EXPERIMENTAL AND INNOVATIVE APPROACHES TO MULTI-TARGET TREATMENT OF PARKINSON'S AND ALZHEIMER'S DISEASES - VOLUME I

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EXPERIMENTAL AND INNOVATIVE APPROACHES TO MULTI-TARGET TREATMENT OF PARKINSON'S AND ALZHEIMER'S DISEASES - VOLUME I

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EDITORIAL
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Editorial: Experimental and Innovative Approaches to Multi-Target Treatment of Parkinson's and Alzheimer's Diseases

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Editorial on the Research Topic

Experimental and Innovative Approaches to Multi-Target Treatment of Parkinson's and Alzheimer's Diseases

Alzheimer's disease (AD) and Parkinson's disease (PD) are incurable and the most common neurodegenerative disorders. Current methods for AD and PD treatment are mostly symptomatic, while a new effective pathogenesis-relevant therapy that would block the disease course and restore all the compromised functions is demanded. AD and PD pathogeneses are largely associated with the accumulation of neurotoxic protein aggregates in the brain, i.e. toxic forms of amyloid-beta (Aβ), alpha-synuclein, and tau protein (Selkoe and Hardy, 2016; Rocha et al., 2018). Hence, enhancing the pathological protein elimination has attracted increasing attention. In June 2021, the FDA approved the drug Aducanumab (brand name-Aduhelm) based on a monoclonal antibody against amyloid. However, the introduction of this drug into widespread clinical practice raises certain skepticism (Knopman and Perlmutter, 2021), mainly due to the uncertain therapeutic effect or clinical benefit. There is also a concern about targeting amyloid or alpha-synuclein directly since Aβ precursor protein and alpha-synuclein are both involved in normal physiological function (Dawkins and Small, 2014; Nellikka et al., 2021) and thus their content should not fall below the critical level. Failure of clinical trials of "Aβ-oriented" drugs may also be related to their use at the late stages of AD, whereas these agents could be effective when pathological aggregation of Aβ just begins preceding the initial signs of cognitive impairment in patients for at least 10–20 years (Frozza et al., 2018). Moreover, neurodegenerative disorders have a multifactorial etiology and involve various pathological processes in addition to neurotoxicity of protein aggregates, such as oxidative stress, neuro-inflammatory response, disturbed neurotrophic function and neurogenesis, synaptic and neurotransmission dysfunction, ion disbalance, etc. that often closely interact and overlap. Therefore, researchers develop multipurpose drug combinations (combination-drugs-multi-targets, CDMT) that do not cause adverse side effects (Sahoo et al., 2018). Multipurpose therapy aimed at various important pathogenetic hubs is a novel trend regarded as a promising strategy for AD and PD therapy.

Currently, a number of research teams work within the CDMT strategy. For example, a recent study from Japan reported on the prevention of neurodegenerative dementia by intranasal rifampicin and resveratrol combination in mice (Umeda et al., 2021). Series of studies revealed that

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antibiotic drug ceftriaxone within a strategy of drug reprofiling produces neuroprotective effects both in AD and PD models through suppressing the glutamate-induced excitotoxicity, modulating the expression of genes related to Aβ metabolism, enhancing neurogenesis, attenuating neuro-inflammatory response, and recovery of neuronal density (Ho et al., 2014; Weng et al., 2016; Tikhonova et al., 2017, 2018). Moreover, a combination of ceftriaxone with erythropoietin allowed reducing the dosage of ceftriaxone by 20 times with maintained efficiency in a PD model (Huang et al., 2015). Finally, the promising results of clinical trials of phase III in China of GV−971 for treating people with mild to moderate AD were reported recently (Xiao et al., 2021). GV-971 is an oligosaccharide derived from marine organisms that affects such pathogenetic mechanisms of AD development as inhibition of Aβ fibril formation, neuroinflammation, and recondition of dysbiosis of gut microbiota (Martins et al., 2020; Ettcheto et al., 2021).

The aim of this Research Topic was to provide an updated overview on the approach of multi-targeted therapy for AD and PD and related issues. Several research groups contributed interesting points of view on this subject and elucidated important current aspects of the problem.

The central theme of the Topic is masterly illustrated by Reich and Hölscher who provided an accurate review of acylated ghrelin as a multi-targeted therapy for AD and PD. The review illustrates the wide-ranging neuroprotective properties of the acylated form of the hormone ghrelin and discusses its potential to ameliorate pathologic changes occurring in AD and PD as well as complications of long-term treatment with the drug.

Tikhonova et al. contributed an original work in which multiple neuroprotective effects of antibiotic ceftriaxone against AD-like pathology are discussed, mainly focusing on mechanisms related to Aβ burden and neuro-inflammatory response.

Komleva et al. reviewed the pathological role of inflamm-aging, brain insulin resistance, and their cross-talk in aging and neurodegeneration. The review summarizes current knowledge on immunosenescence, inflamm-aging, and metainflammation and discusses potential mechanisms of calorie restriction as multi-purpose approach that may effectively break the vicious cycle of metainflammation, improve insulin resistance and slow the onset of neurodegeneration.

Studies on PD have mostly focused on processes and targets in the central nervous system. In clinical practice, neuromodulation techniques such as deep brain stimulation (DBS) are applied to control drug-resistant symptoms of PD. In a case report by Chang et al., a method of bilateral globus pallidus interna (GPi) combined with subthalamic nucleus (STN) variable frequency DBS (bSGC-DBS) implantation was introduced. The case of a young-onset PD patient with refractory dyskinesia explores multi-electrode and multi-target stimulation for the treatment of dystonia disorders.

On the other hand, a review by Ma et al. emphasizes the importance of the peripheral nervous system (PNS) in PD pathology. The paper discusses the use of pathological changes in PNS for clinical diagnosis of PD as well as the application of PNS targets for PD therapy, namely Schwann cell transplantation in the treatment of PD animal models is described. Wichit et al. contributed an original clinical work in which monoamine levels in peripheral body fluids were analyzed in association with clinical profiles in PD patients. The paper points to the involvement of several neurotransmission systems in PD pathology. An original research article by Seo and Yeo focuses on the alterations in muscle proteins that could impair muscle function and add to the bradykinesia and tremor in a pharmacological 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced mouse model of PD.

Two groups contributed original research papers on the effects of electroacupuncture in mouse AD models and discussed underlying mechanisms. Xu et al. focused on the regulation of phospho-tau and glucose metabolism associated with the Akt/GSK3β signaling pathway while Xie et al. concentrated on M2 microglia polarization and glia anti-inflammation.

And last but not least, Ahanger et al. reviewed the role of monosodium glutamate in protein aggregation through a biophysical approach and discussed its potential impact on neurodegeneration.

Taken together, the papers collected in this Issue present the most recent knowledge and experimental evidence about the multi-target approach for therapy of neurodegenerative disorders and offer a new perspective and interesting hypotheses on this topic.

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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ORIGINAL RESEARCH
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Electroacupuncture Protects Cognition by Regulating Tau Phosphorylation and Glucose Metabolism via the AKT/GSK3β Signaling Pathway in Alzheimer's Disease Model Mice

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Background: Alzheimer's disease (AD) is mainly manifested as a continuous and progressive decline in cognitive ability. Neurofibrillary tangles (NFTs) are pathological hallmarks of AD and due to accumulated phosphorylated Tau. Glycogen synthase kinase-3β (GSK3β), as a major Tau kinase and a downstream target of the serine protein kinase B (AKT) signaling pathway, can regulate Tau phosphorylation in AD. Importantly, the AKT/GSK3β signaling pathway is involved in glucose metabolism, and abnormal glucose metabolism is found in the AD brain. Numerous studies have shown that electroacupuncture (EA), which is thought to be a potential complementary therapeutic approach for AD, can protect cognitive ability to a certain extent.

Objective: The purpose of this experiment was to investigate whether the protective and beneficial mechanism of EA on cognition was mediated by the AKT/GSK3β signaling pathway, thereby improving glucose metabolism and Tau phosphorylation in the brain.

Methods: EA was applied to the Baihui (GV20) and Yintang (GV29) acupoints of 6-month-old amyloid precursor protein (APP)/presenilin-1 (PS1) mice for 20 min, and then quickly prick Shuigou (GV26) acupoint. The intervention was performed once every other day for 28 days. The Morris water maze (MWM) test was performed on C57BL/6N (Non-Tg) mice, APP/PS1 (Tg) mice and EA-treated Tg (Tg + EA) mice to evaluate the effect of EA therapy on cognitive function. ^{18}F-FDG positron emission tomography (PET), immunohistochemistry, and western blotting (WB) were used to investigate the possible mechanism underlying the effect of EA on AD.

Results: EA treatment significantly improved the cognition of APP/PS1 mice and the glucose uptake rate in the hippocampus. Furthermore, EA inhibited the phosphorylation of Tau (Ser199 and Ser202) proteins by inducing AKT (Ser473) and GSK3β (Ser9) phosphorylation.

Conclusion: These results demonstrate that EA intervention protects cognition by enhancing glucose metabolism and inhibiting abnormal phosphorylation of Tau protein in the AD model mice, and the AKT/GSK3β pathway might play an irreplaceable role in the regulation process.

Keywords: Alzheimer's disease, electroacupucnture, cognition, glucose metabolism, tau, AKT/GSK3β pathway

INTRODUCTION

According to the World Alzheimer Report 2018, there is one new case of dementia every 3 s across the world. Fifty million people worldwide were living with dementia in 2018, but this number will more than triple to 152 million by 2050. A systematic analysis of the global burden of disease found that dementia was the fastest-growing cause of death between 2005 and 2015, increasing by 40% (GBD 2015 Mortality and Causes of Death Collaborators, 2016). The main clinical manifestations of Alzheimer's disease (AD) are progressive episodic memory disorder, cognitive dysfunction, and decreased ability in activities of daily living. However, treating AD remains challenging. There are currently two types of drugs available, cholinesterase inhibitors and NMDA receptor antagonists, both of which aim to treat some of the symptoms of AD but cannot prevent the progression of the disease (Alzheimer Disease Agents, 2012). AD has become a growing public health problem, and effective treatments are still lacking.

Pathologically, AD is characterized by amyloid plaques and neurofibrillary tangles (NFTs), the main components of which are amyloid-β (Aβ) peptide and Tau protein, respectively, in the brain (Guillozet et al., 2003). Previously, the development of AD therapy mainly focused on Aβ, while Tau has attracted more attention in recent years because of the neurotoxicity of its hyperphosphorylated form (Giacobini and Gold, 2013; Slomski, 2018). Glycogen synthase kinase-3β (GSK3β) is a major Tau kinase. Substantial evidence has shown that GSK3β, which functions as a downstream target of AKT, can regulate both Tau phosphorylation and Aβ production in AD through the PI3K/AKT-dependent pathway (Hernandez et al., 2010). Also, neural activity and function are highly dependent on the continuous supply of glucose. Decreased intake of glucose may be the direct substrate of cognitive impairment in AD (Kuehn, 2020). Interestingly, glucose metabolism is closely related to the activation of AKT/GSK3β pathway, especially involved in the phosphorylation of AKT and GSK3β (Griffith et al., 2019). The above indicated that abnormal changes in phosphorylated Tau and glucose metabolism in AD are closely associated with the AKT/GSK3β pathway (Tokutake et al., 2012).

Acupuncture is a unique therapy used to treat diseases in China. Under the guidance of traditional Chinese medicine (TCM) theory, acupuncture needles are inserted into the body at a certain angle, and acupuncture techniques such as twisting, lifting, and thrusting are used to stimulate specific parts of the body to treat a disease. The insertion point is called the acupuncture point. Electroacupuncture (EA) involves stimulation by a pulsating electrical current through acupuncture needles. In animal and clinical trials, EA have shown unique protective effects in inhibiting neuronal apoptosis and neuroinflammation as well as in promoting cognitive function (Wang et al., 2012; Su et al., 2019). At the same time, Our previous experiments have confirmed that EA could alleviate cognitive impairment by promoting glucose metabolism in the brain of mice (Xu et al., 2020).

As the understanding of AD has advanced, increasing evidences have shown that the mechanism of AD is very complex with the further study of AD (Yu et al., 2018). In the past, we studied the effects of EA on Tau and glucose metabolism separately but neglected to explore the connection between its effects on these processes. Based on the abovementioned findings, the study investigated whether the cognitive protective effect of EA in regulating Tau protein and glucose metabolism is associated with the activation of the AKT/GSK3β signaling pathway. As a good animal model of AD, the amyloid precursor protein (APP)/presenilin-1 (PS1) mouse strain exhibits the pathological characteristics of AD patients to some extent. Therefore, we first assessed changes in the cognitive abilities of these mice with the Morris water maze (MWM) test. To demonstrate that EA can improve the cognitive abilities of mice, the possible mechanism underlying the effect of EA was further studied. Subsequently, we observed glucose metabolism in the brain of mice after EA intervention using ^{18}F-FDG positron emission tomography (PET). Finally, we detected Tau, AKT, GSK3β and their phosphorylation using immunohistochemical staining and western blotting (WB), aiming to elucidate the protective mechanism of EA on cognition.

MATERIALS AND METHODS

Experimental Animals

Amyloid precursor protein/presenilin-1 mice, which overexpress the human APP and PS1 mutations, were obtained from Beijing HFK Bioscience Co., Ltd. [experimental animal license number: SCXK (Jing) 2014-0004]. The mice were housed one per cage in an environment with a temperature of 23 ± 2°C and humidity of 50 ± 10% under a 12-h light/dark cycle (lights on 08:00–20:00 h).

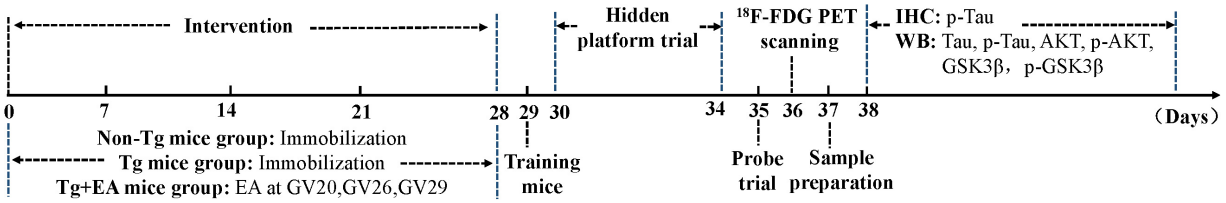

FIGURE 1 | The timeline of the study exploring the effect of EA on the cognition of APP/PS1 mice.

Ad libitum access to water and food was provided. 6-month-old male APP/PS1 transgenic mice were randomly assigned to Tg group or Tg + EA group, with ten mice per group. Age-matched C57BL/6N (Non-Tg) mice were used as controls. After 7 days of acclimation, the mice began to receive EA treatment. All experimental procedures were carried out in strict accordance with the regulations of the National Institutes of Health guide for the care and use of laboratory animals. The timeline of the experimental design is shown in **Figure 1**.

EA Intervention

Based on our previous studies, the acupoint prescription included GV20, GV29, and GV26. According to the Acupoint Standard for Experimental Animals, GV20 is at the middle point of the parietal bone of mice, GV26 is located 1 mm below the tip of the nose of mice, and GV29 is located in the depression between the eyes of mice. The Tg + EA mice were treated with EA. Firstly, we immobilize the mice with special bags based on the size of the mice. Then, one needle was inserted in a backward direction at GV20, and the other needle was inserted toward the tip of the nose at GV29. The insertion depth at the two acupoints was 5 mm. After the needle handles were fixed, the two needles were connected to the EA device. Parameters were set to 2 Hz and 1 mA. After 20 min, turn off the EA apparatus and a quick prick was delivered at GV26. Acupuncture points were stimulated with disposable sterile needles (0.25 mm × 13 mm). The mice in the Non-Tg and Tg groups were immobilized in mouse bags only. The interventions described above were administered once every other day for 28 days.

MWM Test

The MWM test is a classic experiment that assesses cognitive abilities by analyzing rodent behavior. The hidden platform trial of the MWM can be performed to analyze the spatial learning ability, and the probe trial can be used to assess spatial memory ability of mice (Vorhees and Williams, 2006). On day 29 of this study, all the mice were trained to swim in the pool. The hidden platform trials were performed after 24 h (Skelton et al., 2007). A circular platform was placed in a fixed position in the southwest (SW) quadrant of the pool. The mice were placed into the water with their heads facing the pool wall. The experiment was performed 4 times, with each mouse starting from each of the four quadrants. The interval between trials was 20 min, and the training trials were performed for 5 days. The time it took the mice to find the underwater platform (escape latency) was recorded, with the maximum latency being 60 s. The probe trial was performed on day 35. The mice were placed into pool (without the circular platform) in the northeast (NE) quadrant, and their performance was recorded for 60 s. The number of times each mouse passed the platform was recorded, and the time each mouse spent in the platform quadrant and swimming trajectories were analyzed (Dong et al., 2015).

^{18}F-FDG PET

On day 36, ^{18}F-FDG PET imaging was conducted. First, the blood glucose levels of the mice were measured to ensure that the values were 7.0–10.0 mmol/L. Then, the mice were banned from drinking water for 6 h before anesthesia. After completely anesthetized, the mice were injected with 14.8–16.5 MBq ^{18}F-FDG in the tail vein. Waiting for 60 min, micro-PET images were collected for 10 min. Single frame micro-PET images were capture, and then image reconstruction was carried out. The following steps were manually selection the hippocampus regions of interest (ROIs) from transverse, coronal, and sagittal PET/CT images by the experimenter and the uptake rate of ^{18}F-FDG per gram were analyzed (Xu et al., 2020).

Immunohistochemistry

The ABC method of immunohistochemistry was performed. Paraffin slices were dewaxed. 0.1 mol/L citrate buffer for antigen repair for 10 min. The serum of 5% normal sheep were sealed at 37°C for 30 min. A primary antibody against p-Tau (Ser199) (1:600) was added to the tissue and incubated at 4°C overnight. The following day the slices were rinsed with PBS for 3 times, and then the secondary antibody was added and incubated for 10 min. After the slices were rinsed with PBS for 3 times, AB compound were added and incubated for 90 min. After being rinsed again with PBS, the slices were colored, redyed, dehydrated, and made transparent and sealed. The brain slices were observed with a microscope. The information of the primary antibody is listed in **Table 1**.

Western Blotting

The mice hippocampal tissues were quickly stripped after they were sacrificed. The total proteins were extracted from the tissue and the protein concentration were measured by BCA method. After detecting the protein content of the sample, extracted proteins were separated by 10% SDS-PAGE. The voltage of SDS-PAGE electrophoresis separation glue and concentrated glue was 120 and 80 V, respectively. After the proteins were transferred onto PVDF membranes at 200 mA, rinsed the membranes and sealed them at 4°C overnight. The primary antibodies

TABLE 1 | The information of the primary antibodies used in this experiment.

Antibody	Antibody Code	Company
Anti-Tau antibody	ab75714	Abcam, England
Anti-Tau (phospho S199) antibody	ab4749	Abcam, England
Anti-Tau (phospho S202) antibody	ab108387	Abcam, England
Anti-pan-AKT antibody	ab8805	Abcam, England
Anti-AKT1 (phospho S473) antibody	ab8932	Abcam, England
Anti-GSK3 beta antibody	ab93926	Abcam, England
Anti-GSK3 beta (phospho S9) antibody	ab131097	Abcam, England
Anti-GAPDH antibody	ab8245	Abcam, England

against Tau (1:2000), p-Tau (Ser199) (1:500), p-Tau (Ser202) (1:5000), AKT (1:300), p-AKT (Ser473) (1:200), GSK3β (1:800), and p-GSK3β (Ser9) (1:500) primary antibodies were added and incubated at 4°C overnight. The secondary antibody (Shanghai, Jiehao, Haopoly-HRP, 1:5000) were added and incubated at room temperature for 1 h. After rinsed, ECL luminescent solution was added, and the cassette was exposed. An antibody against GAPDH (1: 2000, TA-08, Zibo, China) was used as an internal control. The information of the primary antibodies are listed in **Table 1**.

Statistical Analysis

SPSS 20.0 statistical software was used for data analysis. Data were presented as means ± SD. Multivariate analysis of variance (ANOVA) with repeated measurement design data was used to analyze the difference of escape latency in each group of mice. For the remaining data except escape latency, if the data were normally distributed and had homogeneous variance, one-way analysis of variance was used and LSD test was used for inter-group comparison. If the data were abnormally distributed or the variance was uneven, non-parametric test was used. Statistical significance was set at $P < 0.05$, and high statistical significance was set at $P < 0.01$.

RESULTS

Effect of EA on the Cognitive Abilities of APP/PS1 Mice

In the hidden platform trial of the MWM, the spatial learning abilities of APP/PS1 mice were assessed from day 30 to 34. The escape latency was measured as the time it took a mouse to find the hidden platform fixed in position underwater. The escape latency of the Tg group was maintained at a high level, but that escape latencies of the Non-Tg and Tg + EA groups showed an obvious downward trend over the training phase (**Figure 2A**). A shorter escape latency across training days indicates better learning ability. The results suggested that the mice from the Tg group exhibited learning disabilities. However, compared with Tg group, the escape latency of Tg + EA group decreased gradually and was significantly shortened on days 4 and 5. (**Figure 2A**), suggesting that EA had a protective effect on the learning abilities of APP/PS1 mice.

On day 35, the platform was removed from the southwest (SW) quadrant, and the probe trial was conducted to evaluate the maintenance of memory (Thong-asa et al., 2013). Compared to the Non-Tg, the Tg group exhibited notably fewer platform crossings and spent markedly less time in the SW quadrant. The shorter time spent by the APP/PS1 mice in the SW quadrant, which had been the location of the platform, implies that they exhibited worse memory (Tian et al., 2019). The Tg + EA group stayed obviously longer in the SW quadrant than the Tg group (**Figures 2B,C**). The results suggested that EA treatment significantly promoted memory retention in APP/PS1 mice. Furthermore, we analyzed the swimming trajectories of the mice. It was found that the trajectories of the Tg group were random, whereas the mice from the Non-Tg group and Tg + EA group exhibited trajectories that were mostly concentrated in the SW and northwest (NW) quadrants and passed the platform position several times (**Figure 2D**). Based on the MWM results, EA therapy was beneficial to the cognitive performance of AD model mice, which was related to the protection of spatial learning and memory ability.

Effect of EA on Glucose Metabolism in the Hippocampi of APP/PS1 Mice

Dysregulation of glucose metabolism in the brain are a key sign of the development of AD (Mosconi, 2005). To assess glucose metabolism, ^{18}F-FDG PET was performed after the MWM test. We selected the ROIs in the hippocampus on PET images and further analyzed the glucose metabolism rate by calculating the uptake rate of ^{18}F-FDG per gram in the hippocampus of each group. PET imaging showed that the glucose metabolism rate of the Tg group was lower than the glucose metabolism rates of the Non-Tg and Tg + EA groups. Furthermore, the data obtained from PET imaging confirmed that the uptake rate of ^{18}F-FDG in the hippocampus of the Tg group were obviously lower than that in the Non-Tg group but that significantly increased after EA intervention (**Figure 3**).

Effect of EA on the Expression of Tau in the Hippocampi of APP/PS1 Mice

The formation of NFTs by hyperphosphorylated Tau is considered a crucial event in the pathogenesis of AD (Alonso et al., 1994). We hypothesized that the improvement of cognition by EA is related to the regulation of abnormal Tau phosphorylation. Therefore, we next evaluated the expression of Tau, including phosphorylated Tau and total Tau, in the hippocampus. As expected, we observed an obvious increase in the localization of p-Tau (Ser199) immunopositivity in the hippocampus in the Tg group compared to the Non-Tg group and found that the optical density of p-Tau (Ser199) was significantly higher in the Tg group than the Non-Tg and Tg + EA groups (**Figure 4A**). WB results confirmed that p-Tau (Ser199 and Ser202) levels were obviously increased in the hippocampus in the Tg group compared with the Non-Tg group and were decreased after EA in the Tg + EA group (**Figure 4B**). In brief, the results of WB and immunohistochemistry showed

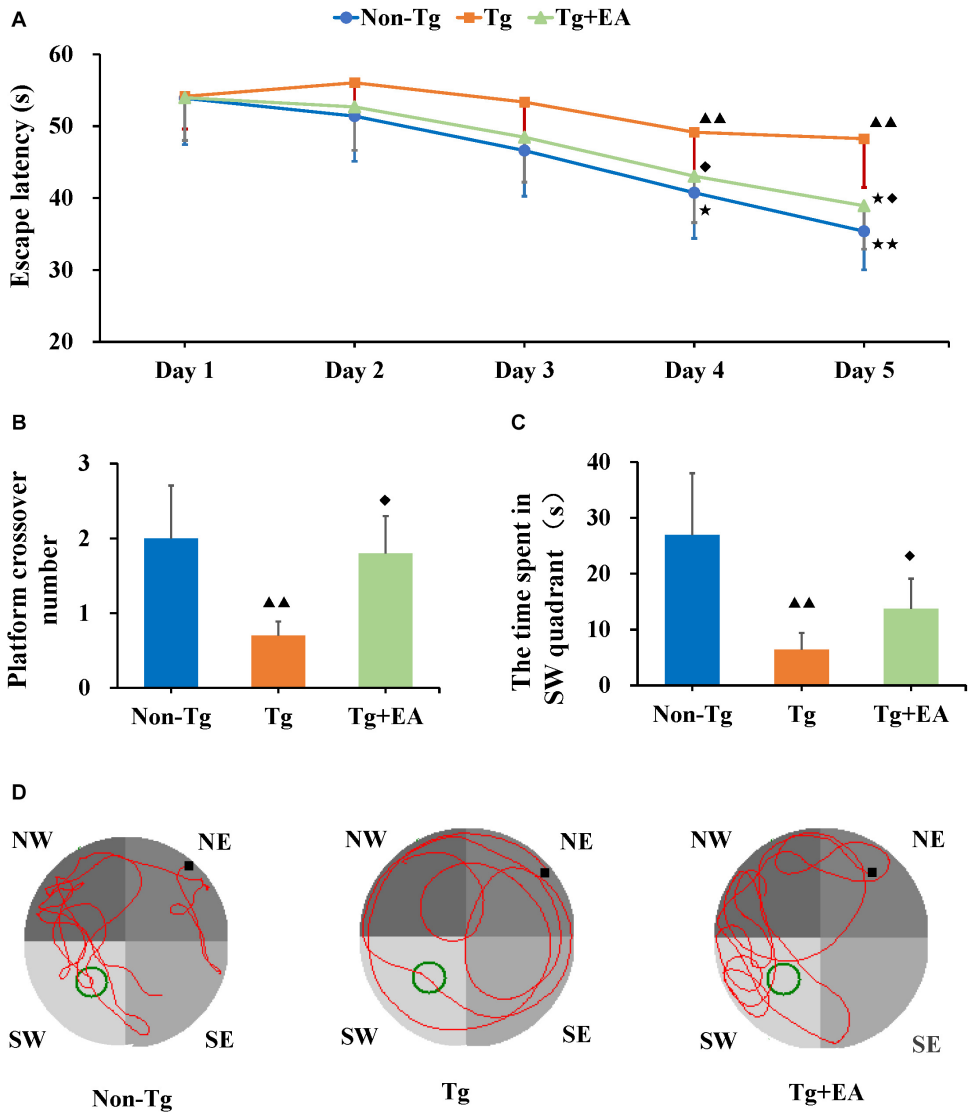

FIGURE 2 | The Results of MWM test. **(A)** The change trend of escape latencies, and comparison of escape latencies between each group. **(B,C)** Comparison of the number of platform crossing and the time spent in the SW quadrant by each group. **(D)** Representative swimming trajectories of mice from each group. $n = 10$, means ± SD. ★★$P < 0.01$, ★$P < 0.05$ compared with day 1. ▲▲$P < 0.01$ compared with the Non-Tg group. ◆$P < 0.05$ compared with the Tg group.

that the neuroprotection of EA was achieved with modulating Tau hyperphosphorylation.

Effect of EA on the AKT/GSK3β Signaling Pathway in the Hippocampi of APP/PS1 Mice

Glycogen synthase kinase-3β is one of the most important kinases for abnormal phosphorylation of Tau protein and is one of the major downstream substrates of AKT (Hernandez et al., 2013). Meanwhile, the phosphorylation of AKT and GSK3β are regulated by integrated signals derived from glucose. Activation of AKT results in a substantial increase in p-AKT (Ser473), which leads to increased phosphorylation of its downstream substrate GSK3β (Ser9), thereby reducing GSK3β activity. We speculated that the effect of EA on glucose metabolism and Tau was related to the AKT/GSK3β pathway. To test this possibility, we next examined the activation of the AKT/GSK3β signaling pathway. The results showed that EA had no significant differences in the total protein expressions of AKT or GSK3β in the hippocampus among groups. However, the levels of both p-AKT (Ser473) and p-GSK3β (Ser9) were significantly increased in the Tg + EA group compared to the Tg group (**Figure 5**). Taken together, these findings suggested that EA treatment activated the AKT/GSK3β signaling pathway of APP/PS1 mice. Activation of AKT results in a substantial increase in p-AKT (Ser473), which leads to

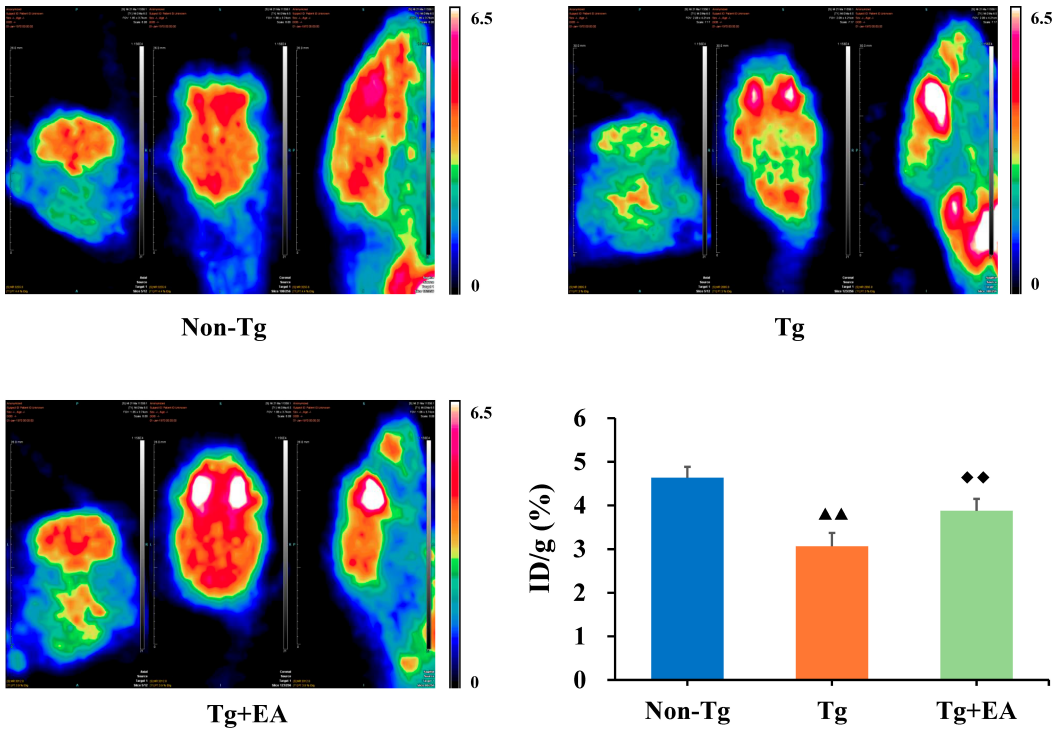

FIGURE 3 | ^{18}F-FDG PET imaging and uptake rate in the hippocampi of APP/PS1 mice. $n = 4$. Data are presented as means ± SD. Color code: min = 0, max = 6.5. ▲▲$P < 0.01$ compared with the Non-Tg group. ◆◆$P < 0.01$ compared with the Tg group.

increased phosphorylation of its downstream substrate GSK3β (Ser9), thereby reducing GSK3β activity.

DISCUSSION

In this study, we investigated the mechanism by which EA intervention affects cognition in the APP/PS1 mouse strain, a rodent model of AD. We found that EA therapy was beneficial in improving cognitive decline by promoting glucose uptake in the hippocampus and that the underlying molecular mechanisms might be associated with phosphorylation of Tau protein through the AKT/GSK3β signaling pathway.

Individuals can experience gradual progressive cognitive decline that results from AD pathology in the brain. When cognitive impairment becomes sufficient to interfere with daily function, the patient is diagnosed with AD (Albert et al., 2011). Cognitive decline is a major issue that affects the quality of life of AD patients (Zhao et al., 2020). Clinical studies have shown that acupuncture reduces cognitive impairment in AD patients (Feng et al., 2012; Tan et al., 2017). In addition, studies have shown that acupuncture is indeed effective in improving cognitive function in AD animal models compared with the placebo effect. According to the theory of TCM, meridian blockage in the brain is the pathological basis of AD and aggravates the abnormal brain function. The governor vessel (GV) is closely related to the central nervous system, and the acupoints on GV are the first choice for the treatment of central nervous system related diseases. In most studies, GV20 is chosen as the main treatment acupoint, and it has been proven to be effective in the treatment of AD mice with EA (Lin et al., 2016). According to the traditional Chinese classics, we proposed a new acupuncture therapy, a "dredge GV and refresh mind therapy," in which EA at GV20, GV26, and GV29 can improve brain function and prevent cognitive decline in AD (Deng et al., 2016; Tang et al., 2019).

Learning and memory are higher neural activities and higher functions of the brain. The MWM test is a classical and the most common method for evaluating memory and learning changes in AD mice. In the MWM test, which assesses spatial learning, the rodents use signs on the walls of the circular swimming pool to find an underwater platform from different falling points (Zhao et al., 2014). Spatial learning ability was assessed by the results of swimming training, and spatial memory level was assessed by analyzing the preference of the mice to the location of the platform after the platform was removed (Neufeld et al., 2019). The MWM has proven to be a reliable test to assess cognitive ability of the rodents, and the behavior of animals in experiments is strongly correlated with hippocampal synaptic plasticity and NMDA receptor function (Pinho et al., 2017). Our results showed that from day 2, the escape latency of the Non-Tg group showed a downward trend, while that of the Tg group did not change, indicating that the learning ability of the Tg mice was significantly impaired. In addition, the mice in the Tg group crossed the platform fewer times and spent less time in the SW quadrant than the mice in the Non-Tg group. Spatial reference memory refers to the ability to complete a spatial

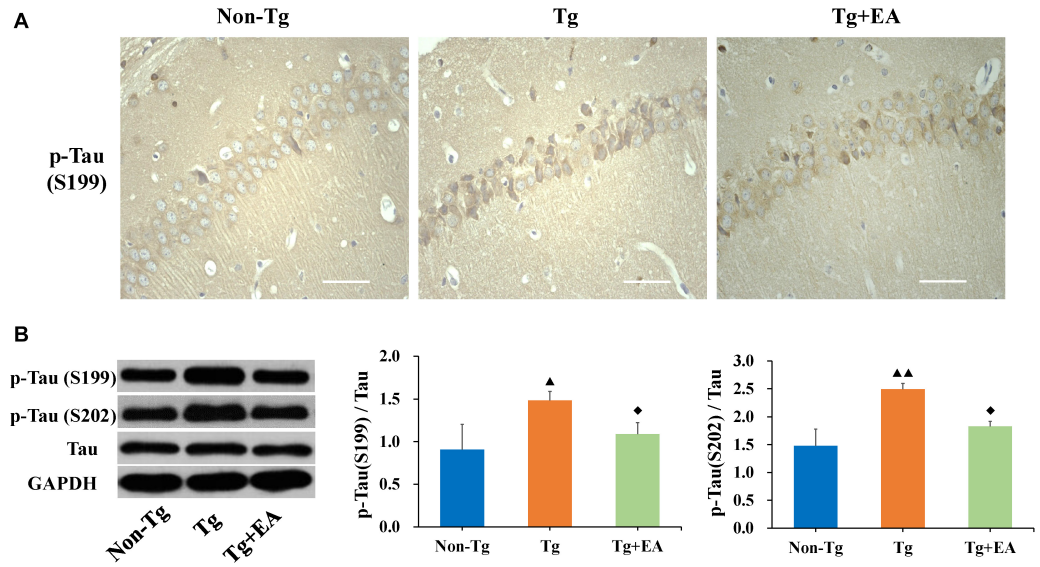

FIGURE 4 | Comparison of the expression of Tau in the hippocampi of APP/PS1 mice. **(A)** Representative immunohistochemistry images of p-Tau (Ser199) in the hippocampus in each group. The scale bar is 50 μm. **(B)** The relative expressions of hippocampal phosphorylated Tau and total Tau and the ratios of p-Tau (Ser199 and Ser202) expression level to the total Tau level in APP/PS1 mice by WB analysis. $n = 6$, means ± SD. ▲$P < 0.05$, ▲▲$P < 0.01$ compared with the Non-Tg group. ◆$P < 0.05$ compared with the Tg group.

