2015: WHICH NEW DIRECTIONS FOR ALZHEIMER'S DISEASE?

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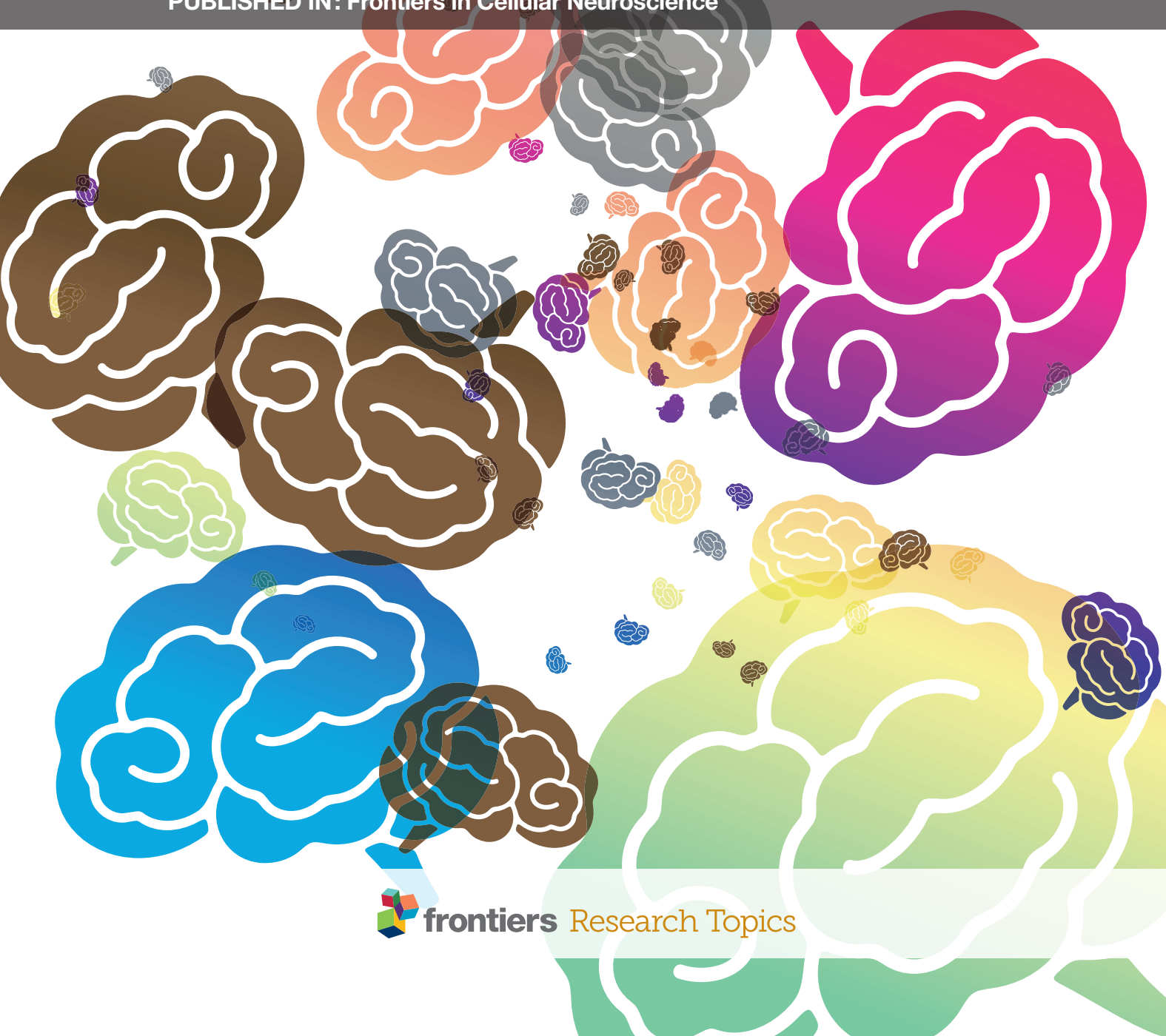

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2015: WHICH NEW DIRECTIONS FOR ALZHEIMER'S DISEASE?

Topic Editor:
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According to the World Health Organization, more than 40 million people in the world are affected with dementia. To date, 60-70% of the cases of dementia are attributed to the Alzheimer's disease (AD). This neurodegenerative disorder gradually takes place over a period of at least 20 years before the onset of symptoms, which are impaired memory, apathy and depression. The characteristics of AD consist in neurofibrillary tangles (intraneuronal aggregates of hyperphosphorylated tau proteins) and senile plaques (dense extraneuronal deposits composed of amyloid β (Aβ)). The other features linked to these two core pathological hallmarks of AD are inflammation, oxidative stress, progressive synaptic and neuronal loss.

In past years, some of the emerging therapeutic strategies against AD were employed to deal with the pathological hallmarks of the disease. Science teams all over the world try to restore the tau phosphorylation equilibrium. Their purpose is to interfere with the aggregation of tau and decrease its amount of proteins per se as well. Furthermore, they are trying either to stimulate the elimination processes of the aggregated tau proteins or to stop the formation of Aβ peptides. This could be reached by the stimulation of the classic techniques of protein degradation such as the autophagic pathway, or by the targeted immunotherapy.

In this Research Topic, we wish to summarize and review the etiology of AD and the related therapeutic opportunities for the next decades. To fully understand the precise mechanisms underlying AD, research findings, reviews, new insights and new approaches include AD and related tauopathies, tau phosphorylation balance, pharmacological compounds against AD, neuroprotection strategies and new therapeutic ways but also risk factors for AD and AD genetic information are included in this issue.

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2015: which new directions for Alzheimer's disease?

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Keywords: Alzheimer's disease (AD), dementia, tau, abeta, therapeutics

Around 44 million people in the world are suffering from dementia including Alzheimer's disease (AD). It is considered as one of the biggest global public health challenges our generation cope with. At the dawn of 2015, AD medical care remains unsuccessful despite the identification of its neuropathological hallmarks one century ago. Being attentive to emerging prospects is essential because the current advances lead to substantive improvement of the medical care. We are pleased to present an issue specifically devoted to AD and to the related therapeutic strategies. The Topic Research in this issue is based on a series of original papers and reviews. The latters focus on the advances in basic and clinical research trends in AD, they provide an up-to-date information and future perspectives on this hot topic as well.

AD is a progressive disease, it occurs over a long period before the onset of symptoms which are impaired memory, apathy, and depression. The characteristics of AD consist in neurofibrillary tangles (intraneuronal aggregates of hyperphosphorylated tau proteins) and senile plaques [dense extraneuronal deposits composed of amyloid β (Aβ)]. The other features linked to these two core pathological hallmarks of AD are inflammation, oxidative stress, progressive synaptic, and neuronal loss. Nowadays, many AD molecular patterns have been screened to identify a potential therapeutic strategy. Although a myriad of evidence shows that the hippocampal volume decrease belongs to the AD earliest signs, as it is pointed out by the authors of the review paper presented in this issue, this element clearly could not be used as a diagnostic criterion (Maruszak and Thuret, 2014).

With the flood of evidence for tau pathology as key event of the disease development, the understanding of diverse tau functions and its molecular behavior is one of the major steps in the progression of our knowledge about the neurodegenerescence detected in AD. The precision of tau role in DNA protection and RNA integrity under physiological conditions or under ROS-producing stress (Violet et al., 2014) provides clarification for a mechanistic model in which tau disturbance initiates an explanation for DNA damages observed in AD. Principally, tau is a phosphoprotein. So, a complex equilibrium between tau kinases and phosphatases activities is one of the main potential therapeutic runways. Abnormal or excessive tau phosphorylation by either kinases such as GSK3β, CDK5, DYRK1A for example or other known and unknown kinases are related to AD pathogenesis. However, the identity and the strict number of tau kinases involved in AD process remain uncertain. In this way, focus at specific tau phosphorylation site(s) by a kinase multitargeting approach as potential AD therapeutic strategy has been proposed to effectively hamper the multifactorial disease progression (Hilgeroth and Tell, 2013). Since diabetes, linked itself to dysregulation of GSK3β activity, is associated in late-life with an increased risk of dementia, epidemiological and experimental studies are summarized in this issue in order to understand the effects of diabetes mellitus on tau pathogenesis. In fact, the authors discuss herein a link between tau, diabetes mellitus and the cognitive impairment (El Khoury et al., 2014). On the other hand, Peptidyl-prolyl cis-trans Isomerase NIMA-interacting 1 (Pin1), which plays a role in the balance of phosphorylation/dephosphorylation of tau, has been suspected of participating in a common mechanism between AD and hypoxia. The pathophysiological relevance of Hypoxia-inducible factor 1α (HIF-1α) pathway regulation in APP amyloidogenic metabolism has been explored in order to clarify the relationship between AD and hypoxia (Lonati et al., 2014). Among tau-based therapeutic strategies, the one which limits the spreading of tau pathology without affecting tau intracellular functions is especially attractive (Medina and Avila, 2014). Strong support for this idea comes from the importance of tau for Aβ-induced synaptotoxicity. In addition to this, high concentration of Aβ inhibits synaptic activity and in turn, it involves toxicity for the targeted synapses (Wang et al., 2013). According to this strategy, synaptic inhibitory systems could help to compensate neurotransmission imbalance (Nava-Mesa et al., 2014). Moreover, natural anti-inflammatory components, as Andrean Compound, has been studied as a preventive or adjuvant agent in AD (Maccioni et al., 2014).

Accumulation of Aβ is induced when the equilibrium between its production and clearance is disrupted. Thus, amyloid-based therapeutic strategies target this balance but numerous trials were unsuccessful for reasons of specificity or biodisponibility. However, the restoration of the microglial homeostasy and the targeting of Triggering Receptor Expressed on Myeloid cells 2 (TREM2) pathway can constitute an elegant therapeutic strategy (Zhao and Lukiw, 2013). Compounds are yet available to improve the microglia-dependent clearance of Aβ, and this strategy would benefit from *in vivo* experiments (Jones et al., 2014). Furthermore, diversity of gut microbiota changes through life stages and alterations of this ecosystem have been associated with diverse diseases. Therapeutic modulation of gut microbiota could constitute a promising adjuvant therapy in central neurodegenerative disorders. An opinion article proposes probiotics as a prophylactic treatment to prevent AD in its early stages (Bhattacharjee and Lukiw, 2013).

Several recent key advances in the field of AD understanding and treatment are presented in this thematic issue. Ultimately, we hope that you will enjoy as us to read these papers and reviews presented in this special issue. The coming years certainly promise a time for completion of a new era in the history of the AD understanding and emerging therapies. AD understanding is within reach. Stay tuned.

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Why looking at the whole hippocampus is not enough—a critical role for anteroposterior axis, subfield and activation analyses to enhance predictive value of hippocampal changes for Alzheimer's disease diagnosis

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The hippocampus is one of the earliest affected brain regions in Alzheimer's disease (AD) and its dysfunction is believed to underlie the core feature of the disease-memory impairment. Given that hippocampal volume is one of the best AD biomarkers, our review focuses on distinct subfields within the hippocampus, pinpointing regions that might enhance the predictive value of current diagnostic methods. Our review presents how changes in hippocampal volume, shape, symmetry and activation are reflected by cognitive impairment and how they are linked with neurogenesis alterations. Moreover, we revisit the functional differentiation along the anteroposterior longitudinal axis of the hippocampus and discuss its relevance for AD diagnosis. Finally, we indicate that apart from hippocampal subfield volumetry, the characteristic pattern of hippocampal hyperactivation associated with seizures and neurogenesis changes is another promising candidate for an early AD biomarker that could become also a target for early interventions.

Keywords: Alzheimer's disease, hippocampus, hippocampal volume, hippocampal asymmetry, hippocampal shape, hyperactivation, dorsal hippocampus, ventral hippocampus

Alzheimer's disease (AD) is the most common type of dementia in people over the age of 65, with a lifetime risk of 10.5% (Sperling et al., 2011). In 2013, there have been 44.35 million people affected by dementia worldwide and their number will reach 75.62 million in 2030 (Alzheimer's Disease International, 2013). AD encompasses pre-dementia and dementia stage. It is characterized by a long asymptomatic period that transforms into mild cognitive impairment (MCI) and later to dementia due to AD.

Individuals with MCI are affected by significant cognitive impairments which are not sufficient to meet AD criteria but are beyond normal healthy aging features. The amnestic subtype of MCI (aMCI) is characterized by a higher likelihood of progressing to dementia (Petersen et al., 2009). However, the annual conversion rate is 5–10% and the majority of MCI individuals even after 10 years will not progress to clinical AD, or some will even improve cognitively (Mitchell and Shiri-Feshki, 2009). Therefore, the possibility to predict who from the MCI group will eventually progress to AD would be invaluable for planning patients' therapy, counselling and clinical trials. Therefore, given that the neurodegenerative process leading to dementia due to AD begins more than a decade before clinical diagnosis is made (Jack et al., 2013), there is a quest for reliable techniques and biomarkers enabling diagnosis at the preclinical stage to provide opportunity for therapeutic intervention. These methods need to be widely available and relatively inexpensive to find application in the diagnosis of the millions of affected individuals worldwide.

With an integrative perspective of molecular and cellular biology as well as neuroanatomy, our review focuses on the recent advancements in hippocampal analyses employing widely available magnetic resonance imaging (MRI) scanners, and describes how it might impact on improved early AD diagnosis.

We discuss why looking solely at the hippocampal volume is not sufficient to diagnose early stage AD. We present the latest data on the hippocampal changes in AD and suggest how they can be employed to diagnose AD earlier than we have been doing so far. To our knowledge, our review is the first one to provide a comprehensive summary of neuroimaging studies focused on hippocampal subfields in the context of AD. We highlight the subfields that might enhance the predictive value of current diagnostic techniques. Moreover, we present how changes in hippocampal volume, shape, symmetry and activation are reflected by cognitive impairment as well as neuropsychiatric symptoms, and how they are linked with neurogenesis alterations. We discuss how measuring these changes might be applied in AD diagnosis. In addition, we demonstrate why both hippocampal neurogenesis and functional differentiation along the anterior-posterior longitudinal axis of the hippocampus deserve more attention in future studies by presenting their relevance for presymptomatic AD diagnosis. Finally, we indicate that apart

from hippocampal subfield volumetry, the characteristic pattern of hippocampal hyperactivation associated with seizures and neurogenesis changes is a very promising candidate for both an early AD biomarker and target for early pharmacological interventions.

The hippocampus is composed of interconnected subfields with distinctive histological characteristics and specialized functions, including four fields of the Cornu Ammonis (CA1-4), dentate gyrus (DG) and the subiculum. The nomenclature of hippocampal subfields and their anatomical definitions vary among different authors, however, there is even less agreement on the location of boundaries between hippocampal head, body and tail.

The hippocampus has two major interconnected roles. It is involved in consolidation of some forms of memory, learning and emotional processing. It encompasses also one of the brain niches where adult neurogenesis occurs, i.e., in subgranular zone of the DG. Neurogenesis is essential for memory, learning and mood (Deng et al., 2010; Eisch and Petrik, 2012; Zainuddin and Thuret, 2012) and there is extensive evidence showing that these processes are altered in AD (Lazarov and Marr, 2013; Maruszak et al., 2013). We discuss further the link between changes in the hippocampus and neurogenesis alterations.

STRUCTURAL CHANGES IN THE HIPPOCAMPUS AS ONE OF AD HALLMARKS

In most cases, AD is diagnosed when the hippocampus is already considerably damaged. The hippocampus is one of the major targets of AD hallmarks: neurofibrillary tangles (NFT), amyloid plaques and neuronal loss. NFT are intraneuronal aggregates of hyperphosphorylated microtubule-associated protein, tau. Their number and spatiotemporal distribution correlates positively with cognitive decline and progression of AD (Braak et al., 1998). First, they affect the brain in the entorhinal/perirhinal cortex, thereby disrupting the origin of the perforant pathway projection to the hippocampus. Then NFT target hippocampal cornu Ammonis (CA) subfields, association cortex, and finally primary neocortex. In the hippocampus NFT target first CA1, then subiculum, later CA2 and CA3 (Braak and Braak, 1991; Braak et al., 1993). NFT are paralleled by neuronal and synapse loss, although it remains unclear whether NFT are causative for these changes or represent a neuroprotective response (Serrano-Pozo et al., 2011). The spatiotemporal spreading of NFT is closely reflected by the progression of brain atrophy, and both NFT and atrophy measures strongly correlate with cognitive decline (Bobinski et al., 2000; Jack et al., 2002; Johnson et al., 2012).

The extracellular amyloid plaques, which are composed of aggregated amyloid β (Aβ) peptides of 39–42 amino acids in length, display less predictable spatiotemporal distribution than NFT. They accumulate mainly in the isocortex and their presence does not correlate with dementia severity (Braak and Braak, 1991; Thal et al., 2002; Giannakopoulos et al., 2003).

The third characteristic feature of AD, the progressive cerebral atrophy, encompasses hippocampal atrophy and can be visualized by MRI. For the majority of AD patients (with the exclusion of those with hippocampal sparing and limbic-predominant AD) hippocampal atrophy is one of the earliest detectable symptoms of ongoing neurodegeneration, thus it has been incorporated in the new diagnostic criteria for AD (Mormino et al., 2009; Dubois et al., 2010; Lo et al., 2011; Jack et al., 2013). Hippocampal atrophy progresses nonlinearly, expressing features of a sigmoidal trajectory as it accelerates during transition from MCI to AD dementia (Sabuncu et al., 2011; Jack et al., 2013). It starts to diverge from normal rate of atrophy around 5.5 years before clinical diagnosis of dementia (Ridha et al., 2006). Already 3 years before diagnosis hippocampal volume is reduced by about 10%, whereas AD patients at the mild disease stage are characterized by the volume reduced by ∼15–20% relative to controls (Johnson et al., 2012).

Hippocampal volume is strongly associated with memory recall performance in the elderly (Reitz et al., 2009). Likewise, in aMCI, MCI and AD hippocampal atrophy positively correlates with measures of cognitive decline, such as Mini-mental state examination (MMSE) or Alzheimer's Disease Assessment Scale-Cognitive Subscale (ADAS-Cog; Pennanen et al., 2004; Jessen et al., 2006; Jak et al., 2007; Schuff et al., 2009; Sabuncu et al., 2011; Frankó and Joly, 2013). AD patients are characterized by higher average rate of hippocampal volume loss than healthy age-matched controls, whereas MCI patients have an intermediate level of volume loss between AD and control individuals (Schuff et al., 2009). Moreover, hippocampal atrophy in MCI converters is more pronounced compared to non-converters (Devanand et al., 2007; Kantarci et al., 2009; Risacher et al., 2009; Costafreda et al., 2011).

It has been proposed that surface-based methods enabling three-dimensional hippocampal shape analysis might serve as a better tool to localize early hippocampal atrophy. This technique estimates surface changes which enable localisation of regions of highest inward and outward transformations. Hippocampal shape is predictive for dementia in the preclinical period, independently of age and gender (Achterberg et al., 2013). Moreover, combining both shape and volume enables better prediction of the risk of dementia (Achterberg et al., 2013). However, the surface-based methods have also some important drawbacks which will be discussed further in this review.

FACTORS AFFECTING HIPPOCAMPAL VOLUME IN AD

Hippocampus is a dynamic structure and there are several factors that influence its volume. First of all, hippocampal volume has been shown to be highly heritable as a recent meta-analysis of genome-wide association studies revealed (Stein et al., 2012). Moreover, there were also suggestions of ApoE epsilon4 (*APOE4*) effect on hippocampal volume. Together with some other AD risk factors, such as elevated homocysteine level, *APOE4* was associated with smaller hippocampal size and changes in hippocampal asymmetry (described below). For instance, Schuff et al. reported that the volume loss in AD patients was significantly higher if they were carriers of *APOE4* allele compared to *APOE4* non-carriers, irrespective of their cognitive impairments (Schuff et al., 2009). *APOE4* status was not associated with higher hippocampal volume loss in healthy controls or MCI patients (Schuff et al., 2009). Mueller and Weiner (2009) have shown that both AD patients and controls that were carriers of *APOE4* exhibit hippocampal volume loss specifically in CA3 and DG compared

to non-carriers (Mueller and Weiner, 2009). Despite the long list of the *APOE4* roles in functional and gray matter changes throughout adulthood as it has been shown to play an important role in neuronal development, neuron migration, axon guidance, microtubule stability, dendritic spine density, synaptic plasticity, and regeneration after injury and in adult neurogenesis (Yang et al., 2011; Dean et al., 2014), there are also studies that do not find positive association of *APOE4* allele with hippocampal volume changes (Reitz et al., 2009; Wang et al., 2012) or that *APOE4* influences not the hippocampal volume but the rate of volume loss over time (Moffat et al., 2000; Cohen et al., 2001; Jak et al., 2007; Lyall et al., 2013). The discrepancies may stem from methodological variability between the studies, such as for instance different sample sizes or different approaches to comparing APOE genotypes.

Among other factors affecting hippocampal volume is age, thus hippocampus is independently affected both by age and AD pathology (Raz et al., 2005; Raji et al., 2009). In addition, many age-related pathological conditions, of which many increase AD risk, such as e.g., hypertension, diabetes mellitus, cardiovascular disease and head trauma, influence hippocampal volume (Fotuhi et al., 2012). Moreover, hippocampal volume might be affected by medications. For instance, antidepressants or cholesterol-lowering treatment have been shown to help reverse hippocampal atrophy (Fotuhi et al., 2012).

Among the factors increasing hippocampal volume is cognitive stimulation, mindfulness and aerobic exercise (for review see Fotuhi et al., 2012). The latter one has been linked with increased both right and left hippocampal volume by around 2% over 1 year and reversing age-related hippocampal shrinkage as well as improving cognition (Erickson et al., 2011). In a 9-year long prospective cohort study, it has been shown that regular physical activity, as simple as walking, increases hippocampal volume, and in addition, larger hippocampal volume was associated with decreased risk of cognitive impairment (Erickson et al., 2010). The influence of aerobic exercise on hippocampal volume might be of particular interest of individuals at risk of AD, as it seems to selectively increase the volume of the anterior hippocampus, which is affected early on in AD course (Erickson et al., 2011). Moreover, there is also extensive evidence of the role of exercise in increasing hippocampal neurogenesis as it increases proliferation and survival of hippocampal progenitor cells (Cotman and Berchtold, 2002; van Praag et al., 2005; Creer et al., 2010). Notably, the remaining above-mentioned factors influencing hippocampal volume have been also implicated in neurogenesis. Recently, another common factor between neurogenesis and hippocampal volume has been revealed—brain-derived neurotrophic factor, which level is decreased in AD as well as in impaired neurogenesis (Honea et al., 2013).

HIPPOCAMPAL ASYMMETRY IN AD

Although hippocampus is structurally and functionally asymmetric, right (R) vs. left (L) hippocampal volume differences have received less research attention. In healthy adults there is hemispheric asymmetry of the whole hippocampus, with larger volume of the right one (Pedraza et al., 2004; Shi et al., 2009). There are also right vs. left differences in the layers' thickness and volumes of different hippocampal subfields. For instance, Lister et al. identified asymmetries in neuronal numbers in rat CA1 and CA3/CA2 subfields, with the right hemisphere containing 21 and 6% fewer neurons, respectively (Lister et al., 2006).

Hippocampal volume asymmetry has been connected with cognitive functions and it has been suggested that hippocampal subfields analysis should be included in these correlation studies. For instance, Woolard and Heckers (2012) in a study of 110 healthy individuals of 32.3 ± 10.7 years of age demonstrated that the R > L asymmetry is limited to the anterior hippocampus and it is correlated with a measure of general cognitive functions (Screen for Cognitive Impairment in Psychiatry) (Woolard and Heckers, 2012). Moreover, they showed that the volume of anterior hippocampus correlates with the volumes of all four cortical lobes, whereas the posterior hippocampus volume was found strongly correlated with the volume of occipital cortex (Woolard and Heckers, 2012).

A meta-analysis indicated that in AD there is a significant R > L hippocampal volume asymmetry as compared to the control group (Barnes et al., 2005; Shi et al., 2009). Given that hippocampal asymmetry might be due to genetic, developmental and environmental factors (Verstynen et al., 2001; Tang et al., 2008), hippocampal asymmetry in AD has been suggested to be influenced by the dose effect of the *APOE4* allele; the R > L asymmetry is progressively reduced and even reversed in *APOE4/4* carriers affected by AD (Geroldi et al., 2000). It has been also postulated that the left hippocampus is more vulnerable than the right one to AD pathology due to already smaller volume (Muller et al., 2005). Nevertheless, hippocampal asymmetry changes with AD progression, with the left hippocampus affected first by dementia, followed by atrophy in the right hippocampus after a time lag (Thompson et al., 2004; Morra et al., 2009b). Finally, one needs to be mindful of the limitations associated with using manual asymmetry measurements. Recently, Maltbie et al. performed a semi-automatic hippocampal segmentation and pointed out that neuroimaging is typically biased to one side due to laterality in visual perception (Maltbie et al., 2012). Although this bias has been reported to be smaller than the true anatomical R > L hippocampal asymmetry values (for further information on this topic and hints on avoiding the potential bias, please refer to work by Rogers et al., 2012), it is worth to take it into consideration while interpreting hippocampal asymmetry data.

THE SUPREMACY OF SUBFIELD ANALYSIS OVER TOTAL HIPPOCAMPAL VOLUME MEASUREMENTS FOR AD DIAGNOSIS

Similarly to other AD biomarkers, application of hippocampal volume changes for AD diagnosis has some limitations. Although there is a higher rate of global hippocampal volume loss in AD patients compared to control individuals and MCI patients, changes in hippocampal volume show moderate sensitivity and low specificity to AD as it is observed also in other conditions such as semantic dementia (Frisoni et al., 2010; Lindberg et al., 2012; Tondelli et al., 2012; La Joie et al., 2013). Sensitivity of total volume analysis is restricted to the stages from MCI to moderate dementia stage (Frisoni et al., 2010). In the asymptomatic AD the markers of Aβ deposition (carbon 11-labeled Pittsburgh Compound B (PiB) positron emission tomography

(PET), cerebrospinal fluid (CSF) $A\beta_{42}$) have been suggested to outperform markers of structural changes but their values plateau by MCI stage (Frisoni et al., 2010).

Given that the hippocampal subfields are differentially vulnerable to neuropathology in AD and their measurements have been shown to be more accurate than global hippocampal volumetry to differentiate prodromal AD (aMCI) from cognitively normal controls (La Joie et al., 2013), it has been proposed that hippocampal subfield volumetry might be a better biomarker for early detection of AD.

AD is characterized by the most prominent neuron loss in CA1 which correlates with dementia severity and Braak staging (Rossler et al., 2002; Mueller et al., 2010). Other studies indicated that apart from smaller volume of CA1, AD patients have decreased size of entorhinal cortex (EC), subiculum and CA1-2 transition zone (Mueller and Weiner, 2009; Mueller et al., 2010; Apostolova et al., 2012) and these findings are consistent with the level of neuronal loss (West et al., 2004; Zarow et al., 2005).

Likewise, owing to differences between studies such as age of participants, applied segmentation protocols, MRI resolution, limited statistical power and small effect sizes, there are still controversies as to which hippocampal subfield is the most reliable to distinguish normal healthy controls from aMCI. For instance, CA1 is the earliest affected hippocampal subfield by NFT and neuronal loss (Braak and Braak, 1991; Braak et al., 1993; West et al., 1994) and it has been shown to remain relatively preserved in healthy aging, thus it has been proposed that CA1 atrophy might be a biomarker of presymptomatic AD (Csernansky et al., 2005; Scher et al., 2007; Frisoni et al., 2008; Gerardin et al., 2009; Raji et al., 2009; Apostolova et al., 2010; La Joie et al., 2010). However, there are other candidates for the area of the largest difference between aMCI and controls, as some indeed point to CA1 (Yassa et al., 2010; Pluta et al., 2012; La Joie et al., 2013), however, others suggest that it is CA1-2 transition area (Mueller and Weiner, 2009; Mueller et al., 2010) but subiculum and CA2-3 (Hanseeuw et al., 2011) or CA3/DG (Yassa et al., 2010) have been also proposed. Some researchers did not focus on aMCI but on the whole MCI instead. Apostolova et al. (2006) showed that MCI patients with smaller hippocampi, particularly in the CA1 and subiculum, are at a higher risk of converting to AD (Apostolova et al., 2006). Moreover, MCI individuals that improve cognitively and revert to control status are characterized by larger hippocampal volumes and relative sparing of CA1 and subiculum (Apostolova et al., 2006). In addition, in a later study Apostolova et al. reported that the most pronounced differences between MCI and AD were in CA1 and CA2-3 bilaterally (Apostolova et al., 2012). Although there is no general agreement about the potential role of CA1 in AD diagnosis, it has been shown that the CSF tau and $A\beta_{42}$ concentrations at baseline correlate with the rate of hippocampal atrophy and progressive inward deformations of the CA1 subfield in the individuals at the very mild stage of AD (Clinical Dementia Rating 0.5; Wang et al., 2012).

In general, the problem with modelling hippocampal volume or hippocampal subfields volume changes in AD is that neuronal loss is detected only in some AD mouse models. Some tg-mice exhibit CA1 pyramidal neuron loss (i.e., Wright et al., 2013; Beauquis et al., 2014), however there are also reports of no change in number of neurons (Schaeffer et al., 2011). For instance, in PDAPP mice at 5 months of age (before the appearance of amyloid plaques in the hippocampus and cortex), a 12% decrease in CA1 and 25% reduction in DG volume was detected (Beauquis et al., 2014). Smaller DG size in PDAPP mice has been reported also by their researchers (Redwine et al., 2003; Wu et al., 2004). Atrophy of CA3/DG has been found in aMCI patients (Yassa et al., 2010; Atienza et al., 2011). However, in MCI and AD patients there have been no reports of sole DG atrophy. Mueller et al. (2010) argue that it might be due to the fact that DG and CA3 are frequently analysed together and owing to the relatively good preservation of CA3 in AD, it is possible that it overshadows subtle effects in DG (Mueller et al., 2010). Indeed, although surface-based methods have similar prognostic performance to other structural neuroimaging approaches, these methods do not provide quantitative information about hippocampal subfields volumes. Moreover, as Pluta et al. argue, that automatic hippocampal subfield segmentation basing on surface-based parcellation does not adequately model the CA4/DG subfield, given that it is to a substantial extent internal to the hippocampal formation (Pluta et al., 2012). Therefore, changes in the hippocampal surface might not truly reflect degeneration in DG.

ANTERIOR VS. POSTERIOR LOCATION OF HIPPOCAMPAL SUBFIELDS AND THEIR ROLE IN AD

Another approach to looking at the hippocampus employs differences in gene expression, behavior and functional connectivity, which were used to divide rodent hippocampus into three gross anatomical subregions, following the longitudinal axis of the hippocampus: dorsal, ventral and intermediate subfields (see Poppenk et al., 2013 for detailed information about connectivity and detailed information about postulated differences along dorsal-ventral axis; Moser and Moser, 1998; Fanselow and Dong, 2010; Zarei et al., 2013). These hippocampal regions correspond to the septal, temporal and caudal poles in rat (Swanson and Cowan, 1977). The septal pole, located dorsally and anteriorly in rodents, corresponds to posterior hippocampus in humans (Tanti and Belzung, 2013). The temporal pole, ventrally and posteriorly located in rodents, is the anterior hippocampus in humans (Tanti and Belzung, 2013). The intermediate subregion in humans is often not distinguished (Poppenk et al., 2013).

These three hippocampal regions are characterized by different patterns of connectivity with prefrontal cortex (PFC), posterior cingulate cortex (PCC) and thalamus (Zarei et al., 2013). For instance, connections between the hippocampus and PCC are thought to be involved in autobiographical and episodic memory (Zarei et al., 2013).

So far, there have been strong discrepancies in defining the boundaries of the dorsal/ventral subregions (see review by Tanti and Belzung, 2013). Frequently, little information regarding the methodology behind distinguishing the dorsal and ventral subfields is provided which prevents comparisons between studies. Nevertheless, the major hippocampal subfields, such as CA1, CA3 and DG are segregated into dorsal, intermediate and ventral.

The most pronounced atrophy in AD has been found in the anterior hippocampus (as well as posterior parahippocampal gyrus and the precuneus) compared to age-matched controls

(Raji et al., 2009). Moreover, hippocampal head (anterior) atrophy has been reported as a predictive marker of conversion to AD (Csernansky et al., 2005; Apostolova et al., 2006; Morra et al., 2009a; Costafreda et al., 2011). Costafreda et al. identified more pronounced changes in the right lateral and medial aspects of hippocampal head in individuals that later converted to AD and his results correspond with previous reports of anterior CA1 (Csernansky et al., 2005; Apostolova et al., 2006; Costafreda et al., 2011). Recently, Franko and Joly demonstrated that regions with the highest atrophy rate in anterio-lateral hippocampus correspond to those with highest tau deposition (Frankó and Joly, 2013). This supports the above-mentioned link between anterior CA1 and conversion to AD.

In addition, AD patients exhibit weaker connectivity with the PCC in the hippocampal body and stronger connectivity with the PFC in the head of the hippocampus compared to the healthy controls (Zhang et al., 2010; Zarei et al., 2013). Previous studies found disrupted functional connectivity with default mode network (DMN) in AD (as it is not in the scope of this review, please refer to Greicius et al., 2004; Mevel et al., 2011). Moreover, diffusion tensor imaging (DTI) provided evidence that structural connectivity between PCC and hippocampus is decreased during the earlier stages of AD (Zhou et al., 2008). The differences in the hippocampal connectivity were proposed to contribute to cognitive deficits in AD (Zarei et al., 2013).

Interestingly, the levels of adult neurogenesis in the dentate gyrus of the hippocampus have been associated with differential functional roles dependant on its dorsal (posterior) or ventral (anterior) location: dorsal neurogenic pool of hippocampal progenitor cells is associated with learning and memory whereas ventral—with motivational and emotional behaviors. The latter one can be exemplified by the studies looking at neurogenesis in neuropsychiatric disorders, such as depression, in which greater vulnerability to stress is reflected by a significant decrease in the ventral hippocampus (Tanti and Belzung, 2013). Given that neurogenesis is altered in the course of AD (for details see review by Lazarov and Marr, 2013), and that dorsal neurogenesis is implicated in memory and learning, it is surprising that only few studies have explored the role of dorsal-ventral differences in AD and in hippocampal neurogenesis in the course of AD.

Fuster-Matanzo et al. identified that overexpression of glycogen synthase kinase-3β (GSK-3β), an enzyme involved in both AD pathogenesis and neurodevelopment, leads to significant decrease in the number of granular neurons and increased astrocytosis in mice DG (Fuster-Matanzo et al., 2011). Notably, these changes are spared in the ventral DG, where reduced GSK-3β activity and less cell death compared with the dorsal area were found (Fuster-Matanzo et al., 2011). Fuster-Matanzo et al. study has elegantly demonstrated that there are regional differences in GSK-3β activity in AD which might explain why dorsal hippocampus is more susceptible to neurodegeneration (Fuster-Matanzo et al., 2011).

In the 3xTg mice the level of proliferation was reduced compared to non-Tg animals, and in the female mice it was present predominantly in the dorsal than ventral hippocampus, whereas the males exhibited first changes in neurogenesis in the ventral hippocampus (Rodriguez et al., 2008). However, the authors did not investigate if the number of new-born neurons is subregion-dependent.

In another report, authors did not analyse neurogenesis but presented interesting findings with regard to dorsal hippocampus and spatial memory, for which the dorsal hippocampus is essential. Yiu et al. found that TgCRND8 mice exhibit severe impairment of spatial memory and presented with decreased CREB activation in dorsal CA1, decreased spine density and dendritic complexity of CA1 pyramidal neurons (Yiu et al., 2011). They exhibited also decreased neuronal network activity.

These three studies point to the role of dorsal hippocampus in AD. However, as mentioned already above, the anterior (ventral in rodents) hippocampus, is the subregion, where the majority of volume differences are reported in aMCI and AD patients. Wolf et al. reported that atrophy of left posterior hippocampus was better in discriminating controls from MCI (Wolf et al., 2001). It is plausible that neurogenesis impairment in AD patients affects predominantly mood, whereas circuits' dysfunction underlies memory impairment and associated symptoms. Hence, the described above dorsal-ventral (posterior-anterior) discrepancies in the hippocampus role in the early stages of AD might be reflected by neuropsychiatric symptoms which receive much less attention than cognitive symptoms, but constitute a common feature of AD (Lyketsos et al., 2011). Around 35–75% MCI (Apostolova and Cummings, 2008) and 75% of AD patients experience emotional symptoms, with depression and anxiety as the most common ones during the prodromal disease stage (Sturm et al., 2013). The most frequently observed neuropsychiatric features in MCI individuals are apathy, depression, anxiety, irritability, whereas in AD patients apart from these same symptoms agitation/aggression is also present (Lyketsos et al., 2002; Wadsworth et al., 2012; Dillon et al., 2013). It has been noticed that these symptoms often precede and accelerate conversion to dementia (Apostolova and Cummings, 2008; Wadsworth et al., 2012; Dillon et al., 2013). For instance, depression has been shown to double risk of conversion to AD (Modrego and Ferrández, 2004). Notably, emotional symptoms accompanying cognitive problems are more common among future converters to AD (Gallagher et al., 2011). However, neuropsychiatric symptoms do not correlate with cognitive impairment (Dillon et al., 2013). Notably, the data from AD animal models frequently does not cover emotional symptoms, thus dorsal-ventral analysis might by biased by focusing exclusively on the cognitive domain.

Another emotion-related AD feature is emotion contagion which is a basic affective mechanism synchronizing physiological and behavioral states with those of another to promote affective simulation and altruistic behavior and is not dependant on higher order cognitive processes. Anterior hippocampus has been associated in both MCI and AD patients with experiencing high levels of emotional contagion (Sturm et al., 2013). Emotional contagion was found to be weakly correlated with depression symptoms (Sturm et al., 2013). It has been proposed that higher level of emotional contagion might be due to less efficient inhibition of emotions and salience network release which are associated with smaller volume in primarily right-hemisphere temporal lobe structures (Sturm et al., 2013).

FUNCTIONAL HIPPOCAMPAL ABNORMALITIES IN AD

In the next section of the review, we focus on the link between functional hippocampal changes and their predictive value for AD, and we demonstrate the link between structural and functional alterations is still unresolved. These changes could occur sequentially or simultaneously and they might influence each other, as we exemplify by showing the association between neurogenesis and seizures.

HYPEREXCITABILITY AND SEIZURES AT EARLY AD STAGES

It has been reported that during memory tasks individuals with mild AD show reduced hippocampal activity, whereas aMCI and MCI patients exhibit hyperactivity in the hippocampus/parahippocampal region (Hämäläinen et al., 2007; Miller et al., 2008; O'Brien et al., 2010; Yassa et al., 2010; Putcha et al., 2011). Task-related hyperactivity has been described in asymptomatic carriers of AD pathological mutations during associative encoding task (right anterior hippocampus; Quiroz et al., 2010); in asymptomatic offspring of autopsy-confirmed AD patients (Bassett et al., 2006); in cognitively intact young and old carriers of *APOE4* (Bookheimer et al., 2000; Dickerson et al., 2005; Filippini et al., 2009) and in low-performing clinically healthy aged individuals (Miller et al., 2008). Conversely, individuals at late aMCI stage and early AD already express the hippocampal hypoactivity pattern (Hämäläinen et al., 2007). During an associative memory task hyperactivation of the anterior hippocampus and EC in MCI patients compared to the controls was observed (Dickerson et al., 2005). Recently, the characteristic pattern of hyperactivity as well as shape and volume changes were detected in the CA3/DG of aMCI patients (Yassa et al., 2010; Bakker et al., 2012). CA3/DG network is essential for a key process for episodic memory, pattern separation (described later in this review).

Initially this type of hyperactivity has been regarded as compensation to deal with a cognitive task by recruiting additional neuronal resources; however it is more likely that it reveals neuronal excitotoxicity and in addition, the excess activation directly contributes to memory impairment (Brewer et al., 1998; Morcom et al., 2007; Putcha et al., 2011). There is an overall negative correlation between hippocampal activity and performance on memory task in the aged individuals (Putcha et al., 2011). Therefore, given that the network hyperexcitability contributes to cognitive impairment, Bakker et al. have demonstrated that by using a low dose of antiepileptic drug, levetiracetam, hippocampal activation in aMCI was reduced to the level observed in the control group and that had an impact on improved memory performance (Bakker et al., 2012).

In AD patients as well as in several transgenic AD mouse models spontaneous epileptic seizures have been observed (Velez-Pardo et al., 2004; Rao et al., 2009; Noebels, 2011) and they are attributed to the above-described increased network hyperexcitability (Hazra et al., 2013). Hazra et al. suggested that the seizures occur due to failure of inhibitory interneurons in DG to generate action potentials which leads to impaired DG function that is analogous to that observed in epilepsy. In addition, there is increased synaptic facilitation in the perforant pathway, leading to increased excitatory synaptic responses and spatiotemporal hyperexcitability of the DG perforant pathway circuit, and as a consequence a runaway excitation affecting hippocampal circuits adjacent to DG, which is called "breakdown of DG" (Hsu, 2007; Busche et al., 2008, 2012; Noebels, 2011; Hazra et al., 2013). These phenomena have been attributed to the Aβ pathology that has been linked with synaptic depression and to LTP inhibition (Busche et al., 2008; Hazra et al., 2013).

The occurrence of seizures in AD might have serious implications for neurogenesis. Seizures have been shown to increase neurogenesis, however they eventually decrease the survival of new-born neurons which also show aberrant migration and form aberrant circuits (see review by Parent and Murphy, 2008). Recently, Hester and Danzer (2013) demonstrated that status epilepticus similarly affects both dorsal and ventral hippocampus integration of new-born granule neurons and mossy fiber sprouting (Hester and Danzer, 2013).

Seizures are followed by comorbidities such as emotional difficulties (including increased risk for depression), cognitive impairment (memory problems) and behavioral problems, and at least some of them originate in disrupted neurogenesis. Surprisingly, there is considerable overlap between these symptoms and AD at an early stage.

PATTERN SEPARATION IN AD

Hyperactivity in DG/CA3 region has been linked with age-related mnemonic discrimination deficits, i.e., an inability to distinguish between items and similar objects—so called lures in memory (Yassa et al., 2011a). Aging is characterized by a shift in bias from pattern separation to pattern completion in the DG/CA3 network and that correlates with changes in perforant pathway integrity (Yassa et al., 2011b). Perforant pathway originates in the layer II of EC and leads to granule cells in the DG which send projections to CA3. That is an essential network for pattern separation—encoding distinctive representations of experiences that have overlapping features with prior memories (Gallagher and Koh, 2011). DG plays an essential role in pattern separation, whereas CA3 in pattern completion (Yassa and Stark, 2011).

As it has been already mentioned above, AD is characterized by a degeneration of perforant pathway connections and compromised pattern separation circuit might lead to episodic memory impairment. Recently, Ally et al. reported that AD patients demonstrate impaired both pattern separation and completion, whereas individuals with aMCI, depending on the lag between tasks, behaved similarly to the controls (if the lag was short) or to AD patients (if the lag was long) which indicates a rapidly degrading visual memory representation in aMCI (Ally et al., 2013). Therefore, the authors proposed that aMCI is characterized by rapid forgetting, whereas dementia due to AD is a disorder of encoding. Moreover, in 12-month old Tg2576 AD mice expression of mutant human APP led to disruption of DG/CA3 network, which is proposed as critical for pattern separation (Palmer and Good, 2011).

There is also accumulating data showing that hippocampal neurogenesis is an important player for pattern separation (Aimone et al., 2010, 2011; Sahay et al., 2011a,b; Tronel et al., 2012). Therefore, it is plausible that neurogenesis alterations in

the course of AD are contributing to impairment of pattern separation, however, it remains to be investigated.

Given that the volume of the posterior hippocampus positively correlates with memory for very detailed contextual information (temporal and spatial relations; representation at fine granularities), whereas anterior hippocampus is responsible for a coarse, global representation (spatial locales or contexts, coarse representation), it has been proposed that posterior hippocampus is biased towards pattern separation, whereas anterior hippocampus is more suited for pattern completion (Poppenk and Moscovitch, 2011; Evensmoen et al., 2013; Poppenk et al., 2013). Moreover, Poppenk and Moscovitch (2011) suggested that the posterior, but not the anterior hippocampus, is closely linked to episodic memory retrieval through its connection with the DMN, including pregenual anterior and PCC, and precuneus (Poppenk and Moscovitch, 2011). It remains to be explored how the reported decrease in the anterior hippocampal volume observed in AD corresponds with these hypotheses.

CONCLUSIONS

There is considerable evidence that hippocampal volume decreases early during AD progression. However, these symptoms are not specific enough to fulfil criteria of AD biomarkers and enable presympromatic AD diagnosis. In this review, we present current data on detailed subfield and subregion analyses of the hippocampus and we demonstrate that so far, there is no consensus on which subfield could become a reliable early-stage disease biomarker. There are discrepancies in the analysed cohorts, methodology (labor-intensive and suffering from intra- and interobserver variability manual segmentation vs. automatic segmentation protocols; using T1-weighted MRI sequences at 1.5–3 Tesla that lack the contrast and resolution to distinguish e.g., CA4/DG region vs. T2-weighted MRI), using single or multiple MRI scans over time, anatomical labelling (e.g., in delineating the border between the anterior and posterior hipocampus), and that all prevents direct comparisons between studies. For instance, Nestor et al. argue that absolute volume differences between different protocols may vary by > 30% (Nestor et al., 2012). Our review indicates that apart from hippocampal subfield volumetry, there is another promising candidate for early AD biomarker which is the characteristic pattern of hippocampal hyperactivation associated with seizures and neurogenesis changes. The relation between these phenomena demands also further in depth investigations as it might unravel a very important early disease target for therapeutic intervention. Finally, we believe that improving harmonization of different MRI protocols and in depth analysis of neurogenesis changes in the course of AD might result in development of new disease cellular and molecular signatures.

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A major role for Tau in neuronal DNA and RNA protection *in vivo* under physiological and hyperthermic conditions

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Nucleic acid protection is a substantial challenge for neurons, which are continuously exposed to oxidative stress in the brain. Neurons require powerful mechanisms to protect DNA and RNA integrity and ensure their functionality and longevity. Beside its well known role in microtubule dynamics, we recently discovered that Tau is also a key nuclear player in the protection of neuronal genomic DNA integrity under reactive oxygen species (ROS)-inducing heat stress (HS) conditions in primary neuronal cultures. In this report, we analyzed the capacity of Tau to protect neuronal DNA integrity *in vivo* in adult mice under physiological and HS conditions. We designed an *in vivo* mouse model of hyperthermia/HS to induce a transient increase in ROS production in the brain. Comet and Terminal deoxyribonucleotidyltransferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) assays demonstrated that Tau protected genomic DNA in adult cortical and hippocampal neurons *in vivo* under physiological conditions in wild-type (WT) and Tau-deficient (KO-Tau) mice. HS increased DNA breaks in KO-Tau neurons. Notably, KO-Tau hippocampal neurons in the CA1 subfield restored DNA integrity after HS more weakly than the dentate gyrus (DG) neurons. The formation of phosphorylated histone H2AX foci, a double-strand break marker, was observed in KO-Tau neurons only after HS, indicating that Tau deletion did not trigger similar DNA damage under physiological or HS conditions. Moreover, genomic DNA and cytoplasmic and nuclear RNA integrity were altered under HS in hippocampal neurons exhibiting Tau deficiency, which suggests that Tau also modulates RNA metabolism. Our results suggest that Tau alterations lead to a loss of its nucleic acid safeguarding functions and participate in the accumulation of DNA and RNA oxidative damage observed in the Alzheimer's disease (AD) brain.

Keywords: Tau, oxidative stress, hyperthermia, DNA damage, RNA damage, γ-H2AX, DNA repair

INTRODUCTION

Altered DNA and RNA integrity is particularly harmful in differentiated neurons. Non-repaired nucleic acids trigger transcriptional and translational deregulation, which leads to reduced protein synthesis, protein mutation, the production of truncated proteins and genomic instability. Oxidative stress generates a wide range of nucleic acid lesions including base modifications, deletions and strand breaks. Neurons in the brain continuously face the harmful effects of oxidative stress due to high oxygen consumption. Therefore, the preservation of nucleic acid integrity from oxidative damage is essential to maintain neuronal functionality and ensure their longevity (Englander and Ma, 2006; Chen et al., 2007; Englander, 2008; Mantha et al., 2013). To decipher the defense mechanisms involved in the protection of neuronal DNA integrity in normal brain is crucial to understand DNA alteration observed in neurodegenerative diseases (Brasnjevic et al., 2008; Coppedè and Migliore, 2009).

Tau plays a well-known role in microtubule assembly and stabilization. It has recently been shown that Tau functions as an essential nuclear player in the protection of neuronal genomic DNA integrity under reactive oxygen species (ROS)-producing heat stress (HS) in primary neuronal cultures (Sultan et al., 2011). We observed that oxidative stress and HS in wild-type (WT) neurons led to Tau nuclear accumulation, which protected DNA integrity from HS-induced damage (Sultan et al., 2011). However, the mechanisms responsible for Tau-mediated DNA protection are unknown. DNA protection may be mediated partially through Tau interactions with the A-T-rich DNA minor groove (Sjöberg et al., 2006; Wei et al., 2008; Sultan et al., 2011; Camero et al., 2013); however, a role for Tau in DNA repair mechanisms cannot be excluded.

As this new and major DNA protective role of nuclear Tau has been described in primary neuronal cultures (Sultan et al., 2011). the major aim of this work was to analyze the capacity of Tau to protect neuronal DNA integrity *in vivo* in adult mice under physiological and HS conditions to overcome any potential artifactual effects related to the embryonic origin of cultured neurons.

Using a novel *in vivo* mouse model of transient hyperthermia/HS, this study demonstrated the efficiency of the DNA protective function of Tau in neurons *in vivo*. We showed that Tau was indispensable for the protection of neuronal DNA integrity in the cortex and hippocampus of adult mice under physiological and HS conditions. Tau deletion did not trigger similar DNA damage under physiological and HS conditions. Tau was involved in the DNA double-strand break repair process specifically under HS. Notably, hippocampal neurons in the CA1 subfield showed a reduced ability to restore DNA integrity after HS than neurons in the dentate gyrus (DG). Surprisingly, our data obtained indicated that Tau deficiency altered the integrity of genomic DNA and cytoplasmic and nuclear RNA, suggesting that Tau could protect both RNA and DNA.

MATERIALS AND METHODS

ANIMALS

Seven month-old homozygous female KO-Tau mice (Tucker et al., 2001) and littermate WT mice were used to assess the role of Tau in DNA protection in aged mice. All animals were maintained in standard animal cages under conventional laboratory conditions (12 h/12 h light/dark cycle, 22°C), with *ad libitum* access to food and water. The animals were maintained in compliance with institutional protocols and all animal experiments were performed in compliance with, and following the approval of the local Animal Resources Committee (CEEA 342012 on December 12, 2012), standards for the care and use of laboratory animals, and the French and European Community guidelines. Three different mice have been used in each group for all experiments.

IN VIVO HYPERTHERMIA MODEL

We designed an *in vivo* mouse model of transient hyperthermic stress based on the rat model described previously by Papasozomenos (1996). The mice were anesthetized using xylazine (20 mg/kg) and ketamine (100 mg/kg) and maintained in a 37°C environment for 30 min to avoid anesthesia related hypothermia and Tau hyperphosphorylation as previously described (Planel et al., 2007). The mice were then maintained at 37°C (control (C) group) or heat stressed (HS group) by being placed in an incubator containing ambient air heated to 44°C for 20 min. The rectal temperature of the mice was monitored every 10 min and did not exceed 41°C. In the (HS+24H) group, mice were subjected to HS during 20 min and then returned to room temperature during 24 h.

OXIDATIVE STRESS-INDUCED PROTEIN DAMAGE

Protein oxidation was analyzed using an OxyIHC oxidative stress-detection kit (Millipore) according to the manufacturer's directions. Protein carbonyl groups generated by oxidative stress were visualized using immunolabeling after reaction with 2,4-dinitrophenylhydrazine (DNPH).

TISSUE COLLECTION FOR IMMUNOBLOTTING AND CONFOCAL MICROSCOPY

The mice were euthanized through cervical dislocation, and their brains were rapidly removed. One hemisphere of each brain was post-fixed for 24 h in 4% paraformaldehyde and embedded in paraffin. The hippocampus and cortex were dissected from the other hemisphere and used for biochemical analyses.

MOUSE BRAIN CYTOPLASMIC AND NUCLEAR FRACTIONATION

Mouse tissues were harvested in ice-cold buffer A (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.15% NP-40) supplemented with protease inhibitors (Complete Mini-Roche) and phosphatase inhibitors (125 nM okadaic acid and 1 mM orthovanadate). The tissues were mechanically homogenized using a 50-ml all-glass homogenizer on ice and centrifuged at 100 g for 1 min. The supernatant was collected, and a second homogenization was conducted. The supernatant was collected as the cytoplasmic fraction after centrifugation at 1000 g for 10 min. The pelleted nuclei were washed three times and lysed in ice-cold radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitors (Complete Mini-Roche) and phosphatase inhibitors (125 nM okadaic acid and 1 mM orthovanadate). The samples were sonicated and centrifuged at 12000 g at 4°C for 20 min to yield the supernatant as the nuclear fraction. The protein concentrations were determined using a bicinchoninic acid assay (BCA) kit. Lamin B and synaptophysin (SYP) were used as specific nuclear and cytoplasmic markers, respectively.

ELECTROPHORESIS AND IMMUNOBLOTTING

Electrophoresis and immunoblotting were performed as described previously (Sultan et al., 2011) using a Tau C-terminal antibody as described previously (Galas et al., 2006). The results are expressed as the mean ± S.E.M. of three different mice. ImageJ software was used for quantification.

IN VIVO COMET ASSAY

The alkaline *in vivo* comet assay was specifically developed in the cortex for this project. The mouse cortices were dissected and mechanical disaggregation of each tissue was performed by using the Medimachine® system (Becton Dickinson). A small piece of cortex was inserted into a Medicon (i.e., a disposable chamber containing an immobile stainless steel screen allowing for efficient cutting) with approximately 1.0 mL of PBS buffer. The Medicon was thus inserted into the Medimachine® which was then run for 5 s. Once the tissue was processed, the cell suspension was recovered and viability was assessed using the trypan blue exclusion method. Cell viability was assessed using the trypan blue exclusion method. The comet assay was performed as described previously (Sultan et al., 2011). The Olive tail moment (OTM; Olive et al., 1990) was used to evaluate DNA damage. The OTM, expressed in arbitrary units, is calculated by multiplying the percent of DNA fluorescence in the tail by the length of the tail in micrometers. The tail length is measured between the edge of comet head and the end of the comet tail.

A major advantage of using the OTM as an index of DNA damage is that both the amount of damaged DNA and the distance of migration of the genetic material in the tail are represented by a single number.

TERMINAL DEOXYRIBONUCLEOTIDYLTRANSFERASE-MEDIATED DEOXYURIDINE TRIPHOSPHATE NICK END LABELING (TUNEL) STAINING

Terminal deoxyribonucleotidyltransferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) staining was conducted on tissue slices using the TUNEL Apoptosis Detection Kit (Millipore) according to the manufacturer's instructions. Tissue slices were pre-treated with low concentration of DNAse (1 μg/mL during 1 h) to perform positive controls. The TUNEL assay is often used to detect late apoptosis-induced DNA breaks; however, it also detects accessible 3′-hydroxyl (3′-OH) groups that are generated from DNA single- or double-strand breaks (DSB) under non-apoptotic conditions (Liu et al., 2005).

DNAse AND RNAse TREATMENTS

Brain slices from heat-stressed KO-Tau mice were incubated with DNAse-free RNAse (0.5 mg/mL, 3 h, Roche), RNAse-free DNAse (0.2 mg/mL, 3 h, Millipore #17-141 h) or a mixture of DNAse/RNAse prior to the TUNEL assay.

IMMUNOFLUORESCENCE

Sagittal (5 μM) brain slices were deparaffinized and unmasked using citrate buffer (3.75 mM acid citrate, 2.5 mM disodium phosphate, pH 6) for 10 min in a domestic microwave. The slices were submerged for 1 h in 1% horse serum (Vector Laboratories), and the primary antibodies were incubated overnight at 4°C in the presence of PBS-0.2% Triton using the following primary antibodies: total Tau (Tau CTer) and Tau1 antibodies (Galas et al., 2006) and the phospho-histone H2A.X (Ser 139) antibody from Millipore.

