NEUROSCIENTIFIC RESEARCH FOR MANAGEMENT OF DEMENTIA

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NEUROSCIENTIFIC RESEARCH FOR MANAGEMENT OF DEMENTIA

Topic Editors:
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The articles in this eBook are divided into three main chapters. Chapter 1 focuses on the role of specific proteins in the pathological processes of neurodegeneration, specifically Alzheimer's disease. Chapter 2 describes novel candidates and risk factors for the diagnosis of Alzheimer's disease. Chapter 3 targets various therapeutic interventions from pharmacological targets to cognitive function. This eBook thus provides an overall overview of the latest research in understanding mechanisms leading to the development of Alzheimer's disease, diagnosis, and therapeutic interventions.

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EDITORIAL
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Editorial: Neuroscientific Research for Management of Dementia

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Editorial on the Research Topic

Neuroscientific Research for Management of Dementia

Each system in our body is valuable for the unique function it carries out. The nervous system for example is easily the most complex system in the human species which is sophistically made up of neurons, synapses, and various important specialized cells for appropriate signaling of neurotransmission. Overall role of CNS is to control whole body functions in a systematic mechanism. Any abnormalities in any part of neuro-mechanism result in physiological to psychological disorder/behaviors. Therefore, researchers and medical health care staff are driven to improve physical health as well as state of psychological health in term of close connection with the nervous and immune system. The purpose of this Research Topic of Frontiers in Aging Neuroscience was to shed light on the latest outstanding discoveries pertained in wide spectrum of aging neuroscience by covering different aspects of nervous system to dementia in their original research articles and reviews. Taking this into consideration, the Research Topic on Frontiers in Neuroscientific Research for Management of Dementia by Frontiers in Aging Neuroscience makes a contribution with updates and different perspective on this important theme, developed over 17 articles (**Table 1**).

We hope that this Frontiers Research Topic will be an enrichment for Neuroscientific Research for Management of Dementia, with the efforts and commitment of all authors to whom we give our acknowledgment as well as to the reviewers who have contributed in improving and clarifying these diverse contributions due to their valuable comments. Finally, a special thanks to Editor in Chief and Frontiers management team for support in publishing process.

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TABLE 1 | Summaries of articles in the Research Topic on Frontiers in Neuroscientific Research for Management of Dementia.

Authors	Title	Summary
Cho et al.	Changes in blood factors and ultrasound findings in mild cognitive impairment and dementia	The present study aimed to assess the changes in blood factors and ultrasound measures of atherosclerosis burden patient with MCI and dementia. Peripheral blood samples and ultrasonography findings were obtained for 53 enrolled participants. Flow cytometry was used to evaluate levels of activated platelets and platelet-leukocyte aggregates (PLAs). The number of platelets expressing p-selectin was correlated with IMT (intima media thickness) and plaque number in both the MCI and dementia groups. The number of platelets expressing p-selectin glycoprotein ligand (PSGL) was strongly correlated with IMT in patients with MCI, whereas the number of platelets expressing PGSL was correlated with plaque number rather than IMT in patients with dementia. PLAs was associated with both IMT and plaque number in patients with MCI but not in those with dementia. Our findings demonstrate that alterations in IMT and plaque number are associated with an increased risk of cognitive decline as well as conversion from MCI to dementia and that blood factor analysis may aid to detect the severity of cognitive decline.
Rahman et al.	Entanglement of UPR^{ER} in Aging driven Neurodegenerative diseases	The endoplasmic reticulum (ER) is an indispensable cellular organelle that remains highly active in neuronal cells. The ER bears the load of maintaining protein homeostasis in the cellular network by managing the folding of incoming nascent peptides; however, the stress imposed by physiological/environmental factors can cause ER dysfunctions that lead to the activation of ER unfolded protein response UPR^{ER}. Aging leads to deterioration of several cellular pathways and therefore weakening of the UPR^{ER}. The decline in functioning ofthe UPR^{ER} during aging results in accumulation of misfolded proteins that becomes intracellular inclusions in neuronal cells, resulting in toxicity manifested as neurodegenerative diseases. With ascension in cases of neurodegenerative diseases, understanding the enigma behind aging driven UPR^{ER} dysfunction may lead to possible treatments.
Xiong et al.	Dl-3-n-butylphthalide treatment enhances hemodynamics and ameliorates memory deficits in rats with chronic cerebral hypoperfusion	Our previous study has revealed that chronic cerebral hypoperfusion (CCH) activates a compensatory vascular mechanism attempting to maintain an optimal cerebral blood flow (CBF). However, this compensation failed to prevent neuronal death and cognitive impairment because neurons die prior to the restoration of normal CBF. Therefore, pharmacological invention may be critical to enhance the CBF for reducing neurodegeneration and memory deficit. Dl-3-n-butylphthalide (NBP) is a compound isolated from the seeds of Chinese celery and has been proven to be able to prevent neuronal loss, reduce inflammation, and ameliorate memory deficits in acute ischemic animal models and stroke patients. In the present study, we used magnetic resonance imaging (MRI) techniques, immunohistochemistry, and Morris water maze to investigate whether NBP can accelerate CBF recovery, reduce neuronal death and improve cognitive deficits in chronic cerebral hypoperfusion (CCH) rats after permanent bilateral common carotid artery occlusion (BCCAO). Rats were intravenously injected with NBP (5 mg/kg) daily for 14 days beginning the first day after BCCAO. The results showed that NBP shortened recovery time of CBF to pre-occlusion levels at 2 weeks following BCCAO, compared to 4 weeks in the vehicle group, and enhanced hemodynamic compensation through dilation of the vertebral arteries and increase in angiogenesis. NBP treatment also markedly reduced reactive astrogliosis and cell apoptosis and protected hippocampal neurons against ischemic injury. The escape latency of CCH rats in the Morris water maze was also reduced in response to NBP treatment. These findings demonstrate that NBP can accelerate the recovery of CBF and improve cognitive function in a rat model of CCH, which suggesting that NBP is a promising therapy for CCH patients or vascular dementia.
Jan et al.	Perspective insights of exosomes in neurodegenerative diseases: A critical appraisal	Exosomes are small membranous entities of endocytic origin. Their production by a wide variety of cells in eukaryotes implicates their roles in the execution of essential processes, especially cellular communication. Exosomes are secreted under both physiological and pathophysiological conditions, and their actions on neighboring and distant cells lead to the modulations of cellular behaviors. They also importantly assist the delivery of disease causing entities, such as, prions, α-syn, and tau, and thus, facilitate spread to non-effected regions and accelerate the progressions of neurodegenerative diseases. The characterization of exosomes, provides information on aberrant processes, and thus, exosome analysis has many clinical applications. Because they are associated with the transport of different cellular entities across the blood-brain barrier, exosomes might be useful for delivering drugs and other therapeutic molecules to brain. Herein, we review roles played by exosomes in different neurodegenerative diseases, and the possibilities of using them as diagnostic biomarkers of disease progression, drug delivery vehicles, and in gene therapy.
Park et al.	Low serum phosphorus correlates with cerebral Aβ deposition in cognitively impaired subjects: results from the KBASE study	Alzheimer's disease (AD), characterized by progressive cognitive decline, is the most prevalent neurodegenerative disease in the elderly. Cerebral β-amyloid (Aβ) deposition is the major pathological hallmark of AD. Recent studies also have shown that the serum level of phosphorus correlates to the risk of incident dementia. To date, the linkage between cerebral Aβ deposition and the serum phosphorus level remains unknown. In this study, we analyzed the levels of serum phosphorus in 109 mild cognitive impairment (MCI) and 73 AD dementia (ADD) subjects. All subjects underwent Pittsburgh compound B positron emission tomography (PiB-PET) imaging to measure cerebral Aβ deposition. The results with Aβ deposition was compared with the serum levels of phosphorus. The subjects with cerebral Aβ deposition showed lower levels of serum phosphorus than those without Aβ deposition. Furthermore, multiple regression analyses showed that a low level of serum phosphorus correlated with cerebral Aβ deposition, even when age, sex, apolipoprotein E ε4 genotype, and MMSE z-score were controlled for. Serum levels of other ions, including calcium, iron, zinc, and copper, showed no such correlation. In conclusion, our results suggest that the serum level of phosphorus may be used as an easily accessible blood biomarker for cerebral Aβ deposition in a cognitively impaired population.
Kuang et al.	Neuroprotective effect of Ligustilide through induction of α-secretase processing of both APP and Klotho in a mouse model of Alzheimer's disease	Emerging evidence suggests that alpha-processing single transmembrane proteins, amyloid precursor protein (APP) and anti-aging protein Klotho, are likely to be involved in the progression of Alzheimer's disease (AD). The natural phthalide Ligustilide (LIG) has been demonstrated to protect against aging- and amyloid-β (Aβ)-induced brain dysfunction in animal models. The present study is to investigate the effects of LIG on cognitive deficits and metabolism of both APP and Klotho and its underlying mechanism in AD double-transgenic (APP/PS1) mice and cultured human cells. Our results show that treatment with LIG significantly ameliorated memory impairment and Aβ levels and plaques burden. Specifically, LIG might act as a potent enhancer of α-secretase, disintegrin, and metalloprotease 10 (ADAM10), leading to upregulation of alpha-processing of both APP and Klotho and subsequent increases in the levels of both soluble APP fragment (sAPPα) and soluble Klotho (sKL) with inhibition of IGF-1/Akt/mTOR signaling in AD mice and cultured cells. Moreover, the specific ADAM10 inhibitor (G1254023X) effectively reversed LIG-induced alpha-processing of both APP and Klotho *in vitro*, while Klotho gene knockdown by small interfering RNA (siRNA) significantly blunted LIG-mediated inhibition of IGF-1/Akt/mTOR

(Continued)

TABLE 1 | Continued

Authors	Title	Summary
		signaling *in vitro*. Taken together with reported neuroprotective effects of both sAPPα and sKL as well as autophagy induction by Akt/mTOR pathway inhibition, our findings suggest that neuroprotection of LIG against AD is associated with induction alpha-processing of APP and Klotho and potential Aβ clearance. Whether LIG might induce Aβ autophagic clearance and the underlying mechanisms need to be further studied.
Yang et al.	Multiple evidences for association between cognitive impairment and dysglycemia in Parkinson's disease: implications for clinical practice	Background and purpose: It remains unclear about the etiopathogenesis of cognitive impairment (CI) in Parkinson's disease (PD). Since diabetes mellitus (DM) has been shown to be associated with CI in several diseases, we examined the association between CI and dysglycemia in PD. Methods: Enrolled PD patients completed a series of clinical and neuropsychological assessments. Motor symptoms were determined by Hohen-Yahr staging (H-Y staging) and Unified Parkinson's Disease Rating Scale—motor score (UPDRS-III). Neuropsychological functions were evaluated by the MiniMental State Examination (MMSE), the Montreal Cognitive Assessment (MoCA), and the Hamilton Anxiety and Depression Scales. Moreover, fasting glucose, fasting insulin, glycosylated hemoglobin A1c (HbA1c) and oral glucose tolerance test were performed to assess glucose metabolism. Results: MoCA and MMSE scores in PD patients with DM group (PD-DM) were significantly lower than those in PD patients without DM group (PD-nDM). Consistently, PD-DM group showed significantly higher constituent ratio of CI than PD-nDM group. In addition, MoCA scores in HbA1c $\geq$ 6.5% group and HbA1c $\geq$ 7% group were significantly lower than those in the corresponding control groups. MoCA score in IR $\geq$ 3 group was significantly lower than that in IR $<$ 3 group. Furthermore, MoCA score was negatively correlated with H-Y staging, HbA1c and insulin resist ance, respectively. Finally, regression analysis indicated that H-Y staging and HbA1c $\geq$ 7% were independent risk factors of CI in PD. Conclusions: CI may be tightly associated with dysglycemia in, at least partially, PD patients. Importantly, H-Y staging and HbA1c $\geq$ 7%, two independent risk factors of CI in PD, may serve as key biomarkers in future PD clinical practice.
Islam et al.	Presence of anticardiolipin antibodies in patients with dementia: a systematic review and meta-analysis	Growing evidences are supporting toward the involvement of antiphospholipid antibodies [aPLs e.g., lupus anticoagulant (LA), anticardiolipin (aCL) and anti-β2-glycoprotein I (anti-β2-GPI) antibodies] in various neurological manifestations including migraine, epilepsy and dementia in the presence or absence of autoimmune diseases such as antiphospholipid syndrome or systemic lupus erythematosus. The aim of this systematic review and meta-analysis was to assess the presence of aPLs in dementia patients without a diagnosis of any autoimmune disease. Electronic databases (e.g., PubMed, Web of Science, Scopus, ScienceDirect, and Google Scholar) were searched without any year or language restrictions and based on the inclusion criteria, nine prospective case-control studies assessing only aCL were included involving 372 dementia patients and 337 healthy controls. No studies were found to assess the presence of both LA or anti-β2-GPI. The study-specific odds ratios (ORs) and 95% confidence intervals (CIs) were calculated using random-effects model. We observed the prevalence of aCL in dementia was higher (32.80%) than that of controls (9.50%) e.g., 3.45 times higher risk of presenting with dementia than the controls, and significant presence of aCL antibodies was detected in dementia patients compared to controls (OR: 4.94, 95% CI: 2.66–9.16, $p < 0.00001$; $I^2 = 32\%$, $p = 0.16$). Publication bias was not observed from Egger's ($p = 0.081$) and Begg's tests ($p = 0.180$). Based on the study quality assessment using modified Newcastle-Ottawa Scale for case-control studies, seven of nine studies were of high methodological quality scoring $\geq$ 7 (median value). In summary, aCL antibodies were significantly present in dementia patients suggesting that aCL antibodies are generated due to the autoimmune-derived effects of dementia or there might be a potential causative role of this autoantibody in dementia pathogenesis.
Moretti et al.	Vitamin D, homocysteine, and folate in subcortical vascular dementia and Alzheimer dementia	Dementia is a worldwide health problem which affects millions of patients; Alzheimer's disease (AD) and subcortical vascular dementia (sVAD) are the two most frequent forms of its presentation. As no definite therapeutic options have been discovered, different risk factors for cognitive impairment have been searched for potential therapies. This report focuses on the possible evidence that vitamin D deficiency and hyper-homocysteinemia can be considered as two important factors for the development or the progression of neurodegenerative or vascular pathologies. To this end, we assessed: the difference in vascular risk factors and vitamin D-OH25 levels among groups of sVAD, AD, and healthy age-matched controls; the association of folate, B12, homocysteine, and vitamin D with sVAD/AD and wether a deficiency of vitamin D and an increment in homocysteine levels may be related to neurodegenerative or vessel damages. The commonly-considered vascular risk factors were collected in 543 patients and compared with those obtained from a healthy old volunteer population. ANOVA group comparison showed that vitamin D deficiency was present in demented cases, as well as low levels of folate and high levels of homocysteine, more pronounced in sVAD cases. The statistical models we employed, with regression models built, and adjustments for biochemical, demographic and neuropsychiatric scores, confirmed the association between the three measures (folate decrease, hyperhomocysteinemia and vitamin D decrease) and dementia, more pronounced in sVAD than in AD.
Serino et al.	A novel Virtual Reality-based training protocol for the enhancement of the "mental frame syncing" in individuals with Alzheimer's Disease: a development-of-concept trial	A growing body of evidence suggests that people with Alzheimer's Disease (AD) show compromised spatial abilities. In addition, there exists from the earliest stages of AD a specific impairment in "mental frame syncing," which is the ability to synchronize an allocentric viewpoint-independent representation (including object-to-object information) with an egocentric one by computing the bearing of each relevant "object" in the environment in relation to the stored heading in space (i.e., information about our viewpoint contained in the allocentric viewpoint-dependent representation). The main objective of this development-of-concept trial was to evaluate the efficacy of a novel VR-based training protocol focused on the enhancement of the "mental frame syncing" of the different spatial representations in subjects with AD. We recruited 20 individuals with AD who were randomly assigned to either "VR-based training" or "Control Group." Moreover, eight cognitively healthy elderly individuals were recruited to participate in the VR-based training in order to have a different comparison group. Based on a neuropsychological assessment, our results indicated a significant improvement in long-term spatial memory after the VR-based training for patients with AD; this means that transference of improvements from the VR-based training to more general aspects of spatial cognition was observed. Interestingly, there was also a significant effect of VR-based training on executive functioning for cognitively healthy elderly individuals. In sum, VR could be considered as an advanced embodied tool suitable for treating spatial recall impairments.

(Continued)

TABLE 1 | Continued

Authors	Title	Summary
Gong et al.	Chronic monoarthritis pain accelerates the processes of cognitive impairment and increases the NMDAR subunits NR2B in CA3 of hippocampus from 5-month-old transgenic APP/PS1 mice	Many factors impact cognitive impairment; however, the effects of chronic pain and the mechanisms underlying these effects on cognitive impairment are currently unknown. Here we tested the hypothesis that chronic pain accelerates the transition from normal cognition to mild cognitive impairment in 5-month-old transgenic APP/PS1 mice, an animal model of Alzheimer's disease, and that neurotoxicity induced by N-methyl-D-aspartic acid receptor (NMDAR) subunits may be involved in this process. Chronic monoarthritis pain was induced in transgenic APP/PS1 mice and 5-month-old wild-type mice by intra- and pre-articular injections of Freund's complete adjuvant into one knee joint. Pain behavior, learning and memory function, and the distribution and quantity of NMDAR subunits (NR1, NR2A and NR2B) in hippocampal CA1 and CA3 regions were assessed. Our results showed that although persistent and robust monoarthritis pain was induced by the Freund's complete adjuvant injections, only the transgenic APP/PS1 mice with chronic monoarthritis pain exhibited marked learning and memory impairments. This result suggested that chronic monoarthritis pain accelerated the cognitive impairment process. Furthermore, only transgenic APP/PS1 mice with chronic monoarthritis pain exhibited an overexpression of NR2B and an increased NR2B/NR2A ratio in the hippocampus CA3. These findings suggest that chronic pain is a risk factor for cognitive impairment and that increased neurotoxicity associated with NMDAR subunit activation may underpin the impairment. Thus, NMDARs may be a therapeutic target for the prevention of chronic pain-induced cognitive impairment.
Alexiou et al.	A Bayesian model for the prediction and early diagnosis of Alzheimer's disease	Alzheimer's disease treatment is still an open problem. The diversity of symptoms, the alterations in common pathophysiology, the existence of asymptomatic cases, the different types of sporadic and familial Alzheimer's and their relevance with other types of dementia and comorbidities, have already created a myth-fear against the leading disease of the twenty-first century. Many failed latest clinical trials and novel medications have revealed the early diagnosis as the most critical treatment solution, even though scientists tested the amyloid hypothesis and few related drugs. Unfortunately, latest studies have indicated that the disease begins at the very young ages thus making it difficult to determine the right time of proper treatment. By taking into consideration all these multivariate aspects and unreliable factors against an appropriate treatment, we focused our research on a non-classic statistical evaluation of the most known and accepted Alzheimer's biomarkers. Therefore, in this paper, the code and few experimental results of a computational Bayesian tool have been reported, dedicated to the correlation and assessment of several Alzheimer's biomarkers to export a probabilistic medical prognostic process. This new statistical software is executable in the Bayesian software Winbugs, based on the latest Alzheimer's classification and the formulation of the known relative probabilities of the various biomarkers, correlated with Alzheimer's progression, through a set of discrete distributions. A user-friendly web page has been implemented for the supporting of medical doctors and researchers, to upload Alzheimer's tests and receive statistics on the occurrence of Alzheimer's disease development or presence, due to abnormal testing in one or more biomarkers.
Vieira et al.	Protein Tyrosine Phosphatase 1B (PTP1B): A Potential Target for Alzheimer's Therapy?	Despite significant advances in current understanding of mechanisms of pathogenesis in Alzheimer's disease (AD), attempts at drug development based on those discoveries have failed to translate into effective, disease-modifying therapies. AD is a complex and multifactorial disease comprising a range of aberrant cellular/molecular processes taking part in different cell types and brain regions. As a consequence, therapeutics for AD should be able to block or compensate multiple abnormal pathological events. Here, we examine recent evidence that inhibition of protein tyrosine phosphatase 1B (PTP1B) may represent a promising strategy to combat a variety of AD-related detrimental processes. Besides its well described role as a negative regulator of insulin and leptin signaling, PTB1B recently emerged as a modulator of various other processes in the central nervous system (CNS) that are also implicated in AD. These include signaling pathways germane to learning and memory, regulation of synapse dynamics, endoplasmic reticulum stress and microglia-mediated neuroinflammation. We propose that PTP1B inhibition may represent an attractive and yet unexplored therapeutic approach to correct aberrant signaling pathways linked to AD.
Zheng et al.	Plasma Exosomes Spread and Cluster Around β-Amyloid Plaques in an Animal Model of Alzheimer's Disease	Exosomes, a type of extracellular vesicle, have been shown to be involved in many disorders, including Alzheimer's disease. Exosomes may contribute to the spread of misfolded proteins such as amyloid-β and α-synuclein. However, the specific diffusion process of exosomes and their final destination in brain are still unclear. In the present study, we isolated exosomes from peripheral plasma and injected them into the hippocampus of an Alzheimer's disease mouse model, and investigated exosome diffusion. We found that injected exosomes can spread from the dentate gyrus to other regions of hippocampus and to the cortex. Exosomes targeted microglia preferentially; this phenomenon is stable and is not affected by age. In Alzheimer's disease mice, microglia take up lower levels of exosomes. More interestingly, plasma exosomes cluster around the amyloid-β plaques and are engulfed by activated microglia nearby. Our data indicate that exosomes can diffuse throughout the brain and may play a role in the dynamics of amyloid deposition in Alzheimer's disease through microglia.
Xu et al.	The Impact of Microbiota-Gut-Brain Axis on Diabetic Cognition Impairment	Progressive cognitive dysfunction is a central characteristic of diabetic encephalopathy (DE). With an aging population, the incidence of DE is rising and it has become a major threat that seriously affects public health. Studies within this decade have indicated the important role of risk factors such as oxidative stress and inflammation on the development of cognitive impairment. With the recognition of the two-way communication between gut and brain, recent investigation suggests that "microbiota-gut-brain axis" also plays a pivotal role in modulating both cognition function and endocrine stability. This review aims to systemically elucidate the underlying impact of diabetes on cognitive impairment.
Wang et al.	YXQN Reduces Alzheimer's Disease-like Pathology and Cognitive Decline in APPswePS1dE9 Transgenic Mice	Alzheimer's disease (AD) is the world's most common form of dementia, in which aggregation of amyloid-β (Aβ) is the hallmark. Unfortunately, few medicines have succeeded to completely cure AD. Yangxue Qingnao (YXQN) is a Chinese traditional medicine, and its pharmacological effect is improving cerebral blood flow. In this study, we firstly demonstrated that YXQN reduced AD-like pathology and cognitive impairment in APPswePS1dE9 (APP/PS1) mice with two months administration. Our data showed that YXQN substantially ameliorated behavioral defects in 10-month old APP/PS1 mice using Morris Water Maze and Y-maze tests, in which the cognitive ability of YXQN high-dose group approaches to wild type mice. Next, we focused on the brain pathological alterations in the YXQN group by three experiments, including thioflavin-S, congo-red, and Aβ-immunohistochemistry staining. The results demonstrated that the high-dose of YXQN

(Continued)

TABLE 1 | Continued

Authors	Title	Summary
		dramatically suppressed amyloid plaques in the hippocampus and cortex of APP/PS1 mice, which showed a 47-72% reduction in plaque deposits, relative to the vehicle group. In addition, our data verified that YXQN decreased the cerebral amyloid load by attenuating β-secretase BACE1 and γ-secretase PS1 in the pathological processing of APP, and promoting the level of α-secretase ADAM10 in the physiological processing of APP to generate more sAPPα, which combats amyloidosis formation, and also carries out neurotropic and neuroprotective effect. Taken together, our results strongly suggest that YXQN could be a potential medicine for AD, and provide new evidence for further AD drug research and development.
Cozac et al.	Increase of EEG spectral theta power indicates higher risk of the development of severe cognitive decline in Parkinson's disease after 3 years	Objective: We investigated quantitative electroencephalography (qEEG) and clinical parameters as potential risk factors of severe cognitive decline in Parkinson's disease. Methods: We prospectively investigated 37 patients with Parkinson's disease at baseline and follow-up (after 3 years). Patients had no severe cognitive impairment at baseline. We used a summary score of cognitive tests as the outcome at follow-up. At baseline we assessed motor, cognitive, and psychiatric factors; qEEG variables (global relative median power spectra) were obtained by a fully automated processing of high-resolution EEG (256-channels). We used linear regression models with calculation of the explained variance to evaluate the relation of baseline parameters with cognitive deterioration. Results: The following baseline parameters significantly predicted severe cognitive decline: global relative median power theta (4–8 Hz), cognitive task performance in executive functions and working memory. Conclusions: Combination of neurocognitive tests and qEEG improves identification of patients with higher risk of cognitive decline in PD.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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frontiers in Aging Neuroscience

MINI REVIEW
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Entanglement of UPR^{ER} in Aging Driven Neurodegenerative Diseases

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The endoplasmic reticulum (ER) is an indispensable cellular organelle that remains highly active in neuronal cells. The ER bears the load of maintaining protein homeostasis in the cellular network by managing the folding of incoming nascent peptides; however, the stress imposed by physiological/environmental factors can cause ER dysfunctions that lead to the activation of ER unfolded protein response (UPR^{ER}). Aging leads to deterioration of several cellular pathways and therefore weakening of the UPR^{ER}. The decline in functioning of the UPR^{ER} during aging results in accumulation of misfolded proteins that becomes intracellular inclusions in neuronal cells, resulting in toxicity manifested as neurodegenerative diseases. With ascension in cases of neurodegenerative diseases, understanding the enigma behind aging driven UPR^{ER} dysfunction may lead to possible treatments.

Keywords: aging, UPR (unfolded protein response), endoplasmic reticulum (ER), neurodegenerative diseases, dementia

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INTRODUCTION

The cellular homeostasis maintains existence of life through integrative communication among various macromolecules working in unity through numerous biochemical pathways. The endoplasmic reticulum (ER) not only maintains Ca^{2+} homeostasis but also controls translation, folding, maturation and trafficking of about one third of cellular proteins. Various environmental insults can disturb proper functioning of ER, leading to accumulation of unfolded/misfolded protein cargo in the ER lumen that gives rise to a condition called ER stress. The cell responds through a highly conserved pathway known as the ER unfolded protein response (UPR^{ER}; Walter and Ron, 2011; Corazzari et al., 2017). UPR^{ER} first focuses on alleviation of the imposed stress by initiating steps of adaptive mechanisms in the secretory pathway for restoration of homeostasis but conditions of prolonged stress and damage provokes UPR^{ER} to succumb through apoptosis (Walter and Ron, 2011).

Aging is notably a process during which the cell witnesses decline in its ability to respond to stress. Age related frailty perturbs the multifarious schematic of UPR^{ER} giving rise to a myriad of pathologies characterized by the presence of disease specific misfolded proteins playing havoc with cellular homeostasis (Nuss et al., 2008). The pathology of neurodegenerative disorders such as Alzheimer's disease (AD), Parkinson's disease (PD) and Huntington's disease (HD) emerge as a consequence of disturbance in proteostasis. This review presents research findings that highlight the mechanism of UPR^{ER} in aging driven neurodegenerative diseases.

THE PATHWAY OF ER STRESS INDUCED UPR^{ER}

The ER lumen docks a range of resident molecular chaperones like glucose regulated protein 78 (GRP78), glucose regulated protein 94 (GRP94), caltericulin (CRT) and protein disulfide isomerase (PDI) that aid in folding of incoming nascent proteins. GRP78, also referred to as BiP/HSPA5, is the master ER chaperone that folds the nascent polypeptides, binds the ER luminal Ca^{2+} and marks misfolded protein cargo for their degradation through ER associated degradation (ERAD; Lee, 2005; Wang et al., 2009; Park et al., 2017). During ER stress, ER lumen is overloaded with misfolded protein, so the molecular programming of UPR^{ER} first tries to alleviate the debilitated homeostasis by upregulating the expression of GRP78 (Zhu and Lee, 2015). Normally, GRP78 rests in association with the luminal components of three ER resident transmembrane proteins referred to as the sensors of UPR^{ER}: PKR-like ER kinase (PERK), inositol requiring enzyme-1 (IRE-1) and activating transcription factor 6 (ATF-6). First, an adaptive response of UPR^{ER} starts where GRP78 disassociates from the luminal components of transmembrane PERK, IRE-1, ATF6 and gets recruited to the misfolded protein cargo. This activates UPR^{ER} signaling pathway that disseminates the information along a cascade of downstream effector molecules in the cytosol (**Figure 1**).

PERK undergoes homo-dimerization and trans-autophosphorylation (PERK-P) upon leaving its association from GRP78. The activated PERK phosphorylates on serine 51 of the cytoplasmic eukaryotic initiation factor 2α (eIF2 α; eIF2 α-P) which inhibits its immediate effector, guanine nucleotide exchange factor for eIF2 complex, thereby preventing the assembly of 43S initiation complex. This leads to attenuation of global protein translation in the cell aiming to reduce the load of new protein cargo in the ER lumen, but paradoxically favors the translation of mRNA with internal ribosome binding sites (IRES) such as activating transcription factor 4 (ATF4; Harding et al., 2000). ATF4 is a cAMP response element binding (CREB) transcription factor that is involved in controlled up-regulation of genes for amino acid metabolism, antioxidant response, autophagy and apoptosis (Ma and Hendershot, 2003; Blais et al., 2004). The ATF4 mRNA has two upstream open reading frames (uORFs), uORF1 and uORF2, in 5′ untranslated region (UTR), whose translation depends upon the concentration of eIF2-GTP- Met-tRNA$_i^{Met}$/40S ribosome ternary complex. In non-stressed condition, low levels of eIF2 α-P and ample ternary complex concentration, leads to the translation of both uORFs and attenuation of coding ATF4 transcript (Vattem and Wek, 2004). Whereas stress induced rise in concentration of eIF2 α-P diminishes ternary complex prompting translation of coding ATF4 transcript (Baird et al., 2014). Under the condition of unresolved ER stress, ATF4 expression is prolonged, which ultimately up-regulates another effector molecule, C/EBP homologous protein (CHOP), thereby stimulating the apoptotic-signaling cascade. PERK arm of UPR^{ER} affects antioxidant pathway, not only through ATF4 activation, but also via another cytoplasmic substrate, the nuclear factor E2 related factor2 (Nrf2), which gets imported into the nucleus for up-regulation of antioxidant genes (Cullinan et al., 2003).

The stimulation of IRE1 after its dissociation from GRP78 during the imposed ER stress leads to homo-dimerization and autophosphorylation of IRE1 (IRE-P). This imposes conformational change in IRE1 that affects its downstream effector molecules. To release the stress imposed on ER lumen due to overload of protein folding, the endoribonuclease activity of phosphorylated IRE1 splices X-box binding protein-1 (XBP1) in an unconventional way producing XBP1(S), which is ensued by the activation of genes for UPR^{ER} like chaperones, ERAD and organelle biosynthesis (Yoshida, 2007). In another attempt to rescue the cell, IRE1 independently degrades specific subsets of mRNA, thereby halting production of new proteins in the ER lumen (Hollien and Weissman, 2006). However, when the imposed stress continues to impede the system, the activated IRE1 arm also participates in triggering pro-apoptotic signaling by forming complex with TNF receptor-associated factor 2 (TRAF2), leading to activation of c-jun-N-terminal kinase (JNK; Urano et al., 2000; Nishitoh et al., 2002).

Upon activation, the third arm of UPR^{ER}, ATF6, is translocated to the golgi apparatus, where it undergoes intramembranous proteolytic cleavage by site-1 and site-2 proteases (S1P and S2P respectively). This event releases N-terminal part of ATF6 that is imported into the nucleus to upregulate the transcription of ER stress responsive elements (ERSE; Lee et al., 2002).

The eIF2 α-P also leads to the activation of another closely related stress response referred to as the integrated stress response (ISR). The PERK arm of UPR^{ER} joins hands with ISR through eIF2 α-P, which is also the target of three other kinases of the pathway: RNA-activated protein kinase (PKR), heme-regulated inhibitor kinase (HRI) and general control non-depressible 2 (GCN2). The attenuation of most of the cellular mRNA translation after phosphorylation of eIF2 α saves the cell by conserving molecular resources in the cytosol (Palam et al., 2015).

UPR^{ER} INTERSECTS WITH INFLAMMATION AND AUTOPHAGY

The master transcriptional regulator of inflammation nuclear factor-κB (NF-κB) has been reported to be upregulated during ER stress (Deniaud et al., 2008). The tripartite arm of UPR^{ER} touches inflammatory signaling cascade directly/indirectly through NF-κB. The PERK/eIF2 α-P induced attenuation of global protein translation triggers NF-κB (57). The IRE1-TRAF2 complex not only activates NF-κB (Hu et al., 2006), but also JNK, during ER stress. In a study of Shiga toxigenic *E. coli*, ATF6 has been shown to cause ascension in the expression levels of NF-κB in reply to UPR^{ER} (Yamazaki et al., 2009).

Autophagy (macro-autophagy), in general, is a conserved survival pathway that canonically triggers degradation of organelles/molecules aimed at retrieving their building molecules back in the cytosol. During ER stress, UPR^{ER} effector molecules

crosstalk with molecular markers of autophagy, the microtubule associated protein1 light chain 3 (LC3) and lysosome associated membrane protein (LAMP; Tanida et al., 2008). The PERK/eIF2 α-P/ATF4 and IRE1/spliced XBP1 pathways stimulate induction of LAMP-3 and LC3, respectively (Mujcic et al., 2009; Margariti et al., 2013). The induction of autophagy during UPR^{ER} potentiates the adaptive trial of the cell by clearing off the load of misfolded protein from ER lumen.

AGING ABATES ACTIVATION OF UPR^{ER} AND ITS EFFECTORS

The process of aging causes decline in the proper functioning of cellular metabolic pathways. The changes in cells undergoing aging weaken UPR^{ER}, causing it to fail to recuperate ER stress. The various molecular chaperones in the ER lumen, such as GRP 78, GRP 94, calreticulin and PDI, undergo oxidative damage in the aging cell that diminishes the efficiency of these molecular chaperones to fold nascent protein; hence, presenting a mass of misfolded protein cargo in the lumen (Rabek et al., 2003; Nuss et al., 2008). As evidenced through studies, the expression levels of GRP78 also become mitigated because of aging in murine cortex, rat hippocampus, cortex and cerebellum (Paz Gavilán et al., 2006; Hussain and Ramaiah, 2007; Naidoo et al., 2008). This causes protein toxicity, leading to derangement in proteostasis, which becomes an underlying cause of age related diseases.

Aging deteriorates the three molecular sensors of UPR^{ER}. RT-PCR studies in aged mice showed significant lowering of PERK mRNA expression in rat hippocampus (Paz Gavilán et al., 2006). During aging cell environment starts favoring apoptotic-signaling cascade via activation of CHOP and caspases-12 (Hussain and Ramaiah, 2007; Naidoo et al., 2008). Furthermore, the IRE1 arm favors upregulation of kinases involved in apoptotic pathway, such as ASK1 and JNK (Ichijo et al., 1997). The molecular pathway of UPR^{ER} also intersects with inflammatory pathways in the cell (Cao et al., 2016). The master molecule NF-κB, is shown to be upregulated during aging (Yalamanchili et al., 2016). Aging associated human pathology has also been shown to involve a decline in autophagy (Caramés et al., 2010). Thus, aging imposes misfolded protein toxicity in the cell through disabled UPR^{ER}, leading to emergence of age related dysfunction and diseases.

THE MOLECULAR SIGNATURES OF UPR^{ER} IN AGING DRIVEN NEURODEGENERATIVE DISEASES

Neurodegenerative diseases find their source of origin in the perturbations that alter proper functioning of ER. Age related frailty disarms the adaptive arm of UPR^{ER} and presents distressing conditions in the brain to promote accumulation of misfolded protein cargo in the ER lumen that later on become inclusions of specific abnormal proteins. Most of the models of aging driven neurodegenerative disease have been marked with the presence of specific protein inclusions because of ER stress in the brain and central nervous system, which are toxic to the post-mitotic neurons. The evoked UPR^{ER} attempts to ameliorate the condition of deranged protein homeostasis, but failing to do so it compromises with the system in the form of neuropathology (**Figure 2**). During such proceedings, the synaptic loss becomes an early event in the pathology that conclusively leads to the death of neurons, which has been studied in cases of AD and PD and frontotemporal dementia (Mallucci et al., 2007; Tampellini, 2015). Here, we discuss neurodegenerative diseases with aging as the prominent risk factor in which the involvement of UPR^{ER} markers has been well studied and shown to be potential targets for therapeutic interventions.

ALZHEIMER'S DISEASE (AD)

The most prominently studied form of dementia in aging patients is AD, which shows a characteristic extracellular buildup of toxic amyloid-β peptide (Aβ), hyperphosphorylated tau protein, which interfere with Ca^{2+} homeostasis and proteostasis, leading to synaptic loss and neuronal degeneration (Singh et al., 2016). Hoozemans et al. (2009) have shown in autopsy samples of brains of AD patients that ER stress markers like PERK-P, eIF2 α-P and IRE1-P markedly increased as a result of UPR^{ER} activation. Studies in AD temporal cortex and hippocampus registered two-fold heightened-expression levels of GRP78 (Milisav et al., 2015; Casas, 2017). Also in mice AD model, 1.5–2 fold increase in GRP78 was found to be associated with accumulation of Aβ (Soejima et al., 2013). Resende et al. (2008) studied the activation of UPR^{ER} in primary rat embryo cortical neurons treated with Aβ oligomers and documented significant increase in levels of GRP78 as well as ER Ca^{2+} release resulting in tau phosphorylation. Moreover, studies on persistent UPR^{ER} have shown the active involvement of glycogen synthase kinase 3β (GSK-3β), an active kinase involved in tau phosphorylation (Kim et al., 2005), which also co-localizes with PERK-P in cortical cells (Hoozemans et al., 2009), is a well-documented target of PERK (McAlpine and Werstuck, 2014). Additionally, tau protein has been shown to be involved in the stimulation of PERK, IRE1 and ATF6, which cooperatively elicit the inflammatory signaling cascade in the brains of AD patients (McAlpine and Werstuck, 2014) The chemical modifications of already prevailing proteins regulate short-term memory, whereas long-term memory requires *de novo* protein synthesis and is under the control of phosphorylation status of eIF2 α (Kandel, 2001). Cases of AD patients have shown increasing levels of eIF2 α-P in their histological samples (Hoozemans et al., 2007).

Studies on knock-in mice expressing mutant presenilin 1 (PS1), which induces early onset of familial AD, showed intensified levels of pro-apoptotic CHOP with concomitant diminishing levels of anti-apoptotic Bcl-2 (Milhavet et al., 2002). The role of active NF-κB in driving inflammatory gene transcription has been well documented in aging cell lines, as well as neuropathology in AD (Lukiw and Bazan, 1998).

Moreover, higher levels of LC3 have been reported in the hippocampal neurons in the brain of AD patients, suggesting the active involvement of autophagy (Nijholt et al., 2011).

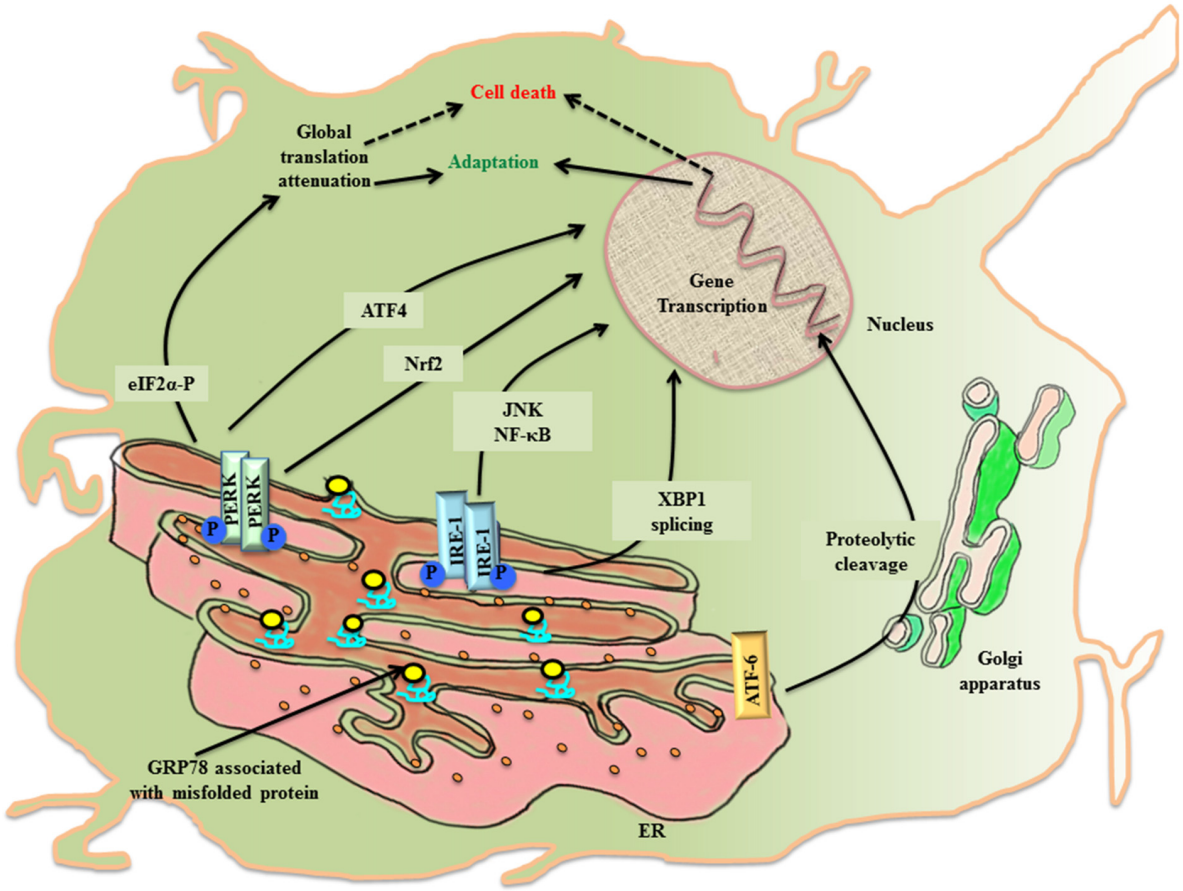

FIGURE 1 | The activation of UPR^{ER} in the neuronal cell. Under the imposed ER stress, a neuronal cell activates UPR^{ER} that starts with the release of GRP78 from its association with the luminal components of the three transmembrane transducers of UPR^{ER}: PERK, IRE1 and ATF6. GRP78 is recruited to the misfolded protein cargo. This stimulates all the three transducers in a series of events that disseminate their effect through transcriptional control of genes. PERK phosphorylates cytoplasmic eIF2 α that causes attenuation of global protein translation, paradoxically favors translation of ATF4 and activates Nrf2. Further IRE1 leads to XBP1 splicing and activation of JNK/NF-κB. ATF6 undergo proteolysis. All working in coherence, first ameliorates the stress, but later under chronic ER stress apoptotic pathway leads to cell death.

PARKINSON'S DISEASE (PD)

The pathology behind PD has been speculated to be due to mutations in three different genes and certain specific transposons; namely, α-synuclein, associated with early onset of familial PD; parkin and ubiquitin C-terminal hydrolase L1, manifesting some rare forms of PD (Shen et al., 2016). The loss of dopaminergic (DA) neurons account for the motion disorder featured in PD (Hirsch et al., 2013). Lewy bodies (LBs), the heavily ubiquitinated cytoplasmic accumulations of α-synuclein, hallmark of PD and accumulations of parkin substrate due to loss of functional Parkin, cause ER stress that evokes UPR^{ER} (Imai et al., 2001). The increasing neuronal death in neurotoxin induced models of Parkinsonism has been reported due to apoptotic pathway, where robust expression of CHOP has been well documented (Silva et al., 2005), demonstrating the involvement of PERK arm of UPR^{ER}. Paradoxically, the other two arms of UPR^{ER}, IRE1 and ATF6, have been shown to be pro-adaptive and neuroprotective for DA neurons in cases of neurotoxin induced PD models through up-regulation of expression of GRP78 and ERAD genes (Egawa et al., 2011; Hashida et al., 2012; Valdés et al., 2014). In *Drosophila* model of PD expressing human α-synuclein, the protective arm of UPR^{ER} invariably coordinates through XBP1 mediated autophagy (Fouillet et al., 2012).

HUNTINGTON'S DISEASE (HD)

HD is characterized by neuronal dysfunction and neurodegeneration in the CNS that leads to dementia. Brain samples from Huntington's patients display accumulation of misfolded mutant Huntingtin protein (mHtt) as intracellular inclusions, where the glutamine residue shows an expansion of more than 40 repeats (Bossy-Wetzel et al., 2008). Reports suggest that UPR^{ER} is stimulated in HD cases. For example, increasing levels of GRP78 and CHOP mRNA have been observed in human autopsy samples (Carnemolla et al.,

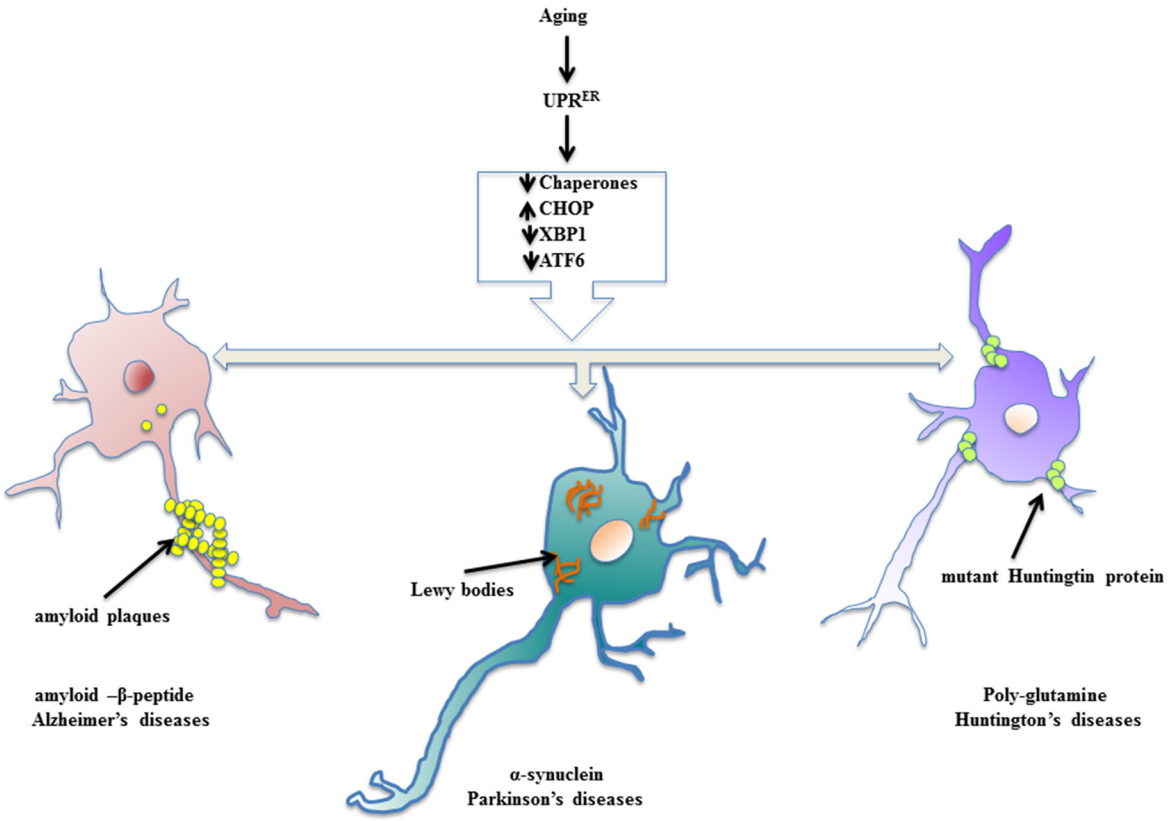

FIGURE 2 | Enervated UPR^{ER} resulting in neuropathologies. Aging declines the function of UPR^{ER} thereby preparing the stage for surfacing of neurodegenerative diseases. The specific toxic, misfolded protein accumulations are characteristic of aging driven neurodegenerative diseases.

2009). There is also an increase in levels of phosphorylated IRE1, GRP78 and XBP1 in striatal tissue of HD patients (Lee et al., 2012). Another study showed that toxic poly-glutamine expanded protein entraps ERAD proteins, leading to impairment of ERAD (Kalathur et al., 2015). Additional studies confirmed the weakened processing of ATF6 in both animal models and human HD patients (Fernandez-Fernandez et al., 2011). The proteinopathy of HD also targets autophagy by making it dysfunctional (Martin et al., 2015). The activation of JNK pathway in studies of cells overexpressing poly-glutamine accumulations further reinforces data showing UPR^{ER} driven neuronal death in HD (Kouroku et al., 2002).

REMEDIATION OF NEURODEGENERATION BY TARGETING UPR^{ER}

Being the underlying cause of aforementioned neurodegenerative diseases, the molecular signatures of UPR^{ER} are striking targets for therapeutic intervention. In mouse models of AD, chemical chaperones, 4-phenylbutyric acid (PBA) or tauroursodeoxycholic acid (TUDCA), have been shown to recuperate ER folding ability thereby rescuing neurons (Ricobaraza et al., 2012; Ramalho et al., 2013). Studies in human tau expressing stable cells (HEK293/tau) with overexpression of nucleotide exchange factor SIL1, a co-chaperone for GRP78, showed reduced tau hyperphosphorylation (Liu et al., 2016). However, a seemingly contrasting study showed that SIL1 might function in GRP78 independent manner for the amelioration of neuronal fitness in AD (Labisch et al., 2017). Dantrolene, licensed for treatment of spasticity, diminishes memory deficit by inhibiting PERK/eIF2α/CHOP in mouse AD models (Peng et al., 2012). Salubrinal (Sal), which selectively activates the levels of eIF2 α-P, causes elevation in GRP78 expression thereby protecting against Aβ neurotoxicity (Lee et al., 2010). In a similar way, Salubrinal also attenuates apoptosis that accentuates neuronal survival in PD mouse models (Colla et al., 2012; Mollereau et al., 2016). Out of the select set of mRNA translated after PERK-mediated phosphorylation of eIF2α, β-site APP cleaving enzyme-1 (BACE1) mRNA encodes for the key secretase that leads to the production of Aβ through the cleavage of amyloid precursor protein (APP; Kimura et al., 2016). Targeting the dephosphorylation of eIF2α-P by arctigenin, a bioactive product from *Arctium lappa*, leads to cessation of Aβ formation (Zhu et al., 2013). The restoration of translation and thereby prevention of neuronal loss was reported in mutant tau-expressing mice after challenging with PERK inhibitor, GSK2606414 (Radford et al., 2015). An attempt to target ISR, where eIF2α competitively inhibits eIF2B, an ISR inhibitor called

ISRIB (affecting eIF2B) when administered in rodents, led to reversal of global translational halt that consequently enhanced long-term memory (Sidrauski et al., 2015). Emerging concepts suggesting different consequences of UPR^{ER} have highlighted advantageous role of XBP1 on memory. The administration of XBP1(S) through adeno-associated virus rescued long-term hippocampus memory in XBP1 knockout mice (Martínez et al., 2016). Quercetin, a flavonol, stimulates IRE1 endoribonuclease activity hence inhibiting tau hyperphosphorylation (Suganthy et al., 2016). Inhibition of autophagy by mammalian target of rapamycin (mTOR) complex (mTORC1) is challenged by AVN-211 (mTOR inhibitor) that concomitantly induces autophagic clearance of toxic aggregates in AD (Cai et al., 2015; Towers and Thorburn, 2016). Activation of XBP1 and ATF6 have also been proven to be protective in PD models (Egawa et al., 2011; Valdés et al., 2014; Mollereau et al., 2016). The suppression of PERK/eIF2α arm also prevented neurodegeneration in PD *Drosophila* mutants (Celardo et al., 2016). Several studies ascribe neuroprotective role of ATF6 (precisely, ATF6 α subtype) in PD mouse models (Hirsch et al., 2013; Voutilainen et al., 2015). One latest report proposes therapy for PD that activates Nrf2 through oral gavage of dimethyl fumarate (DMF) in mouse models (Lastres-Becker et al., 2016). Reports from studies in HD transgenic mice suggest that the selective silencing of XBP1 by small interfering RNA (siRNA) leads to mitigation of neural loss (Vidal et al., 2012). Additionally, XBP1-deficient HD transgenic mice showed augmented clearance of mHtt through autophagy (Vidal et al., 2012). Alleviation of ER stress by using chemical chaperone TUDCA in HD model showed reduction in neural loss and improved motor activity (Keene et al., 2002). The inhibition of eIF2 α-P/PERK rescued striatal neurons from huntingtin cytotoxicity (Leitman et al., 2014). The IRE1/JNK route of UPR^{ER} upregulates an actin binding protein, which is a negative regulator of autophagy, called ectodermal-neural cortex 1 (ENC1). ENC1 knockdown data illustrated relief in mHtt induced neuronal death (Lee et al., 2016). In recent development on transgenic *Drosophila*, treatment with autophagy-enhancing molecule, AUTEN-67, caused impediment in HD symptoms (Billes et al., 2016).

CAN WE INTERFERE WITH AGING TO PREVENT NEURODEGENERATIVE DISEASES BY TARGETING UPR^{ER}?

We can look for the answer to this question by revisiting a remarkable study of the insulin/IGF-like signaling (IIS) pathway mutant of *C. elegans* that revealed lifespan extension was dependent on the active participation of IRE1 and XBP1, as was gaining resistance against ER stress. Additionally, XBP1 coordinated with IIS-activated forkhead box (FOXO) transcription factor DAF-16 to regulated important gene targets of longevity and ER homeostasis (Henis-Korenblit et al., 2010). Dietary restriction (DR) that promoted longevity and slowing of disease onset also showed stimulation of IRE1 in specific neurons of *C. elegans* (Chen et al., 2009). Reports from a forward genetic study of *C. elegans* again strengthen the stand of healthy ERAD and ensuing autophagy in life span extension and amelioration of proteinopathy maladies (Denzel et al., 2014). Thus, we can target neuronal damage that stems from aging.

CONCLUSION

The ER plays an important role in the proper functioning and health of neuronal network. An aging system undergoing diminishing cellular performance enervates the ER, leading to decreased UPR^{ER}, thereby failing to recuperate the imposed stress. Studies of model organisms have reinforced the importance of the activation of UPR^{ER} molecular markers in stimulating longevity. Age related dysfunction in UPR^{ER} weakens the ERAD pathway, thereby potentially promoting accumulation of misfolded protein cargo, which eventually becomes toxic intracellular inclusions. As witnessed by the studies highlighted in this review, the prominent aging driven neurodegenerative diseases share a common pathology of toxic misfolded protein accumulations. This provides an opportunity for therapeutic interventions to prevent the various molecular signatures of UPR^{ER} pathway that can stave off both aging and neuropathologies. Our focus on interfering with the temporal expression patterns of UPR^{ER} molecular markers can help us understand the unsolved issues of aging driven neurodegenerative diseases.

AUTHOR CONTRIBUTIONS

SR, JihK and RM conceived the idea; SR, ATJ, AA, JiwK, JihK and RM contributed to writing of the manuscript.

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MINI REVIEW
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Perspective Insights of Exosomes in Neurodegenerative Diseases: A Critical Appraisal

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Exosomes are small membranous entities of endocytic origin. Their production by a wide variety of cells in eukaryotes implicates their roles in the execution of essential processes, especially cellular communication. Exosomes are secreted under both physiological and pathophysiological conditions, and their actions on neighboring and distant cells lead to the modulations of cellular behaviors. They also assist in the delivery of disease causing entities, such as prions, α-syn, and tau, and thus, facilitate spread to non-effected regions and accelerate the progressions of neurodegenerative diseases. The characterization of exosomes, provides information on aberrant processes, and thus, exosome analysis has many clinical applications. Because they are associated with the transport of different cellular entities across the blood-brain barrier (BBB), exosomes might be useful for delivering drugs and other therapeutic molecules to brain. Herein, we review roles played by exosomes in different neurodegenerative diseases, and the possibilities of using them as diagnostic biomarkers of disease progression, drug delivery vehicles and in gene therapy.

Keywords: diagnostics, drugs, exosomes, neurodegeneration, therapeutics

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INTRODUCTION

Exosomes were first considered to be vesicles produced by mature blood reticulocytes and secreted into extracellular milieu with topically expressed transferrin receptors (Harding and Stahl, 1983; Johnstone et al., 1987). Unlike microvesicles, which arise from outward budding of plasma membrane, apoptotic bodies (fragments of dying cells), and ectosomes, exosomes are produced by the fusion of multivesicular bodies (MVBs) with membranes. Exosomes are homogenous and smaller than other extracellular vesicles, with sizes ranging between 30–100 nm (Raposo and Stoorvogel, 2013; Keerthikumar et al., 2015). They are secreted by reticulocytes, mesenchymal cells, neurons, fibroblasts, epithelial cells, endothelial cells, platelets, allophycocyanins (APCs), tumor cells and other cells (Bobrie et al., 2011; Colombo et al., 2014), and are present in bronchioalveolar lavage, synovial fluid, urine, bile, breast milk, serum and other body fluids (Théry et al., 2009; Vlassov et al., 2012) because they display specific cell-derived components on their surfaces, exosomes with different cellular backgrounds possess same set of molecules, such as, enzymes, nucleic acids, cytokines and bioactive compounds. Following release, they are uptaken by surrounding cells or transported systemically in cerebrospinal fluid (CSF; Grapp et al., 2013; Gui et al., 2015), breast milk (Zonneveld et al., 2014), blood (Baranyai et al., 2015), urine (Royo et al., 2016) and in other biological fluids to provide a means of cell-to-cell communication (Bang and Thum, 2012). Accordingly, exosomes influence physiological and pathobiological conditions.

Exosome discovery has exhibited enormous growth over the past three decades. Once known primarily for their role in eliminating excessive cellular proteins and undesirable molecules, exosomes are now known to be required for many physiological processes, such as, the maintenance of normal physiological functions and cell-to-cell communication, and to play important roles in the progression of diseases, such as, cancer and neurodegenerative diseases. Their involvement in neurodegenerative disease progression are attributed to their abilities to transfer biomolecules and pathogenic entities across biological barriers (Candelario and Steindler, 2014; Thompson et al., 2016). Furthermore, their abilities to transport proteins and nucleic acids (siRNA, miRNA) have been exploited for the delivery of drugs and other encapsidated biomolecules. Here, we describe the roles of exosomes in the progressions of neurogenerative disorders, their diagnostic roles as neurodegeneration markers, and their therapeutic applications for the treatment of diseases and for gene therapy.

EXOSOME COMPOSITIONS

Technological advances have resulted in new ways of isolating exosomes by centrifugation, immunocapture and by using microfluidic methods, based on size, shape, density and surface marker expression. Although no consensus has been reached regarding isolation methods, immunoaffinity capture (IAC), chromatography, density gradient centrifugation and ultracentrifugation are the methods more commonly used (Skog et al., 2008). Exosome compositions are dictated by the functional states of cells (Bang and Thum, 2012), though their compositions are largely dependent on their origins (Cosme et al., 2013). Analyses of their compositions by Western blotting (Raposo et al., 1996), Fluorescence Activated Cell Sorting (FACS; Clayton et al., 2001) and mass spectrometry have revealed the presence of endosome associated proteins (Alix, TSG101), a series of tetraspanins (CD9, CD26, CD53, CD63, CD81, CD82), heat shock proteins (HsP70, Hsp90), cytoskeletal elements (tubulin, actin), clathrin, follitin-1, annexins, lysosomal protein (Lamp2b), RAB proteins, intercellular adhesion molecule-1 (ICAM-1), major histocompatibility complexes (MHCs), and co-stimulatory molecules of T-cells, like CD86 (Théry et al., 2002; Keller et al., 2006; **Table 1**). Proteomic analysis of exosomes also revealed the presence of cell surface anchored sheddases (e.g., ADAM, a disintegrin and metalloproteinase) and cell surface and soluble matrix metalloproteinases (MMPs; Théry et al., 2002). In addition to their extracellular matrix (ECM) remodeling roles, MMPs are critical in cellular interaction, proteosomal processing of exosomal contents and in the intercellular communication. Additionally, protein components, such as, peroxidases and pyruvate kinases, have also been reported in exosomes of enterocyte and human dendritic cells (Théry et al., 2002).

Exosomal membranes are also enriched with lipids, such as, ceramides, phosphatidyl ethanolamine, phosphatidylserine, diacylglyceride, cholesterol, lyso-bis-phosphatidic acid and sphingomyelin (Janas et al., 2015; Haraszti et al., 2016). Although characterization of exosomes depends on the cell type and the protocol used, exosomes have been reported to contain different amounts of DNA (dsDNA, ssDNA, MtDNA), RNA (mRNAs, miRNAs, snRNA, ncRNA) and small cytoplasmic RNAs (van Balkom et al., 2015; Zhang et al., 2015; Kalluri and LeBleu, 2016). Data pertaining to exosomes has been collated in the Exocarta[1] database, which contains 41,860 proteins, 4956 mRNAs and 1116 miRNAs.

[1] www.exocarta.org

TABLE 1 | Table summarizing information on exosomal components.

Constituents	Type	Function
Alix, TSG101, Rab proteins (Rab11, Rab27b), VAMP7	MVB formation	Proteins involved in multivesicular (MVB) biogenesis
CD9, CD26, CD53, CD63,CD81, CD82	Tetraspannins	Morphogenesis, fission and fusion processes
Hsc70, Hsp 90	Heat-shock proteins	Inherent characteristic of exosomes
GTPases, clathrin and flotillin	Membrane transport and fusion proteins	Membrane transport and fusion proteins
Actin, Tubulin and Annexins	Cytoskeletal elements	Structural
Metabolic enzymes (peroxidases, lipid and Pyruvate kinases, etc.)	Metabolism	Regulation of metabolic activity
mRNA, miRNA, SnRNA	Genetic material	Gene expression regulation
Ceramides, phosphatidylserine, diacylglyceride, cholesterol, Phosphatidylcholine, sphingomyelin, etc.	Phospholipids and cholesterol	Required for signaling and fusion to cell membrane
MHC molecules, ICAM-1, CD86, P-selectin, immunoglobulin A33	Immune function	Regulation of immune response (immunosuppressive/immune activation)
Transmembrane proteins such as αM (DCs), α4β1 (reticulocytes)	Signaling	Cell specific exosome recognition and characterization

The table contains information of proteins, nucleic acid and lipid components along with the role they play in exosomes.

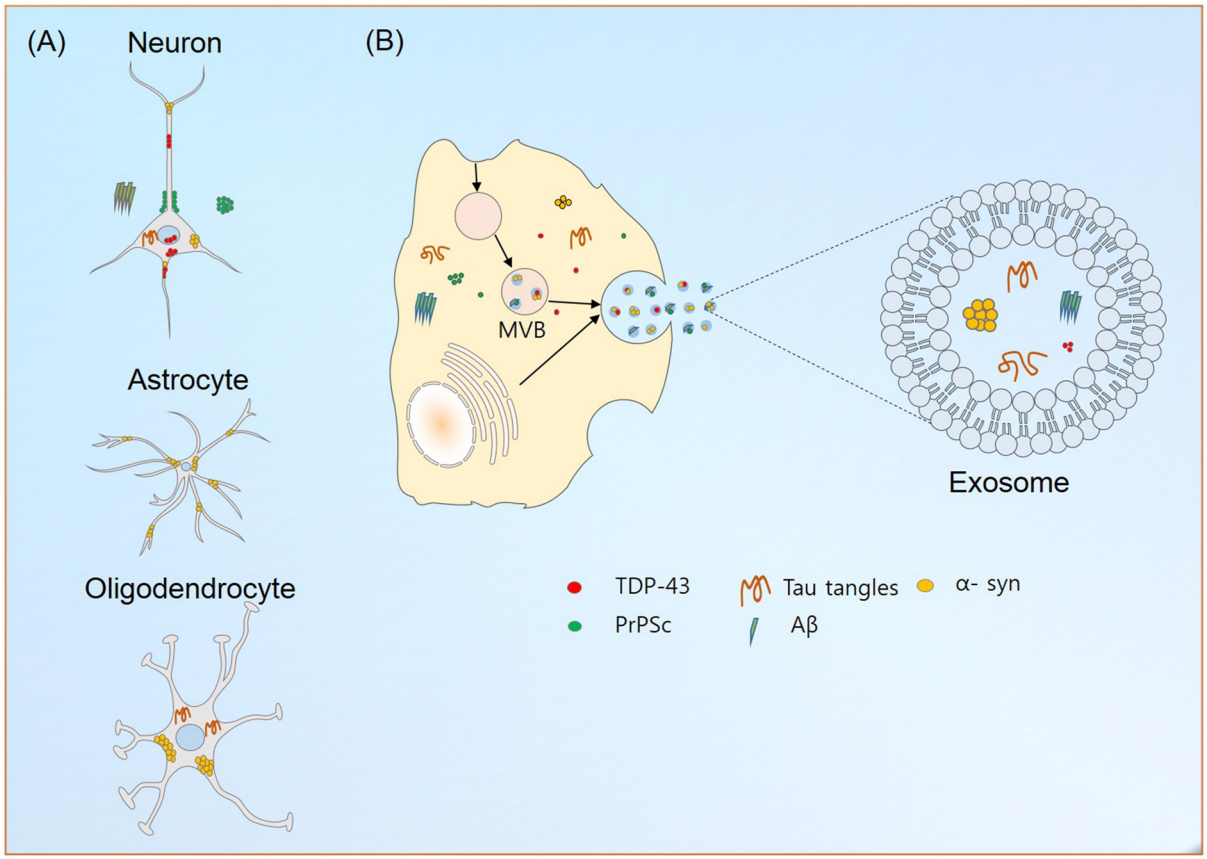

FIGURE 1 | Exosome production and neurodegeneration. **(A)** Localization of aggregated or misfolded proteins credited for their roles in the neurodegeneration in different neuronal cells. **(B)** Exosome production in neuronal cells. The abilities of exosomes to carry disease causing entities to neighboring and distant localized cells contributes to the aggravation of neurological diseases. Similarly, their transport across blood brain barrier (BBB) helps in reducing the possible emergence of different neurological diseases.

EXOSOMES AND NEURODEGENERATION

Exosome secretion has been reported for a number of cells in the nervous system (**Figure 1A**). Though exosomes from neurons display GluR2/3 subunit receptor molecules, microglia exosomes contain pro-inflammatory cytokine, IL-β, astrocytes Hsp70, IL-β and synapsin-1 and exosomes from oligodendrocytes contain myelin lipids: cholesterol, galactocerebrosides (Bianco et al., 2009; Kettenmann et al., 2011; Wang et al., 2011). Exosomes have a great effect on cell-to-cell communication, due to: (1) interactions between topical proteins and receptors on target cells; and (2) proteolysis of their cargoes and internalizations of their contents via endocytosis. Furthermore, they allow intercellular communications, via the transport of protein and nucleic acid entities under both normal and diseased states, which suggests exosomes participate in development, cellular function and associated pathologies.

Aggregation of proteins is a hallmark of neurodegenerative diseases, and their accumulations in the CNS hinder mitochondrial and proteosomal functions, axonal transport and synaptic transmission and enhance endoplasmic reticulum stress (Joshi et al., 2014; Yuyama et al., 2015). The ability of exosomes to carry misfolded or aggregated proteins enhances the progression of neurodegenerative diseases (**Figure 1B**). In line with the prion-like spreading hypothesis (Prusiner, 1982), their implications in the transmission of infectious particles, prions (in Creutzfeldt-Jakob disease, CJD), amyloid precursor protein (APP; in Alzheimer's disease (AD)), α-synuclein (α-syn; in Parkinson's disease (PD)) and superoxide dismutase 1 (SOD1; in amyotrophic lateral sclerosis (ALS)) between cells in the nervous system are currently being explored.

Prion Diseases

Prions are infectious particles that arise due to misfolded or aberrant conformations of proteins (Kupfer et al., 2009; Moore et al., 2009). Given an ability to transfer misfolded states to native forms of similar proteins, the proteopathic state of prions triggers a series of refoldings and aggregations, causing aggregations to oligomers and fibrils (Polymenidou and Cleveland, 2011; Cescatti et al., 2016). Prion-mediated spread of pathophysiological prominence to other cells leads

to neurodegeneration. The best example of this mode of transmissible proteinopathy is CJD. The theory of exosome-mediated propagation of prion disease resulted from the studies of Vella et al. (2007, 2008) which showed exosome-associated protein-rich plasma (PrP; encoded by *PRNP*) in the CSF of sheep (Stahl and Prusiner, 1991). Later, Fevrier et al. (2004) reported release of both normal (PrPC) and pathogenic (PrPSc) prion protein in an exosome secreted by PrP-expressing cells. On one side where alteration of the exosome cargo and structure by PrPSc was reported (Bellingham et al., 2012; Coleman et al., 2012), upregulation of exosome secretion was found increasing the effectiveness of PrPSc (Kovacs, 2016).

Alzheimer's Disease

AD arises as a result of the extracellular deposition of amyloid-β (Aβ; encoded by *AβPP*) fibrils and of abnormally phosphorylated tau protein (encoded by *MAPT*) in neurons (Iqbal et al., 2010; Murphy and LeVine, 2010; Bloom, 2014). Aβ propagation via exosomes to extracellular milieu was reported by Rajendran et al. (2006) who observed cleavage of APP followed by secretion of β-amyloid in exosomes. Subsequently, secretions of the C-terminal part of APP was observed *in vitro* (Sharples et al., 2008) and *in vivo* (Perez-Gonzalez et al., 2012). Though, exosomes have been reported to induce the formation of neurotoxic oligomers of Aβ (Yuyama et al., 2012; Joshi et al., 2014), they have also been reported to have a neuroprotective role of clearing toxic oligomeric species in exosomal lumen (Dinkins et al., 2014). Impaired Aβ clearance in AD patients and the neuronal and microglial exosome disposal of Aβ (Yuyama et al., 2015) hints at the possibility of a dual clearance mode for Aβ in AD.

Neurofibrillary tangles representing hyper-phosphorylated misfolded tau have been observed in the CSF of AD patients (Šimić et al., 2016). Exosomal secretion of tau is considered critical for the spread of tauopathy to different areas of the AD brain. Contrary to the dependence of tau propagation on the exosomal secretory pathway (Asai et al., 2015), recent studies on the role of exosomes in proteinopathies revealed regulation of Tau secretion by neuronal activities (Wu et al., 2016). Additional work is required to clarify the role played by exosomes in the progression of AD.

Parkinson's Disease

Pre-synaptic α-syn (encoded by *SNCA*) protein, which exists as an equilibrium between monomeric and oligomeric states, is a major component of Lewy bodies in PD (Spillantini et al., 1997). Presence of α-Syn in synaptic vesicles suggests its involvement in synaptic vesicle processing (Vargas et al., 2014). α-Syn induced conformational changes facilitates vesicle curvature during their production (Westphal and Chandra, 2013). Lee et al. (2005) reported compartmentalization of α-syn promotes its misfolding, and Grey et al. (2015) reported acceleration of α-syn aggregation in exosomes. Mutagenic and disease like factors favoring fibrillization, a pathogenic process in PD, are thought to propagate in a prion-like process of α-syn misfolding (Olanow and Brundin, 2013). Stuendl et al. (2016) reported the promotion of α-syn aggregation by exosomal loaded α-syn species isolated from CSF of PD patients.

Amyotrophic Lateral Sclerosis

Misfolding of Cu/Zn SOD1 is a characteristic feature of the familiar and sporadic form of ALS (Bosco et al., 2010). Neuronal secretion of exosomes containing mutant SOD1 was found to transmit pathogenic traits to nearby neurons, such as, the misfolding of SOD1 (Gomes et al., 2007). Exosomes of astrocytic origin loaded with SOD1 show selective toxicity to neurons (Basso et al., 2013) and help establish the exosome-mediated pathogenic behavior of mutant SOD1 in ALS.

TDP-43 is a highly conserved nuclear protein (encoded by *TARDBP*), and is another entity involved in ALS. Its pathogenic mechanisms include cytoplasmic mis-localization, misfolding followed by aggregation, and inclusion formation (Scotter et al., 2015). Dipeptide repeat proteins (DPRs) represent unconventional translation product of C9*ORF*72. Studies indicate an association between TDP-43 and DPRs resulting in the release and transport of exosomes, which supports the notion of exosome-based disease spread (Ding et al., 2015; Westergard et al., 2016).

FUNCTIONAL ASPECTS OF EXOSOMES

Exosomes in Disease Diagnosis

The progressive accumulation of protein aggregates (Brettschneider et al., 2013) and lack of sensitive biomarkers (Poste, 2011) hamper the development of disease-specific treatments for neurodegenerative diseases. Though protein aggregates can be detected in CSF and blood, their extremely low levels limit their usefulnesses as potent biomarkers of neurodegenerative diseases (Blennow and Zetterberg, 2015). Furthermore, low amounts of nucleic acid entities also limit their usefulnesses as biomarkers. Given these limitations, the focus for biomarkers in neurodegenerative diseases has shifted from proteins to the vesicular constituents of biofluids. In addition to the surface localizations of specific proteins, the presence of disease-specific molecular signatures in exosomes makes them strong diagnostic candidates. As they protect their cargoes from degradation (Théry et al., 2002; Chiasserini et al., 2014), screening exosomes in CSF and serum offers a means of identifying their cellular origins and thus provides insights of cellular and pathogenic processes going in CNS. Given evidence of their secretion into CNS (Thompson et al., 2016), it is possible that screening vesicular entities of CSF for vesicle associated membrane protein-2 and enolase (neuron specific markers), integrin α-M (microglia specific marker) and transmembrane protein 132D (oligodendrocyte marker) could be used to detect neurodegenerative diseases. A good example of disease-specific molecular signatures in exosomes is provided by exosomes produced by tumor cells in brain that displays of immunosuppressive and oncogenic factors (van der Vos et al., 2011).

Exosomes and Therapeutics

The ability of exosomes to cross the blood-brain barrier (BBB) and pass through interstitial fluid into CSF has highlighted their use as drug delivery vehicles targeting the CNS. Systemic administration of exosomes with an siRNA cargo was utilized for drug delivery to the brains of mice (Alvarez-Erviti et al., 2011; Cooper et al., 2014), and the intranasal administration of exosomes loaded with an anti-inflammatory drug was also used for drug delivery to the mouse CNS (Zhuang et al., 2011). Though actual transport routes remain elusive, exosomes have great potential for the delivery of therapeutic RNAs, proteins and small molecules.

Delivery of Therapeutic RNA

The abilities of exosomes to transport nucleic acid cargoes (siRNA, miRNA) under physiological and pathological condition have increased interest in exploiting them as drug delivery vehicles and for genetic therapy. This ability is adopted to alter the expressions of genes via the bindings of siRNA or miRNA to complementary mRNA sequences thereby controlling gene expression at the post transcriptional level (Bartel, 2009; Ha et al., 2016). Furthermore, exosomes improve the stabilities of their contents in the systemic circulation and extracellular space, and thus, increase efficacies of delivery. Alvarez-Erviti et al. (2011) demonstrated the therapeutic advantages of exosome based siRNA delivery to mouse brain. They engineered surface localized Lamp2b protein to rabies glycoprotein to assist targeted delivery of therapeutic cargo to brain, and successfully delivered therapeutic GAPDH siRNA to neurons, microglia and oligodendrocytes in the brain. In addition, this study also provided insights regarding their utilization for the delivery of therapeutics to other tissues.

Delivery of Therapeutic Proteins

In addition to the delivery of RNA, exosome-based therapeutic protein delivery has also been shown to be beneficial in PD. Characterized by diminished SOD1 and catalase levels (Ambani et al., 1975), reactive oxygen species (ROS) overproduction causes the activation and inflammation of microglia in brain. Therapeutic delivery of catalase loaded into exosomes successfully crossed the BBB and inhibited neurodegeneration (Haney et al., 2015), and improved disease state in PD.

Delivery of Small Therapeutic Molecules

Knowing that efficiency of a drug depends on functional transport across the BBB, strategies, such as, delivery as nano-formulations and PEGylated drugs, were commonly employed for targeted drug delivery (Yoshida et al., 1992; Veronese et al., 2002; Agrawal et al., 2014). However, delivery as nano-formulations was discontinued for toxicity reasons and rapid clearance by the mononuclear phagocyte system (MPS), while PEGylated drugs were discontinued because PEGylation reduced target-drug interaction, and thus, affected distribution in the brain. Exosome employment improves small molecule transport across the BBB transport because MPS clearance rates are lower for nano-delivery systems. Sun et al. (2010) adopted an exosomal strategy to deliver curcumin (an anti-inflammatory that reduces the productions of inflammatory cytokines like tumor necrosis factor-α (TNF- α) and interleukin-6 (IL-6)) in brain. Exosomal curcumin delivery increased the solubility, stability and bioavailability of curcumin. Zhuang et al. (2011) followed this by co-administering curcumin and JS1124 (an activator of Stat3 inhibitor) to reduce production of inflammatory cytokines in brain (Sun et al., 2010), and a similar approach was adopted to deliver doxorubicin and paclitaxel across the BBB (Tian et al., 2014; Yang et al., 2015). In addition, Sun et al. (2010) reported that intranasal administration of endosomal GL26 suppressed glioma growth by increasing their microglial uptake. The therapeutic efficiencies of drugs observed in animal models indicate the potential of exosomes for the delivery of drugs across BBB to combat neurodegenerative disorders and brain tumors.

EXOSOME ENGINEERING

A large number of studies have been performed on the exosomal delivery of therapeutic molecules, but comparatively little effort has been directed toward engineering exosomes for the target-specific deliveries of therapeutic cargoes. The prerequisite for targeted delivery of therapeutic molecules in different diseases is achieved by surface engineering of targeted peptide or protein on exosomes. Bioengineering of exosomes aimed at achieving correct insertion and avoiding peptide cleavage, results in the expressions of target peptides as fusion to signal peptide of lysosomal associated membrane protein-2b (Lamp-2b). A good example for this phenomena is provided by rabies viral glycoprotein (RVG) and iRGD peptides, which when engineered to exosomes of immature dendritic cells aided targeting of brain and tumor tissues (Alvarez-Erviti et al., 2011; Tian et al., 2014). Bioengineering was found to enhance the cellular uptakes of exosomes, and thereby increase the specificity of treatment in tissues of interest. For example, the surface expression of folate receptor-α (FRα) on choroid plexus epithelial cell derived exosomes was found to direct its cargo to brain parenchymal cells through choroid plexus (Grapp et al., 2013). Target peptide engineering facilitating delivery of drugs to brain tissues on crossing BBB or through choroid plexus, holds considerable promise in terms of overcoming the shortcomings of delivering drugs to brain (Alvarez-Erviti et al., 2011; Grapp et al., 2013). The specific cellular characters of exosomes add advantage for target specific delivery of therapeutic cargo.

CONCLUSION

Exosomes are nano-vesicles produced by diverse cell types and perform a multitude of functions in different cellular backgrounds. Although they have been mainly studied in the context of cell-to-cell communication, the surface localizations of specific proteins on exosomes and their disease specific molecular signatures makes them strong candidates for disease diagnosis. Furthermore, their abilities to transport proteins and nucleic acids goes well for their potential exploitation as drugs delivery agents. Because of the range of prospective application offered by exosomes, future work in this area seems necessary;

to formulate robust and reproducible extraction protocols, to provide clearer understanding of the pathways involved in the biogenesis of exosomes and the loading of targeted therapeutic moieties, and to identify strategies for engineering exosomes that are capable target-specific drug delivery.

AUTHOR CONTRIBUTIONS

IC, TSA and ATJ conceived the idea. ATJ and MAM contributed to writing of the manuscript. ATJ, SR, HRY and EJL proofread the contents for upgradation and prepared the figures.

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Plasma Exosomes Spread and Cluster Around β-Amyloid Plaques in an Animal Model of Alzheimer's Disease

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Exosomes, a type of extracellular vesicle, have been shown to be involved in many disorders, including Alzheimer's disease (AD). Exosomes may contribute to the spread of misfolded proteins such as amyloid-β (Aβ) and α-synuclein. However, the specific diffusion process of exosomes and their final destination in brain are still unclear. In the present study, we isolated exosomes from peripheral plasma and injected them into the hippocampus of an AD mouse model, and investigated exosome diffusion. We found that injected exosomes can spread from the dentate gyrus (DG) to other regions of hippocampus and to the cortex. Exosomes targeted microglia preferentially; this phenomenon is stable and is not affected by age. In AD mice, microglia take up lower levels of exosomes. More interestingly, plasma exosomes cluster around the Aβ plaques and are engulfed by activated microglia nearby. Our data indicate that exosomes can diffuse throughout the brain and may play a role in the dynamics of amyloid deposition in AD through microglia.

Keywords: exosomes, Alzheimer's disease, microglia, aging, β-amyloid plaques

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INTRODUCTION

Exosomes are a bioactive vesicle (40–100 nm) secreted by various cells *in vitro* and *in vivo* under physiological and pathological conditions. They have been isolated from biological fluids such as blood, cerebrospinal fluid and urine (Pisitkun et al., 2004; Caby et al., 2005; Vella et al., 2008). A number of proteins related to neurodegenerative disorders, including prion disease, Parkinson's disease and Alzheimer's disease (AD), have been shown to be released by cells in association with exosomes (Coleman and Hill, 2015). Evidence demonstrates that exosomes can facilitate the unique transmissible nature of prions (Fevrier et al., 2004; Vella et al., 2007; Guo et al., 2016); the exosomes from human-derived prions infected cells are efficient initiators of prion propagation in uninfected recipient cells and produce clinical prion disease when inoculated into mice (Vella et al., 2007; Guo et al., 2016). While prion disease has traditionally been thought to be the only neurodegenerative disease that is transmissible, misfolded forms of key proteins involved in other neurodegenerative disorders such as AD, Parkinson's disease and amyotrophic lateral sclerosis, may also spread in a similar way (Bellingham et al., 2012; Coleman and Hill, 2015).

AD, the most common type of dementia, is pathologically characterized by extracellular deposition of senile plaques and intracellular accumulation of neurofibrillary tangles (Kandimalla et al., 2016a,b; Manczak et al., 2016). A growing number of studies suggest that AD, including amyloid deposits and neurofibrillary tangles, develops in a prion-like manner (Eisele et al., 2009; Frost and Diamond, 2010; Coleman and Hill, 2015). Experimental seeding of amyloid-β (Aβ) pathology has been shown in primates and transgenic mice by intracerebral or peripheral inoculation with AD brain homogenate (Baker et al., 1994; Meyer-Luehmann et al., 2006; Eisele et al., 2010; Hamaguchi et al., 2012; Heilbronner et al., 2013). An autopsy study also found marked deposition of gray matter and vascular Aβ in relatively young patients with iatrogenic Creutzfeldt–Jakob disease (Jaunmuktane et al., 2015). Therefore, exosomes have been suspected to participate in the prion-like propagation of lesions in AD (Bellingham et al., 2012; Vingtdeux et al., 2012; Coleman and Hill, 2015). This idea is supported by the observation that exosomes isolated from either neuronal cell cultures or brain contain Aβ precursor protein (APP) and APP-processing products, including C-terminal fragments and Aβ (Vingtdeux et al., 2007; Perez-Gonzalez et al., 2012). Moreover, exosomes can cross the blood–brain barrier and are exploited as drug delivery vehicles in many studies (Alvarez-Erviti et al., 2011; Haney et al., 2015). Exosomes contribute to the long distance transmission of biological information and therefore affect many physiological and pathological processes. However, the specific diffusion process of exosomes in the brain is still unclear. In addition, it is unknown if the peripheral circulation can communicate with the central nervous system via exosomes.

Microglia, the resident immune cells in the brain, phagocytose dead cells and help to clear misfolded protein aggregates such as amyloid plaques in AD (Bard et al., 2003). *In vitro* studies show that neuron-derived and oligodendrocyte-derived exosomes are incorporated into microglia (Fitzner et al., 2011; Yuyama et al., 2012). It is unknown, however, whether microglia are also the final destination of peripheral exosomes. Exosomes from brain can enter the bloodstream and have been explored as potential biomarkers of preclinical AD or other neurodegenerative diseases (Shi et al., 2014; Fiandaca et al., 2015; Goetzl et al., 2015). Exosomes are reported to dramatically stimulate Aβ fibril formation by its surface glycosphingolipids, and mediate Aβ fibrils uptake into microglia in a phosphatidylserine-dependent manner (Yuyama et al., 2012). Intracerebrally injected exosomes result in reduction of Aβ pathology (Yuyama et al., 2014, 2015). Secretion of exosomes is decreased in progranulin-associated frontotemporal dementia (Benussi et al., 2016). However, Dinkins et al. (2014) show that exosomes interfere with the uptake of Aβ by primary cultured astrocytes and microglia *in vitro*. Preventing exosome secretion with GW4869 can reduce amyloid plaque formation *in vivo*. Conversely, increasing the secretion of exosomes enhances plaque formation (Dinkins et al., 2014, 2015). Furthermore, reducing exosome secretion by genetic neutral sphingomyelinase-2 defects in 5XFAD mice ameliorates AD-associated pathology and improves cognition (Dinkins et al., 2016). Further studies are needed to explore the role of exosomes in AD pathology, especially in amyloid deposition.

In the present study, we traced the spread of exogenous plasma exosomes to explore the specific diffusion processes of exosomes in mouse brain and their target cells. In addition, we investigated the role of exosomes in amyloid deposition in an AD transgenic mouse model.

MATERIALS AND METHODS

Animals

hAPP-J20 mice expressing human APP with Swedish and Indiana (KM670/671NL, V717F) mutations were purchased from the JAX MMRRC (Stock # 034836). They were housed in groups of five under a normal 12 h light/dark cycle, kept under standard temperature and humidity and in pathogen free conditions. All experiments were approved by the Institutional Animal Care and Use Committee of Zhejiang University and carried out in accordance with the National Institutes of Health guidelines for the use of laboratory animals.

Cell Cultures

The human neuroblastoma cell line SH-SY5Y (SY5Y) expressing GFP was cultured using Dulbecco's modified Eagles medium (DMEM) supplemented with 10% fetal bovine serum in a 5% CO2 humidified incubator at 37°C.

Blood Collection and Platelet Free Plasma (PFP) Preparation

We collected blood from hearts of 2 months old C57BL/6 mice, then prepared platelet free plasma (PFP) as described previously (György et al., 2014; Osteikoetxea et al., 2015) and in accordance with the standardization of International Society on Thrombosis and Hemostasis on blood sampling and handling for MV analysis (Lacroix et al., 2013). Briefly, in order to prevent release of platelet-derived EVs *in vitro*, blood was collected into acid-citrate-dextrose tubes (Greiner Bio-One; György et al., 2014). Blood was centrifuged (2×15 min, 3000 g at 4°C). PFP was stored at −80°C until use.

Exosome Isolation

We isolated exosomes from PFP using total exosome isolation reagents (Life Technologies). Vesicles were enriched according to the manufacturers' instructions. Briefly, the plasma sample was centrifuged (20 min, 2000× g; 20 min, 10,000× g at room temperature) to remove cells and debris. The supernatant was transferred to a new tube, then 0.5 volumes of 1× PBS were added and mixed, before adding 0.05 volumes of Proteinase K, followed by 10 min incubation at 37°C. Then 0.2 volumes of exosome precipitation reagent was added to the sample following by incubation at 4°C for 30 min. After incubation, the sample was centrifuged at 10,000× g for 30 min at room temperature. Exosomes were contained in a pellet at the bottom of the tube.

Electron Microscopy

Exosomes were resuspended in PBS at a concentration of 100 μg protein/ml. The exosome mixture was then applied to the grid and negatively stained with 2% phosphotungstic acid. Transmission images were acquired using an HT-7700 transmission electron microscope (Hitachi, Tokyo, Japan).

Nanoparticle Tracking Analysis

To quantify exosomes and characterize their dispersion and size distribution, nanoparticle tracking analysis (NTA) was performed using a NanoSight LM10 instrument (Malvern, Instruments) and NTA Version 2.3 Build 0034 software. For NTA, 100 μl of exosomes were diluted in 50 ml PBS, and particle size was calculated automatically for at least 5000 particles. PBS was assessed before the experiment to ensure that it was particle-free.

Western Blotting Analysis

Exosome samples were subjected to SDS-PAGE on 12% gels, followed by Western blotting. Proteins were blotted to PVDF membranes, and membranes were blocked in 5% milk in PBS/0.1% Tween 20 (PBS/Tween). The following primary antibodies were used: rabbit anti-ALIX (1:1000, Abcam, ab88388), rabbit anti-Tsg101 (1:2000, Abcam, ab125011), rabbit anti-GM130 (1:1000, ABclonal, A5344). Blots were visualized using HRP-conjugated secondary antibodies and the ECL Detection Reagent (Thermo Fisher Scientific) and were imaged on a LAS3000 image reader (Raytest, Germany).

Exosome Tracking

Exosomes were labeled with a red fluorescent lipophilic dye DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; Beyotime, C1036), allowing monitoring of exosome movement. DiI emits strong fluorescence when excited by green light and incorporated into membranes, and does not disrupt the membrane properties. Exosomes were resuspended in sterile PBS and incubated with 5 μM DiI for 10 min. DiI-exosomes were then washed and resuspended in sterile PBS three times to remove free DiI and other impurities such as lipoproteins. The control was prepared by DiI incubation with PBS, which was washed as for the DiI-exosome preparation.

Cell Incubation with DiI-Exosomes

DiI-exosomes were administered to SH-SY5Y cells and incubated for 24 h in serum-free conditions at a working concentration of 2 μg protein/ml. Images were acquired using an inverted microscope (Olympus, IX53).

Stereotaxic Injection of Exosomes into the Mouse Hippocampus

The DiI-exosome solution (3 μl at 1 μg protein/μl) and the control (3 μl) was stereotactically injected into the dentate gyrus (DG) of the mouse hippocampus using a stereotactic apparatus (KOPF), at the following coordinates (relative to Bregma): anterior posterior: −2.0, medial lateral: ± 1.6, dorsal ventral: −2.1. The brains were perfused with cold 0.9% saline and fixed in 4% paraformaldehyde for 3–20 days after injection.

Immunofluorescence Staining

Mice were perfused transcardially with 0.9% saline. Brains were removed immediately and immersed in 4% paraformaldehyde. After dehydration in 30% sucrose, coronal brain sections (20 μm) were prepared with a sliding microtome (Leica). Brain slices were stained as previously described (Zheng et al., 2015). In brief, brain slices were blocked with blocking buffer (10% fetal bovine serum, 1% nonfat milk, 0.2% gelatin in PBS containing 0.5% Triton X-100) and incubated with primary antibodies: rabbit anti-MAP-2 (1:600, #4542, CST), rabbit anti-IBA1 (1:600, 019-19741 Wako), mouse anti-6E10 (1:1000, SIG-39320, Covance), monoclonal anti-glial fibrillary acidic protein (GFAP, 1:400, G3893, Sigma). After incubating with primary antibodies overnight at 4°C, the brain sections were washed three times with PBS containing 0.5% Triton X-100 and three times with PBS. Slices were then incubated with the appropriate secondary antibodies: AlexaFluor 488 AffiniPure donkey anti-rabbit lgG (1:300, 711-545-152, Jackson ImmunoResearch), AlexaFluor 647 donkey anti-rabbit (1:1000, A-31571, Invitrogen), AlexaFluor 488 donkey anti-mouse (1:1000, A-21206, Invitrogen). After incubation with secondary antibodies for 2 h at room temperature, the brain sections were washed once with PBS containing 0.5% Triton X-100 and three times with PBS. Free floating brain sections were mounted on glass slides with a drop of 4′,6-diamidino-2-phenylindole (DAPI) Fluoromount-G mounting medium (0100-20, Southern Biotech). Images were taken using confocal microscope (OLYMPUS FV1000) and a large field of view CCD system (Zeiss, Axio Scan.Z1). Then images were processed using Fluoview Viewer (FV10-ASW; Olympus) and the ZEN 2 (blue edition, V1.0 en; Zeiss) software packages, respectively.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism version 6.05 for Windows (GraphPad Software, San Diego, CA, USA). All values in the figures are presented as mean ± standard error of the mean (SEM). Differences between two means were assessed with unpaired two-tailed Student's *t* test. *P* values of less than 0.05 were considered statistically significant.

RESULTS

Characterization of Exosomes

Exosomes were analyzed for morphology and size distribution using a Nanosight system and transmission electron microscope, respectively (**Figures 1A,B**). Consistent with recent reports (Momen-Heravi et al., 2012; Chernyshev et al., 2015), the exosomes were observed as a homogenous population with low dispersity and with a peak in particle size at 137 nm (**Figure 1A**). Streaming, a factor related to Brownian motion of small particles, caused the reported size distribution to be larger than actual size distribution (Scott et al., 2005). Transmission electron microscopy revealed that the media

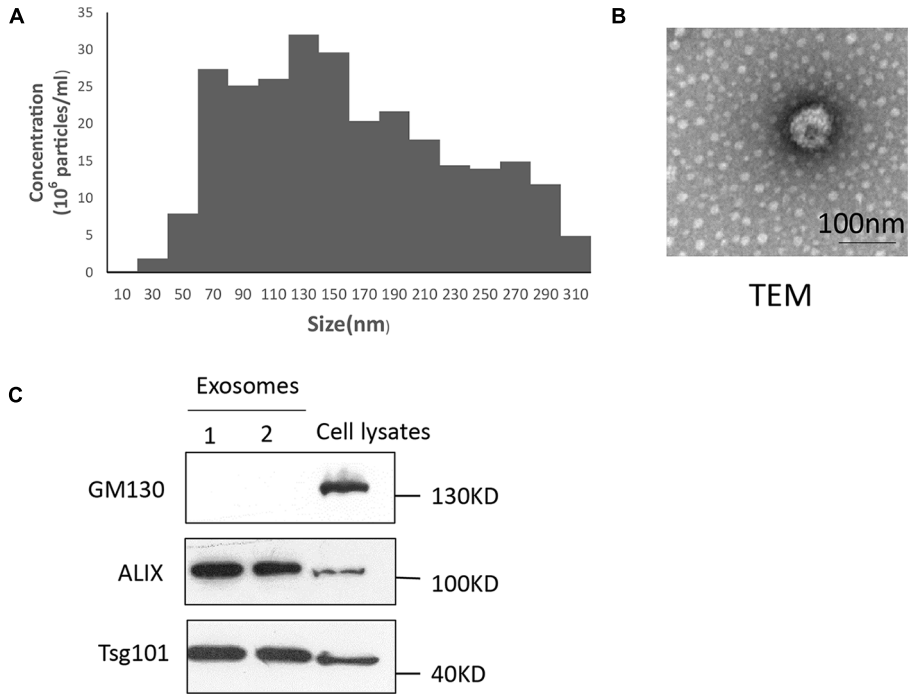

FIGURE 1 | Characterization of plasma exosomes. (A) Exosomes were measured via nanoparticle tracking analysis (NTA) analysis to quantify exosomes and show their size distribution. Exosomes comprise a homogenous population with a peak in diameter at 137 nm and low polydispersity. **(B)** Exosomes (1,00,000× g pellet) underwent negative staining with phosphotungstic acid and were examined by electron microscopy. Scale bars, 100 nm. **(C)** Fractions of exosomes were analyzed by Western blotting to detect the exosomal markers Alix and Tsg101, and the negative marker GM130. All approximate protein masses are represented in kDa.

contain morphologically distinct particles of approximately 60–110 nm diameter that are membrane bound and "cup shaped" (**Figure 1B**) as previously described. The presence of exosomes was confirmed by detecting the exosomal markers Alix and Tsg101, and negative marker GM130 in Western blotting analysis (**Figure 1C**).

