First we evaluated synaptic plasticity in CA1 hippocampal region of NPC1−/− and WT mice because memory loss is one of the pathognomonic symptoms of NPC disease. For this purpose, we induced long term potentiation (LTP) at Schaffer collateral/commissural fiber-CA1 synapses in hippocampal slices using one train of high frequency stimulation (HFS) (1 s 100 Hz). This stimulation induced a sustained enhancement of PS in WT mice (Figure 2(a)): the values of PTP were 323.8 ± 29.8 while the values of the LTP recorded at 10, 20, and 60 min after the tetanus were 250.2 ± 29.9, 222 ± 31.9, and 195.6 ± 29.8, respectively ($n = 12$ from 12 different animals) (Figure 2(c)). The same stimulation in the NPC1−/− mouse slices induced a marked inhibition of the expression of both PTP and LTP and a complete blockade of the LTP maintenance phase. PTP and LTP values at 10, 20, and 60 min after tetanic stimulation were 221.8 ± 42.5, 137.3 ± 11, 117.8 ± 14, and 103.3 ± 20.9, respectively ($n = 11$, $p < 0.05$ and $p < 0.01$ versus untreated WT; see the legend for details). The decrease of PTP was approx. 32% whereas the LTP decrement was about 45%, 50%, and 47% at 10, 20, and 60 min of recording, respectively.

In slices from WT animals treated with saline solution ($n = 5$ from 5 different animals) and with Miglustat ($n = 5$ from 5 different mice), the PTP and LTP values at 10, 20, and 60 min after tetanic stimulation did not significantly change with respect to the untreated mice. The values were 320.3 ± 36.9, 246.7 ± 19.3, 226.9 ± 34.5, and 188.6 ± 24.4, respectively, for the WT saline treated mice and 318.3 ± 31.2, 258.6 ± 21.3, 232.6 ± 23.1, and 200.1 ± 30.8 for the WT Miglustat treated mice. In slices from NPC1−/− mice treated with saline solution ($n = 5$ from 5 different animals), tetanic stimulation induced an inhibition of both PTP and LTP as reported for the NPC1−/− untreated mice. The PS % values were 216.7 ± 40.4 for PTP and 130.3 ± 12.4, 120.8 ± 13.5, and 108.3 ± 21.4 at 10, 20, and 60 min after tetanic stimulation.

Miglustat treatment in the NPC1−/− mice was able to revert the impairment of both PTP and LTP observed in slices from NPC1−/− saline treated mice. The values of PTP and LTP at 10, 20, and 60 min after tetanic stimulation were 355.7 ± 46.8, 295.8 ± 30.5, 249.4 ± 29, and 211.1 ± 29.1, respectively, thus reaching values similar to those of saline treated WT mice ($n = 11$, $p < 0.05$ and $p < 0.01$ versus saline treated NPC1−/−; see the legend for details).

3.3. Effect of Miglustat on ERK Phosphorylation in Hippocampal Slices of NPC1−/− Mice. ERK phosphorylation has been shown to occur following different LTP inducing paradigms in hippocampal slices [16]. Indeed, after LTP induction,

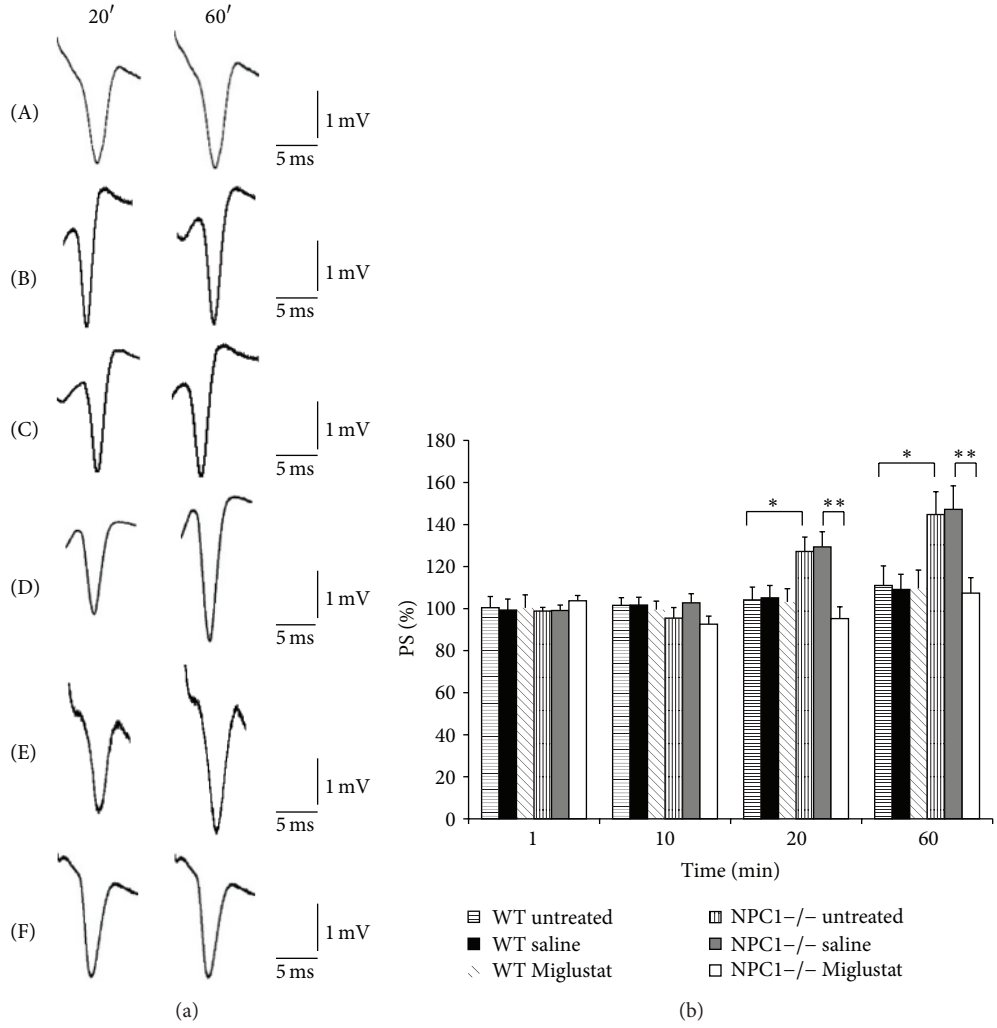

Figure 1: Basal synaptic transmission in CA1 hippocampal subfield in slices obtained from WT and NPC1−/− mice. (a) Recordings were acquired at times $t = 20$ and $t = 60$: curves (A), (B), and (C) refer to WT untreated, WT saline treated, and WT Miglustat treated mice slices, whereas curves (D), (E), and (F) represent the PS of NPC1−/− untreated, NPC1−/− saline, and NPC1−/− Miglustat treated mice. (b) % PS amplitude as a function of time is shown in WT untreated (horizontal pattern bar, $n = 8$), in WT saline treated (black bar $n = 5$), in WT Miglustat treated (diagonal pattern bar, $n = 5$), in NPC1−/− untreated (vertical pattern bar, $n = 8$), in NPC1−/− saline treated (grey bar, $n = 5$), and in NPC1−/− Miglustat treated (white bar, $n = 11$) mouse slices at minutes 1, 10, 20, and 60. PS amplitude corresponds to an average of 6 recordings/min. Bars in the plot are means ± SEM of values obtained from different slices. Note significant statistical differences in PS amplitude during 1-hour recording: at 20 min recording 127.1 ± 6 versus 104.1 ± 6.1, respectively, in NPC1−/− and WT slices of untreated mice ($^{*}p < 0.05$) and 95.2 ± 5.6 versus 129.4 ± 7.2, respectively, in NPC1−/− slices of Miglustat and NPC1−/− slices of saline treated mice ($^{**}p < 0.01$); at 60 min recording 144.7 ± 10 versus 111 ± 9 in NPC1−/− and WT slices of untreated mice ($^{**}p < 0.01$) and 107.3 ± 7.3 versus 147.2 ± 11 in NPC1−/− slices, respectively, of Miglustat and saline treated mice ($^{**}p < 0.01$).

ERKs are considerably phosphorylated and the extent of this phosphorylation depends on the type of the LTP inducing paradigm [17]. Here, we analyzed the phosphorylation state of ERKs at distinct time periods (5, 15, and 30 min) after delivering the high frequency tetanus. To this aim, a single slice for each time point (from a single mouse) was subjected to electrophysiology, rapidly dissected to isolate CA1 region before it was homogenised and protein lysates analyzed by Western Blot (Figure 3). Quantification of the immunoreactive levels of the activated ERK2 kinase, normalized by the amount of the total kinase, revealed that induction of LTP increased ERK2 phosphorylation in slices from WT mice by approx. 47%, 60%, and 80% at 5, 15, and 30 min after LTP, respectively ($n = 6$ from 6 different animals for each time point, $p < 0.01$). The same tetanic stimulation in the NPC1−/− mouse slices failed to induce a significant ERKs phosphorylation at each time point ($n = 6$ from 6 different animals, $p < 0.01$) (Figure 3). We then asked whether Miglustat was able to restore ERKs phosphorylation in stimulated NPC1−/− mouse slices. Therefore, we evaluated ERK2 phosphorylation levels in CA1 region of slices from NPC1−/− mice treated either with Miglustat or with saline at different time periods after LTP induction. We found that Miglustat reestablished ERK2 phosphorylation levels

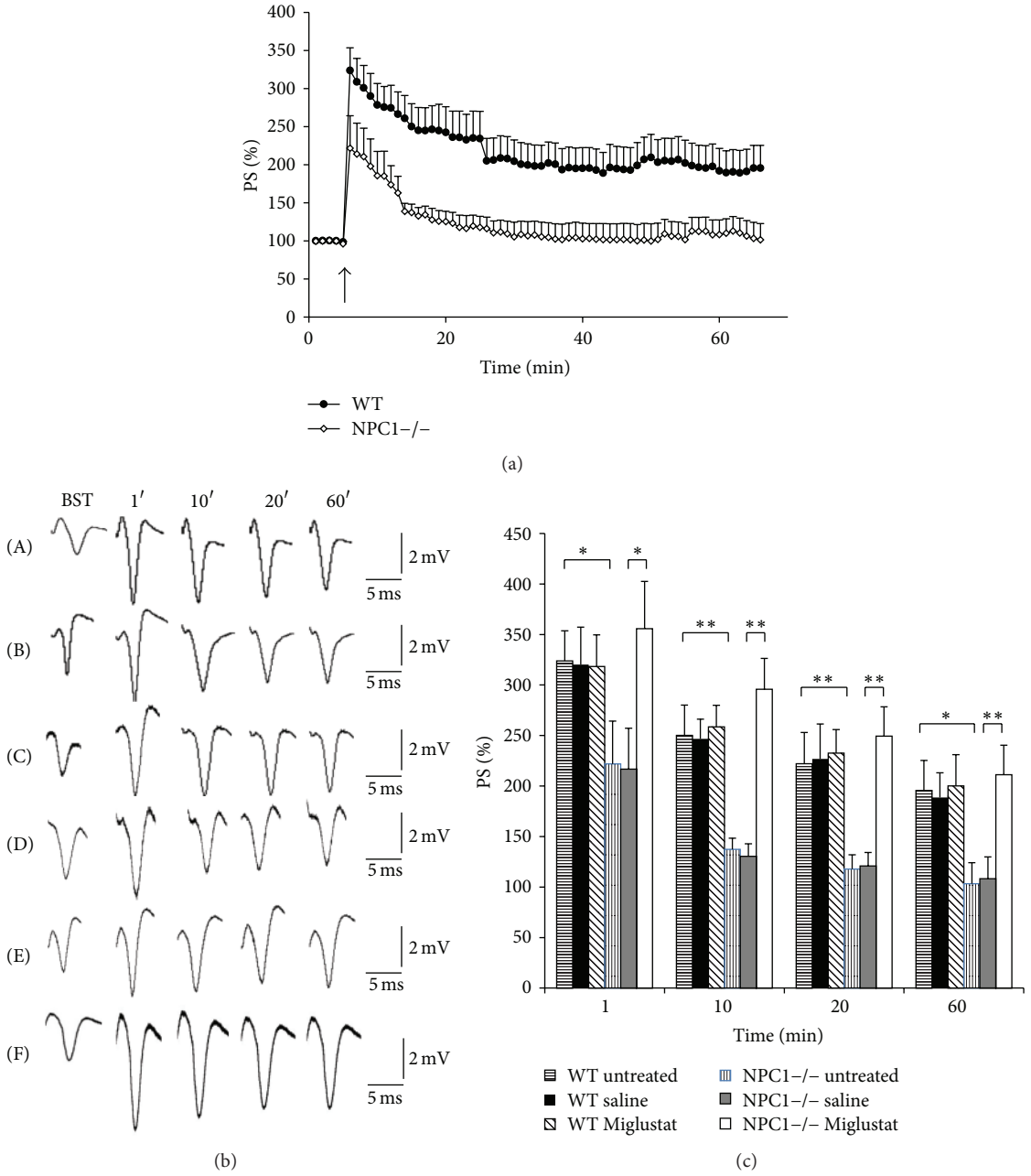

Figure 2: Synaptic plasticity in CA1 hippocampal subfield of WT and NPC1−/− mice. (a) % PS amplitude as a function of time is shown after tetanic stimulation applied at time $t = 5$ (arrow). The PS amplitude, measured every minute, corresponds to an average of 6 recordings/min. Points in the plot are means ± SEM of values obtained from different slices. Note the impairments of both the induction and the expression of LTP in NPC1−/− untreated mice (white rombs, $n = 11$) in comparison to the WT (black circles, $n = 12$) untreated mice. (b) Recordings were obtained from different slices of WT (A) and NPC1−/− untreated (B), WT saline (C) and NPC1−/− saline treated (D), and WT Miglustat (E) and NPC1−/− Miglustat (F) treated mice. The first curve of each group refers to the BST and it was recorded before the application of the tetanic stimulation, while the other curves refer to population spikes at times 1, 10, 20, and 60 min after the HFS. (c) % PS amplitude after HFS as a function of time is shown in WT untreated (horizontal pattern bar, $n = 12$), in WT saline treated (black bar $n = 5$), in WT Miglustat treated (diagonal pattern bar, $n = 5$), in NPC1−/− untreated (vertical pattern bar, $n = 11$), in NPC1−/− saline treated (grey bar, $n = 5$), and in NPC1−/− Miglustat treated (white bar, $n = 11$) mice slices at minutes 1, 10, 20, and 60. Bars in the plot are means ± SEM of values obtained from different slices. Note significant statistical differences in PS amplitude during 1-hour recording in NPC1−/− untreated versus WT untreated mice slices: PTP and LTP values at 10, 20, and 60 min after tetanic stimulation were, respectively, 221.8 ± 42.5, 137.3 ± 11, 117.8 ± 14, and 103.3 ± 20.9 versus 323.8 ± 29.8, 250.2 ± 29.9, 222 ± 31.9, and 195.6 ± 29.8 ($^{*}p < 0.05$ at 1 and 60 min; $^{**}p < 0.01$ at 10 and 20 min). A statistically significant difference is also present in NPC1−/− Miglustat treated versus NPC1−/− saline treated mice slices: the values recorded were 355.7 ± 46.8, 295.8 ± 30.5, 249.4 ± 29, and 211.1 ± 29.1 versus 216.7 ± 40.4, 130.3 ± 12.4, 120.8 ± 13.5, and 108.3 ± 21.4 ($^{*}p < 0.05$ at 1 min; $^{**}p < 0.01$ for all the following minutes).

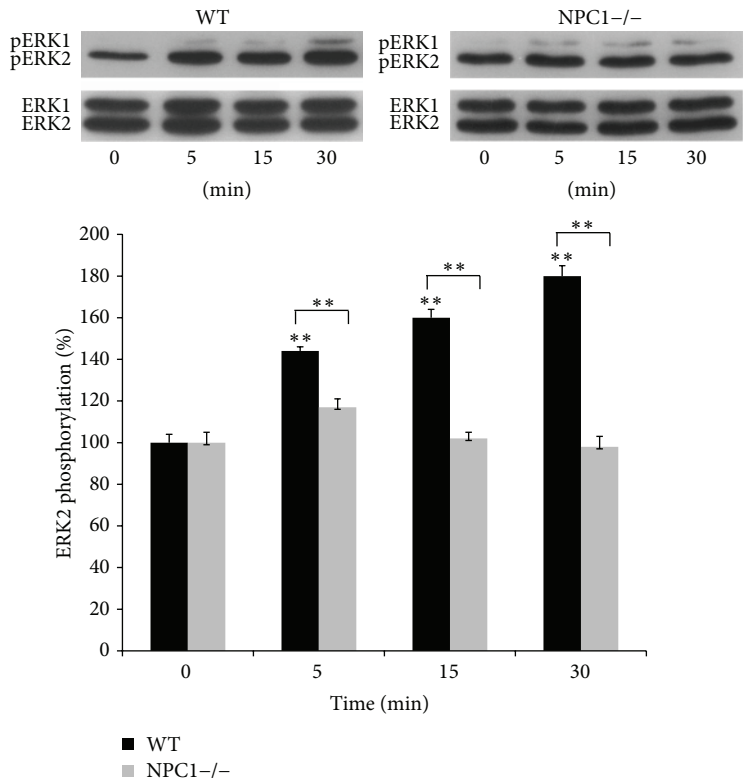

Figure 3: Time-course analysis of phosphorylation changes of ERK1/2 during LTP in WT and NPC1−/− mice. Representative Western Blots of phosphorylated and total form of ERKs are shown in the upper panels. Densitometric quantitation of the immunoreactive bands at different times after LTP induction is illustrated in the lower panel. Values represent the normalized percent changes in ERK2 protein phosphorylation for each time point after LTP. Bars in the plots represent means ± SEM ($n = 6$ for WT mice, $^{**}p < 0.01$ versus control values ($T0$); $n = 6$ for NPC1−/− mice, $^{**}p < 0.01$ versus WT mice at the corresponding time point).

principally at 5 and 15 min after LTP (40% and 45% increment, resp., and 20% after 30 min; $n = 5$ from 5 different animals for each group $p < 0.05$, Figure 4). As control of gavage procedure and to verify the specificity of Miglustat action, we also evaluated ERKs activation in slices from WT mice treated either with saline or with Miglustat. We found that ERKs phosphorylation levels did not significantly change with respect to slices from untreated animals ($n = 4$ from 4 different animals for each group, Figure 4).

4. Discussion

In this study, we analyzed whether synaptic plasticity was altered in NPC1−/− mice, a well-established mouse model of the NPCD [12], and we proposed Miglustat as treatment to counteract the synaptic deficits. The main findings obtained from this study can be summarized as follows: (i) BST, which we previously found enhanced in NPC1−/− mouse slices, returned to normal values after Miglustat treatment; (ii) hippocampal PTP and LTP induction and maintenance were considerably reduced in slices from NPC1−/− mice whereas Miglustat *in vivo* administration was able to revert this impairment; (iii) application of a tetanic stimulation, which induced a rapid and strong increment in ERKs phosphorylation in WT hippocampal slices, failed to induce a significant ERKs phosphorylation in NPC1−/− mouse slices and Miglustat treatment was capable of restoring this activation; (iv) oral treatment of NPC1−/− mice with Miglustat was able to prevent the onset of symptoms *in vivo*.

Tetanic stimulation of Schaffer collaterals in CA3 area of the hippocampus of NPC1−/− mice induced a synaptic plasticity characterized by a partial inhibition of both PTP and LTP induction, whereas the maintenance phase was completely blocked. These results, obtained by recording the PS at the level of pyramidal layer, are partially in line with the observation of Zhou et al. [18], which demonstrated a defect in hippocampal LTP in NPC1 mutant mice with a reduction of field EPSP.

Posttetanic LTP in the hippocampal region has been widely studied and ascribed to the NMDA and AMPA receptor activity [19, 20]. The reduction of both PTP and LTP that we found in NPC1−/− slices could be caused by a malfunction of several different mechanisms correlated with these receptor activations. First, PTP in NPC mice could be affected by a decrease in neurotransmitter release due to a reduced elevation in presynaptic Ca2+ after tetanic conditioning. Indeed, several studies support the important role played by cholesterol and membrane rafts in the neurotransmitter release process. For example, it has been reported

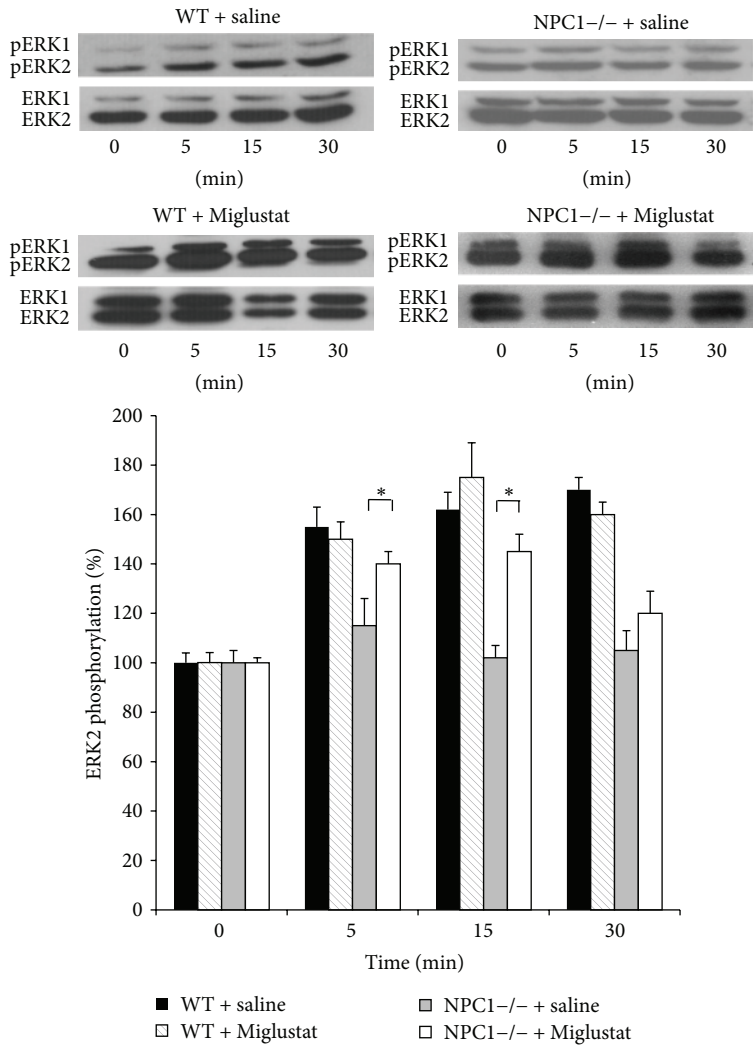

Figure 4: Effects induced by Miglustat treatment on the activation of ERK1/2 during LTP in WT and NPC1−/− treated mice. The immunoreactive levels of activated and total kinases at the indicate times are shown for a representative experiment in the upper panels; normalized values (means ± SEM) of activated kinases are illustrated in the lower panel ($n = 5$ for NPC1−/− mice treated with Miglustat; $^{*}p < 0.05$ versus untreated NPC1−/− mice at the corresponding time point).

that exocytosis of synaptic vesicles, including Ca2-dependent glutamate release, is impaired following a decrease in cholesterol levels [21, 22]. Furthermore, in a previous paper, we provided evidence that agents interfering with plasma membrane cholesterol (methyl-beta-cyclodextrin (Cdex)) inhibit the NMDA-stimulated influx of calcium in hippocampal cells in culture [23]. Indeed, in another paper from our group, we demonstrated that Cdex strongly reduces synaptic transmission and blocks the expression of LTP [11].

The NPC1−/− mouse hippocampal neurons may present an impaired lipid domain organization that affects the NMDAR transduction pathway leading to an impairment of LTP induction. The hindering of the LTP maintenance phase could be due to a defective regulation of GluR1 AMPA receptor trafficking and a reduced exposure of these receptors on the cell surface as a consequence of the gangliosides and/or cholesterol accumulation. These results may be in agreement with Brachet et al. [10] who have recently demonstrated that NMDAR activation during LTP induction leads to a loss of redistribution of intracellular cholesterol in the neuron triggering AMPA receptor synaptic delivery and, in turn, synaptic potentiation.

Overall, these results indicate that a cholesterol dysmetabolism may be responsible for synaptic plasticity phenomena impairment, suggesting that the LTP impairment described in NPC1−/− mice could result from a defect in lipid transport system.

In WT mouse slices, we previously reported a rapid and strong increment in ERKs phosphorylation after delivery of LTP inducing HFS, which lasted for at least 30 min [14]. Application of a tetanic stimulation in NPC1−/− hippocampal slices was unsuccessful to induce a significant ERKs phosphorylation. Given the possibility of a reduced NMDA receptor activation dependent on cholesterol levels and thus

a reduced Ca2+ influx in NPC1−/− slices, it is reasonable to hypothesize that the lack of ERKs phosphorylation may depend on a failure of the transduction mechanism mediated by NMDAR [24].

In the present study, we observed that Miglustat administration is able to revert both the hyperexcitability previously reported in NPC1−/− mouse hippocampal slices [15] and the impairment of PTP and LTP. Following the hypothesis that the mechanism by which Miglustat is able to revert the impairment of synaptic plasticity in NPC1−/− mice involves ERKs phosphorylation, we expected that *in vivo* Miglustat administration could restore ERKs phosphorylation in stimulated NPC1−/− mouse slices. Indeed, Miglustat treatment was capable of reestablishing ERKs activation in slices from NPC1−/− mice suggesting a direct effect of the drug on the signal transduction pathways of synaptic potentiation.

Since 2009 Miglustat has been designated Orphan Medicinal Product by EMA for the treatment of neurological symptoms in NPC patients. Indeed, several advantages have been noticed in clinics during Miglustat therapy and in particular amelioration of neurological impairment of NPC patients, mostly in adolescent or adult onset, before irreversible neurological damage occurs. However, the reason for this amelioration has yet to be determined.

Our observations regarding a protective effect of Miglustat on NPC1−/− mice (Video supplementary material) are in agreement with the data reported by other authors although in our study we used a low dose of the drug (0.2 mg/kg/die by gavage) [25, 26]. The choice of gavage administration was made to give the drug in the exact amount whereas, in other studies, Miglustat treatment was carried out by feeding animals mixing the drug in the pellet diet (50–1200 mg/kg/day).

In vitro, Miglustat acts as an inhibitor of glucosylceramide synthase, the enzyme that regulates the first step of ganglioside synthesis and for this reason has been used to ameliorate several dyslipidosis, including Gaucher and GM1 gangliosidosis. Depletion of glycosphingolipids by Miglustat treatment reduces pathological lipid storage, improves endosomal uptake, and normalizes lipid trafficking in peripheral blood B lymphocytes from NPC patients [27]. The fact that Miglustat, which has no direct effect on cholesterol metabolism, corrects the abnormal lipid trafficking seen in B lymphocytes from NPC patients may indicate that glycosphingolipid accumulation could be an important pathogenetic event in NPC disease.

5. Conclusion

It has been recently proposed that, beyond its effect on glycosphingolipids synthesis, Miglustat could play other roles such as interfering with histones acetylation [28], blocking the oxidative stress (observed in NPC cell patients), or acting as chaperon to give the correct folding of mutated NPC1 [29]. A better understanding of the mechanisms of action of Miglustat is critical to improve its therapeutic role.

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

The Vitamin A Derivative All-Trans Retinoic Acid Repairs Amyloid-β-Induced Double-Strand Breaks in Neural Cells and in the Murine Neocortex

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The amyloid-β peptide or Aβ is the key player in the amyloid-cascade hypothesis of Alzheimer's disease. Aβ appears to trigger cell death but also production of double-strand breaks (DSBs) in aging and Alzheimer's disease. All-trans retinoic acid (RA), a derivative of vitamin A, was already known for its neuroprotective effects against the amyloid cascade. It diminishes, for instance, the production of Aβ peptides and their oligomerisation. In the present work we investigated the possible implication of RA receptor (RAR) in repair of Aβ-induced DSBs. We demonstrated that RA, as well as RAR agonist Am80, but not AGN 193109 antagonist, repair Aβ-induced DSBs in SH-SY5Y cells and an astrocytic cell line as well as in the murine cortical tissue of young and aged mice. The nonhomologous end joining pathway and the Ataxia Telangiectasia Mutated kinase were shown to be involved in RA-mediated DSBs repair in the SH-SY5Y cells. Our data suggest that RA, besides increasing cell viability in the cortex of young and even of aged mice, might also result in targeted DNA repair of genes important for cell or synaptic maintenance. This phenomenon would remain functional up to a point when Aβ increase and RA decrease probably lead to a pathological state.

1. Introduction

DNA damage, such as DNA single- or double-strand breaks (DSBs), is known to occur in aging [1, 2] as well as in Alzheimer's disease (AD) [2–6]. Recently DSBs were shown to be produced by Aβ or amyloid-β peptides [7, 8] through oxidative stress [9]. Furthermore, Aβ peptides not only increase neuronal vulnerability (e.g., apoptosis) in DNA-dependent Protein Kinase (DNA-PK) deficient mice, a key enzyme of the Nonhomologous End Joining (NHEJ) pathway involved in DSBs repair [10], but also reduce the activity of this enzyme [11, 12]. Thus, extracellular Aβ peptides trigger DSBs production and impair DSBs repair.

However, if numerous factors are contributing to the formation or increased levels of Aβ peptide, such as mainly age, the *apoE4* allele, cholesterol rich food, or glucocorticoid stress hormone dexamethasone, others factors, such as the *apoE2* allele and the growth factor BDNF, are neuroprotective [13, 14] or participate in "adaptive cellular responses" [15]. Among them, some even diminishes DNA damage. This is the case of glutamine that reduces etoposide-induced damage [16] and of NAD that attenuates Aβ-induced DNA damage [8].