These antibodies were revealed via secondary antibodies coupled to Alexa 488 or 568 (Life Technologies). The sections were counterstained and mounted with Vectashield/DAPI (Vector Laboratories). 4′,6-diamidino-2-phenylindole (DAPI) was used as a chromatin counterstain.

IMAGING SYSTEMS AND IMMUNOFLUORESCENCE QUANTIFICATION

Mouse hippocampal sections were acquired using an LSM 710 confocal laser-scanning microscope (Carl Zeiss). The confocal microscope was equipped with a 488-nm Argon laser, 561-nm diode-pumped solid-state laser and a 405-nm ultraviolet laser. The images were acquired using an oil 40x Plan-NEOFLUAR objective (1.3 NA) and an oil 63X Plan-APOCHROMAT objective (1.4 NA). All recordings were performed using the appropriate sampling frequency (8 bits, 1024-1024 images and a line average of 4). Serial sections from the three-dimensional reconstruction were acquired using Z-steps of 0.2 μm.

Images is an array of pixel and each pixel contains information about the different light intensity or color. This information is encoded in grid as a gray level. The gray values or gray scale describe the fluorescence intensity of every pixel. Acquisitions in confocal microscope are executed in 8 bits, therefore the different values of gray level of a pixel that can take are from 0 to 255 levels (0 no signal and 255 maximum signal).

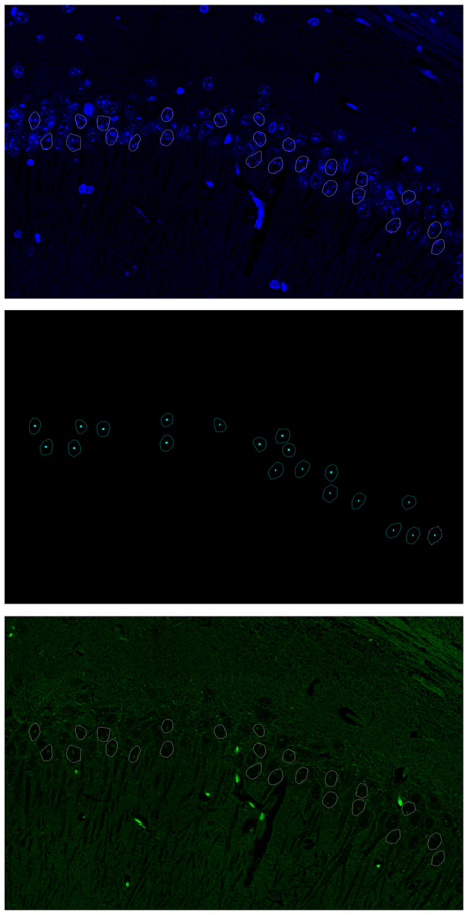


FIGURE 1 | Fluorescence quantification in the nuclei of hippocampal neurons. The elliptical selection tool of ImageJ was used to mark 20–30 representative nuclei based on DAPI staining. The immunofluorescence quantifications from the nuclear mean intensity fluorescence are expressed in gray values, and image analyses of the raw data were obtained with ImageJ and ZEN software programs.

The elliptical selection tool of ImageJ marked 20–30 representative nuclei based on DAPI staining (**Figure 1**). All immunofluorescence quantifications from the nuclear mean intensity fluorescence are expressed in gray values, and image analyses of the raw data were obtained using ImageJ (http://rsb.info.nih.gov.gate2.inist.fr/ij/, NIH, USA) and ZEN (Carl Zeiss) software programs. The results are expressed as the mean ± S.D. of the gray value from 20–30 different nuclei or at least three different areas of the CA1 cell layer.

STATISTICS

Student's *t*-test (BiostaTGV software, Jussieu, France) was used to determine the significance (*p*-value) between groups for immunoblotting and immunofluorescence analysis. A *p*-value < 0.05 was considered to indicate a significant difference.

RESULTS

TAU PROTECTS NEURONAL DNA INTEGRITY IN AN *IN VIVO* MOUSE MODEL UNDER PHYSIOLOGICAL AND HYPERTHERMIC CONDITIONS

We have shown previously that nuclear Tau protects DNA integrity in primary neuronal cultures of embryonic origin (Sultan et al., 2011). We designed an *in vivo* mouse model of transient hyperthermia/HS that induced ROS production in the cortex and the hippocampus, a region in the mouse brain particularly sensitive to oxidative stress, to investigate the physiological relevance of the DNA-protective function of Tau.

The ability of HS to induce oxidative stress (Flanagan et al., 1998) in the cells of 7-month (7 m) WT and KO-Tau mice was qualitatively analyzed through the immunohistochemical detection of carbonyl groups added to proteins (**Figure 2**). HS induced an increase of the carbonyl immunolabeling both in WT and KO-Tau cells in sagittal hippocampal sections.

The data obtained after Western blot analysis indicated that Tau was present in the nuclei of neurons from the cortex and hippocampus of WT mice under physiological conditions (**Figures 3A, B**), which is consistent with previous results in neuronal cultures (Sultan et al., 2011). HS increased Tau nuclear localization, also as observed previously in neuronal cultures (Sultan et al., 2011). The HS-induced nuclear accumulation of Tau in both regions was reversible. Tau in cortical and hippocampal nuclear extracts from WT mice was positively labeled with Tau1 antibody in physiological and HS conditions showing that nuclear Tau was predominantly dephosphorylated at epitope Ser195-202 (**Figures 3A, B**), as previously described in cultured neurons (Sultan et al., 2011).

The degree of DNA damage was monitored using a single-cell gel electrophoresis (Comet) assay in the cortices of 7 m WT and KO-Tau mice under control and HS conditions to analyze the capacity of Tau to protect neuronal DNA integrity *in vivo*. A highly significant enhancement of the median OTM, which reflects DNA fragmentation, was observed in KO-Tau mice compared with WT mice under physiological conditions (2.5-fold in KO-Tau C vs. WT C, $p < 0.001$), which shows that the Tau protein plays a major role in the protection of DNA integrity *in vivo* in the adult mouse brain (**Figure 4A**). HS treatment also selectively increased the OTM in KO-Tau mouse cells (1.4-fold in KO-Tau HS vs. KO-Tau C, $p < 0.05$); however, no significant induction of DNA damage was observed after HS treatment in WT mice (**Figure 4A**).

The TUNEL assay was performed to specifically visualize and quantify DNA breaks *in vivo*. Sagittal hippocampal sections from non-treated or HS-treated WT and KO-Tau mice were subjected to TUNEL assays, and the results were imaged using laser-scanning confocal microscopy (**Figure 4B**). Nuclear TUNEL fluorescence was specifically quantified in the neurons (**Figure 4C**) of two distinct hippocampal areas, the DG and the CA1 subfield. A strong and highly significant difference was observed between WT and KO-Tau mice under control and HS conditions, consistent with the Comet assays. TUNEL nuclear intensity was dramatically higher in the DG and CA1 of KO-Tau mice compared with WT mice under control condition (≈20-fold increase in DG and CA1 KO-Tau C vs. WT C, $p < 0.001$). Altogether, these observations support an essential physiological role for Tau in the protection of neuronal DNA integrity. HS significantly increased TUNEL-positive cells in KO-Tau mice compared with control (C) non-treated cells (CA1, 1.6-fold in KO-Tau HS vs. KO-Tau C DG, $p < 0.001$; DG, 2.5-fold in KO-Tau HS vs. KO-Tau C, $p < 0.001$). Only a fraction of the hippocampal neurons were TUNEL-positive after HS, which reflects the heterogeneity of the stress response between neurons (**Figure 4B**). As a positive control, DG and CA1 sagittal sections from 7-month-old WT mice in control condition were pretreated with a low concentration of DNAse to create substrate for the end-labeling réaction (**Figure 4B**). The level of gray was quantified within the nuclei in cells from DG and CA1 subfields in the positive control and compared to the averaged gray levels in the DG and CA1 regions in HS condition (DG: 70% in KO-Tau HS vs. positive control; CA1: 100% in KO-Tau HS vs. positive control), showing the potent effect of HS to induce nucleic acid breaks in KO-Tau neurons.

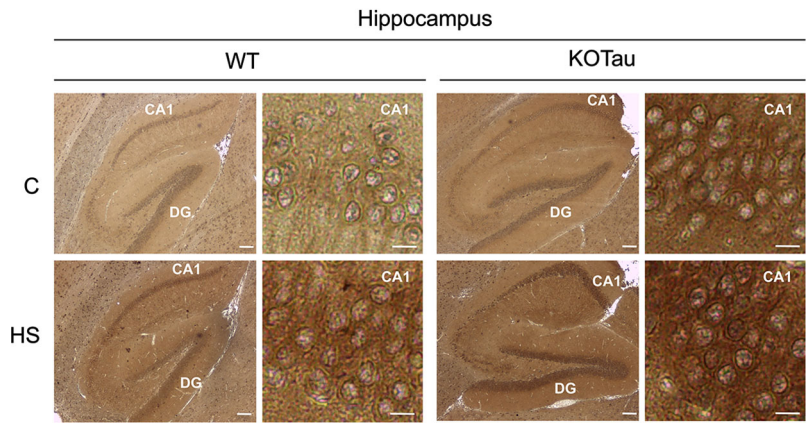
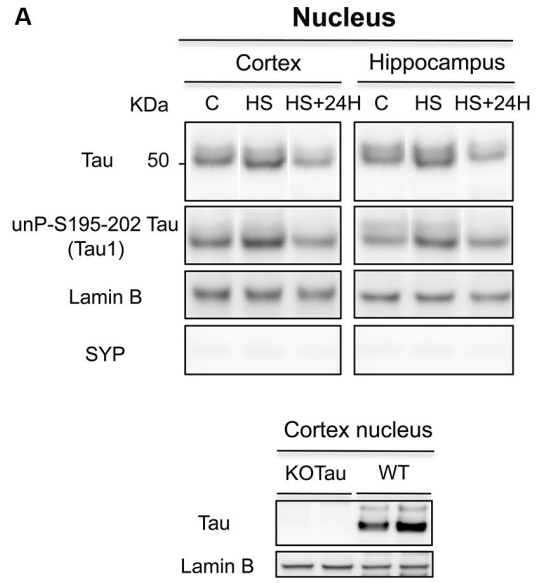


FIGURE 2 | Hyperthermia generates oxidative stress in WT and KO-Tau hippocampi. Protein carbonyl groups generated by oxidative stress were visualized using immunolabeling after reaction with 2,4-dinitrophenylhydrazine (DNPH) in sagittal sections of the hippocampus from WT and KO-Tau mice under control (C) or heat stress (HS) conditions. HS increased DNPH staining in WT and KO-Tau sections. *Scale bars* indicate 200 or 10 μm (zoom).



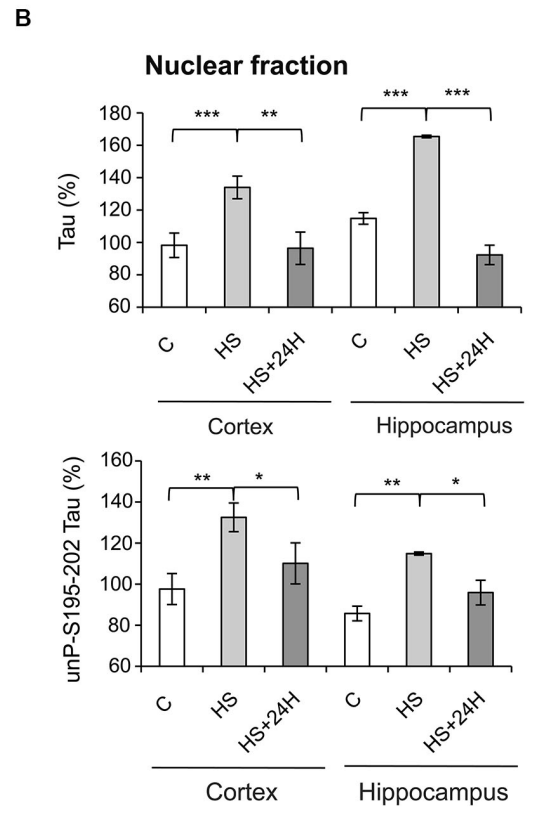


FIGURE 3 | Nuclear Tau protects genomic DNA integrity from hyperthermia-induced damage. (A) Nuclear extracts from the cortex and hippocampus of WT mice in the control (C) condition, after HS or after a 24-h recovery after HS (HS+24 h) were analyzed using immunoblotting for Tau independent of phosphorylation (Tau) and Tau unphosphorylated at epitope S195-202 (Tau1). Lamin B and synaptophysin (SYP) were used as specific nuclear and cytoplasmic markers, respectively.

(*Continued*)

FIGURE 3 | Continued
(B) Densitometric analysis of Tau (normalized to lamin B) and Tau1 (normalized to total Tau) revealed an increase in Tau protein dephosphorylated at epitope S195-202 in the nuclei of neurons under HS. 24 h of recovery restored basal nuclear Tau levels. The data shown are the mean ± S.D. of three different mice. *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$.

KO-Tau mice were allowed to recover for 24 h at room temperature after HS to investigate the later effects of HS-induced DNA damage. The nuclear TUNEL fluorescence in DG neurons returned to basal levels ($p < 0.001$); however, the nuclear TUNEL fluorescence only partially decreased in CA1 cells (1.2-fold in KO-Tau HS+24 h compared with KO-Tau HS, $p < 0.05$; **Figure 4C**). These results demonstrate the persistence of DNA damage selectively in CA1 neurons.

No change in cell density was detected (**Figure 4D**), which demonstrates the absence of HS-induced cell toxicity in KO-Tau mice in our *in vivo* HS model.

H2AX PHOSPHORYLATION IS INDUCED IN KO-TAU NEURONS ONLY AFTER HYPERTHERMIA

The production of DNA DSB leads to H2AX phosphorylation (γ-H2AX) under normal conditions (Kuo and Yang, 2008), which is necessary to initiate DSB repair. We performed fluorescent immunohistochemical labeling using an anti-γ-H2AX antibody in hippocampal sections from WT and KO-Tau mice before and after HS treatment to investigate DSB formation (**Figure 5A**). No significant increase in γ-H2AX labeling was observed between 7 m WT and KO-Tau mice under control conditions. However, a strong increase in nuclear γ-H2AX foci was observed in KO-Tau hippocampal (DG and CA1) cells after HS treatment (**Figure 5A**).

A strong increase in γ-H2AX levels (3.2 fold in HS KO-Tau vs. HS WT, $p < 0.05$) was also specifically observed in lysates from KO-Tau hippocampi after HS treatment (**Figure 5B**) using immunoblotting. These results indicate that only HS-induced neuronal DNA damage in KO-Tau mice led to γ-H2AX foci production.

γ-H2AX fluorescent labeling and TUNEL assays were performed concomitantly in hippocampal slices of 7 m KO-Tau mice (**Figure 5C**). HS specifically evoked γ-H2AX foci formation on chromatin in certain TUNEL-positive neurons, as observed in the TUNEL/γ-H2AX, γ-H2AX-DAPI and TUNEL-DAPI overlays.

γ-H2AX labeling in DG cells from KO-Tau mice decreased to basal levels 24 h after HS (**Figure 5D**), which is similar to the TUNEL staining. This result further confirms the capacity of the DG cells in KO-Tau mice to repair HS-induced DNA damage. In contrast, discrete γ-H2AX foci persisted in the nuclei of CA1 neurons, which indicated the reduced capacity of CA1 neurons to fully restore DNA integrity after HS.

HYPERTHERMIA ALTERS DNA AND RNA INTEGRITY IN KO-TAU NEURONS

The TUNEL-positive staining described in **Figure 3B** was also present in the cytoplasm of KO-Tau neurons (**Figure 6**). Cytoplasmic TUNEL staining was diffuse and, therefore, did not

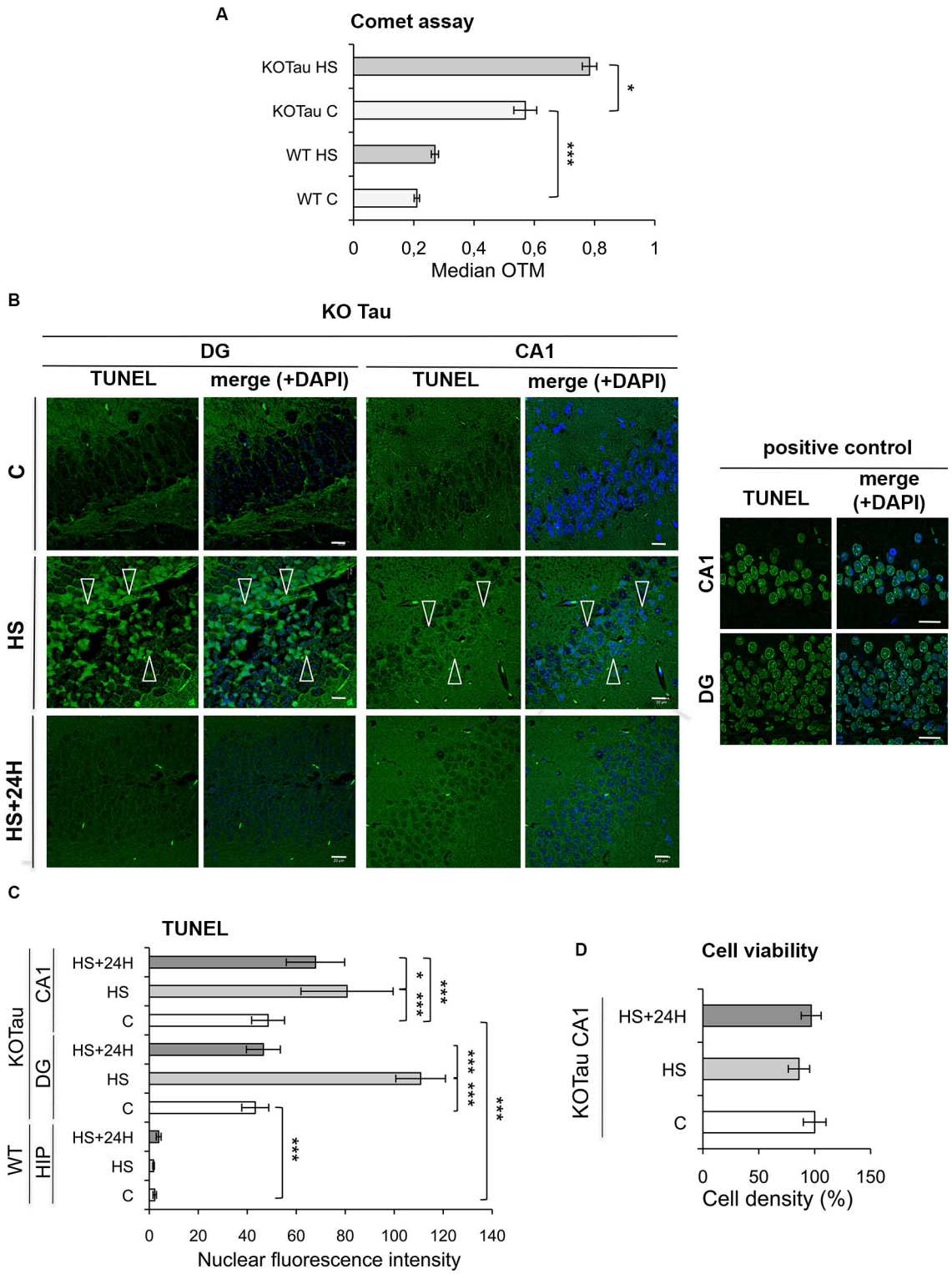


FIGURE 4 | Hyperthermia increases non-cytotoxic nucleic acid damage selectively in KO-Tau neurons. (A) The effect of Tau deficiency on genomic DNA integrity was measured using a Comet assay in control (C) and HS mice. The results are presented as the OTM from WT or KO-Tau cortices under C or HS conditions. Tau deficiency selectively promoted DNA damage accumulation and the majority of fragmentation under C and HS conditions. Each OTM value is the median value of 150–200 cells from three different cortices. *** $p < 0.001$; * $p < 0.05$. **(B)** Representative images of the dentate gyrus (DG) and CA1 sagittal sections from 7-month-old KO-Tau mice subjected to TUNEL assay under C, HS and 24 h recovery after HS (HS+24 h) conditions and analyzed using laser scanning confocal microscopy. Nuclei

(*Continued*)

FIGURE 4 | Continued
were detected with DAPI staining. HS induced a strong positive TUNEL staining selectively in DG and CA1 KO-Tau neurons. The arrows indicate TUNEL-positive neurons. As a positive control, DG and CA1 sagittal sections from 7-month-old WT mice in control condition have been pretreated with a low concentration of DNAse to create substrate for the end-labeling reaction. *The scale bars* indicate 20 μm. **(C)** The effect of Tau deficiency on nuclear nucleic acid integrity was detected using the TUNEL assay under C, HS or HS+24 h conditions. The level of gray (0 = black; 255 = white) was quantified within the nuclei (based on DAPI detection) in cells from whole WT hippocampi (HIP) or DG and CA1 subfields from KO-Tau hippocampi. Tau deficiency clearly increased the averaged gray levels in the DG and CA1 regions in C and HS conditions. 24 h after HS, the gray level fully returned to basal levels in the nuclei from KO-Tau DG neurons but only partially decreased in the CA1 neurons, which shows the selective weakness of CA1 neurons compared with DG cells in the removal of HS-induced damage. The data shown are the mean ± S.D. of 20–30 nuclei. *** $p < 0.001$; * $p < 0.05$. **(D)** Quantification of DAPI-stained nuclei did not show significant changes in cell density in 7-month-old CA1 KO-Tau mice after HS or HS+24 h. These data indicate that HS-generated nucleic acid damage did not promote cell death.

correspond to mitochondrial DNA fragmentation. We hypothesized that the cytoplasmic TUNEL labeling reflected RNA fragmentation because the TUNEL assay is based on fluorochrome labeling of 3′-OH termini after nucleic acid breaks.

Hippocampal sagittal sections from heat-stressed 7 m KO-Tau mice were incubated with high concentrations of DNAse-free RNAse, RNAse-free DNAse or a mixture of DNAse and RNAse to investigate the possibility that the cytoplasmic TUNEL staining corresponded to RNA strand breaks (**Figure 6**).

Pre-treatment with RNAse fully abolished cytoplasmic TUNEL staining and partially abolished nuclear TUNEL staining, which suggested that the cytoplasmic staining and a portion of the nuclear staining was HS-induced RNA damage in KO-Tau neurons. Pre-treatment with DNAse only partially removed nuclear TUNEL staining despite the complete disappearance of DAPI staining, which indicates complete DNA degradation. This result suggests that nuclear RNA is damaged after HS in KO-Tau neurons. Concomitant pre-treatment with DNAse and RNAse fully abolished cytoplasmic and nuclear TUNEL staining, which confirms the specific alterations of these nucleic acids in stressed KO-Tau neurons. These results suggest that DNA and nuclear and cytoplasmic RNA are damaged after HS in KO-Tau neurons.

DISCUSSION

DNA PROTECTIVE FUNCTION OF TAU *IN VIVO*

The present study demonstrates a novel, major physiological role for nuclear Tau in the protection of neuronal DNA integrity *in vivo* in the adult mouse brain under physiological conditions; the absence of Tau rendered neuronal cells abnormally susceptible to HS-induced DNA damage.

Neurons in the brain encounter recurrent oxidative stress throughout their lifespan. The high basal levels of DNA damage in KO-Tau compared with WT neurons in aged mice under physiological conditions likely reflected a loss of the intrinsic protective function of Tau against chronic endogenous oxidative stress in the brain. It may contribute to the different deficits observed with age in Tau KO mice (Ke et al., 2012).

We designed a novel *in vivo* mouse model of transient hyperthermia/HS as a valuable and useful tool to easily investigate the effects of a transient and acute ROS increase in the whole brain of WT or transgenic mice. This model can be used to delineate the role of Tau in DNA protection under oxidative stress conditions. However, we cannot exclude the possibility that effects other than oxidative stress are involved in hyperthermia (Morano et al., 2012). This model demonstrated that hyperthermia potentiated DNA alterations in the absence of Tau *in vivo*, which reproduces the DNA protective role of Tau under HS conditions in primary neuronal cultures (Sultan et al., 2011).

Altogether these results show that Tau plays an essential role to preserve DNA integrity in adult neurons *in vivo* under physiological and HS conditions.

TAU MODULATES DNA DOUBLE-STRAND BREAK REPAIR

DNA damage in neurons triggers a cascade of highly potent DNA repair mechanisms to maintain genome integrity (Canugovi et al., 2013). DNA damage responses can induce highly dynamic post-translational modifications of histones that are critical for the DNA repair process (Lukas et al., 2011). DBS, one of the most toxic forms of DNA damage, quickly induce phosphorylation at serine 139 in the C-terminal sequence of histone H2AX (γ-H2AX), which promotes the recruitment of multiple DNA repair factors.

The absence of strong γ-H2AX accumulation in KO-Tau neurons under control conditions notwithstanding the strong increase in neuronal DNA damage in KO-Tau compared with WT mice observed using Comet and TUNEL assays, indicated that the DNA damage accumulation in Tau-deficient mice under physiological conditions differs from the HS-induced damage. This result suggests that chronic oxidative stress primarily induces single breaks rather than DSB or that the DSB repair process is impaired in KO-Tau neurons under physiological conditions.

Conversely, hyperthermia induced a strong and transient increase in γ-H2AX foci selectively in the nuclei of KO-Tau neurons, indicating that Tau deficiency induced the accumulation of non-repaired DNA DSB through altered DNA damage-induced chromatin post-translational modifications. This result suggests that Tau modulates double-strand break DNA repair responses under hyperthermia.

Hyperthermia-induced nucleic acid damage is reversible, which suggests that a Tau deficiency delays, but is not critical for, the DNA repair process. Tau likely plays a modulating rather than an essential role in double-strand break DNA repair mechanisms, and other proteins could compensate for its loss of function.

These results show an essential role for Tau in the control of DNA breaks under physiological conditions in adult neurons *in vivo* and a modulating role in the DNA double-strand break repair process under hyperthermia. Overall, this study suggests

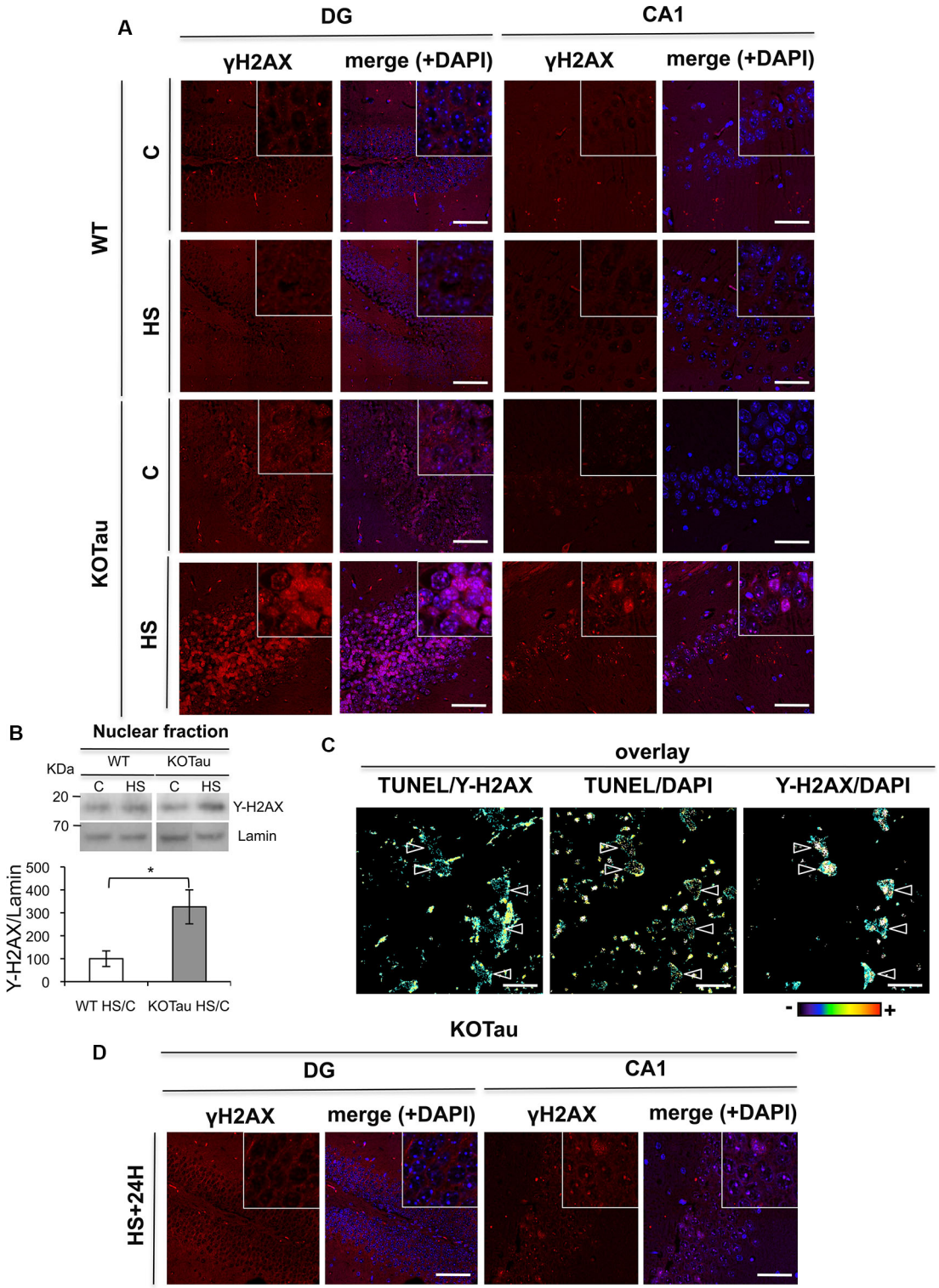


FIGURE 5 | Tau deletion induces γ-H2AX accumulation under hyperthermia. H2AX phosphorylation was detected using an anti-γ-H2AX antibody in hippocampal sections from 7 m WT and KO-Tau mice. **(A)** Representative images of sagittal DG and CA1 sections from WT and KO-Tau mice under control (C) or HS conditions labeled for γ-H2AX and analyzed using confocal microscopy are shown. DAPI stained the nuclear chromatin. HS induced a strong increase in γ-H2AX specifically in the KO-Tau hippocampus. *The scale bars* indicate 50 μm. **(B)** Nuclear extracts of the hippocampus from WT and KO-Tau mice in the C or HS condition were analyzed using immunoblotting for γ-H2AX. Lamin B was used as a specific nuclear loading protein. **(C)** Sagittal DG sections from WT and KO-Tau mice

(*Continued*)

FIGURE 5 | Continued
were subjected to a TUNEL assay, labeled with γ-H2AX and analyzed using confocal microscopy. Comparisons of TUNEL-γ-H2AX, TUNEL-DAPI and ϒ-H2AX-DAPI overlays highlighted the occurrence of double-strand breaks (DBS) only in some nuclei (arrows). *Scale bars* indicate 10 μm. **(D)** Representative images of sagittal DG sections from 7-month-old KO-Tau mice 24 h after HS labeled for γ-H2AX and analyzed using laser scanning confocal microscopy. The nuclei were detected using DAPI staining. Nuclear γ-H2AX labeling returned to control levels in DG neurons, but discrete γ-H2AX foci persisted in the nuclei of CA1 neurons. *The scale bars* indicate 50 μm.

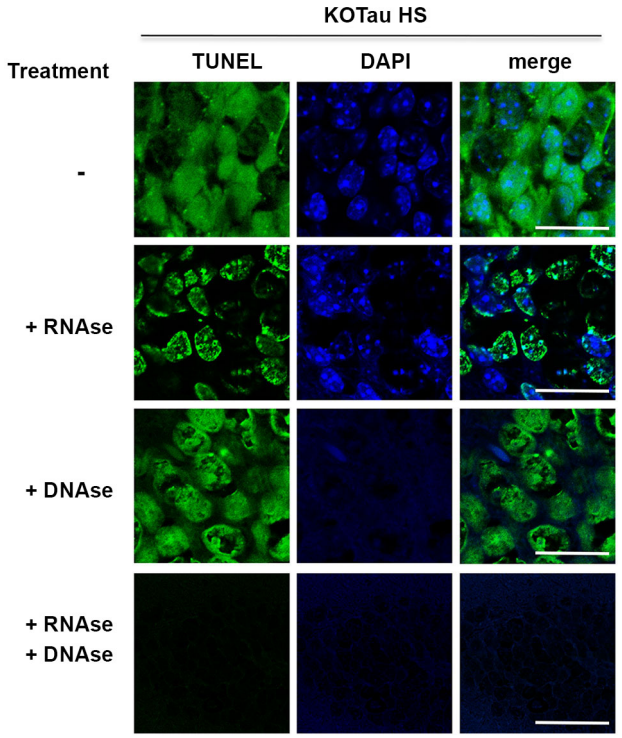


FIGURE 6 | Hyperthermia causes DNA and RNA damage in Tau-deficient neurons. Sagittal hippocampus sections from KO-Tau mice subjected to HS were pre-treated or not with DNAse-free RNAse, RNAse-free DNAse or both before TUNEL staining and imaged using laser scanning confocal microscopy. RNAse pre-treatment fully abolished cytoplasmic staining and reduced nuclear TUNEL staining levels. DNAse pre-treatment fully abolished DAPI staining and reduced nuclear TUNEL staining. These data demonstrated that Tau deficiency induced nuclear DNA and cytoplasmic/nuclear RNA damage under HS.

an important role of Tau in DNA repair mechanisms although we cannot exclude that Tau may only be involved in DNA protection.

DIFFERENTIAL DNA VULNERABILITY AMONG HIPPOCAMPAL NEURONS

DNA from hippocampal neurons in the CA1 subfield showed a higher susceptibility to damage than DG neurons 24 h after hyperthermia. This result highlights the relative weakness of KO-Tau CA1 neurons to restore DNA integrity compared with DG neurons.

Selective deficiency in oxidized DNA repair has been reported in CA1 neurons compared to others hippocampal neurons under oxidative stress conditions, hypoxia, ischemia and neurodegeneration in Alzheimer's disease (AD; Wang and Michaelis, 2010).

Our data suggest that impaired Tau-dependent DNA repair plays a role in the selective vulnerability of CA1 neurons.

RELATIONSHIP BETWEEN TAU AND RNA METABOLISM

The present *in vivo* study suggests that Tau deficiency triggers alterations in RNA integrity in hippocampal neurons under HS.

Indeed, RNA oxidation predominantly leads to strand breaks (Poulsen et al., 2012). Cytoplasmic TUNEL staining has been described, but RNA fragmentation is rarely suggested as a possible cause. Nevertheless, TUNEL protocols often advise the use of RNAse treatment to clear the so-called cytoplasmic background (Zhang et al., 2006).

However, as RNA integrity has been analyzed in an indirect way, further experiments like mass spectrometry would be necessary to confirm the alteration of RNA in KO-Tau mice.

It is generally acknowledged that altered RNA is degraded rather than repaired because very few RNA repair mechanisms have been described in mammalian cells (Aas et al., 2003; Nunomura et al., 2009). Cleaved RNA is particularly harmful for neurons because it can lead to the translation of dysfunctional truncated or mutated proteins. RNA damage accumulation in KO-Tau neurons suggests that Tau plays a role in the RNA quality control process in addition to its previously described genomic DNA protective function. Tau is an RNA-binding protein (Kampers et al., 1996), and Tau may protect RNA partially through direct or indirect interactions. Further experiments are necessary to elucidate the potential role of Tau in RNA metabolism.

CONCLUSIONS

Our data suggest that Tau protection of DNA and RNA integrity plays a key role in nucleic acid integrity under physiological conditions and under ROS-producing stress such as hyperthermia.

Tau is impaired in several devastating neurodegenerative diseases (i.e., tauopathies) such as AD. An increase in oxidative DNA (Brasnjevic et al., 2008; Coppedè and Migliore, 2009; Bradley-Whitman et al., 2014) and RNA (Lovell et al., 2011; Nunomura et al., 2012) damage occurs in a subset of vulnerable neurons that exhibit Tau pathology during the early stages of AD. Therefore, the pathological forms of Tau may have altered nucleic acid protective functions. A loss of Tau-mediated nucleic acid functions may participate in the DNA and RNA damage accumulation observed in tauopathies.

AUTHOR CONTRIBUTIONS

Marie Violet, Lucie Delattre, Alban Chauderlier, Audrey Sultan and Raphaelle Caillierez performed the *in vivo* HS model, immunohistochemistry, TUNEL assay, Western Blot and cell viability. Meryem Tardivel performed the confocal microscopy analysis and quantification. Smail Talahari performed the Comet assay. Fabrice Nesslany designed and supervised the Comet assay. Luc Buée and Marie-Christine Galas designed and supervised the experiments. Eliette Bonnefoy, Bruno Lefebvre, Luc Buée

and Marie-Christine Galas interpreted the data. Marie-Christine Galas wrote the manuscript. Eliette Bonnefoy, Bruno Lefebvre and Luc Buée revised the manuscript critically for important intellectual content. All authors approved the final version of the manuscript and are accountable for all aspects of the work.

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Recent developments of protein kinase inhibitors as potential AD therapeutics

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Present Alzheimer's disease (AD) therapies suffer from inefficient effects on AD symptoms like memory or cognition, especially in later states of the disease. Used acteylcholine esterase inhibitors or the NMDA receptor antagonist memantine address one target structure which is involved in a complex, multifactorial disease progression. So the benefit for patients is presently poor. A more close insight in the AD progression identified more suggested target structures for drug development. Strategies of AD drug development concentrate on novel target structures combined with the established ones dedicated for combined therapy regimes, preferably by the use of one drug which may address two target structures. Protein kinases have been identified as promising target structures because they are involved in AD progression pathways like pathophysiological tau protein phosphorylations and amyloid β toxicity. The review article will shortly view early inhibitors of single protein kinases like glycogen synthase kinase (gsk3) β and cyclin dependent kinase 5. Novel inhibitors will be discussed which address novel AD relevant protein kinases like dual-specificity tyrosine phosphorylation regulated kinase 1A (DYRK1A). Moreover, multitargeting inhibitors will be presented which target several protein kinases and those which are suspected in influencing other AD relevant processes. Such a multitargeting is the most promising strategy to effectively hamper the multifactorial disease progression and thus gives perspective hopes for a future better patient benefit.

Keywords: protein kinase, inhibitor, tau phosphorylation, small molecules, structure–activity relationships, inhibitor binding

INTRODUCTION

Presently Alzheimer's disease (AD) therapies are limited by the availability of just two groups of drugs with one group consisting of just one drug. Acetylcholine esterase inhibitors increase the neuronal acetylcholine amounts, whereas memantine, an antagonist at the neuronal *N*-methyl D-aspartate (NMDA) receptor, reduces a neuronal overstimulation caused by the neurotransmitter glutaminic acid (Birks, 2006; McShane et al., 2006). Both the loss of acetylcholine and the neuronal overstimulation contribute to the decay of neurons and thus the AD progression. However, the patient benefit of the present drugs is poor and limited to the early stage of the disease (Terry and Buccafusco, 2003; Raina et al., 2008). Memory, cognition, and daily behavior for life managing is hardly improved by the drugs in later stages of a severe AD (Terry and Buccafusco, 2003; Raina et al., 2008). It is known that AD is a multifactorial disease which means that different pathophysiological factors all contribute to the AD progression (Iqbal and Grundke-Iqbal, 2002). Most important hallmarks are the protein deposites which are found inside and outside the neuronal cells, namely the extracellular Aβ plaques and the intracellular neurofibrillary tangles (NFTs; Mohandas et al., 2009; Piau et al., 2011).

However, both proteins and their precursors are known to play a central role in the neuronal decay and disease progression. While a toxicity of the Aβ plaques is still under debate, their soluble precursors of non-aggregated Aβ proteins are toxic in various ways also by the formation of NFTs as a result of aggregation of a hyperphosporylated and misfolded tau protein as will be discussed later (Mattson, 2004; Pakaski and Kalman, 2008; Mohandas et al., 2009; Piau et al., 2011). Such tau protein can no longer support the intracellular transport mediated by the microtubules and with the loss of neuronal function the cell is dedicated to undergo apoptosis (Iqbal and Grundke-Iqbal, 2008; Marco et al., 2010). Protein kinases are known to mainly contribute to these toxic events as they play a central role in the cellular pathways of regulated cell function and division.

With the understanding of various protein kinase functions the question of developing inhibitors as potential AD therapeutics has arisen. Protein kinase inhibitors are long established in cancer therapies where they regulate the overactivity of protein kinases which lead to uncontrolled cell divisions, cell migration, and cellular invasion (Krug and Hilgeroth, 2008; Zhang et al., 2009). The toxicity of such anticancer protein kinase inhibitors has always been a critical question of causing toxic or undesired side-effects (Liao and Andrews, 2007). Viewing the years of research in this field multitargeting protein kinase inhibitors established and are well tolerated by patients with only limited side-effects (Krug and Hilgeroth, 2008). So there are certain perspectives that protein kinase inhibitors for AD therapy may show promising effects in the pathophysiological AD process on one hand and for a more effective therapy on the other hand with respect to the knowledge that present drugs are no real perspective drugs to effectively influence

the disease progression. The review will give a short summary of the early protein kinase inhibitors which target early known single target structures. Such early protein kinase inhibitors have been developed to reduce the activity of tau protein hyperphosphorylating kinases which have been found partly overactive or overexpressed in respective neuronal cells in AD brains and thus mainly contribute to the aggregation and loss of function of the hyperphosphorylated tau. Then novel inhibitors will be viewed which address novel targets with evidence for the AD progression and those which are dedicated to a multitargeting of more than one target structure.

EARLY PROTEIN KINASE INHIBITORS

Tau protein has been the main target structure for protein kinase inhibitors since tau protein was found hyperphosphorylated in AD brains. The tau protein hyperphosphorylation results in dissociation from the microtubules the function of which is supported by tau protein (Martin et al., 2013). Moreover, the hyperphosphorylation causes a loss of solubility and leads to the formation of paired helical filaments (PHFs) which further aggregate to NFTs (Avila, 2006; Martin et al., 2011, 2013). The reason for the tau protein hyperphosphorylation is an imbalance of phosphorylation and dephosphorylation of tau. This imbalance is partly driven by a reduced tau protein dephosphorylation or by an overactivity of the phosphorylating protein kinases (Iqbal and Grundke-Iqbal, 2002; Tian and Wang, 2002; Pei et al., 2008; Martin et al., 2013). Glycogen synthase kinase (GSK) 3β plays a central role in the tau phosphorylation process. It has been reported that 31% of the pathological phosphorylation sites of tau protein are phosphorylated by GSK3β (Martin et al., 2013). GSK3β has been found co-localized with NFTs and is found overexpressed in AD brains with increased activity (Pei et al., 1997; Leroy et al., 2007). Toxic Aβ is known to increase the activity of GSK3β which contributes to an increased Aβ production via the tau phosphorylation (Hoshi et al., 1996; Takashima et al., 1996). Therefore GSK3β forms a link of Aβ toxicity and tau pathology (Martin et al., 2013). Lithium as an early GSK3β inhibitor reduced tau phosphorylation and prevented reversed aspects of tau pathology in animal models (Tariot and Aisen, 2009). However, treatment of AD patients with mild AD in the early disease states showed no improvement in cognition (Hampel et al., 2009). The result correlated with unchanged AD biomarkers in the cerebrospinal fluid (CSF) of the patients, namely phosphorylated tau, total tau, and toxic Aβ (Hampel et al., 2009). Several GSK3β inhibitors are under development belonging to the paullone, indirubin, and the maleimide families. However, no representative of these inhibitor groups reached clinical trials so far. Reasons for their failure have been proven cytotoxic effects.

The other important tau protein kinase which is involved in pathophysiological tau protein phosphorylation is the cyclin dependent kinase 5 (cdk5). The normally cdk5 regulating protein p35 is found truncated in AD brains to 25 amino acids. This protein p25 leads to a constitutive activation of cdk5 and thus causes the pathophysiological tau phosphorylation (Patrick et al., 1999). The hyperphosphorylated tau protein dissociates from the microtubules and forms NFTs on cdk5 induction by p25 (Cruz et al., 2003; Noble et al., 2003). Also NFTs are phosphorylated by cdk5 action (Baumann et al., 1993; Hollander et al., 1996). Cdk5 phosphorylated tau becomes a better substrate for GSK3β so that excessive tau phosphorylation proceeds (Sengupta et al., 1998). Moreover, cdk5 promotes apoptosis in AD brains which may follow Aβ toxicity influenced by the hyperphosphorylated tau or by remaining p35 protein (Hamdane et al., 2005; Utreras et al., 2009).

From the number of cdk inhibitors which were all non-selective like flavoperidol a pan-cdk inhibitor investigated for cancer therapy, cdk5-selective so-called CP-inhibitors were reported with nanomolar affinities and the ability to cross the blood brain barrier (Wen et al., 2008). They were described to reduce increased Aβ levels on p25 overexpression. However, as far as known these inhibitors remained in preclinical states.

NOVEL INHIBITORS

SINGLE PROTEIN KINASE TARGETING OF CDK5, P38, JNK, Erk, CK, and DYRK1A

Due to the significant role of cdk5 in tau phosphorylation there have been ongoing efforts to develop novel inhibitors of the kinase which have been structurally based on the cdk inhibitor roscovitine (seliciclib) as a trisubstituted purine compound (**Figure 1**).

Beside cdk5 roscovitine inhibits several cdks like cdk1, cdk2, or cdk9 in micromolar concentrations (Bettayeb et al., 2008). It presently undergoes clinical trials of various types of cancer (U.S. National Institutes of Health, 2010). However, roscovitine crosses the blood brain barrier and this advantage might have driven further studies because only about 2% of small molecule inhibitors are able to penetrate into the CNS (Meijer and Raymond, 2003; Pardridge, 2005). One structurally varied compound has been DRF 53 with a 2-pyridyl residue attached to the 4-position of the benzylamine substituent. With a similar cdk inhibition profile the toxic properties increased compared to roscovitine so that it is suggested to be used as anticancer agent like roscovitine (Bettayeb et al., 2008). In a recent paper the 2-pyridyl residue has been changed by both a 3- and a 4-pyridyl residue (Demange et al., 2013). More favourable cdk5 inhibition data resulted in nanomolar ranges. Docking studies in the ATP binding pocket demonstrated a hydrogen bonding of the 4-pyridyl nitrogen to the Nε of Lys89 of the protein backbone to explain the increased activity. However, beside cdk5 also cdk2 was inhibited and all derivatives were toxic (Demange et al., 2013).

P38 protein kinase belonging to the mitogen activated protein kinases (MAPKs) is a tau protein phosphorylating kinase which contributes to tau protein dissociation from the microtubules and facilitates a further tau aggregation (Martin et al., 2013). In AD brains Aβ activates p38 isoforms p38α und p38β via activated glia (Rojo et al., 2008). In the following proinflammatory cytokines are produced like interleukin-1β (IL-1β) and tumor necrosis factor α (TNFα) (Shaftel et al., 2008). So nanomolar active p38 inhibitor SB-239063 is able to reduce the inflammatory cytokine production (Underwood et al., 2000).

Erk isoforms 1 and 2 contribute to abnormal tau protein phosphorylation including the formation of pathological tau conformations (Pei et al., 2002; Martin et al., 2013). So the inhibition of Erk promises a reduced tau pathology. FR-180204 is the sole selective Erk inhibitor described so far (Ohori et al., 2005).

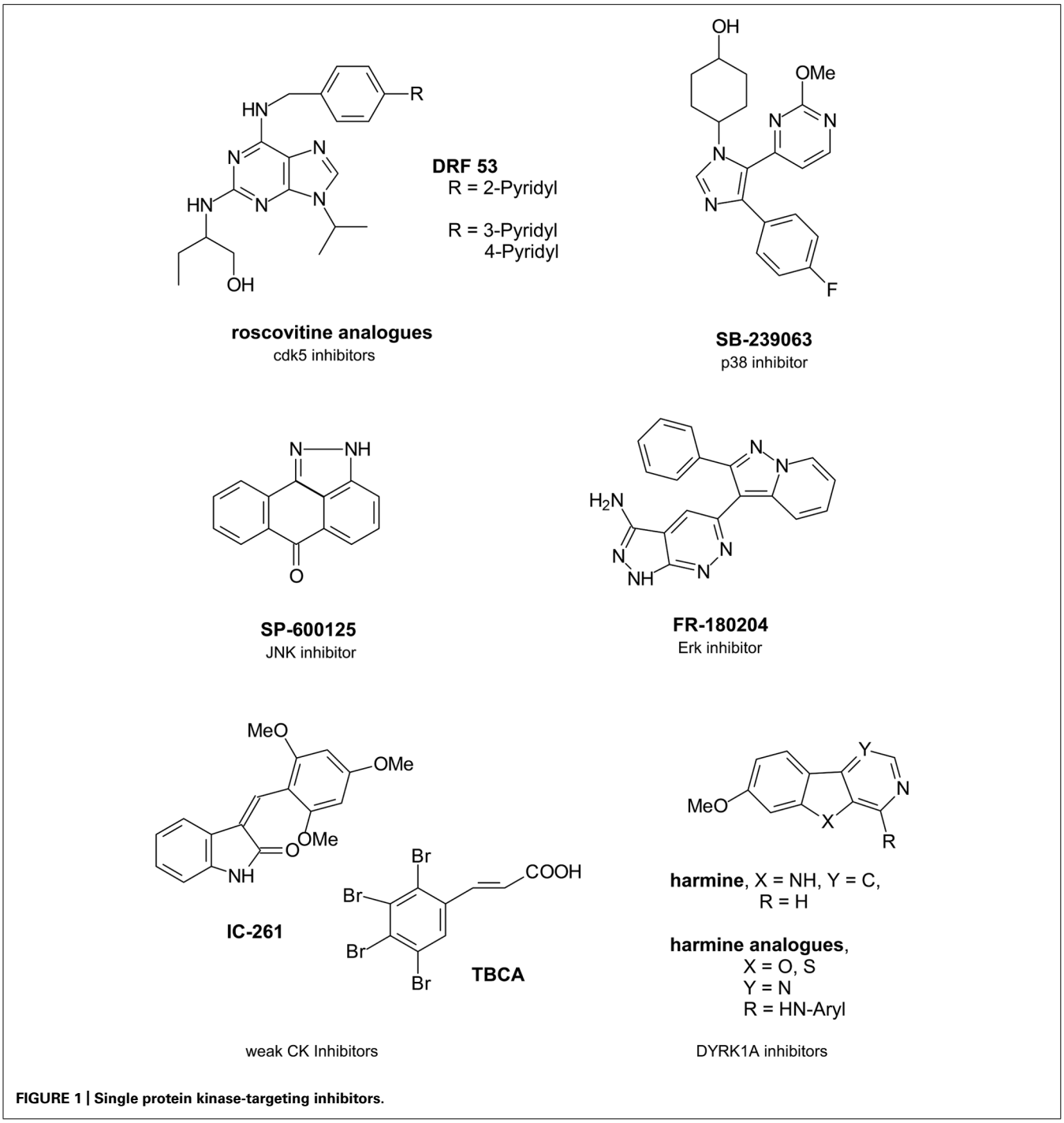


FIGURE 1 | Single protein kinase-targeting inhibitors.

Finally, JNKs of the MAPK family have been interesting target structures because they also contribute to tau phosphorylation. JNK activation is mediated by toxic Aβ fragments (Philpott and Facci, 2008). However, activated JNKs also increase the γ-secretase activity and thus contribute to increased toxic Aβ levels resulting from the amyloid precursor protein (APP) by γ-secretase cleavage (Shen et al., 2008). Both MAPKs p38 and JNK have been co-purified with NFTs in AD brains (Zhu et al., 2000). So JNKs are potential AD-therapeutic target structures. SP-600125 is a potent JNK isoform inhibitor (Bennett et al., 2001). However, JNK3 is the known isoform which mediates the toxic response to Aβ with a greater role in the regulation of toxicity in brain than the other isoforms JNK1/2 (Philpott and Facci, 2008). So it would be of benefit to develop a more exclusive JNK3 inhibitor.

Casein kinase isoforms CK1δ and CK1ε are expressed in brain and are mainly involved in the pathophysiological tau phosphorylation with 26% of those tau amino acids being phophorylatated by CK1 (Knippschild et al., 2005; Martin et al., 2013). CK1δ mRNA

levels as well as the kinase itself are found mainly increased in AD brains up to 30-fold (Yasojima et al., 2000). CK1 has been found co-localized with NFTs like GSK3β being active in the tau phosphorylation of NFTs (Kuret et al., 1997; Schwab et al., 2000). Moreover, CK phosphorylation of tau sites facilitates the dissociation from the microtubules (Martin et al., 2013). Similar to GSK3β Aβ peptides activate CK thus triggering the tau pathology which on the other hand increases Aβ (Takashima et al., 1996; Flajolet et al., 2007). Furthermore, CK1ε is known to play a role in the APP processing, likely by regulating the activity of γ-secretase which contributes to the formation of the toxic Aβ peptides. CK is additionally a priming kinase for GSK3β and has a regulatory role in cdk5 function (Knippschild et al., 2005).

Thus, CK is an interesting target structure for AD relevant protein kinase inhibitors, because the AD overactivity of the kinase may be regulated by an inhibitor without effecting the basic activity in cells. The presently known CK inhibitors IC-261 and tetrabromocinnamic acid (TBCA) are poor inhibitors with activities in partly higher micromolar ranges (Mashhoon et al., 2000; Pagano et al., 2007).

Dual-specificity tyrosine phosphorylation-regulating kinase 1A (DYRK1A) phosphorylates tau and transcription factor cAMP Response Element Binding (CREB) which is involved in learning and memory (Yang et al., 2001). Aβ peptides increase DYRK1A mRNA levels in AD brains (Kimura et al., 2007). However, tau phosphorylation of DYRK1A is reported to be triggered by GSK3β (Kimura et al., 2007). Interestingly, DYRK1A phosphorylates serine 202 of tau protein and this phosphorylation induces a conformational change of tau which is pathological (Martin et al., 2013). Thus, DYRK1A emerged to an interesting protein kinase for a tau-directed therapy. Harmine is an early DYRK1A inhibitor which inhibits not only the enzyme itself but also its tyrosine autophosphorylation (Seifert et al., 2008). While harmine is a promising inhibitor with activities in nanomolar ranges, recent structural changes in the molecular skeleton by replacing the indole nitrogen by an oxygen or a sulfur atom and by replacing the annelated pyridine in the β-carboline scaffold by a pyrimidine led to decreases in activity (Loidreau et al., 2013). Moreover, it remains of doubt whether DYRK1A is a favourable target kinase because an inhibition would have negative consequences for the CREB phosphorylation. This phosphorylation is necessary for learning and memory processes which are both impaired in AD patients.

MULTITARGETING

One GSK3β inhibitor named tideglusib (NP-12) with a thiadiazolidinone scaffold presently undergoes phase 2 of clinical trials (**Figure 2**).

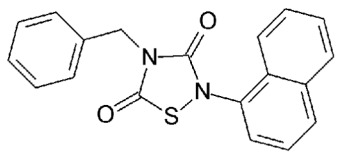
In contrast to the other GSK3β inhibitors which are competitive inhibitors of ATP in the ATP-binding pocket NP-12 is a non-competitive inhibitor which was reported to reduce tau phosphorylation and amyloid depositions in brain and prevent neuronal death and cognitive deficits in animal models (http://clinicaltrials.gov/ct2/results?/term = Noscira, 2010). Moreover, NP-12 was identified as nuclear receptor PPARγ agonist which mediated effective anti-inflammatory and neuroprotective properties (Luna-Medina et al., 2007).

In recent studies two inhibitors of the MAPK family have been investigated in their effects in Aβ-injected rats (Ashabi et al., 2012). U0126 is an inhibitor of MEK1 and 2 which regulates the activity of Erk1 and 2, while PD169316 is a p38 inhibitor. Aβ activates MAPKs p38, Erk, and JNK. This activation causes a mitochondrial dysfunction by disturbance of the mitochondrial biogenesis. In early states of AD this mitochondrial abnormality is responsible for brain energy depletions. Aβ causes deficits in learning, memory and cognition as demonstrated by the behavioral changes of the rats after Aβ-treatment. The inhibitor applications led to increased levels of c-fos as activated gene which contributes to long-term memory processes. Moreover, the levels of CREB increased. The transcription factor is not only involved in early processes of long-term memory as discussed but also regulates the expression of factors PGC-1α and of NRF-1 (Kudo et al., 2005; Vercauteren et al., 2006). Both factors play a role in the biogenesis of mitochondria which are dysregulated in pathological settings like AD (Vercauteren et al., 2006). So beside the role of MAPK inhibitors in a potential tau phosphorylation and tau pathology as discussed both inhibitors may show benefit for an improvement of memory and mitochondrial impairment and thus play a neuroprotective role against Aβ-mediated deficits.