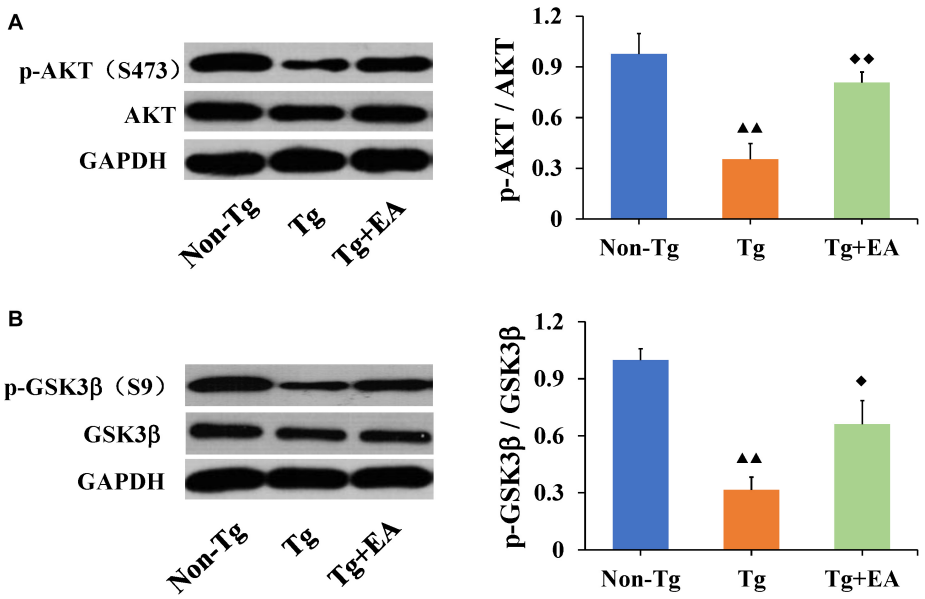

FIGURE 5 | Comparison of the expressions of AKT and GSK3β in the hippocampi of APP/PS1 mice. **(A)** The relative expressions of hippocampal p-AKT (Ser473) and total AKT, and the ratio of p-AKT (Ser473) expression level to the total AKT level in APP/PS1 mice by WB analysis. **(B)** The relative expressions of hippocampal p-GSK3β (Ser9) and total GSK3β, and the ratio of p-GSK3β (Ser9) expression level to the total GSK3β level in APP/PS1 mice by WB analysis. $n = 6$, means ± SD. ▲▲$P < 0.01$ compared with the Non-Tg group. ◆◆$P < 0.01$, ◆$P < 0.05$ compared with the Tg group.

positioning task through multiple learning and is an important measure in learning and memory research and for nervous system function assessment. The results suggested that the Tg mice exhibited memory impairments, which is in accordance with what is observed following AD-related pathological changes. Importantly, the effect of EA treatment from day 1 to 3 showed a good trend. Compared with that of the Tg group, the cognitive ability of the Tg + EA group was significantly improved on days 4 and 5, suggesting that EA had an obvious effect on AD. Based on the "treating pre-disease" theory of TCM, we hypothesized that earlier EA intervention might have a better effect on protecting cognition. Providing intervention

before cognitive impairment develops may be more beneficial (Ding et al., 2020).

Moreover, in adult humans, the brain uses approximately 20% of the energy in the body. Glucose consumption is tightly linked to neuronal activity and neuronal function. Regional metabolic aberrations underlie the functional and cognitive decline seen in patients with AD (Shen et al., 2019; Kuehn, 2020). PET scans provide functional information that is unique and cannot be obtained using other types of imaging. Hence, ^{18}F-FDG PET, which offers acceptable sensitivity and accuracy, is recognized as a potential tool for pre-symptomatic diagnosis of AD (Shen et al., 2019; Blazhenets et al., 2020). In AD, numerous interrelations between abnormal glucose metabolism and the occurrence of brain lesions have been described. First, AD could be a partial consequence of insulin resistance, which affects insulin signaling and favors abnormal deposition of Aβ and phosphorylated Tau accumulation in the brain, leading to cognitive decline (Malkki, 2015). Abnormalities of glucose metabolism occur in the early stages of AD, involving the temporal and parietal lobes. In animal models, patients and people at high risk of AD showed this characteristic (Arrieta-Cruz and Gutierrez-Juarez, 2016). Using ^{18}F-FDG PET, we observed that EA treatment activated the hippocampus, suggesting that EA enhanced glucose metabolism and contributed to energy metabolism, thus improving the cognitive function of the Tg mice (De Santi et al., 2001).

Increasing evidence suggests that Tau protein is one of the most peculiar proteins in the central nervous system (de Calignon et al., 2012). The highly flexible structure of Tau protein allows interactions with multiple partners, suggesting that Tau is involved in numerous signaling pathways. The accumulation of Tau, especially hyperphosphorylated Tau, which is a major component of neurofibrillary lesions characteristic of AD and other brain disorders (Karikari et al., 2020), is more compatible with the clinical severity and progression of pathological findings in AD than Aβ (Rapoport et al., 2002). Studies on the correlation between cognitive impairment and histopathological changes have consistently demonstrated that the number of NFTs, not the number of plaques, correlates best with the presence and/or degree of dementia in AD (Bittar et al., 2020). The neurodegenerative synaptic dysfunction is associated with the abnormal expression Tau. The more phosphorylated Tau deposition in the brain of AD patients, the lower their cognitive score (Hoover et al., 2010). Meanwhile, FDG PET show that pathological Tau is consistent with regions with low glucose metabolism in the brain (Baghel et al., 2019). Tau protein phosphorylation mainly occurs in serine or threonine residues, and there are many phosphorylation sites. We chose Ser199 and Ser202 as the representative phosphorylation sites. Phosphorylation of a number of serine phosphorylation sites of Tau, including Ser199, is elevated in AD mice (Neddens et al., 2018). Our results showed that phosphorylation of Tau at Ser199 and Ser202 were significantly increased in the hippocampus in the Tg group compared to the Non-Tg group, but that the level of total Tau was not significantly changed. This suggested that acupuncture has an effect against AD by efficiently inhibiting Tau phosphorylation.

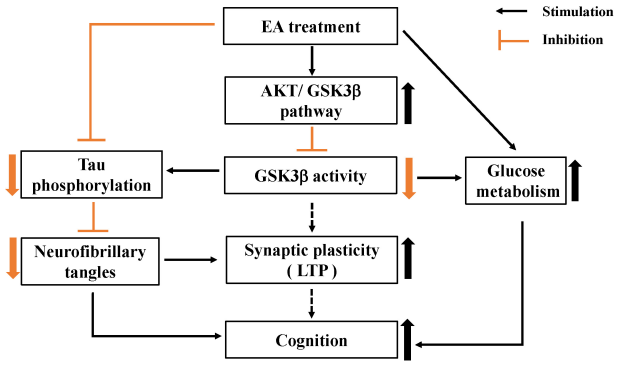

FIGURE 6 | Schematic representation of EA protective effects on cognition in APP/PS1 mice.

Glycogen synthase kinase-3β is a serine/threonine-protein kinase that is essential for energy metabolism and nerve cell development (Hooper et al., 2008). Moreover, GSK3β promotes actin and tubulin assembly, processes required for synaptic reorganization during memory formation, which is critical for the induction of memory formation, switching off LTD, and allowing LTP to occur (Peineau et al., 2007). Besides, our previous study found that EA therapy can improve the expression of NMDARs in hippocampus, and EA may regulate the LTP mediated by NMDARs, enhance cognitive ability. Substantial evidence has revealed that GSK3β, which functions as a major Tau kinase and a downstream target of the PI3K/AKT signaling pathway, regulates both Tau phosphorylation and Aβ production in AD (Qi et al., 2017). In our study, the levels of p-AKT and p-GSK3β in the hippocampus in the Tg + EA group were dramatically increased compared with those in the Tg group, but the total expression of AKT and GSK3β were unaffected. Phosphorylation increased AKT activity but decreased GSK3β activity. Our results suggested that EA activated AKT to promote the phosphorylation of GSK3β (Ser9), by reducing GSK3β activity, and ultimately inhibit the phosphorylation of Tau in the hippocampus, thereby protecting cognitive function. It is important to note that the fluctuations in blood glucose levels can affect the phosphorylation of AKT and GSK3β. In other words, the activities of AKT and GSK3β are regulated by integrated signals from glucose. Conversely, inhibition of GSK3β can also regulate glucose levels in animal models of insulin resistance. In view of this, GSK3β has attracted increasing attention as a potential therapeutic target in the treatment of diabetes (Maqbool and Hoda, 2017). As an integrator of cellular glucose sensors and multiple signals, activation of the AKT/GSK3β signaling pathway may affect neurondysfunction resulting from changes in glucose availability, such AD (Clodfelder-Miller et al., 2005). Besides, GSK3β can inhibit the activity of glycogen synthesis and reduce the synthesis of glycogen *in vivo*. On the other hand, GSK3β can indirectly inhibit the synthesis of glycogen by affecting insulin signaling pathway. Therefore, we speculate that the mechanism by which EA regulates brain glycometabolism might partly involve the AKT/GSK3β signaling pathway. More importantly, GSK3β is

the intersection of Tau phosphorylation and glucose metabolism abnormalities in AD.

In summary, this study provides evidence for the protective effect of EA intervention on cognition, with EA tending to be beneficial for enhancing learning and memory abilities in AD model mice. In addition, this study reveals the mechanism underlying the protective effect of EA on cognition. We found that EA has an effect on the AKT/GSK3β signaling pathway, as reflected by increased phosphorylation of AKT and GSK3β, and that a reduction in GSK3β activity contributes to improvements in glucose metabolism and inhibition of abnormal Tau phosphorylation (**Figure 6**). The mechanisms underlying the protective effect of EA on cognition could involve multiple processes. The multitarget effect of acupuncture is appropriate given the complexity of AD pathogenesis. Future studies clarifying the mechanism underlying the effect of EA in AD should be encouraged.

DATA AVAILABILITY STATEMENT

The data analyzed in this study is subject to the following licenses/restrictions: The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher. Requests to access these datasets should be directed to Anping Xu, xuanping01@163.com.

ETHICS STATEMENT

The animal study was reviewed and approved by the Medicine and Animal Ethics Committee of the Beijing University of Chinese Medicine.

AUTHOR CONTRIBUTIONS

AX: experimental design, data analysis, and manuscript preparation. QZ and YT: experimental design and manuscript preparation. XW and XY: data collection. YZ and ZL: experimental design. All authors contributed to draft the manuscript and have read and approved the final manuscript.

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REVIEW
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Acylated Ghrelin as a Multi-Targeted Therapy for Alzheimer's and Parkinson's Disease

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Much thought has been given to the impact of Amyloid Beta, Tau and Alpha-Synuclein in the development of Alzheimer's disease (AD) and Parkinson's disease (PD), yet the clinical failures of the recent decades indicate that there are further pathological mechanisms at work. Indeed, besides amyloids, AD and PD are characterized by the culminative interplay of oxidative stress, mitochondrial dysfunction and hyperfission, defective autophagy and mitophagy, systemic inflammation, BBB and vascular damage, demyelination, cerebral insulin resistance, the loss of dopamine production in PD, impaired neurogenesis and, of course, widespread axonal, synaptic and neuronal degeneration that leads to cognitive and motor impediments. Interestingly, the acylated form of the hormone ghrelin has shown the potential to ameliorate the latter pathologic changes, although some studies indicate a few complications that need to be considered in the long-term administration of the hormone. As such, this review will illustrate the wide-ranging neuroprotective properties of acylated ghrelin and critically evaluate the hormone's therapeutic benefits for the treatment of AD and PD.

Keywords: growth hormone secretagogue receptor 1 alpha, mitochondrial dysfunction, inflammation, autophagy, insulin resistance, dopamine, neurodegeneration, ghrelin

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INTRODUCTION

Alzheimer's disease (AD) and Parkinson's disease (PD) are multi-faceted neurodegenerative diseases that reach far beyond the accumulation and aggregation of Amyloid Beta (Aß), Tau and Alpha (α)-synuclein. Indeed, the last decades of research have indicated that cognitive decline is driven by the interplay of various pathologic processes, involving insulin-associated bioenergetic impairments and the reduced cerebral metabolization of glucose (Neth and Craft, 2017), mitochondrial defects (Bose and Beal, 2016; Onyango et al., 2016), vascular abnormalities, reduced blood flow, blood brain barrier (BBB) damage (Kisler et al., 2017; Sweeney et al., 2018), dysfunctional autophagy and mitophagy (Kerr et al., 2017; Fujikake et al., 2018; Liu J. et al., 2019), oxidative stress (Cenini et al., 2019), chronic systemic inflammation, pathological immune cell infiltration into the brain (Amor and Woodroofe, 2014; Anderson et al., 2014; Stephenson et al., 2018), inflammasome activation (Ising et al., 2019; Stancu et al., 2019), demyelination (Wang S. S. et al., 2018), the degeneration of axons (Kandan et al., 2013), the development of type 2 diabetes mellitus (T2DM), the cerebral desensitization of growth and neurotrophic factors, in particular insulin (Gault and Holscher, 2018; Holscher, 2019), as well as alterations of the dopaminergic system, including the extensive atrophy of substantia nigra pars compacta

(SNpc)-located dopaminergic neurons and dopamine depletion in the striatum in PD (Martorana and Koch, 2014; Poewe et al., 2017).

Especially in the case of AD, countless Aβ and a handful of Tau-directed therapies have been tested in clinical trials, yet none of them, including a very recent phase II trial with the anti-Tau antibody semorinemab, have come to fruition. Such discouraging findings have come to question the amyloid hypothesis, as reflected by the notably diminished numbers of Aβ-based and elevated quantities of neuroprotection-focussed and anti-inflammatory approaches in the clinic in 2019. Moreover, while Aβ may be the undisputed culprit in familial AD patients with respective genetic mutations (<1%), sporadic AD patients (>99%) may endure several other risk factors, such as secondary inflammatory conditions, head injuries, the $APOE_4$ allele, T2DM/insulin resistance and brain glucose hypometabolism, the presence of metabolic and vascular syndrome and presumably many more. This is paralleled in the varying clinical profile, as sporadic AD patients may exhibit high or low $A\beta_{1-42}$ burden, with or without the prevalence of Tau of Lewy body biomarkers, in the cerebrospinal fluid. This suggests that multiple pathologic, but also protective, factors cooperate in the progression of AD and that that a differential treatment regimen, which commonly necessitates the use of multiple drugs for chronically advancing disorders, might be necessary for individual patients. Therefore, monotherapies are presumably an ineffective way of approaching AD and PD and are more likely to fail, supporting the concept that multi-targeted therapies are more profitable (Iqbal and Grundke-Iqbal, 2010; Adams, 2020; Huang et al., 2020).

THE GHRELIN SYSTEM AND ITS PHYSIOLOGICAL ROLE

Belonging to a group of physiologically secreted hormones, ghrelin serves numerous important functions. Ghrelin is predominantly produced by gastric X/A-like cells that are located in the oxyntic gland of the stomach (Date et al., 2000), although a lower degree of the hormone is also expressed in various peripheral tissues, in lymphocytes and in the CNS (Ferrini et al., 2009). In a serious of catalytic steps, the precursor preproghrelin is expressed, cleaved to proghrelin and transported to the Golgi body, where it may be acylated by the linkage of an O-linked octanoyl lipid group (C:8.0) at Ser^3 via ghrelin O-acyltransferase (GOAT). Ultimately, following translocation to the endoplasmic reticulum (ER), proghrelin is further processed by prohormone convertase 1/3 to generate the 28 amino acid-long anorexigenic hormone ghrelin. Mature ghrelin is stored within secretory granules of X/A-like cells and released into the bloodstream upon fasting to stimulate appetite (Cummings et al., 2001; Yanagi et al., 2018). Conversely, increased circulatory levels of glucose and long-chain fatty acids (LCFA) following meal intake as well as the postprandial release of insulin block the secretion of ghrelin (Gagnon and Anini, 2012; Lu et al., 2012; Sakata et al., 2012). Depending on the presence or absence of the acyl group at Ser^3, mature ghrelin can be further distinguished into its active form, acylated ghrelin (AG), and desacylated ghrelin (DAG) (Hosoda et al., 2000; Yanagi et al., 2018). The acylation state of ghrelin is transient, however, as liberated AG is continually deacetylated by acyl-protein thioesterase 1 and butyrylcholinesterase in the blood stream (De Vriese et al., 2004; Satou et al., 2010; Schopfer et al., 2015). Through the circulatory system, AG is able to reach and cross the blood brain barrier (BBB) in either direction through the recognition of the lipophilic acyl/octanyloid side chain and saturable systems, whereas DAG obtains brain entry through non-saturable diffusion through the BBB (Banks et al., 2002; Diano et al., 2006). Furthermore, although still unidentified, the liberation of fasting-associated plasma factors appear to further stimulate the BBB translocation of AG (Banks et al., 2008). Notably, the presence of GOAT has been detected in human serum, the hippocampus and the temporal gyrus (Gahete et al., 2010; Goebel-Stengel et al., 2013; Murtuza and Isokawa, 2018). It has been verified that DAG can be locally modified by GOAT, which presumably allows ghrelin to exert centralized effects in selected tissues and brain areas, such as the hippocampus (Murtuza and Isokawa, 2018). Ultimately, AG stimulates intracellular downstream signaling through its cognate G-protein coupled receptor (GPCR), known as the growth hormone secretagogue receptor type 1α (GHS-R1α). Importantly, DAG is incapable of interacting with GHS-R1α, yet the existence of distinct DAG-binding receptors has been postulated (Howard et al., 1996; Yanagi et al., 2018).

GHS-R1α is widely transcribed in multiple key areas of the brain, such as the hippocampus, hypothalamus, cortex, ventral tegmental area (VTA), SN, spinal cord, dorsal and median raphe nuclei, sympathetic preganglionic nerves and endothelial cells of the cerebral vasculature, yet it is also expressed by various immune cells and in peripheral tissue (Guan et al., 1997; Hosoda et al., 2000; Gnanapavan et al., 2002; Jiang et al., 2006; Pan et al., 2006; Ferens et al., 2010). Notably, only GHS-R1α, but not its truncated and non-functional splicing variant GHS-R1ß, is capable of interacting with AG. In contrast, a dominant-negative role for GHS-R1ß has been suggested, in which heterodimerization of GHS-R1ß with GHS-R1α encourages receptor endocytosis to obstruct intracellular signaling (Leung et al., 2007).

As a major metabolic hormone, AG elevates the secretion of growth hormone (GH) by the pituitary gland, reduces insulin, yet increases glucagon secretion by pancreatic cells and promotes the hepatic release of glucose into the blood, thus maintaining steady plasma glucose levels during fasting (Mani et al., 2019). Furthermore, AG induces the expression of the orexigenic peptides neuropeptide Y (NPY) and agouti-related protein (AgRP) in the hypothalamus to stimulate appetite, as extensively described in Yanagi et al. (2018). Other physiological processes that are commanded by ghrelin include the regulation of the gastrointestinal motility and acid secretion, cardiac function, osteoblast proliferation, bone maturation and muscular/myoblast outgrowth, the formation of long-term memory, the control of behaviors such as spontaneity, anxiety, food/reward behavior as well as the navigation of the circadian rhythm (Abdalla, 2015; Shi et al., 2017; Yanagi et al., 2018).

Following receptor stimulation in the brain, AG exerts a broad range of neuroprotective effects and has, thus, emerged as a

potential candidate for the treatment of AD and PD. Despite the selectivity of GHS-R1α for AG, DAG has demonstrated its own neuromodulatory effects, although the underlying signaling mechanisms remain a mystery and appear to be limited to the periphery (Yanagi et al., 2018). For the sake of this review and in association to AD and PD, the focus will be placed on the multifarious neuroprotective actions of AG.

MITOCHONDRIA AND THE NEURONAL ENERGY METABOLISM

Impairments in the Mitochondrial Function and Adenosine Triphosphate Production Are Key Events in Alzheimer's and Parkinson's Disease'

Generally, it has been well-established that mitochondrial dysfunction, originating from genetic mutations of key mitochondrial proteins, environmental toxins, excessive oxidative stress, or aging, is a key driver of PD. Similarly, oxidative stress and pathological Aß, which accumulates in mitochondria, depolarizes the mitochondrial membrane potential, inhibits electron transport chain (ETC) enzymes and provokes the production of reactive oxygen species (ROS), trigger mitochondrial and, thus, bioenergetic defects in AD. The mitochondrial pathology in AD and PD is further exacerbated by impaired mitochondrial biogenesis, a mechanism that leads to the generation of additional mitochondria to meet greater energetic demands in cells, and mitophagy, which is a form of autophagy that mediates the degradation of malfunctional, ROS-overproducing mitochondria (covered in chapter 4) (Bose and Beal, 2016; Onyango et al., 2016; Fang et al., 2019; Liu J. et al., 2019).

Importantly, it must be pointed out that all cellular functions necessitate energy and, therefore, the availability of two bioenergetic substrates: ATP and guanosine-5'-triphosphate, which may be converted into ATP. The latter is generated in mitochondria through the ETC. In this oxygen-requiring process, electrons (H^+) are drawn from the reducing agents NADH and $FADH_2$ and funneled through the inner mitochondrial membrane to the outer compartment of the mitochondrion via complex I, III, and IV. This establishes an electrochemical proton gradient (also known as protonmotive force Δp) and, thus, elicits the influx of electrons from the outer to the inner compartment through complex 5 (ATP synthase), which subsequently converts ADP to ATP. To recharge NAD^+ and FAD^+ and to resume ATP generation, the brain relies on glucose as major energy substrate as well as its metabolization in the tricarboxylic acid cycle (TCA) as main bioenergetic pathway (Penicaud et al., 2002; Arun et al., 2016; Martinez-Reyes and Chandel, 2020). Generally, functional deficits in complex 1 are associated with PD and defects in complex IV are implicated in AD (Cottrell et al., 2002; Arun et al., 2016). Given the pivotal role of mitochondrial dysfunction in PD, toxins that are selectively taken up by dopaminergic neurons and impair mitochondrial complex 1, such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), kill SN-located, dopaminergic neurons and induce Parkinsonism in humans and rodents (see **Figure 2**) (Langston et al., 1983; Meredith and Rademacher, 2011).

As reviewed in Muddapu et al. (2020), the neuronal populations in the hippocampal CA1 region as well as the SN are more vulnerable toward metabolic deregulation, which may be linked to the progression of AD (CA1) and PD (SN). Generally, the oxidative phosphorylation of glucose via the TCA poses the primary energy source for neurons and the cellular stress associated with aging and neurodegenerative diseases, for example amyloid aggregation or the prevalence of genetic risk factors, provoke greater bioenergetic demands. As a direct consequence of these higher energetic needs, the neuronal mitochondria are forced to generate excessive amounts of ATP at the cost of the elevated co-production of ROS. The increased oxidative burden, on the other hand, may subsequently spark glial dysfunction and the excessive release of glutamate, NMDA/AMPA receptor activation and aberrant intraneuronal Ca^{2+}-amassment, neuroinflammation and astroglial scar formation, inflammation-driven permeabilization of the BBB and pro-inflammatory cytokine-driven insulin resistance. It is incompletely understood whether mitochondrial defects elicit insulin resistance or vice versa, however. It has also been hypothesized that both factors might negatively influence each other (Neth and Craft, 2017). In any way, the desensitization of insulin in the CNS is linked to reduced cerebral glucose uptake, the diminished liberation of lactate, another pivotal energy source for neurons, by astrocytes and chronic glucose hypometabolism (Muddapu et al., 2020), as evident in the brains of both AD (Lyingtunell et al., 1981; Hoyer et al., 1988; Ogawa et al., 1996; Drzezga et al., 2003; Mosconi et al., 2008) and PD patients (Huang et al., 2008; Hosokai et al., 2009; Liepelt et al., 2009; Borghammer et al., 2010; Berti et al., 2012). It has been postulated that the development of cerebral insulin resistance, in the long-term, enforces the utility of energy sources other than glucose and the TCA cycle. Most notably, this bioenergetic shift is thought to preferentially promote the β-oxidation of ketone bodies (lipids) to produce ATP in the brain (see Neth and Craft, 2017).

More implicit, mitochondrial dysfunction is connected to the impairment of key effectors. As indicated in an AD animal model, APPswe/PS1dE9 mice displayed reduced hippocampal levels of the catalytic α2-subunit of 5' adenosine monophosphate-activated protein kinase (AMPK) (Pedros et al., 2014), which is a master effector that upregulates ATP synthesis, curbs ATP utility, maintains the mitochondrial homeostasis and navigates mitophagy when the cellular energy stores are depleted (Herzig and Shaw, 2018). Additionally, the transcription of the biogenesis-mediators PGC1α and mitochondrial nuclear respiratory factor (NRF)1/2 were reduced in the hippocampi of the APPswe/PS1dE9 mice (Pedros et al., 2014). Strikingly diminished levels of the mitochondrial markers PGC1α, succinate dehydrogenase complex A (which participates in the TCA cycle) and translocase of outer mitochondrial membrane 20 have also been observed in the post-mortem-derived SNpc of PD patients. In the context of PD, as further confirmed by genetic deletion in rodents, PGC1α is crucial for the survival of SNpc-located dopaminergic neurons and, thus, dopamine production

(Jiang et al., 2016). As such, the function of various mitochondrial master modulators, including AMPK and PGC1α, is disturbed in AD and PD. For more information about AG's influence on insulin resistance and glucose hypometabolism in the CNS (chapter 7) as well as the dopaminergic pathology (chapter 8), please see the respective chapters.

Acylated Ghrelin Ameliorates Oxidative Stress and Enhances the Mitochondrial Function, Adenosine Triphosphate Generation and Biogenesis

In the context of mitochondrial dysfunction, AG strengthens the mitochondrial vigor in multiple ways. Notably, as depicted in **Figure 1**, AG drives mitoprotection and autophagy by activating shared key effectors. While mitochondria-based investigations are limited in the field of AD, it was demonstrated that AG guards primary rat and N42 hypothalamic neurons from Aß oligomer-provoked depolarization of the mitochondrial membrane (Martins et al., 2013; Gomes et al., 2014). Further mechanistic insight can be derived from studies in PD models. In the 1-methyl-4-phenyl-1,2,5,6 tetrahydropyridine (MPTP) mouse model of PD, AG protected from neuronal death in the SNpc, as displayed by the normalized B-cell lymphoma 2 (Bcl-2)/Bax ratio and lowered caspase-3 activity, stimulated the neuronal activity, elevated the production of multiple LCFAs, for instance palmitic acyl CoA (C16:0), and improved the mitochondrial respiration by activating the ROS-buffering, mitochondrial uncoupling protein 2 (UCP2).

Furthermore, AG promoted the mitochondrial biogenesis, resulting in increased numbers of nuclear respiratory factor (NRF)1-positive mitochondria (Jiang et al., 2008; Andrews et al., 2009a; Donadelli et al., 2014). Based on previous propositions, AG may support the execution of the mitochondrial ß-oxidation and evoke the generation of LCFAs as a fuel source for ATP production upon mild negative energy balance in the CNS (Andrews et al., 2009a; Horvath et al., 2009). This idea must be addresses with care, however, since AG differentially navigates lipid metabolism in a tissue-specific manner. In the periphery, for example, independent of the hormone's orexigenic effects, AG stimulated the expression of lipogenic enzymes (fatty acid synthase (FAS), lipoprotein lipase and more) and lessened the transcription of carnitine palmitoyltransferase Ia (CPT1a), a rate-limiting effector necessary to induce fatty acid oxidation, in white adipose tissue to promote fat storage (Theander-Carrillo et al., 2006; Perez-Tilve et al., 2011). In stark contrast, AG enhanced fatty acid oxidation and lipolysis in mouse skeletal muscles (Kraft et al., 2019). In the brain, it was discovered that AG selectively diminishes the expression of the lipogenesis-affiliated FAS in the VMH, which appears to be a site-specific process to promote ß-oxidation and induce the expression of anorexigenic NPY. In the other hand, AG does not modulate FAS in other brain areas, including the amygdala, striatum, hippocampus, several cortical regions and others (Lopez et al., 2008; Yanagi et al., 2018). Moreover, under physiological conditions, AG was shown to discourage, rather than elevate, fatty acid oxidation in the hypothalamic ARC and in the cortex (Lage et al., 2010; Gao et al., 2013; Mir et al., 2018). As such, at least under physiological conditions, AG presumably does not induce β-oxidation in brain areas other than the VMH. On the other hand, it must be noted that the prevalence of cerebral insulin resistance during AD leads to defects in the metabolism of glucose, which is believed to provoke the use of β-oxidation and lipids (ketones) as primary energy sources for neurons (Neth and Craft, 2017). Thus, it is plausible that AG may assist the compensatory execution of ß-oxidation in neurons that may occur during more advanced stages of AD and, possibly, PD. As a word of caution, while the β-oxidation of lipids has been proven in astrocytes, its utility by neurons is yet to be verified (Tracey et al., 2018). In any case, given that neurons enter an initial hyperglycolytic state and overproduce ATP plus, inevitably, ROS in their mitochondria to cope with the additional cellular stress in AD and, presumably also, PD (Neth and Craft, 2017; Muddapu et al., 2020), AG may alleviate glucose hypermetabolism and the associated oxidative stress. By activating the ROS-ablating UCP2 and driving mitochondrial biogenesis, AG enhances the functionality and bioenergetic efficiency of mitochondria during AD and PD, as further outlined below.