In this study we were particularly interested in all-trans retinoic acid (RA) [17–20], a derivative of vitamin A [21]. RA is known to be involved in development, neuronal differentiation [22], spine formation through the RARα receptor [23], cell growth arrest in anticancer therapy [24], and memory decline in aging [25–27]. The neuroprotective role of vitamin A and RA in relation to AD and to the β-amyloid cascade—not to DNA DSBs—has intensively been studied,

for example, [28]. Firstly, RA was shown to increase, via its RARα receptor, the expression of the major α-secretase, ADAM10 (A Disintegrin and Metalloproteinase domain-containing protein 10), diminishing the production of Aβ peptides [29]. This effect is mediated by RA-responsive elements upstream of the ADAM10 coding region [30, 31]. RA can also inhibit the γ-secretase activity through activation of the Extracellular Signal Regulated Kinase, ERK1/2 [32]. Secondly, vitamin A and its derivatives appear to inhibit Aβ oligomerisation *in vitro*, Aβ deposition, and tau phosphorylation in AD mouse models [33]. Thirdly, RARα signaling removes Aβ plaques and induces Aβ oligomers clearance via Neprilysin and Insulin Degrading Enzyme [34]. On the contrary, Aβ is increased in the cerebral vasculature while RARα is decreased in the neocortex of rats maintained on a 1-year retinoid-deficient diet [35]. Finally, following a RA treatment in APP/PS1 double-transgenic mice, Aβ deposits, AICDs (the Amyloid precursor protein Internal C-terminal Domains), tau phosphorylation, and glial response were decreased, whereas spatial learning was improved [36].

RAR are major players in the neuroprotective effects of RA. RA by binding to them allows the formation of RAR/RXR heterodimers and the replacement of corepressors, such as HDAC (histone deacetylase), by coactivators, such as CBP (CREB-binding protein). The histone acetyltransferase activity of CBP [37] and the down-regulation of DNA methyltransferases [24] result in RA-dependent transcription. Indeed, RA hypomethylates promoters, such as the one of RARβ2 or of methyltransferases, altering gene transcription [24].

According to these data, we wondered if, as a consequence of local chromosome relaxation, DNA repair proteins [2], such as the catalytic subunit of DNA-PK and Ataxia Telangiectasia Mutated kinase (ATM), could be recruited at chromosome sites of RA-dependent gene expression and if DSBs could be repaired at these sites. We have investigated whether RA and its receptor (RAR) might be involved, not in prevention of Aβ synthesis, Aβ oligomerisation, and plaques removal, as already shown, but in repair of Aβ-induced DSBs. We showed—to our knowledge for the first time—that this is indeed the case by studying neuronal and astrocytic cell lines as well as neocortical tissue from young and old mice. Furthermore, we examined the repair mechanisms involved and the consequences on cell viability, in the search for effective early AD therapies.

2. Materials and Methods

2.1. Mice, Tissue Samples, and Dissections. C57BL/6J male mice (Janvier, Le Genest-St-Isle, France) were sacrificed at 4 months (young adults) and at 16 months (aged adults). Brains were quickly removed and cortices dissected under a binocular microscope, weighted, and homogenized (about 5 sec with a Polytron apparatus, VWR International) in neurobasal medium (Gibco Life Technologies). The homogenized tissue was immediately treated as described below. Animals were handled in accordance with Federal Swiss Veterinary regulations and approval.

2.2. Culture of Neuronal SH-SY5Y Cells and of Astrocytic DI TNC_1 Cells. Human SH-SY5Y cells (European Collection of Animal Cell Culture, UK) were routinely grown in a 37°C incubator containing 5% CO_2/95% humidified air in RPMI-1640 (Gibco Life Technologies) with 10% FCS (Bioconcept, Switzerland), 2 mM L-glutamine (Gibco Life Technologies), 100 IU/mL penicillin G, and 100 μg/mL streptomycin (Invitrogen). Cells were halved every 5 to 6 days. For this purpose, the confluent cells were released in DPBS (150 mM NaCl, 3 mM KCl, 1.5 mM KH_2PO_4, 7.9 mM $Na_2HPO_4{\cdot}2H_2O$, and 0.1 mM EDTA; pH 7.4), centrifuged at 1000 rpm during 5 min, and resuspended in RPMI-1640 and 10% FCS at the desired dilution.

The DI TNC_1 cell line [38, 39] was grown in DMEM medium (Sigma) supplemented with 10% FCS in a 37°C incubator containing 5% CO_2/95% humidified air. Confluent cells were released in trypsin solution (0.25%), centrifuged at 1100 rpm during 5 min, and resuspended in DMEM with 10% FCS at the desired dilution.

Cells were photographed with an inverted phase contrast microscope (ZEISS Telaval 31) using a photo camera (Leica DFC 490) with the FireCam analyst software (Leica).

2.3. Tissue and Cell Treatments. For the cell treatments, two days before experiment, the medium with 10% FCS was replaced by the medium with 1% FCS. The cultured cells and the homogenized cortical tissue (see before) were treated or not with 20 μM monomeric Aβ_{1-42} peptides (Enzo Life Sciences), during the first 30 min, then with or without 5 μM RA (Sigma-Aldrich) for 30 more min, resulting in four combinations of 2 × 30 min treatments (Ø-Ø; Aβ_{1-42}-Ø; Ø-RA; Aβ_{1-42}-RA). Other treatments were carried out with 20 μM Aβ_{42-1}, 20 μM Aβ_{1-40} (Enzo Life Sciences), 10 μM RARα/β agonist Am80 (Santa Cruz Biotechnology), 1–50 μM RAR$\alpha/\beta/\gamma$ antagonist AGN 193109 (Labforce), 50 μM caspase-3 inhibitor z-VAD-FMK (Santa Cruz Biotechnology) [40], 3 μM HDAC inhibitor trichostatin A (TSA; Sigma) [41], 10 μM inhibitor of Ataxia Telangiectasia Mutated kinase or ATM KU 55933 (KU; Labforce), and 150 μM inhibitor II of the catalytic subunit of DNA-PK NU 7026 (NU; Calbiochem), in combination with RA and/or Aβ_{1-42} for 30 min or 24 h. All treatments were carried out in triplicate in a 37°C incubator containing 5% CO_2 and 95% humidified air.

2.4. Neutral Single-Cell Gel Electrophoresis (Comet Assay). DSBs were measured in SH-SY5Y cells, in DI TNC_1 cells, and in the homogenized cortical tissue using Trevigen Comet AssayTM kit (AMS Biotechnology, UK) with the following modifications. After treatment, cells were resuspended in ice-cold Ca^{2+} and Mg^{2+} free PBS to a concentration of 1.0 to 1.5 × 10^5 cells/mL. The same number of cells from the dissociated cortical tissue was resuspended in the same solution with the addition of 20 mM EDTA and processed as for the cell cultures. An aliquot of 50 μL cells was added to 500 μL of 1% molten low-melting agarose (Seaplaque, FMC BioProduct, USA) kept at 42°C. Fifty microliters was immediately spread on a comet slide (AMS Biotechnology), which was incubated at 4°C in the dark for 10 min to accelerate agarose gelling and then transferred to prechilled lysis solution (AMS

Biotechnology) for 60 min at 4°C. Subsequently, the slide was incubated in Neutral Electrophoresis Buffer (500 mM Tris base, 1.5 M sodium acetate, pH 9.0) for 30 min at 4°C. DSBs were separated by electrophoresis at 26 V for 45 min. Then, the slides were immersed in 70% ethanol at room temperature for 30 min and air-dried. DNA was stained 10 min at room temperature with 100 μL SYBR Green I dye (Gibco Life Technologies) diluted 1 : 1000 in water and then rinsed with distilled water. Comets of at least 30 cells per treatments were immediately photographed using an Olympus digital camera attached to an Olympus BX51 epifluorescence microscope (Axio vision rel. 4.6).

2.5. Analysis of DSBs on Agarose Gel Electrophoresis. To visualize the average tail length of a treated cell population, 10 μL of SH-SY5Y cells (1×10^5 cells/mL) was mixed with 5 μL lysis buffer (AMS Biotechnology) for 5 min at 4°C and then 1.5 μL Tris EDTA 10x was added. The whole sample was loaded into a slot of 1% agarose MP gel (Roche Life Science). A 1 kb ladder (Invitrogen) was used as a size marker. Electrophoresis was run for 40 min at 66 mV. The agarose gel was stained by ethidium bromide (10 mg/mL) and analyzed with a GS-700 imaging densitometer (Bio-Rad). Signal intensities were measured with the molecular analyst software program (Bio-Rad).

2.6. Immunocytochemistry. Cortices of 4- ($n = 3$) and 16-month-old C57BL/6J male mice ($n = 3$) were mechanically dissociated and fixed for 30 min at room temperature in 4% paraformaldehyde in PBS on coverslips pretreated with 100% alcohol. After rinsing for 3 × 5 min with PBS, cells were incubated for 1 h30 with the primary antibody diluted in PBS. The mouse monoclonal anti-bIII-tubulin antibody (Sigma), diluted 1 : 1000 in PBS, and the mouse monoclonal anti-glial fibrillary acidic protein antibody (GFAP, Sigma), diluted 1 : 500, were used. After rinsing for 5 min with PBS, coverslips were incubated for 1 h at room temperature with the secondary anti-mouse IgG antibody coupled to Alexa-Fluor 488 (Molecular probes/Invitrogen), diluted 1 : 1000, in presence of Dapi (1.0 μg/mL, Sigma) and rinsed for 3 × 5 min with PBS, and then mounted on glass slides fixed with Fluorsave (Calbiochem).

Nine to twelve pictures of immunostained cells, corresponding to 34 to 69 cells, were taken for each mouse using a Zeiss Axioskop 2 plus microscope (Carl Zeiss, Feldbach, Switzerland) equipped with epifluorescence and were digitalized with an Axiocam camera.

2.7. Cell Viability Assay. For assessing cell viability of SH-SY5Y cells and DI TNC_1 cells, 20000 cells/well were grown in 1 mL RPMI-1640 with 10% FCS for 6–8 days and, two days before the experiment, the medium was replaced by RPMI-1640 with 1% FCS. For the cortical tissue, 1 mg freshly homogenized tissue was resuspended in 1 mL of the same medium. The cells were treated or not during 30 min with 5 μM RA and/or 20 μM $A\beta_{1-42}$ monomers or 3.3 μL digitonin (CellTiter-Glo Luminescent Cell Viability Assay, Promega) used as a positive control to decrease cell viability. 100 μL of cell suspension was shaken for 2 min with 100 μL of CellTiter-Glo Reagent prepared according to the manufacturer (CellTiter-Glo Luminescent Cell Viability Assay, Promega) to induce cell lysis and to release the cell ATP content, as indicator of metabolic activity. After 10 min incubation at room temperature, luminescence (in relative light units) was recorded with a luminometer (GloMax 20/20, Promega). Medium without cells or tissue samples resulted in background luminescence.

2.8. Caspase-Glo. For assessing caspase-3 and caspase-7 activation, SH-SY5Y cells were grown in 1 mL RPMI-1640 with 10% FCS. Two days before the experiment, the medium was replaced by RPMI-1640 with 1% FCS. Cells were treated or not during 30 min or 24 h with 20 μM $A\beta_{1-42}$, 50 μM z-VAD (in DMSO, Merk), or 100 nM staurosporine (Sigma-Aldrich). 100 μL of cell suspension was mixed for 2 min with 100 μL of Caspase-Glo 3/7 Reagent prepared according to the manufacturer (Caspase-Glo 3/7 Assay, Promega) to induce cell lysis and cleavage of a luminogenic substrate of caspase-3/caspase-7. After 1 h30 incubation at room temperature, luminescence was recorded with a luminometer (GloMax 20/20, Promega). A control without cells was also used.

2.9. Statistical Analysis. For the comet assay, values of mean comet tail length were compared for each condition by a one-way analysis of variance (ANOVA) to establish the effects of various treatments. When overall statistically significant differences in treatments effect were obtained by ANOVA, comparisons of means among subgroups were made after Bonferroni corrections. In parallel, the nonparametric Kruskal-Wallis test was used to compare the shape of comet tail distribution also with Bonferroni corrections to compare subgroups. The Kruskal-Wallis analysis is particularly suitable when the number of measures is small (less than 15 per group) or when the distribution is not Gaussian (asymmetric box plot). Level of significance is $P < 0.05$. Analyses were carried out with the Stata 13.1 software (Stat Corp., TX, USA, 2013).

3. Results

3.1. Retinoic Acid Repairs Aβ-Induced DSBs in SH-SY5Y and DI TNC_1 Cells. To demonstrate that RA can repair $A\beta$-induced DSBs, SH-SY5H cells and astrocytic DI TNC_1 cells were treated with $A\beta_{1-42}$ for half an hour before the addition or not of RA for also 30 min. The presence of RA resulted in shorter tail lengths comparable to untreated lysed cells (Figures 1(a) and 1(b)). The mean comet tail length was significantly higher in $A\beta$ treated SH-SY5Y cells as well as DI TNC_1 cells compared to all other treatments (Figures 1(c) and 1(d)). These results were corroborated by an independent experiment showing on an agarose gel comet tails starting from their cell nuclei loaded into the gel's slots. Short DNA fragments—between about 0.85 kb and 3.0 kb—were generated more frequently when $A\beta$ was present and were reduced in number in presence of RA (Figure 1(e)). Apoptotic fragments of $n \times 180$ bp could not be detected,

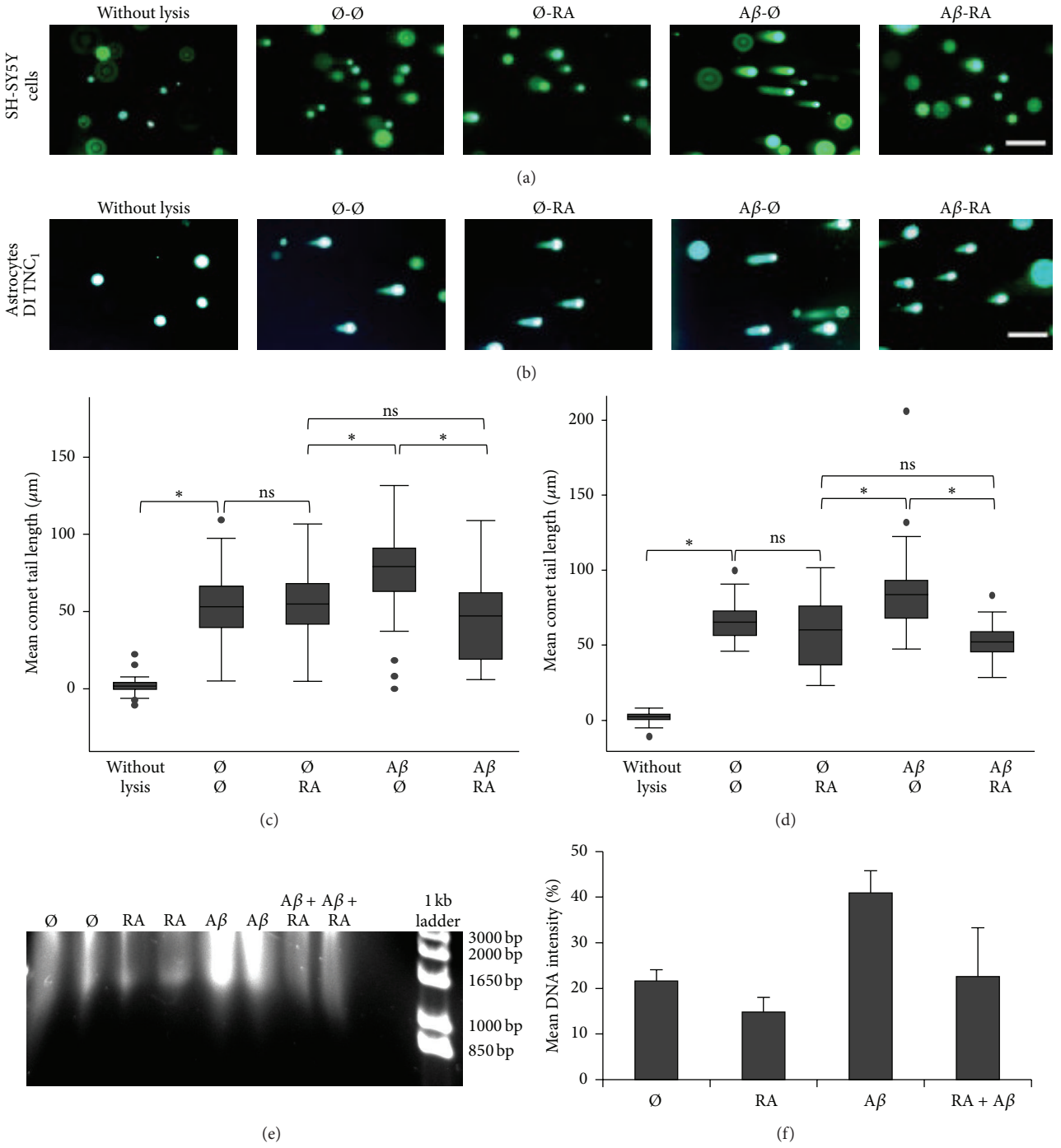

FIGURE 1: RA repairs Aβ-induced DSBs in SH-SY5Y and astrocytic DI TNC_1 cells. (a) Representative pictures of comets with various tail lengths of SH-SY5Y cells or of (b) DI TNC_1 cells following 30 min RA (5 μM) and/or 30 min Aβ (20 μM) treatments. Ø = without treatment for 30 min; scale bar: 200 μm. (c) Box plots of mean comet tail lengths of SH-SY5Y cells (number of cells measured: $31 < n < 53$) and of (d) DI TNC_1 cells ($31 < n < 36$). ANOVA with Bonferroni correction: $^*P < 0.05$; ns = not significant. (e) Agarose gel electrophoresis showing comet tails of about 1×10^3 lysed SH-SY5Y cells per well treated with RA (5 μM) and/or Aβ (20 μM). Each treatment was carried out twice. A 1 kb ladder (Invitrogen) was used. (f) Graphical representation of mean optical DNA intensities for each duplicated treatment in (e). It shows that RA decreases the amount of Aβ-induced DSBs and corroborates Fig. (c). The DNA intensities were measured for 1 kb to about 20 kb DNA.

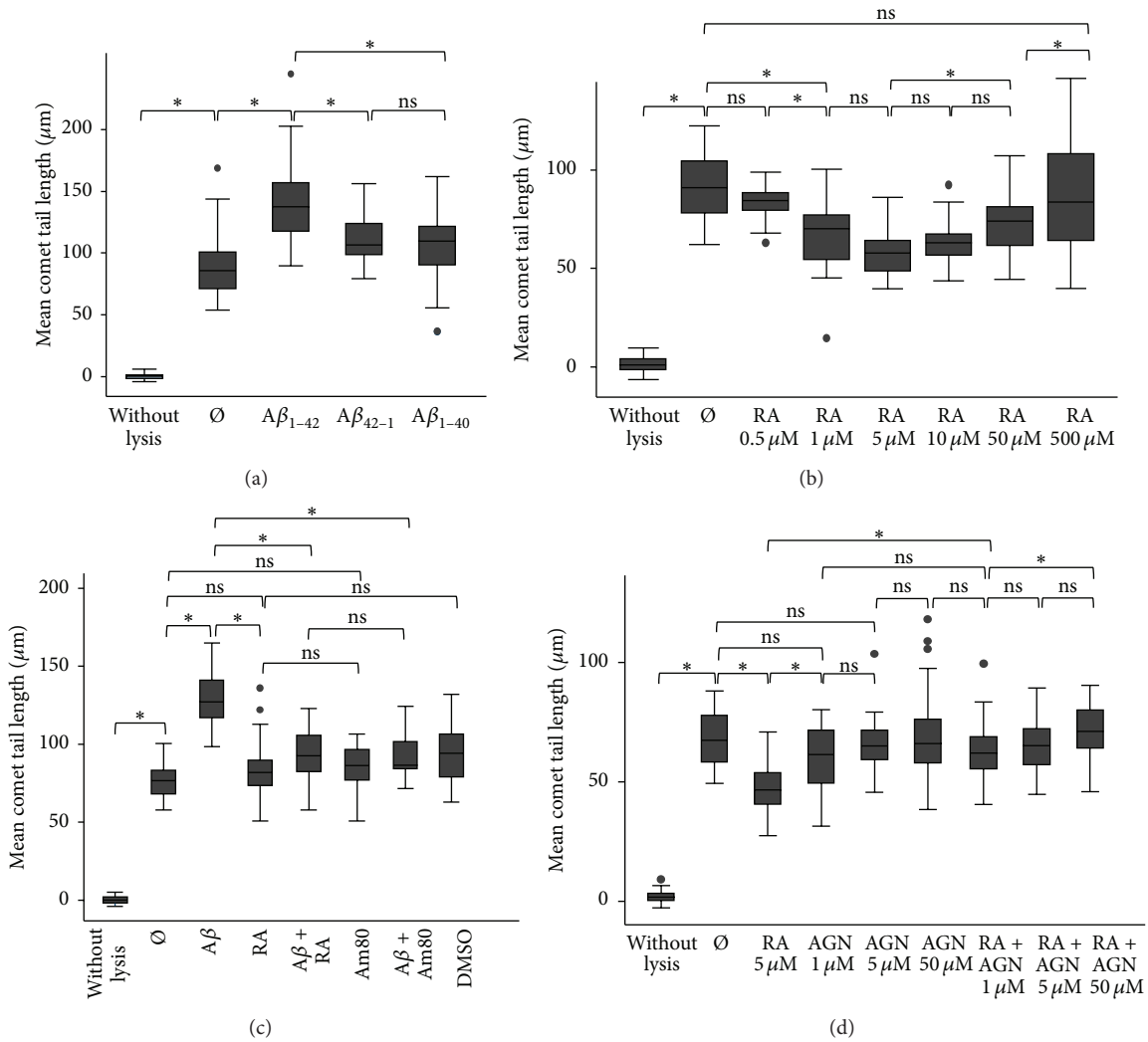

Figure 2: Control experiments for the effects of Aβ and RA on mean comet tail lengths in SH-SY5Y cells. (a) Box plot of mean comet tail lengths according to 30 min treatments with $A\beta_{1-42}$ and control peptides $A\beta_{42-1}$ or $A\beta_{1-40}$ (20 μM Aβ; number of cells measured: $33 < n < 48$). (b) Box plot of mean comet tail lengths depending on 30 min treatments of various RA concentrations ($25 < n < 35$). (c) Box plot of mean comet tail lengths according to 30 min treatments with 20 μM $A\beta_{1-42}$, 5 μM RA, 10 μM RARα/β agonist Am80, and its solvent DMSO (1% v/v), demonstrating a similar specific effect of RA and of Am80 in SH-SY5Y cells ($26 < n < 41$). (d) Box plot of mean comet tail lengths according to the RAR$\alpha/\beta/\gamma$ antagonist AGN 193109 (1–50 μM AGN) in presence or absence of 5 μM RA ($28 < n < 49$). The reduced mean tail length due to RA is abolished in presence of AGN. ANOVA with Bonferroni correction: $^{*}P < 0.05$; ns = not significant; Ø = without treatment for 30 min.

suggesting that apoptosis was not activated under these 30 min treatments. Quantification of signals intensities up to the gel's slots revealed that DSBs were almost halved when RA was added after Aβ (Figure 1(f)).

3.2. Retinoic Acid Repairs $A\beta_{1-42}$-Induced DSBs in a Concentration-Dependent Manner via RARα/β in SH-SY5Y Cells. We demonstrated that DSBs are induced specifically by the $A\beta_{1-42}$ peptide and not by the nonpathological $A\beta_{1-40}$ form or a control peptide with the reverse sequence $A\beta_{42-1}$, all treatments being carried out for 30 min with 20 μM Aβ peptides (Figure 2(a)). A similar result was obtained with DI TNC_1 cells. Furthermore, a dose-response curve (Figure 2(b)) showed that RA repairs DSBs most efficiently at concentrations between 1 μM and 50 μM, with a peak of maximal efficiency at 5 μM, whereas it had no effect at 0.5 μM or 500 μM. Using a cell viability assay, we observed that a treatment with 0.5 μM RA (101.3 ± 15.3%) and 1 μM RA (103.8 ± 8.6) resulted in a similar viability compared to that of the untreated cells (100 ± 10%). However, the viability increased at 5 μM RA (135.8 ± 30.8%), 10 μM RA (152 ± 12.7%), and 50 μM RA (126 ± 30.2) but dropped at 500 μM RA (27.1 ± 2.1) ($n = 3$).

Furthermore, we demonstrated in SH-SY5Y cells that the RARα/β agonist Am80 used at a concentration of 10 μM had the same effect compared to RA (Figure 2(c)). Both significantly reduced Aβ-induced DSBs. Moreover, no significant difference was observed between Am80 and RA suggesting that in SH-SY5Y cells the RARα/β receptor was sufficient to

mediate all DSBs repair activity, thus excluding the involvement of other potential receptors, such as PPARβ/δ [42]. Finally, the addition of 1 μM RAR$\alpha/\beta/\gamma$ antagonist AGN 193109 to 5 μM RA significantly impaired the diminution of DSBs due to the RA treatment. This effect was even increased with a higher concentration of AGN (50 μM compared to 1 μM), clearly demonstrating that the binding of RA to its RAR is needed to repair DSBs (Figure 2(d)).

3.3. Retinoic Acid Repairs Aβ-Induced DSBs in the Neocortex of Young and Aged C57BL/6J Mice. RA added half an hour after the Aβ_{1-42} treatment can also repair DSBs in cells originating from the murine cortex of young ($n = 3$) and aged C57BL/6J mice ($n = 3$). The presence of RA resulted in shorter tail lengths comparable to untreated lysed cells and cells treated with RA alone (Figures 3(a) and 3(b)). The mean comet tail length was significantly higher in Aβ treated cortical cells compared to all other conditions in the young as well as in the aged mice (Figures 3(c) and 3(d)). However, the difference in mean comet tail lengths between the Aβ treatment and the other conditions was less important in the aged compared to the young mice possibly due to a decreased metabolism. Indeed, the difference between the Aβ and the Aβ + RA treatment was statistically different in all 3 young mice, but only in 2 aged mice out of 3. We further showed by immunocytochemistry (Figure 3(e)) that the cells analyzed in the comet assay consist in neurons (68% for pooled age groups) as well as astrocytes (26%) and that the ratio of astrocytes/neurons (0.38) was similar to that reported in the literature [43] (Figure 3(f)).

3.4. Histone Deacetylase Inhibitor Trichostatin A and/or Retinoic Acid Induce DSBs Repair through DNA-PK and ATM in SH-SY5Y Cells. To ascertain the mode of action of RA via its receptor RAR, SH-SY5Y cells were treated with histone deacetylase (HDAC) inhibitor trichostatin A (TSA). We observed that TSA decreased mean tail length produced by Aβ as efficiently as RA (Figure 4(a)). Moreover, DSBs were significantly decreased in cells treated with TSA + RA compared to RA treated cell, suggesting a possible synergetic effect of both chemicals as they act at different sites of the same receptor complex [37].

Moreover, the inhibition of the RA repair activity was shown to be mediated both by ATM using ATM inhibitor KU 55933 and by DNA-PK using DNA-PK inhibitor NU 7026 (Figure 4(b)). The ATM inhibitor induced as many DSBs as the DNA-PK inhibitor or the Aβ treatment. In presence of RA, inhibition of both pathways, but not of only one, produced a similar amount of DSBs as the Aβ treatment. Thus, both ATM and DNA-PK protein kinases need to be present, so that RA is able to repair DSBs in SH-SY5Y cells.

3.5. Effects of Aβ on Apoptosis and of RA on Cell Viability. SH-SY5Y cells were treated with Aβ and/or caspase-3 inhibitor z-VAD. Staurosporine was used as positive control for apoptotic cell death. Only this last compound resulted in cell death after 24 h, but not after 30 min treatment (Figure 5(a)). However, in these cells, caspase-3 was already active after 30 min as demonstrated by z-VAD treatment (Figure 5(b)). Staurosporine was activating caspase-3 and caspase-7 after 30 min and even more after 24 h compared to the untreated cells. However, statistical significance was not reached comparing control treatment to staurosporine treatment, even if a tendency towards significance increased at 24 h ($P = 0.08$) compared to 30 min ($P = 0.92$). After 30 min, caspases were already activated but cell death was not observed, whereas after 24 h cells were dead and caspases could not be activated further. A comet assay carried out under the same conditions (Figure 5(c)) revealed that the mean comet tail length was not diminished by z-VAD in presence of Aβ after 30 min compared to the Aβ treatment. In this case, DSBs were not due to activated caspase-3. After 24 h, the mean comet tail length was significantly reduced by z-VAD in presence of Aβ suggesting the production of DSBs due to activated caspase-3. Overall, Aβ-induced DSBs appear not to be related to proapoptotic events after 30 min treatment.

Furthermore, cell viability was measured by quantification of intracellular ATP in comparison to cells lysed by digitonin. After 30 min Aβ treatment in presence or not of RA, DI TNC_1 cell viability was neither decreased nor increased, whereas it was the case subsequent to the digitonin treatment (Figure 5(d)). An identical observation was made for SH-SY5H cells (not shown). Cells from 4-month-young neocortical tissue showed after 1 h a much higher viability than cells from 16-month-old tissue (Figure 5(e)), suggesting a higher fragility of the latter tissue in the *in vitro* conditions. Overall viability dropped after 30 min Aβ treatment due to decreased intracellular ATP in the young tissue ($P = 0.001$). However, this decrease was less significant when RA was added to Aβ for both young and old cortical tissues ($P = 0.036$). We observed an increase in viability of about 53% for the young tissue and of about 59% for the old tissue, indicating that under the experimental conditions RA enhances viability at a time when DSBs are being repaired.

4. Discussion

Our main observation is that RA is involved in Aβ-induced DSBs repair in neuronal and astrocytic cell lines as well as in the cortical tissue, likely in cortical neurons as well as astrocytes, and in young and even in aged mice. Truly the addition of RA after DSBs production by Aβ_{1-42} treatment can repair DSBs. This was shown by two independent methods: comet assay and gel electrophoresis with SH-SY5Y neuroblastoma cells. Thus, according to these data, RA and its receptor (RAR) are involved not only in the prevention of Aβ synthesis, Aβ oligomerisation, and plaques removal, as already shown [29, 32–34, 36], but also in the Aβ-induced DSBs repair.