Another MAPK family inhibitor MW01-2-069A of the protein kinase p38α further profiled in Aβ-related studies and by molecular properties (Munoz et al., 2007). The selectivity in binding to the p38 isoform α could be reasoned with the favourable binding of the 4-pyridyl-residue in the ATP binding pocket which allows hydrogen bonding to the amide function of Gly110 of the protein backbone. Furthermore, the phenyl residue finds an optimized location in the neighbored hydrophobic binding pocket. P38α inhibitors have been discussed above to reduce the production of proinflammatory cytokines (Kim et al., 2004). MW01-2-069A was demonstrated to attenuate Aβ-mediated decreases of synaptophysin as a presynaptic protein. The downregulation of synaptophysin causes synaptic dysfunctions in the hippocampus and following hippocampal behavioral deficits. Favorably, the compound passes the blood brain barrier and proved to be metabolically stable to an extent of 70% (Munoz et al., 2007). However, the inhibitory activity in submicromolar ranges has to be considered critically with respect to later clinical studies.

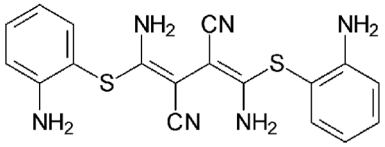
The protein kinase Rock has been demonstrated to be activated in neurites surrounded by amyloid deposites (Petratos et al., 2008). The following outgrowth of the neurites could be protected by Rock inhibitor Y-27632. Moreover, an amyloid-induced loss of synapses could be retracted, so that novel synapse formation were observed. Furthermore the Rock inhibitor interfered with the APP processing by an increase of the α-secretase activity leading to less toxic Aβ (Quin et al., 2006).

1-Aza-9-oxafluorenes discovered as moderate cdk1 inhibitors first have been further developed as selective GSK3β inhibitors with a 3-carbonyl amide function at the 3-position of the molecular skeleton (Voigt et al., 2008) and a loss of the cdk1 inhibition. While the pyridine nitrogen atom of the 3-carbonyl derivatives showed optimized hydrogen bonding to the NH function of amino acid Val135 of the protein backbone, the NH amide function bound to carbonyl amide function of Thr138 via a water



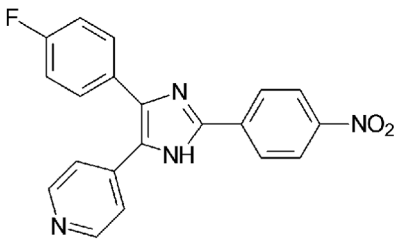
tideglusib (NP-12)

GSK3β inhibitor
PPARγ agonist
- antiinflammatoric
- neuroprotective



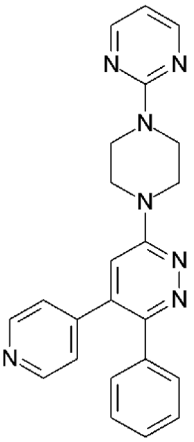
U0126

MEK1,2 inhibitor
neuroprotective



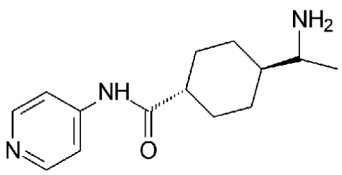
PD169316

p38 inhibitor
neuroprotective



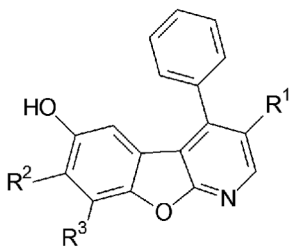
MW01-2-069A

p38α inhibitor
- antiinflammatoric
- neuroprotective



Y-27632

Rock inhibitor
- neurogenerative
- neuroprotective



R^1 = OH, OBn
R^2 = R^3 = H
R^2 = -$(CH{=}CH)_2$-R^3

multikinase inhibitor

FIGURE 2 | Multitargeting inhibitors.

molecule. Further variations at the molecular scaffold led to the benzo-annelated lead compounds which further profiled as cdk1, gsk3β and cdk5 inhibitors (Tell et al., 2012a). The binding mode of those benzo-annelated naphtho compounds to the protein kinase backbone was demonstrated to be inverse compared to the early 1-aza-9-oxafluorenes with an orientation of the 3-substituent within the hydrophobic binding pocket nearby the gatekeeper amino acids (Voigt et al., 2008; Tell et al., 2012a). One nanomolar active 3-hydroxy compound showed exclusive cdk1, gsk3β and cdk5 inhibition properties and proved to be non-toxic in various cellular assays (Tell et al., 2012b). It effectively inhibited tau phosphorylation of various tau amino acids and has been suggested for further tau pathology studies in tau mice models.

CHALLENGES

During the last decades of AD research it has become more obvious that AD is not only a multifactorial disease with various pathological events which contribute to the diseases progression but also a cross-linked disease. Aβ toxicity has been demonstrated to increase tau pathology. The linker has been GSK3β which itself is known to increase the production of Aβ. GSK3β has been known to be primed by cdk5 so that the inhibition of cdk5 reduces Aβ toxicity. Synaptic dysfunctions are not only mediated by a decrease of acetylcholine, but also by Aβ-induced increases of presynaptic proteins. Moreover, Aβ mediates inflammatory brain processes via protein kinases.

The low benefit of drugs which target one single target structure enforced studies for a potential multitargeting. The early strategy in this field concentrated on the combination of differently acting drugs by linkers like alkyl chains. The present outcome of these studies is poor. However, combining of two drugs by a linker leads to new molecules with changed molecular properties. Resulting enlarged molecular weights influence resorption processes and brain entry properties. It will be a challenge to develop a favourable combined drug for AD therapy.

Protein kinases have been identified as mainly disease influencing target structures. Their central role in tau pathology by tau phosphorylation is extended to disease-linking processes and makes them to most interesting target structures.

Limits in the development of inhibitors of such AD-relevant protein kinases have been pointed out: the brain entry of such small molecules, toxic cellular effects which accompany also recent inhibitors and highly active molecules. The latter aspect became obvious by recent studies with GSK3β inhibitors. None of the highly active inhibitors reached clinical trials. A micromolar inhibitor seems to be more promising.

What can be the conclusion from all these points? It will be of great interest to develop a drug which itself has such multitargeting properties. As protein kinases are promising target structures further developments shall concentrate on a multitargeting protein kinase inhibitor which has to be profiled in the various ways like brain entry abilities, low cellular toxicity and finally a sufficient but not a too strong activity just to regulate the protein kinases' overactivities and not to interfere with their activities in normal cells. 1-Aza-9-oxafluorenes from a really perspective compound class on this way of a hopeful AD drug development.

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Insulin dysfunction and Tau pathology

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The neuropathological hallmarks of Alzheimer's disease (AD) include senile plaques of β-amyloid (Aβ) peptides (a cleavage product of the Amyloid Precursor Protein, or APP) and neurofibrillary tangles (NFT) of hyperphosphorylated Tau protein assembled in paired helical filaments (PHF). NFT pathology is important since it correlates with the degree of cognitive impairment in AD. Only a small proportion of AD is due to genetic variants, whereas the large majority of cases (∼99%) is late onset and sporadic in origin. The cause of sporadic AD is likely to be multifactorial, with external factors interacting with biological or genetic susceptibilities to accelerate the manifestation of the disease. Insulin dysfunction, manifested by diabetes mellitus (DM) might be such factor, as there is extensive data from epidemiological studies suggesting that DM is associated with an increased relative risk for AD. Type 1 diabetes (T1DM) and type 2 diabetes (T2DM) are known to affect multiple cognitive functions in patients. In this context, understanding the effects of diabetes on Tau pathogenesis is important since Tau pathology show a strong relationship to dementia in AD, and to memory loss in normal aging and mild cognitive impairment. Here, we reviewed preclinical studies that link insulin dysfunction to Tau protein pathogenesis, one of the major pathological hallmarks of AD. We found more than 30 studies reporting Tau phosphorylation in a mouse or rat model of insulin dysfunction. We also payed attention to potential sources of artifacts, such as hypothermia and anesthesia, that were demonstrated to results in Tau hyperphosphorylation and could major confounding experimental factors. We found that very few studies reported the temperature of the animals, and only a handful did not use anesthesia. Overall, most published studies showed that insulin dysfunction can promote Tau hyperphosphorylation and pathology, both directly and indirectly, through hypothermia.

Keywords: Alzheimer's disease, diabetes mellitus, Tau phosphorylation, kinases, phosphatases

INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by a progressive loss of memory and a decline of cognitive functions. With aging population as a main risk factor (Harman, 2002), this pathology becomes one of the most frequent neurodegenerative diseases and one of the top public health economic concerns since more than 35 million people worldwide were affected in 2010 (Querfurth and Laferla, 2010).

Amyloid plaques and neurofibrillary tangles (NFT) are the two histopathological hallmarks of AD. Amyloid plaques are composed of extracellular aggregates of the β-amyloid peptide (Aβ) (Glenner and Wong, 1984; Selkoe, 2001), while NFT are composed of abnormally phosphorylated Tau protein, assembled into paired helical filaments (PHF) (Brion et al., 1985; Grundke-Iqbal et al., 1986; Buée et al., 2000).

Only rare cases of early onset Familial Alzheimer's Disease (FAD) are caused by mutations in the amyloid precursor protein (APP) or presenilin genes. By contrast, the etiology of the vast majority of cases, which are sporadic late onset AD (LOAD), is still misunderstood due to its multifactorial components involving both genetic and environmental factors. Two other common syndromes in the elderly are associated with aging, i.e., diabetes mellitus (DM) and impaired glucose tolerance. It is estimated that, in 2030, the number of diabetic people over 64 years old will exceed 82 million cases in developing countries, and 48 million cases in developed countries (Wild et al., 2004).

To date, there is increasing evidence supporting a link between AD and insulin dysfunction (Frolich et al., 1999; Gasparini et al., 2002; Craft and Watson, 2004; De La Monte et al., 2009; Sims-Robinson et al., 2010). For example, compared to age-matched controls, AD brains show low concentrations of insulin accompanied by an increase in the number of insulin receptors (Frolich et al., 1999; Craft and Watson, 2004). Moreover, numerous population-based studies have examined the association between AD and insulin dysfunction. Some studies found no association (Arvanitakis et al., 2006; Thambisetty et al., 2013); while, interestingly, others suggest that hyperglycemia, impaired insulin secretion, glucose intolerance and insulin resistance are all associated with increased risk of AD (Luchsinger et al., 2004; Ronnemaa et al., 2008; Crane et al., 2013a). Thus, higher AD incidences rates were detected in elderly diabetic patients (Leibson et al., 1997; Ott et al., 1999; Luchsinger et al., 2004; Xu et al., 2004; Ronnemaa et al., 2008), and reduced mental skills have been observed in diabetic children (Northam et al., 2001; Schoenle et al., 2002; Fox

et al., 2003; Dahlquist and Kallen, 2007; and for review, see Roriz-Filho et al., 2009). All this evidence has prompted the hypothesis that AD might be a form of brain diabetes (Hoyer, 1998; De La Monte et al., 2006).

In this article, we will review both *in vitro* and animal findings reporting a correlation between diabetes and Tau pathology, one of the major neuropathological hallmarks of AD. We will also focus on the mechanisms that might link insulin dysfunction to Tau pathology.

TAU PROTEIN

STRUCTURE AND FUNCTION

Tau is a microtubule-associated protein that is abundant in the central nervous system (CNS) and expressed mainly in axons. In the human brain, Tau proteins constitute a family of six isoforms, ranging from 352 to 441 amino acids, all derived from single gene by alternative splicing (Goedert et al., 1989). The amino-terminal region of Tau, also called the "projection domain," project from the microtubule (MT) space to interact with the plasma membrane and is essential for determining axonal diameter (Chen et al., 1992). The carboxy-terminal region is characterized by the presence of 3 or 4 repeats that mediate the properties of Tau to stabilize MT and promote their polymerization (Weingarten et al., 1975; Cleveland et al., 1977a,b; Himmler et al., 1989; Butner and Kirschner, 1991; Gustke et al., 1994). These functions are negatively regulated by phosphorylation at multiple sites in, and around, the MT binding domain (for review, see Buée et al., 2000; Avila et al., 2004). Indeed, at least 30 Ser/Thr potential Tau phosphorylation sites have been described on Tau (Sergeant et al., 2008). Tau phosphorylation is regulated by numerous Ser/Thr kinases and phosphatases; among them glycogen synthase kinase-3β (GSK-3β) and protein phosphatase 2A (PP2A) are considered to be the major Tau kinase and phosphatase *in vivo*, respectively (Planel et al., 2002; Tian and Wang, 2002).

Intracellular aggregates of abnormally hyperphosphorylated Tau characterize a group of neurodegenerative diseases called "Tauopathies" (for review, see Buée et al., 2000). Tau hyperphosphorylation may lead to its aggregation *in vitro* (Alonso et al., 2001; Sato et al., 2002) and is thought to induce NFT formation and neurodegeneration in AD brains (Trojanowski and Lee, 1994).

INTERACTION BETWEEN TAU AND AMYLOID PATHOLOGY

The second major neuropathological hallmark of AD are amyloid plaques, mainly composed of amyloid-β (Aβ), a peptide of 39–43 amino acids generated *in vivo* by specific proteolytic cleavage of the APP, a transmembrane glycoprotein (Hardy and Higgins, 1992). It appears that soluble oligomeric Aβ forms, rather than amyloid plaques, contribute to the cellular pathology of the AD and correlate with the severity of cognitive impairment in humans (Lue et al., 1999; and for review, see Laferla et al., 2007).

To date, the interaction between Tau pathology and amyloid plaques in AD remains unresolved. Several murine animal models combining the two types of lesions have been investigated to better address this issue.

However, results have shown different possible types of interaction between the two pathologies. Thus, it was reported that injection of Aβ42 peptide potentiates Tau pathology in a mouse model of Tauopathy overexpressing a mutant form of the protein (Gotz et al., 2001). Similarly, double transgenic APP/Tau mice show early Tau pathology in comparison to Tau transgenic mice (Lewis et al., 2001; Perez et al., 2005; Ribe et al., 2005; Terwel et al., 2008). Interestingly, cultured neurons obtained from $Tau^{-/-}$ mice seem to be protected against neuronal death and cytotoxicity induced by Aβ, suggesting that Tau is crucial for Aβ-induced neurotoxicity (Rapoport et al., 2002). These results were further supported *in vivo*, in animal studies combining both $Tau^{-/-}$ and APP transgenic mice and showing that Tau reduction is protective against neurological and behavioral deficits induced by Aβ (Palop et al., 2006; Roberson et al., 2007). More importantly, this hypothesis is further supported by numerous *post-mortem* neuropathological studies showing that the pathway of Tau pathology is progressive, sequential and hierarchical (Bierer et al., 1995; Braak et al., 1999; Delacourte and Buée, 2000), whereas amyloid deposition is diffuse, widespread and extremely heterogeneous (Braak et al., 1999; Delacourte et al., 1999). Moreover, NFT formation was found to be strongly correlated with the degree of dementia and memory loss in AD brains, whereas Aβ was not (Braak and Braak, 1991, 1997; Delacourte et al., 1999). These observations have led some authors to the hypothesis that Tau pathology could be the major cause of cognitive decline in humans (Wilcock and Esiri, 1982; Delaere et al., 1989; Arriagada et al., 1992; Duyckaerts et al., 1997, 1998; Gomez-Isla et al., 1997; Delacourte et al., 2002; Giannakopoulos et al., 2003; Guillozet et al., 2003; Bretteville and Planel, 2008). These findings are in accordance with numerous animal studies showing significant impairment of cognitive functions, synaptic dysfunctions, as well as altered hippocampal synaptic plasticity in different transgenic mouse models of Tauopathies (Polydoro et al., 2009; Hoover et al., 2010; Van Der Jeugd et al., 2011; Burnouf et al., 2013).

INSULIN DYSFUNCTION

BASICS OF INSULIN SIGNALING

The insulin receptor (IR) is a hetero-tetrameric receptor composed of two extracellular α-subunits that bind insulin, and two transmembrane β-subunits that have intracellular tyrosine kinase activity. The binding of insulin to the α-subunits of IR leads to the activation of the β subunit via auto-phosphorylation. When activated, IR phosphorylates insulin receptor substrate (IRS) proteins, which then activate phosphoinositide-3 kinase (PI3K). Two major signaling pathways are further activated by PI3K: the mitogen-activated protein kinase (MAPK) pathway and the Akt pathway (for review, see White, 1997; Taha and Klip, 1999). MAPK pathway is mainly involved in cell differentiation, cell proliferation and cell death, whereas Akt signaling is implicated in cell proliferation, cell growth, and protein synthesis (for review, see Brazil and Hemmings, 2001; Le Roith and Zick, 2001; Tremblay and Giguere, 2008). Akt further induces the phosphorylation of GSK-3β, rendering it inactive, which results in increase of glycogen synthesis in the periphery. Thus, disruption of Akt

signaling leads to the dephosphorylation of GSK-3β, and hence to its activation; (for review, see Taha and Klip, 1999).

In insulin resistance that occurs in T2DM, the capacity of insulin to induce all these phosphorylation cascades is significantly decreased. Muscle biopsies of insulin resistant subjects revealed a reduction in the enzymatic activity and phosphorylation state of kinases and substrates involved in the IR signaling pathway (for review, see Schulingkamp et al., 2000; White, 2003; Pirola et al., 2004; Youngren, 2007). However, the site(s) of the initial perturbation of signal transduction are far from clear.

INSULIN IN THE CNS

In the past, the brain was described as "an insulin insensitive organ" (Goodner and Berrie, 1977). However, it is currently well known that insulin and its receptors are widely present in the CNS (Baskin et al., 1988; Lerorrtt et al., 1988; for review, see Schulingkamp et al., 2000). Although the origin of brain insulin is still a matter of debate, several evidence suggest that central insulin is actively transported from periphery across the blood-brain barrier (BBB) (Banks et al., 1997a,b), and might be also produced locally by neurons in the brain (Devaskar et al., 1994).

The initial evidence of *de novo* insulin synthesis in the CNS came from a report showing insulin immunoreactivity in adult rat brain (Havrankova et al., 1978b). This evidence was later confirmed by other studies showing insulin gene expression and synthesis in cultured mammalian neurons (Schechter et al., 1988, 1990; Devaskar et al., 1994).

Interestingly, it was shown that high peripheral insulin concentrations acutely increase the levels of insulin in the brain and the cerebrospinal fluid (CSF), whereas prolonged peripheral hyperinsulinemia leads to a down-regulation of IRs in the BBB and to a reduction in insulin transport into the brain (Wallum et al., 1987).

IRs are selectively distributed in the brain. Thus, IRs mRNA levels are highly concentrated in several brain areas including the olfactory bulb, pyriform cortex, amygdala, hippocampus, hypothalamus and the cerebellar cortex (Marks et al., 1991; Zhao et al., 1999). Accordingly, immunohistochemistry studies revealed that IRs are highly expressed in the olfactory bulb, hypothalamus, cerebral cortex, amygdala and hippocampus (Havrankova et al., 1978a,b; Baskin et al., 1987; Marks and Eastman, 1990; Marks et al., 1991; Unger et al., 1991; Zhao et al., 1999). Differences between peripheral and central IRs are not well understood, although some structural differences, mainly including the molecular weight and antigenicity, have been noted (Heidenreich et al., 1983; Heidenreich and Gilmore, 1985).

The expression of IRs in specific brain regions, particularly the hippocampus and medial temporal cortex suggests that insulin is implicated in memory processes (Singh et al., 1997; Zhao et al., 1999; Duarte et al., 2012; De Felice, 2013). For example, it was reported that both mRNA and protein levels of IRs are increased in the rat brain following a spatial memory task, suggesting that insulin might regulate normal memory function (Zhao et al., 1999).

By contrast, several findings have showed that insulin dysfunction, e.g., chronic hyperinsulinemia, DM or insulin resistance have a negative impact on memory process and cognitive function (Roriz-Filho et al., 2009; Sims-Robinson et al., 2010; Benedict et al., 2012; Crane et al., 2013b).

Mechanisms by which insulin might influence memory are reviewed elsewhere (Zhao and Alkon, 2001; Craft and Watson, 2004; Gerozissis, 2008; Freiherr et al., 2013). In brief, insulin signaling was shown to regulate neuronal survival, neurotransmission and synaptic activities (Zhao and Alkon, 2001). Insulin might also regulate synaptic plasticity by modulating long-term potentiation (LTP) (Zhao et al., 2010; Nistico et al., 2012), and promoting long-term depression (LTD) (Van Der Heide et al., 2005; Labouèbe et al., 2013), two major mechanisms involved in learning and memory.

In this context, it was demonstrated that treatment with insulin sensitizers (e.g., liragultide, pioglitazone) ameliorates central insulin resistance (Han et al., 2013; Hu et al., 2013; Yang et al., 2013), improves cognitive impairment (Han et al., 2013), enhances synaptic plasticity (Han et al., 2013), and significantly decreases levels of Tau phosphorylation in the rat brain (Hu et al., 2013; Yang et al., 2013).

Interestingly, it was recently established that administration of intranasal insulin is a non-invasive technique that leads to an increase of insulin levels specifically in the CNS, without affecting peripheral glucose and insulin levels (Reger et al., 2008a). Thus, several clinical studies have demonstrated a beneficial effect of intranasal insulin on memory and cognitive function in AD patients (Reger et al., 2008a,b; Craft et al., 2012).

Therefore, although emerging evidence support a role of brain insulin signaling in several complex CNS functions, additional mechanistic studies are warranted to better elucidate the contribution of insulin signaling in normal and diseased brains.

DIABETES MELLITUS

Diabetes mellitus (DM) is a chronic disease, marked by high levels of blood glucose. It occurs in two main forms: type 1 and type 2 diabetes, that respectively represent about 5–10% and 90–95%, of the total diabetes cases (CDC, 2008).

Type 1 diabetes mellitus

Type 1 diabetes mellitus (T1DM) was previously known as Insulin-dependent DM (IDDM) or juvenile diabetes to distinguish it from type 2 diabetes mellitus (T2DM), which generally has a later onset; however, the majority of new-onset type 1 diabetes is seen in adults (Naik and Palmer, 2007).

It is due to autoimmune destruction of the insulin-producing β-cells resulting in low or no production of insulin hormone. Thus, insulin therapy is absolutely required in patients with T1DM (McCrimmon and Sherwin, 2010). However, one major feared side effect of insulin therapy is iatrogenic hypoglycemia, which might occur in almost 90% of all insulin treated patients (Cryer, 1997, 2001, 2004; Cryer et al., 2003).

Type 2 diabetes mellitus

Type 2 diabetes mellitus (T2DM), previously known as non-insulin dependent DM (NIDDM), was usually associated with aging. About 40% of the population over 65 and 50% over 80 years of age have T2DM or impaired glucose tolerance, and half of diabetic people are not diagnosed (Lamberts et al., 1997).

However, T2DM is not a disease exclusively associated with old age, as it is more and more diagnosed in young people as the number of cases has risen in parallel with obesity. Today, there are 382 million people with T2DM, and it is estimated that this number will rise to 592 million by 2035 (IDF, 2013). In T2DM, pancreas produces insulin, but, for reasons that remain unclear, cells are unable to use it, a characteristic of insulin resistance etiology. Thus, in a positive feedback loop, pancreas overproduces insulin to overcome the resistance, leading to a hyperinsulinemia in early phases of T2DM (Festa et al., 2006). Over time, the suffering pancreas becomes unable to produce enough insulin, leading to hyperglycemia and insulin deficiency that accompany T2DM in the end of its natural history (Defronzo, 2004).

IMPACT OF INSULIN DYSFUNCTION ON TAU PHOSPHORYLATION

Over the last decade, there has been considerable interest on the impact of insulin dysfunction and diabetes on Tau pathology. It has been shown that insulin could regulate Tau phosphorylation in neuronal cells (Hong and Lee, 1997; Lesort et al., 1999; Lesort and Johnson, 2000), which was confirmed by observations of hyperphosphorylated Tau in mice showing abnormal insulin levels in the brain (Schubert et al., 2003, 2004; Planel et al., 2004; Schechter et al., 2005). Despite all these data, very little is known about the effects of diabetes on Tau pathogenesis *in vivo*.

IN VITRO STUDIES

Observations from *in vitro* studies strongly suggest that Tau phosphorylation might be regulated by insulin signaling through a biphasic manner. Thus, short insulin treatment (<2–3 min), either in rat primary cortical neurons (Lesort and Johnson, 2000), or in SH-SY5Y human neuroblastoma cells (Lesort et al., 1999), leads to a rapid and transient Tau hyperphosphorylation at the AT8 (Ser^{202}/Thr^{205}), AT180 (Thr^{231}), PHF-1 (Ser^{396}/Ser^{404}) and T3P (Ser^{396}) epitopes (Lesort et al., 1999; Lesort and Johnson, 2000). By contrast, prolonged exposure to insulin, in both human neuroblastoma cells (up to 60 min of treatment) (Lesort et al., 1999), and human neuronal NT2N cells (5 min of treatment) (Hong and Lee, 1997), results in a significant decrease in Tau phosphorylation. Interestingly, all these studies have led to the conclusion that the effects of insulin are mediated by GSK-3β activity (Hong and Lee, 1997; Lesort et al., 1999; Lesort and Johnson, 2000).

Therefore, insulin signaling plays an important role in the regulation of Tau phosphorylation, and this effect is correlated with GSK-3β activity, further suggesting a key role of this kinase in Tau pathology.

IN VIVO STUDIES

The specific effects of insulin dysfunction on Tau pathology are not fully understood. Therefore, several animal models are currently used to investigate the impact of both T1DM and T2DM on Tau phosphorylation.

T1DM and Tau phosphorylation

Models of T1DM used in the literature mainly mimic insulin deficiency that characterizes human T1DM. These models can develop T1DM either spontaneously, due to a specific genetic background, or by treatment with a drug called Streptozotocin (STZ).

STZ (2-deoxy-2-(3-(methyl-3-nitrosoureido)-D-glucopyr-anose)) is a toxin synthesized by *Streptomycetes achromogenes*, that specifically enters pancreatic β cells via the glucose transporter GLUT2, and leads to cell death through DNA alkylation (Delaney et al., 1995). Thus, it is widely used to induce insulin deficiency associated to T1DM (Ganda et al., 1976), both in wild-type background context and AD pathological animal models.

STZ-induced wild-type animal models. Using an extensive panel of phospho-dependent antibodies that detect specific epitopes on Tau protein, several animal studies have investigated the impact of peripheral STZ treatment on Tau phosphorylation in non-transgenic animals (**Table 1**). For example, we have previously reported that STZ administration (200 mg/Kg, i.p.) resulted in a massive Tau hyperphosphorylation at the Tau-1 (Ser^{195}/Ser^{198}/Ser^{199}/Ser^{202}), pS199 (Ser^{199}), PHF-1, AT8, AT180, Ser^{422}, Ser^{262}, Ser^{356} and Ser^{400}epitopes in the brain of non-transgenic mice (Planel et al., 2007b). Other studies have confirmed our results of increased Tau phosphorylation following STZ treatment either in mice (Clodfelder-Miller et al., 2006; Jolivalt et al., 2008; Ke et al., 2009; Kim et al., 2009), or in rats (Qu et al., 2011), and observed a further increase in Tau phosphorylation at the AT270 (Thr^{181}) and Thr^{212} epitopes (**Table 1**).

Some of the phosphoepitopes detected are associated to particular functions during Tau pathology. For example, Ser^{422} is associated with early pre-tangle formation and is characteristic of abnormal, AD-like Tau phosphorylation (Augustinack et al., 2002). AT8 is considered an early marker of Tau dysfunction (Matsuo et al., 1994; Hasegawa et al., 1996; Bussière et al., 1999; Augustinack et al., 2002), whereas PHF-1 is associated with late paired helical filament and NFT formation (Bramblett et al., 1993; Goedert et al., 1993, 1994). In addition, some phosphorylation sites have been linked to specific aspects of Tau pathology such as the sequestration of normal Tau (e.g., Thr^{231}/Ser^{235}) (Alonso et al., 1997, 2004), the inhibition of Tau MT binding (e.g., Ser^{262}) (Biernat et al., 1993; Drewes et al., 1995), and the promotion of Tau aggregation (e.g., Ser^{396}, Ser^{422}) (Gong and Iqbal, 2008).

Interestingly, we have shown that the massive Tau hyperphosphorylation in STZ treated mice was mostly, but not completely, rescued by returning animals to normothermia, suggesting that hypothermia was partially involved in STZ-mediated Tau hyperphosphorylation. We have previously demonstrated that Tau phosphorylation can be induced by hypothermia (Planel et al., 2004, 2007b). Moreover, we have shown that this mechanism is mainly attributable to a direct and rapid inhibition of PP2A activity in the brain (Planel et al., 2004).

In fact, hypothermia is a common outcome in experimental diabetes (Shalaby et al., 1989; Kilgour and Williams, 1996, 1998) and several human diabetic populations (Neil et al., 1986; Scott et al., 1987).

In addition, Tau phosphorylation is known to be exquisitely sensitive to temperature, increasing by 80% per degree Celsius drop under 37°C in mice (Planel et al., 2007a; Papon et al., 2011).

Table 1 | Summary of animal studies showing Tau hyperphosphorylation and its molecular mechanisms in diabetic animal models.

References	Animal model	Diabetes context	Tau phosphorylation sites	Tau kinases	Tau PP	Insulin signaling	Anesthesia prior to sacrifice	T(°C)
	T1DM-WT	**STZ-induced (i.p.)**						
Clodfelder-Miller et al., 2006	C57BL6/J (M)	150 mg/Kg	AT8, AT180, AT270, PHF-1, 12E8, S199, T212 (+), Tau-1 (−)	pJNK, p38 (+), pS9 GSK3β (+)	PP2A (−)	N.D.	N.R.	No
Planel et al., 2007b	C57BL6/J (M)	200 mg/Kg	AT8, AT180, PHF-1, S199, S422, S262, S356, S400 (+), Tau-1 (−)	pCamKII, pJNK (+), pS9 GSK3β (+)	PP2A (−)	N.D.	No	Yes
Kim et al., 2009	C57BL6/J (M)	150 mg/Kg	T231, S199, 202, 396 (+)	N.D.	N.D.	N.D.	Yes	No
Jolivalt et al., 2008	Swiss-Webster (M)	90 mg/Kg	T231, PHF-1 (+)	pS9 GSK3β (−), pGSK3α (−), pAkt (−)	N.D.	pIR, PDK1 (−)	Yes	No
Qu et al., 2011	Sprague-Dawley (R)	55 mg/Kg	PHF-1 (+), Tau-1 (−)	pS9 GSK3β (−), pAkt (−)	PP2A (−)	N.D.	N.R.	No
	T1DM-Tg	**STZ-induced (i.p.)**						
Jolivalt et al., 2010	hAPP (M)	90 mg/Kg	T231, AT8, PHF-1 (+)	pS9 GSK3β (−), pY216 GSK3β (+)	N.D.	pIR (−)	Yes	No
Ke et al., 2009	pR5 (M)	200 mg/Kg	AT8, AT100, AT270, 12E8, PHF-1, S422 (+)	N.D.	N.D.	N.D.	N.R.	No
	T1DM	**Genetic**						
Li et al., 2007	BB/Wor (R)	Spontaneous	S396 (0)	pS9 GSK3β (−), pAkt (−)	N.D.	IRβ (−)	Yes	No
Papon et al., 2013	NOD (M)	Spontaneous	AT8, CP13, TG3, S422, 262, PHF-1 (+), Tau-1 (−)	pAkt (+), pS9 GSK3β (+), pCamKII, Cdk5, p35 (−)	PP2A, PP2B (−), PP5 (+)	N.D.	No	Yes
Schechter et al., 2005	Insulin$^{-/-}$ (M)	KO	AT180 (+)	pJNK (+), pS9 GSK3β (+), pMAPK (−)	N.D.	N.D.	N.R.	No
	T2DM-WT	**HFD-induced (Kcal%)**						
Moroz et al., 2008	C57BL6/J (M)	60%	N.D.	N.D.	N.D.	IRS-1, IRS-4 ARNm (+)	No	No
Becker et al., 2012	C57BL/ 6NTac (M)	∼25%	AT8, AT180, AT270, S199, 396 (0)	pAkt, pMAPK, pJNK, Cdk5, pS9 GSK3β (0)	PP2A (0)	N.D.	N.R.	No
	T2DM-WT	**HFC-induced**						
Bhat and Thirumangalakudi, 2013	C57BL6/J (M)	21% Fat, 1.25% Cholesterol	PHF-1, T231 (+)	pS9 GSK3β (−), pAkt (−)	N.D.	IRS-1 (−)	Yes	No
	T2DM-Tg	**HFD-induced (Kcal%)**						
Julien et al., 2010	3xTg-AD (M)	60%	PHF1, CP13, AT270 (0)	N.D.	N.D.	N.D.	Yes	No
Leboucher et al., 2012	THY-Tau22 (M)	59%	S202, T205, S214, S404, S422 (+)	pAkt (+), pS9 GSK3β (+)	PP2A (0)	IRS1 (+)	N.R.	No

(Continued)

Table 1 | Continued

Ref.	Animal model	Diabetes context	Tau phosphorylation sites	Tau kinases	Tau PP	Insulin signaling	Anesthesia prior to sacrifice	T(°C)
	T2DM	**Genetic**						
Jung et al., 2013	OLETF (R)	Spontaneous	T212 (+), T231 (+), S262 (+), S396 (+)	pS9 GSK3β (0), Cdk5 (0), CamKII (+)	PP2A (−)	N.D.	Yes	No
Li et al., 2007	BBZDR/ Wor (R)	Spontaneous	S396 (+)	pS9 GSK3β (−), pAkt (−)	N.D.	IRβ (0)	Yes	No
Jung et al., 2011	OLETF (R)	Spontaneous	T212 (+), Tau-1 (−)	pS9 GSK3β, pY216 GSK3β, Cdk5 (0)	PP2A, PP2B (0)	IRβ (+)	Yes	No
Kim et al., 2009	db/db (M)	Spontaneous	AT8, S199, 202, 396, 422 (+)	N.D.	N.D.	N.D.	Yes	No
Li et al., 2012a	db/db (M)	Spontaneous	S396 (+)	pJNK (+)	PP2A (−)	N.D.	Yes	No
Schubert et al., 2004	NIRKO (M)	KO	AT180 (+)	pS9 GSK3β (−), pAkt (−)	N.D.	N.D.	N.R.	No
Schubert et al., 2003	IRS-2$^{-/-}$(M)	KO	AT8 (+)	pS9 GSK3β (+)	PP2A (−)	IRβ, pIR, IRS-1, IRS-2 (−)	N.R.	No
	T3DM-WT	**STZ-induced (i.c.v.)**						
Salkovic-Petrisic et al., 2006	Wistar (R)	1 mg/Kg	N.D.	p-GSK3α/β (+), Akt (−)	N.D.	N.D.	N.R.	No
Grunblatt et al., 2007	Wistar (R)	1 mg/Kg	PHF-1 (+)	N.D.	N.D.	pRI (+), IRβ (−)	N.R.	No
Deng et al., 2009	Wistar (R)	1.5 mg/Kg	PHF-1, S199, 396, T212 (+)	pS9 GSK3β (−), pMAPK (−)	N.D.	pPI3K (−)	No	No
Chu and Qian, 2005	Sprague-Dawley (R)	3 mg/Kg	S202, 396, 404 (+)	N.D.	N.D.	N.D.	N.R.	No
Chen et al., 2011	Wistar (R)	3 mg/Kg	S396, T181 (+)	pS9 GSK3β (−), pY216 GSK3β (+), pAkt (−)	N.D.	N.D.	N.R.	No
Li et al., 2012b	Wistar (R)	3 mg/Kg	AT8 (+)	N.D.	N.D.	N.D.	N.R.	No
Chen et al., 2013a	C57BL6/J (M)	3 mg/Kg	S199/202, T205 (+), S214 (−)	pS9 GSK3β (0), pAkt (0)	N.D.	IRS-1, p-IRS-1, PI3K, p-PI3K (+), p-PDK1 (−)	N.R.	No
Du et al., 2013	Sprague-Dawley (R)	3 mg/Kg	T205 (+), S396 (+), Tau-1 (−)	pS9 GSK3β (0), pMAPK (0), pJNK (0)	p-PP2AC (0)	N.D.	N.R.	No
	T3DM-Tg	**STZ-induced (i.c.v.)**						
Plaschke et al., 2010	Tg2576 (M)	1.25 mg/Kg	AT8 (0)	pGSK3-α/β (−)	N.D.	N.D.	Yes	No
Chen et al., 2013b	3xTg-AD (M)	3 mg/Kg	S199/S202 (+), 12E8 (+), S422 (+)	GSK3β (−), pS9 GSK3β (−), GSK3α (−), pGSK3α (+), Akt (−), pAkt (+)	N.D.	IRβ (+), PI3K (+), PDK-1 (−)	N.R.	No

T1DM: Type 1 diabetes mellitus; T2DM: Type 2 diabetes mellitus; T3DM: Type 3 diabetes mellitus; T(°C), temperature; WT, wild-type; Tg, transgenic; M, mouse; R, rat; STZ, streptozotocine; i.p., intra-peritoneal; i.c.v., intra-cerebroventricular; HFD, high-fat diet; hAPP, human Amyloid precursor protein; pR5, human Tau trangenic P301L (pR5 construct); BB/Wor, BioBreeding/Worcester; NOD, non-obese diabetic; BBZDR/Wor, bio-breeding Zucker diabetic rat/Worcester; OLETF, Otsuka long evans Tokushima fatty; KO, knockout; NIRKO, neuronal insulin receptor knockout; 3xTg-AD, triple transgenic Alzheimer's disease; pJNK, phospho-c-Jun N-terminal kinase; pMAPK, phospho-mitogen-activated protein kinase; GSK-3β, glycogen synthase kinase 3β; pS9 GSK-3β, phospho-Serine 9 GSK-3β, it is an inhibitory phosphorylation; pCamkII, phospho-Ca2+/calmodulin-dependent protein kinase; Cdk5, cyclin-dependent kinase 5; PP, protein phosphatase; PP2AC, catalytic subunit of PP2A; IR, insulin receptor; IRβ, β subunit of IR; PDK1, 3-phospho-inositide-dependent protein kinase-1; IRS, insulin receptor substrate; pPI3K, phospho-phosphatidylinositol 3-kinase; (−), decrease; (+), increase; (0), non-significant, N.D., non-determined; N.R., not reported.

It has to be mentioned that previous studies done in other laboratories described above did not monitor the temperature of animals and therefore it is likely that hypothermia could contribute to the observed Tau hyperphosphorylation in these studies (Clodfelder-Miller et al., 2006; Ke et al., 2009; Kim et al., 2009; Qu et al., 2011).

Therefore, temperature control during Tau analysis experiments in animal models is crucial to avoid artifactual hypothermic Tau hyperphosphorylation.

STZ-induced transgenic animal models. Beside studies performed in a physiological context, several investigators have addressed the impact of STZ treatment on Tau phosphorylation and NFT formation in transgenic mouse models of AD (**Table 1**). For example, Ke et al. have shown that insulin depletion increases more significantly Tau phosphorylation at multiple epitopes in mice that overexpress P301L mutant human Tau, and are prone to develop NFT (Gotz et al., 2001). This suggests that experimental diabetes leads to advanced NFT formation, as well as early neurofibrillar deposition in Tau transgenic mice (Ke et al., 2009). Similarly, insulin deficiency enhances the severity of Tau phosphorylation in the hippocampi of APP transgenic mice, which are characterized by the accumulation of the β-amyloid peptide and high levels of Aβ-immunoreactive plaques (Jolivalt et al., 2010).

Therefore, it seems that STZ-induced T1DM recapitulate NFT formation observed during of AD, and this effect is further worsened when the induction is combined to genetic pre-disposition to AD.

However, it is important to note that several studies from those cited above have used anesthesia prior to sacrifice (Clodfelder-Miller et al., 2006; Jolivalt et al., 2008, 2010; Ke et al., 2009; Kim et al., 2009) (**Table 1**), which is known to enhance Tau phosphorylation (Planel et al., 2007a; Papon et al., 2011; Whittington et al., 2011; Le Freche et al., 2012). This increase can be induced either directly, through a pharmacological effect of the drug itself (Whittington et al., 2011; Le Freche et al., 2012), or indirectly, through anesthesia-induced hypothermia (Planel et al., 2007a).

Thus, further animal studies with temperature control are warranted to separate the impact of hypothermia from that of insulin dysfunction on Tau phosphorylation.

Animal models of spontaneous T1DM. Although several studies have reported Tau hyperphosphorylation in STZ-induced animal models, it is important to extend these findings to a model that does not require a drug to induce T1DM, because, as we will see later, if STZ reaches the brain in minute quantities, it can lead to Tau hyperphosphorylation on its own.

Thus, we have investigated Tau hyperphosphorylation in the non-obese diabetic (NOD) mouse, one of the most valuable genetic animal models for T1DM (Leiter, 2001). These mice spontaneously develop T1DM at 13–25 weeks of age as a consequence of selective destruction of insulin-producing β cells (Makino et al., 1980). Interestingly, our data indicated that Tau hyperphosphorylation correlates with the appearance of spontaneous diabetes in adult NOD mice, and this effect was exacerbated when the mice became hypothermic as a consequence of diabetes. Interestingly, even in the absence of any deregulation in the glucose metabolism, we have observed a slight increase in Tau phosphorylation at Tau-1 and Ser^{422} epitopes in non-diabetic adult NOD mice. Moreover, the onset of diabetes (the appearance of hyperglycemia and glycosuria) was correlated with an extensive Tau hyperphosphorylation at the AT8, CP13 (Ser^{202}), Ser^{262} and Ser^{422} epitopes in comparison to control. Furthermore, the appearance of hypothermia further extended Tau hyperphosphorylation to PHF-1 and TG3 (Thr^{231}) epitopes in diabetic NOD mice (Papon et al., 2013), (**Table 1**).

Of note, Li et al. have also investigated Tau phosphorylation in BB/Wor rats, another model of spontaneous T1DM. Although these authors did not find any significant changes in the levels of Tau phosphorylation at the Ser^{396} epitope (Li et al., 2007), it should be mentioned that there was no report of the rats temperature and that they were anesthetized, which could have masked a potential elevation in Tau phosphorylation.

Thus, while our study demonstrated Tau hyperphosphorylation in NOD mice, additional studies should be done to our results in other animal models of spontaneous T1DM.

The insulin knockout animal model. In addition to genetic and STZ-induced animal models, the insulin knockout mouse model ($Insulin^{-/-}$) might also mimic insulin deficiency that characterizes T1DM. These mice rapidly develop DM, show a dramatic glycosuria, and die within 48 h (Duvillie et al., 1997).

Interestingly, Schechter and colleagues have investigated the impact of insulin deficiency on Tau phosphorylation in $Insulin^{-/-}$ mice. Thus, among all phospho-epitopes investigated, these authors have observed a significant increase in Tau phosphorylation at the AT180 epitope, suggesting that insulin signaling might affect Tau phosphorylation *in vivo* (Schechter et al., 2005), (**Table 1**).

T2DM and Tau phosphorylation

Models of T2DM used in animal studies mainly reproduce human T2DM features, including obesity, hyperglycemia, hyperinsulinemia, and insulin resistance. These models develop T2DM either spontaneously, or by using a variety of treatments including special diets.

Animal models of spontaneous T2DM. Several genetic animal models are used in the literature to address the impact of genetic T2DM on AD pathogenesis (Ellis et al., 1998; Li et al., 2007; Jolivalt et al., 2008; Takeda et al., 2010). These models are mainly characterized by an impairment of leptin signaling, by either lacking leptin (ob/ob mice) (Coleman, 1978), or carrying specific mutations in the leptin receptor (Bio-Breeding Zucker Diabetic Rats/Wor (BBZDR/Wor) rats and db/db mice) (Hummel et al., 1966; Tirabassi et al., 2004). Leptin is an adipocyte-specific hormone that plays important role in satiety and energy expenditure. It acts through the leptin receptor, a single-transmembrane-domain receptor of the cytokine receptor family. However, mechanisms by which leptin signaling leads to the development of T2DM are not well understood.

Several studies have reported Tau hyperphosphorylation in rat (Li et al., 2007; Jung et al., 2011, 2013) and mouse models (Kim et al., 2009; Li et al., 2012a) of spontaneous T2DM. For example,

Jung et al. have reported an increase in Tau phosphorylation at the Tau-1, Thr^{212} (Jung et al., 2011, 2013), Thr^{231}, Ser^{262} and Ser^{396} (Jung et al., 2013) epitopes in the chronic Otsuka Long Evans Tokushima Fatty (OLETF) T2DM rat model, and these changes were further increased with age (Jung et al., 2013). In addition, Li and colleagues have also observed Tau hyperphosphorylation at the Ser^{396} site in the cortex of BBZDR/Wor rat strain (Li et al., 2007). These results were also confirmed in db/db mice that showed Tau hyperphosphorylation at the AT8, Ser^{199}, Ser^{202}, Ser^{396}, and Ser^{422} epitopes (Kim et al., 2009; Li et al., 2012a) (**Table 1**). However, since these animals were anesthetized prior to sacrifice and their temperature was not reported, it would be interesting to repeat these experiments in controlled conditions.

Animal models of induced T2DM. High fat diet (HFD) is commonly used to induce T2DM in animals. Moroz et al. have reported that HFD increases body weight, reduces brain weight and leads to brain insulin resistance, since mice fed with HFD showed increased brain insulin gene expression paralleled with decreased IR binding (Moroz et al., 2008). However, levels of both phosphorylated and total Tau were not affected in the brain of non-transgenic animals treated with HFD, although Tau mRNA levels were significantly increased (Moroz et al., 2008). Similarly, levels of total soluble and insoluble Tau, but not phospho-Tau were increased in HFD fed 3xTg-AD transgenic mice, suggesting that effects of HFD are modest on Tau phosphorylation (Julien et al., 2010).

On the other hand, a recent study has demonstrated that HFD leads to Tau phosphorylation in a model of Tauopathy treated with HFD, in a manner independent of insulin resistance, suggesting that, other obesity-related factors, might contribute to Tau pathology (Leboucher et al., 2012).

Interestingly, Bhat and Thirumangalakudi have reported that feeding mice with high-fat/cholesterol (HFC) diet also leads to peripheral insulin resistance. Importantly, these authors showed that HFC results in increased levels of Tau hyperphosphorylation at the PHF-1 and Thr^{231} epitopes in the mice hippocampi (Bhat and Thirumangalakudi, 2013).

It is difficult to draw conclusion from HFD treatments since published results are so divergent. The reasons for these differences could include the composition of the diet, the duration of the treatment, the background strains, the levels of contamination of the animal facilities and, of course, confounding hypothermia.

Insulin signaling impairment animal models. The group of Schubert et al. have generated and explored a brain/neuron insulin receptor knockout (NIRKO) mouse model that exhibit a conditional inactivation of the IR gene in the CNS (Schubert et al., 2004). These mice are mainly characterized by hyperinsulinemia, mild insulin resistance, obesity and reduced fertility (Bruning et al., 2000), and might therefore represent a model of T2DM. Central IR deficiency in these mice has resulted in a significant increase of Tau phosphorylation at the AT180 epitope (Schubert et al., 2004).

Another model of insulin signaling impairment that might represent a model of T2DM is the IRS-$2^{-/-}$ mouse model. These mice are insulin resistant and develop diabetes as a result of impairment in survival and function of pancreatic β-cells (Bruning et al., 1998; Withers et al., 1998, 1999; Kulkarni et al., 1999; Michael et al., 2000). Schubert et al. have demonstrated that IRS-$2^{-/-}$ mice exhibit Tau hyperphosphorylation at the Ser^{202}epitope (Schubert et al., 2003), (**Table 1**).

Therefore, animal models of insulin signaling dysfunction have provided additional evidence that insulin plays crucial role in the regulation of Tau phosphorylation *in vivo*. However, further studies are needed to better understand mechanisms underlying these effects.

T3DM and Tau phosphorylation

Data from T1DM and T2DM animal models strongly suggest that insulin signaling plays a key role in modulating AD pathogenesis. However, in almost all animal models cited above, Tau pathology was investigated in a context of peripheral insulin dysfunction. Therefore, animal models exhibiting specific alteration of the central insulin function might be helpful to address the role of brain insulin disruption in AD pathology. Thus, the group of Hoyer and colleagues have suggested that rats treated with intra-cerebroventricular (i.c.v.) STZ represent a sporadic AD animal model characterized by a specific insulin-resistant brain state (Nitsch and Hoyer, 1991; Duelli et al., 1994; Lannert and Hoyer, 1998; Salkovic-Petrisic and Hoyer, 2007). Interestingly, de la Monte et al have later proposed that AD might represent a specific form of brain diabetes and thus proposed the term of "type 3 diabetes" (T3DM) (De La Monte et al., 2006; Lester-Coll et al., 2006). Importantly, this hypothesis is supported by a recent *post-mortem* study demonstrating a state of brain insulin resistance in human AD patients (Talbot et al., 2012).

In induced T1DM animal models, STZ is administrated peripherally at high doses (55–200 mg/Kg). However, in T3DM rats, STZ is injected in the brain ventricles at doses of up to 100 times lower than those used in systemic injections (Nitsch and Hoyer, 1991; Duelli et al., 1994; Lannert and Hoyer, 1998). Although it was shown that pancreatic architecture, as well as levels of both insulin and glucose are not affected following central STZ treatment (De La Monte et al., 2006; Lester-Coll et al., 2006), numerous studies have reported that T3DM rat models exhibit several neurochemical, structural and behavioral changes that are similar to cellular abnormalities observed in AD brains. These changes include brain atrophy, cell loss, neurodegeneration (De La Monte et al., 2006; Lester-Coll et al., 2006), a decrease in glucose utilization notably in the hippocampi and entorhinal cortices of treated rats (Duelli et al., 1994), a reduction of energy metabolism (Lannert and Hoyer, 1998; Prickaerts et al., 2000; Yun et al., 2000), an impairment in learning and memory, as well as a significant increase in oxidative stress (Sharma and Gupta, 2001; Veerendra Kumar and Gupta, 2003; Ishrat et al., 2006; Pathan et al., 2006).

The mechanism of action of central STZ is far from clear, but some observations suggest that it might be similar to that in the periphery. Indeed, GLUT2, the glucose transporter targeted by peripheral STZ, was found to be regionally distributed in the mammalian brain (Brant et al., 1993; Leloup et al., 1994;

Ngarmukos et al., 2001). In addition, DNA damage was observed in T3DM rat brains (Nitsch and Hoyer, 1991; Lannert and Hoyer, 1998).

The vast majority of studies that have investigated Tau phosphorylation following i.c.v. STZ administration (ranging from 1 to 3 mg/Kg) were performed in non-transgenic animal models. Interestingly, all these studies have observed a significant increase in Tau phosphorylation at several epitopes, notably AT8, PHF-1, Ser^{199}, Ser^{202}, Ser^{396}, Ser^{404}, Thr^{181}, Thr^{205}, Thr^{212}, and Tau-1(Chu and Qian, 2005; Salkovic-Petrisic et al., 2006; Grunblatt et al., 2007; Deng et al., 2009; Chen et al., 2011, 2013b; Li et al., 2012b; Du et al., 2013) (**Table 1**).

Interestingly, Chen et al. have recently investigated the impact of T3DM on Tau phosphorylation in the 3xTg-AD mouse model. These authors demonstrated an increase in Tau phosphorylation at the Ser^{199}/Ser^{202}, 12E8 (Ser^{262}/Ser^{356}) and Ser^{422}epitopes (Chen et al., 2013b).

By contrast, Plascke et al. didn't detect any increase in the level of phosphorylated Tau in the brain of Tg2576 mice (Plaschke et al., 2010).

Therefore, T3DM leads to several alterations resembling to those found in human AD patients, further giving consistent evidence that brain insulin resistance might be a central event in AD. However, it should be noted that it remain difficult to conclude from all studies cited above because they are quite heterogeneous, considering differences in the rat strain used, the dose of STZ delivered, the age at STZ treatment and brain regions investigated (for review, see Salkovic-Petrisic et al., 2006).

MECHANISMS LINKING INSULIN DYSFUNCTION TO TAU PATHOLOGY

KINASES ACTIVATION

The increased phosphorylation of Tau on many residues might be attributable to the activation of multiple kinases. Among the kinases able to phosphorylate Tau *in vitro*, GSK-3β is considered to be the major physiological and pathological Tau kinases (Planel et al., 2002; Hernandez et al., 2013).

Studies that have addressed GSK-3β activation in diabetic animal models (using antibodies detecting the phosphorylation state either at the Ser9 inhibitory site, or the Tyr216 activation site of the kinase) have shown contradictory findings (**Table 1**), thus making it difficult to conclude about the implication of this kinase in the correlation between diabetes and Tau pathogenesis. Of note, we (Planel et al., 2007b), and others (Clodfelder-Miller et al., 2006; Deng et al., 2009), have demonstrated that GSK-3β is inhibited in STZ-induced T1DM mice, and our data demonstrated that this inhibition can be attributed to hypothermia (Planel et al., 2001), as GSK-3β S9 phosphorylation is a constant feature of hypothermia in mouse brain during metabolic stress (Planel et al., 2004) or anesthesia (Planel et al., 2007a). However, it is important to note that, even when inhibited, GSK-3β is still participating to Tau hyperphosphorylation, as we previously demonstrated with lithium, a GSK-3β inhibitor, in mice rendered hypothermic by starvation (Planel et al., 2001).

PHOSPHATASES INHIBITION

Ser/Thr protein phosphatases (PP) are classified into five types, PP1, PP2A, PP2B, PP2C, and PP5, on the basis of their substrate specificities and sensitivity to specific activators and inhibitors (Liu et al., 2005). Biochemical studies have demonstrated that PP1, PP2A, PP2B (Calcineurin), and PP5 are involved in Tau dephosphorylation (Gong et al., 1994; Tian and Wang, 2002). Importantly, it is believed that PP2A is the major Tau phosphatase *in vivo* (Gong et al., 2000; Planel et al., 2001, 2004, 2007a), with PP2A, PP1, PP5, and PP2B contributing to 71, 11, 10 and 7%, respectively, of the total Tau phosphatase activity in the brain (Liu et al., 2005).

The analysis of different Tau phosphatases in diabetic animal models is less documented in comparison to kinases (**Table 1**). However, although several investigators have observed a decrease only in the expression of PP2A in T2DM mice brains (Schubert et al., 2003; Li et al., 2012a), we (Planel et al., 2007b), and others (Clodfelder-Miller et al., 2006; Qu et al., 2011; Jung et al., 2013), have reported that PP2A activity is inhibited in both T1DM and T2DM animal models.

Interestingly, Deters and colleagues demonstrated that co-expression of P301L mutant human Tau and a dominant negative form of PP2A in the brain of transgenic mice significantly increases Tau hyperphosphorylation and NFT formation, suggesting a crucial role for PP2A in regulating Tau pathology (Deters et al., 2009). This finding is particularly important since PP2A is inhibited in human AD patients and seems to be an important factor in the progression of the disease (Gong et al., 1993, 1995; Vogelsberg-Ragaglia et al., 2001).

Thus, PP2A seems to be a key protein in the link between insulin dysfunction and AD. Extending analyses to other diabetic mouse models or genetically modified mice will facilitate the understanding of molecular mechanisms underlying PP2A function, and thus help for the identification of molecules that may compromise the reversibility of Tau hyperphosphorylation.

INFLAMMATION

Several studies have shown that insulin dysfunction is associated with inflammation. In the periphery, insulin was shown to modulate many aspects of the inflammatory process. Thus, at low doses, insulin exerts anti-inflammatory effects (Dandona, 2002); whereas, during chronic hyperinsulinemia, insulin may exacerbate inflammatory responses and increase markers of oxidative stress (Krogh-Madsen et al., 2004). Specifically, it was demonstrated that levels of proinflammatory cytokines, including Interleukin-1 (IL-1), IL-6, and C-reactive protein (CRP) are elevated in diabetic patients (Hak et al., 2001; Spranger et al., 2003).

In the CNS, several animal studies have also associated insulin dysfunction to inflammation. For example, a significant increase in the number of glial fibrillary acidic protein (GFAP)-reactive astrocytes (also known as astrogliosis) was reported in the hippocampi of both NOD and STZ-induced mouse models (Saravia et al., 2002).