Exosomes Can Diffuse Over Great Distances in Mouse Brain

Several reports have revealed that exosomes facilitate the unique transmissible nature of prions (Vella et al., 2007; Guo et al., 2016). Key proteins involved in other neurodegenerative disorders such as AD, Parkinson's disease and amyotrophic lateral sclerosis may also spread via misfolded proteins similar to prions (Bellingham et al., 2012; Coleman and Hill, 2015). To identify the trail of exosomes in the brain, we injected DiI-labeled exosomes into the mouse DG and traced the movement of the exosomes over time (3, 6 and 20 days after injection). We found that exosomes could spread from the injection site to other areas of hippocampus gradually and moved to the cortex as time went on (**Figures 2A–C**). Fluorescent images showed the most abundant exosomes in the cortex on day 20, but none were observed on day 3; a small portion of exosomes began to appear in the cortex on the 6th day after injection (**Figure 2A**). On the 20th day, most of the exosomes had spread from hippocampus to the cortex and only a few exosomes were retained in the hippocampus (**Figures 2B,C**). We prepared 15 coronal brain sections at approximately 200 μm intervals from mice brains 20 days after injection. Exosomes were observed in most brain sections (approximately 12–13 brain sections, Supplementary Figure S1). In the hippocampus, exosomes were concentrated in the CA3 region of mouse hippocampal slices, very little were observed in CA1 or CA2 of the hippocampus (**Figure 2D**). Meanwhile, the control group showed little red fluorescence in the hippocampus and cortex at 3, 6 and 20 days after injection (**Figure 2E**, Supplementary Figure S2). Yuyama et al. (2014) continuously injected exosomes intraventricularly for 14 days and found significant reductions in Aβ levels and Aβ-associated synaptotoxicity in the mouse hippocampus, as well as reductions in Aβ deposition.

Exosomes Target Microglia Preferentially and Stably in Mouse Brains

As the resident immune cells of brain, microglia have been involved in brain injury and various neurological disorders. Recent studies show that oligodendrocyte-derived and N2a-derived exosomes were preferentially internalized by primary microglia *in vitro* (Fitzner et al., 2011). When exosomes were administered into mouse brain, microglia engulfment of exosomes was observed a few hours later. To further follow the fate of exosomes over a period of

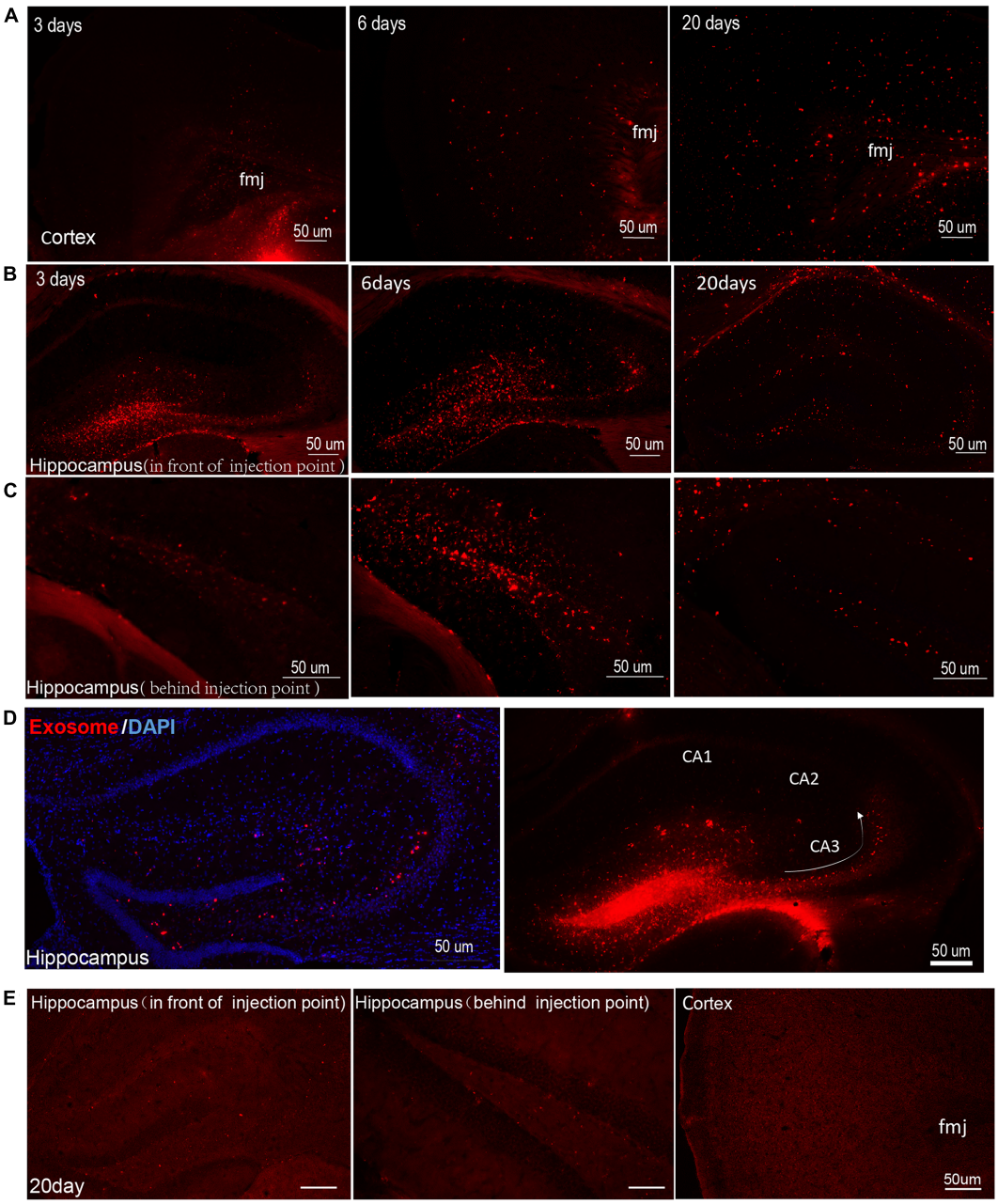

FIGURE 2 | Exosomes diffuse over great distances in mouse brain. Images were captured at 3, 6 and 20 days after injection of DiI-stained exosomes into the hippocampal dentate gyrus (DG) region. DiI-exosomes can emit red fluorescence. **(A)** Representative photomicrographs showing the distribution of exosomes in cortex. In order to show the structure more clearly, we marked the forceps major corpus callosum (fmj) in figures. The content of exosomes increased with time in cortex. **(B,C)** Representative photomicrographs showing the distribution of exosomes in hippocampus in front of **(B)** and behind **(C)** the injection point. Exosomes show better diffusion in hippocampus on day 6 than on day 3. On the 20th day, most of the exosomes spread from the hippocampus to the cortex. **(D)** Images were captured 3 days after injection of exosomes in hippocampal DG region. The figure on the left show DiI-exosomes and counterstain with 4′,6-diamidino-2-phenylindole (DAPI), while the figure on the right shows DiI-exosomes only. The arrow shows the projection direction from the DG region to CA3. Exosomes concentrated in hippocampal CA3 region. In contrast, very few exosomes are observed in area CA1 and CA2 of the hippocampus. **(E)** The control (DiI incubation with PBS and being re-isolated, washed, resuspended in sterile PBS for three times as the DiI-exosomes' preparation) were injected into hippocampal DG region. Representative photomicrographs show images captured at 20 days after injection and the images captured at 3 and 6 days after injection are shown in Supplementary Figure S2. Scale bars, 50 μm.

time in brain, we detected their presence in target cells on the 20th day after intracerebral injection. We observed that exosomes were still predominantly localized in Iba1-positive microglia in cerebral cortex and hippocampus (**Figures 3A,B**). In contrast, very little uptake of exosomes was observed in GFAP-positive astrocytes (**Figure 3C**) or MAP-2-positive

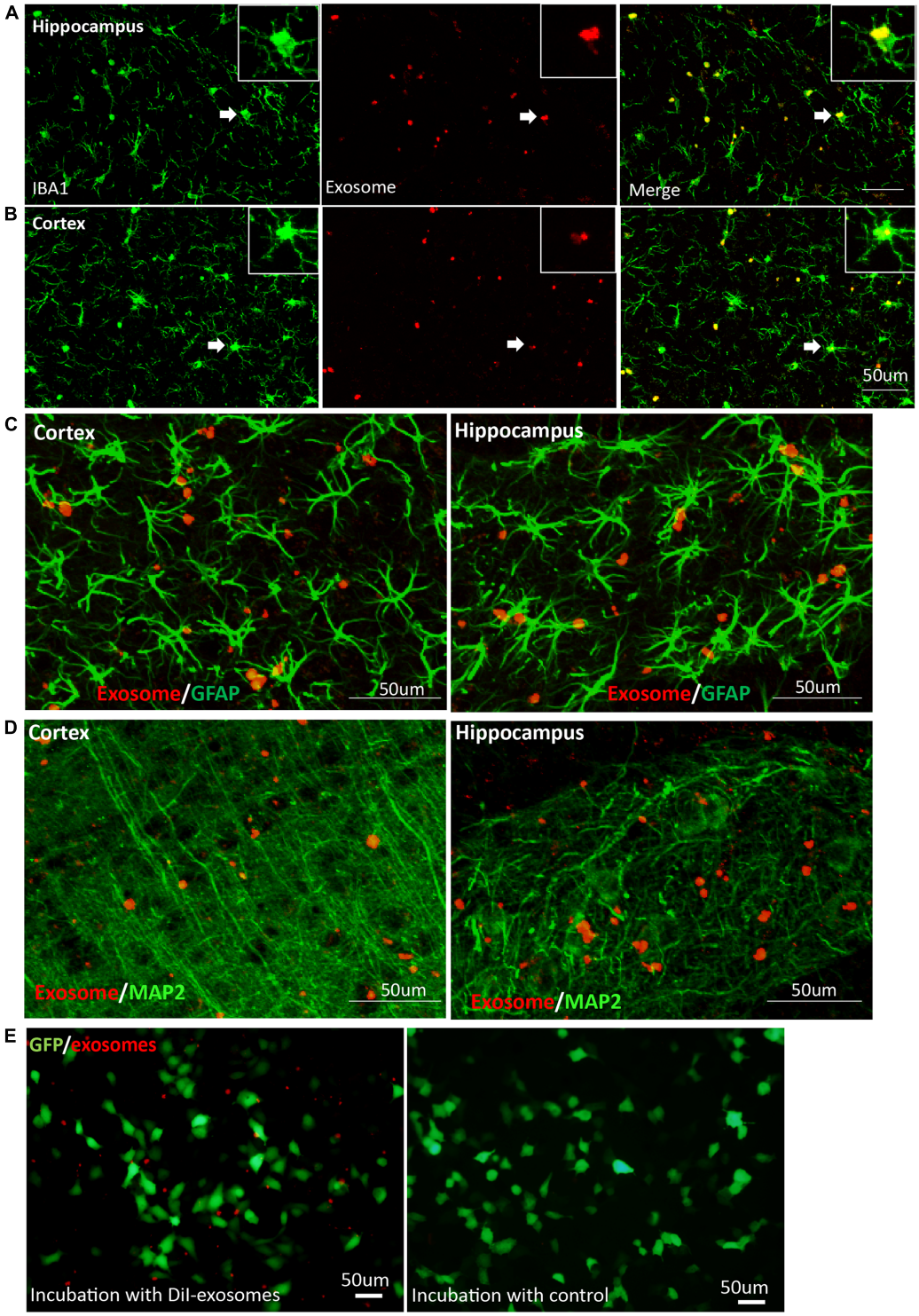

FIGURE 3 | Exosomes target microglia preferentially and stably. Images were captured at 20 days after DiI-exosome injection in non-transgenic mice (17 months old). **(A,B)** Microglia in the hippocampus and cortex were stained with anti-Iba1 antibody. Merged figures show that the majority of exosomes are localized in microglia. Inset, higher magnification of an exosome-containing microglia cell labeled with Iba-1 antibody. **(C,D)** Exosomes (red) are not taken up by astrocytes labeled with anti- glial fibrillary acidic protein (GFAP) antibody (green, **C**) or neurons labeled with anti-MAP2 (green, **D**) in cortex and hippocampus. **(E)** SH-SY5Y cells were incubated with DiI-exosomes *in vitro*. After 24 h incubation, images were acquired using an inverted microscope. Compared with the control group (DiI incubation with PBS and being re-isolated, washed as the DiI-exosomes' preparation and the same volume of control was incubated with sy5y cells), fluorescent signals are observed in the medium incubated with DiI-exosomes but are rarely detected in SH-SY5Y cells and cannot be detected in the control group.

neurons (**Figure 3D**) in cerebral cortex and hippocampus. Weak fluorescent signals could be occasionally detected in neurons of the DG, suggesting limited neuronal uptake of exosomes. Accumulation of exosomes may occur in the hippocampal CA3 region along the projection from the DG to the CA3 region (**Figure 2D**). We next used the neuroblastoma cell line SH-SY5Y with DiI-exosomes *in vitro*. After 24 h incubation with labeled exosomes, fluorescent signals were observed in the medium but rarely detected inside SH-SY5Y cells (**Figure 3E**). Combined with previous experimental results (Fitzner et al., 2011; Yuyama et al., 2012), our data demonstrated that exosomes target microglia preferentially and stably in mouse brains.

Aging Does Not Affect the Phagocytosis of Exosomes by Microglia

Age-associated microglial senescence in the brain leads to abnormal function and may eventually promote neurodegeneration (Luo et al., 2010). Since cellular internalization of exosomes occurs through phagocytosis in microglia, we investigated whether aging could affect the ability of microglia to engulf exosomes. We compared phagocytosis of exosomes by microglia in the hippocampus between young (3.7 months old) and old (17 months old) mice. No significant difference was observed (**Figures 4A–C**, Supplementary Figure S3).

The Ability of Microglial Engulfment of Exosome in AD Transgenic Mouse Brain Is Reduced

Several studies revealed that microglial phagocytic capacity is impaired in AD (Hickman et al., 2008). In this study, we compared microglial phagocytosis of exosomes in hippocampus between 17 month-old hAPP-J20 mice and littermate controls. We observed that microglial phagocytosis of exosomes was lower in hAPP-J20 mice (**Figures 4D–F**, Supplementary Figure S4).

Exosomes Cluster Around the Aβ Plaques and Are Engulfed by Activated Microglia Nearby

One pathological feature of AD is extracellular amyloid deposition and the presence of senile plaques (Beyreuther and Masters, 1991). Continuous intracerebral injection of neuroblastoma-derived or neuronal exosomes into AD transgenic mice results in marked reduction in Aβ pathology (Yuyama et al., 2014, 2015). However, injection of the astrocyte-derived exosomes into the brains of 10-day-old 5XFAD mice stimulates aggregation of Aβ *in vivo* (Dinkins et al., 2014). Here, we injected plasma exosomes into 17-month old AD mice to assess the relationship between plasma exosomes and Aβ plaques. We observed that exosomes clustered around the Aβ plaques, especially the large plaques (**Figure 5**). It has been demonstrated that extracellular Aβ plaques are often surrounded by activated microglia in both humans with AD (Styren et al., 1990) and AD animal models (Frautschy et al., 1998). We confirmed this phenomenon in hAPP-J20 mice (**Figure 5**). It is interesting that most exosomes clustered around Aβ plaques were localized in activated microglia (**Figure 5**), suggesting that microglia may play a role in AD pathogenesis through engulfing exosomes.

DISCUSSION

Neurodegenerative diseases, including AD, begin with dysfunction in a discrete region, and involve much larger areas of the brain at later stages. AD pathology has been proposed to spread through functionally and anatomically connected brain regions (Braak and Braak, 1991; Buckner et al., 2005; Braak et al., 2006; Harris et al., 2010), perhaps by a prion-like mechanism (Eisele et al., 2009; Frost and Diamond, 2010; Acquatella-Tran Van Ba et al., 2013; Marciniuk et al., 2013). Previous studies have demonstrated that exosomes facilitate the unique transmissible nature of prions (Vella et al., 2007; Guo et al., 2016). Many proteins associated with AD including APP and the APP-processing products, C-terminal fragments and Aβ can be found in exosomes from both neuronal cell cultures and brain tissues (Rajendran et al., 2006; Vingtdeux et al., 2007; Perez-Gonzalez et al., 2012). Pathogenic proteins involved in other neurodegenerative disorders also have been shown to be released by cells in association with exosomes (Bellingham et al., 2012).

In our study, we observed that exosomes diffused from the injection site in the DG to other brain regions, which could indicate the potential for exosome-bound pathogenic proteins to travel great distances in the brain. Previous studies have also shown that exosomes can cross the blood-brain barrier. When combined with our result that plasma exosomes can spread in the brain parenchyma, these data suggest that exosomes from peripheral blood may communicate with the CNS in physiological and pathological conditions. In addition, we found that exosomes concentrated in the CA3 region of the hippocampus. In contrast, very few exosomes were observed in the CA1 and CA2 areas of the hippocampus. This may due to projections from the DG to CA3 region. Synaptic connections between the mossy fibers of the granule cells in the DG and CA3 neurons, and the Schaffer collaterals from CA3 to CA1 neurons constitute the forward hippocampal polysynaptic circuit in mice (Harris et al., 2010). Further studies are needed to explore the relationship between exosomal diffusion and axonal projections in the hippocampus. It is reported that approximately 98% of all potent drugs that may be therapeutic for many neurological diseases in the CNS failed in clinic trials because of their inability to cross the blood-brain barrier (Pardridge, 2012). Exosomes have been increasingly used as delivery platforms, encapsulating reagents or siRNAs (Alvarez-Erviti et al., 2011; Haney et al., 2015). Therefore, the exosomal diffusion in brain observed in our study further supports the feasibility of using exosomes as a delivery system from the peripheral circulation to the brain.

Our data are consistent with previous observations that oligodendrocyte-derived exosomes are specifically and

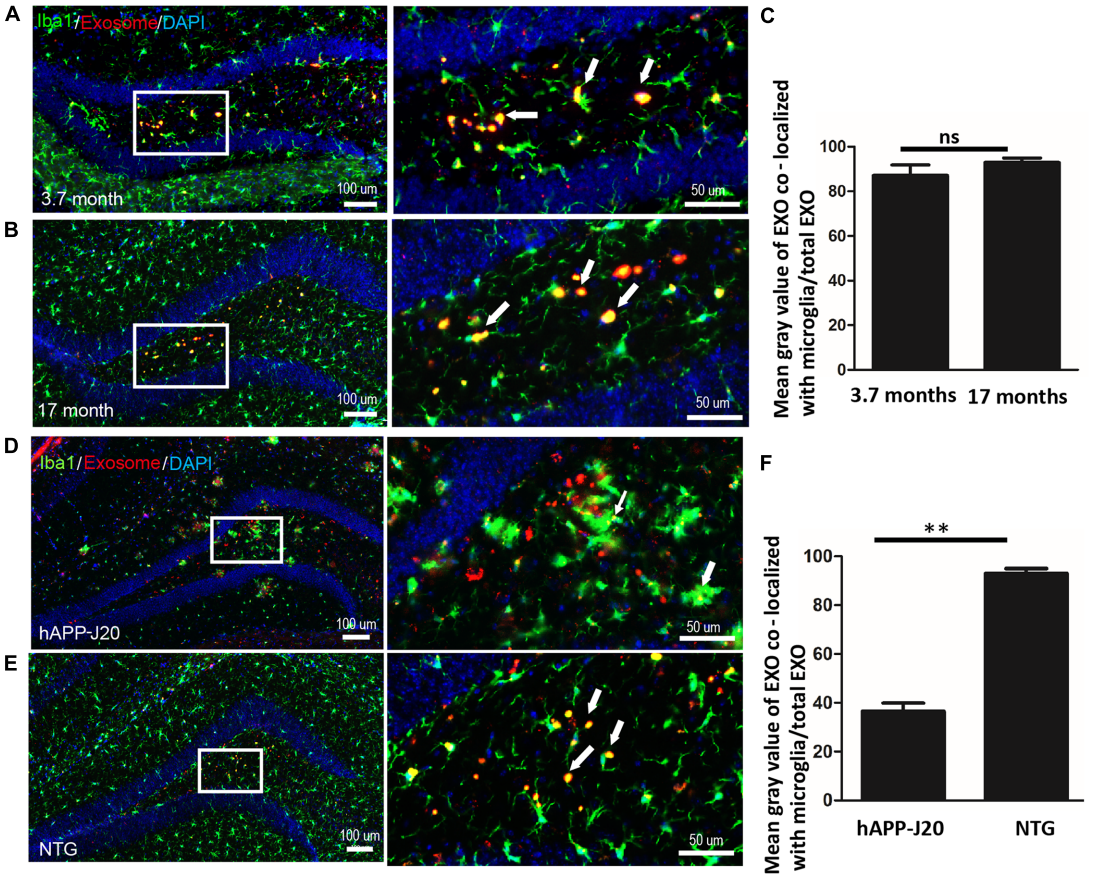

FIGURE 4 | Aging does not affect the phagocytosis of exosomes by microglia DiI-exosomes were stereotactically injected into the hippocampus. Hippocampal immunofluorescence staining was detected after 20 days. **(A,B)** Hippocampal sections from 3.7 **(A)** and 17 **(B)** month old mice were stained with anti-Iba1 antibody (green). **(C)** Quantification of the relative uptake of exosomes by microglia (mean gray value of exosomes co-localized with microglia/total exosomes) in hippocampus of 3.7 and 17 months old mice (double-blinded, ImageJ analysis). ns, no statistically difference. EXO, exosomes. **(D,E)** Exosomes target microglia in hAPP-J20 mice with lower efficiency. Hippocampal sections from hAPP-J20 transgenic mice **(D)** and non-transgenic mice **(E)** were stained with anti-Iba1 antibody. **(F)** Quantification of the relative uptake of exosomes by microglia in hippocampus of hAPP-J20 transgenic mice and non-transgenic mice. $N = 3$ (5 brain slices for each mouse); $^{**}P < 0.01$, two-tailed student's t test was used.

efficiently taken up by microglia (Fitzner et al., 2011). Harris et al. (2010) reported that oligodendrocyte-derived exosomes are selectively taken up by microglia via micropinocytosis; Yuyama et al. (2012) further showed that microglia engulf exosomes in a phosphatidylserine-dependent manner. In agreement with these previous studies, we found that plasma exosomes were efficiently taken up by microglia. Unlike short-term tracking in a previous report, we investigated the diffusion of exosomes in the brain for up to 20 days. Our results showed that exosomes can spread stably in the brain and are largely taken up by microglia. A portion of exosomes not engulfed by microglia were observed in the cortex and hippocampus, which rules out the possibility that the spreading of exosomes is due to the migration of microglia containing labeled exosomes. To evaluate whether neurons and astrocytes also engulf exosomes, we tested fluorescently-labeled exosomes in these cells. In contrast to microglia, very little uptake of exosomes was observed in astrocytes or neurons in cerebral cortex or hippocampus, which is supported by *in vitro* results (Fitzner et al., 2011).

The proteins and nucleic acids carried by exosomes play important roles in signal delivery and material exchange between cells. In a similar fashion, microglia may communicate with the other cells in brain through exosomes. However, exosomes can potentially carry pathogenic proteins, such as prions, propagating their toxic assemblies and promoting the progress of diseases (Vella et al., 2007; Guo et al., 2016). Engulfment of exosomes by microglia may prevent the propagation of exosome-bound pathogenic proteins to other cells. Interestingly, insulin-degrading enzyme and Aβ-degrading enzymes have been found in the exosomes secreted by microglia (Tamboli et al., 2010). Therefore, exosomes may enhance the ability of microglia to clear pathogenic proteins. Further studies are required to clarify the roles of exosomes in the CNS and their specific relationships with microglia.

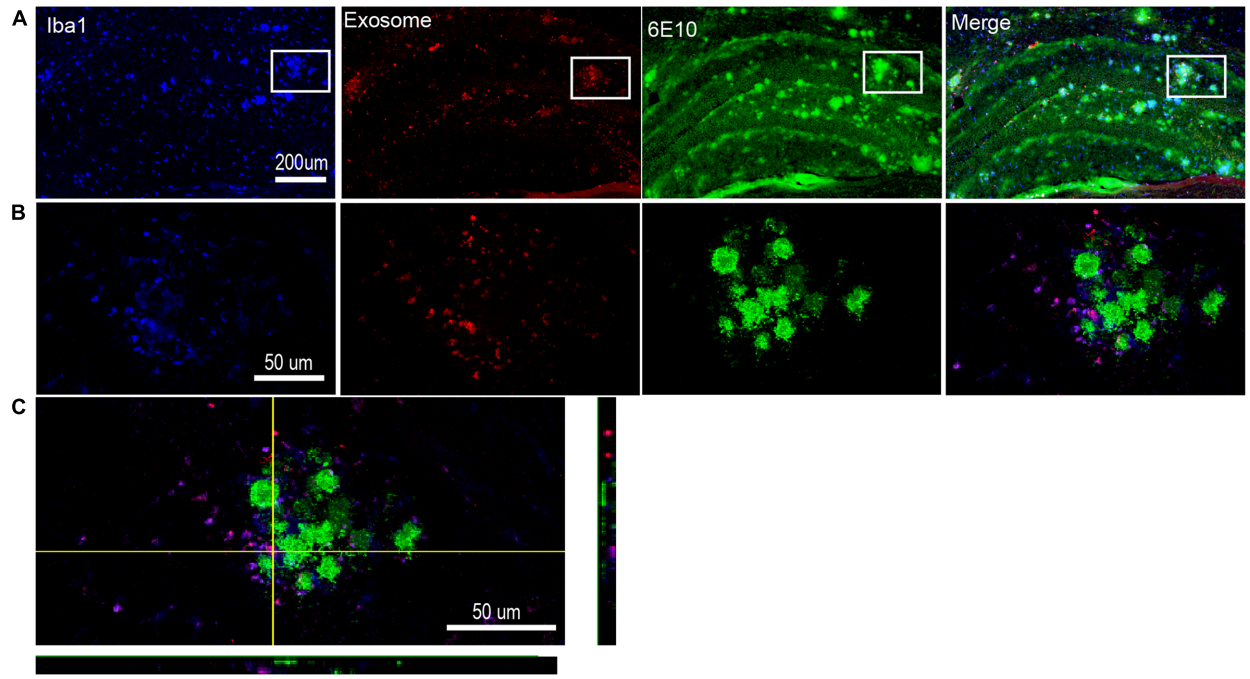

FIGURE 5 | Exosomes cluster around the Aβ plaques and are engulfed by nearby activated microglia. (A) Representative photomicrographs showing the distribution of DiI-exosomes in hippocampus of hAPP-J20 transgenic mice. Microglia are labeled with anti-Iba1 (blue), exosomes are labeled with DiI (red) and anti-6E10 is used to label Aβ plaques (green). **(B)** Higher magnification images of the boxed areas in **(A)** show a single Aβ plaque. Exogenously injected exosomes and microglia cluster around the Aβ plaque. Moreover, the exosomes are largely localized in this microglia. **(C)** Representative confocal image through a single plane of exosome (red) internalized by microglia (green) in the hippocampus.

Aging effects on microglial phagocytosis has been widely studied. A recent report suggests that the ability of primary murine microglia to take up exosome-associated oligomeric α-synuclein is compromised in aged mice (Bliederhaeuser et al., 2016). Moreover, phagocytic deficits are found in human monocytes from elderly individuals (Bliederhaeuser et al., 2016). Our studies in mouse brain show that aging did not alter the ability of microglia to take up plasma exosomes, suggesting that dysregulation of microglia caused by aging may not influence the engulfment of plasma exosomes.

Impaired microglial phagocytic capacity has also been observed in neurodegenerative diseases such as AD. Here we show that exosomes targeted microglia in hAPP-J20 mice with lower efficiency compared with control littermates. Microglia pro-inflammatory activation and dysfunction has been observed in AD brains. However, both the decreased amyloid-clearing ability of microglia (Flanary, 2005) and the damage to microglia by amyloid (Korotzer et al., 1993) have been reported in AD brain. von Bernhardi (2007) proposed that AD is caused by dysfunctional microglia rather than by hyperactive microglia. Microglia from aged PS1-APP mice have a 2–5 fold decrease in the expression of the Aβ-binding scavenger receptors RAGE, scavenger receptor A and CD36 compared to their littermate controls (Hickman et al., 2008), which indicates microglial dysfunction in AD and supports the idea that microglia senescence contributes to the pathogenesis of AD. Therefore, the observation in our study that engulfment of exosomes is reduced in the AD mouse model may due to phagocytic deficits of microglia in AD.

Recently, *in vitro* studies demonstrate that a fraction of Aβ peptide is released in association with exosomes (Rajendran et al., 2006). Exosomes enhance conformational changes in Aβ to form nontoxic amyloid fibrils and promote the uptake and clearance of Aβ by microglia (Yuyama et al., 2012). These findings suggest that exosomes are involved in Aβ metabolism in the brain. Here, we observed that exogenously injected exosomes clustered around Aβ plaques, especially large plaques, in hAPP-J20 mice. Interestingly, exosomal markers are found to be enriched in amyloid plaques in the brains of Tg2576 mice (Kokubo et al., 2005) and postmortem human AD patients (Rajendran et al., 2006). Meanwhile, activated microglia were found to surround the extracellular Aβ plaque as previously reported (Rogers et al., 1988; Frautschy et al., 1998; Stalder et al., 1999; Buckner et al., 2005). The confocal images show that most of the exosomes around the plaques are localized in these activated microglia. Yuyama et al. (2014, 2015) reported that glycosphingolipids are abundant in exosomes and Aβ can bind to the exosome surface via the glycan moieties of glycosphingolipids. Exosome-bound Aβ is then transported into microglia for degradation, resulting in a decrease in Aβ levels, Aβ plaques and Aβ-related pathologies in APP mice. Combined with the observations in our study, these data suggest that exosomes may play a role in trafficking of Aβ aggregates through microglia during disease

progression. However, injection of astrocyte-derived exosomes into the brains of 10-day old 5XFAD mice stimulated aggregation of Aβ in at the site of injection (Dinkins et al., 2014). Increasing serum exosome content by treating 5XFAD mice with ceramide ultimately enhanced plaque formation (Dinkins et al., 2015) The size of amyloid plaques changes over days in brains of AD model mice (Bolmont et al., 2008). Whether the plasma exosomes support this change needs further exploration. It is worth noting that although engulfment of exosomes by microglia is inefficient in AD transgenic mice overall, the activated microglia clustering around plaques can take up exosomes effectively. This may indicate that phagocytosis in microglia clustering around plaques is stronger than other parts of the brain. Whether the plasma exosomes can decrease Aβ plaques and play a role in pathological process of AD, as well as which type of cells these functional exosomes come from, needs further investigation.

Overall, our study described the movement of exosomes across great distances in the brain, which can help to advance medical research of exosomes in neurodegenerative diseases. Moreover, exosomes may play roles in amyloid deposition through microglia in AD.

AUTHOR CONTRIBUTIONS

BZ, BS and TZ conceived and designed the study; TZ, JP, YC, YM, ZG, HP, LZ and HZ performed the experiments; JP and TZ processed and analyzed all data; BS and BZ wrote the manuscript with input from TZ. All authors have read and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/ 10.3389/fnagi.2017.00012/full#supplementary-material

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Changes in Blood Factors and Ultrasound Findings in Mild Cognitive Impairment and Dementia

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The present study aimed to assess the changes in blood factors and ultrasound measures of atherosclerosis burden patient with mild cognitive impairment (MCI) and dementia. Peripheral blood samples and ultrasonography findings were obtained for 53 enrolled participants. Flow cytometry was used to evaluate levels of activated platelets and platelet-leukocyte aggregates (PLAs). The number of platelets expressing p-selectin was correlated with intima media thickness (IMT) and plaque number in both the MCI and dementia groups. The number of platelets expressing p-selectin glycoprotein ligand (PSGL) was strongly correlated with IMT in patients with MCI, whereas the number of platelets expressing PGSL was correlated with plaque number rather than IMT in patients with dementia. PLAs was associated with both IMT and plaque number in patients with MCI but not in those with dementia. Our findings demonstrate that alterations in IMT and plaque number are associated with an increased risk of cognitive decline as well as conversion from MCI to dementia and that blood factor analysis may aid to detect the severity of cognitive decline.

Keywords: dementia, vascular disease, mild cognitive impairment, blood factor analysis, atherosclerosis

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INTRODUCTION

Aging has been regarded as a major risk factor for neurodegenerative disease (Coutu et al., 2017). Previous studies have demonstrated a strong association between aging and vascular diseases (Jeerakathil et al., 2004; Gottesman et al., 2010; Pantoni, 2010). The research has indicated that factors associated with aging and vascular dysfunction exhibit a cross-sectional relationship with mental status as determined based on Mini-Mental State Examination (MMSE) score (Atiya et al., 2003). Recent studies have reported that carotid artery atherosclerosis is associated with a subsequent risk of new or recurrent cerebrovascular diseases, such as stroke, post-stroke vascular dementia and mild cognitive impairment (MCI; Knopman et al., 2016; Dearborn et al., 2017; Meyer et al., 2017). Furthermore, chronic hypoperfusion caused by carotid stenosis has been reported to play a role in cognitive decline (Ruitenberg et al., 2005; Yurkovetsky et al., 2006).

Dementia represents a major public health concern (Moon et al., 2015), as accumulating evidence has demonstrated that the incidence and prevalence of dementia increase rapidly with advancing age (Silvestrini et al., 2009; Petersen et al., 2014; Moon et al., 2015).

Abbreviations: AD, Alzheimer's disease; ANOVA, analysis of variance; CDR, Clinical Dementia Rating Scale; FACS, fluorescence-activated cell sorting; FITC, fluorescein isothiocyanate; HDL, high-density lipoprotein; IgG, immunoglobulin G; IMT, intima-media thickness; LDL, low-density lipoprotein; MCI, mild cognitive impairment; MMSE, Mini-Mental State Examination; PLA, platelet-leukocyte aggregation; PMA, platelet-monocyte aggregation; PRP, platelet-rich plasma; PSGL, p-selectin glycoprotein ligand.

Although it has been difficult to investigate changes in the incidence and prevalence of dementia due to variations in diagnostic criteria and methods, a recent epidemiological study indicated that dementia prevalence and incidence have decreased in some countries (Wu et al., 2017). Moreover, the number of patients with dementia remains stable in the aging population of these countries (Wu et al., 2017). Some evidence has suggested that vascular risk factors are associated with the onset and progression of Alzheimer's disease (AD; Luchsinger et al., 2005; Deschaintre et al., 2009). In addition, increased cerebrovascular risk has been associated with more severe dementia and MCI incidence (Luchsinger et al., 2005; Mioshi et al., 2006; Deschaintre et al., 2009; Li et al., 2011). Considering the role of vascular blood factors in patients with MCI, such factors may also influence the progression of cognitive decline (Iadecola and Gorelick, 2003). However, there are currently no markers for the prediction of prognosis or the risk of conversion from MCI to dementia. Therefore, it is necessary to develop noninvasive diagnostic methods for the assessment of vascular status (de la Torre, 2010).

Recent clinical investigations have focused on the relationship between levels of circulating adhesion factors in peripheral blood and cerebrovascular diseases (Vermeer et al., 2003; Dearborn et al., 2017). Platelets and leukocytes play a major role in atherothrombosis, aggregates of which result in the formation of atherosclerotic plaque (Folsom et al., 2009; Lievens et al., 2010; Dopheide et al., 2016; Gerdes et al., 2016). Although other factors associated with vascular disease can influence cognitive state, few studies have utilized flow cytometry to investigate platelet and leukocyte markers in older adults with cognitive decline. Research has demonstrated a correlation between circulating adhesion molecules in patients with atherosclerosis and atherosclerosis factors such as intima-media thickness (IMT) and the number of plaques, which may aid in determining the presence and/or extent of cognitive decline (Moon et al., 2015). In order to determine the potential usefulness of this correlation for determining diagnoses/prognoses, blood factor analysis is required. Based on the pathophysiological mechanism underlying dementia, most relevant studies have aimed to identify molecular markers based on drug responses (Mioshi et al., 2006; Jellinger and Attems, 2007; Steinhubl et al., 2007; Coley et al., 2008). As such, little is known regarding the potential role of circulating adhesion molecules in patients with vascular diseases during the early and later stages of cognitive dysfunction.

The present study aimed to assess the relationship between changes in blood factors and ultrasound findings in patients with MCI and dementia exhibiting signs of atherosclerosis, and to suggest the possibility of the most appropriate treatment strategy for patients with MCI or multiple diagnoses.

MATERIALS AND METHODS

Participants

The present study enrolled 53 participants who had visited the neurology outpatient clinic of Severance Hospital between August 2016 and February 2017. Participants with an infection such as aspiration pneumonia were excluded to avoid parallel infection, which may also activate platelets or leukocytes. The control group consisted of nine age- and sex-matched individuals with no clinical signs of cerebrovascular or cardiovascular disease such as stenosis or atherosclerosis. Participants of the patient group were categorized into three subgroups: vessel damage, MCI and dementia groups. The "vessel damage" group represents participants who contain one or more cardiovascular and cerebrovascular diseases containing atherosclerosis, stenosis, or stent implanted. "MCI" group is basically classified with based on the Petersen's criteria (Petersen, 2004). In our case, we only considered the amnestic MCI for more homogeneous sample. The basal score of participants are 25–27 on the MMSE adjusted according to age and education as assessed for the Korean population. Clinical Dementia Rating Scale (CDR) values 0.5 and 1.0 were classified into the MCI group. We also used additional cognitive evaluation battery the Korean version of Addenbrooke's Cognitive Examination-Revised (K-ACE; Mioshi et al., 2006). The type of "dementia" group was included AD only which is diagnosed by department of neurology in Severance hospital using neuroimaging and cognitive evaluation (MMSE and CDR) containing Seoul Neuropsychological Screening Battery (SNSB) widely used in South Korea.

No differences in atherosclerotic risk profile (e.g., hypertension, hyperlipidemia and diabetes) were observed. Fasting levels of total and high-density lipoprotein (HDL) cholesterol, triglycerides and low-density lipoprotein (LDL) cholesterol were also measured in each participant. The characteristics of the participants are shown in **Table 1**. All participants underwent cognitive assessment consisting of the CDR scale and MMSE. Patients with MMSE scores from 25 to 27 and CDR values 0.5 and 1.0 were classified into the MCI group. Patients with dementia were diagnosed with dementia and they were classified into the dementia group. All participants provided written informed consent, and the study protocol was approved by the Institutional Review Board of the Severance Hospital (4-2016-0531). All participants gave written informed consent in accordance with the Declaration of Helsinki.

Ultrasonography

Participants underwent measurement of carotid artery IMT, plaque morphology and thickness of carotid plaques via B-mode ultrasonography. Carotid artery plaques were examined using a high-resolution B-mode ultrasound system (Accuvix V10, Seoul, South Korea) equipped with a multi-frequency linear array transducer (5–10 MHz). All measurements were performed by a technician trained in ultrasound research in accordance with a standard scanning and reading protocol.

Carotid artery IMT is defined as a distance from media-adventitia to lumen-intima interface. Carotid artery IMT (longitudinal view) was measured offline in a plaque-free region at the far wall of the common carotid artery (CCA) using a computerized system. The upper limit of normal for IMT was defined as 1.0 mm. IMT measures were obtained from walls of three arterial segments of both carotid arteries; the near and far

TABLE 1 | Patients characteristics.

	Control participants (n = 9)	Vessel damage stenosis patients (n = 29)	Cognitive decline		p-value
			MCI (n = 11)	Dementia (n = 14)	
Age	65.3 ± 8.1	68.1 ± 12.1	66.3 ± 12.2	70.9 ± 9.7	0.632
Gender					0.188§
Male	11.1 (1)	58.6 (17)	54.5 (6)	28.6 (4)	
Female	88.9 (8)	41.4 (12)	45.5 (5)	71.4 (10)	
Hypertension	22.2 (2)	31.0 (9)	45.5 (5)	50.0 (7)	0.821§
Diabetes	9.0 (1)	17.2 (5)	27.3 (3)	14.3 (2)	0.420§
Hyperlipidemia	22.2 (2)	24.1 (7)	27.3 (3)	50.0 (7)	0.250§
Total cholesterol (mg/dL)	168.7 ± 42.9	138.6 ± 43.6	138.9 ± 41.4	152.9 ± 43.0	0.400
LDL cholesterol (mg/dL)	76.9 ± 18.2	69.2 ± 32.4	61.8 ± 38.3	76.9 ± 33.7	0.742
HDL cholesterol (mg/dL)	67.0 ± 40.5	50.3 ± 13.1	54.0 ± 14.9	55.1 ± 17.7	0.344
MMSE score	29 ± 0	25.4 ± 3.2	24.7 ± 2.3	20.5 ± 4.3	0.392

Data are expressed percentage as mean ± SD. The each patient number is presented in parenthesis. §Derived from Chi-squared test.

wall of the proximal 10 mm of the internal carotid artery, the near and far wall of the carotid bifurcation beginning at the tip of the flow divider and extending 10 mm proximally, and the near and far wall of the arterial segment extending 10–20 mm proximally to the tip the flow divider into the CCA (Prati et al., 2008).

In accordance with the Mannheim Consensus, carotid plaques were defined as focal protruding structures into the lumen with a size of at least 0.5 mm or 50% of the IMT where is over 1.5 mm (Touboul et al., 2007). The lesions with an IMT ≥ 1.1 mm were defined as atheromatous plaques. Thickness of carotid atheroma to measure and count the plaques was measured in a longitudinal and vertical view for screening accurate plaque. The plaques were measured everywhere.

Flow Cytometry

Blood samples were collected into Vacutainer tubes containing 0.5 mL of 3.2% buffered sodium citrate, immediately following which the citrated blood sample was added to a fixation solution to minimize *ex vivo* platelet activation and prepared for flow cytometry. Whole blood samples were used to assess platelet activity. Whole blood resuspended in Tyrode's solution was incubated with phycoerythrin (PE)-conjugated anti-CD41a for immunological identification of platelets. The samples were simultaneously incubated with fluorescein isothiocyanate (FITC)-conjugated anti-CD62p or FITC-conjugated anti-CD162 at saturating concentrations for 15 min at room temperature in the dark. Levels of platelet-bound anti-CD62p or -CD162 were determined by analyzing 50,000 platelets for FITC fluorescence. Results were expressed as a percentage of antibody-positive platelets.

Red blood cell-lysed blood samples were used to determine leukocyte levels, which were identified based on the forward and sideward scatter properties of PE-CD45-positive leukocytes. Monocytes and lymphocytes were identified based on strong expression of PE-CD14 and PE-CD154, respectively. The presence of platelet-leukocyte aggregates was assessed during the detection of CD42b-FITC-labeled platelets. FITC-conjugated immunoglobulin G (IgG) and PE-conjugated IgG antibody were used for isotype control experiments. A minimum of 50,000 cell events was analyzed in each assay. The percentages of platelet-monocyte and platelet-lymphocyte complexes were calculated. All antibodies used were purchased from BD Biosciences (New Jersey, NJ, USA). Blood samples were analyzed using LSRII (Becton Coulter, San Jose, CA, USA).

Statistical Analysis

Participant characteristics and plaque thickness are presented as mean ± SD. Fluorescence-activated cell sorting (FACS) and ELISA values are presented as medians. The clinical characteristics of each group were compared using the Mann–Whitney nonparametric test. Statistical significance among groups was determined via one-way analysis of

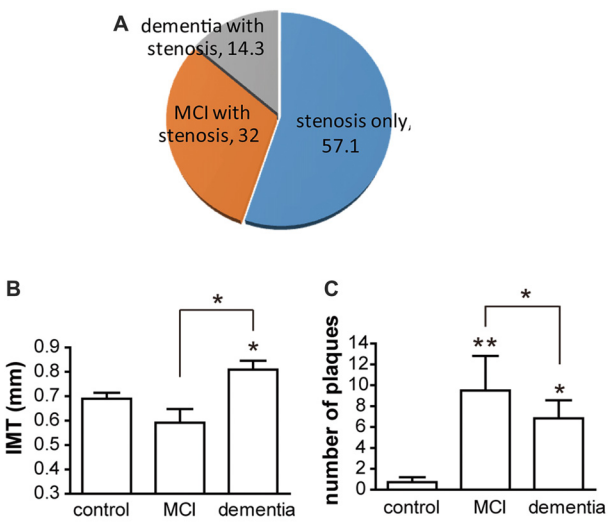

FIGURE 1 | The distribution of participants and atherosclerotic condition. **(A)** Of participant, patients groups are three. Without cognitive decline, patients with atherosclerosis only are 57.1% of total patients. With atherosclerosis, patients with mild cognitive impairment (MCI) are composed in 32.1% and patients with dementia are constituted in 14.3%. **(B)** Intima-media thickness (IMT) is shown as mean ± SD. Differences of IMT is significant between dementia and control group. Also it is significant between dementia and MCI. **(C)** Numbers of plaques are shown as mean ± SD. There are significant differences between control and MCI; control and dementia; and MCI and dementia. *$p < 0.05$ vs. control group, **$p < 0.01$ vs. control group.

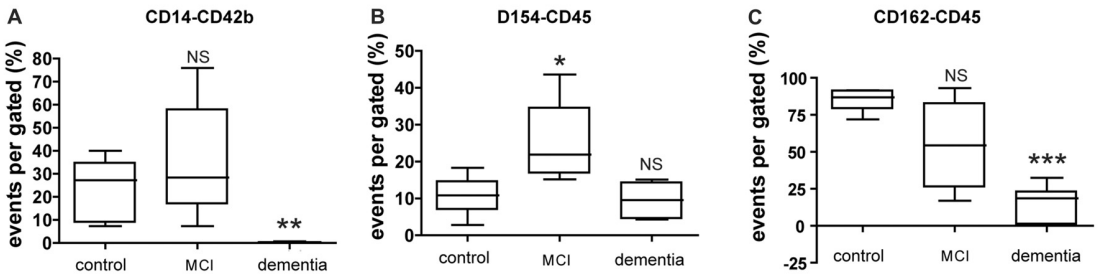

FIGURE 2 | Expression of circulating blood factors by fluorescence-activated cell sorting (FACS) analysis. **(A)** Circulating levels of monocyte aggregated with platelets in each group. **(B)** Circulating levels of leukocyte aggregated with CD40Ligand (CD154) in each group. **(C)** Circulating levels of leukocyte aggregated with p-selectin glycoprotein ligand (PSGL; CD162) in each group. The data presents with % as an event number per gate by FACS analysis. Bars represent median values, and the error bar, the standard deviation. *$p < 0.05$ vs. control group, **$p < 0.01$ vs. control group, ***$p < 0.001$ vs. control group. NS, not significant.

variance (ANOVA). Probability values were two-tailed, and values of $p < 0.05$ were considered statistically significant (SPSS, Windows version 17.0, Chicago, IL, USA). Pearson correlation coefficients were used to assess the association between ultrasound findings (IMT and plaque number) and changes in blood factors associated with atherosclerotic vessel dysfunction. Values of the coefficient constant r and r^2 are provided. A value of $p \leq 0.05$ was regarded as significant (SPSS, Windows version 17.0, Chicago, IL, USA).

RESULTS

Clinical Characteristics and Ultrasound Findings of Control and Patient Groups

In the present study, there was no significant difference in vascular risk factors among the control, vessel damage, MCI and dementia groups. Moreover, there were no significant differences among the three groups with regard to total cholesterol ($p = 0.4$), HDL ($p = 0.7421$) and LDL ($p = 0.3439$). Participant characteristics are detailed in **Table 1**. Of all patients with vessel damage diagnosed with carotid vascular stenosis or atherosclerosis, patients with vessel damage without any cognitive dysfunction comprise 57.1%; patients with MCI is 32.1%; and patients with dementia is 14.3% (**Figure 1A**). Particularly, all of patient with MCI had been also with vessel damage. The IMT was significantly thicker in the dementia (mean thickness: 0.809 ± 0.097 mm) group than in the control group (mean thickness: 0.643 ± 0.123 mm), but not in the MCI group (mean thickness: 0.743 ± 0.226 mm; **Figure 1B**). However, plaque numbers were significantly increased in both the MCI and dementia groups relative to values for the control group (control: 2.5 ± 0.7 vs. MCI: 9.5 ± 6.6, $p = 0.0061$; control vs. dementia: 6.8 ± 4.2, $p = 0.0012$; **Figure 1C**).

Changes in Levels of Circulating Adhesion Blood Factors in Patients with MCI and Dementia

FACS analysis of circulating blood factors associated with platelet-leukocyte aggregation (PLA) revealed that specific changes in PLA level in each MCI or dementia group (**Figure 2**). The levels of platelet-monocyte aggregates (PMA) as determined by CD154-CD14 double-staining is lower in dementia group compared to control, but not significant difference in MCI group (**Figure 2A**). In contrast, the level of PLA (CD154-CD45) is higher in MCI group, but not significant change in dementia group (**Figure 2B**). In case of another PLA (CD162-CD45), there was significant decrease in dementia group compared to control. It was different from the result in MCI group presenting no significant change compared to control (**Figure 2C**).

The number of platelets expressing p-selectin (CD62p) by FACS was increased in dementia group whereas the number of platelets expressing p-selectin glycoprotein ligand (PSGL; CD162) was high both in MCI and dementia group (**Figures 3A,B**). The level of p-selectin detected on the surface of active platelets significantly increased in the vessel damage group and MCI group, based on changes in platelet-rich plasma (PRP) levels as determined via ELISA. The level of p-selectin and PSGL in plasma was higher in the MCI group than in the control group (**Figures 3C,D**).

Associations between Vessel Status and Circulating Blood Factors in Patients with Cognitive Decline

The correlation between cognitive function and circulating adhesion factors was analyzed as shown in **Table 2**. The number of platelets expressing p-selectin was correlated with IMT and plaque numbers in both the MCI and dementia groups. The number of platelets expressing PSGL was strongly correlated with IMT, but not with plaque numbers, in MCI group. In contrast, the number of platelets expressing PSGL was correlated with plaque number rather than IMT in dementia group. Unlike in the dementia group, factors associated with blood aggregation and vessel dysfunction such as CD40Ligand (CD154), PLA and PMA, were correlated with IMT and plaque number in the MCI group (**Table 2**). In the MCI group, IMT was significant correlation with most of circulation blood factors except of the level of PMA. Plaque number in MCI group was also significantly correlated with p-selectin, PLA and PMA (**Table 2**). In the dementia group, IMT was significant correlation with the level of p-selectin only. In contrast, plaque number was a significant correlation with

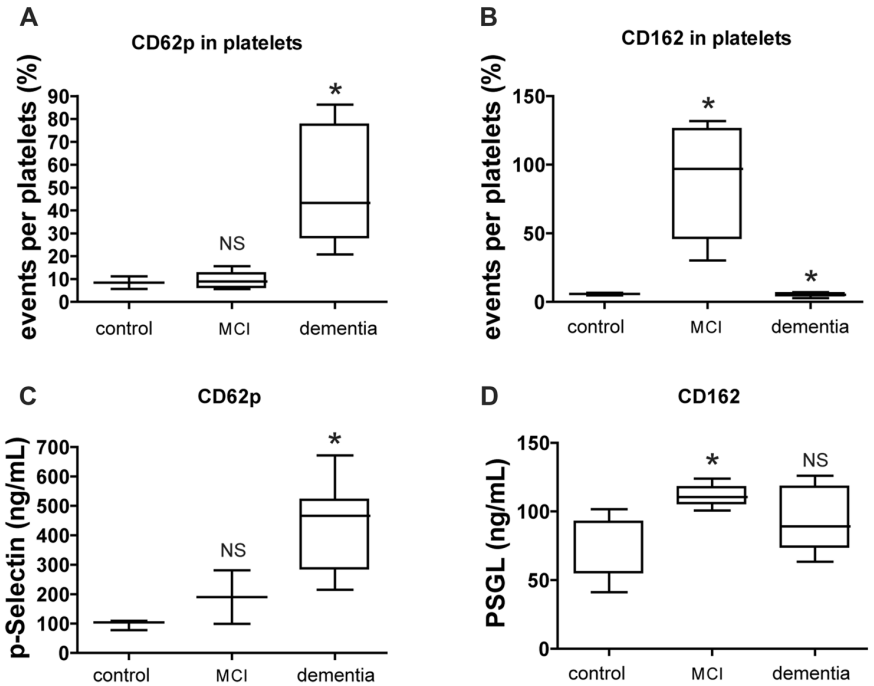

FIGURE 3 | CD62p and CD162 expression in cell surface marker and soluble level. **(A,B)** Level of platelet surface expressing CD62p (p-selectin) or CD162 (PSGL) was evaluated by FACS analysis. **(C,D)** Level of CD62p or CD162 in plasma was evaluated by ELISA. The data of FACS represents with % as an event number per gate. The result of ELISA is presented as ng/ml. Bars represent median values, and the error bar, the standard deviation. *$p < 0.05$ vs. control group. NS, not significant.

p-selectin and strong correlation with PSGL among circulating adhesion factors (**Table 2**).

DISCUSSION

In the present study, we demonstrated that alterations in IMT and plaque number are associated with an increased risk of cognitive decline as well as risk of dementia. Our results suggest that ultrasound findings may aid in identifying older individuals at increased risk for the progression of cognitive decline when morphological impairment of cerebrovascular structures has been identified. Moreover, our findings suggest that the presence of atherosclerotic changes and changes in blood factors such as PSGL, PLA and PMA can be used to predict MCI and dementia.

In the present study, levels of p-selectin in circulating platelets, PSGL and circulating PMA were significantly increased in patients with MCI relative to controls (**Figures 2**, **3**). The changes in circulating blood factors have been reported to relate with vascular diseases like as ischemic stroke or atherosclerosis (Marquardt et al., 2002; Nadar et al., 2004). Based on this association, several noninvasive measures for evaluating subclinical atherosclerosis have received intense attention in clinical and research settings for the predictive diagnosis of cerebrovascular diseases.

TABLE 2 | Correlation between ultrasonic characters and circulating blood factors.

Correlating pair		MCI			Dementia		
Ultrasonic characters	**Circulating blood factors**	***r***	***R^2***	***p* value**	***r***	***R^2***	***p* value**
IMT	p-selectin	0.810	0.656	**0.0149***	0.609	0.371	**0.0273***
	PSGL	0.951	0.904	**0.0036****	−0.207	0.043	0.5653
	CD40 Ligand	0.906	0.822	**0.0019****	−0.035	0.001	0.923
	PLA	0.819	0.671	**0.0243***	0.333	0.111	0.2667
	PMA	0.370	0.137	0.3665	0.191	0.037	0.5317
Number of plaques	p-selectin	0.954	0.911	**0.0116***	0.665	0.442	**0.0255***
	PSGL	0.775	0.600	0.0704	0.921	0.849	**0.0002*****
	CD40 Ligand	0.333	0.111	0.4656	0.565	0.319	0.0887
	PLA	0.808	0.653	**0.0278***	−0.026	0.001	0.9434
	PMA	0.905	0.819	**0.0347***	−0.388	0.150	0.1906

*Pearson's correlation coefficients, R squared, and associated p-values are shown. The bold values are representing the significant circulating blood factors in each ultrasonic character group. *$p < 0.05$ vs. control group, **$p < 0.01$ vs. control group, ***$p < 0.001$ vs. control group.*

Researchers have suggested a relationship between atherosclerotic severity and circulating adhesion blood factors; atherosclerotic severity and cognitive decline in the above mentioned reports. With one step further linking between them, our findings provide insight into the use of blood factor analysis (using FACS) as well as ultrasonographic evaluation of vessel status in both clinical and research settings. Changes in platelet activation and monocyte distribution are observed in the early stages of atherosclerosis. Such changes are strongly associated with stroke onset, as demonstrated by various studies (King et al., 2009; Cardenas et al., 2012; Xiang et al., 2013). The monocyte receptor CD14 and leukocyte antigen CD45 are best known for their crucial role in immunity. In addition, CD14 and CD16 are well-known biomarkers for atherosclerotic disease progression (Folsom et al., 2009). Research has also suggested that (PSGL, CD162) is a pro-atherogenic marker of vascular disease progression (Folsom et al., 2009).

The present study shows that increased IMT was more frequently observed in patients with MCI, whereas increased numbers of carotid plaques were more frequently observed in patients with dementia. The patients with MCI in our study comprise 32% of all patients with atherosclerosis, and all patients of the MCI group in the present study had been diagnosed with carotid vascular stenosis or atherosclerosis (**Figure 1**). These findings suppose that vessel damage is followed by MCI. A lot of findings in previous studies suggest that greater degrees of carotid atherosclerosis are associated with the progression from MCI to dementia (Mioshi et al., 2006; Urbanova et al., 2014; Moon et al., 2015; Knopman et al., 2016; Dearborn et al., 2017). The very recent study reported that up to 50% of patients develop vascular stenosis, and that anterior cerebral artery (ACA) plaques are associated with dementia even after controlling for vascular risk factors (Dearborn et al., 2017). Other researches have suggested that atherosclerosis plays a role in cognitive impairment, particularly in older adults (Jellinger and Attems, 2007; White et al., 2016). Such research has further demonstrated a converging relationship between degenerative vascular dysfunction and cognitive dysfunction. In our study, most patients with MCI exhibit atherosclerotic vessel abnormalities, such as increased IMT and plaque numbers, increasing the risk for progression to dementia. Especially in aged people, it is reported to estimate that 15%–42% of people over the age of 65 years exhibit some form of MCI, and that approximately 5%–15% of patients with MCI go on to develop dementia (Winblad et al., 2004; Petersen et al., 2014). Recent evidence has revealed that vessel dysfunction contributes to AD as well as vascular dementia (Murray et al., 2011). In this previous study, the authors reported an IMT cutoff value of 0.805 for the prediction of MCI development (baseline: 0.825 mm; Murray et al., 2011). Diagnosis of dementia in such patients is required in order to ensure the appropriate therapeutic guidelines and treatment are utilized (Wu et al., 2017).

Our results indicate that intima thickness and plaque number are associated with higher levels of p-selectin, as the evidence that platelets are engaged in the formation of PLAs (Steinhubl et al., 2007). In the dementia group of the present study, which included individuals with dementia, plaque numbers corresponded strongly with levels of PSGL-positive platelets. Control of plaque numbers with appropriate therapy such as statin treatment may thus delay or prevent the progression of cognitive decline to dementia. Our findings also indicated that carotid atherosclerosis correlates with MCI as well as increased numbers of PSGL-expressing platelets. Analysis of blood factors using ultrasonography may aid clinicians in determining the most appropriate treatment strategy for patients with cognitive decline with vessel disease.

The present study has some limitations that need consideration. First, longer follow-up is required to verify our findings. We are currently preparing a 3-year follow-up study including the participants of the present study and additional volunteers. Second, as the sample size was rather small, future studies should enroll a larger population. Future FACS studies should also aim to determine a cutoff value for each blood cell population for the prediction of progression from cognitive decline to dementia in older adults with cardio/cerebrovascular disease. Our simple assessment of vascular risk factors does not seem to be a fully satisfactory approach for adequately counteracting the risk of developing dementia, when compared to other large-scale studies (Coley et al., 2008; Luzzi et al., 2010). Nevertheless, we suggest that analysis of circulating adhesion factors may aid in predicting the risk of progressive cognitive impairment. Additionally, aggressive treatments for vascular disease should be considered for individuals with a predisposition toward dementia. Despite these limitations, our findings provide a basis for further study regarding biomarkers of both cerebrovascular disease and cognitive dysfunction.

In conclusion, our findings demonstrate that circulating adhesion molecules level and interaction between factors present significant differences in patient with MCI or dementia. Alterations in IMT and plaque number are associated with an increased risk of cognitive decline as well as conversion from MCI to dementia. These results suggest that ultrasound findings may aid in identifying older individuals at increased risk for the progression of cognitive decline in when cerebrovascular damaged. Moreover, our findings suggest that the presence of atherosclerotic changes and changes in blood factors such as p-selectin, PSGL, PLA and PMA can be useful candidates to monitor the severity of cognitive decline.

AUTHOR CONTRIBUTIONS

KC composed the manuscript and performed most of laboratory works and data. JK collected blood sample from outpatients and clinical records; and GWK supervised the whole study.

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Corrigendum: Changes in Blood Factors and Ultrasound Findings in Mild Cognitive Impairment and Dementia

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In the published article, the first affiliation was incorrect. Instead of "(Department of Life Science, Kyonggi University, Suwon, South Korea)", it should be "(Department of Neurology, College of Medicine, Yonsei University, Seoul, South Korea)". The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way.

The incorrect affiliation has been removed and the article has been updated.

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Low Serum Phosphorus Correlates with Cerebral Aβ Deposition in Cognitively Impaired Subjects: Results from the KBASE Study

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Alzheimer's disease (AD), characterized by progressive cognitive decline, is the most prevalent neurodegenerative disease in the elderly. Cerebral β-amyloid (Aβ) deposition is the major pathological hallmark of AD. Recent studies also have shown that the serum level of phosphorus correlates to the risk of incident dementia. To date, the linkage between cerebral Aβ deposition and the serum phosphorus level remains unknown. In this study, we analyzed the levels of serum phosphorus in 109 mild cognitive impairment (MCI) and 73 AD dementia (ADD) subjects. All subjects underwent Pittsburgh compound B positron emission tomography (PiB-PET) imaging to measure cerebral Aβ deposition. The results with Aβ deposition was compared with the serum levels of phosphorus. The subjects with cerebral Aβ deposition showed lower levels of serum phosphorus than those without Aβ deposition. Furthermore, multiple regression analyses showed that a low level of serum phosphorus correlated with cerebral Aβ deposition, even when age, sex, apolipoprotein E ε4 genotype, and MMSE z-score were controlled for. Serum levels of other ions, including calcium, iron, zinc, and copper, showed no such correlation. In conclusion, our results suggest that the serum level of phosphorus may be used as an easily accessible blood biomarker for cerebral Aβ deposition in a cognitively impaired population.

Keywords: phosphorus, Alzheimer's disease, mild cognitive impairment, β-amyloid, PiB-PET, KBASE, blood-based biomarker

INTRODUCTION

Alzheimer's disease (AD) is one of the most disastrous neurodegenerative diseases and the most prevalent cause of dementia in elder population. Since methods for early diagnosis and for treatment of AD are insufficient, diverse problems emerge as the aged population increases in modern societies. Cerebral β-amyloid (Aβ) plaques and neurofibrillary tangles (NFT) are characteristics of AD (Hardy and Higgins, 1992; Scheuner et al., 1996; Wisniewski et al., 1997; Murphy and LeVine, 2010; Bloom, 2014). Particularly, the abnormal production and

impaired clearance of Aβ contribute to Aβ accumulation, which is aggregated into plaques (Mawuenyega et al., 2010; Querfurth and LaFerla, 2010). The cerebral accumulation of Aβ is closely associated with cellular toxicity, synaptic dysfunction, aberrant neuronal activity, and destabilization of neural networks (Palop and Mucke, 2010). Even though Aβ pathology may not necessarily correlate with cognitive decline in a linear manner, accumulating evidence indicates that cerebral Aβ plays a critical role in AD pathogenesis and that neuritic plaques are specific characteristics of AD (Hardy and Higgins, 1992; Wisniewski et al., 1997; Nelson et al., 2009). Consequently, detection of Aβ plaques in brains is important not only for early diagnosis of AD but also for the differential diagnosis of AD from other causes of cognitive impairment. As such, it is crucial to determine whether cognitively impaired subjects, including mild cognitive impairment (MCI) or dementia patients, have cerebral Aβ deposition in order to diagnose the underlying AD accurately. Although Aβ deposition can be measured by positron emission tomography (PET) using radioactive ligands, like Pittsburgh compound B (PiB), it is very expensive and not commonly accessible (Kang et al., 2016; Park et al., 2017). Furthermore, although several cerebrospinal fluid (CSF) biomarkers, including CSF Aβ, show correlations with cerebral Aβ, sampling human CSF is an invasive procedure, and the inter-institutional reliability of CSF AD biomarker measurement is suboptimal. Therefore, discovery of easily accessible biomarkers that reflect the degree of cerebral Aβ deposition would be very beneficial.

Since some ions, including phosphorus, magnesium, and calcium, are highly involved in the pathogenesis of age-related neurological abnormality, many researchers have been interested in the relationship between these ions and neurodegeneration (Glick, 1990; Durlach et al., 1997; Andrási et al., 2005; Canzoniero and Snider, 2005; Mattson, 2007). Compared with control brains, various regions of AD brains were shown to contain increased levels of aluminum but decreased levels of magnesium as well as phosphorus (Glick, 1990; Andrási et al., 2005). In addition, AD patients had relatively lower levels of plasma magnesium, copper, zinc, iron, and selenium (Vural et al., 2010). Among these substances, phosphorus is abundant in human brains (Andrasi et al., 1995) and is an integral element essential for controlling numerous physiologic processes of the human body. Studies have shown that various processes such as energy storage, bone and muscle production, hormonal balance, and brain metabolism require appropriate level of phosphorous (Fiske and Subbarow, 1929; Bourgoignie, 1982; Shi et al., 2003; Takeda et al., 2012; Li et al., 2017). In addition, AD pathogenesis involves phosphorus in many key processes such as hyperphosphorylation of tau and accumulation of Aβ (Yumoto et al., 2009; Bloom, 2014). A recent study also showed that phosphorus dendrimer interacts with amyloid peptide and microtubule associated protein (MAP) tau in such a way that it affects the aggregation process of Aβ and tau (Wasiak et al., 2012). Furthermore, the level of phosphorus was significantly reduced in various regions of AD brains, including cortex entorhinalis and cortex frontalis basalis (Andrási et al., 2005). However, the relationship between peripheral level of phosphorus and AD pathogenesis remains unclear.

In this study, we investigated whether serum level of phosphorus is associated with cerebral deposition of Aβ in cognitively impaired subjects. We measured cerebral Aβ deposition in 182 cognitively impaired subjects using PiB-PET and compared the results with their serum levels of phosphorus. We show that the deposition of cerebral Aβ correlates with the decreased serum levels of phosphorus.

MATERIALS AND METHODS

This study was part of the Korean Brain Aging Study for the Early Diagnosis and Prediction of Alzheimer's Disease (KBASE), an ongoing prospective cohort study which started its recruitment in 2014 and aimed to find biomarkers for AD as well as to determine risk factors for AD-related functional and structural brain changes. This work was approved by the Institutional Review Board (IRB) of the Seoul National University Hospital and SMG-SNU Boramae Medical Center, South Korea. All subjects or their legal representatives provided their written informed consent after being fully informed about the study.

Participants

As of August 2016, a total of 182 subjects who were 55 years or older participated in the study. Among the subjects, 109 had mild cognitive impairment (MCI), and 73 had clinically diagnosed AD dementia (ADD). They underwent comprehensive KBASE baseline assessment including clinical examination, neuropsychological assessments, neuroimaging, including magnetic resonance imaging (MRI), PiB-PET, and blood tests. Individuals with MCI (age 55–90 [inclusive]) met the following criteria: (a) memory complaint reported by themselves or reported by an informant or a clinician; (b) presence of objective memory impairment; (c) intact functional activities; and (d) non-demented. All MCI subjects had a global CDR score of 0.5. With respect to criterion (b), MCI individuals scored at least 1.0 standard deviation (SD) below the respective age, education, and gender-specific mean on at least one of the four memory tests that were part of the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) neuropsychological battery (namely, Word List Memory, Word List Recall, Word List Recognition, and Constructional Recall test) (Lee et al., 2004). Inclusion criteria for patients with ADD (age 55–90 [inclusive]) were: (a) meet both criteria of the Diagnostic and Statistical Manual, 4th Edition for dementia (Americal Psychiatric Association, 2000) and the criteria for probable ADD set according to the National Institute of Aging and Alzheimer's Association (NIA-AA) guidelines (McKhann et al., 2011), and (b) have a global CDR score of 0.5 or 1. The exclusion criteria were: (a) presence of any psychiatric or neurological disorders that could affect mental function, (b) severe communication problems that would create difficulty in a assessment or brain scan, (c) contraindications for MRI scanning, (d) absence of a reliable informant, or (e) illiteracy.

Clinical and Neuropsychological Assessment

All participants underwent standardized clinical assessment by trained psychiatrists, based on the KBASE protocol, which corresponded with the Korean version of the Consortium to Establish a Registry for Alzheimer's Disease Assessment Packet (CERAD-K) (Lee et al., 2002). In addition, participants underwent KBASE neuropsychological assessment by trained neuropsychologists while incorporating the CERAD neuropsychological battery (Lee et al., 2004), which contained the Mini-Mental State Examination (MMSE). Due to the influence of gender, age, and education on MMSE score (Piccinin et al., 2013), we used the z-score of MMSE instead (Lee et al., 2004). Body mass index (BMI) was calculated for all participants from their heights and weights. Through a weekly clinical review panel meeting (chaired by DL) with a clinical review panel composed of several geriatric psychiatrists, clinical neuropsychologists, and psychometrists, individuals were allocated to either MCI group or ADD group. Ineligible individuals were excluded.

PiB-PET

Participants underwent three-dimensional PiB-PET imaging and T1-weighted MR using a Biograph mMR scanner (Siemens, Washington DC, USA). Forty minutes after intravenous injection of 555 MBq of ^{11}C-PiB, a 30-min emission scan was obtained. The data were reconstructed into a 256 × 256 image matrix using iterative methods (6 iterations with 21 subsets) and were corrected for uniformity, ultrashort echo time (UTE)-based attenuation, and decay reduction. Sagittal T1-weighted images (repetition time = 1,670 ms; echo time = 1.89 ms; field of view, 250 mm; 256 × 256 matrix with 1.0 mm slice thickness) were acquired. Images were pre-processed using Statistical Parametric Mapping 8 (SPM8) implemented in Smith and Barton (2014) (MathWorks, Natick, MA). PiB-PET data were co-registered to the individual T1 images and transformation parameters to a standard Montreal Neurological Institute (MNI) template were computed. Individual Brain Atlases using Statistical Parametric Mapping Software (IBASPM) software was used for the inverse transformation parameters, which transformed the coordinates from the automatic anatomic labeling (AAL) 116 atlas (Tzourio-Mazoyer et al., 2002) into an individual space for each subject (resampling voxel size = 1 × 0.98 × 0.98 mm). Only the gray matter (GM) was used for extraction by applying GM mask to each individual in order to exclude white matter and CSF space.

Based on previous studies (Jack et al., 2008; Reiman et al., 2009; Choe et al., 2014; Park et al., 2017), the cerebral regional mean ^{11}C-PiB uptake values were calculated using the individual AAL116 atlas from the T1-coregistered PiB-PET images and the cerebellar gray matter ^{11}C-PiB uptake values were used for the quantitative normalization. To determine the regions of interest (ROIs), the AAL algorithm and a region-combining method (Reiman et al., 2009) were applied and brain regions were divided into the frontal, lateral parietal, posterior cingulate-precuneus (PC-PRC), and lateral temporal regions, where prominent ^{11}C-PiB retention has been reported (Klunk et al., 2004). The standardized uptake value ratio (SUVR) of each ROI was obtained by dividing the mean value for all voxels within the ROI by the mean cerebellar gray matter uptake value. Subjects were classified as PiB positive (PiB+) if the SUVR value exceeded 1.4 in at least one of the four ROIs (i.e., frontal, lateral temporal, lateral parietal, and PC-PRC) or as PiB negative (PiB-) if the SUVR values of all four ROIs were equal to or less than 1.4 (Reiman et al., 2009; Choe et al., 2014). Being classified as PiB− would mean that subjects have negative amyloid deposition, while being classified as PiB+ would mean that subjects have positive amyloid deposition.

Blood Sampling and Quantification of Serum Phosphorus, Calcium, Iron, Zinc, and Copper

Blood samples were taken via venipuncture in the morning (around 9 am) after overnight fasting and were collected into serum separator tubes (Becton, Dickinson and Co., Franklin Lakes, NJ, USA). The tubes were stored at room temperature (RT) for 30 min and then were centrifuged at 1,300 g for 10 min at RT. Separated serum was used for assays. Serum phosphorus, calcium, and iron levels were measured at Seoul Clinical Laboratories, using an enzymatic colorimetric method (ADVIA 1800 Autoanalyzer; Siemens, Washington DC, USA). The levels of zinc and copper were determined by inductively coupled plasma-mass spectroscopy (ICP-MS) using a Varian 820-MS ICP mass spectrometer (Bruker, Victoria, Australia). Additionally, apolipoprotein E (APOE) genotyping was done by using the genomic DNA, as previously described (Wenham et al., 1991).

Statistics

To investigate the relationship between serum phosphorus and global cerebral Aβ deposition, partial correlation analysis was used, with correction for the influence by the different covariates (CV; age and sex). Variables were logarithmically transformed to adjust skewness of the distribution and to normalize variance. To compare the serum phosphorus level between PiB− and PiB+ with statistical control for the effects of CV (age and sex), analysis of covariance (ANCOVA) was performed, and *p*-values were obtained by pairwise comparison. For the independent association between serum phosphorus level and global cerebral Aβ deposition, multiple regression analyses were conducted after controlling for age, sex, ApoE ε4 carrier status, calcium, MMSE z-score, and others. To identify the relationship between serum phosphorus level and risk of accumulation of global cerebral Aβ, relative risk (95% confidence interval) tests were conducted. In addition, we carried out logistic regression analysis followed by receiver operating characteristic (ROC) curve analysis to show the discrimination power of ApoE ε4 carrier status and phosphorus on PiB positivity. The predicted probabilities were used to calculate the discrimination power for the prediction of PiB positivity. Comparison of ROC curves was performed according to a previous report, DeLong et al. (1988). For the demographic data of subjects (**Table 1**), independent *t*-tests were performed to compare values (age, education, MMSE z-score, and global amyloid deposition). Chi-squared tests were carried

out to evaluate the intergroup differences of categorical variables (sex, CDR score, and ApoE ε4 carrier status). All statistical analyses were performed using Medcalc 17.2 (Medcalc Software, Ostend, Belgium). P values were stated in each figure or table to indicate statistical significance as appropriate.

RESULTS

Demographic Data of Subjects

A total of 182 subjects (109 MCI subjects and 73 ADD subjects) were included in this study. Table 1 shows the demographic and clinical characteristics of the participants (**Table 1**). Amongst the MCI subjects, 50.5% were PiB+ ($n = 55$), whereas 78.1% of ADD patients were PiB+ ($n = 57$). No differences were observed in age ($p = 0.256$) and sex ($p = 0.830$) between MCI and ADD groups (**Table 1**).

Relationship between the Serum Level of Phosphorus and the Global Deposition of Cerebral Amyloid

The partial correlation analyses revealed a significant correlation between the serum level of phosphorus and the deposition of cerebral amyloid in both MCI ($r = -0.23$, $df = 105$, $p = 0.018$) and ADD ($r = -0.26$, $df = 69$, $p = 0.031$) groups (**Figure 1A**). A similar correlation was observed when the two groups were combined ($r = -0.24$, $df = 178$, $p = 0.001$; **Figure 1B**, left). In the combined (MC1 plus ADD) group, PiB+ subjects had a lower serum level of phosphorus as compared with PiB− subjects ($p = 0.009$; **Figure 1B**, right). To identify the relationship between the level of phosphorus and the deposition risk of cerebral amyloid, we conducted a relative risk (RR) analysis. We categorized subgroups into quartiles (quartile 1, ≤3.4 mg/dL; quartile 2, >3.4 and ≤3.7 mg/dL; quartile 3, >3.7 and ≤4.0 mg/dL; quartile 4, >4.0 mg/dL), as described in previous reports (Dhingra et al., 2007, 2010; Ye et al., 2016). The subjects with low levels of phosphorus (quartile 1, ≤3.4 mg/dL) showed higher risk of PiB positivity (RR = 1.50, $p = 0.028$; 95% CI, 1.04–2.14; **Table 2**). Chi-squared test for trend also showed that the proportion of PiB+:PiB− significantly decreased, as the quartile number increased (first quartile to fourth quartile) ($p = 0.020$, $\chi^2 = 5.447$). Furthermore, the multiple regression analyses were conducted in three models (set 1, MCI only; set 2, ADD only; set 3, both MCI and ADD; **Table 3**). Age, sex, and ApoE ε4 carrier status were included as covariates. Serum phosphorus showed a significant negative correlation with cerebral Aβ deposition in all three models (set 1, $\beta = -0.193$ and $p = 0.037$; set 2, $\beta = -0.260$ and $p = 0.034$; set 3, $\beta = -0.226$ and $p = 0.003$; **Table 3**). In addition, serum phosphorus level was correlated with cerebral Aβ deposition even after controlling for serum calcium levels and/or MMSE score with age, sex, and ApoE ε4 types (age, sex, calcium, and ApoE as covariates, $\beta = -0.066$ and $p < 0.001$; age, sex, MMSE score and ApoE as covariates, $\beta = -0.066$ and $p < 0.001$; age, sex, calcium, MMSE score and ApoE as covariates, $\beta = -0.067$ and $p < 0.001$; **Table 4**).

TABLE 1 | Demographic and clinical characteristics of participants.

	MCI ($n = 109$)	ADD ($n = 73$)	*P*-value
Gender, M/F	36/73	23/50	0.8304[a]
Age, years	73.98 ± 0.6	72.73 ± 0.9	0.2559[b]
Education, years	9.70 ± 0.4	9.37 ± 0.6	0.6628[b]
MMSE z-score	−1.22 ± 0.1	−3.13 ± 0.2	<0.0001[b*]
Global CDR, *N* (%)			<0.0001[a*]
0.5	109 (100%)	24 (32%)	
1	0 (0%)	49 (68%)	
ApoE ε4 carrier, *N* (%)	35 (32%)	41 (56%)	0.0013[a*]
Global Aβ deposition (SUVR)	1.53 ± 0.1	1.87 ± 0.1	<0.0001[b*]
PiB positivity, %	50.5%	78.1%	0.0002[a*]

Data were presented as Mean ± SEM or N (%).
MCI, mild cognitive impairment; ADD, Alzheimer's disease dementia; PiB, Pittsburgh compound B; ±, PiB positivity; SEM, standard error of mean; n, number of subjects; MMSE, mini-mental state examination; MMSE z score, a revised value of the MMSE score with consideration for age, gender, and education level; CDR, clinical dementia rating; ApoE, Apolipoprotein E; SUVR, standardized uptake value ratio.
*[a]P, significance by chi-squared test; [b]P, significance by independent t-test; *p < 0.05.*

Serum Phosphorus Increases Discrimination Power between PiB− vs. PiB+

We performed logistic regression and ROC curve analysis, using independent variables (ApoE ε4 carrier status and serum phosphorus, **Figure 2**). Each of the ROC curves had a significant *p*-value ($p < 0.0001$, **Figure 2A**). AUC value was significantly enhanced by combining the two variables (AUC, 0.757–0.800; **Figure 2A**, gray and black line; **Figure 2B**, $p = 0.0343$, comparison of ROC curves analysis). These results suggest that the serum level of phosphorus may be used as a blood-based biomarker for PiB positivity or AD in conjunction with ApoE ε4 genotype for cognitively impaired population.

Other Serum Ion Levels Have No Influence on Either Serum Phosphorus or Cerebral Amyloid Deposition

Partial correlation plots showed that the serum levels of other ions such as calcium, iron, zinc, and copper levels were not significantly correlated with either cerebral Aβ deposition (SUVR) (**Figures 3B–E**, left graphs; $p > 0.05$) or serum phosphorus levels (**Figures 3B–E**, right graphs; $p > 0.05$) after controlling for covariates, such as age and sex. Body mass index (BMI) showed no significant association with cerebral Aβ deposition either in cognitively impaired subjects (**Figure 3A**). In addition, multiple regression analyses showed that serum phosphorus was significantly associated with cerebral Aβ deposition even after controlling for covariates, including other ions and BMI (age, sex, BMI, calcium, iron, zinc, and copper as covariates, $\beta = -0.072$ and $p < 0.01$; **Table 5**). These results indicate that blood phosphorus level is associated with cerebral Aβ deposition, while other ion levels, including calcium, iron, zinc, and copper, are not correlated with brain Aβ deposition in cognitively impaired subjects.

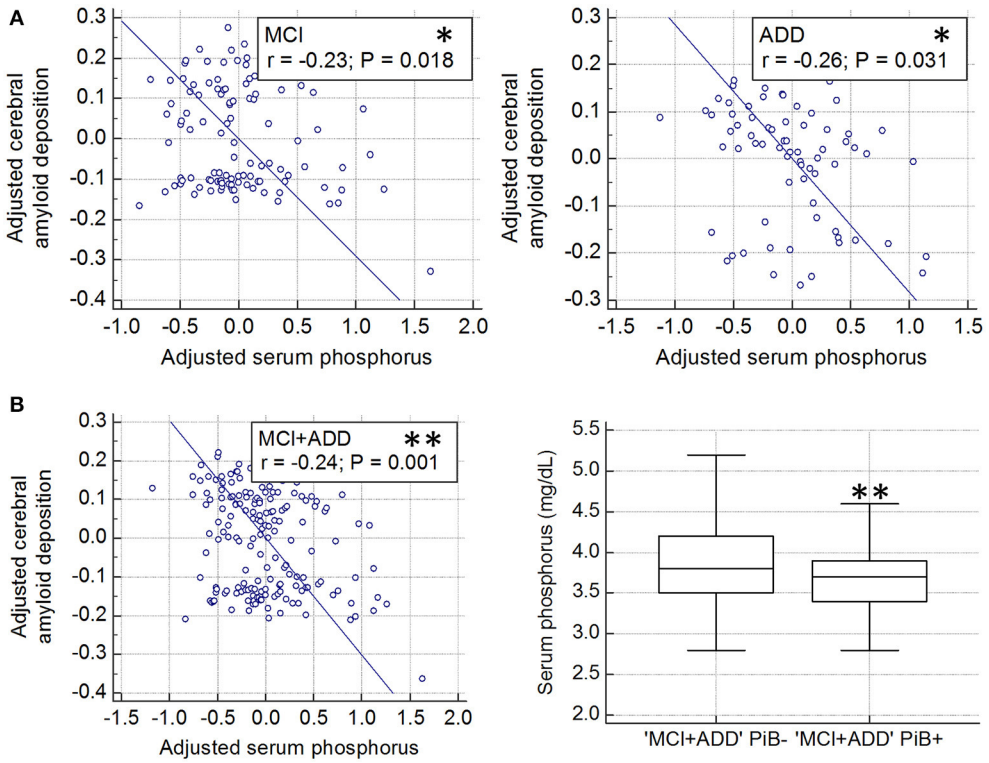

FIGURE 1 | Association cerebral amyloid deposition with serum phosphorus levels in cognitively impaired subjects **(A)** Partial correlation plot shows the relationship between global cerebral amyloid deposition (SUVR) and serum phosphorus after controlling for age and sex, in both MCI and ADD (MCI, $*p < 0.05$; ADD, $*p < 0.05$). Global cerebral amyloid burden was natural log transformed to normalize variance. **(B)** Partial correlation plot in whole cognitively impaired subjects (MCI plus ADD, $**p < 0.01$, left graph). Global cerebral amyloid burden was natural log transformed. Comparison of serum phosphorus levels between PiB+ and PiB− in whole cognitively impaired subjects (MCI plus ADD, $**p < 0.01$; *P*–values were obtained by ANCOVA comparing adjusted mean after controlling for the effect of age and sex, right graph). PiB, Pittsburgh Compound B; MCI, mild cognitive impairment; ADD, Alzheimer's disease dementia; ±, PiB positivity; SUVR, standardized uptake value ratio.

TABLE 2 | Relationship between serum phosphorus and risk of cerebral amyloid deposition in cognitively impaired subjects.

Serum phosphorus level	PiB status		RR (95% CI)	$P^{\ddagger}$	*P*
	PiB−, *n* (%)	PiB+, *n* (%)			
Quartile 1 (≤3.4 mg/dL)	16 (30.2)	37 (69.8)	1.50 (1.04 ~ 2.14)	0.0279*	[a]0.1020
Quartile 2 (>3.4 and ≤3.7 mg/dL)	14 (33.3)	28 (66.7)	1.43 (0.98 ~ 2.09)	0.0648	[b]0.0196*
Quartile 3 (> 3.7 and ≤ 4.0 mg/dL)	16 (38.1)	26 (61.9)	1.33 (0.90 ~ 1.96)	0.1580	–
Quartile 4 (>4.0 mg/dL)	24 (53.3)	21 (46.7)	1	–	–

PiB, Pittsburgh compound B; RR, relative risk; CI, confidence interval.
$P^{\ddagger}$, P of RR; [a]P by chi-square test $\chi^2 = 6.207$; [b]P by chi-squared test for trend $\chi^2 = 5.447$; $*p < 0.05$.

DISCUSSION

Phosphorus is an essential constituent of organic materials, including nucleic acids, organic phosphates, vertebrate teeth, and bones (Bansal, 1990). It plays an essential role in activating enzymes and generating energy as a component of molecules such as adenosine triphosphate (ATP). Diverse pathological states are related to the perturbation in metabolic homeostasis of phosphorus. Examples include not only peripheral muscle weakness and tremor but also neuropathological states such as metabolic encephalopathy, delirium, and seizures (Weisinger and Bellorin-Font, 1998; Subramanian and Khardori, 2000). It is therefore not surprising that phosphorus can affect human brain as well as regulation of peripheral metabolism. For instance, the interaction of phosphorus dendrimer with proteins related to neurodegenerative disease, such as prion, Aβ, and tau, has been shown to influence their aggregation process during pathogenesis (Andrasi et al., 1995; Wasiak et al., 2012). Based on these observations, it has been proposed that phosphorus affects the pathogenesis of neurodegenerative diseases (Andrasi et al., 1995; Wasiak et al., 2012).

TABLE 3 | Multiple regression analyses of phosphorus and global cerebral Aβ deposition.

Covariates	β	SE	*t*	*P*-value	*F (df)*	R^2-*adj*
SET 1: MCI						
[a]Dependent variable: global cerebral Aβ deposition				< 0.001*	6.71 (4, 103)	0.176
Age	−0.001	0.001	−0.867	0.388		
Sex	−0.030	0.092	0.323	0.747		
Phosphorus	−0.193	0.092	−2.111	0.037*		
ApoE ε4 type	0.393	0.086	4.589	< 0.001*		
SET 2: ADD						
[a]Dependent variable: global cerebral Aβ deposition				< 0.001*	5.52 (4, 68)	0.201
Age	−0.009	0.001	−1.392	0.168		
Sex	0.199	0.129	1.541	0.128		
Phosphorus	−0.260	0.120	−2.166	0.034*		
ApoE ε4 type	0.417	0.109	3.842	< 0.001*		
SET 3: MCI + ADD						
[a]Dependent variable: global cerebral Aβ deposition				< 0.001*	15.2 (4, 176)	0.240
Age	−0.008	0.005	−1.812	0.072		
Sex	0.100	0.077	1.298	0.196		
Phosphorus	−0.226	0.074	−3.030	0.003*		
ApoE ε4 type	0.457	0.067	6.813	< 0.001*		

[a]*Natural log-transformed to normalize variance.*
*Statistical significance, *p < 0.05.*
Aβ, beta-amyloid; MCI, mild cognitive impairment; ADD, Alzheimer's disease dementia; SE, standard error; β, regression coefficient; ApoE, Apolipoprotein E.

TABLE 4 | Multiple regression analyses of phosphorus, calcium, MMSE score, and ApoE ε4 genotype with global cerebral Aβ deposition in cognitively impaired subjects.

Covariates	β	SE	*t*	*P*-value	*F (df)*	R^2-adj
[a]Dependent variable: global cerebral Aβ deposition				< 0.001*	14.0 (5, 175)	0.266
Age	−0.002	0.001	−1.848	0.066		
Sex	0.023	0.020	1.158	0.249		
Phosphorus	−0.066	0.019	−3.382	< 0.001*		
Calcium	0.028	0.024	1.173	0.242		
ApoE ε4 type	0.127	0.018	7.226	< 0.001*		
[a]Dependent variable: global cerebral Aβ deposition				< 0.001*	17.3 (5, 175)	0.312
Age	−0.003	0.001	−2.197	0.030*		
Sex	0.014	0.020	0.699	0.485		
Phosphorus	−0.066	0.019	−3.479	< 0.001*		
MMSE score	−0.007	0.002	−3.624	< 0.001*		
ApoE ε4 type	0.115	0.017	6.695	< 0.001*		
[a]Dependent variable: global cerebral Aβ deposition				< 0.001*	14.7 (6, 174)	0.313
Age	−0.003	0.001	−2.225	0.027*		
Sex	0.014	0.020	0.738	0.461		
Phosphorus	−0.067	0.019	−3.540	< 0.001*		
Calcium	0.027	0.023	1.181	0.239		
MMSE score	−0.007	0.002	−3.617	< 0.001*		
ApoE ε4 type	0.115	0.017	6.710	< 0.001*		

[a]*Natural log-transformed to normalize variance.*
*Statistical significance, *p < 0.05.*
Aβ, beta-amyloid; MCI, mild cognitive impairment; ADD, Alzheimer's disease dementia; SE, standard error; β, regression coefficient; ApoE, Apolipoprotein E.

The levels of phosphorus significantly decrease in both the brains (Andrási et al., 2005) and CSFs of AD patients (Subhash et al., 1991) as compared with controls. Thus, it is likely that the down-regulation of phosphorus in the brain is a consequence of the pathological processes rather than normal aging processes (Subhash et al., 1991). Intriguingly, recent studies proposed that

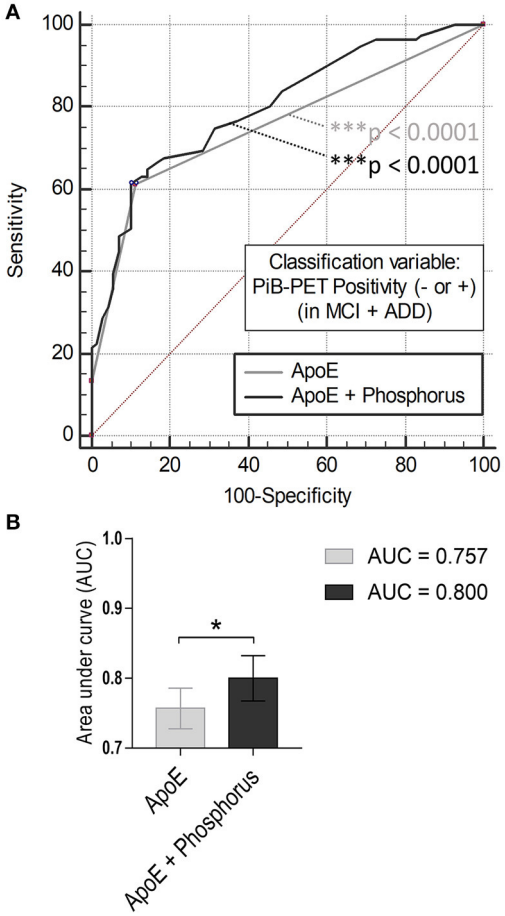

FIGURE 2 | Comparison of ROC curves analysis **(A)** ROC curve models using independent variables (ApoE ε4 and/or serum phosphorus). Gray line (AUC: 0.757, ***$p < 0.0001$), ApoE alone; black line (AUC: 0.800, ***$p < 0.0001$). **(B)** Comparison of ROC curves analysis between the models (*$p < 0.05$, ApoE alone vs. ApoE with phosphorus).

increased levels of serum phosphorus contributed to the risk of ADD (Basheer et al., 2016; Li et al., 2017). In contradiction with these studies, however, we found that the subjects with serum phosphorus below 3.4 mg/dL (normal range 3.4–4.5 mg/dL) had a high risk of cerebral Aβ accumulation (**Table 2**). One explanation for this contradiction would be that the previous results were obtained from people at or under the age of 60. In these studies, no significant difference was observed among people over 60. In our study, the mean age of each cohort was over 70, suggesting that the difference may be attributed to the subjects' ages (**Table 1**). Indeed, Li et al. (2017) proposed that the role of serum phosphorus could be different in people with other dementias under 60 as compared with age-related dementia such as late-onset AD. Consistent with our speculations, when the data presented in the previous study (Li et al., 2017) are narrowed down to people at or over 60, the serum levels of phosphorus are reduced in incident dementia.

Amyloid aggregation and AD pathogenesis can be influenced by BMI as well as various ions such as calcium, iron, zinc, and copper are known to be relevant to amyloid aggregation (Miller et al., 2010; Hane et al., 2013; Han et al., 2016). The BMI value declines at the preclinical stage but not after the onset of AD (Gu et al., 2014). In this study, we found no significant correlation of cerebral Aβ deposition with serum levels of calcium, iron, and zinc (**Figures 3B–E**, left) or with BMIs (**Figure 3A**). The correlation between serum phosphorus level and cerebral amyloid deposition remained significant even after controlling for the effect of other ions and BMI in the multiple regression model (**Table 5**). Further studies are needed with larger sample sizes to examine the effects of more diverse elements and indices involved in phosphorus metabolism and nutritional states such as the diet pattern.

Cerebral Aβ deposition in AD is tightly associated with tauopathy characterized by hyperphosphorylation of tau in postmortem brains of AD (Morishima-Kawashima and Ihara, 2002; Bloom, 2014). Aβ induces tau phosphorylation via MAPK and GSK-3β, triggering microtubules destabilization, axonal transport impairment, and neuronal death (Busciglio et al., 1995; Greenberg and Kosik, 1995; Le et al., 1997; Takashima et al., 1998). Tau phosphorylation also contributes to Aβ-induced neurodegeneration, leading to the loss of septal cholinergic neurons (Zheng et al., 2002). Hyperphosphorylated tau needs tremendous amount of phosphorus (Iqbal et al., 2010), significantly depleting phosphorus from brains and blood (Butner and Kirschner, 1991; Khatoon et al., 1992). The disruption of the brain blood barrier may also contribute to decreases of phosphorus in both brains and blood during AD pathogenesis (Bell and Zlokovic, 2009; Zlokovic, 2011). However, knowledge of tau deposition and CSF tau concentrations would be needed to draw solid conclusions.

In this study, we used a natural log-transformed value of the global cerebral Aβ deposition (SUVR) as the dependent variable of multiple linear regression analyses to resolve non-normal distribution of global Aβ deposition value. However, the patterns of partial regression plots derived from multiple linear regression analyses seemed to roughly show bimodal distribution, even when the dependent variable, global Aβ deposition value, was natural log-transformed. This phenomenon might be related to characteristic bimodal distribution of cerebral Aβ deposition as reported in previous studies (Villain et al., 2012; Chételat et al., 2013; Villeneuve et al., 2015). However, given that our results from multiple logistic regression analyses were consistent with those from multiple linear regression analyses, our finding still supports the inverse relationship between serum phosphorus level and cerebral Aβ deposition. Further experiments are needed to elucidate the underlying mechanism. Following standard practice in PET image analysis, we used AAL to determine ROIs. Single-subject approaches such as AAL can be biased due to the idiosyncrasies of the atlas individual, therefore multi-subject approaches might be preferable (Rohlfing et al., 2004; Svarer et al., 2005; Heckemann et al., 2006). However, our analysis relied on four relatively coarse ROIs, therefore the benefit of using a more accurate segmentation method would have been small.