4.1. Aβ_{1-42} Induces DSBs with a Pathological Potential. We choose to grow SH-SY5Y and DI TNC_1 cells in presence of FCS 1% instead of FCS 10%. In both cases, Aβ induced significantly more DSBs, but in the former condition cells were more differentiated as in the cortical tissue. L-Glutamine was also added as it is already known to protect against DSBs formation [16] and only the effect of RA had to be

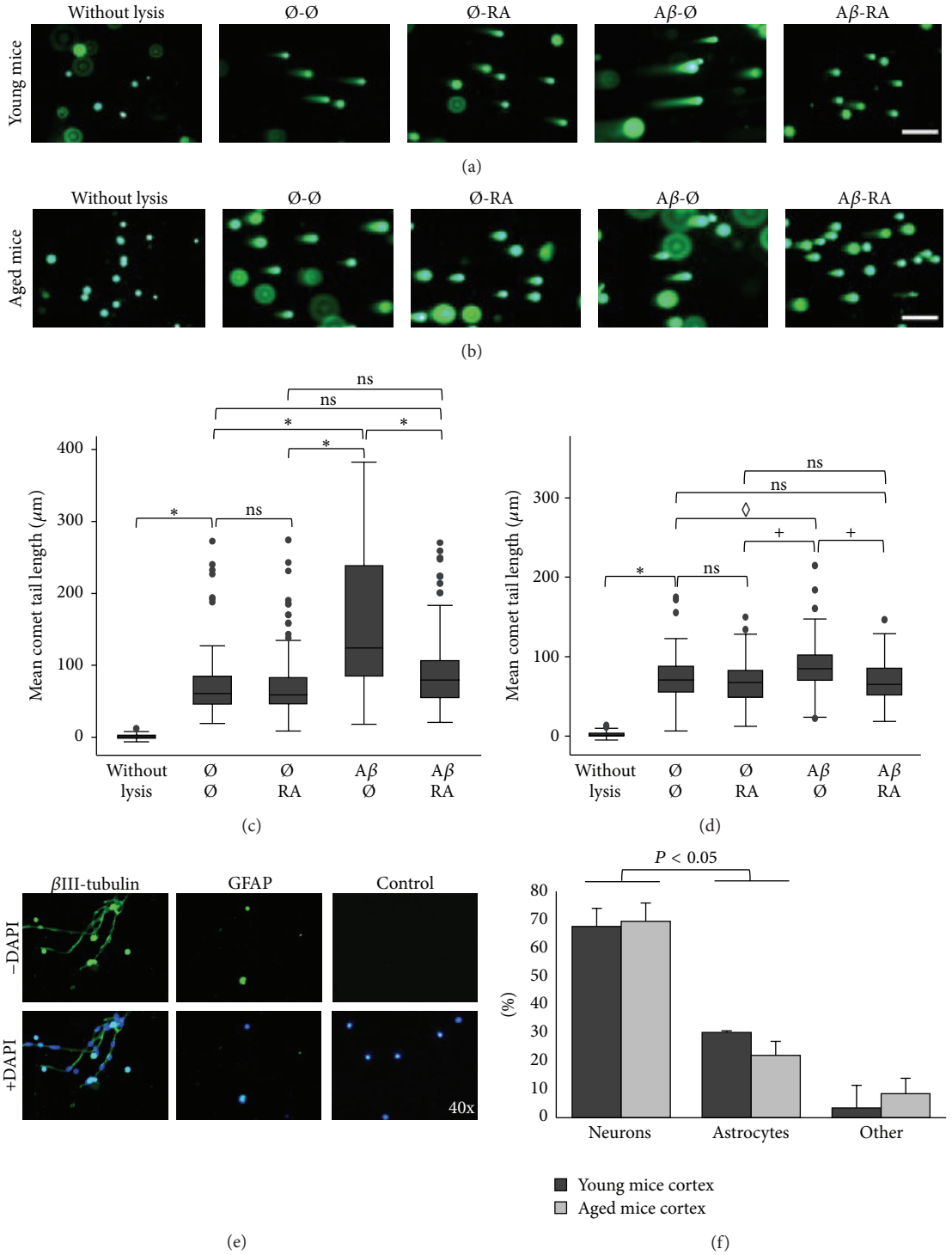

FIGURE 3: RA repairs Aβ-induced DSBs in the neocortex of C57BL/6J mice. (a) Representative pictures of comets with various tail lengths of cortical cells originating from young (4 months; $n = 3$ mice) or (b) aged (16 months; $n = 3$) mice following 30 min RA (5 μM) and/or 30 min Aβ (20 μM) *in vitro* treatments. Ø = without treatment for 30 min; scale bar: 200 μm. (c) Box plots of mean comet tail lengths of 3 young (number of cells measured: $31 < n < 56$) and of (d) 3 aged mice ($30 < n < 51$). Statistical analyses (ANOVA with Bonferroni correction) revealed significant differences in all 3 young mice ($^{*}P < 0.05$), whereas only 2 out of 3 ($^{+}P < 0.05$) or 1 out of 3 aged mice ($^{\Diamond}P < 0.05$) reached statistical significance; ns = not significant. (e) Immunofluorescent pictures of cortical cells of young mice stained with anti-βIII-tubulin to mark neurons, anti-GFAP to label astrocytes, and DAPI as a nuclear marker. A similar study war carried out with cortical cells of aged mice. (f) Proportions of neurons, astrocytes, and other cell types in the cortex of young ($n = 3$) and of aged mice ($n = 3$). Number of cells analyzed for each mouse: $33 < n < 70$. $P < 0.05$: comparison between neurons and astrocytes.

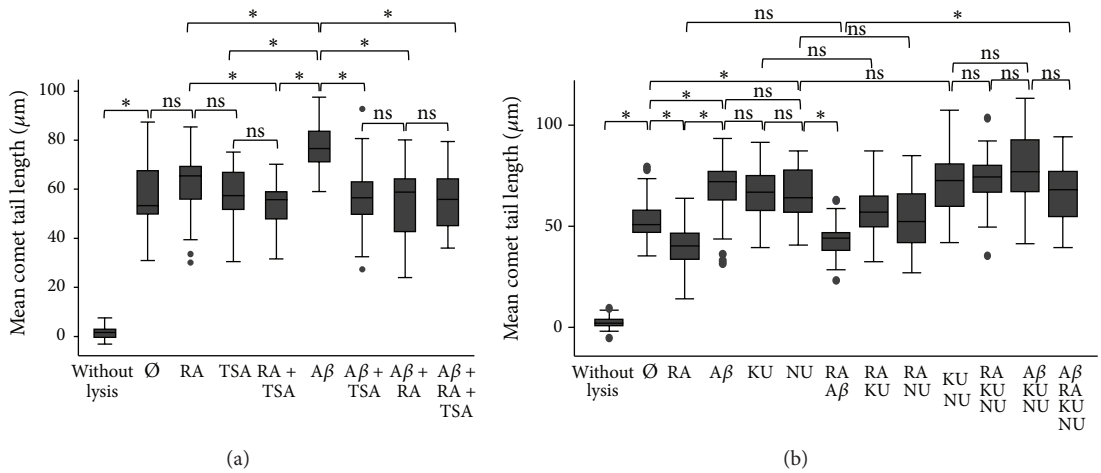

Figure 4: Repair pathways of Aβ-induced DSBs. (a) Box plot of mean comet tail lengths of SH-SY5Y cells (number of cells measured: $31 < n < 47$) after 30 min treatments with RA (5 μM), the HDAC inhibitor TSA (3 μM), and Aβ (20 μM) demonstrating the involvement of HDAC. (b) Box plot of mean comet tail lengths of SH-SY5Y cells ($20 < n < 42$) after 30 min treatments with RA (5 μM), Aβ (20 μM), KU 55933 (ATM inhibitor, 10 μM), and NU 7026 (DNA-PK inhibitor, 150 μM) demonstrating the involvement of ATM and of DNA-PK. ANOVA with Bonferroni correction: $^{*}P < 0.05$; ns = not significant; Ø = without treatment for 30 min.

tested. L-Glutamine was equally added to the homogenized cortical tissue but B27 supplement was avoided as it contains RA. Interesting was the observation that only the most pathological form of Aβ, Aβ_{1-42}, was inducing DSBs in both of the SH-SY5Y and DI TNC_1 cells, suggesting that Aβ_{1-42}-induced DSBs might play a role in Alzheimer's pathology. Noteworthy is also the fact that Aβ_{1-42} treatment appeared to be less efficient in inducing DSBs in the aged than in the young murine cortical tissue. This cannot be due to the state of the Aβ that corresponds to a mixture of monomer and oligomers in both cases [44] but rather to the state of the tissue, such as a lower metabolism of the aged cortical tissue.

4.2. RA Has a Counteracting Effect on DSBs Production. RA has been shown to reduce DSBs in a concentration-dependent manner in SH-SY5Y cells. 5 μM RA appears to be optimal for decreasing DSBs and increasing cell viability, that is, ATP concentration, compared to the untreated cells. Higher or lower RA concentrations resulted in unchanged DSBs levels compared to the control cells, whereas increased RA concentrations only decreased cell viability. This variation of RA effect according to its concentration is compatible with the observation that when neuronal activity is low, endogenous RA synthesis is locally activated in order to maintain the structures of neurons (homeostatic synaptic plasticity) and that conversely RA synthesis decreases when neuronal activity increases [20]. It has to be observed that the DSBs levels following the RA treatments were not consistently lower than the background DSBs levels of the untreated samples. This phenomenon might be due to variations of endogenous RA levels in relation to cell differentiation or division. Furthermore, RA was shown to decrease Aβ-induced DSBs through the RARα/β receptors by using the Am80 agonist and the AGN 193109 antagonist. The agonist being as effective as RA in reducing DSBs, an effect of RA through another receptor, for example, RXR-PPARβ/δ [42], appears excluded in SH-SY5Y cells. The observation that the HDAC inhibitor TSA, which enables derepression of Retinoic Acid Response Element-dependent gene expression, results in a similar decrease of Aβ-induced mean comet tail length as RA strengthens the role of RAR in repair of Aβ-induced DSBs. Reparation might occur either through expression of genes involved in DSBs repair or more directly through chromatin decondensation at sites of RA-dependent gene expression allowing access to proteins involved in DSBs repair. One of these proteins is DNA-PK of the NHEJ repair pathway. However, its inhibitor NU 7026, used at a concentration of 150 μM, resulted only in a partial repair of Aβ-induced DSBs compared to the RA treatment. A concentration effect of NU was excluded as a dose-response curve demonstrated that mean comet tails lengths reached a maximum size at 150 to 250 μM NU and decreased significantly at 50 to 100 μM NU (data not shown). The ATM inhibitor, KU 55933, resulted equally in a partial repair of Aβ-induced DSBs. We showed in SH-SY5Y cells that both of DNA-PK and ATM, known to be complementary [45, 46], enable the complete counteracting effect of RA on DSBs production.

4.3. Adaptive Cellular Response due to RA-Mediated DSBs Repair: The DSB-PRAE Hypothesis. We observed that short-term (not long-term) treatment with Aβ is not decreasing cell viability or causing cell death and that caspase-3 was activated at the same level in presence of Aβ or not in SH-SY5Y grown with 1% FCS. Indeed, even staurosporine is known to induce neurite outgrowth in the short term before inducing cell death and apoptotic DNA fragments in the long term [47]. We also did not observe apoptotic fragments of $n \times 180$ bp after 30 min Aβ treatment, suggesting that apoptosis is not yet activated under these conditions. However, at that time, DSBs are induced by Aβ, and if not repaired in the longer term, they might be deleterious [48]. Furthermore, in the short time, RA can repair DSBs and appears even to increase viability in

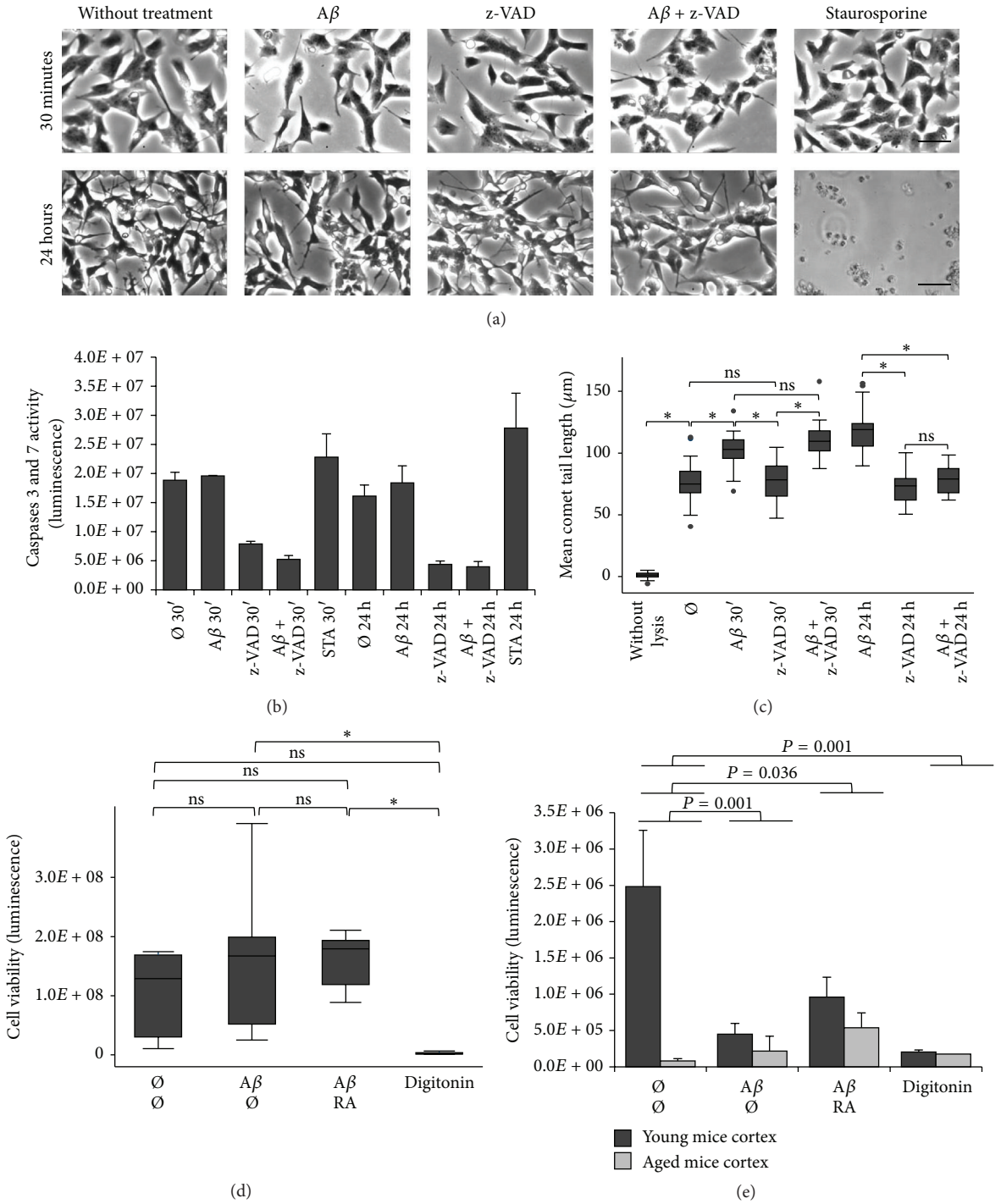

Figure 5: Aβ effects on the apoptotic cascade, on DSBs, and on cell viability in presence of the caspase-3 inhibitor z-VAD or RA. (a) Pictures of phase contrast microscopy with SH-SY5Y cells treated with 20 μM Aβ and/or 50 μM z-VAD did not show cell death, whereas treatment with 100 nM staurosporine resulted in apoptotic cell death after 24 h, not after 30 min. Scale bar = 200 μm. (b) Measures of caspase-3/caspase-7 (CaspaseGlo assay) after 30 min and 24 h treatment with Aβ and/or z-VAD or staurosporine (STA). Caspases were activated in the control cell cultures (Ø = without treatment) and in presence of Aβ after 30 min and 24 h but were not able to induce cell death. A nonsignificant increase of caspases between 30 min and 24 h resulted in cell death (a). (c) Box plot of mean comet tail lengths after treatments with Aβ and/or z-VAD shows that Aβ-induced DSBs in SH-SY5Y are not related to apoptotic DSBs after 30 min, whereas they are related to apoptotic DSBs at 24 h (number of cells measured: $27 < n < 38$). (d) Measures of viability (Glomax assay) of astrocytic DI TNC_1 cells treated for 30 min with RA (5 μM) and/or Aβ (20 μM) or digitonin (3.3 mM) ($n = 9$). A similar result was observed with SH-SY5Y cells, that is, no alteration of viability despite RA and/or Aβ treatments. (e) Measures of viability (Glomax assay) of cells from cortical tissue of young (dark gray; 4 months; $n = 3$) and aged mice (light gray; 16 months; $n = 3$) after treatments with RA and/or Aβ or digitonin during 30 min. Viability is less significantly decreased in presence of RA. ANOVA with Bonferroni correction: $^{*}P < 0.05$; ns = not significant; Ø = without treatment for 30 min.

the cortex of young and to a lesser extent of aged mice. The fact that RA is repairing DSBs as Am80 and trichostatin A and that both of them alter memorization [49, 50] suggests a similar role for RA. We make the hypothesis that whereas Aβ produces DSBs, RA results in targeted error-prone repair of the subset of DNA regions with RA-dependent gene expression, involved in particular in neuronal or synaptic maintenance [23]. This process of selective DNA repair might result in a neuronal state adapted to the metabolic changes occurring with aging. This phenomenon of "DSBs production and repair-dependent adaptive gene expression" (DSB-PRAE hypothesis) would remain functional up to a pathological threshold reached by Aβ increase and RA decreased response with aging and might be an explanation for the role of DSBs in memorization [7].

In conclusion, we have found that RA repairs Aβ-induced DSBs in neural cells of the cerebral cortex. Our data are in favor of a role of RA in increasing neural viability and also possibly in providing a neuronal adaptive response. However, this should be demonstrated notably in an AD mouse model. Furthermore, our data suggest that a better knowledge of the mechanisms involved in Aβ-induced DSBs might provide additional means to target pathological Aβ-induced changes, not just by impairing the Aβ-amyloid cascade but by repairing some of its consequences.

Conflict of Interests

The authors declare no actual or potential conflict of interests.

Authors' Contribution

Emmanuelle Gruz-Gibelli, Natacha Chessel, Clélia Allioux, Pascale Marin, and Françoise Piotton have carried out the experiments; Geneviève Leuba has contributed to the project's design and to the writing of the paper; statistics were made by François R. Herrmann; Armand Savioz has lead the work.

Acknowledgment

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Review Article

Current Research Therapeutic Strategies for Alzheimer's Disease Treatment

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Alzheimer's disease (AD) currently presents one of the biggest healthcare issues in the developed countries. There is no effective treatment capable of slowing down disease progression. In recent years the main focus of research on novel pharmacotherapies was based on the amyloidogenic hypothesis of AD, which posits that the beta amyloid (Aβ) peptide is chiefly responsible for cognitive impairment and neuronal death. The goal of such treatments is (a) to reduce Aβ production through the inhibition of β and γ secretase enzymes and (b) to promote dissolution of existing cerebral Aβ plaques. However, this approach has proven to be only modestly effective. Recent studies suggest an alternative strategy centred on the inhibition of the downstream Aβ signalling, particularly at the synapse. Aβ oligomers may cause aberrant N-methyl-D-aspartate receptor (NMDAR) activation postsynaptically by forming complexes with the cell-surface prion protein (PrPC). PrPC is enriched at the neuronal postsynaptic density, where it interacts with Fyn tyrosine kinase. Fyn activation occurs when Aβ is bound to PrPC-Fyn complex. Fyn causes tyrosine phosphorylation of the NR2B subunit of metabotropic glutamate receptor 5 (mGluR5). Fyn kinase blockers masitinib and saracatinib have proven to be efficacious in treating AD symptoms in experimental mouse models of the disease.

1. Introduction

Alzheimer's disease (AD) is currently incurable neurodegenerative condition which is highly prevalent in old age [1–3]. It was first described in 1906 by Alois Alzheimer, who analysed brain tissue from a patient who had died from an unknown mental illness. According to the Alzheimer's Association, 13% of people over 65 suffer from this disease in developed countries, where it is the fifth leading cause of death in patients at this age. According to the World Health Organization (WHO) estimates, the overall projected prevalence in global population will quadruple in the next decades, reaching 114 million patients by 2050 [2]. Apart from having a great social impact, this would clearly lead to increased economic burden to healthcare systems worldwide [1–3].

AD is classified according to the age of onset and whether it is developed spontaneously or as a result of genetic mutations. Familial AD (FAD) is an early-onset (sometimes as

Table 1

Activity	Compound	Clinical trial
Inhibitors of β-secretase	(i) E2609 (ii) MK-8931 (iii) LY2886721	(i) NCT01600859 (ii) NCT01739348 (iii) NCT01807026 and NCT01561430
Inhibitors and modulators of γ-secretase	(i) Semagacestat (LY450139) (ii) Avagacestat	(i) NCT00762411, NCT01035138, and NCT00762411 (ii) NCT00810147, NCT00890890, NCT00810147, NCT01079819
Selective γ-secretase modulators (SGSM)	(i) Ibuprofen, sulindac, indomethacin, and R-flurbiprofen (Tarenflurbil) (ii) NIC5-15	NCT00322036, NCT00105547
Nonsteroidal inhibitory of cyclooxygenase activity (NSAIDs)	CHF5074	NCT01203384, NCT01303744, NCT00954252
Inhibitors of Aβ aggregation	(i) Glycosaminoglycans 3-amino acid, 1-propanesulfonic synthetic (3APS, Alzhemed, tramiprosate) (ii) Colostrinin (iii) Scyllo-inositol compound (ELND005) (iv) PBT1 (clioquinol) and PBT2	Phase III in 2007
Modulation of β-amyloid transport from the brain to the peripheral circulation	(i) PF-0449470052 (ii) TTP4000 (NCT01548430)	(i) Phase II (ii) Phase I (February 2013)
Active immunotherapy	(i) Anti-Aβ42 vaccine (AN1792) (ii) CAD 106 (iii) ACC-001 (iv) ACI-24, MER5101 and AF205 (v) Bapineuzumab, solanezumab (vi) Gantenerumab (vii) Crenezumab (MABT5102A) (viii) Ponezumab (PF-04360365) (ix) MABT5102A, GSK933776A, NI-101, SAR-228810 and BAN-2401 (x) Gammagard	(i) Phase II (ii) NCT01284387, NCT00479557 and phase II NCT01227564 (rejected) (iii) Preclinical (iv) NCT01127633, NCT02008357 and NCT01900665 phase III (2012) (v) NCT01760005, NCT02051608 and NCT01224106 phase III (vi) NCT01343966, NCT01998841 phase II (April 2013) (vii) Phase I (viii) NCT00818662

early as 40 years of age) disease, which is caused by hereditary mutations and represents approximately 2% of diagnosed cases. The vast majority of patients suffer from the sporadic AD, which is subdivided into early- and late-onset forms. If identified in individuals under 65 years of age, early-onset diagnosis is given (3–5% prevalence), with the rest of the cases referred to as a late-onset AD (95–97% prevalence) [3–7]. In FAD, mutations in genes coding for amyloid precursor protein (APP; chromosome 21), presenilin 1 (PS1; chromosome 14) and presenilin 2 (PS2; chromosome 1), serve as triggers for beta amyloid (Aβ) formation, particularly of the long form of the peptide (Aβ1-42). In case of sporadic AD, a significant number of patients (approximately 25%) are carriers of the e4 allele of the ApoE gene (apolipoprotein E; chromosome 19), a lipid transport protein. The exact mechanism whereby ApoE contributes to increased Aβ levels is currently unknown [6–9].

Aging is considered the principal risk factor for sporadic AD development. Other potential risk factors including hypertension, dyslipidemia, metabolic syndrome and diabetes have also been identified [10–12].

In the present paper, we discuss treatment strategies structured according to a number of existing hypotheses aimed at explaining the origins of AD:

(a) amyloid cascade hypothesis,

(b) cholinergic hypothesis,

(c) dendritic hypothesis,

(d) mitochondrial cascade hypothesis,

(e) metabolic hypothesis,

(f) other hypotheses (oxidative stress, neuroinflammation).

The principal targets and clinical trials of the compounds aimed at reducing Aβ formation and plaques are summarized in Table 1. Relevant data for the molecules developed in the context of cholinergic, dendritic, mitochondrial cascade, metabolic and other hypotheses are presented in Table 2.

Table 2

Activity	Compound	Clinical trial
Inhibitors of Tau hyperphosphorylation: glycogen synthase kinase 3 inhibitors (GSK3β)	(i) Tideglusib (ii) NP031112 (iii) Sodium selenite (VEL015)	(i) NCT01350362 phase II (ii) NCT00948259 (iii) ACTRN12611001200976 phase II
Inhibitors of Tau aggregation	RemberTM, TRx 0237	NCT01626391, NCT01689233, NCT01689246 and NCT01626378
Microtubule stabilizers	(i) Paclitaxel (ii) Epothilone D (iii) TPI 287 (taxane)	(i) Clinical trial 2013 (interrupted) (ii) NCT01966666
Tau-specific immunotherapy	AADvac1 vaccine	NCT01850238 and NCT02031198 phase I trial (2013)
Anticholinesterase inhibitors	(i) Donepezil, rivastigmine, galantamine, (ii) Ladostigil (TV3326)	
PrPC–mGluR5–Fyn signaling	(i) Masitinib (ii) Saracatinib (AZD0530)	(i) NCT00976118 (ii) NCT01864655 and NCT02167256
5-HT6 receptor blockage	Lu-AE-58054 (SGS-518), PF-05212365 (SAM-531), SUVN-502, AVN-322, PRX-07034	Different phases of clinical trials
Antidiabetic drugs	(i) Rosiglitazone and pioglitazone (ii) Intranasal insulin (Humulin R U-100) (iii) Amylin and pramlintide (amylin analog)	(i) NCT00550420, NCT00348140 phase III (ii) NCT01767909 (iii) NCT01429623 and NCT01354691 phase II
Cdk5 inhibitors	Roscovitine and flavopiridol	

2. The Amyloid Cascade Hypothesis

Aβ peptide is derived from proteolysis of APP, an integral transmembrane protein found in different cell types, including neurons and glial cells [1–4]. In humans, alternative splicing produces multiple isoforms of the molecule, with APP695 being the most abundant in the brain [3]. APP is processed into smaller peptide fragments, one of which is Aβ, via cleavage by α-, β-, and γ-secretase enzyme protein complexes, which include presenilin and nicastrin molecules [8]. Under physiological conditions, APP is catabolized by the α-secretase to produce a soluble sAPPα fragment, which remains in the extracellular space, and a carboxy-terminal 83-amino acid (C83) fragment, which is anchored in the plasma membrane [8–10]. sAPPα is involved in the regulation of neuronal excitability, improves synaptic plasticity, learning, and memory, and increases neuronal resistance to oxidative and metabolic stresses [8]. In a neuropathological situation, APP is first preferentially cleaved by β-secretase 1 (BACE), which fragments APP into sAPPβ and a 99-amino acid membrane-bound fraction (C99). Additional processing of the C99 fragment by γ-secretase results in the generation of either Aβ(1-40) or Aβ(1-42) peptides, thought to be responsible for senile plaque formation [8–12]. Whilst sAPPα is beneficial to the organism, Aβ peptides may cause synaptic loss, decrease neuronal plasticity, alter energy metabolism, induce oxidative stress and mitochondrial dysfunction, and may provoke disruptions in cellular calcium homeostasis [8, 9].

The amyloid cascade hypothesis suggests that the formation, aggregation, and deposition of Aβ peptides, and especially Aβ(1-42), are a primary event in AD pathogenesis which triggers neurotoxicity and neurodegeneration [6–8]. Excessive extracellular Aβ may also presumably lead to increased Tau phosphorylation and the formation of neurofibrillary tangles. Molecular genetics studies into the mechanisms of FAD gave credence to this hypothesis, suggesting potential novel therapeutics, such as inhibitors of β- and γ-secretase or enhancers of α-secretase activity. However, in cases of sporadic AD, where Aβ generation does not appear to have a clear genetic basis, amyloid cascade hypothesis cannot fully explain the root causes of the disease [11–13].

2.1. Imbalance in the Generation/Removal of β-Amyloid in Alzheimer's Disease

Role of Neuroinflammation. It is believed that Aβ is generated continuously and its aggregation and subsequent plaque deposition in AD is concentration-dependent. Excessive accumulation of both soluble and insoluble Aβ may occur not only as a result of aberrant APP processing by β- and γ-secretase enzymes but may also be caused by inefficient removal of newly generated Aβ. Reduced activity of Aβ-degrading enzymes, such as neprilysin, insulin degrading enzyme (IDE), and angiotensin converting enzyme I (ACE I), may provoke an imbalance between the amyloid generation and clearance [13–19]. Additional predisposing factors, including the ApoE status and the presence of comorbidities such as metabolic syndrome and diabetes likely contribute to sporadic AD in a manner, which is still poorly understood.

A lack of direct correlation between amyloid plaque burden and memory loss in AD patients demonstrates that neurotoxicity is not solely dependent on the insoluble Aβ [14–16]. In fact, biochemical studies have demonstrated a good correlation between the levels of soluble Aβ oligomers in the brains of patients with AD and the degree of cognitive impairment [19]. It has been suggested that soluble Aβ-driven

synaptic loss may be responsible for neurodegeneration observed in AD. If that turns out to be the case, then central nervous system (CNS) inflammatory processes will likely be implicated [20, 21]. Neuroinflammation is a blanket term used to describe immune response in neurodegenerative diseases. It involves the activation of glial cells, especially microglia and astroglia. Under physiological conditions, microglial cells have a phagocytic function. In AD, activated microglia secrete a large number of molecules [21–23]. Such substances, among which are proinflammatory cytokines, prostaglandins, reactive oxygen species (ROS), and nitric oxide synthase (NOS), contribute to a chronic state of perpetual stress. A prolonged release of all these factors can eventually cause neuronal death [22, 23].

2.2. Antiamyloidogenic Pathway and Amyloidogenic Route as Strategies for Development of Therapeutic Treatments Modifying the Course of Alzheimer's Disease. In the last two decades, the pharmaceutical industry has focused primarily on the amyloidocentric approach, devoting substantial resources to develop effective AD drugs. However, multiple failures of drug candidates in clinical trials have led researchers to question the feasibility of this strategy [10–12]. One possible reason for failure is a lack of biomarkers which could reliably identify AD in relatively early stages. It is entirely possible that the patients currently recruited for phase III trials are in such advanced stages of AD that any attempted intervention is probably useless. Therefore, new diagnostic tools capable of early detection are sorely needed. In the meantime, there is still a number of novel treatments under development, which target the amyloidogenic route. In order to reduce Aβ generation from the APP, γ- and β-secretase inhibition and the potentiation of α-secretase activity have been considered.