In addition, T2DM was shown to aggravate vascular inflammation in an AD mouse model, changes that can be related to impaired central insulin signaling (Takeda et al., 2010). Notably,

a study by Pistell et al. has established that different diet composition results in various inflammatory reactions in the brain. Thus, these authors have demonstrated that HFD consumption leads to a significant increase in the levels of proinflammatory cytokines, chemokines as well as reactive astrocytosis and microgliosis (Pistell et al., 2010). These data suggest that inflammation might be tightly related to Tau hyperphosphorylation observed during T2DM.

Moreover, brains of i.c.v.-STZ treated rats showed increased levels of IL-1 and tumor necrosis factor-α (TNF-α) (Chen et al., 2013a), as well as pronounced astrogliosis and microglial activation (Prickaerts et al., 1999; Chen et al., 2013a).

On the other hand, there is evidence that Tau pathology is associated with neuroinflammation. Thus, IL-1, IL-6 as well as nitric oxide have been shown to exacerbate Tau pathology and NFT formation *in vitro* (Li et al., 2003; Quintanilla et al., 2004; Saez et al., 2004). Moreover, Shepherd et al. have demonstrated that microglial activation might contribute to Tau hyperphosphorylation and NFT formation in *postmortem* AD brains (Shepherd et al., 2000; for review, see Arnaud et al., 2006). Similarly, administration of lipopolysaccharide (LPS), which is a bacterial endotoxin commonly used in animal studies to induce systemic inflammation (Lien et al., 2000), significantly increases Tau hyperphosphorylation in a triple transgenic mouse model of AD (Kitazawa et al., 2005). In addition, Yoshiyama et al. have demonstrated that microglial neuroinflammation precedes Tau pathology in P301S transgenic mice (Yoshiyama et al., 2007).

Mechanisms by which neuroinflammation might contribute to Tau pathology are not well elucidated. However, it is known that one signaling pathway by which neurons and microglia communicate is fractalkine (CX3CL1) and its cognate receptor (CX3CR1). In the CNS, CX3CL1 is highly expressed in neurons, whereas CX3CR1 is exclusively expressed in microglia cells (Harrison et al., 1998). Therefore, Bhaskar et al. have suggested that the CX3CL1/CX3CR1 pathway could modulate Tau pathology, and that this effect might be dependent upon microglial-derived IL-1-p38 MAPK signaling pathway (Bhaskar et al., 2010).

Overall, the link between insulin dysfunction, inflammation (both peripheral and/or central), and Tau hyperphosphorylation is unclear and would benefit from future research focus.

STRESS

Stress might represent another mechanistic link between insulin dysfunction and Tau hyperphosphorylation. Indeed, it was recently reported that stress leads to both peripheral and central insulin resistance, as well as increased Tau hyperphosphorylation in the mouse brain. Notably, these authors have pointed a central role of JNK (c-Jun N-Terminal Kinase), a major stress signaling pathway (Solas et al., 2013). These results suggest that insulin resistance might mediate Tau phosphorylation through a stress-dependent mechanism. Further studies are required to better understand this issue.

INSULIN SIGNALING: THE CONVERGING ROAD

Insulin signaling impairment in diabetic animal models

While impaired peripheral insulin signaling is well known in animal models of diabetes, there is conflicting evidence of central insulin signaling dysfunction.

The phosphorylation levels of IRs were reported to be significantly decreased in mice treated with systemic STZ (Jolivalt et al., 2008, 2010), as well as in the insulin KO mouse model (Schubert et al., 2004) (**Table 1**). By contrast, levels of the β subunit of IR (IRβ) have shown contradictory findings; whereas Jung *et al* have observed that IRβ levels are increased in the brain of the OLETF T2DM rat model (Jung et al., 2011), Li et al. have reported a significant decrease in IRβ levels in the cortex of spontaneous T1DM, but not T2DM rats (Li et al., 2007). Similarly, the insulin KO mouse model has revealed a decrease in the expression of IRβ in the brain (Schechter et al., 2005).

Conflicting results have also been reported for Akt, a key kinase implicated in the insulin-signaling pathway, and also known to phosphorylate Tau *in vitro* (Ksiezak-Reding et al., 2003). Thus, a significant reduction in the phosphorylation levels and the activity of Akt (Li et al., 2007; Qu et al., 2011) was reported in the brain of both T1DM and T2DM animal models (Jolivalt et al., 2008, 2010; Qu et al., 2011; Bhat and Thirumangalakudi, 2013). By contrast, several data showed augmented phosphorylation of Akt and GSK-3β Ser9 (inhibitory phosphorylation) in NOD and STZ-treated mice (Clodfelder-Miller et al., 2005, 2006; Planel et al., 2007b). It is interesting to note that the rise in Akt and GSK-3β Ser9 phosphorylation in both NOD and STZ-treated mice is probably due to the inhibition of PP2A, as inhibitors of the phosphatase upregulate the phosphorylation of the two kinases (Andjelkovic et al., 1996; Planel et al., 2001). However, how peripheral insulin dysfunction results in central PP2A inhibition remains to be elucidated.

Moreover, Moroz et al. have demonstrated that HFD-induced T2DM leads to an increase in mRNA levels of IRS-1 and IRS-4 in the brain of non-transgenic mice (Moroz et al., 2008). By contrast, Bhat and Thirumangalakudi have reported a decrease in IRS-1 levels in the brain of HFC-treated mice (Bhat and Thirumangalakudi, 2013).

Interestingly, a recent study has demonstrated that HFD potentiated Tau pathology in a mouse model of Tauopathy, in a manner independent from insulin resistance, suggesting that other factors, probably linked to obesity, might be implicated in Tau pathology during T2DM (Leboucher et al., 2012).

Several investigators have also reported a deregulation in gene and/or protein expression of the insulin-signaling pathway in T3DM animal models. For example, the phosphorylation level of PI3K was significantly decreased in mice treated with central STZ (1.5 mg/Kg, i.c.v.) (Deng et al., 2009). Moreover, Grunblatt et al. have demonstrated that mRNA levels of both insulin and IRs are decreased, whereas levels of phosphorylated IRβ are increased in the hippocampi of T3DM rats (Grunblatt et al., 2007), (**Table 1**).

In summary, the involvement of the central insulin signaling in Tau pathology is still controversial, with divergent results showing a decrease, an increase, or even no significant changes in the insulin signaling proteins in the brains of diabetic animals (**Table 1**). Therefore, further investigations are crucial to get a more definitive picture of the role of central insulin signaling in the progression of Tau pathology.

Implication of insulin signaling on Tau pathology

Beside its classical function of regulating glucose metabolism, there is increasing evidence supporting a role for the

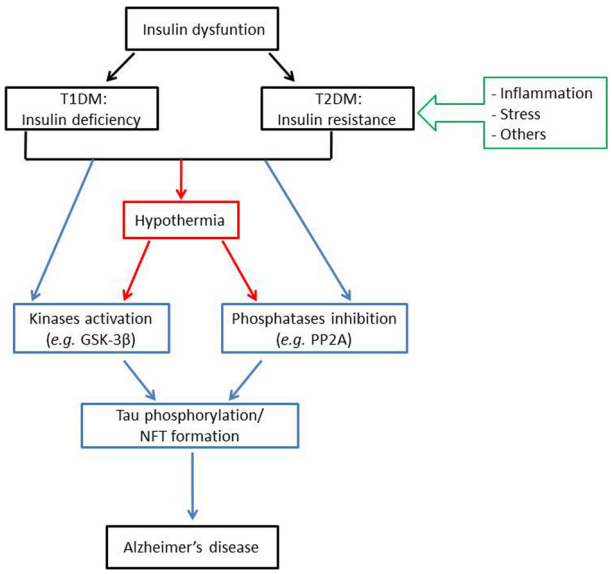

FIGURE 1 | Insulin dysfunction might enhance Alzheimer's disease pathology through distinct mechanisms. Insulin dysfunction, manifested by either T1DM, characterized by insulin deficiency; or T2DM, characterized by insulin resistance, might lead to Tau phosphorylation and NFT formation, one of the major histopathological hallmarks of Alzheimer's disease. This effect can be induced either directly, through kinases activation (such as GSK-3β) and/or phosphatases inhibition (such as PP2A); or indirectly, through the effect of hypothermia on kinases and phosphatases activities. For note, insulin resistance might be related to Tau pathology through several mechanisms, mainly including inflammation and stress. Abbreviations: T1DM: type 1 diabetes mellitus; T2DM: type 2 diabetes mellitus; GSK-3β: glycogen synthase kinase 3β; PP2A: protein phosphatase 2A; NFT: neurofibrillary tangles.

insulin-signaling pathway in neuronal development as well as in learning and memory, therefore suggesting a crucial role of insulin signaling in AD pathogenesis (Zhao and Alkon, 2001; Craft and Watson, 2004; Gerozissis, 2008).

It has been proposed that insulin could affect Tau phosphorylation through the regulation of GSK-3β, since it is a kinase downstream in the IR signaling pathway (Hong and Lee, 1997). Although insulin administration in STZ treated mice completely (Planel et al., 2007b), or partially (Clodfelder-Miller et al., 2006; Jolivalt et al., 2008) rescued Tau phosphorylation in mice, these findings are in contrast with human studies that report a higher risk of AD in patients treated with insulin (Ott et al., 1999; Luchsinger et al., 2001). However, one should be cautious in making direct conclusions from these epidemiological studies, as it might be because the patients were having a more severe stage of diabetes that they required treatment with insulin. Thus, more controlled studies in animals, as well as in human patients are important to better understand the role of insulin in the brain.

Furthermore, several studies have investigated the impact of rosiglitazone treatment on Tau phosphorylation in animal models. For example, Escribano et al. have observed a significant decrease in Tau phosphorylation in the brain of transgenic hAPP mice following rosiglitazone treatment (Escribano et al., 2010). Similarly, Yoon et al. have demonstrated that this drug reduced Tau phosphorylation both in *vitro*, and in the hippocampi of OLETF T2DM rats. Interestingly, these authors suggest that this effect might be due to a decrease in JNK activity (Yoon et al., 2010).

Thus, although brain insulin signaling might have an important effect on Tau phosphorylation, the molecular mechanisms underlying this effect are far from clear. Better understanding of these mechanisms might help to develop therapeutic strategies aiming to reduce Tau hyperphosphorylation in the brain.

CONCLUSION

Overall, numerous preclinical studies examining the correlation between insulin dysfunction and Tau hyperphosphorylation converge to indicate that both T1DM and T2DM might affect Tau pathology, either directly or indirectly (**Figure 1**). Apart from some emerging evidences, mechanisms by which insulin dysfunction and/or other features of diabetes such as obesity and inflammation contributes to Tau pathology are still not fully elucidated. Therefore, further studies using other animal models (e.g., ob/ob mice…) are required to better understand the contributions of these mechanisms to Tau hyperphosphorylation. However, future studies must carefully report and control physiological parameters such as body temperature, and should avoid using anesthesia for the sacrifice of the animals.

In light of the increased incidence of diabetes in the young population, future focus on the correlation between insulin dysfunction and Tau pathology may provide invaluable information for the treatment and prevention of sporadic AD.

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Pin1, a new player in the fate of HIF-1α degradation: an hypothetical mechanism inside vascular damage as Alzheimer's disease risk factor

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Aetiology of neurodegenerative mechanisms underlying Alzheimer's disease (AD) are still under elucidation. The contribution of cerebrovascular deficiencies (such as cerebral ischemia/stroke) has been strongly endorsed in recent years. Reduction of blood supply leading to hypoxic condition is known to activate cellular responses mainly controlled by hypoxia-inducible transcription factor-1 (HIF-1). Thus alterations of oxygen responsive HIF-1α subunit in the central nervous system may contribute to the cognitive decline, especially influencing mechanisms associated to amyloid precursor protein (APP) amyloidogenic metabolism. Although HIF-1α protein level is known to be regulated by von Hippel-Lindau (VHL) ubiquitin-proteasome system, it has been recently suggested that glycogen synthase kinase-3β (Gsk-3β) promotes a VHL-independent HIF-1α degradation. Here we provide evidences that in rat primary hippocampal cell cultures, HIF-1α degradation might be mediated by a synergic action of Gsk-3β and peptidyl-prolyl *cis/trans* isomerase (Pin1). In post-ischemic conditions, such as those mimicked with oxygen glucose deprivation (OGD), HIF-1α protein level increases remaining unexpectedly high for long time after normal condition restoration jointly with the increase of lactate dehydrogenase (LDH) and β-secretase 1 (BACE1) protein expression (70 and 140% respectively). Interestingly the Pin1 activity decreases about 40–60% and Pin1^{S16} inhibitory phosphorylation significantly increases, indicating that Pin1 binding to its substrate and enzymatic activity are reduced by treatment. Co-immunoprecipitation experiments demonstrate that HIF-1α/Pin1 in normoxia are associated, and that in presence of specific Pin1 and Gsk-3β inhibitors their interaction is reduced in parallel to an increase of HIF-1α protein level. Thus we suggest that in post-OGD neurons the high level of HIF-1α might be due to Pin1 binding ability and activity reduction which affects HIF-1α degradation: an event that may highlight the relevance of ischemia/HIF-1α as a risk factor in AD pathogenesis.

Keywords: Alzheimer's disease, cerebrovascular deficiencies, hippocampal neurons, Pin1, HIF-1α, GSK-3β, oxygen glucose deprivation

INTRODUCTION

Alzheimer's disease (AD) is a multifactor neurodegenerative pathology affecting the elderly population. The pathogenesis of sporadic late-onset AD has not been identified, but further studies support that cerebral ischemia/stroke significantly increases AD risk (Kalaria, 2000; Zhang et al., 2007). Indeed, it has been suggested that cerebral hypoperfusion causing neuronal damage in vulnerable brain areas (Koistinaho and Koistinaho, 2005; Zhang et al., 2007) may serve as a basis for some cases of dementia after stroke (Ogunshola and Antoniou, 2009).

At the molecular level, a large percentage of hypoxic responses are controlled by hypoxia-inducible transcription factor-1 (HIF-1; Webb et al., 2009) whose involvement in neurodegenerative disorders is becoming widely accepted, although its role may greatly depend on whether it is the cause or the consequence in disease progression (Ogunshola and Antoniou, 2009; Bulbarelli et al., 2012).

HIF-1 is a heterodimeric protein composed of a constitutively expressed HIF-1β subunit and oxygen-regulated HIF-1α subunit (Wang et al., 2006). Under hypoxic conditions HIF-1α

Abbreviations: PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulphate; SEM, standard error media; TBST, Tris-buffered saline-Tween; OGD, oxygen glucose deprivation; Ara-C, 1-β-D arabinofuranosylcytosine; BSS, balanced salt solution; LDS, lauryl-dodecyl sulphate; ECL, enhanced chemiluminescence; DMSO, dimethyl sulfoxide; pNA, *p*-nitroanilide; juglone, 5-Hydroxy-1,4-naphtoquinone; APP, amyloid precursor protein; PI3K, phosphatidylinositol-3 kinase; Pin1, peptidyl-prolyl *cis/trans* isomerase (PPIase); HIF-1α, hypoxia-inducible transcription factor subunit-α; Gsk-3β, glycogen synthase kinase-3β; BACE1, β-secretase 1; LDH, lactate dehydrogenase; VHL, von Hippel-Lindau protein.

is stabilized, and in complex to the constitutive HIF-1β subunit induces the transcription of a plethora of genes (Semenza, 2003), whereas in normoxic conditions it is rapidly subjected to proteasomal degradation (Webb et al., 2009). HIF-1α degradation system displays enormous plasticity since it can be induced by hydroxylation and phosphorylation events either alone or in combination (Flügel et al., 2007). Indeed, although HIF-1α degradation mainly depends on two prolyl hydroxylation allowing the binding with tumor suppressor von Hippel-Lindau protein (VHL; Salceda and Caro, 1997; Sonenberg and Gingras, 1998; Semenza, 2010), evidences show that glycogen synthase kinase-3β (Gsk-3β), by phosphorylation of Ser551, Thr555, and Ser589 residues in HIF-1α oxygen degradation domain (ODD), can promote its ubiquitination and proteasomal degradation in a VHL-independent manner (Flügel et al., 2007).

In addition, during a brief hypoxic event, the HIF-1α protein stabilization needs the Akt-induced inhibitory phosphorylation of Gsk-3β on Ser9 residue (Mottet et al., 2003). A growing number of reports showed that both hypoxic and non hypoxic stimuli appear to promote HIF-1α stabilization by means of PI3K/Akt pathway (Zhong et al., 2000; Zundel et al., 2000; Hirota and Semenza, 2001; Li et al., 2008) in a cell and tissue specific way (such as in cortical neurons; Zhang et al., 2009).

The Gsk-3β-mediated degradation of HIF-1α implies a scenario similar to that of c-myc and cyclin E (Yeh et al., 2004, 2006) which upon phosphorylation by the kinase and isomerase-mediated conformational change, are ubiquitinated and degraded in the proteasome. Gsk-3β can indeed cooperate synergistically with the peptidyl-prolyl *cis/trans* isomerase (Pin1) in ubiquitination of a wide range of proteins (Liou et al., 2011). Gsk-3β as a proline-directed kinase can selectively phosphorylate Ser/Thr-Pro residues allowing the Pin1 substrate recognition and their *cis* to *trans* isomerization. The *cis* or *trans* conformation of phospho-Ser/Thr-Pro motif, as recently suggested, could be a crucial determinant in regulating protein degradation (Liou et al., 2011) in view of the fact that the ubiquitin E3 ligase complex might have a structural preference for phosphorylated substrates via a *trans* conformation. Pin1-mediated conformational change in phospho-Ser/Thr-Pro motifs, hence, represents a novel molecular switch in a large number of biological processes. Therefore, Pin1 is tightly regulated by multiple levels (Lu et al., 2002), and alterations in its functionality often lead to several pathologies, included cancer and neurodegeneration (such as AD; Lu et al., 2007). In pathological conditions, the Pin1 ability to interact with downstream substrates is inhibited by phosphorylation of Ser16 residue in its binding domain (Eckerdt et al., 2005; Lonati et al., 2011) while oxidative modification in the catalytic domain can abolish the enzymatic activity of isomerase (Butterfield et al., 2006a,b).

Although recent studies highlight indirect link between HIF-1α regulation/activity and Pin1 overexpression in breast cancer (Kim et al., 2008) and in prostate cancer (Yuan et al., 2011), little is known about the relationship of this two proteins in neuronal tissues, under physiological or pathological conditions. Consistent with that we asked whether Pin1 might participate in HIF-1α modulation under normoxic and post-ischemic conditions, such as those mimicked after oxygen glucose deprivation (OGD) treatment, where HIF-1α protein levels are carefully regulated and Pin1 activity might be altered.

Here we show that, in rat primary hippocampal cultures, Pin1 interacts with HIF-1α, and catalyzing its isomerization plays a central role in Gsk-3β-mediated proteasomal degradation of the transcription factor. Moreover in neurons subjected to OGD, Pin1 binding and activity interestingly are partially inhibited affecting HIF-1α ubiquitination and protein level.

Considering that recent studies performed in the central nervous system highlight the pathophysiological relevance of hypoxia/HIF-1 pathways regulation of β-secretase 1 (BACE1) expression and amyloid precursor protein (APP) amyloidogenic metabolism (Zhang et al., 2007; Bulbarelli et al., 2012), alterations in HIF-1α protein levels/degradation pathway may contribute to the cognitive decline and dementia in AD patients influencing the disease course.

MATERIALS AND METHODS

MATERIALS

All commercial chemicals were of the highest available grade: Sprague-Dawley rats were from Charles-River Laboratories (Lecco, Italy). The 5% CO_2: 95% N_2 gas cylinder was from Sapio, Monza, Italy. Complete protease inhibitor cocktail was from Roche Diagnostics S.p.A (Milano, Italy). Hydroxy-1,4-naphtoquinone (juglone), Lactacystin, 1-β-D arabinofuranosylcytosine (Ara-C), lithium chloride solution, SB-216763 Gsk-3 inhibitor, solutions for electrophoresis were from Sigma Chemical Co. (Milano, Italy). All the stock solutions for cell culture were from Euroclone (Celbio Milano, Italy). Gibco Neurobasal medium (NBM) and B27 supplement, Dynabeads® protein G, sodium dodecyl sulphate (SDS) NuPAGE reagents (4–12% Bis-Tris gel; sample buffer; running buffer), Novex Sharp Protein Standard, anti-Tau was from Life Technologies (Milano, Italy).

Anti-Pin1, anti P-Pin1^{S16} and anti-Ubiquitin (PD41) were from Cell Signaling (Beverly, USA). Anti-HIF-1α and anti-lactate dehydrogenase (LDH) antibodies were from Abcam (Cambridge Science Park, UK). Anti P-Ser/Thr-Pro (MPM2) and Anti-Pin1 for immunoprecipitation antibodies were from Millipore S.p.A (Milano, Italy). Anti-BACE1 antibody was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Secondary HRP-conjugated antibodies and ECL SuperSignal detection kit were from Pierce (Rockford, IL, USA). Anti-Actin and anti P-Tau^{T231} antibodies were from Sigma Chemical Co (Milano, Italy).

CELL CULTURE

Hippocampal neurons cultures were prepared from E18-E19 rat hippocampi as previously described, with minor modifications (Brewer et al., 1993; Bulbarelli et al., 2009). Neurons were plated on polylysine coated dishes (60 mm diameter, 5×10^5 cells/dish). The medium for cell culture was NBM containing 2% B27 supplement and 12.5 nM glutamate. After 72 h in culture, half of the cell medium was replaced with NBM w/o glutamate and supplemented with 1-β-D arabinofuranosylcytosine (Ara-C) (5 μM final concentration) to prevent glial proliferation. Cells were maintained at 37°C, 5% CO_2 for 8 days before treatment. All experiments were carried out in accordance with the

guidelines established by the European Community Council and were approved by the Italian Ministry of Health (DL 116/92). Adequate measures were always taken to minimize animal pain or discomfort.

OXYGEN AND GLUCOSE DEPRIVATION TREATMENT

Primary hippocampal neurons were subjected to OGD as previously described (Bulbarelli et al., 2012). Briefly, culture medium was replaced by through exchange with a glucose-free balanced salt solution (BSS; NaCl 130 mM, KCl 5.5 mM, $CaCl_2$ 1.8 mM, $MgCl_2$ 1 mM, HEPES 20 mM). Then cells incubated in a hypoxia chamber (Billups–Rothenberg, Del Mar, CA) saturated for 10 min with 5% CO_2: 95% N_2 were sealed at 37°C for 3 h. After OGD, cells were supplemented with the restoration solution: glucose (final concentration 5 mM) and B27 (final concentration 2%) in NBM.

Cells were maintained in normal culture conditions (37°C in a 5% CO_2 atmosphere) for different times of restoration: one hour (R 1 h) and overnight (R o/n) after the reestablishment of normoxia. Untreated hippocampal cells were incubated in NBM supplemented with 2% B27 in a 5% CO_2 atmosphere.

TREATMENTS WITH INHIBITORS

5-Hydroxy-1,4-naphtoquinone (juglone) was dissolved in dimethyl sulfoxide (DMSO) to obtain a 10 mM stock solution and used as Pin1 catalytic activity inhibitor, accordingly to previous studies (Chao et al., 2001; Galas et al., 2006). Hippocampal cells were incubated with a 10 μM juglone final concentration for 8 h as already described (Bulbarelli et al., 2009), or with 1 μM lactacystin, an inhibitor of ubiquitin-proteasome degradation system, for 16 h (Cazzaniga et al., 2007). According to literature Gsk-3 kinase activity was inhibited by means of lithium chloride administrated to hippocampal cells at 10 mM final concentration for 1 h (Stambolic et al., 1996) or SB-216763 administrated at 10 μM (Facci et al., 2003) for 3 h accordingly to experimental indications obtained in our cellular model.

IMMUNOPRECIPITATION

Cells subjected or not to OGD or to inhibitor treatments for different times, were rinsed twice with phosphate-buffered saline (PBS) and harvested by scraping with a rubber policeman after lysis with non denaturising buffer (1% Nonidet P-40, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl) in presence of complete protease inhibitor cocktail and phosphatase inhibitors (2 mM Na orthovanadate, 1 mM Na fluoride, 1 mM Na pyrophosphate). Then, immunoprecipitation of the endogenous HIF-1α, Pin1 or P-Ser/Thr-Pro (MPM2) proteins was performed from 1 mg of total proteins using anti-HIF-1α (1:100), anti-Pin1 (1:100), anti-MPM2 (1:100) and Dynabeads protein G according to the manufacturer's instruction. Immunoprecipitated proteins were eluted in lauryl-dodecyl sulphate (LDS) lysis buffer with reducing agent (Life Technologies) and boiled for 10 min at 70°C before analysis on NuPAGE Bis-Tris 4–12% polyacrylamide gels.

SDS-PAGE ELECTROPHORESIS AND IMMUNOBLOTTING

Samples were obtained after lysis with denaturising buffer (2% SDS lysis, 50 mM Tris-HCl, pH 6.8 plus protease inhibitor cocktail and phosphatase inhibitors). The total protein amount was evaluated by means of Bicinchoninic acid assay and 25 μg of each sample were subjected to on SDS-polyacrylamide gel electrophoresis (PAGE) on 12.5% polyacrylamide gels. For immunoprecipitation, samples obtained were loaded on NuPAGE Bis-Tris 4–12% gels. After SDS-PAGE electrophoresis samples were transferred to a nitrocellulose membrane (Amersham, GE Healthcare Europe GmbH, Milano, Italy) and proteins revealed by Ponceau staining (Sigma Chemical Co., Milano, Italy). Membranes were blocked in TBS-Tween 0.1% buffer containing 5% non-fat milk or TBS-Tween 0.2% buffer containing 3% bovine serum albumin, and probed with specific antibodies in TBS-T buffer containing 5% non-fat milk or 5–3% bovine serum albumin according to manufacturer's instructions. Immunoblotting was performed using anti-Pin1 (1:1000), anti-P-Pin1^{S16} (1:1000), anti-HIF-1α (1:1000), anti-ubiquitin (PD41) (1:1000), anti LDH (1:2000), anti-BACE1 (1:1000), anti-P-Ser/Thr-Pro (MPM2) (1:1000), anti-P-Tau^{T231} (1:5000), anti-Tau (1:1000) and anti-β-actin (1:1500). Immunoreactive proteins were revealed by enhanced chemiluminescence (ECL) and semi-quantitatively estimated by a KODAK image station 2000R. Normalization was carried out with respect to the amount β-actin in the same sample.

PPIase ASSAY

Pin1 activity was measured according to the methods of Janowski et al. (1997) and Hennig et al. (1998) with slight modifications as follows (Janowski et al., 1997; Hennig et al., 1998). Stock solutions for the protease-free assay was prepared as described: the phosphorylated substrate Ac-AA(pS)PR-pNA was dissolved in 0.47 M LiCl/trifluoroethanol (anhydrous) at 30 mg/ml concentration; trypsin protease was dissolved in 35 mM HEPES, pH 7.8 at 50 mg/ml concentration.

For a typical measurement, to determine the PPIase activity originating from Pin1, the sample buffer (35 mM HEPES, pH 7.5) was incubated in a temperature-controlled cuvette holder at 4°C. Then Ac-AA(pS)PR-pNA (0.02 mg/ml) was added and the reaction was started by the injection of trypsin (0.1 mg/ml). After the initial burst phase where all trans peptides were cleaved, protein samples obtained after lysis, as described for immunoprecipitation, was added to the reaction. The absorption at 390 nM, which detects the formation of free *p*-nitroanilide (pNA), was monitored using a Beckman Coulter DU 800 spectrophotometer. The enzymatic activity of Pin1 in the sample (unit/mL) was calculated as follows: absorbance (OD) ∗ total volume/9620 M^{-1} cm^{-1} (extinction coefficient ϵ) ∗ volume of sample in mL ∗ 1 cm (d). Normalization was carried out with respect to total protein in the same sample.

$$\text{Unit/mL} = \left((\Delta\text{Abs}/\text{ min}) * (V \text{ tot in mL})\right) \Big/ \left(\varepsilon * (V \text{ of sample in mL}) * d\right)$$

The measured activity was normalized to the total protein content of the lysate.

STATISTICAL ANALYSIS

All data are expressed as mean ± SEM of three separate experiments performed in triplicate. The differences were calculated by

means of Student's *t*-test. A p value <0.05 was considered to be statistically significant.

RESULTS

EFFECTS OF THE OXYGEN AND GLUCOSE DEPRIVATION ON HIF-1α PROTEIN EXPRESSION, Pin1 PHOSPHORYLATION AND ACTIVITY IN HIPPOCAMPAL NEURONS CULTURES

Hippocampal cells were subjected to OGD for 3 h. Following the treatment, we investigated the HIF-1α protein levels after 1 hour (R 1 h) and overnight (R o/n) of normal oxygen and glucidic conditions restore. As already demonstrated by our group (Bulbarelli et al., 2012), hippocampal neuron viability is not affected by the OGD treatment.

As shown in **Figure 1A**, HIF-1α protein level increased at R 1 h (60%) remaining significantly higher than control up to R o/n. Concomitantly, we detected that HIF-1α ubiquitination state was decreased about 40% at R 1 h almost unvarying at R o/n (**Figure 1B**).

Relatively to HIF-1α expression increase after normal condition restoration (post-OGD) we examined lactate dehydrogenase (LDH) and BACE1 protein expression, two proteins whose *genes* are under HIF-1α transcriptional regulation. We observed that LDH expression increased about 70% already at R 1 h, slightly decreasing during overnight, while BACE1 protein level significantly increased during o/n, reaching 150% of increment (**Figure 1A**).

Contemporary we observed that Pin1 activity was reduced in a time dependent manner of about 40% at R 1 h, decreasing till 60% during overnight (**Figure 2A**). In addition, in the same conditions we assessed $Pin1^{S16}$ phosphorylation (a state that inhibits Pin1 binding to its substrate): the isomerase was significantly phosphorylated at R 1 h (150%) slightly decreasing at R o/n (110%; **Figure 2B**).

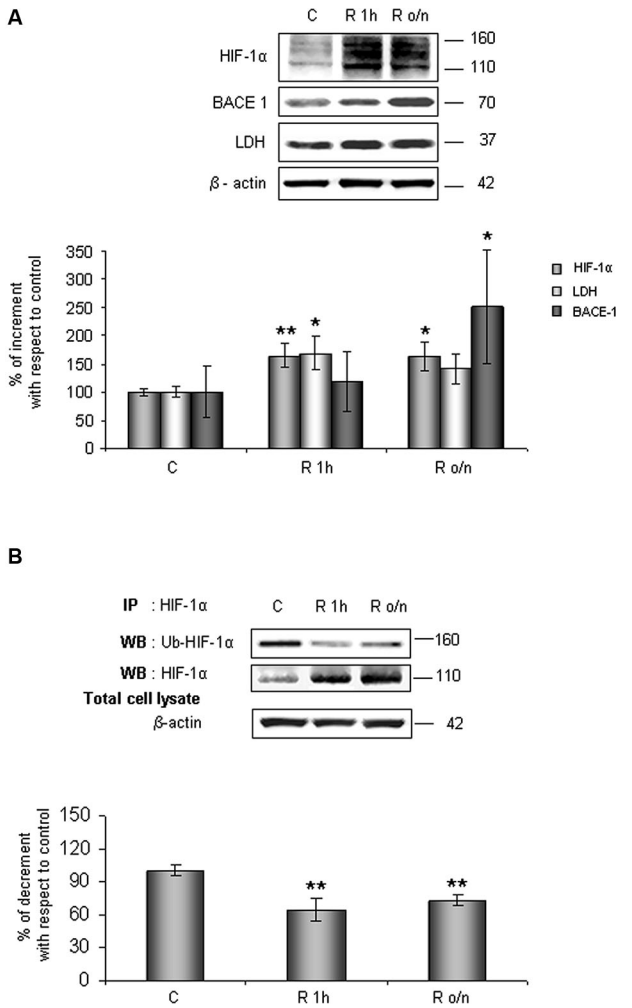

FIGURE 1 | In post-OGD conditions HIF-1α protein levels increases while its ubiquitination state decreases. Cells were subjected to OGD treatment and restoration of normoxic and glucidic conditions for different times (R 1 h, R o/n). **(A)** Cells were collected and lysed with 2% SDS lysis buffer. Equal amounts of samples (as protein content) were subjected to SDS-PAGE and western blot analysis. HIF-1α, LDH, BACE1 levels were detected by specific antibodies, revealed by enhanced chemiluminescence (ECL) and semi-quantitatively determined by a KODAK image station 2000R. Anti-β-actin antibody was employed to confirm equal protein loading in the different lanes. Results obtained are shown on the bar-graphs. Normalization was carried out with respect to the β-actin amount in the same sample. **(B)** HIF-1α ubiquitination state was evaluated after immunoprecipitation using western blot analysis. Cells were collected and lysed in non denaturising NP40 buffer. Western blot analysis performed on immunoprecipitated proteins showed bands corresponding to ubiquitinated HIF-1α protein (140 kDa), HIF-1α protein (110 kDa). Proteins were detected by specific antibodies (anti-HIF-1α; anti-ubiquitin), revealed by ECL and semi-quantitatively determined by a KODAK image station 2000R and the results obtained are shown on the bar-graphs. IP, immunoprecipitation; WB, western blot. Data represent the mean ± SEM of three separate experiments performed in triplicate. Statistical significance is obtained with Student's *t*-test in comparison with controls. * $p < 0.05$, ** $p < 0.01$.

PHOSPHORYLATION OF HIF-1α Ser/Thr-Pro MOTIFS FOR SPECIFIC Pin1 RECOGNITION AND INTERACTION

We examined the amino acid HIF-1α protein sequence to evaluate the presence of conserved Ser/Thr-Pro motifs potentially recognizable by Pin1 when phosphorylated (Flügel et al., 2007), by means of the alignment of human (Q16665) (Iyer et al., 1998) and rat (O35800) HIF-1α protein sequence (Kietzmann et al., 2001; Align tool EXPASY). As shown the analyses revealed a number of these motifs in HIF-1α sequence. In particular, the Gsk-3β target residue Ser589 precedes a proline in one of the above mentioned Ser/Thr-Pro motifs (**Figure 3A**).

Therefore in order to find one or more HIF-1α phosphorylated Ser/Thr-Pro motifs (PSer/Thr-Pro), we employed the monoclonal antibody MPM2, which specifically recognizes the PSer/Thr-Pro motifs. Immunoprecipitated HIF-1α protein was identified by MPM2 antibody, confirming that HIF-1α protein contains PSer/Thr-Pro motifs under normal culture conditions. On the other hand, among proteins immunoprecipitated with MPM2, HIF-1α was identified by a specific antibody (**Figure 3B**).

Given that this phosphorylation might allow Pin1 interaction with its hypothetic substrate, we therefore tested in our neuronal model whether HIF-1α associates with Pin1 by co-immunoprecipitation of endogenous HIF-1α or Pin1. As shown in **Figure 3C**, HIF-1α was detected among proteins immunoprecipitated by the anti-Pin1 antibody as well Pin1 was

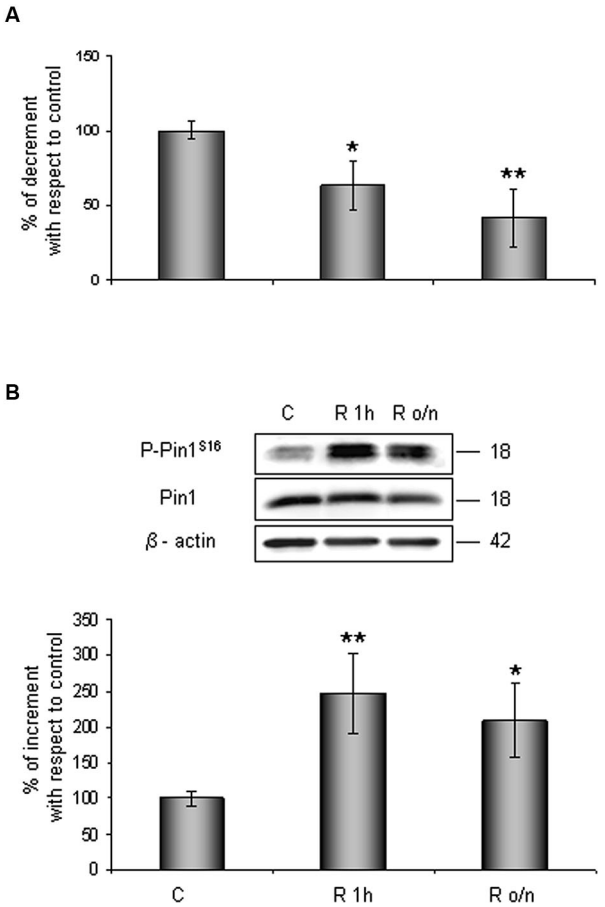

FIGURE 2 | Pin1 inhibition in post-OGD conditions. **(A)** PPIase activity decreases during restoration of normal cultures conditions following OGD. Cells were lysed and collected in non denaturising NP40 buffer at R 1 h and R o/n. Pin1 isomerase activity against Ac-AA(pS)PR-pNA substrate were measured as described in materials and methods. The measured activity was normalized to the total protein concentration of the lysate. **(B)** Pin1^{S16} phosphorylation increases in response to OGD treatment. Cells were lysed in the presence of 2% SDS lysis buffer after OGD treatment and restoration of normoxic and glucidic conditions for different times (R 1 h, R o/n). Total protein content were subjected to SDS-PAGE and Western blot analysis. P-Pin1^{S16} and total Pin1 were detected by specific antibodies and revealed by ECL, and semi-quantitatively determined by a KODAK image station 2000R. Anti-β-actin antibody was employed to confirm equal protein loading in the different lanes. Results obtained are shown on the bar-graphs. The amount of P-Pin1^{S16} correspond to phospho-protein level on Pin1 total protein. Normalization was carried out with respect to the β-actin amount in the same sample. Data represent the mean ± SEM of three separate experiments performed in triplicate. Statistical significance is obtained with Student's *t*-test in comparison with controls. * $p < 0.05$, ** $p < 0.01$.

detected in the protein complex immunoprecipitated by the anti HIF-1α antibody suggesting that HIF-1α and Pin1 might interact each other. Immunoprecipitation with LDH antibody was performed as negative control.

Pin1 INHIBITION AFFECTS HIF-1α DEGRADATION PATHWAY

In order to understand whether Pin1 plays a role in HIF-1α ubiquitination and the following degradation, hippocampal cells were treated for 8 h with 10 μM juglone, an irreversible inhibitor of Pin1 catalytic activity. As expected, juglone treatment triggered a strong inhibition of Pin1 isomerase activity (almost 75%; **Figure 4A**).

The effect of Pin1 inhibition was firstly studied in the whole lysate. As shown in **Figure 4B**, HIF-1α protein level increased of about 70% with respect to control level. Since it is known that Pin1 promotes Tau dephosphorylation (Bulbarelli et al., 2009), a more convincing evidence of the Pin1 inhibition was obtained evaluating Tau phosphorylation on residue Thr231. As expected we observed an increase in Tau phosphorylation.

In parallel, to evaluate the proteasomal degradation of HIF-1α in our cellular model, cells were incubated for 16 h with 1 μM lactacystin an irreversible proteasome inhibitor, revealing HIF-1α protein increment of about 80%. Data obtained are comparable to ones detected after juglone, suggesting that Pin1 might be engaged in the HIF-1α degradation. Contemporary, immunoprecipitated HIF-1α resulted about 35% less ubiquitinated than in untreated cells (**Figure 4C**).

Gsk-3β INHIBITION INFLUENCES Pin1/HIF-1α ASSOCIATION

To assess the involvement of Gsk-3β activity in phosphorylation of Ser/Thr-Pro motifs, hippocampal neurons were incubated in presence of 10 mM LiCl for 1 h, or in presence of 10 μM SB-216763 for 3 h. Then we performed HIF-1α protein immunoprecipitation to analyse phosphorylation state in Ser/Thr-Pro motifs and the amount of Pin1 co-immunoprecipitated.

As shown in **Figure 5A**, phosphorylation of Ser/Thr-Pro motifs was greatly reduced in cells treated with the inhibitors, concomitantly to a considerable decrease in the amount of co-immunoprecipitated Pin1, although total Pin1 amount resulted almost unchanged (**Figure 5B**). In addition, we observed the HIF-1α ubiquitination decrease and a slight enhance of protein level both in the immunoprecipitated fractions (**Figure 5A**) and in the total lysates (**Figure 5B**), indicating a possible reduction in the HIF-1α protein degradation.

In order to confirm the Gsk-3β inhibition, **Figure 5B** shows the increase of Gsk-3β inhibitory phosphorylation on Ser9 after LiCl treatment, accordingly to Martin et al. (2009). Moreover, inhibitory effect on Gsk-3β activity was assessed evaluating Tau phosphorylation on Thr231 residue, a well known target of kinase activity (**Figure 5B**; Lin et al., 2007). As expected, after treatment with both the inhibitors, Tau phosphorylation decreased.

EFFECTS OF THE OXYGEN AND GLUCOSE DEPRIVATION ON Gsk-3β PHOSPHORYLATION AND HIF-1α/Pin1 ASSOCIATION

Considering results above, to investigate whether HIF-1α ubiquitination decrease in post-OGD conditions might correlate to Gsk-3β inhibition, the P-Gsk-3β^{S9} levels were evaluated. As shown in **Figure 6A**, Gsk-3β^{S9} inhibitory phosphorylation increased about 40% at R 1 h and duplicated at R o/n, while total Gsk-3β protein resulted unchanged during the treatment. Concomitantly we detected a significant decrease in Ser/Thr-Pro motifs phosphorylation, revealed by anti-MPM2, on the immunoprecipitated HIF-1α protein at R 1 h (**Figure 6B**). At R o/n we observed a slightly increase of P-Ser/Thr-Pro motifs that however did not reach control levels (**Figure 6B**). Moreover, we also evaluated the

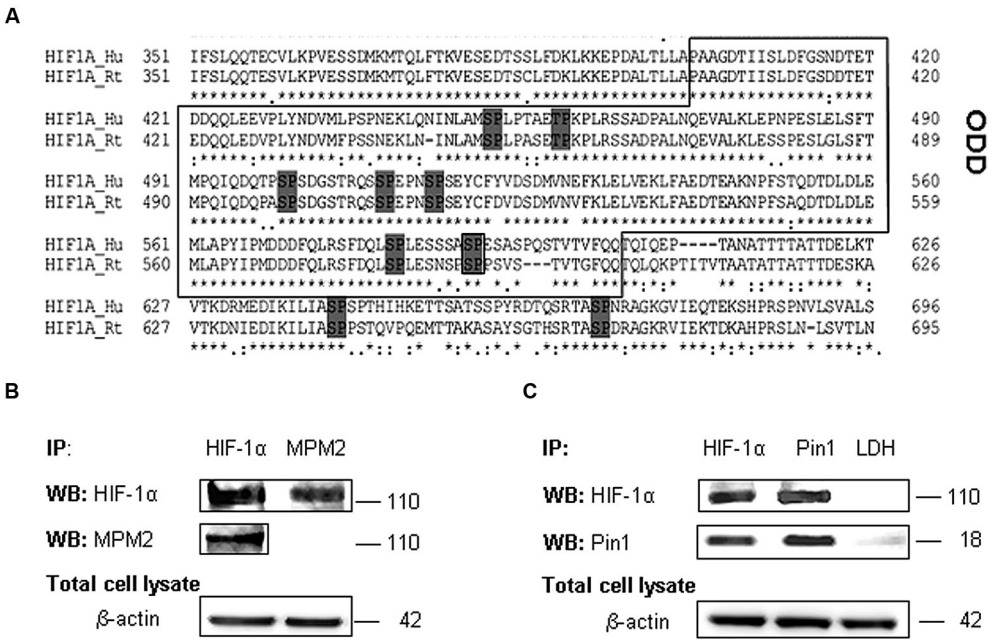

FIGURE 3 | HIF-1α phosphorylation on PSer/Thr-Pro motifs and interaction with Pin1. **(A)** Alignment of amino acid sequences of the HIF-1α oxygen degradation domain (ODD) (aa 402-604) from HIF-1α human and rat homologues. PSer/Thr-Pro motifs are highlighted in dark gray. **(B)** Cells were collected and lysed in non denaturising NP40 buffer. Immunoprecipitated HIF-1α is recognized by MPM2 antibody, vice versa among immunoprecipitated MPM2 epitopes, HIF-1α antibody detects the corresponding protein. **(C)** Co-immunoprecipitation of endogenous HIF-1α and Pin1 proteins in primary rat hippocampal cells lysed in non denaturising NP40 buffer. Immunoprecipitation with LDH antibody was performed as negative control. Western blotting analysis performed on immunoprecipitated proteins showed a 110 kDa and a 18 kDa band corresponding to HIF-1α and Pin1 respectively. Proteins detected by specific antibodies, were revealed by ECL. IP, immunoprecipitation; WB, western blot; MPM2, antibody against phosphorylated Ser/Thr-Pro motifs.

co-immunoprecipitated Pin1 amount: a considerable decrease in HIF-1α/Pin1 association was revealed at R 1 h, while the Pin1 amount at R o/n was quite equivalent to the control (**Figure 6B**).

DISCUSSION

Coexistence of ischemic and neurodegenerative pathology seems to have a deep impact on the expression of the dementia, suggesting common mechanisms interactions. Cerebrovascular diseases such as ischemia leading to cerebral blood flows reduction (hypoperfusion) might indeed initiate and/or accelerate the neurodegeneration cascade *via* amyloid deposition and synaptic neuronal dysfunction (Iadecola, 2010; Kalaria and Ihara, 2012). Depletion of oxygen and glucose sources typical of brain ischemia (Brouns and De Deyn, 2009), results in the activation of highly heterogeneous phenomena in which one of the key component is the transcription factor HIF-1. Therefore protein levels of its hypoxia responsive α subunit (HIF-1α) are finely regulated by degradation through ubiquitin-proteasome system that may be induced by hydroxylation (VHL-mediated) and as recently suggested by phosphorylation (Gsk-3β-mediated; Flügel et al., 2007, 2012). Here we propose that, in hippocampal cellular model, the Pin1 plays an important role in Gsk-3β-mediated HIF-1α ubiquitination/degradation pathway.

In cortical neurons, as already demonstrated, HIF-1α protein levels are maintained higher for long time after reperfusion following OGD by PI3K/Akt signaling (Zhang et al., 2009). Since the PI3K/Akt pathway is known to inhibit Gsk-3β and to regulate HIF-1α in a cell type specific manner (Mottet et al., 2003), we speculate that in neuronal cells HIF-1α degradation might be also regulated by the Gsk-3β pathway working in parallel or in alternative to the well known VHL-mediated mechanism.

Gsk-3β regulates the ubiquitination state of several targets in cooperation with Pin1 (Liou et al., 2011), that mediating prolyl *cis/trans* isomerization can influence the stability of its substrates via phosphorylation-dependent ubiquitin-mediated proteolysis both under physiological and pathological conditions; accordingly, Pin1 might interact and catalyze the isomerization of HIF-1α phosphorylated by the kinase under physiological conditions. Interestingly, here we show, for the first time in neurons, that OGD treatment results in a Pin1 activity reduction, an event that in turn leads to HIF-1α protein levels increase. Indeed, although the transcription factor should be immediately degraded in normoxic conditions by VHL-mediated pathway restoring control levels (Semenza, 2010), in hippocampal cells high levels of HIF-1α protein are detectable at R 1 h and maintained for long time (even up to overnight) after normal oxygen and glucidic conditions restore following OGD. Moreover, HIF-1α high levels are accompanied by a significant decrease of protein ubiquitination state, indicating an impairment in degradation pathway.

Concerning to Pin1, OGD triggers a partial inhibition of its enzymatic activity and also increases Ser16 residue

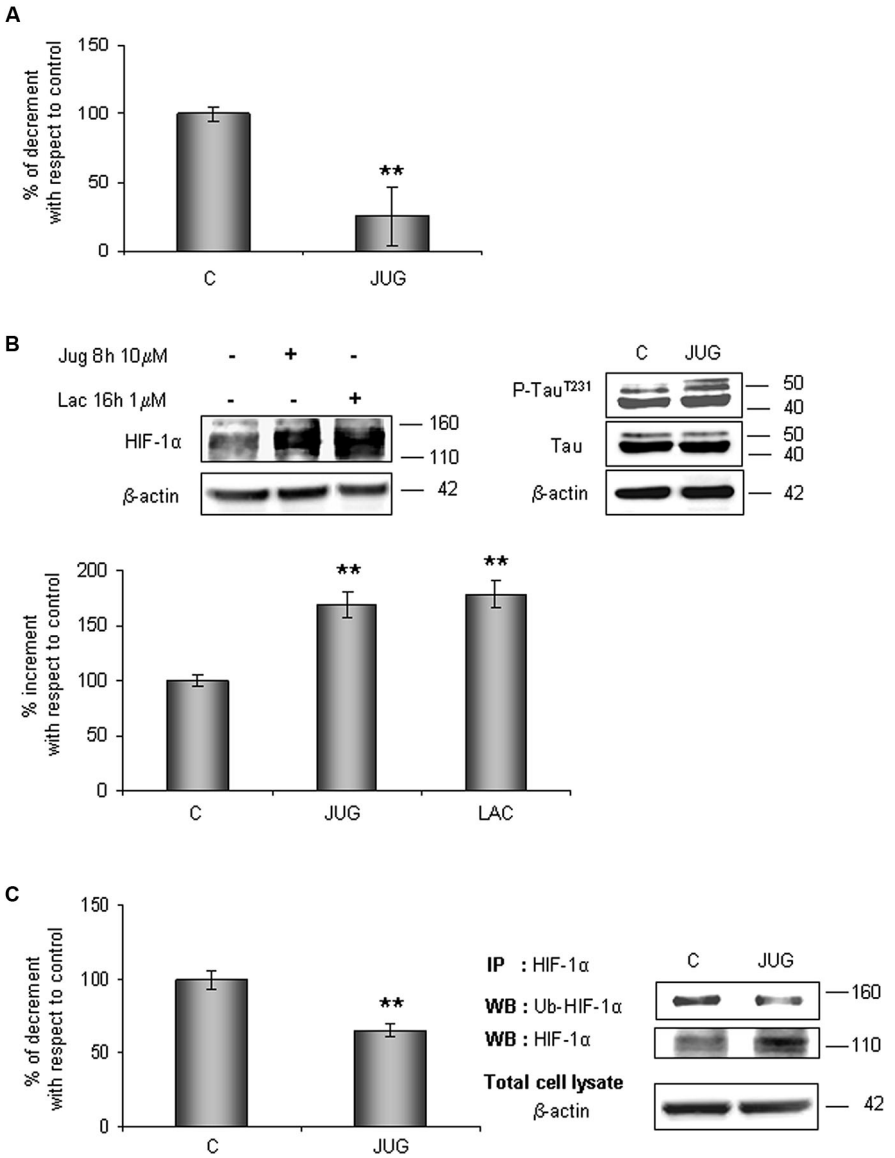

FIGURE 4 | HIF-1α protein levels increases in parallel to a decrease of its ubiquitination state after juglone (jug) treatment. Cells were treated or not with juglone 10 μM for 8 h. **(A)** PPIase activity decreases in cells subjected to juglone treatment. Cells were lysed and collected in non denaturising NP40 buffer and Pin1 isomerase activity against Ac-AA(pS)PR-pNA substrate were measured as described in Section materials and methods. The measured activity was normalized to the total protein concentration of the lysate. **(B)** Neurons were treated or not with juglone or lactacystin (lac) 1 μM for 16 h. Equal amounts of total cell lysates (in the presence of 2% SDS) were subjected to SDS-PAGE and western blot analysis. HIF-1a, Tau, P-Tau^{T231} levels were detected by specific antibodies, revealed by ECL and semi-quantitatively determined by a KODAK image station 2000R. HIF-1α results obtained are shown on the bar-graphs. Anti-β-actin antibody was employed to confirm equal protein loading in the different lanes, normalization was carried out with respect to the β-actin amount in the same sample. **(C)** HIF-1α ubiquitination state decreases after juglone treatment. Western blot analysis showed a 110 kDa and 140 kDa bands corresponding to HIF-1α and to ubiquitinated HIF-1α protein respectively. Proteins were detected by specific antibodies (anti-HIF-1α; anti-ubiquitin), revealed by ECL and semi-quantitatively determined by a KODAK image station 2000R. Results obtained are shown on the bar-graphs. IP, immunoprecipitation; WB, western blot. Data represent the mean ± SEM of 3 separate experiments performed in triplicate. Statistical significance is obtained with Student's *t*-test in comparison with controls. * $p < 0.05$, ** $p < 0.01$.

phosphorylation in the binding domain. Therefore, on one side Ser16 phosphorylation may inhibit Pin1 function in recognizing and binding its substrates, on the other side Pin1 isomerization activity might be diminished by oxidative modification occurring in the catalytic domain (Lu et al., 2002; Lonati et al., 2011). Indeed, as already known oxidative stress might reduce Pin1 function (Butterfield et al., 2006a,b).

Considering data above, we suppose that Pin1 partial inhibition may directly affect the HIF-1α degradation pathway, thus we

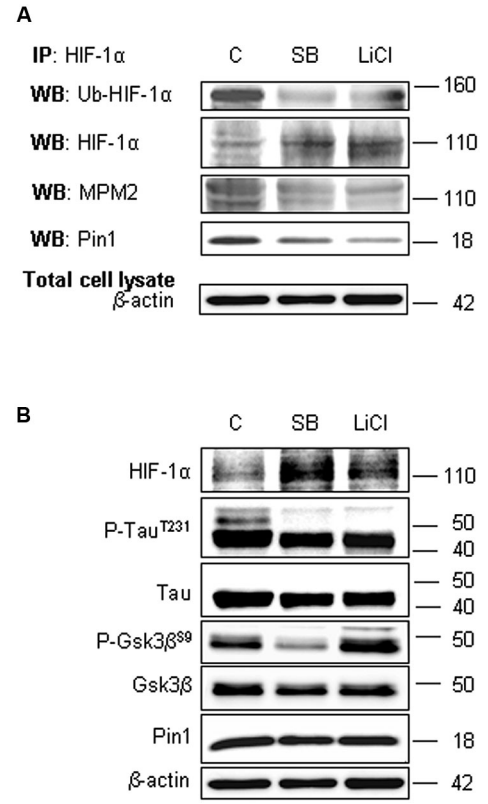

FIGURE 5 | Lithium Chloride (LiCl) or SB-216763 treatment inhibits Gsk-3β leading to a decrease in HIF-1α phosphorylation on PSer/Thr-Pro motifs and in HIF-1α/Pin1 interaction. Cells were treated or not with LiCl 10 mM for 1 h or SB-216763 10 μM for 3 h. **(A)** Western blot analysis performed on HIF-1α immunoprecipitated protein revealed a decrease of the ubiquitination state (anti-ubiquitin), of Ser/Thr-Pro motifs phosphorylation (anti-MPM2) and of Pin1 co-immunoprecipitation (anti-Pin1). **(B)** Equal amounts of total cell lysates (in the presence of 2% SDS) were subjected to SDS-PAGE and Western blot analysis. HIF-1α, Tau, P-Tau^{T231}, Gsk-3β and P-Gsk-3β^{S9} and Pin1 levels were detected by specific antibodies and revealed by ECL. Tau^{T231} phosphorylation as well known Gsk-3β target was assessed to evaluate the kinase inhibition. Anti-β-actin antibody was employed to confirm equal protein loading in the different lanes. IP, immunoprecipitation; WB, western blot; MPM2, antibody against phosphorylated Ser/Thr-Pro motifs.