Of note, mutational studies in UCP2-modified and MPTP-treated mice revealed that UCP2 is a joint key mediator in the protection of SN-VTA dopaminergic neurons from apoptosis, the decrease of ROS as well as the increase of mitochondrial biogenesis (Andrews et al., 2005; Conti et al., 2005). Cell culture studies have implied that AG increases the steady state levels of UCP2 by preventing its ubiquitination and degradation, resulting in the cellular accumulation of this ROS-quenching protein (Zhang, 2017). Mechanistically, AG induces UCP2 by inhibiting acetyl-CoA carboxylase (ACC), leading to the intracellular depletion of malonyl-CoA stores and, thus, the activation of the LCFA-transporter CPT1a in the outer mitochondrial membrane (Yanagi et al., 2018). In conjunction with Acyl-CoA synthases in the outer and acylcarnitine translocase plus CPT2 in the inner mitochondrial membrane, respectively, CPT1a delivers and processes converts LCFAs into acyl-CoA and delivers the latter into the inner mitochondrial compartment for β-oxidation and ATP production (Schlaepfer and Joshi, 2020). As a direct consequence of β-oxidation, nascent fatty acids are generated in the inner-mitochondrial compartment and used as "flip-flopping" proton translocators by UCP2 to shuttle H^+ into the inner-mitochondrial compartment. This process results in mitochondrial uncoupling, partially dispels and reduces the electrochemical proton gradient (Δp) that is maintained by the ETC and forestalls the Δp-dependent generation of ROS at complex I and III. As such, the induction of UCP2 buffers the glucose/TCA-exacerbated production of ROS by the ETC (see Jezek et al., 2018 for an extensive description of UCP functions), which may be protective in AD and PD. Similar to the SNpc (Andrews et al., 2009a), AG also augmented the induction of UCP2, mitochondrial respiration and mitochondrial abundance in hypothalamic NPY/AgRP neurons *in vivo* (Andrews et al., 2009b). Moreover, AG rescued neurons from apoptosis and caspase-3 activation in a UCP2-dependent manner, improved the mitochondrial ATP generation plus total ATP levels and alleviated the ROS load in the brains of rodents that were

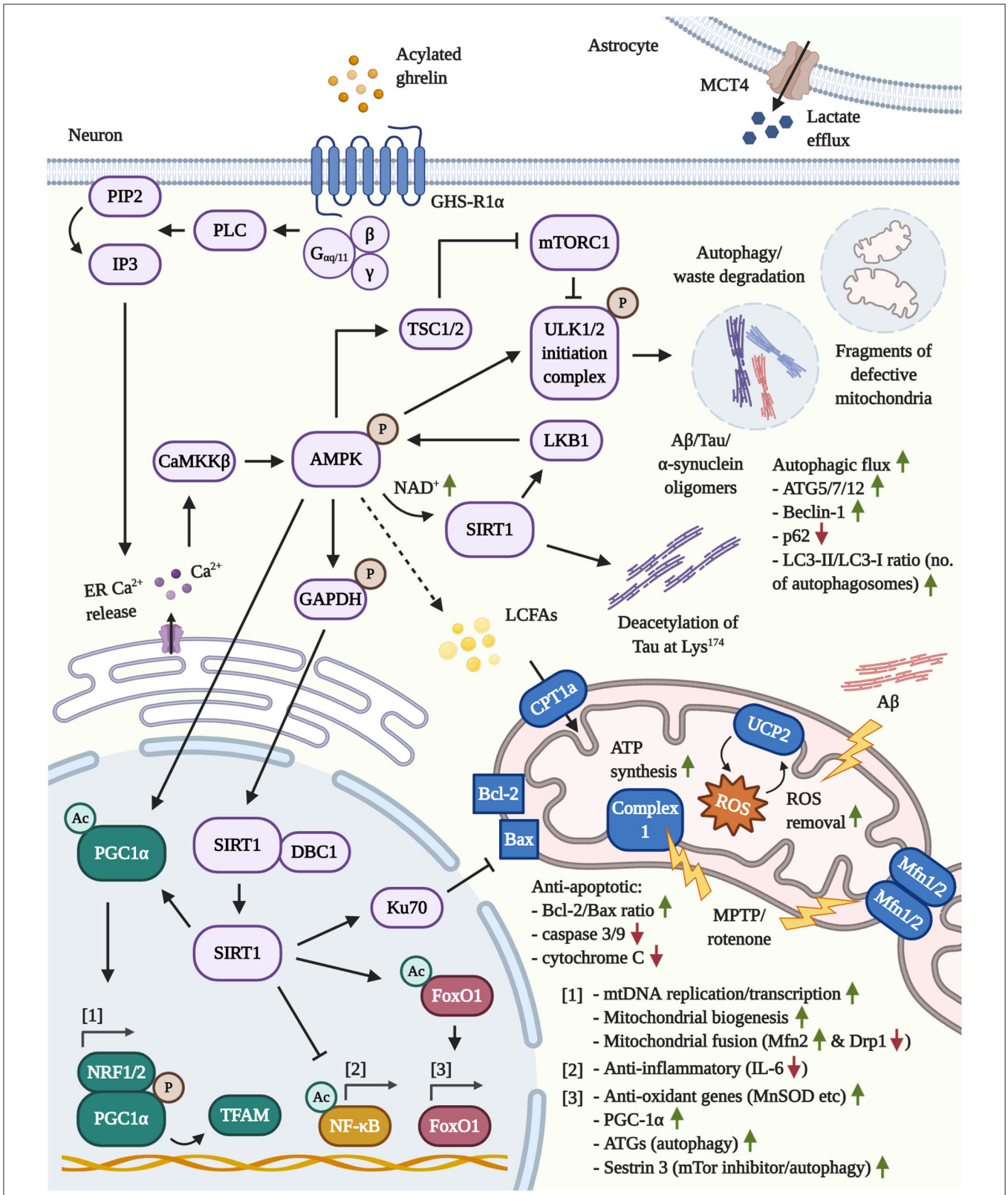

FIGURE 1 | Illustration of the neuroprotective pathways following GHS-R1α activation by AG or ghrelin agonists in neurons and astrocytes. [1] Mitochondrial function: By activating the key mediator AMPK, AG induces the transcriptional co-activator PGC1α. The latter, in concert with NRF1/2, enhances mitochondrial biogenesis, the *(Continued)*

FIGURE 1 | synthesis of TFAM and TFAM-mediated mtDNA replication/transcription. By increasing the transcription of Mfn2, PGC1α protects from MPTP/rotenone-driven mitochondrial fragmentation. In addition, AMPK/GAPDH-mediated phosphorylation of nuclear SIRT1 frees the latter deacetylase and leads to the inactivation of pro-inflammatory NF-κB, the activation of the Bax-sequestrating Ku70 and the stimulation of FoxO1-regulated anti-oxidant and autophagy genes. Lastly, the induction of the AMPK/CPT1a/UCP2 pathway prevents pathological mitochondrial depolarization (such as by Aβ). Furthermore, UCP2-driven mitochondrial uncoupling increases the mitochondrial respiration, bioenergetic efficiency and mitigates the co-generation of ROS by the ETC, which may protect from the stress-induced hyperproduction of ATP and ROS during early stages of AD. Given that more advanced stages of AD are characterized by neuronal glucose hypometabolism and a chronic shift toward other bioenergetic processes, in particular the β-oxidation of lipids, AG may support the compensatory execution of β-oxidation to generate ATP. Notably, AMPK inhibits ACC, thus depleting the intracellular malonyl-CoA pools and, in turn, elevating the activity levels of the malonyl-CoA-regulated CPT1a (not shown). [2] Autophagy: (Macro)autophagy is primarily driven by the GHS-R1α/AMPK/$TSC_{1/2}$-mediated inactivation of mTOR/mTORC1 and the direct phosphorylation of ULK1 via AMPK, resulting in the degradation of cellular waste, amyloids (Aβ/Tau/α-synuclein) and defective mitochondria. Moreover, by raising the intracellular NAD^+ levels, AMPK reinforces its activity through the activation of the cytoplasmic, NAD^+-dependent SIRT1 and the AMPK-kinase LKB1. SIRT1 is also involved in the deacetylation of Tau at Lys^{174}, which was reported to abrogate the pathological propagation of Tau throughout the brain. Besides triggering autophagy, AG upregulates various ATGs and Beclin-1, while promoting autophagosome maturation and the autophagic flux. [3] Astrocytes: The stimulation of GHS-R1α encourages the expression of the lactate-efflux transporter MCT4 by astrocytes, leading to the increased secretion of lactate, a potent energy source for neurons.

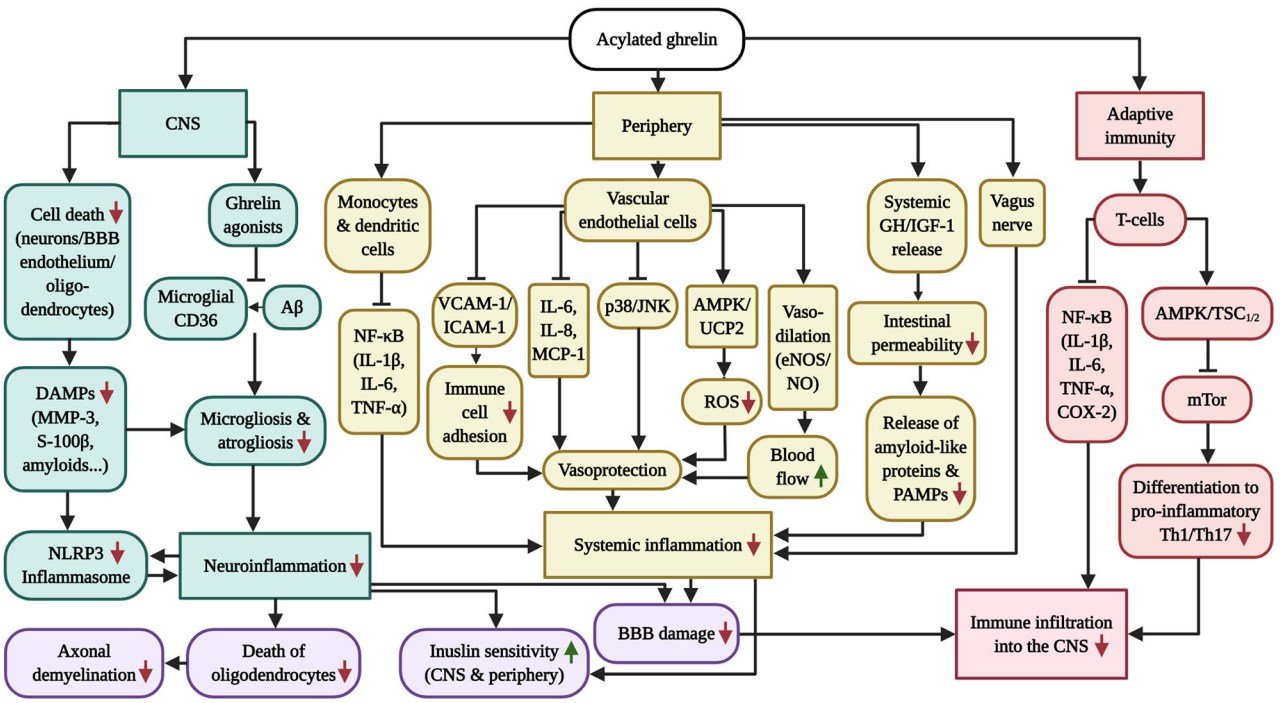

FIGURE 2 | Overview of the anti-inflammatory capabilities of GHS-R1α receptor activation. AD and PD are characterized by chronic systemic inflammation, which includes micro-/astrogliosis and inflammasome activation following the accumulation of amyloids and DAMPs in the CNS, vagus nerve and intestinal (microbiome) inflammation in the periphery as well as pathologic $CD4^+$ T-cell infiltration into the brain, which is exacerbated by the inflammation-driven injury of the BBB and vasculature. While AG has successfully prevented neuroinflammation in AD and PD models, the diagram further illustrates the beneficial effects of AG on inflammasome induction, peripheral inflammation and adaptive immunity in other inflammatory disease models, which culminate in vascular protection as well as enhanced blood flow, BBB stability, insulin sensitivity, oligodendrocyte survival and axonal myelination. Of note, GHS-R1α does not appear to be expressed by microglia, suggesting that the anti-inflammatory benefits of AG in the CNS are indirect. Ghrelin agonists offer the additional benefit of blocking microglial CD36, thus inhibiting Aβ-elicited inflammation.

subjected to cardiac arrest and/or traumatic brain injury (Lopez et al., 2012a; Xu et al., 2019). In concert with the animal experiments, AG decreased the ROS burden, rescued the toxin-induced dysfunction of complex 1 in mitochondria, normalized the mitochondrial transmembrane potential and prohibited apoptosis, as shown with improvements in the Bcl-2/Bax ratio, cytochrome C release and caspase-3/9 activity, in various cell models, including MPTP- and rotenone-stressed MES23.5 dopaminergic cells (Dong et al., 2009; Yu et al., 2016), rotenone-burdened rat retinal ganglion cells (Liu et al., 2018) and primary hypothalamic neurons during oxygen and glucose withdrawal (Chung et al., 2007).

To unravel the pathways that drive mitochondrial biogenesis, the missing links can be derived from related studies that center on aging and caloric restriction, yet only indirectly use AG. In this context, AG has been deemed as the main neuroprotective factor that is secreted upon caloric restriction and is responsible for the survival-promoting activation of AMPK (Bayliss et al., 2016).

In rodents, dietary restriction reduces oxidative stress, increases ATP production at a reduced cost of total oxygen, lowers mitochondrial membrane potential and drives mitochondrial biogenesis via the activation of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α) (Lopez-Lluch et al., 2006). PGC1α acts as a master regulator that induces mitochondrial biogenesis and upregulates the nuclear expression of mitochondria-related genes, such as the transcription factors NRF1, NRF2 and mitochondrial transcription factor A (TFAM). Subsequently, NRF1, NRF2, and TFAM initiate the replication of mtDNA, the transcription of mitochondrial respiratory genes and the synthesis of anti-oxidative proteins, for example glutathione peroxidase 1 and manganese superoxide dismutase (MnSOD). Jointly, the PGC1α-induced transcriptional changes in mitochondria-associated genes protect from MPTP oxidative assault in cell and animal models of PD, whereas the deletion of PGC1α exacerbates MPTP-induced injury and excitotoxicity (Scarpulla, 2002, 2006; Kang and Hamasaki, 2005; St.-Pierre et al., 2006; Mudo et al., 2012; Quan et al., 2020). The pro-mitochondrial impact of PGC1α in PD, whose expression levels were found to be decreased in the brains of PD patients, has been made evident in knockdown studies, in which the suppression or conditional knockdown of PGC1α led to the selective atrophy of dopaminergic neurons in the SNpc and lessened dopamine pools in the striatum of adult rodents (Shin et al., 2011; Jiang et al., 2016).

As illustrated in **Figure 1**, PGC1α is regulated by the coordinated actions of AMPK plus sirtuin 1 (SIRT1). And indeed, the latter 3 effectors are all activated by AG (Bayliss and Andrews, 2013). Initially, the canonical activation of GHS-R1α involves, but is not limited to, $G_{\alpha q/11}$ coupling to GHS-R1α, the induction of phospholipase C (PLC), the PLC-mediated turnover of phosphatidylinositol 4,5-bisphosphate (PIP2) into inositol triphosphate (IP3) and the liberation of calcium (Ca^{2+}) from the intracellular ER stores by IP3. Additionally, Ca^{2+} influx by the non-canonical association of Gαs with GHS-R1α, followed by the activation of cAMP, PKA and opening of N-type Ca^{2+} channels have been reported. The induction of the cAMP/PKA pathway is highly debated and appears to be conditional and cell-type specific, however (Kohno et al., 2003; Yin et al., 2014; Yanagi et al., 2018). Furthermore, cAMP/PKA-signaling is evoked by the physiological release of AG-counteracting and growth-promoting hormones that are associated with nutrient abundance and increased glucose metabolism, such as insulin and leptin (Yang and Yang, 2016). Therefore, in general, AG induces intraneuronal Ca^{2+} accumulation in an IP3-mediated manner, leading to the activation of the AMPK-phosphorylating calmodulin-dependent protein kinase kinase-β (CaMKKß) (Hawley et al., 2005; Anderson et al., 2008). Activated AMPK further elevates PGC1α levels, directly phosphorylates PGC1α to promote promotor binding and raises intracellular nicotinamide adenine dinucleotide (NAD^+) levels, leading to activation of the NAD^+-sensitive deacetylase SIRT1 (Iglesias et al., 2004; Jager et al., 2007; Canto et al., 2009; Fujitsuka et al., 2016). Furthermore, AMPK phosphorylates glyceraldehyde 3-phosphate dehydrogenase (GAPDH) at Ser^{122} during starvation to encourage the nuclear trafficking of GAPDH and the displacement of SIRT1 from its repressor Deleted in Breast Cancer 1 (DBC1) (Chang et al., 2015). On the other hand, SIRT1 deacetylates and activates liver kinase B1 (LKB1), the second major AMPK-targeting kinase besides CaMKKß, indicating a reciprocal relationship between AMPK and SIRT1 (Lan et al., 2008). Moreover, SIRT1 is capable of shuttling between the cytoplasm and nucleus (Tanno et al., 2007) and deacetylates nuclear PGC1α to initiate mitochondrial biogenesis (Lagouge et al., 2006) as well as the transcription factor forkhead box protein O1 (FoxO1) to amplify the expression of PGC1α (Daitoku et al., 2003; Frescas et al., 2005; Nakae et al., 2008). AG was shown to induce the synthesis of FoxO1 in the hypothalamus (Lage et al., 2010) and, under conditions of cellular stress, FoxO1 drives the transcription of various anti-oxidant enzymes, such as the PGC1α-co-activated, mitochondrial MnSOD (St.-Pierre et al., 2006; Hsu et al., 2010; Tong et al., 2012). Besides the activation of PGC1α and FoxO1, SIRT1 also improves stress tolerance through deacetylation of other effector proteins, such as the inflammatory master regulator nuclear factor κB (NF-κB) (Yeung et al., 2004) or DNA repair factor Ku70, which was shown to scavenge pro-apoptotic Bax from mitochondria to support cellular survival (Cohen et al., 2004). The activation of the AMPK/Sirt1/PGC-1α/UCP2 pathway by AG, in a GHSR1α-dependent manner, has also been connected to the amelioration of oxidative stress, neuronal atrophy and functional decline in response to hypoxic-ischemic encephalopathy *in vivo*, emphasizing the neuroprotective impact of this pathway (Huang et al., 2019). As such, AG not only mitigates the stress-provoked ATP hyperproduction and the associated excessive generation of ROS by burdened mitochondria in a UCP2-driven manner, but also re-invigorates mitoprotective and mitochondrial biogenesis-inducing AMPK and PGC-1α signaling in AD and PD.

A Possible Implication of Acylated Ghrelin in the Enhancement of Mitochondrial Fusion and Fission

Unsurprisingly, in accordance with general mitochondrial dysfunction, the efficiency of mitochondrial fusion and fission gradually declines during the aging process and is disturbed in neurodegenerative diseases (Liu et al., 2020). Cell and animal studies in AD models as well as post-mortem examinations of patients, although not always matching perfectly, signify that the transcription of fusion-enhancers (OPA1, mitofusin (Mfn)1/2) is attenuated and the expression or activity of fission-modulators (dynamin-related protein 1 (Drp1), mitochondrial fission 1 protein (Fis1) and S-nitrosylated Fis1) are aberrantly elevated. These alterations provoked mitochondrial hyperfission, neuronal injury and synaptic degeneration *in vitro* and *in vivo* (Wang et al., 2008, 2009; Cho et al., 2009). On the other hand, in Aβ-based AD models, the genetic deletion of the fission-inducer Drp1 rescued from mitochondrial fragmentation, the drop of mitochondrial membrane potential and ATP production, the generation of ROS *in vitro* and prevented the accumulation of lipid peroxidation products, beta-secretase 1 expression, the formation of amyloid plaques and cognitive decline in APPswe/PSEN1dE9 mice (Baek et al., 2017). Similarly, the pharmacological or genetic interference with Drp-1 or the overexpression of the fusion-enhancers Mfn2

and OPA1 ameliorated excessive mitochondrial fission and impaired ATP production in PINK1/Parkin-mutant cells (Lutz et al., 2009) and shielded against MPTP-driven mitochondrial fragmentation, the stimulation of the pro-apoptotic activity of p53, Bax and PUMA, dopaminergic neuron and nerve terminal loss as well as motor deficits, but not micro- and astrogliosis, in the murine SNpc (Filichia et al., 2016). In opposition to Aβ, the role of PD-associated α-synuclein is less evident. While mutant α-synuclein enhances mitochondrial fragmentation, impairs the mitochondrial respiratory activity and induces neuronal death by inducing the displacement of wt α-synuclein from the inner-mitochondrial membrane (Kamp et al., 2010; Nakamura et al., 2011; Guardia-Laguarta et al., 2014), it must be noted that wt α-synuclein, in fact, promotes fusion and its expression may be a compensatory and protective mechanism to prevent hyperfission in PD (Berthet et al., 2014; Guardia-Laguarta et al., 2014; Menges et al., 2017).

Recent reports suggest a possible role for AG in the regulation of the mitochondrial fission and fusion dynamics (Morgan et al., 2018). In general, caloric restriction, which enhances the plasma release of AG, favors mitochondrial fission, leading to an increase in the expression levels of Drp1 and Fis1, while not altering the transcriptional pools of fusion-modulators, such as Mfn1, Mfn2 or OPA1 (Khraiwesh et al., 2013). Moreover, mitochondrial toxins, such as the PD-poison rotenone, and the pharmacological stimulation of AMPK activity, independent of any mitochondrial damage, provoke mitochondrial fission. In the context of AMPK, mitochondrial fission factor (MFF) has recently been identified as a direct downstream target of AMPK and the AMPK-mediated activation of MFF leads to the induction of the fission-promoting Drp1 (Toyama et al., 2016).

Notably, the stimulation of PGC-1α in response to heightened energy expenditure has been linked to the transcriptional upregulation of the mitochondrial fusion-advocate Mfn2 in the skeletal musculature of mice (Soriano et al., 2006). It was also shown that the overexpression of PGC-1α opposed unloading-associated muscular atrophy in the murine hindlimbs and prevented the transcriptional decline of the fusion-imparting proteins Mfn1, Mfn2 and OPA1, therefore restoring mitochondrial defects by improving fusion (Cannavino et al., 2015). Importantly, in the context of PD, the rotenone-evoked mitochondrial fragmentation and dysfunction have been connected to impairments in the mitochondrial biogenesis, the decreased activity of TFAM and PGC-1α as well as deregulated mitochondrial fusion and fission, which was related to transcriptional alterations in Mfn2, OPA1, Drp1, and Fis1 in PC12 dopaminergic neurons. The application of PGC-1α siRNA as well as the overexpression of this mitochondrial effector confirmed that PGC-1α upregulates the synthesis of Mfn2, while suppressing the transcription of Drp1. On the contrary, the neuronal exposure to rotenone augmented p-Drp1 levels and promoted its translocation toward mitochondria to evoke fragmentation, which was exacerbated by the muting of PGC-1α and prevented through the overexpression of PGC-1α. The results of this study imply a primarily fusion-enhancing and fission-inhibiting function of PGC-1α under physiological conditions, while the induction of PGC-1α protects from stress-driven mitochondrial fragmentation in dopaminergic neurons (Peng et al., 2017).

Considerably, AG stimulates the activity of the fusion/fission-regulators AMPK and PGC-1α in GHS-R1α-expressing cells, including neurons (Bayliss and Andrews, 2013; Huang et al., 2019). Moreover, besides an impressive range of other mitoprotective effects, the ghrelin analogs JMV2894 and/or hexarelin suppressed excessive, cisplatin-triggered mitochondrial fission in the skeletal muscles of rats by reversing the upregulation of Drp1 and the downregulation of Mfn2, thus raising the Mfn2/Drp1 index back to the levels of control rodents (Sirago et al., 2017). This is in line with the Mfn2-upregulating and Drp1-impeding function of PGC-1α (Peng et al., 2017), suggesting that AG stimulates the AMPK/PGC-1α axis to ameliorate mitochondrial fragmentation in response to cellular stress (Sirago et al., 2017). Therefore, AG may guard against pathologic hyperfission in AD and PD. Nonetheless, future studies are necessary to confirm a fusion/fission-navigating function of AG in appropriate models of neurodegeneration.

Acylated Ghrelin Navigates the Release of Lactate by Astrocytes

Interestingly, AG may coordinate bioenergetic communication between astrocytes and neurons. Using a combination of rodents and primary hypothalamic astrocyte culture, it was discovered that AG downregulates the expression of glucose transporter (GLUT)2, but not GLUT1 or GLUT3, increases the transcription of glutamate-aspartate transporters in a GHS-R1α-dependent manner, enhanced the expression of lactate dehydrogenase and glycogen phosphorylase, diminished the transcriptional levels of glutamine synthase and upregulated the lactate-transporter monocarboxylate transporter 4 (MCT4). Ultimately, the latter changes led to reduced glucose uptake, elevated glutamate uptake and the steadily rising lactate levels in the cell culture medium (Fuente-Martin et al., 2016). Although the latter study showed some inconsistencies, AG appears to trigger a physiological, metabolic switch in astrocytes to preserve glucose and curb its uptake by astrocytes during fasting. In exchange, AG appears to prime astrocytes toward glutamate and possibly glycogen metabolism to generate ATP, while encouraging the liberation of lactate as a powerful alternative energy source for neurons (Schurr et al., 1988). Thus, AG possibly supports the neuronal activity in face of AD/PD-associated bioenergetic deficiencies and glucose hypometabolism in the brain (Neth and Craft, 2017; Sweeney et al., 2018).

AUTOPHAGY AND MITOPHAGY

Deficiencies in Autophagy and Mitophagy Promote the Accumulation of Amyloids and Defective Mitochondria in Alzheimer's and Parkinson's Disease

Classically, dysfunctional autophagy is a common trait shared by most neurodegenerative diseases. Due to the less efficient removal of waste proteins in neurons, deficits in autophagy are thought to encourage the accumulation of toxic and misfolded proteins, such as Aβ and Tau in AD as well as α-synuclein (Lewy

bodies) in PD (Fujikake et al., 2018). As a side note, the genetic deletion of the autophagy modulators autophagy related (ATG)5 and ATG7 evoked the age-dependent formation of ubiquitinated, diffuse inclusions, severe neuronal atrophy and disturbances in motor function and coordination. Thus, impairments in autophagy induce neurodegeneration independent of amyloid accumulation in affected brain areas (Hara et al., 2006; Komatsu et al., 2006).

Immunohistological investigations in the brain tissue of AD patients suggest that early increases in the neuronal rate of autophagy compensate for the accumulation of waste products, whereas the lysosomal function (proteolytic enzyme activity) and the clearance of lysosomal vacuoles is gradually impaired. This results in the intraneuronal accumulation of non-degraded and amyloid-containing autophagosomes, co-localizing strongly with neurons that display intracellular Tau pathology and the relative loss of mitochondria and other organelles (Cataldo et al., 1994; Nixon et al., 2005). An important distinction to make is that the blockade of autophagy, as achieved with the inhibition of mTor, obviously slowed the rate of degradation, yet showed no major consequences. In contrast, the inhibition of lysosome-associated proteolytic enzymes was capable of producing an AD-like phenotype (Boland et al., 2008). Therefore, AD patients appear to show deficits in the fusion of waste-filled autophagic vacuoles with lysosomes and the intra-autophagosomal degradation process. Nonetheless, AD patients showed a massive decline in the transcriptional levels of the autophagy initiator Beclin-1 during early stages of AD and strategies that have aimed to enhance the degree of autophagy, such as the lentivirus-mediated expression of Beclin 1 or the autophagy-inducing blockage of mTor by rapamycin, have been successful in the purging of Aβ and Tau pathology in *in vitro* and *in vivo* models of AD (Pickford et al., 2008; Jaeger et al., 2010; Spilman et al., 2010; Majumder et al., 2011). Such findings indicate that autophagy-enhancing approaches must ensue early, since the mere upregulation of autophagy is insufficient at an advanced stage of AD, when insoluble and proteolysis-resistant aggregates have already formed in the brain (Majumder et al., 2011).

Analogical to AD, late-stage PD patients showed diminished levels of the LAMP1, LAMP2A, and heat shock cognate 70, which execute chaperone-mediated autophagy, yet displayed elevated LC3-II levels (symbolic for autophagosome accumulation) and α-synuclein inclusions in the SN pars compacta (SNpc) and/or the amygdala. This indicates that, similar to AD, autophagosomal efficiency is lost during PD, leading to the amassment of defective, waste-cluttered lysosomes and the failure of amyloid clearance (Chu et al., 2009; Alvarez-Erviti et al., 2010; Dehay et al., 2010). There is also evidence that proteins involved in autophagosome initiation and formation, for example LC3 or ULK1/2, are sequestered into Lewy bodies in the brain of PD patients (Tanji et al., 2011; Miki et al., 2016). However, the lentiviral overexpression of Beclin 1 or the utility of the autophagy-activator and mTor-inhibitor rapamycin rescued the apoptosis of dopaminergic neurons in response to the loss of proteasomal function or the accumulation of α-synuclein in cells and animals (Pan et al., 2008; Spencer et al., 2009). These findings propose that early pharmacological interventions to potentiate the rate of autophagy may be useful to prevent the harmful accumulation of amyloids, although such approaches, due to dysfunctions in the autophagy machinery, are less likely to succeed at more advanced stages of AD and PD. These later defects in autophagy are likely to be the accumulative result of general impairments in the neuronal metabolism, including mitochondrial defects, heightened oxidative stress and amyloid burden, glucose hypometabolism, diminished growth factor and insulin-signaling etc, indicating that multi-targeted therapeutic approaches are advantageous.

Notably, mitophagy poses a specialized form of autophagy that rids cells from defective, ROS-generating mitochondria. As expected, mitophagy is widely impaired in respective models as well as in the brains of AD and PD patients, while the selective pharmacological enhancement of mitophagy can reverse several other pathological aspects, such as the generation of insoluble Aβ, Tau hyperphosphorylation, neuroinflammation, neuronal atrophy and cognitive impediments (Fang et al., 2019; Liu J. et al., 2019).

A Mitophagy-Enhancing Role of Acylated Ghrelin Has Been Strongly Indicated

Interestingly, ghrelin may improve mitophagy, an autophagy derivate involved in mitochondrial quality control and disposal of damaged mitochondria (Bayliss and Andrews, 2013). While ghrelin is often praised for its ability to promote mitophagy, little mechanistic research has been conducted. To our knowledge, there is only a single study that has truly confirmed a mitophagy-boosting function, showing that the administration of AG enhanced autophagy and led to the emergence of autophagosome-enclosed mitochondria at various stages of degradation in HL-1 cardiac muscle cells (Ruozi et al., 2015). At the time, Bayliss and Andrews also admitted that there is no direct evidence that AG activates or promotes the activity of the main mitophagy modulators Parkin or PTEN-induced kinase 1 (Bayliss and Andrews, 2013). Based on the current lack of studies, it can only be assumed that AG promotes mitophagy indirectly by generally enhancing cellular autophagy (see chapter 4.3 below) and reducing the mitochondrial generation of ROS in a UCP2-conveyed manner, thus avoiding the accumulation of dysfunctional mitochondria in the first place.

Acylated Ghrelin Induces Autophagy in the Periphery and in the CNS

Indeed, AG's autophagy-enhancing features, as summarized in **Figure 1**, have only recently emerged in the literature. Nonetheless, there is abundant evidence that highlights ghrelin's autophagy-triggering and tissue-preserving function in peripheral tissue. AG-driven autophagy is dependent on the stimulation of AMPK, leading to increased levels of ATG5, ATG7, ATG12, and Beclin-1, lessened p62 levels (an autophagy marker that is adversely correlated with autophagy), an elevated microtubule-associated protein light chain 3 (LC3)-II/LC3-I ratio, which serves as a marker to quantify mature autophagosomes (Mizushima and Yoshimori, 2007), and improvements in the autophagic flux (demonstrative of the formation and degradation rate of autophagosomes in a given time frame) (Slupecka et al., 2012; Tong et al., 2012; Mao et al.,

2015; Ruozi et al., 2015; Ezquerro et al., 2016; Wan et al., 2016; Xu et al., 2017).

In contrast, the cerebral induction of autophagy by ghrelin has only sparsely been investigated. Nonetheless, it was demonstrated that SH-SY5Y cells stably expressing mutant amyloid precursor protein (APP) exhibit elevated anti-apoptotic Bcl-2 levels, decreased caspase-3 and caspase-7 activities, increased proteasome activity and improved autophagy, as marked by increased Beclin-1, LC3-II and normalized p62 levels, upon treatment with AG. The cytoprotective effects of AG were attributed to its ability to improve crosstalk between proteasomal and autophagosomal pathways, leading to the enhanced clearance of the overexpressed APP/Aß fragments in this cell model (Cecarini et al., 2016). Another well-constructed study discovered that caloric restriction raises both mRNA and protein levels of NPY as well as ghrelin in rat cortical neurons, resulting in diminished phospho-mTor levels, increased LC3-II levels, decreased p62 pools and enhanced autophagic flux (Ferreira-Marques et al., 2016). Importantly, autophagy was independently achieved through the use of AG or NPY, respectively, whereas the individual administration of either GHS-R1α or Y_1, Y_2, or Y_5 receptor antagonists were able to attenuate autophagy, suggesting a synergistic effect of both peptides. Although we will not further address the neuroprotective properties of NPY (see Li et al., 2019), it must be noted that AG was shown to raise the synthesis of NPY in hypothalamic and cortical neurons (Wren et al., 2002; Ferreira-Marques et al., 2016). As such, there is the need to clarify which peptide acts in what brain region and which autophagy-promoting pathways are activated by NPY or AG, respectively.

As it is the common consensus, (macro)autophagy is primarily controlled by the activity of mTor or, more precisely, mTOR complex (mTORC)1. In the absence of nutrients and in a cellular effort to maintain the status quo, the deactivation of mTORC1 is linked to the decreased activity of ribosomal protein S6 kinase beta-1 (S6K1)/S6 protein, the elevated activation of the transcription-repressor 4E-binding protein 1, the inactivity of eukaryotic translation initiation factor 4E and, thus, the overall decreased expression of proteins. Shut-down of the growth-facilitating mTORC1 pathway, however, promotes the activity of the unc-51 like autophagy activating kinase ($ULK_{1/2}$) initiation complex, which launches autophagosome maturation and the cellular purging of waste products, such as Aß, Tau or α-synuclein (Huang and Manning, 2008; Ma and Blenis, 2009; Lan et al., 2017; Kaleli et al., 2020). Tuberous sclerosis $(TSC)_{1/2}$ acts as a major regulatory switch for mTORC1-mediated growth vs. autophagy and, typically in response to stressful cellular conditions and starvation, the activating phosphorylation of the cytoplasmic energy-sensor AMPK results in the AMPK-mediated phosphorylation of $TSC_{1/2}$ and the inhibition of mTORC1 (Inoki et al., 2003; Manning and Cantley, 2003; Demetriades et al., 2016). Additionally, a reciprocal connection between mTor and AMPK exists, in which the absence of nutrients promotes the AMPK-driven suppression of mTor and the activating phosphorylation of ULK1 at Ser^{317} and Ser^{777}, whereas energetic abundance stimulates the inhibitory phosphorylation of ULK1 at Ser^{757} via mTor (Kim et al., 2011; Lan et al., 2017).

AG-evoked autophagy is mainly connected to the downstream activation of the phospho-AMPK/mTOR axis, as it has been well-described for the initiation of autophagy in peripheral tissue (Tong et al., 2012; Mao et al., 2015; Ruozi et al., 2015; Ezquerro et al., 2016; Xu et al., 2017). In contrast, the limited amount of cerebral studies with AG, at the very least, have verified the induction of autophagy via mTOR inhibition in cortical neurons (Ferreira-Marques et al., 2016). The stimulation of AMPK, which is a highly debated therapeutic option for the treatment of PD, is, in fact, responsible for the large majority of AG's neuroprotective effects, including (macro)autophagy, mitochondrial enhancement as well as the cellular safeguarding from oxidative stress and inflammation (Bayliss and Andrews, 2013; Curry et al., 2018). Besides AMPK, another important positive regulator of autophagy poses SIRT1 (Chen et al., 2020). The activation of SIRT1 via AG has been confirmed in the periphery (Fujimura et al., 2014; Tamaki et al., 2015; Fujitsuka et al., 2016; Yang et al., 2016) as well as the hypothalamus in adult rodents and mouse models of aging (Velasquez et al., 2011; Fujitsuka et al., 2016). Indeed, SIRT1 is not only elevated upon treatment with AG, but interference with AMPK/SIRT1 signaling prevented the induction of autophagy in lymphoblastic leukemia cells (Heshmati et al., 2020). It has also been reported that SIRT1 directly deacetylates Tau protein at Lys^{174} and the viral delivery of SIRT1 to the hippocampus of SIRT1-deficient and P301S Tau transgenic mice attenuated the cerebral propagation of Tau (Min et al., 2018). AG-upregulated and SIRT1-activated and FoxO1 is well-known in aging research, responsible for the transcription of ATG genes and the mTor-suppressor Sestrin 3, therefore encouraging autophagy (see **Figure 1** for an illustration of the discussed pathways) (Lage et al., 2010; Zhang et al., 2015).

Interestingly, in some instances, AG induces counterintuitive signaling pathways and stimulates neuroprotective Akt, which is an mTor-activator. The AG-driven induction of these discrepant signaling cascades seem to be highly conditional for preventing neuronal apoptosis during cerebral ischemia and excitotoxicity, however, and may be linked to the upregulation of other growth factors, such as IGF-1 (Frago et al., 2011; Spencer et al., 2013). In any case, the current evidence suggests that AG augments the neuronal rate of autophagy by inducing AMPK-signaling to inhibit mTor and upregulating the expression of various autophagy-implementing effectors to degrade amyloids, such as Aβ, in AD and PD. Since AG improves other pathologic areas, for instance mitochondrial dysfunction (chapter 3.2), insulin resistance and glucose hypometabolism (chapter 7.2) in neurons, AG may further ameliorate the functional deficits in autophagy that occur during later stages of AD and PD.

INFLAMMATION

Systemic Inflammation in Alzheimer's and Parkinson's Accelerates Disease Progression

The detrimental impact of the neuroinflammatory pathology, which is believed to commence decades before the appearance of any symptoms, is widely acknowledged in AD and PD.