Inhibitors and Modulators of β-Secretase. β-secretase enzyme complex participates in the initial stages of the amyloidogenic APP-processing pathway. The development of β-secretase inhibitors is a challenge because, besides the APP, this complex has many more substrates. To give just one example, neuregulin-1, which is involved in the myelination of CNS axons and synaptic plasticity, is a target of β-secretase [3]. Broad range of substrates can lead to significant side effects, even if the specific inhibition of the enzyme is achieved. Nevertheless, E2609 (clinical trial ID# NCT01600859), MK-8931 (NCT01739348), and LY2886721 (NCT01807026 and NCT01561430) have all shown efficacy in reducing Aβ production by up to 80–90% in the cerebrospinal fluid (CSF) in humans. None of β-secretase inhibitors have reached the market so far [3, 24–27].

Inhibitors and Modulators of γ-Secretase. The γ-secretase complex is responsible for the final stage of amyloidogenesis, leading to the generation of Aβ(1-40) and Aβ(1-42). γ-secretase inhibition was initially considered a promising disease-modifying strategy. However, substrate promiscuity presents similar issues facing β-secretase inhibitors [28–30]. Notch protein, responsible for regulating cell proliferation, development, differentiation, and cellular communication, is one of the targets of the γ-secretase [28]. Just as with the β-secretase inhibitors, off-target secondary effects are a major concern [30].

Semagacestat (LY450139) is a γ-secretase inhibitor that decreased Aβ levels in blood and CSF in humans [31]. The clinical study results, (NCT00762411, NCT01035138, and NCT00762411) which recruited over 3000 patients, are an example of the worst possible outcomes. It was reported that semagacestat treatment was associated with the worsening of cognition and the abilities to carry out the activities of daily living (ADAS-cog scale) in AD patients. Additional side effects included weight loss, increased incidence of skin cancer, and a higher risk of infection. Avagacestat is another γ-secretase inhibitor the development of which was discontinued as a result of a lack of efficacy (NCT00810147, NCT00890890, NCT00810147, NCT01079819) [32–34].

Selective γ-secretase modulators (SGSM) may, in theory, be developed in such a way as to avoid the adverse events associated with general enzyme inhibition. The goal of such treatments is to block APP processing without interfering with other signaling pathways like Notch [35].

SGSM development began with the observation that several nonsteroidal anti-inflammatory drugs (NSAIDs) decreased Aβ(1-42) peptide levels *in vitro* and *in vivo*. Examples of these drugs are ibuprofen, sulindac, indomethacin, and flurbiprofen [36]. The accepted mechanism of action (MOA) of NSAIDs is the inhibition of cyclooxygenase (COX) enzymes. While ibuprofen is a COX inhibitor, R-flurbiprofen (Tarenflurbil) is not, and its effects on the reduction of Aβ levels cannot be attributed to COX inhibition. Unfortunately, Tarenflurbil and Ibuprofen did not show efficacy for AD treatment in their respective clinical trials [36, 37]. CHF5074, just like R-flurbiprofen, is an NSAID devoid of COX inhibitory activity. *In vitro*, CHF5074 inhibited Aβ(1-42) production presumably by blocking γ-secretase complexes [38–41]. Recent studies have reclassified this compound as a microglial modulator based on its ability to reduce both amyloid burden and microglial activation [39]. Results from a phase II trial in patients with Mild Cognitive Impairment (MCI) indicate that CHF5074 treatment leads to improvements of several cognitive measures and reduces inflammatory marker levels in the CSF [38–40].

The idea that the long-term use of NSAIDs could confer some protection against AD generated some interest in NSAIDs as a treatment potentially useful for reducing Aβ(1-42) levels. However, negative results reported in clinical trials with NSAIDs suggest that this hypothesis requires further refinement [37].

Another example of a possible SGSM is NIC5-15, which is a naturally occurring molecule. NIC5-15, also known as pinitol, is a natural cyclic sugar alcohol [41]. This compound supposedly modulates γ-secretase and is reportedly capable of reducing Aβ production, while not affecting the substrate cleavage of Notch. No peer-reviewed data are available for this compound, so any reported results should be considered as a forward-looking statements requiring rigorous scientific proof. However, it is claimed that the compound improves cognitive function and memory in preclinical models of AD neuropathology. If true, these data suggest that NIC5-15 may be a suitable therapeutic agent for the treatment of AD for

two reasons: (a) it preserves Notch activity and (b) also it is potentially an insulin sensitizer. Moreover, it is supposedly being investigated as an anti-inflammatory inhibitor because it may prevent microglia activation. Once again, independent researchers have not yet confirmed these results.

2.3. Inhibition of β-Amyloid Peptide Aggregation. Aβ peptide aggregates give rise to amyloid plaques. The following compounds were developed in order to prevent senile plaque formation.

The only inhibitor of Aβ aggregation that reached phase III trials is the 3-amino-1-propaneosulfonic acid (3-APS, Alzhemed, tramiprosate) [42, 43]. This medication was designed to interfere with or antagonize the interaction of soluble Aβ with endogenous glycosaminoglycans. Glycosaminoglycans have been shown to promote aggregation of Aβ amyloid fibril formation and deposition [43]. However, the disappointing results of the phase III clinical trial in 2007 have led to the suspension of this compound in Europe [44].

Colostrinin, a complex of proline-rich polypeptides present in ovine, bovine, and human colostrum inhibits aggregation of Aβ and its neurotoxicity in cell assays, and improves cognitive performance in mice models [45]. Although a phase II trial showed slight improvements in Mini Mental State Evaluation in patients with mild AD in a treatment period of 15 months, this beneficial effect was not maintained after another 15 months of continuous treatment [45].

Scyllo-inositol (ELND005) is an oral amyloid antiaggregation agent capable of reducing Aβ toxicity in the mouse hippocampus. 18-month long phase II clinical trial with ELND005 was conducted in participants with mild-to-moderate AD. This dose-finding, safety and efficacy trial did not meet its primary clinical efficiency outcomes [46].

Clinical trials for AD treatment were also performed with metal chelating 8-hydroxiquinolines (8-HQ) compounds clioquinol and PBT2 [47]. While their mechanism of action is not completely understood, it is thought that these molecules block the interaction between the base metals and brain Aβ peptide. It was suggested that increased levels of oxidative stress in the brain of AD patients might be partially due to copper ions binding to Aβ, leading to metal-mediated generation of reactive oxygen species (ROS) [48–50]. It was also hypothesized that 8-HQs may prevent Aβ aggregation while simultaneously restoring homeostasis in cellular levels of copper and zinc ions [49, 50]. Unfortunately, these molecules failed in the phases II and III of clinical development due to lack of efficacy.

2.4. Compounds Which Promote the Removal of Amyloid Deposits and Aggregates. Another potential treatment option which is centered on the amyloidogenic pathway is to promote the clearance of existing amyloid aggregates and deposits. To achieve this, three different strategies have been evaluated.

2.4.1. Activation of Enzymes That Degrade Amyloid Plaques. Aggregates and amyloid plaques are degraded by multiple proteases including neprilysin, IDE, plasmin, endothelin converting enzyme, angiotensin converting enzyme, and metalloproteinases. Protein levels of these enzymes decrease in AD, which contributes to the formation and accumulation of Aβ [13–16]. Despite being an attractive strategy for developing disease-modifying drugs, no compounds with this MOA have ever reached advanced clinical development due to the lack of specificity.

2.4.2. Modulation of β-Amyloid Transport between the Brain and the Peripheral Circulation. Aβ transport between the CNS and the peripheral circulation is regulated by (1) apolipoproteins (e.g., Aβ may be transported from the blood to the brain when it is bound to APOE); (2) low-density lipoprotein receptor-related protein (LRP-1), which increases Aβ outflow from the brain to the blood; (3) receptor for advanced glycation end products (RAGE), which facilitates the transport of Aβ across the blood-brain barrier (BBB) [15, 51, 52].

The goal of any treatment, which is focused on this mechanism, is to reduce cerebral amyloid load by attempting to restrict Aβ to the peripheral circulation. To this end, a number of different strategies have been proposed, notably the peripheral administration of LRP-1. However, the only drug candidates that have reached clinical stage are the inhibitors/modulators of RAGE. These include PF-0449470052, which failed in phase II trials, and TTP4000, with the phase I trial completed in February 2013 (NCT01548430). The results of this trial have not been published.

2.4.3. Antiamyloid Immunotherapy

Active Immunotherapy. Immunotherapy strategy aimed to promote Aβ clearance with the objective of reducing the amyloid load in AD. Active immunization (vaccination) with either Aβ(1-42) (predominant form found in senile plaques) or other synthetic fragments has been successfully evaluated in transgenic mouse models of AD. Assays are generally based on the stimulation of B cells, T cells, and immune responses through activation of the phagocytic capacity of microglia. Human tests were initially promising; however treatment with the first-generation vaccine (AN1792) has produced serious adverse events that led to the discontinuation of the phase II trial. AN1792 consisted of a synthetic full-length Aβ(1-42) peptide with a QS-21 adjuvant. As a result of a T cell-mediated autoimmune response, 6% of patients have developed cerebral inflammation which turned out to be aseptic meningoencephalitis [53].

Second-generation vaccines were designed using a shorter Aβ(1-6) peptide segment in an attempt to prevent nonspecific immune response seen with the full-length vaccine. CAD 106, designed by Novartis, was the first second-generation vaccine that reached the clinical phases of development [54]. A recently completed phase II clinical trial have shown a Aβ-specific antibody response in 75% of treated patients, without causing adverse inflammatory reactions. ACC-001, developed by Janssen, has recently completed two-phase II trials (NCT01284387 and NCT00479557) with an additional phase II trial still ongoing (NCT01227564). However, the pharmaceutical company has abandoned the plans for further development of this vaccine. Other vaccines,

including tetra-palmitoylated Aβ(1-15) reconstituted in a liposome (ACI-24), MER5101 and AF205 are currently in various stages of preclinical development [55–58].

Passive Immunization. It is the administration of monoclonal or polyclonal antibodies directed against Aβ. This therapy consists of the intravenous administration of anti-Aβ antibodies to the patient. An advantage of this strategy compared to active immunization is that the proinflammatory T cell-mediated immune reactions should not occur. Studies in transgenic animals have shown that passive immunization reduces cerebral amyloid load and improves cognition, even when the amyloid plaque numbers are not significantly reduced. This could be attributed to the neutralization of soluble amyloid oligomers, which are increasingly recognized to play a fundamental role in the pathophysiologic cascade of AD.

Bapineuzumab and solanezumab are two monoclonal antibodies that have reached advanced stages of clinical development [59]. However, in 2012, two phase III clinical trials had failed because of a lack of efficacy in patients with mild-to-moderate AD. Both bapineuzumab and solanezumab are humanized monoclonal antibodies against Aβ(1-6) and Aβ(12-28), respectively [60, 61]. In case of bapineuzumab, significant reduction in brain amyloid plaques and phosphorylated Tau in cerebrospinal fluid was reported. However, the treatment failed to produce significant improvements of cognitive function. In a solanezumab trial, infusions of 400 mg of solanezumab or placebo were administered once a month for 80 weeks in patients with mild-to-moderate AD. The results suggested that solanezumab may improve cognition in mild AD; however statistical significance was not achieved in that study [61]. Currently solanezumab is in phase III trials in patients with AD (NCT01127633 and NCT01900665) and in older individuals who have normal thinking and memory function but who may be at risk of developing AD in the future (NCT02008357).

Another monoclonal antibody, gantenerumab, is being investigated in people at risk of developing presenile AD due to genetic mutations. NCT01760005 trial is still recruiting participants and will determine the efficacy of both gantenerumab and solanezumab in the prodromal disease stages [62–64]. In parallel, two additional phase III trials of gantenerumab in patients with mild AD (NCT02051608) and prodromal AD (NCT01224106) are ongoing. Gantenerumab is a fully human IgG1 antibody designed to bind with high affinity to a conformational epitope on the β-amyloid fibres. Microglia recruitment and ensuing phagocytosis will presumably lead to amyloid plaque degradation. Experimental studies in transgenic mice support this hypothesis.

Crenezumab (MABT5102A) is a humanized monoclonal antibody which uses IgG4 backbone [65]. A phase II clinical trial to assess the safety and efficacy in patients with mild-to-moderate AD (NCT01343966) was completed in April 2014, although the results are not yet publicly available. The most recent phase II trial aiming to evaluate the safety and efficacy of crenezumab in asymptomatic carriers of E280A autosomal-dominant mutation of PSEN1 commenced in November 2013 (NCT01998841).

Other monoclonal antibodies against Aβ developed so far include PF-04360365 (ponezumab) which targets the free carboxy terminal amino acids 33–40 of the Aβ peptide; MABT5102A, which binds to Aβ monomers, oligomers, and fibrils with equally high affinity; GSK933776A, which is similar to bapineuzumab in that it binds to the N-terminal Aβ(1-5). In addition, other passive immunotherapies mostly in phase I clinical development include NI-101, SAR-228810, and BAN-2401 [57, 58, 61–65].

Gammagard is a preparation of antibodies from human plasma. Its safety for human use had been previously demonstrated in certain autoimmune conditions. Gammagard effects were evaluated in a small number of AD patients (NCT00818662). It is believed that this mixture contains a small fraction of polyclonal antibodies against the Aβ peptide. In addition, this preparation may possess immunomodulatory properties that could potentially enhance microglial phagocytosis [66–68].

3. Strategies Focused on Tau Proteins

Tau proteins are highly soluble and abundant in the neurons where they play a critical role in microtubule stabilization, particularly in axons [69–71]. Tau hyperphosphorylation leads to the formation of insoluble paired helical filaments (PHF) which form neurofibrillary tangles. The loss of microtubule-binding capacity provokes cytoskeleton destabilization, which eventually causes neurodegeneration and neuronal death [70]. As an alternative to amyloidocentric approaches, Tau-centered treatments aim to inhibit the phosphorylation and/or aggregation of Tau protein. In addition, microtubule-stabilizing drugs could be used as a disease-modifying strategy in AD [71]. In recent years, immunomodulation was suggested as a viable option for promoting effective clearance of Tau aggregates.

3.1. Inhibitors of Tau Hyperphosphorylation. All Tau proteins are a product of alternative splicing of a microtubule-associated protein Tau (MAPT) gene. Phosphorylation is the primary mechanism which regulates Tau binding to microtubules. Under physiological conditions the protein remains soluble; however, in AD, pathological hyperphosphorylation of Tau compromises its normal functions [72, 73]. Hyperphosphorylation occurs as a result of an imbalance between the catalytic activity of kinases and phosphatases. Increased expression of active forms of various kinases in the areas proximal to neurofibrillary tangles has been described in AD, including CDK5, GSK3β, Fyn, stress-activated protein kinases JNK and p38, and mitogen-activated protein kinases ERK1 and ERK2 [74]. Some or all of these kinases contribute to the perpetuation of the phosphorylation of Tau in neurofibrillary tangles [73, 75–81]. As a result, significant research efforts have been devoted to the development of kinase inhibitors as a possible treatment strategy for AD. For example, SP600125, a widely used pan-JNK inhibitor, exerts beneficial effects on cognition and reduces neurodegeneration in an APP/PS1 transgenic mouse model of AD [80]. It has been proposed that specific inhibition of JNK3 could be

sufficient to bring similar benefits as seen with SP600125 in rodent models [78–81]. Human data in AD patients indicate a positive correlation between the levels of JNK3 and Aβ(1-42) in the brain [77]. Furthermore, JNK3 upregulation was detected in the CSF and was associated with memory loss. Thus, JNK3 inhibition remains a promising target for future therapies [81].

CDK5 belongs to the family of serine/threonine cyclin-dependent kinases and is responsible for a number of physiological functions within the CNS, including neurite outgrowth and the regulation of axonal development [82]. CDK5 catalytic activity is dependent on its direct association with p35, key regulator of CDK5 signaling. This cofactor is cleaved by a nonlysosomal protease calpain in a calcium-dependent manner [83]. Conversion of p35 to p25 results in prolonged activation and mislocalization of CDK5. Due to the increases in intracellular calcium levels observed in the brains of AD patients, pathological activation of CDK5 occurs, resulting in hyperphosphorylation of Tau and neuronal cell death [83, 84]. CDK5 inhibition may thus also be potentially considered as a possible drug target. Currently existing CDK5 inhibitors roscovitine and flavopiridol have demonstrated neuroprotective properties in *in vitro* and *in vivo* models of excitotoxicity, ischemia, and neurodegeneration [84, 85].

GSK3β inhibitors are arguably in the most advanced stages of clinical development for AD. Among the various drugs that are currently being studied, tideglusib, an irreversible inhibitor of GSK3β, has recently completed phase II trials (NCT01350362). Tideglusib administration for a period of 26 weeks to patients with mild-to-moderate AD did not show clinical efficacy, and the compound has since been discontinued for this indication [86]. Another study (NCT00948259) evaluated the safety and tolerability of a 20-week administration of NP031112 compared with placebo in patients with AD. No data has been reported for this study.

Phosphatase activation has also been considered as a possible drug target. Currently, there is only one protein phosphatase 2 (PP2A) agonist in development. Sodium selenite (VEL015) is undergoing phase II trials in Australia (ACTRN12611001200976). Experimental studies have shown that sodium selenate reduces Tau phosphorylation, both in cell culture and in mouse models of the disease [86–88]. VEL015 administration to rodents have resulted in significant cognitive improvements and substantial reduction of neurodegenerative phenotype.

3.2. Inhibitors of Tau Aggregation. Hyperphosphorylated Tau aggregates contribute to neurotoxicity observed in AD brain. Methylene blue dye derivatives have shown some promise in inhibiting the formation of Tau aggregates. Methylene blue disrupts the aggregation of Tau, has the ability to inhibit amyloid aggregation, improves the efficiency of mitochondrial electron transport chain, reduces oxidative stress, prevents mitochondrial damage, and is also a modulator of autophagy [74, 89]. The first-generation molecule derived from methylene blue (Rember) appeared to stabilize AD progression in a clinical trial which lasted 50 weeks. These results motivated researchers to develop a next-generation version of methylene blue, TRx 0237. This compound is a purified derivative of methylene blue which not only inhibits Tau protein aggregation but also dissolves brain aggregates of Tau [74]. Several clinical trials are currently underway (NCT01626391, NCT01689233, NCT01689246, NCT01626378) to evaluate the potential efficacy of this drug in AD.

3.3. Microtubule Stabilizers. Microtubule stabilization may potentially achieve a similar end-result as that seen with the inhibitors of Tau hyperphosphorylation and aggregation. Paclitaxel is a microtubule-stabilizing drug currently in use in the oncology field. Unfortunately, this compound is incapable of crossing the BBB and its use is associated with serious adverse events, which limits its utility in AD [90, 91]. In addition to paclitaxel, other microtubule-stabilizing compounds such as TPI 287 have been considered as a possible AD therapy. TPI 287 is a derivative of taxane, also used in cancer treatment. TPI 287 stabilizes the microtubules by binding to tubulin. NCT01966666 clinical trial will evaluate TPI-287 safety, pharmacokinetic properties, and tolerability by intravenous infusion in mild-to-moderate AD.

Epothilone D is a microtubule-stabilizing compound which improved axonal transport, reduced axonal dystrophy, decreased Tau neuropathology, and reduced hippocampal neuron loss; however, drug development for AD was discontinued in 2013 after a failed clinical trial [91].

With respect to Tau, additional studies are necessary in order to better understand the exact molecular mechanisms involved in Tau neurotoxicity. Recent studies comparing the neurotoxic profiles of various forms of Tau suggest that a soluble form is likely the most toxic [69]. This has been corroborated by a recent report specifically identifying oligomeric Tau as toxic [92]. Therefore, future therapeutic strategies should be focused on targeting soluble forms of Tau.

3.4. Anti-Tau Immunotherapy. Just as with the immunotherapies targeting Aβ, both passive and active immunization approaches against Tau have been considered. In fact, it was demonstrated that reductions in Tau aggregate formation and improved clearance of Tau oligomers and insoluble aggregates could all be achieved with either active or passive immunotherapies [93]. In rodents, treatment with monoclonal antibodies directed against hyperphosphorylated Tau has led to improvements in cognition and was not associated with significant adverse effects [93].

In 2013 Axon Neuroscience began a phase I trial to evaluate the safety and tolerability of AADvac-1, an active immunotherapy which consists of a synthetic peptide derived from the Tau sequence coupled to keyhole limpet hemocyanin; the precise molecular nature of the antigen has not been disclosed (NCT01850238 and NCT02031198) [94]. AADvac-1 uses aluminum hydroxide as an adjuvant. At the 2014 Alzheimer's Association International Conference (AAIC) in Copenhagen, good preclinical safety profile was reported for the treatment period of up to 6 months in rats, rabbits, and dogs. These early results are encouraging and it remains to be seen whether AADvac-1 will demonstrate acceptable safety and efficacy in human patients.

4. The Cholinergic Hypothesis

AD is a neurodegenerative disease characterized by a progressive loss of learning and memory as well as neuronal death. The hippocampus, the main brain region involved in memory processing, is influenced by cholinergic modulation [95]. One of the well characterized anomalies associated with neurotransmitter alterations is the degeneration of cholinergic neurons in the nucleus basalis of Meynert and the loss of cholinergic inputs to the neocortex and hippocampus. Several studies reported decreases in choline acetyltransferase (ChAT), acetylcholine (ACh) release, as well as reductions in nicotinic and muscarinic receptors in the cerebral cortex and hippocampus of postmortem AD brains [96]. Acetylcholinesterase inhibitors (AChEI), one of the only 2 classes of drugs currently approved for AD treatment, act by increasing ACh bioavailability at the synapse. Unfortunately, none of these drugs are capable of reversing the course of AD nor of even appreciably slowing down the rate of disease progression [97]. Their clinical effect is largely palliative; however, their potential use in combination therapy with other disease-modifying compounds should not be excluded.

Ladostigil (TV3326) is both a reversible inhibitor of AChE and is a selective and irreversible inhibitor of brain monoamine oxidases A and B, the use of which improves extrapyramidal symptoms and provides an antidepressant effect [98, 99]. It also appears to be a potent antiapoptotic, antioxidant, anti-inflammatory, and neuroprotective agent. NCT01429623 and NCT01354691 phase II clinical trials with ladostigil are currently underway.

5. Dendritic Hypothesis (Aβ-PrPC–mGluR5–Fyn Signaling)

Dendritic abnormalities appear in the relatively early stages of AD. While dystrophic neurites, reduced dendritic complexity, and dendritic spine loss are all documented features of AD, it is only recently that we are beginning to understand the underlying molecular changes that occur on the postsynaptic side, in the dendrite [100–102]. Some data suggest that soluble Aβ oligomers are the principal neurotoxic species responsible for dendritic pathology. Aβ oligomers may cause aberrant N-methyl-D-aspartate receptor (NMDAR) activation postsynaptically by forming complexes with the cell-surface prion protein (PrPC). PrPC is enriched at the neuronal postsynaptic density, where it interacts with Fyn tyrosine kinase-metabotropic glutamate receptor 5 complex (Fyn-mGluR5). Fyn activation occurs when Aβ is bound to PrPC-Fyn-mGluR5 complex. Activated in this manner, Fyn can cause tyrosine phosphorylation of the NR2B subunit of this NMDAR. This results in an initial increase and then a loss of cell-surface NMDARs [103]. Fyn overexpression accelerated synapse loss and the onset of cognitive impairment in the J9 (APPswe/Ind) transgenic AD mouse model, while its inhibition produced an opposite effect [100]. In addition, as mentioned earlier, Fyn can also contribute to Tau hyperphosphorylation. Previous studies had reported elevated levels of Fyn in AD brain. Furthermore, Fyn was shown to phosphorylate Tau at Tyr18 residue [101]. Thus, Fyn appears to be a viable target in the treatment of AD pathology. Saracatinib (AZD0530) and masitinib (AB1010) are Fyn kinase inhibitors currently in phase II and phase III clinical trials for mild-to-moderate AD (NCT01864655, NCT02167256, NCT00976118, NCT01872598) [103–105]. Both compounds are capable of blocking Fyn in a nanomolar range.

In a NCT00976118 clinical trial, oral masitinib was administered for a period of 24 weeks, concomitantly with one of the AChEIs (donepezil, rivastigmine, or galantamine) and/or memantine. In that study a significant improvement in the ADAS-Cog test response was reported. These results are encouraging; however, the very small patient pool ($n = 26$) on memantine in this phase II trial is clearly not sufficient to draw conclusions on the potential efficacy of this compound. MOA of masitinib in AD is twofold. Apart from blocking Fyn, masitinib is also a stem cell factor (SCF) receptor (c-KIT) inhibitor. By inhibiting SCF/c-Kit signaling on mast cells (MCs), this compound may prevent neuroinflammation by blocking the activated MCs-microglia interactions [102–105].

6. 5-HT6 Receptors in Alzheimer's Disease

5-HT6 receptors are expressed in areas of the CNS involved in learning and memory. Their inhibition was shown to promote acetylcholine release. In AD, 5-HT6 antagonism may lead to the restoration of acetylcholine levels [106]. This hypothesis is supported by evidence that the 5-HT6 receptor antisense oligonucleotides improve spatial learning and memory in the Morris water maze test in normal rats [107]. 5-HT6 inhibitors may be useful in combination therapy, together with AChEIs. For example, Lu-AE-58054 (SGS-518) and PF-05212365 (SAM-531) are being considered as possible treatments for mild-to-moderate AD. Other compounds that are in various stages of clinical research are SUVN-502, AVN-322, and PRX-07034 [108].

7. Changing the Concept: AD as a Metabolic Disorder

Clinical studies suggest that diabetes is a major risk factor that contributes to AD pathology. Results from published research indicate that there is a close link between insulin-deficient diabetes and cerebral amyloidosis [109]. Peripheral and central insulin signaling impairments are likely present in both diseases. As a result, "type 3 diabetes" hypothesis of AD was developed, which attempts to bridge the observed metabolic phenotypes present in diabetes and AD into a coherent framework. Insulin hormone is at a centerpiece of this hypothesis [110].

Observations made in the "Hisayama Study" indicate that altered expression of genes related to diabetes mellitus in AD brains may be a result of AD pathology and suggest that peripheral insulin resistance, metabolic syndrome, and/or full-blown diabetes may lead to worsening of cognitive symptoms [111]. Impaired central insulin signaling in the hippocampal circuits, a key region involved in learning and memory, is likely present in AD [112]. Glucose toxicity, insulin

resistance, oxidative stress, elevated levels of advanced glycation end products, and cytokine-mediated neuroinflammation are among the proposed mechanisms by which diabetes could increase the risk of AD development. In a recent study, Clarke and colleagues demonstrated that hypothalamic administration of soluble Aβ oligomers initiates neuroinflammatory cascades which eventually cause disturbances in peripheral glucose homeostasis [113]. Tumor necrosis factor α (TNFα) quite possibly plays an important role in this process [114, 115].

As molecular mechanisms causing AD and T2DM pathologies are possibly related, it is logical to assume that drugs used in T2DM treatment may have a neuroprotective effect in AD [116]. Thiazolidinediones (TZDs) are an example of antidiabetic compounds whose possible role in AD was investigated. TZDs are agonists of peroxisome proliferator-activated receptor γ (PPAR-γ), which act by promoting PPAR-γ heterodimerization with the retinoid X receptor (RXR). PPAR-γ/RXR heterodimer is a transcription factor, which regulates expression of genes involved in lipid and glucose metabolism. TZDs improve insulin sensitivity and reduce cytokine-dependent inflammation [117, 118]. Rosiglitazone and pioglitazone are used as antidiabetic drugs, which regulate glucose homeostasis by increasing insulin sensitivity, reducing blood glucose levels, and improving lipid metabolism. Both compounds have also been studied as potential therapeutics for AD treatment, with reported improvements in mitochondrial oxidative metabolism. In animal models, pioglitazone modified various indices of brain aging but did not slow down the cognitive decline [118]. Rosiglitazone and pioglitazone also induce the expression of peroxisome proliferator-activated receptor-γ co-activator 1 alpha (PGC-1α), a molecule that plays multiple roles in mitochondrial biogenesis, energy metabolism, and mitochondrial antioxidants expression. Previous studies have demonstrated that, in the human brain tissues, the expression of PGC-1α decreases with progression of AD dementia [119]. Thus, PGC-1α upregulation may improve the mitochondrial energy metabolism and AD pathology [120].

Pioglitazone treatment improved memory and cognition in patients with mild-to-moderate AD in a small clinical trial [121]. A larger phase II trial demonstrated improvements in memory retention and attention with rosiglitazone treatment (6 months) in patients who did not possess an ApoE4 allele [122]. However, phase III trial using rosiglitazone failed to show efficacy in AD (NCT00550420) [123]. It is important to note that in these trials rosiglitazone was administered alone at dosages that were much lower than those needed to exert a beneficial effect on AD pathophysiology in rodent models of the disease. NCT00348140 is a recently completed clinical trial in which rosiglitazone was administrated in combination with AChEIs in patients with mild-to-moderate AD. No results have yet been reported.

Intranasal insulin had also been considered as a treatment option for AD. This particular route of administration is attractive as it bypasses the BBB. This is very significant because insulin transport to the brain from the periphery is dependent on active transport mechanisms which may become disrupted in AD. In addition, the probability of possible adverse events in peripheral tissues is minimized. In theory, insulin delivery directly to the brain will activate cerebral insulin signaling leading to improvements in memory processing and will result in neuroprotection [124, 125]. A currently ongoing clinical trial NCT01767909 is evaluating long-term (12 months) efficacy of intranasal insulin (Humulin R U-100) in mild AD.

Other pancreatic hormones such as amylin may also play a role in AD. Adler and colleagues reported that patients with AD have reduced concentrations of plasma amylin. In transgenic animal models of AD, amylin and pramlintide (amylin analog) decreased brain Aβ levels and improved cognition. Interestingly, amylin also inhibited β-secretase enzyme, while pramlintide did not [126, 127].