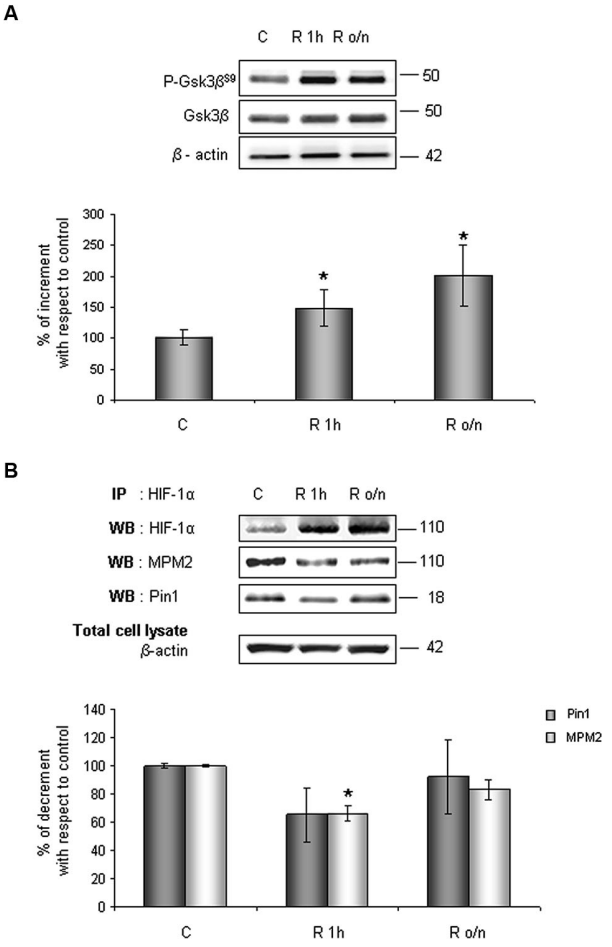

FIGURE 6 | In post-OGD conditions P-Gsk-3β^{S9} levels increases. Cells were subjected to OGD treatment and restoration of normoxic and glucidic conditions for different times (R 1 h, R o/n). **(A)** Cells were collected and lysed with 2% SDS lysis buffer. Equal amounts of samples (as protein content) were subjected to SDS-PAGE and western blot analysis. Gsk-3β and P-Gsk-3β^{S9} levels were detected by specific antibodies, revealed by ECL and semi-quantitatively determined by a KODAK image station 2000R. Anti-β-actin antibody was employed to confirm equal protein loading in the different lanes. Results obtained are shown on the bar-graphs. The amount of P-Gsk-3β^{S9} correspond to phospho-protein level on Gsk-3β total protein. Normalization was carried out with respect to the β-actin amount in the same sample. **(B)** HIF-1α phosphorylation on Ser/Thr-Pro motifs and HIF-1α/Pin1 interaction was evaluated after HIF-1α immunoprecipitation using western blot analysis. Cells were collected and lysed in non denaturising NP40 buffer. Western blot analysis performed on immunoprecipitated proteins showed bands corresponding to Pin1 protein (18 kDa), HIF-1α protein and HIF-1α phosphorylated on Ser/Thr-Pro motifs (110 kDa). Proteins were detected by specific antibodies (anti-HIF-1α; anti-MPM2; anti-Pin1), revealed by ECL and semi-quantitatively determined by a KODAK image station 2000R and the results obtained are shown on the bar-graphs. IP, immunoprecipitation; WB, western blot. Data represent the mean ± SEM of three separate experiments performed in triplicate. Statistical significance is obtained with Student's *t*-test in comparison with controls. * $p < 0.05$, ** $p < 0.01$.

evaluated if Pin1/HIF-1α interaction might be required for HIF-1α ubiquitination.

Firstly, after exploring in HIF-1α amino acid sequence the presence of motifs constituted by serine/threonine residues preceding a proline (Ser/Thr-Pro) that, when phosphorylated, allows Pin1 to specifically recognise its substrates, we found these phosphorylated motifs in HIF-1α protein, employing the anti-MPM2 antibody (which specifically recognizes the PSer/Thr-Pro motifs). Given that this evidence pinpoints the transcription factor as an hypothetic target for Pin1 binding, we provided the evidence of HIF-1α/Pin1 association by co-immunoprecipitation, indicating that the transcription factor and the isomerase may interact each other.

Afterwards, we evaluated the HIF-1α level expression, inhibiting Pin1 catalytic activity by means of juglone treatment. Since we had already established that juglone-mediated Pin1 inhibition leads to Tau^{T231} phosphorylation increase (Bulbarelli et al., 2009), here we show Tau phosphorylation augment after juglone as

positive control of the treatment. Under conditions in which Pin1 enzymatic activity is highly repressed, data obtained showed that HIF-1α levels strongly increase in parallel with a mild decrease of its ubiquitination state, demonstrating that Pin1 affects protein ubiquitination and degradation. Interestingly the HIF-1α levels observed after juglone treatment were comparable to that observed in proteasome inhibited cells suggesting that in our cellular model, Pin1 might play a main role in the fate of the transcription factor. Taking into account that HIF-1α and Pin1 co-immunoprecipitate, it is plausible that the enzyme catalyzes HIF-1α peptydil-prolyl conformational isomerization.

As Liou and colleagues reported (Liou et al., 2011), Pin1 can cooperate synergistically with Gsk-3β in ubiquitination of a wide range of proteins, therefore the role of this kinase in Ser-Thr/Pro phosphorylation and the consequent interaction between Pin1 and HIF-1α have been investigated.

Among the several Gsk-3β phosphorylation consensus motifs in HIF-1α amino acid sequence individuated by Flügel et al. (2007), we observed that the Ser589 is neighboring to a proline constituting a Ser-Thr/Pro motif, which phosphorylated might be the consensus for Pin1 recognition. Therefore we speculate that this sequence could be significant in the HIF-1α stability.

To understand whether decreasing HIF-1α phosphorylation levels might affect Pin1/HIF-1α interaction, we employed two different Gsk-3β activity inhibitors: the well known LiCl and the more specific SB-216763. Since LiCl treatment induces Gsk-3β inhibitory phosphorylation on Ser9 (Martin et al., 2009), here we confirme the kinase inhibition showing an increase in Ser9 phosphorylation. On the contrary, SB-216763 induces the dephosphorylation of Gsk-3β at Ser9 (Jaeger et al., 2013), nevertheless inhibits Gsk-3β activity in an ATP competitive manner (Cross et al., 2001).

Interestingly, data obtained after the treatment with Gsk-3β activity inhibitors showed: (i) a strong reduction of phosphorylated Ser/Thr-Pro motifs of HIF-1α protein; (ii) a considerable decrease of co-immunoprecipitated Pin1 amount; (iii) an higher HIF-1α protein level with respect to control. The reduction of HIF-1α phosphorylated motifs accompanied by protein levels increase, give reason for Pin1 binding decrease, suggesting that Pin1 might be involved in the Gsk-3β/HIF-1α stability regulation independent from the VHL pathway (Flügel et al., 2007). Indeed, Flügel et al. (2007) suggest that the regulation of HIF-1α degradation is not limited by the presence of oxygen, resulting therefore independent of HIF-1α hydroxylation and VHL-E3 ligase complex. Moreover, the F-box protein Fbw7, an E3 ubiquitin ligase, has been recently identified as novel critical component in HIF-1α degradation that is recruited to HIF-1α after its phosphorylation by Gsk-3β (Flügel et al., 2012): in a wider framework we can speculate that Pin1 might be involved in the Gsk-3β/Fbw7 HIF-1α degradation system. Taken together our experiments indicate for the first time that Pin1 interacts with HIF-1α in order to regulate the transcription factor protein levels. Therefore the decrease of Pin1 ability to bind and isomerize PSer/Thr-Pro motif might correlate with high levels of HIF-1α observed in post-OGD conditions (see a schematic representation in **Figure 7**). To deepen the OGD effect on HIF-1α and Pin1 interaction we analyzed Gsk-3β^{S9}, HIF-1α Ser/Thr-Pro motif phosphorylation and HIF-1α/Pin1 association. Interestingly, here we show that, Gsk-3β^{S9} inhibitory phosphorylation increased at R 1 h, correlating both to the Ser/Thr-Pro motifs phosphorylation decrease and HIF-1α/Pin1 association reduction. *Vice versa* at R o/n we observed a slightly increase of P-Ser/Thr-Pro motifs that however did not reach control levels and the co-immunoprecipitated Pin1 amount was quite equivalent to the control. This observation apparently in contrast with the HIF-1α increase might be explained by the fact that although Pin1 might be able to bind the transcription factor, since Pin1^{S16} phosphorylation decreased, the isomerase resulted enzymatically inhibited (see the PPIase assay in **Figure 2**). Hence, we hypothesize that in post-OGD hippocampal neurons, Gsk-3β and Pin1 inhibition lead to a decrease of HIF-1α ubiquitination affecting the consequent degradation of the transcription factor. Since, unconventional VHL-independent HIF-1α degradation pathway involving RACK1 has been already described (Isaacs et al., 2002; Semenza, 2010), here we propose that Pin1/Gsk-3β-mediated HIF-1α degradation may be a functionally predominant pathway in our cellular model, that instead of VHL requires the involvement of the E3 ubiquitin ligase Fbw7.

Furthermore, it has been recently proposed that Pin1 inhibits Gsk-3β activity (Ma et al., 2012) promoting Ser9 phosphorylation, however in our cellular model subjected to OGD this event

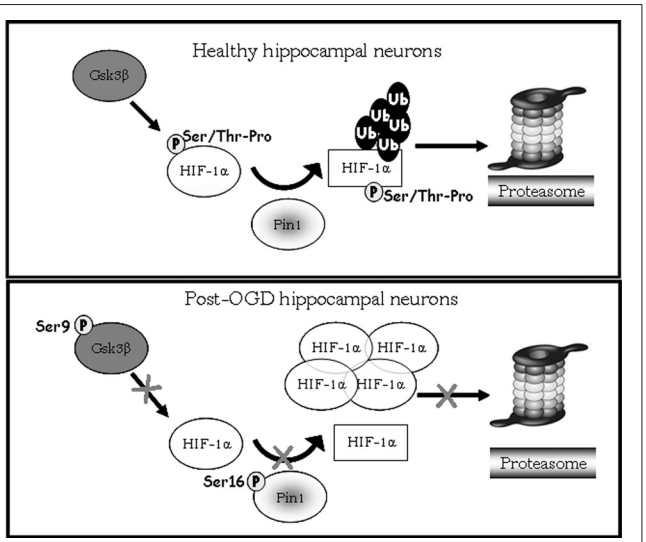

FIGURE 7 | Model showing HIF-1α degradation pathway mediated by Pin1/Gsk-3β. In healthy hippocampal neurons Gsk-3β phosphorylates the HIF-1α transcription factor on Ser/Thr-Pro motif potentially recognizible by Pin1. After phosphorylation Pin1 is able to recognize and bind the *cis* form of HIF-1α (circle form), and catalyzes its isomerization to *trans* conformation (square). The E3 ligase complex having a structural preference for phosphorylated substrates in *trans* conformation to favors ubiquitination and the consequent degradation of the transcription factor *via* proteasome. In post-OGD neurons, instead, Gsk-3β is inhibited by phosphorylation on Ser9, therefore HIF-1α is not phosphorylated on Ser/Thr-Pro motif resulting unrecognizable by Pin1. Moreover Pin1 itself is inhibited by phosphorylation on Ser16, and its enzymatic activity is blocked leading to loss of HIF-1α *cis/trans* isomerization. HIF-1α is not more ubiquitinated and degradated via proteasome, accumulating in its *cis* conformation.

seems to be independent from Pin1, deserving future experiments to deeply investigate.

For all above mentioned, under condition mimicking an ischemic event influencing Pin1 activity might be extremely detrimental causing intracellular molecular mechanisms deregulation, eventually leading to pathophysiological conditions such as in AD. In fact Pin1 has been identified as a common regulator of both Tau and APP pathologies (Lu and Zhou, 2007), regulating Tau phosphorylation levels and the NFTs formation as well as levels of Aβ peptide production (Liou et al., 2003; Pastorino et al., 2006).

Our group has recently demonstrated that after OGD the amount of peptide $A\beta_{42}$ increases both in neurons and in cerebrovascular endothelial cells (Bulbarelli et al., 2012), probably related to the increase of HIF-1α transcriptional activity (Zhang et al., 2007).

Notably, in parallel to HIF-1α high levels we observed the increase of BACE1 protein under our experimental conditions. Hence, in our hypothesis, the alteration in Pin1-mediated HIF-1α degradation resulting from an ischemic event, might accelerate APP amyloidogenic metabolism/$A\beta_{42}$ production in neurons, supporting the theory that cerebral hypoperfusion induces Aβ deposition. As well in AD the Pin1 deregulation might lead to a more rapid HIF-1α stability/activation inducing the expression of genes implicated in pathological intracellular mechanisms involved in vascular diseases and neurodegeneration.

In view of that there could be a pathogenic synergy between the two disease processes, stroke and AD might effectively share common risk factors (Iadecola, 2010): in this scenery the role of Pin1 in HIF-1α isomerization and degradation may outcome as central mechanism in vascular damages *vs.* AD and *vice versa*. Furthermore, considering that it has been recently demonstrated the correlation between Pin1 activity reduction and the essential hypertension (Wang et al., 2013a,b), the isomerase partial loss of function might be detrimental in multiple interrelated disease; hence the role of Pin1 in HIF-1α regulation might contribute to the morbidity of hypertension, becoming in turn a risk factor for stroke and AD.

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The role of extracellular Tau in the spreading of neurofibrillary pathology

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The microtubule-associated protein (MAP) tau plays a critical role in the pathogenesis of Alzheimer's disease (AD) and several related disorders collectively known as tauopathies. Development of tau pathology is associated with progressive neuronal loss and cognitive decline. In the brains of AD patients, tau pathology spreads following an anatomically defined pattern. Mounting evidence strongly suggests that accumulation of abnormal tau is mediated through spreading of seeds of the protein from cell to cell and point at the involvement of extracellular tau species as the main agent in the interneuronal propagation of neurofibrillary lesions and spreading of tau toxicity throughout different brain regions in these disorders. That would support the concept that pathology initiates in a very small part of the brain many years before becoming symptomatic, spreading progressively to the whole brain within 10–20 years. Understanding the precise molecular mechanism underlying tau propagation is crucial for the development of therapeutics for this devastating disorder. In this work, we will discuss recent research on the role of extracellular tau in the spreading of tau pathology, through synaptic and non-synaptic mechanisms.

Keywords: Alzheimer, exosomes, vesicles, neurodegeneration, propagation, spreading, tau, tauopathies

INTRODUCTION

A common pathological feature of many neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), amyotrophic lateral sclerosis (ALS) or prion diseases, among others, is the abnormal deposition of proteins in the brain. Among these pathological proteins, the MAP tau forms intraneuronal filaments in a spectrum of neurological disorders collectively known as tauopathies.

Tau protein is a MAP that under physiological conditions regulates microtubules (MT) assembly, dynamic behavior, and spatial organization, and has also been shown to regulate the axonal transport of organelles, including mitochondria. The gene encoding tau protein *MAPT* is located on chromosome 17q21.3, spans approximately 150 kb and consists of 16 exons (Pittman et al., 2006) from which six major isoforms are expressed in adult brain through alternative splicing (reviewed in Andreadis, 2012). The interaction between tau and tubulin is mediated by four imperfect repeat domains (encompassing 31–32 residues) encoded by exons 9–12 (Lee et al., 1989). Alternative splicing of exon 10 results in the production of isoforms containing 3 or 4 binding domains (3R and 4R tau) (Himmler et al., 1989).

Adult human brain contains equal amounts of 3R and 4R isoforms whereas foetal brain, however, only expresses 3R tau, demonstrating developmental regulation of exon 10 splicing (Goedert et al., 1989). Different brain regions also differ in the relative levels of 3R and 4R isoforms with granule cells in the hippocampal formation reported to have only 3R tau. Disturbances, usually increases, in the 3R/4R ratio are a common feature in most neurodegenerative tauopathies. Furthermore, morphological differences exist among different diseases or disease types as different tau isoforms are accumulated in diseased brains, namely, six tau isoforms in AD, 3R tau isoforms in Pick's disease, and 4R tau isoforms in profressive supranuclear palsy (PSP) and cortical basal degeneration (CBD; Goedert and Spillantini, 2011). Interestingly, a recent study has shown that the 4R/3R ration may have been underestimated in AD brains when compared with PSP or CBD, presumably due to extensive deamidation at Asn279 (Dan et al., 2013).

Within neurons, tau is predominantly found in axons (Hanger et al., 2009) as a highly soluble phosphoprotein (Iqbal et al., 2009). Phosphorylation is also developmentally regulated, with a high tau phosphorylation level during embryogenesis and early development, when only the shortest of the isoforms is being expressed. By contrast, adult brain expresses all six isoforms with relatively reduced phosphorylation levels compared with the foetal one (Liu et al., 2007).

The key discovery directly involving tau protein in neurodegeneration and dementia came from the finding that highly penetrant, dominant mutations in the *MAPT* gene encoding tau cause an inherited form of frontotemporal dementia and parkinsonism (Hutton et al., 1998; Poorkaj et al., 1998; Spillantini et al., 1998). A number of neurodegenerative disorders present prominent tau pathology in the CNS, predominantly within the neuronal compartment, but also within glial cells. Because of this

shared histopathological feature, they are referred collectively as tauopathies, although they constitute a group of etiologically heterogeneous, clinically and neuropathologically overlapping disease entities (Ballatore et al., 2007; Spillantini and Goedert, 2013). In tauopathies, the intracellular soluble tau forms filamentous structures of aggregated, hyperphosphorylated tau, which are associated with synaptic loss and neuronal death. The occurrence of fibrillar tau inclusions in tauopathies strongly supports a key role in the observed clinical symptoms and pathology.

Further insights into the overlapping pathogenic and etiologic aspects of the discrete diseases will help to design (perhaps common) disease-modifying treatment strategies (Medina, 2011). To achieve that goal however, it is critical to understand the normal biological roles of tau, the specific molecular events that induce tau to become neurotoxic, the biochemical nature of pathogenic tau, the means by which pathogenic tau exerts neurotoxicity, and how tau pathology propagates.

As mentioned, the recognition of the MAP tau as a key player in the pathobiology of human neurodegenerative diseases has led to major efforts to understand its biological and pathological function(s). This has resulted in an improved understanding of tau cellular functions beyond its classical role in stabilizing MT (Morris et al., 2011) to unveil novel physiological tau functions that may also play a role in pathogenesis. Such functions include axonal transport (Terwel et al., 2002; Rodríguez-Martín et al., 2013), neuronal polarization (Caceres and Kosik, 1990; Dawson et al., 2010), axonogenesis (DiTella et al., 1994; Klein et al., 2002; Belkadi and LoPresti, 2008), interactions with the plasma membrane (Brandt et al., 1995; Lee et al., 1998; Maas et al., 2000), signal transduction (Lee et al., 2004; Ittner et al., 2010) and cell cycle (Andorfer et al., 2005). Furthermore, despite lacking an identified nuclear localization signal, tau has also been reported in nuclei in a number of cell lines (Loomis et al., 1990; Wang et al., 1993) and human brain (Brady et al., 1995) where it may play a role in DNA protection (Sultan et al., 2011).

EXTRACELLULAR TAU

It has been over 20 years since the original report that intracellular tau levels are increased in the brains of AD patients when compared to non-demented controls (Barton et al., 1990; Khatoon et al., 1992). This increase in the amount of tau could be toxic to neurons since a reduction in the amount of intracellular tau has indeed a protective effect in mouse models of neurodegeneration (Rapoport et al., 2002; Roberson et al., 2007) and it has been suggested that reducing tau levels may be therapeutically beneficial (Götz et al., 2013). However, we must be cautious since other studies in similar tau-deficient mice point in the opposite direction, suggesting that loss of tau function can actually lead to neurodegeneration (Dawson et al., 2010).

Little is known about how tau synthesis is regulated although some factors such as fibroblast growth factor (Tatebayashi et al., 1999), Dyrk1A (Qian et al., 2013), or the haplotype H1 have been involved in increased synthesis whereas the miRNA-34 family (Dickson et al., 2013) seems to downregulate tau levels.

Conventional wisdom has suggested that the presence of tau in the brain parenchyma or in the cerebrospinal fluid (CSF) is a consequence of tau protein being released from dead cells. However, this has recently been challenged by a number of studies showing extracellular tau being released from cell lines and neurons via multiple pathways, strongly supporting the notion that secretion of tau protein may be an important biological function of tau protein, especially in disease. Despite the fact that tau lacks a signal sequence a number of reports have now shown that tau is released into culture medium from neuroblastoma cells, tau-expressing non-neuronal cells, induced pluripotent stem cell-derived human neurons, and mouse primary neurons (Kim et al., 2010; Shi et al., 2012). Thus, tau has been reported to be secreted unconventionally in naked form (Chai et al., 2012) or associated to exosomes (Saman et al., 2012) and/or other membrane vesicles (Simón et al., 2012a). Since increased tau cellular levels are detrimental, secretion has been proposed as a mechanism to eliminate the excess of tau protein thereby avoiding its toxicity (Simón et al., 2012b). Interestingly, while full length tau has been detected in the extracellular space, C-terminal cleavage of tau has been shown to enhance its secretion (Plouffe et al., 2012) which could have pathological relevance since some truncated tau species appears to be characteristic of particular tauopathies whereas other tau fragments may be common to several tauopathies (Hanger and Wray, 2010; Kovacech et al., 2010).

Extracellular tau has also been detected in the brain interstitial fluid of both wild-type and P301S tau-expressing mice in microdialysis studies (Yamada et al., 2011), as it has also been the case in patients following severe traumatic brain injury (Marklund et al., 2009; Magnoni et al., 2012). Actually, exosomal tau secretion has been suggested to account for the elevated CSF tau levels typically observed in early AD (Saman et al., 2012). Interestingly, tau mutations that are associated with the development of tauopathy appears to reduce tau release (Karch et al., 2012). Interestingly, physiological secretion of endogenous tau by cortical neurons appears to be regulated by neuronal activity, as tau release is enhanced by glutamate receptor stimulation induced by the agonist *S*-AMPA (Pooler et al., 2013). This process is calcium-dependent and modulated by phosphorylation and released tau is present in a relatively dephosphorylated state, compared to that of intracellular tau.

Thus, increasing evidence point out to extracellular tau as a physiological process independent of cell death (**Figure 1**), although the precise relationship between tau release under physiological conditions and the propagation of pathology in AD and other tauopathies remains to be determined.

Tau can be toxic when applied extracellularly to cultured cells (Gómez-Ramos et al., 2006; Kopeikina et al., 2012). Several mechanisms for internalization of tau has been proposed, such as internalization of soluble, uncoated ("naked") tau via receptor-mediated endocytosis (Gómez-Ramos et al., 2009), dynamin-driven endocytosis of non-fibrillar, soluble tau aggregates (Wu et al., 2013) or even actin-dependent, proteoglycan-mediated macropynocytosis (Holmes et al., 2013). Furthermore, it has been suggested that extracellular tau might provoke a receptor-activated increase in intracellular calcium through M1/M3 muscarinic receptor stimulation (Gómez-Ramos et al., 2008; Díaz-Hernández et al., 2010) and that such receptor activation could lead to endocytosis of extracellular tau. Remarkably,

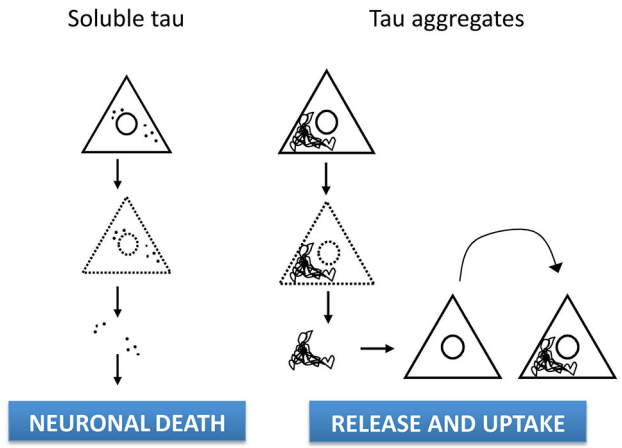

FIGURE 1 | Cell-to-Sources of extracellular tau and -cell spreading. The established view has long considered the presence of tau in the brain parenchyma or in the CSF as a consequence of tau protein being released after cell lysis. However, extracellular tau appears to result from a physiological process independent of cell death, as tau being can be released from cell lines and neurons via multiple pathways, either in naked form or vesicle-associated. In addition, tau misfolding in diseased brain leads to abnormal conformations of tau that can be taken up by surrounding neurons. Thus, pathological progression could involve transmission of tau protein through a prion-like mechanism resulting in neurodegeneration in susceptible brain regions.

tau phosphorylation could inhibit its interaction with M1/M3 receptors and it has been proposed that such alterations might be involved in the transmission of tau pathology (Simón et al., 2013).

The discovery of extracellular tau as a physiological process that is independent of cell death (Pooler et al., 2013), indicates that tau release does not occur only as a result of reduced neuronal viability, and therefore that the increased tau observed in interstitial fluid and CSF in tauopathies may not be due solely to tau release from dying neurons (Yamada et al., 2011; Nedergaard, 2013). It is worth mentioning that tau phosphorylation at threonine 181 and total tau levels in CSF are considered useful biomarkers of neuronal degeneration or injury in the recent National Institute on Aging and Alzheimer Association (NIA-AA) revised criteria for the diagnosis of AD (Jack et al., 2012).

PROPAGATION OF TAU PATHOLOGY

Development of tau pathology is associated with progressive neuronal loss and cognitive decline. In the brains of AD patients, tau pathology propagates following an anatomically defined pattern described by the neuropathological Braak sequential staging (Braak et al., 2011). As a matter of fact, recent NIA-AA guidelines recommend the assessment of Braak and Braak staging of neurofibrillary degeneration as part of the so called "ABC score" for the neuropathological diagnosis of AD (Montine et al., 2012).

The originally staging system (Braak and Braak, 1991) defined six stages based on the presence and density of characteristic argyrophylic inclusions (neurofibrillary tangles (NFT), neuropil threads) in the medial temporal lobe and several brain isocortical regions. This system was subsequently adapted by the authors for routine use in paraffin-embedded tissue based on tau immunohistochemistry (Braak et al., 2006). Stages I–II (transentorhinal) correlate with the lengthy preclinical phase of the disease; whereas stages III–IV (limbic) do so with mild cognitive impairment (loss of episodic memory) or mild dementia; and advanced V–VI stages (isocortical) usually correspond to cases with moderate to severe dementia. Accurate staging of AD-related tau-positive pathology may be particularly important in the classification of preclinical disease and in the identification of atypical AD phenotypes. The above mentioned NIA-AA guidelines allow for standardization for diagnostic and research purposes.

Clinicopathological studies show that tau pathology progression from the entorhinal cortex through the hippocampus and into the limbic and association cortex is the main neuropathological variable that correlates with the clinical cognitive status of the patient (Arriagada et al., 1992; Nelson et al., 2012). Whether that pattern of accumulation reflects cell-to-cell spreading of disease, or simply successive involvement of differentially resistant neuronal populations, has been a matter of debate in recent years. Recent evidence from human studies suggests that tau pathology is actually linked to existing networks of neuronal connectivity. Thus, rather than diffuse, random, or confluent, tau pathology would target specific large-scale distributed networks that in the healthy brain feature convergent intrinsic functional and structural covariance (Seeley et al., 2009). However, the precise molecular and cellular mechanisms by which tau propagates and neuronal networks degenerate are still unknown.

Increasing evidence suggests that synaptic dysfunction is a key pathophysiological hallmark in neurodegenerative disorders, including AD which has been indeed considered a synaptopathy (Selkoe, 2002; Sheng et al., 2012), as synapse density best correlates with the cognitive decline observed in patients. Long regarded primarily as an axonal protein, when hyperphosphorylated tau also accumulates in the somatodendritic compartment during AD (Ballatore et al., 2007). Actually, tau mislocation in dendritic spines has been proposed to lead to synaptic dysfunction by various mechanisms, including regulating the amount of glutamate receptors in spines (Hoover et al., 2010), interacting with post-synaptic signaling complexes, targeting of synaptic mitochondria (Pooler et al., 2014) or destabilizing dendritic spines and dendritic arbor (Koleske, 2013). Presence of tau in the synapse in healthy brains suggests a role for tau in regulating normal synaptic function whereas during neurodegeneration, tau synaptotoxicity seems to be related to soluble forms rather than insoluble aggregates (Pooler et al., 2014).

On the other hand, emerging evidence strongly suggests that tau is essential for Aβ-induced synaptotoxicity (Ittner et al., 2010), a process that may involve EphB2, and NMDA receptors (Cissé et al., 2011; Sheng et al., 2012). Furthermore, studies in mouse organotypic hippocampal slice cultures from amyloid precurssor protein transgenics have demonstrated that extrasynaptic NR2B-containing NMDA receptors are required for tau-induced neurodegeneration (Tackenberg et al., 2013). Could then tau play a role in the transition between synaptic and extrasynaptic NMDA receptors? Although we do not have a definitive answer to that, the improved NMDA receptor antagonist nitromemantine protects against Aβ-induced synaptic dysfunction (Talantova et al., 2013). Nitromemantine selectively inhibits extrasynaptic over synaptic

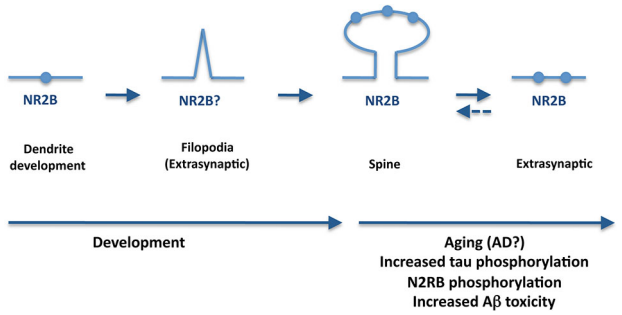

FIGURE 2 | A potential role for tau in synapsis during aging and AD. During AD, tau is hyperphosphorylated and mislocates to the axonal compartment. Furthermore, tau appears essential for Aβ-induced synaptotoxicity whereas extrasynaptic NR2B-containing NMDA receptors are required for tau-induced neurodegeneration. An increase of extrasynaptic receptors with aging and AD may also explain the progression of the disease.

NMDA receptor activity (Kaufman et al., 2012), thus preventing the toxic effect of the activation of extrasynaptic NMDA receptors. Aβ binding to synaptic or extrasynaptic receptors may lead to different signaling and consequences (protection or death) (Li et al., 2011). Increase of extrasynaptic receptors with aging and AD may also explain the progression of the disease (**Figure 2**).

Interestingly, recent *in vivo* studies in tauopathy transgenic mouse models expressing human mutant tau specifically in the entorhinal cortex have shown relocation of tau from axons to the somatodendritic compartment as well as propagation of tau pathology to regions outside the entorhinal cortex, strongly suggesting a trans-synaptic mechanism of spreading of pathology through anatomically connected neuronal networks (de Calignon et al., 2012; Liu et al., 2012). These findings have been further supported by more recent neuropathological studies in post-mortem brains from argyrophylic grain disease (AGD), a sporadic tauopathy mainly involving the medial temporal lobe and the limbic region (Ferrer et al., 2008). This pathology exhibits a short number of closely related tau-positive inclusions and a highly homogeneous pattern of distribution and progression of pathology along several regions of the medial temporal lobe with known connectivity between them and with extra-temporal areas of involvement, leading to its proposition as a natural model for studying tau propagation in human brain (Rábano et al., 2014).

Recently, release and subsequent uptake of tau fibrils that directly contact native protein in recipient cells have been shown to mediate propagation of tau misfolding among cells, at least *in vitro* (Frost et al., 2009; Kfoury et al., 2012). Remarkably, intracerebral inoculation of synthetic preformed tau fibrils induced NFT-like inclusions that propagated from injected sites to connected brain regions in a time-dependent manner (Iba et al., 2013). Furthermore, conformation-specific trans-cellular propagation of tau fibrils after release into the extracellular space and subsequent triggering of aggregation in recipient cells by contacting native protein has been show in co-culture experiments (Kfoury et al., 2012). Thus, newly aggregated intracellular tau can transfer between co-cultured cells (**Figure 1**), thus providing a mechanism for tau-targeted immunotherapies as therapeutic strategy for AD and tauopathies (Gu and Sigurdsson, 2011; Medina, 2011; Golde et al., 2013). Actually, it has been suggested that the most likely mechanism of action for anti-tau antibodies is targeting tau released from cells (Yanamandra et al., 2013). The recent development of imaging-based biomarkers (Maruyama et al., 2013) will enable to track the progression of tau pathology in living patients and greatly facilitate the early phase testing of tau immunotherapy and other tau-based therapeutic strategies.

CONCLUSIONS

In summary, we have highlighted recent developments in tau biology relevant to AD and tauopathies. It has become increasingly clear that, apart from the well-established intracellular functions of tau in microtubule stabilization and axonal transport, intracellular and extracellular tau most likely have important signaling roles that could contribute to the neurodegenerative process in AD and related tauopathies. Furthermore, the presence of tau in synaptic regions of healthy brain suggest that tau may play a role in the regulation of normal synaptic function. In addition, recent studies have suggested that misfolding of tau in diseased brain leads to abnormal conformations of tau that can be taken up by surrounding neurons. Thus, pathological progression could involve transmission of tau protein through a potential prion-like, seeding mechanism resulting in neurodegeneration in susceptible brain regions. However, insufficient evidence exists yet to reliably determine whether there is a direct relationship between the recent identification of a physiological role for extracellular tau and the impairments in tau function associated with disease.

Key questions still remain open, such as the neuronal selectivity, the nature of the extracellular tau species involved, or the precise seeding/templating mechanisms, among many others. More research is needed to identify disease mechanisms driving release and propagation of tau pathology and to determine the impact of extracellular tau on cognitive decline during neurodegeneration. The observation that misfolded tau can be secreted and taken up by adjacent neurons calls for the development of novel strategies to block the propagation of tau pathology in the brain. Despite the fact that the presence of extensive tau pathology is central to the disease, tau-based therapeutic strategies have received little attention until recently (Medina and Avila, 2014). Next few years will certainly bring new insights into the cellular mechanisms underlying tau secretion and uptake, likely identifying novel therapeutic approaches intended to interfere early on in the process of propagation of tau pathology.

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Multiple effects of β-amyloid on single excitatory synaptic connections in the PFC

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Prefrontal cortex (PFC) is recognized as an AD-vulnerable region responsible for defects in cognitive functioning. Pyramidal cell (PC) connections are typically facilitating (F) or depressing (D) in PFC. Excitatory post-synaptic potentials (EPSPs) were recorded using patch-clamp from single connections in PFC slices of rats and ferrets in the presence of β-amyloid (Aβ). Synaptic transmission was significantly enhanced or reduced depending on their intrinsic type (facilitating or depressing), Aβ species (Aβ40 or Aβ42) and concentration (1–200 nM vs. 0.3–1 μM). Nanomolar Aβ40 and Aβ42 had opposite effects on F-connections, resulting in fewer or increased EPSP failure rates, strengthening or weakening EPSPs and enhancing or inhibiting short-term potentiation [STP: synaptic augmentation (SA) and post-tetanic potentiation (PTP)], respectively. High Aβ40 concentrations induced inhibition regardless of synaptic type. D-connections were inhibited regardless of Aβ species or concentration. The inhibition induced with bath application was hard to recover by washout, but a complete recovery was obtained with brief local application and prompt washout. Our data suggests that Aβ40 acts on the prefrontal neuronal network by modulating facilitating and depressing synapses. At higher levels, both Aβ40 and Aβ42 inhibit synaptic activity and cause irreversible toxicity once diffusely accumulated in the synaptic environment.

Keywords: β-amyloid (Aβ), synaptic connection, synaptic dynamics, excitatory post-synaptic potential (EPSP), short term potentiation (STP), synaptic augmentation (SA), post-tetanic potentiation (PTP)

INTRODUCTION

In patients and animal models of the early stages of Alzheimer's Disease (AD), declines in episodic or spatial memory and cognition are correlated with an increase in brain levels of soluble β-amyloid (Aβ) (Lue et al., 1999; Walsh et al., 2002; Rowan et al., 2003). A causal link between the accumulation of Aβ in a soluble, toxic state and impairment of neuronal mechanisms that support memory was demonstrated in a mature βAPP transgenic mouse model wherein a single systemic injection of an antibody to Aβ eliminated the memory deficit (Dodart et al., 2002). There is a major focus on the synapse as the initial site of damage in AD (Selkoe, 2002; Nimmrich and Ebert, 2009). Synaptic dysfunction as a consequence of diffusible Aβ is also inferred from studies showing reduced basal transmission and altered plasticity (Klyubin et al., 2005; Shankar et al., 2008; Minano-Molina et al., 2011). In anatomic terms, synapse numbers are reduced early in some AD brain regions (Davies et al., 1987), especially in the prefrontal cortex (PFC) and medial temporal lobe (Morris and Baddeley, 1988). Meanwhile, additional studies in the recent decade indicate that low levels of Aβ peptides could be essential for the modulation of synaptic plasticity (Parihar and Brewer, 2010).

In AD, limbic and association cortices are selectively involved while primary cortical areas remain relatively preserved. These regions of neuronal vulnerability in fact correspond to the degree to which neuronal plasticity can be demonstrated in them (Arendt, 2001). The PFC is a critical association region associated with executive type cognitive function. PFC also orchestrates a unique form of short-term memory termed "working memory." Working memory is a limited capacity system that supports non-routine types of daily activity. It is a temporary storage system for maintaining and rapidly manipulating information, and is closely connected with attention, strategic information flow and action (Goldman-Rakic, 1996). Experimentally, prefrontal cortical neurons are found to remain persistently active during the delay between sensory cue and an executed response task. The ongoing activity, an electrical correlate of working memory, is stable to the interference from distractors (Goldman-Rakic, 1995). Further, fMRI studies confirm the role of PFC in strategic encoding and goal directed control over the retrieval process in episodic type memory processes (Simons and Spiers, 2003). Less appreciated than episodic memory, working memory is also impaired in the early stages of AD, according to clinical and *in vivo* studies (Morris and Baddeley, 1988). Impairment of PFC function may precede the pathological changes of AD in other cortical association areas (Reid et al., 1996). Correspondingly, soluble Aβ accumulates in the PFC to one of the highest and earliest levels across several cortical regions in the pre-tangle stages of AD (Gouras et al., 2000) and in transgenic mice (Zhuo et al., 2008). Tangle formation in the PFC is also highly correlated with the transition to clearly recognizable dementia (Wang and Al, 2001).

In the PFC network, excitatory synaptic connections in layer V show both facilitated (F-connection) and depressed (D-connection) excitatory post-synaptic potentials (EPSPs) in response to short train stimuli (Wang et al., 2006). F-connections are formed predominantly by complex-type pyramidal cells (PCs) which feature dual apical dendrites, a high degree of interconnectivity and of reciprocity in chemical synaptic connections. In contrast, D-connections are typically formed by simple PCs that are common to primary cortices. A computer simulation study revealed that these facilitating synapses play a crucial role in the formation of persistent neuronal activity, consistent with the properties of working memory (Mongillo et al., 2008). The critical role of the PFC in working memory and early involvement in AD make it a suitable region to examine the electrophysiologic effects of Aβ.

Numerous studies of AD-promoting factors (e.g., Aβ) have examined their effects on field electrophysiological characteristics in the hippocampus and cortex. Because of competing synaptic inputs and influences from other local networks, the use of field recordings may account for seemingly contradictory early reports, where Aβ either increased excitability through membrane depolarization and enhancing long term potentiation (LTP) or depressed both synaptic transmission and LTP induction (Wu et al., 1995; Selkoe, 2002; Walsh et al., 2002; Esteban, 2004; Puzzo et al., 2008; Li et al., 2011). Up until now, a study focused on activity-dependent plasticity specific to the association cortex has not been reported. It is therefore timely to carry out an investigation of an *in situ* neural network, especially at the resolution of individual synaptic connections, within an association cortical area such as the PFC.

In this study, using multi-neuron patch clamp recording from PFC slices, we found that synaptic responses of single excitatory synaptic connections were significantly enhanced or reduced depending on their intrinsic type (facilitating or depressing), the tested Aβ species (40 or 42 amino acids) and concentration (low dose 1–200 nM vs. high dose 0.3–1 μM). Low-doses of Aβ40 enhanced F-connections and inhibited D-connections in the PFC. In contrast, high-doses of Aβ40 and low-doses of Aβ42 inhibited all types of excitatory synaptic connections. Further, the inhibition induced with bath application was commonly difficult to recover or even became worse by washout. However, direct local and brief application of the peptides by pipette at comparable concentrations produced similar inhibitions with a rapid and complete recovery upon washout. Based on the principles of synaptic dynamics that have been well-studied in our previous computer simulation of synaptic responses of single synaptic connections, the effects of Aβ were considered to be produced via both pre- and post-synaptic mechanisms.

MATERIALS AND METHODS

ELECTROPHYSIOLOGICAL RECORDINGS

Prefrontal cortical slices were prepared using a published protocol (Wang et al., 2006). Briefly, brain was dissected from normal adolescent Wistar rats (day 25–35) or young adult ferrets (7–9 weeks old). PFC slices (300 μM) were sectioned using a vibratome (DTK 1000 Zero 1 Microslicer) and then incubated in artificial cerebrospinal fluid (ACSF) before transfer to a recording chamber (at 34°C). Neurons in layer V of the medial PFC were visually identified using infrared differential contrast videomicroscopy (BX50WI, Olympus). An advanced technique consisting of quadruple patch clamp recording was used to record from candidate cell bodies (somata), and single synaptic connections formed between neuron pairs were determined electrophysiologically according to standard characteristics of chemical synaptic transmission (Wang et al., 2006). Somatic whole-cell signals (6–12 mΩ pipette resistance) were amplified using Axoclamp-200B amplifiers (Axon Instruments, USA). Recordings were sampled over real time and filtered using the program Igor (Igor Wavemetrics, Lake Oswego, OR, USA), digitized by an ITC-18 interface (Instrutech, Great Neck, NY, USA) and stored on hard drive (Macintosh G5 computer) for off-line analysis (Igor). Stimulating and voltage recording glass micropipettes were filled with (mM): 100 potassium gluconate, 20 KCl, 4 ATP-Mg, 10 phosphocreatine, 0.3 GTP, 10 Hepes (pH 7.3) and 0.4% biocytin (Sigma). Presynaptic action potentials (AP) were elicited using short (3 ms), suprathreshold, intracellular depolarizing current pulses. The extracellular recording solution consisted of ACSF, containing (mM): 125 NaCl, 2.5 KCl, 25 glucose, 25 $NaHCO_3$, 1.25 NaH_2PO_4, 2 $CaCl_2$, and 1 $MgCl_2$. Only neurons with stable access resistance were included in the statistical analyses. Membrane potentials were routinely voltage-clamped at -70 ± 2 mV to maintain Vm against drift by using small current injections. Neurons were filled with 0.4% biocytin (Sigma) by diffusion at the end of the recordings for later identification of neuronal types.

Once synaptic connections were obtained, the EPSP failure rate, certain dynamic features of EPSPs and short term potentiation components including synaptic augmentation (SA) and post-tetanic potentiation (PTP), were recorded (Wang et al., 2006). For the EPSP failure rate, single APs were repeatedly evoked (0.5 Hz, 15–30 times) in a presynaptic cell and the number of corresponding EPSP failures in a postsynaptic cell were counted. For the synaptic dynamic features, an EPSP train was evoked by 6–8 presynaptic APs at 10–20 Hz followed by a recovery test response (RTR) after a 500 ms delay. SA and PTP were induced by giving a 15 pulse (tetanus) stimulus at 50 Hz. Single test responses (0.5 Hz) were recorded for 20 s before and up to 100 s after the train. This procedure was repeated four times, each preceded by a 2 min interval.

HISTOLOGICAL PROCEDURES AND 3D COMPUTER RECONSTRUCTION

After recording, the slices bearing biocytin-injected neurons were fixed for at least 24 h in cold 0.1 M phosphate buffer saline (PBS, pH 7.4) containing 2% paraformaldehyde, 1% glutaraldehyde, and 0.3% saturated picric acid. Thereafter, the slices were rinsed several times (10 min each) in PBS. To block endogenous peroxidases, slices were transferred into phosphate-buffered 3% H_2O_2 for10–30 min. After five to six rinses in PBS (10 min each), slices were incubated overnight at 4°C in avidin-biotinylated horseradish peroxidase according to the manufacturer's protocol (ABC-Elite, Vector Labs, Petersborough, UK) (2% A, 2% B, and 1% Triton-100). Following incubation and additional rinses, the reaction was developed with diaminobenzidine (DAB) under visual control using a bright-field microscope (Zeiss, NY,

USA) until all cell processes appeared clearly visible (usually after 2–4 min). The reaction was stopped upon transferring the sections into PBS. Slices were mounted in aqueous mounting medium.

3D neuron models were reconstructed from stained cells using the Neurolucida system (MicroBrightField Inc., USA) and a bright-field light microscope (Olympus, BX51, Japan). Reconstructed neurons subsequently underwent quantitative analysis using the NeuroExplorer (MicroBrightField Inc., USA; ×60 magnification, numerical aperture = 0.9; Z-axis resolution = 0.37 μm). Putative synapses were identified according to the criteria as published (Wang et al., 2002).

PREPARATION AND TREATMENT OF SOLUBLE SYNTHETIC Aβ

Soluble synthetic Aβ peptides (Aβ40, Aβ25–35, and Aβ42) were purchased from Biosource (Camarillo, CA) or the Harvard Protein Core laboratory and prepared as 0.1 mM stock following published methods and stocked at −80°C (Stine et al., 2003). The quality of monomer stock solutions were examined periodically using western blots [NuPAGE Novex 4–12% Bis-Tris gels (Invitrogen)] (**Figure A1**). Mouse monoclonal anti-human amyloid beta protein antibody (6E10, purchased from Signet) was used at a 1000X dilution. Horse anti-mouse IgG HRP-linked antibody (Cell Signaling Technology, 2000X dilution) was used as secondary antibody and the development with Chemiluminol that followed was captured on hyperfilm (Amersham Biosciences). Different concentrations of Aβ were freshly prepared before use by defrosting and diluting the stock solution with ACSF. Only monomer predominant stock solution was used in the current study. Soluble Aβ was bath-applied either at low dosage (1–200 nM) or at high dosage (300 nM–1 μM) (see Chen et al., 2000). During recording, brain slices were continuously perfused with oxygenated ACSF at a flow rate of 0.75–1.0 ml/min. The ACSF volume in the tube leading to and including the recording chamber was 1.5–2.0 ml. This enabled quick replacement of the recording solutions (≤3 min.) when switching between experimental procedures. Each recording procedure was repeated under three conditions: (1) pre-application, (2) application, and (3) washout of Aβ. The Aβ application recordings typically lasted for 30 min. whereas washout recordings lasted for 10–30 min.

MODELING ANALYSIS OF SYNAPTIC RESPONSES

The quantitative analysis of basal synaptic dynamic properties of excitatory connections has been carried out using a well-known computer model of a combination of EPSP train and a RTR evoked with a 500 ms delay (Markram et al., 1998; Tsodyks et al., 2000; Wang et al., 2006). The RTR is used to test the recovery of synaptic facilitation or depression, which characterizes the synapse type. The model extracts four key parameters of the connection: *DFUA* (*D*, the time constant of recovery from depression (ms); *F*, the time constant of recovery from facilitation (ms); *U*, utilization of synaptic resources, analogous to the neurotransmitter release probability (*p*); *A*, the absolute strength of a synaptic connection (nA), defined as its maximum response when $p = 1$). This modeling approach is based on fitting the mean output behavior of synaptic connections and therefore requires inputting only averaged responses (i.e., average EPSP traces). Generally speaking, reductions in *A* correspond to the situation when the amplitudes of all EPSPs in the train and RTR become smaller, keeping an unchanging EPSP pattern. In the case of reductions in *U*, the amplitude of the 1st EPSP is reduced whereas subsequent EPSPs are facilitated. When normalized to the first EPSP in such recordings, the subsequent EPSP amplitudes, but not the RTR, are magnified. In the case of a larger *D* value, both EPSPs (subsequent to the 1st) and the RTR show reductions. Oppositely, a high value for the parameter *F* correlates to the situation in which both EPSPs (subsequent to the 1st) and the RTR are increased.

STATISTICAL ANALYSIS

Paired student *t*-test was used to compare EPSP responses between different conditions: [(1) pre-application, (2) application, and (3) washout of Aβ.]. Unpaired student *t*-test was used for the comparison of EPSP responses between different treatments of Aβ. The statistical analyses of EPSP train, SA and PTP were all based on an intrinsic comparison of individual single synaptic connections between the Aβ application or washout condition with the pre-application condition. In order to lessen the influence from the variance of synaptic strength between individual connections, the statistical comparisons were made using normalized values. In the analysis of EPSP train, all EPSP values of a connection were normalized to the mean of EPSPs recorded under the pre-application condition. In the analysis of SA and PTP, all EPSP values of a connection were normalized to the mean of pre-tetanus EPSPs obtained in the pre-Aβ application condition. Furthermore, an EPSP pattern defined by a certain "8-EPSP train + RTR" configuration for each type of synapse, allowed each EPSP value to be treated as an independent outcome value in the statistical comparison. Multiple outcome values per connection were therefore used in the EPSP train analysis. The same principle was also applied to the analyses of SA and PTP.

RESULTS

Quadruple patch clamp recording was performed to record synaptically coupled pairs ($n = 100$) in layer V of the PFC of rats ($n = 92$, age P25–P35) and ferrets ($n = 8$, age 7–9 weeks old). Since synaptic dynamics (i.e., depressing and facilitating types) of synapses are consistent across species (Wang et al., 2006), the data of the two species were pooled together in order to maximally utilize the obtained data. The studied connections comprise synapses formed between PCs (PC–PC, $n = 86$ pairs) and those formed by a PC onto an interneuron (PC–IN, $n = 14$ pairs) (**Figure 1**). The neuronal type was identified according to the morphology (PCs and interneurons—mainly basket cells and Marttinoti cells) combined with the firing pattern of APs evoked by depolarizing current steps injected into neuronal somata (Wang et al., 2002, 2006). EPSP responses of a postsynaptic cell were induced by APs evoked by brief depolarizing current injections delivered into the presynaptic neuronal soma. We previously characterized the excitatory neuronal network in layer V of the PFC (Wang et al., 2006). Facilitation-dominant synapses (F-connections) are abundant in the PFC while depression-dominant synapses (D-connections), typically common in primary cortical areas, form a minor population in

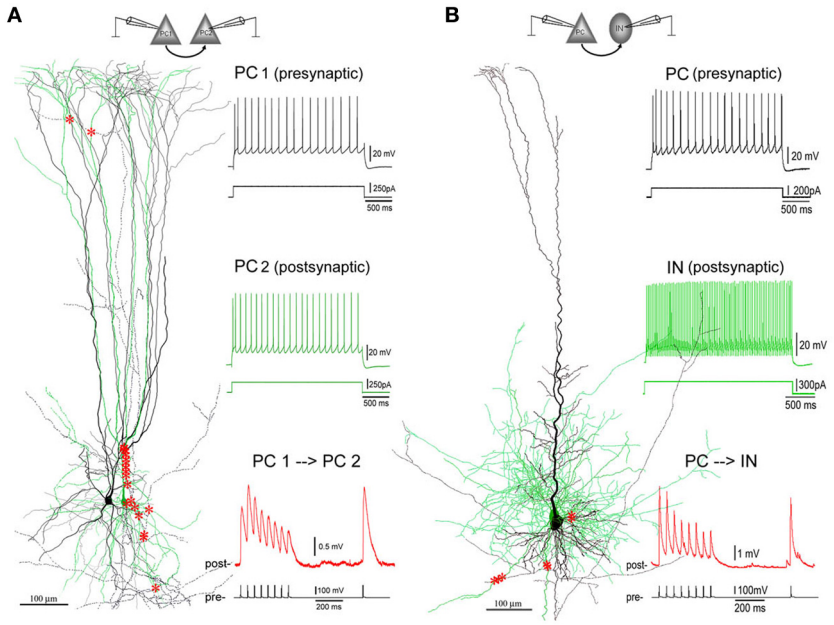

FIGURE 1 | Excitatory synaptic connections in layer V of PFC. (A) A facilitating (F-type) PC-PC connection in layer V of the PFC of an 8-weeks old ferret. Left panel: 3D computer reconstruction of the connection: Both pre- (PC1 in black) and post-synaptic (PC2 in green) cells are complex PCs featured by an apical dendrite with multiple early-bifurcated major branches. A total of 20 putative synapses are marked with red stars onto the basal, apical, oblique and tuft dendrites of PC2. Right panel: Physiological responses of pre- (upper, in black) and post-synaptic (middle, in green) cells were induced by injections of depolarization currents into their somata. Excitatory postsynaptic potentials (EPSPs, down, in red) were recorded from PC2 by giving brief current injections to induced action potentials (APs, bottom, in black) in PC1. The postsynaptic response train is composed of 8 EPSPs at a 20 Hz frequency followed by a recover test response (RTR) with 500 ms delay. **(B)** A depressing (D-type) PC-IN connection in layer V of the PFC of a P30 rat. The color coding for the reconstructed pre- and post-synaptic cells and their physiological and synaptic responses are the same as the PC-PC connection in **(A)**. A total of 7 putative synapses are marked with red stars onto basal dendrites of the postsynaptic interneuron. Note: The postsynaptic interneuron appears to be a fast-spiking basket cell according to its axonal and dendritic morphologies and fast AP firing induced by depolarization current injection to its soma.

the PFC. In order to examine effects of beta-amyloid peptides (Aβ40, Aβ25–35, Aβ42) on single F- and D-connections, low- (1–200 nM) and high-dosages (0.3–1 μM) of Aβ were continuously bath-applied while recording of EPSPs. The failure rate, synaptic dynamics and STP were investigated. The experimental procedure of applying Aβ was successfully performed in 48% of recorded connections (48 out of 100 pairs: rats, PC–PC, $n = 35$ pairs and PC–IN, $n = 7$ pairs; ferrets, PC–PC, $n = 6$ pairs). Unfortunately, synaptic responses of the other 52 connections became unstable or even disappeared in the middle of Aβ application. These unstable connections were excluded from the data analysis.

INFLUENCES OF Aβ ON FAILURE RATES OF F- AND D-CONNECTIONS

Presynaptic APs can fail to induce the release of neurotransmitter resulting in failures of evoked EPSPs. F-connections generally display higher failure rates than do D-type connections because the initial release probability of F-connections is usually lower. Single APs in presynaptic cells were generated at 0.5 Hz and EPSP failure rates of F- and D-connections were observed, respectively, under conditions of pre-application (in ACSF only), Aβ application and washout. An example of widening transmission failure in an F-type synapse exposed to 1 μM Aβ40 and moderate recovery upon washout is given in **Figure 2A**. Surprisingly, we found that lower doses of Aβ40 tended to reduce the synaptic failure rate in F-connections. In contrast, the transmission failure rate became increased under all other studied conditions, including low-dose Aβ40-bathed D-connections, high-dose Aβ40 or low-dose Aβ42 applied to F- or D-connections (**Figure 2B**). The increase in failure rate was statistically significant for the cases of low and high doses of Aβ40 to D-connections ($P = 0.01$ and $P = 0.024$, respectively). A trend toward higher failure rate, although not statistically significant, was clearly visible in the cases of high-dose Aβ40 to F-connection ($n = 3$) and low-dose Aβ42 to F- ($n = 3$) and D- ($n = 4$) connections. The opposing directions in failure of synaptic responses was present in **Figure 2C**, wherein, the net failure reduction in the case of low-dose Aβ40 to F-connections was opposite in direction to the net failure increase in all other cases. After washout for 10–30 min, these contrasting net changes in failure rates virtually remained (**Figure 2D**). Note the failure rate was further increased in the case of low-dose Aβ42 to F- and D-connections following washout (**Figure 2B**, far right set: compared with pre-application, $P = 0.04$; compared with Aβ application, $P = 0.05$). This phenomenon indicates that Aβ42 is selectively more toxic to synaptic connections in the PFC.