While beneficial in the healthy brain, neurodegenerative conditions provoke a chronic shift of microglia as well as astrocytes from the supportive M2 to the pro-inflammatory M1 state, resulting in the release of various pro-inflammatory cytokines, including TNF-α, IFN-γ, IL-1ß, IL-6, and IL-12, and chemokines, for example the immune cell-recruiting monocyte chemoattractant protein 1 (MCP-1), the generation of excessive amounts of ROS and nitric oxide (NO) as well as the secretion of glutamate. Over time, prolonged neuroinflammation encourages various other secondary complications, such as impairments in protein degradation, amyloid misfolding, Tau hyperphosphorylation (in conjunction with the inflammation-perpetuating activation of the inflammasome), permeabilization of the BBB, peripheral immune cell infiltration into the CNS, mitochondrial dysfunction, cerebral insulin resistance, injury of the axonal myelin sheath and oligodendrocytes (evident in AD, yet less clear in PD), axonal transport deficiencies, synaptic damage, and, ultimately, widespread neuronal apoptosis (Gonzalez et al., 2014; Najem et al., 2014; Chen et al., 2016; Wang S. S. et al., 2018; Ising et al., 2019). Microglia may be stimulated by the Toll-like receptor (TLR)-mediated recognition of bacterial and viral particles, for example lipopolysaccharides (LPS) (Boche et al., 2013), the TLR2-driven interaction with α-synuclein (Kim et al., 2013), TLR2/4-binding to Aß (Reed-Geaghan et al., 2009), and serum-derived or locally released TNF-α and IFN-γ, whose combinatorial action was shown to be a crucial inflammatory mediator of dopaminergic cell death in a rodent model of PD (Mir et al., 2008; Barcia et al., 2011). In this context, some genetic variants of TLR4 have been linked to AD and the increased expression of TLR2 has been identified in AD models (Balistreri et al., 2009; Letiembre et al., 2009), whereas the enhanced transcription of TLR2 and TLR4 have been detected in α-synuclein and MPTP mouse models of PD (Panaro et al., 2008; Letiembre et al., 2009), indicating that immune regulation is harmfully altered in AD and PD. Likewise, astrocytes may be provoked by TLR2/4/5/6 receptor ligands, Aß or α-synuclein as well as microglia-derived cytokines, in particular the key stimulatory agents IFN-γ and TNF-α (Johnstone et al., 1999; Bezzi et al., 2001; Lee H. J. et al., 2010; Barcia et al., 2011; Ma et al., 2013).

Notably, there are other inflammatory triggers besides amyloids in AD and PD. More precisely, fragments derived from apoptotic neurons, termed damage-associated molecular patterns (DAMPs), are capable of stimulating inflammatory cascades via interaction with TLRs or receptors for advanced glycation endproducts on microglia. DAMPs, of course, include Aß, Tau and α-synuclein, but also encompass many more, such as myelin debris, neuron-specific enolase (a glycolytic enzyme), S100 calcium-binding protein β (S-100ß) (an astroglial modulator), advanced glycation end products and many more. Furthermore, pathogen-associated molecular patterns (PAMPs) that originate from cerebral infections, such as LPS, or, in the case of AD, infections with members of the Herpesviridae family and Hepatitis C virus, may further potentiate neuroinflammation (Morales et al., 2014; Sochocka et al., 2017; Cortes et al., 2018; Stephenson et al., 2018).

Importantly, inflammation is not limited to the brain in AD and PD, but is potentiated by multiple inflammatory mechanisms in the periphery. First, the presence of heightened levels of pro-inflammatory cytokines in the blood stream can be sensed by the CNS through the so-called gut-brain axis, also known as the "vagal reflex." The latter involves the intestinal monitoring of the peripheral inflammatory status by the efferent ends of the vagus nerve. In the presence of abnormally elevated levels of pro-inflammatory cytokines in the blood stream or following gut microbial inflammation, the vagal nerve signals to the nucleus tractus solitarius (NTS), a major signaling hub located in the brain stem, that receives input from multiple peripheral organs. The NTS, on the other hand, further projects across the entirety of the CNS, ultimately leading to the intestinal return of immune-suppressing signals through the afferent ends of the vagal nerve. It has been proposed that chronic inflammation provokes NTS dysfunction, which, in turn, propagates neuroinflammation and death across the brain in AD (Daulatzai, 2012; Wang J. T. et al., 2018). Moreover, intestinal inflammation and injury are strikingly pronounced prior to the onset of AD and PD, which, besides the additional inflammatory burden, induce the leakage of Aβ- and α-synuclein-like amyloids that may cross the enteric nervous system (ENS), enter the brain and stimulate cross-seeding (Ambrosini et al., 2019). Second, blood-borne pro-inflammatory cytokines/chemokines, PAMPs and DAMPs may access the CNS directly or indirectly, by promoting BBB damage and leakage. In cooperation, the cerebral and blood stream-derived inflammatory agents induce neuronal death, kill oligodendrocytes, injure the axonal myelin sheath, evoke atrophy of the neuronal projections and lead to the assassination of astrocytes, further weakening the integrity of the BBB (Sankowski et al., 2015). Notably, metabolic and vascular disorders provoke chronic low-grade inflammation in the peripheral system that, as anticipated, contribute to the development of AD and PD (Chen et al., 2016). Third, as a consequence of BBB permeabilization, immune cell infiltration is encouraged. DAMPs, such as aggregated amyloids or fragments of apoptotic neurons, may reach the circulatory stream through the lymph nodes or following BBB breaching, while Aß may also be drained at perivascular and leptomeningeal spaces. Subsequent peripheral inflammatory responses by antigen-presenting cells and lymphocytes (T-cells) then induce immune entry into the CNS in AD and PD (Fisher et al., 2011; Anderson et al., 2014).

Regarding immune infiltration, $CD4^+$ T-cell-deleted mice were shown to be protected from MPTP-triggered neurodegeneration, proposing that the adaptive immune system is heavily involved in PD pathology (Brochard et al., 2009). Further *in vivo* studies in the MPTP model support the idea that, in conjunction with BBB injury and the loss of tight junction proteins in the nigrostriatal area, the pathological infiltration of lymphocytes and other immune cells as well as the T-cell-driven induction of microglia occur in the SN (Kurkowska-Jastrzebska et al., 1999; Chao et al., 2009; Reynolds et al., 2010; Depboylu et al., 2012). Moreover, it has been strongly implied that $CD4^+$ T helper (Th)1 and Th17 cells, for instance immunoreactive against α-synuclein, are the main lymphocyte populations that contribute to the death of dopaminergic neurons (Brochard et al., 2009; Reynolds et al., 2010). Moreover, the invasion of $CD4^+$ and $CD8^+$ T-cells has been confirmed in post-mortem brain tissue of

PD patients, co-localizing with lesioned brain regions (Brochard et al., 2009). A clinical investigation concluded that the quantity of serum $CD4^+$ T-cells was correlated to the PD disease score and the functional impairment of T-cell suppressing regulatory T-cells (Tregs) was identified in the blood of PD patients (Saunders et al., 2012). In addition, heightened numbers of partially α-synuclein-reactive Th17 cells were discovered in the blood of PD patients (Sulzer et al., 2017); to the degree that PD has been postulated as an α-synuclein-reactive autoimmune disease (Benner et al., 2008; Hu, 2011). Unsurprisingly, in line with the encouraged cerebral trespassing of lymphocytes, BBB leakage has been confirmed in the brains of PD patients (Kortekaas et al., 2005; Pisani et al., 2012). A similar pathologic role for T-cells has been implied in AD. For example, the long-term administration of low-doses of IL-2, believed to assist the activity of Tregs (Klatzmann and Abbas, 2015), enhanced the levels of Tregs in the rodent brain, improved the $Aß_{42/40}$ ratio, stimulated the clearance of Aß plaques, elevated LTP, attenuated spinal degeneration and reversed memory impediments in the APP/PS1ΔE9 mouse model of AD (Alves et al., 2017). Additionally, altered adaptive immune mechanics have been observed in the CNS of several Aß-based *in vivo* models of AD, displaying hippocampal BBB disruption, the infiltration of peripheral monocytes/macrophages, neutrophils and $CD4^+$ T-cells (predominantly Th1 and Th17) as well as the increased transcription of pro-inflammatory cytokines, such as IFN-γ and IL-17, and chemokines, including MIP-1α (a macrophage attractant) plus CXCL1 (implicated in neutrophil recruitment) (Browne et al., 2013; Zhang J. et al., 2013; Minogue et al., 2014). In accord with the animal studies, blood profiling of AD patients indicated heightened adaptive immune responses, such as a total reduction in naive T-cells, a tendency of T-cells to differentiate into $CD4^+$ subsets or the elevated activity of pro-inflammatory $CD4^+$ Th17 cells (Shalit et al., 1995; Richartz-Salzburger et al., 2007; Speciale et al., 2007; Larbi et al., 2009; Saresella et al., 2011). Another study further concluded that $CD4^+$ T-cell counts might be correlated to AD severity (Shalit et al., 1995).

Intriguingly, AG is capable of preventing the latter described, AD/PD-associated cerebral, peripheral and adaptive immune alterations, as condensed in **Figure 2**. The following chapters will investigate these anti-inflammatory characteristics of AG in greater detail. Although many of the subsequent studies were not conducted in AD or PD models, they serve as a proof of principle to emphasize that AG functions as a potent systemic immunosuppressant, independent of the underlying inflammatory context.

Neuroinflammation

Acylated Ghrelin Abrogates Neuroinflammation Indirectly by Preventing the Apoptosis of Cerebral Cells

Indeed, there is abundant evidence that the utility of AG curbs pro-inflammatory responses in the CNS. A series of *in vivo*/*ex vivo* studies has given clear indication that AG prevents microgliosis, astrogliosis and/or the cerebral expression of pro-inflammatory cytokines in animal models of AD, in which Aβ was the primary inflammatory stimulator (Moon et al., 2011; Dhurandhar et al., 2013; Santos et al., 2017; Jeong et al., 2018), in the MPTP-induced PD rodent model (Moon et al., 2009a), following various forms of ischemic CNS/spinal cord injury (SCI) in rodents (Ersahin et al., 2010, 2011; Lee J. Y. et al., 2010; Cheyuo et al., 2011; Kenny et al., 2013; Lee et al., 2014b, 2015), as well as drug-induced excitotoxicity (Lee J. et al., 2010; Lee et al., 2012). However, despite AG's well-established, anti-inflammatory actions in the CNS, *in vitro* and *in vivo* investigations have confirmed that neither brain- or spinal cord-resident microglia, cultured BV-2 microglial cells nor primary microglia express GHS-R1α (Moon et al., 2009a; Lee J. Y. et al., 2010; Lee and Yune, 2014). In the case of astrocytes, it was reported that AG directly decreases the secretion of tumor necrosis factor alpha (TNF-α) by cultured hypothalamic astrocytes, although the hormone stimulated the liberation of interleukin (IL)-6 (Garcia-Caceres et al., 2014).

Instead of a direct, immunosuppressive effect on microglia and astrocytes, the majority of studies suggest that AG operates in an indirect manner and restrains neuroinflammation through its cytoprotective properties in neurons and other cerebral cells. For instance, in the MPTP mouse model of PD, AG attenuated microglial induction, reduced the expression of IL-1 and TNF-α and diminished nitrotyrosine and NO levels in the SNpc, which protected local dopaminergic neurons and striatal projections from neurotoxic assault (Moon et al., 2009a). Importantly, while the authors verified the absence of GHS-R1α on microglia, the use of GHS-R1α-antagonists fully ablated the anti-inflammatory and protective effects of AG, highlighting that there must be a GHS-R1α-attributed, yet indirect, mechanism at work that inhibits pro-inflammatory immune responses. Interestingly, a cell culture study demonstrated that the reduced microglial activation following AG-treatment was linked to the downregulation of matrix-metalloproteinase 3 (MMP-3) by co-cultured, dopaminergic neurons (Moon et al., 2009a). Likewise, AG guarded bEnd.3 microvascular endothelial cells from oxygen-glucose deprivation/reoxygenation *in vitro,* hippocampal neurons from kainic acid as well as spinal cord neurons from mechanical injury *in vivo*, thus forestalling the release of MMP-3 by apoptotic cells. This, in a MMP-3-dependent manner, resulted in diminished microglial inflammation (Lee J. et al., 2010; Lee et al., 2015). Indeed, MMP-3, typically originating from apoptotic neurons, but also dying astrocytes and endothelial cells, is a well-known inflammatory stimulator of microglia that evokes superoxide production and the microglial secretion of TNF-α, IL-1β and IL-6. In a reciprocal manner, inflammation incites the microglial expression and liberation of MMP-3, initiating a wicked cycle of neuronal degeneration and neuroinflammation (Kim et al., 2005; Kim and Hwang, 2011). Besides MMP-3, *in vitro* studies have demonstrated that AG suppressed the LPS-induced secretion of IL-6 in mouse dopaminergic SN4741 cells and the MPTP-enforced induction of the inflammatory master regulator NF-κB in mouse dopaminergic MES23.5 cells. The latter was further accompanied by the attenuated formation of the oxidative stress marker malonaldehyde, the normalization of the transcriptional levels of the anti-oxidative enzymes SOD and catalase as well as the upregulation of the Bax/Bcl-2 ratio, symbolic for the protection from neuronal apoptosis (Liu et al.,

2010; Beynon et al., 2013). As such, the existing evidence points toward an inflammation-suppressing and survival-enhancing function in non-microglial cerebral cells that is indirectly linked to the reduced liberation of inflammation-stimulating damage associated molecular patterns (DAMPs), such as MMP-3.

The Acylated Hormone Ghrelin Rescues Oligodendrocytes and Prevents Demyelination

It must be mentioned that AG guards oligodendrocytes, the exclusively myelinating cell type in the brain. As confirmed with the utility of GHS-R1α, ERK and p38 inhibitors, an *in vitro* study showed that the interaction of AG with GHS-R1α on oligodendrocytes shields the cells from hydrogen peroxide (H_2O_2) and apoptosis by potentiating oligoprotective ERK signaling, while attenuating the pro-apoptotic activation of p38 (Lee et al., 2011). Another cell culture study emphasized that AG is capable of blocking the LPS-provoked inflammatory stimulation of the p38 and c-Jun N-terminal kinase (JNK) stress kinase pathways, the release of pro-nerve growth factor and the generation of ROS by BV-2 microglial cells, thus protecting co-cultured oligodendrocytes from death by oxidative assault (Lee and Yune, 2014). Collectively, previous research has demonstrated that AG rescued oligodendrocytes from inflammatory and oxidative damage, therefore protecting the integrity of myelinated axons in *in vivo* models of SCI and MS (Lee J. Y. et al., 2010; Lee et al., 2011; Liu F. et al., 2019). As such, the utility of AG may be useful to ameliorate the age-associated myelin pathology in neurodegenerative diseases (Wang S. S. et al., 2018), yet further investigations in the context of AD and PD are necessary.

Ghrelin Agonists May Suppress Microglial Inflammation by Binding to CD36

Notably, human fetal microglia, N9 microglial cells as well as microglia resident in the AD and non-AD brain, along with monocytes, macrophages and endothelial cells, were shown to express a GPCR known as cluster of differentiation 36 (CD36). This receptor has been reported to act as an inflammatory conductor for Aß, leading to the production of ROS and pro-inflammatory cytokines upon the interaction of fibrillar Aß with microglial or macrophage CD36 (Coraci et al., 2002; Bamberger et al., 2003; El Khoury et al., 2003; Demers et al., 2004). Interestingly, a receptor binding site for hexarelin, a synthetic DAG analog, was identified on CD36 (Demers et al., 2004). Furthermore, a study uncovered that DAG, but not AG, was capable of binding to CD36 receptors on cultured N9 cells, preventing fibrillar $Aß_{25-35}$-triggered release of IL-1ß and IL-6 (Bulgarelli et al., 2009). Since anti-CD36 antibodies strongly attenuated N9 microglial H_2O_2 production (Coraci et al., 2002), it is likely that the binding of DAG sterically hinders the pro-inflammatory interaction of CD36 with Aß (Bulgarelli et al., 2009).

Intriguingly, some ghrelin agonists show affinity toward both GHS-R1α and CD36, for example hexarelin or GHRS-6 (Demers et al., 2004; Berlanga-Acosta et al., 2017). Hexarelin was demonstrated to interact with both GHS-R1α and CD36 on cultured THP-1 monocytes and primary peritoneal macrophages derived from $apoE^{-/-}$ mice (Avallone et al., 2006). Moreover, the prolonged daily injection of the CD36-favoring ghrelin derivate EP 80317 dramatically ameliorated the development of vascular lesions in the $apoE^{-/-}$ animal model of arteriosclerosis by lessening the CD36-driven endocytosis of oxidized low density lipoprotein (oxLDL) by macrophages (Marleau et al., 2005). Thus, in direct comparison to the GHS-R1α-binding AG, it is tempting to speculate that GHS-R1α/CD36 co-binding ghrelin analogs may be a superior choice for the amelioration of Aβ-driven microglial inflammation and ROS-production in AD.

Evidence that Acylated Ghrelin Opposes the Activation of the Inflammasome in the Brain

The stimulation of the inflammasome and the associated pyroptosis, the "fiery death" of microglia, oligodendrocytes and other cells, a relatively recent upbringing, have been identified as major drivers of neuroinflammation, demyelination and degeneration of the spinal cord during MS (McKenzie et al., 2018). The nod-like receptor protein 3 (NLRP3) inflammasome-associated propagation of neuroinflammation has also recently been identified in AD and PD, believed to sequentially involve Aß accumulation, the Aß-triggered inflammasome activation, inflammasome-induced cytokine production and the onset of Tau pathology in AD (Mamik and Power, 2017; Ising et al., 2019; Stancu et al., 2019).

Interestingly, in the experimental autoimmune encephalomyelitis (EAE) mouse model, AG not only inhibited microglial immunoreactivity, the activating phosphorylation of NF-κB and the associated synthesis of various pro-inflammatory cytokines in the spinal cord, but also prevented the activation of the NLRP3 inflammasome complex and pyroptosis in the spinal cord of EAE mice. Indeed, the transcriptional levels of the inflammasome components NLRP3 and caspase-1, the pyroptosis-inducer gasdermin D as well as the inflammasome-derived cytokines IL-1ß and IL-18 were drastically reduced in AG-treated EAE mice, resulting in ameliorated behavioral symptoms (Liu F. et al., 2019). In this context, AG was reported to obstruct the activation of NF-κB in the spinal cord of the EAE animal model, in cultured dopaminergic neurons and in primary human T-cells (Dixit et al., 2009; Liu et al., 2010; Liu F. et al., 2019), with NF-κB driving the synthesis of the inflammasome sensor NLRP3 as well as the pro-inflammatory cytokines pro-IL-1/IL-1, pro-IL-18, TNF-α and many more (Afonina et al., 2017). Moreover, AG downregulated the transcription of IL-1 and/or the inflammasome-activating cytokine TNF-α in face of MPTP-injury (PD), threohydroxyaspartate (THA)/kainic acid-assault (excitotoxicity), subarachnoid hemorrhage and SCI (Moon et al., 2009a; Ersahin et al., 2010; Lee J. et al., 2010; Lee et al., 2012, 2014b; Alvarez and Munoz-Fernandez, 2013). As such, AG is adept in blocking the initial steps necessary for NLRP3 inflammasome induction, as observed in the EAE-based study of Liu F. et al. (2019). While it is not entirely evident how AG inhibits NF-κB signaling in GHS-R1α-negative microglia (Moon et al., 2009a; Lee J. Y. et al., 2010; Lee and Yune, 2014), it can be assumed that the prevention of neuronal and oligodendrocyte death, leading to the reduced liberation of DAMPs, indirectly avert inflammatory processes, the sensing of DAMPs by NLRP3

and other inflammasome conductors and, thus, inflammasome formation (see also chapter 5.2.1).

Peripheral Inflammation

Acylated Ghrelin Suppresses Inflammatory Responses in Mononuclear Phagocytes and Quenches Peripheral Inflammation *in vitro* and *in vivo*

Cell culture studies have indicated that AG exerts direct anti-inflammatory actions in the peripheral mononuclear phagocyte system. The expression of GHS/R1α has been confirmed in the murine RAW264.7 macrophage-like cell line as well as in primary immature and mature monocyte-derived dendritic cells of human origin (Dixit et al., 2004; Waseern et al., 2008). Furthermore, *in vitro* studies have shown that the administration of AG downregulated the synthesis of IL-1β, IL-6, and TNF-α in human peripheral blood mononuclear cells following irritation with the mitogen phytohemagglutinin (Dixit et al., 2004). Also, AG dose-dependently blocked the transcription of pro-inflammatory cytokines via the inhibition of NF-κB in LPS-induced RAW264.7 mononuclear cells in a GHS-R1α-dependent manner. Interestingly, AG evoked NF-κB-independent p38 signaling in these cells as well, promoting the secretion of the anti-inflammatory cytokine IL-10 (Waseern et al., 2008). As such, AG dampens the production of pro-inflammatory mediators by mononuclear cells, while encouraging the liberation of anti-inflammatory cytokines. In concert, AG ameliorated the LPS-driven systemic accumulation of pro-inflammatory IL-1β, IL-6, and TNF-α in the plasma, spleen, liver, lungs and lymph nodes, thus protecting mice from endotoxic shock (Dixit et al., 2004).

Over the previous two decades, AG has consistently performed well in animal models of various inflammatory conditions, guarding against endotoxemia/sepsis, pancreatic, hepatic and kidney disease, cardiovascular conditions, arteriosclerosis, colitis, arthritis, age-induced inflammation and more (Baatar et al., 2011; Deboer, 2011). For instance, the administration of AG succeeded in the animal model of colitis, showing downregulated local and systemic release of pro-inflammatory modulators, reduced inflammatory Th 1 activity, elevated action of immunosuppressive regulatory T-cells (Tregs), diminished oxidative stress, ameliorated intestinal tissue loss and reinvigorated mucosal vitality (Gonzalez-Rey et al., 2006; Konturek et al., 2009; Pamukcu et al., 2013; Matuszyk et al., 2015; Ceranowicz et al., 2017). Anti-inflammatory properties of AG have also been confirmed in various clinical studies (Kodama et al., 2008; Takata et al., 2015; Farokhnia et al., 2020). As concluded elsewhere in the context of colitis, the inflammation-ameliorating mechanisms of AG include (i) the attenuation of systemic innate and adaptive immune responses, which is dependent on the direct suppression of leukocytes; (ii) the AG-stimulated liberation of tissue-strengthening GH and insulin-like growth factor 1 (IGF-1) and (iii) the elevation of the intestinal blood flow and motility, thus reducing the contact time of inflammatory irritants with the intestinal mucosa (Baatar et al., 2011; Deboer, 2011). Considerably, intestinal damage, leakiness and inflammation co-occur in AD and PD, preceding the manifestation of neurodegenerative processes. The early inflammatory shift in the gut encourages the release of pro-inflammatory cytokines and chemokines, bacterial stimulants (i.e. LPS) as well as aggregation-prone amyloid-like proteins into the blood stream. The inflammatory stress, combined with the suspected trafficking of intestinal Aβ and α-synuclein seeds across the ENS into the CNS, is thought to instigate amyloid deposition and neuronal atrophy (Ambrosini et al., 2019). As such, AG's beneficial actions in the gut must not be underestimated.

The Vasoprotective and Blood Flow-Enhancing Properties of Acylated Ghrelin

In addition to modulating the monocyte system, AG protects the endothelial vasculature and stimulates vasorelaxation to enhance blood flow. Immunohistochemical examinations of human tissue have confirmed the plentiful presence of GHS-R1α on endothelial cells of various myocardial, but also pulmonary, renal and adrenal blood vessels, whereas the receptor is sparsely expressed by the blood vessel endothelium that supplies nerves and connective tissue (Kleinz et al., 2006). Although to a low degree, GHS-R1α is also expressed throughout the cerebral vasculature and a markedly high density of GHS-R1α has been detected in the microvasculature of the granular layer of the cerebellum (Katugampola et al., 2001; Ku et al., 2015, 2016).

In cell culture studies using human umbilical vein endothelial cells (HUVECs), it was demonstrated that AG inhibited the nuclear translocation of NF-κB even in the absence of inflammatory stimuli, quenched the basal and H_2O_2-triggered release of IL-8 and oxLDL-encouraged release of IL-6, blocked the endothelial expression of the immune cell-recruiting monocyte chemoattractant protein 1 (MCP-1) and reduced the TNF-α-incited adhesion of co-cultured monocytes/macrophages to the vascular endothelial cells, which was presumably related to the endothelial downregulation of vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1) (Li et al., 2004; Zhang, 2017). Since DAG failed to modify inflammatory reactions by HUVECs, it was implied that the described inflammation-dampening effects were reliant on GHS-R1α (Li et al., 2004). Moreover, a recent study revealed a cytoprotective function of AG in palmitate- and glucose-stressed human microvascular endothelial cells, in which AG rescued apoptosis and caspase-3 activity by inhibiting the stress kinases p38 and JNK1/2, diminishing the mitochondrial generation of ROS and normalizing the rate of oxygen consumption and ATP production (Liao et al., 2017). Additionally, clinical studies indicate that AG, in an endothelial nitic oxide synthase (eNOS)-mediated and GH-independent manner, enhances the bioavailability of NO and elicits vasorelaxation, hence improving blood flow and decreasing blood pressure (Nagaya et al., 2001, 2004; Shimizu et al., 2003; Tesauro et al., 2005; Kleinz et al., 2006; Virdis et al., 2015). Since AG enhances AMPK activity in endothelial cells (Fang et al., 2013) and the stimulation of AMPK was proven to trigger the AMPK-conveyed activating phosphorylation of eNOS at Ser^{1177} in cultured human and rat endothelial cells, thus strengthening vasodilation *in vivo* (Morrow et al., 2003; Suzuki et al., 2008), it is highly likely that the AG-evoked liberation of NO is

AMPK-mediated. Thus, in GHS-R1α-expressing blood vessel endothelial cells in the periphery, AG protects the vasculature by inducing the mitochondrial ROS-scavenger UCP-2 to ameliorate oxidative stress, inflammatory responses and vascular insult by hyperglycemia and hyperlipidemia. Moreover, by increasing blood flow, AG might ameliorate the pathologically diminished cerebral blood flow and deficits in the CNS delivery of glucose that have been detected in the brains of AD (Lyingtunell et al., 1981, Eberling et al., 1992; Ogawa et al., 1996; Roher et al., 2012; de Eulate et al., 2017) and PD (Huang et al., 2008; Hosokai et al., 2009; Liepelt et al., 2009; Borghammer et al., 2010; Berti et al., 2012) patients.

Acylated Ghrelin Stimulates Anti-inflammatory Signaling Across the Vagus Nerve

Importantly, various investigations have indicated that AG controls peripheral inflammation via the vagal nerve system. In concert, the area postrema, the nucleus tractus solitarius and the dorsal motor nucleus of the vagus (DMV) form the dorsal vagal complex (DVC) that serves as a commanding platform for the autonomic nervous system, navigating gastrointestinal motility, secretory activity and pancreatic hormone release (Price et al., 2008; Mussa and Verberne, 2013). Interestingly, GHS-R1α is expressed in the DVC and the plasma GH pools and c-Fos immunoreactivity in the DVC were found to decline with age in Fischer344 rats. The injection of GH, on the other hand, was capable of raising the transcriptional levels of GHS-R1α and partially re-established c-Fos immunoreactivity in the DVC of these aged rodents, suggesting that the GH-induced expression of GHS-R1α regulates the vagal sensitivity toward AG. Moreover, the administration of LPS into older animals, which display lessened expression of GHS-R1α in the DVC, evoked the excessive release of TNF-α and IL-6, far greater than in younger littermates (Wu et al., 2009b). In agreement, the utility of a GHS-R1α antagonist exacerbated the endotoxemia-induced liberation of pro-inflammatory cytokines into the blood stream in young rats (Wu et al., 2009b), implying an important immunosuppressive function of AG within the vagal nerve system that gradually deteriorates with age.

Besides the DVC, the presence of GHS-R1α was also discovered on the nodose ganglion of the vagus nerve as well as the nerve terminals of the outgrowing afferent vagal fibers, which innervate the digestive tract and sense the systemic conditions and circulatory hormone levels. The binding of plasma AG to GHS-R1α on the afferent vagal ends mutes vagal firing, contributing to the initiation of feeding and GH-release (Date, 2012). In the context of inflammation, it is broadly accepted that the afferent vagal nerves exert anti-inflammatory (cholinergic) signaling via multiple mechanisms following stimulation (see Bonaz et al., 2016). As confirmed with vagotomy, the interaction of administered AG with the vagal nerve not only quenched systemic inflammation during sepsis (Wu et al., 2007, 2009a), but also suppressed inflammation in *in vivo* models of traumatic brain injury, focal cerebral ischemia and gut ischemia/reperfusion injury, thus attenuating the accumulation of plasma and cerebral inflammatory cytokines (Wu et al., 2008; Bansal et al., 2010, 2012; Cheyuo et al., 2011), inflammation-driven intestinal permeabilization and atrophy (Wu et al., 2008, 2009a; Bansal et al., 2010).

Adaptive Immunity and CNS Infiltration

Acylated Ghrelin Suppresses Pro-inflammatory T-Cells and Blocks Immune Cell Invasion Into the Brain

AG is also involved in the regulation of the adaptive immune system, which is based on the modulation of T-cells. It was confirmed that primary human blood mononuclear cells and human T-cells express GHS-R1α and the treatment with AG counteracted the leptin-induced secretion of the pro-inflammatory cytokines IL-1, IL-6 and TNF-α by these cells *in vitro* (Dixit et al., 2004). In agreement with this, the injection of AG prevented the LPS-stimulated and T-cell-instructed production of IL-1β, IL-6, and TNF-α in various organs and the blood plasma, thus ameliorating anorexia in the *in vivo* endotoxemia model (Dixit et al., 2004). In a follow-up study, the same group demonstrated that AG restrains the production of various cytokines by inhibiting the nuclear translocation of NF-κB and the expression of pro-inflammatory genes. Interestingly, AG was found to be endogenously expressed by T-cells (Dixit et al., 2004) and its synthesis by T-cells declined with age, whereas the infusion of AG reversed the age-correlating increase in a large number of pro-inflammatory cytokines and chemokines in old rats (Dixit et al., 2009). This suggests that the loss of GHS-R1α/AG-signaling in immune cells contributes to the process of immune-senescence during aging, also known as "inflamm-aging." Briefly, the process of inflamm-aging describes the gradual manifestation of an asymptomatic, chronic, systemic and low-grade inflammatory phenotype in the entire physiological system with age that contributes to development of aging-related diseases, such as insulin resistance, T2DM, AD, and PD (Xia et al., 2016).

Besides managing the inflammatory state, it was shown that AG dose-dependently inhibited the differentiation of isolated lymphocytes into the pro-inflammatory T helper cell (Th)17 subset, while GHS-R1α knockout mice exhibited heightened splenic levels of Th17 cells. It was confirmed that T-cell differentiation is coupled to the induction of the mTor/S6K1 and mTor/signal transducer and activator of transcription (STAT)3 pathways and AG blocked Th17 differentiation by inhibiting mTor activity (Xu Y. H. et al., 2015). This proposes that, as observed in neurons, AG drives the mTor-inactivating AMPK/$TSC_{1/2}$ pathway in T-cells (Bayliss and Andrews, 2013; Peixoto et al., 2017). In agreement, AG elicits AMPK-evoked autophagy in the lymphoblastic Jurkat and Molt-4 cell lines (Heshmati et al., 2020), also considering that autophagy is initiated by the inactivation of mTOR (Lan et al., 2017). Generally, the differentiation toward the major, pro-inflammatory Th1 and Th17 subpopulations is dependent on mTORC1/STAT3 signaling as well as the presence of the cytokines IL-6 and transforming growth factor beta (Th17) and mTORC1/STAT4 plus IL-12 (Th1) (Saleiro and Platanias, 2015). As such, AG induces an anti-inflammatory phenotype in lymphocytes by downregulating the production of pro-inflammatory cytokines and, presumably

in an AMPK/$TSC_{1/2}$-mediated manner, limiting mTor-driven differentiation and proliferation.

To investigate the impact on T-cell instructed immune infiltration into the brain, studies in EAE models serve well in the assessment of AG's immunosuppressive capabilities. In the EAE mouse model, AG, but not DAG, improved the overall disease score, reduced lesion size, demyelination, microgliosis, inflammasome induction as well as iNOS and NF-κB activity in the spinal cord and downregulated the production of IL-1ß, IL-6, TNF-α and cyclooxygenase-2 (COX-2) by microglia and spinal cord-invading T-cells. Immune cell invasion into the spinal cord was only blocked by AG in some of these studies, however, which might have been related to the choice of the EAE-initiating antigen used for immunization (Theil et al., 2009; Souza-Moreira et al., 2013; Liu F. et al., 2019). Therefore, while highly implied, a T-cell suppressing function of AG needs to be confirmed in animal models of AD and PD.

Acylated Ghrelin Guards Against Blood Brain Barrier Damage by Reducing Inflammation

The BBB is a continuous, selective cell barrier in cerebral microvessels that separates the periphery (the circulating blood) from the brain. The vascular BBB is composed of an initial layer of endothelial cells, which seal off the paracellular gaps through the expression of tight junction proteins, and is further strengthened by pericytes and astrocyte end-feet. The breaching of the BBB/blood vessels, the leakage of peripheral material into the brain, fluid influx (edema), ion disbalance, the trespassing of peripheral immune cells as well as interrupted cerebral blood flow, are not only a concern in response to mechanical CNS injury, but are also major pathological features of AD and PD (Sweeney et al., 2018).

Importantly, inflammation augments the cerebral entry of peripheral immune cells by provoking the disruption of the BBB. For example, the genetic deletion of TNF-α and the utility of the microglial inhibitor minocycline attenuated the MPTP-induced permeabilization of the BBB in this PD animal model (Zhao et al., 2007). Furthermore, the cerebral infusion of LPS, in a mostly MMP-3-conyeyed manner, injured the BBB through the upregulation of MMP-3, MMP9, and the MMP-driven degradation of various tight junction proteins in rodents (Gurney et al., 2006). MMP-3 is also implicated in BBB damage in PD (Chung et al., 2013) and activates MMP-9 (Lee et al., 2014a), the MMP family member that directly proteolyzes BBB components (Lakhan et al., 2013).

AG not only quenches cerebral and systemic inflammation, as exemplified in the previous pages, but also protected endothelial cells of the BBB and neurons from apoptosis in various contexts, leading to the reduced release of the microglial inflammatory activator MMP-3 (Kim et al., 2005; Moon et al., 2009a; Lee J. et al., 2010; Lee et al., 2015). During various forms of CNS injury, AG was further shown to suppress systemic inflammation by stimulating anti-inflammatory signaling across the vagus nerve (Cheyuo et al., 2011), which led to reduced BBB damage and permeabilization, the transcriptional maintenance of the BBB tight junction proteins occludin and zonula occludens by vascular endothelial cells, the decreased death of neurons and astrocytes as well as the reduced spillage of DAMPs by apoptotic cells, such as neuron-specific enolase and S100β, into the blood stream. Thus, by diminishing total inflammation, the secretion of pro-inflammatory DAMPs and inflammation-driven BBB injury, AG was capable of preventing neutrophil infiltration into the CNS (Ersahin et al., 2010, 2011; Lopez et al., 2012a,b, Mohaddes et al., 2017).