8. Future Strategies

AD is a complex multifactorial disorder which may require equally complex approaches to treatment. Early disease detection, combination therapies, and lifestyle choices are all likely contributors to the successful eradication of the pathology (Figure 1) [128–132]. A broad range of studies show that inadequate nutrition can increase the risk of disease development [131]. A healthy diet can certainly improve your chances of not developing AD. However, neither the Mediterranean-type diet, caloric restriction, nor antioxidant diet alone can prevent or delay AD. We believe that carefully developed nutrition regimens coupled to combination pharmacotherapies targeting multiple pathways involved in AD are a way forward.

Biomarker identification, indicative of prodromal stages of AD, can lead to early diagnosis and improve prognostic outcomes. Currently existing diagnostic approaches are focused on the detection of Aβ(1-42) and total and phosphorylated Tau levels in the CSF and in the brain. Imaging techniques such as brain MRIs are also used [133–135]. As both Aβ and Tau increases likely appear when the disease had already taken hold, we would welcome the discovery of diagnostic markers which could predict the likelihood of AD development at earlier stages.

Growth factors (GFs) are yet another set of molecules which can potentially improve AD pathology. Transforming growth factor β family, insulin-derived GFs (insulin-like growth factor 1, IGF-1 and insulin-like growth factor 2, IGF-2), basic fibroblast growth factor (bFGF), and neurotrophins (nerve growth factor, NGF; brain-derived growth factor, BDGF; glial-derived neurotrophic factor, GDNF) all participate in neurogenesis and neurodevelopment and may be considered as potential targets for AD treatment [135, 136].

9. Concluding Remarks

In summary, accumulated evidence suggests that AD neuropathology shows a multifactorial nature and involves multiple biological pathways. Amyloid cascade hypothesis has dominated the field for over 20 years, as a result of which a large number of studies have focused on inhibition and removal of Aβ and senile plaques. Unfortunately, the amyloidocentric approaches have failed to demonstrate

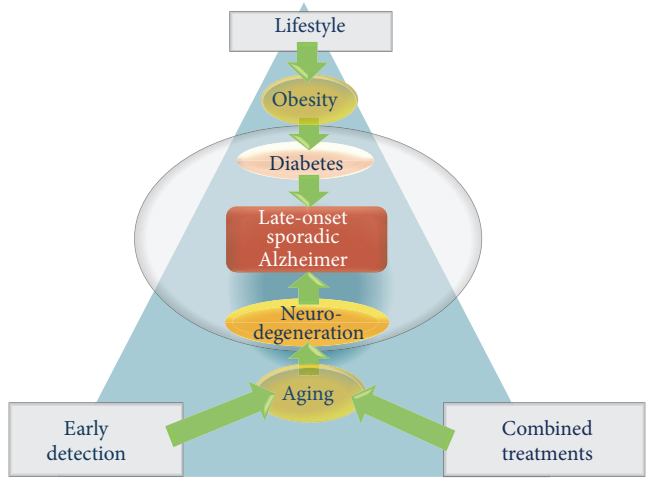

Figure 1: Future strategies in the treatment of late-onset and sporadic forms of AD could be centered on three main points: avoidance of habits and lifestyle leading to obesity and diabetes; early detection of AD biomarkers or structural alterations in presymptomatic individuals; and combined therapies in early phases of cognitive loss.

improvements in cognition in patients. Dendritic spine defects clearly contribute to cognitive decline observed in AD. These defects are considered an early event in memory circuit's destabilization and should be taken into account for future development of investigational drugs. Novel pharmacotherapies should not be limited to the postulates of the amyloid cascade hypothesis. Events occurring at the synapse may prove to be instrumental in understanding the underlying pathology of this devastating disease.

Abbreviations

Aβ:	β-amyloid
ACE I:	Angiotensin converting enzyme I
Ach:	Acetylcholine
AD:	Alzheimer's disease
ApoE:	Apolipoprotein E
APP:	Amyloid precursor protein
AB1010:	Masitinib
AZD0530:	Saracatinib
BACE1:	PPAR and aspartyl protease β-site AβPP-cleaving enzyme
Cdk's:	Serine/threonine cyclin dependent
CNS:	Central nervous system
CSF:	Cerebrospinal fluid
IDE:	Insulin degrading enzyme
LRP:	Low-density lipoprotein
RemberTM:	Methylene blue
MABT5102A:	Crenezumab
MAPT:	Microtubule-associated protein Tau
MOA:	Mechanism of action
MCs:	Mast cells
NOS:	Nitric oxide synthase
NSAIDs:	Nonsteroidal anti-inflammatory drugs
PBT1:	Clioquinol
PGC-1α:	Peroxisome proliferator-activated receptor-γ coactivator 1 alpha
PrP:	Prion protein
PS1/2:	Presenilin 1/2
RAGE:	Receptor of advanced glycation end products
ROS:	Reactive oxygen species
SGSM:	Selective γ-secretase modulators of the enzyme
TV3326:	Ladostigil
VEL015:	Sodium selenite
WHO:	World Health Organization.

Disclosure

Jaume Folch and Antoni Camins are senior co-authors.

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

Neuroprotective Transcription Factors in Animal Models of Parkinson Disease

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A number of transcription factors, including En1/2, Foxa1/2, Lmx1a/b, Nurr1, Otx2, and Pitx3, with key roles in midbrain dopaminergic (mDA) neuron development, also regulate adult mDA neuron survival and physiology. Mouse models with targeted disruption of some of these genes display several features reminiscent of Parkinson disease (PD), in particular the selective and progressive loss of mDA neurons in the substantia nigra pars compacta (SNpc). The characterization of these animal models has provided valuable insights into various mechanisms of PD pathogenesis. Therefore, the dissection of the mechanisms and survival signalling pathways engaged by these transcription factors to protect mDA neuron from degeneration can suggest novel therapeutic strategies. The work on En1/2-mediated neuroprotection also highlights the potential of protein transduction technology for neuroprotective approaches in PD.

1. Introduction

Parkinson disease (PD) is the second most common neurodegenerative disorder. The disease is characterized by a loss of midbrain dopaminergic (mDA) neurons in the substantia nigra pars compacta (SNpc) and the presence of α-synuclein-containing protein aggregates and termed Lewy bodies (and/or Lewy neurites) in affected neurons [1, 2]. Apart from certain familial monogenic forms of the disease, in which mutated genes (e.g., *SNCA*, *LRRK22*, *PINK1*, *PARKIN*, *DJ-1*, and *ATP13a2*) have been identified, the molecular bases of sporadic idiopathic PD remain largely unknown [3, 4]. As for other neurodegenerative diseases, such as Alzheimer's disease and Huntington's disease, ageing is considered a major risk factor for PD development [5].

The current view is that the slow and progressive death of SNpc mDA neurons remains asymptomatic until 30% of mDA neuron cell bodies and 50–60% of axonal terminals are lost [6]. Over time, this loss results in severe dopamine (DA) deficiency in the striatum, leading to the cardinal motor symptoms including rest tremor, bradykinesia, rigidity, and postural instability. No therapies are yet available to prevent the loss of mDA neurons or even delay the course of the disease [7]. One remarkable feature of the disease is that nonnigral dopaminergic neurons including mDA neurons in the ventral tegmental area (VTA), located in the vicinity of the SNpc mDA neurons, are relatively spared. The molecular determinants for the selective vulnerability of the SNpc mDA neurons in PD are not known [8, 9].

A number of studies have pointed to oxidative stress, mitochondrial dysfunction, protein misfolding and aggregation, impaired proteasomal and lysosomal degradation pathways, altered vesicular trafficking, and neuroinflammation as possible culprits in PD pathogenesis [1, 2, 8]. Many PD-linked genes affect mitochondrial activity or integrity [10] and a potential link between mitochondrial dysfunction and PD is supported by the ability of complex I-specific neurotoxins such as MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) to induce PD-like symptoms in rodents and primates, including humans. Recent studies also suggest that DNA damage or repair dysfunction [11–13] and nucleolar stress [14–17] play important roles in PD

pathogenesis and other neurodegenerative diseases [18–23]. Mouse lines expressing PD-linked gene mutations recapitulate several features of PD pathogenesis but most of them do not present the selective and progressive loss of mDA neurons in the SNpc [24–26].

Major progress has recently been made in dissecting the genetic and signalling networks that control the generation of mDA neurons [27–29]. These studies have revealed the crucial role of several transcription factors. Interestingly, a number of these transcription factors (e.g., Engrailed-1/Engrailed-2, Foxa1/2, Lmx1a/b, Nurr1, Otx2, and Pitx3) remain present in adult mDA neurons and are required for their maintenance throughout life [30–32]. The elucidation of the roles and mechanisms of action of these transcription factors during development and adulthood could bring important insights into PD pathogenesis and suggest new therapeutic strategies. It is noteworthy that possible genetic links between these transcription factors and PD have been reported [33–46]. This review summarizes some aspects of the function of these developmental transcription factors in relation to PD.

2. Transcription Factors as Key Players in mDA Neuron Development

Midbrain DA neuron development starts around embryonic day E8 in the mouse with the induction of neurons in the floor plate and the specification of mDA progenitors. These progenitors give rise to mature mDA neurons following successive steps of proliferation, maturation, migration, axonal pathfinding, and synaptogenesis [27–29]. Midbrain DA neurons of the SNpc project mainly to the dorsal striatum to form the nigrostriatal pathway involved in the control of voluntary movements, whereas VTA mDA neurons project to the nucleus accumbens, the amygdala, the hippocampus, and the prefrontal cortex to form the mesolimbic and mesocortical pathways involved in motivation, reward, addiction, cognition, and memory. Mature SNpc mDA neurons express elevated levels of the highly active glycosylated form of the dopamine transporter (DAT) glyco-DAT and the inwardly rectifying potassium channel GIRK2, whereas VTA mDA neurons are more enriched in the calcium binding protein calbindin D28K (CALB1) [47].

Induction and specification of DA progenitors are governed by the concerted action of secreted factors (e.g., SHH, FGF8, Wnt1, and TGF-β) and of key transcription factors including En1/2, Otx2, Lmx1a/b, and Foxa1/2 [27–29]. Early loss of function of any of these transcription factors has dramatic consequences in the ontogenesis of mDA neurons. The subsequent steps of mDA neuron development are accompanied by the expression of additional transcription factors such as Nurr1 and Pitx3, which participate in the differentiation of mDA progenitors into mature mDA neurons. Hence, Nurr1 and Pitx3 are required for the expression of several genes encoding proteins that determine mature mDA neuron identity such as TH (tyrosine hydroxylase), DAT and VMAT2 (vesicular monoamine transporter 2), AADC (dopa decarboxylase), DRD2 (dopamine receptor D2), or ALDH1A1 (aldehyde dehydrogenase 1 family, member A1). Furthermore, Nurr1/Pitx3 are persistently required for maintaining the adult expression of these genes and mDA neurons with a conditional adult ablation of *Nurr1* degenerate progressively [48]. Interestingly, functional interactions between pairs of transcription factors (e.g., Nurr1 and Pitx3, Pitx3 and En1/2, or Nurr1 and Foxa1/2) have been reported [49–51].

Earlier work demonstrated that En1/2 are required for the survival of mature mDA neurons during late embryonic life in a dose-dependent manner [52–54]. This might be achieved through the activation of the Erk1/2 MAPK survival pathways and suppression of the proapoptotic activity of the proneurotrophin receptor p75NTR [55]. It was also shown that En1/2 is involved in the acquisition of a mature mDA neuron identity [50]. For example, in *En1* homozygous mutants (viable on a C57BL/6 background), expression at E13.5 of *Pitx3*, *Th*, *Dat*, *Vmat2*, and *Ddc* (encoding AADC) is reduced in the rostral-lateral mDA domain [50]. Otx2, together with Sox6, also controls mDA neuron subtype identity [56] and in the course of development, its expression becomes restricted to a specific subset of dorsal-lateral VTA mDA neurons [47]. In addition to transcription regulation, the importance of epigenetic mechanisms in all these processes must also be recalled [57].

3. Developmental Transcription Factors Required in Adult mDA Neuron Maintenance

As mentioned above, many developmental transcription factors remain expressed in mDA neurons throughout life and are required for their survival and physiological functions. We shall now briefly describe the effects of loss or gain of function of some of these transcriptions factors and their relevance to PD in adult mDA neurons.

3.1. Manipulating the Expression of Nurr1, Otx2, Foxa1/2, and Pitx3 in Adult mDA Neurons. *Nurr1* expressed in adult mDA neurons of the SNpc and VTA is critical for the maintenance of their phenotype [58, 59]. *Nurr1*-deficient mice die shortly after birth. *Nurr1* haplodeficient young animals present a normal number of mDA neurons and have no abnormal motor phenotype, but the number of mDA neurons decreases in old mice (after 15 months) in parallel with a decreased locomotor activity [48]. *Nurr1*+/− mice also exhibit increased vulnerability to MPTP [60] and show an exacerbated sensitivity to the toxicity of repeated methamphetamine exposure [61]. *Nurr1* ablation in adult mDA neurons using AAV-Cre leads to mDA neuron dysfunction and to the progressive loss of mDA neuron markers [48]. Finally, tamoxifen-induced conditional deletion of *Nurr1* in mDA neurons in 5-week-old mice results in a progressive pathology, associated with loss of or reduced striatal DA, impaired motor behaviour, and dystrophic axons and fragmented dendrites containing varicosities [62]. However, no major loss of mDA neurons was reported in these mice.

Otx2 is expressed in a subset of mDA neurons in the central and mediolateral area of the VTA in the adult [47]. Conditional knockout of *Otx2* in the adult leads to selective loss of the axonal projections from VTA mDA neurons

[63, 64]. Otx2 is also a negative regulator of DAT and there is an inverse correlation between *Otx2* expression and glyco-DAT levels in mDA neurons [47]. Otx2 gain of function in SNpc mDA neurons decreases glyco-DAT levels, thus conferring protection against MPTP toxicity [47, 65]. *Foxa1/2* also continue to be expressed in adult mDA neurons and *Foxa2* heterozygous mice present late-onset, spontaneous degeneration of mDA neurons [66]. Conditional tamoxifen-inducible deletion of both *Foxa1* and *Foxa2* in early adulthood results in a decline of striatal DA content along with locomotor deficits and progressive loss of ALDH1A1, AADC, and DAT, ultimately leading to a reduction of mDA neurons in the SNpc of aged animals [67]. Finally, the spontaneous deletion of *Pitx3* in the Aphakia mouse or global Pitx3 gene inactivation leads to rapid and preferential loss of mDA neurons in the SNpc of neonatal mice [68, 69]. Dorsal SNpc mDA neurons, which do not express Pitx3, are spared in mutant mice similar to what is observed in PD [70].

3.2. En1 Heterozygous Mice as a Model for PD. *En1/2* are expressed in SNpc and VTA mDA neurons from early development on into adulthood [52]. Although *En1*−/− pups die (OF1 background) at birth [71], *En1* heterozygous mice are viable. *En1*+/− mice display a normal number of mDA neurons until 6 weeks after birth when SNpc mDA neurons start to die progressively [72]. The extent of cell death reaches about 40% in the SNpc at 48 weeks of age and is correlated with a decreased DA content in the striatum. Midbrain DA neurons in the VTA are affected to a much lesser extent. *En1*+/− mice present PD-like motor symptoms such as decreased spontaneous locomotor activity (distance travelled, rearing), increased amphetamine-sensitization, and decreased motor coordination and sensorimotor learning (rotarod). The loss of mDA neurons in the VTA, albeit less pronounced, also leads to some nonmotor behaviour alterations such as increased depressive-like behaviour (forced swimming test), increased anhedonic-like behaviour (saccharine preference), and poor social interaction [72]. This suggests that the mesolimbic system is also affected in these mutants. The death of adult mDA neurons from *En1* haploinsufficiency has now been observed in several independent studies [50, 73, 74] and follows the retrograde degeneration of axons [50, 73, 74]. *En1*+/−; *En2*−/− mice (C57BL/6 background) are normal at birth but present a massive loss of mDA neurons in the SNpc of young adult, illustrating an En1/2 dosage effect on survival [75]. Midbrain DA neurons in these mice are also more responsive to MPTP-induced cell death.

A more detailed characterization of *En1* heterozygous mice revealed early signs of degeneration of mDA axon terminals in the striatum [74], prior to neuronal cell loss in the SNpc. Dopaminergic terminals become dystrophic and swollen, contain autophagic vacuoles, and present deficits in DA release and uptake. The nigral dopaminergic cell bodies exhibit signs of decreased autophagy accompanied by an increase in mTOR activity and a decrease of the autophagic marker LC3B [74]. These findings illustrate a retrograde degeneration of the nigrostriatal system in *En1*+/− mice, akin to what occurs in PD [6, 76, 77]. Retrograde degeneration may be a common feature of many progressive neurodegenerative disorders [78]. Individual axons in the nigrostriatal pathway of *En1*+/− mice undergo fragmentation supporting the idea that axonal transport failure might be an early feature of PD [79]. The possible role of autophagy in PD pathogenesis [80, 81] was recently assessed in a mouse model generated by the conditional deletion of the autophagy-related gene *Atg7*, which recapitulates many pathologic features of PD, including age-related loss of mDA neurons [82]. *En1* heterozygous mice thus represent a valuable model to gain further insights into PD pathogenesis.

A recent study shows that mDA neurons in *Lmx1b* conditional knockout mice are progressively lost, in both the SNpc and the VTA. These mice also present abnormally large nerve terminals in the striatum and these terminals are filled with autophagic and lysosomal vesicles, before the onset of mDA cell loss. Very much in analogy with the *En1*+/− mouse phenotype, these findings suggest a retrograde degeneration of mDA neurons. Alteration of the autophagy/lysosomal pathway could be due to increased mTOR activity of mDA neurons in *Lmx1b* mutants and in *En1* heterozygous mice. In this context it is of note that rapamycin treatment of conditional *Lmx1b* knockout mice normalizes the phenotypic alterations [83]. Finally, gene expression profiling in the MN9D dopaminergic cell line identified nuclear-encoded mitochondrial subunits of the respiratory chain as potential *Lmx1a* targets, suggesting a possible link also between *Lmx1a* and mitochondria [84].

4. Developmental Transcription Factors and Neuroprotective Approaches for PD

4.1. Protection of mDA Neurons by En1/2 Protein Transduction in Experiential PD Models. It is now well established that several homeoproteins, including En1/2 and Otx2, are endowed with the ability to transduce cells [85, 86]. This property was exploited to examine the therapeutic potential of En1/2. It was first shown that mDA neuronal loss in *En1*+/− mice can be stopped by infusing recombinant En1/2 proteins (En1 and En2 are biochemically equivalent) in the SNpc [72]. Subsequently, En1/2 protein transduction was shown to protect mDA neurons in various experimental models of PD *in vitro* and *in vivo*, including the MPTP, rotenone, 6-OHDA (6-hydroxydopamine), and mutated a-synuclein (A30P) models [87]. Interestingly, unilateral Engrailed infusion in naive mice increases ipsilateral striatal DA content. This results in amphetamine-induced turning behaviour contralateral to the side of infusion indicating an activation of the nigrostriatal pathway upon Engrailed infusion in the SNpc [87]. Thus, En1/2 is able not only to protect mDA neurons against various PD-related insults but also to increase their physiological activity. Finally, it was shown that forced expression of *Otx2* in mDA neurons in the SNpc of *En1*+/− mice can prevent the progressive loss of mDA neurons caused by *En1* haploinsufficiency [73]. Ectopic expression of *Otx2* in SNpc mDA neurons also protects them against MPTP toxicity (see above). Otx2 protein transduction could thus also be of

potential therapeutic interest for neuroprotection in PD. It is noteworthy that Otx2 protein transduction has previously been shown to protect retinal ganglion cells (RGCs) against NMDA (N-methyl-D-aspartate) toxicity in a mouse model of glaucoma [88].

4.2. Use of Developmental Transcription Factors for Cell Replacement Strategies. Although the feasibility of cell replacement therapy for PD has been demonstrated with embryonic ventral midbrain tissue transplantation [89], the scarcity of the material for transplantation remains a major hurdle [90]. The knowledge gained from the genes and mechanisms involved in mDA neuron development has been very valuable for the generation of midbrain DA progenitors from embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs) [91]. ESCs and iPSCs converted into DA progenitors expressing *Lmx1a* and *Foxa2* by exposure to SHH and FGF8 (with or without Wnt1/TGF-β1/retinoic acid) can then differentiate into DA neurons *in vitro* and/or *in vivo* [92–94]. Similarly, *Nurr1* expression together with that of the transcription factor Ascl1 (also important for mDA progenitor specification) is sufficient to drive DA differentiation of forebrain embryonic rat neural precursors [95–97]. Proper innervation of target areas and functional recovery upon transplantation of *in vitro* generated DA progenitors has now been demonstrated in both rodents and nonhuman primate PD models [98–101]. To further analyse the functionality of transplanted cells, two recent studies used optogenetic tools or "designer receptor exclusively activated by designer drug" (DREADD) technology to stimulate the function of engrafted DA neurons *in vivo* by illumination or by injecting specific drugs, respectively [102–104]. Such approaches will be particularly useful to assess the long-term function of transplanted cells in PD models.

From a safety point of view, as an alternative to DNA or RNA mediated gene delivery, a few studies have considered protein transduction as a means for reprogramming through the delivery of cocktails of recombinant proteins fused to cell penetrating peptides (CPPs) [85, 105]. It was reported that protein-based human iPSCs can efficiently generate functional dopamine neurons and can treat a rat model of Parkinson disease [106]. The neuroprotection achieved by transduction of En1/2 or Otx2 [72, 87, 88], two homeoproteins naturally containing the "penetratin" sequence, should encourage more direct protein delivery-based strategies for neuroprotection or neurorepair.

5. Mechanisms of Action of Developmental Transcription Factors in Adult mDA Neurons

Our knowledge concerning the mechanisms of action of these developmental transcription factors in the survival and maintenance of adult mDA neurons is still limited. However, it has emerged from recent studies that these transcription factors engage several neuroprotective mechanisms and are linked to several survival pathways in adult mDA neurons (Figure 1). Since Otx2 gain of function in the SNpc can prevent cell loss in *En1*+/− mice [73], it is likely that Otx2 and En1/2 share some common neuroprotective mechanisms.

Engrailed survival activity relies on several mechanisms and signalling pathways. It is noteworthy that in addition to being a transcription factor Engrailed is also a translation regulator [31] that guides retinal axons through the translation of local mRNAs [107–110]. This property is explained by the fact that Engrailed, like many other homeoproteins, interacts with the translation initiation factor eIF4E and regulates cap-dependent translation [111, 112]. In the context of neuron survival, it was shown that Engrailed protects mDA neurons against MPTP by upregulating the translation of a subset of nuclear-encoded mitochondrial complex I subunits (e.g., Ndufs1/Ndufs3), thus enhancing complex I activity and ATP synthesis [87, 109]. The importance of translational regulation of nuclear-encoded mitochondrial mRNAs coding for respiratory chain components has also been underscored recently for the function of some PD-linked genes [113]. All these studies support the idea that a failure to sustain the high-energy demand of mDA neurons may be critical in PD pathogenesis [114, 115].

Mitochondria are critically required for long-term axonal survival and maintenance [116]. Thus, decreased mitochondrial activity might contribute to retrograde cell death in *En1* heterozygous mice. As mentioned above, Engrailed plays a role in the activation of the mTOR pathway and the regulation of autophagy [74, 107]. The search for En1/2 translation targets in retinal axons of the Xenopus also identified Lamin B2, which is a major constituent of the nuclear envelope. It was shown that Lamin B2 translation in axons regulates mitochondrial size and mitochondrial membrane potential and supports axon survival [110]. En1/2 might thus play a role in mitochondrial activity and axon maintenance throughout adulthood.

Nurr1-mediated survival might involve neurotrophic GDNF/Ret signalling since Ret is a Nurr1 target gene [117, 118]. Nurr1 is downregulated by mutated α-synuclein [119, 120] and this could compromise GDNF/Ret survival signalling in PD. Absence of Ret signalling in mice causes progressive and late degeneration of the nigrostriatal system [121]. A recent study shows that Parkin cooperates with GDNF/Ret signalling to improve mitochondrial function through activation of the prosurvival NF-κB pathway and prevents mDA neuron degeneration [122]. Gene expression profiling in adult conditional *Nurr1* knockout mice also identified several nuclear-encoded mitochondrial genes as potential Nurr1 transcriptional targets [62]. Thus the mitochondria appear to be a target of Nurr1 activity for mDA neuron maintenance in the adult. In addition, Nurr1 was reported to be a downstream target of the cAMP response element binding protein (CREB) mediated neuroprotection [123]. Another function of Nurr1 in the nucleus might be related to DNA double strand break repair [124]. Interestingly, Nurr1 expression is induced in microglia and astrocytes under inflammatory conditions. Nurr1 activity in these cells suppresses proinflammatory NF-κB target gene expression through recruitment of the CoREST corepressor complex [125]. A more recent study shows that forced expression of Nurr1 and Foxa2 in glial cells markedly protects mDA

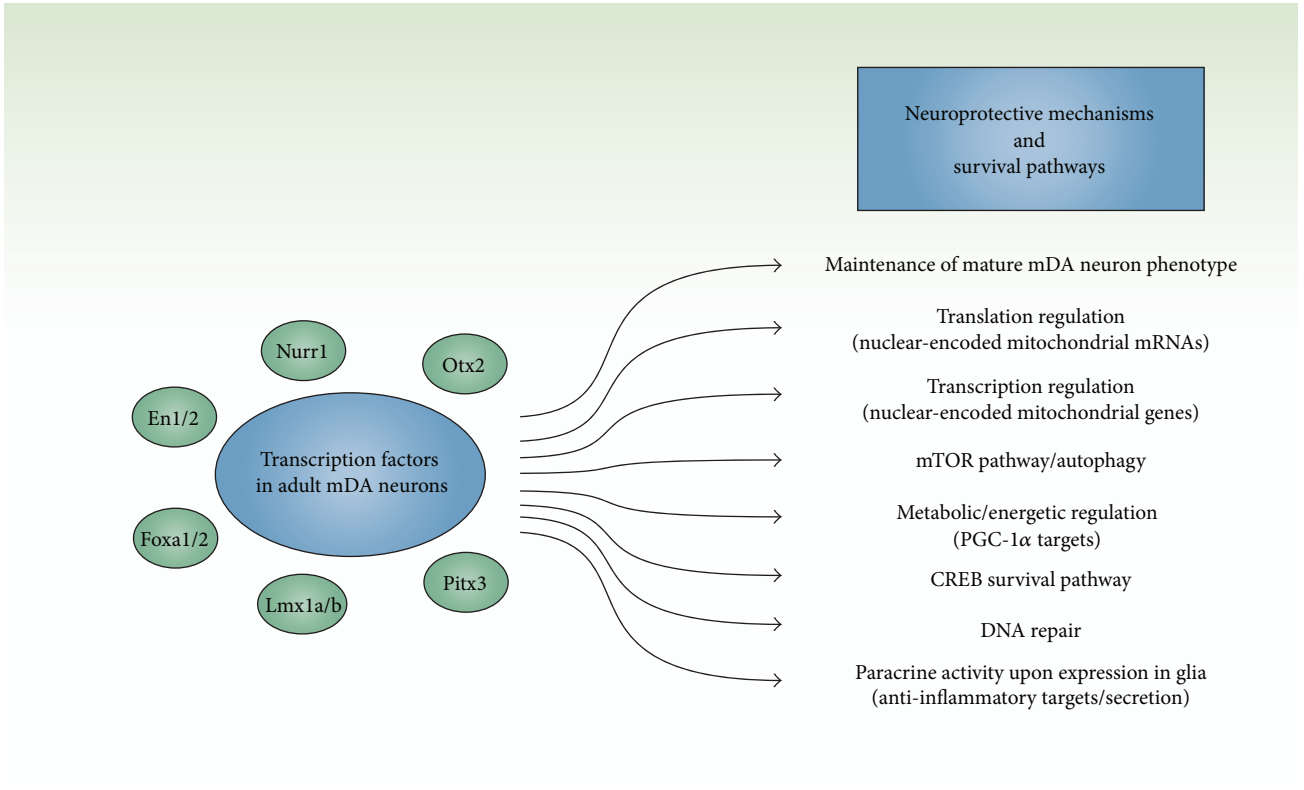

Figure 1: Multiple mechanisms and signalling pathways engaged by transcription factors for neuroprotection of adult mDA neurons.

neurons in the MPTP mouse model of PD [126]. Nurr1 and Foxa2 act synergistically in microglia to decrease the production and release of proinflammatory cytokines and enhance the synthesis and secretion of neurotrophic factors (e.g., GDNF, BDNF, NT3, SHH, erythropoietin, thioredoxin, TGF-β, and IGF-1) with paracrine action on mDA neurons [126]. In view of the ability of homeoproteins to be secreted and internalized, it will be interesting to examine if En1/2 and Otx2 play similar roles in glial cells in a non-cell-autonomous manner. The role of non-cell-autonomous signalling by these homeoproteins has been extensively demonstrated in axon guidance in the visual system for En1/2 [107–109] and in visual cortex plasticity for Otx2 [127, 128].

Pitx3 targets are also linked to several survival pathways in mDA neurons. The Pitx3 target *Aldh1a1* is crucial for the production of retinoic acid that exerts antiapoptotic and antioxidant activities. *Aldh1a1* is expressed in a subpopulation of mDA neurons in the SNpc and VTA. As already mentioned, the dependence on Pitx3 is not uniform for all mDA neurons. In *Pitx3* hypomorphic *Aphakia* mutants, a subpopulation of Pitx3-deficient neurons persists and these neurons are less vulnerable to MPTP-induced degeneration [129]. It has been suggested that striatal uptake and retrograde axonal transport of GDNF maintains proper expression of *Pitx3* and its target *Bdnf* in SNpc mDA neurons [130]. BDNF functions in synaptic transmission, plasticity, and growth and might contribute to synaptic maintenance of the nigrostriatal mDA neurons throughout adulthood [131]. A potential link between Pitx3 and PGC-1α (peroxisome proliferator-activated receptor gamma, coactivator 1 α), which is a positive regulator of genes required for mitochondrial biogenesis and cellular antioxidant responses, has also been recognized [132]. Overexpression of *PGC-1α* disrupts mitochondrial activity end energy balance and this might partly be due to downregulation of *Pitx3* by *PGC-1α* [133]. Finally, Pitx3 also regulates microRNA miR-133b expression, which in turn downregulates *Pitx3* [134]. *miR-133b* was shown to be downregulated in PD patients but the exact role of miR-133b in mDA neuron survival is not known [134].