DIFFERENTIAL EFFECTS OF Aβ ON SYNAPTIC DYNAMICS OF F- AND D-CONNECTIONS

EPSP trains generated by 5–8 APs and RTR 500 ms later were used in a phenomenological modeling strategy to estimate dynamic

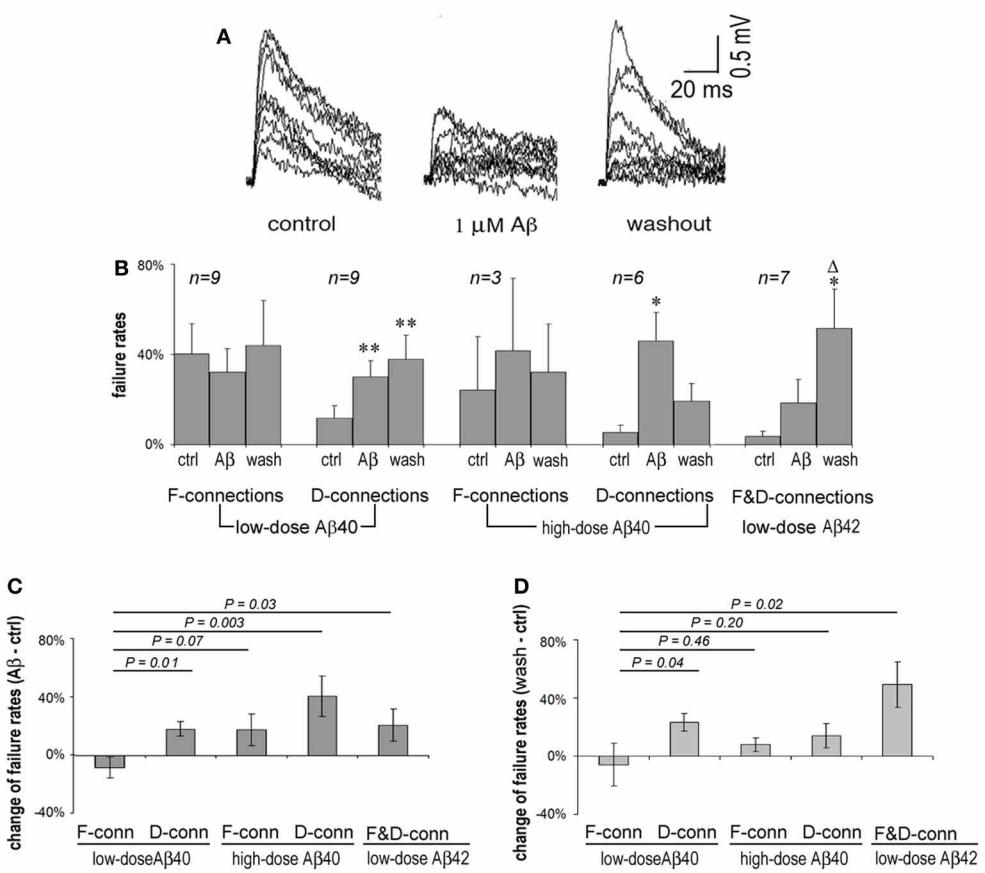

FIGURE 2 | Synaptic failure rates vary according to synaptic type and upon Aβ species and concentration. (A) Superimposed 15 single EPSP traces recorded at 0.5 Hz from an F-type connection in pre-application, application and washout phases of 1 μM Aβ40. EPSPs were generally reduced and the number of failures increased during application of Aβ for 20 min, which tended to recover on washout for 10 min. **(B)** Average failure rates of F- and D-connections in pre-application, application and washout phases in the presence of Aβ were charted according to Aβ species and concentration, and connection type. The synaptic failure rate tended to decrease in F-connections after low-dose Aβ40 applied. In contrast, the failure rates appear to increase in all other cases. Low and high doses of Aβ40 applied to D-connections reached significance ($P = 0.01$ and $P = 0.024$, respectively). A trend to enhance failure rates is shown in the case of high-dose Aβ40 to F-connection ($n = 4$) and low-dose Aβ42 to F- ($n = 3$) and D-($n = 4$) connections. After washout for 10–30 min., the failure rate are further exacerbated in applications of low-dose Aβ42 to F- and D-connections (compared with pre-application, $P = 0.04$; compared with Aβ application, $P = 0.05$). Note: *compared with pre-application, $^{\Delta}$compared with Aβ application; * or $^{\Delta}$ $P < 0.05$; ** $P < 0.01$. **(C)** Net changes in average failure rates following exposure to Aβ (the failure rate in Aβ application - the failure rate in pre-application). The net rate change in low-dose Aβ40 to F-connections was opposite in direction to that of the other cases. The difference between the net rate changes corresponding to low-dose Aβ40 vs. high-dose Aβ40 to F-connections did not quite reach statistically significance possibly due to the low n ($n = 3$) in the latter case. **(D)** Net changes to average failure rates by washout of Aβ (the failure rate in washout of Aβ - the failure rate in pre-application). The differential change in failure rates remained virtually similar to that in **(C)**. Notably, the net rate change became smaller (from 41 to 14%) in high-dose Aβ40 to F-connection, but became bigger (from 21 to 49%) in the cased of low-dose Aβ42 to F-and D-connections.

synaptic parameters—*AUDF* (Markram et al., 1998; Tsodyks and Markram, 1997; Wang et al., 2006). Dynamic synaptic responses are due to the interplay between *U*, *D*, and *F*. *U* represents the probability of synaptic transmitter release, *p*. *D* is the time constant to recover from synaptic depression; *F* is the time constant to recover from synaptic facilitation. The absolute strength, *A*, of a synaptic connection is defined as the maximum synaptic response when *p* equals 1. This approach is based on the mean output behavior of synaptic connections and therefore requires analyzing only average responses (**Figure 3** upper row graphs). Fitting average responses of an EPSP train into the model yields values for *AUDF* parameters of a synaptic connection (**Figure A2** and **Table A1**) (Tsodyks and Markram, 1997). According to the principle as verified in our previous studies (Tsodyks and Markram, 1997; Markram et al., 1998; Wang et al., 2006), changes in the synaptic parameters *AUDF* are essentially evaluated based on the amplitudes and the amplitude pattern of average EPSP train and RTR. Generally speaking, in the case of *A* reduction, amplitudes of all EPSPs become smaller but the EPSP pattern remain unchanged. In the case of *U* reduction, the amplitude of 1st EPSP is reduced while subsequent EPSPs, but not RTR, are facilitated. In the case of *D*

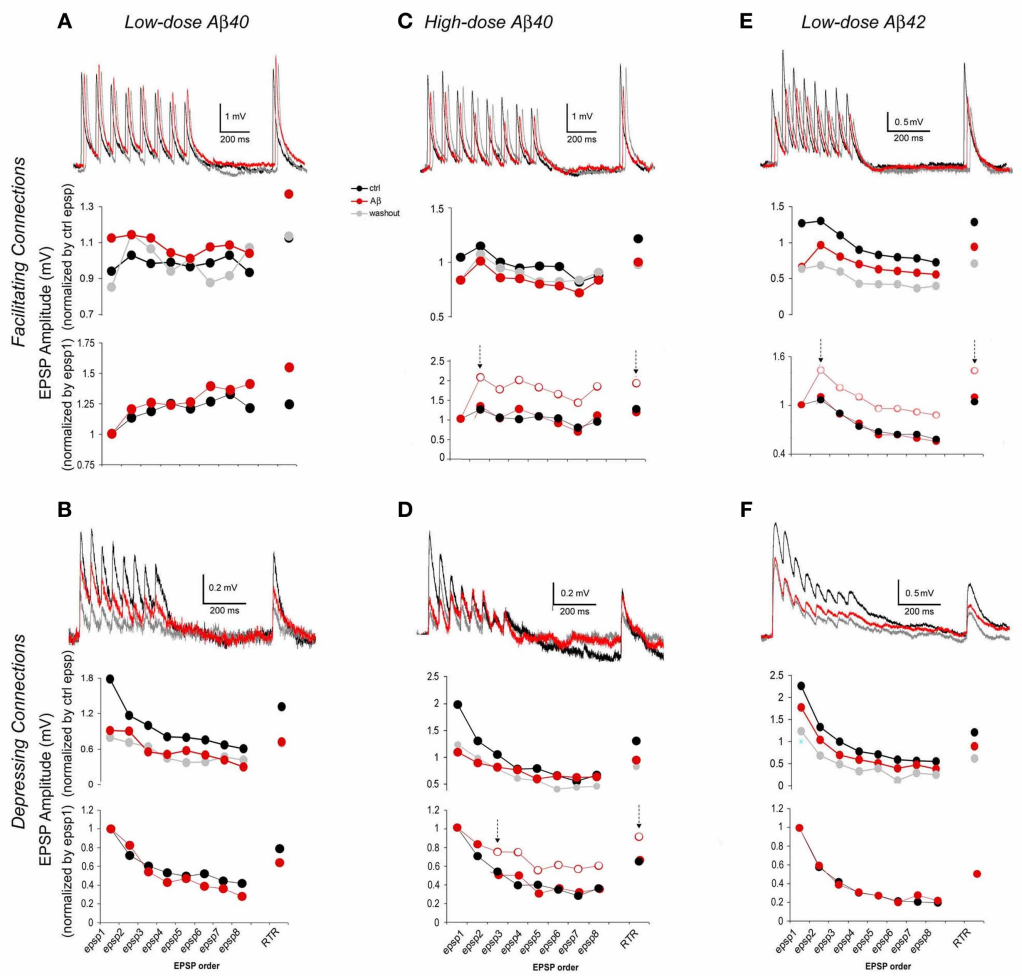

FIGURE 3 | EPSP trains of F- and D- connections change differentially depending upon the synapse type and the Aβ species and concentration. In each case, representative traces (each was an average of 15–30 individual traces) from pre-application, Aβ application and washout phases are presented at the top of each graph. The chart in the middle gives average EPSP amplitudes that were normalized to the mean of pre-application EPSPs for the comparison between pre-application and Aβ application and washout. The chart at the bottom alternatively shows average EPSP amplitudes that were normalized instead to the 1st EPSP of their intrinsic run in order to access changes in EPSP patterns (for clarity, traces of washout were not plotted). **(A)** Low-dose Aβ40 enhanced F-connections. The overall increase in the EPSP train was followed by a comparably larger increment in the RTR. The enhancement tended toward recovery after washout. **(B)** Low-dose Aβ40 inhibited D-connections. The EPSP amplitudes were all significantly diminished while the EPSP pattern remained virtually similar to that in pre-application. **(C)** High-dose Aβ40 inhibited F-connections. EPSPs were unevenly reduced, in which the decrement of the 1st EPSP was greater. In the chart at the bottom, the empty circles between two arrows showed the real pattern of subsequent EPSPs as they relate to the 1st EPSP. The red dots between two arrows that were disassociated with the 1st EPSPs, show a match up of patterns of subsequent EPSPs and RTR between pre-application and Aβ application conditions. **(D)** High-dose Aβ40 inhibited D-connections. The 1st EPSP was notably reduced while the amplitudes of steady state EPSPs (4th through 8th EPSPs) remained unchanged, followed by a reduced RTR. Between two arrows in the chart at the bottom, the empty circles show the pattern of 3rd through 8th EPSPs + RTR as they relate to the 1st and 2nd EPSPs, and the red dots between two arrows were disassociated with the 1st and 2nd EPSPs, giving the matching patterns of 3rd–8th EPSPs and RTR between pre-application and Aβ applications. **(E)** Low-dose Aβ42 inhibited F-connections. EPSPs were unevenly reduced, also indicating that the decrement of the 1st EPSP was greater (The same chart presentation was made as in **C**). **(F)** Low-dose Aβ42 inhibited D-connections. EPSPs were all significantly diminished while the EPSP pattern remained almost the same as in pre-application. Note: Upon washout of Aβ (see the middle charts, also see **Table 1**), the enhancement of low-dose Aβ40 to F-connections (in **A**) appeared to recover to the pre-application level while EPSPs inhibited by Aβ did not recover in **B–D** or even further diminished in **E** and **F**.

enhancement, both subsequent EPSPs and RTR are reduced. In the case of *F* enhancement, both subsequent EPSPs and RTR are increased.

In accordance to this model (Tsodyks and Markram, 1997; Markram et al., 1998; Wang et al., 2006), EPSP trains evoked by 5–8 presynaptic APs and a RTR recorded after a 500 ms delay were analyzed for the estimation of synaptic dynamics based on their amplitudes and patterns of EPSPs, respectively, in pre-application, various Aβ application and washout conditions (**Figure 3**). For comparison between pre-application and Aβ

Table 1 | Comparison results of EPSP trains recorded in pre-application, application and washout of Aβ.

	low-dose Aβ40		high-dose Aβ40 and Aβ25–35		low-dose Aβ42	
	F-connection	**D-connection**	**F-connection**	**D-connection**	**F-connection**	**D-connection**
ctrl vs. Aβ	$P = 0.02$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$
ctrl vs. washout	$P = 0.96$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$

Paired student t-test was used with multiple outcome values per connection.

application and washout, average EPSP amplitudes of individual synaptic connections were first normalized to the mean of EPSPs in pre-application conditions (**Figure 3** middle row graphs) of either low dose Aβ40 (**Figures 3A,B**) or high dose Aβ40 (**Figures 3C,D**) or low dose Aβ42 (**Figures 3E,F**) applications, respectively. Next, in order to better present changes in EPSP patterns, the same responses were alternatively normalized to the 1st EPSP of their own trains (**Figure 3** lower row graphs).

Compared with the pre-application, EPSP amplitudes were significantly increased in F-connections exposed to low-dose Aβ40 (**Figure 3A**, $n = 12$ pairs). The amplitude increase of the EPSP train was followed by a comparably larger increment in the RTR (**Figure 3A** lower row graph), which indicated the enhanced facilitation, *F*. In all other conditions examined (low-dose Aβ40 to D-connections, high-dose Aβ40 to F- or D-connections and low-dose Aβ42 to F- or D-connections) (**Figures 3B–F**), the EPSP amplitudes were all significantly diminished compared to their own pre-applications, respectively, (**Table 1**). In both low-dose Aβ40 and low-dose Aβ42 to D-connections (**Figure 3B**, $n = 7$ pairs; **Figure 3F**, $n = 5$ pairs), the amplitudes of EPSPs were evenly reduced and the EPSP pattern virtually remained the same as in the pre-application. This change is represented as a typical reduction in the absolute synaptic strength, *A*. Interestingly, high-dose Aβ40 and low-dose Aβ42 in F-type connections (**Figure 3C**, $n = 4$ pairs; **Figure 3E**, $n = 8$ pairs) similarly resulted in an uneven reduction of EPSP trains and RTR, in which the decrements of the 1st EPSPs were greater. This result indicated a reduction in absolute synaptic strength, *A* (according to a decline in all EPSPs) accompanied by a reduced release probability, *U* (according to the greater decrements of the 1st EPSPs). In the high-dose Aβ40 to D-connections (**Figure 3D**, $n = 7$ pairs), the 1st EPSP was notably reduced while the amplitudes of steady state EPSPs (4th–8th EPSPs) remained unchanged. The notable decrement of the 1st EPSP represented a *U* reduction, which typically leads to an immediate facilitation of subsequent EPSPs of the train. However, such an immediate facilitation was not visible. Instead, the unchanged steady state EPSPs was followed by a reduced RTR. This phenomenon could be attributed to an interplay of the reduction in both *U* and *A*. The immediate facilitation of subsequent EPSPs due to the notable *U* reduction, would counterbalance the reduction of these EPSPs due to the reduction of parameter *A*, keeping them unchanged.

High-dose Aβ25–35 was used in a few test recordings considering the fact that this short peptide has neurotoxic action and aggregating property (Chen et al., 2000). The high-dose Aβ25–35 showed inhibiting effects on EPSP trains of F- ($n = 2$ pairs) and D-connections ($n = 3$ pairs). The changes in EPSP train induced by Aβ25–35 were similar to those induced by the high-dose Aβ40.

EFFECTS OF Aβ ARE FULLY REVERSIBLE WHEN APPLIED BRIEFLY AND LOCALLY

It is noteworthy that upon washout of the various Aβ-containing mediums, only the enhancement of F-connections by low-dose Aβ40 recovered ($P > 0.05$ in **Table 1**, **Figure 3**). The reductions in EPSP train and RTR in all the other cases did not ($P < 0.05$ in **Table 1**). Rather than reflecting inefficient washout, we suspect a damaging effect on synapses was induced by prolonged Aβ conditions such as in the presence of high concentrations of Aβ40 or low-dose Aβ42. To test this, we briefly applied 1nM Aβ42 ($n = 2$) or 1 μM Aβ40 ($n = 1$) locally to a synaptic connection via a "puff" using a 3rd pipette (a representative recording is shown in **Figure 4**, estimating that the local concentration of Aβ remained close to the concentration in the pipette). Recordings were obtained prior to and at the end of peptide application (only 2 min) and bath washout phases (10 min). The synaptic

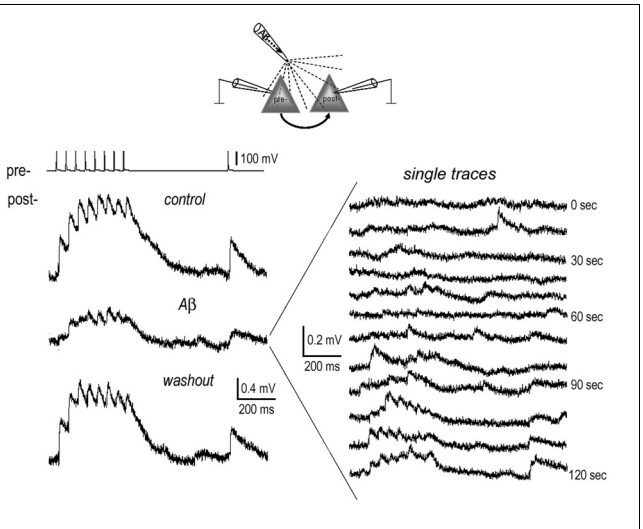

FIGURE 4 | Full recovery of EPSPs from inhibition by brief local application of Aβ. 1 nM Aβ42 was briefly applied near the somata of the connected neurons (diagram) for 2 min. Recordings were carried out before (pre-application) and at the end of application of Aβ, and lastly after washout of Aβ for 10 min. The average EPSP traces display full recovery from the inhibition of Aβ (**left panel**). In the single traces recorded at the end of Aβ application (**right panel**), the EPSPs almost completely disappeared. These started to recover at ∼1 min. after terminating the Aβ "puff."

responses almost completely disappeared at the end of application of Aβ. The recovery of EPSPs, however, began quickly at ∼1 min after the application phase of Aβ had terminated and was largely recovering from inhibition within 2 min into the washout (**Figure 4**, right panel). A full recovery was observed 10 min into the washout (**Figure 4**, the bottom trace in left panel). These results suggest that brief, highly local exposures of synapses to Aβ (even at a high level of concentration) produce reversible inhibition. With this evidence, the aforementioned irreversible inhibitory effects of bath-applied peptides becomes understandable if either modest diffuse accumulations of Aβ42 or abnormally high levels of Aβ40, may be enough to damage synapse function.

OPPOSITE EFFECTS OF LOW NANOMOLAR Aβ40 AND Aβ42 ON SHORT TERM POTENTIATION OF F-CONNECTIONS

In the excitatory neuronal network of the PFC, the F-type connections prominently exhibit forms of short term potentiation (STP) termed SA and PTP (Wang et al., 2006). We next examined effects of low-dose Aβ40 and Aβ42 on the SA and PTP in F-connections. Compared with pre-application, the low-dose Aβ40 to F-connections significantly enhanced synaptic responses during all recording phases, i.e., pre-tetanus baseline, SA induction and PTP induction (**Figure 5A1**, paired t-test with multiple outcome values per connection: all $P < 0.01$, $n = 4$ connected pairs). After washout for 10–30 min, the pre-tetanus EPSPs recovered ($P = 0.557$), however, the enhanced EPSPs still remained at a significantly higher level during the SA and PTP phases (**Figure 5A1** inset table, both $P < 0.01$). Thus, on average, the low-dose Aβ40 enhanced pre-tetanus EPSP by 23 ± 6% (**Figure 5A2,** $P = 0.01$), which recovered after washout for 10–30 min ($P = 0.556$). Meanwhile, the induction of SA was enhanced nearly 2-fold by the low-dose Aβ40 (30 ± 13% vs. 16 ± 9% in pre-application, $P = 0.141$) and the enhancement to nearly 4-fold persisted after washout for 10–30 min (58 ± 16% vs. 16 ± 9%, $P = 0.05$). Similarly, the induction of PTP was enhanced

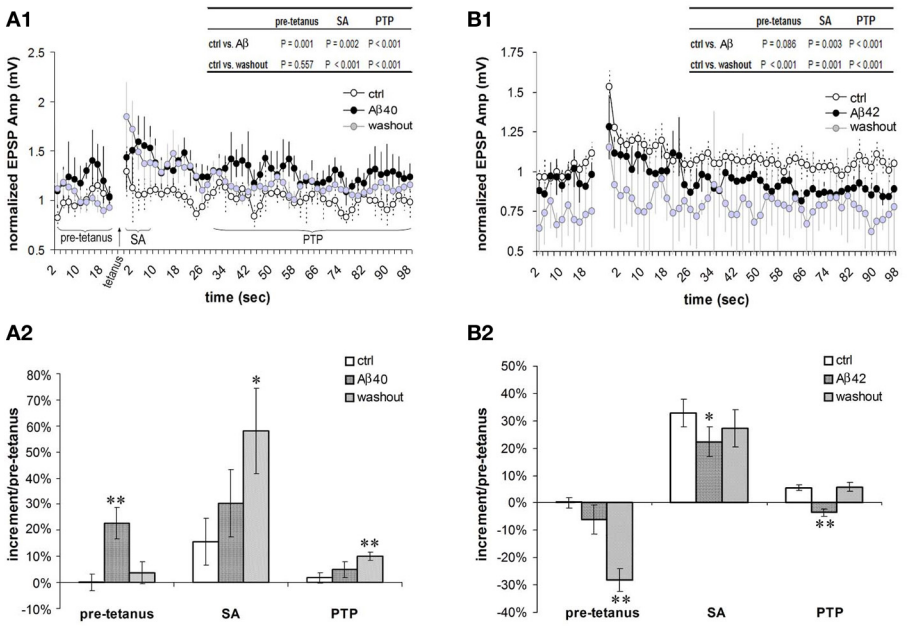

FIGURE 5 | Differential effects of low nanomolar Aβ40 and Aβ42 on the SA and PTP. (A1) Low-dose Aβ40 enhanced synaptic responses (i.e., EPSPs) under all the recording phases (pre-tetanus baseline, SA induction and PTP induction) in F-connections (all $P < 0.01$, $n = 4$ pairs). After washout for 10–30 min, the EPSPs during the pre-tetanus phase recovered to the pre-application level ($P = 0.557$), but still remained significantly higher during the SA and PTP induction phases (both $P < 0.01$, inset table). EPSP amplitudes were normalized to the mean of pre-tetanus EPSPs in pre-application. Paired t-test with multiple outcome values per connection was performed between pre-application and Aβ application, and between pre-application and washout phases. **(A2)** Comparison of increments during pre-tetanus, SA and PTP induction in the case of low-dose Aβ40 application. Compared with the baseline level (0 ± 3%) of increment during pre-tetanus phase of pre-application condition, low-dose Aβ40 enhanced the average baseline EPSP by 23 ± 6% ($P = 0.01$), recovering after a 10–30 min washout (4 ± 4%, $P = 0.556$). Compared with 16 ± 9% in pre-application, the SA appeared to be enhanced by low-dose Aβ40 to 30% ± 13% ($P = 0.141$), and remained enhanced at an average level of 58 ± 16% after a 10–30 min. washout ($P = 0.05$). Similarly, compared with 2 ± 2% in pre-application condition, the PTP appeared to be enhanced by low-dose Aβ40 to 5 ± 3% ($P = 0.284$), and remained enhanced to a statistically significant level after a 10–30 min. washout (10 ± 2%, $P = 0.01$). **(B1)** Low-dose Aβ42 depressed synaptic responses at the pre-tetanus baseline, and significantly at the SA and PTP inductions (both $P < 0.01$, $n = 6$ pairs). After a 10–30 min. washout, the EPSPs under all the recording phases (pre-tetanus, SA induction and PTP induction) became significantly depressed ($P < 0.01$, inset table). **(B2)** Comparison of increments during pre-tetanus, SA and PTP inductions in low-dose Aβ42 applications. Compared with the baseline level (0 ± 2%) of increment during the pre-tetanus phase of pre-application condition, low-dose Aβ42 depressed the average baseline EPSP by −6 ± 5% ($P = 0.08$). This became statistically significant after washout (−28 ± 4%, $P < 0.01$). Compared with 33 ± 5% in pre-application, the SA was significantly depressed by low-dose Aβ42 to 22 ± 5% ($P = 0.01$), recovering after washout (27 ± 7%, $P = 0.530$). Compared with 6 ± 1% of the increment in pre-application, the PTP was significantly depressed by low-dose Aβ42 to −4 ± 1% ($P = 0.01$), again recovering after washout (6 ± 2%, $P = 0.154$). $^*P < 0.05$; $^{**}P < 0.01$.

more than 2-fold by the low-dose Aβ40 (5 ± 3% vs. 2 ± 2% in pre-application, $P = 0.284$) and the enhancement to 5-fold persisted after washout (10 ± 2% vs. 2 ± 2%, $P < 0.01$). In the comparison, the borderline absence of statistical significance was likely due to the low sampling of these difficult-to-obtain recordings.

In contrast, low-dose Aβ42 significantly depressed synaptic responses corresponding to the SA and PTP inductions (**Figure 5B1**, both $P < 0.01$, $n = 6$ connected pairs). After washout for 10–30 min, the EPSPs comprising the SA and PTP phases of STP were further depressed (both $P < 0.01$). Meanwhile, the EPSPs of the pre-tetanus baseline were also significantly depressed (**Figure 5B1** inset table, $P < 0.01$). In **Figure 5B2**, the opposing actions of low-dose Aβ42 highly contrast the actions of low-dose Aβ40 in **Figure 5A2**. Compared with the pre-tetanus baseline level in pre-application (0 ± 2%), the average EPSP during pre-tetanus recordings became progressively depressed into the recording procedure beginning with the application of low-dose Aβ42 and into washout (depressed by −6 ± 5%, $P = 0.08$ and by −28 ± 4%, $P < 0.01$, respectively). The average EPSP increment of SA was originally 33 ± 5% in pre- Aβ application. This was significantly reduced to 22 ± 5% following bath application of low-dose Aβ42 ($P = 0.05$). The average EPSP increment of PTP was 6 ± 1% in pre-application, which became depressed to −4 ± 1% after low-dose Aβ42 was bath applied ($P < 0.01$). Taken together, effects of low nanomolar concentrations of Aβ40 and Aβ42 on EPSP amplitudes are already opposite each other in the pre-tetanus condition, presaging their beneficial and depressing effects on SA and PTP, respectively. These actions on STP induction match their aforementioned influences on isolated EPSP failure rates and on EPSP trains.

DISCUSSION

The experiments in the current study have explored the effects of soluble monomer predominant extracellular Aβ peptides on synaptic failure rates, synaptic dynamic properties and STP (including SA and PTP) of single excitatory connections in normal PFC. The PFC is highly vulnerable to the effects of aging and neurodegeneration but is relatively understudied in AD. To our knowledge, these are the first whole-cell patch clamp recordings from pairs of individual connections formed by pyramidal neurons in PFC that examine Aβ modulation and toxicity on chemical synaptic transmissions. The advantage of this technique over more conventional field studies is that influences from other afferents and reverberant circuits and influences by exciting neuromodulatory fibers are virtually avoided. In addition, the results are highly repeatable based on single synaptic connections that are classified according to their unitary synaptic dynamics.

We found that the transmission involving individual synaptic connections was significantly enhanced or reduced depending on their intrinsic type (facilitating or depressing), the tested Aβ species (40 or 42 amino acids) and concentration (low dose 1–200 nM vs. high dose 0.3–1 μM). Our main findings are that bath applications of low nanomolar Aβ40 have opposite actions on basal and STP properties of F-connections compared with high nanomolar Aβ40 or low nanomolar Aβ42. Specifically, when applied to F-connections, low nanomolar Aβ40 reduces failure rate and enhances EPSP trains and SA and PTP, whereas higher nanomolar Aβ40 and low nanomolar Aβ42 alike inhibit them. Interestingly, low nanomolar Aβ40 inhibits D-connections, acting similarly thereon as high nanomolar Aβ40 or low nanomolar Aβ42. In addition, the inhibitory effects of these bath-applied peptides often appeared irreversible despite long-time washout. Nevertheless, reversibility could be demonstrated when Aβ was applied very locally, briefly and followed with a thorough washout.

Normal concentrations of Aβ in CSF and plasma are in the picomolar range (Bohrmann et al., 1999; Teunissen et al., 2002; Lewczuk et al., 2004), but likely higher in the synaptic cleft. Our differential results in the nanomolar range may add new insight into the modulatory role of Aβ40 by balancing facilitation and depression of synaptic connections to influence activity of synaptic networks in the PFC. Specifically, Aβ40 at physiological levels moderately reduced EPSP failure rate and significantly enhanced EPSP trains and STP of F-connections while dampening D-connections. The net functional result would be to enhance network activity relevant to working memory while limiting incoming distracting signals, respectively. The first study to show that Aβ40 actually increased LTP was Wu et al. (1995), moreover the effect was noticed at 200 nM, same as our "low dose" upper limit. In addition, our results add to the notion from other work that Aβ40 could actually have a beneficial role to moderate Aβ42 effects (Kim et al., 2007). They are also in line with the differential effects of Aβ42 and Aβ25–35 peptides on hippocampal network activation, specifically on θ, β, and γ oscillations (Adaya-Villanueva et al., 2010). However, the possibility is not excluded that Aβ42 at much lower levels such as in the picomolar range also plays a similar physiological role as does Aβ40 in synaptic modulation. In a former study of recordings from hippocampal slices, low picomolar concentrations of Aβ42 caused a marked increase of long-term potentiation in excitatory cells, whereas high nanomolar concentrations lead to the reduction of the potentiation (Puzzo et al., 2008). It may be necessary to study the effects of picomolar Aβ42 on single synaptic connections in future experiments.

In recent years, it has been found that Aβ is physiologically released from synaptic terminals depending on the levels of synaptic activity (Kamenetz et al., 2003; Cirrito et al., 2005). In turn, Aβ may play an inhibitory feedback role to balance the homeostatsis of neuronal networks (Kamenetz et al., 2003; Hsieh et al., 2006; Venkitaramani et al., 2007). A feature of this feedback loop is that Aβ peptides are eventually cleared by endocytosis and diffusion (Venkitaramani et al., 2007). Our results imply that Aβ40 at high nanomolar concentrations or Aβ42 at concentrations as studied here induced an inhibition that might serve as feedback to limit synaptic activity and Aβ production. This is supported by our observation that inhibition of synaptic responses fully recovers when Aβ (40 or 42) is applied briefly and locally followed by a prompt washout (which may be closer to the physiological processes

of endocytosis and diffusion). Conversely, inhibition becomes more difficult to recover from after longer-time bath applications of these peptides. These considerations make it likely that the toxic effects of these peptides on synaptic functions become irreversible once they accumulate near synapses to concentrations that overload endocytic and enzymatic removal mechanisms.

The potential toxicity to synaptic function as evidenced by the resistance to recovery following washout could result from the formation of aggregated Aβ oligomers around synapses aided by long bath application times and high concentrations. The Aβ aggregation is dependent on protein concentration and time (Harper and Lansbury, 1997). Aggregated Aβ oligomers may suppress synaptic responses by disrupting synaptic vesicle endocytosis (Kelly and Ferreira, 2007), inhibiting NMDA receptors (Chen et al., 2002) and P/Q-type calcium currents (Nimmrich et al., 2008) and/or via forming artificial ion pores on neuronal membranes (Small et al., 2009). Aβ40 on the other hand may enhance synaptic facilitation by acting on P-type calcium channels, but once forming oligomers appears to lose the facilitating effect, turning to suppressing synaptic functions (Ramsden et al., 2002). This could explain the opposite effects of Aβ40 at low vs. high concentrations as observed in the current study. Since Aβ42 aggregates more readily than the other Aβ species (Snyder et al., 1994), it is not surprising that Aβ42 might only enhance synaptic activity at picomolar levels (Puzzo et al., 2008). Otherwise, Aβ42 induces synaptic depression at concentrations at or above the low nanomolar range. In recent years, several studies reported that extrasynaptic NMDA receptors are activated by Aβ oligomers, leading to synaptic dysfunction. Soluble Aβ oligomers increase activation of extrasynaptic NR2B receptors inhibiting NMDAR-dependent LTP (Li et al., 2011). Aβ oligomers also reduce baseline synaptic transmission and spontaneous neuronal network activity and induce retraction of synaptic contacts (Ronicke et al., 2011), some of which may be dependent on extrasynaptic sites of action. Prolonged activation of extrasynaptic NMDAR by Aβ oligomers may also play a key role in pathogenic mechanisms of glutamate excitotoxicity (Stanika et al., 2009), and cell death (Hardingham et al., 2002; Papadia and Hardingham, 2007). While our results pertain to synaptic dysfunction at the resolution of single synaptic connections, future studies are foreseeable to address any extrasynaptic contributions.

An important factor to consider is whether such effects of Aβ peptides on synaptic transmission and plasticity occur at the pre- or post-synaptic element. With our research scheme, this can be speculated upon according to changes in the synaptic dynamic parameters, *D-F-U-A*. The enhancement to F-connections by low-dose Aβ40 occurs via an increase in the parameter *F*, a presynaptic mechanism. This is further supported by the enhancement to the SA and PTP by the low-dose Aβ40. It is well-known that synaptic facilitation is mediated by presynaptic residual calcium (Kamiya and Zucker, 1994; Mongillo et al., 2008) and the induction of SA and PTP relies on pre-synaptic mechanisms (Hempel et al., 2000; Zucker and Regehr, 2002). It has also been reported that Aβ acts via presynaptic mechanisms as a positive endogenous modulator for hippocampal synapses in rodent hippocampal cultures and slices (Abramov et al., 2009). The enhancement of F-connections we observe could therefore be related to an effect of Aβ40 acting on P-type calcium channels, a pre-synaptic calcium channel that mediates synaptic facilitation (Ramsden et al., 2002; Tamse et al., 2003; Iegorova et al., 2010). Both low-dose Aβ40 and Aβ42 inhibit D-connections via reducing synaptic strength, reflected in parameter *A*. The *A* parameter represents the synaptic response when the probability of synaptic transmitter release equals 1 at the maximal level. Therefore, changes in synaptic strength, *A*, basically represents alterations in the postsynaptic elements. High-dose Aβ40 inhibits both F- and D-connections via reducing the release probability, *U*, and the synaptic strength, *A*, which involves both pre- and post-synaptic mechanisms. With respect to the same mechanisms, low-dose Aβ42 inhibits F-connections. Experimentally, an inhibition on presynaptic transmission is reported after Aβ42 injection through a block of vesicle fusion in the terminal (Moreno et al., 2009), and the inhibition by Aβ on postsynaptic sites has previously been verified to occur at multiple molecular structures such as AMPA receptors and metabotropic glutamate receptors (Puchtler and Sweat, 1962; Wang et al., 2004; Hsieh et al., 2006; Shemer et al., 2006; Minano-Molina et al., 2011).

Acting on both pre- and post-synaptic sites, Aβ peptides are likely to have multiple actions on multi-synaptic activity in neuronal networks. Low nanomolar concentrations of Aβ40 significantly enhance synaptic facilitation and both SA and PTP forms of STP of F-connections, meanwhile, inhibiting the synaptic strength (*A*) of D-connections. This finding suggests that Aβ40 differentially enhances F-connections via presynaptic sites and inhibits D-connections via postsynaptic sites. High nanomolar Aβ40, on the other hand, inhibits both F- and D-connections through reductions in both *U* and *A* parameters. Aβ42, even at low nanomolar concentrations, inhibits not only D-connections by reducing *A*, but also to F-connections by reducing both *U* and *A*. These peptides at relatively higher concentrations are therefore expected to play a physiological negative feedback role and/or to produce toxic effects on synaptic functions via both pre- and post-synaptic mechanisms. In addition to pre- and post- synaptic regulation of synaptic activity by physiological levels of Aβ and the depression of excitatory transmission by pathological levels, Aβ peptides are also shown to trigger aberrant synchronous circuit activity, even epileptic discharges, at the network level (Minkeviciene et al., 2009; Palop and Mucke, 2010). Our previous work shows that high levels of soluble Aβ may be involved in aberrant synchronous circuit activity via enhancing neuronal excitability and acting on electrical networks. Here again, physiological levels of Aβ act oppositely playing a negative feedback role to dampen electrical network activity by reducing neuronal excitability (Wang et al., 2009). Future patch clamp recording of inhibitory synaptic connections formed between interneuron and PC pairs can further address this issue from another point of view.

The PFC network has the capacity to support persistent activities during recurrent weak inputs, without resorting to the metabolic expenditure of AP generation, precisely because of some special built-in functions such as synaptic facilitation and predominant STP (Hempel et al., 2000; Wang et al., 2006; Mongillo et al., 2008). Facilitation lasting hundreds of milliseconds (and outlasting depression), SA lasting up to 10 s, and PTP lasting up to minutes are each likely to be important mechanisms to sustain network activity during short-term storage and manipulation processes such as working memory tasks (Magleby, 1987; Fisher et al., 1997; Mongillo et al., 2008). STP is an especially important correlate in the PFC to its integrative functions of working memory as well as in the organization of sequential behavior, mental flexibility and planning (Grafman, 1995; Hempel et al., 2000; Mongillo et al., 2008). Understanding endogenous modulators of the working memory network and its processes is increasingly important to the cognition and neurodegeneration fields. At the resolution of single excitatory synaptic connections, our results show that Aβ may be a homeostatic modulator and play multiple roles depending on intrinsic synapse types, soluble Aβ species and their levels in the synaptic environment. Thus, we predict that Aβ influences persistent neuronal activity during working memory tasks in the PFC. High concentrations and mild accumulation of Aβ around synapses likely lead to declines in memory and cognition such as in the early stages of AD.

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APPENDIX

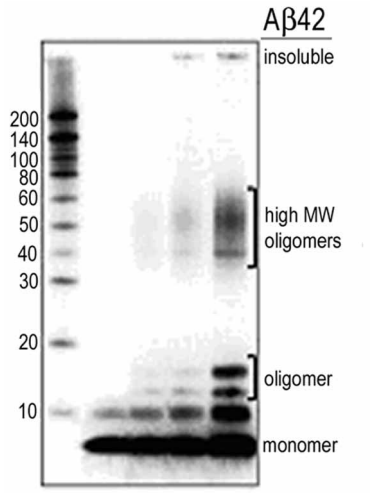

FIGURE A1 | Western blot monitoring of Aβ stock solutions. Monomer predominant preps as shown in lane 1 were used for the recording of single synaptic connections. Synthetic Aβ peptides were solubilized in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP), aliquoted, dried and stored at −80°C. A working stock solution (0.1 mM) was then prepared and stored at −80°C for dilution immediately before use. Aged or oligomerized preps as appear in lane 5 were not used in the current work.

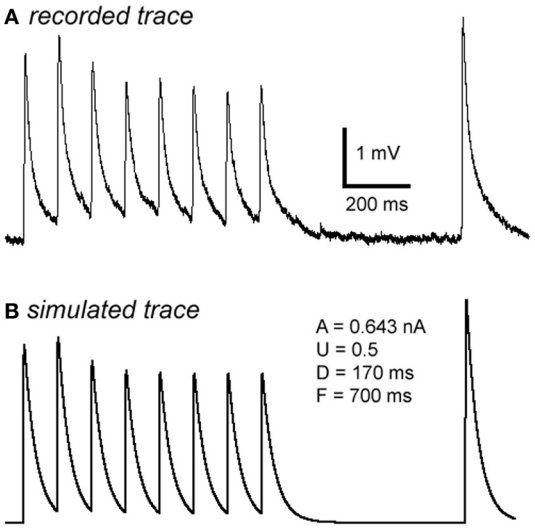

FIGURE A2 | Computer simulation of synaptic responses. The 10 Hz EPSP train followed by a RTR with 500 ms delay was recorded from a PC-IN connection **(A)**. Using a model of dynamic synaptic transmission, the synaptic dynamics were assessed by fitting the EPSPs **(B)**. The modeling extracts four key parameters of the connection: *DFUA* (*D*, the time constant of recovery from depression; *F*, the time constant of recovery from facilitation; *U*, utilization of synaptic resources used analogously to release probability, *p*; *A*, the absolute synaptic strength). If samplings were big enough, these parameters could be quantitatively analyzed enabling a quantitative comparison of basal synaptic dynamic changes induced by Aβ. Trends of changes induced by Aβ were revealed despite low samplings in most data sets (see **Table A1**).

Table A1 | Changes of synaptic dynamic properties in the modeling analysis.

				A	*U*	*D*	*F*	*F/D*
Low-doseAβ40	**Figure 3A**	F-connection	control ($n = 11$)	3.68	0.24	300	589	1.97
			Aβ ($n = 11$)	3.17	0.29	248	926	3.73
			washout ($n = 9$)	3.86	0.16	291	1071	3.69
	Figure 3B	D-connection	control ($n = 6$)	2.61	0.50	359	165	0.46
			Aβ ($n = 6$)	2.21	0.39	623	457	0.73
			washout ($n = 3$)	1.50	0.42	594	382	0.64
High-dose Aβ40&Aβ25–35	**Figure 3C**	F-connection	control ($n = 3$)	4.68	0.32	235	807	3.43
			Aβ ($n = 3$)	3.92	0.26	243	905	3.73
			washout ($n = 3$)	9.75	0.28	200	896	4.49
	Figure 3D	D-connection	control ($n = 6$)	1.83	0.48	679	149	0.22
			Aβ ($n = 5$)	1.36	0.42	430	465	1.08
			washout ($n = 5$)	1.72	0.34	902	223	0.25
Low-dose Ab42	**Figure 3E**	F-connection	control ($n = 7$)	5.94	0.39	382	588	1.54
			Aβ ($n = 7$)	5.84	0.26	428	1093	2.55
			washout ($n = 3$)	4.97	0.31	451	964	2.14
	Figure 3F	D-connection	control ($n = 5$)	3.71	0.55	646	119	0.18
			Aβ ($n = 5$) 2.90	0.45	826	114	0.14	
			washout ($n = 3$)	2.54	0.47	825	142	0.17

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GABAergic neurotransmission and new strategies of neuromodulation to compensate synaptic dysfunction in early stages of Alzheimer's disease

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Alzheimer's disease (AD) is a progressive neurodegenerative disease characterized by cognitive decline, brain atrophy due to neuronal and synapse loss, and formation of two pathological lesions: extracellular amyloid plaques, composed largely of amyloid-beta peptide (Aβ), and neurofibrillary tangles formed by intracellular aggregates of hyperphosphorylated tau protein. Lesions mainly accumulate in brain regions that modulate cognitive functions such as the hippocampus, septum or amygdala. These brain structures have dense reciprocal glutamatergic, cholinergic, and GABAergic connections and their relationships directly affect learning and memory processes, so they have been proposed as highly susceptible regions to suffer damage by Aβ during AD course. Last findings support the emerging concept that soluble Aβ peptides, inducing an initial stage of synaptic dysfunction which probably starts 20–30 years before the clinical onset of AD, can perturb the excitatory–inhibitory balance of neural circuitries. In turn, neurotransmission imbalance will result in altered network activity that might be responsible of cognitive deficits in AD. Therefore, Aβ interactions on neurotransmission systems in memory-related brain regions such as amygdaloid complex, medial septum or hippocampus are critical in cognitive functions and appear as a pivotal target for drug design to improve learning and dysfunctions that manifest with age. Since treatments based on glutamatergic and cholinergic pharmacology in AD have shown limited success, therapies combining modulators of different neurotransmission systems including recent findings regarding the GABAergic system, emerge as a more useful tool for the treatment, and overall prevention, of this dementia. In this review, focused on inhibitory systems, we will analyze pharmacological strategies to compensate neurotransmission imbalance that might be considered as potential therapeutic interventions in AD.

Keywords: septohippocampal system, amyloid-β peptide, excitatory and inhibitory neurotransmission, learning and memory, Alzheimer's disease

INTRODUCTION

Along last three decades, dementias are becoming a worldwide epidemiological problem. The importance of understanding the molecular basis of dementias and designing rational therapies for its treatment is of growing interest for populations where life expectancy along with concerns for a better quality of life are increasing. In December 2005, it was estimated that there were 24.3 million people living with dementia, there would be 31 million in 2010, and people affected by dementia will double every 20 years, rising to 81.1 million in 2040. But the reality is even worse than those approximations. People with dementia need a great amount of support and care that imply a high cost in terms of emotional, social, and financial resources that are mainly provided by their families with the help of local governments or insurance companies. Being ailments that run over a significant time period, the direct and indirect cost of medical care, employment of domestic caregivers, lost productivity in the immediate family, etc., is enormous. A joint effort from researches and health authorities needs to be made to deepen understanding of the etiology and physiopathology of these diseases and therefore develop therapies that improve health and welfare of people with dementia.

Alzheimer's disease (AD) is the most prevalent cause of dementia among more than a hundred dementia types, and is the major cause of dementia in the elderly (around 50% for age range of 80–89 years old). According to the World Alzheimer Report 2010–2012 (Alzheimer's Disease International), in 2010 there were about 36 million cases of AD and other dementias in the world, which will increase to 115.4 million in 2050. AD is a devastating progressive neurodegenerative disease characterized by cognitive decline, brain atrophy due to neuronal and synapse loss, and two neuropathological lesions firstly described

by Alois Alzheimer in 1907: extracellular amyloid plaques and neurofibrillary tangles formation, composed of amyloid-beta peptide (Aβ) and intracellular aggregates of hyperphosphorylated tau protein, respectively (Goedert and Spillantini, 2006). Together with Parkinson's disease, Huntington's disease, transmissible spongiform encephalopathies and amyotrophic lateral sclerosis, AD is one of the neurodegenerative diseases that presents a pathological common mechanism (Soto, 2003) consistent on conformational disorders of a particular protein which can fold into a stable alternative conformation. In most cases, this alteration results in its aggregation and accumulation in tissues as fibrillar deposits that finally induce neuronal death (Bucciantini et al., 2002).

The events that trigger the main pathological changes in AD take place in regions of the temporal lobe, including the medial septum, hippocampus, amygdala, and entorhinal cortex. The early onset of AD is manifested as an inability to form new memories. However, the multiple structural and biochemical changes which are already documented in the mid-to-late stages of AD (such as synapse loss, plaque accumulation, tangle formation, and neurodegeneration) do not explain the memory deficits observed in the early stages of the illness (Selkoe, 2002). For example, the loss of synapses appears to be the best morphological correlate for functional deficits observed in the middle and late stages of AD, but many patients in early stages do not show a significant decline in number of synapses (Terry, 2004; Kelly et al., 2005; Spires-Jones and Knafo, 2012).

Based on these findings, attempts have been made to find an explanation for cognitive deficits observed at early stages of the disease when no significant decline in the synapse and cell number has been detected. It has been proposed that misfolded oligomeric forms or small Aβ aggregates that are not deposited in the tissue might induce an initial state of *synaptic dysfunction* in early AD patients. Numerous genetic, biochemical, and animal model studies have implicated the gradual contribution of Aβ, as a medium for AD. In this sense, it has also been suggested that insoluble amyloid plaques would also have a pathogenic role serving as relatively inert reservoirs of soluble toxic Aβ aggregates that could readily be activated and disassembled by exposure to biological lipids (Martins et al., 2008). This synaptic dysfunction scenario could explain the cognitive deficits observed in the early stages of AD and, thus, precede synapse loss, plaque accumulation, tangle formation, and neurodegeneration (Klein, 2002; Selkoe, 2002; Soto, 2003). However, the mechanisms underlying functional deficits are not known yet.

During the last decade it has been suggested that an imbalance between excitatory and inhibitory neurotransmission systems might underlie the synaptic dysfunction caused by Aβ (Palop et al., 2007; Sun et al., 2009; Palop and Mucke, 2010a; Verret et al., 2012). Pharmacological treatments based on modulating excitatory and/or inhibitory neurotransmission have shown to improve AD symptoms (Farlow, 2009; McKeage, 2009), so that strategies aimed to reestablish the balance between both systems, particularly in early stages of the disease, seem to be the most appropriate to act on the functional deficits caused by Aβ (Huang and Mucke, 2012; Mucke and Selkoe, 2012; Verret et al., 2012).

In this regard, the present paper will review the state of the art of Aβ interactions on excitatory and mainly inhibitory neurotransmission in memory-related brain systems such as amygdaloid complex and septohippocampal system. These regions have shown to be critical in cognitive functions and their neurotransmission systems, particularly the inhibitory one, emerge as pivotal targets for drug design studies to improve learning processes and cognitive dysfunctions that manifest with age.

Aβ AND EXCITATORY NEUROTRANSMISSION

Several hypotheses have been postulated to explain the neurotoxicity of soluble Aβ aggregates on excitatory neurotransmission systems. Some of these proposals include a cascade of reactions that could involve the blockade of the glutamate recruitment by microglia (Hickman et al., 2008), alteration of the glutamatergic neurotransmission (Ashenafi et al., 2005; Santos-Torres et al., 2007), or modification of both glutamate N-methyl-D-aspartate (NMDA) and/or α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid/Kainate (AMPA/Kainate) receptors endocytosis process (Hsieh et al., 2006; Uemura et al., 2007). Other authors consider intracellular calcium increase as the neurotoxic mechanism (Rovira et al., 2002; Resende et al., 2007). Data from Gu et al. (2003) support the cholinergic AD theory and therefore affectation of muscarinic receptors (Kar et al., 1996), suggesting alterations of potassium channels as Aβ action mechanism (Zhang and Yang, 2006). None of these possible mechanisms have completely been discarded at the moment.

The hypothesis explaining Aβ neurotoxic effects through actions on glutamatergic receptors have received important supports. Neuroprotection against Aβ toxic effects has been described by NMDA receptor blockade with MK801. This result supports the idea that a persistent hyperpolarization can reduce the Aβ neurotoxicity due to inactivation of NMDA receptors (Harkany et al., 1999). In accordance, reduction of clinical deterioration in the initial AD phases has also been described using the NMDA glutamatergic non-competitive antagonist *memantine* (O'Mahony et al., 1998). Depression of glutamatergic response by Aβ perfusion has been reported using electrophysiological recordings in amygdala and septum (Ashenafi et al., 2005; Santos-Torres et al., 2007). Other authors support that Aβ alters glutamatergic transmission affecting both metabotropic (Shankar et al., 2008; Um et al., 2013) or AMPA/Kainate receptors endocytosis (Hsieh et al., 2006). It has also been proposed that Aβ effect would be mediated by increased NMDA receptors endocytosis (Uemura et al., 2007) and other authors even postulate that Aβ acting on metabotropic receptors (mGluRI) interferes with the regulation of GABAergic transmission (Tyszkiewicz and Yan, 2005).

On the other hand, results supporting the *cholinergic theory* to explain the mechanisms that underlie AD have also been shown by many authors (Langmead et al., 2008). Muscarinic receptors, specifically M1 subtype, have been broadly related to AD. M1 subtype receptor is widely distributed in the brain and is expressed postsynaptically in cortex and hippocampus (Levey et al., 1991, 1995), important areas for learning and memory. *In vitro* studies have demonstrated that activation of muscarinic receptors induces an alternative pathway for amyloid precursor protein (APP) processing which increases secretion of APP soluble fraction and then

reduces Aβ toxicity (Fisher et al., 2002). In particular, selective M1 agonist, AF267B, attenuates the major hallmarks of AD and reverses deficits in cognition (Caccamo et al., 2006, 2009). However, recent results suggest that a decrease in I_M (the potassium current activated by muscarinic receptor stimulation) may be an integral part of AD pathophysiology (Leao et al., 2012; Duran-Gonzalez et al., 2013), explaining why I_M blockers fail to improve cognition in AD clinical trials (Rockwood et al., 1997). Evidence also points out that the initial injurious effects of the fragment of Aβ, $A\beta_{1-42}$, on M1 muscarinic receptor-mediated transmission is due to compromised coupling of the receptor with $G_{q/11}$ G-protein (Janickova et al., 2013). Nicotinergic neurotransmission has also been involved in AD early stages, not only through an activation of presynaptic α7-nicotinic acetylcholine receptors (α7-nAChR; Dougherty et al., 2003) but also by interaction with GABAergic (Spencer et al., 2006) and glutamatergic (Wang et al., 2009) systems.

Then in synaptic dysfunction processes, Aβ has been found to present differential effects on AMPA and NMDA receptors. NMDA has been related to Aβ neurotoxicity phenomena. However, cholinergic disruption induced by Aβ can be established at different levels, including cholinergic neurodegeneration, alterations in acetylcholine release, direct modulation of muscarinic receptors and associated effectors, or nicotinergic system.

GLIAL CELLS IN THE PHYSIOPATHOLOGY OF AD

Recent reports support the new concept that cognitive function arises from a cooperative activity between both neurons and glia (Perea et al., 2009; Fields et al., 2014). This neuron–glia network integrates information and controls synaptic transmission and plasticity in an active way. The term "tripartite synapse" was proposed in order to describe this cellular configuration which involves presynaptic neuron, postsynaptic neuron, and astrocytes (Araque et al., 1999; Perea et al., 2009). Astrocyte-induced neuromodulation has been described in particular brain structures such as hippocampus (Araque et al., 1998; Jourdain et al., 2007), cortex (Ding et al., 2007), and hypothalamus (Gordon et al., 2005). Several gliotransmitters released from astrocytes modulate synaptic plasticity in those brain structures (Yang et al., 2003; Pascual et al., 2005; Panatier et al., 2006) and participate in learning and memory processes.

Disruption of astrocytic functions and therefore in gliotransmission may underline several brain disorders (i.e., depression, schizophrenia, and epilepsy; Rajkowska et al., 1999; Cohen-Gadol et al., 2004; Fellin et al., 2004; Webster et al., 2005), as well as specific neurodegenerative diseases (i.e., parkinsonism; Forman et al., 2005; Halassa et al., 2007). In fact, enhanced astrocytic *tau* expression in aged transgenic animals results in glutamate-transporter activity reduction and consequent neurodegeneration (Komori, 1999; Dabir et al., 2004). In the context of AD, Aβ can disrupt both astrocytic calcium signaling and glutamate uptake capacity (Vincent et al., 2010; Matos et al., 2012). It has been recently shown in an *ex vivo* astrocyte preparation, that $A\beta_{1-42}$ reduces the expression of the two major glutamate transporters in astroglia, GLT-1 and GLAST, through Adenosine A2A receptors (de Vivo et al., 2010; Matos et al., 2012). Taking into account that glutamate transporters are necessary for the clearance of excitatory neurotransmitters, the resulting excitotoxic neuronal damage induced by higher levels of Aβ through this mechanism is reasonable in AD. On the other hand, the neuromodulatory function of astrocytes in particular brain structures may explain specific vulnerability to excitotoxicity and neurodegeneration.

Aβ pathological increases induce multiple glial morphological changes. In fact, astrocytes and microglia become activated close to senile plaques in order to internalize and degrade Aβ (Mohamed and Posse de, 2011). Oxidative stress and inflammatory response induced by astrocytes and microglia activation may have a dual role in pathophysiology of AD with neuroprotective and detrimental consequences (Maccioni et al., 2001; Schipper et al., 2006). This is the basis of the therapeutic use of non-steroidal anti-inflammatory drugs (NSAID) in order to delay AD onset, as well as to reduce the rate of disease progression (Cudaback et al., 2014). Unfortunately, no clinical trials are available to support and recommend its use to prevent AD. However, a novel compound (CHF5074) with both anti-inflammatory and gamma-secretase (an enzyme involved in APP processing) modulatory activities in animal models may have a possible therapeutic role to prevent AD (Calza et al., 2013).

As mentioned previously, AMPA and NMDA receptors have been widely implicated in the physiopathology of AD (for review Parameshwaran et al., 2008). Several studies reported that astrocytes express functional NMDA receptors (Kommers et al., 2002; Lalo et al., 2006; Verkhratsky and Kirchhoff, 2007) which are involved in neuronal–glial signaling, synaptic transmission and cerebral vasodilation (Lalo et al., 2006; Palygin et al., 2010; Parfenova et al., 2012). Therefore, Aβ-induced dysfunction of glutamate receptors might affect NMDA receptors expressed in glial cells and, as a consequence, disrupt neuron–glial signal transmission (Mota et al., 2014). NMDA receptor antagonists, MK801 and *memantine*, might attenuate glutamate mediated cell excitotoxicity by excessive stimulation of NMDA receptors in astrocytes and neurons (Lee et al., 2010). In addition, regarding differences between glial and neuronal NMDA receptors, a new selective antagonist (UBP141) of astroglial NMDA receptors with potential therapeutic role in neurodegenerative diseases has been developed (Palygin et al., 2011). Finally, Talantova et al. (2013) showed that Aβ was able to induce astrocytic glutamate release which led to extrasynaptic NMDA receptor activation. In this case, *nitromemantine*, improved NMDA receptor antagonist which selectively inhibits extrasynaptic over physiological synaptic NMDA receptors activity, may protect against Aβ-induced synaptic dysfunction in hippocampus through selective extrasynaptic NMDA receptors blocking. In addition, Aβ has been shown to disrupt gliotransmission by enhancing calcium signaling through astrocytic α7-nAChRs which could as well underlie glial-based AD pathology (Lee et al., 2014).

Thus, devolvement of novel drugs targeting glial signaling may have a possible therapeutic role in AD. In fact, antiepileptic drugs such as *levetiracetam* reversed synaptic dysfunction and learning and memory deficits in human APP (hAPP) transgenic mice (Sanchez et al., 2012). One of the action mechanisms of *levetiracetam* is glutamate and GABA transporters increase in neurons and astrocytes (Ueda et al., 2007). Reduction in glutamate excitotoxicity and enhancement of inhibitory neurotransmission after chronic *levetiracetam* administration demonstrates a molecular

mechanism which involves glial cells, to attenuate cognitive abnormalities in AD.