In conclusion, the combination of phosphorus with other biomarkers may help detect cerebral Aβ deposition more

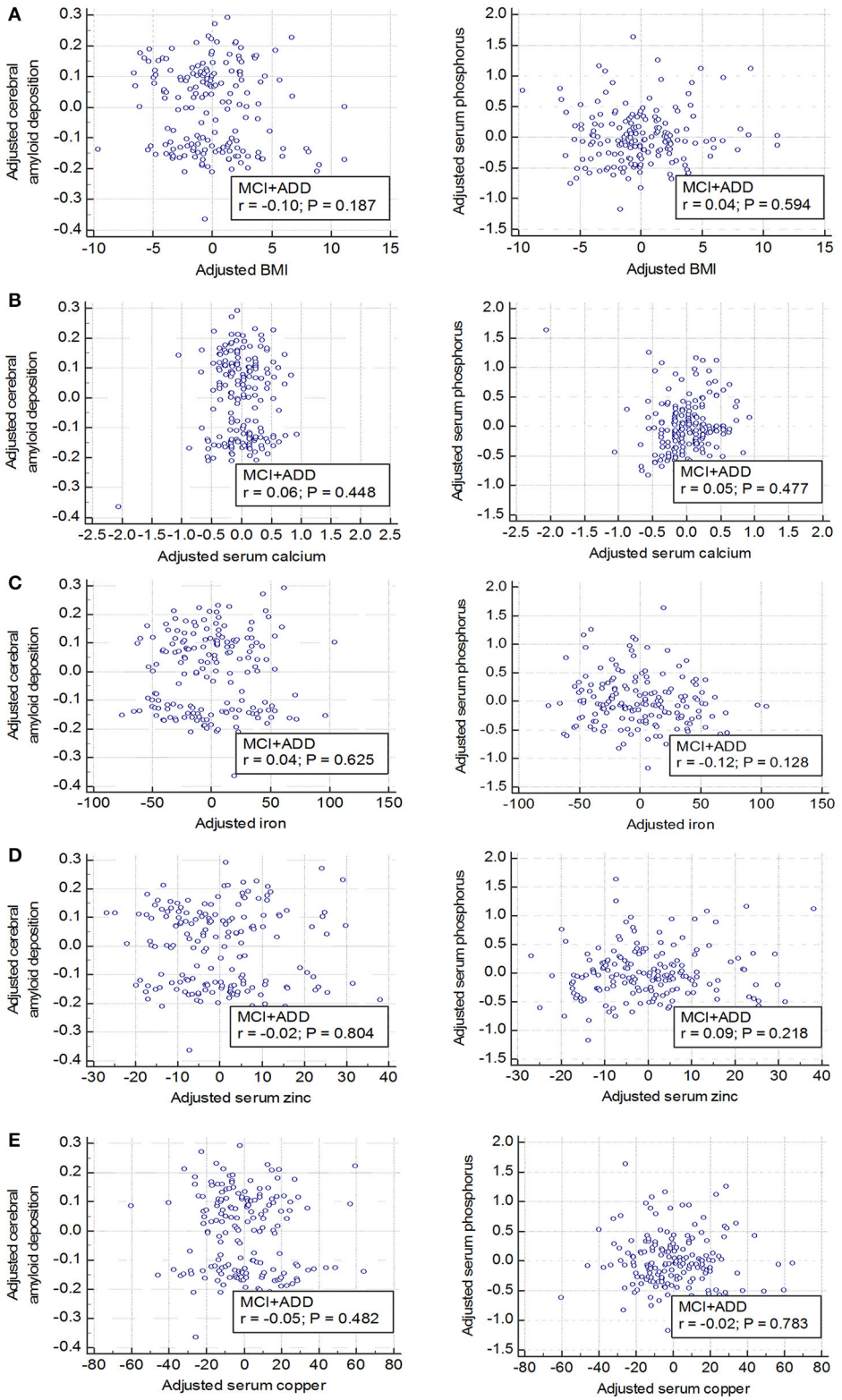

FIGURE 3 | No relationship between elements and body mass index (BMI) and brain Aβ deposition or serum phosphorus **(A–E)** Elements (calcium, iron, zinc, and copper) and an indicator (BMI) which can reflect the health conditions are not associated with both cerebral amyloid deposition (left graphs) and serum phosphorus levels (right graphs). Age and sex were adjusted and global cerebral amyloid burden (SUVR) was natural log transformed to normalize variance. MCI, mild cognitive impairment; ADD, Alzheimer's disease dementia; SUVR, standardized uptake value ratio.

TABLE 5 | Multiple regression analyses of phosphorus, elements, and body mass index (BMI) with global cerebral Aβ deposition in cognitively impaired subjects.

Covariates	β	SE	*t*	*P*-value	*F (df)*	R^2-adj
[a]Dependent variable: global cerebral Aβ deposition				< 0.001*	14.0 (8, 156)	0.037
Age	−0.002	0.001	−1.611	0.109		
Sex	0.033	0.026	1.283	0.202		
Phosphorus	−0.072	0.024	−3.020	0.003*		
BMI	−0.004	0.003	−1.158	0.249		
Calcium	0.034	0.028	1.199	0.233		
Iron	0.001	0.001	0.258	0.797		
Zinc	0.001	0.001	0.105	0.917		
Copper	0.001	0.001	−0.736	0.463		

[a]*Natural log-transformed to normalize variance.*

*Statistical significance, *p < 0.05.*

Aβ, beta-amyloid; SE, standard error; β, regression coefficient; BMI, body mass index.

efficiently in cognitively impaired individuals with MCI and ADD. Our result suggests that the serum level of phosphorus may be used as an easily accessible blood biomarker for cerebral Aβ deposition in a cognitively impaired population.

ETHICS STATEMENTS

The protocol was approved by the Institutional Review Board (IRB) of the Seoul National University Hospital and SMG-SNU Boramae Medical Center, Seoul, South Korea, and was carried out in accordance with the recommendations of the current version of the Declaration of Helsinki. All subjects or their legal representatives gave written informed consent in accordance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

Study concept and design by J-CP, S-HH, DL, and IM-J. Acquisition of data by J-CP, S-HH, MB, DY, and JL. Analysis and interpretation of data by J-CP, S-HH, MB, DY, JL, DL, and IM-J. Critical revision of manuscript for intellectual content by KP, MB, DL, and IM-J. DL and IM-J supervised and coordinated all of the data in the study. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnagi.2017.00362/full#supplementary-material

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Multiple Evidences for Association between Cognitive Impairment and Dysglycemia in Parkinson's Disease: Implications for Clinical Practice

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Background and purpose: It remains unclear about the etiopathogenesis of cognitive impairment (CI) in Parkinson's disease (PD). Since diabetes mellitus (DM) has been shown to be associated with CI in several diseases, we examined the association between CI and dysglycemia in PD.

Methods: Enrolled PD patients completed a series of clinical and neuropsychological assessments. Motor symptoms were determined by Hohen-Yahr staging (H-Y staging) and Unified Parkinson's Disease Rating Scale – motor score (UPDRS-III). Neuropsychological functions were evaluated by the Mini Mental State Examination (MMSE), the Montreal Cognitive Assessment (MoCA), and the Hamilton Anxiety and Depression Scales. Moreover, fasting glucose, fasting insulin, glycosylated hemoglobin A1c (HbA1c) and oral glucose tolerance test were performed to assess glucose metabolism.

Results: MoCA and MMSE scores in PD patients with DM group (PD-DM) were significantly lower than those in PD patients without DM group (PD-nDM). Consistently, PD-DM group showed significantly higher constituent ratio of CI than PD-nDM group. In addition, MoCA scores in HbA1c $\geq$ 6.5% group and HbA1c $\geq$ 7% group were significantly lower than those in the corresponding control groups. MoCA score in IR $\geq$ 3 group was significantly lower than that in IR $<$ 3 group. Furthermore, MoCA score was negatively correlated with H-Y staging, HbA1c and insulin resistance, respectively. Finally, regression analysis indicated that H-Y staging and HbA1c $\geq$ 7% were independent risk factors of CI in PD.

Conclusion: CI may be tightly associated with dysglycemia in, at least partially, PD patients. Importantly, H-Y staging and HbA1c $\geq$ 7%, two independent risk factors of CI in PD, may serve as key biomarkers in future PD clinical practice.

Keywords: cognitive impairment, dysglycemia, Parkinson's disease, glycosylated hemoglobin A1c, risk factors

INTRODUCTION

Parkinson's disease (PD) has been recognized as a multisystem neurodegenerative disorder with typical motor symptoms, including static tremor, bradykinesia, rigidity, postural instability, and gait difficulty. Although movement symptoms are the main clinical characteristics of PD, increasing evidence has shown that PD patients often experience a series of non-motor symptoms, including cognitive impairment (CI), dysfunction of autonomic nervous system, and behavioral disturbances.

Cognitive dysfunction is one of the most devastating and common non-motor symptoms of PD. There are approximately 20–40% of patients suffering from CI in the early stage of PD (Aarsland et al., 2010). The average incidence of mild CI has shown to be 26.7% (range, 18.9–38.2%) in PD (Litvan et al., 2011). More than 75–80% of the patients will eventually develop to dementia (Litvan et al., 2011). The cumulative incidence of dementia in PD approached to 80% in a community-based study (Hely et al., 2008). A prospective study suggested that individuals with PD had a three–suxfold higher risk of developing dementia than people without PD at the same age (Aarsland et al., 2001). PD severely impacts on the patients' life quality, imposing a huge burden on the patients, their family and society. CI makes this hard situation even worse in PD patients.

According to a nationally cross-sectional survey in China, the overall prevalence of diabetes mellitus (DM) was estimated to be 11.6% in the Chinese adult population (Xu et al., 2013). Diabetes is frequently accompanied with a variety of complications. Many studies have shown a relationship between diabetes and cognitive decline, and this correlation is particularly significant in patients over 60 years old (Xu et al., 2009). Several epidemiological studies have suggested that diabetes is associated with the development of PD. For example, diabetes has been shown to be one risk factor for the occurrence of PD (Cereda et al., 2011). Diabetes may aggravate movement symptoms of PD patients (Cereda et al., 2012; Kotagal et al., 2013).

However, little is known about the association between CI and dysglycemia in PD. Therefore, in the present study, we investigated the association between cognitive function and dysglycemia in PD with the following aims: to compare clinical characteristics of Parkinson's disease patients with (PD-DM) or without (PD-nDM) diabetes mellitus, to compare cognitive function at different levels of HbA1c and insulin resistance in Parkinson's disease patients, and finally to investigate risk factors of CI in Parkinson's disease.

MATERIALS AND METHODS

Participants

A total of 282 PD patients were recruited in the department of Neurology at Xinhua hospital affiliated to Shanghai Jiao Tong University from October 2013 to October 2016. All the participants were examined by a movement disorder neurologist

TABLE 1 | Clinical characteristics of PD-DM and PD-nDM patients.

	PD-nDM ($n = 197$)	PD-DM ($n = 85$)	*P*
Gender (male: number/percentage)	108/54.8%	44/51.8%	0.697[c]
Age (year)	69.10 ± 8.21	70.79 ± 7.63	0.121[a]
Duration (year)	5.02 ± 5.48	5.05 ± 5.22	0.430[b]
Onset age (year)	64.08 ± 9.46	65.74 ± 8.12	0.171[a]
Current smoking (number/percentage)	65/33.0%	23/27.1%	0.332[c]
Body mass index (BMI, kg/m^2)	22.56 ± 2.28	23.17 ± 1.57	**0.010[a]**
Degree of education (year)	11.62 ± 3.36	10.92 ± 4.29	0.064[b]
Form of onset			
Tremor (number/percentage)	114/57.9%	42/49.4%	0.195[c]
Dominant side involved (number/percentage)	103/52.3%	50/58.8%	0.362[c]
Drugs			
Levodopa intake (number/percentage)	177/89.8%	80/94.1%	0.268[c]
levodopa equivalent dosage (mg/day)	469.54 ± 289.15	540.15 ± 319.34	0.092[a]
levodopa dosage (mg/day)	409.14 ± 258.46	475.29 ± 295.97	0.128[a]
DRA (number/percentage)	76/38.6%	36/42.4%	0.597[c]
MAOBI (number/percentage)	38/19.3%	17/20.0%	0.890[c]
COMTI (number/percentage)	5/2.5%	5/5.9%	0.291[c]
Movement symptoms			
UPDRS-III	21.91 ± 13.85	23.78 ± 14.41	0.279[b]
H-Y staging			0.126[c]
0–1 (number/percentage)	65/33.0%	18/21.2%	
1.5–2 (number/percentage)	73/37.1%	39/45.9%	
2.5–3 (number/percentage)	59/29.9%	28/32.9%	

PD-nDM, Parkinson's disease patients without diabetes mellitus; PD-DM, Parkinson's disease patients with diabetes mellitus; DRA, dopamine receptor agnoists; MAOBI, Monoamine oxidase type B inhibitors; COMTI, Catechol-O-methyltransferase inhibitors; UPDRS-III, Unified Parkinson's Disease Rating Scale motor score; H-Y staging, Hohen-Yahr staging; [a]two-sample t- test; [b]Mann–Whitney U-test; [c]Chi-square test.

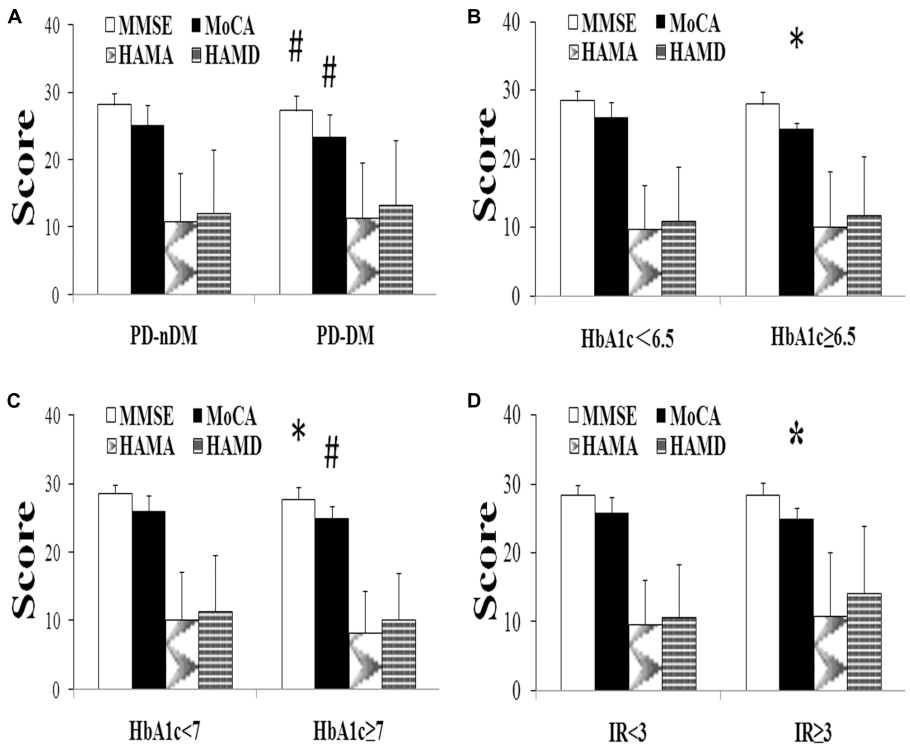

FIGURE 1 | Neuropsychological assessment in Parkinson's disease (PD). **(A)** Comparison of neuropsychological function of PD patients with (PD-DM, $n = 85$) or without (PD-nDM, $n = 197$) diabetes mellitus. **(B)** Comparison of neuropsychological function of PD patients with HbA1c < 6.5 ($n = 90$) or HbA1c ≥ 6.5 ($n = 29$). **(C)** Comparison of neuropsychological function of PD patients with HbA1c < 7 ($n = 95$) or HbA1c ≥ 7 ($n = 24$). **(D)** Comparison of neuropsychological function of PD patients with IR < 3 ($n = 104$) or IR ≥ 3 ($n = 15$). HbA1c, glycosylated hemoglobin A1c; IR, insulin resistance; MMSE, Mini Mental State Examination; MoCA, Montreal Cognitive Assessment; HAMA, Hamilton Anxiety Scale; HAMD, Hamilton Depression Scale. *$p < 0.05$, compared with the corresponding control group; #$p < 0.01$, compared with the corresponding control group.

and met United Kingdom PD Society Brain Bank Research Center clinical criteria, and completed a series of clinical and neuropsychological assessment at the "on" status. The exclusion criteria were (1) patients with parkinsonism syndrome secondary to drugs, cerebrovascular disease, encephalitis, metabolic disease except diabetes, malignant tumor, carbon monoxide poisoning, or any other neurological and psychiatric disorders; (2) patients had prior or planned neurosurgical treatment (e.g., deep brain stimulation); (3) patients had anticholinergic medications (e.g., trihexyphenidyl, memantine); other medical or neurological causes of CI (e.g., seizures, strokes, head trauma, and neurosyphilis); (4) patients suffered from a serious chronic physical disease or diseases that affect glucose metabolism (e.g., cardiac insufficiency, renal failure, thyroid dysfunction) and; (5) patients with fasting glucose <3.9 mmol/L; (6) patients were unable to complete the questionnaire. This study was carried out in accordance with the recommendations of the Ethics Committee of Xinhua Hospital affiliated to Shanghai Jiao Tong University School of Medicine with written informed consent from all patients or their legal guardians. All patients or their legal guardians were given the informed consent form in accordance with the Declaration of Helsinki. The protocol was approved by the Ethics Committee of Xinhua Hospital affiliated to Shanghai Jiao Tong University School of Medicine.

Clinical Data Collection

The detailed demographics characteristics of study participants included age, gender, body mass index (BMI), disease-related variables, medications, and educational background (measured as the total number of school years). All the participants underwent Hohen-Yahr staging (H-Y staging) and Unified Parkinson's Disease Rating Scale – motor score (UPDRS-III) to evaluate motor symptoms. All participants underwent neuropsychological assessment by an experienced neuropsychologist. General cognitive function was measured using the Mini Mental State Examination (MMSE), which has a maximum score of 30. Dementia severity was assessed

TABLE 2 | Comparison of constituent ratio of cognitive impairment (CI) in PD-nDM and PD-DM patients.

	PD-nDM ($n = 197$)	PD-DM ($n = 85$)	*P*
PD-nCI	110/55.8%	22/25.9%	**0.000[c]**
PD-CI	87/44.2%	63/74.1%	

PD-nDM, PD patients without diabetes mellitus; PD-DM, PD patients with diabetes mellitus; PD-nCI, PD patients without cognitive impairment; PD-CI, PD patients with cognitive impairment. [c]Chi-square test.

using Montreal Cognitive Assessment (MoCA), which includes the following seven domains: visuospatial/executive (5′), naming (3′), delayed memory (5′), attention (6′), language (3′), abstraction (2′), and orientation (6′). If the informed years of education were ≤12 years, then 1′ was added to correct the bias for education level. Patients with MoCA scores <26′ were considered to have CI (Dalrymple-Alford et al., 2010; Litvan et al., 2012; Marras et al., 2013). Emotion evaluations were based on Hamilton Anxiety Scale (HAMA, 14 items) and Hamilton Depression Scale (HAMD, 24 items) (Hamilton, 1967; Xiao et al., 2011; Kandiah et al., 2014).

Biochemical Tests

Biochemical tests for fasting glucose, HbA1c, fasting insulin and oral glucose tolerance test (OGTT) were performed in 119 participants. Procedure for 2h-OGTT required administration of 75 g oral glucose load within a 5-min period, and 2-h postprandial glucose was subsequently drawn at 120 min, timed from the beginning of the glucose load (Hu et al., 2004; Cukierman-Yaffe et al., 2009; Bosco et al., 2012; Cereda et al., 2012; Yaffe et al., 2012). A fasting glucose ≥6.1 mmol/L was considered as high fasting glucose (Hu et al., 2004; Cukierman-Yaffe et al., 2009; Cereda et al., 2012; Yaffe et al., 2012). Patients were considered to have an impaired glucose tolerance (IGT) if 2-h postprandial glucose was between 7.8 and 11.1 mmol/L (Hu et al., 2004; Cukierman-Yaffe et al., 2009; Cereda et al., 2012; Yaffe et al., 2012). Insulin resistance (IR) was calculated by the homoeostasis model assessment (HOMA) formula [HOMA-Index: Basal Glucose Plasma (mmol/L) × Basal Insulin Plasma (mU/L)/22.5] (Matthews et al., 1985; Bosco et al., 2011, 2012). Patients were considered to have insulin resistance if their HOMA-Indexes ≥3 (Matthews et al., 1985). DM was defined by one of the following criteria: participants reported diabetes history, discharge diagnosis of DM, a tested fasting glucose level higher than 7.0 mmol/L, or a 2-h postprandial glucose level higher than 11.1 mmol/L (Boon et al., 1997; Hu et al., 2004; Cukierman-Yaffe et al., 2009; Cereda et al., 2012; Yaffe et al., 2012).

Statistical Analyses

All statistical procedures were conducted using Statistical Package for the Social Sciences (SPSS) version 20.0 for Windows. Comparison between groups was performed using a two-sample *t*-test (normal distribution) or Mann–Whitney *U*-test (abnormal distribution) as appropriate. Categorical variables were examined using the Chi-square test. Correlation tests were performed using spearman correlation analysis, and the statistically significant indicators were included in the logistic regression analysis. Statistical significance was set at $p < 0.05$.

TABLE 3 | Clinical characteristics of HbA1c < 6.5 and HbA1c ≥ 6.5 groups in PD.

	HbA1c < 6.5 (*n* = 90)	HbA1c ≥ 6.5 (*n* = 29)	*P*
Gender (male: number/percentage)	51/56.7%	15/51.7%	0.672[c]
Age (year)	70.47 ± 7.79	68.69 ± 7.31	0.267[a]
Duration (year)	4.15 ± 4.18	4.59 ± 3.21	0.181[b]
Onset year (year)	66.32 ± 8.80	64.10 ± 8.55	0.162[a]
Current smoking (number/percentage)	30/33.3%	6/20.7%	0.249[c]
Body mass index (BMI, Kg/m^2)	22.67 ± 1.34	23.28 ± 1.67	0.070[a]
Degree of education (year)	11.90 ± 2.53	12.00 ± 2.36	0.795[b]
Form of onset			
Tremor (number/percentage)	42/46.7%	14/48.3%	1.000[c]
Dominant side involved (number/percentage)	49/54.4%	17/58.6%	0.830[c]
Drugs			
Levodopa intake (number/percentage)	81/90.0%	29/100%	0.111[c]
levodopa equivalent dosage (mg/day)	490.42 ± 295.83	474.14 ± 211.45	0.970[a]
levodopa dosage (mg/day)	436.67 ± 270.64	394.83 ± 173.38	0.143[a]
DRA (number/percentage)	38/42.2%	16/55.2%	0.284[c]
MAOBI (number/percentage)	17/18.9%	5/17.2%	1.000[c]
COMTI (number/percentage)	3/3.3%	1/3.4%	1.000[c]
Movement symptoms			
UPDRS-III	20.66 ± 12.06	19.93 ± 10.22	0.995[b]
H&Y			0.861[c]
0–1 (number/percentage)	32/35.6%	9/31.0%	
1.5–2 (number/percentage)	37/41.1%	12/41.4%	
2.5–3 (number/percentage)	21/23.3%	8/27.6%	

HbA1c, glycosylated hemoglobin A1c; DRA, dopamine receptor agnoists; MAOBI, Monoamine oxidase type B inhibitors; COMTI, Catechol-O-methyltransferase inhibitors; UPDRS-III, Unified Parkinson's Disease Rating Scale motor score, H-Y staging, Hohen-Yahr staging; [a]*two-sample t- test;* [b]*Mann–Whitney U-test;* [c]*Chi-square test.*

RESULTS

Clinical Characteristics of PD-DM and PD-nDM Patients

A total of 282 PD patients were recruited in this study, 85 of them with DM (30.1%) (PD-DM) and 197 of them without diabetes (69.9%) (PD-nDM). As shown in **Table 1**, there was no significant difference in age, gender, education, levodopa equivalent dosage, various types of drug ratio, onset age, duration, onset form, UPDRS-III, H-Y staging, HAMA score, or HAMD score between PD-DM and PD-nDM groups. Body mass index (BMI) in PD-DM group was significantly higher than that in PD-nDM group (23.17 ± 1.57 vs. 22.56 ± 2.28 kg/m^2, $p = 0.010$) (**Table 1**). Interestingly, MoCA score (23.35 ± 3.41 vs. 25.12 ± 2.99, $p = 0.000$) and MMSE score (27.32 ± 2.28 vs. 28.18 ± 1.74, $p = 0.003$) in PD-DM group were significantly lower than those in PD-nDM group (**Figure 1A**). In addition, PD-DM group showed significantly higher constituent ratio of CI compared to PD-nDM group ($x2 = 21.400$, $p = 0.000$) (**Table 2**).

Comparison of Cognitive Function at Different Levels of HbA1c and Insulin Resistance in Parkinson's Disease Patients

In our current study, HbA1c and HOMA-index were used as parameters to evaluate diabetes control and insulin resistance in PD, respectively. As shown in **Figures 1B,C**, MoCA scores in HbA1c $\geq$ 6.5% group (24.41 ± 0.91 vs. 26.02 ± 2.21, $p = 0.016$) and HbA1c $\geq$ 7% group (24.96 ± 1.85 vs. 26.03 ± 2.21, $p = 0.007$) were significantly lower than those in the corresponding control groups. Furthermore, MoCA score in IR $\geq$ 3 group was significantly lower than that in IR $<$ 3 group (24.93 ± 1.58 vs. 25.94 ± 2.23, $p = 0.049$) (**Figure 1D**). In addition, none of HbA1c $\geq$ 6.5%, HbA1c $\geq$ 7% or IR $\geq$ 3 groups showed significant difference in age, gender, education, levodopa equivalent dosage, drug ratio, onset age, duration, onset form, UPDRS-III, H-Y staging, MMSE score, HAMA score, or HAMD score compared with the corresponding control groups (**Tables 3**–**5**).

Risk Factors of Cognitive Impairment in Parkinson's Disease

As shown in **Table 6**, MoCA score was negatively correlated with H-Y staging ($r = -0.243$, $p = 0.008$), HbA1c ($r = -0.207$, $p = 0.024$), and insulin resistance ($r = -0.498$, $p = 0.000$). However, MMSE score had no correlation with either H-Y staging or glucose metabolic indicators (**Table 6**). To identify the risk factors of CI, logistic regression analysis was conducted, and our data indicated that H-Y staging (OR = 1.844, 95% confidence interval:1.162 $\sim$ 2.927, $p = 0.009$) and HbA1c $\geq$ 7% (OR = 4.253, 95% confidence interval:1.596 $\sim$ 11.342, $p = 0.004$) were independent risk factors of CI in patients with PD (**Table 7**).

TABLE 4 | Clinical characteristics of HbA1c $<$ 7 and HbA1c $\geq$ 7 groups in PD.

	HbA1c < 7 ($n = 95$)	HbA1c ≥ 7 ($n = 24$)	P
Gender (male: number/percentage)	55/57.9%	11/45.8%	0.360[c]
Age (year)	70.41 ± 7.71	68.54 ± 7.54	0.239[a]
Duration (year)	4.17 ± 3.05	4.28 ± 4.17	0.578[b]
Onset year (year)	66.13 ± 8.64	64.38 ± 9.23	0.303[a]
Current smoking (number/percentage)	31/32.6%	5/20.8%	0.326[c]
Body mass index (BMI, Kg/m^2)	22.73 ± 1.37	23.18 ± 1.67	0.156[a]
Degree of education (year)	12.01 ± 2.53	11.58 ± 2.26	0.509[b]
Form of onset			
Tremor (number/percentage)	44/46.3%	12/50.0%	0.821[c]
Dominant side involved (number/percentage)	51/53.7%	15/62.5%	0.496[c]
Drugs			
levodopa equivalent dosage (mg/day)	503.29 ± 298.44	419.79 ± 153.40	0.286[a]
levodopa dosage (mg/day)	441.05 ± 268.17	368.75 ± 153.09	0.370[a]
DRA (number/percentage)	43/45.3%	11/45.8%	1.000[c]
MAOBI (number/percentage)	19/20.0%	3/12.5%	0.560[c]
COMTI (number/percentage)	4/4.2%	0	0.582[c]
Movement symptoms			
UPDRS-III	20.64 ± 11.79	19.83 ± 11.03	0.827[b]
H&Y			0.840[c]
0–1 (number/percentage)	33/34.7%	8/33.3%	
1.5–2 (number/percentage)	40/42.1%	9/37.5%	
2.5–3 (number/percentage)	22/23.2%	7/29.2%	

HbA1c, glycosylated hemoglobin A1c; DRA, dopamine receptor agnoists; MAOBI, Monoamine oxidase type B inhibitors; COMTI, Catechol-O-methyltransferase inhibitors; UPDRS-III, Unified Parkinson's Disease Rating Scale motor score; H-Y staging, Hohen-Yahr staging; [a]two-sample t- test; [b]Mann–Whitney U-test; [c]Chi-square test.

TABLE 5 | Clinical characteristics of IR < 3 and IR ≥ 3 groups in PD.

	IR < 3 ($n = 104$)	IR ≥ 3 ($n = 15$)	*P*
Gender (male: number/percentage)	58/55.8%	8/53.3%	0.859[c]
Age (year)	70.23 ± 7.54	68.67 ± 7.79	0.532[a]
Duration (year)	4.18 ± 4.08	4.73 ± 3.04	0.215[b]
Onset year (year)	66.04 ± 8.53	63.93 ± 10.31	0.374[a]
Current smoking (number/percentage)	33/31.7%	3/20.0%	0.394[c]
Body mass index (BMI, Kg/m^2)	22.73 ± 1.42	23.18 ± 1.67	0.578[a]
Degree of education (year)	11.93 ± 2.56	11.87 ± 1.92	0.922[b]
Form of onset			
Tremor (number/percentage)	49/47.1%	7/46.7%	1.000[c]
Dominant side involved (number/percentage)	57/54.8%	9/60.0%	0.786[c]
Drugs			
Levodopa equivalent dosage (mg/day)	480.65 ± 280.49	526.67 ± 255.73	0.482[a]
Levodopa dosage (mg/day)	424.52 ± 257.32	440.00 ± 202.84	0.275[a]
DRA (number/percentage)	48/46.2%	6/11.1%	0.784[c]
MAOBI (number/percentage)	18/17.3%	4/26.7%	0.475[c]
COMTI (number/percentage)	4/3.8%	0	1.000[c]
Movement symptoms			
UPDRS-III	19.97 ± 11.37	24.00 ± 12.96	0.220[b]
H&Y			0.647[c]
0–1 (number/percentage)	37/35.6%	4/26.7%	
1.5–2 (number/percentage)	41/39.4%	8/53.3%	
2.5–3 (number/percentage)	26/25.0%	3/20.0%	

IR, insulin resistance; DRA, dopamine receptor agnoists; MAOBI, Monoamine oxidase type B inhibitors; COMTI, Catechol-O-methyltransferase inhibitors; UPDRS-III, Unified Parkinson's Disease Rating Scale motor score; H-Y staging, Hohen-Yahr staging; [a]*two-sample t- test;* [b]*Mann–Whitney U-test;* [c]*Chi-square test.*

DISCUSSION

In this study, we investigated clinical features of PD patients with or without dysglycemia, examined risk factors of CI in PD, and explored the relationship between CI and dysglycemia in patients with PD.

TABLE 6 | Correlation of CI with H-Y staging, HbA1c and IR in PD.

	MMSE		MoCA	
	r	*P*	*r*	*P*
H-Y staging	−0.070	0.448[d]	−0.243	**0.008**[d]
HbA1c	−0.114	0.218[d]	−0.207	**0.024**[d]
IR	−0.027	0.772[d]	−0.498	**0.000**[d]

MMSE, Mini Mental State Examination; MoCA, Montreal Cognitive Assessment; H-Y staging, Hohen-Yahr staging; HbA1c, serum glycosylated hemoglobin A1C; IR, insulin resistance; [d]*spearman correlation analysis.*

TABLE 7 | Regression analysis on risk factors of CI in PD.

	B	*SE*	*P*	OR	95%CI
H-Y staging	0.612	0.236	**0.009**[e]	1.844	(1.162 ∼ 2.927)
HbA1c ≥ 7%	1.448	0.500	**0.004**[e]	4.253	(1.596 ∼ 11.342)
HbA1c ≥ 6.5%	0.044	1.059	0.967[e]	1.045	(0.131 ∼ 8.329)
IR ≥ 3	0.508	0.768	0.509[e]	0.369	(0.777 ∼ 14.279)

H-Y staging, Hohen-Yahr staging; HbA1c, serum glycosylated hemoglobin A1C; IR, insulin resistance; [e]*logistic regression analysis.*

Cognitive impairment is frequently found in PD, especially in the late stage of the disease (Hely et al., 2008; Aarsland et al., 2010; Litvan et al., 2011). It is well known that diabetes is one of the risk factors of cognitive dysfunction (Xu et al., 2009) in several diseases, but it remains unclear about the role of dysglycemia in CI of PD. In the present study, we observed that PD-DM patients had significant higher BMI compared to PD-nDM patients, which was in accordance with the clinical features of DM (Hu et al., 2004). There was no significant difference in onset form, dominant side involvement, levodopa equivalent dosage, doses of levodopa, UPDRS-III, or H-Y staging between PD-DM and PD-nDM patients. However, previous studies have suggested PD patients with diabetes may have worse motor symptoms compared to those without diabetes. A case-control study indicated that patients with PD and DM had higher motor scores, and needed larger doses of dopaminergic treatment compared to patients with PD only (Cereda et al., 2012). Another cohort study showed that diabetes could aggravate the movement symptoms of PD, especially gait disturbance and postural stability (Kotagal et al., 2013). The disparity between our results and other studies may be due to the difference of sample size and enrolled patient population. In line with the previous study (Profenno et al., 2010), our data showed significant differences in MMSE and MoCA scores were observed between PD-DM and PD-nDM patients. Moreover, the prevalence of cognitive dysfunction is significantly higher

in PD-DM patients than that in PD-nDM patients, suggesting that diabetes may be one risk factor for cognitive dysfunction in, at least partially, PD patients. It has been shown that α-synuclein, amyloid-β (Aβ) peptides, and tau protein may be involved in the pathologic process of CI in PD (Weintraub et al., 2012; Kang et al., 2013). Laboratory studies suggested that CI was caused by aberrant glycation of α-synuclein, Aβ peptides and tau protein (Vicente Miranda et al., 2016), which was induced by glycation agent produced by dysglycemia (Sousa Silva et al., 2013). Insulin resistance may also lead to the abnormal regulation of tau, resulting in Aβ peptides deposition (Bosco et al., 2011). It is necessary to further investigate the specific pathological mechanism for CI in PD in our future research.

HbA1c, a commonly used parameter reflecting the mean glucose concentration during the past 8–12 weeks, is a superior indicator to assess long-term glycemic control (Miedema, 2005). Although several methods have been developed to diagnose human insulin resistance, the HOMA-index method has been recognized as the mostly employed approach in both clinical practice and epidemiological studies due to its simplicity (Matthews et al., 1985; Salgado et al., 2010). Therefore, in our current study, HbA1c and HOMA-index were used as parameters to evaluate diabetes control and insulin resistance in PD, respectively. We observed that MoCA scores in HbA1c $\geq$ 6.5%, HbA1c $\geq$ 7% and insulin resistance $\geq$3 groups were significantly lower than that in the corresponding control groups, suggesting CI is associated with the control situation of diabetes in, at least partially, PD patients. In our study, HbA1c $\geq$ 6.5, HbA1c $\geq$ 7 and IR $\geq$ 3 groups were significantly lower than the corresponding control groups, which is in agreement with actual clinical conditions and also observed in previous studies (Bohnen et al., 2014; Ong et al., 2017).

Furthermore, correlation analysis showed MoCA score was negatively correlated with H-Y staging, HbA1c and insulin resistance, respectively. However, MMSE score was not correlated with either H-Y staging or glucose metabolic indicators. The potential reason could be that MoCA has the higher sensitivity and specificity (Dalrymple-Alford et al., 2010), better evaluation reliability (Gill et al., 2008), and higher discriminant validity (Hoops et al., 2009) in the assessment of cognitive function in PD, whereas there is a ceiling effect of MMSE in cognitive evaluation (Hoops et al., 2009; Marras et al., 2013). Based on the previous studies (Dalrymple-Alford et al., 2010; Litvan et al., 2012; Marras et al., 2013), we set MoCA 26' as a discriminator to differentiate CI, which was shown to be a convenience and reliable approach for clinicians to evaluate patients' cognitive function.

Finally, logistic regression analysis showed that H-Y staging and HbA1c $\geq$ 7% were risk factors of CI in PD. The correlation of CI with H-Y staging is in accordance with the development characteristics of PD. With the progression of disease severity, cognitive dysfunction gradually deteriorated (Hely et al., 2008). Among all the glucose metabolic indicators, HbA1c $\geq$ 7% was a significant risk factor of CI in PD. Some large scale cross-sectional (Cukierman-Yaffe et al., 2009; Nguyen et al., 2010) and prospective studies (Kanaya et al., 2004; Yaffe et al., 2006, 2012) have indicated that lower score of MoCA is associated with higher level of HbA1c. The American Diabetes Association (ADA) proposed that HbA1c $\geq$ 6.5% can be used to diagnose DM, and keeping an HbA1c lower than 7% reduces the occurrence of diabetic microvascular lesion (Yaffe et al., 2012). Based on our study and previous studies, H-Y staging and HbA1c may serve as key parameters for controlling CI in PD clinical practice.

There are still some limitations in this study. First of all, the sample size of our study was relatively small, and the participants were mainly from Shanghai, China, therefore, our results may have a certain area or racial bias. Secondly, our study was a cross-sectional research, lacking of comparative support from prospective study. Thirdly, our study lacked cerebral vascular disease risk factors (such as drinking, hypertension, stroke) assessment, and there was no further analysis of the influence of diabetic medicines on patients' cognitive function. Lastly, all the patients participated in our study had a H-Y staging 3 or less than 3, namely early stage PD patients. Therefore, our results may not apply to the whole PD group.

CONCLUSION

Our study showed that CI may be tightly associated with dysglycemia in, at least partially, PD patients. Importantly, H-Y staging and HbA1c $\geq$7% may be independent risk factors of CI in PD, and may serve as key biomarkers in future PD clinical practice. Further studies are needed to investigate the specific mechanism for CI of PD.

AUTHOR CONTRIBUTIONS

XW conceived the project and designed the study. LYa, ZC, and BL contributed to participant recruitment, data collection and data analysis. MW and LYu contributed to participant recruitment and data collection. YW, JG, YZ, and ZL contributed to supervise the study. LYa, BL, and XW wrote the paper together.

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Presence of Anticardiolipin Antibodies in Patients with Dementia: A Systematic Review and Meta-Analysis

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Growing evidences are supporting towards the involvement of antiphospholipid antibodies [aPLs e.g., lupus anticoagulant (LA), anticardiolipin (aCL) and anti-β2-glycoprotein I (anti-β2-GPI) antibodies] in various neurological manifestations including migraine, epilepsy and dementia in the presence or absence of autoimmune diseases such as antiphospholipid syndrome or systemic lupus erythematosus. The aim of this systematic review and meta-analysis was to assess the presence of aPLs in dementia patients without a diagnosis of any autoimmune disease. Electronic databases (e.g., PubMed, Web of Science, Scopus, ScienceDirect and Google Scholar) were searched without any year or language restrictions and based on the inclusion criteria, nine prospective case-control studies assessing only aCL were included involving 372 dementia patients and 337 healthy controls. No studies were found to assess the presence of both LA or anti-β2-GPI. The study-specific odds ratios (ORs) and 95% confidence intervals (CIs) were calculated using random-effects model. We observed the prevalence of aCL in dementia was higher (32.80%) than that of controls (9.50%) e.g., 3.45 times higher risk of presenting with dementia than the controls, and significant presence of aCL antibodies was detected in dementia patients compared to controls (OR: 4.94, 95% CI: 2.66 – 9.16, $p < 0.00001$; $I^2 = 32\%$, $p = 0.16$). Publication bias was not observed from Egger's ($p = 0.081$) and Begg's tests ($p = 0.180$). Based on the study quality assessment using modified Newcastle–Ottawa Scale for case-control studies, seven of nine studies were of high methodological quality scoring ≥ 7 (median value). In summary, aCL antibodies were significantly present in dementia patients suggesting that aCL antibodies are generated due to the autoimmune-derived effects of dementia or there might be a potential causative role of this autoantibody in dementia pathogenesis.

Keywords: dementia, Alzheimer's disease, antiphospholipid antibodies, anticardiolipin antibodies, systematic review, meta-analysis

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INTRODUCTION

Dementia is a clinical syndrome that encompasses a set of neurologic symptoms involving difficulties in memory, speaking, problem solving, and thinking abilities, leading to the impairments of personal and social life (Román, 2003; Burns and Iliffe, 2009). It is most common in elderly people where advanced age being the strongest risk factor. A prevalence of 7.1% among the aged population (>65 years old) has been reported (Prince et al., 2014), and the number of people with dementia worldwide is estimated at 47 million and is projected to increase over 131 million by 2050 (Prince et al., 2016). Worldwide, the total number of new cases of dementia each year amounts to approximately 7.7 million, indicating one new case every 4.1 s (Prince et al., 2015).

Among several types of dementia, Alzheimer's disease (AD) and vascular dementia (VD) are most commonly observed (Dening and Babu Sandilyan, 2015; Robinson et al., 2015). AD accounts for 60% whereas VD accounts for almost 30% of the prevalence (Kalaria et al., 2008). In AD, neurodegeneration occurs due to abnormal extracellular deposition of insoluble plaques consisting of Aβ peptides and intraneuronal aggregates of twisted fibers consisting of tau proteins (Dening and Babu Sandilyan, 2015). VD occurs when blood circulation to the brain is compromised due to arterial disease resulting in reduced neuronal function and eventually neurons cell death (Dening and Babu Sandilyan, 2015). In AD patients, the synthesis of intra-blood-brain barrier (BBB) IgG was observed which indicates an involvement of immune-mediated mechanisms in the pathogenesis of AD (Blennow et al., 1990).

In previous years, researches have been conducted on autoimmune diseases including antiphospholipid syndrome (APS) which may have links with the risk of dementia development (Gomez-Puerta et al., 2005; Lin et al., 2016). A recent study conducted on 1.8 million hospital cases reported that patients with autoimmune disorders including APS and systemic lupus erythematosus (SLE) were 20% more likely to develop dementia (Wotton and Goldacre, 2017), suggesting an autoimmune-mediated pathogenesis of dementia. In APS, presence of antiphospholipid antibodies (aPLs) (autoantibodies which react against anionic phospholipids and proteins on plasma membranes) namely anticardiolipin (aCL) antibody, anti-β2-glycoprotein I (β2GPI) antibody and lupus anticoagulant (LA) are found persistently in high titers (Miyakis et al., 2006; Giannakopoulos and Krilis, 2013). Presence of aPLs in high titers was also observed in APS patients suffering from different neurologic disorders including dementia (Islam et al., 2016, 2017a). Dementia has been observed in up to 56% APS patients (Chapman et al., 2002; Gomez-Puerta et al., 2005), and a study on non-SLE patients with neurological symptoms showed that over 50% of the patients with high levels of aPLs developed dementia (Inzelberg et al., 1992). Furthermore, aPLs are associated with impaired cognitive function (Schmidt et al., 1995) and the frequency of cognitive dysfunction is high ranging between 19 and 40% in aPLs-positive asymptomatic patients (Jacobson et al., 1999; Kozora et al., 2013).

The pathogenesis of aPL-mediated dementia in APS is not entirely understood. Suggested mechanisms include aPLs-induced BBB disruption (Katzav et al., 2010), aPLs-related microvascular thrombosis (Asherson et al., 1987; Denburg et al., 1997), or a direct effect of aPLs on brain tissues (Appenzeller et al., 2012). Thrombotic events triggered by aPL might contribute to the multiple cerebral thrombotic symptoms and greater aggression to the brain (de Godoy et al., 2000). Besides thrombotic effects, inflammatory and immune effects may contribute to the development of cognitive dysfunction in the presence of aPLs (Katzav et al., 2011).

To date, the association of aPLs in patients with dementia remains inconclusive based on the primary studies conducted on small number of dementia subjects. Certain studies have shown that aCL was significantly ($p < 0.05$) present in dementia patients versus healthy controls, 27% vs. 0% (Juby and Davis, 1998) or 28% vs. 3% (Tan et al., 2001). However, other studies did not report such significant association of aCL positivity in dementia versus healthy subjects, 29% vs. 26.4% (de Godoy et al., 2012). Thus, a systematic review and meta-analysis on all the primary studies was conducted to bring together all evidences in this topic and synthesize a conclusive information about the presence of aPLs in dementia patients. In addition, subgroup analyses were performed to evaluate the presence of aCL in different types of dementia, distinct age ranges and patients in different geographical continents.

MATERIALS AND METHODS

To conduct this meta-analysis, we followed the guidelines published by the Meta-analysis of Observational Studies in Epidemiology (MOOSE) group (Supplementary Table S1) (Stroup et al., 2000).

Study Selection Criteria

Studies were included if: (1) Study design was prospective case-control; (2) The aim of the study was to evaluate the existence of aPLs (LA, aCL, and anti-β2-GPI antibodies) in patients with dementia; (3) Dementia subjects were of any age, sex or race without any underlying autoimmune disorders such as APS or SLE.

Literature Search

A systematic literature search using 'Advanced' and 'Expert' search strategies of PubMed, Web of Science, Scopus, Science Direct, and Google Scholar databases was independently conducted by two researchers (MAI and FA), and the shortlisted studies were independently verified by KKW. There were no search year or language restrictions. Review articles, case reports, clinical trials, editorials, letters, and comments were excluded. Studies were also excluded if overlapping of identical study subjects was observed with other included studies from similar research group. To ensure that there were no potential papers overlooked, we examined the reference list of selected studies and reviewed publications that had cited

the selected studies (via Google Scholar). The electronic search included both Medical Subject Heading (MeSH) in addition of appropriate keywords and combined with the Boolean operators ('AND' and 'OR'). The following search terms were used: (antiphospholipid antibody *OR* antiphospholipid antibodies *OR* anticardiolipin antibody *OR* anticardiolipin antibodies *OR* lupus anticoagulant *OR* β2GPI *OR* β2-GPI *OR* β2glycoprotein *OR* β2-glycoprotein) *AND* (dementia *OR* Alzheimer *OR* Alzheimer's). The final systematic search was conducted on 12th March 2017 (Supplementary Table S2).

Data Extraction, Management and Quality Assessment

Two researchers (MAI and FA) independently extracted the following data from each of the selected studies: first author and year (study ID), study design, country, number of dementia patients and controls (number of female patients and controls), types of dementia, mean age of dementia patients and controls, types and isotypes of tested aPLs, dementia diagnostic criteria, aPLs measurement techniques and cut-off values. To resolve any discrepancies such as unclear or missing data presentation, all authors took part in the discussion. If not resolved, we then contacted either the corresponding or the first author of the respective study for further clarifications. By using a modified version of the Newcastle–Ottawa Scale (NOS) (Islam et al., 2017b), quality of each of the selected studies was assessed; studies scoring above the median NOS value were considered as high quality (low risk of bias) and those scoring below the median value were considered as low quality (high risk of bias) (Wu et al., 2016).

Exploration of Heterogeneity and Publication Bias

Heterogeneity across studies was tested based on I^2 statistics which indicates the percentage of variance attributable to study heterogeneity. Studies with $I^2 < 40\%$, $I^2 = 40$–75% or $I^2 > 75\%$ was considered to have low, moderate or high heterogeneity (Harris et al., 2015). Three independent subgroup analyses were conducted as follows: (1) VD vs. dementia of the Alzheimer's type (DAT); (2) Age ranged from 60 to 70 years vs. above 70 years old; (3) Subjects from Asia and Europe vs. North and South America. Additionally, L'Abbé plot was generated for the visual inspection of heterogeneity by using RStudio (version 1.0.136) software (metafor package, version 1.9-9) (Viechtbauer, 2010).

Publication bias was visually assessed by using funnel plots. Moreover, Egger's regression test (Egger et al., 1997) and Begg's test (Begg and Mazumdar, 1994) was conducted to further assess publication bias with random-effects model. Publication bias was considered significant if $p < 0.05$. Funnel plot was illustrated with the metafor package, version 1.9-9 (Viechtbauer, 2010).

Statistical Analyses of Meta-Analysis

Random-effects model was used to conduct this meta-analysis. Odds ratio (OR) was used to evaluate the comorbid association of the presence of aPLs in dementia patients compared to controls where $p < 0.05$ was considered significant. RevMan (Cochrane Collaboration, software version 5.3.5) (The Cochrane Collaboration, 2014) was used to generate the forest plot.

RESULTS

Study Selection

Our initial search yielded 367 articles where 189 studies were excluded and the remaining 198 articles were evaluated based on title and abstract. Ten studies were shortlisted following the inclusion criteria, and one article (Juby et al., 1995) was excluded due to overlapping of identical study subjects with another eligible study (Juby and Davis, 1998). Therefore, nine studies were included in this meta-analysis (**Figure 1**). Although initially the aim was to evaluate the presence of aPLs in dementia patients, based on our search strategies and systematic review, we could not find studies evaluating LA or anti-β2GPI except for aCL.

Study Characteristics and Quality Assessment

Among the included studies, four were from China (Tan et al., 2001; Zhao and Tan, 2004; Zeng et al., 2006; Qian et al., 2015), two were from Brazil (de Godoy et al., 2005, 2012), and the remaining three studies were from Israel (Mosek et al., 2000), Canada (Juby and Davis, 1998), and United States (Lopez et al., 1992). Across the nine studies, there were 709 subjects (dementia patients: $n = 372$; controls: $n = 337$) in total. All of the studies were designed as case-control and evaluated the presence of aCL as the only aPLs in dementia patients compared to controls. In particular, six of the studies were on VD (Lopez et al., 1992; Tan et al., 2001; Zhao and Tan, 2004; de Godoy et al., 2005; Zeng et al., 2006; Qian et al., 2015) and two on DAT (Juby and Davis, 1998; de Godoy et al., 2012) and one study with both VD and DAT patients (Mosek et al., 2000). The age range of the dementia patients and controls was 65–80.5 and 50.1–78.3 years, respectively. **Table 1** summarizes the major characteristics of the included studies.

Quality assessment of the included studies by using NOS for case-control studies is shown in **Table 2**. The median score of NOS was 7. Among the nine studies, seven studies were of high quality (low risk of bias) scoring ≥ 7 (Lopez et al., 1992; Juby and Davis, 1998; Mosek et al., 2000; Tan et al., 2001; de Godoy et al., 2005, 2012; Qian et al., 2015) and two studies were of low quality (high risk of bias) scoring < 7 (Zhao and Tan, 2004; Zeng et al., 2006).

Assessment of aCL Presence by Meta-Analysis

Presence of aCL in dementia patients was highly significant compared to controls (OR: 4.94, 95% CI: 2.66–9.16, $p < 0.00001$; $I^2 = 32\%$, $p = 0.16$) (**Figure 2**). aCL was present in 32.80% of dementia patients and 9.50% in controls corresponding to 3.45 times higher probability to present with dementia than controls.

Subgroup Analyses on VD and DAT

Our meta-analysis on studies that evaluated the presence of aCL in patients with VD ($n = 472$) (Lopez et al., 1992; Juby and

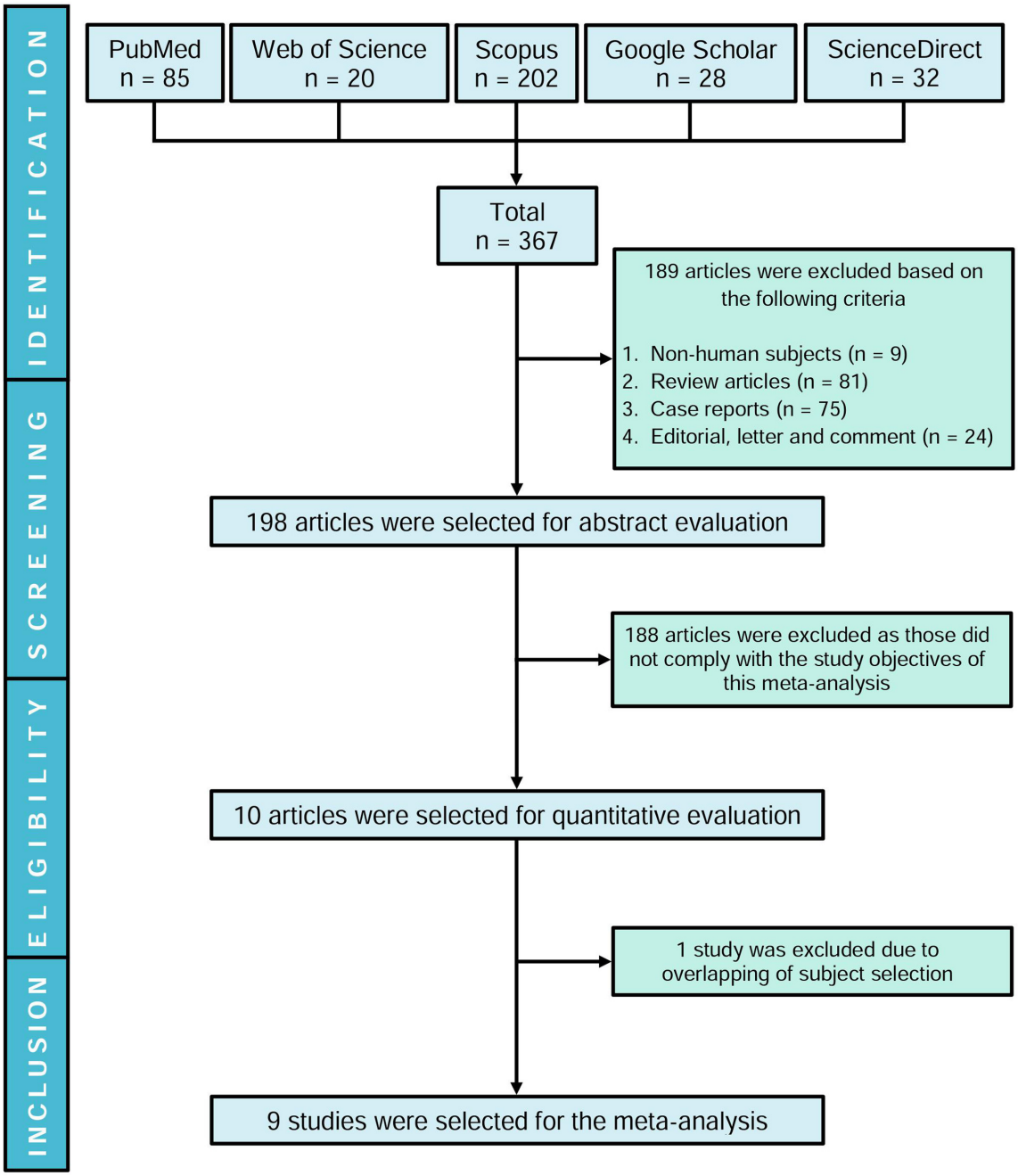

FIGURE 1 | Flow chart illustrating the study selection process in the meta-analysis.

Davis, 1998; Mosek et al., 2000; Tan et al., 2001; de Godoy et al., 2005; Zeng et al., 2006; Qian et al., 2015) indicated that aCL was significantly present in VD patients as compared to healthy controls (OR: 6.89, 95% CI: 3.73–12.74, $p < 0.00001$; $I^2 = 0\%$, $p = 0.41$) (**Figure 3A**). For studies that measured aCL antibodies in DAT patients ($n = 291$) (Juby and Davis, 1998; Mosek et al., 2000; de Godoy et al., 2012), aCL was not significantly associated with DAT (OR: 5.13, 95% CI: 0.52–50.22, $p = 0.16$; $I^2 = 67\%$, $p = 0.05$) (**Figure 3B**).

Subgroup Analyses on Different Age Ranges

Anticardiolipin was significantly present in dementia subjects of age groups between 60 and 70 years old ($n = 293$) (Tan et al., 2001; de Godoy et al., 2005; Zeng et al., 2006; Qian et al., 2015) (OR: 5.99, 95% CI: 3.16–11.35, $p < 0.00001$; $I^2 = 0\%$, $p = 0.67$) (**Figure 4A**) and those more than 70 years old ($n = 316$) (Lopez et al., 1992; Mosek et al., 2000; Zhao and Tan, 2004; de Godoy

TABLE 1 | Major characteristics of the case-control studies mentioned in the meta-analysis.

No.	Study ID	Country	Number of dementia patients (number of female)	Types of dementia (n)	Mean age of dementia patients (years)	Number of controls (number of female)	Mean age of controls (years)	Types of tested aPLs (isotype)	Dementia diagnostic criteria	aCL measurement (test; cut-off)
1	Qian 2015	China	32 (17)	VD (32)	68.7	31 (15)	67.2	aCL (IgG)	DSM-III	ELISA; NR
2	de Godoy 2012	Brazil	31 (23)	DAT (31)	71.2	34 (25)	68.0	aCL (IgG and IgM)	NINCDS-ADRDA	ELISA; NR
3	Zeng 2006	China	75 (31)	VD (75)	65.5	30 (15)	50.1	aCL (IgG and IgM)	NR	ELISA; IgG > 20 GPL, IgM > 20 MPL
4	de Godoy 2005	Brazil	30 (13)	VD (30)	67.2	34 (25)	68.0	aCL (IgG and IgM)	NINCDS-ADRDA	ELISA; IgG > 10 GPL, IgM > 7 MPL
5	Zhao 2004	China	39 (15)	BD (39)	80.5	36 (14)	78.3	aCL (NR)	NR	ELISA; NR
6	Tan 2001	China	31 (11)	VD (31)	66.8	30 (12)	66.2	aCL (IgG)	DSM-I	ELISA; IgG > 20 U/mL
7	Mosek 2000	Israel	87 (48)	DAT (68) MD (3) VD (16)	74.0	69 (40)	78.0	aCL (IgG)	DSM-IV	ELISA; IgG > 20 GPL
8	Juby 1998	Canada	37 (NR)	DAT (26) VD (11)	>65.0	63 (NR)	>65.0	aCL (INR)	NR	ELISA; NR
9	Lopez 1992	United States	10 (3)	VD (10)	72.4	10 (6)	72.1	aCL (IgG)	NINCDS-ADRDA	ELISA; NR

VD, vascular dementia; BD, Binswanger's disease; DAT, dementia of the Alzheimer's type; MD, mixed dementia; aPLs, antiphospholipid antibodies; NINCDS-ADRDA, National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association; DSM-IV, Diagnostic and Statistical Manual of Mental Disorders IV; NR, not reported; ELISA, enzyme-linked immunosorbent assay; IgG, immunoglobulin G; IgM, immunoglobulin M; GPL, immunoglobulin G phospholipid; MPL, Immunoglobulin M phospholipid.

TABLE 2 | Risk of bias assessment of the included studies according to the modified Newcastle-Ottawa Scale (NOS).

Quality assessment	Qian 2015	de Godoy 2012	Zeng 2006	de Godoy 2005	Zhao 2004	Tan 2001	Mosek 2000	Juby 1998	Lopez 1992
Selection									
(1) Is the case definition adequate?	★	★	○	★	○	★	★	○	★
(2) Representativeness of the cases	★	★	★	★	★	★	★	★	★
(3) Selection of controls	○	★	○	★	○	○	★	★	○
(4) Definition of controls	★	○	★	○	★	○	★	★	★
Comparability									
(5) Study controls for the most important factor	★	★	○	★	★	★	★	★	★
(6) Study controls for the second important factor	★	★	○	★	○	★	★	★	○
Exposure									
(7) Was the measurement method of aPLs described?	★	★	★	★	★	★	★	★	★
(8) Were the methods of measurements same for cases and controls (e.g., ELISA)?	★	★	★	★	★	★	★	★	★
(9) Non-response rate	★	★	★	★	★	★	★	★	★

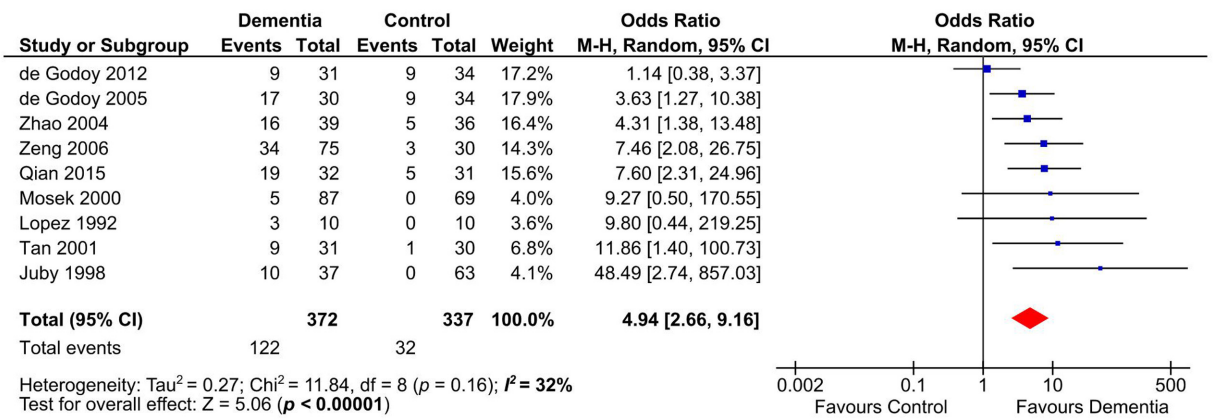

FIGURE 2 | Forest plot representing the presence of anticardiolipin (aCL) antibodies in dementia patients compared to controls.

et al., 2012) (OR: 2.92, 95% CI: 1.06–8.06, $p = 0.04$; $I^2 = 33\%$, $p = 0.21$) (**Figure 4B**).

Subgroup Analyses on Patients in Different Continents

Anticardiolipin antibody was significantly present in Asian and European dementia patients ($n = 460$) (Mosek et al., 2000; Tan et al., 2001; Zhao and Tan, 2004; Zeng et al., 2006; Qian et al., 2015) (OR: 6.64, 95% CI: 3.50–12.62, $p < 0.00001$; $I^2 = 0\%$, $p = 0.91$) (**Figure 5A**), as well as in North and South American dementia subjects ($n = 249$) (Lopez et al., 1992; Juby and Davis, 1998; de Godoy et al., 2005, 2012) (OR: 4.06, 95% CI: 1.04–15.84, $p = 0.04$; $I^2 = 62\%$, $p = 0.05$) (**Figure 5B**).

Heterogeneity and Publication Bias

Low heterogeneity was observed ($I^2 = 32\%$) in assessing aCL in dementia patients compared to controls. Additionally, visual inspection of L'Abbé plot (**Figure 6**) demonstrated no substantial heterogeneity.

Visual assessment of funnel plot (**Figure 7**) showed that the studies were distributed asymmetrically, suggesting the presence of some publication bias. Begg's test was not significant ($p = 0.180$), however, there was a trend toward significance for Egger's regression ($p = 0.081$).

DISCUSSION

In this study, based on the meta-analysis of nine shortlisted studies (372 dementia patients and 337 healthy controls), we validated the fact that aCL antibodies were significantly present in dementia patients as compared to healthy subjects, thus resolving previous conflicting reports on their associations. Our observation based on the meta-analysis on case-control studies was also supported by some cohort studies demonstrating the association of aCL positivity with cognitive decline and impaired

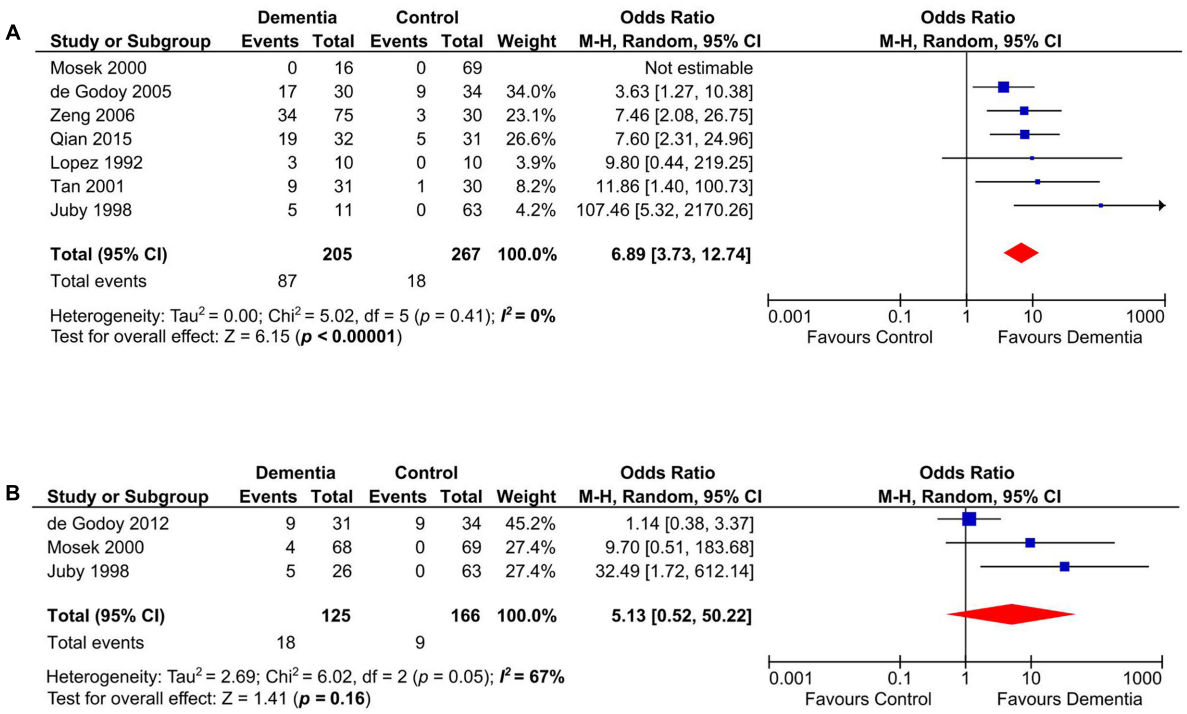

FIGURE 3 | Subgroup analysis of aCL in patients with vascular dementia (VD) **(A)** and DAT **(B)**.

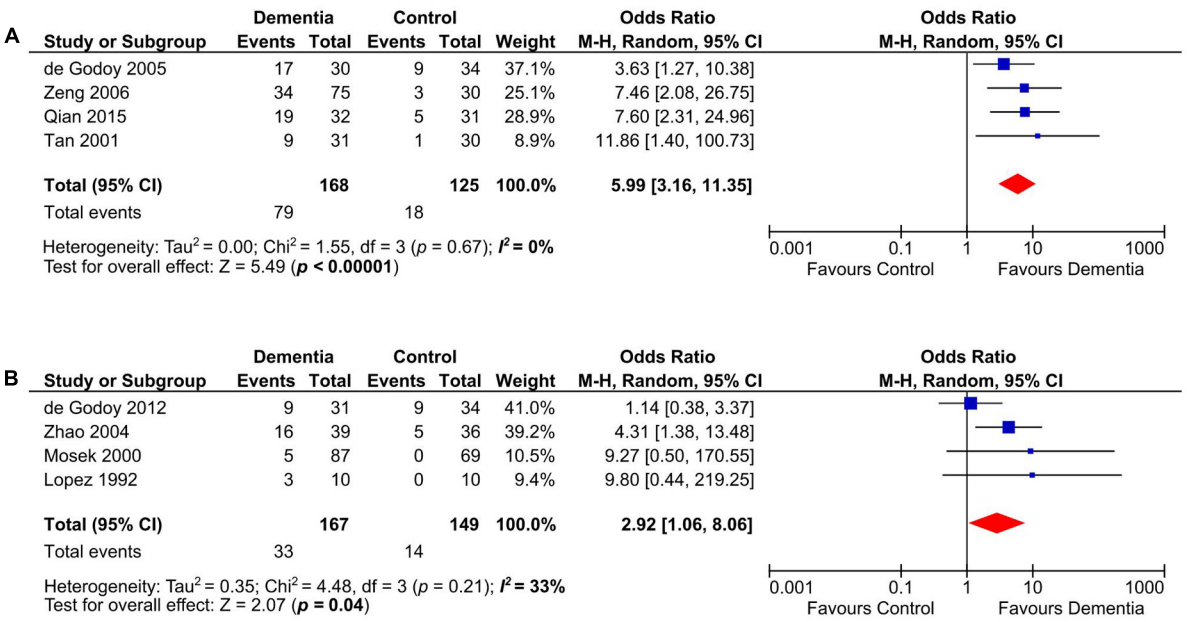

FIGURE 4 | Subgroup analysis of aCL in demetia patients with age range of 60–70 years **(A)** and above 70 years old **(B)**.

motor function as follows: (1) Jacobson et al. (1999) reported that the frequency of impaired neuropsychologic performance was significantly higher among young individuals with aPLs ($n = 27$) as compared with controls ($p < 0.01$). (2) Primary data of a longitudinal study demonstrated that aCL was positive up to 19% of subjects with impaired cognitive and motor function (Arvanitakis et al., 2012); (3) Another cohort study on normal population ($n = 1895$) without neurological disease including dementia indicated that aCL-positive subjects performed worst on the Mini Mental State Examination (MMSE) cognitive scale,

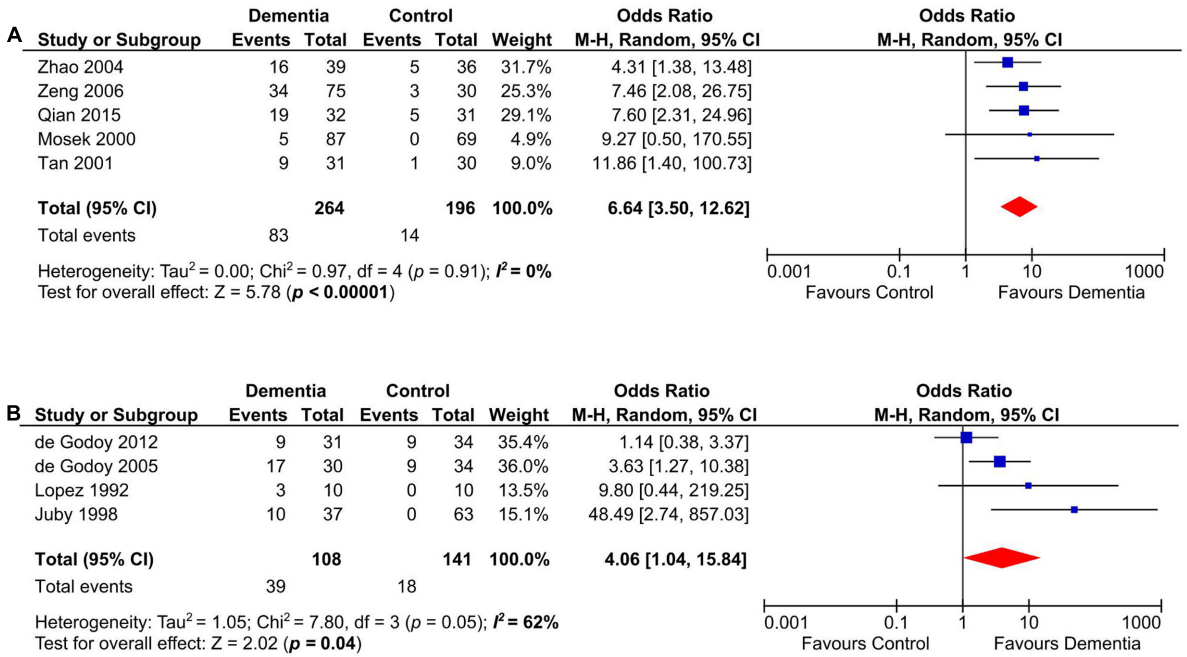

FIGURE 5 | Subgroup analysis of aCL in Asian and European **(A)**, North and South American **(B)** dementia populations.

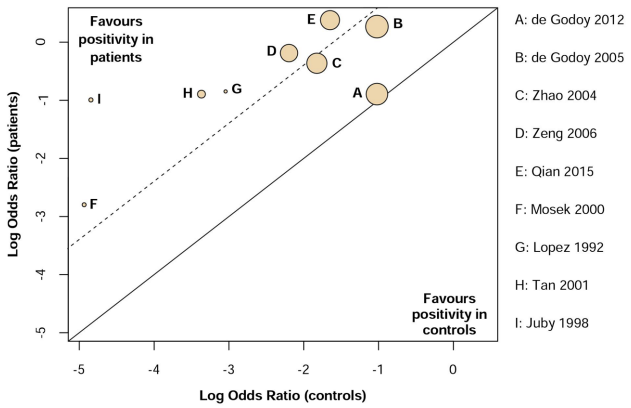

FIGURE 6 | L'Abbé plot suggests no substantial heterogeneity for the assessment of aCL antibodies.

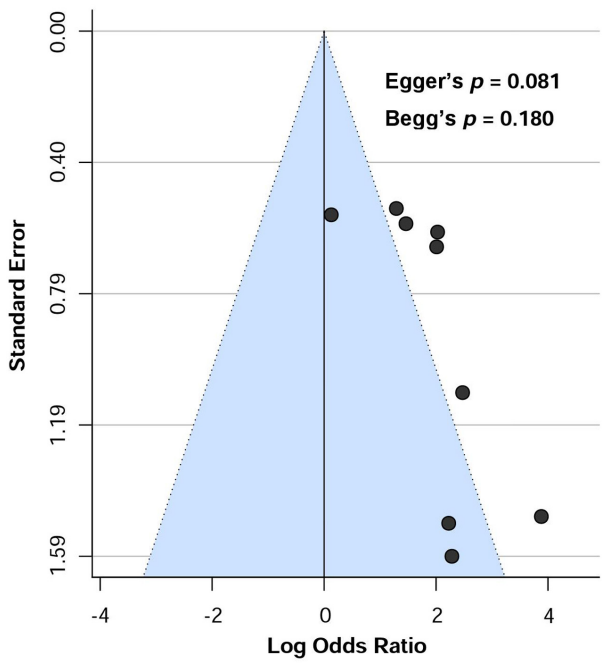

FIGURE 7 | Funnel plot showing the risk of publication bias assessing the presence of aCL antibodies in dementia patients.

suggesting an aCL-mediated mechanism in cognitive decline (Homayoon et al., 2014).

In addition, our subgroup analyses showed that aCL was significantly present in patients with VD but not DAT. aCL has been observed to be associated with stroke development (Levine et al., 1987; Muir et al., 1994; Janardhan et al., 2004). It has been hypothesized that aCL-induced thrombotic events may contribute to multiple cerebral thrombotic symptoms and exert greater aggression to the brain (de Godoy et al., 2000). aCL antibodies may exert VD via vascular events similar with that seen in aCL-associated strokes (Tan et al., 2001).

Although dementia has been observed to comorbid with aPLs, the causative role of aPLs in dementia is still inconclusive. The BBB is an interface comprising of endothelial cells, astrocyte

end-feet and pericytes, separating the brain involving neurons, blood vessels and glial cells from the circulatory system (Ballabh et al., 2004; Capani et al., 2016). BBB protects the central nervous system (CNS) by blocking the entry of harmful substances while allowing the transport of essential molecules (Lee et al., 2016). Interestingly, aCL antibodies have shown a direct binding affinity towards astrocytes in an *in vivo* study (Sun et al., 1992). aCL could inhibit the proliferation of astrocytes that ultimately distort the structure and function of BBB in addition of eliciting thrombus formation in the brain's blood vessels (Yu et al., 1991; Sun et al., 1992). Direct binding of aPLs to brain tissues (Kent et al., 1997; Caronti et al., 1998a,b; Kent et al., 2000) after BBB disruption might be a potential pathogenic mechanism of dementia development.

aPLs mainly react with phospholipids, phospholipid-protein complexes, and phospholipid-binding proteins (Fischer et al., 2007; Misasi et al., 2015). Brain tissues comprising of gray and white-matter contain high proportion of phospholipids (especially on brain cell membranes) such as phosphatidylcholine, phosphatidylserine, phosphatidylinositol, and sphingomyelin (Hamberger and Svennerholm, 1971; Kwee and Nakada, 1988; Calderon et al., 1995) which may become the target of aCL antibodies. In an *in vivo* study, aCL antibodies were found to bind with only brain tissues when compared with liver tissues (Sun et al., 1992). In addition, through a damaged BBB, aCL antibodies were also found to diffuse from blood circulation to CNS as aCL antibodies were simultaneously found in the cerebrospinal fluids of different neurologic disorders such as multiple sclerosis, neurosyphilis and Guillain–Barré syndrome (Harris et al., 1985). Thus, there might exist a selective mechanism of aCL antibody binding with phospholipids of brain tissues, contributing to dementia pathogenesis.

Past studies have shown that aPL-positive subjects for more than 20 years had higher risk of developing dementia (Mosek et al., 2000; Chapman et al., 2002). In transgenic animal model of AD, prolonged exposure of aPLs in the brain was found to generate AD-like pathology including accumulation of amyloid peptides, formation of mature amyloid plaque as well as development of behavioral and cognitive changes (Katzav et al., 2011). The researchers proposed that BBB break down might occur via inflammation, coagulation and direct antibody binding such as aPLs (Katzav et al., 2011).

In patients with VD, cognitive impairments occur due to cerebrovascular disease and ischemic or hemorrhagic brain injury (Iemolo et al., 2009). Increasing evidences of BBB dysfunction have been observed in stroke patients and cerebrovascular incidents are believed to play significant roles in VD development (Ueno et al., 2015; van de Haar et al., 2015). On the other hand, altered BBB was also observed in VD without brain infarcts, suggesting infarction-beyond pathogenesis of VD via BBB disruption (Wallin et al., 1990). Cerebrovascular events have been observed in patients exhibiting aCL antibodies and thought to contribute in the pathogenesis via triggering thrombotic events (Kushner, 1990; de Godoy et al., 2000; Brey et al., 2002). Chapman et al. (1999) reported that IgG aCL antibodies could disrupt neuronal function via permeabilization and depolarization of brain synaptoneurosomes by direct action on nerve terminals. Therefore, subsequent chronic permeabilization could lead to irreversible damage and neuronal loss which might explain our findings of significant presence of aCL in patients with dementia. Therefore, in terms of VD, aCL antibodies might have indirect pathogenic contributions via either developing cerebrovascular events or direct immune-mediated mechanisms.

Blood-brain barrier dysfunction was reported in a group of demented patients (AD = 56; VD = 29) with white-matter changes without evidences of stroke (Wallin et al., 2000). This study concludes that BBB dysfunction might be linked with vasculature and tissue damage. Interestingly, a significant association was observed ($p < 0.05$) between the presence of aCL antibodies and cerebral damage in white-matter of neuropsychiatric SLE patients (Steens et al., 2006). Another study reported that reduced white-matter volume was associated with the presence of aPLs in SLE patients with cognitive impairment (Appenzeller et al., 2007). Therefore, besides BBB, white-matter region could be a potential target of aCL antibodies in the pathogenesis of dementia.

In both AD and VD, oxidative stress is an established phenomenon to be involved in the pathogenesis (Cervellati et al., 2014; Luca et al., 2015; Alam et al., 2016; Islam et al., 2017c). In an experimental mouse model, aCL antibodies were significantly associated with decreased paraoxonase activity and reduced nitric oxide levels (Alves et al., 2005), which suggests the involvement of aCL in inducing oxidative stress.

Several limitations should be noted in this meta-analysis. Firstly, the number of included studies ($n = 9$) in the meta-analysis was relatively low. However, aCL was significantly associated with dementia patients regardless of age ranges (60–70 vs. above 70 years old) nor patients from different geographical continents (Asian and European vs. North and South Americans), suggesting the reproducibility of aCL-dementia association across different age groups and nationalities. Secondly, the cut-off values of aCL antibody positivity were different from one study to another. Thirdly, the diagnostic criteria followed to confirm dementia varied across the studies. Finally, although neither Begg's nor Egger's tests showed significant publication bias, visual inspection of the funnel plot demonstrated asymmetrical distribution of included studies showing a trend towards publication bias. This discrepancy was possibly due assessment of heterogeneity using lower number of studies (<10).

Although a few case-reports and cohort studies reported the presence (Inzelberg et al., 1992; Kurita et al., 1994; Van Horn et al., 1996; Ciubotaru et al., 2002) or absence (Friedman, 2011; De Maeseneire et al., 2014) of anti-β2-GPI and LA in dementia patients, to the best of our knowledge, no case-control studies assessing anti-β2-GPI and LA in dementia patients have been conducted. In addition, the role of aCL antibodies in the pathogenic mechanisms of dementia remains unclear, and further research is required to establish the potential involvement of aCL antibodies in the pathogenesis of dementia.

CONCLUSION

Anticardiolipin antibodies were significantly present in dementia patients compared to healthy controls, underscoring the potential to screen aCL-positive subjects for early symptoms of neurological impairment and dementia, as well as suggesting the important role of aCL antibodies in the pathogenesis of dementia.

AUTHOR CONTRIBUTIONS

MAI and KKW conceived and designed the study. MAI and FA searched the databases and KKW participated in the study selection process. MAI, FA and KKW analyzed and interpreted the data. MAI, FA and KKW drafted the manuscript. THS, SHG and MAK critically edited, reviewed and approved the final version of the submitted manuscript.

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SUPPLEMENTARY MATERIAL

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Vitamin D, Homocysteine, and Folate in Subcortical Vascular Dementia and Alzheimer Dementia

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Dementia is a worldwide health problem which affects millions of patients; Alzheimer's disease (AD) and subcortical vascular dementia (sVAD) are the two most frequent forms of its presentation. As no definite therapeutic options have been discovered, different risk factors for cognitive impairment have been searched for potential therapies. This report focuses on the possible evidence that vitamin D deficiency and hyper-homocysteinemia can be considered as two important factors for the development or the progression of neurodegenerative or vascular pathologies. To this end, we assessed: the difference in vascular risk factors and vitamin D-OH25 levels among groups of sVAD, AD, and healthy age-matched controls; the association of folate, B12, homocysteine, and vitamin D with sVAD/AD and whether a deficiency of vitamin D and an increment in homocysteine levels may be related to neurodegenerative or vessel damages. The commonly-considered vascular risk factors were collected in 543 patients and compared with those obtained from a healthy old volunteer population. ANOVA group comparison showed that vitamin D deficiency was present in demented cases, as well as low levels of folate and high levels of homocysteine, more pronounced in sVAD cases. The statistical models we employed, with regression models built, and adjustments for biochemical, demographic and neuropsychiatric scores, confirmed the association between the three measures (folate decrease, hyperhomocysteinemia and vitamin D decrease) and dementia, more pronounced in sVAD than in AD.

Keywords: sVAD, AD, homocysteine, vitamin D-OH 25, neurodegeneration, inflammation

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INTRODUCTION

Dementia is a major clinical condition, which increases in prevalence and incidence, more rapidly with advancing age. The consequence is the macroscopic alteration of the daily living abilities. However, the different types of dementia have various causes and pathogenesis (Reitz et al., 2011). It is accepted that many cerebrovascular alterations coexist or are determinant even in neurodegenerative disorders such as AD, and the two different conditions share many different risk factors (Jellinger, 2013).

Whereas, the operative definition of AD is established, it is more difficult to define the criteria for subcortical vascular dementia, which is a clinical entity related to small vessel muscle cells disease with consequent hypo-perfusion, diffuse ischemic white matter lesions, and incomplete

ischemic damage (Rockwood, 2003; Korczyn et al., 2012; Jellinger, 2013). These are more frequently localized in the white matter, basal ganglia, thalamus and pons, with surrounding astrocytes and oligodendrocytes (Chui, 2001; Moretti et al., 2005). Clinical characteristic features of sVAD are progressive signs of a dysexecutive syndrome, reduced planning, and cognitive flexibility, decreased processing speed, and behavior alterations, such as depression and apathy (Meyer et al., 2000; Moretti et al., 2011; Roh and Lee, 2014; Shi and Wardlaw, 2016).

Risk factors have been debated in dementia, especially metabolic features, hormonal changes, and vitamin alterations; these aspects have been related to a molecular pathogenesis in all the forms of cognitive decline, and hopefully, to a more effective management. Therefore, the target of many investigations has been to identify nutrition and lifestyle-based risk factors related to dementia, in order to develop possible future primary prevention efforts (Karakis et al., 2016). Many studies have been devoted to the increase of homocysteine, due to low folate and vitamin B12 deficiency in cognitive altered process; these studies focused on homocysteine effects on endothelium and neuronal disruption acting as a promoter of neurodegenerative events (Nelson et al., 2016; Smith and Refsum, 2016), but results are quite confounding, sometimes puzzling, and unclear (Malouf and Grimley Evans, 2008; Hainsworth et al., 2016; Moretti et al., 2017).

In the recent years, an increasing evidence supports a role for the fat-soluble vitamin D in brain function and development (Holick, 2004; McGrath et al., 2004; Schlogl and Holick, 2014; Granic et al., 2015). Vitamin D deficiency has been related with AD due to (1) a neurodegenerative accelerating property (Afzal et al., 2014; Prabhakar et al., 2015); (2) vascular acute damage, due to a presumed endothelium modification caused by a vitamin D direct effect (Sakurai et al., 2014; Prabhakar et al., 2015); and (3) to white matter hyper intensities (WMHs), lacunas and microbleeds in elderly with amnestic mild cognitive impairment (Sakurai et al., 2014; Chung et al., 2015). However, very few data are available regarding the vitamin D status in patients with pure sVAD (Prabhakar et al., 2015). Prabhakar et al. (2015) reported that hypertension and vitamin D deficiency considerably increase the odds of VaD (Prabhakar et al., 2015). On the other hand, a very recent study (Olsson et al., 2017) failed to show an association between baseline vitamin D-status and long-term risk of dementia or cognitive impairment over an 18 years period of time.

Therefore, the purposes of our cross-sectional study are to assess:

1. The differences in vascular risk factors and vitamin D-OH25 levels among groups of sVAD, AD, and healthy controls.
2. The association of folate, B12, homocysteine, and vitamin D with sVAD/AD.

SUBJECTS' CHARACTERISTICS

We conducted a cross-sectional study in a neurological group of patients, affected by AD and sVAD and compared their results with a normal healthy age population. From June 1st 2012 to June 1st 2015 a total of 543 cases were included in the study. 87 men and women had, suffering from Alzheimer's Disease, according to NINDCS-ADRDA (McKhann et al., 1984) criteria and the DSM-V (Fifth Edition), and 456 patients suffering from subcortical vascular dementia, in accordance with the NINDS-AIREN criteria (Chui et al., 1992; Román et al., 1993, see data and literature in Olsson et al., 2017). AD subjects had to show on brain MRI the pattern of hippocampal atrophy, and of the temporoparietal and precuneus regions (Weinstein et al., 1993). sVaD was diagnosed when the CT/MRI scan showed moderate to severe ischemic white matter changes and at least one lacunar infarct (Erkinjuntti et al., 1987; Marshall et al., 2006). As well accepted by literature (see data in Kim et al., 2014) all the patients had severe white matter hyperintensities on MRI, defined as peri-ventricular white matter hyperintensities, localized around the lateral ventricles or white matter hyperintensities, within the deep white matter (see data in Fazekas et al., 1987; Cleutjens et al., 2017). Brain CT-scans or MRI images were assessed independently by the neurologist (RM), after the radiologist's opinion. Brain CT-scans or MRI images were available for all the 543 patients; 362 patients did MRI studies, 181 did CT scans; 298 patients did CT plus MRI. A neurologist (RM) revised all the imaging, employing the Blennow scale for CT scans (Blennow et al., 1991; Wallin and Blennow, 1991) and the Scheltens scale for MRI imaging (Scheltens et al., 1993) in accordance with parameters of recent literature (Kim et al., 2014). There was 93.8% inter-rater agreement for the independent assessment of the scans (kappa = 0.79). Patients were not included in the study if they showed signs of normal pressure hydrocephalus, previous brain tumors, and previous diagnosis of major cerebrovascular disease, white matter lesions, caused by different specific etiologies, such as multiple sclerosis, collagen vascular disease, and genetic forms of vascular dementia (such as CADASIL or CARASIL). Patients with previous major psychiatric illness (i.e., schizophrenia, bipolar disorders, psychosis, compulsive-obsessive disorders, etc.) or central nervous system disorders and alcoholism were excluded too.

Exclusion criteria were the absence of an informed caregiver, unavailability of neuro-radiological examination, and/or the assumption of psychotropic drugs within 2 months prior to the clinical assessment. Seven patients were excluded by the lack of a sufficiently informed caregiver and 18 because they had assumed psychotropic drugs during the 2 months prior to our assessment.

Our control group was composed by healthy subjects, relatives, or caregivers of the patients, with no history of cerebrovascular diseases or degenerative disorders, who voluntarily accepted to take part in the study, matched for age, gender and educational level.

Study subjects underwent a standardized baseline assessment that included a detailed history, a physical examination, laboratory tests, and psychiatric evaluations. The physical examination included cardiac and blood pressure examination, peripheral pulses, retinal vessel, electrocardiographic evaluation. All patients were followed-up with periodical neurological and neuropsychological examinations. The present study was conducted in accordance with the Declaration of Helsinki and with the Ethics

Guidelines of the Committee of the University-Hospital of Trieste, which approved it, and written informed consent was obtained from all the participants or from their caregivers.

METHODS

Patients with AD were grouped in Group A while patients with sVAD were assigned to Group B; controls were Group C. The main outcomes of the study for the AD and sVAD patients were: (1) global performance, assessed using the Mini-Mental State Examination (Folstein et al., 1975); (2) Frontal Assessment Battery (FAB; Dubois et al., 2000); (3) global behavioral symptoms, assessed by the Neuropsychiatric Inventory, NPI (Cummings et al., 1994); and (4) the caregiver stress, assessed by the Relative Stress Scale, RSS (Kinney and Stephens, 1989). Demographic details were registered for all the study subjects. All the patients and controls attended the LABS tests at the Hospital service, in order to reduce potentially different lab methods or different value parameters.

Hypertension was defined as diastolic blood pressure (DBP) $\geq$ 90 mm Hg and/or systolic blood pressure (SBP) $\geq$ 140 mm Hg. Diabetes mellitus was defined as venous plasma glucose concentration of $\geq$ 120 mg/dl after an overnight fast (Cummings et al., 1994). Glycated Hemoglobin (HbA1c) results have been aligned to the assay used in the Diabetes Control and Complications Trial (DCCT), expressed as a percentage (DCCT-HbA1c); non-diabetic "normal" range being 4–5.6%; 5.5–6.5 high risk of diabetes; more than 6.6% as having diabetes (WHO, IDF, 2006; Nathan et al., 2008; Weykamp, 2013). Fasting venous blood samples were collected, centrifuged immediately and stored at −80°C for further laboratory analysis. Clinical laboratory measurements, including serum total cholesterol, triglycerides, and high-density lipoprotein (HDL) cholesterol, have been determined enzymatically and low-density lipoprotein (LDL) cholesterol was calculated using Friedewald's formula (Friedewald et al., 1972). Serum levels of 25(OH)D were measured using enzyme immuno-assay kits (DIAsource Immunoassay S.A. Belgium) and quality control materials provided by the manufacturer. As Prabhakar et al. (2015) we employed the National Osteoporosis Society (NOS; Aspray et al., 2014) and therefore, subjects were categorized into Vitamin D deficiency [25(OH)D: $\leq$ 12 ng/ml], insufficiency [25(OH)D: 12–20 ng/ml] and sufficiency [25(OH)D: N 20 ng/ml] groups.

In order to have a complete evaluation of calcium metabolism, we tested at the same time the level of calcium and PTH. The level of folate, vitamin B12 levels and Homocysteine were also tested to obtain specific measures of vascular risk factors.

Statistical Analysis

Statistical analysis was performed with SPSS statistics 17.0 (SPSS, version 17.0). The difference in baseline characteristics between AD and sVAD and controls was assessed by ANOVA test for categorical variables; in case the ANOVA results were found significant, the multiple comparison analysis was also done by and the Tukey Test, to examine those two groups which were significantly different for each other. The multinomial logistic regression method was applied to analyze the relationship between disease status (AD, sVAD and control) considering them as dependent variable (non-metric) and age (metric), sex (non-metric), Hb1Ac, Cholesterol, and lipid parameters, calcium, PTH, vitamin D-OH25, folate, vitamin B12, and Homocysteine (all metric) as independent variable. The utility of present analysis (Multinomial Logistic regression) was assessed by classification accuracy, which compares the predicted disease group based on logistic model to the actual disease group (which is the value for dependent variable). Univariate odds ratios and 95% confidence intervals were estimated by binary logistic regression analysis. Spearmann's rank correlation analysis was calculated for the demographic variable. $P \leq 0.05$ were considered statistically significant. Results are presented as mean with standard deviations, and *p*-values are presented where appropriate.

RESULTS

Eighty-seven AD patients and 456 sVAD patients were enrolled in the study. One AD patient and five sVAD patients died during the 12-months follow-up; therefore, 86 AD patients and 449 sVAD patients completed the study. Baseline neuropsychological characteristics of the study groups are presented in **Table 1**.

The demographic variables i.e., age and gender were not significantly associated with the dementia status in both AD and sVAD. The demographic variables (age and gender) were not significantly associated with the dementia status in both AD and sVAD. One way analysis of variance (ANOVA) method was applied to explore the statistical significant difference among mean value in three groups (AD, sVAD, and control, **Table 2**). Four of the different biochemical variables (low folate and vitamin B12, low vitamin D-OH25, and high homocysteine) studied were significantly different ($p < 0.001$) in three groups (**Table 2**), which suggested that at least one average out of the three was statistically different than the other (**Table 3**); to explore such group, the multiple comparison analysis was done by Tukey test (**Table 4**). In AD group, mean vitamin D-OH 25 values (10.8 $\pm$ 1.05)were significantly lower than control (18.7 $\pm$ 3.05), mean folate levels (2.4 $\pm$ 0.3) were significantly lower than control (6.4 $\pm$ 0.2), mean vitamin B12 levels (129 $\pm$ 23.2) were significantly lower than controls (249 $\pm$ 13.2) and mean homocysteine levels were significantly higher (18.3 $\pm$ 3.5) than controls (11.1 $\pm$ 3.5).

In sVAD group, mean vitamin D-OH 25 values (9.1 $\pm$ 1.09) were significantly lower than control (18.7 $\pm$ 3.05), mean folate levels (1.9 $\pm$ 0.5) were significantly lower than control (6.4 $\pm$ 0.2), mean vitamin B12 levels (111 $\pm$ 26.2) were significantly lower than controls (249 $\pm$ 13.2) and mean homocysteine levels were significantly higher (23.5 $\pm$ 3.4) than controls (11.1 $\pm$ 3.5).