6. Perspectives: From Basic Science to Potential New Therapeutic Avenues for PD

The characterization of mDA neuron populations in the SNpc and VTA, based on the expression of selected markers [9] and more recent single cell gene expression profiling [135], has revealed a substantial heterogeneity across neurons. This suggests that selected sets of transcription factors expressed in mDA neuron subpopulations might determine the degrees of vulnerability in PD. These developmental transcription factors also have adult functions through the regulation of mitochondrial activity and several survival signalling pathways. Disruption of postmitotic neuron maintenance through an alteration of their transcriptional/translational

regulation may lead to neurodegeneration [136, 137], often marked by cell cycle entry prior to death [138, 139]. As suggested above, many physiological processes participating in neuronal health and survival are controlled by developmental transcription factors. Possible sites of action are DNA repair and chromatin remodelling as well as pathways controlling genome stability. A recent example is Tau-mediated promotion of neurodegeneration through global heterochromatin relaxation [140]. Indeed these are homeostatic processes that can be regulated by classical signalling pathways as shown in the case of SHH [141]. From a more practical point of view, the successful use of homeoproteins, which have the innate ability to transduce, in the protection of mDA neurons has emphasized the potential of protein transduction-based strategies to deliver proteins directly into the cells of interest [142, 143]. The development of therapeutic proteins endowed with their own transduction domain, as is the case for homeoproteins, or made cell-permeable by the addition of a CPP-tag, could thus be seen as an alternative to cell grafting or gene therapy, provided that their effects be long-lasting.

Abbreviations

AADC: Dopa decarboxylase (aromatic L-amino acid decarboxylase)
AAV: Adenoassociated virus
ALDH1A1: Aldehyde dehydrogenase 1 family, member A1
Ascl1: Achaete-scute family bHLH transcription factor 1
Atg7: Autophagy-related 7
ATP13a2: ATPase type 13A2
BDNF: Brain-derived neurotrophic factor
CALB1: Calbindin 1, 28 kDa
CPP: Cell penetrating peptide
CREB: cAMP-responsive-element-binding protein
DA: Dopamine
DAT: Dopamine transporter (SLC6A3)
DDC: Gene encoding AADC
DJ-1: Parkinson protein 7 (PARK7)
DRD2: Dopamine receptor D2
DREADD: Designer receptors exclusively activated by designer drugs
eiF4E: Eukaryotic translation initiation factor 4E
En1/2: Engrailed-1/Engrailed-/2
Erk1/2: Extracellular signal-regulated-kinase 1/2
ESC: Embryonic stem cell
FGF8: Fibroblast growth factor 8
Foxa1/2: Forkhead box A1/2
GDNF: Glial cell derived neurotrophic factor
GIRK2: Potassium inwardly rectifying channel, subfamily J, member 6 (KCNJ6)
IGF-1: Insulin-like growth factor 1
iPSC: Induced pluripotent stem cells
LC3B: Microtubule-associated protein 1 light chain 3 beta (MAP1LC3B)
Lmx1a/b: LIM homeobox transcription factor 1, alpha/beta
LRRK2: Leucine-rich repeat kinase 2
MAPK: Mitogen-activated protein kinase
mDA: Midbrain dopaminergic
MPTP: 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mTOR: Mechanistic target of rapamycin (serine/threonine kinase)
Ndufs1/3: NADH dehydrogenase (ubiquinone) Fe-S protein 1/3
NF-κB: Nuclear factor of kappa light polypeptide gene enhancer in B-cells
NMDA: N-Methyl-D-aspartate
Nurr1: Nuclear receptor subfamily 4, group A, member 2 (Nr4a2)
NT3: Neurotrophin 3 (NTF3)
6-OHDA: 6-Hydroxydopamine
Otx2: Orthodenticle homeobox 2
p75NTR: Nerve growth factor receptor (NGFR)
PARKIN: Parkin RBR E3 ubiquitin protein ligase (PARK2)
PD: Parkinson disease
PGC-1α: Peroxisome proliferator-activated receptor gamma, coactivator 1 α
PINK1: PTEN induced putative kinase 1
Pitx3: Paired-like homeodomain 3
Ret: Ret protooncogene
RGC: Retinal ganglion cells
SHH: Sonic hedgehog
SNCA: Synuclein, α
SNpc: Substantia nigra pars compacta
Sox6: SRY- (sex determining region Y-) box 6
Tfam: Transcription factor A, mitochondrial
TGF-β: Transforming growth factor, β 1
TH: Tyrosine hydroxylase
VMAT2: Vesicular monoamine transporter 2 (SLC18A2)
VTA: Ventral tegmental area
Wnt1: Wingless-type MMTV integration site family, member 1.

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

Identification and Characterization of the V(D)J Recombination Activating Gene 1 in Long-Term Memory of Context Fear Conditioning

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An increasing body of evidence suggests that mechanisms related to the introduction and repair of DNA double strand breaks (DSBs) may be associated with long-term memory (LTM) processes. Previous studies from our group suggested that factors known to function in DNA recombination/repair machineries, such as DNA ligases, polymerases, and DNA endonucleases, play a role in LTM. Here we report data using C57BL/6 mice showing that the *V(D)J recombination-activating gene 1* (*RAG1*), which encodes a factor that introduces DSBs in immunoglobulin and T-cell receptor genes, is induced in the amygdala, but not in the hippocampus, after context fear conditioning. Amygdalar induction of *RAG1* mRNA, measured by real-time PCR, was not observed in context-only or shock-only controls, suggesting that the context fear conditioning response is related to associative learning processes. Furthermore, double immunofluorescence studies demonstrated the neuronal localization of RAG1 protein in amygdalar sections prepared after perfusion and fixation. In functional studies, intra-amygdalar injections of *RAG1* gapmer antisense oligonucleotides, given 1 h prior to conditioning, resulted in amygdalar knockdown of *RAG1* mRNA and a significant impairment in LTM, tested 24 h after training. Overall, these findings suggest that the *V(D)J recombination-activating gene 1*, *RAG1*, may play a role in LTM consolidation.

1. Introduction

Studies suggest that LTM consolidation depends on the morphological establishment, maintenance, and rearrangement of specific neural networks, which includes the strengthening of synapses or the formation of new connections within specific brain areas involved in learning and memory [1–4]. Importantly, concurrently with morphological changes of synaptic connections, transient induction of new gene transcription and protein synthesis are required for LTM formation [5–7]. Indeed, it has been shown that pharmacological blockade of transcription or translation, as well as the targeted mutation of transcription and translation factors, inhibits LTM consolidation [8–11].

One limitation with the current model used to explain LTM consolidation is that at the cellular level synaptic connections and electric patterns are highly dynamic and unstable, while memories can endure for months, years, and even decades. Similarly, at the molecular level, mRNA and proteins undergo molecular turnover. Some have suggested

that epigenetic modulation may explain the permanence of memories [12–15]; however, histone modifications are highly dynamic and reversible [16, 17]. In addition, the rapid turnover rate of transcriptionally active chromatin is a common feature in all nonproliferating cells, including neurons [18–20]. Similarly, DNA methylation and demethylation are dynamic and reversible even in nondividing cells, such as neurons [17, 21]. Hippocampal DNA methylation changes following learning are rapid, but these changes are plastic, not permanent [14, 22]. Moreover, epigenetic mechanisms that chemically modify histones or genomic DNA both regulate transcription. Hence, epigenetic mechanisms most probably function by temporarily regulating transcription during memory and plasticity processes.

In order to assess these questions, our group, as well as others, has been carrying out alternative studies in order to evaluate the possible role of other potential mechanisms that might also be relevant to LTM consolidation. Specifically, we initially postulated that mechanisms involved in DNA recombination/repair may contribute to LTM processes [23]. In the immune system, DNA recombination of gene segments is a well-controlled process involving the activation of DNA endonucleases, which in turn generate DNA DSBs, as well as activation of DNA ligases and DNA repair factors for rejoining new gene segments [24–26]. Interestingly, a recent study reported that when mice explored a novel environment, DNA DSBs were accumulated throughout the brain, particularly in the hippocampus, a region involved in learning and memory [27]. Moreover, these DNA DSBs were repaired within 24 h, suggesting that a physiological machinery for the introduction and repair of these DNA lesions may be related to learning and memory processes. Furthermore, subsequent studies reported that DNA DSBs are introduced in the promoters of a subset of immediate early genes including Fos, Npas4, and Egr1 in response to neuronal activity, synaptic plasticity processes, and learning [28]. Consistent with these findings, we previously reported that Fen-1 endonuclease [29], terminal deoxynucleotidyl transferase (TdT), a template-independent DNA polymerase involved in V(D)J recombination [30], DNA ligase [31, 32], and Non-Homologous End Joining (NHEJ) activity [32, 33] are DNA recombination/repair factors or machineries regulated by and/or required for learning and memory processes.

Here, we report that the *V(D)J recombination-activating gene 1* (*RAG1*), which is key to initiating V(D)J recombination in lymphocytes [34–37], is a factor modulated by context fear conditioning in young adult mice and that its amygdalar expression is required for LTM. Quantitative real-time PCR indicated that *RAG1* mRNA is induced in the amygdala, but not in the hippocampus, after conditioning. Such induction is related to associative learning, rather than to the nonassociative behavioral experiences related to context fear conditioning, as determined with Naïve, context-only, and shock-only controls. Additional control experiments confirmed the sequence identity between amygdalar and thymus *RAG1* PCR products, both showing 100% match to *Mus musculus RAG1* in BLAST analyses. Moreover, double immunofluorescence studies indicated that RAG1 protein is expressed within amygdalar neurons. The functional relevance of *RAG1* was examined using gapmer antisense, versus random oligonucleotides infused directly into the amygdala either immediately prior to or 5 h after conditioning. Pretraining infusions resulted in amygdalar knockdown of *RAG1* mRNA and a significant impairment in LTM, while posttraining infusions did not affect LTM. Together, these findings suggest that *RAG1* plays a role in LTM consolidation.

2. Materials and Methods

The Institutional Animal Care and Use Committee (IACUC) of the Río Piedras Campus of the University of Puerto Rico in compliance with National Institutes of Health (NIH) guidelines for the care and use of laboratory animals (Department of Health and Human Services NIH publication number 86-23) approved all procedures involving animals.

2.1. Contextual Fear Conditioning

2.1.1. Apparatus. Our conditioning chamber (30 × 20 × 18 cm) was made of transparent Plexiglas on two sides and stainless steel on the other two sides. Each of the steel sides had a speaker and a 24 V light. The chamber had a 36-bar-insulated shock grid floor made of stainless-steel rods (Coulbourn Instruments, Allentown, PA). The system included a white-noise generator to provide background noise (70 dB). The floor was removable and was cleaned with 70% ethanol after each subject was trained, reexposed, or tested. Each bar (1.5 cm in diameter) was connected through a harness to a programmable Master Shocker (model 82404SS; Coulbourn Instruments) that delivered scrambled foot shocks to each of the bars in the grid floor. A mini camera (Silent Witness Enterprises, Surrey, British Columbia, Canada) installed directly behind one of the two Plexiglas sides of the conditioning chamber was connected via a processor to a computer system for video recording and scoring of freezing using the Xpress SDK software, which is a PCI bus mastering wavelet video compression/decompression and capture board (Integral Technologies, Indianapolis, IN).

2.1.2. Subjects and Training. Context conditioning was done essentially as previously described [11, 32, 38]. Male C57BL/6 mice of 8–10 weeks of age from Harlan Sprague Dawley, Indianapolis, IN, were used. Food and water were available at all times, and the animals were kept on a 12 h light/dark cycle. In contextual fear conditioning, animals were placed in the conditioning chamber (conditioned stimulus, CS) and allowed to explore for 2 min (habituation). Animals then received three foot shocks of 0.75 mA for 2 s (unconditioned stimulus, US) delivered at 2, 3, and 4 min. Mice remained in the chamber 30 s after the last shock and were then immediately moved to their home cages.

2.2. RNA Extraction, Quantification, and Quality Evaluation. Once trained, animals were decapitated at 15 min, 30 min, or 1 h after conditioning. Some animals were also used as Naïve, 15 min context-only (CO) and 15 min shock-only (SO) controls. CO mice were exposed to the conditioning context for 4 m without receiving any shocks and SO mice

received a rapid single shock and were immediately removed from the conditioning chamber. After removal from the conditioning chamber, both CO and SO mice were returned to their homecages and sacrificed 15 min after the end of their exposure to the conditioning chamber. Brains were rapidly obtained, chilled in ice-cold Phosphate Buffered Saline (PBS), and then transferred to a mouse brain matrix to obtain bilateral amygdalar tissue punches and dissection of the dorsal hippocampus, both between −0.82 and −2.70 mm from bregma points, based on the mouse brain in stereotaxic coordinates, third edition [39]. All tissues were kept in RNA*later* (Ambion, Cat. number 7020) solution on dry ice and later stored at −86°C until RNA extraction. RNA was extracted from individual dorsal hippocampi using the Qiagen RNeasy Mini Kit (Cat. number 74104), while bilateral amygdalar punches were RNA extracted using the Qiagen RNeasy Micro Kit (Cat. number 74004). Extracts were treated using DNase (Qiagen, 79254) and the kit's protocol was performed. RNA samples were quantified using the NanoDrop ND-1000 spectrophotometer and quality was evaluated using the Agilent 2100 Bioanalyzer system.

2.3. Quantitative Real-Time PCR

2.3.1. Primer Design for Real-Time PCR. cDNA sequences from *Mus musculus* genes analyzed [*RAG1*, accession number NM_009019.2; and *glyceraldehyde 3-phosphate dehydrogenase* (*gapdh*), accession number NM_008084.2] were obtained from GenBank. We used the Integrated DNA Technologies PrimerQuest and Oligo Analyzer bioinformatics tools to design specific primers suitable for real-time PCR and also avoid possible hairpins, self/homodimers, and hetero/cross-dimers. A BLAST (basic local alignment search tool) search was done on all primers to ensure that they would not potentially anneal to other targets. The following forward and reverse primers were used: *RAG1*, forward, 5′-TGA GCA CAG GCA AGC TGA TGA-3′ and *RAG1* reverse, 5′-TTG ACA CGG ATG GCC AAG CAA-3′; for *gapdh* forward 5′-ACC CAG AAG ACT GTG GAT GG′-3 and *gapdh* reverse 5′-ACA CAT TGG GGC TAG GAA CA-3′. All primers were synthesized by Integrated DNA Technologies.

2.3.2. cDNA Synthesis and Quantitative Real-Time PCR. Briefly, cDNA was synthesized using the TaqMan Reverse Transcription (RT) Reagents kit (N8080234, Applied Biosystems/Roche). 250 ng of RNA, 2.5 μL of RTBuffer (10x), 6.0 μL $MgCl_2$ (25 mM), 5.0 μL dNTPs (10 mM, 2.5 mM, each nucleotide), 2.0 μL OligodT (50 μM), 0.5 μL RNase inhibitor (20 U/μL), and 3.0 μL RT Enzyme (50 U/μL) in a total volume of 25 μL were used. Thermal cycler conditions were as follows: 25°C for 10 min, 48°C for 30 min, 95°C for 5 min, and 4°C forever. Real-time PCR was performed using the QuantiTect SYBR Green PCR Master Mix Kit (Qiagen, 204163). Real-time PCR amplification conditions were optimized for each gene and we obtained the best results under the following master mix conditions: for housekeeping control gene *gapdh* we used 12.5 μL of SYBR Green Master Mix, 2.5 μL of each primer (5 μM), and 2 μL of cDNA in 25 μL of reaction. For *RAG1*, we used 12.5 μL of SYBR Green Master Mix, 3.5 μL of each primer (5 μM), 1.5 μL of $MgCl_2$ (25 mM), and 2 μL of cDNA in 25 μL of reaction. Real-time PCR amplifications were run in triplicate for each gene per sample in a thermal cycler (Bio-Rad C1000 Touch CFX96 Real-Time PCR System). Amplification conditions were optimized for both genes at 95°C 15 min (hot start), followed by 40 cycles at 95°C for 15 s, 30 s at 58°C, and 72°C for 30 s. Finally, a melt/peak curve analysis was performed from 55°C to 95°C at increasing temperature rate of 0.5 degrees.

2.3.3. Real-Time PCR Analysis. We used the comparative threshold cycle (Delta Ct) method of relative quantification to calculate gene expression levels [40]. Delta Ct method involves comparing the Ct values of the genes of interest with a reference or housekeeping gene: Ct = Ct Target − Ct Reference gene ($Ct_t - Ct_r$). In this case, the Ct value of *RAG1* primers' amplification product is subtracted from the Ct of *gapdh* primers' amplification product ($Ct_{RAG1} - Ct_{gapdh}$). First, the Ct-average of triplicates per gene per sample from PCR amplification was obtained. Importantly, our analyses only used samples displaying triplicate results with high reproducibility, that is, those showing triplicate differences in Ct of no more than 0.05%. In the case that the real-time PCR experiments of a particular sample resulted in low reproducibility, such specific experiments were repeated or in some cases discarded, always making sure that the final analyses could be made with the result of triplicate reactions per sample. As Ct is proportional to the logarithm of initial amount of target in a sample, the relative concentration of one target contextual fear conditioning-trained with respect to a reference (Naive) is reflected in the difference in cycle number (Ct) necessary to achieve the same level of fluorescence. Delta Ct data is then normalized by $2^{-(\text{deltaCt trained}-\text{deltaCt naive})}$.

2.4. Amplification and Molecular Cloning of RAG1 PCR Products. We amplified a *RAG1* mRNA fragment of approximately 48 bp (the same from the real-time PCR experiments described above) from amygdalar, hippocampus, and thymus tissue and also cloned the resulting PCR products for sequence analysis. Briefly, following RNA extraction and purification (as above), cDNA was synthesized using TaqMan Reverse Transcription Reagents kit (N8080234, Applied Biosystems/Roche). 500 ng of RNA from each sample was used and mixed with 2.5 μL of RT Buffer (10x), 4.5 μL $MgCl_2$ (25 mM), 4.0 μL dNTPs (10 mM, 2.5 mM each nucleotide), 2.0 μL OligodT (50 μM), 0.5 μL RNase inhibitor (20 U/μL), and 3.0 μL of RT Enzyme (50 U/μL) in a total volume of 25 μL. cDNA synthesis was performed in a thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) under the following conditions: 25°C for 10 min, 48°C for 30 min, 95°C for 5 min, and 4°C forever. PCR was performed using the PCR Master Mix (2x) (2 × 50 reactions) Kit (Promega, M7502). PCR amplification conditions were performed under the following master mix conditions: 12.5 μL of master mix, 3.5 μL of each primer (5 μM, the same primers used for real-time PCR experiments), 1.5 μL of $MgCl_2$ (25 mM), and 2 μL of cDNA in 25 μL of reaction. Amplifications were performed by an initial denaturation at 95°C for 30 s, followed by 40 cycles at 95°C for 15 s, 58°C for 30 s, and 72°C for 30 s, and final extension

at 72°C for 7 min followed by 4°C forever. PCR amplifications were evaluated by agarose (1%) gel electrophoresis in TAE1X and stained with ethidium bromide. PCR products were purified from the gel using the QIAquick Gel Extraction Kit (50), following the kit protocol manual (Qiagen, 28704). Purified PCR products were cloned using the pGEM-T Easy Vector System I (Promega, A1360). DNA ligation and plasmid transfection were performed as recommended by the kit's protocol. For plasmid transfection we used *E. coli* (from Invitrogen, 18258-012) MAX Efficiency DH5α Competent Cells. Cells were plated onto LB/ampicillin agar medium containing IPTG and X-Gal.

2.5. Plasmid DNA Extraction and Purification for Sequencing. Minipreps for sequencing were performed using a modification of standard methods [41]. Briefly, a single white bacterial colony was transferred into 3 mL of Terrific Broth medium containing ampicillin in a loosely capped 15 mL tube. Cells were cultured overnight at 37°C with vigorous shaking. Harvest and extraction was performed as indicated in standard methods [41], but we also did additional successive purification steps, including RNase A treatment, exonuclease digestion, and proteinase K incubation. After two chloroform extractions and isopropanol precipitations, pellets were dried, dissolved in 50 μL of H_2O, and mixed with 12 μL of NaCl 4 M and 60 μL of freshly made polyethyleneglycol 13% (PEG 8000). The mixture was incubated on ice for 30–60 min and centrifuged for 15 min and the pellet was rinsed with ethanol 70%. Finally, pellets were dried and dissolved in 50 μL of H_2O. Samples were kept in −20°C until used. Sequencing reactions were performed using the BigDye Terminator Chemistry v.3 in an ABI 3130xl Genetic Analyzer. DNA sequences were analyzed by BLAST against the mouse genome (RefSeq RNA) and aligned using ClustalW2.

2.6. Histology: Brain Perfusion and Tissue Preparation. One hour after context fear conditioning, animals received a lethal dose of avertin and were immediately followed by transcardial gentle perfusion with PBS buffer for 5 min. After that, we switched to the fixing buffer (4% paraformaldehyde, pH 7.4) for 5 min. Brains were extracted, fixed in 4% paraformaldehyde solution for 24 h, and later cryoprotected overnight in a 30% sucrose solution and finally frozen at −86°C for storage until future use. Frozen brains were used to collect 20 μm thick coronal sections selecting specifically those containing the amygdala region between −1.06 and −2.30 mm bregma points, based on the mouse brain in stereotaxic coordinates, third edition [39]. The collected sections were placed onto positively charged slides (Probe-On Plus Slides, Fisher, PR) and kept at −86°C until used for immunofluorescence.

2.7. Immunofluorescence. To examine the cellular localization of RAG1 protein expression, slides were dried up at rt for 30 min. Using a heat block set at 200°C, a beaker flask containing the buffer (800 mL ddH_2O, 4 mL 1 M Tris pH 8, and 1.6 mL 0.5 M EDTA) was heated until boiling. Once the buffer's bubbles were moving slowly, slides were slowly immersed into the buffer using a slide-rack. The beaker was immediately covered with a saran wrap containing holes punched with a pipet tip and was allowed to sit for 20 min. The beaker containing the slides was then transferred into an ice bath and allowed to cool down in a cold room (4°C) for 30 min. Slides were partially dried and borders drawn with PAP-Pen. Then, 250 μL of blocking solution (BS) (1x PBS, 10% normal goat serum, and 0.1% Tween-20) was added per slide and incubated for 1 h at rt. Double immunofluorescence was performed, incubating the sections with primary rabbit polyclonal antibody against human RAG1 (Sigma-Aldrich: SAB2106610) diluted at 1 : 100 in 1% goat serum/PBS together with primary anti-NeuN mouse monoclonal antibody (Millipore, MAB377) diluted at 1 : 100. Incubation with primary antibodies was done overnight at 4°C in a moist chamber. Slides were washed with PBS 3 times for 5 min each. Slides were then incubated for 2 h at rt in the dark moist chamber with Alexa Fluor 488-conjugated goat anti-rabbit IgG secondary antibody (Invitrogen) (for detection of RAG1) and Alexa Fluor 568-conjugated goat anti-mouse IgG secondary antibody (Invitrogen) (for detection of NeuN), both diluted at 1 : 200 in 1% goat serum/PBS, followed by 3 PBS washes for 5 min each. Slides were mounted using permanent mounting medium (Vector Laboratories). All slides were first scanned at low magnification (10x) to locate the amygdala, which was subsequently analyzed at higher magnification (40x) using a Zeiss LSM-5 Pascal scanning confocal microscope. Final image composites were created using Zeiss LSM5 PASCAL Image software, version 3.2.

2.8. Protein Extraction. Mice were decapitated 1 h after training and their brains were obtained, chilled on ice-cold PBS, and used to dissect the amygdala as previously explained, between −0.82 and −2.7 mm bregma points, based on the mouse brain in stereotaxic coordinates [39]. Bilateral amygdalar tissue punches from three animals were combined yielding one pool sample per group. Thymus, bone marrow, and muscle tissues were also dissected as controls. Tissues were stored at −86°C until used for protein extraction. Protein extracts were prepared as described by us previously [29, 31–33]. Briefly, tissues were homogenized using a sonic dismembrator in extraction buffer [30 mM HEPES/KOH, pH 7.9, 0.5 M KCl, 5 mM $MgCl_2$, 1 mM EDTA, 2 mM dithiothreitol (DTT), 20% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 μg/mL of each of leupeptin and aprotinin] and incubated for 1 h on ice. Extracts were centrifuged at 14,000 rpm for 1 h at 4°C. The supernatant was then dialyzed for 4 h in dialysis buffer (30 mM HEPES/KOH, pH 7.9, 50 mM KCl, 2 mM EDTA, 5 mM $MgCl_2$, 1 mM DTT, 10% glycerol, 1 mM PMSF, and 1 μg/mL of each of leupeptin and aprotinin). Dialyzed fractions were centrifuged at 14,000 rpm for 30 min at 4°C. Protein extracts were stored at −86°C until used for Western blots. The protein concentration was determined in the NanoDrop ND-1000 spectrophotometer.

2.9. Western Blotting. For Western blotting, protein samples (50 μg) and 4 μL of the Odyssey Prestained Molecular Weight Marker (LI-COR Biosciences, Nebraska, USA) were first separated on a 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins in the gels were transferred to a nitrocellulose membrane using

a semidry electroblotter system at 15 V for 1 h and 30 min. Then, the membranes were blocked overnight using a mixture of 5% nonfat milk and Odyssey Blocking Buffer (LI-COR Biosciences, Nebraska, USA) on an orbital shaker. After 3 washes of 15 min each with PBS Tween-20 (PBS-T), the membrane was incubated with a mixture of two primary antibodies: 1 : 2,000 dilution of a rabbit polyclonal antibody raised against a synthetic nonphosphopeptide derived from human RAG1 (Sigma-Aldrich: SAB2106610) and 1 : 3,000 dilution of a mouse monoclonal anti-actin antibody (Sigma-Aldrich: A4700) at 4°C overnight. After 3 washes of 15 min each with PBS-T, the membranes were incubated with a mixture of two secondary fluorescent antibodies (1 : 10,000 dilution of donkey anti-rabbit IRDye680 and 1 : 12,000 dilution of donkey anti-mouse IRDye800) (LI-COR Biosciences, Nebraska, USA) for 2 h at rt. The membranes were washed 3 times as previously mentioned and scanned for analysis using the Odyssey Infrared Imaging System.

2.10. Antisense Knockdown Experiments

2.10.1. RAG1 Antisense Oligonucleotides for Gene Knockdown. Gapmer antisense oligonucleotides were designed to target the start codon of mouse *RAG1* mRNA. This 20 bp gapmer contains a central block consisting of ten phosphorothioate-deoxynucleotides, sufficient to induce RNaseH cleavage (and thus targeted RNA:DNA hybrid degradation), and it is flanked by two blocks, each consisting of five 2′-*O*-methyl-modified ribonucleotides that protect the internal phosphorothioate-deoxynucleotide block from nuclease degradation, thus increasing stability. As a control, a random sequence was also designed with the same backbone modifications and base composition as the *RAG1* antisense, but in a scrambled sequence order and without homology to any known mouse gene. *RAG1* antisense and random oligonucleotide sequences were, respectively, as follows: 5′-mGmCmC mAmCA* G*A*G* A*T*A* G*C*A* mAmCmA mUmA-3′ and 5′-mCmAmG mAmUA* A*C*C* G*T*A* G*A*G* mCmAmA mCmA-3′ (where "m" represents 2′-*O*-methyl RNA and "*" represents phosphorothioate DNA). Antisense and random sequences were synthesized by Integrated DNA Technologies. All batches of antisense and random oligonucleotides used to complete these studies were received lyophilized and fully purified by RNase Free HPLC. Oligonucleotides were dissolved in sterile 1x TE buffer pH 7.5 solution to a final concentration of 0.2 nmol/μL.

2.10.2. Surgeries. Several surgeries were conducted to determine the proper coordinates for implantation of cannulae into the amygdala, based on the mouse brain in stereotaxic coordinates [39]. For surgery, animals were anesthetized with avertin and placed into a stereotaxic apparatus (David Kopf Instruments), with the nose angled at 0°. After a scalp incision was made, lambda and bregma were located, and holes were drilled in the skull above the target region. Bilateral cannulae (23 gauges) guide (6.8 mm wide) was implanted above the amygdala in order to avoid damaging the amygdala tissue complex with cannulae and injectors while at the same time ensuring amygdala-enriched distribution. The following coordinates were used: anterior-posterior, +1.0 mm from bregma; medial-lateral, −3.4 mm from midline; and dorsoventral, −2.5 mm from skull. The cannulae were secured to stainless-steel screws with dental cement and a light-curable resin. Wire stylets (33 gauges) were inserted into the cannulae guides and checked every day to ensure clean and functional cannulae.

2.10.3. Intra-Amygdalar Oligonucleotide Microinfusions, Behavioral Training, and Memory Testing. After surgery, animals were given a two-day period to recover. Afterwards, animals were handled during 3 days, 3 min each. The fourth day, animals were bilaterally microinfused with 1 μL of 1x TE buffer pH 7.5 (2 min at 0.5 μL/min), as a handling/stress control, and then we proceeded with the regular 3 min manual handling. Microinfusions were accomplished by inserting a 33-gauge stainless-steel injector into the guide cannulae so that it extended 1 mm beyond the tip of the guide, right above the targeted amygdalar complex (see above). Functionality of injectors was verified before every microinfusion between animals and replaced when necessary. After infusion, the injectors were removed, and the stylets were replaced. The fifth day, animals were bilaterally microinfused (2 min at 0.5 μL/min) with 0.2 nmol of *RAG1* antisense or random sequence oligonucleotides, handled for 3 min, and returned to their home cages. One hour after the microinfusions, animals were trained in context fear conditioning and video recorded as described above. One day (24 h) after training mice were reintroduced into the same conditioning context for LTM testing by measuring freezing in the video recordings for 4 m, but they did not receive shocks. For the posttraining injection studies, a different set of animals was treated as above except that the antisense or random oligonucleotide treatment was given 5 h after context fear conditioning instead of 1 h prior to training. For the reconsolidation studies, another set of mice was subjected to surgery cannulation and received handling as above, but in the fifth day, mice were microinfused with saline (as a stress control) and trained in context fear conditioning 1 h later. Next, 24 h after conditioning, animals were microinfused with either random or antisense oligonucleotides 1 h prior to reexposure to the conditioning chamber and video recorded for 90 s for memory retrieval without receiving any shocks. Finally, 48 h after conditioning (24 h after context reexposure), mice were again reexposed to the conditioning chamber for 2 min to measure freezing of LTM in the reconsolidation test.