Finally, astrocytes and neurons work together through several metabolic pathways in order to perform new synthesis of glutamate and GABA (Bak et al., 2006). At inhibitory synapses this pathway is called the GABA–glutamine cycle and it depends on GABA transporters and a multi-enzyme machinery that coordinates this process (i.e., GABA transaminase, glutamate decarboxylase, and glutamine synthetase; Bak et al., 2006; Hertz, 2013). Several studies indicate that the activity of glutamine synthetase is decreased in AD. Dysfunction of astrocyte metabolism and therefore glutamate and GABA–glutamine cycles may underlie cognitive impairment in AD (Le Prince et al., 1995; Robinson, 2000; Nilsen et al., 2014). Drugs targeting GABA-metabolizing enzyme and neurotransmitter transporters are of therapeutical interest in GABA-related neurological disorders (Sarup et al., 2003). However, taking into account the different functional roles of glial and neuronal neurotransmitter transporters and the overlapping in GABA/glutamate metabolic pathways, developing of high selective cell-specific drugs is necessary in order to avoid pharmacological interactions and unpredictable side effects.

In summary, glial cells are dramatically affected in AD. Aβ-induced dysfunction of glutamate receptors (NMDA) in astrocytes disrupts neuron–glial signal transmission. On the other hand, Aβ interaction with cholinergic receptors (i.e., α7-nAChR) and glutamate transporters in glial cells may explain neurotoxicity and selective neurodegeneration. Reducing the activation of astrocytes and microglia is the basis of anti-inflammatory drugs in AD. Finally, metabolism and new synthesis of GABA in glial cells might be an interesting target to selective pharmacological modulation.

Aβ AND EXCITATORY NEUROTRANSMISSION: AMYGDALOID COMPLEX

Amyloid depositions are found not only in the hippocampus but in other subcortical brain structures. In fact, brain amyloidosis in subjects with higher vulnerability to AD pathology (i.e., individuals with mild cognitive impairment, MCI) includes structures such as parietal association cortices, posterior cingulate, precuneus, amygdale, and caudate (Tosun et al., 2013). Among these brain regions, the number of senile plaques has been reported to be the highest in amygdala (Arriagada et al., 1992). Accordingly, a recent diffusion-tensor imaging study has revealed significant decrease in the relative volume of amygdale in early stage AD subjects (Li et al., 2013). The amygdala has long been known to be vulnerable to Alzheimer-type pathology (Hopper and Vogel, 1976), and it has been described as one of earliest locations to develop Alzheimer pathology in Down syndrome (Mann et al., 1986). AD subjects develop brain pathology similar to that of Down syndrome, including Aβ depositions (Nardone et al., 2006). Asymmetrical neuronal loss in the amygdala ranges from 35% to 70% in AD (Scott et al., 1992; Vereecken et al., 1994). In addition, it has been reported that neuronal loss was more severe in the corticomedial regions than in the basolateral region of the amygdala (Tsuchiya and Kosaka, 1990). The distribution pattern of neuronal loss was similar to that of neurofibrillary tangles instead of the distribution of senile plaques (Tsuchiya and Kosaka, 1990). Severity of amygdala pathology correlates with disease duration in AD (Arriagada et al., 1992), and amygdala pathology has been associated with emotional and memory disturbances (Zald, 2003). Despite the importance that amygdaloid complex seems to have, few studies have investigated how Aβ induces injury and may contribute to underlie the emotional and cognitive symptoms typically observed in AD patients.

Based on the amygdala's cytoarchitecture its subnuclei can be classified in superficial amygdaloid nuclei, centromedial group, and the basolateral complex (Heimer et al., 1999; Amunts et al., 2005). The basolateral amygdaloid complex is formed by the lateral, basolateral, and basomedial nuclei (Swanson and Petrovich, 1998), and innervated by cortical projections across the external capsule. In the amygdala, the excitatory synaptic activity evoked by stimulation of the external capsule is fundamentally mediated through the action of glutamic acid on AMPA/Kainate and NMDA receptors (Rainnie et al., 1991; Smith and Dudek, 1996). The amplitude of these responses is significantly depressed by Aβ without changes in membrane resistance values, which confirms that Aβ effect is localized at synaptic level (Ashenafi et al., 2005). Specifically, the Aβ effect seems to be located at presynaptic level since it could be prevented by calcicludine or nifedipine, both selective antagonists of presynaptic L-type calcium channels (Ashenafi et al., 2005).

Regarding cholinergic neurotransmission, the magnocellular division of the basal nucleus presents a high density of acetylcholinesterase positive fibers (Amaral and Bassett, 1989). The activation of these fibers generates depolarization of long duration in pyramidal cells, which is blocked by atropine (a competitive muscarinic receptor antagonist; Washburn and Moises, 1992; Moises et al., 1995). Muscarinic agonists, such as carbachol, mimic this type of response in the amygdaloid pyramidal neurons (Washburn and Moises, 1992; Yajeya et al., 2000). This effect is mediated by the closure of potassium channels and/or opening of non-specific cationic channels (Yajeya et al., 1997, 1999, 2000). On the other hand, data from Wang et al. (2000) showed that $A\beta_{1-42}$ may block presynaptic α7-nAChR in neurons derived from human brain tissues and neuroblastoma cells. It can be assumed that as a consequence of such blocking, the concentration of calcium in the synaptic terminal diminishes, producing a decrease in the amount of neurotransmitter released when the terminal is activated. Although the existence of these receptors has been verified in presynaptic terminals of the amygdala (Girod et al., 2000), a previous study in our group has shown that α7-nAChR was not involved in the $A\beta_{25-35}$ short-term neuromodulatory effects in basolateral amygdaloid complex (Ashenafi et al., 2005). In addition, nicotine stimulates mRNA expression of APP in the amygdala (Gutala et al., 2006). However, its functional implications in amygdaloid complex have not been deeply studied.

Aβ AND EXCITATORY NEUROTRANSMISSION: SEPTOHIPPOCAMPAL SYSTEM

Septum and hippocampus are structures dense and reciprocally interconnected through fimbria–fornix complex, and are functionally coupled to form the septohippocampal system, which shows a critical involvement in generating certain oscillatory activity, such as *theta* rhythm, necessary for fundamental processes in learning and memory (Stewart and Fox, 1990; Bland and Oddie, 2001; Buzsaki, 2002; Sotty et al., 2003; Colom, 2006; Colom et al.,

2010; Rubio et al., 2012). *Theta* oscillation coordinates septo-hippocampal network and depends on interconnections, which include well known cholinergic and GABAergic components (Lynch et al., 1977; Kohler et al., 1984; Bland and Colom, 1993) and recently described glutamatergic projections (Sotty et al., 2003; Huh et al., 2010).

The initial symptoms of AD involve memory impairment and disorientation (McKhann et al., 1984; Swanberg et al., 2004). Damages found in septum and hippocampus could explain those cognitive deficits (Moreno et al., 2007; Palop et al., 2007; Villette et al., 2010; Rubio et al., 2012). Functional images applied in AD subjects have detected defects in the hippocampal formation, a brain structure where the disease begins (Bland and Colom, 1993; Gonzalez et al., 1995; Harris et al., 1998; Wu and Small, 2006). It has been shown that cholinergic cells of the medial septum/diagonal band of Broca (MS–DBB) and the enzymes necessary for the synthesis of acetylcholine, are particularly susceptible to disturbance, with consequent dysfunction in cognitive processes (Yamaguchi and Kawashima, 2001). Intracerebroventricular (i.c.v.) injection of $A\beta_{25-35}$: (i) reduces the activity of acetylcholinesterase in the medial septum, cortex, and hippocampus of rats (Yamaguchi and Kawashima, 2001); (ii) decreases performance in passive avoidance and water maze tests (well established learning and memory tests); (iii) and also reduces neuronal loss and appearance of Aβ deposits in cortex, hippocampus, and caudate nucleus (Maurice et al., 1996). Moreover, the septal injection of different fragments of Aβ ($A\beta_{18-28}$, $A\beta_{25-35}$, or $A\beta_{1-40}$) produces a marked reduction in basal or induced release of acetylcholine (Kar et al., 1996). Inhibition of acetylcholine release is also observed in the hippocampus and cortex in *in vitro* rat brain slices using different Aβ fragments (Kar et al., 1996). It has also been shown that Aβ inhibits some of the effects mediated by acetylcholine through septohippocampal muscarinic receptors (Kar et al., 1996; Santos-Torres et al., 2007). This capability could contribute to the particular Aβ vulnerability of cholinergic neuronal populations. However, the low concentrations (nanomolar) with which such effects are obtained, along with the fact that nervous system cells actively secrete Aβ product, suggest the possibility that this peptide may have physiological activity, acting as a neuromodulator not only on the cholinergic, but also on other neurotransmission systems.

In vitro studies in septum slices have found that Aβ induces a deficit in glutamatergic synaptic transmission (Santos-Torres et al., 2007). It has recently been observed *in vivo* that septal glutamatergic neurons are vulnerable to Aβ through excitotoxic mechanisms (Colom et al., 2010). The two characteristics of the *theta* rhythm, frequency and amplitude, are affected by modulation of septal NMDA receptors (Puma and Bizot, 1999; Bland et al., 2007). Furthermore, injection of NMDA antagonists in MS–DBB decreases the amplitude of hippocampal *theta* rhythm (Leung and Shen, 2004). This indicates that glutamatergic MS–DBB circuits are also affected by Aβ and are important for the generation and maintenance of septohippocampal rhythmic activity amplitude at *theta* frequencies (Colom et al., 2010).

Aβ has also shown to induce dysfunction of septal glutamatergic neurons involving muscarinic receptor effectors, the potassium voltage-gate channels, KCNQ (Leao et al., 2012). In this case, Aβ diminishes septal rhythmicity by decreasing KCNQ conductance, which negatively affects hippocampal rhythmogenesis and could underlie the memory loss observed in AD (Leao et al., 2012). In this sense, it has been reported that in the septohippocampal system Aβ reduces not only KCNQ2 subunit expression and then, KCNQ conductance, altering the neuronal excitability but also the expression of the oxidative stress-related genes superoxide dismutase 1 (SOD1), 8-oxoguanine DNA glycosylase (OGG1), and monamine oxidase A (MAOA). This situation leads to a neuronal dysfunction and damage that could not be fixed because of the decreased expression of repairing genes (Duran-Gonzalez et al., 2013).

Recent studies have shown that Aβ binds to α7-nAChR in several brain structures including the hippocampus (Spencer et al., 2006; Soderman et al., 2008). On the other hand, it has been proposed that α7-nAChR is functionally blocked in hippocampal CA1 neurons due to an interaction between the receptor and Aβ (Soderman et al., 2011). The functional consequences of such interaction may lead to impairments in both cognitive function (Soderman et al., 2008) and synaptic plasticity (Soderman et al., 2011). α7-nAChR inhibition has also been explained by sustained increase in presynaptic Ca^{2+} evoked by Aβ which may underlie disruption of neuronal signaling via nAChRs in the early stages of AD (Dougherty et al., 2003). Since nicotine is able to induce LTP in CA1 hippocampal region probably due to reducing GABAergic inhibition and therefore, increasing the excitability of pyramidal neurons (Fujii et al., 2000), is plausible a functional interaction between α7-nAChR and GABAergic system. However, hippocampal GABAergic interneurons exposed to high levels of amyloid still presented α7-nAChR-mediated activity (Spencer et al., 2006). On the other hand, α7-nAChR and NMDA glutamatergic receptor activities are impaired in synaptosomes derived from AD post-mortem tissue and in presence of high $A\beta_{1-42}$ levels (Wang et al., 2000). Hence it will be essential to advance in the knowledge of α7-nAChR as therapeutic target for the treatment of Aβ-induced pathology and AD (Kem, 2000; Chen et al., 2006; Dziewczapolski et al., 2009).

Aβ AND GABAergic NEUROTRANSMISSION

The regulation of many physiological and cognitive processes is depends on a fine tuning between excitatory and inhibitory systems. In order to maintain neural network stability, GABA, the main inhibitory neurotransmitter in the mammalian central nervous system (Cardinali and Golombek, 1998), is known to regulate excitatory activity preventing neuronal hyperexcitation as well as oscillatory activity and firing rate impairments in neural networks (Oren et al., 2006; Zemankovics et al., 2013).

GABAergic neurons are the principal inhibitory neurons and one of the major local circuit neurons (Moore, 1993) which has been implicated in the regulation of a variety of behavioral functions such as learning and memory (Chapouthier, 1989; Vinogradova et al., 1998). In fact, GABAergic influence is the key to generate rhythmic synchronization of neurons during *theta* and *gamma* activity in different brain regions, contributing to neuronal communication and memory processing (Somogyi and Klausberger, 2005; Gong et al., 2009). Cortical and hippocampal function depends on optimum levels of inhibition (Borhegyi et al., 2004; Kaifosh et al., 2013; Xu et al., 2013; Bissonette et al., 2014) to

maintain an adequate synaptic plasticity activity. For a long time, GABAergic neurotransmission has been considered well preserved in AD (Rissman et al., 2007). However, cumulative evidence indicates that changes in GABAergic neurotransmission are involved in the physiopathology of AD and may be very important as a possible target to pharmacological intervention previous to cognitive dysfunction in early AD. Below, we have reviewed the effect of Aβ on inhibitory GABA system in the different brain structures which relate to cognitive deficits in AD.

It has been already discussed in this review that excitatory neurotransmission contributes to the pathogenesis and progression of AD and as a result could serve to disrupt the excitatory/inhibitory balance in brain structures, participating in memory processing and therefore taking part of mechanisms that could explain cognitive dysfunction. It is important to consider that alterations on inhibitory neurotransmission or GABA receptors may also induce a significant impact on brain structures and functions, and might also participate in the dysregulation of the balance between excitatory and inhibitory neurotransmission seen in AD patients (Palop et al., 2007; Palop and Mucke, 2010b; Mucke and Selkoe, 2012). In fact, increased epileptiform activity and non-convulsive seizure induced by Aβ in both, animals models of AD (Palop et al., 2007) and AD in elderly people (Palop and Mucke, 2009, 2010a; De Simone et al., 2010) suggests that disruption of excitoratory/inhibitory balance by Aβ involves different neurotransmitter systems including the GABAergic.

Cognitive deficits in AD are explained by selective vulnerability, neurodegeneration, and loss of function of neuronal populations and neurotransmitter systems in particular brain regions such as hippocampus, septohippocampal system, and amygdala. Acetylcholine-releasing neurons and glutamatergic neurons in basal forebrain and hippocampus, respectively, are particularly vulnerable to Aβ neurotoxic effects (Coyle et al., 1983; Emre et al., 1992; Danysz et al., 2000; Giannakopoulos et al., 2009) while GABAergic neurons are relatively resistant to neurodegeneration in AD (Rissman et al., 2007). Recently, in a mouse model of AD it has been shown that glutamatergic hippocampal terminals decrease after $A\beta_{1-42}$ perfusion while no significant changes in GABAergic terminals were observed (Canas et al., 2014). GABAergic synapses are preserved in human AD and APP/PS1 transgenic mice (Mitew et al., 2013). However, Aβ might have indirect effects on the inhibitory GABAergic transmission as a result of the dynamic GABAergic balance modulation of the other two excitatory systems (cholinergic and glutamatergic neurotransmission). It has recently been suggested that the imbalance between excitatory and inhibitory systems underlies the synaptic dysfunction caused by Aβ (Sun et al., 2009; Palop and Mucke, 2010a). As a consequence of relative sparing of $GABA_A$ receptors in AD, GABAergic sprouting in cortical and hippocampal networks enhance synaptic inhibition in order to compensate aberrant increases in network excitability (Palop et al., 2007). Hypersynchronous neuronal activity on those networks and Aβ-induced neurological deficits before neurodegeneration can be explained by this mechanism. Increase on glutamic acid decarboxylase (GAD, the rate-limiting enzyme synthesizing GABA) activity and consequently on the tone of the GABAergic system in AD brains has also been reported (Reinikainen et al., 1988). According to previous GABAergic hypothesis, this increase on extracellular GABA levels leads to alterations in neuronal membrane functions, abnormally enhanced synthesis of APP and facilitates neurodegeneration in basal forebrain system (Marczynski, 1998).

Controversially, selective somatostatin/NPY inhibitory interneurons neurodegeneration has been described in the hippocampus of a transgenic presenilin 1 PS1/APP AD model, with preservation of GABAergic mRNA synaptic markers (Ramos et al., 2006). In the same animal model, hyperactive neurons in cortical circuits are linked with a relative decrease in synaptic inhibition rather than increase in excitatory glutamatergic neurotransmission, suggesting impairments in GABAergic function (Busche et al., 2008; Palop and Mucke, 2010a). A recent report also shows that in mice expressing hAPP, the network dysfunction (hypersynchrony and reduced *gamma* oscillatory activity) and memory deficits in AD might arise from inhibitory interneuron deficit (Verret et al., 2012).

In line with the above scenario, therapies aimed at increasing GABAergic activity may reduce network/synaptic dysfunction on brain structures which participate in memory processing in AD subjects. Besides potential benefits of drugs which attenuate the Aβ-induced synaptic dysfunction, several previous studies have shown that selective $GABA_A$ receptor agonists (i.e., muscimol) are able to protect against Aβ-induced neurotoxicity in retinal, hippocampal, and cortical neurons in rodents (Gu et al., 2003; Paula-Lima et al., 2003; Louzada et al., 2004; Lee et al., 2005). Some neuroprotective effects of GABA modulators (i.e., *Etazolate*, selective $GABA_A$ receptor modulator) could be blocked by $GABA_A$ receptor antagonists (Marcade et al., 2008), indicating that these neuroprotective effects were due to $GABA_A$ receptor signaling and opening new therapeutic possibilities for AD treatment (Vandevrede et al., 2013). In fact, $GABA_A$ receptor agonists to treat age-related cognitive deficits were proposed as a new therapeutical approach in the 11th Alzheimer's Disease Drug Discovery International Conference (Wolfe, 2010a,b). However, none precise action mechanism has been well described. Stimulation of GABA receptors by *pentobarbital* apparently restores neuronal maturation and neurogenesis in apolipoprotein E (APOE)4 knocking mice (Li et al., 2009). Apolipoprotein E4 is considered as a major genetic risk factor for early onset AD perhaps by accelerating Aβ plaque formation, or by impairing neuron repair (Baum et al., 2000; Lopez, 2011; Seppala et al., 2012). Despite the plausible neuroprotective effects of GABA agonists, the widely described side effects limit its long-term use (Lanctot et al., 2004, 2007). Furthermore, there is evidence of long-term effects of benzodiazepines ($GABA_A$ agonists) and their relationship with increased risk of dementia (Gallacher et al., 2012).

Cerebrospinal GABA studies as well as neuroimaging and postmortem studies have been useful to show the relationship between GABA system and AD (Jimenez-Jimenez et al., 1998; Yew et al., 1999; Lanctot et al., 2004). Postmortem autoradiographics and benzodiazepine binding studies of GABA receptors in cortex and hippocampus have been controversial to demonstrate changes in $GABA_A$ receptors levels; however, most of them have shown a relative decrease in $GABA_A$ receptors expression in frontal, temporal, and parietal cortical regions and limbic structures. $GABA_A$ subunits specific susceptibility could explain those dissimilar results. However, alteration in $GABA_A$ receptor subunits has shown

paradoxical results probably due to compensatory mechanisms which are not well described (Mizukami et al., 1997, 1998; Howell et al., 2000; Rissman et al., 2003, 2007; Iwakiri et al., 2009). It is plausible that some effects on GABA dysfunction in AD induced by Aβ are not necessarily associated with a significant damage on GABA neurons or reduced expression of $GABA_A$ receptors, and could be explained by functional $GABA_A$ receptor activity changes. In fact, a conventional voltage-clamp study showed that Aβ may increase neuronal excitability by inhibiting GABA-induced Cl^- current in the neurons of central nervous system (Sawada and Ichinose, 1996). This result suggests that GABA modulators and agonists can normalize Cl^- flux and possibly restore the functional properties and excitability of theses neurons. In a series of elegant studies, the microtransplantation of functional receptors and channels from the human AD brain to frog oocytes showed an amplitude reduction of the currents elicited by GABA application, indicating that receptor-channel function was impaired (Miledi et al., 2004) or resulted from a diminished number of $GABA_A$ receptors in the membranes of AD brains (Bernareggi et al., 2007). Finally, GABA currents from AD brains have a faster and less sensitive rate of desensitization than those from control brains (Limon et al., 2011), which was explained by down regulation of α1 and γ2 receptor subunits while a compensatory up-regulation of α2, β1, and γ1 receptor subunits took place. Selective pharmacological modulators of $GABA_A$ subunits (i.e., α5-selective inverse agonist) may be effective to increase cognitive performance in memory disorders (Atack, 2010). Age-dependent reduction of GABA currents in AD brain from human postmortem tissue indicates a reduction of principal GABA receptors subunits (Limon et al., 2012). However, one of the major problems when GABAergic drugs are chronically used is the desensitization. It has been suggested that such phenomenon could be removed with phosphatase inhibitors or neurotrophic factors which positively modulate GABA currents (Palma et al., 2005; Limon et al., 2011) and both could be potential therapeutic targets for new AD drugs.

Hence, although some AD models have shown that the GABAergic system is relatively well preserved, Aβ might have indirect effects on GABAergic neurotransmission and induce inhibitory interneuron deficits, which could underlie neuronal hyperexcitability observed in AD. On the other hand, long-term Aβ exposition generates increased GABAergic activity and up/down regulation of specific $GABA_A$ subunits, as a compensatory mechanism. The understanding of those acute and chronic differential effects of Aβ on inhibitory systems is a pivotal point to develop novel therapeutical strategies to reduce cognitive impairment in early AD.

Aβ AND GABAergic NEUROTRANSMISSION: AMYGDALOID COMPLEX

As previously discussed in this review, there is general agreement to state that amygdala participates in emotional behavior processing. Afferent and efferent specific connections of the amygdala with a large variety of cortical and subcortical structures are the basis of cognitive functions and affective behaviors such as stress, defense, escape, pain, motivation, emotional discrimination, learning, and memory (Swanson and Petrovich, 1998; LeDoux, 2000; Gill and Grace, 2013). GABAergic afferents originating from amygdaloid interneurons of the basolateral complex which synapse on pyramidal cells probably modulate its function (McDonald, 1985; Carlsen, 1988; Carlsen and Heimer, 1988; McDonald and Augustine, 1993). In fact, altered signaling in GABAergic systems in amygdala produces impairments in emotional learning and memory tasks (Bolton et al., 2012). Regarding the above scenario, it has been proposed that inhibitory GABAergic activity in the basolateral amygdala cooperates to promote amygdala–hippocampal synchrony involved in emotional memory formation (Bienvenu et al., 2012). Amygdala dysfunction has been related to both AD (Bienvenu et al., 2012) and Aβ pathology in rodents (Devi and Ohno, 2010; Huang et al., 2010). Previously, our group demonstrated that $GABA_A$ inhibitory evoked responses decreased in amplitude after Aβ perfusion in basolateral amygdaloid complex through a presynaptic mechanism (Ashenafi et al., 2005). A transgenic mice study has shown that accumulation of Aβ in GABA neurons of the basolateral amygdaloid complex was related to enhanced innate and conditioned fear symptoms and spatial memory deficits (España et al., 2010). These results suggest that Aβ-induced dysfunction of GABAergic activity in key brain structures as amygdala might explain the emotional symptoms in AD such as anxiety and fear, as well as faster cognitive decline in memory processing.

A single neurotransmitter imbalance or Aβ-induced neurotoxic effects in a specific brain nucleus would not explain the pathology which involves whole brain regions and circuits. However, some psychological symptoms in AD subjects have been associated with GABAergic changes (Lanctot et al., 2007) and $GABA_A$ agonists have been widely used in the treatment of some behavioral and psychological symptoms of AD, such as aggression and agitation (Lanctot et al., 2004). Aβ-induced changes in GABAergic neurotransmitter system in amygdala would help us to understand the pathophysiology mechanism of emotional symptoms in AD subjects.

Aβ AND GABAergic NEUROTRANSMISSION: SEPTOHIPPOCAMPAL SYSTEM

The function of septal neurons of the basal forebrain is to modulate hippocampus and neocortex circuits' activity in order to maintain sensory information and memory processes (Colom, 2006; Colom and Garrido-Sanabria, 2007). GABA neurons have been well described in that region in close proximity to cholinergic neurons in the MS–DBB complex (Kimura et al., 1980; Castañeda et al., 2005). In the same complex, burst-firing GABAergic neurons contribute to hippocampal *theta* rhythm *in vivo* (Sotty et al., 2003). Similarly, inhibitory neurons of the medial septum provide rhythmic drive to the hippocampus independently of intrahippocampal *theta* genesis (Hangya et al., 2009). GABA-containing afferents originating in the septum innervate most of the inhibitory interneurons in the hippocampus, which, in turn, control the activity of large populations of excitatory pyramidal cells (Freund and Antal, 1988). As a result, these septohippocampal GABAergic projections have a main role in modulating electrical rhythmic activity in the hippocampal formation.

In vivo experiments in rats have shown that cholinergic and GABAergic neurons from medial septum are involved in generating *theta* rhythmicity in the hippocampus (Simon et al., 2006). Predictive modeling of hippocampal microcircuits has shown that inhibitory interneurons from septum have a main role in spatial

memories as well as in the maintenance of *theta* phase precession phenomenon of principal cells (Cutsuridis et al., 2010; Cutsuridis and Hasselmo, 2012). Because *theta* and *gamma* activity play a functional role in memory formation and retrieval (Bastiaansen and Hagoort, 2003; Hasselmo, 2005; Lisman, 2005), disruption of septohippocampal projection might explain cognitive deterioration in neurodegenerative diseases. Actually, several studies have demonstrated the relationship between Aβ-induced pathology and septohippocampal dysfunction as follows. In an interesting study, after single injection of $A\beta_{1-40}$ into the medial septum, a significant reduced hippocampal *theta* rhythm was reported, associated with damage on cholinergic and glutamatergic neurons activity controlled by the GABAergic system (Colom et al., 2010). Similarly, $A\beta_{1-42}$ injection into the MS–DBB complex preferentially injures septal cholinergic neurons but not inhibitory cells (Harkany et al., 1995). Despite these studies have proven that septal GABAergic neurons are spared after acute Aβ injection in septum, this consideration would not mean that Aβ induced septohippocampal dysfunction is not associated with inhibitory functional changes. By using *in vivo* preparations, it has been proposed that reduction of septal cholinergic and glutamatergic inputs onto GABAergic septal neurons may reduce the population of rhythmically bursting GABAergic neurons and suggest that GABAergic neurons are dysfunctional in Aβ-treated rats (Colom et al., 2010). Similarly, hippocampal $A\beta_{1-40/31-35}$ injections induce a significant impairment of spatial memory in rats and concomitant reduction in the hippocampal *theta* rhythm (Liu et al., 2013). Aβ effects are associated with GABAergic neurons dysfunction and greatly weakened septal *theta* transmission to the hippocampus rather than interfere with its generation. Therefore, hippocampal Aβ-induced pathology reduces the bursting activity of septohippocampal GABAergic neurons (Villette et al., 2010). As previously stated, these neurons contact with hippocampal GABAergic interneurons, which in turn, control the pyramidal neurons rhythm. Since GABAergic septohippocampal neurons and hippocampal interneurons are relatively spared, these effects are probably due to functional regulation changes rather than neurodegeneration or reduction in GABA receptors expression.

Conversely, a study with a triple-transgenic mouse model of AD (TauPS2APP) revealed a significant neurodegeneration of GABAergic septohippocampal projection neurons as well as GABAergic hippocampal neurons (Loreth et al., 2012). Accordingly, loss of GABAergic septohippocampal axon terminals in the mouse model hAPP has been described (Rubio et al., 2012). However, some of these changes are not caused by neuronal loss. Recently, it has been reported *in vivo* that Aβ injection in dorsal hippocampus induces a selective death of GABAergic neuronal subpopulations projecting to the medial septum (Villette et al., 2012). In this sense, even with differences in the currently available models of AD, GABAergic decline in the septohippocampal projections may explain loss of hippocampus oscillatory activity necessary for learning and memory processes.

Hippocampal *theta* and *gamma* rhythms changes occur during the early stages of AD as a product of excitation–inhibition imbalance (Goutagny and Krantic, 2013). As described above, these changes may be related with Aβ effects on MS–DBB and afferents which in turn modulate hippocampus activity. However, hippocampal formation itself is a particularly vulnerable region to Aβ peptide accumulation (Moreno et al., 2007; Double et al., 2010). As a consequence, hippocampal atrophy and neurodegeneration are common features found in AD (Hof and Morrison, 1991; Cummings and Cole, 2002; Casas et al., 2004). Acute Aβ rise induces neurotoxic damage and synaptic dysfunction affecting GABAergic neurotransmitter system in the hippocampus and associative cortex by numerous mechanisms, affecting their function before neurodegeneration and accumulation of Aβ in senile plaques occurs (Selkoe, 2002; Klingner et al., 2003). In fact, cumulative evidence shows that soluble forms of Aβ can interfere with hippocampal synaptic plasticity responsible for learning and memory processing, inhibit long-term potentiation (LTP), and enhance long-term depression (LTD; Chen et al., 2000; Freir et al., 2001; Shankar et al., 2008; Ondrejcak et al., 2010). Hippocampal LTP in an AD animal model, the transgenic APP/PS1 mice, is larger after perfusion with $GABA_A$ receptor antagonist as a product of changes in synaptic protein levels. These data suggest that reduced LTP is associated to an enhanced $GABA_A$ receptor-mediated inhibition (Fernandez et al., 2007; Yoshiike et al., 2008). Several studies previously reported cognitive improvement in the presence of chronic systemic treatment with $GABA_A$ antagonists (i.e., flumazenil or picrotoxin; Marczynski, 1995, 1998; Fernandez et al., 2007; Yoshiike et al., 2008). According to these authors, those blockers are plausible useful therapeutic agents for age-related loss of cognitive functions in AD animal models. Nevertheless, other studies affirmed that Aβ-impairment effects on LTP appear to be independent of $GABA_A$ receptor-mediated synaptic inhibition because perfusion with picrotoxin had no effect on the inhibition of LTP (Raymond et al., 2003). Additionally, GABAergic synaptic transmission onto the hippocampus was affected by i.c.v. injection of $A\beta_{1-40}$ and $A\beta_{1-42}$ (Cullen et al., 1997) or $A\beta_{25-35}$ (Sun and Alkon, 2002). Finally, several studies have reported specific GABAergic interneurons decrease (parvalbumin, calretinin, and somatostatin/NPY immunoreactive neurons) in the hippocampus of the APP/PS1 mice (Ramos et al., 2006; Baglietto-Vargas et al., 2010; Takahashi et al., 2010). Therefore, changes in the hippocampal inhibitory systems could explain the cognitive dysfunction in early stages of AD, but it is not clear whether is a consequence of functional decline in GABAergic neurotransmission, specific interneuron degeneration, or an imbalance in the excitatory/inhibitory activity, or a compound of these factors. Differences in the methodological approach (i.e., neuronal recording, genetically modified animal models, data analysis or *in vitro* vs. *in vivo* studies) may explain these paradoxical results. On the other hand, no clinical trial on $GABA_A$ receptor antagonists in AD or MCI has been successful or even performed, and therefore new studies are necessary in order to provide a link between basic science and clinical applications.

In summary, since septohippocampal GABAergic projections as well as intrahippocampal inhibitory interneurons have a main role in hippocampal oscillatory activity, inhibitory neurotransmission is an excellent candidate to be affected by Aβ in AD.

Aβ AND $GABA_B$ RECEPTORS

Despite the existence and widespread distribution of the GABA metabotropic type receptors ($GABA_B$) in the central nervous

system (Emson, 2007), their association with Aβ-induced pathology in AD have not been examined deeply yet. $GABA_B$ are coupled to intracellular signal transduction mechanisms via G proteins (Mott and Lewis, 1994; Kaupmann et al., 1998). These channels mediate slow and prolonged synaptic inhibition mainly by postsynaptic G protein-coupled activated inwardly rectifying potassium (GirK) channels (Luscher et al., 1997; Kaupmann et al., 1998). At presynaptic level, $GABA_B$ receptors also modulate (rather than generate) rhythmic activity in the MS–DBB (Henderson and Jones, 2005) and therefore have a possible role on memory function. $GABA_B$ receptor-mediated inhibition regulates the slow oscillation during *gamma* and *theta* oscillations in the control of cortical network activity (Kohl and Paulsen, 2010). *Gamma* oscillations have been associated with sensory processing (Singer, 1993), memory (Fell et al., 2001; Sederberg et al., 2007), attention (Fries et al., 2001), and finally, with consciousness (Llinas et al., 1998). $GABA_A$ and $GABA_B$ receptors are essential to learning and memory processes (Lasarge et al., 2009; Heinrichs et al., 2010; Mizoguchi and Yamada, 2011) and their pharmacological modulation affect the cognitive function. Since redistribution of hippocampus-dependent memories to neocortical sites is depending on slow oscillations (Diekelmann and Born, 2010), is plausible a role of $GABA_B$ receptors on this cognitive functions (Kohl and Paulsen, 2010). Autoradiography studies of hippocampus and cortex of postmortem AD brains (Chu et al., 1987; Young, 1987) have shown a significant reduction of $GABA_B$ receptors. In the same way, up-regulation of a novel non-coding RNA named 17A, RNA polymerase III-dependent embedded in the human G-protein-coupled receptor 51 gene (GPR51, $GABA_{B2}$ receptor), affect $GABA_{B2}$ intracellular signaling and enhances the secretion of Aβ (Massone et al., 2011). Therefore, the association between $GABA_B$ receptor dysfunction and Aβ-pathological metabolism is credible in AD.

On the other hand, the expression of $GABA_B$ receptors subunits may differ in accordance with the progression of AD. An immunohistochemical study in AD hippocampus demonstrated that in early stages of neurofibrillary tangle pathology the $GABA_B$ receptor subunit R1 expression could be stable or increased, and then decrease as the disease progresses (Iwakiri et al., 2005). These changes indicate that compensatory mechanisms are limited and the dysfunction of hippocampal inhibitory circuitry could involve $GABA_B$ receptors.

From a therapeutic point of view, some studies suggest that $GABA_B$ antagonists are more likely to have neuroprotective effects than agonists (Lafon-Cazal et al., 1999; Bowery, 2006). In fact, $GABA_B$ receptor antagonist may increase the expression of both neurotrophins nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) in rat hippocampus (Heese et al., 2000). Similarly, $GABA_B$ receptor agonist failed to inhibit Aβ-induced neuronal death (Lee et al., 2005; Marcade et al., 2008), while $GABA_B$ antagonist may improve cognitive performance in several animal models (Farr et al., 2000; Genkova-Papazova et al., 2000). New $GABA_B$ receptor antagonists have been developed for numerous *in vitro* and *in vivo* studies (Froestl, 2010). However, there is no clear evidence for its application in AD or any other cognitive dysfunction. Finally, high doses of $GABA_B$ receptor antagonists as well as $GABA_B$ receptor knock-out interrupt hippocampal and cortical oscillations leading to epileptiform activity and spontaneous seizures, respectively (Vergnes et al., 1997; Prosser et al., 2001; Schuler et al., 2001; Leung et al., 2005). In this sense, $GABA_B$ receptors modulation must have an important function in order to reach both, an effective neuroprotection and avoid epileptiform activity.

Aβ AND GirK CHANNELS

$GABA_B$ receptors and GirK channels are coupled and co-expressed in the postsynaptic membrane of pyramidal neurons in the hippocampus (Luscher et al., 1997; Kulik et al., 2003; Lujan et al., 2009) conforming an oligomeric stable molecular complex (Lujan et al., 2009; Ciruela et al., 2010). GirK channels act as key players in the control of cellular and network excitability by modulating synaptic activity in brain structures which participate in cognitive functions (Ciruela et al., 2010). GirK channels exhibit a tonic basal activity, even without receptor signaling, due to their direct binding to the G_a subunit of G proteins (Lujan et al., 2009). Therefore, it is plausible an Aβ-induced intracellular signaling impairment which compromises this effector system. In fact, Aβ has been shown to disrupt G protein-coupled receptors function (Thathiah and De, 2011), and compromise coupling of the receptor with G protein (Janickova et al., 2013) as well as several different secondary messenger systems (Thathiah and De, 2009; Yang et al., 2011; Fu et al., 2012; Zhang et al., 2014). In addition, we have recently showed that Aβ decrease $GABA_B$ currents in CA3 pyramidal neurons, a putative mechanism of Aβ-induced synaptic dysfunction observed in the septohippocampal system, which likely occur directly on GirK channels (Nava-Mesa et al., 2013).

GirK channels activity alteration may have multiple implications for synaptic activity, neuronal network function and cognitive processes. Numerous studies have emphasized its role in several pathological processes in the central nervous system such as epilepsy, pain, addiction, Parkinson or Down syndrome (Luscher and Slesinger, 2010). Deletion studies have revealed GirK channels role in learning and memory processes. GIRK4 knockout mice exhibited impaired performance in a spatial learning and memory task (Wickman et al., 2000). Moreover, mutations in GIRK2 subunit reduced LTP and increased LTD in hippocampus (Sago et al., 1998; Siarey et al., 1999; Wickman et al., 2000) and it is especially relevant in Down syndrome, where cerebral Aβ accumulation is greatly accelerated and leads to invariant early onset AD neuropathology as well as learning and memory impairment (Lott and Head, 2005; Moncaster et al., 2010; Cooper et al., 2012).

Since *loss-of-function* of GirK channels might take part in the mechanisms that lead to excessive neural excitability and epilepsy that can be observed in hAPP transgenic mice model (Palop et al., 2007; Palop and Mucke, 2009, 2010a,b; Mucke and Selkoe, 2012), this type of potassium channels emerge as an interesting potential target to be studied, particularly now that its crystal structure has just been resolved (Whorton and MacKinnon, 2011).

GirK channels may be activated in a G protein-independent manner by different compounds (Kobayashi et al., 1999; Lewohl et al., 1999; Yamakura et al., 2001; Yow et al., 2011) and are blocked by different types of antidepressants (Kobayashi et al., 1999).

However, there is little evidence for GirK subtype-specificity or pharmacokinetic advantages of most of those compounds, as they have other primary molecular targets (Lujan et al., 2013). However, a new class of subtype-selective agonists and antagonists has been identified (Kaufmann et al., 2013; Ramos-Hunter et al., 2013; Wen et al., 2013). An example is ML297, which has been found to be a potent, effective, and selective activator of GirK channels via a $G_{i/o}$-coupled receptor with preference for GIRK1/GIRK2 subunit combination (Days et al., 2010). This drug displays antiepileptic properties in animals models (Kaufmann et al., 2013) and might have a possible therapeutic potential for MCI or AD. As already discussed, seizures and epileptiform activity in AD subjects support the hypothesis that aberrant network activity contributes causally to synaptic and cognitive deficits (Palop and Mucke, 2009). Thus antiepileptic drugs (i.e., *levetiracetam*) might ameliorate those deficits (Sanchez et al., 2012; Vossel et al., 2013).

In summary, tonic GirK channel activity is necessary to control neuronal excitability and synaptic function. Therefore, GirK channels emerge as an interesting potential target to understand the physiopathology of the early stages of AD. A new class of selective GirK agonists with antiepileptic properties appears as a novel therapeutic tool to be tested in further studies.

FUTURE CLINICAL DIRECTIONS

The currently available therapy for AD (memantine, acetylcholinesterase inhibitors) may slow the progression of symptoms, but there are no existing treatments that reverse or stop disease progression even though the multiple advances in clinical and basic research.

As mentioned previously, $GABA_A$ receptor agonists have shown neuroprotective effects against Aβ-induced neurotoxicity in animal and *in vitro* models. Tramiprosate is an anti-amyloid compound for the treatment of AD. This drug has shown to reverse Aβ-induced synaptic plasticity dysfunction through activation of $GABA_A$ receptors (Aisen et al., 2006; Gervais et al., 2007). A phase III clinical trial showed a significant reduction in the hippocampus volume loss, but non-significant reduction in cognitive impairment (Aisen et al., 2011). Apparent divergence between neuroimaging lesions and cognitive deficits make difficult to determine reliable markers of AD. Other type of $GABA_A$ agonist (i.e., benzodiazepines) may have a significant role to manage psychological symptoms associated with AD. However, the undesirable side-effects associated and plausible receptor desensitization limit their chronic use (Lanctot et al., 2007). Furthermore, cognitive decline with lorazepam in individuals with higher risk for AD (carriers of APOE-epsilon4 allele, a common AD susceptibility gene) has been reported in a double-blind crossover study (Stonnington et al., 2009).

On the other hand, $GABA_B$ receptor is involved in the physiopathology of AD, and then is a pharmacological potential target. In a phase II trial, the GABAB receptor antagonist SGS742 improved cognition and memory performance in MCI possibly by up-regulation of GABAB receptors (Froestl, 2010) or through specific hippocampal protein expression (Sunyer et al., 2008; John et al., 2009). Nevertheless, in a more extensive phase IIb clinical trial in subjects with mild to moderate AD (http://www.clinicaltrials.gov/ct2/show/NCT00093951), the same drug was unsuccessful (Sabbagh, 2009).

Cumulative evidence indicates that the imbalance between excitatory and inhibitory neurotransmitter systems may underline the early cognitive deficits in AD. In this sense, antiepileptic drugs (i.e., *levetiracetam* and *topiramate*) may reverse the synaptic dysfunction associated with learning and memory impairment in animal models (Sanchez et al., 2012; Shi et al., 2013). A retrospective observational study, patients with amnestic MCI or early AD, treatment with *lamotrigine* and *levetiracetam* has shown clinical benefits and good tolerability (Vossel et al., 2013). In contrast, chronic treatment with *valproate* was associated with significant toxic effects including morphological brain changes in patients with moderate AD (Tariot et al., 2011).

Several clinical studies, including meta-analysis and clinical trials (Wei et al., 2007; Alvarez et al., 2011; Allegri and Guekht, 2012) reported the cognitive benefits of a mixture of neurotrophic factors (i.e., the nootropic agent *cerebrolysin*) as a therapeutical alternative for AD. In fact, those neurotrophic peptides can act as a neuroprotective agent or by synergically enhancing the effects of cholinesterase inhibitors (Alvarez et al., 2011). Finally, *cerebrolysin* may also regulate synaptic activity via presynaptic $GABA_B$ receptors on hippocampus (Xiong et al., 1996) and it also could improve cognitive performance in patients with mild to moderate AD according to several clinical trials (Wei et al., 2007).

In conclusion, since cholinergic or glutamatergic treatments in AD have shown limited success, therapies combining modulators of different neurotransmission systems seem to be a more useful tool for the treatment, and overall prevention, of this dementia. Pharmacological strategies to recover the unbalance between excitatory and inhibitory neurotransmitters have to take into account the GABAergic system. In this sense, recent data suggest that $GABA_B$ activity modulators which may control the neuronal excitability, as well as neurotrophic factors, are very interesting targets to be considered for further studies.

CONCLUDING REMARKS

It is reasonable to consider that Aβ-induced pathology on inhibitory synaptic activity might be explained by both their effects on specific inhibitory circuits or indirect effects on excitatory afferents to GABA neurons (**Figure 1**). Because of the availability of Aβ on synaptic cleft and the particular vulnerability of each neuronal circuit, some Aβ effects depend on the brain structure under study. On the other hand, there is increasing evidence to suggest that GABAergic neurons are relative spared in animal models of AD-like amyloid pathology. However, Aβ modulates inhibitory GABA activity through functional compensatory up-regulation mechanisms or neurodegeneration on cholinergic and glutamatergic neurons, which in turn innervate GABA interneurons. So GABAergic neurons are responsible for brain rhythmic activity necessary for learning and memory processing. It is necessary to note that many of the differences between studies are related to the experimental model used. The Aβ peptide fraction and also the exposure time or differences between acute, sub-acute and chronically models should be highlighted. In this manner, the effects of Aβ in the short-term tend to be more functional than structural while in the long or chronic –term effects induce activation of

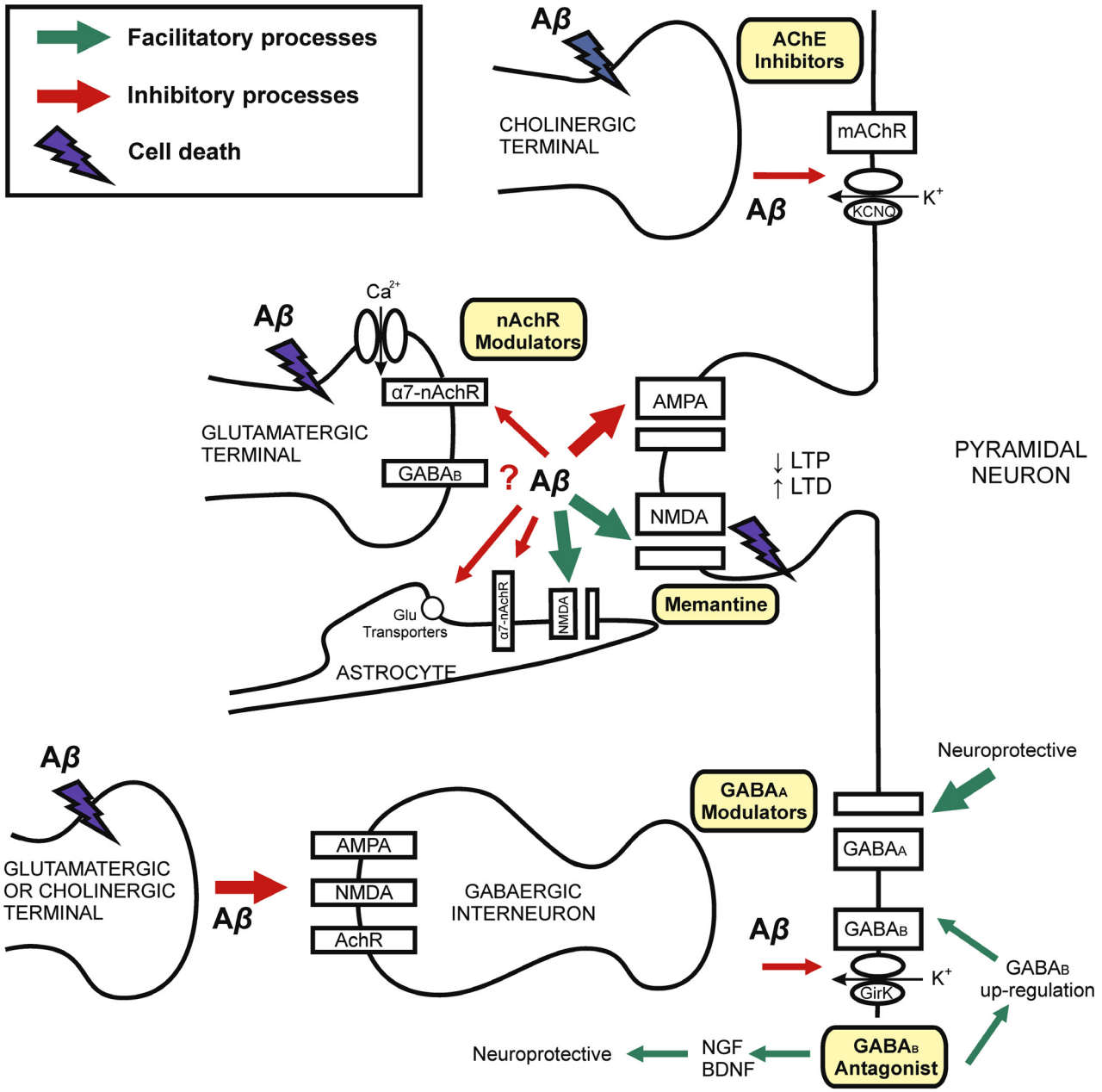

FIGURE 1 | Mechanisms of synaptic dysfunction in Alzheimer's disease. Schematic drawing showing the role of Aβ in the physiopathology of AD and different targets for pharmacological modulation. AD is explained by Aβ-induced neurodegeneration, therefore with decline in synaptic terminals (left) on both principal cells and inhibitory interneurons. Chronic exposition to Aβ induces neuronal death. Synaptic loss is related with cognitive impairment in AD. Early memory decline is correlated with Aβ-synaptic dysfunction on excitatory and inhibitory receptors and neurotransmission. Current evidence for the treatment of AD involve both glutamatergic (NMDA-type glutamate receptors antagonist, i.e., *memantine*) and cholinergic (i.e., *cholinesterase inhibitors*, AChE inhibitors) transmission. The GABAergic system is a possible target for developing pharmacological interventions. $GABA_B$ receptors may be blocked by Aβ. However, $GABA_B$ antagonists induce $GABA_B$ receptors up-regulation and neurotrophic factors expression. $GABA_A$ modulation (i.e., by $GABA_A$ receptor agonist and selective inverse agonist) may have nootropic and neuroprotective effects. Although GABA interneurons are spared in several AD models, the imbalance between excitatory and inhibitory neurotransmission might underlie early AD cognitive dysfunction. *Abbreviations*: Aβ, Amyloid-β; AchR, acetylcholine receptor; AChE, acetyl-cholinesterase; α7-nAChR, α7-nicotinic acetylcholine receptor; BDNF, brain-derived neurotrophic factor; GirK, G protein-gated inwardly rectifying potassium channel; KCNQ, Kv7 family of voltage-gated potassium channels; LTP, long-term potentiation; LTD, long-term depression; NGF, nerve growth factor.

compensatory mechanisms that possibly involve increased expression of specific receptors subunits, which would be interesting molecular targets for drug development.

Hence, new therapeutical approaches must take into account the different drug's action mechanisms, diverse neurotransmitters systems involved and finally, selective different cell targets in order to produce a better clinical results.

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Neuroinflammation in the pathogenesis of Alzheimer's disease. A rational framework for the search of novel therapeutic approaches

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Alzheimer disease (AD) is the most common cause of dementia in people over 60 years old. The molecular and cellular alterations that trigger this disease are still diffuse, one of the reasons for the delay in finding an effective treatment. In the search for new targets to search for novel therapeutic avenues, clinical studies in patients who used anti-inflammatory drugs indicating a lower incidence of AD have been of value to support the neuroinflammatory hypothesis of the neurodegenerative processes and the role of innate immunity in this disease. Neuroinflammation appears to occur as a consequence of a series of damage signals, including trauma, infection, oxidative agents, redox iron, oligomers of τ and β-amyloid, etc. In this context, our theory of Neuroimmunomodulation focus on the link between neuronal damage and brain inflammatory process, mediated by the progressive activation of astrocytes and microglial cells with the consequent overproduction of proinflammatory agents. Here, we discuss about the role of microglial and astrocytic cells, the principal agents in neuroinflammation process, in the development of neurodegenerative diseases such as AD. In this context, we also evaluated the potential relevance of natural anti-inflammatory components, which include curcumin and the novel *Andean Compound*, as agents for AD prevention and as a coadjuvant for AD treatments.

Keywords: neuroinflammation, Alzheimer disease, microglia, astrocytes, nutraceuticals

INTRODUCTION

Alzheimer's disease (AD) is the most common type of dementia and usually affects people over 65. AD is characterized by accumulation of intra and extracellular protein aggregates. Extracellular deposits correspond to amyloid plaques, mainly composed of a 39 to 42 amino acids peptide called β-amyloid (Aβ), generated by proteolytic cleavage of amyloid precursor protein (APP) by beta and gamma secretases. Intracellular protein deposits are named to as neurofibrillary tangles (NFTs) and are composed of hyperphosphorylated τ protein assembled in oligomeric structures called paired helical filaments (PHF). PHF and NFT deposition causes loss of synaptic function and finally neuronal death (Giannakopoulos et al., 2003). This process of neurodegeneration self amplifies when τ aggregates are released into the extracellular environment, since there is an important body of evidence supporting neurotoxicity of τ aggregates (Neumann et al., 2011). Among that evidence we can mention cell culture studies showing that overexpression of τ alters cell morphology, delays cell growth, and changes the distribution of organelles that are transported through the axis by motor axonal microtubule-associated proteins (reviewed in Cambiazo et al., 1995). Moreover, in transgenic mice overexpressing a human τ isoform with four repeated sequences, axonal degeneration develops in the brain and dorsal root ganglia, and is related to accumulation of neurofilaments (Spittaels et al., 1999).

Numerous therapeutic targets to eradicate the AD or lessen its symptoms have been used through years of research. Among the most studied mechanisms for AD treatment is beta amyloid clearance by passive or active immunization but this methodology has been unsuccessful and even deleterious so far (Citron, 2010). On the other hand, neuroinflammation in central nervous system (CNS) appears as a central event in AD pathophysiology. There are promising targets for AD treatment in relation to neuroinflammation and the mechanisms of cross talks between microglia and neurons (Fernández et al., 2008; Morales et al., 2010; Maccioni, 2011; Neumann et al., 2011). In this review, we will address on how neuroinflammatory processes are directly related to cognitive decline and neurodegenerative processes. Moreover, we will describe the implications of the involvement of both astrocytes and microglia in both the inflammatory and neuroimmunomodulatory processes.

NEUROINFLAMMATION

The inflammatory response is almost always a secondary response caused by an initial event after another, like the response to trauma or infections. However, this means it is a central mechanism in the neurodegenerative processes. It is this secondary response that will ensue and probably cause a greater loss of neurons over time as compared to the initial injury (Akiyama et al., 2000). Inflammation plays a key role as a driving force that can modulate the development of various neuropathologies.

Currently the term "neuroinflammation" is used to describe the inflammatory response originated in the CNS after suffering an injury, where there is an accumulation of glial cells. Particularly astrocytes and microglia responses converge immediately after the injury occurs. In this process, cellular and molecular immune components such as cytokines, complement and pattern-recognition are contributing players, and they can lead to the activation of the glial cells, i.e., microglia and astrocytes.

Innate immunity is the first line of defense of the organism against different pathogens. Among the components of the response we can mention pattern-recognition receptors (PRRs), such as toll-like receptors (TLRs), nucleotide-binding, Scavenger receptors (SRs), among others. These receptors recognize not only exogenous pathogen-associated molecular pattern (PAMP) but also endogenous modified molecules called damage-associated molecular pattern (DAMP). Throughout the body, the innate immune system launches inflammatory and regulatory responses via PRRs, phagocytes (macrophages), complement system, cytokines, and chemokines in order to counteract infection, injury, and maintain tissue homeostasis. Agents involved in innate immunity, are directly related to agents involved in the development of neuroinflammation. Cells of the CNS such as neurons, astrocytes, and microglia along with pattern recognition receptors, cytokines, chemokines, complement, peripheral immune cells, and signal pathways constitute the basis for neuroinflammation (Shastri et al., 2013).

An acute inflammatory response in the CNS is caused by the immediate and early activation of the glial cell in response to noxious stimuli, which is basically a defensive response that leads to repair of the damaged area. But, if the "stimulus" remains persistent in time, an inflammatory condition develops, causing a phenomenon of cumulative damage over time due to the chronic inflammatory reaction (Streit et al., 2004). All these events precede and cause neuronal degeneration, generating complex interactions and feedback loops between glial and neuronal cells, leading to cell damage and to the development of a neurodegenerative disease. Thus, neuroinflammation has beneficial or deleterious results in the brain mainly depending on the duration of the inflammatory response (**Figure 1**).

It has been possible to associate a number of neurodegenerative disorders of the CNS to neuroinflammatory events, for example, based on the appearance of high levels of several pro-inflammatory cytokines: AD, Parkinson's disease (Ferrari et al., 2006), Huntington's disease (Hsiao et al., 2013), multiple sclerosis (MS), amyotrophic lateral sclerosis (ALS; Kassa et al., 2009) among others. In all these diseases neuropathological and neuroradiological studies have been performed providing evidence that neuroinflammatory responses could start prior to a loss of neuronal cells. In this regard, increasing evidence has been obtained on the role of certain cytokines in the direct activation of the cellular cascade leading to neurodegeneration and AD (**Table 1**). It would be interesting to identify as correlate the neuroinflammation levels that leading to release of these cytokines, which have neurotoxic effects and are involved in the progression of this disorder pathophysiological process (Frank-Cannon et al., 2009).