According to the NOS criteria (Aspray et al., 2014) we have found that there is a very significant difference in the prevalence of vitamin D-OH 25 deficiency and insufficiency between the three groups (**Table 3**). In AD population, 14% is deficient of vitamin D-OH25, 79% is insufficient, and only 7% is sufficient; in sVAD population, 11% is deficient of vitamin D-OH25, 86%

TABLE 1 | Neuropsychological characteristics of study population (mean and SD in brackets).

Characteristics	Group A	Group B	Group C	*F* chi^2 value	DF	*p*-value
Age	77.9 ± 2.01	75.65 ± 6.54	76.4 ± 2.3	2.66	2.24	0.73
MMSE score	19.4 (2.3)	24.2 (3.5)	27.9 (1.1)	0.71	2.157	0.01
FAB score	9.3 (2.5)	8.4 (1.3)	10.6 (1.2)	0.87	2.3	0.01
NPI score	20.3 (4.6)	16.1(3.2)	7.2 (3.1)	0.75	2.43	0.01
RSS score	42.3 (6.7)	27.3 (3.5)	8.1 (3.2)	0.89	2.02	0.01

TABLE 2 | Comparison of mean value of age, gender, educational level, various biochemical parameters in AD, sVAD, and controls.

Variable (Normal values)	Group A (86)	Group B (449)	Group C (567)	*F* chi^2 value	DF	*p*-value
Age	77.9 ± 2.01	75.65 ± 6.54	76.4 ± 2.3	2.66	2.24	0.73
Gender M/F	41/45	193/256	197/370	0.79	2	0.68
Educational level (years)	7.7 ± 3.4	8.1 ± 1.22	8.0 ± 0.9	0.67	3.11	0.45
Hb1ac (%)	6.1 ± 0.3	6.7 ± 0.7	5.6 ± 0.4	0.79	2.31	0.79
Cholesterol (mg/dl)	197.4 ± 34.6	207.5 ± 21.3	186.4 ± 24.6	0.67	2.251	0.75
Trygliceridis (mg/DL)	139.5 ± 23.9	129.3 ± 25.6	99.5 ± 34.2	0.87	2.13	0.67
HDL (mg/dl)	35.7 ± 12.1	31.1 ± 7.2	39.1 ± 14.1	0.67	2.45	0.43
LDL (mg/dl)	133.8 ± 13.4	150.5 ± 12.2	127.4 ± 7.4	0.87	2.65	0.37
Calcium (8.610.5 mg/dl)	9.8 ± 1.1	10.1 ± 0.4	11.2 ± 2.5	0.65	2.31	0.43
PTH (12–72 pg/ml)	39 ± 2.3	41.3 ± 2.7	37 ± 1.6	0.76	2.11	0.34
Vitamin D-OH 25 (30–100 ng/ml)	10.8 ± 1.05	9.1 ± 1.09	18.9 ± 3.05	2.66	2.251	0.01
Folate (3.89–26.0 ng/ml)	2.4 ± 0.3	1.9 ± 0.5	6.4 ± 0.2	0.71	2.157	0.01
Vitamin B12 (205–870 pg/ml)	129 ± 23.2	111 ± 26.2	249 ± 13.2	2.09	2.35	0.04
Homocysteine (3–15 mcmol/l)	18.3 ± 3.5	23.5 ± 3.4	11.1 ± 3.5	5.77	2.41	0.01

is insufficient and only 3% is sufficient; whereas, in a healthy old population, even considering the declared cases of osteoporotic patients, only 0.17% is deficient of vitamin D-OH25, 24% is insufficient and 76% is sufficient. According to our laboratory cut-off scores, we have found that there is very significant difference in the prevalence of high values of homocysteine (16–20 mcmol/L) and very high levels of homocysteine (21–30 mcmol/L) between the three groups (**Table 3**). In AD population, 74.8% has high levels of homocysteine, 15.1% has very high levels of it and 11% is in normal range; in sVAD population, 67.1% has high levels of homocysteine, 21.8 % has very high values, and only 5.1% is in normal range; whereas in a healthy old population, only 15.1% has high levels of homocysteine, 84.8% is in normal range and 0% has very high levels of it. Considering our laboratory cut-off scores, we have found that there is a very significant difference in the prevalence of low levels of folate (2–3.88 ng/ml) and very low levels of folate (0.5–2.0 ng/ml) between the three groups (**Table 3**). In AD population, 90.7% has low levels of folate, 2.3% has very low levels of it and 7% is in normal range; in sVAD population, 47.1% has low levels of folate, 49.6% has very low values of it, and only 8.7% is in normal range; whereas, in a healthy old population, only 11.1% has low levels of folate, 0.2% has very low levels of it, and 88.7% is in normal range. Considering our laboratory cut-off scores, we also have found that there is a very significant difference in the prevalence of low levels of vitamin B12 (100–204 pg/ml) and very low levels of vitamin B12 (50–99 ng/ml) between the three groups (**Table 3**). In AD population, 88.4% has low levels of vitamin B12 and 11.6% is in normal range; in sVAD population, 70.7% has low levels of vitamin B12 and 29.3% is in normal range; whereas, in a healthy old population, only 25.9% has low levels of vitamin B12, and 74.1% is in normal range. Nobody reported very low levels of vitamin B12.

The univariate regression analysis reveals crude odds ratio for the association between AD and vitamin D insufficiency of 3.4 (95% CI: 10.93–13.56), $p = 0.05$ and vitamin deficiency of 5.6 (95% CI: 8.93–11.56), $p = 0.023$; there is an odd ratio for the association between AD and low levels of folate of 3.7 (95% CI: 2.1–3.4), $p = 0.047$ and very low levels of folate of 4.9 (95% CI: 1.1–2.1), $p = 0.027$; there is a odds ratio for the association between AD and low levels of vitamin B12 of 3.1 (95% CI: 112.1–203.4), $p = 0.046$; there is odds ratio for the association between AD and high levels of homocysteine of 4.5 (95% CI: 18.1–19.4), $p = 0.036$ and of 5.9 with very high levels of homocysteine (95% CI: 20.1–23.4), $p = 0.016$.

The univariate regression analysis reveals crude odds ratio for the association between sVAD and vitamin D insufficiency of 4.1 (95% CI: 11. 3–12.5), $p = 0.034$ and vitamin deficiency of 6.7 (95% CI: 7.3–9.6), $p = 0.01$; there is an odd ratio for the association between sVAD and low levels of folate of 4.1 (95% CI: 2.0–2.9), $p = 0.03$and very low levels of folate of 5.9 (95% CI: 1.1–2.1), $p = 0.01$; there is a odds ratio for the association between sVAD and low levels of vitamin B12 of 3.9 (95% CI: 101.1–163.4), $p = 0.041$; there is odds ratio for the association between sVAD

TABLE 3 | Comparison of mean value of vitamin D-OH, folate, vitamin B12, and homocysteine (divided by different levels) parameters in D, sVAD, and controls.

	Group A (86)	Group B (449)	Group C (567)	*F* chi^2 value	DF	*P*-value
Vitamin D-OH25 deficiency (≤ 12 ng/ml)	12 (14%) pts 8.1 (2.9) ng/ml	49 (11%) pts 4.5 (2.1) ng/ml	1 (0.17%) pts 11.1 (1.1) ng/ml	0.67	2.25	0.01
Vitamin D-OH25 insufficiency (12-20 ng/ml)	68 (79%) pts 15.7 (2.1)	387 (86%) pts 12.1 (9.33)	134 (24%) pts 19.1 (2.1)	0.87	2.13	0.01
Vitamin D-OH25 sufficiency (≥20 ng/ml)	6(7%) pts 23 (2.7)	13 (3%) pts 21 (1.9)	432 (76%) pts 29.4 (2.6)	0.71	2.6	0.43
Homocysteine (3-15 mcmol/l)	10 (11%) pts 10.7 (2.9)	23 (5.1%) pts 11.1 (1.3)	481 (84.8%) pts 8.4 (2.9)	0.81	2.1	0.47
Homocysteine (16–20 mcmol/l)	63 (74.8%) pts 18.5 (2.1)	301 (67.1%) pts 19.3 (1.7)	86 (15.1%) pts 17.1 (2.1)	0.84	2.6	0.05
Homocysteine (21–30 mcmol/L)	13 (15.1 %) pts 24.3 (2.1)	125 (21.8%) pts 26.7 (2.5)	0 pts 0	0.8	2.1	0.01
Folate (3.89–26 ng/ml)	6 (7%) pts 9.7 (1.1)	39 (8.7%) pts 8.5 (2.3)	503 (88.7%) pts 9.1 (1.7)	0.67	2.11	0.47
Folate (2–3.88 ng/ml)	78 (90.7%) pts 2.9 (1.2)	187 (41.7%) pts 1.7 (2.3)	63 (11.1%) pts 3.1 (1.0)	0.81	2.27	0.01
Folate (0.5–2.0 ng/ml)	2 (2.3%) pts 1.1 (20.7)	223 (49.6%) pts 0.8 (1.1)	1 (0.2%) pts 1.2 80.3)	0.81	2.1	0.01
Vit. B12 (205–870 PG/ML)	10 (11.6%) pts 264 (12.5)	132 (29.3%) pts 251 (13.1)	420 (74.1%) pts 305 (23.1)	0.86	2.6	0.45
Vit. B12 (100–204 PG/ML)	76 (88.4%) pts 125.4 (11.3)	317 (70.7%) pts 102.6 (23.1)	147 (25.9%) pts 167.4 (23.1)	0.59	2.6	0.05
Vit. B12 (50–99 PG/ML)	0 pts 0	0 pts 0	0 pts 0	na	na	na

and high levels of homocysteine of 5.9 (95% CI: 17.1–19.7), $p = 0.01$ and of 6.1 with very high levels of homocysteine (95% CI: 23.1–33.4), $p = 0.016$.

Table 5 shows the relationship of the disease state (AD and sVAD) with age, gender, vitamin D-OH25, folate, vitamin B12, and homocysteine levels. The presence of a relationship between them was checked based on the statistical significance of the final model chi-square and existence of a relationship was established. Out of the six considered independent variables, vitamin D-OH 25 levels, folate, and homocysteine had significant contribution toward AD and sVAD groups. Moreover, the regression coefficient (B) for vitamin D-OH25 was −0.46 for AD, indicating that the increase of vitamin D-OH 25 decreased the likelihood of dementia in AD group, with an exponential B value of 0.82 (95% CI: 2.5–10.11), which implies that for an increase of vitamin D-OH 25 levels the odds of having AD decreased by 18%; the regression coefficient (B) for folate was −0.88 for AD, indicating that the increase of folate decreased the likelihood of dementia in AD group, with an exponential B value of 0.91 (95% CI: 2.1–9.1), which implies that for an increase of folate levels the odds of having AD decreased by 9%; the regression coefficient (B) for homocysteine was + 0.57 for AD, indicating that the decrease of homocysteine decreased the likelihood of dementia in AD group, with an exponential B value of 0.85 (95% CI: 2.1–9.1), which implies that for a decrease of homocysteine levels the odds of having AD decreased by 15%.

The regression coefficient (B) for vitamin D-OH25 was −0.39 for sVAD, indicating that the increase of vitamin D-OH 25 decreased the likelihood of dementia in AD group, with an exponential B value of 0.79 (95% CI: 2.0–17.1), which implies that for an increase of vitamin D-OH 25 levels the odds of having sVAD decreased by 21%; the regression coefficient (B) for folate was −0.77 for sVAD, indicating that the increase of folate decreased the likelihood of dementia in sVAD group, with an exponential B value of 0.89 (95% CI: 2.1–8.7), which implies that for an increase of folate levels the odds of having sVAD decreased by 11%; the regression coefficient (B) for homocysteine was +0.23 for sVAD, indicating that the decrease of homocysteine

TABLE 4 | Multiple comparison analysis (Tukey test) of various biochemical parameters in AD, sVAD, and controls.

Variable	Mean Diff.	SE of mean Diff.	*p*-value
VIT. D-OH-25			
A vs. C	−7.9	−2.01	0.01
B vs. C	−9.6	−1.96	0.01
FOLATE			
A vs. C	−4	−0.1	0.01
B vs. C	−4.5	−0.3	0.01
VIT. B12			
A vs. C	−120	−10.1	0.01
B vs. C	−138	−13-0	0.01
HOMOCYSTEINE			
A vs. C	+7.2	0.4	0.01
B vs. C	+12.4	0.1	0.01

decreased the likelihood of dementia in sVAD group, with an exponential B value of 0.8 (95% CI: 2.1–13.4), which implies that for an increase of homocysteine levels the odds of having sVAD decreased by 20%.

In present analysis, the classification accuracy rate of logistic model was 57.1% which was greater than the proportional by chance accuracy; the criteria for classification accuracy was satisfied.

In addition, serum 25(OH)D levels were found to be significantly and directly correlated with low levels of folate in the vitamin D-deficient group (Spearmann's correlation coefficient, $r = 0.7$ and 0.9, $p < 0.01$, respectively for Group A and B), and there is significant correlation between low levels of vitamin B12 in the vitamin deficient group (Spearmann's correlation coefficient, $r = 0.8$ and 0.9, $p < 0.01$, respectively for Group A and B), and finally, there is significant correlation with higher levels of homocysteine in the vitamin deficient and in the vitamin insufficient groups (Spearmann's correlation coefficient, $r = 0.75$ and 0.89, $p < 0.01$, respectively for Group A and B in the deficiency; Spearmann's correlation coefficient, $r = 0.69$, $p < 0.05$ and 0.71, $p < 0.05$, respectively for Group A and B in the insufficient).

DISCUSSION

This study was aimed to answer the questions specified in the introduction and namely whether alteration on the serum levels of folate, B12, homocysteine, and vitamin D may be related to neurodegenerative or vascular damage and our results are in line with previously reported studies (Friedewald et al., 1972; Folstein et al., 1975; McKhann et al., 1984; Erkinjuntti et al., 1987; Fazekas et al., 1987; Kinney and Stephens, 1989; Blennow et al., 1991; Wallin and Blennow, 1991; Chui et al., 1992; Román et al., 1993; Scheltens et al., 1993; Weinstein et al., 1993; Cummings et al., 1994; Dubois et al., 2000; Marshall et al., 2006; Moretti et al., 2006; WHO, IDF, 2006; Nathan et al., 2008; Weykamp, 2013; Aspray et al., 2014; Kim et al., 2014; Sakurai et al., 2014; Chung et al., 2015; Prabhakar et al., 2015; Cleutjens et al., 2017; Olsson et al., 2017).

We found that both A and B groups have lower folate and vitamin B12 levels and a higher homocysteine, even if more pronounced in sVAD than in AD. Of notice was the data obtained with vitamin D-OH 25 level: we have established that 93% of our AD patients and 97% of our sVAD patients suffered either from deficiency or from insufficiency of vitamin D-OH 25. Moreover, we found that for an increase of vitamin D-OH 25 levels the odds of having AD decreased by 18%, for an increase of folate levels the odds of having AD decreased by 9% and for a decrease of homocysteine levels the odds of having AD decreased by 15%. In sVAD we found that for an increase of vitamin D-OH 25 levels the odds of having sVAD decreased by 21%, for an increase of folate levels the odds of having sVAD decreased by 11% and for an increase of homocysteine levels the odds of having sVAD decreased by 20%.

Our study has several limitations:

1. It is a single-center study
2. It has been designed as a cross-sectional study
3. The number of patients is small to interfere
4. It has no pathological confirm

It has some strengths:

1. All the patients can be fully examined
2. All the patients attended neuroimaging and neuropsychological evaluation
3. We have examined two distinct dementing conditions, mainly degenerative (AD) and vascular, related to small vessel disease pathologies (sVAD)
4. We have a healthy old control group
5. For the first time, we produced data concerning the superimposing effects of homocysteine high levels and low vitamin D-OH 25.

Our results seem to have some accordance with many other in literature (Afzal et al., 2014; Sakurai et al., 2014; Chung et al., 2015; Prabhakar et al., 2015) as far as vitamin D-OH low levels, but not with a recent one (Olsson et al., 2017). It also seems in accordance with many other as far as homocysteine and folate (see data and literature in McCully, 1969; Ueland et al., 2000; Hogervorst et al., 2002; Moretti et al., 2017) but not with many other (Homocysteine Studies Collaboration, 2002; Obeid and Herrmann, 2006; Miles et al., 2016).

The novelty of our work is that we report the parallel effect of two controversial factors: homocysteine and vitamin D in two different dementing condition, AD and sVAD, and it seems that the two variables create a detrimental effect, which seems to promote the neural pathology, more evident in vascular condition.

How can vitamin D-OH and homocysteine influence the neural system? Do they act as neurodegenerative promoters or as pure vascular damage factors?

Although homocysteine and vitamin D-OH 25 are different from biochemical and structural properties, their activity is surprisingly similar. The accumulation of homocysteine and the deficiency of vitamin D-OH are both toxic to neuronal

TABLE 5 | Summary of multinomial logistic regression analysis.

Variable		Univariate association			Regression coefficient (B)	SE	*p*-value	EXP (B)	95% CI for exponential (B)
		OR	95% CI	*p*-value					
Dependent	**Independent**								
AD	Age	1.1	1.1–2.3	0.76	0.023	0.015	0.6	1.006	0.9–1.03
	Sex	1.3	1.1–2.1	0.67	0.066	0.28	0.9	1.1	0.5–0.93
	Vit-D-OH-25	4.5	4.5–7.1	0.023	−0.46	0.12	0.01	0.82	2.5–10.11
	Folate	4.2	2.1–5.7	0.05	−0.98	0.17	0.01	0.91	2.1–9.1
	B12	1.8	1.2–4-5	0.45	−0.97	0.65	0.7	0.98	1.3–4.1
	Homocysteine	5.2	2.1–3.4	0.01	+0.57	0.12	0.01	0.85	2.7–10.4
sVAD	Age	1.3	1.4–2.7	0.81	0.016	0.022	0.45	0.98	0.94–1.07
	Sex	1.2	1.6–2.2	0.61	0.297	0.43	0.37	1.2	0.57–0.93
	Vit-D-OH-25	5.7	2.5–6.1	0.01	−0.39	0.12	0.01	0.79	2.0–17.1
	Folate	5.2	2.1–5.9	0.01	−0.77	0.23	0.01	0.89	2.1–8.7
	B12	2.1	1.2–7.1	0.074	−0.67	0.02	0.8	0.7	1.2–4.7
	Homocysteine	5.9	1.9–8.4	0.01	+0.23	0.01	0.01	0.8	2.1–13.4

cells. They might affect neuronal plasticity (Streck et al., 2003), with different mechanisms in early or in adult life, and improve neurodegeneration (Obeid and Herrmann, 2006), alter brain energy productions mechanisms (Streck et al., 2003), potentiate inflammation (Lazarewicz et al., 2003; Herrmann et al., 2006) and reduce the endothelium response to oxidation processes (Lazarewicz et al., 2003; Streck et al., 2003; Herrmann et al., 2006). Homocysteine regulates calcium inflow, via the activation of group I metabotropic glutamate receptors (Lipton et al., 1997; Lazarewicz et al., 2003; Robert et al., 2005; Herrmann et al., 2006; Obeid and Herrmann, 2006), and this has a relevance in the induction of brain lipid peroxidation process, and expanding the neural calcium-related apoptosis mechanism (Blom and Smulders, 2011). Moreover, homocysteine accumulation has an amyloidogenic effect by inducing the endoplasmic reticulum protein HERP, which potentiates the c-secretase activity and enhances the accumulation of AB1-40 in the brain (Mok et al., 2002; Seshadri et al., 2002). Homocysteine also increases the neural vulnerability to the damage created by amyloid accumulation (Morris, 2003). Hyper-homocystenemia upregulates PS1 genes, promoting a hypomethylation of PPM1 (Leulliot et al., 2004), causing, therefore, a hyperphosphorilation of tau protein, which seems to potentiate the microtubules transport mechanism. The activation of caspase-3 by hyperhomocystenemia promotes the accumulation of amyloid on smooth muscle cells of small vessel disease (Leulliot et al., 2004; Chun et al., 2016), which leads to a dysregulation of cerebral blood flow, commonly observed in AD and in vascular dementia. Higher homocysteine levels can be considered as an independent risk factor for moderate to severe leukaraiosis in patients with AD (Pushpakumar et al., 2014; Zhou et al., 2014). Moreover, homocysteine metabolism is regulated by the redox potential in the cell (Blom and Smulders, 2011; Pushpakumar et al., 2014; Zhou et al., 2014), by disruption of the trans-sulfuration pathway (Seshadri et al., 2002; Obeid and Herrmann, 2006; Blom and Smulders, 2011). Homocysteine is a precursor of hydrogen sulfide (H2S), which revealed to be a potent vasodilator and it regulates the vessel diameter, it seems to protect the endothelium from redox stress and chronic inflammation (Pushpakumar et al., 2014; Zhou et al., 2014). The accumulation of homocysteine can cause a reduction in nitric oxide synthesis and potentiate oxidative endothelium stress (Pushpakumar et al., 2014; Zhou et al., 2014).

When we consider vitamin D, the results are surprisingly similar. Animal models demonstrated alterations in fetal and adult animal models induced by a deficiency of vitamin D-OH 25. Vitamin D modulates different processes, such as neurogenesis, cell proliferation, differentiation, and neurotransmitter metabolism (Eyles et al., 2013, 2014; Cui et al., 2015). By exposing fetal animals to vitamin D deficiency, an evident alteration of dopamine and NMDA circuitries can be observed, with clear consequences for altered behavior, memory and motor assessment (Becker et al., 2005; Turner et al., 2013; Eyles et al., 2014; Cui et al., 2015; Overeem et al., 2016). On the other hand, the exposition of previously normally developed brains to vitamin D deficiency seems to result in memory impairment, with major conduct alterations and with less executive possibilities (Byrne et al., 2013). These brains have a reduction of the glutamic acid decarboxylase (key enzymes in gamma-aminobutyric acid (GABAergic inter-neurons), and show decreased levels of glutamate and glutamine in brain tissue (Byrne et al., 2013). In a key study (Brewer et al., 2001), it was found that vitamin D protected rat primary hippocampal cultures from excitotoxicity insults (i.e., glycine and NMDA). Patch clamp studies found that, L-type voltage-dependent calcium currents were reduced following incubation with vitamin D (Brewer et al., 2001; Carlberg et al., 2005; Balden et al., 2012; Byrne et al., 2013; Gezen-Ak et al., 2013; Suzuki et al., 2013; Cui et al., 2015); therefore it has been hypothesized that vitamin D-OH 25 might influence calcium inflow, and consequently it can regulate dendritic cell functions and neural apoptosis (Carlberg et al., 2005; Gezen-Ak et al., 2013; Suzuki et al., 2013;

Cui et al., 2015). On the other hand, vitamin D deficiency has been related to altered myocardial functions, associated with a ventricular dilation and impaired electromechanical coupling (O'Connell et al., 1997; Xiang et al., 2005; Simpson, 2011). Much more stimulating is the endothelial cells expression of the receptors for vitamin D; these receptors are up-regulated under inflammation condition in endothelial cells (Xiang et al., 2005; Bodyak et al., 2007; Wong et al., 2008) and vitamin D analogs decrease endothelium adhesion molecules, and protect against advanced glycation products (Young et al., 2011), reduce vascular smooth muscle contractions and vascular tone in hypertensive models, modulating the calcium influx across endothelial cells (Manolagas et al., 1986; Danielsson et al., 1996; Somjen et al., 2005; Wong et al., 2008; Oh et al., 2009).

However, it cannot be denied that results in Literature, concerning the two above-mentioned biochemical variables, are wide-ranging, controversial and sometimes even contradictory.

In accordance with modern and recent approaches in Literature, neuro-inflammation is the possible point in common between the two factors. We support this idea and speculate on the fact that low levels of vitamin D and high levels of homocysteine, in co-existence, might lead to an altered response to inflammation, and therefore predispose to microvascular and endothelium damages. Different reports documented the Th1 induced homocysteine inflammation response (Murr et al., 2001), and it appears that higher levels of homocysteine can be detected in chronic inflammatory conditions, even if vitamin B12 and folate are in range. Many studies documented that higher levels of homocysteine are related to an increment of neopterin and Il-6 (Bleie et al., 2007), which can be partially modulated only by a correct implementation of folate. Thus, it has been suggested that "an optimal folate status over-ride the influence of immunostimulation on Th1 by Hcy" (Bleie et al., 2007). Li et al. (2015) showed that an animal model induced hyper-Hcy produced higher plasma levels of tumor necrosis factor alpha (TNF-α) and Interleukin 1 beta (IL-1β) and therefore induced a trigger of inflammation. These results have been confirmed by many other reports (Yi-Deng et al., 2007; Krishna et al., 2013; Zhou et al., 2014) which showed that higher levels of Hcy promotes, in many different experimental conditions, the activity of specific but different genes, implicates in methylation process, and caused inflammation of the endothelium matrix and atherosclerosis (Yi-Deng et al., 2007; Krishna et al., 2013; Zhou et al., 2014). In different chronic medical conditions, like Rheumatoid Arthritis (Essouma and Noubiap, 2015) higher levels of homocysteine are more frequent than in the general population, and that hyper-homocysteine in RA creates a chronic condition of oxidative stress, prothrombotic induction, and, indirectly, by the excess of ROS released, it up-regulates the Nuclear Factor Kappa B, considered as one of "the master regulator of the expression of inflammatory genes" (Essouma and Noubiap, 2015; Ying et al., 2015). A very recent work has demonstrated that cytokines released by microglia can activate NF-KB signaling resulting in an enhanced expression of the pro-inflammatory system, such as Toll-like receptors (TLR), in particular, TLR2, 4 and 9 (Zhou et al., 2016). They are hyper-expressed by microglia and directly activate NF-KB in "*in vitro*-BV2 model" of PD (Zhou et al., 2016). Different new studies pointed out the topic of inflammation as a strong basis of many different neurodegenerative pathologies, like PD, where *in vitro* specific models, like 6-OHDA- lesioned PC12 cells highly expressed COX2, IL-2 and TNF-alpha (Rong et al., 2003; Xu et al., 2013). In the same *in-vitro* PD model, it has been established the inflammatory mediator participation of a nuclear receptor subfamily, the so-called NUR, involved as a transcriptional factors, in all the process involving neuronal development and in the response to inflammatory attacks (Rong et al., 2003). In particular, Nur 77 acts as a potent modulator of the macrophage and T cell response (as pointed out by Wei et al., 2016), and indirectly promotes inflammation and mitochondrial dysfunction and therefore causes apoptosis. In particular, there is a well-documented increase of the cytosolic level of Nur 77, with a well-described translocation from the nucleus to the cytosol, following the oxidative stress in the PD *in vitro*-model (Gao et al., 2016; Wei et al., 2016). Even in the vascular dementia model, inflammation is claimed as a determinant factor: different biomarkers have been claimed to be over-expressed, such as CysC (Jonsdottir et al., 2013), which seems to be increased in AD and in VAD patients, compared to healthy subjects, and correlates with the severity of the disease (Chen et al., 2015). In the same line, many other biochemical variables, such as HDL, have found to be increased in plasma in AD and in VAD patients. HDL probably promotes an anti-oxidative stress response in damaged brain structures (Wen et al., 2017). Even uric acid acts as a natural anti-oxidative stress factor (acting as a possible disease modifier in MSA and PD) (Jin et al., 2010; Yilmaz and Granger, 2010). It has been inflammatory process plays a dominant role in the pathogenesis of brain ischemia and contributes to stoke formation (Broughton B. R. et al., 2013). Anoxia induces the production of reactive oxygen species and an induction of ICAM1, VCAMS, Selectins, integrins on endothelium leukocytes and platelets (Broughton B. R. S. et al., 2013; Azizieh et al., 2016).

In the same line go all the most recent studies on vitamin D-OH25 deficiency, in order to promote inflammation and endothelium degeneration (Mangin et al., 2014; Na et al., 2014).

When considering all these results, it can be strongly supported the hypothesis of combined and shared roles of vitamin D and homocysteine in neurodegeneration and in vascular and endothelium disruption.

CONCLUSIONS

The questions which we made at the very beginning have been finally answered.

Many doubts remain; how do homocysteine and vitamin D act to create damage? Do they potentiate neurodegeneration or microvascular alteration?

We have observed that low levels of vitamin D are more present in dementia populations, in degenerative and in small-vessel types; these two groups share hyperhomocystenemia, too; the combined presence of

both factors is significantly higher in these two groups. More studies will be needed to implement further knowledge.

AUTHOR CONTRIBUTIONS

RM designed the study and is the responsible of the data. PC and CC analyzed the data. SG, MD, and CT revised the paper, contribute to its written parts and to the analysis of Literature.

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Chronic Monoarthritis Pain Accelerates the Processes of Cognitive Impairment and Increases the NMDAR Subunits NR2B in CA3 of Hippocampus from 5-month-old Transgenic APP/PS1 Mice

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Many factors impact cognitive impairment; however, the effects of chronic pain and the mechanisms underlying these effects on cognitive impairment are currently unknown. Here we tested the hypothesis that chronic pain accelerates the transition from normal cognition to mild cognitive impairment (MCI) in 5-month-old transgenic APP/PS1 mice, an animal model of Alzheimer's disease (AD), and that neurotoxicity induced by N-methyl-D-aspartic acid receptor (NMDAR) subunits may be involved in this process. Chronic monoarthritis pain was induced in transgenic APP/PS1 mice and 5-month-old wild-type (WT) mice by intra- and pre-articular injections of Freund's complete adjuvant (FCA) into one knee joint. Pain behavior, learning and memory function, and the distribution and quantity of NMDAR subunits (NR1, NR2A and NR2B) in hippocampal CA1 and CA3 regions were assessed. Our results showed that although persistent and robust monoarthritis pain was induced by the FCA injections, only the transgenic APP/PS1 mice with chronic monoarthritis pain exhibited marked learning and memory impairments. This result suggested that chronic monoarthritis pain accelerated the cognitive impairment process. Furthermore, only transgenic APP/PS1 mice with chronic monoarthritis pain exhibited an overexpression of NR2B and an increased NR2B/NR2A ratio in the hippocampus CA3. These findings suggest that chronic pain is a risk factor for cognitive impairment and that increased neurotoxicity associated with NMDAR subunit activation may underpin the impairment. Thus, NMDARs may be a therapeutic target for the prevention of chronic pain-induced cognitive impairment.

Keywords: chronic pain, learning and memory, cognitive impairment, N-methyl-D-aspartic acid receptor (NMDAR), neurotoxicity, hippocampus, mouse model

INTRODUCTION

Mild cognitive impairment (MCI) is dysfunction in memory, learning and attention that is not dementia but is not normal for the given age and does not amount to significant impairments in functional activities (Petersen, 2004). MCI affects 15% of nondemented persons aged more than 70 years (Roberts et al., 2008), and those who have MCI progress to dementia in higher proportions than cognitively normal individuals (Mitchell and Feshki-Shiri, 2009; Petersen et al., 2009). Chronic pain is also common in older people and often coexists with cognitive impairment with or without dementia (Peisah et al., 2014; Sampson et al., 2015; van Kooten et al., 2015). An epidemiological study has shown that approximately 50% of patients with dementia experience pain (Corbett et al., 2012), and pain frequently decreases cognitive performance (Scherder et al., 2008). Although a link between chronic pain and cognitive dysfunction has not been fully clarified, evidence shows that chronic pain in patients with temporomandibular disorder (Moayedi et al., 2012), fibromyalgia (Kuchinad et al., 2007), and back pain (Apkarian et al., 2004) accelerates whole brain gray matter atrophy.

The N-methyl-D-aspartate receptor (NMDAR) is an important ionotropic glutamic acid receptor, and it is formed by combinations of different subunits, including NMDAR1 (NR1), NMDAR2 (NR2A, NR2B, NR2C, NR2D), and NMDAR3 (NR3A, NR3B). These NMDARs subunits exhibit not only differential distribution but also variable functions in the nervous system. Although their normal expression levels and functions are critical for normal functioning of the central nervous system (CNS), abnormalities in NMDARs can cause excitotoxicity leading to subsequent neurodegeneration, as is observed in patients with Alzheimer's diseases (AD). Recent studies indicate that excessive activation of extrasynaptic NR2B participates in the amyloid (A)β-induced loss of synapses and synaptic proteins in neuronal cell cultures (Liu et al., 2010; Rönicke et al., 2011; Chang et al., 2016). Tau phosphorylates NMDARs, which enhances the downstream neurotoxic effects (Ittner et al., 2010). Peripheral (Du et al., 2003; Chen W. et al., 2014), spinal (Lefèvre et al., 2015; Haley and Dickenson, 2016) and supraspinal (Ghelardini et al., 2008; Wei et al., 2008; Da Silva et al., 2010; Cheng et al., 2011; Ho et al., 2013; Ohsawa et al., 2014) NMDARs together contribute to the development and maintenance of chronic pain, but supraspinal NMDAR overexpression in particular enhances responsiveness to noxious stimulation and aggravates chronic pain conditions. Because abnormal expression levels and altered functions of NMDARs are involved in AD and chronic pain, we hypothesized that chronic pain accelerates the progression of cognitive impairment in patients at high risk for dementia, and that the neurotoxicity induced by NMDAR subunits in the hippocampus may be a mechanistic link between AD and chronic pain. Several previous preclinical studies using inflammatory pain and neuropathic pain models have investigated the effects of pain on cognition, and their results confirm that pain worsens attention (Boyette-Davis et al., 2008; Pais-Vieira et al., 2009), emotional decision-making (Ji et al., 2010) and spatial learning and memory (Leite-Almeida et al., 2009; Hu et al., 2010). However, it is still unclear whether chronic pain alters the processes involved in the progression from normal cognition to dementia. No studies have yet explored the relationship between pain and cognitive impairment by examining the mechanisms of action for the involved NMDAR subunits in transgenic animal models of AD.

Therefore, the aim of the present study was to evaluate the effects of chronic pain on cognitive dysfunction by determining pain behavior and cognitive status of 5-month-old transgenic APP/PS1 mice, which are considered a murine model of AD, subjected to a chronic monoarthritis model of pain. Because localization and subunit composition of NMDARs appear to produce paradoxical actions and because NR1, NR2A and NR2B appear to be particularly important for nociception and AD, we also investigated the distribution and expression of these subunits in the hippocampus.

MATERIALS AND METHODS

Animals

APP/PS1 transgenic mice (C57BL/6J) were supplied by the Institute of Laboratory Animal Science at Peking Union Medical College. These mice express a mouse-human hybrid transgene containing the extracellular and intracellular regions of the mouse sequence and a human sequence within the Aβ domain containing Swedish mutations (K594N/M595L), and they also express the exon 9-deleted variant of human presenilin 1 (Zhang et al., 2011; Zong et al., 2011). The animals were housed individually under standard conditions (23–25°C; humidity 50%–60%; 12-h light/dark cycle with lights on at 8:00 a.m.; *ad libitum* access to water and food). All animals were given health checks and acclimatized for 1 week before the experiment. The study was conducted following the recommendations of the Animal Care and Use Committee at Capital Medical University, and the experimental procedures were approved by the Animal Care and Use Committee at the Capital Medical University.

All experiments were performed in 5-month-old wild-type (WT) male C57BL/6J and congenic male APP/PS1 transgenic mice, also 5 months old. The APP/PS1 transgenic mouse strains have been previously described and characterized in multiple studies (Zhang et al., 2011; Wang D. et al., 2013; Wang D. M. et al., 2013). Experiments were conducted using 24 WT mice and 24 APP/PS1 mice. The animals were divided into the following four treatment groups (n = 12 per group): animals injected with Freund's complete adjuvant (FCA; thus, FCA-WT and FCA-APP groups) and saline-injected animals (Sham-WT and Sham-APP groups). Body weight (expressed in grams), as an important health indicator, was measured before treatment and 1, 7 and 14 days and 1 and 2 months after treatment. Six animals were excluded for failure to achieve spatial acquisition. Thus, body weights, knee joint diameters, pain scores and spatial acquisition and probe test scores were obtained from a total of 42 animals as follows: FAC-APP (n = 11), FAC-WT (n = 11), Sham-APP (n = 10) and Sham-WT

(n = 10). After completion of the behavioral experiments, immunofluorescence was performed using five animals from each group, and western blotting was performed using the following additional numbers of animals: FAC-APP (n = 6), FAC-WT (n = 6), sham-APP (n = 5) and sham-WT (n = 5). All behavioral measurements and morphological evaluations were conducted by an experimenter who was blinded to the experimental groups.

Induction of Chronic Monoarthritis Pain

Animals were anesthetized using 2%–3% isoflurane mixed with air at a flow rate of 2 L/min. A model of chronic monoarthritis pain was produced by giving one intra-articular injection of FCA (20 μL, 10 mg/mL) and four peri-articular injections of FCA (four injections of 20 μL each) into the right knee joint (Kelso et al., 2007). One month after the first injection, the same knee joint was reinjected with the same protocol to maintain chronic pain. Animals in the sham groups received the same injection protocol but saline was used rather than FCA. The bilateral hind knee joint diameter (expressed in mm) was measured using a vernier caliper before injection and 1, 7 and 14 days and 1 and 2 months after the initial injection.

Behavioral Measures of Arthritic Joint Pain

Using previously published methods (Ghilardi et al., 2012; Jimenez-Andrade and Mantyh, 2012), behavioral measures of arthritic joint pain, including spontaneous pain (flinching) and stimulus-evoked pain (limb use), were performed before injection and 1, 7 and 14 days and 1 and 2 months after the intital injection. Flinching was defined as the animal spontaneously raising its hind paw in an act representative of spontaneous nocifensive behavior. To evaluate resting pain levels, the number of spontaneous flinches was recorded over a 2-min period. Normal limb use during spontaneous ambulation in an open field apparatus was used as an indicator of stimulus-evoked pain and was scored on a scale of 0–5 as follows: 0, complete lack of limb use; 1, partial non-use of limb during locomotor activity; 2, limping and guarding behavior; 3, pronounced limp; 4, partial but not pronounced limp; 5, normal behavior.

Morris Water Maze (MWM) Task

The Morris water maze (MWM) was used to assess spatial learning and memory. The apparatus and protocol for the MWM task used in this study were based on those described in a previous report (Vorhees and Williams, 2006). Briefly, the maze consisted of a white circular pool (122 cm in diameter and 51 cm in height) filled with opaque water at a temperature of approximately 22°C. The pool was conceptually divided into four quadrants and had four points designated as starting positions: north, east, southeast and northwest. A white circular platform (10 cm^2) was submerged 1.0 cm below the water surface and was located in the center of southwest quadrant. The room temperature was constant, the light levels were even, and experimenters remained behind a visual barrier. Monitoring was performed with a video-tracking system (Ethovision XT, Noldus Ltd, Holland).

The spatial acquisition trial was performed as four trials per day for five consecutive days. The animals were released into the water at water-level at one of four starting positions, facing the pool wall. The video-tracking system started recording the moment that the animals were released and stopped when the animals reached the platform within 60 s. Animals that failed to find the platform within the time limit were guided to the platform. All animals were allowed to stay on the platform for 15 s and then performed in the next trial with a 15-min intertrial interval. The starting position changed in each trial. Following the completion of the trials, the mice were dried and returned to their home cages. The time to reach the platform (escape latency), the length of the path taken to find the platform (escape path), and the swimming velocity were measured as indices of learning the spatial task.

Twenty-four hours after the final spatial acquisition trial, a probe trial was performed to assess the animal's spatial memory. The animals were placed in the water at the northeast starting position, 180° from the original platform position, and swam freely without the platform for 60 s. The number of platform-site crossings, the time spent in the target quadrant, the swimming velocity, and the path length were analyzed.

Western Blotting

The method reported by Gozal et al. (2002) was followed to conduct western blotting. The animals were anesthetized and their brains rapidly removed and cut into several sections (500 μm/slice) on ice under a surgical microscope. Hippocampal CA1 and CA3 regions were microdissected (**Figure 3C**) and stored at −80°C until needed. Western blotting was conducted for three proteins, NR1, NR2A and NR2B subunits (Petralia et al., 2010). The hippocampal tissues were homogenized in ice-cold RIPA buffer (0.1% phenylmethylsulfonyl fluoride) and centrifuged at 14,000 g for 25 min at 4°C. The protein concentration in the supernatant was determined using a BCA kit (Beyotime Biotechnology, Nanjing, China). Supernatants were mixed with an equal volume of Laemmli buffer and denatured at 100°C for 5 min. Protein samples (40 μg/well) were loaded on 8% polyacrylamide gels according to the manufacterer's recommendation (Yu Xi Biotechnology Co., Ltd, Jiangyin, China), and then electrophoresed and transferred to nitrocellulose membranes at 4°C. The membranes were then blocked with 5% fat-free milk in Tris-buffered saline containing 0.1% Tween-20 (TBS/T) for 2 h at room temperature and incubated overnight at 4°C with the following primary antibodies at a dilution of 1:1000: mouse monoclonal anti-NR1 (Abcam, Cambridge, UK, ab134308); rabbit monoclonal anti-NR2A, (Abcam, ab133265); and mouse monoclonal anti-NR2B (Abcam, ab93610). The next day, membranes were washed three times for 10 min each in TBST and probed with secondary antibodies diluted 1:1000–2000 (goat anti-mouse IgG or goat anti-rabbit IgG; Santa Cruz, Dallas, TX, USA) for 1 h at room temperature. The membranes were washed again with TBS/T, and immunoreactivity was detected by application of the ECL Plus chemiluminescence reagent for 5 min. Protein levels

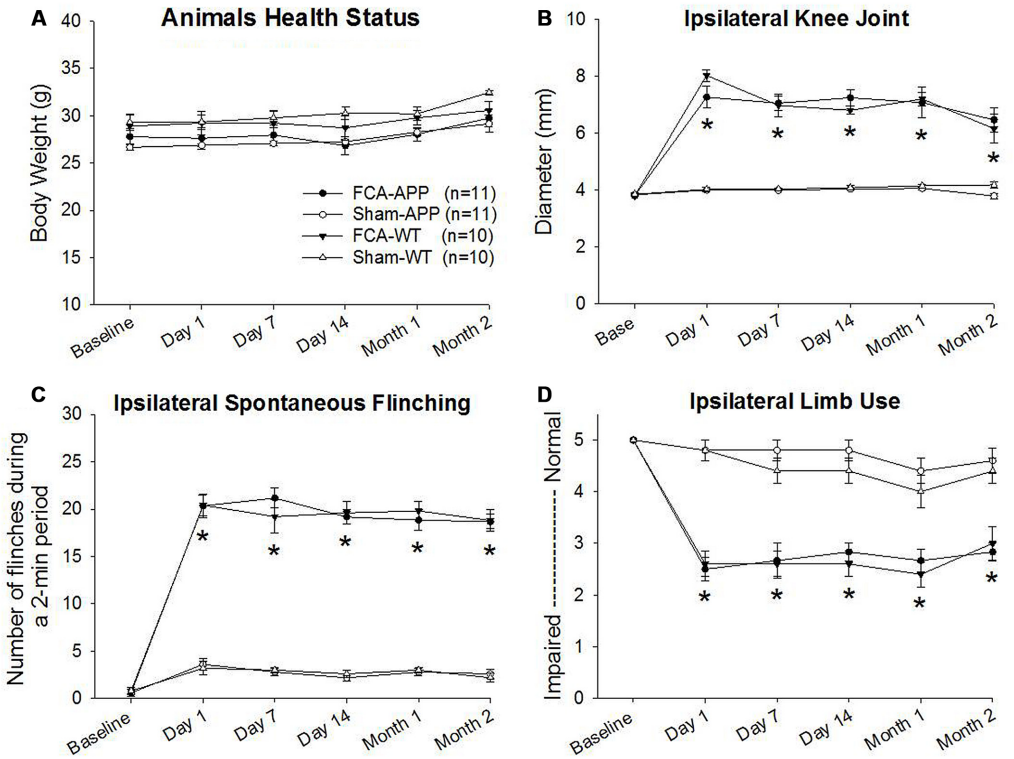

FIGURE 1 | Significant unilateral knee joint inflammation and pain-related behaviors develop in animals with chronic monoarthritis pain. Right knee joints of mice were injected twice with a 1-month interval with Freund's complete adjuvant (FCA) to induce chronic monoarthritis and long-term pain behavior. Saline, instead of FCA, was injected as a control. **(A)** No significant differences are detected in body weights among the mice in the four groups throughout the duration of the experiment. **(B)** Ipsilateral knee joint diameters in FCA-treated animals are significantly greater than those in saline-treated animals, and the swelling remains at significantly higher levels following the initial injection with FCA. **(C)** The number of spontaneous flinches in FCA-treated animals significantly increases following the initial injection and remains at a significantly higher level over time as compared with those in saline-treated animals. **(D)** Ipsilateral hind limb use in FCA-treated animals significantly decreases following the initial injection and remains significantly lower during the experimental period. *$P < 0.001$ compared with saline-treated animals. Data are presented as the mean ± SEM. FCA-APP (n = 11), Sham-APP (n = 11), FCA-wild-type (WT) (n = 10) and Sham-WT (n = 10).

were quantified by determining their optical densities (with arbitrary units) using ImageJ software (National Institutes of Health, Bethesda, MD, USA) and expressed as a ratio relative to β-actin.

Immunofluorescence and Imaging

Animals were anesthetized with sodium pentobarbital (50 mg/kg, Sigma-Aldrich, St. Louis, MO, USA) at the end of the experiment, and perfused intracardially with 20 mL of 0.1 M phosphate-buffered saline (PBS; pH 7.4 at 4°C) followed by 4% paraformaldehyde (pH 7.4) for fixation. The brains were harvested, post-fixed, and then embedded in paraffin.

Paraffin-embedded tissues were cut into 4-μm-thick sections and collected on polylysine-coated slides. All slides were processed on the same day and were exposed to freshly made reagents at the same time to minimize variations in conditions among groups. The sections were deparaffinized in xylene, rehydrated in a graded series of ethanol, and rinsed with running cold tap water. After antigen retrieval was performed using citrate buffer (0.01 mol/L, pH 6.0) for 15 min in a microwave oven, the sections were blocked with 10% normal goat serum in 0.1% Triton X-100, and then incubated with a primary antibody to mouse monoclonal anti-NR1 (diluted 1:200; Abcam, ab134308) in a humidified chamber at 4°C overnight. For double immunostaining, sections were simultaneously incubated with primary antibodies to rabbit monoclonal anti-NR2A (diluted 1:200; Sigma-Aldrich, St. Louis, MO, USA, SAB4501304) and mouse monoclonal anti-NR2B (diluted 1:100; Abcam, ab93610).

The following day, these slides were rinsed and incubated with a secondary antibody (goat anti-mouse IgG labeled with Alexa Fluor 488 for NR1) for 2 h at room temperature. For double immunostaining, sections were rinsed and simultaneously incubated with secondary antibodies (goat anti-rabbit IgG labeled with Alexa Fluor 594 for NR2A, and goat anti-mouse IgG labeled with Alexa Fluor 488 for NR2B; both diluted 1:200 in TBS/T). After being rinsed three times in PBS for 5 min each, all sections were mounted with Vectashield mounting medium containing DAPI (Vector Laboratories, Burlingame, CA, USA). The primary antibody incubation was omitted in one section as a negative control.

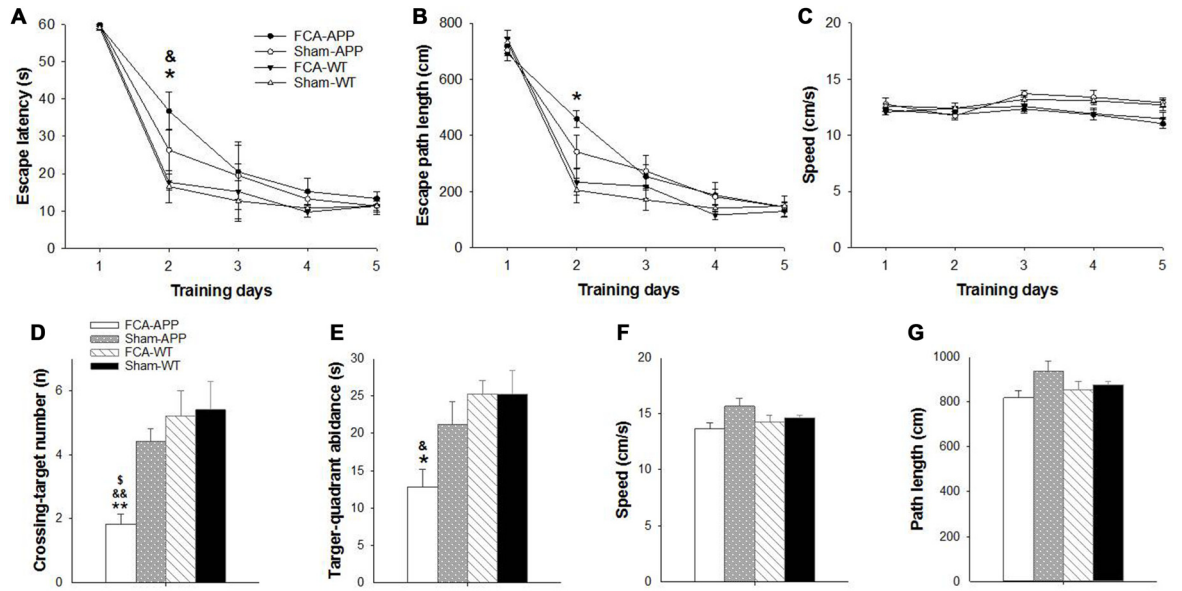

FIGURE 2 | Significant learning and memory impairments are exhibited in the Morris water maze (MWM) task by APP/PS1 transgenic animals with chronic monoarthritis pain. Animals were trained to find the platform under the water surface in spatial acquisition trials **(A–C)** and then tested in a probe trial **(D–G)** without the platform 24 h after the last training trial. **(A)** FCA-treated APP transgenic animals show a greater escape latency than FCA-treated and saline-treated WT animals on the second day. **(B)** The FCA-treated APP transgenic animals show a longer escape path length than saline-treated WT animals on the second day. **(C)** No significant difference in swimming velocity is detected among the four groups. **(D)** The FCA-treated APP transgenic animals cross the platform site in the target quadrant fewer times than the saline-treated transgenic animals and the FCA-treated and saline-treated WT animals. **(E)** The FCA-treated APP transgenic animals spend less time in the target quadrant than the FCA-treated and saline-treated WT animals. **(F,G)** No significant difference is detected in swimming velocity and path length traveled among the four groups. $^{*}P < 0.05$, $^{**}P < 0.001$ for FCA-APP animals compared with Sham-WT; $^{\&}P < 0.05$, $^{\&\&}P < 0.01$ for FCA-APP animals compared with FCA-WT; $^{\$}P < 0.05$ for FCA-APP animals compared with Sham-APP. Data are presented as the mean ± SEM. FCA-APP (n = 11), Sham-APP (n = 11), FCA-WT (n = 10) and Sham-WT (n = 10).

All sections were viewed under a Nikon Eclipse 80i microscope (Nikon Instruments INC, Tokyo, Japan), and images were captured using a Nikon D40 camera system (Nikon Corporation, Tokyo, Japan) using Northern Eclipse software (Empix Imaging, Mississauga, ON, Canada) at magnifications of 10×, 20× and 40× in three channels, blue DAPI (cell nuclei), green Alexa Fluor 488 (NR1 or NR2B), and red Alexa Fluor 594 (NR2A). The photomicrographs were saved as TIFs and quantitatively analyzed using ImageJ software. The mean immunofluorescence intensities of NR1, NR2A and NR2B in hippocampal CA1 and CA3 regions were measured by one experimenter blinded to the conditions to ensure consistency. The CA1 and CA3 boundary was determined based on the method reported by Gozal et al. (2002); **Figure 3C**.

Data Presentation and Statistical Evaluations

Analysis of the data was performed using SPSS Version 22.0 (SPSS Inc., Chicago, IL, USA). All data were tested using the Lilliefors correction to the Kolmogorov-Smirnov test for normal distribution and by Levene's test for homogeneity of variance. After the means were compared using one-way analysis of variance (ANOVA), *post hoc* tests using Bonferroni's correction were performed for detection of the differences among groups. A value of $P < 0.05$ was considered statistically significant. Data are presented as the mean ± SEM.

RESULTS

Progressive and stable body weights were exhibited throughout the duration of the experiment, and there were no significant differences in body weights among the four groups ($P > 0.05$), indicating that all animals were healthy (**Figure 1A**).

Knee Joint Inflammatory and Pain Behaviors

Significant ipsilateral knee joint inflammation and pain-related behaviors developed in the groups of mice subjected to the chronic monoarthritis pain model protocol. Compared with those in saline-treated animals, the ipsilateral knee joint diameters of FCA-treated animals increased significantly after the initial injection throughout the period of the experiment ($P < 0.001$; **Figure 1B**). No significant change with time was detected in the contralateral knee joint ($P > 0.05$). Pain-related behaviors, including spontaneous pain (flinching) and evoked pain (limb use), were assessed throughout the duration of the experiment. FCA-treated animals exhibited a greater number of ipsilateral spontaneous flinches than saline-treated animals after

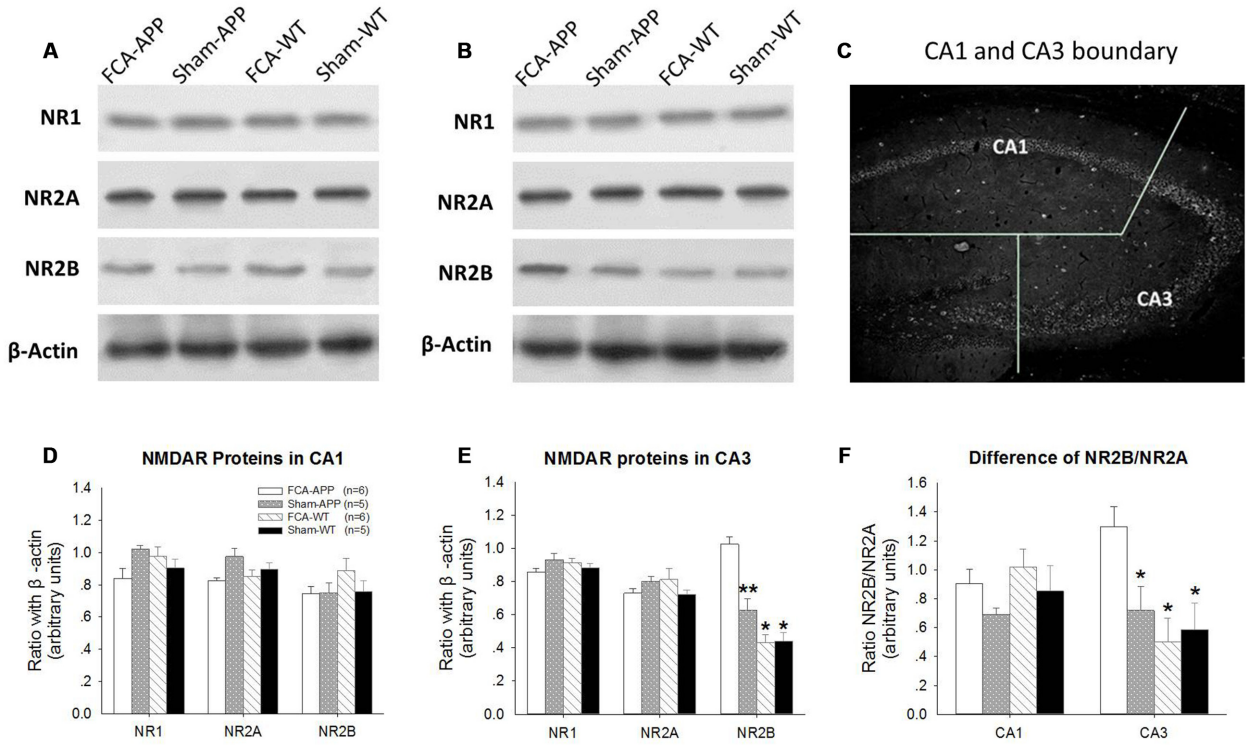

FIGURE 3 | NR2B protein and ratio of NR2B/NR2A significantly increase in CA3 tissue obtained from APP/PS1 transgenic animals with chronic monoarthritis pain. Western blotting analysis was performed to quantify NR1, NR2A and NR2B protein expression in hippocampus CA1 and CA3 of APP/PS1 transgenic animals and WT animals with or without chronic monoarthritis pain. **(A)** Representative images of NR1, NR2A, NR2B and β-actin in CA1. **(B)** Representative images of NR1, NR2A, NR2B and β-actin in CA3. **(C)** Schematic illustration of the dissection procedure used for regional analyses. **(D,E)** Quantitative analysis revealed no significant difference in NR1, NR2A and NR2B protein expression in CA1 among the four groups. **(E)** Quantitative analysis revealed no significant difference in NR1 and NR2A in the CA3 among the four groups, but markedly increased NR2B protein expression in CA3 of APP/PS1 transgenic animals with chronic monoarthritis pain. **(F)** No significant difference is detected in the ratio of NR2B/NR2A in the CA1 among the four groups, but the NR2B/NR2A ratio in the CA3 of APP/PS1 transgenic animals with chronic monoarthritis pain markedly increases. $^{*}p < 0.001$, $^{**}p < 0.05$ compared to FCA-APP. Data are presented as the mean ± SEM. FCA-APP (n = 6), Sham-APP (n = 6), FCA-WT (n = 5) and Sham-WT (n = 5).

the initial injection ($P < 0.001$, **Figure 1C**). The evaluation of ambulatory pain revealed significantly reduced ipsilateral limb use scores in FCA-treated animals compared with those in saline-treated animals ($P < 0.001$, **Figure 1D**). By contrast, the contralateral knee joint presented no significant differences in spontaneous flinches and limb use between these four groups ($P > 0.05$). Together, these data indicated that the mice subjected to the chronic monoarthritis pain protocol showed consistent and robust ipsilateral pain for the 2-month duration of this study.

Morris Water Maze Test

The MWM task was used to examine spatial learning and memory 2 months after chronic monoarthritis pain was induced. We found significant learning and memory impairments in the APP/PS1 transgenic animals with chronic monoarthritis pain.

During the spatial acquisition trials, all animals exhibited similar escape latencies (**Figure 2A**) and length of escape paths (**Figure 2B**), and there was no significant difference in swimming velocity among the four groups (**Figure 2C**). However, the APP/PS1 transgenic animals with chronic monoarthritis pain took more time to locate the platform compared with the WT animals both with and without chronic monoarthritis pain (**Figure 2A**) and traveled a longer path on the second day compared with the WT animals without chronic monoarthritis pain (**Figure 2B**). In the probe trial, the APP/PS1 transgenic animals with chronic monoarthritis pain crossed the platform site fewer times (**Figure 2D**) and spent less time in the target quadrant (**Figure 2E**) than animals in the other three groups, but there was no significant difference in swimming velocity and path length among the four groups (**Figures 2F,G**).

Western Blotting Analysis

We found that NR2B protein expression was significantly increased in hippocampal CA3 tissue obtained from APP/PS1 transgenic animals with chronic monoarthritis pain. Protein expression levels of NR1, NR2A and NR2B in the CA1 (**Figures 3A,D**) and CA3 areas (**Figures 3B,E**) were measured by western blotting analysis to investigate the

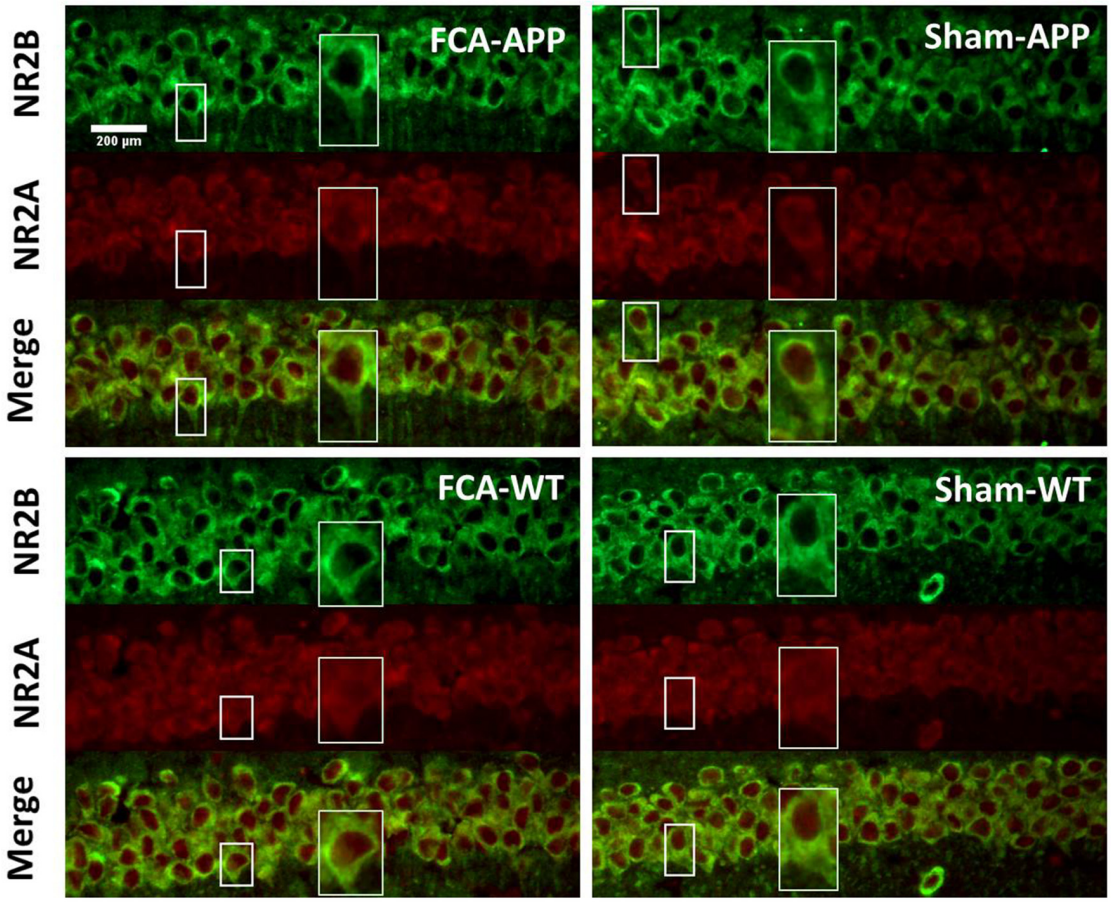

FIGURE 4 | Representative images of NR2A and NR2B colocalization in the CA1 of the hippocampus (40×). Green fluorescence indicates NR2B-positive neurons, and red fluorescence indicates NR2A-positive neurons; yellow fluorescence represents the colocalization of NR2A and NR2B after both images are merged. NR2B is located only on the membrane of neurons, but NR2A is diffusely expressed throughout the entire neuron, including the membrane and interior. Thus, NR2A and NR2B are colocalized on neuronal membranes. Scale bars, 200 μm.

response of NMDARs in APP/PS1 transgenic and WT animals with and without chronic monoarthritis pain. The ratios of NR2B/NR2A were also calculated (**Figure 3F**). As also shown in the representative images of the CA1 NR1, NR2A and NR2B western blots (**Figure 3A**), the quantification results indicated that there was no significant difference among the four groups for CA1 region NR1, NR2A and NR2B protein expression ($P > 0.05$, **Figure 3D**). Similarly, there was no significant difference among the four groups in NR1 and NR2A protein expression levels in the CA3 area ($P > 0.05$, **Figures 3B,E**). However, the NR2B protein expression levels in the CA3 area of APP/PS1 animals with chronic monoarthritis pain were significantly higher than those from APP/PS1 animals without chronic monoarthritis pain and from WT animals with or without chronic monoarthritis pain ($P < 0.05$, **Figure 3E**). No significant difference was found in the ratio of NR2B/NR2A in the CA1 area among the four groups ($P > 0.05$, **Figure 3F**). However, the NR2B/NR2A ratio in the CA3 of APP/PS1 animals with chronic monoarthritis pain was significantly higher than that from APP/PS1 animals without chronic monoarthritis pain and WT animals with or without chronic monoarthritis pain ($P < 0.05$, **Figure 3F**).

Immunofluorescence and Photomicrograph Analysis

Immunoreactivity of NR1, NR2A and NR2B in the hippocampal CA1 and CA3 areas was assessed using immunofluorescence, and photomicrographs were quantitatively analyzed to determine any differences in NR1, NR2A and NR2B levels or distribution. **Figures 4**, **5**, **6A** show images of double immunofluorescence staining for NR2A and NR2B alone or colocalized in merged images captured in the CA1 and CA3. **Figure 7A** shows the images of NR1 and DAPI-labeled cell nuclei in the CA1 and CA3.

The immunohistochemical results indicated that NR2A and NR2B were colocalized in the CA1 area (**Figure 4**). While NR2B was located on neuronal membranes, NR2A was expressed not only on the membrane but also in the interior of neurons. **Figure 5** shows representative images of NR2A and NR2B double immunostaining in the CA3 area. The distribution of NR2A and NR2B in the CA3 was the same as that in the CA1.

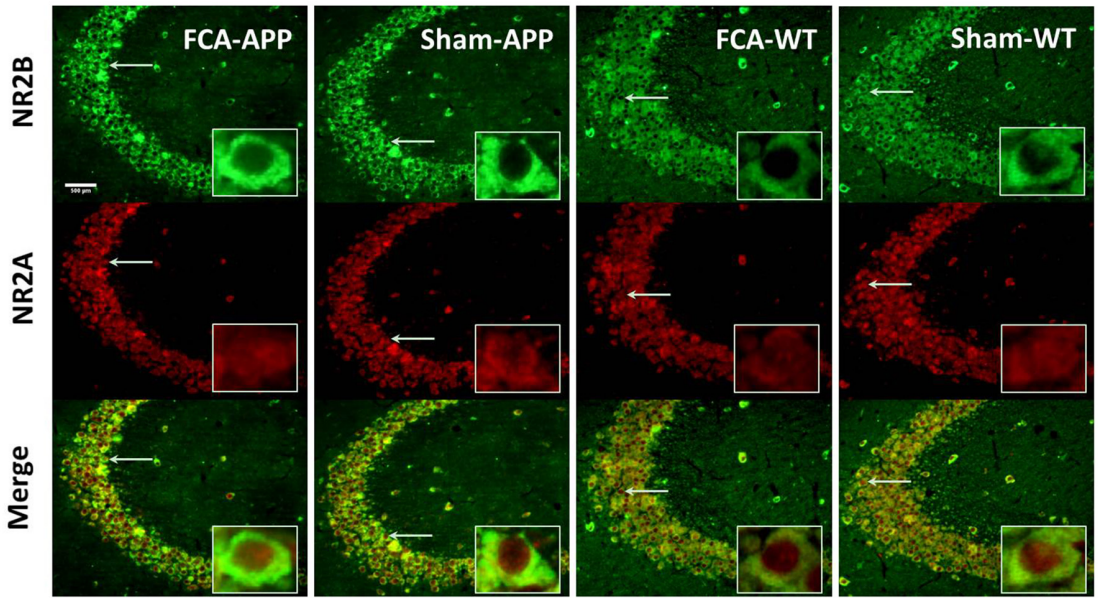

FIGURE 5 | Representative images of NR2A and NR2B colocalization in hippocampus CA3 (20×). Green fluorescence indicates NR2B-positive neurons, and red fluorescence indicates NR2A-positive neurons; yellow fluorescence represents the colocalization of NR2A and NR2B after both images are merged. NR2B is located only on the membrane of neurons, whereas NR2A is diffusely expressed throughout the entire neuron, including the membrane and interior; thus, NR2A and NR2B are colocalized on the membranes of neurons. Scale bars, 500 μm.

The representative images and quantitative analysis results for NR2A and NR2B are shown in **Figure 6**. NR2A and NR2B were double-immunostained in CA1 and CA3 neurons (**Figure 6A**). No qualitative differences were noted among the four groups for NR2A in the CA1 and CA3 (**Figure 6B**), NR2B in the CA1 (**Figure 6C**) and the fluorescence density ratio of NR2B/NR2A in the CA1 (**Figure 6D**). However, the immunofluorescence signal intensity of NR2B (**Figure 6C**) and the fluorescence density ratio of NR2B/NR2A (**Figure 6D**) in the CA3 of APP/PS1 transgenic animals with chronic monoarthritis pain were higher than those in any other group, and those in the APP/PS1 transgenic animals without chronic monoarthritis pain were higher than those from WT animals with or without chronic monoarthritis pain. The representative images and quantitative analysis of NR1 are shown in **Figure 7**. NR1 was widely distributed on neurons in both the CA1 and CA3 regions (**Figure 7A**), and no qualitative differences were noted in these regions among the four groups (**Figures 7B,C**).

DISCUSSION

Transgenic APP/PS1 mice on a C57BL/6J background at 5 months age were used in this study because these animals naturally develop learning and memory impairments as assessed in the MWM task until 12 months of age (Lalonde et al., 2005; Volianskis et al., 2010). The cognitive impairment exhibited in the MWM is unrelated to locomotor effects because learning and memory performances are insensitive to swimming velocity (Fitzgerald and Dokla, 1989). Mice of the C57BL/6J strain have been used to developed a model of robust chronic monoarthritis, as described in a previous publication (2-Kelso et al., 2007), with pain behaviors demonstrating a unilaterally painful condition. Thus, transgenic APP/PS1 and congenic C57BL/6J mice subjected or not to a model of chronic monoarthritis pain were well suited for use to test our hypothesis.

Chronic Monoarthritis Pain Accelerates the Transition from Normal Cognition to Mild Cognitive Impairment

Chronic pain is a persistently negative condition, and its impacts are far-reaching in every way, including emotional changes and cognitive deficits. Especially in elderly patients, chronic pain frequently coexists with cognitive impairment, and patients experiencing pain display pain-related working memory deficits (Buckalew et al., 2008, 2010). Patients with chronic back pain and complex regional pain syndrome also show significantly less bilateral hippocampal volume (Mutso et al., 2012). Although the cerebral cortex and medial temporal lobe play key roles in memory and attention, the hippocampus is essential for the transference of short- to long-term memory and in the control of spatial memory.

The MWM task used in the current study is a valid mean for measuring hippocampus-dependent spatial navigation and reference memory (Morris, 1984). Our results using this task demonstrated that chronic monoarthritis pain impaired the hippocampal-related learning and memory

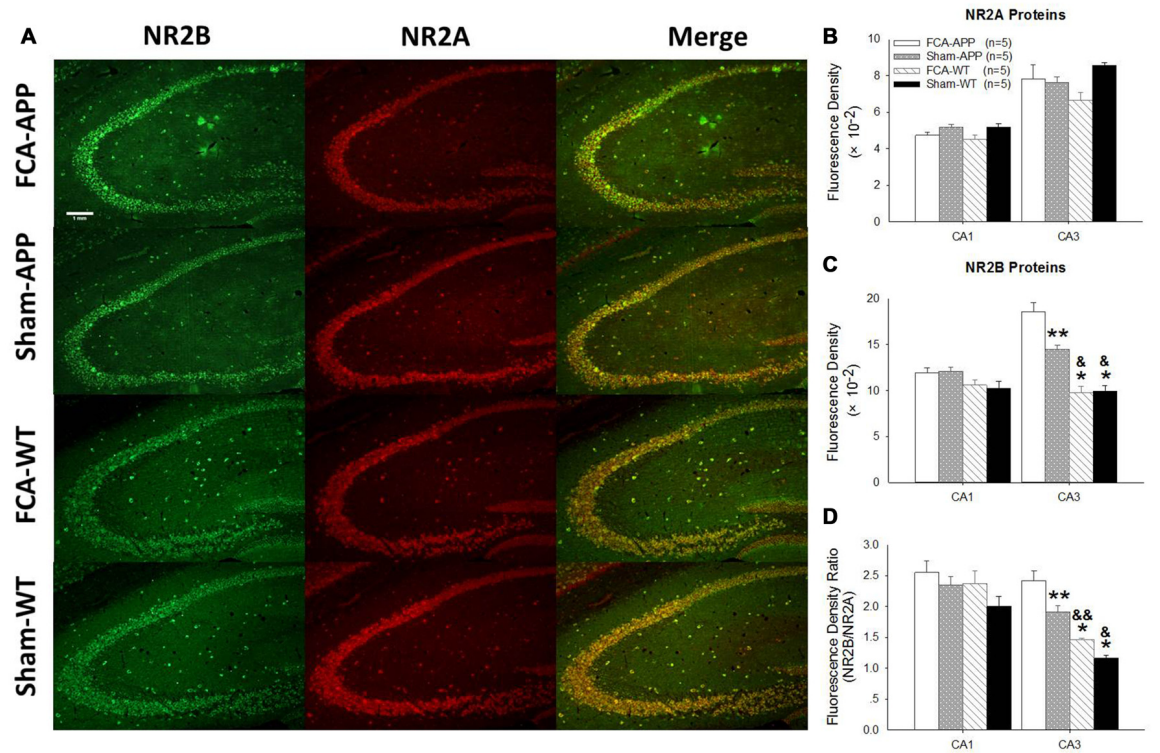

FIGURE 6 | The mean immunofluorescence intensity of NR2B significantly increases in CA3 tissue of APP/PS1 transgenic animals with chronic monoarthritis pain. Representative images of NR2A and NR2B in the hippocampus at 10×. **(A)** Quantitative analysis of NR2A and NR2B densities in CA1 and CA3 **(B,C)** and the ratio of NR2B/NR2A **(D)**. **(A)** Green fluorescence indicates NR2B-positive neurons and red fluorescence indicates NR2A-positive neurons; yellow fluorescence represents the colocalization of NR2A and NR2B. **(B)** Quantitative analysis reveals no significant difference in NR2A fluorescence intensity in the CA1 and CA3 among the four groups. **(C)** Quantitative analysis reveals no significant difference in NR2B fluorescence intensity in the CA1 among the four groups. However, in CA3, the NR2B fluorescence intensity in APP/PS1 transgenic animals with chronic monoarthritis pain is the highest of all groups, while that in APP/PS1 transgenic animals without chronic monoarthritis pain was higher than that in WT animals with or without chronic monoarthritis pain. **(D)** Calculations reveal no significant difference in the NR2B/NR2A ratio in the CA1 among the four groups. However, the NR2B/NR2A ratio in the CA3 of APP/PS1 transgenic animals with chronic monoarthritis pain was the highest among all groups, and the ratio in APP/PS1 transgenic animals without chronic monoarthritis pain was higher than that in WT animals with and without chronic monoarthritis pain. $^{*}P < 0.001$, $^{**}P < 0.05$ compared with FCA-APP; $^{\&}P < 0.01$, $^{\&\&}P < 0.05$ compared with Sham-APP. Data are presented as the mean ± SEM; $n = 5$. Scale bars, 1 mm.

function of APP/PS1 transgenic mice at 5 months of age. This is consistent with other studies that have shown that pain-induced structural and functional abnormalities in the hippocampus contribute to cognitive impairment. A study by Arai et al. (2013) showed that peripheral pain significantly decreases rat hippocampal miRNA. Animals with neuropathic pain also exhibit reduced hippocampal neurogenesis and altered short-term synaptic plasticity (Mutso et al., 2012), deficits in long-term potentiation (LTP; Kodama et al., 2011) and impaired enriched-environment neurogenesis (Terada et al., 2008).

Chronic Monoarthritis Pain Increases the Expression of NR2B in the Hippocampal CA3 Region of APP/PS1 Transgenic Mice Aged 5 Months

It is generally agreed that tissue injury contributes to the hyperalgesia associated with nociceptor sensitization and central sensitization by peripheral and spinal activation of NMDARs. Recent evidence has further revealed that chronic pain induces the overexpression of NMDARs in the rostral ventromedial medulla (Wei et al., 2008), the midbrain ventrolateral periaqueductal gray (Ho et al., 2013), and the anterior cingulate cortex (Wu et al., 2005), which then contributes to the development and maintenance of hyperalgesia. However, the effect of pain on the expression of NMDARs in the hippocampus remains controverial. Hippocampal NR2B protein levels significantly increase in irritable bowel syndrome-like rats, a model of chronic visceral pain (Chen Y. et al., 2014). Postoperative pain upregulates the levels of NMDAR2 subunits in the hippocampus (Chi et al., 2013). By contrast, the partial sciatic nerve ligation model of neuropathic pain reduces the expression of NR1 and NR2B in the hippocampus of injured rats (Wang et al., 2015). Our results provide the first evidence that chronic pain induces the overexpression of NR2B in the CA3 of hippocampus in transgenic APP/PS1 mice.

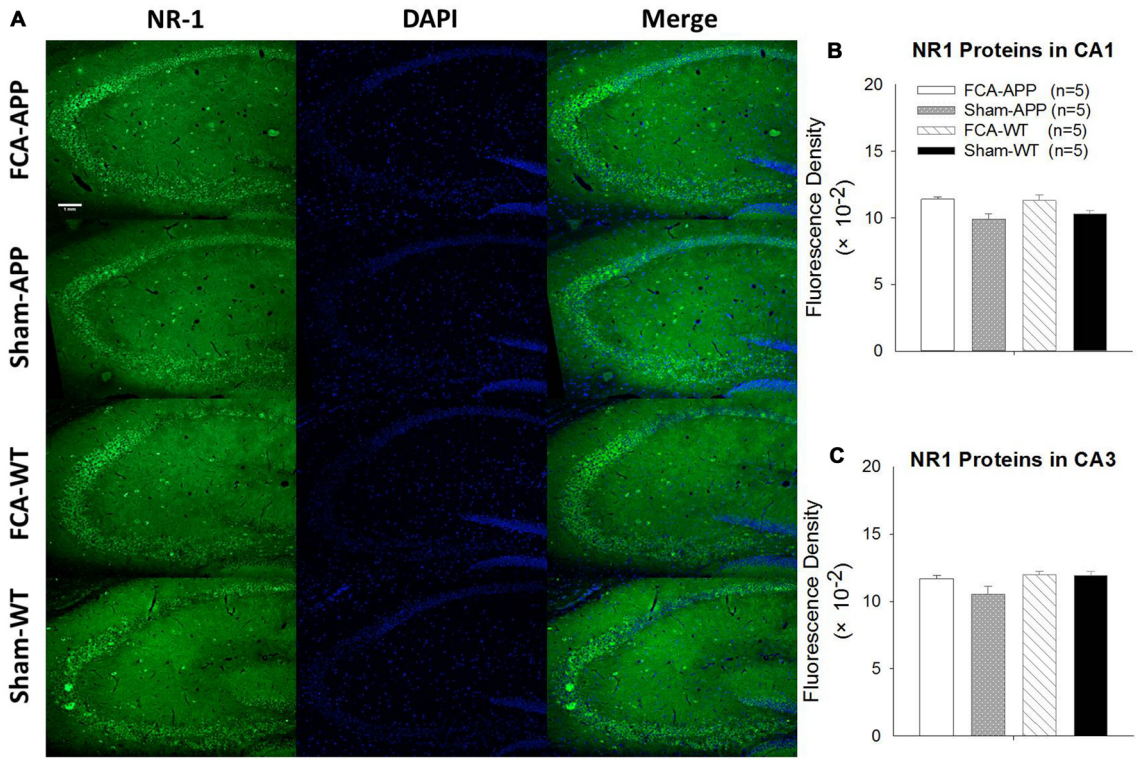

FIGURE 7 | No qualitative differences are detected in the NR1 expression of the hippocampus CA1 and CA3 regions among the four groups. Representative images at a magnification of 10× **(A)**, and quantitative analysis of NR1 immunofluorescence signal intensities in the CA1 and CA3 **(B,C)**. **(A)** Green fluorescence indicates NR1-positive neurons, and blue (DAPI) fluorescence indicates cell nuclei. Quantitative analysis reveals no significant difference in NR1 fluorescence signal intensity in the **(B)** CA1 or **(C)** CA3 among the four groups. Data are presented as the mean ± SEM; $n = 5$. Scale bars, 1 mm.

Chronic Pain Accelerates the Transition from Normal Cognition to Mild Cognitive Impairment through NR2B-Induced Neurotoxicity in the CA3

The results of our study indicated that chronic pain may have provoked the neurotoxic effects of NR2B in the hippocampus and that this neurotoxicity may be responsible for the impaired learning and memory exhibited in APP/PS1 transgenic mice. Chi et al. (2013) also determined that postoperative pain upregulates the levels of NMDAR2 subunits in the hippocampus and contributes to the development of memory deficits. However, the effects of NMDAR are complex and can promote either neuroprotection or excitotoxicity. The distinct subunit expression, trafficking and localization of NMDARs also lead to different activity levels and functions in neurons. Two models, the localization model and the subunit composition model, explain the neurotrophic and excitotoxic effects of NMDARs. Activation of extrasynaptic NMDARs contributes to neurotoxic effects, whereas activation of synaptic NMDARs leads to neuroprotective effects (Hardingham et al., 2002; Hardingham and Bading, 2010). Activation of NR2B is excitotoxic, while activation of NR2A is neurotrophic (Liu et al., 2007; Terasaki et al., 2010; Lai et al., 2011). Furthermore, NR2As are largely concentrated within synapses, while NR2Bs are largely extrasynaptic (Tovar and Westbrook, 1999; Traynelis et al., 2010). Thus, the chronic pain-induced increases observed in the present study in NR2B expression (and presumably function, though this was not directly explored in the present study) could mediate excessive Ca^{2+} influx into the neuron and result in a mitochondrial overload of Ca^{2+}, which would promote dendritic and synaptic damage, cell necrosis or apoptosis. Through this mechanism, the APP/PS1 transgenic mice subjected to chronic monoarthritis pain may have exhibited poor MWM performance.

Other factors upregulating NR2Bs in the hippocampus have been shown to induce cognitive impairment. Chronic early postnatal scream sound stress upregulates NR2B levels in the CA1 and CA3, downregulates the NR2A/NR2B ratio, and impairs spatial learning and memory in male mice (Hu et al., 2016). Social isolation stress exacerbates aggressive behaviors and increases NR2A and NR2B levels in the hippocampus (Chang et al., 2015). The activation of extrasynaptic NR2B is involved in the Aβ-induced impairment of LTP in hippocampal slices (Ondrejcak et al., 2010; Li et al., 2011; Rönicke et al., 2011). NR2B antagonists rescue the impairment of LTP (Li et al., 2011; Rönicke et al., 2011), loss of synapses and synaptic proteins (Liu et al., 2010; Rönicke et al., 2011) and facilitation

of long-term depression (Li et al., 2009) induced by Aβ. But a contradictory result was found using chronic visceral pain: the higher hippocampal NR2B protein levels induced in irritable bowel syndrome-like rats facilitate the LTP of CA1 via tyrosine phosphorylation (Chen Y. et al., 2014). Thus, many unsolved questions will need to be further explored in future studies.

STUDY LIMITATIONS AND FUTURE DIRECTIONS

Some limitations existed in the preliminary study. First, pathology, including amyloid plaque deposition and neurofibrillary tangle formation, may be modified when animals are subjected to chronic pain. Thus, we will explore the effects of chronic pain, analgesia and NMDAR blockers on pathology in our next study. Second, although a previous study failed to show the superiority of memantine in their sample of patients having moderate-to-severe AD with significant baseline agitation and aggression (Herrmann et al., 2013), we will apply methods in a future study to block NMDARs and examine their effects on cognitive impairment and the NR2B response induced by chronic monoarthritis pain. Third, because we did not distinguish synaptic from extrasynaptic NMDARs in this study, we will also examine this in future studies.

CONCLUSION

The results of this study demonstrated that chronic monoarthritis pain accelerated the appearance of impaired spatial learning and memory in a transgenic mouse model of AD, supporting our hypothesis that chronic pain accelerates cognitive impairment processes. The levels of the NR2B NMDAR subunit in the hippocampal CA3 region increased most in transgenic mice modeling AD and subjected to chronic monoarthritis pain. We speculated that the neurotoxicity generated by this NMDAR subunit induced by chronic pain is responsible for the cognitive impairment observed in the current study. Our results suggest that sufficient analgesia is essential for patients with dementia or at risk of dementia. The exact mechanisms underlying the NMDAR involvement in the cognitive impairment induced by chronic pain warrants further investigation and may provide insight into preventive strategies and therapeutic targets for MCI associated with chronic pain.

AUTHOR CONTRIBUTIONS

W-YG contributed to the experimental design, established the animal model, interpreted the data and prepared the manuscript. RW co-designed the study, interpreted the data, prepared the manuscript and provided scientific leadership. YL performed the behavioral experiments, including evaluation of the pain behaviors and the Morris water maze task. HJ assisted in performing the behavioral experiments and establishing the animal model. Z-WZ and Y-LW performed the immunofluorescence and western blot experiments. H-YL and XZ captured the images and analyzed the immunofluorescence and western blot images. J-XN co-designed the experiments and provided practical organization of the study.

FUNDING

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METHODS
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A Bayesian Model for the Prediction and Early Diagnosis of Alzheimer's Disease

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Alzheimer's disease treatment is still an open problem. The diversity of symptoms, the alterations in common pathophysiology, the existence of asymptomatic cases, the different types of sporadic and familial Alzheimer's and their relevance with other types of dementia and comorbidities, have already created a myth-fear against the leading disease of the twenty first century. Many failed latest clinical trials and novel medications have revealed the early diagnosis as the most critical treatment solution, even though scientists tested the amyloid hypothesis and few related drugs. Unfortunately, latest studies have indicated that the disease begins at the very young ages thus making it difficult to determine the right time of proper treatment. By taking into consideration all these multivariate aspects and unreliable factors against an appropriate treatment, we focused our research on a non-classic statistical evaluation of the most known and accepted Alzheimer's biomarkers. Therefore, in this paper, the code and few experimental results of a computational Bayesian tool have been reported. Moreover, major attention was dedicated to the correlation and assessment of several Alzheimer's biomarkers to export a probabilistic medical prognostic process. This new statistical software is executable in the Bayesian software Winbugs, based on the latest Alzheimer's classification and the formulation of the known relative probabilities of the various biomarkers, correlated with Alzheimer's progression, through a set of discrete distributions. A user-friendly web page has been implemented for the supporting of medical doctors and researchers, to upload Alzheimer's tests and receive statistics on the occurrence of Alzheimer's disease development or presence, due to abnormal testing in one or more biomarkers.

Keywords: Alzheimer's disease, early diagnosis, medical decision systems, Bayesian statistics, Markov Chain Monte Carlo, Metropolis-Hastings Algorithm, Gibbs Sampling, Winbugs

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INTRODUCTION

A precise etiology of Alzheimer's disease (AD) is still unclear while several risk factors have been recognized to catalytically affect the early onset and the progression of the disease (Abbott and Dolgin, 2016). According to latest studies (Dubois et al., 2007), AD can be categorized according to potential risk factors, symptoms and pathophysiological lesions into eight different categories

(**Table 1**). Furthermore, these eight categories can be analyzed in depth by adding potential biomarkers in each category (**Figure 1**) which have been proved to affect the severity of the disease (Mantzavinosa et al., 2017). While several attempts at reducing AD severity have already been presented targeting mainly the symptomatic treatment (Ashraf et al., 2015) until now, there is no holistic therapy available that can efficiently reverse AD. For many scientists and pharmaceuticals companies, there are several and different treatment approaches for AD such as cholinesterase inhibitors, NMDA receptor antagonist, β-secretase inhibitors, γ-secretase inhibitors, α-secretase stimulators, tau inhibitors, immunotherapy, nutraceuticals, and nano drugs (Ashraf et al., 2015; Soursou et al., 2015) even though the more secure solution seems to be the early diagnosis of neurodegeneration signs, in order to facilitate the early diagnosis or prediction.

In this regards, Bayesian Statistics constitutes a powerful tool for Science and especially for Biomedical Informatics and Medical Decision Systems. Markov Chain Monte Carlo (MCMC) theory was provided as a solution several times, targeting environmental' s or diseases' evaluations with satisfactory results (Tzoufras, 2009). Bayesian statistics uses all the unknown parameters as random variables, to pre-define the prior distribution of the model and calculate the posterior distribution f(θ|y), which can be expressed as:

$$f(\theta|y) = \frac{f(y|\theta)f(\theta)}{f(y)} \propto f(y|\theta)f(\theta),$$

or including both the prior and the observed data by the expression of the prior distribution f(θ) and the likelihood f(y|θ) as follows:

$$f(y|\theta) = \prod_{i=1}^{n} f(y_i|\theta).$$

In this research paper, a new probabilistic model was created, describing the relationship between AD biomarkers, which may reveal and influence the disease's development, presence or progression. The algorithmic approach to AD prediction coded with WinBUGS biostatistics software (Lunn et al., 2000) for Bayesian inference, data analysis, and modeling. The model, the initial data and few examples are described in the Experimental section of this paper.

MATERIALS AND METHODS

A Probabilistic Approach to AD

Let us recall some basic mathematical notations concerning the Bayesian approach (Congdon, 2005; Vidakovic, 2011; Højsgaard, 2012). Assume a random variable Y known as a response, which follows a probabilistic path f(y|θ), where θ is a parameter vector. We consider a sample $y = [y_1, y_2,....,y_n]$ of size n. If we assume two possible events A, B where $A = A_1 \cup A_2 \cup .... \cup A_n$, $A_i \cap A_j = \varnothing \; \forall \; i \neq j$, Bayes Theorem calculates the probability to occur an event A_i given B,

$$(Ai|B) = \frac{P(B|Ai)P(Ai)}{P(B)} = \frac{P(B|Ai)P(Ai)}{\sum_{i=1}^{n} P(B|Ai)P(Ai)}.$$

In general,

$$P(A|B) = \frac{P(B|A)P(A)}{P(B)} \propto P(B|A)P(A).$$

Finally, given the observed data $y_1, y_2,...,y_n$, the posterior distribution $f(\theta|y_1,...,y_n)$ could be calculated from the prior distribution. Bayesian Inference is based on the p(θ|y) factor which is used by MCMC methods. Markov Chain Monte Carlo methods are based on iterative sampling from the posterior distribution, using various chain probabilities of the sample parameters and resulting posterior means and variances of the parameters or functions of the parameters $\Delta = \Delta(\theta)$ as follows:

$$E\left(\theta_k|y\right) = \int \theta_k p(\theta|y)d\theta,$$

$$Var\left(\theta_k|y\right) = \int \theta_k^2 p(\theta|y)d\theta - [E(\theta_\kappa|y)]^2 = E\left(\theta_k^2|y\right) - [E(\theta_k|y)]^2,$$

TABLE 1 | Alzheimer's disease classification according to symptoms and lesions based on the "Research criteria for the diagnosis of Alzheimer's disease: revising the NINCDS-ADRDA criteria" (Abbott and Dolgin, 2016).

Categories	Description
Prodromal AD (Category1)	Clinical Symptoms, memory disorders, Hippocampal volume loss and biomarkers of CSF that lead to AD pathology
AD dementia (Category2)	The social function, the composite activities of the daily life are obstructed. This state is the threshold between memory changes and in one more cognitive factor
Typical AD (Category3)	Progressive memory loss, cognitive disorders, and neuropsychiatric modifications
Atypical AD (Category4)	Progressive aphasia, Logopenic aphasia, frontal AD morphology and cortical atrophy at the posterior section. Also, is supported from amyloidosis biomarkers in brain or CSF
Mixed AD (Category5)	Incidents that validate the diagnostic AD requirements for typical AD and there are disorders such as cerebrovascular disease or Lewy Bodies disease
Preclinical states of AD (Category6)	This state includes an *in vivo* amyloidosis evidence of the brain, or individuals whose families have the autosomal dominant mutation of AD
Alzheimer's Pathology (Category7)	Senile Plaques and Neurofibrillary tangles, loss of neuronal synapses, amyloid deficits in the cerebral vascular cortex
Mild cognitive impairment (Category8)	Individuals that abstain from the clinic biological character of AD and also have measurable MCI. Those individuals may suffer from AD, but there is no evidence for AD

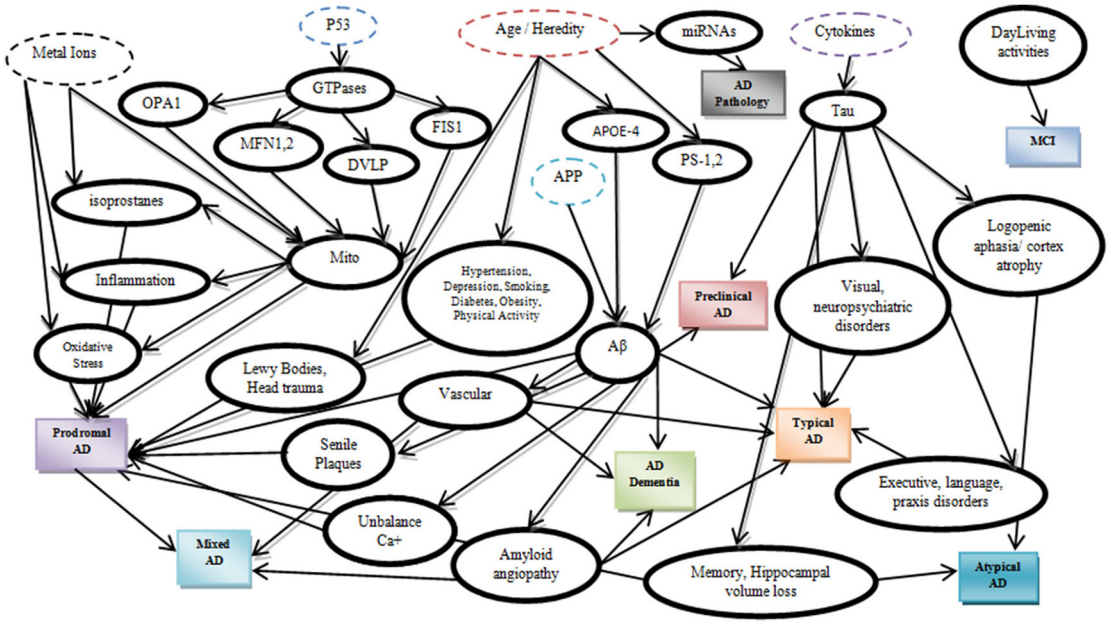

FIGURE 1 | Alzheimer's disease biomarkers expressed through a Bayesian Network.

$$E\left[\Delta(\theta)|y\right] = \int \Delta(\theta)p(\theta|y)d\theta,$$

$$Var\left[\Delta(\theta)|y\right] = \int \Delta^2 p(\theta|y)d\theta - [E(\Delta|y)]^2 = E(\Delta^2|y) - [E(\Delta|y)]^2.$$

The most popular MCMC methods are the Metropolis-Hastings Algorithm (Metropolis et al., 1953; Hastings, 1970) and its particular case, the Gibbs Sampling (Geman and Geman, 1984). In 1988, Lauritzen and Spiegelhalter presented for the first time a Bayesian expert system, the "ASIA model," introducing a fictitious medical decision system for the explanation of dyspnea due to a patient's recent visit to Asia and the presence of several other symptoms (Lauritzen and Spiegelhalter, 1988).