2.10.4. Diffusion Studies. After cannulae implantation, injectors were inserted and animals ($n = 4$) were infused with FITC-*RAG1* antisense oligonucleotides to estimate the area of the antisense diffusion within the amygdala. An infusion of 1 μL of FITC-*RAG1* antisense oligonucleotide (0.2 nmol) was delivered bilaterally into the amygdala during a 2 min period at a rate of 0.5 μL/min. Animals were decapitated 3 h after infusion, and their brains were isolated and stored at −86°C. Coronal amygdalar sections, 20 μm thick, were scanned at low magnification (10x) to locate the amygdala, which was subsequently analyzed at higher magnification (20x and 40x) using a Zeiss LSM-5 Pascal scanning confocal microscope.

Images were processed using Zeiss LSM5 PASCAL Image software, version 3.2.

2.11. Statistical Analysis. All statistical analyses were performed with Prism 4 software (GraphPad Software). Statistical significance was assumed at $P < 0.05$. Real-time PCR experiments of *RAG1* mRNA levels were analyzed by One-Way ANOVA and Newman-Keuls posttests to compare *RAG1* mRNA expression between behavioral groups. Real-time PCR experiments of perfused and nonperfused mice for *RAG1* levels were analyzed using Student's t-test. Knockdown validation of *RAG1* antisense mRNA levels in the amygdala compared to random was analyzed by Student's t-test. Memory acquisition behavioral data was subjected to Two-Way Repeated Measures (RM) ANOVA and Bonferroni posttesting. LTM behavioral tests analyses were analyzed by Student's t-test. Memory retrieval and reconsolidation tests were analyzed by Two-Way ANOVA coupled to Bonferroni posttesting.

3. Results

3.1. Context Fear Conditioning Learning Specifically Induces RAG1 mRNA Expression in the Amygdala. DNA recombination/repair processes involve the activation of endonucleases as well as DNA ligases and polymerases, among other factors. We focused our present studies on *RAG1*, the gene encoding the specialized recombinase of V(D)J recombination, which initiates V(D)J recombination *in lieu* of its site-specific endonuclease activity that targets highly specific recombination signal sequences (RSSs) introducing DNA DSBs in antigen receptor genes [34, 36, 37, 42] and which we identified in a preliminary DNA microarray screen as a potential candidate gene involved in context fear conditioning. For the experiments reported here, we initially trained C57BL/6 male mice in context fear conditioning, sacrificed at 15, 30, or 60 min after training, and obtained dorsal hippocampi and amygdalar tissues. We used quantitative real-time PCR to amplify a fragment of *RAG1* mRNA and determine whether the expression of this gene is modulated in the amygdala or the hippocampus in association with context fear conditioning learning. The results of these experiments are shown in Figure 1. As seen in Figure 1(a), when examining hippocampal *RAG1* mRNA no significant differences were observed between the Naïve (Naive, $n = 6$) and the conditioned (C) groups sacrificed at either 15 ($n = 7$), 30 ($n = 7$), or 60 ($n = 7$) min after training (One-Way ANOVA: $F(3, 23) = 0.8966$, $P > 0.05$). The results demonstrate that the basal levels of hippocampal *RAG1* mRNA do not change significantly after training. In contrast to our findings with the hippocampus, we found that context fear conditioning training results in a significant, rapid, and transient induction in *RAG1* amygdalar mRNA levels (Figure 1(b): One-Way ANOVA, $F(3, 30) = 4.753$, $^{**}P < 0.01$; Multiple Comparison Testing: Naïve versus C15 min, $^{*}P < 0.05$; Naïve versus C30 min, $^{\#}P < 0.05$; Naïve versus C60 min, $P > 0.05$; C60 min versus C30 min, $^{+}P < 0.05$; and C60 min versus C15 min, $P > 0.05$). Overall, these results show that *RAG1* mRNA is induced rapidly and transiently after context fear conditioning training in the amygdala, but not the hippocampus, of young adult C57BL/6 mice. For our next set of experiments, we aimed to determine if such amygdalar induction is specific to associative context fear conditioning, using nonassociative context-only (CO, $n = 6$) and shock-only (SO, $n = 8$) controls, in which the conditioned stimulus (CS) (for CO controls) or unconditioned stimulus (US) (for SO controls) was presented individually, rather than paired. We sacrificed animals from the C ($n = 9$), CO, or SO groups 15 min after their respective associative or nonassociative training. Brains were obtained, amygdalar tissue punches were dissected, and RNA was extracted. Naïve animal controls were also used (Naive, $n = 8$). The results can be seen in the bar graph in Figure 1(c). The results again confirmed the induction at 15 min of amygdalar *RAG1* mRNA after context fear conditioning compared to Naïve, CO, or SO controls and showed no statistical significant difference between any of these controls (One-Way ANOVA, $F(3, 27) = 5.943$, $^{**}P < 0.005$; Multiple Comparison Testing: Naive versus C15 min, $^{**}P < 0.01$; SO15 min versus C15 min, $^{\#}P < 0.05$; CO15 min versus C15 min, $^{++}P < 0.01$; SO15 min versus Naive, $P > 0.05$; SO15 min versus CO15 min, $P > 0.05$; CO15 min versus Naive, $P > 0.05$). Finally, since the results presented so far were obtained from tissue samples obtained from nonperfused brains, we carried out an additional control experiment to determine if the observed changes in *RAG1* mRNA levels could be attributed to the presence of blood cells in the brain or not, since immune cells in the blood are a known biological site of *RAG1* expression [43, 44]. We trained two groups of mice in context fear conditioning and sacrificed animals of both groups 30 min after training, a time corresponding to the peak for amygdalar *RAG1* mRNA induction observed after training (see Figure 1(b)). The brains of animals in one group were obtained as described above. For the second group of animals, mice were injected with a lethal dose of avertin 25 min after training and perfused for 3 min with PBS-1X in order to remove the blood from their brains. Brains from both groups of animals were extracted and amygdalar tissues ($n = 4$ nonperfused; $n = 4$ perfused) were dissected. Amygdalar tissues were used for further RNA isolation, cDNA synthesis, and quantitative real-time PCR analysis of *RAG1* mRNA. The results showed no significant difference between the levels of amygdalar *RAG1* mRNA 30 min after context fear conditioning training of nonperfused (1.304 ± 0.3377) or perfused (1.274 ± 0.2751) mice (Student's t-test; $t_{(7)} = 0.1473$; $P > 0.8$), ruling out the possibility that the observed induction of *RAG1* mRNA (see Figure 1(b)) could be due to residual blood, and thus blood cells, in the examined brain tissues.

3.2. Sequence Analysis of PCR Products Amplified from the Hippocampus, Amygdala, and Thymus. We next set out to confirm the sequence identity of the PCR products from amygdalar and hippocampal tissues amplified using the set of primers designed to target *RAG1* mRNA in our experiments presented above. In addition to amygdalar and hippocampal RNA samples, we also utilized thymus RNA, since this tissue is known to physiologically express *RAG1* due to its role in the immune system [45–48]. Supplementary Figures 1A and B

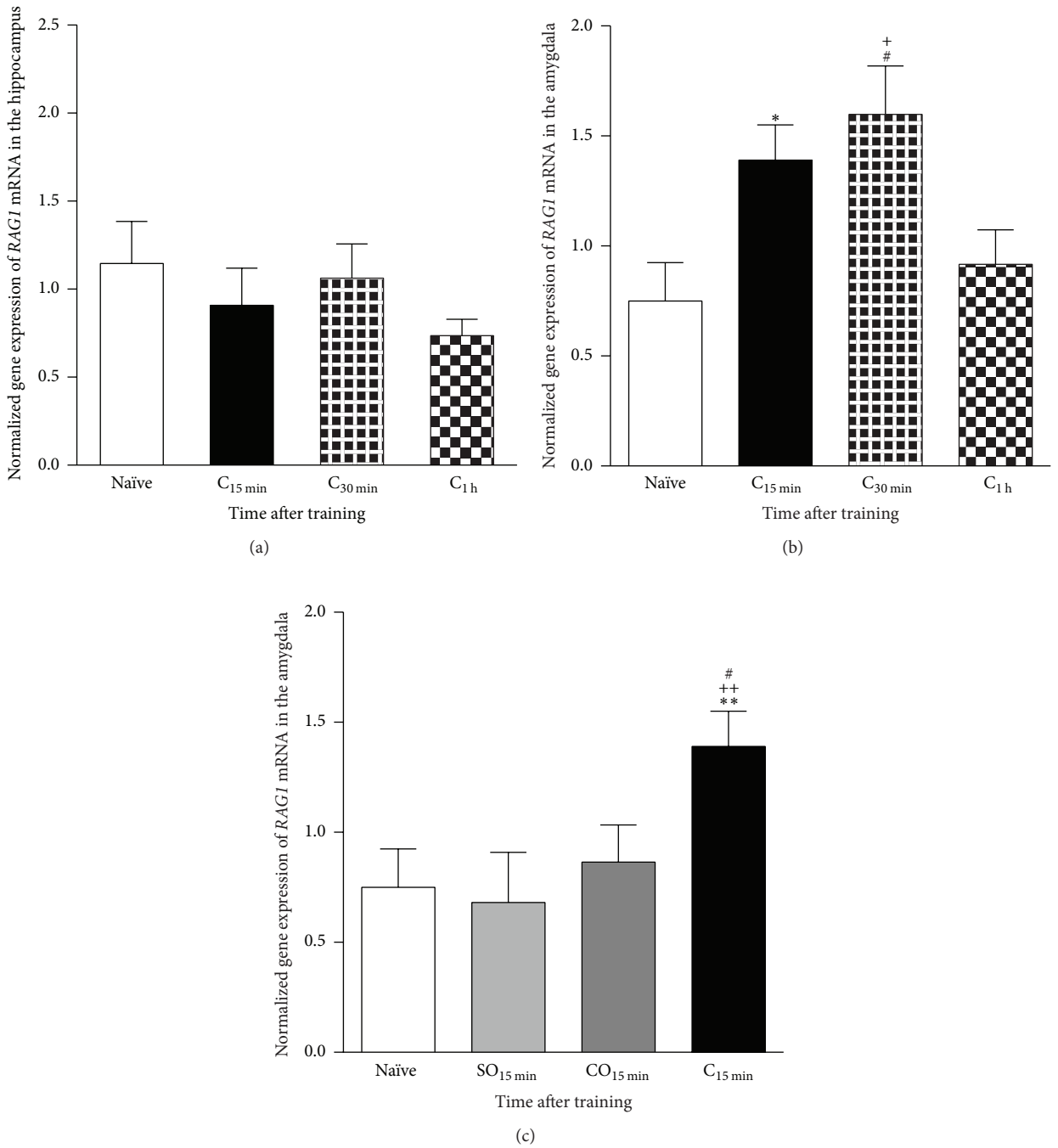

Figure 1: Context fear conditioning induced upregulation of *RAG1* mRNA in the amygdala. *RAG1* mRNA levels were measured in the hippocampus and the amygdala on a time course at 15 min, 30 min, and 1 h after conditioning. (a) Normalized mRNA data showed no significant differences when examining hippocampal *RAG1* mRNA Naïve (N) or the conditioned (C) groups sacrificed at 15, 30, or 60 min after training. (b) In contrast, context fear conditioning results in a significant, rapid, and transient induction in *RAG1* mRNA levels in the amygdala (Naïve versus C15 min, $^{*}P < 0.05$; Naïve versus C30 min, $^{\#}P < 0.05$; Naïve versus C60 min, $P > 0.05$; C60 min versus C30 min, $^{+}P < 0.05$; and C60 min versus C15 min, $P > 0.05$). (c) We sacrificed animals from the conditioned (C), CO, or SO groups 15 min after their respective associative or nonassociative training. Normalized expression confirmed the significant induction at 15 min of amygdalar *RAG1* mRNA after context fear conditioning compared to Naïve, CO, and SO groups and showed no statistical difference between Naïve, CO, or SO controls (SO15 min versus C15 min, $^{\#}P < 0.05$; SO15 min versus Naive, $P > 0.05$; SO15 min versus CO15 min, $P > 0.05$; CO15 min versus C15 min, $^{++}P < 0.01$; CO15 min versus Naive, $P > 0.05$; and Naïve versus C15 min $^{**}P < 0.01$).

(in Supplementary Material available online at http://dx.doi.org/10.1155/2016/1752176) depict the representative results of amplification curves, as well as our melting curve analysis, respectively, for *RAG1* and *gapdh* mRNAs. Results of amplification and melting temperature curves of *RAG1* and *gapdh* are depicted to show the cycle thresholds (Ct) for both genes and the specificity of the amplification products, respectively. Importantly, the results of the melting curve analyses consistently demonstrated that only one specific product per primer-gene set was generated. The *RAG1* amplification products amplified by standard PCR were also visualized by agarose gel electrophoresis (Supplementary Figure 1C). Cloning and sequencing of these PCR products amplified from amygdala, hippocampus, and thymus confirmed their identity as *RAG1* (Supplementary Figures 2D–F) or *gapdh* (data not shown). Sequencing electropherograms from *RAG1* PCR products are shown in Supplementary Figure 2D. PCR fragment sequences from amygdala, hippocampus, and thymus were aligned using ClustalW2 (Supplementary Figure 2E) and compared with *Mus musculus RAG1* reference sequence NM_009019.2 (Supplementary Figure 2F). BLAST analysis confirmed the molecular identity of *RAG1* PCR products from amygdala, hippocampus, and thymus showing 100% matched identity to *Mus musculus RAG1* (Ref|NM_009019.2) in mouse genome BLAST analyses with an *E*-value of 2e − 19.

3.3. RAG1 Protein is Localized within Amygdalar Neuronal Cells. RAG1 is the key endonuclease of V(D)J recombination in immune cells introducing, together with RAG2, DSBs in antigen receptor genes at their RSSs [34, 36, 37, 42]. Previous reports have also suggested that the transcript encoding RAG1 is expressed in nervous cells [34–37, 49]. We therefore considered it important to determine whether RAG1 protein expression is localized to amygdalar neurons. Double immunofluorescence of RAG1 antibody with neuronal nuclear marker, NeuN, was performed on brain coronal sections from animals perfused 1 h after context fear conditioning. Representative images obtained from confocal microscopy examination of the amygdalar areas of brain sections double-labeled with RAG1 (Alexa Fluor 488; green signal) and NeuN (Alexa Fluor 568; red signal) are shown in Figure 2(a). These findings indicate that RAG1 appeared to be expressed predominantly in neurons, as suggested by the colocalization between RAG1 and NeuN. It is also important to mention that while all RAG1 positive cells were neurons, not all neurons showed RAG1 reactivity, suggesting that only a subset of cells expressed RAG1. The molecular specificity of the RAG1 antibody was confirmed by Western blot analyses (Figure 2(b)). Tissue punches from amygdala were obtained 1 h after context fear conditioning. Additionally, reference tissues from thymus and bone marrow, known to express high levels of RAG1 [45–48], and muscle (negative control) were dissected. All tissues were subjected to protein extractions for Western blot analyses. RAG1 protein expression from the amygdala was compared by comigration with bone marrow and standard molecular weight (MW) ladder (Figure 2(b), Panel 1), and thymus (Figure 2(b), Panel 2) extracts, respectively. Both sets of experiments confirmed comigration of a band of approximately 120 KD corresponding to RAG1 protein. In contrast, amygdalar protein extracts compared with muscle extracts (Figure 2(b), Panel 3) showed no comigration of bands indicating, as expected, that RAG1 is specifically expressed in the amygdala (Figure 2(b), Panel 1), as well as bone marrow (Figure 2(b), Panel 1) and thymus (Figure 2(b), Panel 2), but not in muscle tissue (Figure 2(b), Panel 3). Furthermore, RAG1 antibody preabsorption assays with either bone marrow or muscle protein extracts, which display either detectable or undetectable RAG1 expression, respectively (see Figure 2(b), Panel 1 versus Figure 2(b), Panel 3, resp.), showed that only bone marrow protein extracts (known to express RAG1; see Figure 2(b), Panel 1) were able to block the ~120 KD band from amygdalar protein extracts in the Western blots (Figure 2(b), Panel 4). These results indicate that RAG1 antibody was preabsorbed (blocked) only by RAG1 protein expressing tissue (bone marrow).

3.4. RAG1 Plays a Functional Role in LTM Consolidation of Context Fear Conditioning. Our gene expression studies demonstrated that *RAG1* mRNA is specifically induced in the amygdala between 15 min and 30 min as a result of context fear conditioning and it returns to basal levels 1 h after training (see Figure 1(b)). This suggested that RAG1 might play a role in LTM processes. To examine the possible functional role of *RAG1* in LTM consolidation of context fear conditioning, we used an antisense approach to knock down *RAG1* expression in the amygdala (the brain region where induction was observed) and examined the effects of such knockdown on LTM of context fear conditioning. Animals were implanted with bilateral cannulae directed to the amygdala. Cannulae placement confirmation using thionine was highly precise and consistent, showing that injectors specifically targeted regions just above the amygdalar complex. Importantly, only the behavioral data of animals for which thionine staining confirmed correct cannulae localization were used to determine the effects of the oligonucleotides treatment. Representative schematics illustrating the distribution of cannulae placements throughout the amygdala for animals used in our antisense behavioral experiments are depicted in Figure 3(a). Additionally, we examined the diffusion and incorporation of *RAG1* antisense oligonucleotides in the amygdala (Figures 3(b) and 3(c)) through consecutive rostrocaudal sections. Diffusion of the microinfused FITC-*RAG1* antisense oligonucleotide was concentrated within the anterior, posterior, and ventral basolateral amygdala (BLA). It was also observed that FITC-*RAG1* antisense oligonucleotide was clearly incorporated into the cells within these amygdalar regions (Figure 3(d)).

We next evaluated the effects of amygdala *RAG1* antisense treatment on LTM of context fear conditioning. Male C57BL/6 mice were microinfused bilaterally into the amygdala with *RAG1* antisense or random oligonucleotides (n = 16, each) 1 h before conditioning training. The top panel of Figure 4 depicts the experimental design of Figures 4(a)–4(c) and 4(d), respectively. We used the 1 h pretraining infusion time point in order to allow for the antisense oligonucleotides to be diffused into the brain parenchyma and be taken up by neuronal cells prior to subjecting the animals to context

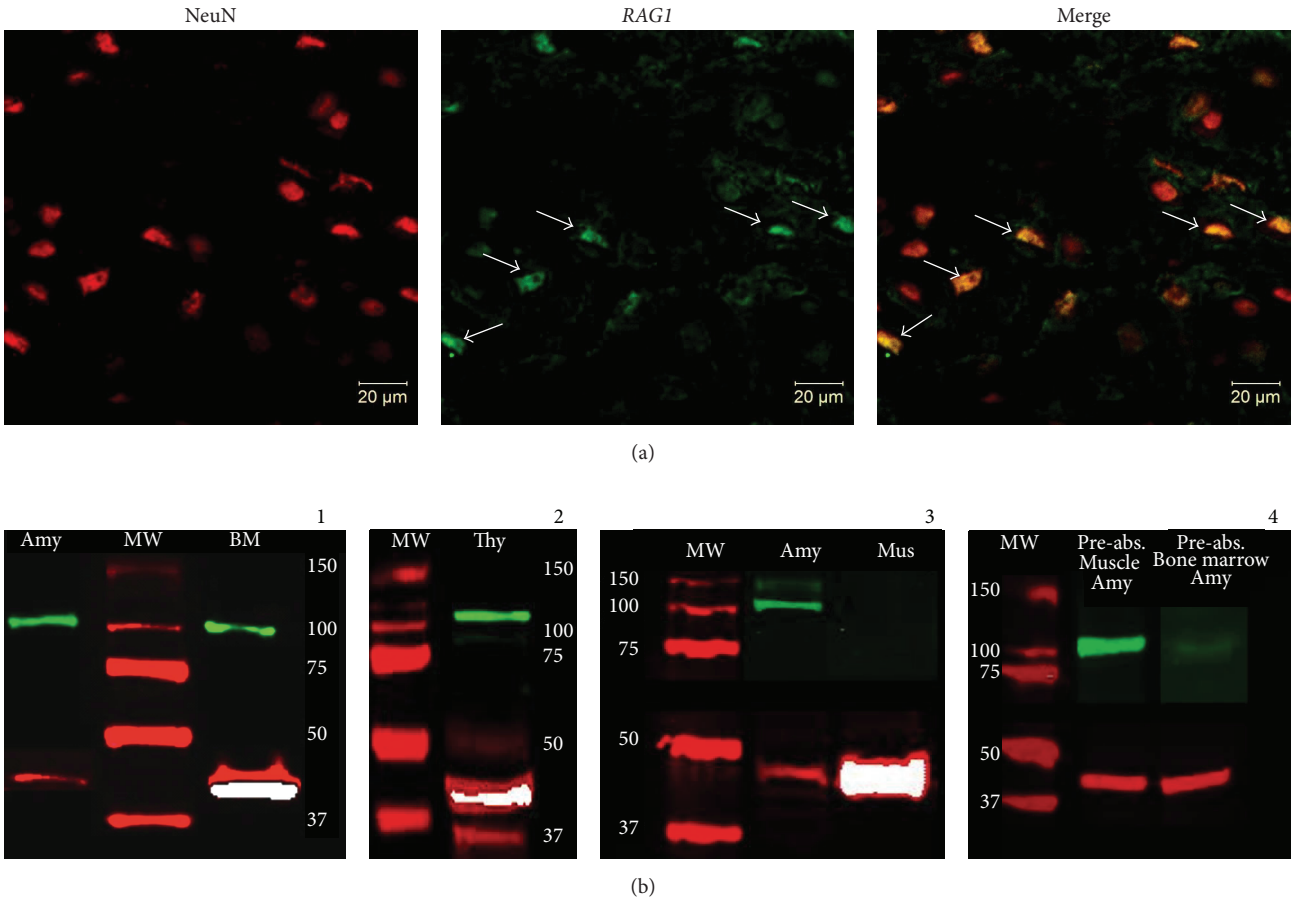

Figure 2: RAG1 protein expression in amygdalar neuronal cells. Amygdalar coronal sections of context fear conditioning-trained mice, perfused 1 h after conditioning, were used for immunofluorescence and analyzed by confocal microscopy. Antibodies from immunofluorescence were validated by Western blot analysis. (a) Amygdalar area representative images of a double immunostaining using RAG1 antibody labeled with Alexa Fluor 488, green channel signal, and NeuN antibody labeled with Alexa Fluor 568, red channel signal. The left panel shows the NeuN positive neuronal nuclei, while the middle panel depicts RAG1 immunopositive cells. The right panel is the merge image showing colocalization of the NeuN neuronal nuclei marker and RAG1. Arrows point to some of the RAG1 immunopositive neurons. These immunofluorescent images revealed colocalization of RAG1 protein expressing cells with those expressing NeuN, suggesting the presence of RAG1 in neurons, although not all neurons expressed RAG1. (b) Tissue punches from amygdala (Amy) were obtained 1 h after context fear conditioning and analyzed in Western blot by comparative comigration with a standard molecular weight (MW) marker and protein extracts from bone marrow (BM) ((b)-1) and thymus (Thy) ((b)-2). Both sets of experiments consistently showed comigration between the tissues with a band corresponding to ~120 KD of RAG1 protein (green channel corresponding to RAG1 and red channel corresponding to beta-actin, ~42 KD); prestained molecular weight (MW) marker (ladder) was included in all the Western blots. ((b)-3) Additionally, tissue protein extracts from leg muscle (Mus) (negative control) were analyzed compared to amygdalar extracts with respect to RAG1 expression. As expected, RAG1 was not expressed in muscle compared to amygdala ((b)-3), bone marrow ((b)-1), and thymus ((b)-2). ((b)-4) RAG1 antibody preabsorption assays, either with muscle or with bone marrow extracts, showed that only bone marrow extracts, which express RAG1 as opposed to muscle, were able to block the ~120 KD band from amygdalar protein extracts in the Western blots, indicating that RAG1 antibody was preabsorbed (blocked) only by RAG1 protein expressing tissue (bone marrow).

fear conditioning. For context fear conditioning, mice were placed inside a conditioning context (the chamber, CS) before receiving three consecutive foot shocks (US). As seen in Figure 4(a), mice receiving either antisense or random acquired the task normally, displaying no significant differences in acquisition of fear conditioning, measured as the progressive enhancement of freezing behavior during a 60 s after-shock period. As stated, Two-Way RM ANOVA followed by Bonferroni posttesting found no effect by treatment, although animals in both groups acquired the task, demonstrating that the infusions did not impair the animals' response in developing and expressing fear during the conditioning experience (Treatment Factor: $F(1, 0.8457) = 0.007142$, $P > 0.9$; Training Factor $F(3, 7863) = 109.8$, $^{***}P < 0.0001$;

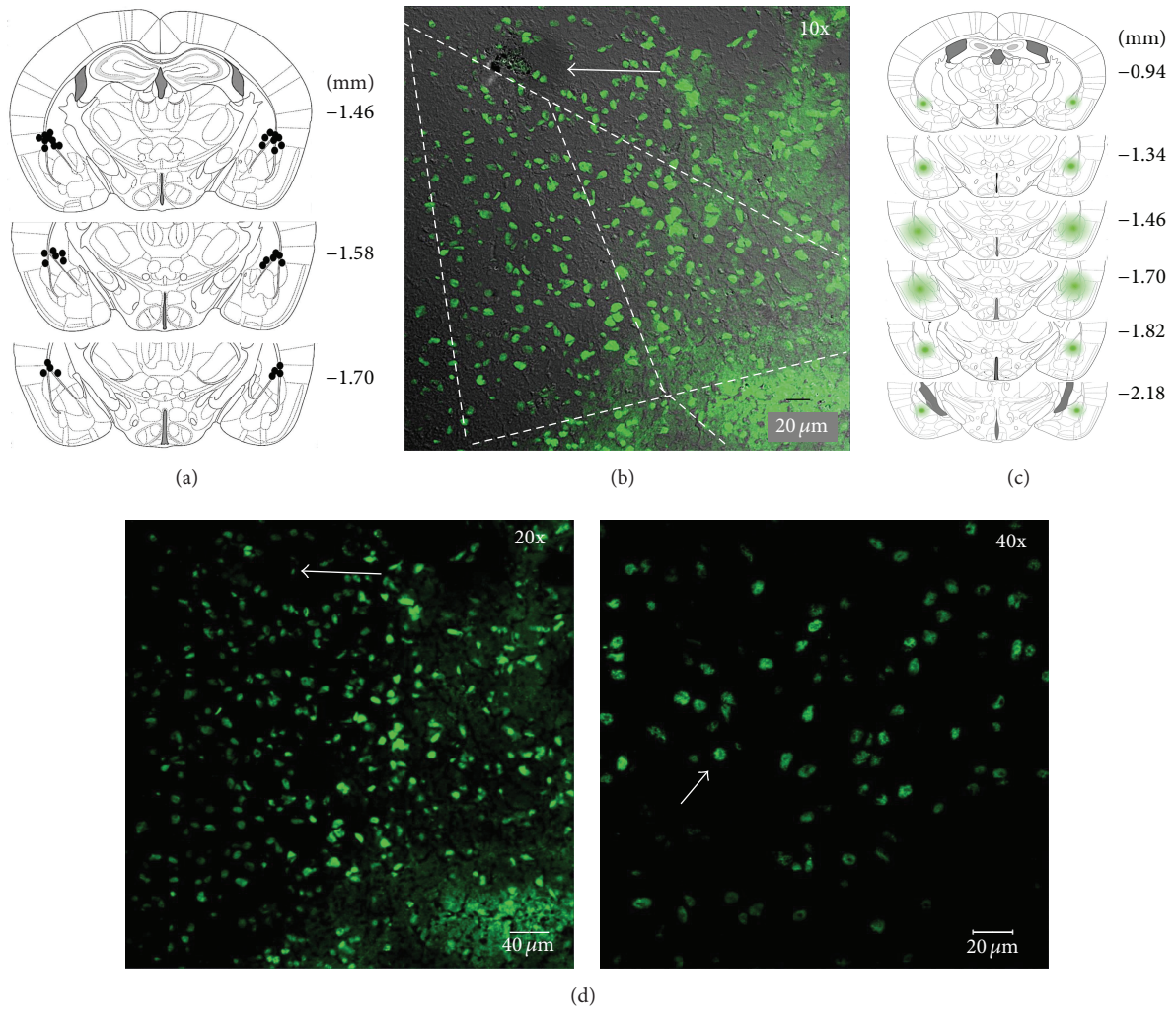

Figure 3: Distribution of cannula placements and *RAG1* antisense oligonucleotide diffusion within the amygdala. After behavioral treatments with *RAG1* antisense or random oligonucleotides, animals were microinfused the next day with thionine to verify cannulae injectors' placement. Another set of animals was used to observe FITC-labeled *RAG1* antisense diffusion. (a) Schematic representation of the amygdala at different rostrocaudal planes illustrating the position of cannulae injectors determined by thionine microinfusion. Injector tips for each cannula are represented by dark spots. (b) FITC-*RAG1* antisense diffusion within the amygdalar complex; arrow indicates the injector's tip. (c) Schemes of coronal sections showing the diffusion of FITC-*RAG1* antisense diffusion into the amygdala of animals decapitated 3 h after fluorescent oligonucleotide infusion. FITC-*RAG1* antisense diffusion is represented by green shading from anterior to posterior areas of the amygdalar complex. The numbers in (a) and (c) indicate the distance from bregma in millimeters. A total of 4 mice were used in these studies. (d) Photomicrograph at higher magnification of FITC-*RAG1* antisense diffusion showed clearly incorporation into the cells (depicted by the arrows) within amygdalar regions.

Interaction: $F(3, 7.457) = 0.1041$, $P > 0.9$; and Subject Matching: $F(25, 118.4) = 1.653$, $^{*}P < 0.05$). Posttesting analysis did not identify any specific significant differences between the groups during the habituation or the 1st, 2nd, or 3rd trials of training ($P > 0.05$, each comparison). These results indicate that both groups were similarly capable of learning the task. LTM was then tested 24 h after conditioning by placing animals back into the conditioning chamber. During the LTM test, mice remained in the chamber for 4 min in order to measure their freezing response to the context (CS). The bar graph in Figure 4(b) shows that during LTM testing mice treated with *RAG1* antisense gapmer oligonucleotides displayed significantly less percent freezing to the conditioning context than random oligonucleotide controls (Student's t-test; $t_{(25)} = 2.602$; $^{*}P < 0.05$). Thus, pretraining antisense microinfusion into the amygdala significantly impaired LTM as tested 24 h after conditioning.