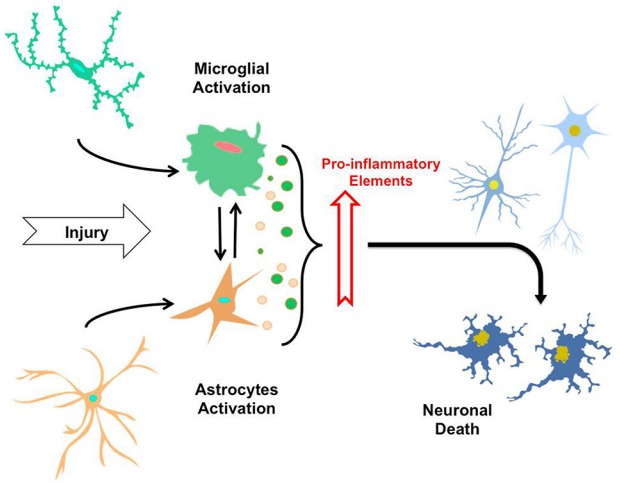

FIGURE 1 | The neuroinflammatory process. By sensing signals of damage or injury, astrocytes and microglia suffer a gradual activation process, leading to morphological changes and secretion of pro-inflammatory elements (i.e., cytokines, cytotoxic elements, ROS). Thus, the constant exposure of astrocytes and microglia to factor causing injuries and secretion of these elements induce mutual activation of microglial cells and astrocytes, along with neuroinflammatory process that eventually trigger neuronal death.

Specifically in AD, it has been demonstrated that there is a high expression of inflammatory mediators in the vicinity of Aβ peptide deposits and neurofibrillary tangles, which in turn are associated with areas of high neurodegeneration (Akiyama et al., 2000); exemplifying the relationship between neuroinflammation and neurodegeneration (**Figure 1**).

Moreover, epidemiological studies have established a link between chronic use of non-steroidal anti-inflammatories (NSAIDs) and reduced risk of AD (Vlad et al., 2008). These studies reported that the use of long-term NSAID has a protective effect against the development of the disease, delaying the onset of the symptoms or reducing the risk of its occurrence. Although the mechanism behind this phenomenon is still unknown, some hypotheses are inclined to the effects of these anti-inflammatory on the regulation of COX-1 and COX-2 protein, whose levels are elevated in individuals with AD (Vlad et al., 2008). It has also been observed that the regulation and blockade of the COX-1 in microglia, by effect of NSAIDs induced an improvement in the symptomatology of AD (McGeer and McGeer, 2007). Results in transgenic animal models of AD, show that NSAIDs reduce in a

Table 1 | Pro-inflammatory elements secreted by astrocytes and microglia during the process of neuroinflammation.

Pro-Inflammatory elements	Effect
Chemokines	Dysfunction, apoptosis and necrosis of neuron, microglia and astrocytes
IL-1β, IL-6, IL-12 INF-γ, TNFα	Astrocytes and microglia activation, dysfunction, apoptosis and necrosis of neuron, microglia and astrocytes
NO, ROS, O_2^-	Oxidative stress in cells; dysfunction, apoptosis and necrosis of neuron, microglia and astrocytes

dose-dependent manner behavioral deficits and the population of activated microglia (McGeer and McGeer, 2007).

Comparative analyses performed in the brains of cognitively normal patients chronically using NSAIDs over age versus those not using NSAIDs revealed no changes in the appearance of senile plaques, but there was a 3-fold decrease in the number of activated microglia in the brains of chronic users of NSAIDs (McGeer and McGeer, 2007). AD patients who used NSAIDs compared with another group of patients who did not use NSAIDs, showed a significantly slower progression of disease (Rich et al., 1995). These findings suggest that the protection provided by the chronic use of NSAIDs in patients with AD may partly be derived from the attenuation of microglial activation.

Lim et al. have conducted studies of cerebral amyloidosis in transgenic mouse models, and gave evidence of ibuprofen effect on amyloid plaque deposition (Lim et al., 2000). After 6 months of treatment with ibuprofen, amyloid plaque deposits were reduced significantly in 10 months old Tg2576 transgenic mice. Also there was a reduction in markers of astroglial and microglial activation (Lim et al., 2000). Moreover, in double transgenic animals ibuprofen reduced microglial activation and decreased the number of amyloid deposits (Yan et al., 2003; Heneka et al., 2005). In addition, Choi et al. demonstrated that the treatment of 20-month-old triple transgenic AD (3 X Tg-AD) mice with the COX-1 inhibitor SC-560, significantly improved memory deficits and reduced amyloid deposition and τ phosphorylation in the hippocampus (Choi et al., 2013). An explanation is that the mice SC-560 treatment alters the phenotype of activated microglia, reducing the expression of pro-inflammatory factors. Authors postulated that this changes in microglial cells may play a role in the reduction of amyloid charge and τ pathology and in rescuing impaired memory in aged 3 X Tg-AD. As stated above, there is important evidence that early treatment in transgenic animals with NSAIDs may reduce neuroinflammation and Aβ peptide deposition in the brain (Jantzen et al., 2002). In spite of that it cannot be overlooked that not all results have been favorable, for example, in transgenic mice models of AD, COX-2 selective inhibitors failed to reduce the inflammatory reaction and showed an increase in the appearance of Aβ42 peptide (Kukar et al., 2005). Recent findings suggest that alterations in microglia and the production of cytokines and chemokines may be an early feature that precedes Aβ deposition in a mouse model of AD (Varvel et al., 2009). This early microglial activation may well play a role in the appearance of vulnerable neuronal populations, similarly to the situation in AD.

On the other hand, clinical trials on the effects of NSAIDs treatments of cognitive decline in Alzheimer's disease patients did not provide clear-cut results, data varied depending on the cognitive test used. For example, results of trials with Naproxen indicate that this NSAID attenuate cognitive decline, but accelerated cognitive decline in fast decliner patients. Conversely, Celecoxib (another NSAID) appears to have similar effects, but attenuated changes in fast decliners (Leoutsakos et al., 2012). Thus, it is premature to make clinical recommendations, but the findings to date, open several potential avenues of research, and possibly the clinical trials should be replicated in one or more large observational studies.

In this context, we can conclude that some NSAIDs are able to reduce the inflammatory response caused by microglial and astroglial cells, but some others are not as effective or may even produce opposite results. This suggests that microglia have different responses after exposure to different types of NSAIDs according to specific mechanism of action of the molecule and also to the source of the primary insult that induces the onset of an inflammatory response. It is also plausible that the response may vary during the course of a given therapy (Jantzen et al., 2002).

ASTROCYTES

Astrocytes are the most abundant glial cells of the nervous system and constitute about 25% of the cerebral volume (Tower and Young, 1973). They have multitude of functions: (i) inducing the formation of neuronal synapses and influencing their development (synaptogenesis); (ii) formation and maintenance of blood-brain barrier; (iii) neurotransmission; (iv) metabolic regulation; (v) ion balance maintenance, and finally (vi) as a component of the "tripartite synapse" model of neurotransmission, in this model of neurotransmission, synapse consists of three functional elements: pre-and postsynaptic neurons and surrounding astrocytes. Then in addition to communication between neurons, there is a bidirectional communication between neurons and astrocytes, implying a predominant role of glial cells in the physiology of the nervous system (Matyash and Kettenmann, 2009; Chaboub and Deneen, 2013). Astrocytes play a key role in the development of the nervous system, since the growing of axons is guided to the target by molecules derived from astrocytes, such as tenascin C and proteoglicans (Powell and Geller, 1999).

Astrocytes are actively involved in synaptogenesis, not only during development but also after CNS injury. In 1997, studies conducted by Pfrieger observed that retinal ganglion cells synaptic activity was 100 times major in the presence of astrocytes (Pfrieger and Barres, 1997). This increase in synaptic activity mediated by astrocytes is precisely due to the increased number of synapses, which is seven times higher in retinal ganglion cells cultured with astrocytes in the absence of astrocytes (Ullian et al., 2001). This increase in the number of synapses is mediated by a matrix-associated protein named thrombospondin (Christopherson et al., 2005; Risher and Eroglu, 2012), which belong to a family of five homologous proteins, and at least four of them are expressed in these cell types during development and after brain damage, inducing synaptogenesis. These proteins

induce ultrastructurally normal synapse formation, both presynaptic and postsynaptic (Barres, 2008).

On the other hand, the metabolic support given by astrocytes, provides active neurons with metabolic substrates through a glucose-lactate shuttle. Increased neuronal activity leads to an increase in glutamate release, which in turn activates astroglial Na^+-dependent glutamate transporters, thus increasing cytosolic Na^+ concentration in astrocytes. In turn, increased Na^+ stimulates glycolysis and lactate synthesis. The lactate is subsequently transported to neurons through specific transporters (Magistretti, 2009). The astrocytes are involved in the maintenance of homeostasis of brain neurotransmitters, being of particular importance for homeostasis and turnover of glutamate by being the main sink of glutamate in the brain. From the bulk of glutamate released during synaptic transmission, several studies have shown that only a minimum percentage of glutamate (∼20%) accumulates in the neurons, while the largest amount of this neurotransmitter is absorbed by perisynaptic astrocytes. This process of eliminating extracellular glutamate by astrocytes, it is extremely critical to prevent excitotoxicity (Verkhratsky and Kirchhoff, 2007).

Numerous records show that astroglial cells possess highly important functions within the brain. However, pathological modifications of astrocytes have been associated with several neurodegenerative disorders. These include ALS, MS, AD, Parkinson's disease, Alexander's disease, epilepsy and Rett syndrome (Okabe et al., 2012). Pathological astrocytes observed in the brains of patients with dementia were initially analyzed by Alois Alzheimer, which found abundant glial cells in the neuritic plaques. Subsequent studies have confirmed that this is a morphological characteristic of reactive astrogliosis in AD that can be found both in brain tissue of patients with AD, and transgenic animal models (Verkhratsky et al., 2010). In studies of post mortem brain tissue from patients with AD a generalized astrogliosis—manifested by cell hypertrophy and an increase in the expression of Glial fibrillary acidic protein (GFAP) in astroglial S100B protein—can be found (Verkhratsky et al., 2010). A more detailed analysis of astrogliosis in brains obtained from elderly patients (with and without AD confirmed) has shown a correlation between the degree of astrogliosis and cognitive impairment. However, a direct relation between changes in astrocytes and increase in senile plaques has been found (Simpson et al., 2010). Morphological data show that reactive astrocytes associate with some Aβ plaques, but not with all of them, while astrogliosis can also been found in areas without Aβ deposits. This may result from the fact that astrocytes may also respond to other pathological factors in the ageing brain (Simpson et al., 2010). In the meantime, no significant difference was found in the expression of GFAP in brain tissue samples from patients with and without dementia (Wharton et al., 2009). Furthermore, it has been shown that fragments of Aβ promote marked inflammatory response in the brain, causing the synthesis of different cytokines and proinflammatory mediators (Lim et al., 2013). Within this inflammatory response, astrocytes express a repertoire of receptors for inflammatory cytokines (IL-1β and TNFα), chemokines and damage signals (including TLR ligands) (Krasowska-Zoladek et al., 2007), while other receptors and other mediators of inflammation, may be induced after appropriate activation signals from other brain cells (Meeuwsen et al., 2003). Studies conducted by van Kralingen, found that a number of inflammatory cytokines were elevated in the CNS following injury. In turn, in various neurological conditions there are elevated levels of specific cytokines (in serum or CSF), correlating with poor results in neurological evaluations. These include TNFα and IL-1β, which have proven to affect the function of the blood-brain barrier. A secondary inflammatory response to IL-1β and TNFα, leads to astrocyte activation, being the long-term effect of these cytokines detrimental to the survival of astrocytes. This reveals a potential new target cell, which may help explain some of the negative effects of these cytokines on brain tissue during neuroinflammation (van Kralingen et al., 2013).

MICROGLIAL CELL

Microglia are widely distributed throughout the brain and spinal cord (Lawson et al., 1990). These cells can be found in brain, spinal cord, retina and optic nerve, but mainly in the hippocampus and substantia nigra (Venneti et al., 2009), and correspond to approximately 5–20% of the total population of glial cells in the CNS (Perry, 1998). These cells are considered as a representative of the immune system in the CNS, since they possess the ability to perform phagocytosis, release cytotoxic factors and behave as antigen presenting cells (Hanisch and Kettenmann, 2007).

Microglia plays a key role in embryonic development as they can secrete growth factors important for the formation of the CNS, and also contribute to the maturation, regeneration and neuronal plasticity. Furthermore, in their resting form they also are involved in other functions such as neurogenesis, neuroprotection and synaptic pruning, which has been found to be complement dependent (Sierra et al., 2010; Vinet et al., 2012). Moreover, these cells are also involved in a number of key processes for the maintenance of homeostasis of brain microenvironment, showing various functions. For example, microglia act as activated macrophages and they respond to any type of tissue injury (Nimmerjahn et al., 2005). Thus, the suitable and appropriate function of microglial cells is essential for the homeostasis of the CNS in both diseased and in normal health frame (Perry et al., 2010).

Microglia under physiological conditions are usually found in an inactivated state (or resting state) which is characterized by a ramified morphology, small and low expression of macrophage related molecules. When activated, drastic changes in morphology of microglia occurs. Activated microglia are not defined by a particular morphology, but are characterized by having few branchings, and a larger cell body with ameboidal form (Hanisch and Kettenmann, 2007).

Numerous signals represent a threat to the homeostasis of the CNS, including structures and/or residues from bacteria, viruses and fungi. Abnormal endogenous proteins, complement factors, antibodies, cytokines and chemokines, among others, are also sensed by the microglia elements and subsequently induce activation (Venneti et al., 2009). Thus, there are two main functional aspects of microglial cells: immune defense and maintenance of CNS homeostasis.

Activation of microglia by TLRs and NOD-like receptors (NLRs) is considered to be "classical" form of microglial

activation where innate immune responses include production of proinflammatory cytokines like TNF-α, IL-1 and IL-6, and chemokines. Classical activation also leads to adaptive immune response by expressing major histocompatibility class II (MHCII) molecules and interaction with T cells (Olson and Miller, 2004). Under inflammatory conditions, there is an increase in active immune response and microglia should moderate the potential damage to the supporting tissues, repair and remodeling of the CNS (Ginhoux et al., 2013). In this state the cells regulate the expression of different surface markers, such as MHCII, growth factors (Harms et al., 2013), PPRs, produce more pro-inflammatory cytokines, such as IL–1β, IL-6, IL-12, interferon gamma (INF-γ) and TNF-α (Xiao et al., 1996). Moreover, activated microglia increase their proliferation (Venneti et al., 2009), synthesize and release cytotoxic factors such as superoxide radicals (O_2^-), nitric oxide (NO) and reactive oxygen species (ROS) (Colton and Wilcock, 2010; Ha et al., 2012). Therefore, it becomes clear that microglials cell have an important role in innate immunity and are the main source of pro-inflammatory factors in brain.

Microglial activation is a phenotypically and functionally different process, since depending on the type, intensity and context of the stimulus; microglial response has a potential neuroprotective or pro-inflammatory effect (Hanisch and Kettenmann, 2007). It is precisely this delicate balance between the neurotoxic and neuroprotective and between pro-inflammatory and anti-inflammatory which determines the role of microglia in a disease or condition. So based on the current research, microglial activation should not be considered as an all or nothing event or single process, and we must realize that the answers to the pathological events depends on context and adapt as changes in the microenvironment occurs.

Studies by Nimmerjahn revealed that microglial processes and arborizations are highly mobile (Nimmerjahn et al., 2005). These are continuously being reconstructed by *de novo* formation and removal of processes, similar to the movements of the filopodia. Such a dynamic and thorough reorganization may allow microglia to fully explore their environment in any situation without disturbing the structures of near neurons. Thus, it is estimated that the entire brain parenchyma could be monitored in a few hours. This is possible because neighboring microglial cells take turns scanning shared regions, ensuring comprehensive detection, avoiding their own contact. This exploration generated by random processes that change quickly, can lead to the translation of microglial cells into a particular site induced for microdamage. Moreover, microglia cells also have many receptors for a large number of molecules, which can immediately detect signs of disruption in the structural and functional integrity of nervous tissue.

In-vitro studies have shown that microglial cells participate in the removal of Aβ peptide in culture (Hardy and Selkoe, 2002). But there is also possible that Aβ protofibrils activate microglia, triggering an inflammatory response and the subsequent release of neurotoxic cytokines. On the other hand, studies in patients receiving NSAIDs in long term treatment revealed a decrease in the incidence of AD, suggesting that attenuation of the inflammatory response may help prevent or maintain a lower probability of developing AD (McGeer et al., 1996, 2006; Fernández et al., 2008; Town, 2010). Even in mice, NSAIDs directly affect the development of amyloid in the brain, reducing Aβ-42 peptide levels (Weggen et al., 2001). This highlights the important role of pro-inflammatory cytokines released as a result of microglial activation and the effect of the damage signs, as major players in the development of AD (**Figure 1**).

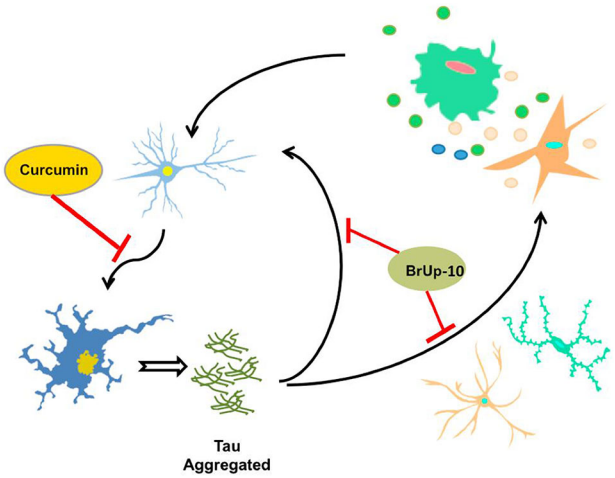

FIGURE 2 | τ aggregates potentiate the neuroinflammatory process. Activated microglia and astrocytes, induce neuronal death through neuroinflammatory process, allowing the release of τ protein aggregates from the dead neuron. These aggregates of τ are capable of inducing activation of microglia and astrocytes, as well as neuronal death, thus producing a circuit of constant neuroinflammation. Considering the possible protective effects of curcumin, neuronal death could be delayed or inhibited by this compound, halting this circuit of neuroinflammation. Furthermore, the nutraceutical complex Brain Up-10 acts on τ aggregates, and may prevent neuronal death and activation of microglia and astrocytes, stopping the circuit and the development of neuroinflammation.

In this context, authors Fernandez and Maccioni, have hypothesized the central role of neuroimmunomodulation in the pathogenesis of AD. In this model a number of innate damage signals, which manifest persistently over time, are able to induce microglial activation, triggering an overactivation state in the cells (Fernández et al., 2008; Maccioni et al., 2009; Morales et al., 2010).

As prolonged exposure to damage signals generate overactivation of microglial cells, the steady release of cytotoxic factors and pro-inflammatory cytokines causes a neuroinflammatory phenomenon which is directly related to neuronal degeneration, mainly as a result of pro-inflammatory molecules (Li et al., 2007; Colton and Wilcock, 2010; Morales et al., 2010), positioning directly microglia and cytokines as key elements in the development of neurodegenerative disorders such as AD (Mrak, 2009; **Figure 2**).

Pathological τ aggregates are able to induce microglial activation with the subsequent events related to the neuroinflammatory cascade. After neuronal death, pathological τ protein aggregates are released into the extracellular medium causing activation of microglia and generating a cascade of constant feedback of damage signals (Morales et al., 2013).

NEW RESEARCH AVENUES

Today, as a result of the lack of effectiveness of current treatments for AD, a lot of effort has been invested to enhance the search for new therapeutic targets. Based on the results obtained in patients taking anti-inflammatory drugs, a new possibility has been opened studying the association of inflammatory processes and AD pathophysiology.

An important strategy to prevent brain impairment is based on dietary changes and nutritional supplements, functional foods and nutraceuticals. In this regard, there is interesting information coming from studies with the antioxidant and antiinflammatory *Andean Compound* (called initially as Andean Shilajit). *Andean Compound* is a very complex mixture of humic substances generated by natural millenary decomposition of vegetal material and is originated as an endemic natural product from the Andes Mountains. *Andean Compound* and its major active principle—fulvic acid—emerge as novel nutraceutical with potential uses against neurodegenerative brain disorders (Carrasco-Gallardo et al., 2012a). This natural compound is a potent anti-inflammatory substance, and a very safe dietary supplement (Carrasco-Gallardo et al., 2012b). In fact, according to studies by Cornejo et al. (2011), fulvic acid is able to block τ self-aggregation affecting the length and morphology of PHFs generated *in vitro*. Additionally, after exposure of preformed τ fibrils to fulvic acid, a decrease in length of PHFs can be detected (Cornejo et al., 2011).

Therapies based on τ protein, appear as an interesting target because tangle formation has been identified as a major event involved in the neurodegenerative process. Currently, our group is working in a compound that contains *Andean Compound* plus complex B vitamins (i.e., B6, B9 and B12 vitamins) named as Brain-Up 10®, underwent a pilot clinical trial and showed a tendency toward less cognitive deterioration, a reduction on neuropsychological symptoms and less distress for the caregiver of treated patients.

Another compound of natural origin, which is currently under study, is curcumin. Curcumin is a natural phenolic compound derived from the perennial herb *Curcuma longa* (turmeric), and is well known to exhibit anti-inflammatory and antioxidant activities (Aggarwal and Harikumar, 2009; Lu et al., 2014). In India, turmeric has traditionally been used for the treatment of diseases associated with injury and inflammation. The information about this compound reported that it may be capable of preventing the death of neurons in animal models of neurodegenerative disorders, but its possible effects on development and neuroplasticity are unknown (Kim et al., 2008). Studies led by the author Kim revealed that curcumin has a dual action in cell cultures of NPC (multi-potent neural progenitor cells): at low concentrations stimulates cell proliferation, whereas at high concentrations it becomes cytotoxic. On the other hand, the administration of curcumin to adult mice resulted in a significant increase in the number of newly generated cells in the dentate gyrus of hippocampus, indicating that this compound enhances adult hippocampal neurogenesis (Kim et al., 2008). Then, curcumin would stimulate developmental and adult hippocampal neurogenesis, with a biological activity that may improve neural plasticity and repair. Recent studies have shown that curcumin was able to prevent damage from Aβ, because it induced decrease in CaMKII function in organotypic hippocampal slices, attenuating synaptic dysfunction, inducing the development of more robust and synaptically efficient neurons, and this is reflected in the inhibition of synaptic dysfunction and neuronal death (Hoppe et al., 2013). These results expand the neuroprotective role of curcumin to a synaptic level, enhancing this compound as an alternative or add-on treatment for AD (**Figure 2**). In both examples—i.e., Brain-Up 10® and curcumin—we show that by looking at natural compounds it is possible to find new alternatives in the search of treatments for AD, so it is important not only to continue generating new synthetic compounds, but also to revisit old traditional medicine as well.

Table 2 | Natural compounds recommended in the treatment AD.

Compound	Active principle	References
Ginkgo biloba	Ginkgo extract EGb761	Canevelli et al. (2014) Vellas et al. (2012)
Resveratrol	3,4,5-trihydroxystilbene	Lu et al. (2012) Kang et al. (2009)
Cerebrolysin	Porcine brain-derived peptide	Wei et al. (2007) Rockenstein et al. (2006) Álvarez and Fuentes (2011)

Other compounds have been used for supplementary treatment of AD (**Table 2**). *Ginkgo biloba* has been highly investigated but the data are confusing. Thus, Canevelli proposes that *Ginkgo biloba* may provide some cognitive benefits in AD patients, but only in cases under cholinesterase inhibitors treatment, but the clinical output of such effects remains to be clarified (Canevelli et al., 2014). On the other hand, in 2012 Vellas and coworkers conducted several clinical trials to study the effect of *Ginkgo biloba* extract in AD patients and healthy elderly subject that used this compound for longer periods of time. They aimed to assess the efficacy of 5 years' administration of a standardized *Ginkgo biloba* extract, widely used as coadjuvant in the treatment of patients with cognitive disorders. However, the results failed to show a protective effect in this type of cognitive disorders (Vellas et al., 2012).

Resveratrol is a powerful antioxidant that is present in many plants, including grapes, peanuts and plums, that protects against environmental stress. This compound has been extensively investigated for their potential properties in cardioprotection, anti-inflammatory effects, anticancer, and antiaging effects. It was also shown recently that it inhibits Aβ aggregation *in vitro* (Lu et al., 2012). The problem is that it exhibits a low bioavailability in the organism (Kang et al., 2009). On the basis of knowledge that resveratrol possesses a variety of bioactivities, a novel series of derivatives have been generated and tested as multi-target agents for AD treatment (Lu et al., 2012).

Another compound used in AD treatment is Cerebrolysin. This compound is a neuropeptide preparation consisting of low-molecular-weight peptides and free amino acids. This compound mimics the action of endogenous neurotrophic factors, and it is postulated that a mixture of this peptide with neurotrophic effects may reduce neurodegenerative pathologies (Rockenstein et al., 2006). Wei et al. proposed that the main effects of Cerebrolysin

include neurotrophic stimulation, neuroimmunological regulation and the improvement of glucose transportation across blood-brain barrier (Wei et al., 2007). After conducting a meta-analysis of six clinical trials, these authors, have postulated that Cerebrolysin could improve the clinical condition of AD patients, but large-scale trials are needed to provide convincing evidences of the efficacy of this compound on cognitive function and activities of daily living in AD (Wei et al., 2007). Further experimental research is needed to elucidate the molecular mechanism involved in some of the pleiotropic activities of Cerebrolysin, and particularly its influence on neuroinflammation, as well as to identify their possible interactions with neurotrophic factors and brain receptors (Álvarez and Fuentes, 2011). Main features of these agents are summarized in **Table 2**.

CONCLUSION

The development of AD involves a series of perturbations and imbalances that systemically affect the normal functioning of the CNS, triggering a condition of dementia. Despite scientific advances and knowledge that exists regarding the AD, still it has not been possible to develop effective treatment options. As it is now, most available treatments are designed against AD symptoms, and serve only as palliative treatments. In this context, Neuroimmunomodulation hypothesis appears as a guide in the search for new targets that have not been considered before, for developing effective treatments. This is the case of τ protein, which under pathogenic conditions self-aggregates and becomes one of the most important actors in the neurodegenerative cascade.

Based on the results of studies on long-term exposure to anti-inflammatory drugs, that show that these drugs are associated with a decreased risk of developing AD, a new interesting therapy may be available. NSAIDs may protect people from Alzheimer through several potential mechanisms that are associated with the disease based on the neuroinflammatory theory (Akiyama et al., 2000; Szekely et al., 2004). For example, they can reduce the inflammatory processes in the brain, because these drugs can inhibit the inflammatory response of microglial and/or astrocytes, reducing cell death due to excitotoxicity mediated by glutamate (Casper et al., 2000).

But the use of these type of drugs requires definitely more exploration and analysis, especially of the mechanism of action leading to an improvement in the patient after prolonged use. The same applies to any neuroprotective effect, since this is a very sensitive issue that should be considered to estimate the effect of these drugs in the body.

The appearance of new compounds that can open the way to new treatments becomes a necessity. In this search we can mention compounds such as curcumin and Andean Compound, which, because of their natural origin and the lack of adverse effects, appears as promising preparation for AD prevention. Studies that have been made on these compounds are very recent, but give strong evidence that its effects are mediated by disruption of the inflammatory response and self-aggregation of the τ protein, positioning them as a future therapeutic option for neuronal injury.

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TREM2 signaling, miRNA-34a and the extinction of phagocytosis

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The triggering receptor expressed in myeloid/microglial cells 2 (TREM2; encoded at chr6p21.1) is a glycosylated type 1 transmembrane sensor-receptor of the immunoglobulin-lectin-like gene superfamily expressed in the human central nervous system (CNS). TREM2 normally functions in immune surveillance, sensing and phagocytosis, including the homeostatic clearance of deleterious extracellular debris. Perhaps not too surprising, TREM2 deficiencies have been associated with pathological deficits in phagocytosis, amyloidogenesis and a compromised innate immune system in the inflammatory, neuro-degenerative illnesses polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy (PLOSL) and more recently with late onset Alzheimer's disease (AD; Forabosco et al., 2013; Golde et al., 2013; Guerreiro et al., 2013; Jonsson et al., 2013; Neumann and Daly, 2013; Zhao et al., 2013). Meta-analysis from multiple genome-wide association studies (GWAS) in AD have recently identified an rs75932628 (R47H; loss of function) variant in TREM2 as a strong AD risk factor, conveying an increase in AD with an odds ratio of 1.3–8.8-fold ($p = 0.0076$) in recent studies, an effect size comparable to that of the APOEe4 allele (Gonzalez Murcia et al., 2013). However, TREM2 R47H mutations appear to be relatively rare in the human populations so far studied (Gonzalez Murcia et al., 2013; Guerreiro et al., 2013; Hampel and Lista, 2013; Jonsson et al., 2013; Lattante et al., 2013).

Not so rare in AD, however, are significant focal increases in the abundance of a pro-inflammatory, NF-κB-regulated miRNA-34a (encoded at chr1p36.22) in virtually all AD cells and tissues examined compared to age-matched controls, as well as in amyloid overexpressing transgenic murine models for AD (Schipper et al., 2007; Wang et al., 2009; Zhao et al., 2013). For example, miRNA-34a was recently shown to be up-regulated, and TREM2 was found to be significantly down-regulated, in short post-mortem interval (mean ∼2 h) samples of sporadic AD hippocampal CA1 compared with age-matched controls. This novel epigenetic mechanism appears to be mediated by virtue of an unusually strong miRNA-34a recognition feature within the 299 nucleotide TREM2 mRNA 3′-untranslated (3′-UTR) region (energy of association, $E_A \leq 16$ kcal/mol; **Figure 1**) (Zhao et al., 2013). The stress- and inflammation-induced transcription factor NF-κB, a driver for miRNA-34a expression, is also strongly up-regulated in the hippocampal CA1, and both NF-κB inhibitors and stabilized anti-miRNA-34a are effective in restoring TREM2 back to homeostatic levels (Kaltschmidt and Kaltschmidt, 2009; Lukiw, 2013; Zhao et al., 2013). Interestingly, a pathologically up-regulated miRNA-34a has been strongly associated with progressive neurotrophic deficits (Wang et al., 2009), altered synaptogenesis (Agostini et al., 2011) and deficient immune and phagocytotic responses in inflammatory degenerative disorders such as cardiovascular disease (Boon et al., 2013), multiple sclerosis (Junker et al., 2009), and in sporadic AD mononuclear cells (Schipper et al., 2007) as well as in AD brain (Zhao et al., 2013).

Abundant evidence indicates that multiple genes, through multiple genetic processes, initiate and propagate AD-type change. Collectively, emerging observations indicate that an epigenetic mechanism involving an NF-κB-mediated, miRNA-34a-regulated down-regulation of TREM2 expression may shape innate immunity, inflammation and the extinction of the phagocytic response that contributes to amyloidogenesis and inflammatory neurodegeneration. Pro-inflammatory transcription factors and miRNAs, such as NF-κB and miRNA-34a, and their target mRNA 3′-UTRs appear to form a highly interwoven genetic regulatory network that may escape classical GWAS- and SNP-based detection. Interestingly, AD-relevant stress-mediated up-regulation of miRNA-34a in cultured microglial cells, subsequent down-regulation in the expression of TREM2-3′-UTR reporter vectors, and rescue by stabilized anti-miRNA-34a indicates that this type of pathogenic signaling can be effectively quenched, at least *in vitro* (Lukiw, 2013; Zhao et al., 2013). Totally novel anti-miRNA strategies involving miRNA-34a mimics (i.e., MRX34) that normally induce senescence and apoptosis, and utilizing liposome delivery technologies are just now appearing in the clinic for the treatment of metastatic liver cancer (Bouchie, 2013). In the near future these approaches may have considerable potential in also directing novel, combinatorial anti-NF-κB- and/or anti-miRNA-based AD therapeutic strategies that target the multiple pathogenic pathways which lie at the core of the AD process.

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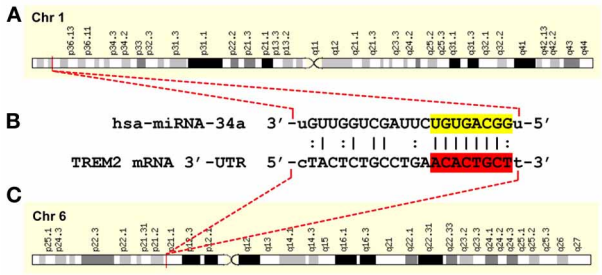

FIGURE 1 | A hsa-miRNA-34a-TREM2-mRNA-3′-UTR complementarity map; gene products on two independent chromosomes orchestrate a down-regulation of TREM2 and a progressive deficit in cellular debris sensing, phagocytosis and clearance in human neurodegenerative disease. (A) an NF-κB-sensitive miRNA-34a (encoded at chr1p36.22) and up-regulated in AD has been found to target **(B)** the central domain of the 299 nucleotide human TREM2 mRNA 3′-untranslated region (3′-UTR) of the TREM2 gene **(C)** encoded at chr6p21.1; thus the functional interaction of 2 independent gene products may be responsible for TREM2 deficits in sporadic AD; in **(B)** the miRNA-34a seed sequence 3′-UGUGACGG-5′ is highlighted in yellow; the complementary TREM2-3′-UTR recognition sequence 5′-ACACTGCT-3′ is highlighted in red; an "|" indicates a full hydrogen bond between miRNA-34a and the TREM2-mRNA-30-UTR and a ":" indicates a partial hydrogen bond; the hsa-miRNA-34a recognition feature is located about midway in the TREM2 mRNA-3′-UTR; other miRNA recognition features located within the TREM2-3′-UTR may also affect TREM2 mRNA stability and regulate its expression; other miRNA-mRNA pairings may also be involved in TREM-2 function; the TREM2 gene has no strong NF-κB binding site within at least 11 kb of its transcription start site (Zhao et al., 2013 and unpublished observations); ribonucleotide sequences and alignment derived using miRBASE algorithms (European Bioinformatics Institute, Wellcome Trust Genome Campus, Hinxton UK; http://www.ebi.ac.uk/enright-srv/microcosm/cgi-bin/targets/v5/detailview.pl?transcript _id=ENST00000373113; Lukiw, 2013; Neumann and Daly, 2013; Zhao et al., 2013).

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Regulating amyloidogenesis through the natural triggering receptor expressed in myeloid/microglial cells 2 (TREM2)

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Amyloidogenesis, the progressive accumulation of amyloid-beta (Aβ) peptides into insoluble, toxic, senile plaque lesions is one of the major defining features of the Alzheimer's disease (AD) brain. Normally, Aβ42 peptides are cleared from the extracellular space by natural phagocytic mechanisms, but when this intrinsic sensing and clearance system is functionally compromised or defective, Aβ42 peptides accumulate. Largely due to their intensely hydrophobic character, under physiological conditions Aβ42 peptides have a strong tendency to self-aggregate into higher order neurotoxic and pro-inflammatory fibrillar aggregates. While the Aβ peptide-clearing mechanisms are highly complex, one particularly important molecular sensor for Aβ42 peptide clearance is the triggering receptor expressed in myeloid/microglial cells 2 (TREM2; encoded at chr6p21.1), a variably glycosylated 230 amino acid transmembrane spanning stimulatory receptor of the immunoglobulin/lectin-like gene superfamily strongly associated with innate-immune, pro-inflammatory, and neurodegenerative signaling in AD. TREM2 is highly and almost exclusively expressed on the outer plasma membrane of microglial cells, the resident phagocytic and scavenging neuroimmune macrophages of the human central nervous system (CNS). As AD progresses, microglia appear to become progressively dysfunctional; TREM2 becomes down-regulated and microglia lose their ability to clear Aβ42 peptides while producing and releasing neurotoxins, reactive oxygen species (ROS), and pro-inflammatory cytokines that further promote Aβ42 production and pathological aggregation (Schmid et al., 2002; Alexandrov et al., 2013; Boutajangout and Wisniewski, 2013; Hickman and El Khoury, 2013).

Recently, much interest in the molecular biology, genetics, and epigenetics of TREM2 expression, and its potential for sensing and scavenging Aβ42 peptides in AD and other progressive neurodegenerative diseases has arisen. TREM2's critical importance is underscored by seven recent observations: (1) that relatively rare mutations of TREM2 (or of its coupling protein, DAP12, also known as TYROBP; see **Figure 1**) are currently associated with the progressive, pre-senile dementing illnesses Nasu-Hakola syndrome, polycystic lipomembranous osteodysplasia with sclerosing leucoencephalopathy (POSL), sporadic amyotrophic lateral sclerosis (ALS), and AD (Nimmerjahn et al., 2005; Neumann and Takahashi, 2007; Guerreiro and Hardy, 2013; Zhao and Lukiw, 2013; Zhao et al., 2013; Cady et al., 2014); (2) that down-regulation in the phagocytic ability of microglia to degrade Aβ42 peptides in AD, and down-regulation in TREM2 expression, has been reported in sporadic AD brain tissues (Hickman and El Khoury, 2013; Zhao et al., 2013); (3) that TREM2 knockdown has been shown to exacerbate age-related neuro-inflammation and enhance cognitive deficiency in senescence accelerated mouse prone 8 (SAMP8) mice (Jiang et al., 2014); (4) that microglial TREM2 gene expression in cell culture, both at the level of mRNA and protein, have been shown to be remarkably sensitive to external cytokine stressors such as tumor necrosis factor alpha (TNFα; a pro-inflammatory adipokine known to be up-regulated in AD brain; Zhao et al., 2013; unpublished observations); (5) that AD-relevant pro-inflammatory neurotoxins such as bacterial lipopolysaccharide (LPS) and environmentally abundant toxic metals such as aluminum strongly down-regulate TREM2 and the ability of microglial cells to phagocytose extracellular debris (Hickman and El Khoury, 2013; Zhao et al., 2013; unpublished observations); (6) that down-regulation in the expression of TREM2 appears to be regulated in part by the up-regulation of the microglial-enriched, NF-kB-sensitive microRNA-34a (miRNA-34a), and perhaps other NF-kB-sensitive miRNAs, and (7) that both anti-NF-kB and anti-microRNA (AM-RNA) strategies have been shown to be useful in the restoration of homeostatic TREM2 gene expression levels and the neutralization of pro-inflammatory signaling and amyloidogenesis, at least *in vitro* (Hill et al., 2009; Pogue et al., 2009, 2010; Alexandrov et al., 2012, 2013; Zhao et al., 2013; unpublished observations). From what we know so far it is tempting to speculate that (1) loss-of function of TREM2 due to genetic mutations in familial AD may have the

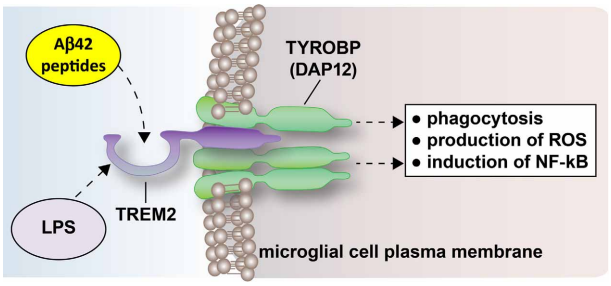

FIGURE 1 | The triggering receptor expressed in myeloid/microglial cells type 2 (TREM2) is a 230 amino acid, ~25.4 kDa integral trans-membrane glycoprotein sensor spanning the lipid bilayer of CNS microglial cells. Very recently, TREM2 has been shown to act as a phagocytic receptor of bacterial lipopolysaccharide (LPS), Aβ42 peptides and other cellular end-stage noxious cellular products (N'Diaye et al., 2009; Guerreiro and Hardy, 2013; Jonsson et al., 2013). TREM2-LPS or TREM2-Aβ peptide recognition may be achieved in part through a pathogen-associated molecular pattern (PAMP) characteristic of highly specific molecular features located on LPS or Aβ42 molecules (Boutajangout and Wisniewski, 2013; Zhao et al., 2013; unpublished observations). Transmembrane signaling via TREM2 is in part accomplished through a trans-membrane adapter, tyrosine kinase binding protein called TYROBP, also known as the DNAX-activation protein 12 (DAP12) or the polycystic lipomembranous osteodysplasia with sclerosing leukoencephalopathy (PLOSL) protein (Schmid et al., 2002; Jonsson et al., 2013). TREM2 signaling triggers the phagocytic uptake of cellular debris and is associated with the further down-stream induction of reactive oxygen species (ROS) and the pro-inflammatory transcription factor NF-kB (Charles et al., 2008; N'Diaye et al., 2009). Up-regulation of ROS and NF-kB are a characteristic feature of inflammatory neurodegeneration and increasing Aβ42 peptide load in AD brain (Hickman and El Khoury, 2013; Jonsson et al., 2013; Zhao et al., 2013). Interestingly, TREM2 expression is critical for the clearance of neural debris of the injured or lesioned CNS, and loss-of-function mutations in TREM2 or TYROBP (DAP12) are linked to presenile dementias characteristic of ALS or AD-type neocortical degeneration (Charles et al., 2008; Guerreiro and Hardy, 2013; Cady et al., 2014; Jiang et al., 2014). This highly schematicized figure was adapted in part from Nimmerjahn et al. (2005), Neumann and Takahashi (2007), Guerreiro and Hardy (2013), and Neumann and Daly (2013).

same end effects on phagocytosis as down-regulation of a fully functional TREM2 in sporadic AD; and that (2) modest TREM2 over-expression might be useful in enhancing the scavenging and removal of cellular debris in the CNS, including neurotoxic and self-aggregating Aβ42 peptides. Importantly, TREM2 signaling has been recently shown to be selectively inducible and manipulated from outside of the cell, suggesting that the modulation of TREM2 expression may be effectively regulated using highly specific targeting via drug-based pharmacological strategies exogenously supplied (Alexandrov et al., 2013; Lukiw, 2013; Zhao et al., 2013).

AD represents a highly complex, insidious, progressive, multi-factorial brain dysfunction whose incidence is reaching epidemic proportions. Despite the billions of dollars already spent on AD research, including multiple Aβ immunization and immunotherapy strategies, there is still no adequate treatment or cure for AD, and the development and implementation of novel, more effective treatment strategies are critical. Recruitment and harvesting of the TREM2 mechanism as a potent, endogenous Aβ42-peptide scavenging activity may represent a singularly attractive new direction for the clinical management of AD. Indeed, as a natural sensor and scavenger of noxious cellular debris TREM-2 stimulation may turn out (1) to be remarkably neuroprotective against both amyloidogenesis and age-related neuro-inflammation; while (2) significantly reducing the progressive cognitive impairment associated with amyloidogenesis and inflammatory neurodegeneration in the CNS (Hickman and El Khoury, 2013; Zhao et al., 2013; Cady et al., 2014; Jiang et al., 2014). Put another way, a decline in TREM2's contribution to the innate immune response, in part driving amyloid-clearance deficits and progressive degeneration characteristic of the AD process, suggest novel therapeutic targets and treatment strategies directed at maintaining natural and homeostatic TREM2 functions. This may be accomplished not only through the direct stimulation of TREM2 itself but also through the poorly understood down-stream TREM2-linked TYROBP (DAP12) signaling pathways responsible for; (1) the actual phagocytosis of extracellular molecules; (2) the production of damaging quantities of ROS; (3) the induction of pro-inflammatory signals via NF-kB; and (4) the maintenance of homeostatic microglial function that together may diminish amyloidogenesis and the intercellular propagation of pathogenic signaling in the AD affected brain (Boutajangout and Wisniewski, 2013; Guerreiro and Hardy, 2013; Zhao and Lukiw, 2013; Jiang et al., 2014; **Figure 1**).

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Alzheimer's disease and the microbiome

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> "Microbial colonization of mammals is an evolution-driven process that modulates host physiology, many of which are associated with immunity and nutrient intake"—Heijtz et al. (2011)

The recognition of the human microbiome (HM) as a substantial contributor to nutrition, health and disease is a relatively recent one, and currently, peer-reviewed studies linking alterations in microbiota to the etiopathology of human disease are few. Emerging studies indicate that the HM may contribute to the regulation of multiple neuro-chemical and neuro-metabolic pathways through a complex series of highly interactive and symbiotic host-microbiome signaling systems that mechanistically interconnect the gastrointestinal (GI) tract, skin, liver, and other organs with the central nervous system (CNS). For example, the human GI tract, containing 95% of the HM, harbors a genetically diverse microbial population that plays major roles in nutrition, digestion, neurotrophism, inflammation, growth, immunity and protection against foreign pathogens (Forsythe et al., 2012; Collins et al., 2013; Douglas-Escobar et al., 2013; see below). It has been estimated that about 100 trillion bacteria from up to 1000 distinct bacterial species co-inhabit the human GI tract, albeit in different stoichiometries amongst individuals, and the varying combinations and strains of bacterial species amongst human populations might contribute, in part, to "***human-biochemical***" or "***genetic-individuality***" and resistance to disease (Aziz et al., 2013; Lukiw, 2013). Interestingly, HM participation in human physiology may also help explain the genome-complexity conundrum—for example why the 26,600 protein-encoding transcripts in *Homo sapiens* are far fewer in number, than for example, the rice genome (*Oryza sativa*; which has about 46,000 functional genes). One thousand different strains of bacteria might be expected to contribute up to 4×10^6 potential mRNAs to the human transcriptome, thus making the human host-plus-microbiome genetic complexity closer to 4,026,600 mRNA transcripts, and a clear "winner" of human genetic complexity over that of rice and other species (Venter et al., 2001; Foster and McVey Neufeld, 2013; Lukiw, 2013). The very recent observation of microbiome-derived small non-coding RNA (sncRNA) and micro RNA (miRNA) translocation and signaling across endothelial barriers, between cells and tissues, and even perhaps between individual species indicates that human neurobiology may be significantly impacted by the actions of HM-mediated sncRNA or miRNA trafficking, and the integration of a cell, tissue or an entire organism into its local environment (Zhao et al., 2006; Alexandrov et al., 2012; Sarkies and Miska, 2013; Reijerkerk et al., 2013; unpublished). This opinion paper encompasses what we know concerning the contribution of the HM to neurological disease, with specific emphasis on Alzheimer's disease (AD) wherever possible.

Firstly, the microbiome of the human GI tract is the largest reservoir of microbes in the body, containing about 10^{14} microorganisms; over 99% of microbiota in the gut are anaerobic bacteria, with fungi, protozoa, archaebacteria and other microorganisms making up the remainder. There is currently an expanding interest in the ability of intestinal bacteria to influence neuro-immune functions well beyond the GI tract. Since mitochondria are believed to originate from bacteria via endosymbiotic relationships that formed very early in the evolutionary history of eukaryotes, cross-reactivity of mitochondria and immunological responses to intestinal bacterial constituents could have deleterious effects on mitochondrial function through some form of molecular mimicry; this is partially evidenced by the inflammatory basal ganglia disorder Sydenham's chorea, rheumatic fever and the link to the facultative anaerobe *Streptococcus* (Carrasco-Pozo et al., 2012; Douglas-Escobar et al., 2013; Hayashi, 2013; Hornig, 2013 see below). Established pathways of GI-CNS communication currently include the autonomic nervous system (ANS), the enteric nervous system (ENS), the neuroendocrine system, and the immune system (Camfield et al., 2011; Heijtz et al., 2011; Forsythe et al., 2012; Aziz et al., 2013; Collins et al., 2013; Foster and McVey Neufeld, 2013; Schwartz and Boles, 2013). Stress further influences the composition of the HM, and reciprocal communication between the CNS and the HM also influences stress reactivity (Forsythe et al., 2012; Foster and McVey Neufeld, 2013). Surprisingly, neuronal signaling pathways along the bidirectional GI-CNS axis remain poorly understood despite their important roles: (i) in coordinating metabolic- and nutritive-functions, and (ii) in their functional disruption in chronic diseases such as metabolic syndrome, diabetes, obesity, anxiety, autoimmune-disease and stress-induced neuropsychiatric disease (Lukiw and Bazan, 2006; Bravo et al., 2012; Foster and McVey Neufeld, 2013; Hornig, 2013; Udit and Gautron, 2013). Studies of the ENS in "germ-free" mice, i.e., those

missing their microbiome, indicates that commensal intestinal microbiota are absolutely essential for passive membrane characteristics, action potentials within the ENS, and the excitability of sensory neurons, thus providing a potential mechanistic link for the initial exchange of signaling information between the GI tract microbiome and the CNS (Foster and McVey Neufeld, 2013; Hornig, 2013; McVey Neufeld et al., 2013). Indeed, secretory products of the GI microbiome and translocation of these signaling molecules via the lymphatic and systemic circulation throughout the CNS are just beginning to be identified. For instance, the GI tract-abundant gram-positive facultative anaerobic or microaerophilic *Lactobacillus*, and other *Bifidobacterium* species, are capable of metabolizing glutamate to produce gamma-amino butyric acid (GABA), the major inhibitory neurotransmitter in the CNS; dysfunctions in GABA-signaling are linked to anxiety, depression, defects in synaptogenesis, and cognitive impairment including AD (Aziz et al., 2013; Hornig, 2013; Mitew et al., 2013; Paula-Lima et al., 2013; Saulnier et al., 2013). To cite another important example, brain-derived neurotrophic factor (BDNF) has pleiotropic effects on neuronal development, differentiation, synaptogenesis and the synaptic plasticity that underlies circuit formation and cognitive function, and has been found to be decreased in brains and serum from patients with schizophrenia, anxiety and AD (Carlino et al., 2013; Lu et al., 2013; Mitew et al., 2013). In experimental infection models known to lead to alterations in the microbiota profile, BDNF expression was found to be reduced in the hippocampus and cortex of "germ free" mice, and this reduction in the expression of BDNF was found to associate with increased anxiety behavior and progressive cognitive dysfunction (Carlino et al., 2013; Foster and McVey Neufeld, 2013; Lu et al., 2013).

Equally interesting are microbiome interactions with the N-methyl-D-aspartate (NMDA) glutamate receptor, a prominent CNS device that regulates synaptic plasticity and cognition (Lakhan et al., 2013). For example, the NMDA-, glutamate-targeting, glutathione-depleting and oxidative-stress-inducing neurotoxin β-N-methylamino-L-alanine (BMAA), found elevated in the brains of patients with amyotrophic-lateral sclerosis (ALS), Parkinson-dementia (PD) complex of Guam and AD, has been hypothesized to be generated by cyanobacteria of the intestinal microbiome, and stress, GI tract disease or malnutrition may further induce BMAA abundance to ultimately contribute to neurological dysfunction (Brenner, 2013). Other HM-resident cyanobacteria-generated neurotoxins such as saxitoxin and anatoxin-α may further contribute to human neurological disease, especially during aging when the intestinal epithelial barrier of the GI tract becomes more permeable (Tran and Greenwood-Van Meerveld, 2013). Interestingly, BMAA, a neurotoxic amino acid not normally incorporated into protein, has been linked with intra-neuronal protein misfolding, a hallmark feature of the amyloid peptide-enriched senile plaque lesions, and resultant inflammatory neurodegeneration, that characterize PD, AD and prion disease (He and Balling, 2013; Hornig, 2013; Mulligan and Chakrabartty, 2013; Schwartz and Boles, 2013). Hence, besides potentially altering CNS neurochemistry and neurotransmission, HM-bacteria not only secrete molecules that potentially modulate systemic- and CNS-amyloidosis, they also widely utilize their own amyloid peptides as structural materials, adhesion molecules, toxins, molecules that function in the protection against host defenses and auto-immunity. The specific contribution of host bacteria and bacterial amyloid, however, to misfolding, amyloidogenic diseases such as AD remain to be more clearly defined (Schwartz and Boles, 2013). The HM further appears to condition host immunity to foreign microbes, including viral infection and xenobiotics, while regulating autoimmune responses that can impact homeostatic metabolic- and neural-signaling functions within the CNS (Ball et al., 2013; Douglas-Escobar et al., 2013; Hornig, 2013). An increased prevalence of autoimmunity, exposure to pathogens both pre- and post-natally, and findings of anti-brain antibodies, common in disorders as diverse as anxiety, schizophrenia, obsessive-compulsive disorder, depression and autism, together suggest that differences in exposure and genetic vulnerability toward HM-mediated autoimmunity may be significant determinants of age-related neurological disease course and outcome (Ball et al., 2013; Douglas-Escobar et al., 2013; Hornig, 2013).

Regarding potentially pathogenic micobiota stationed outside of the GI tract, about 95% of all humans harbor the highly neurotrophic herpes simplex-1 (HSV-1) in their trigeminal ganglia, but whether this is a neutral or symbiotic relationship, or detrimental to the host, remains open to speculation. Induction of HSV-1, and other forms of endogenous viral reactivation are certainly stress-related, but whether GI tract HM-derived metabolites are involved in these kinds of pathogenic activation mechanisms is not well understood (Hill et al., 2009; Prasad et al., 2012). Recent studies suggest that activation of endogenous HSV-1 or other neurotrophic microorganisms, including host-embedded prions, are intimately linked to neurological stressors linked to amyloidogensis, inflammatory neurodegeneration and progressive cognitive impairment, and may be a contributor to the early development of, or predisposition to, schizophrenia and AD (Hill et al., 2009; Prasad et al., 2012; Ball et al., 2013; Manuelidis, 2013). Indeed, correlation of metabolic and neurological phenotypes with the GI tract HM and other specific endogenous bacterial or viral profiles derived from independent molecular analytical technologies should be increasingly useful for deciphering complex host-microbiome relationships in healthy human brain aging and in neuropsychiatric disease (Xie et al., 2013).

Further studies of symbiotic HM-CNS communication intrinsically suggests the potential for microbial-based therapeutic strategies that may aid in the augmentation of the HM, for the treatment of human disease, including neurological disorders (Forsythe et al., 2012; Collins et al., 2013). The original observation of the health-promoting benefits of GI tract bacteria and the HM was first introduced in 1907 by the Russian biologist Ilya Metchnikoff (Nobel Prize in Medicine 1908, shared with Paul Erlich; Buryachkovskaya et al., 2013). Metchnifoff's works focused on prokarytoic immunology, phagocytosis, the

anti-aging properties instilled by host bacteria, inflammation as a protective adaptation against injury, and early ideas on neurogastroenterology (Buryachkovskaya et al., 2013; Saulnier et al., 2013). Hence, for well over 100 years, host-beneficial GI tract bacteria, collectively known as probiotics, have been proposed to be useful to human health, and more recently have been added to various foods and diets because of their positive health-promoting effects (Singh et al., 2013). The beneficial actions of bacterial-based probiotics are highly inter-related, and are thought to function, in part: (i) to aid in complex carbohydrate fermentation and absorption; (ii) to provide a significant source of a range of essential vitamins, particularly those of the vitamin B and K group; (iii) to compete with pathogenic microorganisms in the GI tract; (iv) to antagonize and neutralize enteric pathogens; (v) to metabolize and neutralize dietary carcinogens; and (vi) to favorably modulate the host's immune response to resist infection and disease. Besides the potential application of probiotics in the prevention and treatment of various health conditions and diseases such as allergies, GI and urogenital tract infections, inflammatory disease, cystic-fibrosis and certain cancers there is increasing interest of positive microbiome effects toward the CNS via neural, neuroendocrine, neuroimmune and humoral links (Duncan and Flint, 2013; He and Balling, 2013; Saulnier et al., 2013). For example, there is preliminary research on the influence of probiotics and nutritional factors on the prognosis of multiple sclerosis (von Geldern and Mowry, 2012), cognition (Camfield et al., 2011), neurogastroenterology in general (Saulnier et al., 2013), and stress-related psychiatric conditions including anxiety, autism, depression and schizophrenia (Bravo et al., 2012; Prasad et al., 2012; Douglas-Escobar et al., 2013). Advances in probiotic technologies in CNS disease research are already raising a number of ethical, legal, and socioeconomic concerns (Slashinski et al., 2012).

Lastly, the US NIH "Roadmap" program has recently initiated the HM project (HMP; http://commonfund.nih.gov/Hmp/), using recently discovered genomic technologies with the specific aims (i) to characterize the microbial communities at several different sites on the human body, including nasal, oral and otic cavities, the skin, GI and urogenital tracts; (ii) to analyze the role of these microbes in homeostatic human physiology; (iii) to catalog specific microbiome speciation, composition and correlation with disease; and (iv) to generate resources enabling comprehensive characterization of the HM by multiple independent research groups. These investigations present a highly significant and exciting avenue for future study, and suggest new and perhaps unconventional directions for AD research in 2013 and beyond. Future AD therapies may well, in part, involve probiotic approaches, especially as a prophylactic tactic before mild-cognitive impairment (MCI) or AD is first diagnosed. The implications of altered host-HM interactions in neurological disease would be far-reaching indeed, and these may engender novel microbiome manipulative strategies, tailored to the host, for the more effective therapeutic management of AD and related neuropsychiatric disorders.

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