ACYLATED GHRELIN INDUCES THE RELEASE AND SYNTHESIS OF NEUROPROTECTIVE INSULIN-LIKE GROWTH FACTOR 1

It must not be neglected that AG is involved in the expression and release of other powerful agents, such as the neuroprotective growth factor IGF-1 (reviewed in Costales and Kolevzon, 2016). In the periphery, AG has been deemed as the most powerful stimulator of the GH/IGF-1 axis (Nass et al., 2011). A clinical trial, although slightly underpowered, has indicated that the injection of the ghrelin analog MK-677 proved to sustain IGF-1 release in the long-term, which led to enhancements in the lean body mass of healthy, aged and non-obese subjects after a 1-year treatment period (Nass et al., 2008). Interestingly, AG equally appears to elevate the synthesis of IGF-1 in some brain regions. The injection of the ghrelin agonist GHRP-6 into healthy, adult rodents elevated the transcription levels of IGF-1 in the hypothalamus, the cerebellum and the hippocampus, but not the cortex (Frago et al., 2002). Furthermore, in IGF-1-positive brain areas, the increased phosphorylation of Akt, enhanced levels of the apoptosis-suppressing protein Bcl-2 as well as the inactivation of the apoptosis-mediator Bad were detected (Datta et al., 1997). This suggests that the AG-driven upregulation of IGF-1 in various brain areas occurs in the absence of any toxic insults, encouraging anti-apoptotic signaling in neurons. And indeed, the administration of GHRP-6 to old rats ameliorated the age-associated decline in IGF-1 levels in the cerebellum, inhibited caspase 9/3 and reduced cerebellar apoptosis (Paneda et al., 2003). The elevated hypothalamic synthesis of IGF-1 was also observed in AG or GHRP-6-injected obese rodents that were placed on a high fat dietary regiment (Garcia-Caceres et al., 2014).

INSULIN RESISTANCE

Early Cerebral Insulin Resistance Is Linked to Glucose Hypometabolism, Amyloid Pathology, and Cognitive Decline in Alzheimer's and Parkinson's Disease

While insulin is well-known for its metabolic role in the periphery, the insulin receptor is also widely expressed in the CNS. Indeed, insulin regulates various pivotal processes in neurons, such as the expression of glycolysis-associated enzymes and, thus, glucose metabolism, mitochondrial function and biogenesis, memory, gene and protein synthesis, cellular growth, functional autophagy, the protection from oxidative and ER

stress and the induction of survival pathways. Given the pivotal and neuroprotective role of insulin-signaling in the brain and that T2DM is one of the greatest known risk factors for AD and PD, it is no surprise that its early desensitization in the CNS, believed to predominantly occur in response to chronic inflammation, promotes the development of AD and PD (see Holscher, 2019 for further information) (Blazquez et al., 2014; Werner and LeRoith, 2014; Holscher, 2020).

The negative effects of desensitized insulin on the brain are very apparent, especially in AD. For instance, a high-fat diet, which induces systemic insulin resistance, was shown to attenuate neuroprotective brain-derived neurotrophic factor levels, long-term potentiation (LTP) and dendritic spine density in the hippocampus of previously healthy mice, while accelerating Aβ plaque formation and memory loss in Aβ-transgenic animals (Ho et al., 2004; Stranahan et al., 2008). In addition, insulin suppresses the activity of GSK-3ß, a well-known Tau kinase (Lei et al., 2011), hence the loss of insulin signaling initiates Tau hyperphosphorylation and aggregation in AD (Hong and Lee, 1997; Schubert et al., 2003, 2004). Most importantly, insulin resistance results in pronounced glucose hypometabolism in the CNS. Studies in AD patients have confirmed the reduced sensitivity of the post-mortem-derived hippocampal and cortical brain tissue toward insulin. Moreover, the rate of inhibitory serine phosphorylation of IRS-1 (as a marker of insulin resistance), independent of even T2DM or the APOEε4 allele, rose gradually from previously healthy suspects to mild cognitive impairment (MCI) to AD patients, correlated with the quantity of Aß deposits and was inversely associated with episodic and working memory (Talbot et al., 2012). Such and related investigations led to the designation of AD as "type 3 diabetes" (Steen et al., 2005; Moloney et al., 2010). Furthermore, quantitative microarray RNA studies have revealed that AD patients, prior to the appearance of other neuropathological hallmarks, including Aß plaques and Tau neurofibrillary tangles, exhibit a decline in the cerebral expression of insulin-regulated genes that drive TCA and pyruvate metabolism (Zhao et al., 2015). In accord with insulin resistance, glucose hypometabolism in the CNS, for instance within the cortex, has been linked to the transition from MCI to AD and cognitive dysfunction (Lyingtunell et al., 1981; Hoyer et al., 1988; Ogawa et al., 1996; Drzezga et al., 2003; Mosconi et al., 2008). These early impediments in the neuronal insulin and glucose metabolism have been proposed to trigger a detrimental bioenergetic shift from glucose to alternative and less efficient energy substrates (reviewed in Neth and Craft, 2017) and have been suggested to precede any other pathological alteration, including even mitochondrial dysfunction, in AD patients (Zilberter and Zilberter, 2017; Holscher, 2019). This is further exacerbated by general reductions in the rate of blood flow and, therefore, cerebral glucose delivery in AD patients (Lyingtunell et al., 1981, Eberling et al., 1992; Ogawa et al., 1996; Roher et al., 2012; de Eulate et al., 2017). The latter may be another consequence of insulin resistance, since insulin promotes the cerebral blood flow by enhancing NO-driven vasoconstriction and endothelin 1-mediated capillary recruitment (Craft, 2009).

Impaired neuronal insulin signaling has also been identified in PD patients, with post-mortem analysis indicating that the prevalence of insulin receptors is reduced in the SNpc, the amygdala and the frontal white matter. Furthermore, components of the insulin pathway were found to be deactivated by inhibitory serine phosphorylation in the SN and basal ganglia, which appeared to precede dopamine neuron death, implying that the functional deterioration of insulin signaling manifests prematurely (Moroo et al., 1994; Takahashi et al., 1996; Tong et al., 2009; Morris et al., 2014). Interestingly, various studies have indicated that PD patients also exhibit cortical glucose hypometabolism and diminished blood flow in this area (Huang et al., 2008; Hosokai et al., 2009; Liepelt et al., 2009; Borghammer et al., 2010; Berti et al., 2012), a decreased rate of glucose consumption in the frontal lobe and caudate putamen (Xu Y. Q. et al., 2015) as well as the reduced expression of glycolytic enzymes in the putamen and the cerebellum (Dunn et al., 2014). Indeed, the bioenergetic alterations, in particular when present in the cortex, were associated with cognitive decline in PD patients, thus posing a potential predictor for the onset of PD-related dementia (Huang et al., 2008; Hosokai et al., 2009; Liepelt et al., 2009). In resemblance to AD, it has been postulated that these impairments in the cerebral turnover of glucose manifest prior to the appearance of other pathologic changes, for example the formation of Lewy bodies, in the PD brain (Zilberter and Zilberter, 2017).

Acylated Ghrelin Prevents the Pathology-Associated Development of Insulin Resistance

Intriguingly, AG appears to preserve the cerebral insulin sensitivity. For example, The $A\beta_{25-35}$-induced mouse model of AD displayed pathologic weight loss, decreased energy expenditure, deregulated insulin secretion and elevated HOMA-IR scores, which signified the presence of peripheral insulin resistance, whereas centrally administered AG restored these metabolic alterations. In the brains of these AD-like mice, AG further suppressed glycogen synthase kinase 3β (GSK-3β) activity and Tau hyperphosphorylation (Kang et al., 2015). GSK-3β is a Tau-phosphorylating kinase whose activity is aberrantly enhanced in response to the desensitization of insulin and the associated loss of Akt-signaling during diabetes mellitus and AD (Jolivalt et al., 2008; Zhang et al., 2018). Aβ, in turn, induces insulin resistance and GSK-3β by trapping insulin receptors in the neuronal cytoplasm and promoting inhibitory serine phosphorylation of insulin pathway components, for example IRS-1. As such, Aβ weakens insulin and Akt-signaling in the CNS (Zhao et al., 2008; Najem et al., 2016). In the MSG-generated rat model of obesity and cognitive decline, the administration of the ghrelin analog GHRP-6 normalized the plasma concentrations of various hormones, decreased the abnormally elevated hippocampal pools of Aβ and acetylcholine and enhanced the spatial memory of these rodents (Kutty and Subramanian, 2014). Also, in APPSwDI mice that were fed with a high glycemic index diet, AG, in fact, promoted weight loss, motor activity and spatial memory, while decreasing the degree

of Ser^{636} phosphorylated IRS-1 in the mouse hippocampus, indicating that AG prevented the desensitization of insulin (Kunath et al., 2015). Lastly, the combinatorial application of AG and the insulin-re-sensitizing drug liraglutide, a glucagon-like peptide-1 (GLP-1) analog, was tested in the R6/2 Mouse Model of Huntington's disease (HD), resulting in the normalization of the chronically raised blood glucose levels as well as improved peripheral insulin sensitivity (HOMA-IR) and pancreatic ß-cell function (HOMA-ß) (Duarte et al., 2018). Notably, the co-injection of liraglutide and AG was more beneficial than liraglutide alone and prevented hyperinsulinemia in the cortex, forestalled the accumulation of pro-inflammatory triglycerides and cholesterol, increased IGF-1 levels, decreased lactate and AMP pools and doubled the cortical energy charge (Duarte et al., 2018). Therefore, the latter *in vivo* studies suggest that, via the elevated clearance of Aβ or related amyloids, AG fosters the cerebral insulin sensitivity and prevents insulin resistance-associated bioenergetic impairments, thus elevating cognition during AD.

Mechanistically, it is likely that AG prevents the desensitization of insulin through its potent anti-inflammatory properties (see also **Figure 2**). In the high-fat diet *in vivo* model of obesity, which shows low-grade systemic inflammation, AG counteracted the diet-driven rise in pro-inflammatory plasma free fatty acids and attenuated the amassment of triglycerides, the nuclear translocation of NF-κB and pro-inflammatory TNF-α production (Barazzoni et al., 2011, 2014). According to Barazzoni et al. and García-Cáceres et al., the administration of AG results in a phenotype that, despite exhibiting weight gain, displays low systemic inflammation and diminished triglyceride burden (Barazzoni et al., 2011; Garcia-Caceres et al., 2014). Additional studies indicated that AG, despite its orexigenic effects, blunted the amount of circulatory cytokines, such as IL-1β and IL-6, and oxidative stress markers in T1DM/T2DM animal models (Kyoraku et al., 2009; Garcia-Caceres et al., 2014). Indeed, AG acts as a potent immunosuppressor in the periphery and in the brain (as expounded in chapter 5 and shown in **Figure 2**). Given that inflammation is the driving factor in the maturation of insulin resistance during obesity, T2DM and neurodegenerative diseases, including AD and PD (Tateya et al., 2013; Holscher, 2019), it is implied that AG enhances insulin sensitivity by suppressing systemic inflammation, hyperlipidemia and oxidative assault.

The Orexigenic Effects of Ghrelin May Encourage Secondary Metabolic Deregulation

Despite the latter promising studies, the long-term metabolic effects of AG are questionable. Generally, as concluded by a recent meta-analysis in diabetic patients suffering from gastropareses, the long-term clinical use of AG seems to be safe and is well-tolerated, even in metabolically susceptible populations (Hong et al., 2019). However, clinical studies in healthy, non-obese subjects have shown that the infusion of AG impairs the glucose-stimulated secretion of insulin, diminishes glucose tolerance and worsens insulin sensitivity (Gauna et al., 2004; Vestergaard et al., 2007; Tong et al., 2010, 2014). Furthermore, GHS-R1α antagonists, GOAT inhibitors and the genetic deletion of GHS-R1α enhanced the release of insulin, glucose tolerance, insulin sensitivity and weight loss in *in vivo* models of obesity (Sun et al., 2006; Esler et al., 2007; Longo et al., 2008; Barnett et al., 2010; Qi et al., 2011). Indeed, as an appetite-stimulating hormone, the long-term administration of a ghrelin analog promoted weight gain, which led to increased fasting blood glucose levels and deteriorated insulin sensitivity in healthy, aged adults (Nass et al., 2008). On the other hand, the injection of AG worsened glucose tolerance directly after administration, yet had rather beneficial metabolic long-term consequences, including weight loss, in T1DM, AD, and HD *in vivo* models (Granado et al., 2009; Kyoraku et al., 2009; Kunath et al., 2015; Duarte et al., 2018). Likewise, chronically increased plasma ghrelin levels did not lead to any complications in adult rodents, although it might promote hyperglycemia with age (Iwakura et al., 2005; Reed et al., 2008).

In conclusion, the injection of AG is safe, ameliorates the insulin resistance-driving inflammatory pathology and does not induce metabolic deregulation per se. Nonetheless, the orexigenic effects of the hormone, which encourage weight gain, may negatively affect the systemic insulin sensitivity and glucose tolerance in the long-term, favoring AD and PD.

DOPAMINE

Acylated Ghrelin Protects Nigrostriatal Dopaminergic Neurons and Boosts Dopamine Release in Parkinson's Disease

According to a previous analysis, ∼30% of the dopamine-producing neurons in the SNpc are lost at the time at which clinical motor symptoms, including tremor, rigidity and bradykinesia, manifest in PD. Furthermore, neuronal death is accompanied by the independent destruction of axonal terminals in the SNpc and the degeneration of around 50–60% of neuronal projections from the SNpc toward other brain regions, in particular the striatum. Ultimately, these adverse alterations result in an estimated 50–70% reduction of total dopamine levels in the striatum/putamen (Cheng et al., 2010). Typically, symptomatic relief is provided through the supplementation of the lost striatal dopamine, yet these medications desensitize over time (Armstrong and Okun, 2020).

In the context of PD, there is evidence that AG not only supports the survival of SNpc-located neurons, but also boosts the availability of dopamine. As demonstrated *in vitro*, AG ameliorated the neuronal viability, cell death, caspase-3 activity and Bcl-2/Bax ratio, normalized the mitochondrial membrane potential, attenuated the production of ROS and malonaldehyde, stimulated the antioxidant enzymes MnSOD and catalase and inhibited the pro-inflammatory master transcription factor NF-κB in MPTP-stressed and GHS-R1α-expressing dopaminergic MES23.5 cells (Dong et al., 2009; Liu et al., 2010). Another study in the SN-derived SN4741 cell line, as confirmed with a GHS-R1α antagonist, showed that AG suppresses the LPS-provoked secretion of the pro-inflammatory cytokine IL-6 (Beynon et al., 2013). Similar to MPTP, AG was also capable of reversing the rotenone-induced blockade of mitochondrial

complex 1 and prevented the toxin-induced drop in the mitochondrial membrane potential, resulting in the lessened leakage of the apoptosis-prompting cytochrome C, reduced caspase-3 activation and diminished cell death (Yu et al., 2016). *In vivo*, multiple independent studies in the MPTP rodent model of PD have shown that AG binds to GHS-R1α and activates SNpc dopaminergic neurons, rescues from neuronal death and prevents the depletion of dopamine in the striatum (Jiang et al., 2008; Andrews et al., 2009a; Moon et al., 2009a). Intriguingly, AG preserved the neuronal projections from the SNpc toward the striatum, indicating that AG has axoprotective capabilities (Moon et al., 2009a). Mechanistically, AG is responsible for the neuroprotective effects of caloric reduction and stimulates AMPK activity in the SN of MPTP-treated rodents (Bayliss et al., 2016). Furthermore, AG strengthens the neuronal resilience toward oxidative stress in an AMPK/UCP2-mediated manner, while improving the mitochondrial respiration, ATP production and the number of functional mitochondria through the induction of biogenesis (see chapter 3.2 for further insight). On the other hand, the deletion of ghrelin or GHS-R1α potentiated the neuronal loss and striatal dopamine deprivation in the MPTP mouse model (Andrews et al., 2009a). In addition, Andrews et al. showed that AG reversed the MPTP-provoked downregulation of TH in SNpc neurons *in vivo*. This effect was presumably a combination of the improved mitochondrial ATP generation and, thus, neuronal metabolism (Andrews et al., 2009a), the shielding from ROS that are generated through mitochondrial intoxication with MPTP (Andrews et al., 2009a), the reduction of microglial activation, the decreased release of pro-inflammatory cytokines, such as TNF-α and IL-1β, the diminished formation of NO metabolites (Moon et al., 2009a) as well as the anti-apoptotic effects of AG, as evident by the normalization of the Bcl-2/Bax ratio and the inhibition of caspase-3 (Jiang et al., 2008). Moreover, the stimulation of mitophagy via AG may prevent the accumulation of defective mitochondria and the associated generation of ROS (Bayliss and Andrews, 2013). For an overview of the neuroprotective pathways of AG, please refer to **Figure 1**.

Besides safeguarding SNpc neurons, AG stimulates dopamine release in a physiological manner, as depicted in **Figure 3**. In an extensive study, it was demonstrated that AG binds to nigral neurons, triggers neuronal firing and enhances dopamine turnover, achieving an impressive three-fold increase in total dopamine levels in the striatum of healthy mice (Shi et al., 2013). The same group further discovered that the unilateral injection of AG into the SNpc ameliorated the cataleptic symptoms induced by the dopamine D2 receptor antagonist haloperidol, thus immediately improving the posture of the mice. The intra-SN injection of AG also improved the motor skills of haloperidol-treated DAT^{SN}::DTA rodents in a recent study (Suda et al., 2018).In line with AG's role in dopamine metabolism, the injection of a GHS-R1α-antagonist into the SNpc was sufficient to cause motor disturbances and catalepsy in healthy rodents (Suda et al., 2018). Furthermore, the reduced expression of GHS-R1α was verified in induced pluripotent stem cells with a mutated or disrupted PARK2 gene, which is a mitochondrial gene implicated in mitochondrial quality control, mitophagy, and the development of early-onset PD (Pickrell and Youle, 2015), and in the SNpc of an *in vivo* model of PD (Suda et al., 2018). This implies that mitochondrial and bioenergetic deficits in dopaminergic neurons, which obviously impede the cellular gene transcription, culminate in the downregulation of GHS-R1α. Additionally, the co-emergence of insulin resistance, leading to reductions in PI3K/Akt/mTORC1-driven protein translation in dopaminergic neurons (Athauda and Foltynie, 2016; Anandhan et al., 2017; Holscher, 2019), presumably aggravates dopaminergic dysfunction and neuronal insensitivity toward AG.

As a side note, the combinatorial administration of the ghrelin agonist HM01 or AG with the dopamine-replacing drugs L-dopa or L-dopa/levodopa prevented the L-dopa- and 6-OHDA-associated slowing of the gastric motility and constipation in the 6-OHDA rodent model of PD (Wang et al., 2012; Karasawa et al., 2014). This resulted in markedly enhanced plasma levels of L-dopa and dopamine, indicating that ghrelin may improve the gastrointestinal absorption of dopamine replacement drugs (Wang et al., 2012). On the other hand, in a phase II clinical trial to investigate the constipation-mitigating effects of the ghrelin analog relamorelin, only 18 of the originally recruited 56 PD patients were able to finish the trial. In the vast majority of PD patients, relamorelin potentiated the frequency of incomplete bowel movements to a degree that the prolonged participation of the patients was impossible (Parkinson Study Group, 2017). As such, while AG can evidently improve gastrointestinal dysfunction (Hong et al., 2019), the occurrence of unwanted gastrointestinal side-effects in some PD patients may restrict the administration of ghrelin agonists in the long-term.

Acylated Ghrelin Induces Dopamine Transmission in the Ventral Tegmental Area and Stimulates Locomotor Activity and Memory

Noteworthy, GHS-R1α was found to be expressed by ∼60% of dopaminergic neurons in the VTA of rats (Abizaid et al., 2006). Collectively, animal experiments indicate that the injection of AG stimulates the mesolimbic transmission of dopamine from the VTA to the nucleus accumbens, thus heightening the release of dopamine in the nucleus accumbens and increasing food intake. When injected intracerebroventrically or intra-VTA, AG further enhanced the locomotor activity of rodents (see **Figure 3**) (Abizaid et al., 2006; Jerlhag et al., 2007, 2012; Quarta et al., 2009; Skibicka et al., 2011, 2012; Cornejo et al., 2018). Accordingly, knockout or pharmacological inhibition of GHS-R1α diminish mesolimbic dopamine transmission from the VTA to the nucleus accumbens, disheartening locomotion and the willingness of rodents to work for food rewards (Abizaid et al., 2006; Jerlhag et al., 2010, 2012; Skibicka et al., 2011, 2012). Given that dopamine deprivation in the VTA is linked to hypokinesia and bradyphrenia in PD patients (Yokochi, 2007), AG-associated improvements in this brain area might provide symptomatic relief. Moreover, even though the VTA predominantly projects dopamine toward the nucleus accumbens, it is also connected to the hippocampus (mesolimbic system) and the prefrontal cortex (mesocortical route) (Serrenho

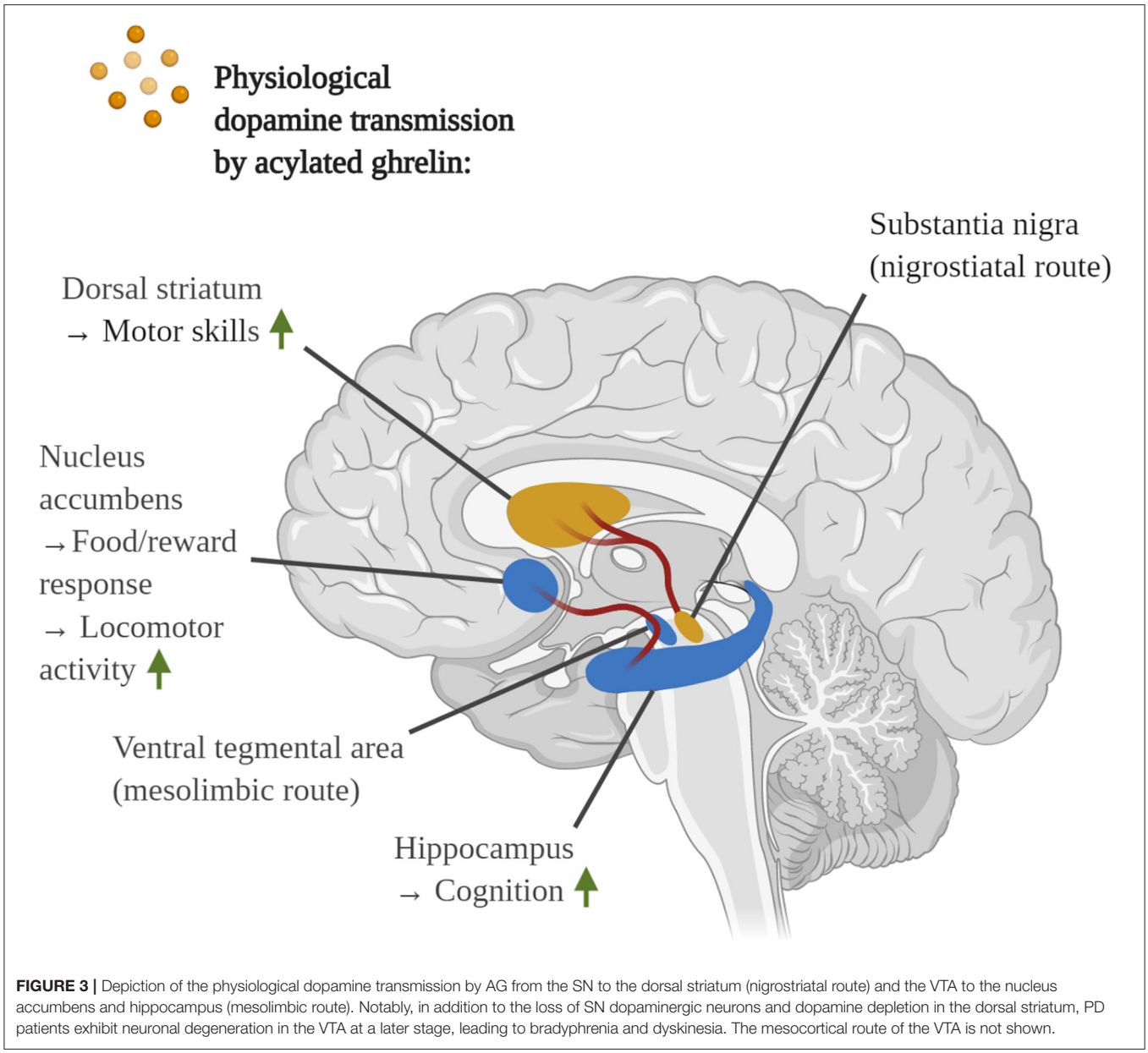

FIGURE 3 | Depiction of the physiological dopamine transmission by AG from the SN to the dorsal striatum (nigrostriatal route) and the VTA to the nucleus accumbens and hippocampus (mesolimbic route). Notably, in addition to the loss of SN dopaminergic neurons and dopamine depletion in the dorsal striatum, PD patients exhibit neuronal degeneration in the VTA at a later stage, leading to bradyphrenia and dyskinesia. The mesocortical route of the VTA is not shown.

et al., 2019). Intriguingly, a study demonstrated that AG improved the object recognition memory of rats only when administered alone, but not when co-given with the D_1R antagonist SKF 83566. Moreover, the injection of SKF 83566 itself did not negatively affect the behavioral performance of the rodents (Jacoby and Currie, 2011). Indeed, the presence of GHS-R1α and D_1R heterodimers has been confirmed in the mouse hippocampus. While we will not further discuss this topic, cross-talk between ghrelin/GHS-R1α and dopamine/D_1R-signaling was responsible for synaptic modifications as well as enhanced glutamate transmission, hippocampal plasticity and memory in rodents (Kern et al., 2015). Therefore, AG may elicit dopaminergic neurotransmission from the VTA to the hippocampus to improve cognition.

ACYLATED GHRELIN ENHANCES MEMORY IN HEALTHY RODENTS AND IN AD ANIMAL MODELS

AG also plays a major role in the retention of long-term memory. Various groups have reported that that the intracerebroventricular, intrahippocampal, or peripheral injection of AG or ghrelin agonists resulted in the binding of AG to GHS-R1α on hippocampal neurons, indicating that AG readily crosses the BBB. Furthermore, the administered AG increased the density of dendritic spines and synapses, enhanced LTP in the hippocampal CA1 region and led to improved learning and memory in healthy rodents (Carlini et al., 2002, 2004; Diano et al., 2006; Atcha et al., 2009). Impressively,

the intracerebroventricular administration of AG restored the undernutrition-induced drop in the cognitive aptitude of rodents (Carlini et al., 2008), which is in line with ghrelin's physiological role as survival hormone (Mani and Zigman, 2017). On the other hand, the genetic deletion of ghrelin or knockdown of GHS-R1α lessened the numbers of hippocampal synapses, worsened LTP and impaired long-term memory (Diano et al., 2006; Davis et al., 2011), although discrepancies between spatial and contextual memory have been reported in GHS-R1α-null mice (Albarran-Zeckler et al., 2012). Albeit in the context of feeding, for a review of the physiological regulation of the hippocampal circuits via AG please see (Serrenho et al., 2019).

Additionally, by ameliorating the underlying cerebral pathology, AG and ghrelin agonists enhanced the cognitive performance of AD-like animals in multiple behavioral paradigms. In such AD *in vivo* models, AG rescued from hippocampal atrophy, synaptic damage and the degeneration of cholinergic projections, heightened brain glycogen levels, diminished Aß aggregation and deposition, blocked Aβ-imparted deficits in LTPs, normalized plasticity-associated p-CREB levels, improved insulin sensitivity, ameliorated microglial and astroglial immunoreactivity, augmented AMPK and suppressed cerebral GSK-3ß activity as well as Tau hyperphosphorylation (Moon et al., 2011; Dhurandhar et al., 2013; Kutty and Subramanian, 2014; Kang et al., 2015; Kunath et al., 2015; Ortega-Martinez, 2015; Bartolotti et al., 2016; Santos et al., 2017; Eslami et al., 2018; Jeong et al., 2018). In line with these findings, ghrelin knockout mice displayed deficits in spatial and recognition memory, worsened olfactory distinction and heightened micro- and astrogliosis in the rostral region of the hippocampus (Santos et al., 2017). The latter *in vivo* models of AD strongly imply that, at least in part, AG raises cognition by countering Aß and Tau toxicity. Direct cytoprotective properties were also observed in primary hippocampal and mHypoE-N42 hypothalamic neurons, in which AG opposed the Aß oligomer-induced cell death in a GHS-R1α-driven manner, diminished superoxide production, restored the neuronal Ca^{2+} homeostasis, rescued from mitochondrial membrane depolarization and reduced the activation of the Tau-kinase GSK-3ß (Martins et al., 2013; Gomes et al., 2014).

ACYLATED GHRELIN INDUCES NEUROGENESIS IN THE HIPPOCAMPUS

Various studies have testified that the administration of AG boosts adult hippocampal neurogenesis in healthy rodents (Zhao et al., 2014; Kent et al., 2015; Hornsby et al., 2016), dwarf rats (Li et al., 2013), in the 6-OHDA rodent model of PD, although neurogenesis was only enhanced in the non-lesioned brain hemisphere (Elabi et al., 2018), and the 5xFAD animal model of AD (Moon et al., 2014). Within the hippocampus, mice and dwarf rats were shown to possess Ki-67-positive, GHS-R1α-expressing immature neuroblasts in the granule cell layer of the DG (Moon et al., 2009b; Li et al., 2013; Hornsby et al., 2016). It was confirmed that, in an IGF-1-independent manner, AG stimulates the proliferation of neuroblasts, leading to an enlarged, doublecortin (DCX)-positive progenitor cell population in the DG of healthy mice and dwarf rats (Moon et al., 2009b; Li et al., 2013; Kent et al., 2015). There is also evidence that the injection of AG or overnight fasting raise the expression levels of the neurogenic transcription factor early growth response 1 (Hornsby et al., 2016). On the other hand, the antibody-mediated depletion of ghrelin decreased the DCX-expressing population of neuroblasts in the DG (Moon et al., 2009b). In concert, a study in depression-prone GHS-R1α$^{-/-}$ mice revealed that the deletion of GHS-R1α exacerbated neuronal loss in response to chronic social defeat stress and diminished the proliferation of progenitor cells in the ventral DG (Walker et al., 2015). Interestingly, caloric restriction not only failed to demonstrate anti-depressive effects in these GHR-R1α knockout mice, but also provoked apoptosis within the neurogenic domain of the DG, as opposed to the growth-stimulating effects that were observed in control mice.

Notably, it has been postulated that GHS-R1α is, in fact, not present on immature neuroblasts and that AG possibly drives neurogenesis by stimulating the release of neurogenic factors by GHS-R1α-expressing adult dentate granule cells (Buntwal et al., 2019). Additional studies are needed to confirm these propositions in the hippocampus of healthy rodents and *in vivo* models of AD, however.

SIGNS OF GHRELIN RESISTANCE DURING OBESITY, AGING, AND ALZHEIMER'S DISEASE

Unfortunately, age- and disease-associated deteriorations in ghrelin-signaling have been implied. For instance, in elderly individuals, the plasma ghrelin levels, along with GH, were found to be decreased (Rigamonti et al., 2002). Similarly, obese individuals and patients with metabolic syndrome displayed reduced plasma ghrelin pools (Tschop et al., 2001; Rigamonti et al., 2002; Shiiya et al., 2002; Tesauro et al., 2005). While no relevant alterations in the blood levels of ghrelin have been observed in AD patients (Proto et al., 2006; Theodoropoulou et al., 2012), the basal plasma levels of ghrelin and the postprandial secretion of the hormone were diminished in PD patients (Fiszer et al., 2010; Unger et al., 2011). It has been proposed that the PD-related Lewy body pathology in the myenteric plexus of the stomach and in the DMV, which innervates the gut and navigates the gastrointestinal motility, might impair the release of ghrelin (Stoyanova, 2014). Strikingly, it was revealed that the locally synthesized levels of ghrelin, its recently discovered splicing analog ln2-ghrelin, GOAT and GHS-R1α were markedly downregulated in the temporal gyrus of AD patients. In contrast, the transcriptional levels of the GHS-R1α-trapping GHS-R1ß were significantly increased, indicating the desensitization of ghrelin in cognition-processing brain areas during AD (Gahete et al., 2010). Thus, the reduced secretion of ghrelin in PD as well as the emergence of cerebral ghrelin resistance in AD have been indicated.

Based on the latest evidence, obesity and T2DM seem to encourage the development of ghrelin resistance (Zigman et al., 2016). Interestingly, obese rodents not only display chronically

lowered plasma levels of AG and total ghrelin as well as decreased gastric synthesis of ghrelin and GOAT, yet also fail to secrete the hormone post-prandially and do not respond to the appetite-stimulating effects of administered AG (Martin et al., 2004; Perreault et al., 2004; Briggs et al., 2010; Gardiner et al., 2010). Ghrelin resistance has also been postulated to blunt VTA and dopamine-regulated food/reward processing in obese rodents (Lockie et al., 2015). Indeed, diet-induced obesity has been shown to result in cerebral ghrelin resistance in rodents, which was marked by the attenuated expression of hypothalamic GHS-R1α, NPY and AgRP as well as the loss of Fos-immunoreactivity in ARC neurons in response to peripheral and central injections of AG (Briggs et al., 2010; Naznin et al., 2015). Additionally, it was unraveled that aged and overweight rodents display impaired translocation of plasma ghrelin across the BBB (Banks et al., 2008).

Since leptin functions as a physiological and anorexic counteragent to ghrelin in the hypothalamus, blocking intraneuronal AG/GHS-R1α-signaling, some studies have suggested that the elevated plasma pools of leptin during obesity weaken the sensitivity toward AG (Hewson et al., 2002; Kohno et al., 2007; Briggs et al., 2014). Thus, obesity and T2DM-associated hyperleptinemia (Maffei et al., 1995; Considine et al., 1996; Okumura et al., 2003; Pandey et al., 2015) may contribute to the desensitization of AG in the hypothalamus. It must be noted that GHS-R1α and leptin receptors strongly co-localize in >90% of neurons in the ARC of the hypothalamus, explaining the rapid neuronal desensitization in this region, whereas receptor co-synthesizing neurons are rarely found elsewhere in the CNS (Perello et al., 2012). Additionally, a high-fat diet, in an inflammation-mediated manner, promotes hypothalamic leptin resistance as well (El-Haschimi et al., 2000; Zhang et al., 2008). This implies that hyperleptinemia might initially attenuate cerebral ghrelin-signaling during obesity, yet is not sufficient to trigger chronic ghrelin resistance.

Concerningly, it has been indicated that inflammation might desensitize ghrelin signaling throughout the CNS. In this context, the systemic liberation of GH was shown to be impaired in obese mice (Briggs et al., 2010). Generally, somatotrophs manage the systemic release of GH, which is triggered by GHS-R1α-mediated mechanisms in the hypothalamus, the vagal afferent nerves and the anterior pituitary, hinting that these areas might desensitize to AG (Khatib et al., 2014). In particular, the vagal nodose ganglion has been shown to desensitize, exhibiting the reduced expression of GHS-R1α, diminished AMPK activation and reduced electric current flow upon exposure to AG (Naznin et al., 2015). Importantly, the exacerbated macrophage/microglial immunoreactivity and expression of TLR4, IL-6 and TNF-α were identified in the hypothalamus and vagal nerves of high-fat diet subjected mice, proposing a possible link between neuroinflammation and ghrelin resistance. In line with this theory, caloric restriction and the associated weight loss ameliorated these inflammatory changes, thus restoring the sensitivity toward AG (Naznin et al., 2018). As such, metabolic and cerebral inflammation possibly induce ghrelin resistance. On the other hand, it is plausible that the initial, inflammation-driven desensitization of insulin and other growth factors during obesity, T2DM, AD and PD (Maldonado-Ruiz et al., 2017; Holscher, 2019, 2020), which negatively affect the rate of cellular protein translation though the loss of Akt/mTORC1-signaling (Holscher, 2019; Liu and Sabatini, 2020), might be responsible for the reduced cerebral expression of GHS-R1α and GOAT in the cortex, hypothalamus and, potentially, further brain regions (Briggs et al., 2010; Gahete et al., 2010; Naznin et al., 2015).