The proposed in this paper AD prediction model was established based on the Bayesian Networks (BN). According to BN theory, if we assume a directed graph G with N nodes, each node $n \in N$ has a number of paternal nodes pa(n) that may be linked with "child" nodes and the joint distribution for such a network given as follows:

$$P(N) = \prod_{n \in N} p(n|pa(n)).$$

By taking into consideration the latest calculations for the relative probabilities of AD progression due to certain brain lesions (**Table 2**) (Christen, 2000; de la Torre, 2002; Praticò et al., 2002; Modrego and Ferrández, 2004; Hooper et al., 2007; Cheung et al., 2008; Stone, 2008; Schuff et al., 2009; Snider et al., 2009; Wang et al., 2009; Israeli-Korn et al., 2010; Barnes and Yaffe, 2011; Nazem and Mansoori, 2011; Serrano-Pozo et al., 2011; Bird, 2012; Alzheimer's Association, 2015; Chakrabarty et al., 2015) and the majority of the published AD biomarkers (Albert et al., 2010, 2011; Besson et al., 2015; Cabezas-Opazo et al., 2015; Dong et al., 2015; Duce et al., 2015; Eskildsen et al., 2015; Jansen et al., 2015; Madeira et al., 2015; Michel, 2015; Nakanishi et al., 2015; Ossenkoppele et al., 2015; Østergaard et al., 2015; Quiroz et al., 2015; Ringman et al., 2015; Risacher et al., 2015; Sastre et al., 2015; Schindler and Fagan, 2015; Sutphen et al., 2015; Thordardottir et al., 2015; Cauwenberghe et al., 2016; Counts et al., 2016; Gaël et al., 2016; Yang et al., 2016) or calculating indirectly the relative probabilities, we designed a Bayesian model for the prediction of AD based on the abnormal testing of one or more biomarkers. The described probabilities were exported through major clinical trials globally and are continuously subject to updating and redefinition. The proposed model includes the main AD categories formulated by the categorical prior distribution.

$$r \sim dcat(p[]),$$

the majority of biomarkers that underlie AD severity and are represented as an acyclic graph.

The Winbugs software requires all the parent knots of the acyclic graph to be initialized as True, something that does not affect the model execution. In the second step of the initialization mode, the "parent" knots Metal_Ions, p53, Age/Heredity, APP, Cytokines are defined with their probabilistic values that indicate the True value, and then all the "child" knots are simply set to False/True. An exception is proposed and occur in the case of LewyBodies existence, while the only way to conclusively diagnose the Dementia with Lewy bodies is through a postmortem autopsy and it is quite difficult to be recognized as a no Alzheimer's Disease case (**Figure 2**). When a biomarker is finally selected as True, then the probabilistic

TABLE 2 | Alzheimer's disease biomarkers, biomarkers' probabilistic impact on Alzheimer's disease presence and the corresponding bibliographic reference.

Biomarker	Relative probability related to AD progression	References
Age (>85)	38%	Alzheimer's Association, 2015
Age (75–84)	43%	Alzheimer's Association, 2015
Age (65–74)	15%	Alzheimer's Association, 2015
Age (<65)	4%	Alzheimer's Association, 2015
Lewy Body disease	10-20% The only way to conclusively diagnose the Dementia with Lewy Bodies is through a postmortem autopsy, and it is quite difficult to be recognized as no Alzheimer's Disease	Alzheimer's Association, 2015
APP	10%,15%,50%	Bird, 2012
Hypertension	20%	Israeli-Korn et al., 2010
GTPases	<1%	Alzheimer's Association, 2015
Depression	13.2%	Modrego and Ferrández, 2004; Barnes and Yaffe, 2011
Smoking	27.4%	Barnes and Yaffe, 2011
Diabetes	6.4%	Barnes and Yaffe, 2011
Obesity	3.4%	Barnes and Yaffe, 2011
Physical Activity	17.7%	Barnes and Yaffe, 2011
APOE4	30-70%	Bird, 2012
PS 1,2	5%	Bird, 2012
Amyloid Angiopathy	80%	Serrano-Pozo et al., 2011
Oxidative Stress	25-30%	Christen, 2000
Inflammation	30-40%	de la Torre, 2002
Isoprostanes	50%	Praticò et al., 2002
P53	75%	Hooper et al., 2007
Cytokines	50%	Chakrabarty et al., 2015
miRNAs	60%	Wang et al., 2009
DVLP	74.3%	Wang et al., 2009
OPA1	61.4%	Wang et al., 2009
MFN1	27.8%	Wang et al., 2009
MFN2	33.6%	Wang et al., 2009
FIS1	60%	Wang et al., 2009
Visual, neuropsychiatric disorders	5%	Alzheimer's Association, 2015
Executive, language, praxis disorders	40%	Alzheimer's Association, 2015
DayLiving disorders	10-20%	Alzheimer's Association, 2015
Metal Ions	24%	Nazem and Mansoori, 2011
Unbalance Ca	5%	Shilling et al., 2014
Senile plaques	Over 60% until the Age of 80 and increases linearly on the Age	Stone, 2008
Amyloid Beta	Over 50% in Ages>85	Snider et al., 2009
Hippocampal volume loss/Memory Impairment	Approximately 10% of elders over the age of 70 years have significant memory loss and more than half of these individuals have AD	Schuff et al., 2009

impact value is attributed to the related knot, according to **Table 2** and the following rule: for the "parent" knots first we assign the probability to be False and then the probability to be True. For the "child" knots we assign probabilities in the form of False|False, False|True, True|False, True|True (**Figures 3–6**).

Experimental

While a single biomarker can be related to more than one AD types, the probabilistic model consists of categorical variables-nodes (∼dcat) where each variable node can be linked with two or more parent variables-nodes or can be presented as a single and independent variable-node. In the case where a node is linked to more than two parent nodes, another similar variable-node is created at the same level within the model. The proposed BN has been designed according to the latest "Research criteria for the diagnosis of Alzheimer's disease: revising the NINCDS-ADRDA criteria" (Dubois et al., 2007) and the model exports for every AD category the maximum probability value given by the biomarkers' evaluation, as it is described below

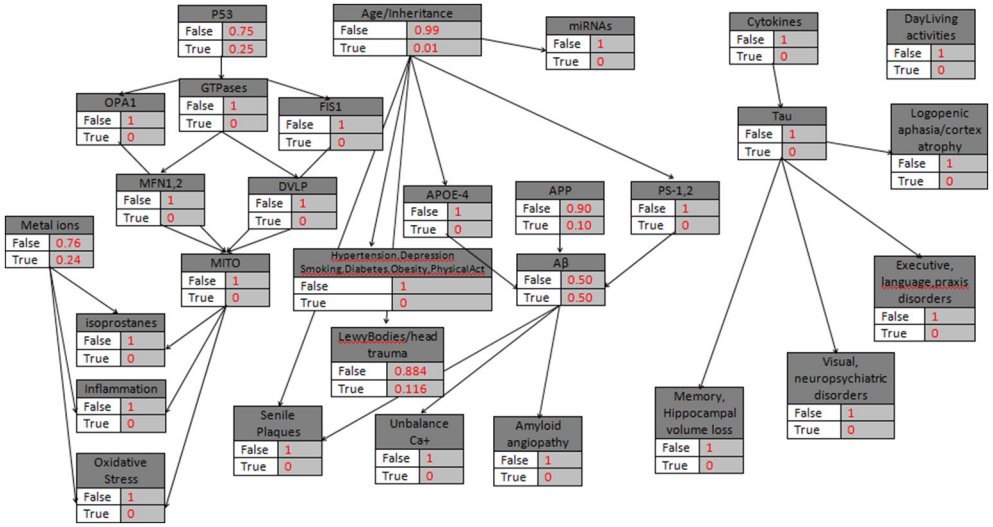

FIGURE 2 | The general probabilistic model with the knots initializations. APP is set to 10%, Age>85, the "parent" knots and the LewyBodies are set to their probabilistic values.

along with the lists of initial values and data from the Winbugs Software.

The General Form of the Model{
Age∼ dcat([1:2])
Ab∼dcat(APOE4, PS1-2, APP[1:2])
Tau, Phospho ∼ dcat(Cytokines 1:2])
MetalIons ∼ dcat([1:2])
LewyBodies ∼dcat(Age[1:2])
Hypertension ∼ dcat(p.Hypertension[Age_Inheritance,1:2])
Depression ∼ dcat(p.Depression[Age_Inheritance,1:2])
Smoking ∼dcat(p.Smoking[Age_Inheritance,1:2])
Diabetes ∼dcat(p.Diabetes[Age_Inheritance,1:2])
Obesity∼dcat(p.Obesity[Age_Inheritance,1:2])
PhysicalActivity∼dcat(p.PhysicalActivity[Age_Inheritance, 1:2])
APP ∼ dcat([1:2])
GTP ∼ dcat(p53[1:2])
APOE4 ∼ dcat(Age[1:2])
PS1-2 ∼ dcat(Age[1:2])
Cytokines ∼ dcat([1:2])
SenilePlaques ∼ dcat(Ab[1:2])
UnbalanceCa ∼ dcat(Ab[1:2])
Vascular ∼ dcat(Ab, Tau_Phospho[1:2])
LogopenicAphasia, CortexAtrophy∼dcat(Tau_Phospho [1:2])
Memory, HippocampalLoss∼dcat(Tau, Phospho[1:2])
ExecLangPrax∼dcat(Tau_Phospho[1:2])
Visual, Neuropsychiatric∼dcat(Tau_Phospho[1:2])
DailyActivities ∼ dcat([1:2])
OxidStress, Inflamation, Isoprostanes ∼dcat(Mito, MetalIons [1:2])
Mito ∼dcat(MetalIons,OPA1, MFN1,DVLP, FIS1 [1:2])
MFN1∼dcat(GTP[1:2])
OPA1∼dcat(GTP[1:2])
DVLP∼dcat(GTP[1:2])
FIS1∼dcat GTP[1:2])
p53 ∼ dcat([1:2])
miRNAs∼dcat(Age[1:2])
MCI∼dcat(DailyAct[1:2])
max1 ← max(Ab, LewyBodies, Mito, OxidStress,
  Memory_Hippocampal_loss, SenilePlaques,
  Unbalance_Ca, Hypertension_depression, Inflamation,
  Isoprostanes, Mito)
ProdromalAD ← max(max1,OxidStress)
ADdementia ← max(Ab, Vascular)
max2 ← max(Ab, Tau, Phospho, Vascular, ExecLangPrax)
TypicalAD ← max(max2, Visual, Neuropsychiatric)
AtypicalAD ← max(LogopenicAphasia, CortexAtrophy, Memory, HippocampalLoss)
MixedAD ← max(Vascular,Category1)
PreclinicalAD ← max(Ab, Tau, Phosph)
ADPathology ← miRNAs
MildCognitiveImpairment ← MCI
}

The model can be extended or adjusted to new biomarkers or relations between the symptoms, the lesions and the exported AD categories. Additionally, the relative probabilities can be updated or even more replaced by the biomarkers values when a secure protocol for AD diagnosis will be verified or proposed by the international health associations. Four examples are provided below concerning cases of abnormal biomarkers tests, revealing potential AD presence.

RESULTS

Example 1

In the first hypothetical case study, a patient is assumed to be diagnosed with problems in daily living activities but with

no other results of abnormal AD biomarkers. Additionally, the patient belongs to a risk group due to the age factor (>85). Therefore, while there is evidence only for abnormal Daily-Living activities, the corresponding node becomes "True," and all the other nodes take the "False" value (**Figure 3**). The model calculates the $P(MCI|DailyLivingActivities)$, the probability that Mild Cognitive Impairment is characterized 'True' given the DailyLivingActivities variable, which can be written as follows:

$$P\left(MCI|DailyLivingActivities\right) = \frac{P(MCI|DailyLivingActivities)P(MCI)}{P(DailyLivingActivities)}$$
$$P(MCI|DailyLivingActivities) = 0.999.$$

Data List
(Age_Inheritance =2, MetalIons=2, APP=2, Cytokines=2, DailyActivities=2, p53=2,
p.Age_Inheritance = c(0.99,0.01),
p.Ab= structure(.Data = c(0.50,0.50,0.50,0.50,0.50,0.50,0.50,0.50), .Dim = c(2,2,2)),
p.Tau_Phospho =structure(.Data = c(1,0,1,0), .Dim = c(2,2)),
p.MetalIons = c(0.76, 0.24),
p.LewyBodies=structure(.Data = c(0.884,0.116,0.884,0.116), .Dim = c(2,2)),
p.Hypertension=structure(.Data = c(1,0,1,0), .Dim = c(2,2)),
p.Depression=structure(.Data = c(1,0,1,0), .Dim = c(2,2)),
p.Smoking=structure(.Data = c(1,0,1,0), .Dim = c(2,2)),
p.Diabetes=structure(.Data = c(1,0,1,0), .Dim = c(2,2)),
p.Obesity=structure(.Data = c(1,0,1,0), .Dim = c(2,2)),
p.PhysicalActivity=structure(.Data = c(1,0,1,0), .Dim = c(2,2)),
p.APP = c(0.90,0.10),
p.GTP = structure(.Data = c(1,0,1,0), .Dim = c(2,2)),
p.APOE4 = structure(.Data = c(1,0,1,0), .Dim = c(2,2)),
p.PS1_2 = structure(.Data = c(1,0,1,0), .Dim = c(2,2)),
p.Cytokines = c(1,0),
p.SenilePlaques = structure(.Data = c(1,0,1,0,1,0,1,0), .Dim = c(2,2,2)),
p.Unbalance_Ca = structure(.Data = c(1,0,1,0,1,0,1,0), .Dim = c(2,2,2)),
p.Vascular = structure(.Data = c(1,0,1,0,1,0,1,0), .Dim = c(2,2,2)),
p.LogopenicAphasiaCortexAtrophy =structure(.Data = c(1,0,1,0), .Dim = c(2,2)),
p.MemoryHippocampalLoss = structure(.Data = c(1,0,1,0), .Dim = c(2,2)),
p.ExecLangPrax=structure(.Data = c(1,0,1,0), .Dim = c(2,2)),
p.VisualNeuropsychiatric =structure(.Data = c(1,0,1,0), .Dim = c(2,2)),
p.DailyActivities = c(0,1),
p.OxidStress1=structure(.Data = c(1,0,1,0,1,0,1,0), .Dim = c(2,2,2)),
p.OxidStress2=structure(.Data = c(1,0,1,0,1,0,1,0), .Dim = c(2,2,2)),
p.Inflamation1=structure(.Data = c(1,0,1,0,1,0,1,0), .Dim = c(2,2,2)),
p.Inflamation2=structure(.Data = c(1,0,1,0,1,0,1,0), .Dim = c(2,2,2)),
p.Isoprostanes1=structure(.Data = c(1,0,1,0,1,0,1,0), .Dim = c(2,2,2)),
p.Isoprostanes2=structure(.Data = c(1,0,1,0,1,0,1,0), .Dim = c(2,2,2)),
p.Mito1=structure(.Data = c(1,0,1,0,1,0,1,0), .Dim = c(2,2,2)),
p.Mito2=structure(.Data = c(1,0,1,0,1,0,1,0), .Dim = c(2,2,2)),
p.Mito3=structure(.Data = c(1,0,1,0), .Dim = c(2,2)),
p.MFN1 = structure(.Data = c(1,0,1,0), .Dim = c(2,2)),
p.OPA1= structure(.Data = c(1,0,1,0), .Dim = c(2,2)),
p.DVLP= structure(.Data = c(1,0,1,0), .Dim = c(2,2)),
p.FIS1= structure(.Data = c(1,0,1,0), .Dim = c(2,2)),
p.p53 = c(0.75,0.25),
p.Ab_APP= structure(.Data = c(1,0,1,0), .Dim = c(2,2)),
p.miRNAs=structure(.Data=c(1,0,1,0), .Dim = c(2,2)),
p.MCI_due_to_DayLiving=structure(.Data=c(0.001,0.999, 0.001,0.999), .Dim = c(2,2)))

Executing the Winbugs code, the result for MCI category is the same as calculated above.

For each stochastic variable of the generated probabilistic model, Winbugs defines the categorical interval (Dubois et al., 2007; Abbott and Dolgin, 2016) for the categorical distribution ~dcat, which receives only positive values. The MCMC results, posterior summary estimations, mean, standard deviation and the estimation of the error is implemented by the batch mean method (**Tables 3, 4**). After 3000 and 10000 iterations of the current MCMC Winbugs algorithms, the mean value of MCI category can be similarly calculated as:

$$EMCI = 2^{*}p_{MCI} + 1.\ \left(1 - p_{MCI}\right)$$
$$= 2\,P\left(MCI|DailyLivingActivities\right)$$
$$= p_{MCI} = 2 - 1.999 = 0.999.$$

Example 2

In a similar case (age>85) where miRNAs' biomarker is assumed to be "True", and there is no other evidence of heredity concerning AD (**Figure 4**), the model calculates the $P\left(ADPathology|miRNAs\right)$. However, while miRNAs' node is also linked to the Age/Heredity node, there is a probabilistic relation between the Age/Heredity and miRNAs' nodes (**Tables 5, 6**).

$$P\left(ADPathology|miRNAs\right) = \frac{P(ADPathology|miRNAs)P(ADPathology)}{P(miRNAs)}$$
$$P(ADPathology|miRNAs) = 1.0.$$

Thus, importing the adjusted data below to the Winbugs, in the case of *ADPathology* given that the miRNAs' variable is "True", the exported probability is 1.

Data List
(Age_Inheritance =2, MetalIons=2, APP=2, Cytokines=2, DailyActivities=2, p53=2,
p.Age_Inheritance = c(0.99,0.01),
p.Ab= structure(.Data = c(0.50,0.50,0.50,0.50,0.50,0.50,0.50,0.50), .Dim = c(2,2,2)),
p.Tau_Phospho =structure(.Data = c(1,0,1,0), .Dim = c(2,2)),
p.MetalIons = c(0.76, 0.24),
p.LewyBodies=structure(.Data = c(0.884,0.116,0.884,0.116), .Dim = c(2,2)),
p.Hypertension=structure(.Data = c(1,0,1,0), .Dim = c(2,2)),
p.Depression=structure(.Data = c(1,0,1,0), .Dim = c(2,2)),
p.Smoking=structure(.Data = c(1,0,1,0), .Dim = c(2,2)),
p.Diabetes=structure(.Data = c(1,0,1,0), .Dim = c(2,2)),
p.Obesity=structure(.Data = c(1,0,1,0), .Dim = c(2,2)),
p.PhysicalActivity=structure(.Data = c(1,0,1,0), .Dim = c(2,2)),
p.APP = c(0.90,0.10),
p.GTP = structure(.Data = c(1,0,1,0), .Dim = c(2,2)),
p.APOE4 = structure(.Data = c(1,0,1,0), .Dim = c(2,2)),
p.PS1_2 = structure(.Data = c(1,0,1,0), .Dim = c(2,2)),
p.Cytokines = c(1,0),
p.SenilePlaques = structure(.Data = c(1,0,1,0,1,0,1,0), .Dim = c(2,2,2)),
p.Unbalance_Ca = structure(.Data = c(1,0,1,0,1,0,1,0), .Dim = c(2,2,2)),
p.Vascular = structure(.Data = c(0,1,0,1,0,1,0,1), .Dim = c(2,2,2)),
p.LogopenicAphasiaCortexAtrophy =structure(.Data = c(1,0,1,0), .Dim = c(2,2)),
p.MemoryHippocampalLoss = structure(.Data = c(1,0,1,0), .Dim = c(2,2)),
p.ExecLangPrax=structure(.Data = c(1,0,1,0), .Dim = c(2,2)),
p.VisualNeuropsychiatric =structure(.Data = c(1,0,1,0), .Dim = c(2,2)),
p.DailyActivities = c(0,1),
p.OxidStress1=structure(.Data = c(1,0,1,0,1,0,1,0), .Dim = c(2,2,2)),
p.OxidStress2=structure(.Data = c(1,0,1,0,1,0,1,0), .Dim = c(2,2,2)),
p.Inflamation1=structure(.Data = c(1,0,1,0,1,0,1,0), .Dim = c(2,2,2)),
p.Inflamation2=structure(.Data = c(1,0,1,0,1,0,1,0), .Dim = c(2,2,2)),
p.Isoprostanes1=structure(.Data = c(1,0,1,0,1,0,1,0), .Dim = c(2,2,2)),
p.Isoprostanes2=structure(.Data = c(1,0,1,0,1,0,1,0), .Dim = c(2,2,2)),
p.Mito1=structure(.Data = c(1,0,1,0,1,0,1,0), .Dim = c(2,2,2)),
p.Mito2=structure(.Data = c(1,0,1,0,1,0,1,0), .Dim = c(2,2,2)),
p.Mito3=structure(.Data = c(1,0,1,0), .Dim = c(2,2)),
p.MFN1 = structure(.Data = c(1,0,1,0), .Dim = c(2,2)),
p.OPA1= structure(.Data = c(1,0,1,0), .Dim = c(2,2)),
p.DVLP= structure(.Data = c(1,0,1,0), .Dim = c(2,2)),
p.FIS1= structure(.Data = c(1,0,1,0), .Dim = c(2,2)),
p.p53 = c(0.75,0.25),
p.Ab_APP= structure(.Data = c(1,0,1,0), .Dim = c(2,2)),
p.miRNAs=structure(.Data=c(0,1,0,1), .Dim = c(2,2)),
p.MCI_due_to_DayLiving=structure(.Data=c(1,0,1,0), .Dim = c(2,2)))

After 10000 iterations, the mean value of *ADPathology* is calculated as:

$$EADPathology = 2 * p_{ADPathology} + 1.\left(1 - p_{ADPathology}\right) = 2,$$
$$P\left(ADPathology|miRNAs\right) = p_{ADPathology} = 2 - 1 = 1.$$

Example 3

In the third example, without the age being a risk factor (<60) the most common case is presented, where both Amyloid-beta and Tau proteins' abnormalities occur, with additional 'True' values in the Age_Inheritance, APP, APOE4 and Vascular variables of the probabilistic model (**Figure 5**).

Data List
(Age_Inheritance =2, MetalIons=2, APP=2, Cytokines=2, DailyActivities=2, p53=2,
p.Age_Inheritance = c(0.57,0.43),
p.Ab= structure(.Data = c(0,1,0,1,0,1,0,1), .Dim = c(2,2,2)),
p.Tau_Phospho =structure(.Data = c(0,1,0,1), .Dim = c(2,2)),
p.MetalIons = c(0.76, 0.24),
p.LewyBodies=structure(.Data = c(0.884,0.116,0.884,0.116), .Dim = c(2,2)),
p.Hypertension=structure(.Data = c(1,0,1,0), .Dim = c(2,2)),
p.Depression=structure(.Data = c(1,0,1,0), .Dim = c(2,2)),
p.Smoking=structure(.Data = c(1,0,1,0), .Dim = c(2,2)),
p.Diabetes=structure(.Data = c(1,0,1,0), .Dim = c(2,2)),
p.Obesity=structure(.Data = c(1,0,1,0), .Dim = c(2,2)),
p.PhysicalActivity=structure(.Data = c(1,0,1,0), .Dim = c(2,2)),
p.APP = c(0.50,0.50),
p.GTP = structure(.Data = c(1,0,1,0), .Dim = c(2,2)),
p.APOE4 = structure(.Data = c(0.30,0.70,0.30,0.70), .Dim = c(2,2)),
p.PS1_2 = structure(.Data = c(1,0,1,0), .Dim = c(2,2)),
p.Cytokines = c(1,0),
p.SenilePlaques = structure(.Data = c(1,0,1,0,1,0,1,0), .Dim = c(2,2,2)),
p.Unbalance_Ca = structure(.Data = c(1,0,1,0,1,0,1,0), .Dim = c(2,2,2)),
p.Vascular = structure(.Data = c(0,1,0,1,0,1,0,1), .Dim = c(2,2,2)),
p.LogopenicAphasiaCortexAtrophy =structure(.Data = c(1,0,1,0), .Dim = c(2,2)),
p.MemoryHippocampalLoss = structure(.Data = c(1,0,1,0), .Dim = c(2,2)),

p.ExecLangPrax=structure(.Data = c(1,0,1,0), .Dim = c(2,2)),
p.VisualNeuropsychiatric =structure(.Data = c(1,0,1,0), .Dim = c(2,2)),
p.DailyActivities = c(0,1),
p.OxidStress1=structure(.Data = c(1,0,1,0,1,0,1,0), .Dim = c(2,2,2)),
p.OxidStress2=structure(.Data = c(1,0,1,0,1,0,1,0), .Dim = c(2,2,2)),
p.Inflamation1=structure(.Data = c(1,0,1,0,1,0,1,0), .Dim = c(2,2,2)),
p.Inflamation2=structure(.Data = c(1,0,1,0,1,0,1,0), .Dim = c(2,2,2)),
p.Isoprostanes1=structure(.Data = c(1,0,1,0,1,0,1,0), .Dim = c(2,2,2)),
p.Isoprostanes2=structure(.Data = c(1,0,1,0,1,0,1,0), .Dim = c(2,2,2)),
p.Mito1=structure(.Data = c(1,0,1,0,1,0,1,0), .Dim = c(2,2,2)),
p.Mito2=structure(.Data = c(1,0,1,0,1,0,1,0), .Dim = c(2,2,2)),
p.Mito3=structure(.Data = c(1,0,1,0), .Dim = c(2,2)),
p.MFN1 = structure(.Data = c(1,0,1,0), .Dim = c(2,2)),
p.OPA1= structure(.Data = c(1,0,1,0), .Dim = c(2,2)),
p.DVLP= structure(.Data = c(1,0,1,0), .Dim = c(2,2)),
p.FIS1= structure(.Data = c(1,0,1,0), .Dim = c(2,2)),
p.p53 = c(0.75,0.25),
p.Ab_APP= structure(.Data = c(0,1,0,1), .Dim = c(2,2)),
p.miRNAs=structure(.Data=c(0,1,0,1), .Dim = c(2,2)),
p.MCI_due_to_DayLiving=structure(.Data=c(1,0,1,0), .Dim = c(2,2)))

Given the initial data set above, after 10000 iterations the estimated probabilities of the eight AD categories (**Tables 7, 8**) reveals high risk for AD presence. The results highlight the role of Amyloid-beta and Tau proteins and emphasize their importance and effectiveness in AD aggravation.

Example 4

In the fourth example, the hypothetical patient (age<60) is a Smoker with an Obesity problem and Depression symptoms (**Figure 6**). The Bayesian model calculates the probabilities respectively,

$$P(ProdromalAD|Depression,\ Obesity,\ Smoking)$$
$$and\ P(MixedAD|Depression,\ Obesity,\ Smoking).$$
$$P(ProdromalAD|Depression, Obesity,\ Smoking)$$
$$= P(MixedAD|Depression, Obesity,\ Smoking) =$$
$$P(ProdromalAD, MixedAD|Depression, Obesity,\ Smoking)$$
$$= \frac{P(ProdromalAD, MixedAD)}{P(Depression, Obesity,\ Smoking)}$$
$$= 0.464.$$

Data List
(Age_Inheritance =2, MetalIons=2, APP=2, Cytokines=2, DailyActivities=2, p53=2,
p.Age_Inheritance = c(0.57,0.43),
p.Ab= structure(.Data = c(1,0,1,0,1,0,1,0), .Dim = c(2,2,2)),
p.Tau_Phospho =structure(.Data = c(1,0,1,0), .Dim = c(2,2)),
p.MetalIons = c(0.76, 0.24),
p.LewyBodies=structure(.Data = c(0.884,0.116,0.884,0.116), .Dim = c(2,2)),
p.Hypertension=structure(.Data = c(1,0,1,0), .Dim = c(2,2)),
p.Depression=structure(.Data = c(0.868,0.132,0.868,0.132), .Dim = c(2,2)),
p.Smoking=structure(.Data = c(0.726,0.274,0.726,0.274), .Dim = c(2,2)),
p.Diabetes=structure(.Data = c(1,0,1,0), .Dim = c(2,2)),
p.Obesity=structure(.Data = c(0.966,0.034,0.966,0.034), .Dim = c(2,2)),
p.PhysicalActivity=structure(.Data = c(1,0,1,0), .Dim = c(2,2)),
p.APP = c(0.50,0.50),
p.GTP = structure(.Data = c(1,0,1,0), .Dim = c(2,2)),
p.APOE4 = structure(.Data = c(1,0,1,0), .Dim = c(2,2)),
p.PS1_2 = structure(.Data = c(1,0,1,0), .Dim = c(2,2)),
p.Cytokines = c(1,0),
p.SenilePlaques = structure(.Data = c(1,0,1,0,1,0,1,0), .Dim = c(2,2,2)),
p.Unbalance_Ca = structure(.Data = c(1,0,1,0,1,0,1,0), .Dim = c(2,2,2)),
p.Vascular = structure(.Data = c(0,1,0,1,0,1,0,1), .Dim = c(2,2,2)),
p.LogopenicAphasiaCortexAtrophy =structure(.Data = c(1,0,1,0), .Dim = c(2,2)),
p.MemoryHippocampalLoss = structure(.Data = c(1,0,1,0), .Dim = c(2,2)),
p.ExecLangPrax=structure(.Data = c(1,0,1,0), .Dim = c(2,2)),
p.VisualNeuropsychiatric =structure(.Data = c(1,0,1,0), .Dim = c(2,2)),
p.DailyActivities = c(1,0),
p.OxidStress1=structure(.Data = c(1,0,1,0,1,0,1,0), .Dim = c(2,2,2)),
p.OxidStress2=structure(.Data = c(1,0,1,0,1,0,1,0), .Dim = c(2,2,2)),
p.Inflamation1=structure(.Data = c(1,0,1,0,1,0,1,0), .Dim = c(2,2,2)),
p.Inflamation2=structure(.Data = c(1,0,1,0,1,0,1,0), .Dim = c(2,2,2)),
p.Isoprostanes1=structure(.Data = c(1,0,1,0,1,0,1,0), .Dim = c(2,2,2)),
p.Isoprostanes2=structure(.Data = c(1,0,1,0,1,0,1,0), .Dim = c(2,2,2)),
p.Mito1=structure(.Data = c(1,0,1,0,1,0,1,0), .Dim = c(2,2,2)),
p.Mito2=structure(.Data = c(1,0,1,0,1,0,1,0), .Dim = c(2,2,2)),
p.Mito3=structure(.Data = c(1,0,1,0), .Dim = c(2,2)),
p.MFN1 = structure(.Data = c(1,0,1,0), .Dim = c(2,2)),
p.OPA1= structure(.Data = c(1,0,1,0), .Dim = c(2,2)),
p.DVLP= structure(.Data = c(1,0,1,0), .Dim = c(2,2)),
p.FIS1= structure(.Data = c(1,0,1,0), .Dim = c(2,2)),

```
p.p53 = c(0.75,0.25),
p.Ab_APP= structure(.Data = c(1,0,1,0), .Dim = c(2,2)),
p.miRNAs=structure(.Data=c(0,1,0,1), .Dim = c(2,2)),
p.MCI_due_to_DayLiving=structure(.Data=c(1,0,1,0), .Dim
= c(2,2)))
```

Given the initial dataset above, after 10000 iterations the estimated probabilities of the eight AD categories (**Tables 9**, **10**) reveals a medium risk for AD presence due Depression, Smoking and Obesity and a set of risk factors for related comorbidities. The results in general, highlight the role of Hypertension, Depression, Smoking, Diabetes, Obesity, and Physical Inactivity as potential AD biomarkers and emphasize their importance and effectiveness in AD aggravation. The calculated probabilities verify the latest clinical findings (Modrego and Ferrández, 2004; Barnes and Yaffe, 2011) where the combination of Mild Cognitive Impairment and Depression in patients, doubles the risk of Alzheimer Dementia development compared with those without depression.

DISCUSSION

While AD is a hardly curable disease, few computational diagnostic tools have been published during the last years, for

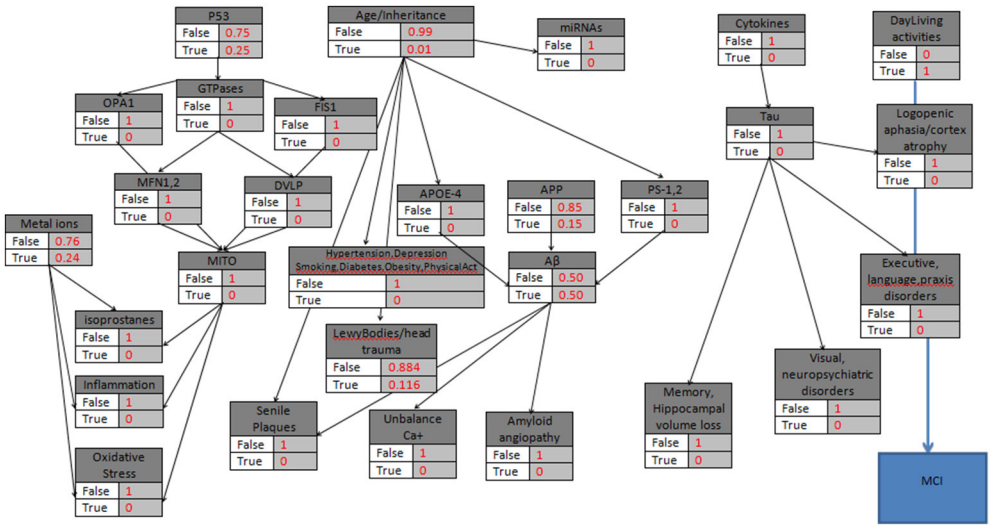

FIGURE 3 | The probabilistic model that can be used for MCI validation with the knots initializations. APP is set to 15%, Age>85, the "parent" knots and the LewyBodies are set to their probabilistic values, and the DailyAcivities have a "strong" probability equal to 1.

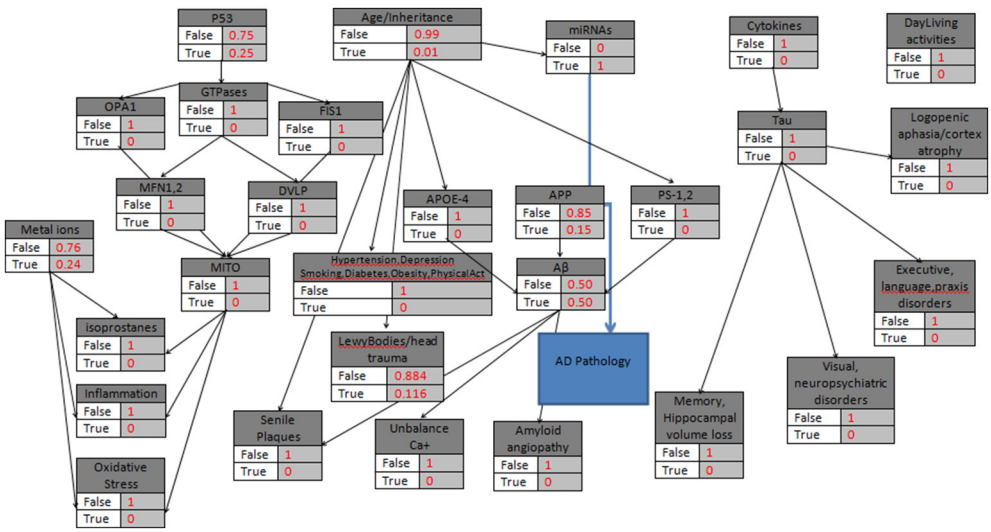

FIGURE 4 | The probabilistic model that can be used for AD Pathology validation with the knots initializations. APP is set to 15%, Age>85, the "parent" knots and the LewyBodies are set to their probabilistic values, and the miRNAs have a "strong" probability equal to 1.

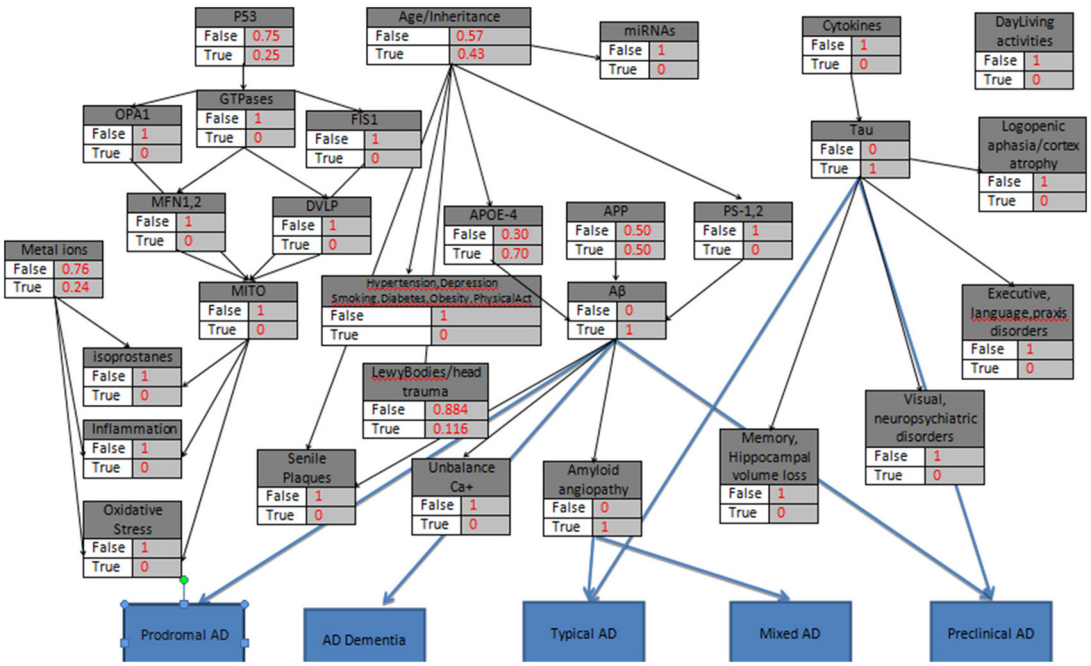

FIGURE 5 | The probabilistic model referring to several categories of Alzheimer's disease simultaneously, with the knots initializations. APP is set to 50%, Age<60, the "parent" knots and the LewyBodies are set to their probabilistic values, and the biomarkers Tau, Aβ, APOE4, Amyloid Angiopathy have a "strong" probability equal to 1.

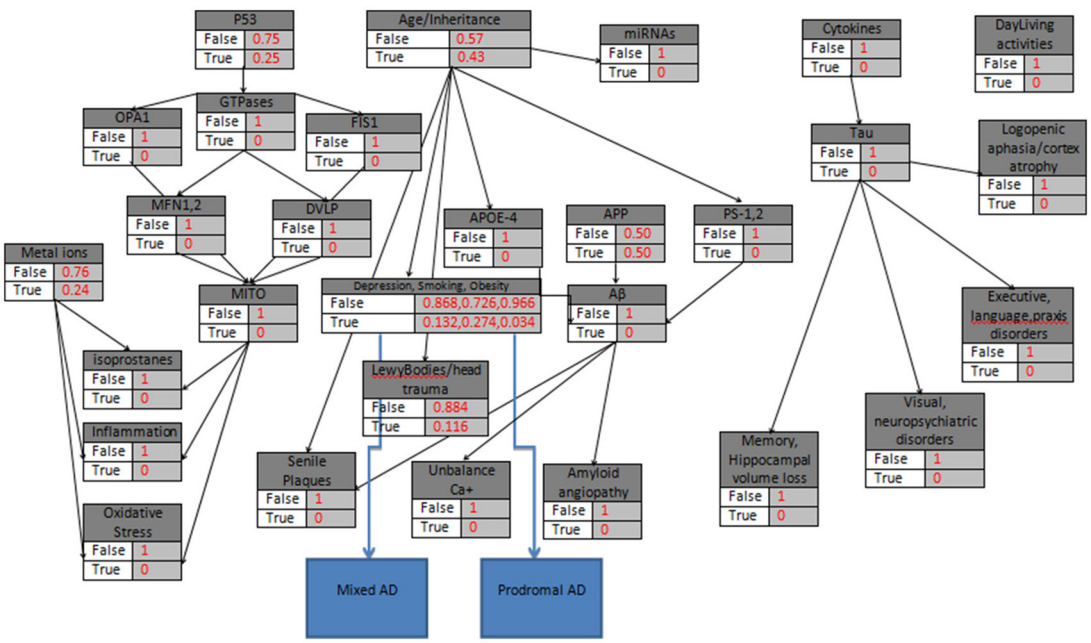

FIGURE 6 | The probabilistic model that can be used for Prodromal AD and Mixed AD validation due to Depression, Obesity and Smoking, with the knots initializations. APP is set to 50%, Age<60, the "parent" knots, the LewyBodies and the Depression, Obesity and Smoking Biomarkers are set to their probabilistic values.

TABLE 3 | WINBUGS statistics for Alzheimer's disease categories according to Example 1.

Node	Mean (After 3000 Iterations)	Mean (After 10000 Iterations)	Standard deviation (After 3000 Iterations)	Standard deviation (After 10000 Iterations)	MC error (After 3000 Iterations)	MC error (After 10000 Iterations)
Prodromal AD	1.566	1.562	0.4957	0.4961	0.007927	0.00473
AD dementia	1.506	1.502	0.5	0.5	0.008313	0.00458
Typical AD	1.506	1.502	0.5	0.5	0.008313	0.00458
Atypical AD	1.0	1.0	0.0	0.0	1.826E-12	1.0E-12
Mixed AD	1.566	1.562	0.4957	0.4961	0.007927	0.00473
Preclinical states of AD	1.506	1.502	0.5	0.5	0.008313	0.00458
Alzheimer's Pathology	1.0	1.0	0.0	0.0	1.826E-12	1.0E-12
Mild Cognitive Impairment	1.999	1.999	0.03649	0.03603	6.423E-4	3.667E-4

TABLE 4 | The total probability value for Alzheimer's disease presence due to alterations in DayLiving Activities.

Alzheimer's disease classification	Probability of Alzheimer's disease presence (in response to DayLiving Activities biomarker, after 3000 Iterations)	Probability of Alzheimer's disease presence (in response to DayLiving Activities biomarker, after 10000 Iterations)
Prodromal AD	0.566	0.562
AD dementia	0.506	0.502
Typical AD	0.506	0.502
Atypical AD	0.0	0.0
Mixed AD	0.566	0.562
Preclinical states of AD	0.506	0.502
Alzheimer's Pathology	0.0	0.0
Mild Cognitive Impairment	0.999	0.999

The results revealed the highest probability 0.999 for the case of Mild Cognitive Impairment, while Prodromal AD and Mixed AD show also high scores.

TABLE 5 | WINBUGS statistics for Alzheimer's disease categories according to Example 2.

Node	Mean	Standard deviation	MC error after 10000 iterations in WinBugs
Prodromal AD	1.562	0.4961	0.00473
AD dementia	2.0	0.0	1.0E-12
Typical AD	2.0	0.0	1.0E-12
Atypical AD	1.0	0.0	1.0E-12
Mixed AD	2.0	0.0	1.0E-12
Preclinical states of AD	1.502	0.5	0.00458
Alzheimer's Pathology	2.0	0.0	1.0E-12
Mild Cognitive Impairment	1.0	0.0	1.0E-12

TABLE 6 | The total probability value for Alzheimer's disease presence due to alterations in *miRNAs* biomarker of the patient.

Alzheimer's disease classification	Probability of Alzheimer's disease presence (in response to miRNAs biomarker)
Prodromal AD	0.562
AD dementia	1.0
Typical AD	1.0
Atypical AD	0.0
Mixed AD	1.0
Preclinical states of AD	0.502
Alzheimer's Pathology	1.0
Mild Cognitive Impairment	0.0

The results revealed the highest probability 1 for the case of AD Pathology, while Prodromal AD and Mixed AD show also high scores.

the evaluation of biomarkers and symptoms and the automated prediction of the disease. There are algorithms for an automated Dementia identification based on MRI, PET and SPECT imaging analysis using Bayes classifiers, support vector machines, and artificial neural networks (Zheng et al., 2016). According to these specific methods, the systems have to be trained with as many cases as possible to improve accuracy in a clinical dataset. There is also a tool for the automatic diagnosis of AD via the combination of PET Images and Neuropsychological Test Data (Segovia et al., 2014). According to its documentation, authors using a multi-kernel classification approach trained a mixed data set to improve the accuracy of their diagnosis in compare with other methods that evaluate imaging results exclusively. It is important to mention another latest clinical decision support system for AD that combines a Rule-Based System with a Clinical Guideline-Based System, and it is modeled through a Bayesian Network (Seixas et al., 2014). This is another case of a decision trained system that accesses a specific dataset of biomarkers to provide an accurate diagnosis of Dementia, Alzheimer's and MCI.

In the current method, all the known AD biomarkers are combined in a complex Bayesian Network to establish

TABLE 7 | WINBUGS statistics for Alzheimer's disease categories according to Example 3.

Node	Mean	Standard deviation	MC error after 10000 iterations in WinBugs
Prodromal AD	2.0	0.0	1.0E-12
AD dementia	2.0	0.0	1.0E-12
Typical AD	2.0	0.0	1.0E-12
Atypical AD	1.0	0.0	1.0E-12
Mixed AD	2.0	0.0	1.0E-12
Preclinical states of AD	2.0	0.0	1.0E-12
Alzheimer's Pathology	1.0	0.0	1.0E-12
Mild Cognitive Impairment	1.0	0.0	1.0E-12

TABLE 8 | The total probability value for Alzheimer's disease presence due to alterations in Ab, Tau/TotalTau, age/inheritance, APP, APOE4 and Vascular disorders of the patient.

Alzheimer's disease classification	Probability of Alzheimer's disease presence (in response to Ab, Tau/TotalTau, age/inheritance, APP, APOE4 and Vascular disorders biomarkers)
Prodromal AD	1.0
AD dementia	1.0
Typical AD	1.0
Atypical AD	0.0
Mixed AD	1.0
Preclinical states of AD	1.0
Alzheimer's Pathology	0.0
Mild Cognitive Impairment	0.0

As it expected, the results revealed high probabilities for the cases of Prodromal AD, AD dementia, Typical AD, Mixed AD, Preclinical states of AD.

TABLE 9 | WINBUGS statistics for Alzheimer's disease categories according to Example 4.

Node	Mean	Standard deviation	MC error after 10000 iterations in WinBugs
Prodromal AD	1.464	0.4987	0.004383
AD dementia	1.0	0.0	1.0E-12
Typical AD	1.0	0.0	1.0E-12
Atypical AD	1.0	0.0	1.0E-12
Mixed AD	1.464	0.4987	0.004383
Preclinical states of AD	1.0	0.0	1.0E-12
Alzheimer's Pathology	1.0	0.0	1.0E-12
Mild Cognitive Impairment	1.0	0.0	1.0E-12

TABLE 10 | The total probability value for Alzheimer's disease presence due to Obesity and Depression problems in a smoker patient.

Alzheimer's disease classification	Probability of Alzheimer's disease presence (in response to Depression, Smoking and Obesity biomarkers)
Prodromal AD	0.464
AD dementia	0.0
Typical AD	0.0
Atypical AD	0.0
Mixed AD	0.464
Preclinical states of AD	0.0
Alzheimer's Pathology	0.0
Mild Cognitive Impairment	0.0

The results revealed medium probabilities for the cases of Prodromal AD and Mixed AD.

a medical diagnostic decision system for AD, not as a generic diagnostic result but mainly as a more sophisticated probabilistic outcome referred to all the eight categories of AD classification. The proposed statistical model is multi-parametric, targeting the convergence of several independent data like plasma and CSF tests with behavioral or imaging tests and their representation through prior categorical distributions. The proposed AD Bayesian model uses the WinBUGS 1.4.3 software, and all the experiments have been executed in a personal computer with medium performance. While the WinBUGS program cannot be used as an online software, a friendly website (http://alzheimers.edu.gr) has also been designed for individual users and medical staff, for the submission and analysis of anonymous AD tests results. External users can upload biomarkers' results in the form of "True" or "False" and receive the personalized exported statistics in their email account. Medical staff can use the prognostic tool even for individual cases, having in mind that in the Bayesian Inference thousands of sample iterations are automatically executed to predefine the unknown prior distribution of the model and calculate the posterior distribution of the heterogeneous data with high accuracy. Since the proposed probabilistic model is based on conditional probabilities, it must be noted that the calculated error is only the Monte Carlo Error that measures the variability of each estimation due to simulation, increasing the accuracy of the model almost to the 100%. Besides the categorical values, the medical staff is prompted to upload in the webpage, the analytic test results, any medications or other special conditions that refer to the under consideration patient, anonymously or even more to ask for an upgrade of the model, if new dynamic relations occur between the biomarkers, or new biomarkers being identified. The authors of this computational method are in the process of designing, organizing and implement an open biological database for the data sharing of biomarkers assessment (Frasier, 2016), the dissemination of accurate clinical practices and the validation of the current method. In this way, we could replace in the future the categorical values of the current model with real datasets from observational studies improving the cooperation between scientists, targeting a holistic solution against AD. Including a large set of multilevel biomarkers, the proposed diagnostic method has not been validated yet. Therefore we will ask and embed in our system every time, the final diagnosis of the clinicians as a feedback for the evaluation and improvement of our model.

We strongly believe and work in this direction, that an international open biological database for hosting AD clinical results, could benefit the research against the disease helping scientists to re-evaluate their diagnostic models and treatments or even more consider alternative solutions.

Apparently, the proposed Bayesian approach can be extended to several other related neurodegenerative disorders where the early recognition of symptoms is a crucial factor for an efficient treatment procedure and in similar cases of unknown etiology such as the hypothesis of Developmental Origins of Health and Disease and the research on epigenetic mechanisms in epidemiological studies (Barker and Osmond, 1986; Barker et al., 1989, 1993).

AUTHOR CONTRIBUTIONS

AA study concept and design, analysis and interpretation of data, study supervision, preparation of the final manuscript, critical revision of manuscript for intellectual content. VM study concept and design, acquisition of data, analysis and interpretation of data, writing of the first draft. NG critical revision of manuscript for intellectual content. MK critical revision of manuscript for intellectual content.

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frontiers in Aging Neuroscience

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Increase of EEG Spectral Theta Power Indicates Higher Risk of the Development of Severe Cognitive Decline in Parkinson's Disease after 3 Years

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Objective: We investigated quantitative electroencephalography (qEEG) and clinical parameters as potential risk factors of severe cognitive decline in Parkinson's disease.

Methods: We prospectively investigated 37 patients with Parkinson's disease at baseline and follow-up (after 3 years). Patients had no severe cognitive impairment at baseline. We used a summary score of cognitive tests as the outcome at follow-up. At baseline we assessed motor, cognitive, and psychiatric factors; qEEG variables [global relative median power (GRMP) spectra] were obtained by a fully automated processing of high-resolution EEG (256-channels). We used linear regression models with calculation of the explained variance to evaluate the relation of baseline parameters with cognitive deterioration.

Results: The following baseline parameters significantly predicted severe cognitive decline: GRMP theta (4–8 Hz), cognitive task performance in executive functions and working memory.

Conclusions: Combination of neurocognitive tests and qEEG improves identification of patients with higher risk of cognitive decline in PD.

Keywords: EEG, cognitive tests, Parkinson's disease, cognitive decline, cohort study

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INTRODUCTION

The progression of Parkinson's disease (PD) is associated with cognitive decline and dementia (Aarsland et al., 2005; Goldman et al., 2012). Dementia in PD reaches about 30% of all cases with PD (Emre, 2003). The risk of dementia is about 80% for the patients living for more than 20 years with PD (Hely et al., 2008). Early and correct identification of the patients with the risk of severe cognitive decline is a challenging problem of neurology, which has led to the suggestion of various markers of cognitive decline in PD (Mollenhauer et al., 2014). Quantitative electroencephalography (qEEG) – digital processing of EEG recordings to obtain numerical and graphical data – showed that the power (the square of amplitudes of electrical activity) of the brain in PD patients with cognitive impairment is increased in the frequency range below 8 Hz, and decreased in the range above 8 Hz (Caviness et al., 2007; Fonseca et al., 2009; Babiloni et al., 2011). Additionally, in cohort

studies qEEG produced promising results in predicting progression to dementia in PD (Bonanni et al., 2008; Klassen et al., 2011; Gu et al., 2014; Olde Dubbelink et al., 2014).

The purpose of our study was to investigate clinical and qEEG parameters as predictors of severe cognitive decline in PD, using high-resolution EEG with 256 electrodes and with fully automated removal of artifacts (Hatz et al., 2015). Our hypothesis was that qEEG variables at baseline are able to predict severe cognitive decline, and these qEEG variables are not influenced by clinical and demographic parameters. To address this research question a prospective (3 years) cohort of PD patients was assessed for potential neurological, psychological, and neurophysiological risk factors.

MATERIALS AND METHODS

Enrollment of the Patients

Patients were recruited from the outpatient clinic of the Department of Neurology and Neurophysiology of the Hospital of the University of Basel (City of Basel, Switzerland) in the period from 2011 to 2012. Selection criteria: PD according to United Kingdom Parkinson's Disease Society Brain Bank criteria (Gibb and Lees, 1988). The patients who had dementia (Diagnostic and Statistical Manual of Mental Disorders, 4th Edition), history of stroke, epilepsy, multiple sclerosis and surgical interventions to the brain, or/and insufficient knowledge of German language, were excluded. Included patients underwent neurological, cognitive and qEEG examinations on inclusion (baseline) and after a mean interval of 3 years (follow-up). Specialists who performed the assessment of the patients (neurologists, neuropsychologists, and technicians) were unaware of the details of this study.

Standard Protocol Approvals, Registrations, and Patient Consents

The research ethics committee of the Cantons of Basel (*Ethikkommission beider Basel*) approved this study. All patients were fully informed of the nature of the study and provided written consent to participate.

Neurological Assessment

Subsection III (motor examination) of the Unified Parkinson's Disease Rating Scale (UPDRS-III) was filled out. Levodopa equivalent of the daily dose of the antiparkinsonian medication was calculated (LEDD, Tomlinson et al., 2010). Disease duration was assessed since the first symptoms of PD reported by the patient or caregiver.

Cognitive Assessment

Cognitive evaluation was performed in individual sessions divided in three parts; each part with duration of approximately 90 min per day. The interval between the parts of each session was between 24 and 48 h. Mini-Mental State Examination and a battery of 14 cognitive tests were applied. Test variables were normalized with reference to a normative data base of 604 healthy controls from the Memory Clinic, Felix Platter Hospital of Basel, Switzerland (Berres et al., 2000). Cognitive tests were grouped in six cognitive domains (Zimmermann et al., 2015): "attention," "executive functions," "fluency," "long-term memory," "working memory," and "visual-spatial functions" (**Table 1**). A score reflecting cognitive performance in each domain comprised mean of the constituent test variables. Additionally, an overall cognitive score comprised a mean of all 14 cognitive tests. Mood and behavior were assessed with tests: Beck Depression Inventory-II, and compartment "Emotional well-being" (six items with five-step gradation) of the Parkinson's disease Questionnaire with 39 items.

Neurophysiological Assessment

Continuous EEG with 256 electrodes (214 active electrodes) was recorded in relaxed eyes-closed state of the patients (Net Station 300; Electrical Geodesics, Inc). Electrode located at C_Z was used as reference. The sampling rate was set at 1000 Hz, oscillations were filtered with 2500 order least-square filter with band-pass 0.5–70 Hz, and notch 50 Hz. Spectral analysis was performed with "TAPEEG" toolbox (Hatz et al., 2015) by multitaper method (Percival and Walden, 1993). Detection and removal of artifacts (e.g., eye blinks) was fully automated, by an independent component analysis. Channels with bad activations were automatically detected and interpolated by spherical spline method. Global relative median power (GRMP) was calculated in frequency ranges: delta (1–4 Hz), theta (4–8 Hz), alpha1 (8–10 Hz), alpha2 (10–13 Hz), and beta (13–30 Hz). Additionally, median value in the frequency range 4–14 Hz was calculated as the 50% quantile of the overall power spectrum from occipital electrodes – occipital median frequency (**Figure 1**).

Statistics

Statistical calculations were performed with R tool for statistical calculations (R Core Team, 2015). The normality of the distribution of the data was tested with Shapiro–Wilk test. The

TABLE 1 | Cognitive tests and cognitive domains.

Domain	Tests within a domain
(1) Attention	• Stroop Color-Word: time for color naming • Trail-Making: time for part A • Digit Span: correct backward
(2) Executive functions	• Trail-Making: time for part B divided by time for part A • Stroop Color-Word: time for interference task divided by time for color naming • Wisconsin Card Sorting: number of errors
(3) Fluency	• Phonemic verbal fluency: correct answers • Semantic verbal fluency: correct answers
(4) Long-term memory	• Verbal Learning – long-delayed recall • Verbal Learning – discrimination
(5) Working memory	• Corsi blocks: correct forward • Divided attention: omissions
(6) Visual-spatial functions	• Block design • Rey-Osterrieth complex figure copy

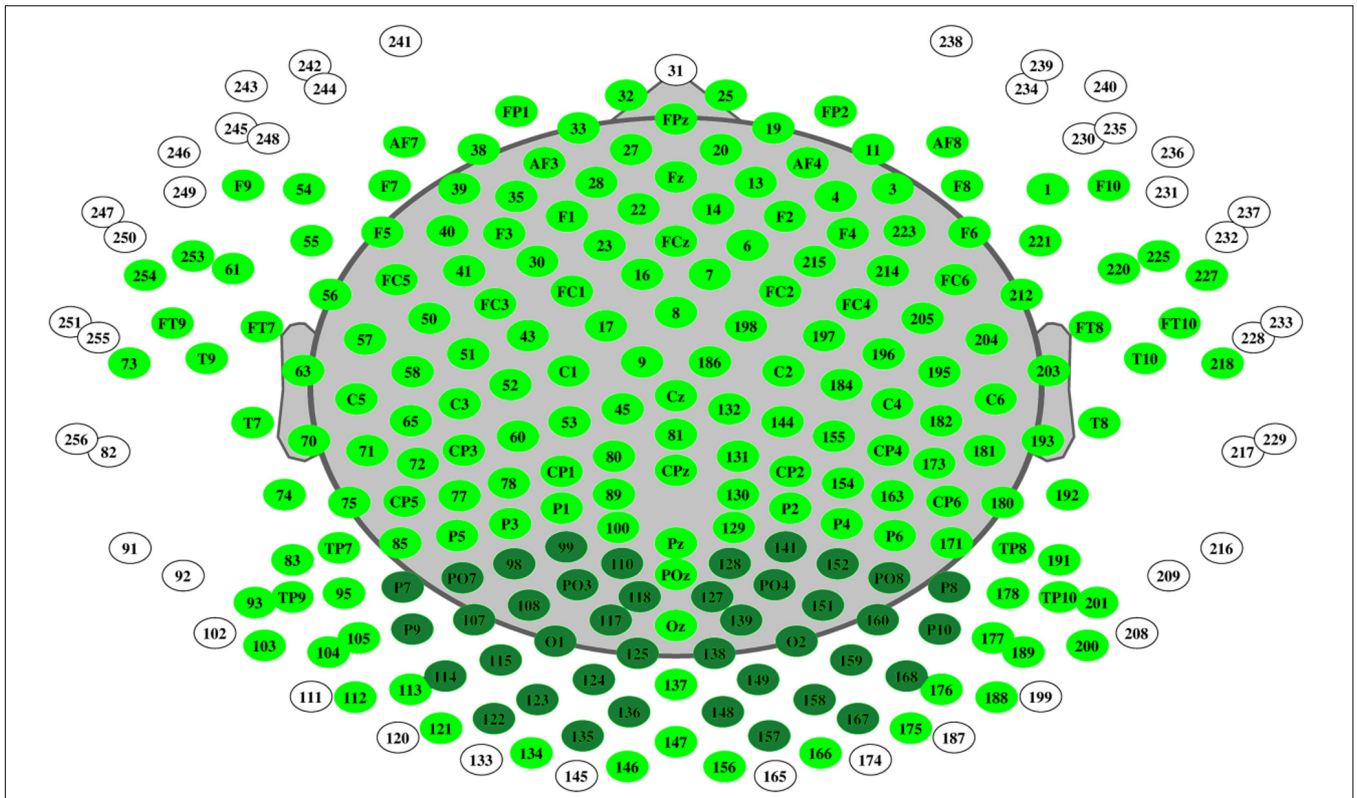

FIGURE 1 | Electrode mapping of 256 electrodes. Active electrodes colored in dark and light green, occipital electrodes – dark green.

influence of the baseline parameters on cognitive state at follow-up was checked with univariate and multivariate linear regression models with backward elimination. Prediction accuracy was checked with receiver operating characteristic (ROC) curves. The results were additionally checked with Random Forest method with regression. The level of statistical significance was set at 0.05.

Cognitive Outcome

A change index in overall cognitive score (CI-OCS) was used as outcome. The CI-OCS was calculated as difference in overall cognitive score between follow-up and baseline, divided by the standard error of the difference (Jacobson and Truax, 1991).

Regression Models

The following baseline variables were considered as predictors: GRMP in ranges delta, theta, alpha1, alpha2, and beta, MF, cognitive domains: "attention," "executive functions," "fluency," "long-term memory," "working memory," and "visual-spatial functions," age, sex, highest educational level (measured in years), disease duration (years), duration of observation (years), LEDD, and UPDRS-III. Significant variables from the univariate regression models were included in multivariate models. To check the added value of the significant predictors to the cognitive task performance, non-normalized to 100% explained variance of the models was calculated. The relative importance of the variables was calculated with the R package "relaimpo" (Grömping, 2006) with method "LMG" and plotted in a bar diagram.

ROC-Curves

The ROC-curve analyses were performed with the R package "pROC" (Robin et al., 2011). We categorized the sample on the basis of MMSE score at follow-up (cut off <24).

Random Forest with Regression

Random Forest (Hastie et al., 2009) is an ensemble machine learning method used for classifying data with high accuracy and for regression analysis. The goal of this method is to reduce the variance in the data and get a higher predictive performance of the model. This is done by using several decision trees, which are constructed based on subsets of the same training data, and then getting predictions on the test set based on the training. Each variable included in the model is evaluated based on its effect on the overall accuracy of the model and is ranked higher up if its exclusion results in a drop in the model accuracy. The role of each variable in the classification process is reflected in output measures called mean decrease accuracy (MDA) and mean decrease gini coefficient (MDGC). MDA is the increase in mean squared error of predictions after predictor variables being randomly shuffled. Higher the MDA, the more important is the variable. MDGC relates to the decrease of node impurity in the decision tree after each split, summed over all splits and trees. Higher the MDGC

is, the more important the variable. Random Forest analysis was performed with the R package "randomForest" (Liaw and Wiener, 2002).

RESULTS

Enrollment of the Patients

Between January 2012 and December 2013, 55 patients were selected in the study and assessed for neurological, psychological and qEEG parameters (Supplement 1). At follow-up, cognitive outcome along with the other clinical data was obtained for 37 patients. Thus, these 37 patients were included in the analysis (**Table 2**). Changes in neurological and cognitive features of the sample are shown in Supplement 2.

Influence on CI-OCS

Regression analyses (Supplement 3, Tables 3–6) identified three baseline parameters which had significant influence on CI-OCS: GRMP theta ($\beta = -3.16$, $p < 0.001$), cognitive domain "executive functions" ($\beta = 0.54$, $p < 0.001$), cognitive domain "working memory" ($\beta = 0.19$, $p < 0.05$), adjusted R squared $= 0.64$, $p < 0.001$. Explained variance of the overall model was 66.9%, of which "executive functions" made 27.5%, GRMP theta – 25.8%, and "working memory" – 13.6% (Supplement 3, Table 6).

TABLE 2 | Sample at baseline.

Factors	Values
Sex, males/females	25/12
Age, years	67 [31, 84]
Disease duration, years	8 [1, 20]
Duration of observation, months	37 [30, 44]
Education, years	14 [9, 20]
Beck Depression Inventory-II	6 [0, 15]
Obsessive Compulsive Inventory	6 [0, 25]
PDQ39 – emotional well-being	17 [0, 50]
UPDRS, Subscale III	14 [0, 50]
Levodopa equivalent, mg per day	691 [150, 2129]
Attention	−0.02 [−2.07, 1.13]
Executive functions	−0.03 [−3.73, 1.17]
Fluency	−0.18 [−1.93, 1.61]
Long-term memory	−0.13 [−1.62, 2.60]
Working memory	−0.23 [−1.50, 2.37]
Visual-spatial functions	−0.23 [−2.60, 1.79]
Overall cognitive score	−0.10 [−2.05, 0.96]
Mini Mental State	29 [24, 30]
GRMP delta, %	22 [9, 42]
GRMP theta, %	18 [10, 46]
GRMP alpha1, %	18 [5, 33]
GRMP alpha2, %	13 [5, 33]
GRMP beta, %	20 [10, 38]
Median frequency, Hz	8.71 [7.14, 9.99]

For continuous variables presented as median and range (M [min, max]). GRMP, global relative median power; PDQ39, Parkinson's disease questionnaire with 39 items; UPDRS, unified Parkinson's disease rating scale.

Additionally, we checked if age, sex, and education had confounding effect on each of the three significant variables (GRMP theta, "executive functions," and "working memory"). No confounding effects were identified (**Figure 2**).

ROC-Curve Analyses

Receiver operating characteristic were built using variables: GRMP theta, "executive functions," and "working memory." Best accuracy was identified in GRMP theta: AUC = 75%, specificity = 63%, specificity = 77% (**Figure 3**; Supplement 3, Table 7).

Random Forest

Global relative median power theta was classified as the most important variable (MDA = 7.49, MDGC = 1.63) (Supplement 3, Table 8).

DISCUSSION

In our observation, increase of GRMP theta (4–8 Hz), and decrease of cognitive performance in domains "executive functions" and "working memory" significantly predicted worse CI-OCS after 3 years.

Our findings in GRMP theta are in line with the data from cohort studies with semi-automated processing of EEG (Klassen et al., 2011; Olde Dubbelink et al., 2014). Additionally, in cross-sectional comparisons between Parkinson's disease patients with dementia and matched healthy controls, spectral power in the frequency range below 8 Hz was significantly increased in demented patients (Babiloni et al., 2011; Fonseca et al., 2013). From a pathophysiologic perspective, EEG slowing in severe cognitive decline may be explained by disruption of thalamo-cortical circuits, and pathological synchronization of the brain motor systems with slow frequencies related to the sensory motor integration (Steriade et al., 1990; Rossini et al., 1991). We can speculate that these pathological changes precede clinical manifestation of cognitive decline in PD.

With regard to cognitive factors, we found that worse scores in domains "executive functions" and "working memory" are significant predictors of cognitive decline. Zimmermann et al. (2015) in a cross-sectional analysis showed significant correlation of occipital median frequency with overall cognitive score, domains "executive functions," "long-term memory," "attention," and "fluency" in dementia-free patients with PD. Olde Dubbelink et al. (2014) found that fronto-executive (spatial span score) and posterior (pattern recognition memory) significantly predicted dementia in Parkinson's disease. Impairment of the executive functions is common in the early stage of PD (Kehagia et al., 2010). However, the cognitive profile of early stage PD is heterogeneous, and the significance of domain-specific cognitive deficits in identifying patients with a risk of dementia is still studied (Robbins and Cools, 2014).

Our findings demonstrate that a combination of neurocognitive tests with qEEG improves identification of patients with PD and higher risk of cognitive decline. While it is important for practical reasons to identify strong risk

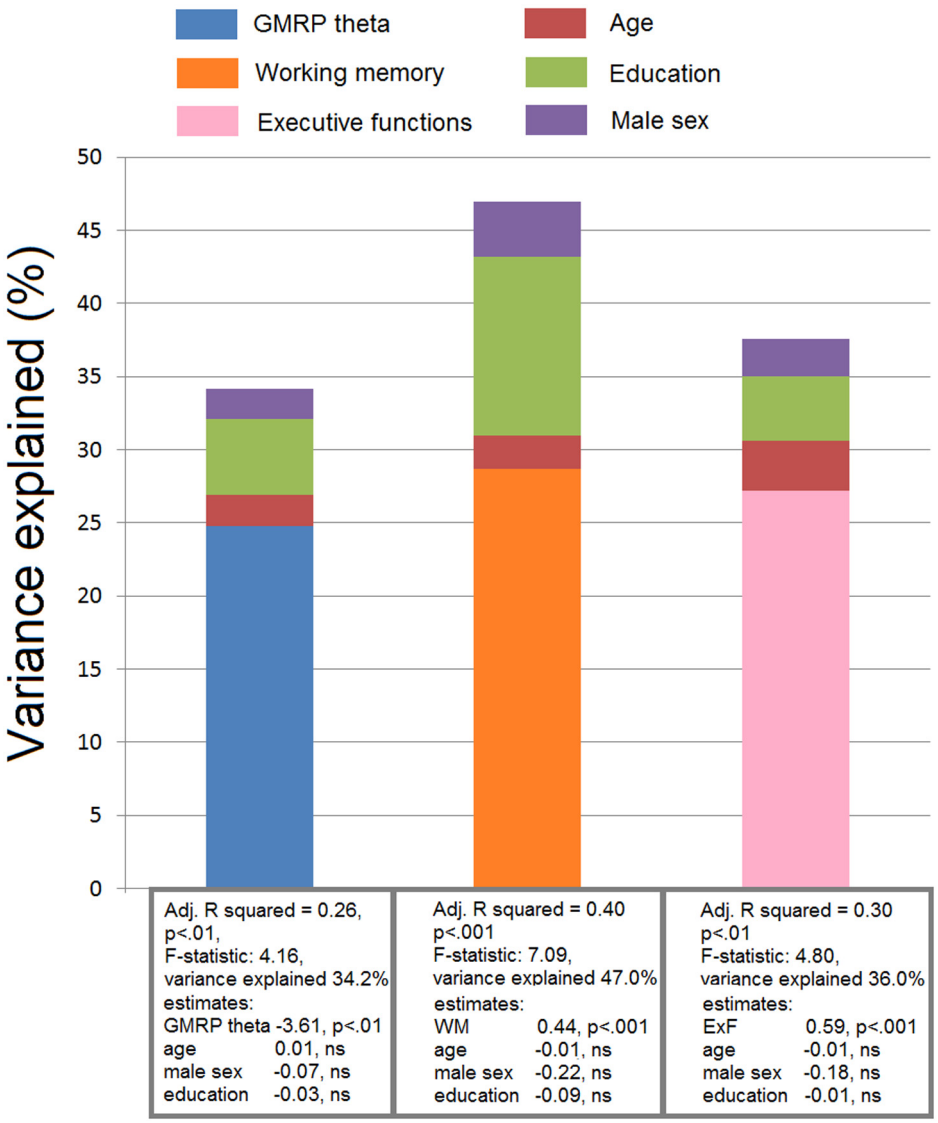

FIGURE 2 | Results of the linear regression analyses. Confounding effect of age, male sex, and education on the significant predictors of cognitive decline (GMRP theta, executive functions, and working memory). The variance of the models, that is explained by these predictors, is shown.

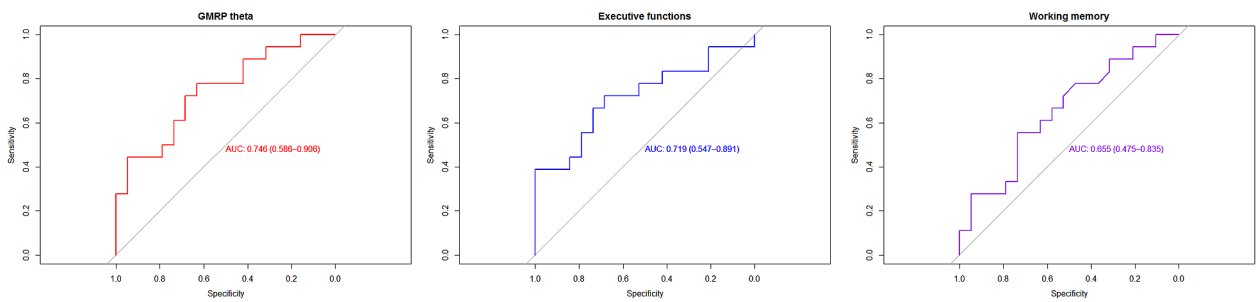

FIGURE 3 | Receiver operating characteristic (ROC)-curves analyses.

factors for dementia developing within 1 or 2 years, the short mean observation period of 3 years is a limitation of the study, and a longer follow-up is warranted. Another limitation is the relatively small sample size. Strengths of the study include the comprehensive neuropsychological and psychiatric assessments as well as the fully automated processing of high-resolution EEG, which could be implemented in clinical practice as a universally available technique. Additionally, automated processing of EEG facilitates the application of advanced analyses of the cognitive function.

CONCLUSION

High GRMP theta, especially when combined with poorer cognitive scores in "executive functions" and "working memory," identifies patients with PD who are at a higher risk of progression to dementia.

AUTHOR CONTRIBUTIONS

VC study concept and design, acquisition of data, writing of the first draft. MC analysis and interpretation of data. FH analysis and interpretation of data, critical revision of manuscript for intellectual content. AM analysis and interpretation of data, critical revision of manuscript for intellectual content. PF analysis and interpretation of data, study supervision, critical revision of manuscript for intellectual content. UG acquisition of data, analysis and interpretation of data, study supervision, critical revision of manuscript for intellectual content.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fnagi.2016.00284/full#supplementary-material

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Neuroprotective Effect of Ligustilide through Induction of α-Secretase Processing of Both APP and Klotho in a Mouse Model of Alzheimer's Disease

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Emerging evidence suggests that alpha-processing single transmembrane proteins, amyloid precursor protein (APP) and anti-aging protein Klotho, are likely to be involved in the progression of Alzheimer's disease (AD). The natural phthalide Ligustilide (LIG) has been demonstrated to protect against aging- and amyloid-β (Aβ)-induced brain dysfunction in animal models. The present study is to investigate the effects of LIG on cognitive deficits and metabolism of both APP and Klotho and its underlying mechanism in AD double-transgenic (APP/PS1) mice and cultured human cells. Our results show that treatment with LIG significantly ameliorated memory impairment and Aβ levels and plaques burden. Specifically, LIG might act as a potent enhancer of α-secretase, disintegrin, and metalloprotease 10 (ADAM10), leading to upregulation of alpha-processing of both APP and Klotho and subsequent increases in the levels of both soluble APP fragment (sAPPα) and soluble Klotho (sKL) with inhibition of IGF-1/Akt/mTOR signaling in AD mice and cultured cells. Moreover, the specific ADAM10 inhibitor (G1254023X) effectively reversed LIG-induced alpha-processing of both APP and Klotho *in vitro*, while Klotho gene knockdown by small interfering RNA significantly blunted LIG-mediated inhibition of IGF-1/Akt/mTOR signaling *in vitro.* Taken together with the reported neuroprotective effects of both sAPPα and sKL as well as autophagy induction by Akt/mTOR pathway inhibition, our findings suggest that neuroprotection of LIG against AD is associated with induction alpha-processing of APP and Klotho and potential Aβ clearance. Whether LIG might induce Aβ autophagic clearance and the underlying mechanisms need to be further studied.

Keywords: Ligustilide (LIG), Klotho, APP, α-secretase processing, β-amyloid

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INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disease characterized by progressive memory loss. Extracellular senile plaques and intracellular neurofibrillary tangles are the main neuropathological hallmarks of AD (Xie et al., 2017). Amyloid beta (Aβ) is a 39–43 amino acid peptide that makes up the core of senile plaques and these Aβ oligomers have been shown to play a central role in the progression of AD (Walsh et al., 2002).

Amyloid beta is proteolytically produced from a single transmembrane amyloid precursor protein (APP), which can be cleaved through two different pathways: amyloidogenic and non-amyloidogenic. Cleavage through the amyloidogenic pathway occurs by the proteolytic enzymes beta and gamma secretase at the N and C termini, and results in the release of Aβ into the extracellular space (Kowalska, 2004). Cleavage through the non-amyloidogenic pathway involves the activation of α-secretase, a member of the disintegrin and metalloprotease (ADAM) family, which cleaves APP within the sequence of the Aβ domain. This action precludes the generation of the Aβ peptide and releases a soluble APP fragment, sAPPα (Lopez-Font et al., 2015). The sAPPα fragment has been shown to have both neurotrophic and neuroprotective effects (Chasseigneaux and Allinquant, 2012; Harti et al., 2013).

Aging of the central nervous system (CNS) is one of greatest risk factors in the development of neurodegenerative pathologies such as AD. Thus, maintaining a healthy neuronal population for as long as possible is a promising therapeutic strategy to combat neurodegeneration of the aging brain (Mohajeri et al., 2015). There have been several reports describing the proteins and signaling pathways involved in regulating the aging process. For example, Klotho is a putative aging-suppressor gene which encodes a type-I transmembrane protein. It is predominantly expressed in the distal convoluted tubules of the kidney and the choroid plexus of the brain, with weaker expression in the pituitary and parathyroid glands, as well as the hippocampus (Kuro-o et al., 1997; German et al., 2012). Similar to the other type-I-transmembrane proteins such as APP and Notch, Klotho is processed by α-secretase (ADAM10 and ADAM17) as well as β and γ-secretases (Bolch et al., 2009). During its processing, the shed ectodomain of Klotho is released into the blood and cerebrospinal fluid and also named as soluble Klotho (sKL). Accumulating evidence indicates that this sKL may play important roles as a neuroprotective factor, thereby supporting healthy brain aging (Hensel et al., 2016; Cararo-Lopes et al., 2017). While it has been shown that mutation of Klotho leads to systemic aging and reduced longevity in mice (Kuro-o et al., 1997; Shiozaki et al., 2008), increasing Klotho expression has been shown to promote healthier aging and prolongation of life (Kuro-o, 2011).

Ligustilide (LIG; 3-butylidene-4,5-dihydrophthalide) is the main lipophilic constituent of the *Umbelliferae* family of medicinal plants, including *Radix angelicae sinensis* and *Ligusticum chuanxiong*, and readily crosses the blood-brain barrier (Chen et al., 2010). Our previous studies have shown that LIG exerts marked neuroprotective effects against several CNS pathologies including forebrain ischemic injury in ICR mice, permanent forebrain ischemia in rats, focal cerebral ischemia/reperfusion in rats, scopolamine induced memory impairment in mice, hydrogen peroxide-induced injury in PC12 cells, and lipopolysaccharide-induced inflammation in primary rat microglia (Kuang et al., 2006, 2008, 2014a; Yu et al., 2008; Wang et al., 2010; Cheng et al., 2011).

In this study, we examined the effect of LIG on cognitive impairment in the APP/PS1 double-transgenic mouse model of AD. Specifically, we investigated the mechanisms underlying the efficacy of this compound, including α-secretase catalytic activities, APP processing, and signaling pathway regulation by the sKL protein.

MATERIALS AND METHODS

Animals and Treatments

For the production of LIG, a well-established procedure in our laboratory was followed as previously described (Kuang et al., 2006). Male transgenic mice APPswe/PS1dE9 [B6/J-Tg (APPswe, PSEN1dE9) 85Dbo/Nju], hereafter "APP/PS1", and C57 BL/6 mice, hereafter "C57", were purchased from Beijing HKF Bioscience Co. Ltd. APP/PS1 mice were intragastrically administered either LIG (30 mg/kg, in 3% Tween 80) or vehicle control (3% Tween 80 alone) for 14 weeks ($n = 12$ per group), starting at 8.5 months of age. C57 mice were administered vehicle control only ($n = 12$). Treatments were administered by oral gavage one time each day and body weights were recorded weekly. After behavioral testing was completed, the mice were killed by a sodium pentobarbital overdose (50 mg/kg, i.p.) and perfused through the heart with cold saline. The brain was removed: one hemisphere was frozen in liquid nitrogen and stored at −80°C until further analysis, and the other hemisphere was fixed in 4% paraformaldehyde for 24 h, dehydrated through an ethanol series (70, 95, and 100%), penetrated with chloroform, and embedded in paraffin. All procedures were approved by the Animal Research Committee of West China School of Pharmacy. All animal studies were conducted in accordance with the Regulations of Experimental Animal Administration issued by the State Committee of Science and Technology of the China.

Y-Maze Test

Short-term working memory of mice was assessed using a Y-maze alternation task as previously described (Cheng et al., 2011). Briefly, each mouse was placed at the end of one arm and allowed to move freely throughout the maze over the course of 5 min. The series of arm entries was recorded visually. Alternation was defined as the mouse entering different arms of the maze in succession as a result of consecutive arm entering. Each successive non-overlapping entrance sequence (e.g., ABC, CAB, or BCA, but not BAB) was defined as one alternation. The percentage of spontaneous alternations was calculated by dividing the total number of alternations by the total number of entries minus 2, and the percentage of alternation was used as an index of short-term memory (Hiramatsu et al., 2010).

Step-Down Passive Avoidance Test

Long-term working memory of mice was assessed using a step-down type of passive avoidance task as previously described (Hiramatsu et al., 2010). Briefly, each mouse was placed on a wooden platform (4 cm × 4 cm × 4 cm). When the mouse stepped down from the platform and placed all of its paws on the grid floor, an electric shock was delivered to the grid floor for 15 s. A retention test was carried out 24 h after the training session in a manner similar to the training, except that no electric

TABLE 1 | Primary antibodies used in this study.

Antibody	Host	Dilution	Application	Source
Aβ1-42	Rabbit	1:100	IHC	Abcam
Klotho	Rat	1:100	IHC	Abcam
NeuN	Rabbit	1:100	IHC	Millipore
APP	Mouse	1:100	IHC	Boster
Akt	Rabbit	1:1000	WB	CST
p-Akt	Rabbit	1:1000	WB	CST
mTOR	Rabbit	1:1000	WB	CST
p-mTOR	Rabbit	1:1000	WB	CST
IGF-1R	Rabbit	1:500	WB	CST
p-IGF-1R	Rabbit	1:500	WB	CST

shock was delivered to the grid floor. Each mouse was placed on the platform again and the latency to step-down was recorded. An upper cut off time of 300 s was set.

Immunohistochemistry

Five micrometer sections of mouse brain were mounted on glass slides. The primary antibodies used in the study are summarized in **Table 1**. The sections were incubated with antibody diluted in PBS at 37°C for 3 h and then 4°C overnight. Immunoperoxidase staining was performed with an avidin-biotin complex kit (Boster Biological Technology). Immunoreactions were visualized using 3,3′-diaminobenzidine tetrahydrochloride and nuclei were lightly counterstained with Meyer's hematoxylin (except NeuN staining). Negative control sections were only stained with secondary antibody to control for non-specific background. Semi-quantitative analysis of the immunostaining results was performed for three sections of each brain, with each section containing three microscopic fields from the cerebral cortex, hippocampal CA1 areas, and the choroid plexus for Klotho. The stereotaxic locations of the area selected was from bregma –1.34 to –1.94 mm. Immunoreactivity was determined based on the integrated optical density (IOD) of immunostaining per field using Image Pro Plus 6.0.

Measurements of ADAM Enzymatic Activity

The method used for measuring ADAM enzymatic activity is based on the cleavage of a secretase-specific peptide conjugated to a fluorescent reporter molecule. Briefly, the brain tissue of APP/PS1 mice was homogenized in RIPA buffer (Beyotime, Jiangsu, China) by a hand-held motor and kept on ice for 1 h. The homogenates were centrifuged at 12,000 × *g* at 4°C for 20 min and supernatants collected. Lysate protein concentrations were adjusted to 2 mg/ml and lysates were then incubated with the fluorogenic ADAM-peptide substrate Mca-Pro-Leu-Ala-Gln-Ala-Val-Dpa-Arg-Ser-Ser-Ser-Arg-NH_2 (10 μM, final concentration; R&D Systems) for 20 min at 37°C. Secretase cleavage results in a fluorescent signal that can be measured on a microplate reader (excitation wavelength 355 nm and emission wavelength 510 nm) and corresponds to the level of secretase enzymatic activity in each sample.

Western Blot Analysis

For the tissue-based assay, the brains of three mice in each group were homogenized in RIPA buffer (Beyotime, Jiangsu, China) by a hand-held motor and kept on ice for 30 min to lyse the cells completely. The homogenates were then centrifuged at 14,000 × *g* at 4°C for 15 min. The supernatants were collected and protein concentration was determined using a BCA protein assay kit (Boster, Wuhan, China). Equal protein was mixed with 5 × SDS-PAGE sample loading buffer (Beyotime, Jiangsu, China) and boiled for 5 min at 99°C.

For the cell-based assay, cells were lysed with RIPA buffer (Beyotime, Jiangsu, China) according to the manufacturer's instructions. Protein concentration was determined by a BCA protein assay kit (Boster, Wuhan, China) and equal protein concentrations were mixed with 5 × SDS-PAGE sample loading buffer (Beyotime, Jiangsu, China) and boiled for 5 min at 99°C.

All tissue and cell samples were fractionated using 10% SDS-PAGE and transferred to a PVDF membrane (Millipore). Membranes were incubated with primary antibodies overnight at 4°C, detected with HRP-conjugated secondary antibodies (Zhongshan Jinqiao Biology) and developed using an ECL chemiluminescence kit (Millipore). The optical density (OD) of each band was determined using Gel Pro Analyzer 6.0 (Media Cybernetics, Bethesda, MD, United States).

Real-Time Quantitative PCR Analysis (RT-PCR)

Total RNA was isolated from either mouse brains or cells using TRIZOL reagent (Invitrogen) and cDNA was synthesized using Revert Aid First Strand cDNA Synthesis Kit (Ferments, Vilnius, Lithuania). RT-PCR was performed using SsoFast Eva Green Supermix (Bio-Rad, Hercules, CA, United States) according to the manufacturer's instructions. The cycling conditions were as follows: initial denaturation at 95°C for 30 s, followed by 40 reaction cycles (95°C for 5 s, 60°C for 10 s, and 72°C for 10 s). The specific primer pairs (Invitrogen Trading, Shanghai, China) are listed in **Table 2**. Gene expression was normalized to β-actin and results are expressed as a fold change of the threshold cycle value relative to control using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001).

ELISA Assay

The levels of soluble and insoluble Aβ in the brains of APP/PS1 mice were quantified as previously described (Deng et al., 2006). Briefly, brains were homogenized in 4 × TBS buffer (w/v) and then centrifuged at 8000 × *g* for 1 h. The supernatants were used for measuring soluble Aβ. To measure insoluble Aβ, the tissue pellets were re-suspended in 5 M guanidine (2×) and sonicated. Homogenates (20 μl) were diluted with 10 × loading buffer and centrifuged at 8000 × *g* for 30 min; supernatants were loaded for ELISA. The soluble and insoluble Aβ levels in all samples were determined using the commercially available mouse Aβ40 and Aβ42 ELISA kit (CUSABIO) according to manufacturer's instructions. Data obtained from brain homogenates is expressed as pg/mg total protein.

TABLE 2 | The specific primer pairs used in polymerase chain reaction.

Gene	RNA Sequence	Sense	Antisense	Product
huKlotho	NM_004795.3	gctctcaaagcccacatactg	gcagcataacgatagaggcc	104 bp
huAPP	NM_201414	accgctgcttagttggtgag	ggtgtgccagtgaagatgag	113 bp
huβ-actin	NM_001101.3	ggagcccgtcggtaattttaa	tctgcatgtgcggttggtt	382 bp
msKlotho	NM_013823.2	tggggtcccattggatagag	actcagggtagtcgccgtc	129 bp
msAPP	NM_007471.2	ccgttgcctagttggtgagt	gctcttctcgctgcatgtc	142 bp
msADAM10	NM_007399.3	catgatgactactgcttggcctat	gcaccgcatgaaaacatcaca	1011 bp
msADAM17	NM_001277266.1	gctgaacctaacccctgaag	ttagcactctgtttctttgctgtc	1839 bp
msβ-actin	NM_00739.3	ctgaccctgaagtaccccattgaaca	ctggggtgttgaaggtctcaaacatg	198 bp

To determine the level of sKL and soluble APP (sAPPα) in the brains of APP/PS1 mice, tissue was homogenized in 10 × PBS and then stored at −20°C overnight. Cell membranes were disrupted through two freeze-thaw cycles and then centrifuged at 5000 × *g* for 5 min. Supernatants were used to measure sKL and sAPPα with the commercially available ELISA kits (CUSABIO) according to manufacturer's instructions.

For the cell-based assays, cells were incubated with 1 or 5 μM LIG in DMSO for 24 h and then collected and centrifuged at 3,000 rpm, 4°C for 30 min. The supernatants were used to measure sKL and sAPPα by ELISA (CUSABIO).

Cell Culture, Transfections, and Drug Treatments

Ligustilide was prepared as a 1 mM stock solution in DMSO and diluted with cell culture media before use. The final concentration of DMSO in the culture medium was 0.1%, which had no effect on cell viability. The same condition of DMSO was also added in controls during the cell treatment.

HEK293T cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, United States) and cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY, United States) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT, United States) in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. For detection of cell viability, the cells were incubated with LIG (1 or 5 μM) for 24 h. For detection of sKL by ELISA and full-length Klotho by RT-PCR, cells were pre-incubated with either TAPI-O (10 μM, Santa Cruz, CA, United States) or G1254023X (10 μM, Sigma, St. Louis, MO, United States) for 30 min and then treated with LIG (5 μM) plus inhibitor for 24 h.

Klotho knock-down in HEK293T cells was achieved using siRNA. Briefly, proliferating cells were transfected with 10 μM control siRNA or Klotho siRNA (Santa Cruz Biotechnology) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, United States) according to the manufacturer's instructions. To determine cell viability, cells were incubated with LIG at 5 μM for 24 h following transfection. To measure Klotho gene expression or protein expression, cells were incubated with LIG at 5 μM for 24 or 48 h following transfection, respectively.

Human neuroblastoma SH-SY5Y cells were obtained from the ATCC (Manassas, VA, United States). Cells were maintained in DMEM (Gibco, Grand Island, NY, United States) supplemented with 10% FBS (Hyclone, Logan, UT, United States) and cultured in a humidified atmosphere of 95% air and 5% CO2 at 37°C. SH-SY5Y cells were transiently transfected with mutant human APP695 cDNA (App_{swe}, kindly provided by Rong Zhang, the Third Military Medical University) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Cells were grown until nearly confluent, washed with serum-free medium, and then incubated with LIG at 5 μM for 24 h prior to the detection of cell viability. Prior to measuring sAPPα by ELISA and full-length APP gene expression by RT-PCR, cells were pre-incubated with G1254023X (10 μM) for 30 min and then treated with LIG at 5 μM plus inhibitor for 24 h.

Cell Viability

The viability of cultured SH-SY5Y and HEK293T cells was measured using an MTT colorimetric assay (Sigma). Briefly, cells were plated overnight in a 96-well plate and then treated with LIG (0.1, 1, 5, and 10 μM) or control for 24 h, respectively. MTT was added to each well at a final concentration of 0.5 mg/ml and cells were incubated for 4 h at 37°C. The culture medium was removed and DMSO was added to each well to dissolve the resulting formazan crystals. The absorbance was measured at a wavelength of 570 nm using a microplate reader (Bio-Tek Instruments). Percent viability was defined as the relative absorbance of treated versus untreated control cells.

Statistical Analysis

The data are expressed as mean ± SEM. Statistical analyses were performed using SPSS V.16.0. The data were analyzed using one-way ANOVA for repeated measures followed by a Tukey *post hoc* test. Values of $p < 0.05$ were considered statistically significant.

RESULTS

LIG Treatment Improves Memory Deficits and Ameliorates AD-Induced Neuronal Loss in APP/PS1 Mice

It has been described that APP/PS1 mice exhibit early AD symptoms at around 6–7 months of age, which worsen within another 3 or more months (Reiserer et al., 2007). Therefore, we opted to treat mice with LIG for 14 weeks starting at 8.5 months of age. During this time, we found no significant effect of drug treatment on mouse weight compared to vehicle treated mice, indicating a lack of toxicity (data not shown).

To investigate the effect of LIG on short- and long-term working memory, APP/PS1 mice were evaluated in a Y-maze spontaneous alternation task and a step-down passive avoidance task. As expected, the percentage of alternation in the Y-maze test was reduced in the vehicle-treated APP/PS1 mice compared to the wild-type C57 mice, indicating a reduction in short-term memory. Interestingly, chronic administration of LIG (30 mg/kg, 14 weeks) significantly rescued this effect, indicating a significant enhancement in short-term memory ($p < 0.05$; **Figure 1A**). Similarly, vehicle-treated APP/PS1 mice showed a significant reduction in step-down latency during the passive avoidance test compared to the C57 group ($p < 0.01$), and this effect was rescued in mice treated with LIG ($p < 0.01$; **Figure 1B**), indicating a significant enhancement in long-term memory. These results show that LIG therapy significantly improves the memory deficits seen in APP/PS1 mice.

Consistent with our behavioral test results, immunohistochemistry analyses revealed a marked decrease in neuronal staining in both the hippocampal CA1 region and the cerebral cortex in vehicle-treated APP/PS1 mice compared to the wild-type C57 mice (**Figure 1C**). Interestingly, this loss was rescued following chronic LIG administration in the aged APP/PS1 mice compared to the vehicle-treated APP/PS1 mice (**Figures 1C,D**), with neuronal staining at a similar level as the C57 control group. Collectively, these results imply that LIG therapy improves short- and long-term memory through its prevention of neuronal loss in the APP/PS1 mouse model of AD.

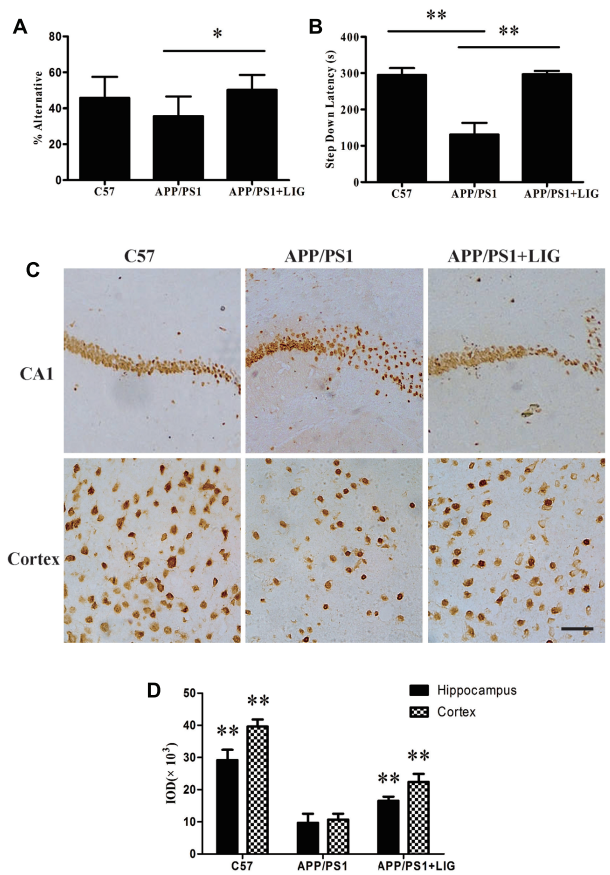

FIGURE 1 | Effect of Ligustilide (LIG) on memory impairment and neuronal loss in amyloid precursor protein (APP)/PS1 transgenic mice. C57 mice were treated with vehicle control and APP/PS1 mice treated with either vehicle control or LIG (5 μM) for 14 weeks starting at 8.5 months of age. **(A)** Effect of LIG on Y-maze test. LIG treatment significantly increased the percentage of alternations used as an index of short-term memory. **(B)** Effect of LIG on the passive avoidance test. LIG treatment increased the step-down latency in comparison to vehicle-treated APP/PS1 mice. **(C)** Representative immunostaining of NeuN in the hippocampal CA1 region and cerebral cortex. Scale bar, 25 μm. **(D)** Quantitative image analysis of NeuN based on the integrated optical density (IOD) of positive immunostaining. The data are expressed as mean ± SEM; $n = 12$ for behavior tests, $n = 4$ for immunohistochemistry; $^*p < 0.05$, $^{**}p < 0.01$.