To confirm the molecular effectiveness of our knockdown by gapmer antisense oligonucleotides of *RAG1* in the amygdala, we performed quantitative real-time PCR experiments.

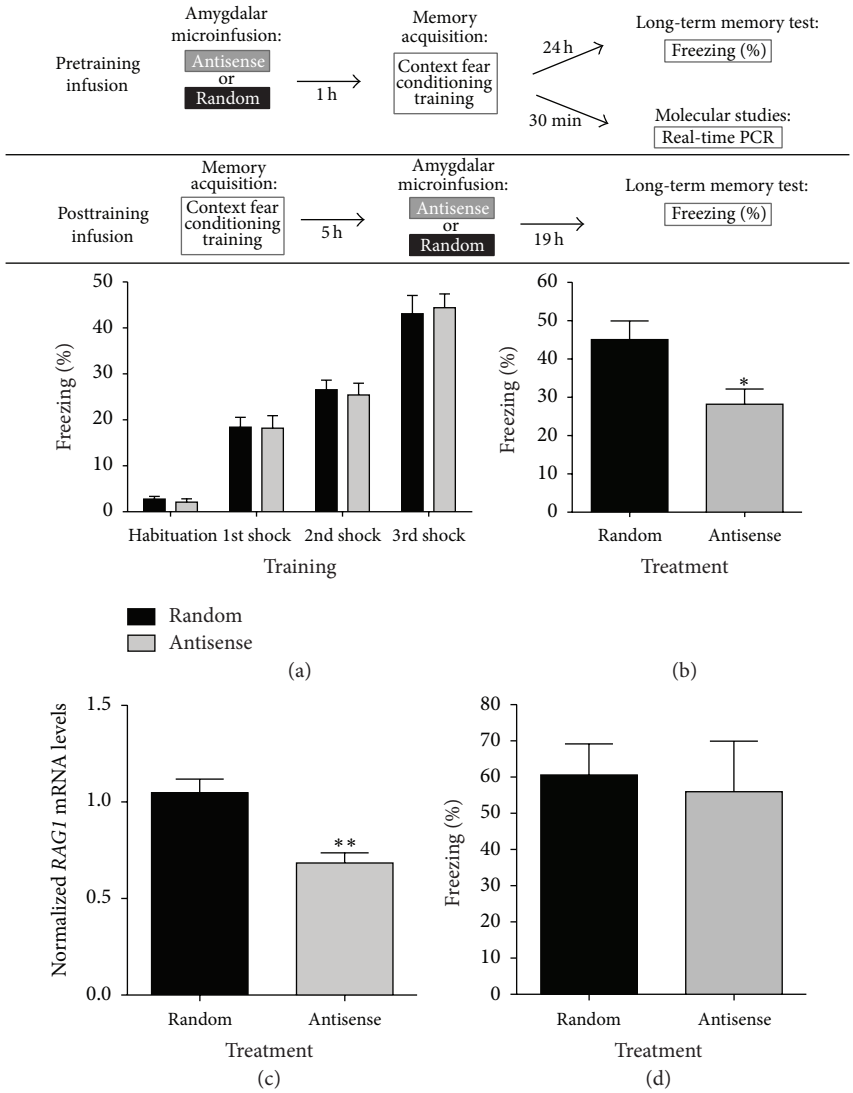

Figure 4: *RAG1* antisense amygdalar treatment impaired consolidation of context fear conditioning. Top panel: diagram depicting the experimental design of these experiments for *pretraining* or *posttraining* amygdalar antisense or random oligonucleotide microinfusion experiments. In the pretraining microinfusion experiments, mice received *RAG1* antisense or random bilateral oligonucleotide microinfusions directed at the amygdala 1 h before conditioning followed by either LTM testing or molecular evaluation. LTM was tested 24 h after conditioning. For molecular evaluation of antisense treatment effectiveness, another group of mice was sacrificed 30 min after conditioning and amygdalar RNA was used for real-time PCR. In the posttraining microinfusion experiments, mice were conditioned, returned to their home cages, and received microinfusions of antisense or random oligonucleotides 5 h after training and returned to their home cages until next day. Nineteen (19) hours later (24 h after conditioning), mice were reexposed to the conditioning chamber without any shocks in order to test LTM. (a) Mice receiving either *RAG1* antisense or random oligonucleotide treatment displayed no significant differences during memory acquisition measured as the progressive enhancement of freezing behavior (Two-Way ANOVA, Treatment Factor: $F(1, 0.8457) = 0.01015$, $P > 0.9$; Training Factor $F(3, 7863) = 94.37$, $^{***}P < 0.0001$; Interaction: $F(3, 7.457) = 0.08950$, $P > 0.9$). Bonferroni posttesting analysis did not identify significant differences between the groups during the habituation or the 1st, 2nd, or 3rd trials of training ($P > 0.05$, each comparison), indicating that both groups were similarly capable of learning the task. (b) LTM was tested 24 h after conditioning. The bar graph shows that, unlike the results obtained for acquisition, mice treated with *RAG1* antisense gapmer oligonucleotides displayed significantly less percent freezing to the conditioning context than random oligonucleotide controls during the LTM test (Student's t-test; $t_{(25)} = 2.602$; $^{*}P < 0.05$). (c) The molecular effectiveness of our knockdown by gapmer antisense oligonucleotide of *RAG1* in the amygdala was determined by quantitative real-time PCR. Mice were infused 1 h before context fear conditioning with bilateral *RAG1* antisense or random oligonucleotides and decapitated 30 min after conditioning. *RAG1* mRNA normalized against *gapdh* mRNA showed that treatment with *RAG1* antisense gapmer oligonucleotides effectively knocked down the levels of *RAG1* amygdalar mRNA compared to the random controls (Student's t-test; $t_{(16)} = 3.947$; $^{**}P < 0.005$). No significant differences in the levels of *gapdh* were observed between treatments (data not shown). (d) We used 5 h posttraining amygdalar microinfusions of *RAG1* antisense oligonucleotides or random controls with a different set of animals without any pretraining infusion and LTM was tested 24 h after training. Unlike in the pretraining microinfusion experiments, both the antisense and random posttraining-infused mice displayed similar levels of conditioned freezing during the LTM test (Student's t-test; $t_{(12)} = 2.835$; $P > 0.7$).

The antisense approach is a well-established technique and has been extensively used in the brain to assess memory function [50–54]. Our *RAG1* antisense gapmer targets the translation initiation codon, thus causing a knockdown of RAG1 protein by translational repression [55–58]. Additionally, the gapmer oligonucleotide contains a central block of deoxynucleotides sufficient to induce the endogenous mechanism of RNA:DNA duplex degradation by ribonuclease H (RNase H) cleavage and thus targeted degradation of mRNA hybridized with the antisense oligonucleotide. Moreover, the antisense gapmer is flanked by blocks of 2′-*O*-methyl modified ribonucleotides that protect the internal block from exonuclease degradation [58–61], which increases the stability and half-life of the unhybridized gapmer oligonucleotide itself. Testing the effectiveness of antisense treatment in the brain has been used by us and others by measuring knockdown of target gene mRNA levels using real-time PCR [62–64]. To test the molecular effectiveness of the *RAG1* gapmer antisense oligonucleotide, mice were infused 1 h before context fear conditioning with bilateral *RAG1* antisense ($n = 8$) or random ($n = 10$) oligonucleotides. Amygdalar *RAG1* mRNA expression was analyzed in trained mice sacrificed 30 min after conditioning, the time point of highest expression seen in the time course studies (see Figure 1(b)). *RAG1* mRNA was normalized against *gapdh* mRNA as above. As seen in Figure 4(c), treatment with *RAG1* antisense gapmer oligonucleotides effectively knocked down the levels of *RAG1* amygdalar mRNA compared to the random controls (Student's t-test; $t_{(16)} = 3.947$; $^{**}P < 0.005$). No significant differences in the levels of *gapdh* were observed between treatments (data not shown). These results show the effectiveness and selectivity of the antisense treatment on knocking down *RAG1* expression.

The results presented so far suggest that RAG1 is required in the early phase of LTM consolidation. Molecular events leading to LTM consolidation occur within the early time window during the first 6 h after training, although consolidation processes may last from hours to days and even the transfer of memories to other cortical regions might take longer time periods [65–67]. For instance, it is reported that, in rodents, fear memories require distinct molecular and temporal transcriptional/translational events lasting up to 6 h [65, 66, 68, 69] after learning experiences. More related to this work, our previously reported findings suggested that DNA ligase-dependent NHEJ events, which are associated in general with the repair of DNA DSBs, but also with V(D)J recombination processes in the immune system [26, 70, 71], are also induced rapidly in the hippocampus after context fear conditioning [32]. In addition, we also reported that the function of the flap structure-specific DNA endonuclease 1 (Fen1), known to be involved in DNA recombination/repair processes [29, 72], is induced in the amygdala 3 h after conditioned taste aversion (CTA) learning and is required for LTM consolidation [29]. Hence, for our next set of experiments, we used delayed posttraining amygdalar infusions of *RAG1* antisense oligonucleotides or random controls in order to better assess whether RAG1 is specifically involved in the early stages of consolidation. Animals assigned to the antisense or the random treatments were implanted with cannulae as the animals in the pretraining infusion experiment (Figures 4(a) and 4(b)). Mice of both groups were subjected to context fear conditioning as above, except that these animals had not received pretraining infusions of gapmer antisense or random oligonucleotides before exposing them to the task. Immediately after training, mice were returned to their home cages and then subjected to bilateral intra-amygdalar infusions of either *RAG1* antisense or random oligonucleotides 5 h after training. LTM was tested 24 h after conditioning. The results depicted in Figure 4(d) show that, unlike in the pretraining microinfusion experiments (see Figure 4(b)), both the antisense and random posttraining-infused mice ($n = 7$, each group) displayed similar levels of conditioned freezing during the LTM test (Student's t-test; $t_{(12)} = 2.835$; $P > 0.7$). Overall, these behavioral results suggest that, for LTM of context fear conditioning to be established, RAG1 is required at early, rather than later, time points following the time of learning experiences.

Finally, in additional control experiments we tested the effects of *RAG1* gapmer antisense treatment on memory reconsolidation of context fear conditioning. We used this additional control because our previous studies using the DNA ligase inhibitor ara-C, which blocks DNA repair by blocking NHEJ activity [31, 73, 74], showed that treatment with this inhibitor blocked LTM consolidation, but not reconsolidation, of context fear conditioning [32]. Such results also suggested that DNA recombination/repair mechanisms are specific to the initial stages of LTM consolidation and are not involved in memory reconsolidation processes activated after the retrieval of memories that have already been established. For these experiments (see the top panel of Figure 5 for depiction of the experimental design), a set of animals was bilaterally implanted with cannulas to target the amygdala (see Section 2). This protocol was performed similar to [32, 38]. On day 1, mice were microinfused with saline (as a stress control) and were trained 1 h later in context fear conditioning. All animals were returned to their home cages immediately after training. On day 2 (24 h after training), animals were microinfused with either random ($n = 5$) or antisense ($n = 6$) gapmer oligonucleotides. Microinfusions were given 1 h prior to a 90 s reexposure period to the conditioning chamber in order to induce memory retrieval. Next, all animals were returned to their home cages. Finally, on day 3 (48 h after training), all mice were again exposed to the conditioning chamber (CS) for 2 min to measure freezing responses. As seen in Figure 5(a), no significant differences were observed during acquisition between the animals assigned to antisense or random *RAG1* gapmer oligonucleotide treatment. Accordingly, Two-Way RM ANOVA followed by Bonferroni posttesting found no effect by treatment assignment, although animals in both groups acquired the task normally, demonstrating that the infusions did not impair the animals' response in developing and expressing fear during the conditioning experience (Treatment Factor: $F(1, 8.194) = 3.979$, $P > 0.05$; Training Factor $F(3, 3134) = 1725$, $^{***}P < 0.0001$; and Interaction: $F(3, 1.069) = 0.5881$, $P > 0.6$). Posttesting analysis did not identify any specific significant differences between the groups during the habituation or the 1st, 2nd, or 3rd trials of training ($P > 0.05$, each comparison).

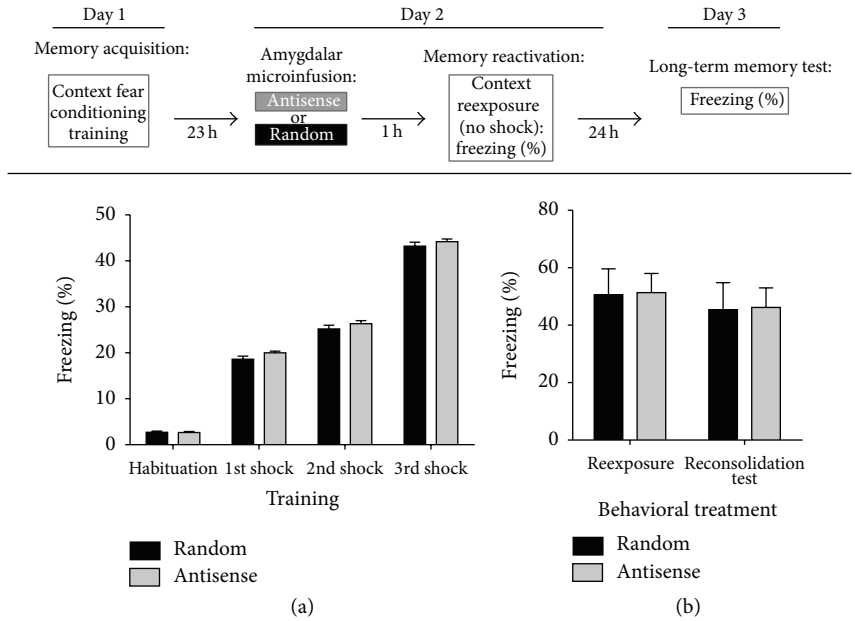

Figure 5: *RAG1* antisense amygdalar treatment does not interfere with reconsolidation of context fear conditioning. To test the effects of *RAG1* gapmer antisense treatment on memory reconsolidation of context fear conditioning, another set of animals was bilaterally implanted with cannulas to target the amygdala. Top panel: diagram depicting the experimental design. On day 1, mice were trained in context fear conditioning and immediately returned to their home cages. Antisense or random oligonucleotides were microinfused into the amygdala 1 h before memory reactivation on day 2. The effect of antisense or random oligonucleotide treatment on LTM reconsolidation was assessed on day 3, 48 h after conditioning. (a) On day 1, mice were microinfused with saline 1 h before training and returned to their home cages immediately after conditioning. Two-Way RM ANOVA and Bonferroni posttesting demonstrated that the infusions did not impair the animals' response in developing and expressing fear during the conditioning experience (Treatment Assignment Factor: $F(1, 8.194) = 3.979$, $P > 0.05$; Training Factor $F(3, 3134) = 1725$, $^{***}P < 0.0001$; and Interaction: $F(3, 1.069) = 0.5881$, $P > 0.6$). (b) On day 2, animals were microinfused with either random or antisense gapmer oligonucleotides 1 h prior to a 90 s reexposure period to the conditioning chamber in order to induce memory retrieval and returned to their home cages. For the reconsolidation test, on day 3 (48 h after training), mice were reexposed to the conditioning chamber (CS) for 2 min to measure freezing responses. No significant differences between the freezing responses of antisense or random gapmer oligonucleotide treated animals on day 2 (b) or on day 3 (b) were observed (Two-Way ANOVA: Treatment Factor: $F(1, 3.068) = 0.009017$, $P > 0.9$; Training Factor $F(1, 146.5) = 0.4307$, $P > 0.5$; Interaction: $F(1, 0.001515) = 0.00004453$, $P > 0.9$). Bonferroni posttesting identified no difference between treatments in the reexposure and reconsolidation tests, respectively ($P > 0.05$, each comparison).

These results indicate that both groups were similarly capable of learning the task. More importantly, as seen in Figure 5(b), with respect to our question of whether amygdalar *RAG1* antisense treatment impaired memory reconsolidation or not, the results identified no significant differences between the freezing responses of antisense or random gapmer oligonucleotide treated animals on day 2 (memory retrieval/reexposure test) or on day 3 (reconsolidation test) (Two-Way ANOVA: Treatment Factor: $F(1, 3.068) = 0.009017$, $P > 0.9$; Training Factor $F(1, 146.5) = 0.4307$, $P > 0.5$; Interaction: $F(1, 0.001515) = 0.00004453$, $P > 0.9$). Bonferroni posttesting identified no difference between treatments in the reexposure and reconsolidation tests, respectively ($P > 0.05$, each comparison). Overall, these results are congruent with our previous findings suggesting that DNA DSB repair mechanisms are possibly relevant only for the initial stages of LTM consolidation and are not utilized when established memories are retrieved or reactivated [32].

4. Discussion

We previously reported that a DNA repair system involving DNA ligase function and NHEJ activity is induced and required for memory consolidation [32]. Additionally, studies with TdT, a specialized polymerase involved in V(D)J recombination [71, 75, 76], showed that TdT expression is induced as a result of new experience in brain regions involved in memory formation and is required for normal learning and memory in mice [30]. Our subsequent studies identified the endonuclease Fen1, known to be involved in DNA recombination/repair processes, as being induced after associative learning and being required for LTM formation [29]. Moreover, in more recent studies, mice exposed to a novel environment [27] were shown to accumulate DNA DSBs in the brain, including the hippocampus. These DNA lesions were shown to be transient, as they were repaired after 24 h, highlighting the importance of the DNA repair of

such DSBs, which if left unrepaired could ensue in neuronal dysfunction, and also suggesting that they could be related to learning processes associated with exposure to novel environments. In addition, more recent studies showed that DNA DSBs are introduced in the promoters of early-response genes and are required for their induction in response to neuronal activity, synaptic plasticity processes, and context fear conditioning [28]. Accordingly, these studies are reminiscent of our previous findings showing that NHEJ activity repairing DNA DSBs is rapidly induced in the hippocampus, but not the insular cortex, following context fear conditioning in mice [32].

Our present studies further support the notion that DNA recombination/repair machineries particularly involving DNA endonucleases might be involved in learning and memory processes. Specifically, we identified and characterized the expression of *RAG1* in wild type C57BL/6 mice brain during context fear conditioning and confirmed that *RAG1* expression is required in the amygdala for early consolidation of fear memory. These findings further confirm previous reports suggesting the role of *RAG1* in some behavioral paradigms and support the notion that DNA recombination/repair mechanisms may be required in LTM.

4.1. RAG1 Induction in the Amygdala Is Associated with Context Fear Conditioning Learning. Using quantitative real-time PCR, we compared hippocampal and amygdalar *RAG1* expression of context fear conditioning-trained animals during a time course of 15 min, 30 min, and 1 h after conditioning. The levels of hippocampal *RAG1* mRNA after training remained similar to Naïve basal levels. On the other hand, we did observe a rapid and transitory induction of *RAG1* mRNA in the amygdala between 15 and 30 min, which returned to baseline at 1 h, suggesting that *RAG1* is tightly regulated at the level of transcription in the amygdala compared to the hippocampus. This is consistent with previous reports supporting the major role of the amygdala in fear memory, compared to the hippocampus [77–79]. Additionally, we compared amygdalar expression of *RAG1* in conditioned animals and mice subjected to the individual components of this aversive learning paradigm: context-only and shock-only. Interestingly, we found that *RAG1* mRNA levels are significantly higher only in context fear conditioning-trained animals compared with those trained with the individual, unpaired, components of the associative paradigm, as well as with naive. The specific induction of *RAG1* in conditioning paired stimuli suggested that such induction was not merely a correlate of fear itself, but it could be involved in associative learning and LTM formation. In contrast, previous reports have found that immediate early genes involved in general neuronal activation such as *c-fos* are induced in context fear conditioning, but also in context-only (hippocampus) and shock-only (amygdala) animal groups [80–83]. However, the specific induction of *RAG1* to conditioned animals in the amygdala suggests that it does not correspond to the response of a general pattern of neuronal activation.

Because of the widely established role of RAG1 during DNA rearrangement, a process thought to be highly specific to nuclei of immune cells, we wanted to determine the cellular localization of this endonuclease within the amygdalar tissue after context fear conditioning. Thus, after ruling out the possibility that the observed changes in *RAG1* mRNA after conditioning could be due to the presence of residual blood cells in the tissues examined, we performed immunofluorescence analysis of the RAG1 protein. Importantly, for immunofluorescence, brains from trained animals sacrificed 1 h after conditioning were also perfused with PBS1X to remove residual blood and subsequently with paraformaldehyde for tissue fixation. Results demonstrated that NeuN, a marker for neuronal nuclei, was colocalized with RAG1 expression, suggesting that RAG1-positive cells in the amygdala after training are predominantly neurons. Interestingly, not all neurons marked with NeuN were colabeled with RAG1, suggesting that DNA recombination/repair machineries associated with this factor might be restricted to only a subset of cells in the amygdala after context fear conditioning.

4.2. RAG1 Is Required for Consolidation, but Not Reconsolidation, of Context Fear Conditioning. The amygdala plays a critical role for LTM consolidation and for the representation of the US component of context fear conditioning [78, 79, 84–86]. We found that *RAG1* mRNA is specifically induced in the amygdala, but not in the hippocampus, after training. Interestingly, some reports have demonstrated that the effect of amygdalar lesions on fear memory impairment is stronger than the effect of hippocampal lesions, suggesting a major role of the amygdala in fear memory [78, 79]. Hence, we decided to extend our studies by using experiments addressing the functional role of amygdalar *RAG1* on LTM of context fear conditioning. We found that *RAG1* antisense, but not random, oligonucleotides were effective in selectively suppressing the levels of amygdalar *RAG1* mRNA and also impaired LTM of context fear conditioning without affecting acquisition of the task. Animals microinfused with antisense or random oligonucleotides 5 h after conditioning showed no effects in LTM by the antisense targeting, suggesting that *RAG1* is only required in the early phase of LTM formation. Furthermore, *RAG1* antisense or random oligonucleotides infused into the amygdala 1 h prior to memory reactivation of previously trained untreated mice resulted in no effect in either memory retrieval or memory reconsolidation. Consistent with these findings, we previously found that administering ara-C, an inhibitor of DNA ligase and of NHEJ activity shown to block LTM consolidation of context fear conditioning, just prior to memory reactivation had no effect in memory reactivation or memory reconsolidation [32]. Altogether, these results suggest that, unlike CREB inactivation and general protein synthesis inhibition [38, 87, 88], blockade of DNA recombination/repair processes during memory reactivation does not interfere with reconsolidation of fear conditioning [32]. The fact that mechanisms associated with the introduction of DNA DSBs are restricted to the early phases of LTM consolidation and are not activated as a result of memory retrieval might represent a mechanism for a balance that would allow neurons to maintain their integrity by not overriding their mechanisms for DNA repair, which could occur if these lesions were to be introduced every time an established memory is retrieved.

The results presented above are consistent with previous studies using *RAG1*-knockout ($RAG1^{-/-}$) and *RAG1*-deficient mice ($RAG1^{-/+}$). $RAG1^{-/-}$ mice showed reduced levels of fearfulness for some measures of fear-motivated behavior in both the open-field behavior test and the elevated-plus maze [36]. Additionally, $RAG1^{-/+}$ exhibited impaired social recognition memory [37]. Moreover, $RAG1^{-/-}$ mice showed memory impairment compared with wild type in the Morris water maze [89]. These findings with $RAG1^{-/-}$ and $RAG1^{-/+}$ mice are interesting; however, it is known that, in some cases, such gene targeting models might be masked by compensatory mechanisms, or developmental and physiological side effects, sometimes undetectable, because the mutation targets all cells [50, 90–92]. For instance, mice presenting inactivating mutations or deletion of the *RAG1* gene show severe combined immunodeficiency (SCID) caused by small lymphoid organs, impaired development of B and T lymphocytes, and inability to perform V(D)J recombination, but with no obvious neuroanatomical abnormalities [93–97]. In contrast, $RAG1^{-/+}$ mice in which one copy of the *RAG1* gene is deleted and thus which are heterozygous for the *RAG1 gene* (−/+), are immunocompetent and indistinguishable from wild type mice, displaying normal lymphocytes differentiation and V(D)J recombination [93, 95]. Nevertheless, neither of the knockout or heterozygous models mentioned above have regional or temporal control for *RAG1* gene inactivation. The antisense oligonucleotide approach has the advantage of brain region specificity in the region of interest (the amygdala) at a specific time point. For instance, knockout mice targeting the transcription factor encoded by the immediate early gene *c-fos* show normal acquisition and LTM [50]. However, acute knockdown with antisense oligonucleotide to specific brain regions involved in the learning paradigm inhibits LTM of wild type mice [50], suggesting that compensatory mechanisms are activated in the absence of Fos-mediated transcription in knockout models. Conversely, CREB mutants and knockouts display memory disruption in fear conditioning and in a wide range of behavioral paradigms [11, 98, 99]. Similarly, targeted injection of CREB antisense into the hippocampus or into the amygdala results in disruption of LTM in a variety of tasks [53, 100]. Similar to our studies reported here with gapmer antisense oligonucleotides, a study using shRNAs targeting *RAG1* delivered with lentiviral vectors into the CA3 region of the rat hippocampus was used to assess the effects of suppressing *RAG1* expression on spatial learning in the Morris Water maze [101]. These studies demonstrated that suppressing *RAG1* expression in the hippocampal CA3 region does impair spatial learning in rats. The authors also examined the effects of such suppression on context fear conditioning. Similar to our findings here with respect to *RAG1* amygdalar knockdown in mice, their findings suggest that hippocampal expression of *RAG1* is necessary for LTM of context fear conditioning, although no experiments were done to examine whether changes in *RAG1* expression occurred either in the hippocampus or in the amygdala as a result of learning in either of the paradigms. With respect to the findings on context fear conditioning, it is important to state that in our studies reported here we did not observe hippocampal induction of *RAG1* mRNA levels after conditioning (see Figure 1(a)); that is, *RAG1* mRNA levels remained at their basal Naïve levels at each of the time points examined. We cannot rule out, however, and in light of the study mentioned above, that the basal constitutive expression of *RAG1* in the hippocampus after context fear conditioning may play a role in LTM, as well as the induced amygdalar expression. Finally, as suggested previously [102] it is possible that different molecular mechanisms operate in consolidation of distinct learning paradigms, either spatial learning or context fear conditioning, which might involve regulated or constitutive *RAG1* expression and function in distinct regions of the brain.

Our findings on *RAG1* induction and functional knockdown studies with antisense, together with previous reports on $RAG1^{-/+}$ [36, 89] and $RAG1^{-/+}$ [58] mouse models, support the possibility that this DNA endonuclease, which introduces DNA DSBs, is required for associative memory formation. Additional support for the required role of *RAG1* in LTM is provided by the observation that *RAG1* induction is specifically related to the pairing of individual conditioning stimuli (Figure 1(c)) and the fact that the amnesic effect of amygdalar knockdown of RAG1 is evident 24 h after acquisition of context fear conditioning (Figure 4(b)). The crucial question at this time is whether RAG1 indeed is involved in introducing DNA DSBs in response to learning and whether the repair of such DNA lesions is required for LTM. Although the data presented here and that reported previously concerning the experience-dependent expression of factors specifically related to V(D)J recombination in brain regions involved in memory formation further strengthen this view [27–32], additional published data could suggest that such changes may not be associated with the introduction of DNA DSBs and DNA repair. For example, a cytoplasmic form of the P13 kinase member ataxia-telangiectasia mutated (ATM) [103], known to play a key role in DNA DSB repair, was shown to play an important role in synaptic plasticity. However, the studies also suggested that this form of cytoplasmic ATM is not involved in the key role of DNA DSB repair ascribed to the nuclear form of the protein. The authors suggested that cytoplasmic ATM plays cellular roles in neurons that are independent of its role in responding to DNA lesions. Thus, we cannot rule out at this time that our findings with RAG1 may be unrelated to its role in introducing DNA DSBs that is well documented in the immune system. However, in the studies presented here localization of RAG1 after conditioning was concentrated in neuronal nuclei in the amygdala, colocalizing with the neuronal nuclear marker NeuN, suggesting that the role of RAG1 in the amygdala after learning is associated with nuclear machineries such as DNA recombination/repair. Moreover, our previous reports on experience-dependent induction of NHEJ activity and DNA repair machineries suggest that DNA DSBs are generated as a result of learning, perhaps as a consequence of induced endonuclease activity such as RAG1, and subsequent DNA repair by induction of NHEJ/DNA ligase pathway. Overall, based on these findings we propose that DNA recombination/repair pathways [23], involving a V(D)J-like mechanism using *RAG1*, DNA ligase/NHEJ [31–33], Fen1 [29], and TdT [30], possibly operate

together with epigenetic and transcriptional/translational regulation for LTM formation. Such a mechanism could be responsible not only for regulating the activity of gene promoters and thereby gene expression, but also for increasing the diversity of the repertoire of genes and proteins required for synaptic plasticity processes and the establishment of specific connectivity of neuronal networks and/or the specific patterns of gene expression involved in the establishment of specific long-term memories.

5. Conclusion

The present studies have identified and characterized, *RAG1*, a gene encoding a key endonuclease that introduces DNA DSBs in recombination processes of the immune system, required for LTM formation of aversive experiences. In immune cells, somatic DNA rearrangement is initiated by RAG1, which exerts endonuclease activity upon the RSSs of V(D)J gene segments resulting in enhanced diversity of antigen receptor genes [42, 71, 104, 105]. Because of the well-known molecular function of RAG1 as the DNA endonuclease initiating V(D)J recombination of T-cell and immunoglobulin receptors in lymphoid cells, our findings support the proposed role of DNA recombination/repair mechanisms in LTM processes [23, 27–32]. Introduction of DNA DSBs, DNA repair, and DNA rearrangement are not inconsistent with synaptic plasticity models and mechanisms previously described. Thus, an integrated control of the introduction of DNA DSBs, DNA repair, DNA rearrangement, epigenetics, and transcriptional and translational mechanisms may orchestrate gene regulation in memory formation.

Conflict of Interests

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

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