Interestingly, a recent study demonstrated that klotho-deficient mice, which are *in vivo* models of accelerated aging, were unresponsive to the anorexigenic and life-extending effects of AG, indicating an age-associated development of ghrelin resistance. On the other hand, the ghrelin signaling potentiator rikkunshito, a herbal extract, was capable of enhancing the physiological function and rodent life-span (Fujitsuka et al., 2016). The decreased sensitivity toward AG could be related to an age-induced decline in the transcriptional levels of GHS-R1α, as reported for the anterior pituitary in 24-month-old Lou C/Jall rats, the vagal nerve in aged Fischer-344 rats and the brainstem in rats and dwarf rats (Katayama et al., 2000; Kappeler et al., 2004; Wu et al., 2009a). It was also verified that the age-associated decrease in the vagal expression of GHS-R1α potentiates LPS-triggered inflammation (Wu et al., 2009b). Considering the vast neuroprotective properties of AG, it is likely that the insufficient availability of ghrelin and the reduced ghrelin sensitivity in the CNS, such as the temporal lobe (Gahete et al., 2010), contribute to age-related cognitive decline.

CONCLUSION

AG is a multi-talented hormone that has demonstrated great therapeutic potential. Synoptically, AG is neuroprotective, anti-oxidative, enhances the mitochondrial function, prevents mitochondrial hyperfission, induces autophagy and, possibly, mitophagy to dispose of amyloids and defective, ROS-generating mitochondria, suppresses systemic inflammatory responses and, possibly, the inflammasome, defies inflammation- and Aβ-triggered insulin resistance and the associated bioenergetic impairments, heightens the production of dopamine, promotes hippocampal neurogenesis and strengthens cognition in a direct and indirect manner in AD and PD. Notably, deacetylation half-life times ($t_{1/2}$) of 4 h or 6.4 h, respectively, have been reported for AG in human plasma and ∼27 min in rat serum, whereas the degradation $t_{1/2}$ of total circulatory ghrelin has been estimated to be as short as ∼9-11 min (De Vriese et al., 2004; Liu et al., 2008; Tong et al., 2013). Fortunately, synthetic ghrelin analogs offer prolonged stability, significant plasma release for up to 24 h, oral bioavailability and the ability to co-bind CD36, which may be useful to diminish Aβ-driven microglial inflammation (Bulgarelli et al., 2009; Muller et al., 2015; Berlanga-Acosta et al., 2017). To further enhance efficacy, the combination of ghrelin agonists with other growth factors, such as EGF (Barco et al., 2011, del Barco et al., 2011, del Barco-Herrera et al., 2013; Subiros et al., 2016), GH (Wu et al., 2009b; Zhou et al., 2017), insulin (Granado et al., 2011) and GLP-1 (Duarte et al., 2018), or DAG, to prevent AG-encouraged glucose intolerance and hypoinsulinemia (Gauna et al., 2004; Kiewiet et al., 2009), may be profitable. On the

other hand, due to the possible development of ghrelin resistance in AD and reports of gastrointestinal complications in some PD patients, clinical studies are warranted to monitor the long-term effectiveness of AG. Considering the many intertwined pathologic processes in AD and PD, the varying clinical profile as well as the many historical failures of monotherapies, especially for Aβ-based therapies in AD, multi-targeted therapies, such as the application of the powerful hormone AG and related growth factors, deserve higher recognition. "Perhaps there is a field of treasures right there, waiting to be discovered" (Gault and Holscher, 2018).

CONTRIBUTION TO THE FIELD

Over the last decades, strategies to reduce the cerebral load of harmful monomeric, oligomeric, or insoluble amyloid deposits, such as Amyloid Beta, have repeatedly failed to produce any cognitive or motor improvements in patients of Alzheimer's (AD) and Parkinson's disease (PD). The many failures of these amyloid monotherapies indicate that novel therapeutic strategies are necessary. In addition, the multi-factorial pathology of AD and PD, ranging far beyond amyloid toxicity, suggests that targeting multiple pathologic factors might be more a more promising strategy to achieve clinical success. Interestingly, ghrelin, a peptide hormone that is released during fasting, has been shown to activate an impressive range of neuroprotective pathways that have the potential to ameliorate the majority of these pathologic alterations in AD and PD. Therefore, the review compiles the existing evidence, integrates information from other disease models to illustrate less discussed pathologic matters in AD and PD, outlines the neuroprotective functions of ghrelin and describes the underlying molecular mechanisms in great detail. Additionally, the manuscript evaluates the often disregarded clinical challenges, adverse effects and limitations of a possible pharmacological intervention with ghrelin analogs in AD and PD patients. Given the manifold promising and neuroprotective effects of ghrelin in the brain, but also to monitor the possible loss of effectiveness and the frequency and severity of undesirable side-effects, long-term clinical studies in AD and PD patients are warranted.

AUTHOR CONTRIBUTIONS

The manuscript was written and the figures were made by NR. CH reviewed and revised the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Inflamm-Aging and Brain Insulin Resistance: New Insights and Role of Life-style Strategies on Cognitive and Social Determinants in Aging and Neurodegeneration

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Over the past decades, the human life span has dramatically increased, and therefore, a steady increase in diseases associated with age (such as Alzheimer's disease and Parkinson's disease) is expected. In these neurodegenerative diseases, there is a cognitive decline and memory loss, which accompany increased systemic inflammation, the inflamm-aging, and the insulin resistance. Despite numerous studies of age-related pathologies, data on the contribution of brain insulin resistance and innate immunity components to aging are insufficient. Recently, much research has been focused on the consequences of nutrients and adiposity- and nutrient-related signals in brain aging and cognitive decline. Moreover, given the role of metainflammation in neurodegeneration, lifestyle interventions such as calorie restriction may be an effective way to break the vicious cycle of metainflammation and have a role in social behavior. The various effects of calorie restriction on metainflammation, insulin resistance, and neurodegeneration have been described. Less attention has been paid to the social determinants of aging and the possible mechanism by which calorie restriction might influence social behavior. The purpose of this review is to discuss current knowledge in the interdisciplinary field of geroscience—immunosenescence, inflamm-aging, and metainflammation—which makes a significant contribution to aging. A substantial part of the review is devoted to frontiers in the brain insulin resistance in relation to neuroinflammation. In addition, we summarize new data on potential mechanisms of calorie restriction that influence as a lifestyle intervention on the social brain. This knowledge can be used to initiate successful aging and slow the onset of neurodegenerative diseases.

Keywords: aging, inflammation, inflammasome, metaflammasome, insulin resistance, Alzheimer's disease, anti-inflammatory strategies, calorie restriction

INTRODUCTION

It is a known fact that over the past decades, human life expectancy has greatly increased (Costantini et al., 2018). As a result, the population is aging, and this determines the development of geriatric medicine. Since aging is the main risk for the development of age-associated diseases, the field of geriatrics and geroscience has been developing very actively recently. The main goal of studies is to avoid age-related diseases before it is too late. Recently, the number of publications on anti-aging technologies and interventions has been increasing. This topic is certainly very popular not only in the medical community but also in society (Scapagnini et al., 2016).

Aging may be a complex process that happens under the influence of genetic, epigenetic, and environmental factors. Changes in an aging organism occur at the molecular, cellular, and tissue levels (Khan et al., 2017). In this regard, the question naturally arises on what factors possibly influence it. The most promising and effective approaches are nutritional strategies, physical activity, and hormone therapy (Scapagnini et al., 2016). In addition, these approaches can be used not only as anti-aging strategies but also as preventive directions. Preventive technologies will slow down aging and have a greater impact on quality of life than disease-specific approaches.

In order to understand the basis for development the directions of preventive and anti-age medicine, it is necessary to understand what basic pathological processes underlie the aging process. Some of these processes that determine aging include inflammation, cellular senescence, and senescence-associated secretory phenotype (SASP) development, altered glucose tolerance, and insulin resistance (IR) following dysregulated nutrient sensing and impaired cell–cell communication (De Souto Barreto et al., 2020). All these pathophysiological processes underlie age-associated neurodegenerative disorders.

It is predicted over the subsequent years that the incidence of age-related neurodegenerative diseases will increase dramatically. One of the most important factors in brain aging is the extremely high energy demand of neurons for maintaining neuronal work and preserving mental abilities (Davinelli et al., 2016).

With age, there is an increase in systemic inflammation, the inflamm-aging, and peripheral immunosenescence. Due to reciprocal interactions between the nervous and immune systems, chronic aseptic inflammation within central nervous system (CNS), called neuro-inflamm-aging, develops. Immunosenescence and inflamm-aging accompany brain aging and the loss of mental, cognitive, and other complex behaviors characteristic of Alzheimer's disease (AD) and Parkinson's disease (PD) (Khan et al., 2017; Costantini et al., 2018).

Recently, much research has been focused on the consequences of nutrients, and adiposity- and nutrient-related signals in brain aging and cognitive decline. Previously, it has been shown that insulin signaling affects the molecular cascades that underlie hippocampal functions, cognition, and memory (Spinelli et al., 2019). Our previous results have shown that a significant contribution to the development of brain IR is caused by neuroinflammation due to the overproduction of proinflammatory cytokines, astroglial and microglial activation, and disruption of the processes of reparative neurogenesis (Komleva et al., 2018).

The purpose of this review is to discuss current knowledge in interdisciplinary field of geroscience—immunosenescence, inflamm-aging, and metainflammation—which make a significant contribution to aging. A substantial part of the review is devoted to frontiers in the brain IR in relation to neuroinflammation. In addition, in this article, we summarize new data on potential mechanisms of calorie restriction (CR) influence as a lifestyle intervention on the social brain. This knowledge can be used to initiate successful aging and slow the onset of neurodegenerative diseases.

INFLAMM-AGING AND IMMUNOSENESCENCE IN ALZHEIMER'S DISEASE

Inflamm-Aging and Immunosenescence

Immunosenescence is a phenomenon of irreversible loss of the ability to divide, as a result of which damage to the immune defense is observed, which contributes to the progression of susceptibility to disease in the elderly. Immunosenescence occurs when the ability to respond to new antigens is reduced (Pawelec, 2017). In addition, a variety of factors affect the progression of immunosenescence—genetics, environment, lifestyle, and nutrition—leading to infections and progression of disease pathology (**Figure 1**) (Costantini et al., 2018).

Franceschi et al. (2000) first used the term "inflamm-aging," proposing a hypothesis based on a series of observations showing that aging of many organisms, including humans, is accompanied by an increase in the level of inflammatory markers in the blood, cells, and tissues. This is chronic, sterile, not associated with the presence of an infectious agent, and primarily due to endogenous signals, or subclinical (asymptomatic), mild, or basal inflammation, which is associated with aging. Already today, more and more studies indicate that "inflammatory" aging or inflammation associated with age is a risk factor for many chronic non-communicable diseases, such as cardiovascular (coronary heart disease and arterial hypertension), metabolic [diabetes mellitus 2 type (T2D)], musculoskeletal (osteoarthritis, osteoporosis, and sarcopenia), neurological (depression, dementia, and AD), and hematologic (malignant neoplasms and anemia) diseases, which leads to adverse effects on human health (Giunta et al., 2008). Many researchers agree that the number of nosologies is not limited to this list (Deleidi et al., 2015; Pawelec, 2017). The approaches for the treatment of diseases in which inflammation predominates in the pathogenesis may include, in addition to limiting caloric intake and increasing physical activity, the use of drugs. The drug action is aimed at limiting it by interfering with the processes of intracellular and extracellular signaling at different stages, and not only involves the impact on the main clinical manifestations or targets (blood pressure, cholesterol, blood sugar, etc.) (Zotkin et al., 2020).

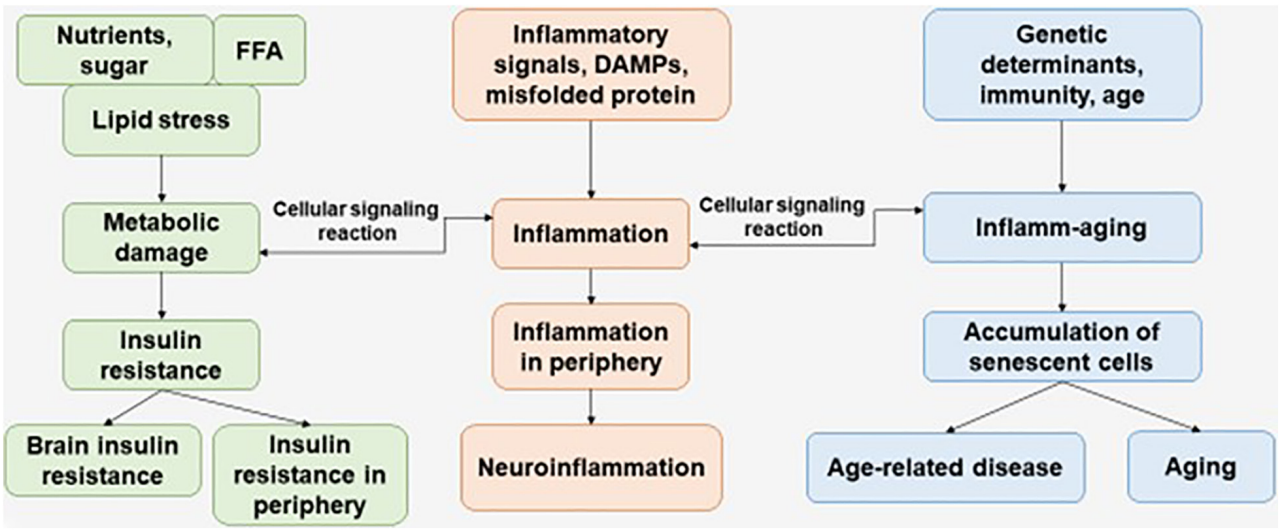

FIGURE 1 | The interaction of factors initiating the development of metabolic damage, inflammation, and inflamm-aging. The aging process and age-related disease can be accompanied with central and peripheral insulin resistance, inflammation, and accumulation of senescent cells. FFAs, free fatty acids.

Alzheimer's Disease and Neuroinflammation

AD is a fatal neurodegenerative disorder that is pathologically defined by extensive neuronal loss and the accumulation of intracellular neurofibrillary tangles (NFTs) and extracellular amyloid plaques in the brain. Studies since the discovery of amyloid beta (Aβ) and tau protein have provided detailed information on molecular pathological events, but little is known about the causes of AD, as well as about the possible effective treatment of this pathology (Silva et al., 2019; Tiwari et al., 2019).

It is well known that the risk of late-onset AD is partially due to genetics. In 2019, the results of a large meta-analysis of associations across the genome of clinically diagnosed late-onset AD were published. Currently, 25 risk loci are affected, five of which were identified in the latest study. Another confirmation of the contribution of the immune system to the late onset of AD confirms that the neurological and immune-mediated disease haplotype HLA-DR15 (human leukocyte antigen) is a risk factor for AD. In addition to tau-binding proteins and amyloid precursor protein (APP) metabolism, pathway analysis includes immunity and lipid metabolism, which are also associated with late onset of AD (Kunkle et al., 2019).

Newly discovered evidence proposes that inflammation is an important feature of AD, diabetes mellitus, and other pathologies; and it is believed that this process plays an important role in the pathogenesis of these syndromes. Therefore, understanding the interactions between the nervous system and the immune system may be key to preventing or delaying the onset of most diseases of the CNS. Neuroinflammation is an important part of the brain's defense mechanisms against a variety of pathological agents, such as infections and injuries, and includes both the formation of soluble factors and the activation of specialized cells that are mobilized to respond and restore the brain's normal physiology. Neuroinflammation is characterized by neuronal death in certain areas of the CNS (**Figure 2**) (Salmina et al., 2015; Calsolaro and Edison, 2016).

In AD, central events seem to combine the inflammasome, NF-κB pathway, and the microglial activation by a variety of factors, including Aβ and proinflammatory cytokines (Harms et al., 2015; Rea et al., 2018). Aβ in the brain microenvironment causes the glial activation that leads to microgliosis and astrocytosis around pathological proteins. Thus, glial cells are chronically activated in the brain before the onset of AD, which is associated with the development of chronic inflammation and contributes to the pathogenesis of AD. In the AD brain, microgliosis and astrocytosis because of the presence of senile plaques and NFTs can be detected immunohistochemically, and these glial cells exhibit pathologically specific morphology. Although the degree of gliosis correlates with cortical thickness and neurodegeneration, the role of various glial cells in neurodegenerative processes remains unknown (Saito and Saido, 2018).

Microglia, the main immune cells of the brain's innate immunity, perform movements similar to macrophages to remove pathogens and protect neurons from various factors. At the same time, microglial cells secrete reactive oxygen species (ROS) and nitric oxide, which are neurotoxic. They also release proinflammatory cytokines and chemokines in response to danger signals. Dysregulation of microglial activity is associated with the pathogenesis of AD during aging (Salmina et al., 2015; Clayton et al., 2017). Triggering receptor expressed on myeloid cells-2 (TREM2) plays an important role in supporting microglial cell survival. Previously, it was shown that TREM2 promotes microglial clustering around fibrillar Aβ plaques in AD mouse model and postmortem human brain sections (Ulrich et al., 2017). Furthermore, TREM2 is a Aβ receptor that mediates microglial function and removal of Aβ (Zhao et al., 2018). An increase in soluble TREM2 fragments in cerebrospinal fluid

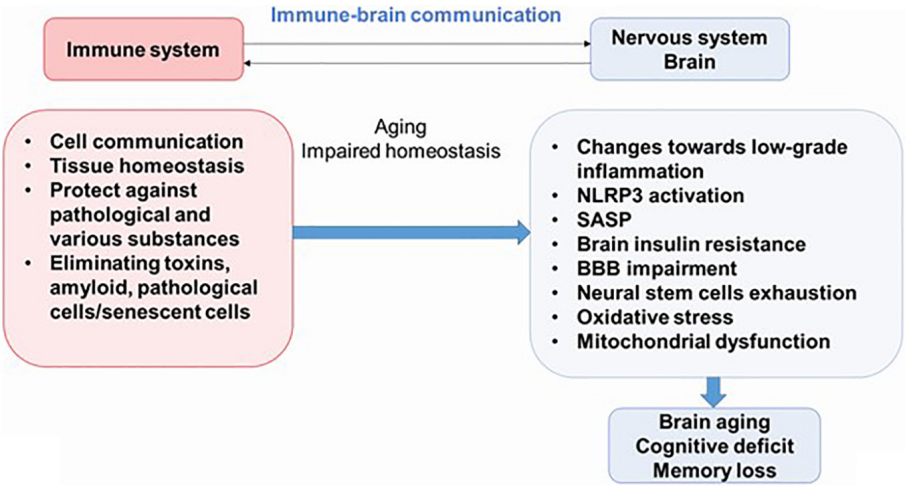

FIGURE 2 | The immune–brain communication. The main functions of immune system are cell communications, tissue homeostasis, protecting against pathogens and various substances, and eliminating pathological cells. During aging, increased systemic inflammation leads to impaired homeostasis and could result to cognitive deficit. NLRP3, NLRP3 inflammasome; SASP, senescence-associated secretory phenotype; BBB, blood–brain barrier.

indicates coincidence with markers of neuronal damage and onset of clinical dementia in AD (Ulrich et al., 2017).

It should be noted that recent human positron emission tomography (Aβ-PET) data indicate that Aβ deposition begins years before memory impairment and cognitive decline (Hatashita and Wakebe, 2019). Given the fact that Aβ acts as strong damage-associated molecular patterns (DAMPs), it seems that the interval between early accumulation of Aβ and later signs of disease progression, such as tau pathology and brain atrophy, is influenced by innate immune responses (**Table 1**). One of the canonical pathways of this Aβ-induced innate immune response is the activation of the NOD-like receptor (NLR) family, a pyrin domain-containing 3 (NLRP3) inflammation, which has been the subject of intense research (Heneka et al., 2018) (**Figure 3**).

Studies have shown that Aβ oligomers can trigger the expression of the NLRP3 inflammasome and thus promote inflammation and intensify association between T2D and AD (Rea et al., 2018). Inflammasome is involved in the progression of metabolic syndrome due to impaired adipose tissue sensitivity. It has been conclusively demonstrated that obesity triggers NLRP3 activation and that secreted IL-1β that impairs insulin signaling, which contributes to IR in mice (Mori et al., 2011; Rea et al., 2018). Another study found that obesity was associated with NLRP3 activation in adipose tissue (Mori et al., 2011; Vandanmagsar et al., 2011; Rea et al., 2018).

The role of NLRP3 inflammasomes in the pathogenesis of obesity has been supported by data showing that $Nlrp3^{-/-}$ and $Asc^{-/-}$ knockout mice are protected from obesity and IR induced by a high-fat diet. In addition, NLRP3 activation by inflammasomes/caspase-1 appears to be a key regulator of adipocyte differentiation and directs adipocytes to an insulin-resistant phenotype (Stienstra et al., 2010).

Consequently, CR and weight loss in obese diabetic subjects decrease the expression of the Nlrp3 and IL-1 genes in adipocytes, improving insulin sensitivity (Vandanmagsar et al., 2011). However, some studies have failed to find an association between NLRP3 inflammasome formation and obesity or IR (Nishimoto et al., 2016). Understanding the molecular mechanisms of chronic inflammation remains a major medical problem (Nishimoto et al., 2016; Rheinheimer et al., 2017). Thus, further research is required to understand the relationship between NLRP3 inflammasome formation and IR.

Moreover, the inflammasome pathway is activated by a variety of intracellular processes and associated with increased age and age-related diseases. Both the inflammatory pathway and the senescent cell-related SASP activate the inflammasome through the NF-κB and IL-α cascade, causing the inflammatory response and cytokine production that delays resolution and healing (Chien et al., 2011; Mori et al., 2011; Rea et al., 2018).

Therefore, caspase-1 or inflammasome inhibitors have been proposed as novel treatments for pathologies associated with aging and metabolism deterioration (Stienstra et al., 2010; Kanbay et al., 2019).

METABOLIC HALLMARKS OF AGING AND THEIR ROLE IN THE COGNITIVE RESERVE

One of the most urgent tasks of modern gerontology is the search for various pathogenic factors that worsen the health and well-being of the elderly. Loss of function over time is distinctive for aging. Usually, the deterioration of the physical and mental condition occurs gradually. Incidentally, it is still not known whether this diminishment could be a result of physiological or pathological processes (Akintola and van Heemst, 2015).

Cognitive reserve is the determining factor in the difference between physiological and pathological brain aging. Cognitive reserve is related to the brain's ability to maintain cognitive

TABLE 1 | Damage-associated molecular patterns (DAMPs), their receptors and molecular action in insulin resistance and Alzheimer's disease.

DAMP	Receptors or sensors	Molecular action and effects	References
High mobility group box 1 (HMGB1) (alarmin)	RAGE, TLR4	Signal to the NF-κB signaling pathway and thus contributes to the inflammatory responses in type 2 diabetes mellitus, in the genesis and pathophysiology of IR and neurodegeneration.	Gonelevue et al., 2018; Paudel et al., 2020
Aβ (amyloid)	TLR4, TLR2, NLRP3, CD36, CD14 receptor	Aβ activates the NLRP1 and NLRP3 inflammasomes. The oligomers can disturb the functions of K^+ channels, decreasing the intracellular K^+ concentration and thus activating caspase-1. Increasing K^+ efflux with valinomycin led to activated caspase-1 and IL-1β secretion from neurons. Aβ can also activate microglial cells in the brain through interaction with the surface receptor CD36, which induces the formation of a TLR2–TLR6 heterodimer and subsequently leads to NF-κB signaling.	Stewart et al., 2010; Heneka et al., 2018; Venegas and Heneka, 2019
Chromogranin A (CGA) (an acidic protein localized in secretory vesicles)	TLR4, CD14, or class A scavenger receptor	The stimulation of target receptors promotes the uptake of Aβ and phagolysosome formation. Upon lysosomal rupture, cathepsin B release is instrumental in the activation of procaspase-1 that ultimately produces IL-1β.	Lechner et al., 2004; Venegas and Heneka, 2019
ATP	P2 × 7R (an ATP-gated ion channel supporting Na^+ and Ca^{2+} influx into and K^+ efflux out of the cell)	The decrease in intracellular K^+ leads to P2 × 7R-mediated NLRP3 inflammasome formation. Together with IL-1β release, NLRP3 inflammasome activation in the brain through the P2 × 7 receptor induces an increase of tau secretion in exosomes and its subsequent transmission to neurons.	Muñoz-Planillo et al., 2013; Asai et al., 2015; Venegas and Heneka, 2019
Ceramide (a sphingosine-based, lipid- signaling molecule that is formed from serine and 2 fatty acids)	NLRP3	Ceramide can act as an endogenous signal to caspase-1 cleavage and IL-1β secretion	Shin et al., 2015; Venegas and Heneka, 2019
S100	RAGE	Stimulate cell proliferation and migration and inhibit of apoptosis and differentiation, which participate in neurodegenerative processes. RAGE receptor activation leads to the activation the p38 MAPK cascade NF-κB.	Cristóvão and Gomes, 2019; Venegas and Heneka, 2019
mt-DNA and cf-DNA	TLR9 AIM2	Induce the release of interferon type 1 and TNF-α. Exogenous mtDNA fragments induced TLR9-mediated NF-κB activation in primary muscle cells. mtDNA increased TLR9 content in muscle cells. When cf-DNA binds to TLR, signaling occurs through MyD88, which leads to a type I IFN response. When cf-DNA binds to AIM2, caspase-1 is activated, and subsequently, IL-1β is released.	Shin et al., 2015; Venegas and Heneka, 2017; Yuzefovych et al., 2018
HSPs—heat shock proteins	PRRs (pattern recognition receptor). TLR2 and TLR4	Interaction with receptors leads to the induction of inflammatory cytokines such as TNF-α, IL-1β, IL-12, and GM-CSF.	Campanella et al., 2018; Venegas and Heneka, 2019
Homocysteine (Hcy)	NLRP3	Activation of the inflammasome with the subsequent release of interleukins. Hcy mediates the development of insulin resistance.	Smith et al., 2018; Zhang et al., 2018
Glucose	NLRP3	Induction of IL-1β secretion followed by increased apoptosis triggered by Fas via NF-κB and JNK and/or inhibiting insulin signaling.	Shin et al., 2015
IAPP [islet amyloid polypeptide (IAPP)–amylin]	NLRP3, CD36, and RAGE	IAPP has cytotoxic effects; assembly of the inflammasome leads to the formation of mature IL-1β.	Fawver et al., 2014; Shin et al., 2015
FFAs and their metabolites (palmitate)	NLRP3, TLR4	The production of inflammatory cytokines through activation of TLR and NLRP3 contributes to the development of insulin resistance by suppressing insulin signaling.	Shin et al., 2015

function despite being constantly under the influence of stressors and degenerative events associated with aging and the AD development. It is known that hippocampal neurogenesis is a lifelong process of continuous inclusion of functionally active new neurons into neuronal circuits. Accordingly, neurogenesis in the adult hippocampus is increasingly seen as a key factor in the sustainability of the cognitive reserve (Dainikova and Pizova, 2014). In addition, it was determined that the decisive factor in determining the resistance of nerve tissue to neurodegeneration is age-related decline in glial function and metabolic coupling. Thus, impairment of neuroglia and cell metabolism promotes the transition from physiological to pathological aging (Verkhratsky et al., 2015, 2020).

Risk factors that have been considered in relation to brain aging include metabolic disorders. The aging process of the brain can be accompanied with impaired glucose metabolism or decreased glucose supply to the brain. Moreover, brain IR has been associated with an increased risk of both cognitive decline and dementia, including AD and vascular dementia (Hughes and Craft, 2016; Yin et al., 2016; Baranowska-Bik and Bik, 2017) (**Figure 1**).

Currently, research has made significant progress in understanding the pathogenesis of AD but, unfortunately, without any disease-modifying therapeutics or proven prevention strategies. One of the most relevant and promising areas in terms of therapeutic effects is the study of brain

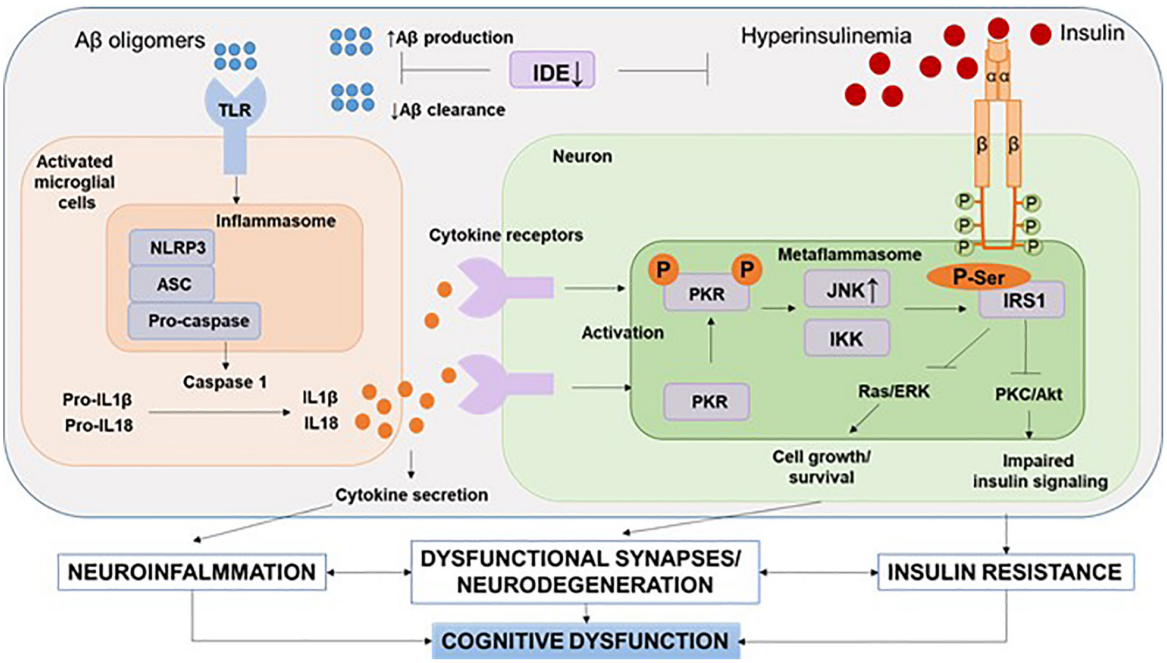

FIGURE 3 | NLRP3 inflammasome and metaflammasome activation in response to DAMPs. In the brain, activation of toll-like receptors leads to the assembly of NLRP3 inflammasomes and the maturation and release of IL-1β. Through cytokine receptors, kinases are activated—components of metaflammasome—JNK and IKK. This leads to impaired insulin signaling and the development of insulin resistance, which leads to dysfunction of synapses and the development of neurodegeneration. Aβ, amyloid beta; Akt, protein kinase B; ASC, apoptosis-related speck-caspase recruitment domain; DAMPs, damage-associated molecular patterns; IDE, insulin degrading enzyme; IKK, IkBa kinase; IRS, insulin receptor substrate; JNK, c-Jun N-terminal kinases; NLRP3, NOD-like receptor pyrin domain-3 inflammasome; PKC, protein kinase C; PKR, double-stranded RNA-dependent protein kinase; Ras/ERK, kinase regulated by extracellular signals; TLR, toll-like receptors.

metabolism. The leading opinion postulates that glucose metabolism is reduced in almost every neurological and psychiatric condition (Mosconi et al., 2009; Bélanger et al., 2011; Neth and Craft, 2017).

Brain Insulin, Glucose, and Other Energy Sources for Thoughts During Aging

Until now, the production of insulin in the brain remains a controversial issue. There is a lot of conflicting evidence about the production of insulin in brain structures and cell types. The initial hypothesis was that insulin is able to cross the blood–brain barrier (BBB) through a saturable transport system. However, this mechanism is limited and ineffective. Later, data appeared on the possible production of insulin in the brain. Thus, the expression of mRNA and insulin protein was found in the hippocampus, olfactory bulb, striatum, hypothalamus, and entorhinal and prefrontal cortices (Mehran et al., 2012). Insulin secretion has also been reported in cultured astrocytes (Takano et al., 2018). In a recent study, the authors describe the presence of not only insulin mRNA but also the protein itself in the epithelial layer of the choroid plexus of mice and humans, along with proteins associated with the processing and secretion of insulin (Mazucanti et al., 2019).

Since the stability of the cognitive reserve is largely determined by neurogenesis, the study of metabolic trophic factors that influence this process is important. Insulin at moderate concentrations is known to play a neurotrophic role. Insulin has a pivotal role in the brain development, functioning of neurogenic niches, and aging. Activation of the insulin/IGF-I (insulin-like growth factor) signaling pathway regulates the exit of neuroblasts from the quiescence state. This signaling cascade, insulin and IGF-I, has been shown to promote neurogenesis by modulating proliferation, differentiation, and survival of neural stem cells (NSCs) (rev. in Spinelli et al., 2019). In healthy metabolic conditions, acute increases in insulin levels have a valuable effect on cognition. Nevertheless, chronically elevated insulin significantly reduces the level of its mediated effects (Neumann et al., 2008). Moreover, chronic hyperactivation of the insulin/IGF-I pathway can cause premature depletion of the stem cell pool (Spinelli et al., 2019). In contrast, high insulin levels may be associated with increased Aβ deposition in the brain, as insulin and Aβ compete for the same enzyme that provides their clearance, namely, the insulin-degrading enzyme (Hölscher, 2019). Thus, insulin can have a trophic or deleterious effect on neurogenesis (Spinelli et al., 2019). This conclusion can be confirmed by studies demonstrating impaired learning in animals with a model of type 2 diabetes mellitus (T2DM), as well as the observed cognitive deficit in clinical studies among patients with this pathology (Zilliox et al., 2016).

Recently, the term "type 3 diabetes mellitus" has often been used to denote AD, since the pathological events accompanying

this pathology are pathogenetically associated with central IR (Kandimalla et al., 2017). Aβ suppresses insulin expression in astrocytes (Pitt et al., 2017; Spinelli et al., 2019). These data show bi-directional changes between impaired brain insulin signaling and Aβ deposition in AD. According to these results, local IR and changes in central glucose metabolism may be considered as early markers for the diagnosis of AD (Hölscher, 2019).

It was shown that central glucose hypometabolism can be detected decades before the clinical onset of AD (Mosconi et al., 2009; Sperling et al., 2011; Neth and Craft, 2017). However, in the last few years, it is becoming increasingly obvious as a condition of reactive or compensatory glucose hypermetabolism in neurologic diseases as an initial reaction to trauma and developing pathological processes (Ashraf et al., 2015; Neth and Craft, 2017). According to Neth and Craft (2017), a glucose hypermetabolism could be a temporary solution to the injury problem with a permanent reduction in glucose utilization. If additional data confirm the occurrence of an initial increase and a final decrease in glucose metabolism in the brain, then this shift can be visualized at an early stage (before the onset of clinical symptoms) and work to prevent the pathology. This hypermetabolic glucose shift at the early stages is likely complemented by increased use of other fuels as well. As the disease progresses, a bioenergy shift may occur due to a decrease in glucose dependence and an increase in the use of alternative energy sources (Neth and Craft, 2017).