LIG Increases α-Secretase Catalytic Activity by Increasing ADAM10

Our results thus far have demonstrated that LIG therapy attenuates cognitive impairment and reduces neuronal loss. To determine the effect of LIG on APP and Klotho processing, we first investigated the effect of LIG on Klotho and APP gene expression as well as its effect on sKL and sAPPα secretion by RT-PCR and ELISA, respectively. Using HEK293T cells and genetically modified SH-SY5Y cells engineered to express the human APP695 (hAPP), we show that LIG treatment plays a significant role in both the secretion and gene expression of these proteins. **Figures 2A,B** show that LIG treatment of HEK293T cells results in a dose-dependent increase in sKL secretion and Klotho gene expression. Interestingly, although LIG treatment significantly increased sAPPα secretion in the SH-SY5Y cells, we found a striking reduction in expression of the full-length gene (**Figures 2C,D**), indicating an increase in APP processing in the presence of LIG. Cell viability was examined after 24 h of incubation with LIG at 0.1–10 μM and we saw no effect of LIG (**Supplementary Figure S1**). Thus, LIG is non-toxic to HEK293T cells and hAPP-transfected SH-SY5Y cells.

Studies have shown a significant role for the ADAM family of enzymes in the catalysis and ectodomain shedding of APP, Notch, and Klotho among other cell surface proteins (Allinson et al., 2003; Bolch et al., 2009). To understand how LIG may be affecting the relative contributions of ADAM enzymes, we examined ADAM10 and ADAM17 in this study, as they have been shown to be relevant in AD (Lammich et al., 1999; Vingtdeux and Marambaud, 2012). RT-PCR analysis revealed a significant reduction in ADAM10 gene expression in APP/PS1 vehicle-treated control mice compared to the C57 control mice, with no effect on ADAM17 gene expression. Interestingly, we found a strong induction of ADAM10 gene expression in APP/PS1 mice following chronic administration of LIG, with little effect on ADAM17 (**Figure 2E**). To further investigate the functional relevance of ADAM10 in this model, we measured α-secretase activity in the brains of mice using a secretase-specific peptide conjugated to a fluorescent reporter molecule (see "Materials and Methods"). **Figure 2F** shows a significant increase in α-secretase activity in APP/PS1 mice treated with

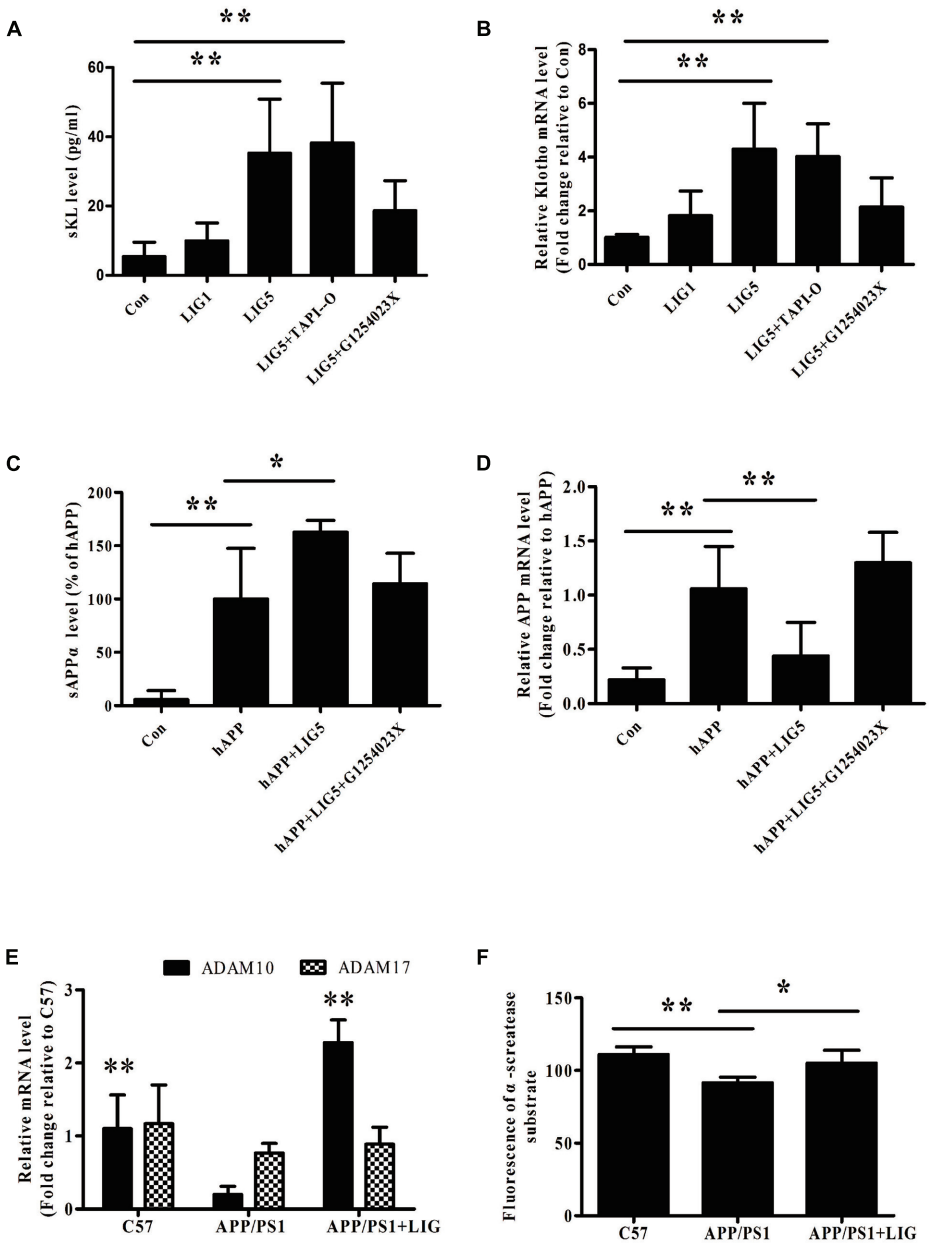

FIGURE 2 | Effect of LIG on α-secretase catalytic activity *in vitro* and *in vivo*. **(A,B)** Effect of LIG (1 or 5 μM) on **(A)** soluble Klotho (sKL) secretion as measured by ELISA and **(B)** full-length Klotho gene expression as measured by RT-PCR in HEK293T cells. Effect of α-secretase processing was determined by culturing cells in the presence or absence of inhibitors specific to ADAM enzymes (TAPI-O, ADAM17 inhibitor; G1254023X, ADAM10 inhibitor). **(C,D)** Effect of LIG (1 or 5 μM) on **(C)** sAPPα secretion as measured by ELISA and **(D)** full-length APP gene expression as measured by RT-PCR in SH-SY5Ycells expressing hAPP. Effect of α-secretase processing was determined by culturing cells in the presence or absence of ADAM10 inhibitor G1254023X. **(E)** Effect of LIG on ADAM10 and ADAM17 gene expression in APP/PS1 mice as measured by RT-PCR. **(F)** Effect of LIG on ADAM enzymatic activity in APP/PS1 mice as measured by the fluorescence of an ADAM peptide-specific fluorescent reporter substrate. The data are expressed as mean ± SEM; $n = 4$ for RT-PCR, $n = 6$ for biochemical analysis in mice, and $n = 8$ for analyses in HEK293T and SH-SY5Y cells; $^{*}p < 0.05$, $^{**}p < 0.01$.

LIG compared with vehicle-treated APP/PS1 group ($p < 0.05$), indicating a role for LIG in ADAM10 α-secretase activity.

Thus far we have shown that LIG treatment increases Klotho and APP processing and also increases ADAM10 expression and enzymatic activity. Next, we used inhibitors specific to ADAM10 and ADAM17, G1254023X and TAPI-O, respectively, to examine the role of these enzymes in LIG-induced Klotho and APP processing. **Figures 2A,B** show that when HEK293T cells are pre-treated with G1254023X to inhibit ADAM10 prior to LIG there is a significant reduction in LIG-induced sKL secretion and Klotho

gene expression, while inhibition of ADAM17 had no effect. Additionally, inhibition of ADAM10 resulted in a significant decrease in sAPPα, while rescuing APP gene expression in the hAPP-tranfectedSH-SY5Y cells (**Figures 2C,D**). Collectively, the results from these studies indicate that the effect of LIG on α-secretase enzymatic activity and downstream Klotho and APP processing is mediated by ADAM10.

LIG Reduces Aβ Levels and Directs APP Processing toward the Non-amyloidogenic Pathway in APP/PS1 Transgenic Mice

To further study the effect of LIG on APP processing *in vivo*, we examined full-length APP expression and Aβ plaque deposition in the brains of C57 and APP/PS1 mice by immunohistochemistry. **Figure 3A** shows that while there is a significant increase in APP expression and Aβ plaque deposition in the APP/PS1 mice treated with vehicle control compared to C57 mice, chronic administration of LIG significantly rescues this effect, almost to the level of the C57 control mice ($p < 0.01$, quantification in **Figure 3B**). Additionally, compared to C57 mice, APP/PS1 mice show higher levels of full-length APP gene expression and reduced sAPPα secretion. However, treatment with LIG resulted in a 30% reduction in APP gene expression by RT-PCR ($p < 0.05$) and also stimulated the release of sAPPα ($p < 0.05$) (**Figures 3C,D**), suggesting that LIG not only affects APP synthesis but also directs APP processing toward the non-amyloidogenic pathway.

Lastly, we examined the contribution of LIG to Aβ40 and Aβ42 expression in TBS-soluble (soluble) and guanidine-soluble (insoluble) brain fractions. Elevated Aβ42 has been identified as an important event in the early pathogenesis of AD (Findeis, 2007), moreso than the relative expression of Aβ40. This is important to note as we did not see any changes in Aβ40 expression in the APP/PS1 transgenic mouse model used in this study, and yet we found much higher levels of Aβ42 expression in both soluble and insoluble brain fractions of APP/PS1 mice (**Figures 3E,F**). Along these same lines, chronic LIG therapy only slightly reduced the Aβ40 expression found in the soluble brain fraction of APP/PS1 mice compared to vehicle treated controls, an effect which did not reach significance (**Figure 3E**). However, when we examined Aβ42 expression following LIG treatment, we show that it is significantly rescued in both the soluble and insoluble brain fractions compared to control, indicating the relevance and specificity of this compound. Collectively, these data indicate that LIG enhances APP processing through the α-secretase pathway, thus inhibiting production of Aβ42.

LIG Increases Klotho Expression and α-Secretase Processing in APP/PS1 Transgenic Mice

To study the effect of LIG on Klotho processing *in vivo*, we examined its expression in the choroid plexus of mice by immunohistochemistry. **Figure 4A** shows that while there is a significant reduction in Klotho staining in APP/PS1 vehicle-treated mice compared to C57 control mice, LIG significantly reversed this effect; indeed, quantitative analysis of our immunostaining reveals an increase in Klotho expression by more than 100% ($p < 0.01$, **Figure 4B**). Along these same lines, LIG treatment significantly increased Klotho gene expression in APP/PS1 mice as measured by RT-PCR ($p < 0.05$) and also stimulated the release of sKL as measured by ELISA ($p < 0.05$) (**Figures 4C,D**). These results indicate that LIG not only increases expression of full-length Klotho in the brains of APP/PS1 mice, but also increases the production of α-secretase processed sKL, which is then released into circulation.

Upregulation of Klotho by LIG Leads to an Inhibition of IGF-1/Akt/mTOR Signaling *in Vitro* and *in Vivo*

Accumulation of misfolded Aβ in the brain is believed to be a net result of imbalance between its production and removal (Zuroff et al., 2017). Therefore, we investigated if LIG-mediated Klotho upregulation might have an effect on Aβ clearance. As described previously, Klotho is a transmembrane protein whose cleavage results in the formation of sKL. Once in circulation, sKL acts as a hormone which is able to inhibit the insulin growth factor 1 (IGF-1) signaling pathway, a prominent regulator of stress resistance and aging (Cohen et al., 2009; Abramovitz et al., 2011). The IGF-1 signaling pathway induced activation of PI3K and thereby the Akt/mTOR. Down-regulate mTOR activity permitting activation of autophagy that clears misfolded proteins including Aβ plaques (O'Neill et al., 2012; Zhu et al., 2013). Therefore, we specifically investigated the effect of LIG-mediated Klotho regulation on Akt/mTOR signaling in APP/PS1 mice using western blot analysis. **Figure 5A** shows a significant increase in phosphorylation of both Akt and mTOR in APP/PS1 vehicle treated mice compared to C57 control mice and this was significantly reduced in mice chronically treated with LIG (quantification shown in **Figure 5B**). Next, we asked if the reduction in p-Akt and p-mTOR by LIG is an effect of its ability to regulate Klotho processing, with sKL acting as a ligand for the IGF-1 signaling pathway. Transient transfection of HEK293T cells with an siRNA against Klotho reduced its gene expression by approximately 75% compared to cells transfected with a control siRNA, regardless of LIG treatment (**Figure 5C**). Using western blot analysis 48 h following siRNA transfection, we examined the role of Klotho in Akt/mTOR and IGF-1 signaling in the presence and absence of LIG. Knocking down Klotho gene expression by siRNA significantly increased IGF-1R phosphorylation and increased phosphorylation of Akt and mTOR (**Figures 5D,E**). Interestingly, inhibition of Klotho gene expression resulted in the abrogation of LIG-mediated IGF-1 pathway inhibition, revealing that inhibition of this signaling pathway by LIG is mediated by its upregulation of endogenous Klotho.

DISCUSSION

The formation of Aβ fibrils is associated with a cascade of neuropathogenic events which induce brain neurodegeneration

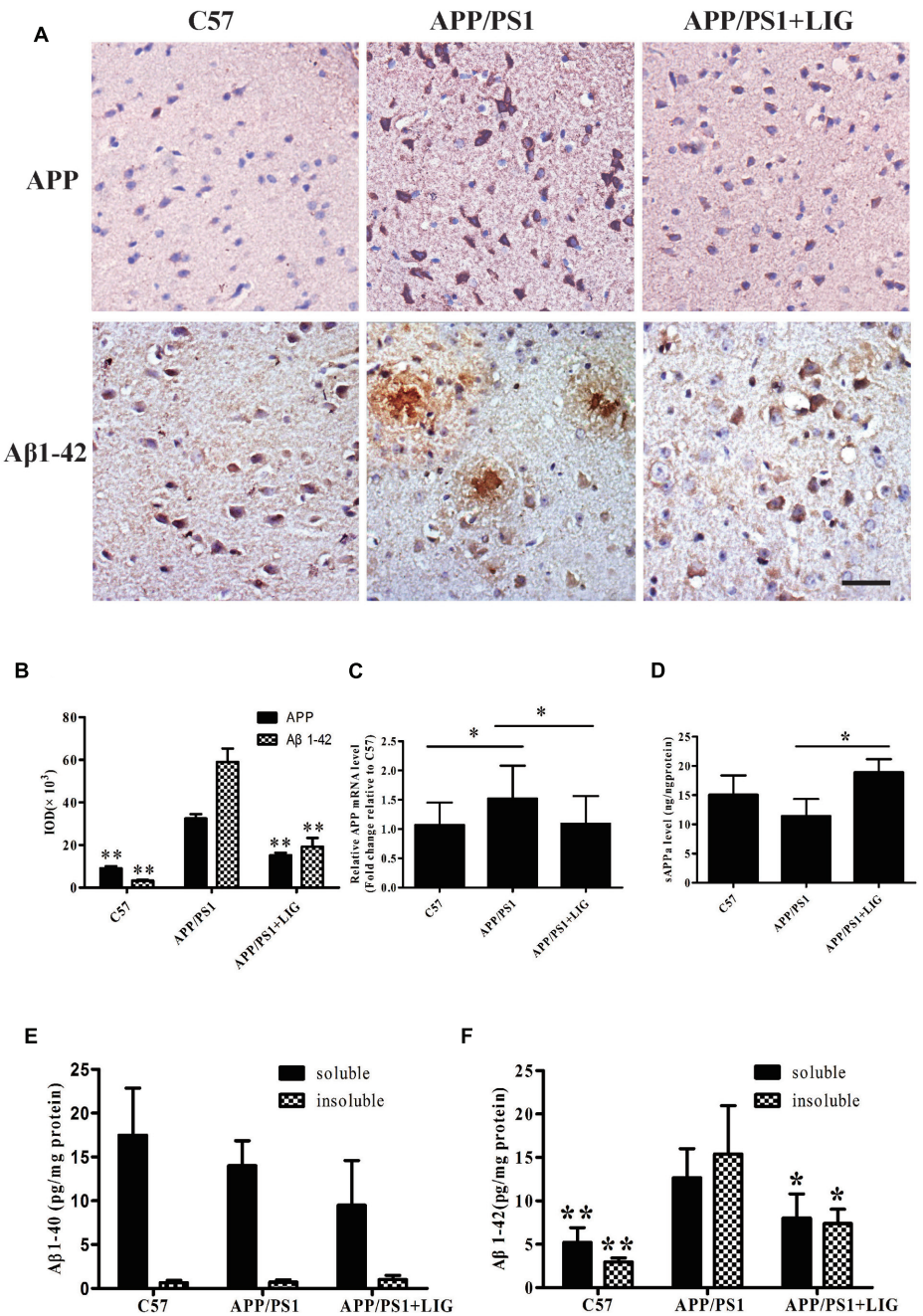

FIGURE 3 | Effect of LIG on APP processing and amyloid-β generation in APP/PS1 transgenic mice. **(A)** Representative immunostaining of APP and Aβ42 in the cerebral cortex of mice treated ± LIG or vehicle control. Scale bar, 25 μm. **(B)** Quantification of immunostaining from **(A)** based on the IOD of positive immunostaining. **(C)** Effect of LIG on full-length APP gene expression in APP/PS1 mice as measured by RT-PCR. **(D)** Effect of LIG on secreted sAPPα in APP/PS1 mice as measured by ELISA. **(E,F)** Effects of LIG on soluble and insoluble Aβ40 peptides **(E)** and Aβ42 peptides **(F)** in brain homogenates of APP/PS1 transgenic mice as measured by ELISA. The data are expressed as mean ± SEM; $n = 4$ for immunohistochemistry and RT-PCR, $n = 6$ for biochemical analysis; $^{*}p < 0.05$, $^{**}p < 0.01$.

and lead to the progression of AD (Kirkitadze and Kowalska, 2005). Disruption of the Aβ clearance machinery and an increase in Aβ accumulation gives rise to neurotoxic assemblies. Therapeutic strategies aimed at lowering cerebral Aβ by inhibiting its production and/or enhancing its clearance are currently under development (Lemere, 2009; Kurz and Perneczky, 2011). LIG has been proposed to be a potentially promising candidate for the treatment of AD based on its efficacy shown in our previous studies. But until now, the actual therapeutic value and mechanism of action of LIG on

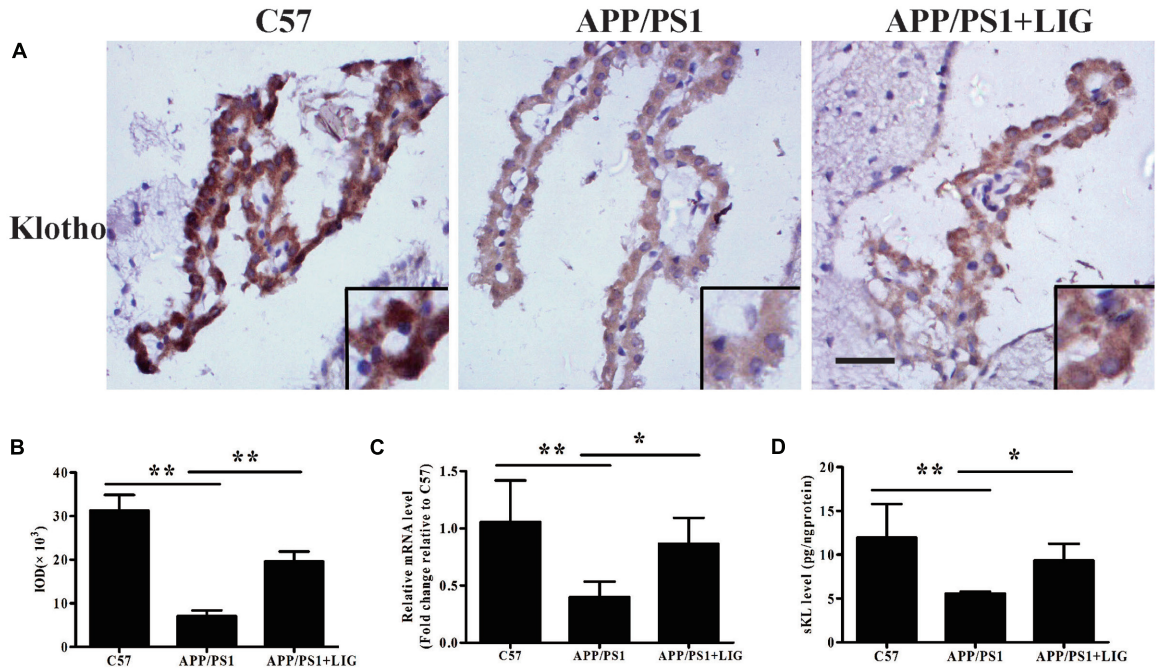

FIGURE 4 | Effect of LIG on Klotho expression and processing in APP/PS1 transgenic mice. **(A)** Representative immunostaining of Klotho in the choroid plexus of APP/PS1 mice in the presence or absence of LIG or vehicle control. Scale bar, 25 μm. **(B)** Quantification of immunostaining from **(A)** based on the IOD of positive immunostaining. **(C)** Effect of LIG on gene expression of Klotho as measured by RT-PCR in APP/PS1 transgenic mice. **(D)** Effect of LIG on sKL secretion as measured by ELISA in APP/PS1 transgenic mice. The data are expressed as mean ± SEM; $n = 4$ for immunohistochemistry and RT-PCR, $n = 6$ for biochemical analysis; $^{*}p < 0.05$, $^{**}p < 0.01$.

AD pathology and cognitive deficit had not fully been clarified. In the current work, we demonstrated that LIG treatment upregulates the α-secretase processing of APP and Klotho, playing a role in Aβ production and clearance in APP/PS1 transgenic mice.

Previous studies have shown that Aβ plaque deposition occurs before and/or in the early stages of neurodegeneration and behavioral changes in AD patients (Selkoe, 1994). Our results showed that LIG significantly improves the neurobehavioral deficits of APP/PS1 mice in both the passive avoidance and Y-maze tests for working memory. Our studies also showed that LIG treatment decreases aggregated soluble and insoluble Aβ42 levels and also decreases Aβ plaque deposition in the brains of APP/PS1 mice. Based on these results, we hypothesized that the ability for LIG to decrease cerebral Aβ accumulation may be attributable to two effects: directing APP processing toward a non-amyloidogenic pathway thus inhibiting Aβ production, and directing Klotho processing toward releasing sKL and thus acting as a regulator of Aβ clearance. Our studies confirmed that LIG significantly enhances sAPPα release, precluding Aβ generation. sAPPα has been shown to be beneficial for memory function and also to possess both neuroprotective and neurotrophic properties (Mattson, 1997). Results from our study indicate the potential role for LIG-mediated production of sAPPα to serve as a neuroprotective agent and contribute to the long-term benefit of LIG on memory loss in APP/PS1 mice.

Members of the ADAM family are considered likely α-secretase candidates responsible for alpha-cleavage in cells (Vingtdeux and Marambaud, 2012), and ADAM10 and ADAM17 specifically have been considered the likely candidates for α-secretase mediated APP cleavage (Nunan and Small, 2000) and Klotho processing. Results from our study showed that long-term LIG administration promotes non-amyloidogenic processing of APP in addition to sKL processing *in vitro* and *in vivo*, by increasing α-secretase cleavage of full-length APP and Klotho. In the brains of APP/PS1 mice, only ADAM10 protein levels were significantly elevated following LIG treatment, with no effect of LIG on ADAM17. To more fully understand the effect of LIG on ADAM-regulated processing of APP and Klotho, we used the selective, competitive inhibitors G1254023X and TAPI-O against ADAM10 and ADAM17, respectively. We found that ADAM10 inhibition significantly rescued the effect of LIG with no effect of ADAM17 inhibition, indicating the involvement of ADAM10 in LIG-induced APP and Klotho processing.

Apart from inhibition of Aβ production, promotion of Aβ clearance is also regarded as a valuable strategy in treating AD (Wostyn et al., 2011). Recently, it has been shown that impairment of autophagy contributes to an abnormal accumulation of Aβ protein in several age-dependent neurodegenerative diseases, including AD, Huntington's disease, and Parkinson's disease. And indeed, several studies have indicated that autophagy is involved in the clearance of Aβ under

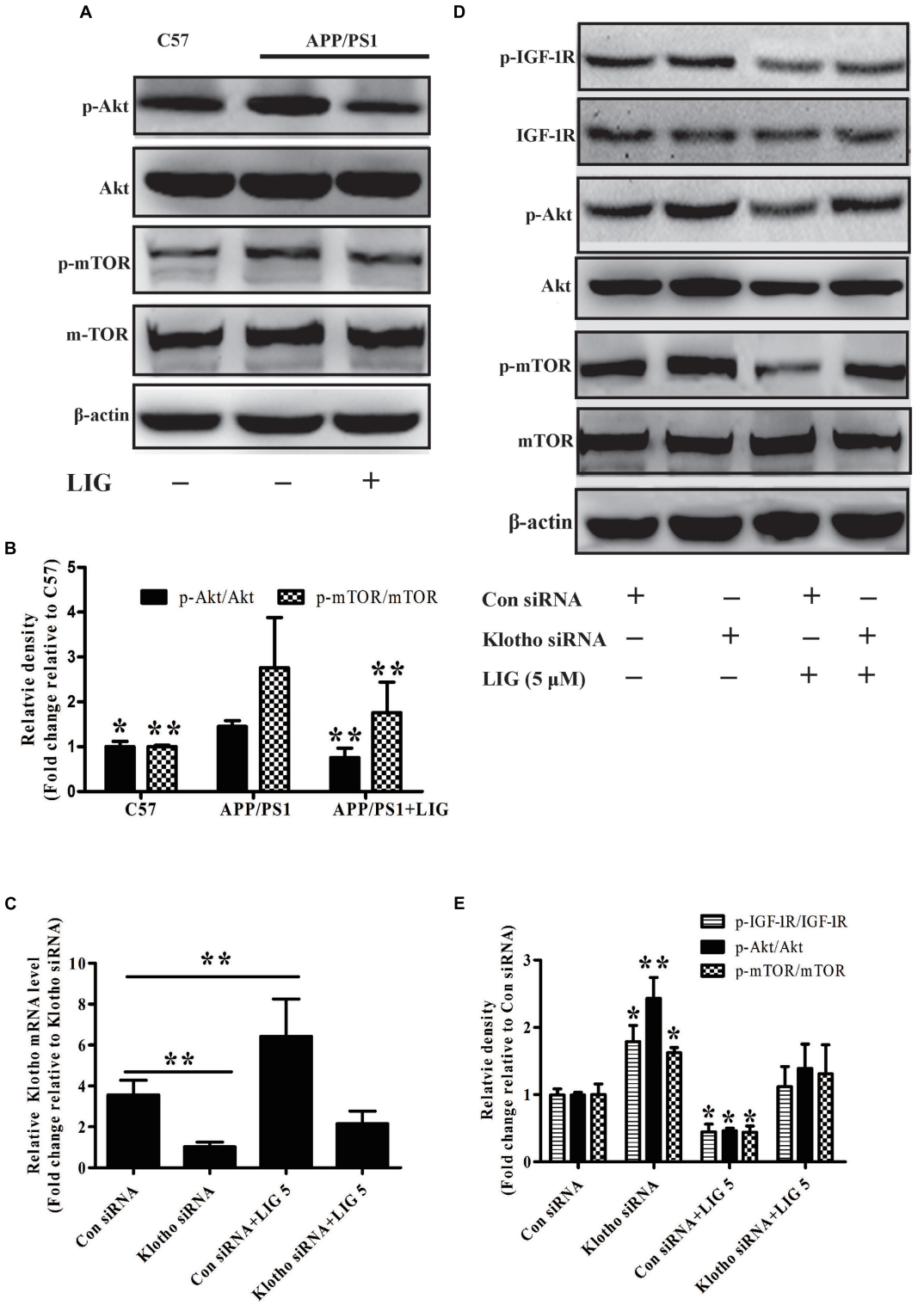

FIGURE 5 | Effect of LIG on insulin growth factor 1 (IGF-1)/Akt/m-TOR signaling pathways *in vivo* and *in vitro*. **(A)** Representative western blot analysis of brain homogenates from C57 or APP/PS1 mice treated ± LIG. **(B)** Quantification of western blot from **(A)** normalized to control C57 mice. **(C)** Full-length Klotho gene expression as measured by RT-PCR in HEK293T cells transfected with either control or Klotho-specific siRNA in the presence or absence of LIG. **(D)** Representative western blot analysis of HEK293T cells from **(C)** indicating the effect of Klotho inhibition on LIG induced IGF-1/Akt/mTOR signaling. **(E)** Quantification of western blot from **(D)** normalized to control C57 mice. The data are expressed as mean ± SEM; $n = 4$ for western blot and RT-PCR; $^{*}p < 0.05$, $^{**}p < 0.01$.

physiological conditions, effectively maintaining Aβ homeostasis in the healthy brain (Hung et al., 2009; Tian et al., 2011; Li et al., 2013). However, emerging evidence has suggested that autophagy is reduced in the brains of AD patients and animal models of AD, and this reduction leads to the accumulation of Aβ, subsequently contributing to its pathogenesis (Pickford et al., 2008; Nixon and Yang, 2011; Tan et al., 2014). Further evidence has suggested that compounds which inhibit mTOR signaling can increase Aβ clearance and rescue memory impairment by enhancing autophagy in AD mouse models (Spilman et al., 2010; Vingtdeux et al., 2011).

A previous study from our lab showed that Klotho alleviated oxidative stress by negatively regulating IGF-1 signaling pathway and thereby prevented the development of aging-associated AD (Kuang et al., 2014b). Consistent with these results, it has been reported that Klotho-induced mTOR inhibition primarily depends on its ability to inhibit the IGF-1/Akt/mTOR signaling cascade (Lin et al., 2013; Xie et al., 2013). Based on these studies, we speculated that sKL upregulated by LIG might have effect on promoting Aβ clearance via enhancing autophagy through inhibition of the IGF-1/Akt/mTOR pathway, an important pathway associated with Aβ autophagic clearance (Steele and Gandy, 2013). Our results showed that LIG resulted in significant inhibition of Akt/mTOR signaling in APP/PS1 mice as measured by western blot. Furthermore, we revealed the necessity of Klotho for this effect of LIG by target siRNA to knockdown Klotho expression in HEK293T cells.

CONCLUSION

Our data demonstrate for the first time that LIG is able to reduce cerebral Aβ burden and improve cognitive impairment in the APP/PS1 transgenic mouse model of AD via enhancing ADAM10 activity. The possible underlying mechanism of LIG may include two aspects: (1) LIG promotes the non-amyloidogenic processing of APP resulting in increased sAPPα levels and decreased Aβ production; (2) LIG upregulates sKL generation with inhibition of IGF-1/Akt/mTOR signaling. Taken together with the reported neuroprotective effects of both sAPPα and sKL as well as autophagy induction by Akt/mTOR pathway inhibition, our findings suggest that LIG may benefit for AD treatment through Aβ generation inhibition and potential clearance promotion via induction alpha-processing of APP and Klotho. Whether LIG may induce Aβ autophagic clearance and the underlying mechanisms need to be further investigated in future. In addition, in order to know the potential effects of LIG on alpha-processing single transmembrane proteins under physiological conditions, a new study with LIG-treated wild-type mice needs to be done in future.

AUTHOR CONTRIBUTIONS

Study design: J-RD and XK. Data collection and analysis: XK, H-JZ, X-NC, L-JL, and J-RD. Study supervision: J-RD. Paper drafting: XK, AT, and J-RD. Paper revising: J-RD, XK, and AT. Final approval of the version to be published: All authors.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnagi.2017.00353/full#supplementary-material

FIGURE S1 | Effect of LIG on cell viability. **(A)** Analysis of cell viability using MTT assay in HEK293T cell after 24 h of incubation with LIG at 0.1–10 μM. **(B)** Analysis of cell viability using MTT assay in SH-SY5Y cells after 24 h of incubation with LIG at 0.1–10 μM. **(C)** Analysis of cell viability using MTT assay in hAPP-transfected SH-SY5Y cells 24 h after transfection. **(D)** Analysis of cell viability using MTT assay in HKE293T cells 24 h after Klotho siRNA transfection.

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A Novel Virtual Reality-Based Training Protocol for the Enhancement of the "Mental Frame Syncing" in Individuals with Alzheimer's Disease: A Development-of-Concept Trial

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A growing body of evidence suggests that people with Alzheimer's Disease (AD) show compromised spatial abilities. In addition, there exists from the earliest stages of AD a specific impairment in "mental frame syncing," which is the ability to synchronize an allocentric viewpoint-independent representation (including object-to-object information) with an egocentric one by computing the bearing of each relevant "object" in the environment in relation to the stored heading in space (i.e., information about our viewpoint contained in the allocentric viewpoint-dependent representation). The main objective of this development-of-concept trial was to evaluate the efficacy of a novel VR-based training protocol focused on the enhancement of the "mental frame syncing" of the different spatial representations in subjects with AD. We recruited 20 individuals with AD who were randomly assigned to either "VR-based training" or "Control Group." Moreover, eight cognitively healthy elderly individuals were recruited to participate in the VR-based training in order to have a different comparison group. Based on a neuropsychological assessment, our results indicated a significant improvement in long-term spatial memory after the VR-based training for patients with AD; this means that transference of improvements from the VR-based training to more general aspects of spatial cognition was observed. Interestingly, there was also a significant effect of VR-based training on executive functioning for cognitively healthy elderly individuals. In sum, VR could be considered as an advanced embodied tool suitable for treating spatial recall impairments.

Keywords: allocentric, egocentric, spatial memory, Alzheimer's Disease, virtual reality

INTRODUCTION

It is traditionally accepted that humans are able to represent space and recall spatial information with two fundamental spatial representations: one comprising information about the position of the individual in relation to the surrounding objects (i.e., egocentric), and the other one including information about the position of the objects relative to each other in the environment (i.e., allocentric). For example, to recall the spatial position of a supermarket, individuals may simply refer to turning "left" or "right" since these directions strictly depend first of all on the spatial position of their own body (i.e., egocentric reference frame). However, since the position of an object "changes" according to the spatial position of our own body in the space, individuals, in order to refer to the supermarket, also need to have a stored spatial layout that includes the relationships between two external landmarks (i.e., allocentric reference frame). A neurocognitive model was recently advanced to explain how spatial recall works (Burgess et al., 2001; Byrne et al., 2007). Burgess and his colleagues explained that, in the presence of a spatial cue, allocentric representation is retrieved through a process of pattern completion. Though initially allocentric, this representation is translated to egocentric for navigation purposes in the medial parietal areas via information provided by other cells (Hartley et al., 2014). More specifically, the retrosplenial cortex (RSC) is responsible for the transformation of long-term hippocampal allocentric representations into egocentric parietal representations to account for the rotational offset between the different spatial coordinates (Maguire, 2001; Vann et al., 2009). While parahippocampal regions are involved in processing the visuo-spatial structure of the spatial scene, RSC supports the process of spatial recall thanks to the retrieval of reference that allows the scene to be localized within the wider spatial environment (Epstein et al., 2007; Ekstrom et al., 2014).

Studies on spatial recall provide a unique opportunity to better understand topographical disorientation that may occur in both physiological and pathological aging (Moffat, 2009; Gazova et al., 2012), especially in Alzheimer's Disease (AD; Lithfous et al., 2013; Serino et al., 2014b). In patients with AD, there is a main impairment in episodic memory functioning due to neurodegeneration in medial temporal structures (e.g., entorhinal cortex, hippocampal formation, parahippocampal gyrus; for review, see Tromp et al., 2015). This episodic memory disorder is temporally and spatially related to both the distribution of neurofibrillary tangles within the medial temporal lobe and the volumetric loss of the hippocampus (Braak and Braak, 1995; Dubois et al., 2007). However, due to these neurodegenerative processes, episodes of topographical disorientation are also common since they are related to an early deficit in allocentric recall both in patients with amnestic mild cognitive impairment (aMCI) and with AD (for review, see Serino et al., 2014b). In addition, there exists from the earliest stages of AD the presence of a specific impairment in the ability to encode and store an allocentric hippocampal representation and then to translate it into an egocentric parietal representation. Serino and Riva (2013, 2014) proposed that there is a specific cognitive process (i.e., the "mental frame syncing") underlying this egocentric-allocentric transformation that may be useful in supporting the recall of spatial scenarios. The mental frame syncing may be conceived as a cognitive process that permits the transformation from a stored allocentric viewpoint-independent representation (including the above mentioned object-to-object information) to an egocentric one by computing the bearing of each relevant "object" in the environment in relation to the stored heading in space (i.e., information about our viewpoint contained in the viewpoint-dependent representation). Serino and Riva (2013, 2014) argued that patients with AD might experience a break in the "mental frame syncing" due to neurodegenerative processes that affect the medial temporal lobe and the hippocampus in particular. Indeed, neurofibrillary tangle deposition leads to degeneration of hippocampal subfields, first the CA1 and then the subiculum, CA2, CA3, and CA4 (Bartsch and Wulff, 2015). Padurariu et al. (2012) found neuronal loss especially in the CA1 and CA3, which elaborates allocentric representations containing pure object-to-object information of the spatial scene and those containing information about the individual's viewpoint within the spatial scene, respectively (Behrendt, 2013).

In this context, Virtual Reality (VR) could be considered a new advanced tool for specifically assessing and treating spatial recall impairment (Burgess et al., 2002; Bohil et al., 2011; García-Betances et al., 2015; Serino et al., 2015b).

A virtual town center where subjects can wander around, find an object and memorize its spatial position using an HMD and a gamepad is an example of how a VR environment can be used to assess and treat spatial recall deficiencies among subjects with AD. Indeed, in virtual environments it is possible to easily implement a "reorientation task," which is characterized by two phases. In the *encoding phase*, participants are instructed to memorize the position of an object. Then, in the *retrieval phase*, participants have to indicate the position of the object, starting from another position. As suggested by Bosco et al. (2008), this strategy induced interference in the egocentric representation of the object with respect to the participants' view (i.e., "virtual disorientation"). To indicate the position of the object, this technique forced the participants to refer to their allocentric viewpoint-independent representation and sync it with the allocentric viewpoint-dependent representation. In a recent study, Serino et al. (2015b) used a virtual task based on this paradigm and found that patients with AD have specific impairment in the synchronization of allocentric viewpoint-independent and viewpoint-dependent representation. In this study, participants were asked to memorize the position of an object after having entered a virtual room. Then they were asked to recall its position in two different tasks. The first task involved an aerial map of the room (i.e., a task that measures the ability to store an allocentric viewpoint-independent representation); the second task involved entering the empty version of the virtual room again, but this time from another starting point (i.e., a task that measures the ability to sync the stored allocentric viewpoint-independent representation with the viewpoint-dependent one). Results revealed that while aMCI patients showed a deficit only in the first task, a more profound deficit was found for patients with

AD; namely, they were not able to synchronize the two different representations.

As concerns rehabilitation, some recent systematic reviews and meta-analyses have provided support for the efficacy of cognitive training in improving the quality of life and careers of patients with AD. However, conclusions drawn from existing studies must be viewed with caution due to the limited number of randomized controlled trials and their methodological limitations (Clare and Woods, 2004; Olazarán et al., 2010; Bahar-Fuchs et al., 2013).

As reviewed by García-Betances et al. (2015), VR can also be used as a rehabilitative tool for patients with AD, but very little work has been done on this topic so far. In general terms, the first great advantage offered by VR is its possibility to develop tailored cognitive exercises within meaningful environments (Riva et al., 2006), which is particularly relevant since cognitive training might be particularly demanding for patients with AD. More specifically, in virtual environments it is possible to implement cognitive training based on specific rehabilitative mechanisms. For example, in our cognitive training we exploited the technique known as "virtual disorientation" (as described before), which is difficult to set up in a real-life situation.

An interesting example was offered by Kober et al. (2013), who found that VR can be a useful tool to implement a cognitive training program for spatial abilities when also used with passive navigation. In a study in which eleven neurologic patients with focal brain lesions and topographical disorientation performed a route finding task, results showed that a VR-based verbally guided passive navigation training program was able to improve general spatial abilities in neurologic patients. Caglio et al. (2012) showed that a virtual navigation task led to improvements in memory function in a 24-year-old man with a traumatic brain injury; this improvement corresponded to increased activity in cerebral structures crucial for both navigation and memory, such as the hippocampus and parahippocampus. However, no studies have explored the potentiality of VR for training the cognitive ability to synchronize different spatial representations in patients with AD.

Based on these premises, the main objective of this development-of-concept trial[1](Dobkin, 2009) is to evaluate if a VR-based training protocol specifically focused on the enhancement of the "mental frame syncing" between allocentric viewpoint-dependent and viewpoint-independent representation would be able to improve general spatial abilities in a sample of patients with AD. In order to preliminarily evaluate the efficacy of the novel VR-based training, two groups of patients with AD (i.e., "VR-based training" vs. "Control Group") will be compared using a traditional neuropsychological battery before and after both training programs. Moreover, eight cognitively healthy elderly individuals were recruited to participate in the VR-based training in order to have a different comparison group.

[1]The development-of-concept trial (which is in Phase I in the context of the Food and Drug Administration's classification of clinical trials for the pharmaceutical industry to test drugs) was aimed at preliminarily testing the efficacy of new interventions in randomized and masked pilot studies with control groups. In this kind of study, small groups of patients were usually recruited.

MATERIALS AND METHODS

Participants

We recruited 20 elderly subjects (age > 65 years old) from a social senior center located in Milan (Italy) from individuals referred as meeting the NINCDS-ARDRA criteria (McKhann et al., 1984). They were evaluated with the Milan Overall Dementia Scale (Brazzelli et al., 1994) and only individuals who had a score under 85.5 (i.e., the clinical cut-off for probable dementia) were included in the study. Participants were randomly assigned to "VR-based training" ("VR Group-AD"; $n = 10$) or "Control Group" ("Control Group-AD"; $n = 10$). We also recruited eight cognitively healthy elderly subjects (age > 65 years old) from the same setting (i.e., a social senior center located in Milan, Italy) to participate in the VR-based training ("VR Group-Normal Aging"; $n = 8$). They were evaluated with the Milan Overall Dementia Scale (Brazzelli et al., 1994) and only individuals who had a score over 85.5 were included in the study.

Exclusion criteria for the three groups were: (1) presence of visual and balance deficits which may interfere with the use of VR technology; and (2) the additional presence of psychiatric disorders or other neurological conditions, such as traumatic brain injuries or strokes.

The VR Group-AD included nine women and one man, while the Control Group-AD included eight women and two men ($\chi^2 = 0.392$; $p = 0.531$). The mean age for the VR Group-AD was 86.60 ($SD = 6.13$), with mean years of education of 9.80 ($SD = 3.97$); the mean age for the Control Group-AD was 88.70 ($SD = 3.59$), with mean years of education of 7.00 ($SD = 5.00$). There were no significant differences between two groups in terms of age [$t_{(18)} = 1.675$; $p = 0.111$] or education [$t_{(18)} = -0.934$; $p = 0.362$]. The VR Group-Normal Aging comprised four women and four men. The mean age for this group was 86.62 ($SD = 6.19$), with mean years of education of 9.12 ($SD = 5.05$). There were no significant differences between the VR Group-AD and VR Group-Normal Aging in terms of age [$t_{(16)} = 0.318$; $p = 0.755$] or education [$t_{(16)} = -0.009$; $p = 0.993$]. All participants provided written informed consent, which was approved by the Ethical Committee of IRCCS Istituto Auxologico Italiano. The study was conducted in compliance with the Helsinki Declaration of 1975, as revised in 2008.

VR-Based Training Program

A Virtual Reality (VR)-based protocol was developed to train the ability in syncing between allocentric viewpoint-dependent and allocentric viewpoint-independent representations (Serino and Riva, 2013, 2014). The training program consisted of 10 sessions for 3–4 consecutive weeks, with approximately three sessions a week. Each session contained an "encoding phase" and a "retrieval phase" (see Supplementary Material). The first and last sessions were also devoted to the administration of a neuropsychological assessment (see **Figure 1**).

Each VR-based treatment session had the same structure. All the sessions were carried out in a quiet room in the social senior center; participants were invited to enter and take a seat in front of a table where the PC was positioned (see the Section Procedure for other technical details). They were then asked to

take a gamepad and to navigate inside the virtual environment, thus starting the session. After initial training in VR technology (i.e., a different virtual city where participants were assisted in learning how to navigate within virtual environments using the gamepad), the encoding phase started. A virtual city had been developed as the rehabilitation environment. It was built around a central square with a fountain and a bar with some tables, which represents the starting point of the navigation (see **Figure 2**). There were buildings and shops spread out in the city; no human characters were present in order to avoid possible interference in the rehabilitation process. The neuropsychologist asked participants to enter this virtual city starting from its center

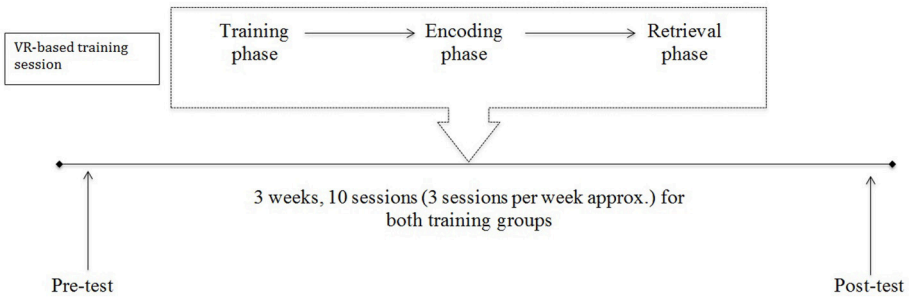

FIGURE 1 | Timeline of the VR-based training program. The training consisted of 10 sessions for 3–4 consecutive weeks, with approximately three sessions a week. After brief training in VR technology (about 2 min), each session comprised two parts: an "*encoding*" and a "*retrieval phase.*" Participants were assessed with a comprehensive neuropsychological assessment before and after participation in the training.

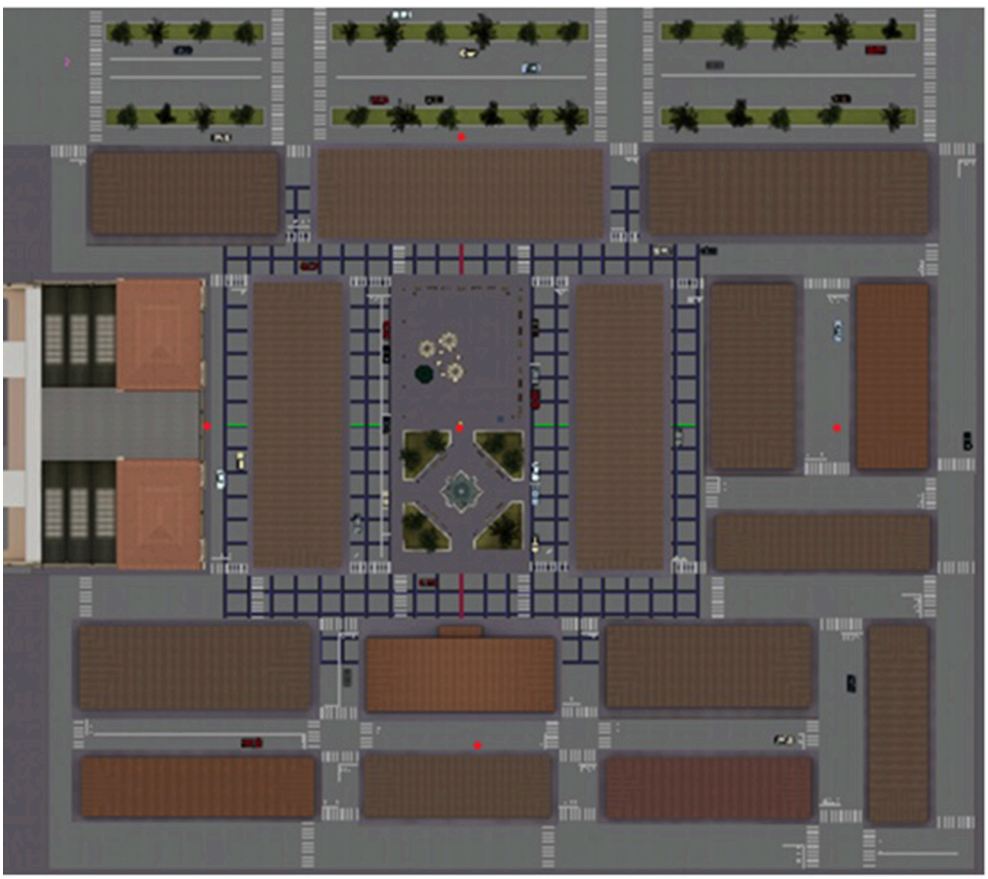

FIGURE 2 | The map of the virtual city. The city was built around a central square with a fountain and a bar with some tables, which represents the starting point of the navigation. There were buildings and shops spread out in the city. In the northern part of the city, there was a large street surrounded by trees with some cars, whereas the southern part was more residential.

to discover one, two or three hidden objects (i.e., a bottle of milk, a plant in a vase and a trunk; see **Figure 3**). Participants were specifically instructed to memorize the positions of these objects, which were placed at different parts of the city (see **Table 1**).

The number of the objects to be memorized depended on the level reached by each participant; if the patient was not able to locate the first object, the other objects were not presented. There was no time limit in the encoding phase, but all participants found the object(s) in ∼10–15 min. Next, in the retrieval phase, they were asked to retrieve the position of the objects identified in the first phase (which were now absent) once they entered the virtual city from another starting point. There was no time limit in the retrieval phase. Also in this case, all participants retrieved the position of the object(s) in ∼10–15 min; they were instructed to stop and tell the neuropsychologist when they thought they had reached the correct position. If participants were not able to retrieve the correct position of the object, the neuropsychologist helped them by guiding them in the virtual environment in order to strengthen the amnestic trace.

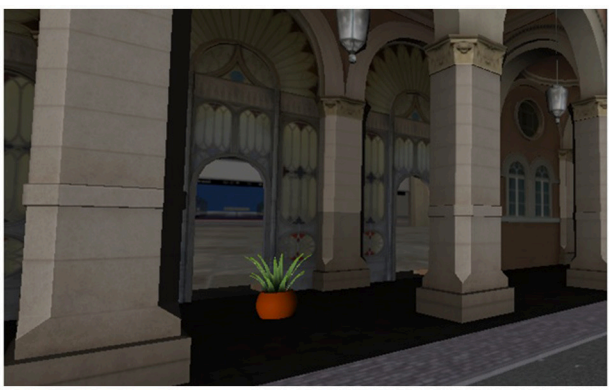

FIGURE 3 | One of the objects to be found during the VR-based training program. During the encoding phase, participants were asked to locate one, two or three hidden objects (i.e., a bottle of milk, a plant in a vase and a trunk). They received the specific instruction of memorizing the position of these objects, which were positioned at different parts of the city since in the retrieval phase they were asked to retrieve their spatial positions. The training was personalized according to the level reached by each participant, so that if a patient was not able to locate the first object, the other objects were not presented.

The entire VR-based session treatment lasted about 20 min. For each session, the neuropsychologist filled in a grid indicating which object(s) was/were found and briefly describing the progress reached during the session. It was essential to record the number of objects to be included in each session because, as previously explained, if patients were not able to find the first object, the other objects were not presented. Because patient response to VR-based training can vary greatly, cognitive exercises should be personalized according to a patient's specific needs.

In the first five VR treatment sessions, an interactive aerial view of the virtual city was displayed during the retrieval phase; its display was oriented depending on the participant's movement. The aerial view of the city was on the left part of the screen of the PC, and the participants could see in every direction for a distance of 25 m, which was the maximum distance at which any object could be seen (see **Figure 4**).

Moreover, when needed, the neuropsychologist helped patients with the gamepad used to navigate in the virtual city or provided little clues in order to avoid errors. Indeed, de Werd et al. (2013) underlined the importance of "errorless learning" in people with AD and severe memory impairments.

This VR-based training was developed using the open-source based software NeuroVirtual 3D, a recent extension of the software NeuroVR (Riva et al., 2011; Cipresso et al., 2014). The software (http://www.neurovr3.org/) is composed of two main modules: the Editor, which permits the customization of pre-designed virtual environments (e.g., a city, an apartment, a supermarket, etc.) to the specific needs of an experimental setting; and the Player, which allows the administration of the configured virtual environments. Thanks to the Editor, researchers can customize virtual environments by choosing the appropriate stimuli from a database of objects (both 2D and 3D objects, videos, and sounds). No programming skills are necessary since there is a user-friendly icon-based interface that allows researchers to easily put the stimuli in the pre-designed virtual environments. When ready, the configured virtual environments can be visualized via the Player both in immersive or not-immersive modalities.

Outcome Measures

To obtain detailed information across a wide range of cognitive domains before and after both training programs, all participants underwent a detailed neuropsychological assessment conducted

TABLE 1 | The objects with their spatial location in the virtual city in the encoding phase and the starting point of the participants, after the virtual disorientation, in the retrieval phase.

	Session 1	Session 2	Session 3	Session 4	Session 5	Session 6	Session 7	Session 8	Session 9	Session 10
ENCODING PHASE										
Objects' Position	West	West; East	West; East	West; East	South; North; East	West; East	West; East	West; East	North; South; West	South; East; West
RETRIEVAL PHASE										
Starting point	East	North	South	East	West	South	North	South	East	Center

After the fifth session, the number of possible objects to be memorized remains fixed to two for three sessions (which means that there was not an increase of difficulty) because of the absence of the facilitation offered by the interactive aerial view during the retrieval phase.

FIGURE 4 | During the retrieval phase, to facilitate the retrieval of spatial information, an interactive aerial view of the virtual city was presented during the navigation and the display was oriented depending on the participant's movement in the virtual world.

by an experienced neuropsychologist. These measures included (1) the phonemic verbal fluency and categorical verbal fluency test (Novelli et al., 1986) and Frontal Assessment Battery (FAB; Appollonio et al., 2005) for the evaluation of executive functions; (2) the Attentional Matrices Test (Spinnler and Tognoni, 1987) for measuring selective attention; (3) the Digit Span Test (Monaco et al., 2012) for assessing short-term memory abilities; (4) and the Corsi Block Test (Corsi, 1973; Monaco et al., 2012) in both its versions (i.e., Corsi Span and Corsi Supraspan) for the assessment of short and long-term spatial memory abilities. All scores obtained from the neuropsychological battery were corrected for age and education level according to Italian normative data.

Procedure

After participants gave their informed consent to be included in the study, one group of patients with AD was randomly assigned to the "VR-based training" ("VR Group-AD"), while the other group of patients with AD was randomly assigned to the "Control Group-AD" ("Control Group-AD"). Control Group-AD underwent the traditional cognitive rehabilitative activities with the neuropsychological staff at the social senior center (i.e., cognitive stimulation programs, such as cards games, naming, fluency, and music listening). A group of cognitively healthy individuals were recruited to participate in the VR-based training ("VR Group-Normal Aging").

All participants were then given the Mini-Mental State Examination (MMSE; Folstein et al., 1983) and the neuropsychological battery in order to obtain an overview of their general cognitive functioning. As specifically regards the VR group, participants were asked to sit in front of a portable computer (ACER ASPIRE with CPU Intel® Core™i5 and graphic processor Nvidia GeForce GT 540M, 1024 × 768 resolution). The participants were also given a gamepad (Logitech Rumble F510) that allowed them to explore and interact with the environment. After the training phase in VR technology (∼2 min), the VR-based treatment session started. Both training programs consisted of 10 sessions for 3–4 consecutive weeks, with approximately three sessions a week. At the end of both training programs, the same neuropsychological assessment was administered to all participants.

Data Analysis

To evaluate the efficacy of the novel VR-based training, we first compared the performances on the neuropsychological assessment between the two groups of patients with AD. To ensure that there were not differences in performance on the neuropsychological tests before the participants underwent the training programs, a series of Mann–Whitney *U*-tests were carried out on participants in the "VR Group-AD" and "Control Group-AD" on these variables. Then we computed the differences between pre- and post-test scores on the neuropsychological assessment (delta scores). Then, a Mann–Whitney *U*-test between the "VR group-AD" and "Control Group-AD" on each computed delta score was carried out.

Moreover, we compared the performances on the neuropsychological assessment between the patients with AD who participated in the VR-based training program with those of cognitively healthy elderly individuals. After the comparison between the two groups on the neuropsychological assessment before participating in VR-based training, a Mann–Whitney *U*-test between "VR group-AD" and "VR Group-Normal Aging" on computed delta scores for each neuropsychological test was carried out.

Given the small sample sizes, comparisons between groups were most suited to non-parametric testing for all our analyses (Siegel and Castellan, 1988). All statistical analyses were

performed using the Statistical Package for the Social Sciences for Windows (SPSS Inc., Chicago, IL, USA), version 23. A $p < 0.05$ was considered statistically significant.

RESULTS

Comparison between the Two Groups of Patients with AD—Baseline Characteristics

Participants' scores obtained from the neuropsychological assessment and the statistical comparisons are shown in **Table 2**.

Results from the Mann–Whitney *U*-test indicated no significant differences between the two groups of patients with AD on the neuropsychological tests administered before the beginning of both training programs. Accordingly, the VR Group-AD and Control Group-AD were comparable as concerns the baseline characteristics.

Comparison between the Two Group of Patients with AD—Pre- And Post-Neuropsychological Assessment

To investigate the efficacy of the novel VR-based training, the differences between pre- and post-test scores on the neuropsychological assessment (delta scores) between the two groups were evaluated using the Mann–Whitney *U*-Test.

As shown in **Table 3**, VR Group-AD scores were significantly better than Control Group-AD scores on the Corsi Block Test—Supraspan. Regarding all of the other neuropsychological measures administered, VR group-AD performance did not differ significantly from that of Control Group-AD. Although not significant, a great improvement for VR Group-AD was noticeable in attentional abilities, as underlined by the higher delta score on the Attentional Matrices Test.

Comparison between Patients with AD and Cognitively Healthy Controls Assigned to VR-Based Training- Baseline Characteristics

Cognitive abilities evaluated with the neuropsychological assessment before participation in the VR-based training and the statistical comparisons between the two groups are shown in **Table 4**.

Results from the Mann–Whitney *U*-test showed that patients with AD manifested greater difficulties in general cognitive functioning and in long-term spatial memory, as measured

TABLE 2 | Baseline characteristics.

	VR group-AD	Control group-AD	z^c	p^d	r^e
MMSE[a]	22.05 (1.62)	20.79 (1.80)	−1.592	0.111	0.355
Verbal fluency test	19.90 (4.86)	15.90 (5.84)	−1.290	0.197	0.288
Verbal categorical test	23.20 (3.22)	19.90 (5.90)	−1.367	0.172	0.306
FAB[b]	11.18 (2.75)	10.36 (3.34)	−0.832	0.406	0.186
Attentional matrices test	20.80 (8.19)	18.38 (3.90)	−0.756	0.406	0.169
Digit span test	6.30 (1.51)	5.57 (0.57)	−0.683	0.495	0.153
Corsi block test—span	3.91 (1.02)	4.08 (0.57)	−0.456	0.648	0.102
Corsi block test—supraspan	6.83 (1.71)	7.97 (1.89)	−0.756	0.450	0.169

Scores obtained from the first neuropsychological assessment for patients with AD assigned to the VR-based training ("VR Group- AD") and for patients with AD assigned to the "Control Group-AD" ("Control Group-AD"). Data are shown as means and standard deviations (SD).

[a] *MMSE, Mini Mental State Examination.*

[b] *FAB, Frontal Assessment Battery.*

[c] *Mann–Whitney U testing.*

[d] *p-value.*

[e] *effect size (|r| > 0.40 can be considered as a medium effect size, Cohen, 1988).*

TABLE 3 | Pre-post assessment.

	VR group-AD	Control group-AD	z^b	p^c	r^d
Verbal fluency test	−1.60 (4.00)	0.20 (4.21)	−0.836	0.403	0.187
Verbal categorical test	0.70 (4.71)	0.50 (4.21)	−0.038	0.970	0.008
FAB[a]	0.84 (4.26)	−0.66 (1.94)	−0.725	0.468	0.162
Attentional matrices test	4.71 (9.52)	0.70 (2.89)	−1.067	0.286	0.239
Digit span test	0.01 (0.82)	−0.33 (0.90)	−0.728	0.466	0.162
Corsi block test—span	0.17 (1.20)	0.14 (1.21)	−0.084	0.933	0.019
Corsi block test—supraspan	1.56 (2.53)	−0.01 (1.43)	−2.120	0.035	0.474

Delta scores obtained from the pre-post neuropsychological assessment for patients with AD assigned to the VR-based training ("VR Group-AD") and for patients with AD assigned to the "Control Group-AD" ("Control Group-AD"). Data are shown as means and standard deviations (SD).

[a] *MMSE, Mini Mental State Examination.*

[b] *Mann-Whitney U testing.*

[c] *p-value.*

[d] *effect size (|r| > 0.40 can be considered as a medium effect size, Cohen, 1988).*

TABLE 4 | Baseline characteristics.

	VR group-AD	VR group-normal aging	z^c	p^d	r^e
MMSE[a]	22.05 (1.62)	27.73 (2.02)	−3.560	<0.001	0.839
Verbal fluency test	19.90 (4.86)	22.87 (10.16)	−0.801	0.423	0.189
Verbal categorical test	23.20 (3.22)	25.75 (9.42)	−0.490	0.624	0.155
FAB[b]	11.18 (2.75)	13.12 (3.34)	−1.422	0.155	0.335
Attentional matrices test	20.80 (8.19)	29.42 (10.11)	−1.599	0.110	0.379
Digit span test	6.30 (1.51)	5.63 (0.55)	−0.489	0.625	0.115
Corsi block test—span	3.91 (1.02)	4.62 (0.60)	−1.780	0.075	0.419
Corsi block test—supraspan	6.83 (1.71)	9.25 (1.89)	−2.577	0.010	0.607

Scores obtained from the first neuropsychological assessment for patients with AD assigned to the VR-based training ("VR group- AD") and for cognitively healthy elderly individuals assigned to the VR-based training ("VR Group-Normal Aging"). Data are shown as means and standard deviations (SD).

[a] *MMSE, Mini Mental State Examination.*

[b] *FAB, Frontal Assessment Battery.*

[c] *Mann–Whitney U testing.*

[d] *p-value.*

[e] *Effect size (|r| > 0.40 can be considered as a medium effect size, Cohen, 1988).*

by MMSE and Corsi Block Test—Supraspan, respectively. It is important to note that although there were not significant differences between the two groups in the other cognitive domains, the mean scores of the cognitively healthy elderly individuals appeared above the clinical cut-off (Verbal fluency test: 17; Verbal categorical test: 25; Corsi Block Test—Span: 3.75; Digit: 3.75) or near the clinical cut-off (FAB: 13.50 and Attentional Matrices: 30).

Comparison between Patients with AD and Cognitively Healthy Controls Assigned to VR-Based Training—Pre- and Post-Neuropsychological Assessment

Differences between pre- and post-test scores on all neuropsychological tests (delta scores) were evaluated using the Mann–Whitney *U*-Test (**Table 5**).

Findings indicated a significant improvement in Verbal Fluency Test scores for cognitively healthy elderly individuals after the VR-based training. As concerns the other cognitive domains measured, although it is possible to note a general increase in the other tests evaluating executive functioning (i.e., Verbal Categorical Test and FAB), there were no other significant differences.

DISCUSSION

In this study, the efficacy of a novel VR-based training protocol to train the ability to sync allocentric viewpoint-dependent and viewpoint-independent representation in a sample of patients with Alzheimer's disease (AD) was evaluated. A group of cognitively healthy elderly individuals also participated in the VR-based training in order to have a different comparison group. Our results indicated a clear improvement in long-term spatial memory (as measured by the Corsi Block Test—Supraspan) after VR-based training for patients with AD; this means that transference of improvements from the VR-based training to more general aspects of spatial cognition was observed. Interestingly, VR-based training also had a significant effect on executive functioning (as measured by the Verbal Fluency Test) in cognitively healthy elderly individuals.

The first possible interpretation of our findings is that the improvement in long-term spatial memory for patients with AD may have resulted from the information provided by the interactive aerial view of the virtual city during the first phase of the training. Indeed, a recent study has found that the recall of spatial information is facilitated by the presence of an interactive aerial view of the experienced virtual environments because it provides a real-time allocentric viewpoint-dependent representation that helps individuals to place the current egocentric heading in space to compute the bearing of each relevant stored object (Serino and Riva, 2015). This "external aid" may help patients with AD improve their compromised ability to synchronize allocentric viewpoint-dependent and viewpoint-independent representation. Both active navigation and goal-directed spatial decision-making have a beneficial effect on spatial performance. In our VR-based training, participants were asked to use a gamepad to actively navigate in the virtual environments and complete a task. There was a virtual world where patients could perform actions at concrete objectives. Several studies have demonstrated the benefit of active navigation on memory performance (Brooks, 1999; Gaunet et al., 2001; Péruch and Wilson, 2004; Plancher et al., 2012). For instance, Plancher et al. (2012) asked older adults, patients with amnestic mild cognitive impairment and patients with AD to memorize a series of elements in two experimental conditions: as the driver of a virtual car (i.e., active exploration) and as the passenger of that car (i.e., passive exploration). Interestingly, for all groups, active exploration led to an enhanced recall of allocentric spatial information.

These results were generally interpreted as suggesting the role of action in enriching memory trace thanks to reinforcement of item-specific processing (Engelkamp, 1998). However, interacting with a complex environment provided participants not only with a sensorimotor trace essential for

TABLE 5 | Pre-post assessment.

	VR group-AD	VR group-normal aging	z^b	p^c	r^d
Verbal fluency test	−1.60 (4.00)	3.50 (3.89)	−2.240	0.025	0.527
Verbal categorical test	0.70 (4.71)	3.87 (5.03)	−1.206	0.228	0.284
FAB[a]	0.84 (4.26)	1.49 (2.13)	−0.670	0.503	0.157
Attentional matrices test	4.71 (9.52)	1.25 (8.53)	−0.357	0.721	0.084
Digit span test	0.01 (0.82)	−0.12 (0.83)	−0.369	0.466	0.086
Corsi block test—span	0.17 (1.20)	−0.12 (0.84)	−0.272	0.712	0.064
Corsi block test—supraspan	1.56 (2.53)	1.88 (4.21)	−0.267	0.790	0.059

Delta scores obtained from the pre-post neuropsychological for patients with AD assigned to the VR-based training ("VR group-AD") and for cognitively healthy elderly individuals-assigned to the VR-based training ("VR Group-Normal Aging Data are shown as means and standard deviations (SD).

[a] *MMSE, Mini Mental State Examination.*

[b] *Mann–Whitney U testing.*

[c] *p-value.*

[d] *Effect size (|r| > 0.40 can be considered as a medium effect size, Cohen, 1988).*

memory recall, but also with the opportunity to implement goal-directed spatial decision-making, which is another key element of active navigation. Planning spatial routes in a complex environment to achieve different objectives and, consequently, accomplish different intentions improved memory performance and appeared to be supported by the hippocampus (Viard et al., 2011).

These theoretical considerations on goal-directed spatial decision-making may offer an interesting explanation for the other findings of our work. Indeed, we also found an improvement in executive functioning of cognitively healthy elderly subjects after participation in VR-based training. By "executive function," we refer to a complex set of cognitive abilities that includes planning, sequencing, problem-solving, and monitoring (Chan et al., 2008), strictly dependent on frontal lobe activity. According to the so-called "frontal aging hypothesis," frontal lobes are particularly vulnerable to age-related decline (Greenwood, 2000), which leads to consequent and progressive decreases in executive functioning.

The role of frontal lobes has been demonstrated in other high-level cognitive functions, such as episodic memory (Lepage et al., 2000; Habib et al., 2003) and spatial memory (Maguire et al., 1998). In particular, as anticipated, the prefrontal cortex contribution was shown in spatial learning (Dahmani and Bohbot, 2015), and its involvement in navigation is thought to be linked to decision-making, planning and working memory (Spiers and Maguire, 2006; Moffat et al., 2007; Viard et al., 2011). For instance, Korthauer et al. (2017) found that performance on the virtual version of the Morris Water Task was associated with better verbal fluency, set switching, response inhibition, and the ability to mentally rotate objects.

It is possible that our VR-based training stimulated the two groups in different ways. In patients with AD, the training specifically stimulated the "mental frame syncing," leading to a better spatial performance. For cognitively healthy subjects, the training enhanced frontal functioning, stimulating the implementation of goal-directed spatial decision-making to solve the proposed task.

These findings are particularly promising for AD patients because they provide support for the feasibility of VR as an effective medium for delivering spatial training programs on specific spatial skills that can be generalized to general aspects of spatial processing, which appear to become compromised early in this population. As such, VR-based interventions could be included in the wider panorama of non-pharmacological and non-invasive treatments for AD (Olazarán et al., 2010; Bahar-Fuchs et al., 2013) that promote neuroplasticity and neural reorganization through environmental enrichment (Durlach et al., 2000; Rose et al., 2005; Bohil et al., 2011; García-Betances et al., 2015; Morganti, 2015; Repetto et al., 2016). It has been demonstrated that mice exposed to environmental enrichment have shown improvements in hippocampal long-term potentiation and changes in the neuroplasticity in cerebral regions associated with learning and memory (Alwis and Rajan, 2014). More specifically, it was observed that hippocampal place cell activity was present during a virtual navigation (Harvey et al., 2009). In addition, Clemenson and Stark (2015) have recently found that 3D video games were able to improve performance on memory tasks known to be dependent on hippocampal activity.

On the other hand, our results indicated that our training also led to improvements in executive functioning, allowing the possibility to set up specific protocols for healthy elderly people aimed at improving the traditional cognitive empowerment approaches for this population (Serino and Pedroli, 2016).

Limitations must be taken into account when considering our findings. First of all, although this is a development-of-concept trial and small groups of patients are usually sufficient for this type of study, the small sample size of may limit the generalizability of our findings. Another limit of the current study is that a measure of the ability to synchronize the two described allocentric representations was not included as an outcome measure, which prevents us from investigating the relationship between neuropsychological assessments and the ability to conduct the "mental frame syncing" and from showing the progress of the patients throughout training in a graph. Indeed, only two studies introduced novel VR-based tests aimed at evaluating the presence of a deficit in "mental frame syncing,"

and neither of those tests was standardized (Serino et al., 2014a, 2015b). However, it is interesting to note that preliminary results from these studies showed that general cognitive functioning (measured with MMSE) was associated with the ability to conduct the "mental frame syncing" (Serino et al., 2014a).

In sum, our results concerning the efficacy of a novel VR-based training program to improve synchronization of different spatial representations for AD are surely encouraging, but also preliminary. We have demonstrated the feasibility of our approach, but this study should be viewed as a proof-of-concept that requires further development. Summarizing researches conducted so far on patients with AD using VR technologies, García-Betances and colleagues noted that the majority of works in this field had privileged not-immersive VR-based solutions (García-Betances et al., 2015). This is not only a "technological issue," but as future step of our approach it would be crucial to investigate its efficacy using a computer-assisted virtual environment (CAVE). In this system, virtual environments are usually projected onto the screen of a small room completely surrounding the users, thus enhancing the sense of presence (i.e., the sense of "being there;" Riva et al., 2014) and the possibility of interacting with the objects in the scene. Although there are more user-friendly, immersive and low-cost rehabilitative technologies (e.g., Oculus Rift, for a review see Castelvecchi, 2016), it is possible to develop a training program in which the interactive aerial view rotates according to the orientation of the participants' heading direction. In our previous study carried out in a CAVE, we found that the presence of a small interactive aerial view of a virtual city, including a visualized larger arrow indicating the heading direction, was able to facilitate spatial recall (Serino et al., 2015a). These outcomes emphasized the crucial role of a larger visualized arrow in order to retrieve the correct path; this arrow worked as a "external aid" in reinforcing participants' "mental frame syncing" by giving them information about their heading direction in the environment. These findings were compatible with studies elucidating the role of retrosplenial cortex in heading retrieval (Epstein, 2008; Vann et al., 2009; Marchette et al., 2014), a specific cognitive process dissociable by place recognition (Julian et al., 2015).

With these developments, controlled clinical trials involving a greater number of participants are required to provide adequate support to our proposal. Other future challenges include the inclusion of a follow-up to provide more consistent results and the elaboration of a more complex cognitive training program focusing on other cognitive skills beyond the spatial one (Clare and Woods, 2004; Bahar-Fuchs et al., 2013). Indeed, current increasing evidence on the efficacy of non-pharmacological approaches in patients with AD is sufficiently encouraging to justify additional clinical studies on this population.

AUTHOR CONTRIBUTIONS

SS, EP, and GR developed the study concept. All authors contributed to the study design. EP, CT, and NM were involved in the data collection. SS performed the data analysis and interpretation under the supervision of GR. SS, EP, and CT wrote the first draft of the manuscript. GD, MS, KG, and GR were involved in the critical revision of the manuscript for important intellectual content. All the authors approved the final version of the manuscript for submission.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fnagi.2017.00240/full#supplementary-material

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Dl-3-n-Butylphthalide Treatment Enhances Hemodynamics and Ameliorates Memory Deficits in Rats with Chronic Cerebral Hypoperfusion

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Our previous study has revealed that chronic cerebral hypoperfusion (CCH) activates a compensatory vascular mechanism attempting to maintain an optimal cerebral blood flow (CBF). However, this compensation fails to prevent neuronal death and cognitive impairment because neurons die prior to the restoration of normal CBF. Therefore, pharmacological invention may be critical to enhance the CBF for reducing neurodegeneration and memory deficit. Dl-3-n-butylphthalide (NBP) is a compound isolated from the seeds of Chinese celery and has been proven to be able to prevent neuronal loss, reduce inflammation and ameliorate memory deficits in acute ischemic animal models and stroke patients. In the present study, we used magnetic resonance imaging (MRI) techniques, immunohistochemistry and Morris water maze (MWM) to investigate whether NBP can accelerate CBF recovery, reduce neuronal death and improve cognitive deficits in CCH rats after permanent bilateral common carotid artery occlusion (BCCAO). Rats were intravenously injected with NBP (5 mg/kg) daily for 14 days beginning the first day after BCCAO. The results showed that NBP shortened recovery time of CBF to pre-occlusion levels at 2 weeks following BCCAO, compared to 4 weeks in the vehicle group, and enhanced hemodynamic compensation through dilation of the vertebral arteries (VAs) and increase in angiogenesis. NBP treatment also markedly reduced reactive astrogliosis and cell apoptosis and protected hippocampal neurons against ischemic injury. The escape latency of CCH rats in the MWM was also reduced in response to NBP treatment. These findings demonstrate that NBP can accelerate the recovery of CBF and improve cognitive function in a rat model of CCH, suggesting that NBP is a promising therapy for CCH patients or vascular dementia.

Keywords: DL-3-n-butylphthalide, bilateral common carotid artery occlusion, MRI, cerebral blood flow, vertebral artery, angiogenesis, cognition impairment

INTRODUCTION

Chronic cerebral hypoperfusion (CCH) occurs in the early stages of senile dementia including Alzheimer's disease (AD), vascular dementia (VaD) and mixed dementia (Pappas et al., 1996; Yoshikawa et al., 2003; Schuff et al., 2009; Zhao and Gong, 2015), which has been considered to play an important role in neurodegeneration in the development of dementia and AD. It has been reported that many diseases such as heart disease (de la Torre, 2012), hypertension (Farkas et al., 2002; Choi et al., 2015), diabetes (Kwon et al., 2015) and atherosclerosis (McColl et al., 2009) contribute to vascular constriction and cause CCH.

The mechanisms underlying dementia caused by CCH involve neuronal loss (Pappas et al., 1996; Bang et al., 2013), white matter lesion (Tomimoto et al., 2003; Chida et al., 2011), glial activation (Simpson et al., 2007), synaptic dysfunction (Simpson et al., 2007), oxidative stress (Ritchie et al., 2004; Kašparová et al., 2005), accumulation of Aβ (Li et al., 2009b; Okamoto et al., 2012) and aggravation of progressive cognitive deficits (de la Torre, 2012; Choi et al., 2015).

Bilateral common carotid artery occlusion (BCCAO) is a widely used model of vascular dementia due to its instigation of CCH (Pappas et al., 1996; Farkas et al., 2007; Jing et al., 2015). Our previous study showed that BCCAO reduced cerebral blood flow (CBF) until 3 weeks when the CBF returned to the baseline level; however, neuronal death and cognitive impairment occurred at 2 weeks post-BCCAO in rats (Jing et al., 2015). Therefore, it is critical to recover the CBF earlier to prevent neurodegeneration and memory deficits. Unfortunately, there is currently no effective therapies available to accelerate the recovery of CBF and improve cognitive function in CCH.

L-3-n-Butylphthalide (l-NBP) was first isolated from the seeds of Chinese celery and was discovered within the last few decades. Based on the therapeutic property of this compound, dl-3-N Butylphthalide (dl-NBP, or NBP) was synthesized later in China and approved by the China Food and Drug Administration (CFDA) as an acute stage anti-ischemia treatment (Abdoulaye and Guo, 2016). NBP is a synthetic chiral compound containing L- and D-isomers of butylphthalide. Studies have demonstrated that NBP can reduce blood glucose level in diabetes (Zhang et al., 2011; Wang et al., 2016), decrease glial activation in a mouse model of amyotrophic lateral sclerosis (Feng et al., 2012), protect dopamine neurons in a rotenone model of Parkinson's disease (Xiong et al., 2012), prevent neural loss in experimental ischemic stroke (Liu et al., 2007; Li et al., 2010), ameliorate vascular cognitive impairment in ischemic and dementia patients (Zhu et al., 2014) and AD models (Peng et al., 2010). Studies also demonstrated that NBP is safe and is extensively metabolized by multiple enzymes (Diao et al., 2013). However, its effects on CBF, glial cell reactivity and neuron degeneration following CCH remain unclear.

Based on the discovery of the effects of NBP in an acute ischemic stroke (AIS) model and in patients, we hypothesized that NBP may be also effective for CCH. In the present study, we used magnetic resonance imaging (MRI) techniques, immunochemistry and Morris water maze (MWM) to explore the effect of NBP on dynamic changes in CBF, glial cell reactivity, neurodegeneration and cognitive function in CCH rats induced by BCCAO.

MATERIALS AND METHODS

Animals and Drug Administration

Adult male Sprague-Dawley rats (420–450 g, approximately 6 months old) were obtained from the Animal Experiment Center of Southern Medical University (Guangzhou, China). All rats were housed in cages under controlled temperature ($21 \pm 1°C$) and humidity ($55 \pm 10\%$), with a 12 h light/12 h dark cycle. Food and water were available *ad libitum* throughout the study. All animal procedures were performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by competent ethics committees at Jinan University.

Rats (94 total) were randomly divided into seven groups. Two groups: the vehicle group and the NBP-treated group, were assigned for measurement of CBF and vertebral artery (VA) diameters, and five groups (Sham, Veh- 2 weeks, NBP- 2 weeks, Veh- 4 weeks, and NBP- 4 weeks) for detection of behavior and pathology. The allocation of animals in each group is shown in **Table 1**.

NBP-treated rats received daily tail-vein injection of NBP solution (5 mg/kg) in HP-β-CD and 9% saline for 14 days (from day 1 to 14 after BCCAO). Observation on the pathological changes in rats was made at 2 weeks (NBP- 2 weeks) and 4 weeks (NBP- 4 weeks) after BCCAO. Rats in the vehicle group received a daily tail-vein injection with the solvent (HP-β-CD and saline) in the same dose as the NBP- treated group (but without NBP) for 14 days after BCCAO. For sham group, rats were undergone the same surgical and manipulation procedures without BCCAO.

DL-3-n-butylphthalide was provided by Shijiazhuang Pharmaceutical Group Co. Ltd. NBP was dissolved in 0.9% saline with a Hydroxypropyl-β-cyclodextrin (HP-β-CD: 0.9% saline, 1:3), and prepared for intravenous injection (i.v.). The chemical structure of dL-3-n-butylphthalide  is shown as the figure on the left. NBP is a novel synthetic chiral compound containing L- and D-isomers of butylphthalide.

Chronic Cerebral Hypoperfusion

CCH was induced by the modified permanent BCCAO (2VO) method, with initial occlusion of the right common carotid artery (RCCA) followed by occlusion of the left common

TABLE 1 | Information of animal numbers.

Animal groups	Beginning	Died during experiment	End	Experiment
Vehicle	12	4	8	CBF
NBP	12	2	10	CBF
Sham	10	0	10	Behavior
Veh- 2 weeks	15	5	10	Behavior
NBP- 2 weeks	14	4	10	Behavior
Veh- 4 weeks	16	6	10	Behavior
NBP- 4 weeks	15	5	10	Behavior

carotid artery 1 week later, as described previously (Sarti et al., 2002). Briefly, rats were anesthetized with 10% chloral hydrate (0.35 mL/100 g). The animal was placed facing up on a surgery table, and a ventral midline incision was performed on the neck to expose the common carotid arteries. The RCCA was carefully separated from the vagus nerve and surrounding tissues, then ligated permanently with double sutures (3-0). During the procedure, the operating room was maintained at a temperature of 28.0 ± 2.0°C. The left common carotid artery occlusion (LCCAO) was performed in the same manner 1 week after RCCA occlusion (RCCAO). Sham-operated rats underwent the same surgery procedure except occlusion of common carotid artery.

Magnetic Resonance Imaging

MRI experiments were conducted with a Discovery 750 3.0T scanner with an 8-channel wrist coil (GE Healthcare, Milwaukee, WI, USA). NBP-treated and vehicle group rats were scanned with MRI at six time points starting at pre-occlusion, then after BCCAO, followed by 1, 2, 3 and 4 weeks after BCCAO. Following anesthesia with 10% chloral hydrate (0.3 mL/100 g), animals were placed in a supine position prior to scanning. All imaging parameters for the 3D ASL series were described in our previous study (Jing et al., 2015). Briefly, 15 slices acquired in ascending order (slice thickness = 4 mm), no gap between slices; field of view = 120 mm × 120 mm, matrix = 512 (points) × 12 (arms); number of excitations = 5, bandwidth = 62.5 kHz, scan duration was 9 min 14 s, labeling duration = 1650 ms, post labeling delay = 1025 ms, repetition time = 4132 ms and echo time = 11 ms.

MRI images of blood vessels were captured by time-of-flight angiography with a 3D Fast SPGR. Scan parameters were as follows: echo time = 3.9 ms, repetition time = 20 ms, field of view = 80 mm × 60 mm, matrix = 320 × 224, number of excitations = 1, bandwidth = 31.2 kHz, and scan duration was 231 s.

3D Arterial Spin Labeling Technique

CBF in different brain regions of the rats was measured using the 3D Arterial Spin Labeling (ASL) technique. The processes and the parameters of this technique were used as described in our previous study (Jing et al., 2015).

Morris Water Maze Task

To evaluate deficits in learning and spatial memory caused by cerebral chronic hypoperfusion, rats in each group (n = 10) were evaluated using a MWM at 2 weeks and 4 weeks after BCCAO using methods described by Morris (1984). Escape latency of rats to find and stand on a platform under water in navigation trials and travel path around and within the quadrant of the platform in probe trials were monitored by a video camera linked to an animal behavioral recording system (Ethovison XT, Noldus Information Technology Co., The Hague, Netherlands).

Tissue Preparation

At 2 weeks and 4 weeks after BCCAO, rats (n = 4 in each group) were anesthetized as described above and transcardially perfused with physiologic saline followed by 4% paraformaldehyde in 0.1 mol/L phosphate-buffered saline (PBS, pH 7.4). The brain was dissected out immediately and post-fixed in the same fixative overnight at 4°C. Coronal blocks from the optic chiasm to the posterior level of hypothalamus, which includes the hippocampus and parietal cortex (PC), were prepared, and processed for dehydration with an increasing alcohol gradient and three xylene exposures in an Automated Tissue Processor (LYNX II, Hatfield, PA, USA). The blocks were embedded with paraffin in an embedding machine (Thermo Scientific HistoStar, Kalamazoo, MI, USA). Coronal sections at 10-μm-thickness were cut with a paraffin microtome (Leica, RM2235, Wetzelar, Germany). Sections were mounted on slides for staining, and one representative photomicrograph showing PC and hippocampus of these sections was taken from hematoxylin and eosin (HE) stain as shown in **Figure 1**.

Immunofluorescent Labeling

Immunofluorescence was performed as previously described (Jing et al., 2015). Briefly, sections were processed for immunofluorescence labeling with CD34 antibody (for endothelial cells of microvessels), glial fibrillary acidic protein (GFAP) antibody (for astrocytes), NeuN antibody (for neurons) and cleaved caspase-3 (C-caspase 3) antibody (for apoptotic cells). First, sections were immersed in blocking solution (5% normal goat serum or donkey serum in PBS) at room temperature for 2 h, then incubated overnight at 4°C with CD34 antibody (Rabbit, 1:200, Boster, Wu Han, China), GFAP antibody (Rabbit, 1:1000, Abcam, United Kingdom) separately, and NeuN antibody and cleaved caspase-3 antibody (goat or mouse, 1:1000, Abcam, Cambridge, MA, USA) for double-labeling. After three washes with 0.01 M PBS, the sections were then incubated with secondary antibody (Alexa Fluor 488-conjugated goat anti-rabbit, donkey anti-rabbit, or donkey anti-mouse IgG and Alexa Fluor 546-conjugated donkey anti-goat, Jackson Immunoresearch, West Grove, PA, USA) for 2 h at room temperature. The concentration of all secondary antibodies was 1:1000. After three washes in PBS, sections were covered with anti-quenching fluorescence mounting medium.

Quantitative Analysis

Detection of Cerebral Blood Flow

CBF was automatically calculated from 3D-ASL images by a scanner software (Functool 3D ASL, Software version 4.5, GE Medical Systems, Milwaukee, WI, USA). Approximately 2 × 2 mm regions from the PC, basal ganglion and hippocampus areas were selected for CBF measurement. A total of 48 regions from these areas in each group were analyzed at each time point.

Measurement of Vertebral Arteries (VAs)

Images showing VAs were captured by 3D-TOF angiography. The diameter of both VAs were measured at the middle of the cervical region. A total of 16 diameter values in each group were analyzed at each time point.

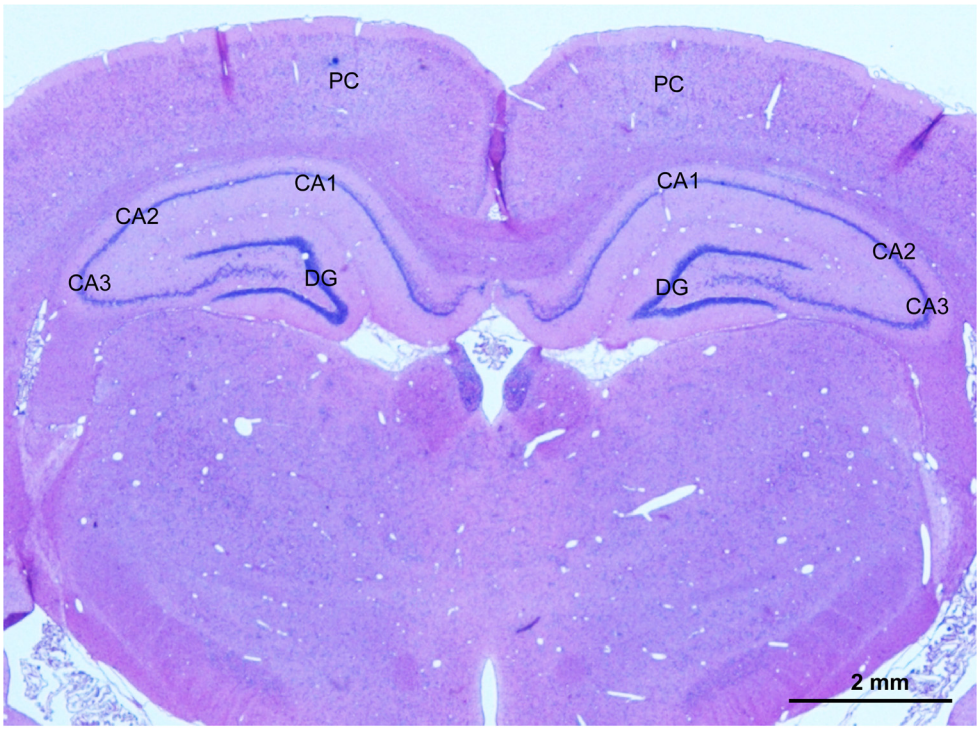

FIGURE 1 | A representative photomicrography of hematoxylin and eosin (HE) stained section showing selected areas for evaluation of the angiogenesis, glial activation, apoptosis and neuronal injury in the parietal cortex (PC) and CA1 area and CA3 area in the hippocampus. Scale bar is 2 mm.

Analysis of Numbers of CD34, GFAP, C-Caspase 3 and NeuN Positive Cells

For pathological detection, five rats in each group were used. In each group, a total of 40 sections at 100-μm intervals at one time point were selected for immunofluorescent labeling. Digital images were captured under 400× magnifications from the CA1 and CA3 subfields of the hippocampus and PC. Forty photos were taken from both side of the CA1 region, CA3 region or PC in each group at one time point separately. The number of CD34, GFAP, C-caspase 3 and NeuN positive cells in an area (45 μm^2) of each image was counted with ImageJ. The exposure time for imaging was consistent in each photo. Measurements were performed by two individuals blind to the study parameters.

Analysis of Spatial Memory Function

The data concerning escape latency and frequency of rats crossing the original platform were transferred from the animal behavioral recording system to Software Origin for analysis. A total of 40 numerical values in each group at different time points were analyzed.

Statistical Analysis

Values presented in the study were expressed as mean ± standard error. CBF and MWM data were input to Software Origin and analyzed by repeated measures two-way ANOVA with Bonferroni *post hoc* test. The remaining non-repeated data were input into StatView software (Version 5.1) and analyzed using an unpaired-test. Differences were considered statistically significant when p was less than 0.05.

RESULTS

NBP Promoted Recovery of CBF in the Parietal Cortex, Hippocampus and Striatum

Using the 3D ASL technique, we detected CBF in the PC, hippocampus and striatum of rats in the NBP-treated and vehicle groups at the time of pre-occlusion, beginning of BCCAO, and 1, 2, 3 and 4 weeks after BCCAO. Color signals from the imaging system range from green to red and represent an increasing gradient of CBF level. Before BCCAO, red signal appeared in the brain areas including the cortex, hippocampus and striatum in both groups in the pre-occlusion condition (**Figure 2A**). After BCCAO, signals were shifted to the green end of the spectrum in vehicle-treated rats indicating low CBF. This persisted until 3 weeks post-occlusion when the red signal reappeared in the cortex, hippocampus and striatum and reached pre-occlusion levels at 4 weeks after BCCAO. These results demonstrated the success of this CCH model, and were consistent with our previous study (Jing et al., 2015). But in NBP-treated animals, red signal reappeared at 1 week following BCCAO and gradually became more prominent until 4 weeks after BCCAO (**Figure 2A**).

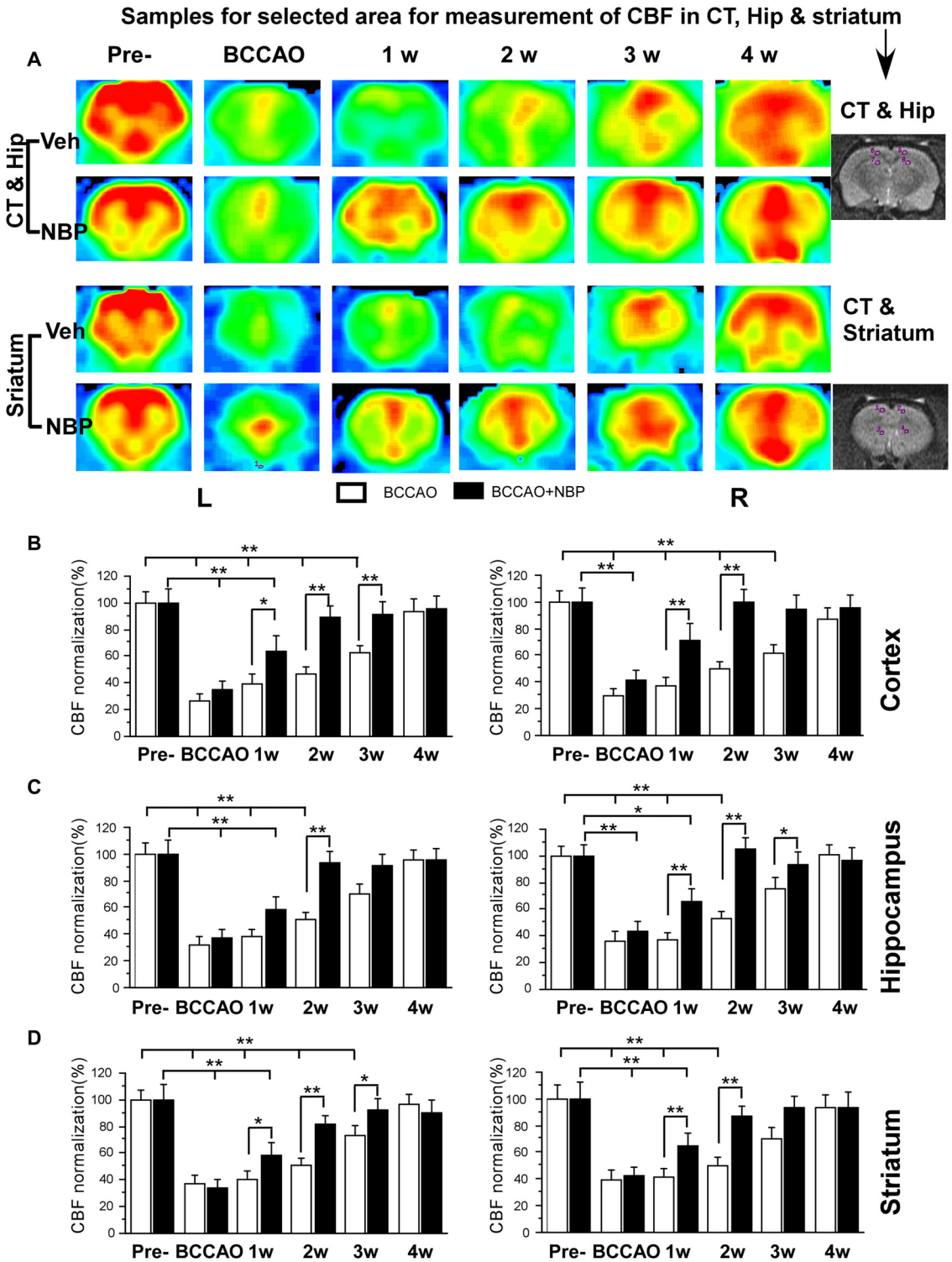

FIGURE 2 | Magnetic resonance imaging (MRI; 3D ASL) analysis showing changes in cerebral blood flow (CBF) in the vehicle and NBP-treated rat brains at different time points following bilateral common carotid artery occlusion (BCCAO). **(A)** CBF of the PC and hippocampus in the vehicle group is shown in the top row and the NBP-treated group in the second row. Likewise, CBF in the striatum with vehicle treatment is displayed in the third row, and the NBP-treated group is shown in the fourth row of images. The red color labeling represents increased CBF level, while green indicates lower CBF level. CBF quantitative analysis results in the cortex **(B)** hippocampus **(C)** and striatum **(D)** in the vehicle and BNP-treated groups at different time points after BCCAO. $^{**}p < 0.01$; $^{*}p < 0.05$. CT, cortex; Hip, hippocampus; Pre, pre-occlusion; BCCAO, bilateral common carotid artery occlusion; L, left; R, right.