As it has already been mentioned, the most preferred energy substrate for the brain, except the prolonged fasting, is glucose. For normal functioning of the brain, a constant supply of glucose is necessary. In small concentrations, glycogen was also found in the brain, which is stored in astrocytes. Glycogen provides lactate as an energy source for neurons through monocarboxylate transporters to support neural functions such as hippocampal-regulated memory formation and learning (Rich et al., 2019). Under conditions of reduced glucose intake, ketone bodies, mainly formed as a result of fatty acid (FA) oxidation, are an alternative main source of energy. In addition, FAs and their metabolites are capable of influencing many brain functions. This also allows them to be considered as potential targets for pharmacological and/or dietary interventions in certain brain pathologies (Romano et al., 2017).

Nevertheless, glucose is of the greatest importance as a brain energy substrate, and therefore, disturbances in glucose metabolism have significant consequences on the functioning of the brain. Decreased hippocampal volumes have been described in elderly with impaired glucose tolerance. It accompanied with a lower cognitive test performance. Similarly, another study confirmed that patients with higher fasting glucose and glycosylated hemoglobin experienced decreased memory and learning ability (Grabenhenrich, 2014). Glucose hypometabolism was most obviously recorded in the frontal, parietal, and temporal cortices (Tondo et al., 2020). This suggests that IR affects similar areas of the brain as in AD, supporting the notion that central IR may contribute to neurodegeneration. Moreover, diabetes has been repeatedly shown to be a strong predictor of cognitive dysfunction in the elderly (Kong et al., 2018).

Cognitive decline is not limited to impairments in learning and memory; there are other impairments as well. Brain IR has been confirmed to be associated with decreased task processing speed, cognitive flexibility, and motor skills. Current evidence confirmed that IR should be considered as an pivotal risk factor for the progression of cognitive dysfunction (Moheet et al., 2015).

Thus, clinical work and experimental studies in animals propose that IR has destructive effects on cognitive functions, in particular on learning and memory. Therefore, it becomes the principal aim to study the metabolic pathways and their association with the progression of AD and other neurodegenerative disorders (Spinelli et al., 2019).

Brain Insulin Resistance in the Pathological Aging

Changes in brain insulin signaling, and in particular in the hippocampus, can alter molecular pathways involved in synaptic plasticity and neurogenesis in adults, thereby leading to a decrease in cognitive reserve, an increased risk of neurodegeneration, and a shortened life span (Epel, 2020). Long-term persistent excess of nutrients is the cause of stress acceleration of aging. However, an excess of nutrients causes hyperactivation of insulin signaling and leads to desensitization of IR-dependent molecular cascades. Because of this influence, the brain stops responding to insulin and eliminates both the metabolic and cognitive effects of this hormone (Spinelli et al., 2019).

IR makes it difficult for cells to maintain energy homeostasis. The brain in AD neurodegeneration is accompanied by changes similar to those observed in peripheral tissues in diabetes mellitus, including metabolic stress and neuroinflammation (Talbot et al., 2012). Thus, it can be assumed that such mechanisms explain IR in T2DM and impair central insulin transduction in patients with AD. Significant similarities between neuropathogenic mechanisms are induced by Aβ oligomers and cause the loss of neurons and synapses, as well as mechanisms associated with peripheral IR in diabetes (Craft, 2012).

Many explanations have been proposed for the impaired insulin transduction in the AD brain. One of them is decreased extracellular insulin assessed in cerebrospinal fluid, decreased total or cell surface of insulin receptor expression, and decreased affinity of insulin receptors for insulin (Talbot, 2014). The deficiency of extracellular insulin in the AD brain remains unclear and identified the opposite results obtained in cerebrospinal fluid (Molina et al., 2002). Similarly, deficits in total insulin receptors in AD brain tissues were not found in studies using age-matched controls, and cell fractionation did not reveal deficiencies in insulin receptor levels on the cell surface. Although the binding of insulin to the insulin receptor may be reduced in the brain tissue in AD, insulin still manages to activate the catalytic domain of the insulin receptor at a level of 71–74% of normal levels even in the hippocampal formation in AD. As noted above, a significantly greater decrease in insulin sensitivity is observed already after binding to the insulin receptor in the brain in AD. In the hippocampus, which is responsible for memory and learning, insulin activated only

10% of the normal level of insulin receptor substrate (IRS) (Talbot et al., 2012).

Thus, the most likely cause of decreased insulin signaling in the brain in AD is IR due to dysfunctional IRS-1. This appears to reflect Aβ-induced secretion of proinflammatory cytokines by glial cells. Among the early changes in AD, there is an increased solubility of Aβ, the monomers of which combine to form oligomers, which can later assemble into fibrils and form amyloid plaques (Sengupta et al., 2016). Also at the AD onset, Aβ oligomers activate microglia, which leads to the secretion of proinflammatory cytokines such as IL-1, IL-6, and tumor necrosis factor α (TNF-α) (Hemonnot et al., 2019). Such activation of microglia may play a key role in the AD pathogenesis, given the recent discovery that disabling a gene in AD model encoding a microglial receptor (i.e., NOD-like receptor 3) and perceiving inflammatory pathogens, including Aβ, prevent the development of AD and cognitive abnormalities that usually occur in this AD animal model (Heneka et al., 2013). Through neuronal receptors, microglial IL-1, IL-6, and TNF-α activate serine kinases IRS-1, known as IkBa kinase (IKK), C-Jun N-terminal kinase (JNK), and extracellular signal-regulated kinase (Erk2) (Talbot and Wang, 2014). Thus, Aβ oligomers injected into neuronal cultures or into the cerebral ventricles markedly increase the phosphorylation of IRS-1 serine (IRS-1 pS) at several sites, namely, S312, S616, and/or S636 (S307, S612, and S632 in rodents' Erk2) (Talbot and Wang, 2014).

Elevated neural IRS-1 pSer is significant in the cortex and hippocampal formation in AD and, apparently, is the main cause of IRS-1 dysfunction in AD (Tanokashira et al., 2019). The most common cause of IR is inhibition of downstream signaling due to serine phosphorylation of the IRS1 (Boucher et al., 2014). Similar changes occur in the AD brain. Insulin activation of IRS-1 is consistently decreased in tissues with significantly increased levels of IRS-1 pS616 and IRS-1 pS636. These molecules may act as potential markers of central IR (Talbot et al., 2012; Talbot and Wang, 2014). As expected, the levels of these candidate biomarkers correlate significantly with the Aβ deposition and are associated with cognitive decline (Kong et al., 2018).

This may explain crossroad peripheral IR due to obesity and/or diabetes mellitus and brain IR in AD (Liu et al., 2011). Obesity and T2DM are, in fact, risk factors for AD and are associated with increased vascular proinflammatory cytokines (Pugazhenthi et al., 2017). With the development of AD, the impaired integrity of the BBB promotes the penetration of cytokines. They, in turn, activate IRS-1 serine kinases in the same way as cytokines obtained from microglia (Ferreira et al., 2018).

Frontiers in Insulin Resistance Markers

The relationship between IR and AD may be enhanced due to a common etiology leading to an increased risk of AD (Spinelli et al., 2019). IR is a potentially modifiable risk factor for AD; in this regard, early diagnosis of IR remains highly relevant. Previous research has relied on measurements of systemic IR based on blood glucose and insulin values, such as the Homeostatic Model of Insulin Resistance (HOMA-IR) assessment (da Silva et al., 2019). Peripheral and central IR overlap to some extent, and this may explain why associations between HOMA-IR and glucose hypometabolism in the brain have been observed. Currently, a variety of proteins have been isolated that are consistently significantly related to IR and AD pathology. They are discoidin, CUB, and LCCL domain-containing protein 2 (DCBLD2); Ephrin-B2 (ENFB2); ciliary neurotrophic factor receptor subunit alpha (CNTFR); neuronal growth regulator 1 (NEGR1); leucine-rich repeat-containing protein 4B (LRRC4B); and SLIT and NTRK-like protein 4 (SLITRK4). However, a search for markers specific to central IR is also required (Westwood et al., 2017).

For these reasons, in recent years, research has focused on assessing glucose metabolism in the brain and analyzing extracellular brain vesicles extracted from blood as biomarkers of IR and early phase of cognitive decline. In a study in patients with IR, but without loss of cognitive functions, IR was associated with hypometabolism in the hippocampus and higher levels of blood pressure biomarkers in the cerebrospinal fluid (Westwood et al., 2017). Cerebral glucose metabolism is closely related to neuronal activity, and a decrease in the cerebral metabolic rate for glucose (CMRglc) is one of the main hallmarks of AD. *In vivo* imaging using 2-[^{18}F]fluoro-2-deoxy-D-glucose PET (FDG-PET) demonstrates a consistent and progressive decrease in CMRglc in patients with AD. A decrease in CMRglc in preclinical stages of AD, with mild cognitive impairment (MCI) was also indicated, as well as in carriers of the allele of apolipoprotein E-epsilon-4, a strong genetic risk factor of AD (Mosconi et al., 2008).

In a recent study, Mullins et al. (2017) demonstrated that pSer312-IRS-1 (which stimulates uncoupling of IRS-1 and leads to its degradation) and p-panTyr-IRS-1 (which promotes insulin-stimulated responses) are biomarkers of AD. Based on these data, a methodology for the isolation of exosomes from plasma was developed, followed by immunoprecipitation against the cell surface adhesion protein L1-CAM to enrich neural origins. It was demonstrated that pathogenic and signal peptides in plasma exosomes expressing L1-CAM/NCAM effectively distinguish between AD and control group and can predict the diagnosis of the disease. In addition, it was confirmed that the peripheral IR is separated from the brain IR that occurs in AD (Mullins et al., 2017; Kapogiannis et al., 2019; Spinelli et al., 2019). These data indicate the presence of IR of the brain in patients with AD and, to a lesser extent, in people with diabetes (Mullins et al., 2017; Spinelli et al., 2019).

Neuroinflammation and Brain Insulin Resistance

Inflammation is a feature of diabetes mellitus and AD, and it is believed that this process plays an important role in the pathogenesis of these two pathologies. Inflammation is an important part of the body's defense mechanisms against many pathological agents, such as infections and trauma, and includes both the formation of soluble factors and the activation of specific cells that are mobilized to respond and maintain the normal physiology and homeostasis (Chatterjee and Mudher, 2018).

It is believed that similar inflammatory processes occur in the CNS and periphery. The presence of inflammatory markers in AD in the brain tissue, including increased levels of cytokines/chemokines, which accompany with gliosis, was confirmed in many studies (Cao, 2015). In addition, an increase

of inflammatory mediators in the blood, such as TNF-α, IL-6, and IL-1β, was observed in patients suffering from AD (Ng et al., 2018). Moreover, increased production of proinflammatory cytokines in adipose tissue is a key feature of the pathogenesis of metabolic disorders. A recent study has shown that an elevated level of TNF-α expressed in adipose tissue of obese individuals is the reason of peripheral IR. Therefore, in both the central and peripheral tissues, uncontrolled or chronic inflammation accompanies IR (Saltiel and Olefsky, 2017). Interestingly, inflammation also underlies hypothalamic dysfunction in obesity (Samodien et al., 2019). New evidence indicates that it is the inflammation and stress of the endoplasmic reticulum that are critical pathogenetic events in the central and peripheral IR in metabolic disorders (Arruda et al., 2011). In an obese and diabetic animal model, the neuroinflammation, especially through activation of TNF-α and the IkBa kinase (IKK)-b/nuclear factor-kB pathway, is a principal mechanism underlying the disease pathogenesis (Milanski et al., 2009). Consequently, the pathology of the hippocampus in AD and the pathology of the hypothalamus in obesity have common pathogenetic pathways associated with inflammation (De Felice and Ferreira, 2014).

Our own data have shown the protective phenotype of Nlrp3 knockout mice in the development of brain IR (Chernykh et al., 2018). Using an experimental approach to modeling AD, we investigated new molecular mechanisms of insulin signaling dysregulation in the amygdala in association with neuroinflammation and emotional disorders. It has been established that experimental AD is accompanied by impaired expression and functional activity of molecules–components of insulin-mediated signaling pathways and the development of IR together with up-regulation of neuroinflammation in the brain amygdala. This cascade of pathological reactions is reflected in emotional behavior disorder. NLRP3-dependent mechanisms have been demonstrated in the basolateral amygdala in normal conditions and during the development of neurodegeneration. It has been experimentally proven that preventing the development of local IR by blocking NLRP3 inflammasomes should be considered an approach to correcting BIR and emotional disorders in AD. The studied molecular mechanisms linking the development of local IR with neuroinflammation (with the participation of pIRS, GLUT4, IRAP, and NLRP3) and impaired cognitive and emotional spheres open up new possibilities for the prevention and correction of neurodegeneration in AD.

Thus, suppression of neuroinflammation by preventing the expression of NLRP3 inflammasomes in *Nlrp3*-knockout mice has a protective role in the development of AD, accompanied by IR, due to modulation of the expression of pIRS-Ser and downstream of insulin signaling cascade (Gorina et al., 2019).

Activation of Proinflammatory Factors and Signaling Pathways in a Cell Upon Damage to Insulin Signaling in Neurons in Alzheimer's Disease

In peripheral IR, impaired TNF-α signaling results in JNK activation (Chen et al., 2015). Activation of the TNFa/JNK pathological pathway is associated with the main inflammatory and stress-signaling mechanisms, including tension of the endoplasmic reticulum and activation of stress kinases IKK (IkBa kinase) and PKR (double-stranded RNA-dependent protein kinase) (Nakamura et al., 2010). In T2DM, high levels of TNF-α initiate serine phosphorylation of IRS-1 by stress kinases, blocking insulin signaling (Nakamura et al., 2010). TNF-α levels increase in microvessels of the brain and cerebrospinal fluid in AD (Ruan et al., 2009). Initial information that impaired insulin signaling in neurons in AD is associated with proinflammatory signaling was based on the fact that oligomers of Aβ induce inhibition of IRS-1 through TNF-a/JNK activation (Bomfim et al., 2012). These ideas were confirmed, and it was shown that common mechanisms underlie damaged peripheral insulin transduction in diabetes mellitus and central local IR in the AD brain. Namely, it was shown that IKK and PKR were increased in AD in the brain, and they mediate the inhibition of IRS-1 in hippocampal neurons induced by oligomers of Aβ (Lourenco et al., 2013). IKK mediates neuronal inhibition of IRS-1 by Aβ oligomers (Bomfim et al., 2012).

METAFLAMMATION AND METAFLAMMASOME

As already described, assembly of the multiprotein complex inflammasome occurs not only in neurodegenerative diseases but also in metabolic disorders. The terms "metabolic inflammasomes" or "metaflammasomes" encompass metabolic disorders and the inflammation they cause. In other words, metaflammasome is a cascade of a signaling response in a cell caused by DAMPs followed by a metabolic pathway response and cytokine release (**Table 1** and **Figure 3**) (Kanbay et al., 2019; Kuryłowicz and Koźniewski, 2020).

The expression of the four main components of the metaflammasome complex has been confirmed in the human brain. It includes phosphorylated forms of IKKβ, IRS1, JNK, and PKR (Taga et al., 2017).

C-Jun N-terminal kinases (JNKs) play an important role in a wide range of different stress-induced pathways. Thus, they are involved in neuronal cell death, migration, neuronal plasticity, autophagy, regeneration, metabolism, and regulation of cellular aging. Various stressors, including cytokines, ROS, growth factors, and Aβ oligomers, initiate the JNK signaling pathway. The role of JNK has been confirmed in studies of the relationship between neuronal death in AD and amyloid plaques. JNKs have been shown to increase Aβ production and are involved in the maturation and development of NFTs. In addition, it is currently considered a promising area of study of potential JNK inhibitors as a potential target for the treatment of neurodegenerative changes in AD (Yarza et al., 2016).

Studies have experimentally confirmed that low levels of JNK in T2DM are not accompanied by the development of cognitive impairments, including dementia. Conversely, a high level of C-Jun N-terminal kinases expression was recorded in patients with dementia and comorbid AD. In this regard, it has been convincingly demonstrated that JNK inhibition in the liver improves insulin signaling and reduces glucose tolerance. Kinase

inhibition leads to a decrease in obesity and an increase in insulin sensitivity, while with the development of obesity, a pathological increase in expression is noted (rev. in Taga et al., 2017).

Another component of the so-called metaflammasome, which is involved in the regulation of inflammation, is IKKβ. IKKβ has a neuroprotective function by inhibiting neuronal NF-κB. This in turn protects neurons from Aβ and oxidative stress (Liu et al., 2017; Taga et al., 2017).

A recent study investigated the relationship of these four kinases. The data on the strength of the relationship between the components of the metaflammasome are compelling. Therefore, it was shown that there is no relationship between IKKβ and JNK kinases in the absence of dementia. At the same time, with the progression of AD, an inverse relationship is observed between IKKβ and JNK. There is no connection between IKKβ and IRS1 and PKR. This shows the special role of the relationship between the components of the metaflammasome, depending on the state, the presence of pathology, and the brain environment (Taga et al., 2017).

Another of the kinases of the so-called metaflammasome complex is a proapoptotic enzyme—eukaryotic initiation factor 2α kinase 2 (PKR). PKR inhibits translation and participates in cellular signaling, which leads to brain damage in AD and impaired memory consolidation. Aβ results in the activation of PKR and its accumulation in degenerating neurons. PKR modulates Aβ synthesis through the induction of beta-site APP-cleaving enzyme 1 (BACE 1). An elevated level of PKR was observed in the cerebrospinal fluid in AD patients and patients with an MCI. PKR activation leads to downstream cascade resulting in TNFα and IL1-β production. It was also shown experimentally that PKR inhibits molecular processes of memory consolidation. This kinase is also currently considered as a potential target for inhibition, can reduce neuronal death, and can facilitate cognitive decline in neurodegeneration (Hugon et al., 2017).

It was reported by Taga et al. (2017) that high expression of IRS1 and PKR is associated with cognitive impairment but not dementia. There are suggestions that the components of the metaflammasome can be activated precisely in the early stages of AD or in MCIs, which also makes these kinases promising for use as marker molecules for early diagnosis of degenerative events. This is confirmed by experimental animal studies, where after the intervention of a high-fat diet with the development of IR, an increase in IRS1 in the mice hippocampus was noted, which was accompanied by a deficit in spatial working memory due to postsynaptic impairment (Arnold et al., 2014; Spinelli et al., 2019).

In general, the metaflammasome hypothesis is based on the concept that dysfunction of the endoplasmic reticulum (due to the accumulation of unfolded proteins) leads to the expanded protein reaction and increased inflammation (Taga et al., 2017).

In addition, immunometabolic pathways are sensitive to lipids and are associated with lipotoxicity, which in turn causes metaflammation and changes in lipid metabolism (Ertunc and Hotamisligil, 2016). Since insulin is an important regulator of lipid metabolism as well, dyslipidemia is one of the main features of IR. The characteristic of peripheral IR is an increased content of free FAs (FFAs), an increase in the level of very-low-density lipoprotein (VLDL), and a decrease in high-density lipoprotein (HDL) (Kamagate et al., 2008; Neth and Craft, 2017). The dyslipidemia plays a role in amyloid deposition in AD, probably due to the effect of cholesterol on Aβ processing in the brain (Berti et al., 2015; Neth and Craft, 2017). This was confirmed by PET imaging. In addition, various genetic studies have identified several genes involved in lipid and cholesterol metabolism as increasing the risk of AD. This is primarily apolipoprotein-E (ApoE), followed by apolipoprotein-J (APOJ or clusterin, CLU), ATP-binding cassette subfamily A member 7 (ABCA7), and sortilin-like receptor. These results suggest a potential link between dyslipidemia and the accumulation of cerebral amyloid, which, in turn, may be mediated by IR, as well as other causes of lipid metabolism disorders, such as carriage of the Apoe4 allele (Neth and Craft, 2017).

ApoE is the main apolipoprotein, produced in the CNS, and directly increases the risk, progression, and pathogenesis of AD. Allele differences in ApoE confer specific effects on Aβ deposition, degradation and clearance, tau phosphorylation, neuronal damage, and inflammation (Stukas et al., 2015). There is evidence that the carriage of the ApoE4 allele can contribute to a decrease in insulin signaling by directly interacting with insulin receptors, leading to the uptake of insulin receptors within endosomes (Zhao et al., 2017). The role of clusterin (apolipoprotein J) in the risk and severity of AD was confirmed in relation to both cognitive function and Aβ metabolism. ApoA-I can also influence the pathology of AD, potentially by modulating cerebrovascular integrity and function, aiding in the removal of Aβ peptides from the cerebrovascular smooth muscle cells and reducing inflammation (Stukas et al., 2015).

Anti-inflammatory Strategies Targeting Neurodegeneration and Metaflammation

Since the important role of inflammation in the pathogenesis of neurodegeneration and IR in various pathologies, including diabetes mellitus and AD, has been convincingly shown, approaches based on an anti-inflammatory strategy can be used to treat symptoms and to interrupt the vicious circle of metaflammation. As visceral fat is strongly linked to metabolic disorders, strategies for correcting IR in metabolic-cognitive states are very promising approaches as well (Kullmann et al., 2020).

There is epidemiological evidence that some anti-inflammatory approaches, in particular the use of non-steroidal anti-inflammatory drugs (NSAIDs), reduce the risk of AD. However, this category of drugs does not affect cognitive function (Rivers-Auty et al., 2020). In clinical trials, no evidence of the effectiveness of NSAIDs was found. The ineffectiveness of anti-inflammatory strategies may be due to inappropriate non-steroidal anti-inflammatory drugs or due to epidemiological results caused by confounding factors. However, there is evidence that, for example, the use of diclofenac is associated with a decrease in morbidity, as well as with a slower decline in cognitive function. However, this requires further research into the potential therapeutic effects

of diclofenac in AD. Some antidiabetic drugs, which are aimed at lowering blood sugar levels, also have anti-inflammatory effects. This action is also associated with the hypolipidemic effect and direct modulation of immune responses. Despite promising results from clinical trials of anti-inflammatory drugs, salicylates, no clear guidelines have been established regarding the recommendation of these compounds for the prevention or treatment of T2DM. The use of other NSAIDs to combat metaflammation also requires clinical trials. However, most of the known methods of treating T2DM exhibit anti-inflammatory properties to varying degrees, which arise because of triggering various pathways and their effects, depending on many factors. Therefore, further clinical studies are needed to test new drugs and identify specific molecular pathways that could be therapeutically targeting metainflammation (Kuryłowicz and Koźniewski, 2020; Rivers-Auty et al., 2020).

There is evidence of a decrease in systemic inflammation with the applying of dietary protocols in clinical trials, associated primarily with a reduction in calorie intake (Lopez-Garcia et al., 2004; Kuryłowicz and Koźniewski, 2020). High-fat diets and high calories cause metainflammation, so the idea that dietary intervention can help reduce inflammatory the response in IR is very promising. Although the studies were not uniform in design, all reported that weight loss, improved glycemic control, and hepatic steatosis were associated with varying degrees with serum C-reactive protein (CRP) reduction (Kuryłowicz and Koźniewski, 2020).

Since it is a well-known fact that excess of nutrients in the course of obesity and IR impairs metabolism leading to the endoplasmic reticulum stress, possible CR may have a protective role (Park et al., 2012; Kuryłowicz and Koźniewski, 2020; Ma et al., 2020).

Calorie restriction is one of the most promising approaches for reducing the negative effects of metabolic disorders, age-related diseases, and pathologies associated with metaflammation (Park et al., 2012; Ma et al., 2020). In animal studies, it has been shown that reducing calorie intake increases life span and helps to lower blood glucose and insulin levels (Redman and Ravussin, 2011; Kim et al., 2020). There is currently evidence that CR for 2 years by 15% in healthy, non-obese people leads to a decrease in systemic oxidative damage (Redman et al., 2018). The main effects of CR in mammals include weight loss, improved insulin signaling by increasing hormone receptor sensitivity, normal lipid profiles, and increased adiponectin levels (Balasubramanian et al., 2017).

Thus, research data demonstrate the beneficial effects of CR in conditions such as diabetes, inflammation, obesity, and cardiovascular disease (Redman et al., 2018). However, the mechanisms underlying such changes remain unclear. An increase in adipose tissue is associated with the development of age-related metabolic changes, including the development of IR. In contrast, a decrease in adipose tissue during prolonged CR led to an improvement in age-related IR (Escrivá et al., 2007; Fabbiano et al., 2016; Corrales et al., 2019). CR slows down and restores age-related immunosenescence by regulating energy metabolism and oxidative stress and decreasing the production of proinflammatory cytokines and neuroendocrine homeostasis (Costantini et al., 2018).

CALORIE RESTRICTION AND COGNITIVE AND SOCIAL DETERMINANTS OF AGING: CD38 SIGNALING MECHANISM IN AGING AND NEURODEGENERATION

Inflammation, metainflammation, central and peripheral IR are determinants of aging along with behavioral, social, environmental, toxic, and other factors (Vidaček et al., 2018; Lever-van Milligen et al., 2019; Epel, 2020) (**Figure 4**). Different types of stressors can potentially lead to adaptive changes or accelerated aging. It depends on the nature of the stressors, stress resilience, and the stress response. At the same time, one of the promising strategies of slowing aging is an increase in stress resistance due to boosting stress resilience (Epel, 2020).

In relation to age and aging (pathological and physiological), many intervention approaches are considered. There are many potential approaches that may improve stress resilience: lifestyle interventions and CR are thought to work in part through adaptive response. Excess nutrients, high-fat diets, excessive calorie intake, and the traditional American diet can act as a stress acceleration of aging and lead to defective behavioral health. Therefore, CR can be considered as a stress rejuvenescence. The various effects of CR on metainflammation, IR, and neurodegeneration have been described (Hotamisligil, 2017; Epel, 2020). Less attention has been paid to the social determinants of aging and the possible mechanism by which CR might influence social behavior (Pifferi et al., 2018).

The positive effect of CR on cognitive longevity has been described, including through the effect on the morphological and functional properties of astroglia. Experiments on mice have shown that CR increases astroglial complexity and improves synaptic plasticity. Accordingly, this approach can increase neural compensation and cognitive reserve contributing to the healthy aging (**Figure 4**) (Verkhratsky et al., 2020).

One possible mechanism for mediated neuroprotection of CR is by regulating Ng-associated Ca^{2+} signaling. It causes a decrease in CaMKII and calpain activity, as well as downstream signaling that regulates neuronal metabolism, survival, and plasticity (Kim et al., 2016). CR significantly enhances cerebral blood flow and BBB function in young mice by reducing rapamycin expression, enhancing endothelial nitric oxide synthase signaling, and increasing ketone body utilization. This promotes memory formation and learning ability during aging and reduces anxiety in aging mice (Parikh et al., 2016). In a recent published study, the effect of CR on the social brain function was determined. In the long-term study CALERIE 1, CR did not change mood, but CR enhanced mood in patients in CALERIE 2, as well as improvements in tension anxiety were detected (Dorling et al., 2020). At the same time, there is also evidence that CR accelerated gray matter atrophy in old mouse lemurs but protected old animals from

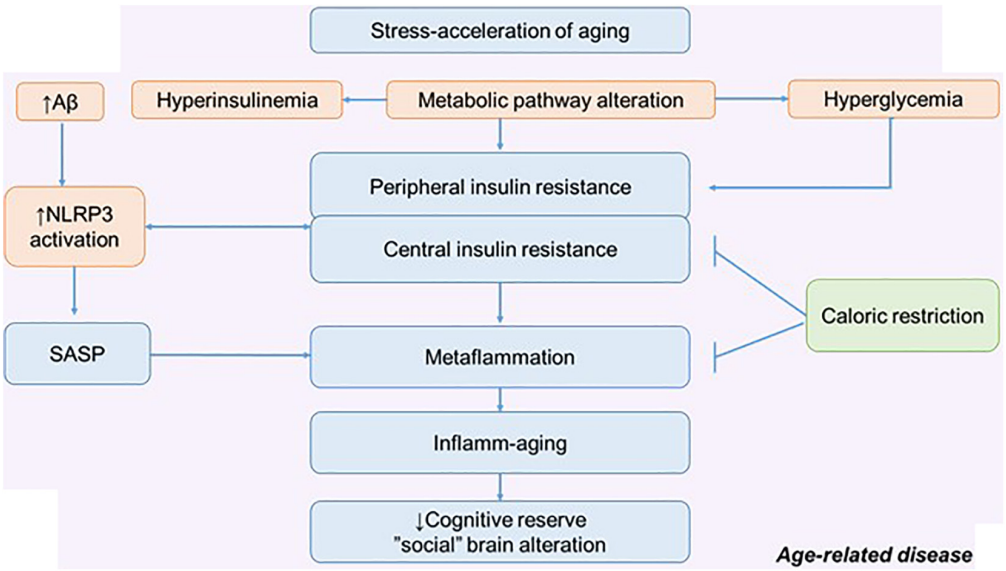

FIGURE 4 | The metabolic pathway alteration in the development of metaflammation and inflamm-aging. Possible role of calorie restriction in preserving cognitive reserve and social behavior. NLRP3, NLRP3 inflammasome; SASP, senescence-associated secretory phenotype.

white matter atrophy in comparison with old control animals (Pifferi et al., 2018).

Currently, the mechanism of the CR influence on social behavior remains relevant and unexplored. A potential mechanism of this action may be the restoration of NAD^+ through the activation of sirtuins and changes in the expression of NADase-CD38 (Tarragó et al., 2018). It was revealed that CD38 could act as a potential pharmacological target to reverse age-related NAD^+ decline. NAD^+ is an energy metabolism booster. CR affects AMP-activated protein kinase (AMPK) activity, which can modulate the bioavailability of NAD^+ (Connell et al., 2019).

Calorie restriction activates sirtuins, suppresses signaling of growth hormone/insulin-like growth factors and mTORC1 (mammalian target of rapamycin), and enhances mitochondrial redox regulation (Zullo et al., 2018). Sirtuins are a family of proteins with NAD^+-dependent enzymatic activities. Sirtuins regulate various cellular processes including glucose production, insulin sensitivity, inflammation, DNA repair, fat differentiation, FA oxidation, neurogenesis, and aging (Lee et al., 2019).

Previous research has reported a link between sirtuins and mitochondrial function and abnormal tau proteins and amyloid. It was confirmed that SIRT1, 3, and 6 are involved in age-related disease and regulation of life span, as well as AD progression (Hoshino et al., 2018). In mice model of AD, accompanied by impaired DNA repair, the precursor of NAD^+, nicotinamide riboside (NR), increases SIRT3 and SIRT6 (Hou et al., 2018). The role of sirtuins has been proven not only in the development of inflammation but also in IR. SIRT1 activation leads to the suppression of metaflammasome components, namely, JNK and IKK (Yoshizaki et al., 2010).

As already noted, neurons have a high-energy demand, and therefore, they are very sensitive to a decrease in NAD^+ and disruption of ATP production. NAD^+, by increasing the sirtuins, affects neuronal survival, contributing to the maintenance of a balance between mitochondrial biogenesis and mitophagy (Kerr et al., 2017). These arguments are also supported by the fact that selective overexpression of SIRT1 and SIRT6 in transgenic mice increases the life span of animals. The application of NAD^+ precursors [NR, nicotinamide mononucleotide (NMN), and nicotinamide] has a positive effect in neurodegenerative diseases and aging. The restoration of the NAD^+ level led to an increase in life expectancy in different animal species (Zhang et al., 2016; Hou et al., 2018). In this regard, it is logical to assume that the restoration of NAD^+ can act as a promising approach to the treatment of AD by influencing the pathology of tau protein and inflammation, as well as DNA repair (Hou et al., 2018).

Moreover, it is considered that NAD^+ depletion not only is characteristic of AD but also occurs during physiological aging; the NAD^+ precursor suppliers to medical nutrition leads to an improvement in cognitive functions and synaptic plasticity in AD (Gong et al., 2013). It is known that the enzymes involved in the degradation of cellular NAD^+ are CD38 and PARP. It is believed that CD38 is the main NADase in mammalian tissues; in addition, CD38 and its homolog BST-1/CD157 degrade both NMN and NR. Thus, CD38 and BST-1 are involved in the regulation of cell metabolism, regulation of sirtuin activity, and signaling. It should be noted that genetic or pharmacological ablation of CD38 protects against metabolic dysfunction caused by a diet high in fat and calories by modulating SIRT1 activity (Chini et al., 2017). The studies in animals suggest that increasing tissue NAD^+ levels by genetic CD38 ablation can significantly alter energy homeostasis in animals fed a calorie-excessive diet (Chiang et al., 2015).

At the same time, the physiological functions of CD38 in the brain have also been established. Thus, CD38 has been shown to play an important role in the secretion of oxytocin (OT)

in the hypothalamus and in the regulation of social memory and social interactions (Higashida et al., 2019). In addition, recently, our group, together with Japanese colleagues, showed that NR corrects social deficits, as well as anxious behavior in CD157 knockout mice. These results suggest that increasing NAD^+ levels with NR may allow animals with a deficiency of cyclic ADP-ribose and oxytocin to overcome this deficiency and function normally (Gerasimenko et al., 2020). This is supported by evidence that peripheral OXT administration improves social recognition, object recognition, and depressive behavior in high-fat-treated mice (Hayashi et al., 2020).

Therefore, diet-based strategies toward to CRs could be a promising therapeutic approach against AD, by influencing many signaling pathways, resulting in increasing cognitive reserve and maintaining social function (Esposito et al., 2015; Alkhatib et al., 2017; Wakabayashi et al., 2019; Kuryłowicz and Koźniewski, 2020).

CONCLUSION

Thus, inflamm-aging is one of the manifestations immune aging and is a risk factor of morbidity and mortality among the elderly people. Since various chronic diseases associated with age are directly associated with inflammatory response, the approaches to decrease metaflammation could considered as intervention in age-related pathology. Despite numerous studies in age-associated pathologies, studies of the contribution of the components of congenital immunity in healthy aging are insufficient. It remains unclear whether the inflammatory phenotype is a manifestation of healthy aging or is associated with development age-related pathology. Moreover, given the role of metainflammation in neurodegeneration, lifestyle interventions such as CR may be an effective way to break the vicious cycle of metainflammation and have a role in social behavior.

AUTHOR CONTRIBUTIONS

YK: conception and design. YK and AS: literature review and drafting the article. AC, OL, YG, and IL: critical revision for relevant intellectual content. YK, AS, and MG: final approval of the version to be published. All authors have read and agreed to the published version of the manuscript.