Quantitative analysis showed dynamic changes in CBF of different brain areas (**Figures 2B–D**). In the left cortex, CBF in vehicle-treated rats dramatically reduced to 27.02% ± 4.92 upon BCCAO, 39.45% ± 6.99 at 1 week, 46.68% ± 5.65 at 2 weeks and 62.37% ± 6.21 at 3 weeks after BCCAO (all $p < 0.01$ vs. the pre-occlusion level). Left cortical CBF returned to normal level at 4 weeks post-BCCAO ($p > 0.05$ vs. the pre-occlusion level; **Figure 2B**). However, in the NBP-treated group, CBF in the left cortex returned to 88.71% ± 8.81, 91.72% ± 8.65, 95.60% ± 9.02 from 2 weeks, 3 weeks to 4 weeks after BCCAO, respectively (all $p > 0.05$ vs. the pre-occlusion level). CBF significantly increased at these time points in the NBP-treated group compared with vehicle-treated animals ($p < 0.05$ at 1 week, $p < 0.01$ at 2 weeks and 3 weeks). In general, changes in the right cortical CBF were similar to the left cortex. In this brain region, CBF for NBP-treated animals had returned to 71.25% ± 12.68 at 1 week which was similar to the pre-occlusion level ($p > 0.05$ vs. **Figure 2B**). Changes in hippocampal CBF are shown in **Figure 2C**. In the left hippocampus, CBF was also markedly decreased to 37.26% ± 6.80 during the period of BCCAO in the vehicle group ($p < 0.01$ vs. the pre-occlusion level) and gradually recovered to 72.99% ± 8.05 at 3 weeks ($p > 0.05$ vs. the pre-occlusion level). However, after NBP treatment, CBF recovered to the normal level at 2 weeks (81.65% ± 6.46, $p > 0.05$ vs. the pre-occlusion level, and $p < 0.01$ vs. the vehicle group). The changing pattern of CBF in the right hippocampus of the vehicle group was similar with that in the left hippocampus. However, NBP treatment induced more effective outcome for the CBF, which was 64.28% ± 10.32 at 1 week and 87.45% ± 7.53 at 2 weeks (both $p < 0.01$ vs. the vehicle group). The CBF level has reached to the normal level at 2 weeks ($p > 0.05$ vs. the pre-occlusion group). For the striatum (**Figure 2D**), CBF changes were similar to those observed in the cortex. CBF in the left striatum dramatically reduced to 32.21 ± 5.58 during BCCAO, and gradually recovered to pre-occlusion levels (95.54% ± 7.27) at 4 weeks after BCCAO. However, after NBP treatment, the CBF level was significantly higher than that in vehicle group at 1 week (57.88% ± 9.96 vs. 38.56% ± 4.92), at 2 weeks (93.62% ± 8.18 vs. 50.77% ± 5.86) and at 3 weeks (91.7% ± 0.17 vs. 70.4% ± 6.97). The CBF of the right striatum underwent similar changes as the left striatum. Although the recovery of CBF to the normal level in the right striatum in vehicle-treated rats occurred earlier than that in the left striatum at 3 weeks after BCCAO. Therefore, the effect of NBP treatment occurred at 1 week (65.35% ± 9.65 vs. 37.27% ± 5.28) and 2 weeks (93.97% ± 8.87 vs. 53.02 ± 5.72), both $p < 0.01$ between NBP-treated rats and vehicle-treated rats.

NBP Stimulated the Dilation of Bilateral Vertebral Arteries after BCCAO

In our previous study, we found that the VA diameter increased after BCCAO (Jing et al., 2015). In the present study, we further investigated whether NBP would influence VAs. The results showed morphological changes of VAs in both the vehicle and NBP-treated groups at different time points after BCCAO (**Figures 3A,B**). Quantitative analysis indicated that the diameter of normal left VAs was 0.2 mm ± 0.01 in the vehicle-treated rats (**Figure 3C**), but increased to 0.32 mm ± 0.01 in response to BCCAO and gradually increased to 0.65 mm ± 0.01 at 4 weeks after BCCAO. When treated with NBP, the VAs of rats exhibited much greater dilation than that observed in the vehicle treatment group. The diameter of the left VAs of NBP-treated rats was significant larger than the vehicle group at 1 week (0.52 mm ± 0.03 vs. 0.39 mm ± 0.01), 2 weeks (0.66 mm ± 0.02 vs. 0.45 mm ± 0.01), 3 weeks (0.73 mm ± 0.01 vs. 0.52 mm ± 0.02) and at 4 weeks (0.76 mm ± 0.01 vs. 064 mm ± 0.01) post-BCCAO (all $p < 0.01$). Alteration in the value and the pattern of diameter of the right VAs in the vehicle group and NBP-treated group were similar to those observed for the left side (**Figure 3D**).

NBP Induced Angiogenesis in the Cortex and Hippocampus after BCCAO

To investigate the effect of NBP on angiogenesis after BCCAO, we used CD34 (an endothelial cell marker) immunofluorescence labeling to detect changes in angiogenesis in the PC, the CA1 area and the CA3 region of the hippocampus at 2 weeks and 4 weeks after BCCAO (**Figure 4A**). Quantitative analysis data is shown in **Figure 4B**. In these three areas, changes in the number of CD34 positive cells exhibited a similar trend that hypoperfusion induced a significant decrease in the number of CD34 positive cells significantly at 2 weeks ($p < 0.01$, vs. sham) but returned it to the normal level at 4 weeks ($p > 0.05$, vs. sham) after BCCAO. However, NBP treatment prevented the decrease of CD34 positive cells at 2 weeks after BCCAO ($p > 0.05$, vs. sham).

NBP Reduced Astrocyte Activation in the Hippocampus in CCH Rats

To investigate the neuroprotective effects of NBP, we employed GFAP single labeling or NeuN and cleaved-caspase-3 double labeling techniques to identify astrocytes, neurons and apoptosis cells, respectively, in the hippocampus. The temporal changes in GFAP immunofluorescent labeling in each group are shown in **Figure 5A**. Quantitative analysis demonstrated that the number of GFAP positive cells/45 μm^2 was 8.75 ± 1.44 (LCA1) and 11.87 ± 1.05 (RCA1) in the sham group (**Figure 5B**). This number was increased to 23.00 ± 1.03 (LCA1) and 22.75 ± 0.92 (RCA1) in the vehicle group at 2 weeks, and 40.75 ± 2.87 (LCA1) and 38.75 ± 1.21 (RCA1) at 4 weeks after BCCAO (all $p < 0.01$ vs. the sham group). Following NBP treatment, the number of GFAP positive cells markedly decreased to 10.75 ± 1.42 (LCA1) and 9.50 ± 1.01 (RCA1) at 2 weeks and 19.50 ± 0.86 (LCA1) and 20.00 ± 1.41 (RCA1) at 4 weeks following BCCAO (both $p < 0.01$ vs. the vehicle group). For the CA3 area, the number of GFAP positive cells and temporal pattern of change in both sides were similar to that in the LCA1 area after BCCAO among the sham, the vehicle and NBP-treated groups (**Figure 5B**).

NBP Reduced Apoptosis and Protected Neurons in the Hippocampus in CCH Rats

Representative images of double labeled neuronal (NeuN positive, green) and apoptotic markers

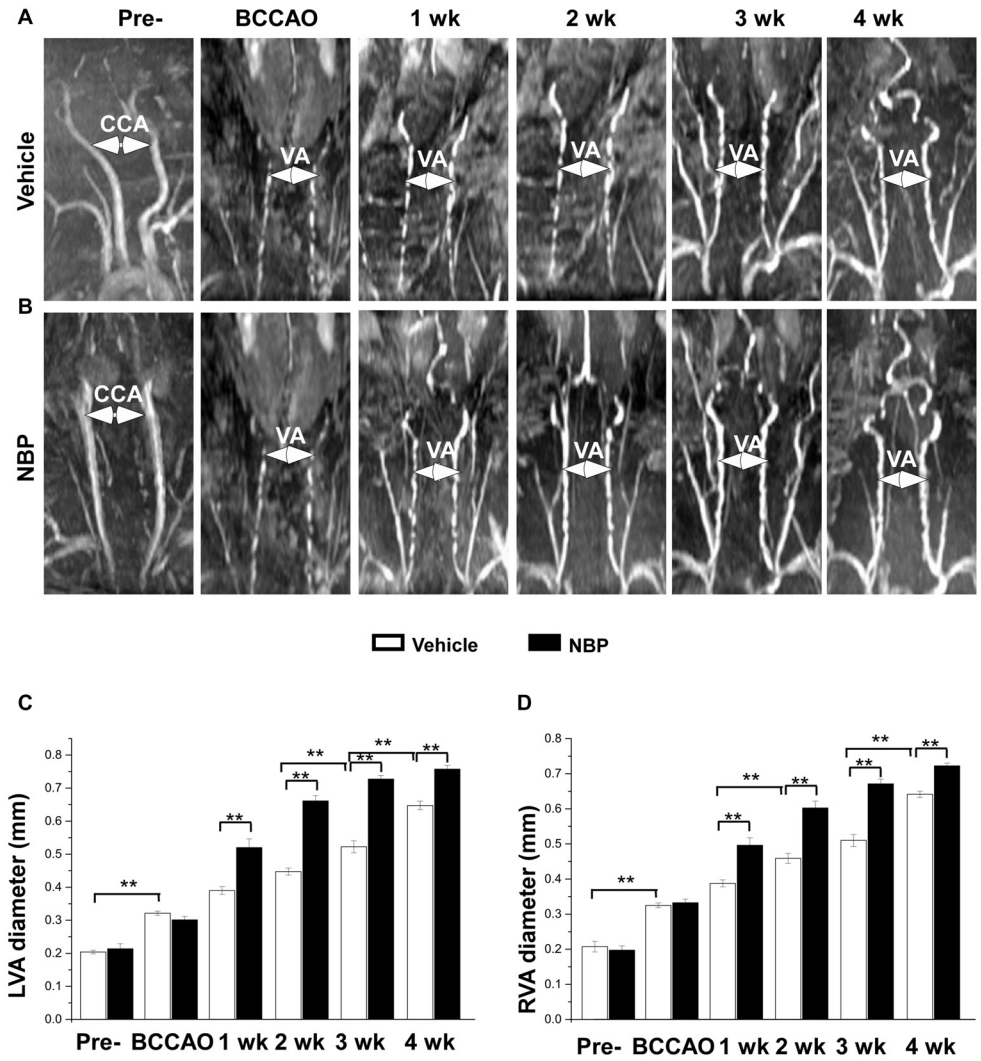

FIGURE 3 | MRI (3D-TOF) angiography showing morphological changes in diameter of vertebral arteries (VAs) in the vehicle and NBP-treated groups. Morphological changes in VAs are shown in the vehicle group **(A)** and NBP-treated group **(B)**. Both CCAs were visible pre-occlusion but were not after BBCAO. However, the diameter of both VAs gradually increased until 4 weeks after BCCAO. VA diameter progressively increased after NBP treatment. **(C,D)** Quantitative analysis showing the changes of left **(C)** and right **(D)** VA diameter from 1 to 4 weeks in the NBP-treated group and the vehicle-treated group. $^{**}p < 0.01$.

(cleaved-Caspase-3 positive, red) in the left CA1 and CA3 area are shown in **Figure 6A**. Quantitative analysis indicated that the number of caspase-3 positive cells/45 μm^2 markedly decreased to 2.5 ± 0.50 (LCA1) and 3.87 ± 0.71 (RCA1) at 2 weeks after BCCAO in the NBP-treated group, which was near sham level in the LCA1 (2.00 ± 0.65) and RCA1 (3.87 ± 0.82) but significantly less than that observed in the vehicle group in the LCA1 (10.25 ± 0.67, $p < 0.01$) and the RCA1 (11.00 ± 0.71, $p < 0.01$, **Figure 6B**). Although the number of caspase-3 positive cells in the LCA1 (9.12 ± 0.61) and RCA1 (9.00 ± 1.28) was still greater than the sham level (both $p < 0.01$) at 4 weeks after BCCAO in the NBP-treated group, it was significantly reduced compared to the numbers in the LCA1 (21.00 ± 3.85) and RCA1 (22.62 ± 1.28) in the vehicle-treated group at this time point (both $p < 0.01$). Changes in number and pattern of caspase-3 positive cells over time in both sides of the CA3 area were similar to the LCA1 area in both the vehicle and NBP-treated groups at different time points post-BCCAO (**Figure 6B**).

Change in the number of neurons (NeuN positive cells) in the CA1 and CA3 regions was shown in **Figure 6C**. The number of NeuN positive cells in the left CA1 area in vehicle-treated rats significantly decreased when compared to sham rats ($p < 0.01$) but significantly increased after NBP treatment when compared to the vehicle-treated rats ($p < 0.01$) at 2 weeks and at 4 weeks after BCCAO. In addition, there was no difference between the NBP-treated rats and the sham rats ($p > 0.05$) at these postischemic time points. In the right CA1 area, the number of NeuN positive cells markedly decreased at 4 weeks after BCCAO but it was significantly recovered to the normal level in NBP-treated rats at the same postischemic time point.

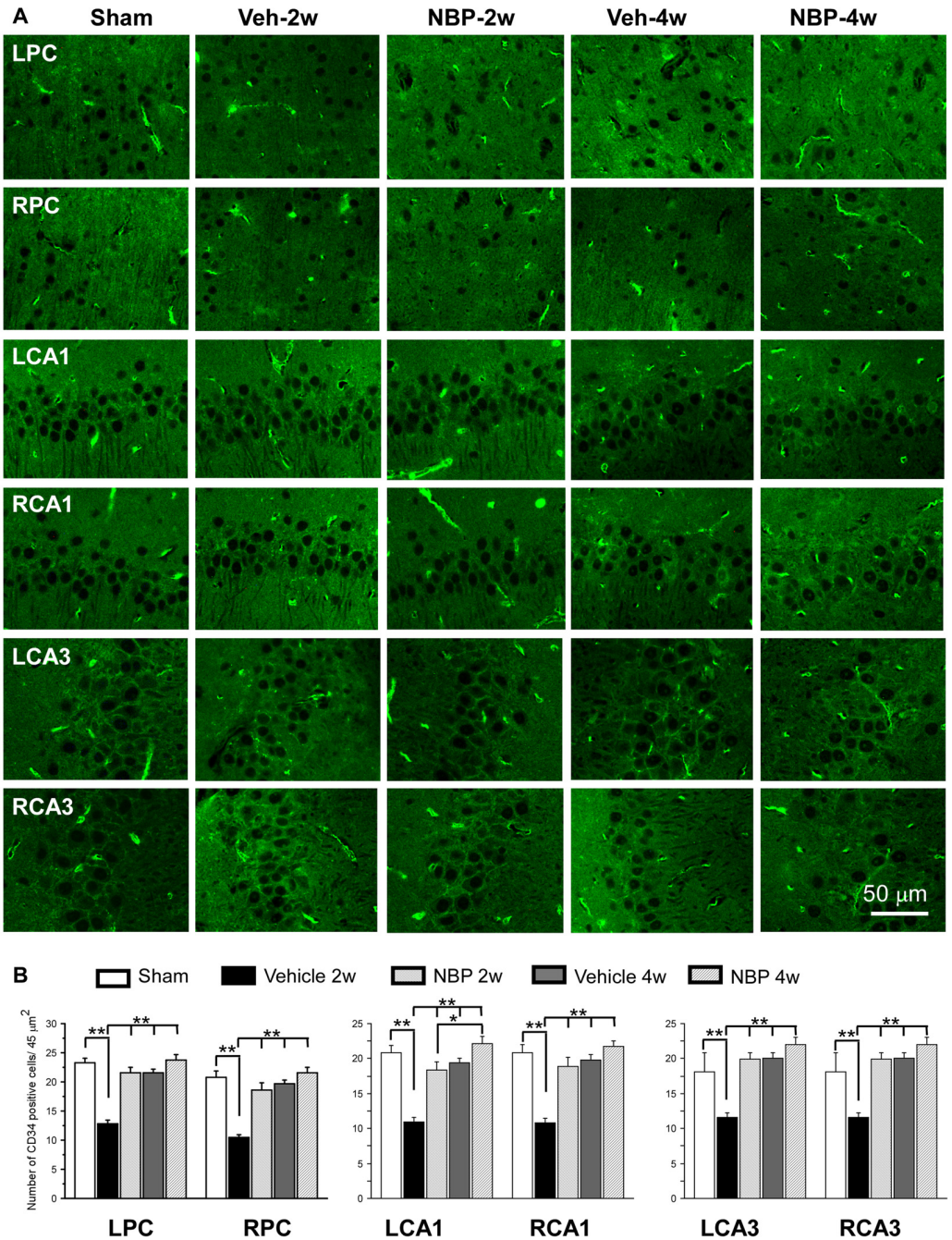

FIGURE 4 | Changes in the density of immunolabeled microvessels in response to vehicle and NBP treatment. **(A)** Immunofluorescent labeling showing the density of CD34-positive microvessels in the left parietal cortex (LPC), right parietal cortex (RPC), left CA1 area (LCA1), right CA1 area (RCA1), left CA3 area (LCA3) and right CA3 area (RCA3) in the sham group, the vehicle group at 2 weeks after BCCAO (Veh-2 weeks), vehicle group at 4 weeks after BCCAO (Veh-4 weeks), BNP-treated group at 2 weeks after BCCAO (NBP-2 weeks) and NBP-treated group at 4 weeks after BCCAO (NBP-4 weeks). **(B)** Quantitative analysis showing changes in microvessel density in these areas at different time points after BCCAO. The scale bar in the image of the lower right corner is also contributed to other images in **(A)** $^{**}p < 0.01$; $^{*}p < 0.05$.

Also, there were no difference in the number of NeuN positive cells between the sham rats and BNP-treated rats at 2 weeks or 4 weeks following BCCAO. Both left and right CA3 areas shared a similar changing pattern that the number of NeuN positive cells significantly decreased in vehicle-treated rats ($p < 0.01$ vs. the sham rats) but returned to the sham level in NBP-treated rats ($p < 0.01$ vs. the vehicle rats) at 2 weeks and 4 weeks following BCCAO.

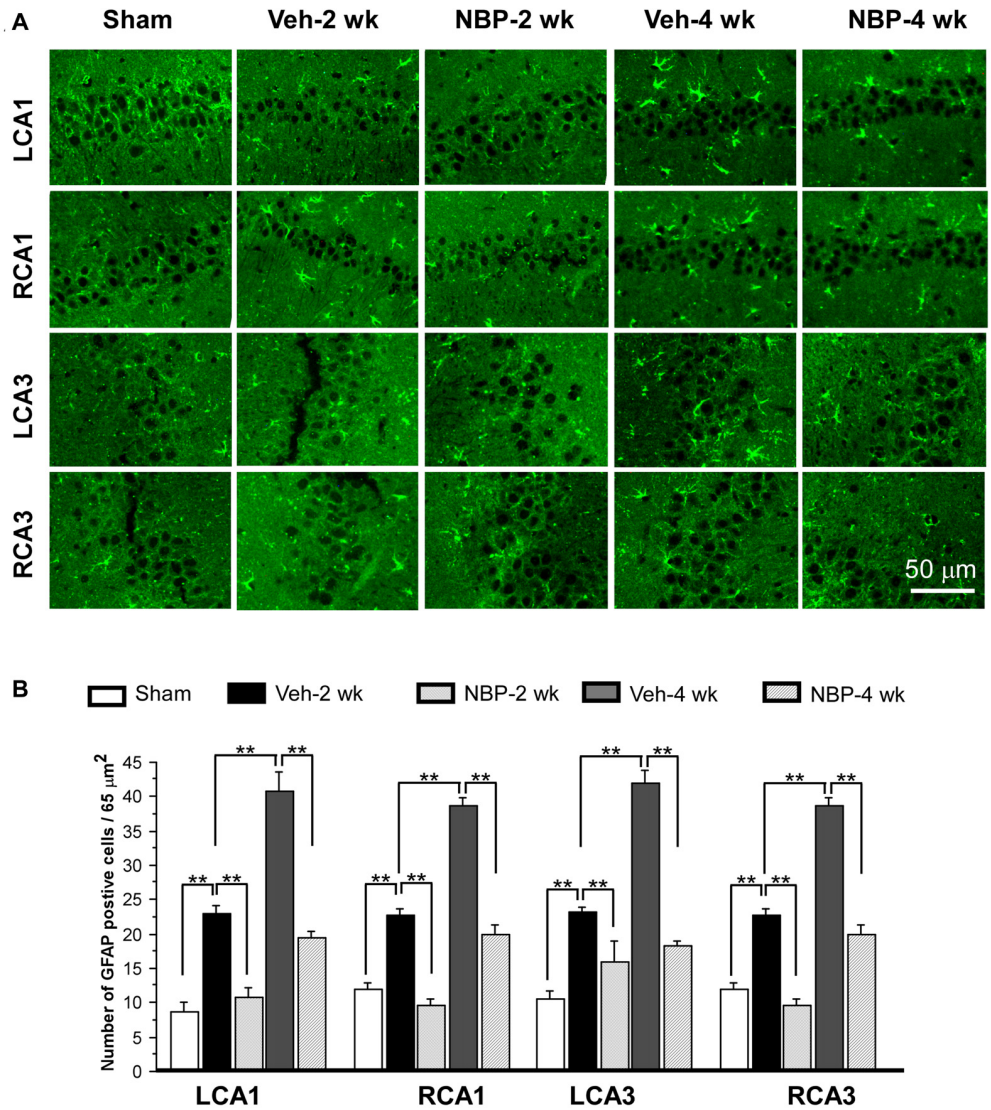

FIGURE 5 | Changes in astrocyte reactivity in response to vehicle and NBP treatment. **(A)** Immunofluorescent labeling shows glial fibrillary acidic protein (GFAP)-positive cells in LCA1, RCA1, LCA3 and RCA3 in sham animals, as well as Veh-2 week, Veh-4 week, NBP-2 week and NBP-4 week groups. **(B)** Quantitative analysis indicates changes in astrocyte density in these areas at different times following BCCAO. The scale bar in the image of the lower right corner is also contributed to other images in **(A)**. $^{**}p < 0.01$.

NBP Ameliorated Learning and Memory Deficits after BCCAO

Finally, we investigated whether NBP could improve cognitive impairment induced by BCCAO using the MWM. For this assessment, we analyzed the escape latency and the frequency in the platform quadrant. Plots of escape latency in each group are shown in **Figure 7A**, which illustrates a gradual decreased latency pattern in the final days of training. However, the escape latency was notably reduced in NBP-treated rats at 2 weeks and 4 weeks after BCCAO from day 1 to day 4 when compared to the vehicle-treated group (all $p < 0.01$). In addition, the escape latency for vehicle-treated rats was significantly longer than that for the sham group from day 2 to day 4 in the 2 weeks and 4 weeks groups (all $p < 0.01$). The frequency of time in the platform quadrant at day 6 was 3.90/min ± 0.67 in the sham group but significantly less in the vehicle-treated group at 2 weeks (1.90/min ± 0.38, $p < 0.05$) and 4 weeks (1.70/min ± 0.34, $p < 0.01$) after BCCAO when compared to the sham group, respectively (**Figure 7B**). However, after treatment with NBP, the frequency increased to 3.2/min ± 0.47 at 2 weeks and 3.60/min ± 0.31 at 4 weeks after BCCAO ($p < 0.05$ and $p < 0.01$, respectively, vs. vehicle-treated group) after BCCAO. At day 6, the swimming path of rats was different between the different groups (**Figure 7C**). The swimming path in the platform quadrant was less in the Veh-2 week or Veh-4 week groups than that in the sham, NBP- 2 week or NBP- 4 week groups.

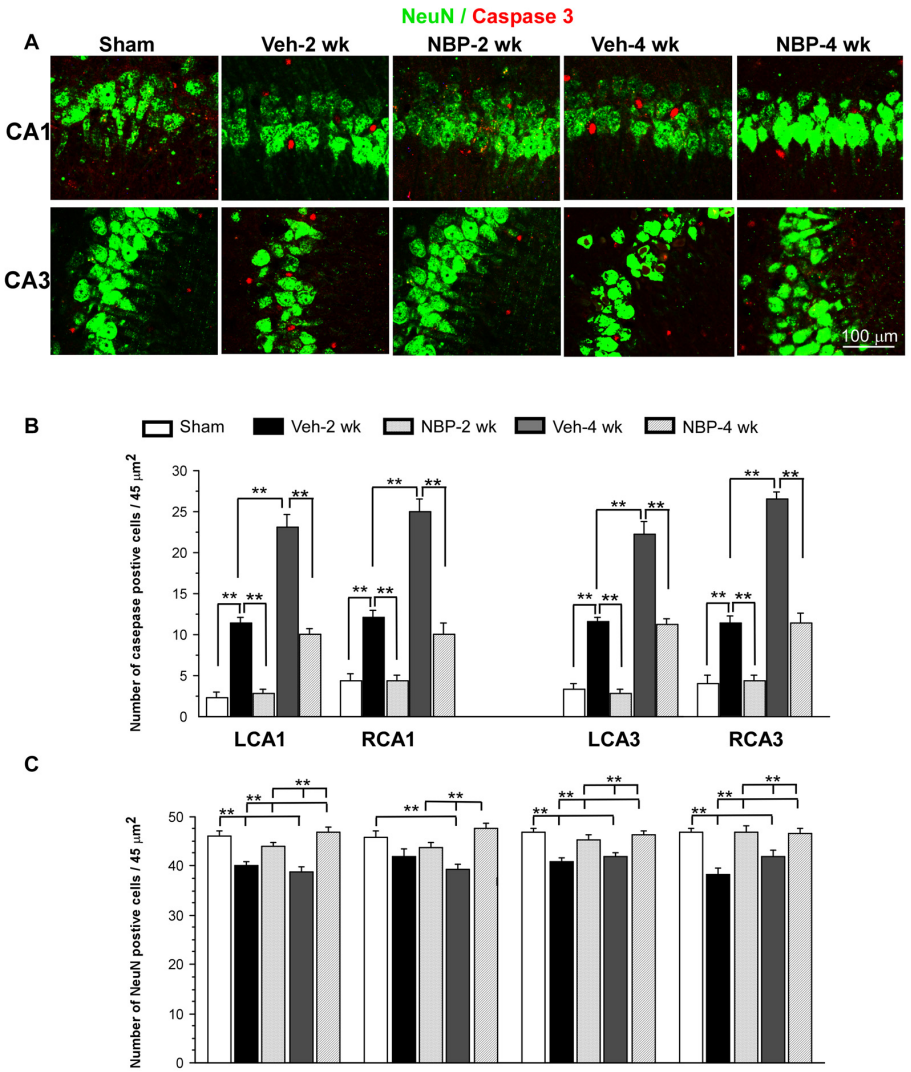

FIGURE 6 | Effect of NBP treatment on neurodegeneration. **(A)** Representative images of cleaved caspase-3 and NeuN positive cells in the left CA1 and CA3 areas in the sham, Veh-2 week, Veh-4 week, NBP-2 week and NBP-4 week groups. **(B,C)** Quantification revealed changes in the number of Caspase-3 and NeuN positive cells in the LCA1, RCA1, LCA3 and RCA3 regions at different time points post-BCCAO. The scale bar in the image of the lower right corner is also contributed to other images in **(A)**. $^{**}p < 0.01$.

DISCUSSION

The present study provides the first report of NBP's effects on MRI-documented CBF dynamic changes in an experimental CCH model. In addition, NBP treatment also protects neurons against neuronal degeneration, reduces apoptosis and glial reactivity and cognitive impairment in CCH caused by BCCAO.

Effects of NBP on Dynamic Changes in CBF and Vascular Plasticity in CCH Rats

Our previous research has shown that cortical and striatal CBF dramatically decreased acutely and returned to the sham level from 3 weeks after BCCAO (Jing et al., 2015). In addition, we also found that BCCAO triggered gradual dilation of VAs and increased microvascular density in the cortex, striatum and cerebellum at 4–6 weeks after BCCAO. Although this phenomenon is part of a compensatory response to insufficient CBF in the forebrain, this compensatory ability is limited and fails to prevent neurodegeneration and amelioration of memory impairment (Jing et al., 2015). In the present study, however, we found that NBP was able to significantly elevate CBF level in the cortex, striatum and hippocampus from an early stage (1 week) and returned it to a normal level at 2 weeks following BCCAO. Meanwhile, in the vehicle group, it took 4 weeks post-BCCAO for CBF to reach the pre-occlusion level. The mechanism underlying recovery of CBF by NBP treatment may be related to a subsequent increase in VA diameter and promotion of angiogenesis following BCCAO (**Figures 3**, **4**). Also, NBP treatment prevented neuronal loss and improved memory deficits, which may be related to the earlier recovery

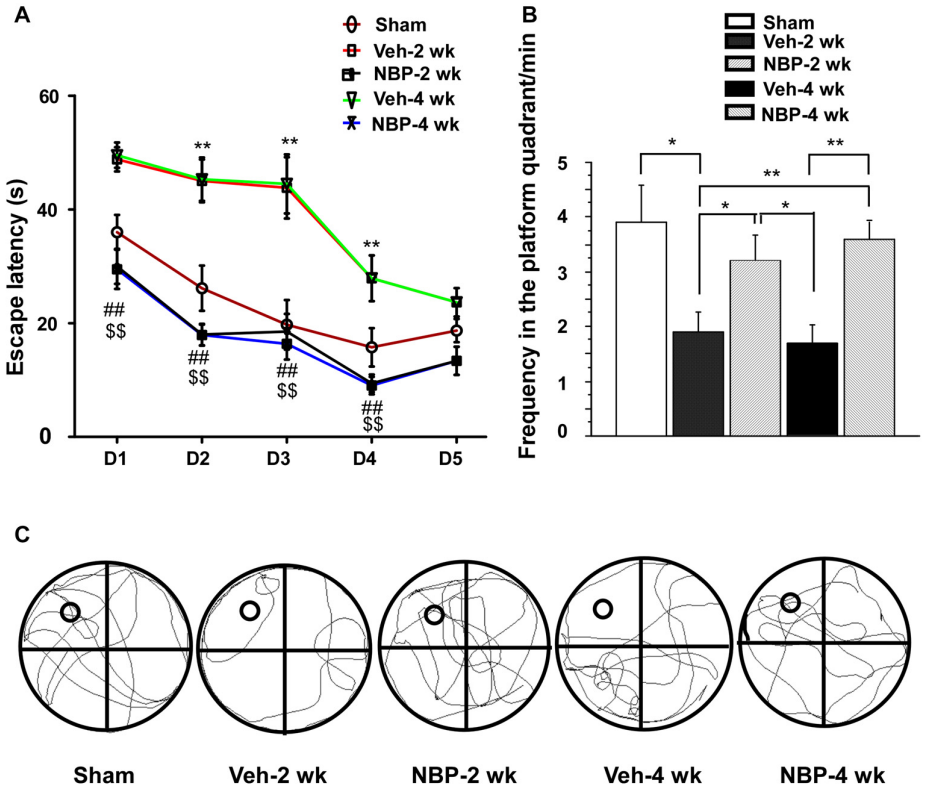

FIGURE 7 | Alteration of cognitive impairment as measured by the Morris water maze (MWM) in response to vehicle and NBP treatment. **(A)** Escape latency changes in the water maze in the different groups from day 1 to day 5. (**$p < 0.01$, Veh-2 week group or Veh-4 week group vs. the sham group; $^{\#\#}p < 0.01$, NBP-2 week group vs. Veh-2 week or Veh-4 week groups; $^{\$\$}p < 0.01$, NBP-4 week group vs. Veh-2 week or Veh-4 week groups). **(B)** Changes in the frequency of time in the platform quadrant between the different groups. **$p < 0.01$; *$p < 0.05$. **(C)** Swimming path of rats at day 6 in the different groups. The small empty circle represents the platform in one quadrant of the swimming pool (the large circle). The swimming path distance in the platform quadrant was reduced in the Veh-2 week or Veh-4 week groups compared to the sham, NBP- 2 week or NBP- 4 week groups.

of CBF by NBP (**Figure 2**). It has been reported that NBP can prevent cold-induced ischemic stroke via improvement of cerebral microvasculature (Liu et al., 2007). The underlying mechanism by which NBP promotes angiogenesis under CCH conditions is not completely clear. Studies have shown that NBP can up-regulate expression of VEGF in diabetic rats (Zhang et al., 2010) and Alzheimer's rats (Hou et al., 2010) and expression of VEFG and BFGF in focal cerebral ischemic rats (Li et al., 2008). Therefore, it is plausible that the effect of NBP on angiogenesis in CCH rats may be regulated by increasing expression of VEGF and BFGF. In addition, NBP can also promote microcirculation by inhibiting activation of platelet-forming pathways (Ye et al., 2015). Further investigation is required to clarify this question.

Effects of NBP on Neuroprotection, Glial Reactivity and Memory Amelioration in CCH Rats

Many studies have shown that CCH causes neurodegeneration, glial relativity and memory deficits in experimental models as well as in patients with subcortical ischemic vascular dementia (SIVD) and AD [32, 2]. AD and SIVD patients showed marked CBF reductions in the forebrain (Schuff et al., 2009). Using CCH rats induced by BCCAO, we have previously found that neuronal degeneration, inflammation and cognitive impairment are prominent in animals from 2 weeks to 6 weeks following BCCAO, and the extent of cognitive dysfunction is dependent on the duration of ischemia (Jing et al., 2015). This further highlights the critical need for effective therapies for patients suffering from chronic hypoxic/ischemic stroke.

NBP has been proven to play a critical role in neuroprotection, anti-inflammation, anti-apoptosis and amelioration of memory deficits in ischemic animal models and stroke patients. It has been reported that NBP protects immortalized human umbilical vein endothelial cells (HUVECs) and rat brain microvascular endothelial cells from death by blocking oxidative/nitrosative stress and mitochondrial damage when these cells are exposed to oxygen glucose deprivation (OGD) *in vitro* (Li et al., 2009a; Yang et al., 2012). NBP also reduces neuronal cell death, significantly lowers neurological deficit scores and reduces infarct volume in the penumbra of MCAO rats (Li et al., 2010; Zhang et al., 2012). In a transient cardiac arrest rat model, NBP shows protective benefits for the majority of CA1 neurons against death (Zhang et al., 2016). Based on multiple reports of effective outcomes in animal studies, NBP has been proven to be effective in

treating stroke patients in several clinical trials in China. One study showed that patients (20) suffering from AIS exhibit lower NIHSS scores than the placebo group (20 AIS patients) but the NIHSS scores are similar to those in Cerebrolysin group (20 AIS patients) after receiving NBP treatment within 12 h after AIS for 10 days (Xue et al., 2016). This study also demonstrates the safety of usage of NBP. Another large scale clinical study covered 38 hospitals from 2007 to 2009, during which 573 patients suffering from AIS within 48 h of stroke onset were enrolled to receive a 90 day treatment with NBP alone, NBP plus aspirin, or ozagrel plus aspirin (Cui et al., 2013). The best outcome reported from this study was achieved from the group treated only with NBP. Furthermore, patients with AIS have higher level of circulating progenitor cells after NBP treatment (Zhao et al., 2016). Therefore, from the results of AIS in experimental animals and patients, NBP treatment has achieved satisfying outcomes in reducing neuronal death, ameliorating neurological deficits and stimulation of microcirculation. The mechanisms of neuroprotection mediated by NBP in acute ischemic injury may occur via multiple pathways including anti-oxidation, anti-apoptosis and anti-inflammation (Li et al., 2009a, 2010; Yang et al., 2012).

The outcomes of the present study further demonstrate that NBP treatment can also protect hippocampal neurons, reduce apoptosis and astrocyte reactivity and improve spatial memory deficits in CCH rats. These results suggest that NBP may be also a promising drug for CCH or vascular dementia. It would be interesting to explore the additional effects of NBP on other diseases such as vascular dementia or AD.

Neurodegeneration in Acute and Chronic Global Ischemia Models

Transient global ischemia can be achieved by occlusion of bilateral common carotid arteries and VAs (4-VO ischemia model; Pulsinelli et al., 1982; Yamaguchi et al., 2005). It has been well established that transient global ischemia model induces selective neuronal death (Pulsinelli et al., 1982; Traystman, 2003; Yamaguchi et al., 2005). The highest mortality of neurons is found in the CA1 of the hippocampus followed by cortical and striatal spiny neurons after transient global ischemia (Pulsinelli et al., 1982). In Pulsinelli et al.'s (1982) study, 10 min ischemia causes approximately 50% CA1 neurons with severe damage, whereas approximately 50% neurons with moderate damage are found in the CA3 area after 20 min ischemia. Therefore, CA1 pyramidal neurons are ischemia-sensitive, whereas CA3 pyramidal neurons are relatively resistant in the acute transient global ischemia. In contrast, permanent occlusion of bilateral common carotid arteries (2-VO) causes chronic hypoperfusion (Farkas et al., 2007). Neurodegeneration in 2-VO model is not as severe as that in the acute 4-VO model which may be due to hemodynamic compensatory responses of the brain. It has been reported that the hippocampal size did not change in CCH brain, but the neuronal density as determined by NeuN staining in the CA1 area is significantly reduced at 7 days after BCCAO (Cechetti et al., 2012). Surprisingly, in the present study, we found that the number of NeuN positive neurons is reduced not only in the CA1 area, but also in the CA3 areas at 2 weeks and 4 weeks after BCCAO, suggesting that the neurodegenerative mechanisms induced by acute transient global ischemia and CCH are distinct, and further studies are obviously important in order to appropriately use these ischemic models and to identify specific and effective therapeutic agents for treating different types of ischemia.

AUTHOR CONTRIBUTIONS

LH, YR designed and organized the project, revised and approved the manuscript. ZX, WL and LHZ performed experiment, collected and analyzed data and wrote the manuscript. CKT revised and approved the manuscript. LZ, CS, ZJ, YX and WL performed experiment, collected and analyzed data.

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YXQN Reduces Alzheimer's Disease-Like Pathology and Cognitive Decline in APPswePS1dE9 Transgenic Mice

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Alzheimer's disease (AD) is the world's most common form of dementia, in which aggregation of amyloid-β (Aβ) is the hallmark. Unfortunately, few medicines have succeeded to completely cure AD. Yangxue Qingnao (YXQN) is a Chinese traditional medicine, and its pharmacological effect is improving cerebral blood flow. In this study, we firstly demonstrated that YXQN reduced AD-like pathology and cognitive impairment in APPswePS1dE9 (APP/PS1) mice with 2 months administration. Our data showed that YXQN substantially ameliorated behavioral defects in 10-month old APP/PS1 mice using Morris Water Maze and Y-maze tests, in which the cognitive ability of YXQN high-dose group approaches to wild type mice. Next, we focused on the brain pathological alterations in the YXQN group by three experiments, including thioflavin-S, congo-red, and Aβ-immunohistochemistry staining. The results demonstrated that the high-dose of YXQN dramatically suppressed amyloid plaques in the hippocampus and cortex of APP/PS1 mice, which showed a 47–72% reduction in plaque deposits, relative to the vehicle group. In addition, our data verified that YXQN decreased the cerebral amyloid load by attenuating β-secretase BACE1 and γ-secretase PS1 in the pathological processing of APP, and promoting the level of α-secretase ADAM10 in the physiological processing of APP to generate more sAPPα, which combats amyloidosis formation, and also carries out neurotropic and neuroprotective effect. Taken together, our results strongly suggest that YXQN could be a potential medicine for AD, and provide new evidence for further AD drug research and development.

Keywords: Yangxue Qingnao, Alzheimer's disease, APP/PS1 mice, amyloid-β, APP processing

INTRODUCTION

Alzheimer's disease (AD) accounts for a large number of dementia cases and afflicts more than 48 million individuals worldwide (Alzheimer's Association, 2015). It is a degenerative disease of the central nervous system, with amyloid-β (Aβ) deposition in the brain as a crucial pathological hallmark (Campion et al., 2016), which antedates any other triggered pathological changes of

AD, as has been demonstrated by positron emission tomography (PET) (Forsberg et al., 2008; Brendel et al., 2015). Unfortunately, all the medicines designed to prevent the production and aggregation of Aβ have invariably failed in their clinical III trials, including the most anticipated BACE1 inhibitor and Aβ monoclonal antibody.

As an AD triggering molecule, Aβ is a proteolytic product of the amyloid precursor protein (APP) via the amyloidogenic pathway, in which APP is cleaved by β-secretase (BACE1) to produce extracellular release part-soluble APP peptide-β (sAPPβ), and C-terminal fragment-β (CTFβ)—also known as C99. Subsequently, cleavage of C99 by γ-secretase complex [mainly presenilin 1 (PS1)] releases Aβ, mainly Aβ42 and Aβ40 (Oddo et al., 2004; Dong et al., 2006). While, in the dominant pathway of APP (non-amyloidogenic pathway) under normal conditions *in vivo*, initial processing of APP by α-secretase (ADAM10) within the Aβ domain generates a secreted form of sAPPα and CTFα (C83), which in turn precludes Aβ generation (Kim and Tsai, 2009; Portelius et al., 2011). In addition, sAPPα provides neuroprotection and promotes neuron outgrowth (Pimplikar and Ghosal, 2011; Milosch et al., 2014).

For AD research and medicine development, APPswe/PS1dE9 double transgenic mice (APP/PS1 for short) expressing a chimeric mouse/human APP695 with the Swedish mutation (KM594/595NL), together with a mutant human PS1 protein with E9 deletion were constructed by Jankowsky's lab (Jankowsky et al., 2001). Compared with WT-APP, APPswe is more easily cleaved by BACE1, and PS1-dE9 shows higher γ-secretase activity to generate Aβ (Li et al., 2016). The APP/PS1 mice develop amyloid pathology in the brain at an age of 6–7 months and closely recapitulate the pathological characteristics and progressive course of AD (Perez et al., 2005; Puig et al., 2016).

Recent data showed that augmentation of cerebral blood flow (CBF) could be a new approach to the treatment of Alzheimer's disease (Goldsmith, 2011). Further, CBF measured by arterial spin labeling MRI was reported as a preclinical marker of Alzheimer's disease (Wierenga et al., 2014). Yangxue Qingnao (YXQN) extract is a famous Chinese medicine to improve CBF and brain nourishment. It is composed of 11 Chinese herbs, and is a patent medicine used for alleviating headache and dizziness treatment in clinics for 20 years. Four of them come from Siwu Tang, Four Herbs Decoction, one of the most famous prescriptions for activating blood circulation, which is recorded in the Chinese first national pharmacopeia, Prescriptions People's of the Welfare Pharmacy. The other seven herbs additionally activate blood circulation, and play the role of anti-oxidation, protection of neurons and regulation of the enzymes targeting the nervous system (Wang et al., 2013).

In this study, we explored the effects of YXQN extract on AD pathology and cognitive function in APP/PS1 transgenic mice. We assessed the amyloidosis changes by YXQN administration, and also detected the proteases involved in the proteolytic process of APP, including ADAM10, BACE1, and PS1, in order to develop the potential Chinese medicine for AD treatment.

MATERIALS AND METHODS

Drug Supplementation

Yangxue Qingnao is a widely applied Chinese traditional patent medicine, consisting of 11 active components, including Angelica sinensis, Ligusticum chuanxiong hort, White peony root, Prepared radix rehmanniae, Uncaria, Spatholobus suberectus, Prunella vulgaris, Cassia seed, Nacre, Rhizoma corydalis, and Asarum, effects of which are shown in **Table 1**. YXQN extract was provided by the TIANJIN TASLY Pharmaceutical co., LTD, the processing of the product followed strict quality control, and the ingredients were subjected to standardization. YXQN extract was dissolved in distilled water at 0.069 g, 0.208 g, and 0.624 g per mL for use. Positive control Aricept donepezil (HCl salt) tablets (commonly referred to as donepezil; Eisai (China) Pharmaceutical co., LTD) was prepared with a concentration of 0.103 mg/mL for use. The diluted YXQN and donepezil were used for oral administration by 0.1 mL/10 g of weight in mice. In all, the drug dosages are YXQN low-dose at 0.69 g/kg, YXQN middle-dose at 2.08 g/kg, YXQN high-dose at 6.24 g/kg, and donepezil at 1.03 mg/kg. Besides, the dosages of YXQN middle-dose and donepezil are equal with clinical application doses for patients in Pharmacology.

Animal Treatment and Experiment Schedule

Amyloid precursor protein/presenilin 1 mice (B6C3-Tg (APPswe, PSEN1dE9) 85Dbo/J) were purchased from the Model Animal Research Center of Nanjing University (Nanjing, China). This study was carried out in accordance with the recommendations of the Chinese Council on Animal Care Guidelines, the Model Animal Research Center of Nanjing University. The protocol was approved by the Model Animal Research Center of Nanjing University.

In the AD model of APP/PS1 mice, degenerate cognitive function and Aβ deposits could be observed at 6–7 months (Jankowsky et al., 2004). Thus, we used equal numbers of female and male APP/PS1 mice at age of 8 months to explore the effects of YXQN and assessed AD pathology at the age of 10 months after 2 months administration. After 1 week of acclimatization to the cages, the 8-month APP/PS1 mice were randomly divided into five groups (vehicle group, $n = 16$; YXQN low-dose group, $n = 17$; YXQN middle-dose group, $n = 18$; YXQN high-dose group, $n = 18$; donepezil group, $n = 16$) and then orally administered with attenuated donepezil, YXQN (low-, middle-, and high-dose), or water (0.1 mL/10 g weight) for 2 months. Littermates were used as WT vehicle control ($n = 16$) throughout the study and were given distilled water for 2 months as well. Mice were housed in standard laboratory cages with a 12 h light and dark cycle along with free access to food and water.

The experimental design of behavior and biochemical analysis is shown in **Figure 1**. During the 2-month administration period, cognitive function of the five groups of APP/PS1 mice was measured by Y-maze at 30th and 60th day, and measured by Morris Water Maze (MWM) at the termination of drug supplementation. After that, a set of the biochemical index in the

TABLE 1 | Characterization of the herbs included in YXQN.

Herbs	Percentage content (%)	Identified compounds	Effects
Siwu Tang (Four herbs decoction)			
Angelicae sinensis	6.76	Ferulic acid	**Increasing cerebral blood flow and improving blood circulation:** anti-oxidant, neuroprotection, anti-anginal, anti-apoptotic, synergistic promotion of blood vessel regeneration, decreasing the brain infarct size, inhibiting neutrophil adhesion to endothelial cells, protective effect of vascular dysfunction and hypertension, maintaining blood-brain barrier integrity, et al. (Xu et al., 2009; Panth et al., 2016; Wang et al., 2016)
Ligusticum chuanxiong hort	6.76	Ligustrazine	
White peony root	5.41	Peoniflorin	
Prepared radix rehmanniae	5.41	Rehmannioside	
The seven modified portion			
Uncaria	13.51	Rhynchophylline	**Activating blood circulation, anti-oxidation, neuroprotection and enzyme regulation:** preventing neurotoxicity during ischemia, blocking of calcium channel, attenuating oxidative stress and neuronal damage, inhibiting the production of nitric oxide, radical scavenging effect, inhibiting myocardial infarction, up-regulation of Bcl-2, dopaminergic antagonist, inhibiting histamine release, anti-inflammatory, attenuates pro-inflammatory responses through down-regulation of MAPK/NF-κB signaling pathways, et al.(Xu et al., 2009; Song et al., 2012; Huang et al., 2014; Miao et al., 2016)
Spatholobus suberectus	13.51	Genistein	
Prunella vulgaris	13.51	Ursolic acid and 2-alpha-hydroxyursolic acid	
Nacre	13.51	Water-soluble extract	
Cassia seed	13.51	Naphthopyrones and Alaternin	
Rhizoma corydalis	6.67	L-Tetrahydropalmatine	
Asarum	1.35	Methyleugenol	

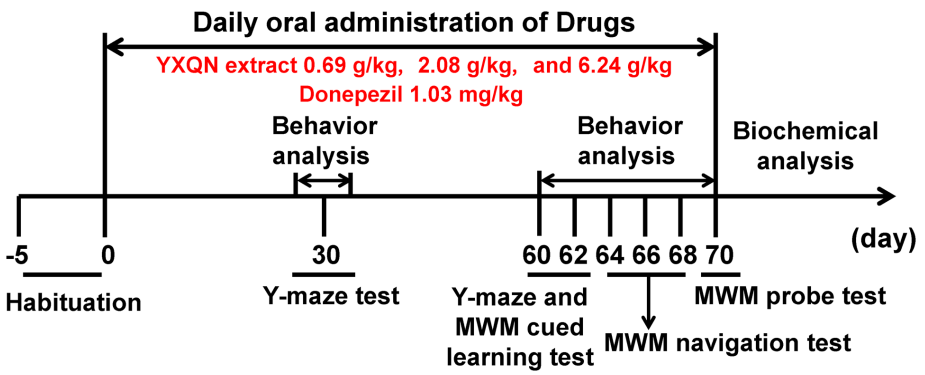

FIGURE 1 | Behavior and biochemical analysis schedule to study the effects of YXQN on APP/PS1 mice.

brain was investigated. Therefore, all AD pathological indices in this research were determined in 10-month-old APP/PS1 mice.

Behavioral Assessments

The short-term spatial memory ability for five groups of APP/PS1 mice was tested by Y-maze spontaneous alternation. In brief, we placed mice separately into a radially symmetric Y-maze with three arms (arms: 40 cm long, 4 cm wide; walls: 30 cm tall). The number and sequence of arm entries were scored over 8 min. Alternations were calculated when a mouse consecutively traveled to all three arms in any order without re-entering the previous arms. Percent of alternation was formulated as the ratio of the number of alternations to the number of total arm entries minus two (Lalonde, 2002; Town et al., 2008).

The spatial learning-memory ability was assessed by the MWM tests, which consists of the orientation navigation tests and the spatial probe tests. Mainly, the MWM contains a circular tank (diameter: 120 cm) filled with water at 24°C and a hidden platform (diameter: 15 cm) positioned 1–2 cm below the opaque water in the middle of the northeast quadrant. Before the measurement, mice were trained to find the platform for 3 days, orienting by cues on the wall of the tank as spatial references (Vorhees and Williams, 2006). For orientation navigation tests, mice were allowed to search for the platform for 120 s and to

stay on the submerged platform for 30 s, before they were placed back in the cage under a heater to dry. Mice were tested four times a day for six consecutive days. The escape latency and the swim path tracking until the mice landed on the platform were recorded on videotape. For the probe trials, which were performed to determine memory retention on the next day (day 7), the platform was removed, and mice were placed into the pool from the opposite quadrant where the platform had been located. They were allowed to swim for 120 s, and the number of platform crossings, the percent of time spent in each quadrant, and the swim path tracking were recorded on videotape.

Histological Examinations

After behavioral analysis, mice were euthanized with pentobarbital sodium and fixed in 4% paraformaldehyde after myocardial perfusion. Brains were dissected and embedded in paraffin for preparing sagittal sections and further staining analysis. The sections from each mouse were separately stained according to the following procedures:

For thioflavin-S staining, brain sections were stained with 0.01% thioflavin-S in 50% ethanol, and following differentiation in 50% ethanol (Heneka et al., 2013). Then, stained sections were analyzed with the Hg-Lamp for fluorescence excitation.

Congophilic amyloid staining by congo-red conformed to standard protocols (Vom Berg et al., 2012). Briefly, sections were incubated with 0.5% congo-red in 80% methanol and 20% glycerol for 20 min, and following differentiation with 0.2% KOH in 80% ethanol. Subsequently, nuclei were stained blue with hematoxylin.

In addition, brain sections were subjected to EnVision system immunohistochemistry to detect Aβ, ADAM10, BACE1, and PS1. The primary antibody 6E10 (Covance) was used for assessing Aβ deposition at 1:500 dilution. Moreover, the specific antibodies ADAM10, BACE1, and PS1 (Bioss) antibodies were used for staining and analyzing the expression of ADAM10, BACE1, and PS1 at 1:200 dilution.

Finally, all stained sections were analyzed using a BX41 or IX71 microscope (Olympus) and collected using DP Controller software. We performed the quantitative assessment of three defined regions per mouse brain. Plaque number and staining area were calculated by Image-Pro Plus 6.0 software. The staining area fraction was determined by dividing total plaque area by the area of the microscopic field.

Western Blot

The homogenized brain tissues were eluted by boiling in SDS-sample buffer. Then, we performed SDS-PAGE to assess brain proteins using the Bio-Rad mini gel system (Vom Berg et al., 2012). In particular, Aβ and APPct levels were measured with urea-based electrophoresis, and then transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were then probed with antibodies at the appropriate dilutions, including Aβ and full-length APP (6E10, Covance) and APPct (A8717, Sigma–Aldrich) at 1:1000 (Sarajarvi et al., 2009; Heneka et al., 2013), ADAM10 (Bioss), BACE1 (Bioss), and PS1 (Bioss) at 1:500. We used β-actin (Abcam) as internal controls and Image-Pro Plus software for densitometric analysis.

ELISA for Human Aβ

For ELISA test of Aβ40 and Aβ42 peptides from soluble and insoluble fractions, we homogenized frozen cerebral hemispheres (150 mg/mL wet weight) in PBS containing 1% SDS with protease inhibitors (Roche), followed by centrifugation at 100,000 g for 60 min at 4°C (Kawarabayashi et al., 2001; Vom Berg et al., 2012). The supernatant was removed as the soluble fraction, and the pellets (insoluble fraction) were dissolved in 70% formic acid. Then the insoluble fraction was neutralized by 1 M Tris buffer (pH 11). We used the Human Amyloid-β (aa1-40) or (aa1-42) Quantikine ELISA Kit to analyze Aβ40 and Aβ42 according to the manufacturer's instructions (R&D Systems).

Statistical Analysis

Data presented as means ± standard errors of the mean (SEM). All quantitative results including histology staining, Western blot, ELISA, and behavioral tests were analyzed by ANOVA with Dunnet's *post hoc*. All analyses were carried out using SPSS 17.0 statistics software (Chicago, IL, United States).

RESULTS

YXQN Counteracts Cognitive Decline in APP/PS1 Mice

Utilizing 8 month old APP/PS1 mice and their littermates (WT), we evaluated the possible effects of YXQN on cognitive function of mice after 2 months of drug administration by two kinds of behavioral test, including MWM tests and Y-maze tests. Firstly, the APP/PS1 mice were administrated with vehicle, YXQN low-dose 0.69 g/kg, middle-dose 2.08 g/kg, and high-dose 6.24 g/kg (equivalent to 33, 100, and 300% clinical application dose), or donepezil 1.03 mg/kg (equivalent to 100% clinical application dose) per day from 8 to 10 months of age. At the time of termination of drug supplementation, mice were subjected to the navigation tests in the MWM to assess their spatial learning-memory formation. The search time to find the platform (escape latency) and path tracking were recorded. As shown in **Figure 2A**, compared with WT mice, the typical path tracking of the vehicle APP/PS1 mice was disorganized, indicating the mice searched for the hidden platform by a random trajectory. YXQN administration APP/PS1 mice showed shorter path lengths and selective search tracking, similar to WT mice. These data suggested an improvement in the spatial memory of AD mice from YXQN groups. Moreover, the average escape latencies of 6 consecutive days of each group were displayed in curves (**Figure 2B**). By statistics, escape latencies were demonstrated tend to decrease over time, and overall, latencies were significant different among groups ($F = 23.475$ day: $p < 0.0001$ group: $p = 0.002$; RM-ANOVA). The vehicle APP/PS1 showed the longest latencies, and the vehicle WT mice showed the shortest latencies on each day. Compared with vehicle AD mice, YXQN low-, middle-, and high-dose APP/PS1 mice and donepezil APP/PS1 mice showed different degrees of shortened latencies, and YXQN high-dose group most closely approached the latencies of the WT group, and presented significant differences with vehicle group on day 3

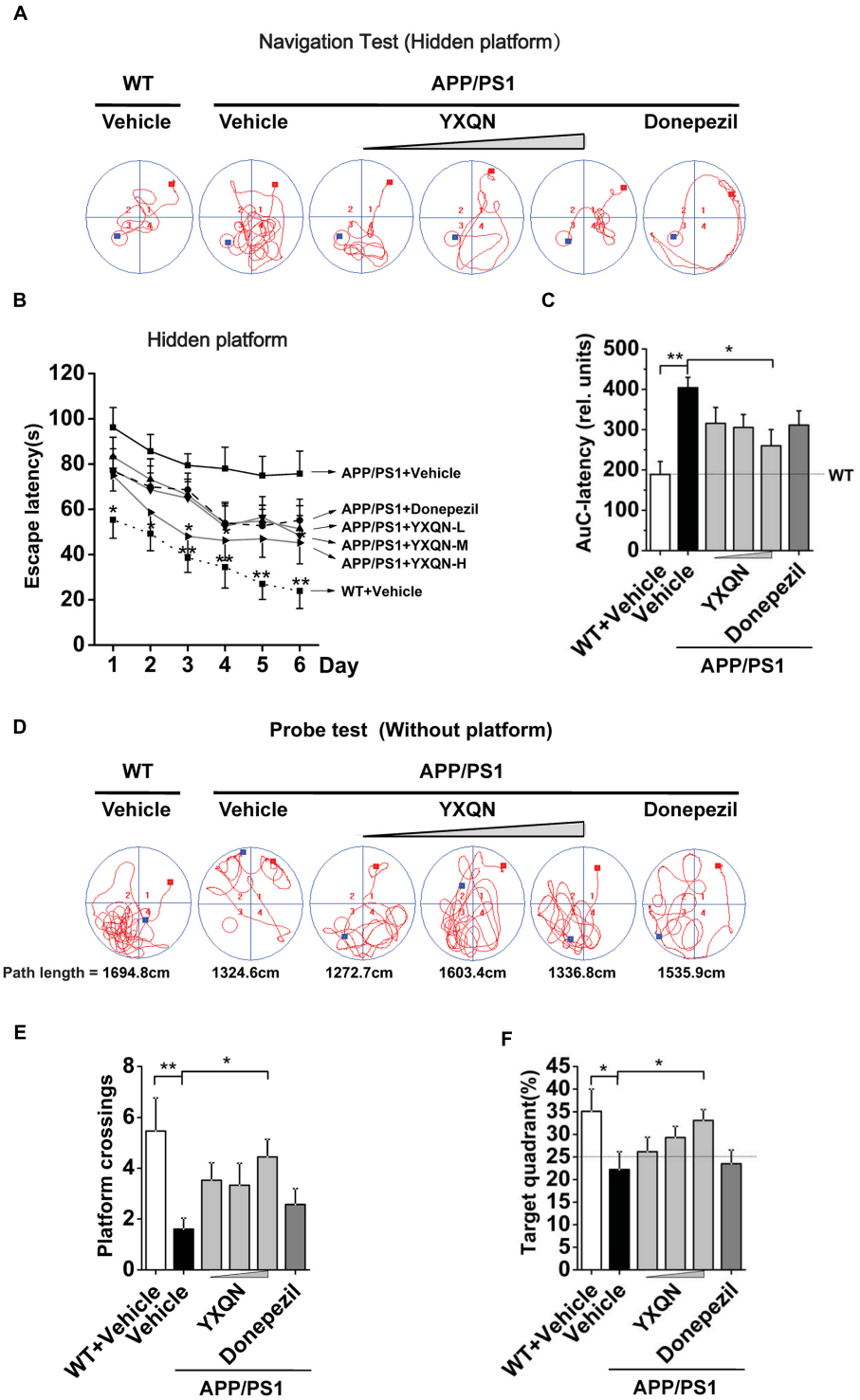

FIGURE 2 | Yangxue Qingnao improves the spatial and long-term memory decline in APP/PS1 mice by MWM. (A–C) Navigation tests analysis in MWM by escape latencies for WT mice (n = 16) and the APP/PS1 mice with vehicle, YXQN extract or donepezil supplementation for 2 months (n = 14–17). **(A)** Representative path tracking in the navigation tests with hidden platform. **(B)** Average latencies curve of four trials per day in the six consecutive days. **(C)** The area under the curve (AuC) of the escape latency was calculated for each group for statistical comparison. **(D–F)** Probe tests analysis of individual groups utilized MWM on day 7. **(D)** Representative path tracking in the probe tests without hidden platform. **(E)** The average times that the mice crossed platform location in 120 s. **(F)** The percentage of searching time that the mice of individual groups spent in the target quadrant where the platform has been located in days 1–6. The drug dosages are YXQN low-dose (YXQN-L) at 0.69 g/kg, YXQN middle-dose (YXQN-M) at 2.08 g/kg, and YXQN high-dose (YXQN-H) at 6.24 g/kg. Data are represented as group mean ± S.E.M. All *post hoc* statistical comparisons are versus the vehicle APP/PS1 mice, $*p < 0.05$ and $**p < 0.01$ (Dunnet's *post hoc*).

and 4 ($F = 2.948$ and 2.572; $p = 0.036$ and 0.036). Especially, on day 6, the latencies of YXQN low-, middle-, and high-dose APP/PS1 mice were remarkably shorter by 32.10, 36.06, and 40.32%, respectively, relative to vehicle APP/PS1mice. Next, for quantificational evaluation, the area under the escape latency curve were calculated, which represented the general cognitive level over six consecutive days. As shown in **Figure 2C**, compared to WT mice, the vehicle APP/PS1 mice presented a substantial increase in AuC-latency ($F = 4.015$; $p < 0.0001$), suggesting the declining spatial memory. The reduction in the escape latency was observed in the different dosages of YXQN and donepezil groups compared with the vehicle group. Particularly, the AuC-latency of the high-dose YXQN group showed the most significant decrease ($F = 4.015$; $p = 0.011$), approximating the AuC-latency of the WT group. Thus, YXQN, especially in high-dose, could substantially ameliorate the severe deficit in spatial and long-term memory formation in aged APP/PS1 mice.

The above results of the navigation tests with hidden platform were supported by a subsequent probe trial without the platform. Again, the typical path tracking of each group in 120 s was shown in **Figure 2D**. Within the similar total path length, the vehicle APP/PS1 mice swam randomly throughout the tank, indicating their poor memory retention of the location of the platform. However, the WT mice and also the YXQN administrated APP/PS1 mice used a spatially biased search strategy to locate the platform, indicating their good memory retention. Further, we calculated the platform location crossing times and the percent of target quadrant search time, both of which evinced the memory retention of the location where the hidden platform had been placed. The data suggested that there were more platform location crossing times and a higher percent of target quadrant search time in the YXQN high-dose group than the vehicle group in APP/PS1 mice ($F = 2.300$; $p = 0.012$; $F = 2.779$; $p = 0.023$). The platform location crossing times and percent of target quadrant search time of YXQN high-dose group were both approximate to the outcomes of WT group ($F = 2.300$; $p = 0.001$; $F = 2.779$; $p = 0.010$) (**Figures 2E,F**). These results provided evidence on the significant compensating effect of YXQN high-dose on cognitive deficits.

To further confirm the above results, the Y-maze alternation tests were performed for the detection of short-term spatial memory ability. As shown in **Figure 3A**, compared with the vehicle APP/PS1 group, the percentage alternation of YXQN high-dose showed a notable increase in the memory test after 1 month of drug treatment ($F = 1.490$; $p = 0.024$). When 2 months of the drug treatment finished, again the YXQN high-dose group showed the most significant effect on increasing alternation ($F = 2.183$; $p = 0.015$), implying the enhancement of short-term memory by YXQN high-dose treatment. Further, in Y-maze alternation tests, the amelioration of cognitive ability was independent of motor ability (Reiserer et al., 2007), as manifested by the unchanged numbers of arms entered in each group (**Figure 3B**).

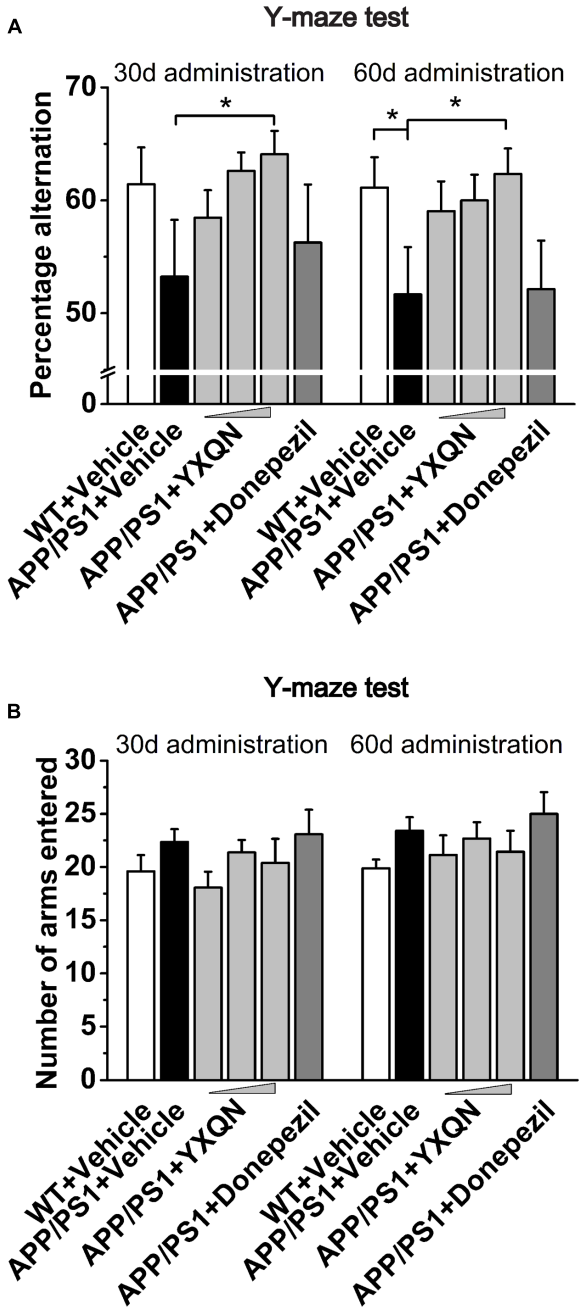

FIGURE 3 | Yangxue Qingnao improves the short-term spatial memory decline in APP/PS1 mice by Y-maze. (A) Short-term spatial memory observed twice (administration 1- or 2-month) by percentage alternation among arms in WT mice ($n = 16$) and the APP/PS1 mice with vehicle, YXQN or donepezil administration ($n = 14$–17). **(B)** Total arm entries of individual groups. The drug dosages from low to high of YXQN extract are 0.69, 2.08, and 6.24 g/kg. Data are represented as group mean ± S.E.M. All *post hoc* statistical comparisons are versus the vehicle APP/PS1 mice, $^*p < 0.05$ (Dunnet's *post hoc*).

Above all, our results indicated that YXQN significantly improved cognitive deficits of APP/PS1 mice in a dose-dependent manner. Remarkably, the effect of decreased cognitive impairment was greater in the YXQN middle- and high-dose groups than the donepezil group. Intriguingly, with YXQN high-dose supplemented for 2 months, the spatial short- and

long-term memory formation and retention of the APP/PS1 mice was similar to that of the littermates WT mice, suggesting that YXQN counteracts cognitive decline in APP/PS1 mice.

YXQN Decreased Amyloid Burden in the Hippocampus and Cortex of APP/PS1 Mice

Based on the fact that YXQN extract supplementation may ameliorate cognitive decline in the AD mouse model, we next investigated the core pathology of the AD-amyloid burden in APP/PS1 mice of each group. Firstly, using specific antibody 6E10, the Aβ plaques were stained in the sagittal brain sections of each group. As a result, substantial cerebral amyloidosis could be observed by Aβ-immunoreactive in vehicle APP/PS1 mice at 10 months of age (**Figure 4A**). However, YXQN low-, middle-, and high-dose groups all presented a forceful reduction in the Aβ deposition when compared with the vehicle group. The Aβ covered areas were reduced by approximately 50% in the APP/PS1 mice treated with the three dose levels of YXQN extract ($F = 9.212$; $p < 0.0001$ and $p < 0.0001$) (**Figure 4C**). Next, to corroborate the finding of the reduction of amyloid burden in YXQN groups, congo-red staining was performed, which specifically stains the amyloid plaque. Again, YXQN extract significantly decreased the Aβ burden in the brain of APP/PS1 mice (**Figure 4B**). Particularly, compared with the vehicle group, the YXQN high-dose group showed a 72% decrease in plaque covered area ($F = 12.080$; $p = 0.002$, $p = 0.001$, and $p < 0.0001$) (**Figure 4D**).

The hippocampus (HC), entorhinal cortex (EC), and cingulate cortex (CC) form the CC-EC-hippocampus, which is the most important element in a brain for organizing spatial memory and transforming short-term memory to long-term memory. This region is the first area to suffer damage in the brain of AD patients and is closely associated with neurodegeneration (Khan et al., 2014; Lopez et al., 2014; Chang et al., 2016). We therefore performed thioflavin-S staining on the brain sections to investigate whether YXQN administration alters Aβ deposition in the hippocampus and cortical areas, including the EC and the CC. Thioflavin-S is a kind of fluorochrome specifically binding to amyloid deposits, and can be excited to produce green fluorescence. As shown in **Figure 5A**, the YXQN middle- and high-dose groups both significantly decreased the amount of thioflavin-S positive plaques in the hippocampus, EC, and CC, compared with the vehicle group. By quantifying, YXQN middle- and high-dose groups, respectively, reduced the Aβ plaques by 32 and 44% in hippocampus areas ($F = 5.938$; $p = 0.028$ and $p = 0.002$) (**Figure 5B**), and also by 39 and 57% in the cortex areas, relative to the vehicle group ($F = 15.051$; $p = 0.003$ and $p < 0.0001$) (**Figure 5C**). Taken together, YXQN treatment groups had substantially reduced Aβ deposition in the hippocampus and cortical regions in a dose-dependent manner, suggesting that YXQN extract ameliorated cognitive impairment in MWM and Y-maze probably through reducing the Aβ deposition in the hippocampus and cortical areas of APP/PS1 mice.

YXQN Decreases Brain Aβ Levels by Altering APP Process in APP/PS1 Mice

Accumulating evidence indicates that soluble Aβ oligomers and Aβ fibrils participate in the pathological and cognitive symptoms of AD through different processes (Larson and Lesne, 2012; Zahs and Ashe, 2013). To define which kind of Aβ assemblies YXQN is involved in, we examined the amount of Aβ40 and Aβ42 in the soluble (SDS-soluble) or in the insoluble (formic acid-soluble) fractions in cerebral homogenate by ELISA. The majority of extracellular aggregations of Aβ40 and Aβ42 were detected in the formic acid-soluble fractions. Of the two, Aβ42 is the more amyloidogenic form of the peptide, due to its more hydrophobic nature (Kayed et al., 2003; Sadigh-Eteghad et al., 2015). As shown in **Figure 6A**, compared with the vehicle group, there was a significant decrease in the levels of soluble Aβ40 ($F = 9.069$; $p = 0.008$, $p = 0.001$, and $p = 0.001$) and Aβ42 ($F = 15.569$; $p < 0.0001$ and $p < 0.0001$) in the YXQN middle- and high-dose groups. Moreover, compared with the vehicle group, the APP/PS1 mice in YXQN groups showed a 50–70% reduction in highly aggregated forms (insoluble, formic acid extract) both of Aβ40 ($F = 43.774$; $p = 0.001$, $p < 0.0001$, and $p < 0.0001$) and Aβ42 ($F = 19.671$; $p < 0.0001$, $p < 0.0001$, and $p < 0.0001$).

To further confirm the above results of ELISA that YXQN reduced the levels of Aβ, the amount of total cerebral Aβ were tested by Western blot. As shown in **Figure 6B**, the low-, middle-, and high-dose YXQN dramatically reduced the amount of Aβ in the brain, by 42, 62, and 77%, respectively, relative to the vehicle group ($F = 6.339$; $p = 0.011$, $p = 0.003$). To investigate APP processing involved in the reduction of Aβ levels, we further analyzed the expression of full-length APP and APP-derived C-terminal fragments (CTFα and CTFβ) in the brain of APP/PS1 mice in each group by immunoblot with the specific antibody. CTFα, one of the α-secretase-derived fragments from the non-amyloidogenic processing of APP, is a physiological product. Meanwhile, β-secretase-derived CTFβ from the amyloidogenic processing of APP is the pathologic product that further generates Aβ. As shown in **Figure 6C**, full-length APP was unchanged among different treatment groups. However, in YXQN treated APP/PS1 mice, the levels of CTFβ were remarkably decreased, along with an obvious increase of CTFα. Notably, the ratio of CTFα to CTFβ in the YXQN middle- and high-dose groups showed a 1.6∼1.8-fold elevation above that of the vehicle group by quantification ($F = 3.593$; $p = 0.039$ and $p = 0.017$). Thus, the results indicate a modulation effect of YXQN on suppressing amyloidogenic and promoting non-amyloidogenic processing of APP.

YXQN Improves sAPPα Production by Promoting α-Secretase Expression

Subsequently, to corroborate the effect of YXQN extract on non-amyloidogenic processing, we examined the expression of N-terminal α-secretase-derived sAPPα in cerebral hemispheres of each group, which has been reported as antagonizing amyloidogenic processing of APP and playing a neurotrophic

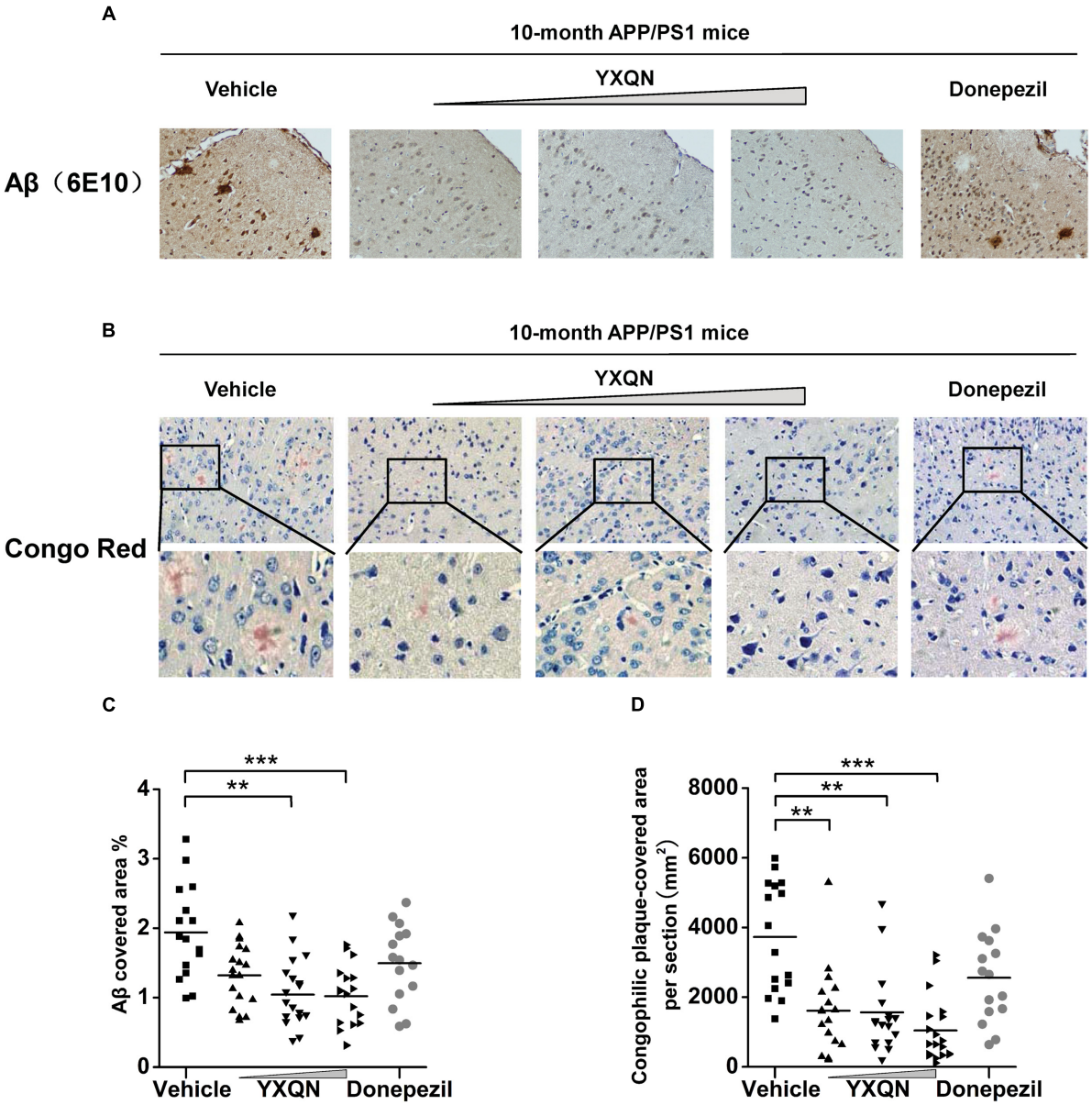

FIGURE 4 | Yangxue Qingnao reduces amyloid deposits in the brain of APP/PS1 mice. (A–D) Aβ plaque load in 10-month-old APP/PS1 mice after 2 months of vehicle, YXQN, and donepezil administration assessed by Aβ reactive antibody 6E10 **(A,C)** or congo red **(B,D)**. **(A)** Analysis of Aβ deposition levels by immunohistochemical staining in APP/PS1 mice with vehicle, YXQN or donepezil supplementation (the percent of the cover area quantified in C, n = 15–18). **(B)** Congo red positive plaques in groups show representative cortical areas and higher-magnification images. **(D)** Quantification of the cover area of congophilic plaques (n = 15–18 mice per experiment group). The drug dosages from low to high of YXQN extract are 0.69, 2.08, and 6.24 g/kg. Mean ± S.E.M. For statistical analyses, one-way analysis of variance (ANOVA) was used **(C,D)**. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.0001$.

role. Our results showed that the expression of sAPPα in YXQN middle- and high-dose groups presented a robust augmentation compared with the vehicle group (**Figure 7A**), which was consistent with the increase of CTFα. Given that ADAM10 as a common α-secretase cleaves APP to generate sAPPα and CTFα, the expression of ADAM10 in each group was detected by immunoblot. As shown in **Figure 7A**, a similar notable augmentation of ADAM10 was observed in YXQN middle- and high-dose groups, compared with the vehicle group. Then, we immunostained brain slices of the five groups with ADAM10 antibody and analyzed the total staining area. As shown in **Figure 7C**, compared with the vehicle group, the degree of ADAM10 staining in the cortex of YXQN middle- and high-dose groups strikingly increased. There was a meaningful difference between middle- or high-dose YXQN and vehicle APP/PS1 mice in the total positive stain cover area of ADAM10 ($F = 16.433$; $p = 0.006$ and $p < 0.0001$) (**Figure 7D**), which is in accordance with the results from the Western blot (**Figure 7A**). These data

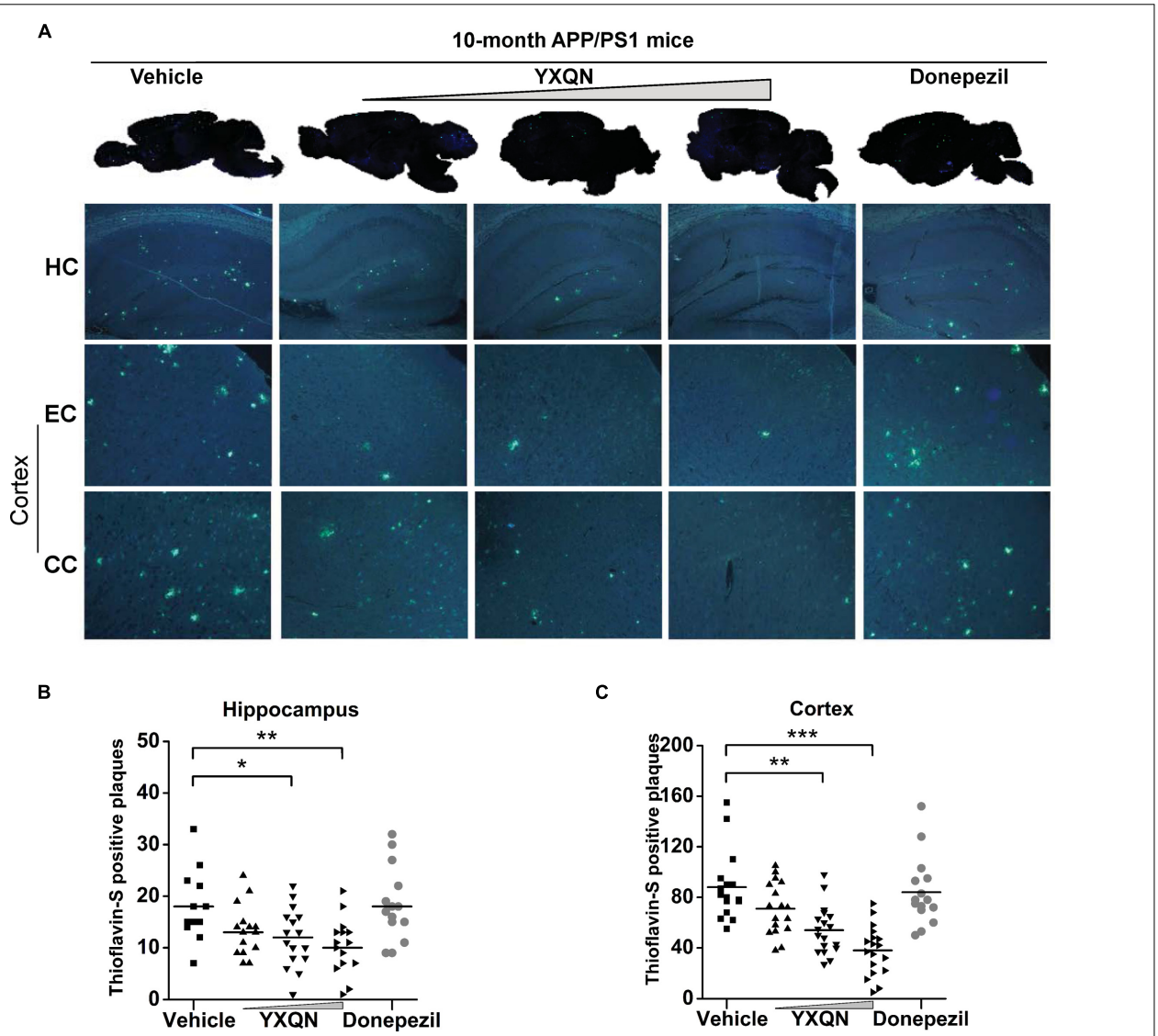

FIGURE 5 | Yangxue Qingnao decreases Aβ deposition that presents both in hippocampus and cortex of APP/PS1 mice. (A) In the APP/PS1 mice with vehicle, YXQN, and donepezil supplementation, Aβ deposition was quantified in whole-brain sagittal plane and hippocampus (HC), entorhinal cortex (EC) or cingulate cortex (CC) area using thioflavin S. **(B,C)** Quantitative analysis of the number of Aβ plaques loaded in cortex region **(B)** and hippocampus region (**C**; n = 12–18). The drug dosages from low to high of YXQN extract are 0.69, 2.08, and 6.24 g/kg. Mean ± S.E.M. ANOVA with Dunnet's *post hoc* analysis was used. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.0001$.

provided evidence that YXQN improved non-amyloidogenic processing of APP by promoting ADAM10 expression.

YXQN Inhibits APP Pathological Process by Reducing BACE1 and PS1 Expression

BACE1 and PS1 as β- and γ-secretase play a core role in the pathological processing of APP (amyloidogenic processing) (Oddo et al., 2004; Willem et al., 2015). Based on the effects of YXQN in reducing the production of CTFβ and Aβ, the expression of BACE1 and PS1 were further detected in the brain of each group by both Western blot and immunohistochemistry, to confirm the roles of YXQN in the inhibition of APP pathological processing. As shown in **Figure 7B**, compared with the vehicle group, the expressions of BACE1 and PS1 in the brain tissues of YXQN groups were greatly reduced in a dose-dependent manner. A reduction of the BACE1 level was observed in the donepezil group as well. Moreover, in the analysis of the staining area of BACE1 and PS1 by immunohistochemistry, we found the BACE1 and PS1 levels markedly reduced in all three YXQN dosage level groups (**Figure 7C**). The total staining areas of BACE1 in low-, middle-, and high-dose groups were substantially decreased by 45, 57, and 79%, relative to the vehicle group ($F = 15.754$; $p = 0.005$, $p = 0.001$, and $p < 0.0001$); and that in the donepezil group also showed a slight reduction ($F = 15.754$; $p = 0.032$) (**Figure 7E**). Likewise, the three groups

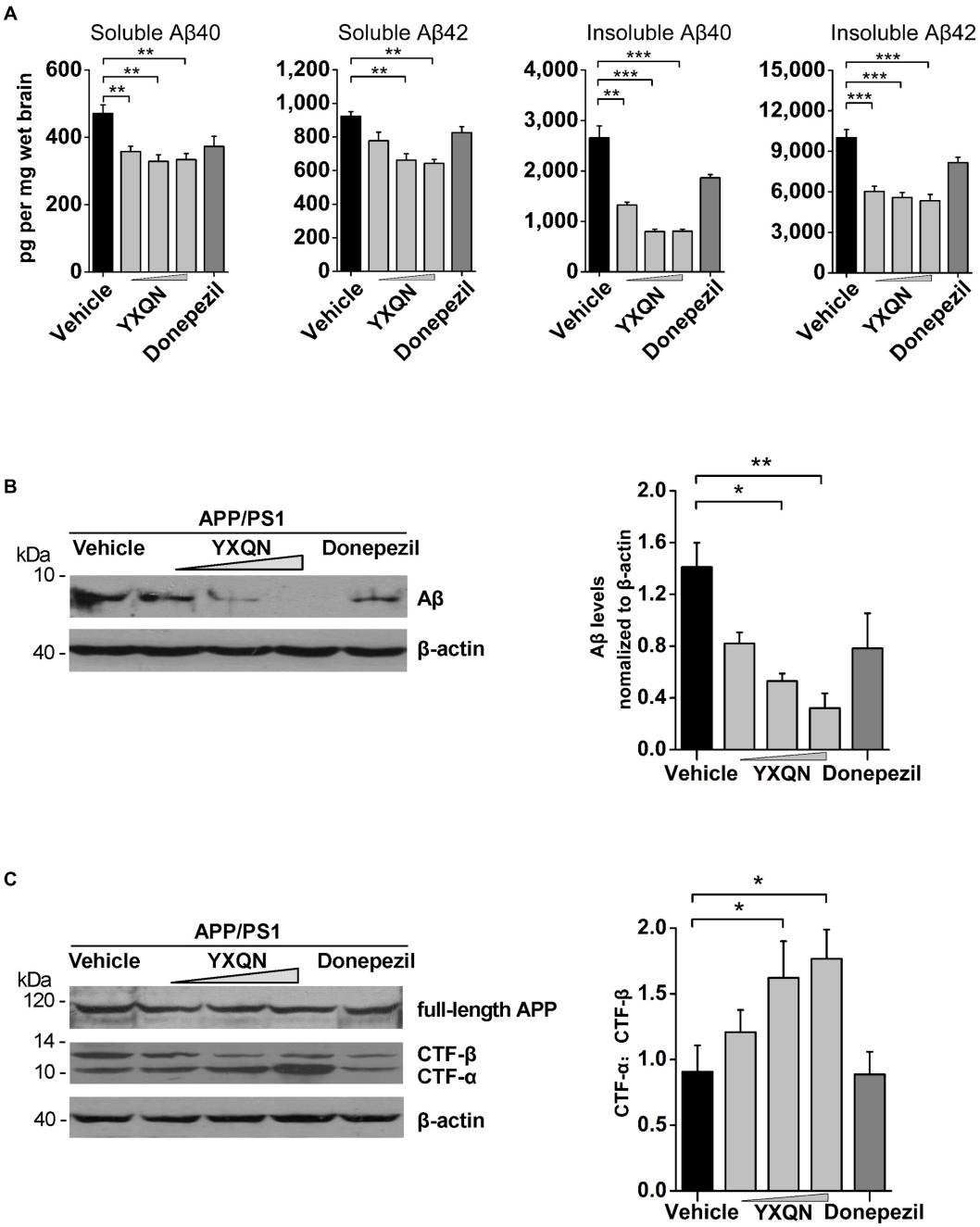

FIGURE 6 | Effects of YXQN on cerebral Aβ levels and APP process in APP/PS1 mice. (A) The levels of formic acid-soluble (insoluble) and SDS-soluble (soluble) Aβ40 and Aβ42 in brain homogenates from YXQN administration from 8- to 10-month old using ELISA kits (n = 16–18). **(B)** The levels of total Aβ in the RIPA brain extracts of 10-month-old APP/PS1 mice analyzed by Western blot with antibody 6E10 (β-actin as internal controls). **(C)** The expression of full-length APP and carboxyl-terminal fragments (CTFs) analyzed by immunoblot with the APPct antibody and densitometric scanning, and quantified by ratio of C-terminal α-cleavage product (CTF-α) and β-cleavage product (CTF-β). Western blot images and densitometric quantification of blots is from at least three independent experiments (n = 6 mice per group). The drug dosages from low to high of YXQN extract are 0.69, 2.08, and 6.24 g/kg. The numbers presented are mean ± S.E.M. (Dunnet's *post hoc* analysis). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.0001$.

of YXQN, respectively, showed a 31, 45, and 54% reduction of PS1 expression in APP/PS1 mice, compared with vehicle APP/PS1 mice ($F = 7.506$; $p = 0.005$) (**Figure 7F**), suggesting the diminished production of Aβ peptide. Taken together, these data might indicate that YXQN extract inhibited the expression of BACE1 and PS1, and leaded to the reduction of Aβ in the brain of APP/PS1 mice. These results therefore addressed the suppression of amyloidogenic pathological processing of APP by YXQN.

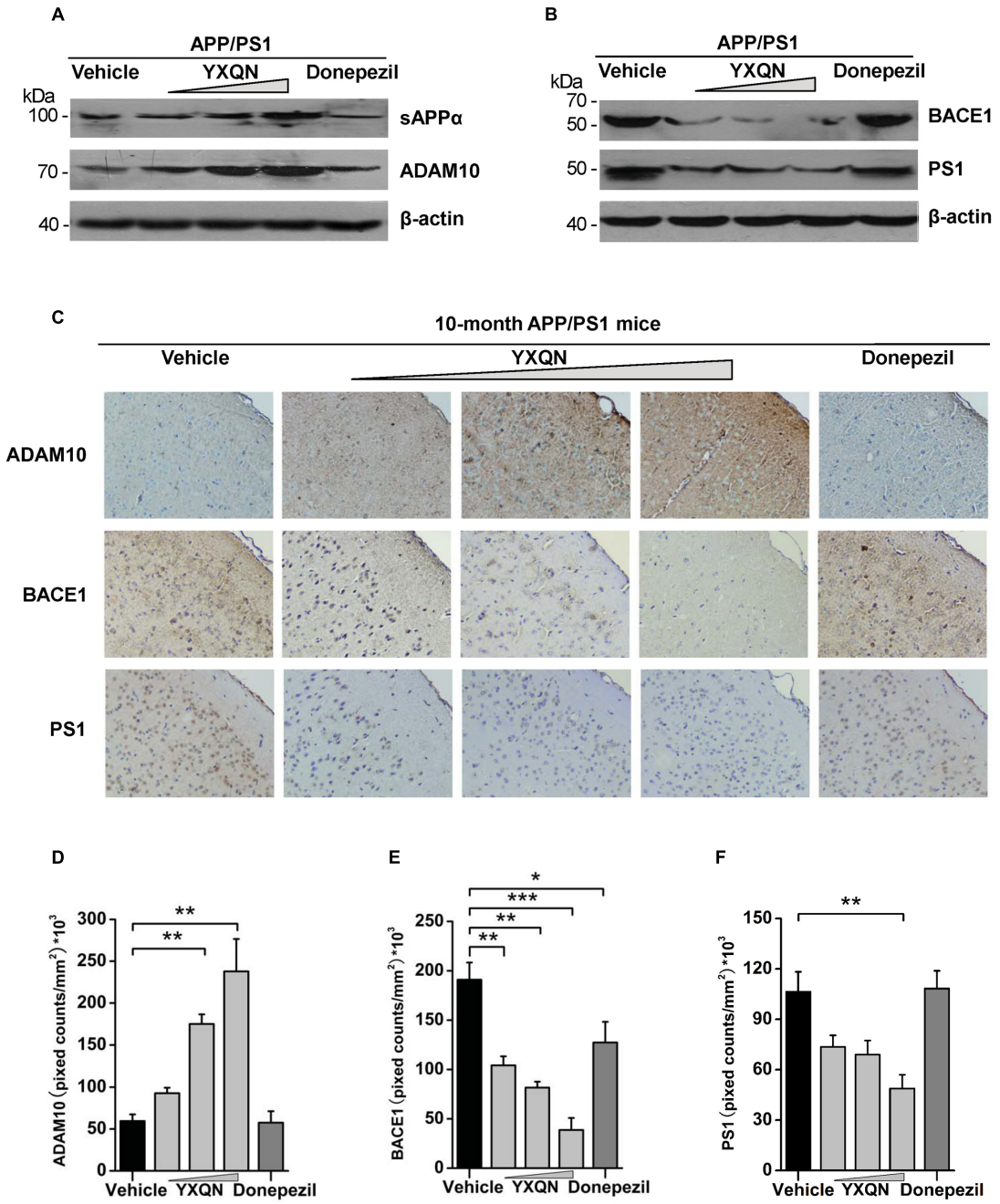

FIGURE 7 | The action of YXQN activates sAPPα and ADAM10, and inhibits BACE1 and PS1 expression demonstrated by Western blot and immunohistochemistry. (A) Immunoblot analysis of α-cleavage product sAPPα and α-secretase (ADAM10) in the brain homogenates of 10-month-old APP/PS1 mice with vehicle, YXQN or donepezil administration. **(B)** BACE1 and PS1 from mouse brains were subjected to immunoblot analysis. Western blot images of at least three independent experiments are shown (n = 6 mice per group). **(C)** Staining for ADAM10, BACE1, and PS1 using specific antibody in sagittal sections of brain, and quantified by pixed counts (n = 6). The drug dosages from low to high of YXQN extract are 0.69, 2.08, and 6.24 g/kg. Mean ± S.E.M. ANOVA with Dunnett's *post hoc* analysis was used **(D–F)**. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.0001$.

All in all, our results confirm a significant role of YXQN against cognitive decline and Aβ aggregation in AD, through up-regulating the level of α-secretase ADAM10 in the physiological processing of APP, and down-regulating β-secretase BACE1 and γ-secretase PS1 in the pathological processing of APP.

DISCUSSION

Designing drugs to protect neurons from AD is very challenging, and large numbers of therapeutic drugs focusing on reducing Aβ levels have failed (Liu et al., 2014; Wisniewski and Goni, 2015).

However, more and more clinical data show that the causes of AD are closely associated with CBF and brain nourishment. One traditional Chinese medicine, YXQN is a formula based on a famous decoction, Siwu Tang, which promotes blood circulation to alleviate headaches and dizziness, and which has been used clinically for 20 years. Our results demonstrated the pronounced effects of YXQN extract, not only on ameliorating cognitive and memory impairment, but also on mitigating the critical pathology in APP/PS1 mice.

A classical AD model, APP/PS1 mice were used in the present research. These mice were structured based on Aβ pathology, learning-memory deficit accompanied with detectable cerebral Aβ at 4 months (Jankowsky et al., 2004; Perez et al., 2005), significant amyloidosis at 6–7 months, and further aggravated at 10 months. Hence, 8-month APP/PS1 mice were administrated with diluted YXQN extract for 2 months, when the mice normally presented severe cognitive deficits and significant Aβ deposits, equivalent to moderate to severe AD patients. Excitingly, after 2 months of YXQN administration, YXQN APP/PS1 mice showed a substantial decrease in Aβ levels, compared to vehicle or donepezil APP/PS1 mice, in which the high-dose group showed a 47–72% reduction in plaque deposits relative to the vehicle group (**Figures 4**, **5**). While donepezil has been used widely to improve symptoms of AD, it functions not by Aβ-dependent pathogenic mechanisms, but by inhibiting cholinesterase. The significant Aβ reduction using YXQN compared to donepezil, revealed its specific attenuation of the Aβ deposition.

We focused on the morphometric analyses of decreased Aβ aggregation by YXQN in the CC-EC-hippocampus system. As a central part of the limbic system, the CC is closely associated with emotion formation and processing, learning, and memory (Frankland et al., 2004). As the key connection between the hippocampus and neocortex, the EC plays an essential role in spatial memories, including memory formation and consolidation (Lopez et al., 2014). The CC receives signals from the thalamus and the neocortex and sends them to the EC via the cingulum. The EC is one of the first regions of the brain to suffer from Alzheimer's disease, with significantly decreased volume (Khan et al., 2014). Responsible for central spatial memory and navigation, the damage of the hippocampus is associated with memory loss and disorientation in AD (Takeda and Tamano, 2014; Chang et al., 2016). Our data shows that YXQN extract, especially in high doses, markedly reduces Aβ deposition in the AD associated regions, CC-EC-hippocampus system, accompanied with the ameliorative effect on spatial learning memory. The behavioral assessments, including MWM tests and Y-maze spontaneous alternation, showed that in a dose-dependent manner, YXQN extract substantially improved both long and short term spatial memory. The middle-dose of our experiments (2.08 g/kg for mouse) is equal with the clinical dose for patients (0.168 g/kg for human, about 10 g/per day), where there is certain clinical evidence of cognitive improvement with YXQN; in 60 patients with amnestic mild cognitive impairment (aMCI), the memory decline was delayed by a 3-month per oral course of YXQN, while in 35 patients with senile dementia it occurred by a 2-month course (Wang et al., 2005; Zhao et al., 2007).

The aggregation and deposition of Aβ is a foremost causative factor in AD pathogenesis. The cleavage of APP in both amyloidogenic or non-amyloidogenic pathways is regulated by three secretases, α-secretase (ADAM10) for the non-amyloidogenic processing of APP to CTFα and sAPPα, β-secretase (BACE1) and γ-secretase (PS1 mainly) to generate the Aβ fragment (Hoey et al., 2009; Portelius et al., 2011). Importantly, sAPPα shows an antagonistic action on amyloidogenic processing (Obregon et al., 2012; Tyan et al., 2012; Bailey et al., 2013). Our Western blot and immunohistochemistry results proved that YXQN increased the levels of CTFα and sAPPα by the higher expression of ADAM10 (**Figures 6C**, **7**), to play a neurotropic function against amyloidosis formation in AD mice. Further, the augmented ADAM10 were confirmed by the 6 times induction of mRNA transcriptional level (data not shown).

Because β- and γ-secretase are responsible for the amyloidogenic pathological processing of APP to generate Aβ fragments, altering their activity will change the production of Aβ (Esler and Wolfe, 2001; Oddo et al., 2004). Lower expressions of β- and γ-secretase could reduce the β-pathology process (Dewachter and Van Leuven, 2002; Willem et al., 2015). In this light, investigating the impact of YXQN extract on BACE1 and PS1 is of great interest. Western blot and immunohistochemistry verified that YXQN presented a dose-dependent inhibition of BACE1 and PS1expressions (**Figures 7B–F**), thus inhibiting the β-pathology process. We found YXQN decreased BACE1 through the inhibition of mRNA transcriptional level, but PS1 reduction may be associated with degradation pathway (data not shown). More importantly, unlike the direct inhibitors of β- or γ- secretase *in vitro*, YXQN reduced the levels of BACE1 and PS1 without altering the average lifespan and athletic ability of APP/PS1 mice. In short, YXQN significantly decreases amyloid plaques through two major aspects of the molecular mechanism: up-regulating the level of α-secretase ADAM10 in the physiological processing of APP, and down-regulating β-secretase BACE1 in the pathological processing of APP.

In addition to seven other Chinese medicines, the chief compounds of YXQN are Angelica sinensis, Ligusticum chuanxiong hort, White peony root, and Prepared radix rehmanniae, which have been used clinically as Siwu Tang for replenishing, nourishing, and increasing blood flow from as early as the Song dynasty (1000 years ago). Studies suggest that their probable molecular mechanism on AD treatment includes activating the neurotrophin signaling pathway and increasing CBF. The structure and function of neurons is maintained by the release of trophic factors. A new APP knock-in mouse model proved a direct and positive link between vascular and parenchymal Aβ, both of which can be modulated by CBF (Li et al., 2014; Sun et al., 2016). Findings indicate that decreased CBF might have implications for aMCI (Jefferson et al., 2015; Zamolodchikov et al., 2016). Another study reports that both amyloid plaques and decreased CBF are primarily associated with deficits in cognitive function (McDade et al., 2014). Moreover, neurotrophin related proteins such as the brain-derived neurotrophic factor have a protective role against Aβ toxicity (Lim et al., 2015). Using whole-genome DNA microarray to compare YXQN treated and vehicle

APP/PS1 mice, we found 7 differentially expressed genes in the neurotrophin signaling pathway (data not shown). It appears that the 11 compounds of YXQN perform the functions of stimulating blood flow, anti-oxidation, protection of neurons, and regulation of the enzymes targeting the nervous system (**Table 1**); in which increasing CBF may relate to the effects of YXQN on enhancing ADAM10 and sAPPα; the anti-oxidation, neuroprotection and enzyme regulation may be associated with the effects of YXQN on inhibiting BACE1 and PS1, and activating ADAM10. Interestingly, neuronal overexpression of ADAM10 in transgenic mice reduces BACE1 processing of APP and amyloid deposition, that means the up-regulated α-secretase could promote anti-amyloidogenic processing of APP (Postina et al., 2004). In addition, our research about the effects of each component of YXQN on AD pathology related events in SH-SY5Y cell line presented that Angelicae sinensis, Ligusticum chuanxiong hort, Uncaria and Spatholobus suberectus were responsible for the increased sAPPα and decreased Aβ. Of them, Spatholobus suberectus also significantly increased the expression of ADAM10. While, the block of BACE1 expression was associated with Prunella Vulgaris or Cassia seed treatment. Further study is required to clarify the relative contribution of the individual ingredient of YXQN to the effects observed both *in vitro* and *in vivo*.

To date, YXQN has been commonly used for improving headaches, dizziness, giddiness, irritability and insomnia over 20 years. As YXQN formulated on the theoretical and clinical foundations of traditional Chinese medicine, it is effective toward multiple targets, especially, against the cognitive impairment caused by various cerebral vessel-related lesions. Data from 273 patients with chronic cerebral vascular insufficiency (CCI) at 9 hospitals in China demonstrated that, after 8 weeks of treatment, YXQN was as effective as nimodipine in improving the symptoms of CCI, including baseline in severity of headache, heavy-headed feeling, dizziness and sleep disorder (Wu et al., 2013); likewise, research in 83 patients showed that 12-week clinical YXQN treatment could effectively improve the CCI symptoms by reducing the vertigo score, and increasing middle cerebral artery mean velocity and vertebral artery mean velocity (Gu et al., 2005). Further, animal experiments also provided another line of evidence that YXQN increased CBF and attenuated cerebral microcirculatory disturbance in the ischemia-reperfusion injury, and therefore elevated model group rats' memory performance (Gu et al., 2005; Xu et al., 2009; Xiong et al., 2011). In all, YXQN as a clinical medicine for vascular diseases by improving CBF is definite. Moreover, our work provides direct evidence on YXQN counteracts cognitive decline and decreases Aβ aggregation in AD mouse model. Overview, these studies indicate the multiple protective functions of YXQN on CBF associated disease, including CCI and AD.

In summary, our data provides lines of evidence that YXQN extract plays remarkably effective roles in reducing amyloid plaques in the brain, and in improving the cognitive decline of AD. In addition, at the clinical dose of YXQN and donepezil, YXQN shows more significant effect than the cholinesterase inhibitor, donepezil, on improving the cognitive decline of APP/PS1 mice by 2 months administration. Moreover, YXQN transfers APP processing from amyloidogenic to non-amyloidogenic by the activation of ADAM10 to enhance sAPPα levels. We have shown that YXQN could be a safe prospective anti-AD therapy directly addressing Aβ-dependent pathogenic mechanisms.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: XW, RS, WL, ZL, LW, and XL. Acquired data: XW, RS, and ZL. Analyzed and interpreted the data: XW, XZ, YL, ZS, JL, and XL. Wrote the manuscript: XW and XL.

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REVIEW
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The Impact of Microbiota-Gut-Brain Axis on Diabetic Cognition Impairment

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Progressive cognitive dysfunction is a central characteristic of diabetic encephalopathy (DE). With an aging population, the incidence of DE is rising and it has become a major threat that seriously affects public health. Studies within this decade have indicated the important role of risk factors such as oxidative stress and inflammation on the development of cognitive impairment. With the recognition of the two-way communication between gut and brain, recent investigation suggests that "microbiota-gut-brain axis" also plays a pivotal role in modulating both cognition function and endocrine stability. This review aims to systemically elucidate the underlying impact of diabetes on cognitive impairment.

Keywords: advanced glycation end products, diabetic encephalopathy, hypothalamic-pituitary-adrenal axis, inflammation, microbiota, gut

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INTRODUCTION

Diabetic neuropathy refers to a series of neurological dysfunction caused by diabetes. With the duration of the disease, the nerve damage may occur throughout the body of diabetic patient. In Miles and Root (1922) noticed that diabetes can cause central nervous system lesions which will finally result in cognitive dysfunction. In Reske-Nielsen et al. (1966) proposed the concept "diabetic encephalopathy (DE)" when they studied the brain tissues from 16 young diabetic patients who died of vascular complications. Due to the lack of uniform diagnostic criteria, epidemiological survey concerning DE is very difficult, but it is suggested that the incidence of cognitive impairment in the diabetic population may reach as high as 40%. A clinical cohort study suggested that brain atrophy is significantly and positively correlated with type 2 diabetes (Wisse et al., 2014). Progressive cognitive impairment is a central characteristic of DE. A recent meta-analysis that included 1,148,041 cases found that diabetes can increase the risk of cognitive impairment by about 2 times (Gudala et al., 2013). To date, progressive cognitive dysfunction has been recognized as a typical signs and symptoms in diabetic population (van den Berg et al., 2010).

With an aging population, the incidence of diabetes is rising; more importantly, more and more DE cases are found in younger population. The damage attributed to diabetes on cognitive function has become a major threat that seriously affects the quality of life. Therefore, speeding up the study of the pathogenesis of diabetic cognitive impairment (DCI) and establishing an effective prevention strategy is urgent. There is a study found that cognitive dysfunction may occur in the early stage of diabetes and will progress with the disease, and this progression is much faster in type 2 diabetes than that in type 1 patients (Brands et al., 2007). In this sense, early intervention is very important.

As a metabolic disease, the development of diabetes is dually controlled and regulated by neuroendocrine factors and digestive system. With the deepening of the study, it is suggested that gut-brain crosstalk may play a key role in this process. Gut-brain crosstalk is a very complex network system; it maintains the stability of gastrointestinal tract on the one hand and affects the emotion and cognition function on the other hand, and this network is known as "gut-brain axis (GBA)" (Rhee et al., 2009). Recent studies indicated that there is a two-way communication between gut and brain. Bercik et al. (2011) demonstrated that gut microbes can affect the level of rat brain-derived neurotrophic factor; Dinan and Cryan (2012) found stress may in turn activate the hypothalamus by affecting the intestinal microbial activity—pituitary—adrenal axis which will lead to depression. Another research conducted in animals also observed that germ-free can impair memory function (Gareau et al., 2011). At present, it is believed that gut **m**icrobes-**g**ut-**b**rain **a**xis (MGBA) may be an ideal target for understanding and treating DCI (Foster and McVey Neufeld, 2013).

THE PATHOGENESIS OF DIABETIC ENCEPHALOPATHY

Diabetic cognitive impairment (DCI) refers to cognitive impairment and brain physiological and structural changes caused by diabetes. Both type 1 and type 2 diabetes can induce and promote DCI development (Biessels et al., 2002a). There is a study observed that the characteristics of neurobiology and neuroradiological imaging of DCI is very similar to that of brain aging (Biessels et al., 2002b), suggesting DCI shares similar mechanisms with the brain aging process.

The pathogenesis of DCI has not yet been entirely clarified, but it is found that cerebral ischemia, oxidative stress, and non-enzymatic protein glycosylation, low grade inflammation and calcium homeostasis changes, etc., may play a role in the development of DCI.

Cerebral Vascular Dysfunction

Blood-brain barrier (BBB) is located on the nerve endometrial capillaries, including peripheral nerve microvascular endothelial cells (PnMECs), pericytes of endoneurial microvascular origin, and basement membrane (Poduslo et al., 1994; Abbott et al., 2006). The physiological function of BBB is realized by physical barrier and the ionic charge on the cerebral vascular endothelial cells, and any changes will lead to BBB dysfunction. At present, the hypertrophy of basement membrane and the lysis of BBB have been recognized as characteristic changes in diabetic neuropathy (Giannini and Dyck, 1995; Shimizu et al., 2011).

In fact, BBB damage has now been considered as a key factor for DCI (Biessels et al., 2008). It has been demonstrated that pathological changes associated with type 2 diabetes can damage BBB integrity, increase the permeability of BBB and lead to the easy permeation of limited substances into brain parenchyma (Dai et al., 2002; Hawkins et al., 2007; Kamada et al., 2007). A recent research demonstrated that BBB breakdown promotes the macrophage infiltration and cognition impairment in mice (Stranahan et al., 2016), suggesting BBB dysfunction is closely related with diabetic cerebral inflammation.

Physical Barrier of BBB

The physical barrier of BBB is composed of two components, i.e.,: vascular endothelial cells and basement membrane. Studies have demonstrated that diabetes and continuous high blood glucose will directly and finally induce dramatic damage of endothelial cells in both cerebral and peripheral vascular system (Xu et al., 2010; Li et al., 2016), and chronic untreated diabetes would also impair BBB by reducing tight junction proteins (e.g., ZO-1 and claudin-5) expressions and thus cause a series of cerebral dysfunction (Yoo et al., 2016). This is confirmed from a research that diabetes will significantly increase cerebral vascular permeability (Fouyas et al., 2003).

Enough blood supply plays a pivotal role in maintaining the normal function of brain. Diabetes can significantly induce cerebral vascular endothelial dysfunction and increase platelet aggregation, reduce cerebral blood flow, and cerebral vascular surface area, and finally lead to vascular endothelial proliferation and plasma viscosity increment (Dalal and Parab, 2002; Fouyas et al., 2003). Recently, Yu et al. (2016) found in diabetic patients that the disruption of BBB is significantly associated with acute stroke. Therefore, protecting the integrity of cerebral vascular endothelial cells should have important effects on reducing diabetic damage to the brain.

The basement membrane of BBB is composed of extracellular matrix adhesion proteins (e.g., type IV collagen) and fibronectin, and is strictly regulated by matrix metalloproteinases (MMPs); studies found MMP-2 and MMP-9 can degrade type IV collagen and fibronectin (Tilling et al., 2002; Chang, 2016). In a most recently published clinical study, Garro et al. (2017) found that the blood MMP-2 is lower while MMP-9 is higher in children with diabetic ketoacidosis (DKA) compared with levels in children without DKA, strongly suggesting the important role of BBB in the development of DE.

Charge Barrier Changes in BBB under Diabetic Condition

Concerning the charge barrier changes under diabetes, amounts of studies have demonstrated that there is negative relation between blood glucose and anionic charge levels on the cell membrane. This relation is most obvious in patients with diabetic nephropathy (Márquez et al., 2015). As well known, heparan sulfate is a negatively charged polysaccharide that is abundantly expressed in all layers of the glomerular filtration barrier (GFB), therefore, it is believed to play a central role in the development of diabetic proteinuria (Garsen et al., 2014). In fact, more and more studies have also reported this correlation in DCI. Previously, Briani et al. (2002) found that titers of heparin sulfate antibodies are elevated in neurological associated disease and concluded that it might associate with the breakdown of BBB. Recent studies confirmed that heparan sulfate proteoglycan agrin accumulation is related with the maturation of BBB during embryogenesis, and agrin contributes to brain endothelium tight junctions (Steiner et al., 2014) and endfoot membrane integrity of astrocytes (Noell et al., 2009).

Nitrogen/Oxygen Stress and Non-enzymatic Glycosylation

Oxidative Stress Damage of DCI

Glucose metabolism disorder is one of the basic reasons for diabetic damage. Blood glucose is the main energy source of the brain and mitochondria is the most important place for glucose aerobic oxidation in the brain. Under chronic and persistent high glucose condition, mitochondria will produce large amounts of reactive oxygen species (ROS) and leads to the oxidative stress, which will impair mitochondrial function and finally affect brain function (Liu et al., 2006; Cardoso et al., 2013). Reports have confirmed that hippocampus and cerebral cortex show a significant oxidative stress in DCI (Grillo et al., 2003; Mastrocola et al., 2005) and anti-oxidative stress treatment is believed to have a positive effect on ameliorating cognitive impairment (Kuhad and Chopra, 2007).

Chronic and sustained high glucose and ROS stimulation can directly stimulate apoptosis of neuronal cells (Liu et al., 2003). As discussed above, changes in BBB, including the integrity of the BBB damage and increased permeability, may cause diabetic cognitive dysfunction. In fact, ROS can increase the BBB permeability by down-regulating expression of tight junction proteins and remodeling cerebral vascular structure. A research demonstrated in human-source highly immortalized brain endothelial cell line hCMEC/D3 that ROS can activate PI3K-PKB pathway, induce cytoskeletal actin rearrangement and spatial redistribution, suppress tight junction proteins expression, and finally increase cerebral endothelial cell permeability and alter the integrity of BBB (Schreibelt et al., 2007).

On the other hand, oxidative stress can also impair neurogenesis. There are two locations existing immortalized neural stem cells in the brain of mammalian species: namely the subventricular zone (SVZ) and the subgranular zone (SGZ). The nerve cells generated from these two sites can be integrated into the local nervous loop and participate in learning and memory processes. Study from Edgardo and colleagues (Alvarez et al., 2009) confirmed that the learning and memory ability is dramatically decreased in STZ-induced diabetic mice compared with normal mice; the DCX-positive cells, which reflect the amounts of new-born neurons, are significantly reduced in diabetic animal; and the lipofuscin deposition in SVZ and SGZ is dramatically increased, indicating oxidative stress contributed to the development of neurogenesis disorders.

Non-enzymatic Protein Glycosylation

As discussed above, the excessive oxidative stress has been recognized as one of the most important pathogenesis of diabetes. Studies indicated that the process of oxidative stress is strictly associated with protein glycosylation, and these two synergic factors contribute to the worsening of diabetes and diabetic complications, including diabetic cerebral vascular damage and cognitive obstacles (Vlassara and Palace, 2003). It has been well recognized that the severity of diabetic neuropathy is closely related with the history of diabetes and the level of hyperglycemia (Dahl-Jørgensen et al., 1986). Chronic and sustained high glucose environment will increase the generation of advanced glycation end products (AGEs). Studies suggest that AGEs participate in the whole process of the pathophysiology of diabetic neuropathy. In a prospective clinical study that lasted for 27 years, researchers found that the degree of neuropathy is related with HbA1c and AGEs (Sveen et al., 2013).

AGEs are a broad class of non-enzymatic products of reactions between proteins or lipids and aldose sugars (Singh et al., 2001) characterized by fluorescence, brown color, and intra- and inter-molecular cross-linking and are formed by the process of nonenzymatic glycation, in which reducing sugars such as glucose react non-enzymatically with amino groups of proteins and other macromolecules. In addition to glucose, other reactive dicarbonyls, such as methylglyoxal (Mgx), glyoxal (Gx), and deoxyglucosones, are also known to generate AGEs (Brownlee et al., 1984; Wautier and Guillausseau, 1998). So far, only a few AGE structures have been identified *in vivo*, such as Nε-(carboxymethyl)lysine (CML), pentosidine, imidazolones, and oxalic acid monolysinylamide (OMA) et al. Although AGEs can be continuously produced and accumulated under high glucose circumstance in the body, western diet which is accompanied with high AGEs in the food will dramatically accelerate this process, as about 10% of oral consumed AGEs can be absorbed into the circulation system (Uribarri et al., 2011; Vlassara and Striker, 2011; Illien-Jünger et al., 2015). When studying the relation between Maillard reaction products and Alzheimer's disease, Smith et al. (1994) observed that the level of AGEs at neurofibrillary tangles and senile plaques is significantly increased in the patients. More importantly, AGEs are found to be co-localized with astrocytes and microglia in these patients (Takeda et al., 1998), strongly suggesting the important role of AGEs in DCI development. The composition of AGEs is very complex. There is a study demonstrated that CML, one of the major components of AGEs, is accumulated in the nervous system of diabetic patients (Sugimoto et al., 1997).

Recently, the *in vivo* effect of D-ribose (Rib) on glycosylation has attracted more and more interests (Wei et al., 2012). Rib exists in all kinds of cells and is a key component of many important biological molecules (Keller et al., 1988). There is a clinical study involving type 2 diabetes patients reported that the urine level of Rib in these patients is abnormally high (Tao et al., 2013), and this elevation participates in cognitive dysfunction in these patients (Han et al., 2014). This finding is further demonstrated by animal experiments that intraperitoneal injection of Rib to mice can significantly increase the plasma glycated proteins and AGEs content, while with less impact on blood sugar (Wei et al., 2012); moreover, this treatment significantly increases brain levels of AGEs, and contributes to learning and memory decline (Han et al., 2011).

AGEs can alter protein features and affect cell function via multiple pathways (Duran-Jimenez et al., 2009). Although the underlying pathogenesis is very complex, receptor pathway may play a major role in it. The receptor for AGEs, namely RAGE, is an immunoglobulin superfamily which can combine with a plurality of ligands. RAGE is expressed in different cell types, including neurons in the whole nervous system. The accumulation of AGEs and the activation of RAGE can lead

to oxidative stress, activate NF-κB pathway and up-regulate the target genes expression, trigger inflammation, and result in neuronal cell damage. Animal experiments show that oxidative stress can hinder neurogenesis, increase AGEs production and promote the neuron cell apoptosis (Jing and Zhang, 2011). Therefore, oxidative stress and AGEs accumulation constitute a vicious cycle. There are studies found in STZ-induced diabetic rats that the level of plasma ROS is as high as two times of that in normal rats, which accompanies with AGEs accumulation and RAGE up-regulation; and anti-oxidative stress treatment significantly reduces levels of AGEs and RAGE, thereby prevents neuron damage (Aragno et al., 2005). Toth et al. (2006) reported that knockout of RAGE can dramatically ameliorate neurodegenerative changes in diabetic rats, indicating the significant role of AGEs and RAGE in the development of DCI. Besides RAGE-mediated damage, a recent study reports that the accumulation of AGEs can cause hypertrophy of BBB basement cells, stimulate the production, and secretion of transforming growth factor-β (TGF-β) from the outer membrane, promote the release of vascular endothelial growth factor and MMP-2 from cerebral vascular endothelial cells, and lead to the destruction of BBB (Shimizu et al., 2013).

Nitric Oxide Stress

Nitric oxide (NO) is considered to be a bridge that connects diabetic neuropathy and the organism metabolism. Studies found in DM rats that diabetes will increase nitric oxide synthase (NOS) activity in the brain, and excessive NO will lead to learning and memory dysfunction (Xue et al., 2009; Talarowska et al., 2012). There are three types of NOS, namely neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS). The activation of nNOS and eNOS depends on Ca^{2+} and calmodulin (CaM), and small amount of NO can be generated under normal circumstances; iNOS is a non-calcium-dependent enzyme with little or no expression under normal conditions, however, factors including hyperglycemia, AGEs, oxidative stress, ischemia, and hypoxia etc. can activate the enzyme and induce a large amount of NO production. Under physiological conditions, NO can act as a vasodilator and a messenger that mediates information transmission; but at pathological state, as a free radical, it can damage the biomolecules and lead to neurons apoptosis or necrosis (Tokuno et al., 2002; Kim et al., 2010).

There are further studies found that NO stress will influence the synaptic plasticity of the hippocampus. As well known, hippocampus is an important sites that managing learning and memory via long-term potentiation (LTP) induction and maintenance. NO plays an important role in LTP and NOS inhibition can lead to learning and memory dysfunction. Research indicated that sustained hyperglycemia can cause an increase of glutamate, N-methyl-D-aspartate receptor (NMDA) receptor dysfunction, and Ca^{2+}/CaM-dependent nNOS activity increase, and the overproduction of NO would result in the enhancement of LTP (Yang et al., 1999; Biessels et al., 2002a). Liu et al. (2003) found that the content and activity of hippocampal NOS is negatively correlated with learning and memory ability in diabetic rats, but this relationship is converse in healthy rats. This can be explained in that excessive NO will hide its LTP enhancing effects by directly inducing neuron damage, thus damaging memory process.

Inflammation

There is a saying that hyperglycemia is a major cause of DCI, while chronic inflammation can build a bridge between them (Kamboj et al., 2008). Studies have demonstrated that type 2 diabetes is actually a low level chronic inflammatory disease. In Hotamisligil et al. (1993) found in diabetic animals for the first time that the inflammatory cytokines are abnormally increased, and neutralizing inflammatory cytokines by antibodies can increase glucose uptake in peripheral tissues and reduce insulin resistance. Thereafter, amounts of studies found in both animal disease model and diabetic population that type 2 diabetes is accompanied by the elevation of blood lipopolysaccharides (LPS), C-reactive protein (CRP), interleukin (IL)-6, and IL-1, etc. (Yudkin et al., 1999; Cani et al., 2007a; Zhao et al., 2012), further confirmed the low-grade inflammation nature of diabetes. With study progressing, this nature has been recognized to play an important role in the development of DCI. There is a study demonstrated that macrophage activation and infiltration in the nervous system can lead to chronic degenerative disease of the central nervous system (Kierdorf et al., 2010), and blockade the activation of macrophage can significantly decrease levels of malondialdehyde, catalase and superoxide-positive cells in the brain (Wang et al., 2015).

There is currently lack of large cohort study involving inflammatory factors and cognitive impairment risk in diabetic population. A cohort study that included 5,217 cases and followed up for 10 years found that high IL-6 levels in middle age will increase the risk of cognitive decline by as high as 1.81 times (Singh-Manoux et al., 2014), and this was further demonstrated by another study that the negative effects of high IL-6 on cognition will not change concerning use or non-use of statins (Wichmann et al., 2014). TNF-α is closely related with the activity of hippocampus. The increase of TNF-α would specifically damage the spatial memory capacity of animal and decrease the expression of nerve growth factor, thus interference the growth and function of hippocampus (Golan et al., 2004); it was demonstrated in African American patients who have high risk of cardiovascular events that elevated TNF-α dramatically reduces the processing and acting speed of the brain (Windham et al., 2014). Adhesions molecules play an important role in mediating inflammatory cell infiltration and activation. Baydas et al. (2003) reported that the expression of adhesion molecules are significantly increased in the hippocampus of STZ-induced diabetic rats and this elevation is related with memory and learning defects in rats. Study found that the presence of inflammatory cytokines and the activation of NF-κB can directly lead to neuronal dysfunction (Mattson and Camandola, 2001; Liu et al., 2013), and this dysfunction can be relieved by the inhibition of inflammatory signaling pathways (Hofmann et al., 1999).

Besides inflammatory cytokines, amounts of evidences have indicated that metabolic factors also contribute to inflammatory DCI. As discussed above, Rib is elevated in diabetic patients, recent study observed that it can activate RAGE, thereafter activate NF-κB pathway and damage the brain (Han et al., 2014).

Meta-analyses have proposed that overweight will increase the risk of cognitive dysfunction (Anstey et al., 2011; Loef and Walach, 2013); in fact, obesity will also induce a low-grade inflammation state and aggravate cognitive impairment in these patients (Nguyen et al., 2014).

A key feature during the inflammatory process is the appearance of Danger Associated Molecular Patterns (DAMP) (Bianchi, 2007). Among DAMPs, a particular molecule that is associated with nerve damage and deserves concern is high mobility group protein box-1 (HMGB-1) (Andersson and Tracey, 2011). HMGB-1 is a nuclear protein that can bind to DNA and regulate gene expression. Abundant evidences suggest that HMGB-1 plays a pivotal role in the tissue repair response, involves in inflammation process, and actively participates in the process of chronic neuropathic disorders (Feldman et al., 2012). Although HMGB-1-related cell signal transduction mechanism is not so clear, it has been recognized that RAGE and TLR2/4 are important receptors mediating its function. Ligand binding studies showed that the affinity of HMGB-1 to bind with RAGE is about 7 times higher than that of AGEs. When HMGB-1 is released into the cytoplasmic, it will exist at all-thiol state (at-HMGB-1) and bind with RAGE to function (Huttunen et al., 2002); there is report suggesting that at-HMGB-1 can also form a complex with CXCL12 and play a role via CXCR4 (Venereau et al., 2013). While in oxidative stress environment, HMGB-1 may experience disulfide reaction and produce the disulfide isoform of HMGB-1 (ds-HMGB-1); ds-HMGB1 mainly participates in the generation of inflammatory cytokine via toll-like receptor 4 (TLR4) (Venereau et al., 2013).

Glucose and Lipid Metabolic Disorder

High blood glucose is a characteristic of diabetes. The prolonged latency of neuron evoked potentials is a common phenomenon in both type 1 and type 2 diabetes. Study found that the course of diabetes and the level of HbA1c can extend the latency period and insulin therapy can alleviate this change (den Heijer et al., 2003). Moreover, diabetes can also damage the hippocampus structure and result in its dysfunction (Kamal et al., 1999; Gaspar et al., 2010a). A prospective cohort study that included 127,209 people and followed up for 8 years observed that the risk of occurring cognitive impairment in diabetic patients who did not underwent oral hypoglycemic agents treatment will increase to 2.41 times compared with healthy population, while oral hypoglycemic agents treatment can decrease this risk to 1.61 (Hsu et al., 2011). Although hyperglycemia has no effect on the number and concentration of mitochondria and in neurons of hippocampus, it will increase KIF1A, VGluT-1, and synaptotagmin-1 expression, while decrease KIF5B, SNAP 25 and synaptophysin expression (Gaspar et al., 2010b; Baptista et al., 2013), indicating diabetes may have an impact on the neuronal axonal transport at hippocampus.

Diabetic patients are often accompanied with lipid metabolism disorders, manifesting with high cholesterol, high triglycerides, high density lipoprotein and low high-density lipoprotein in the blood. As well known, the brain is an organ rich in cholesterol; however, the brain cholesterol levels are relatively independent of blood cholesterol levels due to the existence of BBB. Although the risk of blood high cholesterol on cognitive impairment is still controversial (Wood et al., 2014), amounts of studies from animal experiments concluded that elevated blood cholesterol levels will promote Aβ amyloid precursor protein production (Posse de Chaves, 2012; Maulik et al., 2013). Brain cholesterol transport between neurons and glial cells is mainly through clusterin/apolipoprotein J and apolipoprotein E (ApoE). However, as ApoE participates in the clearance of Aβ, it is now believed to be involved in the genesis of cognitive impairment. Liao et al. (2014) found that when the Alzheimer's disease mice are treated with ApoE monoclonal antibody, the behavior is improved and accompanies with brain Aβ deposition reduction. In this respect, anti-ApoE antibody may be developed as a potential treatment toward cognitive impairment. Low-density lipoprotein receptor families (LDLR) also play an important role in the pathogenesis of cognitive dysfunction in the brain. Abnormal endocytosis, abnormal lipoproteins signaling pathways and synaptic dysfunction caused by abnormalities of LDLR will impair brain function (Lane-Donovan et al., 2014). In addition, there are a large number of clinical studies have found reduced HDL will increase the risk of cognitive impairment. Therefore, lipid metabolic disorder in diabetic patients plays a role in the pathogenesis of brain cognitive dysfunction.

Calcium Homeostasis Imbalance

As well known, calcium (Ca^{2+}) homeostasis plays a pivotal role in maintaining the normal function of the organism. It has been well recognized that diabetes and its complications can damage Ca^{2+} homeostasis in neurons, induce degenerative changes of the neuron, and eventually lead to neuronal dysfunction and cell death. Biessels and Gispen (1996) pointed out that ischemia, oxidative stress, and non-enzymatic protein glycation etc. will finally induce Ca^{2+} homeostasis imbalance and lead to nerve degeneration.

Plenty of studies have investigated the involvement of Ca^{2+} homeostasis in DCI. Researchers found that the learning and memory ability of diabetic mice are significantly decreased, and the mRNA and protein expressions of calcium channel protein CaV1.2 in the brain are increased, indicating the synaptic calcium uptake capacity is enhanced; and L-type calcium channel blocker nimodipine can reverse the abnormal expression, improve the Ca^{2+}- dependent changes in synaptic plasticity, and ameliorate cognitive dysfunction of the mice (Manschot et al., 2003; Singhal and Sandhir, 2015).

There are many mechanisms that mediate diabetic Ca^{2+} influx, the most important mechanisms lie in the following two pathways: (1) calcium channel excitability enhancement. Calcium channel is commonly activated via the activation of G protein; report demonstrated that the dysfunction of Ca^{2+} channels mediated by G proteins is an important mechanism that leads to Ca^{2+} influx in diabetes (Hall et al., 2001). (2) Ca^{2+}-Mg^{2+}-ATP enzyme, which is an important regulator that control intracellular Ca^{2+} concentration, the activity of the enzyme in diabetic neuropathy patients is reported to be abnormally enhanced (Migdalis et al., 2000).

The abnormal Ca^{2+} influx would induce Ca^{2+} overloading, and finally result in the apoptosis of the concerned cells. Besides inducing apoptosis process, Ca^{2+} influx can also activate phospholipase, prevent mitochondrial electron transport, release free radicals, and finally lead to cell death (Muranyi et al., 2003).

As discussed above, the pathogenesis of DCI is very complex, and its mechanism is not yet entirely clear. At present, it is recognized that the pathogenesis of DCI is closely related with risk factors of diabetes (**Figure 1**).

GUT DYSBACTERIOSIS CONTRIBUTES TO DCI

Microbiota-Gut-brain axis (MGBA) is a two-way adjustment shaft. Amounts of studies have demonstrated that MGBA plays a key role in maintaining the body's metabolism and neuroendocrine stability (Rhee et al., 2009). Although the adjustment factors of MGBA axis is very complex, recent studies indicated that the microbiota and neuroactive peptides are the core, and they may closely related with the development of DCI. Gut dysbacteriosis has been demonstrated to play a role in many psychiatric disorders (Collins et al., 2012; Cryan and Dinan, 2012; Bienenstock et al., 2015).

The specific composition of microbiota is very complex and differs from individuals, but the relative abundance and distribution of the microbiota in healthy population is similar, and the most important two are *Firmicutes* and *Bacteroides*, which account for at least three-quarters of the total microbiota in the organism (Eckburg et al., 2005). The importance of microbiota on health has been recognized in this decade, however, the dialogue pathway and the specific mechanism between gut bacteria and distant organs (such as the brain) has just started. Most recent studies observed that major changes concerning the composition of the microbiota are associated with the occurrence of diabetes (Qin et al., 2012; Forslund et al., 2015), and systematic studies concerning the relationship between microbiota and diabetes have confirmed that gut dysbacteriosis and type 2 diabetes have a direct relationship (Qin et al., 2012; Karlsson et al., 2013), and microbiota may deeply

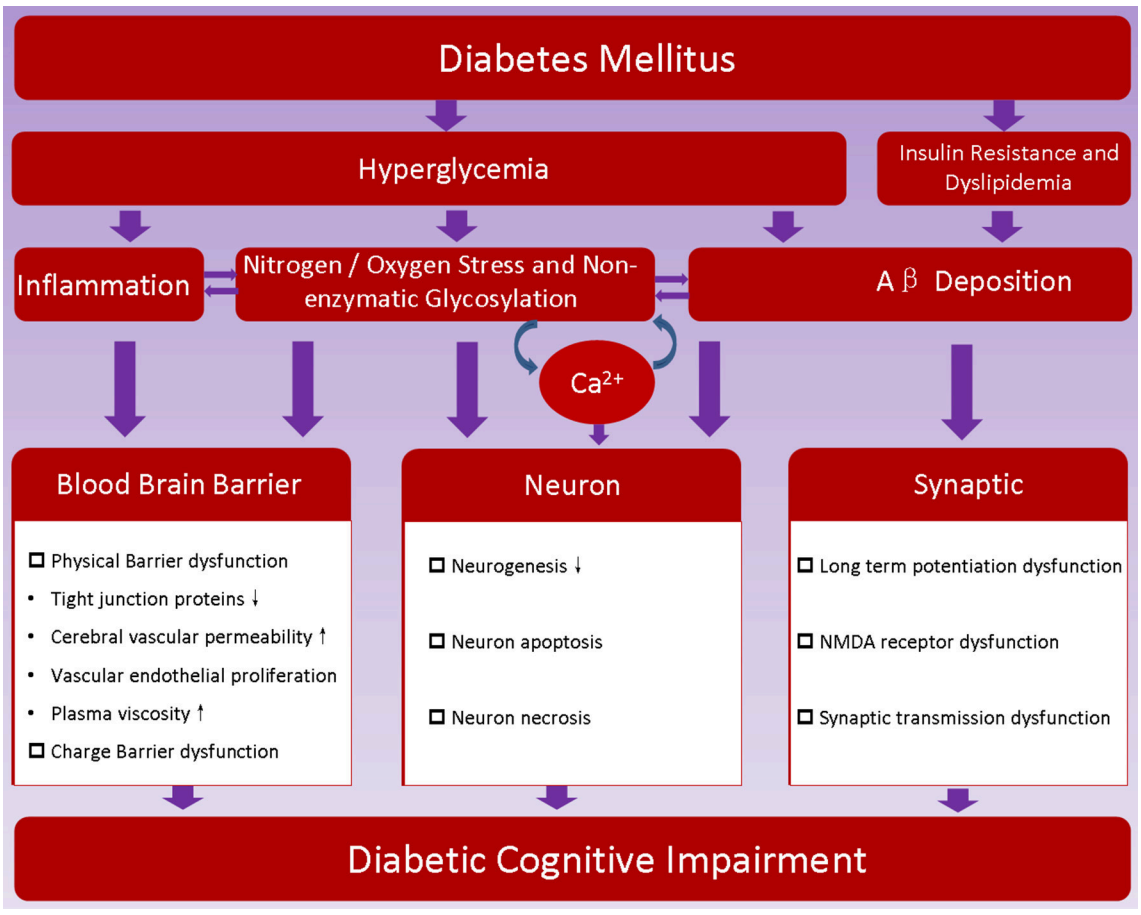

FIGURE 1 | Proposed pathogenesis of diabetic encephalopathy. Hyperglycemia, insulin resistance and metabolic disorder are characteristics of diabetes; they may promote dysfunction of blood brain barrier, induce neuron loss, hamper synaptic transmission, and thereafter contribute to the development of diabetic cognitive impairment.

influence the development of diabetes and DCI. Mechanisms including endocrine, immune and neural signaling etc. have been indicated to connect microbiota and brain function (Cryan and Dinan, 2012). Studies observed that germ free mice exhibit a series of spontaneous brain changes including hyperactivity of hypothalamus-pituitary-adrenal axis (Sudo et al., 2004), blood-brain barrier (BBB) permeability increment (Braniste et al., 2014), axon hypermyelination (Hoban et al., 2016), and cognition impairment (Gareau et al., 2011).

Mass Effect of Microbiota

The specific mechanism of microbiota imbalance involves diabetes has not been fully elucidated. A study from Cani et al. (2007a) found in mice that high fat diet will increase the amount of toxin-productive Gram-negative bacteria and decrease probiotics (e.g., *Lactobacillus* and *Bifidobacterium*) numbers; on the other hand, stimulating the proliferation of probiotics by adding oligofructose in the food can ameliorate diabetic symptoms (Cani et al., 2007b). In Wu et al. (2010) investigated the stool samples from type 2 diabetic patients and found that the amounts of probiotics is negatively correlated with the level of blood glucose, and hypoglycemic treatment can dramatically increase probiotics level to normal. It is believed that the decrease of gut probiotics will induce the impaired glucose tolerance, reduce sugar-induced insulin secretion, increase the symptoms of endotoxemia, and finally lead to type 2 diabetes (Cani et al., 2007b).

Co-metabolism Products between Microbiota and Gut Play a Pivotal Role in Maintaining the Body in a Healthy State

It is conventionally believed that probiotics may protect the intestinal mucosa against pathogen's attack and guarantee normal intestinal permeability mainly through its "mass effect." However, recent studies unveiled that the "mass effect" may play only a small role and most of the probiotics' effects are achieved through the "co-metabolism" mechanism between probiotics and the body (**Figure 2**). Microbiota derived metabolic products have been observed in the blood and in central nervous system, and these products are indicated to be important regulators in gut-brain cross-talk (Leclercq et al., 2017).

The most important metabolic products are small chain fatty acids (SCFAs), including butyric acid, propionic acid, and acetic acid et al. (Miles and Root, 1922; Macfarlane and Macfarlane, 2003). SCFAs are mainly derived from dietary fibers. It is found that SCFAs have neuroactive properties that could directly influence brain function and behavior (MacFabe et al., 2011), promote the development and stability of the nervous system (Stilling et al., 2014) and strengthen the tight connections of intestinal epithelial cells (Reske-Nielsen et al., 1966; Ait-Belgnaoui et al., 2014). Dietary factors can seriously affect the production of gut SCFAs. There are reports demonstrated that rodents fed with western food had reduced levels of SCFAs (Berger et al., 2014; Ojo et al., 2016), compared with controls fed with low-fat diet; and if the body lacks a butyrate-producing bacteria, it is easy to develop type 2 diabetes, while healthy population have more butyrate-producing bacteria than that of diabetic population (Karlsson et al., 2013). More and more studies indicate that SCFAs may influence the body from multiple pathways:

(1) Receptor-mediated pathway: SCFAs can regulate the energy balance of the host via the G protein coupled receptor GPR41 and GPR43 (Brown et al., 2003). The combination between SCFAs and GPR41/43 can promote the release of PYY from the intestine, thereby inhibiting intestinal peristalsis and increase SCFAs absorption (Samuel et al., 2008). Another report demonstrated that SCFAs can stimulate GLP-1 secretion via GPR41/43 pathway (Tolhurst et al., 2012), which was shown to help in attenuating pancreatic islet hypertrophy and improving sensitivity (Hwang et al., 2015).

(2) Energy source and anti-inflammatory effects: SCFAs can also be utilized by the intestinal epithelial cells as important energy sources, thus affect the epithelial cell proliferation, differentiation and apoptosis. On the other hand, SCFAs also impact functions of dendritic cells function, influences the proliferation of T cell, inhibits NF-κB activation (Hamer et al., 2008; Maslowski et al., 2009; Qin et al., 2012; Trompette et al., 2014; Andrade-Oliveira et al., 2015), therefore show anti-inflammatory property.

(3) Neuroprotective effects: SCFAs reach the brain through circulation (Macfabe, 2012) and posses neuroprotective effects (Sun et al., 2015). Reports demonstrate that butyrate, an important component of SCFAs, can improve the age-related memory decline (Reolon et al., 2011) and possess anti-anxiety and anti-depressant effect (Gundersen and Blendy, 2009; Zhu et al., 2009), indicating the positive role of SCFAs in the regulation of central nervous system dysfunction. Studies indicated that SCFAs may influence brain function by regulating neuropeptides secretion. It is found that SCFAs can stimulate the sympathetic nervous system (Kimura et al., 2011), promote the secretion of GABA, serotonin, and dopamine (Grider and Piland, 2007; Lyte, 2011), affect angiogenesis and neurogenesis in the brain (Yoo et al., 2011), influence the cognitive process of learning and memory (Levenson et al., 2004; Li W. et al., 2009; Stefanko et al., 2009) and improve memory performance in the novel object recognition task (Yoo et al., 2015). It is well-known that PYY and glucagon-like peptide type 1 (GLP-1) can not only inhibit intestinal motility and improve glucose metabolism, but also induce satisfy feeling and behavior changes. Researchers found that SCFAs can promote the neuropeptide PYY release from intestinal mucosal epithelial type L cells, and increase GLP-1 and GLP-2 production (Holst, 2007; Samuel et al., 2008; Holzer et al., 2012). Study from Li and colleagues indicated that GLP-1 signaling can promote hippocampal neural plasticity and improve memory function (Li et al., 2012). Besides promoting neuropeptides secretion, SCFAs also play a role in neuron proliferation and differentiation. For instance,

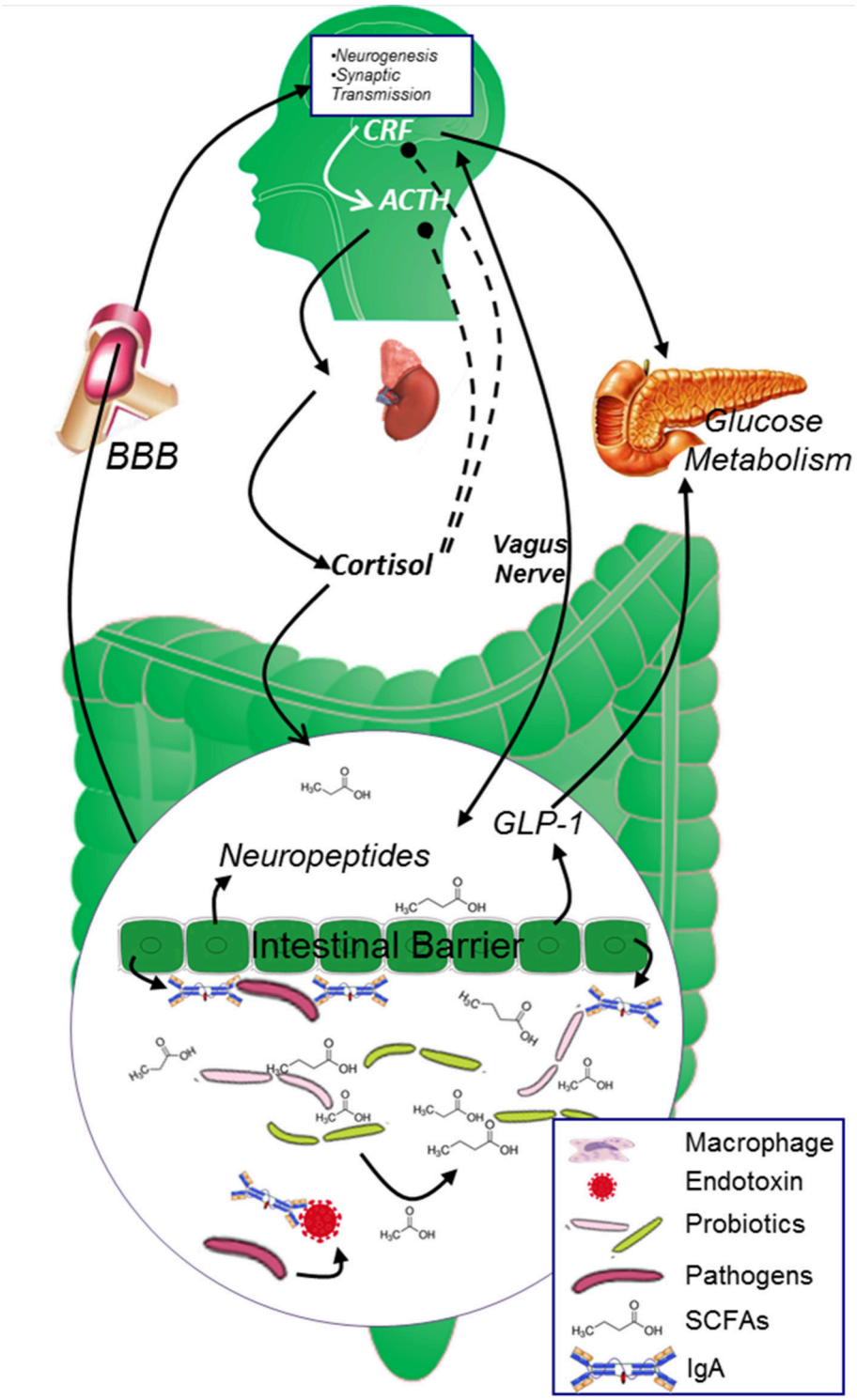

FIGURE 2 | The two-way cross-talk of MGBA. In healthy population, the glucose metabolism is strictly controlled by both neuropeptides originated from the brain and endocrine factors secreted from the digestive system. CRF and cortisol modulates the secretion of insulin from islet β cells and maintains blood glucose at reasonable level. Microbiota in the gut digest dietary fiber and "co-metabolize" with gut epithelial cells to keep gut epithelial cell barrier and promote the secretion of neuropeptides, SCFAs originated from Microbiota and neuropeptides generated from the epithelial cells will further circulate to the brain and affect brain function. ACTH, adrenocorticotrophin hormone; AGEs, advanced glycation end products; BBB, blood brain barrier; CRF, corticotropin releasing factor; GABA, γ-aminobutyric acid; ROS, reactive oxygen species; SCFAs, short chain fatty acids.

SCFAs can directly increase the expression of brain-derived neurotrophic factor (BDNF) and glia-derived neurotrophic factor (GDNF) and inhibit histone deacetylase (HDAC) (Wu et al., 2008).

(4) Hippocamp preserving effects: By investigation on the relation between dietary factors and microbiota (Noble et al., 2017), the influence of microbiota on cognition health has been further defined (Fröhlich et al., 2016). Evidences from animal (Kanoski and Davidson, 2011) and human (Baym et al., 2014) have observed the positive relation between SCFAs withdrawn and the impairment of hippocampal-dependent learning and memory function as early as 3 days (Kanoski and Davidson, 2010) after the experiment beginning.

Due to the important role of SCFAs, it has become a hot research topic in the field of diabetes, and some scholars indicated that the co-metabolism between microbiota and the organism may be a potential target for drug treatment and design (Huang et al., 2016; Obrenovich et al., 2016).

Dysbacteriosis Induced Endotoxemia Leads to Inflammatory State of the Body and Brain

In 2007, Remy Burcelin research group from France firstly proposed that "endotoxemia" originated from intestinal flora is an important factor that triggers inflammatory responses in type 2 diabetes (Cani et al., 2007a). They observed that the composition of intestinal flora in diabetic mice is significantly changed compared with normal mice, beneficial bacteria (e.g., *Bifidobacterium*) which plays a pivotal role in protecting gut barrier is strikingly decreased, the intestinal permeability is increased, and blood toxins (e.g., LPS) are significantly increased (Cani et al., 2007b). Exogenous LPS administration not only lead to weight gain in mice, but also increase the levels of other inflammatory factors in animal (Cani et al., 2007a); and once the animals are treated with antibiotics, with the composition of intestinal flora changes, the intestinal permeability is restored, blood endotoxins are decreased, and the body's inflammatory response is ameliorated (Cani et al., 2008). Although the mechanism of intestinal flora mediated "endotoxemia" is not fully understood, the two interrelated factors may be involved: (1) intestinal dysbacteriosis induced production of endotoxins and inflammatory cytokines, and (2) intestinal dysbacteriosis induced intestinal epithelial permeability increase.

Diabetes has been recognized as a low-grade inflammatory disease, and gut dysbacteriosis will appear under this setting. Inflammatory cells play a pivotal role against gut-derived bacterial products entering the blood circulation. Reports revealed that LPS can stimulate the activation of inflammatory cells (Steenbergen et al., 2015). On the other hand, gut microbiota can also directly stimulate the generation of inflammatory cytokines (Heumann et al., 1994). It is found that intravenous injection of LPS to healthy humans will increase serum levels of inflammatory cytokines and cortisol and decrease memory performance (Krabbe et al., 2005). The findings that inflammatory cytokines are able to reach the brain (Schedlowski et al., 2014) further demonstrate these cytokines may be an important regulator between gut-brain cross-talk. Studies demonstrated that cytokines can increase the expression of serotonin and GABA within the hippocampus (Wang et al., 2012; Jin et al., 2013), thus inhibit brain function. Clinical reports have indicated a positive association between inflammatory cytokines and cognitive decline (Sellbom and Gunstad, 2012). In fact, SCFAs have been demonstrated to inhibit diabetic inflammation. It is found that butyrate has anti-inflammatory actions by preventing LPS-induced activation of inflammatory cells and suppresses nuclear translocation of NF-κB (Segain et al., 2000).

Dysbacteriosis Induced Intestinal Barrier Dysfunction

An important mechanism of microbiota-mediated GBA lies in its effects on intestinal barrier, mainly including the cell barrier and immune barrier. Patients with dysbacteriosis have been observed with higher intestinal permeability, while healthy population without microbial alterations did not (Leclercq et al., 2014), indicating the importance of microbiota in preserving gut barrier.

(1) Cell barrier: The cell barrier is mainly composed of enterocytes and tight junctions between neighboring cells (Gallo and Hooper, 2012). Toll like receptors (TLRs) involves in the proliferation and repair process of intestinal epithelial cells (Rakoff-Nahoum et al., 2004). A most recent study reported that microbiota can also affect the activation of TLRs (Caesar et al., 2015), showing the importance of microbiota on the integrity of epithelial cell. LPS and pro-inflammatory cytokines have been demonstrated to down-regulate tight junctions expression and cause disruption of the gut barrier (Al-Sadi et al., 2009, 2014; Guo et al., 2016). The importance of microbiota on protecting the intestinal barrier has been verified in human patients. Previously, a study showed that when the hepatic encephalopathy patients are administrated with oral antibiotics, their brain dysfunction can be ameliorated (Morgan, 1991), indicating antibiotics treatment rebalances the composition and enhances the integrity of intestinal barrier and BBB, thus decreases the permeability of harmful substances across gut epithelial cells and BBB. Recently, there is a research demonstrated that butyrate can stabilize hypoxia-inducible factor (HIF; Kelly et al., 2015), which is critical for preserving gut barrier integrity.

(2) Immune barrier: Numerous immune cells in the gut lumen play a key role in defending the body against invading bacteria (Gallo and Hooper, 2012). Studies in sterile animals demonstrated that the microbiota is crucial for the occurrence of gut associated lymphoid tissue (GALT), which plays an pivotal role in the normal secreting of immunoglobulin IgA and effective controlling of the inflammatory response (Quigley, 2008).

Gut Dysbacteriosis Increased the Permeability of BBB

The direct influence of microbiota on BBB has not yet been fully explored. But findings have indicated the importance of normal gut flora on the stability of BBB. Recently, Braniste et al. (2014) demonstrated that gut microbiota plays an important role in modulating the integrity of BBB; they observed that the permeability of BBB is strikingly increased in germ-free mice, and this is attributed to the down-regulation of tight junction proteins (e.g., occludin and claudin 5) in the brain endothelial cells; once the GF mice are "conventionalized" with flora from pathogen-free mice, the integrity of the BBB is dramatically enhanced, and the authors attributed this to microbiota metabolites, i.e.,: SCFAs. A study from Kanoski et al. (2010) found in rats that low-fiber diet will increase the leaky of BBB in the hippocampus by reducing the tight junction proteins expression; and BBB damage was positively associated with cognitive impairment (Davidson et al., 2012), strongly indicating the importance of microbiota and its metabolic products SCFAs on the integrity of BBB. Besides the effects of SCFAs as discussed above, it is found that SCFAs can also increase the electrical resistance on the epithelial cells and decrease the paracellular permeability (Suzuki et al., 2008); however, the role of SCFAs on ionic charge on BBB still needs to be clarified, the exact mechanisms underlying SCFAs-modulated BBB integrity remains unknown, and study in this area may lead to a new direction toward the treatment of neuro-dysfunction diseases.

Gut Dysbacteriosis Related with Insulin Resistance

Insulin, produced in pancreatic beta cells, plays a central role in modulating blood glucose metabolism, and insulin resistance is one of the characteristic of diabetes. Circulating insulin can cross the BBB and insulin receptors are found to be expressed in synapses (Zhao and Alkon, 2001) and particularly concentrated in the hippocampus (Havrankova et al., 1978). Recent studies revealed insulin signaling in the central nervous system participated in the cognition and neuronal plasticity (Biessels and Reagan, 2015). A research demonstrated that SCFAs deficiency can induce peripheral insulin resistance and cognition impairment in both animals (Gao et al., 2015) and humans (Rönnemaa et al., 2008). The mechanisms are complex. It is found that insulin can activate α-amino-3-hydroxy-5-methyl-4- isoxazolepropionic acid (AMPA) receptors and leads to increased hippocampal long-term potentiation (LTP; Adzovic and Domenici, 2014). Another mechanism involving insulin modulated cognition is via inflammation pathway. Besides possessing peripheral anti-inflammatory effects against endotoxin (Jeschke et al., 2004), recent observation indicated insulin in the central nervous system can attenuate brain inflammation and preserve memory function (Adzovic et al., 2015).

Due to the above important findings, healthy microbiota transfer has been experimentally applied to patients with insulin resistance and received satisfactory outcome (Vrieze et al., 2012). This is demonstrated in low-fiber diet fed rats that antibiotic treatment to these rats helps to improve insulin sensitivity (Suárez-Zamorano et al., 2015) by depleting bad micribiota in the gut.

CROSS-TALK BETWEEN MICROBIOTA AND HPA AXIS IN THE DEVELOPMENT OF DCI

As discussed above, the development of diabetes is dually controlled and regulated by neuroendocrine factors and digestive system. The most important regulatory pathway of human neuroendocrine pathway is hypothalamic-pituitary-adrenal (HPA) axis (Dinan et al., 2006). It was found that HPA is excessively activated in germ-free animal (Sudo et al., 2004). As well known, corticotropin releasing factor (CRF) released from the hypothalamus plays a key role in the HPA axis. More recently, a research reported that diabetes can lead to hyperactivity of the HPA axis and cause functional hypercortisolism (Tirabassi et al., 2016). In fact, CRF has now been believed to be a messenger that modulates microbiota-gut-brain axis (MGBA) (Holzer and Farzi, 2014). Studies found that in germ-free animals the body's CRF and adrenocorticotrophin hormone (ACTH) levels are significantly increased, while brain-derived neurotrophic factor (BDNF) and N-methyl-D-aspartic acid receptor subtypes 2α (NMDAR-2α) are strikingly reduced (Sudo et al., 2004; Crumeyrolle-Arias et al., 2014), indicating microbiota plays an important role in modulating HPA axis and in the process of the neural development. Recent studies indicate hypercortisolism under diabetic state may exacerbate dysbacteriosis-attributed DCI.

CRF includes large family protein peptides, mainly including CRF, Urocortin 1 (UCN1), UCN2, and UCN3, etc. CRF family peptides mainly function through their receptors, namely CRF-R1 and CRF-R2, in which UCN and CRF can simultaneously bind with the two receptor types while UCN3 and UCN2 are highly selective for CRFR2. It has been well studied that CRF family peptides have a wide function on the organism. Recently, studies found that they play an important role in the regulation of diabetes and its complications. For instance, they can inhibit the apoptosis of pancreatic islet cells (Blaabjerg et al., 2014), regulate the release of insulin from islet β cell (van der Meulen et al., 2015), and adjust glucose uptake and utilization of target cells and organs (Chen et al., 2006; Roustit et al., 2014). In the whole animal study, CRF family peptides are found to have a positive effect on diabetes and its complications. Previously, research observed that UCN1 can decrease the content of AGEs in diabetic animals, ameliorate plasma levels of creatinine, and urea nitrogen, reduce the accumulation of glomerular extracellular matrix in the kidney, inhibit the expression of TGF-1β and VEGF, and improve renal injury (Li et al., 2008; Li X. et al., 2009). Recently, it is found that UCN1 can ameliorate diabetic cardiomyopathy via Akt/GSK-3β pathway (Liu et al., 2015). Therefore, converging lines indicate that CRF family peptides play a protective role in the development of diabetes. However, concerning the development of DCI, a depressive conclusion may be

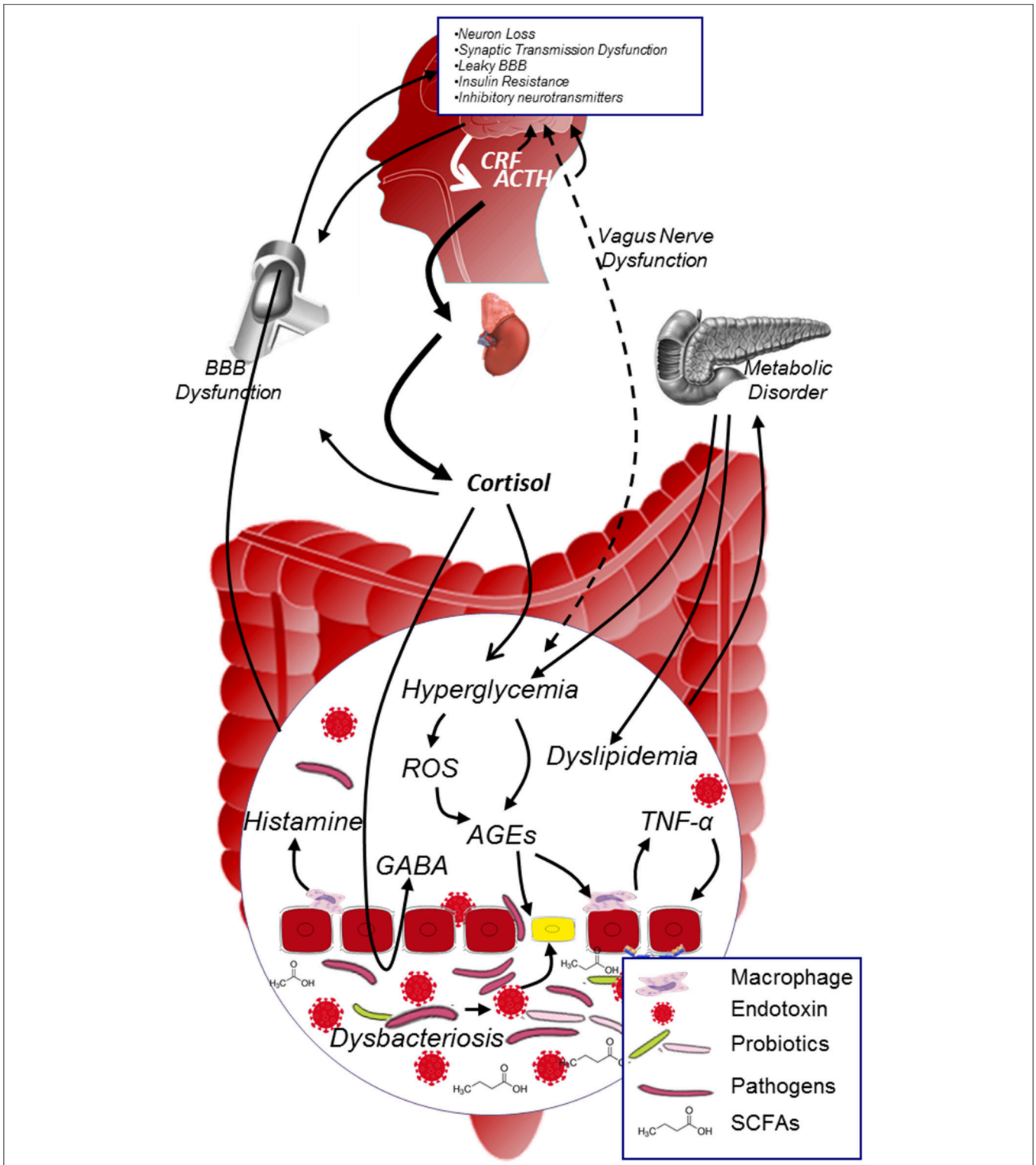

FIGURE 3 | MGBA modulated DCI. In diabetic population, metabolic disorder and hypercortisolism will exacerbate hyperglycemia, promote the generation of AGEs and inflammatory cytokines in the internal environment; on the other hand, dysbacteriosis in the gut will dramatically increase the production of endotoxin and decrease the level of SCFAs. Both dysbacteriosis and internal environment disorder will lead to the intestinal barrier and BBB dysfunction, facilitate harmful substance (e.g., AGEs, endotoxin, pathogens etc.) to access to neurons, and thus contribute to the development of DCI. ACTH, adrenocorticotrophin hormone; AGEs, advanced glycation end products; BBB, blood brain barrier; CRF, corticotropin releasing factor; GABA, γ-aminobutyric acid; ROS, reactive oxygen species; SCFAs, short chain fatty acids.

drawn, and this conclusion can be traced from the following clues:

Hypercortisolism Contributed Microbiota Changes

A most recent finding observed that diabetes can lead to hyperactivity of the HPA axis and cause hypercortisolism (Tirabassi et al., 2016). Hypercortisolism can result in a series of dysfunction of or damage to the body, including the composition changes of microbiota and cognitive dysfunction. Evidence from a recently published research showed that the changes of HPA axis in autism patients can lead to a particular structure and composition transform of microbiota, and the extent of this change is closely related to the severity of the disease (Mayer et al., 2014). Another study showed that 2 h of the environmental stress can change the microbiota (Galley et al., 2014), indicating the important function of CRF family peptides on the microbiota. On the other hand, diabetic dysbacteriosis leads to the dysfunction of intestinal barrier and BBB and promotes the development of DCI, hypercortisolism should obviously facilitate effects of dysbacteriosis on DCI.

Hypercortisolism Aggravates Intestinal Barrier Abnormal

As discussed above, the intestine and microbiota play an important role in the development of diabetes. CRF is a neuropeptide which is closely related to the initiation of stress. Studies found that the activation of peripheral CRF receptors may lead to stress-associated physiological function changes of the intestine (Kiank et al., 2010). Amounts of evidences indicate that the integrity of the intestinal epithelium, intestinal mucosal immune system, and microbiota composition can be affected by the CRF family peptides. The activation of CRF receptors can directly cause the damage of intestinal barrier function (Söderholm et al., 2002), increase the trans-intestinal epithelium ability of the bacteria, and promote the infiltration of inflammatory cells in the intestinal lamina (Rhee et al., 2009). Previous study also observed that the activation of CRF receptors can increase the permeability of the blood vessels and induce tissue edema via the activating mast cells and histamine H1 receptor (Wu et al., 2006).

Hypercortisolism Leads to the Secretion of Inhibitory Neurotransmitter

The balance between excitatory and inhibitory neurotransmitters is very important in maintaining the normal function of brain. Studies found that hypercortisolism induced imbalance of microbiota may promote the abnormal secretion of neurotransmitter such as serotonin (also known as 5-HT) (Diaz Heijtz et al., 2011), γ-aminobutyric acid (GABA), and histamine (Saulnier et al., 2013) et al., and finally results in anxiety symptoms.

Study concerning the role and mechanism of HPA on DCI is rare. Evidence from existed references depicts us with an image that the involvement of HPA on DCI is very complex: it is beneficial to blood metabolism on the one hand, and hypercortisolism is harmful to brain function on the other hand. Therefore, strictly controlling the stable state of HPA is very important, and study concerning this area may bring us with new understandings of DCI.

Converging lines have suggested that MGBA plays a role in modulating DCI (**Figure 3**). On the one hand, microbiota possess various effects on protecting the organism: it enhances the integrity of the gut epithelial cells, limits the low grade inflammation in many chronic disease, improves the glucose metabolism, reduces the oxidative stress and AGEs production, mediates the secretion of neuropeptides, influences the neurogenesis, and affects the activation of HPA. On the other hand, HPA plays a key role in regulating the neuroendocrine pathway: it interacts with microbiota (although the specific mechanism deserves to be elucidated), participates in the stress process, regulates glucose metabolism, and affects the permeability of blood vessels etc. The interaction between microbiota and HPA is complex, the balance between these two factors is pivotal in maintaining normal function of the body. Investigating microbiota-HPA mediated MGBA should have great impact on understanding the development of DCI.

NERVE CONNECTION BETWEEN GUT AND BRAIN

Vagus nerve, which is widely distributed in the gut, has also been well-established as a connection among microbiota, the peripheral nervous system, and the central nervous system (Layé et al., 1995; Forsythe and Kunze, 2013). It is observed that gut microbiota can directly activate the enteric nervous system (Furness, 2012) and further transmit information into brain via vagus nerve. Besides this "by-pass" pathway, vagus nerve endings also express receptors for inflammatory cytokines including IL-1 and prostaglandins (Ek et al., 1998), therefore can be directly activated by gut-derived inflammatory cytokines. However, studies concerning this area are relatively few, and further investigations deserve to be carried out to unveil the direct connection between microbiota and brain in this aspect of view.

CONCLUSION

With an aging population worldwide, the incidence of diabetes and diabetic complications has dramatically increased. One of an important complication accompanied with diabetes is cognitive impairment. Evidences within this decade strongly suggest that risk factors such as oxidative stress, inflammation, and AGEs, which accounts for the development of diabetes, may also seriously affect brain function. The most recent studies concerning gut microbiota elucidated one more possible mechanism on the progression of DCI. From the discussions above, we may predict that intervention targets such as reducing ROS and AGEs generation, inhibiting over-inflammation impairment or re-balancing gut-sourced health-preserving effects etc. should help to delay the development of DCI. In view of irreversible development of diabetes, early intervention and therapeutic strategies targeting the gut microbiota may be effective on reducing or ameliorating the progression of DCI.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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Protein Tyrosine Phosphatase 1B (PTP1B): A Potential Target for Alzheimer's Therapy?

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Despite significant advances in current understanding of mechanisms of pathogenesis in Alzheimer's disease (AD), attempts at drug development based on those discoveries have failed to translate into effective, disease-modifying therapies. AD is a complex and multifactorial disease comprising a range of aberrant cellular/molecular processes taking part in different cell types and brain regions. As a consequence, therapeutics for AD should be able to block or compensate multiple abnormal pathological events. Here, we examine recent evidence that inhibition of protein tyrosine phosphatase 1B (PTP1B) may represent a promising strategy to combat a variety of AD-related detrimental processes. Besides its well described role as a negative regulator of insulin and leptin signaling, PTB1B recently emerged as a modulator of various other processes in the central nervous system (CNS) that are also implicated in AD. These include signaling pathways germane to learning and memory, regulation of synapse dynamics, endoplasmic reticulum (ER) stress and microglia-mediated neuroinflammation. We propose that PTP1B inhibition may represent an attractive and yet unexplored therapeutic approach to correct aberrant signaling pathways linked to AD.

Keywords: Alzheimer's disease, protein tyrosine phosphatase 1B, diabetes, synaptic plasticity, neuroinflammation, insulin signaling, leptin signaling, endoplasmic reticulum stress

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INTRODUCTION

There are currently no disease-modifying therapies for Alzheimer's disease (AD), and treatments offer limited, temporary improvement in quality of life (Rafii and Aisen, 2015). This scenario drives scientists and pharmaceutical companies into an intense search for effective therapies for AD. Unfortunately, most if not all therapeutics that showed promise in preclinical models failed to translate into effective therapies, as evidenced by numerous unsuccessful clinical trials. Because AD comprises a broad range of deregulated processes taking place concomitantly, drugs acting on multiple aberrant processes hold promise as candidates for AD therapeutics. Herein, we discuss recent evidence indicating that protein tyrosine phosphatase 1B (PTP1B) inhibitors fulfill this criterion.

A pivotal event in AD pathogenesis is the buildup in the brain of amyloid-β oligomers (AβOs), neurotoxins that trigger synapse failure and lead to cognitive impairment (Ferreira and Klein, 2011; Ferreira et al., 2015; Selkoe and Hardy, 2016). In neurons, AβOs attack synapses (Lacor et al., 2004), altering membrane receptor composition

(Lacor et al., 2007; De Felice et al., 2009; Jürgensen et al., 2011), impairing synaptic plasticity (Lambert et al., 1998; Walsh et al., 2002), and ultimately leading to synapse loss. Several signaling pathways germane to learning and memory are affected by AβOs. Some of those are initiated by receptor tyrosine-kinases (RTKs) such as the insulin receptor (IR; De Felice et al., 2009; Ma et al., 2009), the leptin receptor (LepR; Marwarha et al., 2011; Maioli et al., 2015) and the brain-derived neurotrophic factor (BDNF) receptor, TrkB (Tong et al., 2004; Echeverria et al., 2007). Additionally, AβOs activate microglia, triggering exacerbated release of proinflammatory cytokines implicated in memory impairment and mood alterations in mouse models of AD (Ledo et al., 2013, 2016).

PTP1B emerged recently as a regulator of a variety of processes within the central nervous system (CNS), many of which therapeutically relevant for AD. Increased PTP1B activity is associated with defective neuronal insulin and leptin signaling (Zabolotny et al., 2002; Pandey et al., 2013, 2014), pathways that are impaired in AD (Bomfim et al., 2012; Bonda et al., 2014). Significantly, down-regulation of PTP1B restores hypothalamic insulin and leptin signaling (Chiarreotto-Ropelle et al., 2013; Lindtner et al., 2013; Pandey et al., 2013, 2014; Yu et al., 2013). PTP1B down-regulates neuronal BDNF-TrkB pathway, whereas PTP1B inhibition boosts BDNF signaling (Ozek et al., 2014; Krishnan et al., 2015). Importantly, mice lacking PTP1B in the hippocampus and cortex displayed improved performance in the Barnes maze (Fuentes et al., 2012), posing this phosphatase as a negative regulator of spatial memory. PTP1B negatively regulates hippocampal store-operated calcium entry (nSOC; Koss et al., 2013), an essential process for the stabilization of mushroom spines that is impaired in transgenic AD mice (Sun et al., 2014; Zhang H. et al., 2015). Furthermore, PTP1B is up-regulated by endoplasmic reticulum (ER) stress (Agouni et al., 2011; Popov, 2012; Hakim et al., 2015), a neuronal response activated by AβOs and implicated in synapse loss and cognitive decline in AD (Kam et al., 2013; Lourenco et al., 2013). Finally, PTP1B is highly expressed in hippocampal microglia (Pei et al., 1994), and was recently described as a positive regulator of neuroinflammation (Song et al., 2016).

In the following sections, we review evidence suggesting that PTP1B modulates several CNS processes relevant to the physiopathology of AD, making it an attractive target to be explored in AD pharmacotherapy. Inhibiting PTP1B appears as a promising, yet neglected strategy to combat multiple aspects of AD.

INSULIN SIGNALING

Insulin signaling is initiated by activation of IR autophosphorylation at tyrosine residues upon insulin binding (Guo, 2014). The immediate effectors IRS-1 and IRS-2 (IR substrate 1 and 2) are then recruited and activated by tyrosine phosphorylation to propagate intracellular signaling (Guo, 2014). PTP1B dephosphorylates tyrosine residues in IR and IRS-1 (**Figure 1**), reducing insulin sensitivity and shutting down signaling (Goldstein et al., 2000; Bakke and Haj, 2015). PTP1B deficient mice are hypersensitive to insulin and present low basal glycemia and insulinemia (Elchebly et al., 1999), and inhibiting PTP1B improves insulin signaling and reverses T2D phenotypes (Malamas et al., 2000; Zinker et al., 2002; Gum et al., 2003; Tamrakar et al., 2014). Conversely, exacerbated PTP1B activity underlies insulin resistance in T2D (Zabolotny et al., 2002; González-Rodríguez et al., 2010).

An epidemiological correlation between AD and T2D exists, with each disease increasing the risk of developing the other (Ott et al., 1996; Craft and Watson, 2004; De Felice, 2013a,b). However, the mechanisms underlying this connection remain elusive. A breakthrough discovery that contributed to current understanding of such mechanisms was that neurons exposed to AβOs become insensitive to insulin (Zhao et al., 2008; De Felice et al., 2009). Further studies showed that AβOs impair insulin signaling by increasing IRS-1 inhibitory serine phosphorylation and decreasing activating tyrosine phosphorylation (Bomfim et al., 2012). Importantly, defective insulin signaling was confirmed in *post mortem* AD brains (Bomfim et al., 2012; Talbot et al., 2012). Conversely, boosting insulin signaling protects synapses against AβOs toxicity (De Felice et al., 2009). These discoveries paved the way for a whole new aspect of AD, which has provided important advances of therapeutic relevance. For instance, anti-diabetic drugs developed to treat insulin resistance in T2D have shown promising preclinical results, protecting synapses, preventing inhibition of IRS-1 and, most importantly, ameliorating cognitive phenotypes in animal models of AD (McClean et al., 2011; Bomfim et al., 2012; Hansen et al., 2015; Qi et al., 2016). Those studies have provided molecular grounds for on-going clinical trials aimed at testing the efficacy of intranasal insulin and glucagon-like peptide 1 (GLP-1) analogs in AD (De Felice and Ferreira, 2014).

Thus, it seems reasonable to predict that PTP1B inhibitors—which restore insulin sensitivity in T2D models (Malamas et al., 2000; Zinker et al., 2002; Gum et al., 2003; Panzhinskiy et al., 2013; Tamrakar et al., 2014)—may rescue neurons from defective insulin signaling in AD. Although this hypothesis has not yet been tested directly, there is evidence from non-AD models of neuronal insulin resistance validating PTP1B inhibition as an effective approach to rescue neuronal insulin signaling (Krishnan et al., 2015; Qin et al., 2015a; Zhang Z. Y. et al., 2015).

LEPTIN SIGNALING

In obesity, defective hypothalamic leptin signaling impairs sensing and processing of satiety signals, leading to increased caloric intake and decreased energy expenditure (Halaas et al., 1995; Farooqi et al., 1999; Morton et al., 2006). Ob/Ob mice, which do not produce leptin, exhibit increased food intake and become profoundly obese (Zhang et al., 1994). Leptin signaling is initiated by binding of leptin to LepR, leading to tyrosine autophosphorylation of LepR and subsequent phosphorylation of Janus kinase 2 (JAK2), which propagates downstream intracellular signaling (Iida et al., 1996; Fei et al., 1997).

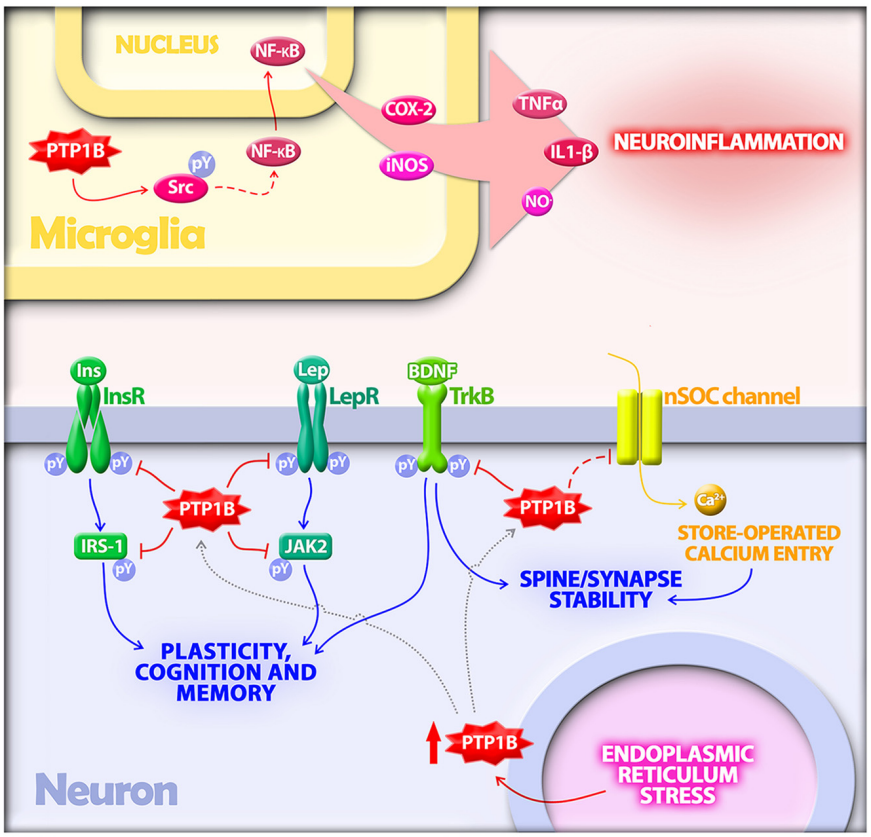

FIGURE 1 | Protein tyrosine phosphatase 1B (PTP1B) regulates multiple mechanisms implicated in the pathogenesis of Alzheimer's disease (AD). In microglia, PTP1B is a positive regulator of neuroinflammation. PTP1B activates Src via dephosphorylation at a negative regulatory site. Src, in turn, indirectly activates NF-κB, a transcriptional regulator of proinflammatory mediators including TNF-α, IL-1β, COX-2 and inducible nitric oxide synthase (iNOS). In neurons, PTP1B is upregulated by endoplasmic reticulum (ER) stress, a cellular response activated by amyloid-β oligomers (AβOs) in AD. Elevated PTP1B inhibits signaling by receptor tyrosine kinases germane to synaptic plasticity, cognition and memory. Substrates for PTP1B in neurons that have been implicated in AD include the insulin receptor (InsR) and its substrate IRS-1, the leptin receptor (LepR) and its immediate downstream effector Janus kinase 2 (JAK2), and the brain-derived neurotrophic factor (BDNF) receptor (TrkB). PTP1B further regulates neuronal store-operated calcium entry (nSOC), a mechanism required for spine/synaptic stability found to be impaired in models of AD.

Strong evidence implicates PTP1B in obesity-associated hypothalamic leptin resistance (Cheng et al., 2002; Zabolotny et al., 2002). PTP1B dephosphorylates LepR and JAK2, functioning as a negative regulator of leptin signaling (**Figure 1**). PTP1B-null mice are resistant to weight gain induced by high-fat diet (HFD) or by deletion of the leptin gene, suggesting PTP1B inhibition as a strategy to rescue leptin signaling in food intake disorders and obesity (Elchebly et al., 1999; Cheng et al., 2002).

Beyond hypothalamic signaling, leptin plays important roles in the CNS. LepRs are highly expressed in the hippocampus (Huang et al., 1996; Mercer et al., 1996; Scott et al., 2009) where leptin signaling is important for cognition and memory (Irving and Harvey, 2014). Aβ down-regulates hippocampal leptin and LepR expression (Marwarha et al., 2010; Bonda et al., 2014). Interestingly, leptin prevents hippocampal synaptic disruption and neuronal death induced by Aβ (Doherty et al., 2013). Leptin also modifies Aβ levels (Fewlass et al., 2004) and reduces tau phosphorylation in neuronal cells (Greco et al., 2008, 2009a,b). Importantly, neuronal leptin resistance has been described in the AD hippocampus (Bonda et al., 2014; Maioli et al., 2015), further underlining the relevance of defective leptin signaling in AD.

Leptin signaling has been proposed as a neuroprotective target in AD (Gomes et al., 2014; Johnston et al., 2014). Because direct administration of leptin or LepR agonists in conditions of leptin resistance may not result in the desired biological effect, a more attractive approach to boost leptin signaling in AD would be to reverse neuronal leptin resistance. The evidence described above suggests that recovery of leptin sensitivity could be achieved by PTP1B inhibitors.

ENDOPLASMIC RETICULUM STRESS

ER stress and activation of the unfolded protein response (UPR) are important toxic mechanisms in AD (Lourenco et al., 2015). We recently demonstrated that AβOs trigger ER stress in hippocampal neurons in a mechanism that requires TNF-

α receptor activation (Lourenco et al., 2013). ER stress triggered by TNF-α has also been linked to peripheral insulin resistance in obesity and diabetes (reviewed in Hotamisligil, 2010).

PTP1B localizes predominantly to the cytoplasmic surface of the ER (Haj et al., 2002) and mediates ER stress signaling (Wang et al., 2009). Downregulation of PTP1B ameliorates ER stress in obesity and diabetes models (Delibegovic et al., 2009; Agouni et al., 2011; Owen et al., 2013). Two of the UPR branches activated upon ER stress involve inositol-requiring enzyme 1 (IRE-1) and activating transcription factor 6 (ATF6). Interestingly, PTP1B potentiates IRE-1-mediated ER stress response and its expression is regulated by ATF6 (Gu et al., 2004; Wang et al., 2009). Moreover, recent evidence links hypothalamic ER stress and activation of the UPR to development of PTP1B-mediated leptin resistance and increased food intake following chronic sleep fragmentation in mice (Hakim et al., 2015).

Collectively, data suggest that PTP1B mediates the toxic consequences of neuronal ER stress (**Figure 1**). It has been hypothesized that PTP1B may be a key link between insulin signaling and ER stress (Popov, 2012). This raises the possibility that PTP1B inhibitors may be able to compensate the detrimental impact of ER stress on synapse stability and cognition in AD.

SYNAPTIC PLASTICITY, STABILITY AND MEMORY

The functions of PTP1B in neurophysiology are gaining traction as novel roles for PTP1B in the brain are discovered. PTP1B has been implicated in a variety of neuronal processes, including some related to synapse biology that are potentially relevant to the pathogenesis of AD.

BDNF is a major regulator of synaptic plasticity. BDNF signaling through its receptor, TrkB, modulates synapse structure and function to produce long-term potentiation (LTP), a form of activity-dependent synaptic plasticity thought to underlie learning and memory (Leal et al., 2015). AD brains display reduced BDNF levels in clinical (Phillips et al., 1991; Connor et al., 1997; Soontornniyomkij et al., 1999) and preclinical disease stages (Peng et al., 2005). Conversely, BDNF is neuroprotective in animal models of AD (Arancibia et al., 2008; Nagahara et al., 2009). Therefore, stimulating BDNF signaling represents an attractive approach in AD therapy.

Recent studies showed that PTP1B downregulates BDNF signaling through dephosphorylation of TrkB (Ozek et al., 2014; Krishnan et al., 2015; **Figure 1**). *Ptpn1*$^{-/-}$ mice are hypersensitive to BDNF, and pharmacological inhibition of PTP1B increases neuronal responsiveness BDNF (Ozek et al., 2014). PTP1B inhibition may, thus, represent a means to enhance BDNF signaling, improving synaptic plasticity and cognition in AD.

PTP1B has further been implicated in hippocampal synapse formation and learning (Fuentes et al., 2012). PTP1B is present in dendritic spines of hippocampal neurons, and functional or genetic PTP1B deficiency affects spine morphology and leads to disorganization of pre- and post-synaptic terminals. Interestingly, mice lacking PTP1B in the hippocampus and cortex exhibit improved performance in the Barnes maze compared to wild-type controls, posing this phosphatase as a negative regulator of spatial memory (Fuentes et al., 2012).

Neuronal store-operated calcium entry (nSOC) is essential for stabilization of mushroom spines in hippocampal neurons (Sun et al., 2014). AD mice exhibit impaired nSOC, which causes destabilization and loss of spines through a mechanism involving overactivation of metabotropic glutamate receptor 5 (mGluR5; Sun et al., 2014; Zhang H. et al., 2015). PTP1B is a negative regulator of hippocampal nSOC (Koss et al., 2013; **Figure 1**), suggesting PTP1B inhibition may restore deficient nSOC.

Collectively, these findings suggest PTP1B inhibition may result in improved synapse plasticity, function and stability, ultimately enhancing cognitive performance.

MICROGLIA-MEDIATED NEUROINFLAMMATION

Chronic neuroinflammation is an important feature of AD (Heneka et al., 2015; Heppner et al., 2015). Evidence for activated microglia has been described in transgenic AD mice (Frautschy et al., 1998; Bornemann et al., 2001) and in AD brains (Cagnin et al., 2001; Edison et al., 2008). Microglia are implicated in cognitive impairment in AD through sustained secretion of neurotoxic cytokines (Wang et al., 2015) and synapse pruning (Hong et al., 2016; Lui et al., 2016). Injection of AβOs in mouse brains induces microglia-mediated neuroinflammation (Xu et al., 2016), leading to memory impairment and mood alterations (Ledo et al., 2013, 2016). Direct activation of microglia by AβOs was recently demonstrated in primary microglial cultures (Ledo et al., 2016).

Activated microglia are the main source of proinflammatory cytokines such as TNF-α and IL-1β in the brain (Wang et al., 2015). TNF-α is implicated in memory impairment caused by AβOs (Bomfim et al., 2012; Lourenco et al., 2013). Activation of TNF-α signaling is associated with inhibition of IRS-1 in hippocampal neurons (Bomfim et al., 2012). TNF-α is also implicated in peripheral insulin resistance in diabetes (Hotamisligil et al., 1993; Hotamisligil and Spiegelman, 1994), and it has been proposed that neuronal insulin resistance induced by TNF-α may underlie the connection between diabetes and AD (De Felice and Ferreira, 2014). IL-1β has been described as a mediator of cognitive impairment associated with peripheral and central inflammation by disrupting hippocampal synaptic plasticity (Di Filippo et al., 2013; Erion et al., 2014). These lines of evidence support dampening microglia-mediated neuroinflammation as an attractive therapeutic approach in AD (Ransohoff, 2016; Santos et al., 2016; Wes et al., 2016).

PTP1B is regulated by proinflammatory signals and is highly expressed in hippocampal microglia in AD (Pei et al., 1994). Interestingly, a recent study unraveled a novel role for PTP1B as a positive regulator of neuroinflammation (Song et al., 2016). PTP1B levels are increased in LPS injected brain, and PTP1B overexpression potentiates microglial responses via dephosphorylation/activation of Src and nuclear

translocation of NF-κB, leading to increased expression of proinflammatory molecules including TNF-α, IL-1β, cyclooxigenase-2 and inducible nitric oxide synthase (iNOS; **Figure 1**). Significantly, LPS-induced neuroinflammation is attenuated by pharmacological PTP1B inhibitors (Song et al., 2016). Conversely, TNF-α increases PTP1B expression via NFκB in adipose tissue of HFD mice (Zabolotny et al., 2008) and in organotypic hypothalamic cultures (Ito et al., 2012), further exacerbating inflammation in a feed-forward mechanism. This suggests that pharmacological inhibition of PTP1B may constitute a therapeutic strategy to counteract neuroinflammation.

PTP1B INHIBITORS AS DRUG CANDIDATES FOR NEUROLOGICAL DISEASES

PTP1B has long been pursued as a therapeutic target in human diseases, particularly in diabetes and obesity (Zhang and Lee, 2003). Multiple PTP1B inhibitors have been developed and tested in preclinical models, validating the concept of PTP1B inhibition as an effective therapeutic approach for diabetes (Tamrakar et al., 2014). Nevertheless, certain structural features of PTP1B complicate the development of small molecule inhibitors with fundamental characteristics required for drug candidates, namely, specificity/selectivity and bioavailabilty. First, the active sites of protein tyrosine phosphatases (PTPs) are highly conserved among the more than 100 family members (Tonks, 2013). Therefore, inhibitors designed to bind to the active site of PTP1B often inhibit other PTPs as well, leading to off-target effects (Tamrakar et al., 2014; Tautz, 2015). Second, most inhibitors developed so far are phosphotyrosine-mimicking molecules bearing a charged group, which drastically affects pharmacokinetics (Tautz, 2015). For those reasons, few PTP1B inhibitors have reached clinical trials, and none have made it through phase II tests (Tamrakar et al., 2014; Tautz, 2015).

In AD, the blood-brain barrier (BBB) represents an additional obstacle drugs need to overcome to reach the CNS. Fortunately, however, significant advances have been achieved in developing effective PTP1B inhibitors with potential for clinical use. For example, an important recent study provided compelling evidence that potent and selective PTP1B inhibitors administered peripherally (e.g., intraperitoneally or subcutaneously) inhibit PTP1B activity in the brain (Krishnan et al., 2015). *MeCP2* deficient mice, a model of Rett syndrome, exhibit increased PTP1B expression, leading to defective brain insulin signaling and impaired glucose metabolism. This was associated with neurological phenotypes in female mice and reduced lifespan in male mice, recapitulating Rett syndrome in humans. Remarkably, long-term systemic treatment with two distinct PTP1B inhibitors, CPT157633 and UA0713, rescued disease phenotypes in *MeCP2* deficient mice. Moreover, PTP1B inhibitors increased tyrosine phosphorylation of TrkB, leading to enhanced signaling in response to BDNF in the forebrains of *MeCP2* deficient mice (Krishnan et al., 2015).

Another recent study showed that intraperitoneal administration of trodusquemine, a selective BBB-permeant (Ahima et al., 2002; Lantz et al., 2010) PTP1B inhibitor, relieved anxiety in *LMO4* knockout mice exhibiting impaired endocannabinoid signaling due to increased PTP1B activity in amygdala (Qin et al., 2015b). Those studies support the feasibility of using peripherally administered PTP1B inhibitors to treat CNS disorders.

CONCLUSION

Compelling recent findings suggest PTP1B holds potential as a therapeutic target in AD. Results indicate that PTP1B regulates distinct CNS responses depending on cell type: while PTP1B modulates insulin and leptin signaling in neurons, it regulates astrocyte differentiation (Yamada et al., 2013) and proinflammatory responses in microglia. Because PTP1B participates in several cellular/molecular processes linked to AD pathogenesis (**Figure 1**), compounds capable of reaching the CNS and inhibiting PTP1B activity in neurons and/or glial cells may rescue multiple aberrant processes associated with cognitive decline and neurodegeneration in AD.

Detailed preclinical studies are warranted to validate the potential benefits of PTP1B inhibition in AD models. Because PTP1B may interfere with pathological mechanisms that operate at different disease stages, it will be important to investigate whether PTP1B inhibition delays, prevents or slows down disease progression or temporarily ameliorates symptoms.

Finally, the above described pathological mechanisms in which PTP1B has been implicated are not exclusively associated with AD, but also with other neurological disorders. This raises the possibility that PTP1B inhibition may be useful for treatment of other brain disorders related to metabolic deregulation, and perhaps even in normal, age-related cognitive decline.

AUTHOR CONTRIBUTIONS

MNNV: development of the subject matter, drafting of the article, conception and design of the figure, critical revision of the article, final approval of the version to be published; NMLS and FGF: development of the subject matter, drafting of the article, critical revision of the article, final approval of the version to be published; STF: discussion of contents, critical revision of the article, final approval of the version to be published.

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