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REVIEW
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Rationalizing the Role of Monosodium Glutamate in the Protein Aggregation Through Biophysical Approaches: Potential Impact on Neurodegeneration

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Monosodium glutamate (MSG) is the world's most extensively used food additive and is generally recognized as safe according to the FDA. However, it is well reported that MSG is associated with a number of neurological diseases, and in turn, neurological diseases are associated with protein aggregation. This study rationalized the role of MSG in protein aggregation using different biophysical techniques such as absorption, far-UV CD, DLS, and ITC. Kinetic measurements revealed that MSG causes significant enhancement of aggregation of BSA through a nucleation-dependent polymerization mechanism. Also, CTAB-BSA aggregation is enhanced by MSG significantly. MSG-induced BSA aggregation also exhibits the formation of irreversible aggregates, temperature dependence, non-Arrhenius behavior, and enhancement of hydrodynamic diameter. From the isothermal titration calorimetry measurement, the significant endothermic heat of the interaction of BSA-MSG indicates that protein aggregation may be due to the coupling of MSG with the protein. The determined enthalpy change (ΔH) is largely positive, also suggesting an endothermic nature, whereas entropy change (ΔS) is positive and Gibbs free energy change (ΔG) is largely negative, suggesting the spontaneous nature of the interaction. Furthermore, even a low concentration of MSG is involved in the unfolding of the secondary structure of protein with the disappearance of original peaks and the formation of a unique peak in the far-UV CD, which is an attention-grabbing observation. This is the first investigation which links the dietary MSG with protein aggregation and thus will be very instrumental in understanding the mechanism of various MSG-related human physiological as well as neurological diseases.

Keywords: monosodium glutamate, protein aggregation, nucleation-dependent polymerization, isothermal titration calorimetry measurement, neurodegeneration

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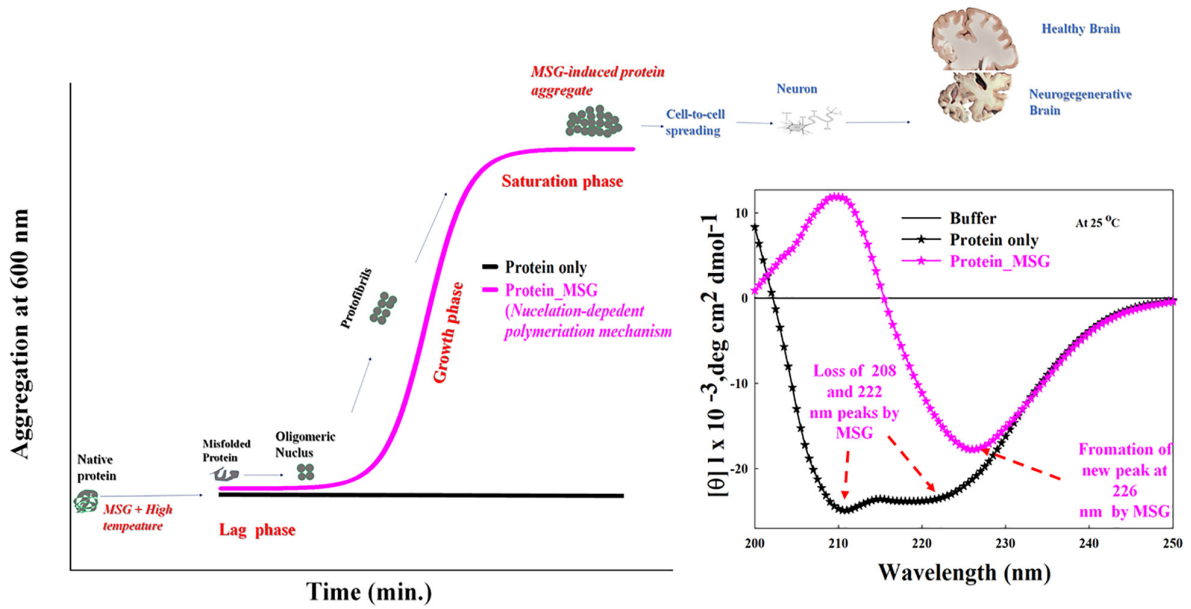

GRAPHICAL ABSTRACT 1 | Kinetic measurements indicating that monosodium glutamate causes significant enhancement of aggregation of protein through nucleation-dependent polymerization mechanism. Even low concentration of MSG is involved in the unfolding of secondary structure of protein with the disappearance of original peaks (208 and 222 nm) and formation of unique peak (226 nm) in the far-UV CD.

INTRODUCTION

Monosodium glutamate (MSG), chemical formula $C_5H_8NO_4Na$, is the sodium salt of glutamic acid and is naturally found in cheese, tomatoes, mushroom, and grapes (Jinap and Hajeb, 2010; **Figure 1A**). MSG is also known as Ajinomoto or Chinese salt and sodium 2-aminopentanedioate (IUPAC name). Structurally, it is composed of 12% sodium cations (Na^+), 78% glutamate anions ($C_5H_8NO_4^-$), and 10% water. Sodium cations and glutamate anions in MSG are held by an ionic bond. It acts as a powerful flavor enhancer due to its unique fifth taste called umami or savory or meaty taste, so MSG is one of the world's most extensively used food additives and is generally recognized as safe according to the Joint FAO/WHO Expert Committee on Food Additives (JECFA), the Food and Drug Administration (FDA), and the European Food Safety Association (EFSA) (Yadav, 2010). However, there are numerous reports regarding the harmful effects of food containing excessive amounts of MSG and how it causes headache, migraine, tingling or burning in the face, sweating, facial pressure or tightness, numbness, flushing in the neck and other areas, rapid fluttering heartbeats (heart palpitations), chest pain, nausea, and weakness, according to the International Classification of Headache Disorders (ICHD), third edition. Such physiological responses are referred to as MSG symptom complex or Chinese restaurant syndrome (Yang et al., 1997).

Structurally, the core component of MSG is glutamate, which is a negatively charged, polar, and nonessential amino acid (i.e., can be synthesized by the human body and so is not essential to human diet). It has been suggested that glutamate bound to other amino acids in proteins does not show any taste; however, glutamate can show taste when it is unbound or in free form (Löliger, 2000). In the free or unbound form, glutamate stimulates the glutamate receptors (TAS1R1, TAS1R, mGluR4, and mGluR1), which are located in the taste buds and are responsible for inducing the flavor profile called umami (Conn and Pin, 1997). There are various studies which suggested that thymocytes, lymphocytes, and thymic stromal cells also possess the receptors for glutamate (Pavlovic et al., 2006). Further, it is reported that MSG comprises two isomers, i.e., L-glutamate and D-glutamate enantiomers, but only L-glutamate enantiomer is responsible for enhancing the flavor (Okoye et al., 2016). Processed MSG comprises 99.6% L-glutamate form which is a greater percentage of L-glutamate than that observed in the free glutamate ions of naturally occurring foods (Halpern, 2000). When ingestion of MSG occurs orally in humans, it splits into glutamate and sodium ions in the digestive tract. In the gut and intestine, glutamate is absorbed by active transport. The absorbed glutamate is carried from the intestine to the lumen across the apical membrane with the help of transporters like EAAC-1 (excitatory amino acid transporter) and NaDC-1 (sodium carboxylate transporter) (Kanai and Hediger, 2003). These transporters transport glutamate into the blood stream and circulate throughout the body. In the intestinal enterocyte, catabolism of glutamate initiates in the cytosol and mitochondria through the phenomenon of transamination in the presence of aspartate aminotransferase, alanine aminotransferase, branched-chain aminotransferase, and glutamate dehydrogenase (GDH) enzymes, resulting in the formation of end product α-ketoglutarate. α-Ketoglutarate enters into a TCA cycle with the release of carbon dioxide (Windmueller and Spaeth, 1976). Moreover, it has been reported that consumption of MSG

results in the formation of nine urinary metabolites, which include glutamate, alpha-ketoglutrate, malonate, citrate, 5-aminovalerate, 5-hydroxymethyl-4-methyluracil, dimethylamine methylamine, and beta-hydroxyisovalerate. Thus, the elimination of MSG in the form of these urinary metabolites occurs through the kidneys (Nahok, 2019).

Glutamate is also an excitatory neurotransmitter present in the central nervous system (brain and spinal cord) and by means of the (vGlut) vesicular glutamate transporter family; glutamate is transported into the synaptic vesicle. It is well reported that the excitotoxic behavior of glutamate results in neurodegenerative diseases (Terlisner, 2009). Likewise, MSG has been observed in connection with neurotoxicity, which refers to damage of the central or peripheral nervous system. The damage of neurons by the overstimulation or overactivation of glutamate receptors by excitatory neurotransmitter glutamate may lead to the death of neurons by an act of excitotoxins; the process is called excitotoxicity (Choi, 1988). During excitotoxicity, there is excessive discharge of glutamate from glutamatergic nerve terminals, which leads to the overactivation of glutamate receptors (NMDA receptor and AMPA receptor), resulting in an increase in the level of intracellular calcium ions (Ca^{2+}). This increase in the level of calcium ions causes the activation of enzymes like endonucleases, phospholipases, and proteases (such as calpain), which are responsible for the damage of cytoskeleton, membrane, DNA, and especially the structure of neurons. Even the elevation of Ca^{2+} is involved in mitochondrial stress, causing extreme oxidative phosphorylation and production of reactive oxygen species (ROS) by triggering nitric oxide synthase, eventually resulting in cell death (Mehta, 2013). It has been observed that a rise in glutamate beyond the optimum concentration of about 1 mM in the synaptic cleft leads to a degeneration of neurons (Clements et al., 1992).

There are numerous reports which suggests that excessive use of MSG in the food is linked with FM (fibromyalgia) (Smith et al., 2001), hyperaggregation of platelets in rats (H.M.Youssef et al., 2010), atrial fibrilization (Shi et al., 2011), hypatoxicity (Egbuone, 2009), nephrotoxicity of endocrine disruptors, hyperglycemia consequently with diabetes mellitus (Ogbuagu, 2019), and overweight (Zanfirescu, 2019). Furthermore, in rats it is revealed that the platelet count, bleeding, and coagulation time increase by the dietary ingestion of MSG (Ajibola, 2012). There are numerous recent reports which highlights that the orally taken MSG increases the free radical formation, escorts DNA damage and triggers the apoptotic signaling cascade, which subsequently onset repair mechanism in stem cells and leads to neural cell necrosis (Mathew, 2019). Thus, it is a promoter of neurodegenerative diseases such Alzheimer's diseases, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, and multiple sclerosis (Fuchsberger, 2019).

Generally, it is a well-known fact that neurological diseases are related with the phenomenon of misfolding and protein aggregation, and this phenomenon may be defined as the cellular event by which proteins are unable to fold into functional form resulting in misfolded proteins, which in turn polymerizes into aggregates, thereby amalgamating together intracellularly as well as extracellularly, forming a structurally and functionally abnormal assembly and triggering a wide variety of pathological and neurological diseases (Chiti and Dobson, 2006; Ahanger et al., 2021). Protein aggregate is the protein in the nonnative state with at least twice size of native protein, i.e., 100 to 1000 nm. It shows fewer properties like reduced activity or no biological activity (Dobson, 2003; Chiti and Dobson, 2006). Protein misfolding and aggregation are currently the primary source of apprehension in biology and medicine due to their association with various debilitating human neurodegenerative proteinopathies, including Alzheimer's, Parkinson's disease, amyotrophic lateral sclerosis, and Huntington's disease. The mutations, errors during protein synthesis, aging, environmental stress, and formation of ROS (reactive oxygen species) (Tyedmers et al., 2010) are some possible causes that can result in the formation of protein misfolding and aggregation. Furthermore, pH (Katayama et al., 2006), temperature, protein concentration (Ghosh et al., 2018), additives (Hamada et al., 2009), and viscosity (Barnett et al., 2015) are some factors which can influence the protein aggregation. Despite having numerous aforementioned reports regarding the association of MSG with various neurological disorders, still there are no reports available about the role of MSG in protein misfolding and aggregation, or the role of MSG has not been explored yet in protein misfolding and aggregation. Thus, there is a need for vigorous investigations regarding the role of MSG in protein misfolding and aggregation.

Investigations of various aspects such as human complications and proteinopathies including neurodegenerative diseases can be executed and studied by promoting and inhibiting protein aggregation in *in vitro* experiments (Bhattacharyya and Das, 1999). Bovine serum albumin (BSA) has been often used as the best model protein for studying *in vitro* protein misfolding and aggregation by virtue of its strong ligand-binding capacity; being a carrier for vitamins, fatty acids, hormones, iron, drugs, and trace minerals; having a structural similarity with human serum albumin; and osmotic pressure and pH of blood being also controlled through serum albumin in circulation (Carter and Ho, 1994). Therefore, the fundamental purpose of this study was to induce the aggregation in bovine serum albumin using high temperature in the presence of MSG and to unfold the effective role of MSG in delaying or increasing protein aggregation. This study will provide some explanation for various MSG-induced neurodegenerative and physiological complications. Different spectroscopic techniques such as absorption spectroscopy (Moosavi-Movahedi et al., 1996), fluorescence spectroscopy (De et al., 2005), circular dichroism (Gelamo and Tabak, 2000), dynamic light scattering (Kelley and McClements, 2003), and isothermal titration calorimetry (Parray et al., 2019) were used for this purpose.

MATERIALS AND METHODS

Materials

Bovine serum albumin (BSA, UniProtKB-A0A140T897_BOVIN: A0A140T897) used was lyophilized, which was obtained from Sigma-Aldrich. Dietary MSG (MSG, CAS number: 142-47-2) and cetrimonium bromide (CTAB) were obtained

from Sigma-Aldrich. Merck (India) provided chemicals like monobasic, i.e., (NaH_2PO_4) sodium dihydrogen phosphate, and dibasic, i.e., disodium hydrogen phosphate (Na_2HPO_4) and sodium dihydrogen phosphate (NaH_2PO_4), which were used to prepare the solution of phosphate buffer. The phosphate buffer system in which all experiments were done after calculating its strength was kept 25 millimolars with 7.0 pH, and a suitable amount of both monobasic and dibasic was added in Milli-Q water which was obtained from the Millipore system. All the chemicals received were directly used with no more extra purification. The stock of BSA protein solution was formulated with the concentration of 15 milligrams per milliliter. By using the Varian Cary 100 Bio double-beam spectrophotometer, the absorbance of protein aliquot from the stock at the wavelength of 278 nm and 44,000 molar extinction coefficient as well as the concentration of protein stock was measured. A stock of 5 molars of MSG and 25 millimolars of surfactant CTAB was also formulated in the phosphate buffer at pH 7.0. The pH's of all the above solutions were measured with the help of Toshcon Digital pH Meter CL-54+.

Methods

Kinetic Measurements

The kinetic aggregation of BSA protein solution was carried out by monitoring its turbidity in the presence of MSG through time course measurement using the Jasco V-660 UV-vis spectrophotometer (JASCO Corporation 2967-5, Ishikawa-machi, Hachioji-shi, Tokyo, Japan) at the wavelength of 600 nm. The spectrophotometer was coupled with setup of a Peltier temperature regulator (ETCS61) to control the temperature. The study of aggregation kinetics of BSA was carried out in the presence of varying concentrations of MSG ranging from 0.1 M to 4.5 M and also at different temperatures ranging from 60, 70, 75,78, 81, 82, 84 to 85°C. To avert and tackle the hindrance created by absorbance of chromophoric groups when the light interacts with protein, the turbidity measurements of protein solution are generally executed above the higher wavelength 400 nm. It has been suggested that aggregates of proteins are characterized with high optical density and high turbidity. The curve fitting was done to data acquired after the experiments of kinetic measurements by applying a four-parameter logistic curve in the above mathematical statement (Sharma et al., 2010a,b; Ahanger et al., 2021):

$$y = y_0 + \frac{a}{1 + \exp(-(t - t^1/2)/b)} \quad (1)$$

where y is the absorbance at any time t, y_o is the initial absorbance value, a is the maximum absorbance, $t_{1/2}$ is the time at which absorbance is half of its maximum, b is $1/k_{app}$ (reciprocal apparent rate constant), apparent rate constant k_{app} is $1/b$, and lag time, $t_{lag} = t_{1/2} - 2b$.

Kinetic measurements of BSA in the presence of fixed concentration of monosodium glutamate at various temperatures

The effect of temperature on kinetic measurements of BSA in the presence of fixed concentrations of MSG was also checked. The rate constants, i.e., the rate constant coupled with nucleation phase (1/tlag) and the apparent rate constant coupled with growth phase (k_{app}) calculated from this experiment, were applied to evaluate the activation energy of aggregation through using equations (Sharma et al., 2010a,b) (2) and (Sharma et al., 2010a,b) (3):

$$\left(\frac{(\Delta ln\,(1/tlag)}{\Delta T}\right) = \frac{-Ea, nuc}{RT^2} \quad (2)$$

$$\left(\frac{(\Delta ln\,(k)}{\Delta T}\right) = \frac{-Ea, elong}{RT^2} \quad (3)$$

Structural Measurements

Dynamic light scattering measurements

To measure the size distribution profile particle and the values of hydrodynamic radius (R_h) in the solution (in which agglomerates of BSA in the presence of MSG -induced BSA aggregates were removed by centrifugation), dynamic light scattering (DLS) was performed using RiNA Laser Spectroscatter, Model-201 (RiNA GmbH Berlin, Germany), at 25 ± 0.1°C. The protein solutions were filtered through Millipore syringe filters with the size of 0.22 μm. Polystyrene was applied to make the disposable cell with the scattering angle of 90°. The measuring light scattering intensity of fluctuations is the basic principle of analysis of particle size distribution profile in the protein solution. Therefore, the size distribution profile of BSA-MSG was acquired through determining the time-dependent fluctuations of scattered light which are caused by particle proteins moving in the solution under the influence of Brownian motion. From these fluctuations, diffusion coefficients of BSA-MSG can be determined mathematical formula (Parray et al., 2019) (4).

$$D = \frac{\mathrm{kT}}{6\pi\eta\mathrm{Rh}} \quad (4)$$

where D is the diffusion, k is Boltzmann's constant, T is the temperature, η is the shear viscosity of solvent, and R_h is the hydrodynamic radius of the particle. The data was analyzed by using the PMgr version 3.01 software.

Circular dichroism spectroscopy measurements

Far UV-CD analysis of a protein solution with a concentration of 5 micromolars, in a cuvette with path length 0.1 cm recorded with J-1500 Circular Dichroism Jasco Spectropolarimeter (JASCO International Co. Ltd, Tokyo, Japan). The machine is connected with a mini-Jasco circulatory water bath (MCB-100) and a very effective nitrogen purge system. The CD experiment was performed at room temperature with wavelength 200 nm. To calibrate the machine regularly, D-10 camphorsulfonic acid (CSA) was used. The data obtained from the CD are averages of three scans for a sample, and this acquired data in t units of millidegree was converted into mean residual ellipticity.

Isothermal titration calorimetry studies

Isothermal titration calorimetry (ITC) is a biophysical method applied to evaluate the binding interaction and thermodynamic parameters of protein with ligand in the solution by calculating the heat that is either liberated or consumed. A VP ITC

Calorimeter (MicroCal, 22 Industrial Drive East, Northampton, MA 01060, United States) instrument was used for ITC measurement, at 25°C in 25 mM phosphate buffer (pH 7.0), in which the calorimeter cell was injected with a fixed concentration of 30 μM protein solution. During the ITC measurement, the ligands with the concentration of 600 μM MSG were titrated against the cell having 30 μM BSA. Each ligand solution was loaded with 10-microliter aliquots in each 260-second step through the syringe, and each ligand was loaded into control, i.e., phosphate buffer. The normalization of data was done with the results of titration of respective ligands, and MicroCal Origin ITC software was used for fitting the data to produce the profile of heat change. From the measured heat changes, the stoichiometry (N), binding enthalpy (ΔH), and association constant (K_a) were calculated on binding of MSG with BSA. Gibbs-free energy changes (ΔG) were also elevated from the calculated heat changes by means of the following equation (Parray et al., 2019; Ahanger et al., 2021):

$$\Delta G = -RT\, lnKa = \Delta H - T\Delta S \quad (5)$$

where R is the gas constant and T is the absolute temperature.

RESULTS

Kinetic Measurements of BSA in the Presence of Increasing Concentrations of Monosodium Glutamate at 60°C Temperature

By means of time course measurement using the UV-visible spectrophotometer, the aggregation kinetics of bovine serum albumin in the presence of dietary MSG was done by assessing the turbidity of the protein solution. The aggregation kinetics was performed on a protein solution with a fixed volume of 5 μM by titrating with different concentrations of MSG starting from 0.1 molar to 4.5 molar. The experiment was done at a wavelength of 600 nm and at 60°C in 25 millimolar phosphate buffer at pH 7.0. It is clear from **Figure 1B** that dietary MSG increases the BSA aggregation denoted as a function of time with the enhancement in absorbance at the wavelength of 600 nm. There was no aggregation observed in 5 μM BSA (in buffer as control), but as the concentration of MSG was varied from 0.1 to 4.5 M, the tendency of aggregation also increased with the increase in absorbance from 0.00289 to 0.2110. This MSG-induced BSA aggregation is represented by a sigmoidal curve, marked by the absence of a nucleation phase with insignificant absorbance, steeply followed by a growth phase which was stabilized by a saturation phase.

Kinetic Measurements of BSA in the Presence of Increasing Concentrations of Monosodium Glutamate at 70°C Temperature

All the parameters were the same in the aggregation kinetics as the 5-μM protein concentration was taken with varying concentrations of MSG starting from 0.1 molar to 4.3 molar. The experiment was done at wavelength of 600 nm at 70°C of temperature in 25 millimolar phosphate buffer with pH 7.0. It is clear from **Figure 2** that dietary MSG increases the BSA aggregation denoted as a function of time with the enhancement in absorbance at the wavelength of 600 nm. There was no aggregation observed in 5 μM alone (taken as control), but as the concentration of MSG was increased from 0.1 to 4.3 M, there was a significant increase in the absorbance observed from 0.04 to 0.88 nm. Furthermore, an increase in the 10-degree temperature form, from 60 to 70°C, MSG was involved in the induction of a well-established lag phase in the process of aggregation which was not observed at 60°C temperature. This MSG-induced BSA

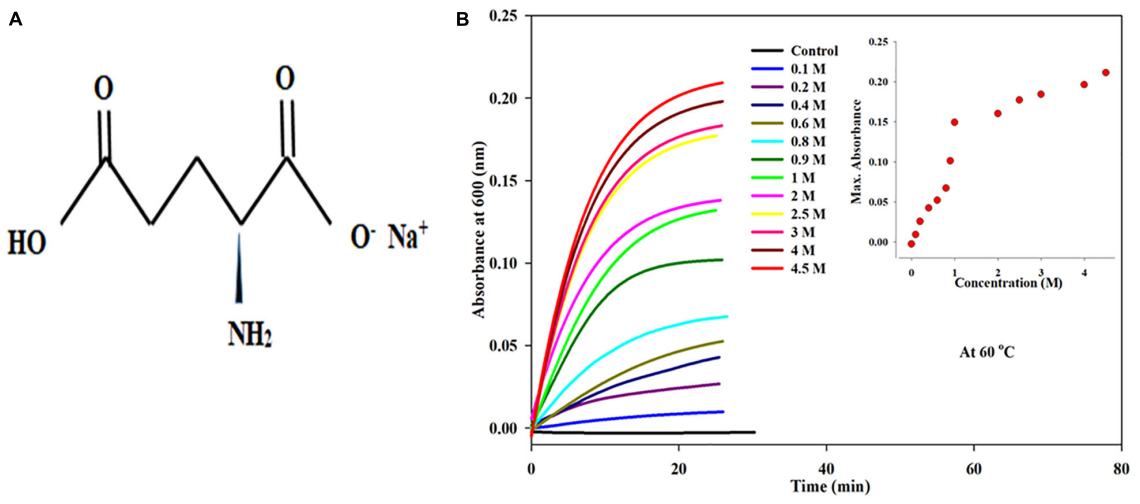

FIGURE 1 | (A) Chemical structure of monosodium glutamate (MS). **(B)** Kinetic measurements of BSA in presence increasing concentrations of monosodium glutamate (0.1 to 4.5 Molar) at 60°C temperature, 25 mM phosphate buffer, pH. 7.0 and 600 nm. Inset of **Figure 1** inset shows the influence of concentration on (A_{max}) maximum absorbance of MSG-induced BSA aggregation at 60°C temperature.

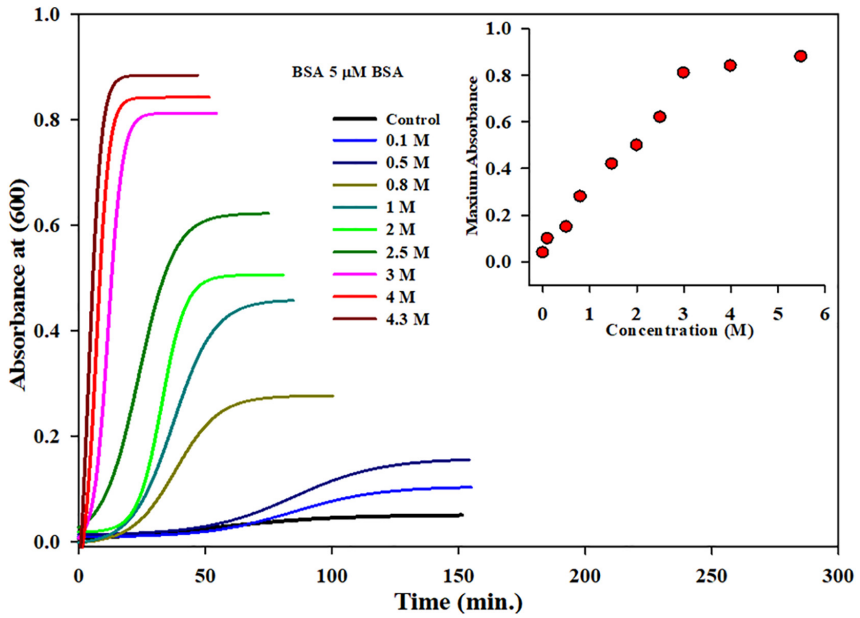

FIGURE 2 | Kinetic measurements of BSA in presence increasing concentrations of monosodium glutamate (0.1 to 4.5 Molar) at 70°C temperature, 25 mM phosphate buffer, pH. 7.0 and 600 nm. Inset of **Figure 2** inset shows the influence of concentration on (A_{max}) maximum absorbance of MSG-induced BSA aggregation at 70°C temperature.

aggregation is represented by a sigmoidal curve, marked by the well-established lag or nucleation phase, steeply followed by the growth phase which was stabilized by the saturation phase.

The insets of **Figures 1B**, **2** represent the plot of maximum absorbance versus concentration (M) which suggests that MSG-induced aggregation of BSA is nonlinearly dependent on the concentration. Through applying the log–lin plot of log absorbance versus time and the log–log plot of log absorbance versus log time, the mechanism of MSG-induced protein aggregation was described as depicted in **Figures 3A,B**.

Kinetic Measurements of BSA in the Presence of Monosodium Glutamate at Various Temperatures

In this experiment, the aggregation kinetics of 5 µM BSA were carried out in the presence of a fixed concentration (1 molar) of MSG at various temperatures (60°C, 63°C, 65°C, 70°C, 75°C, 78°C, 80°C, 81°C, 82°C, 84°C, and 85°C) depicted in **Figure 4A**. It is certain from this figure that, as the temperature increases from 60°C to 85°C, the MSG-induced aggregation of BSA is significantly enhanced, with the increase in the absorbance from 0.120 to 0.4 94. The turbidity of the solution was noticed to be lesser at 60°C; however, there is significant rise in the turbidity of the solution after 60°C temperature.

The plots between $\ln t_{lag}$ and $\ln(k)$ versus the reciprocal of temperature for MSG-induced aggregation of BSA are shown in **Figures 4Bi,ii**. The **Figure 4Bi** plot illustrates the nonlinear relationship between the reciprocal of temperature and $\ln t_{lag}$, and with the drop in temperature, t_{lag} gets prolonged. Further, the activation energies (Ea) of kinetics aggregation of BSA in the presence of MSG were calculated from the plots of $\ln t_{lag}$ vs. 1000/T and $\ln(k)$ vs. 1000/T which largely show a nonlinear behavior. The nonlinear fit curve made up of two linear segments and the complete curve was fitted with two linear equations, which in turn gives two activation energies, i.e., 60 to 80°C which is 0.60 kcal/mol and 80 to 85°C which is 107.16 kcal/mol. Also, the activation energy from 60°C to 80°C is 9.90 kcal/mol and that from 80°C to 85°C is 4.44 kcal/mol, calculated from the linear fit of the plot of $\ln(k)$ vs. 1000/T. The activation energy calculated from the plot of ln tlag vs. 1000/T is accompanied with the nucleation phase of aggregation whereas the activation energy calculated from the plot of $\ln(k)$ vs. 1000/T is accompanied with the growth phase of aggregation. Thus, the temperature is an important factor on which MSG-induced BSA aggregation is dependent.

Influence of Monosodium Glutamate on the Kinetics of CTAB-Induced Aggregation of BSA

The aggregation kinetics was carried out in the presence of MSG and surfactant cetyltrimethylammonium bromide (CTAB) at 70°C shown in **Figure 5**. In this experiment, the ratio of BSA and CTAB was kept fixed at 1:30, i.e., the concentration of the protein solution was maintained at 40 micromolars of BSA and the concentration of CTAB was maintained at

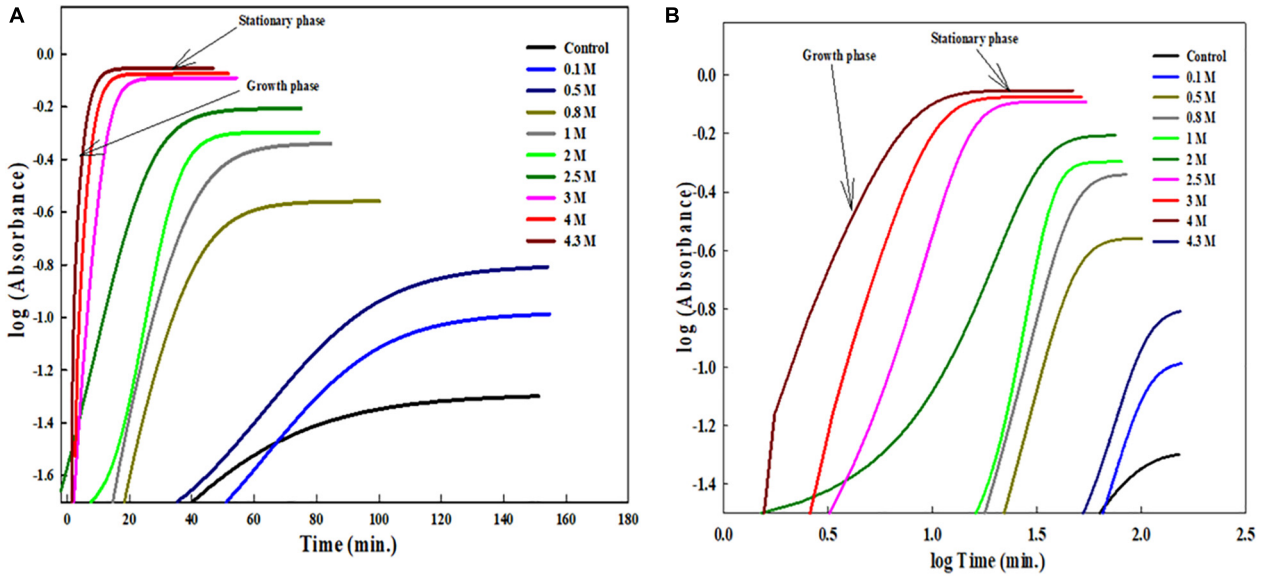

FIGURE 3 | (A) Shows the log-lin plot of absorbance versus time and **(B)** shows the log-log plot of absorbance versus time for BSA aggregation at 70°C temperatures in the presence of increasing concentration of MSG.

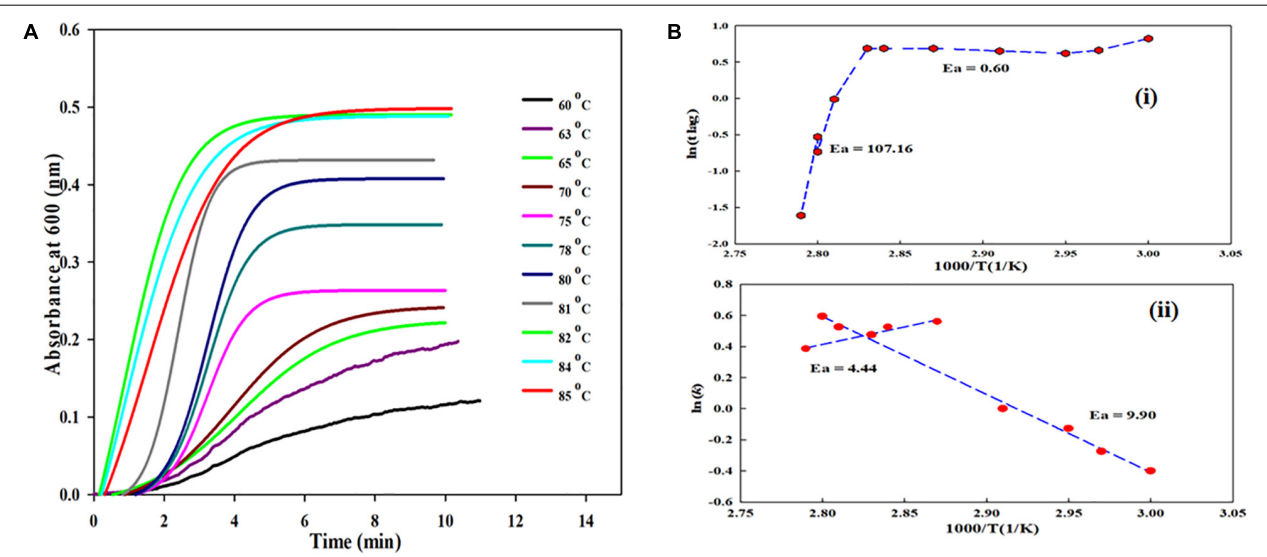

FIGURE 4 | (A) Kinetic measurements of BSA in presence fixed concentrations of monosodium glutamate at various temperatures (60°C, 63°C, 65°C, 70°C, 75°C, 78°C, 80°C, 81°C, 82°C, 84°C, and 85°C). **(B) (i)** shows plot between $\ln t_{lag}$ versus the reciprocal of temperature for MSG-induced aggregation of BSA and **(B) (ii)** shows plot between ln*(k)* versus the reciprocal of temperature for MSG-induced aggregation.

1200 micromolars and only the concentration of MSG was varied from 2.5 to 4.5 M. **Figure 5** indicates that BSA at 40 μM does not show the aggregation with the absorbance of 0.00076. Once 1200 μM CTAB was added to the reaction mixture, there was a marked increase in the aggregation with the absorbance of 3.15.

After that, this CTAB-induced aggregation was carried out in the presence of increasing concentration of MSG from 2.5 to 4.5 molars. From **Figure 5**, it is clear that with the increase in concentration of MSG from 2.5 to 4.5, there is further increase in the aggregation of BSA in the presence of CTAB. CTAB-induced BSA aggregation in the presence of increasing concentration of MSG from 2.5 to 4.5 molars with the absorbance of 3.08 was found very close to CTAB-induced BSA aggregation (i.e., absence of MSG) with the absorbance of 3.1. This indicates that MSG is not involved in the inhibition of aggregation of BSA even in the presence of CTAB. **Supplementary Figure 1** shows that both MSG and

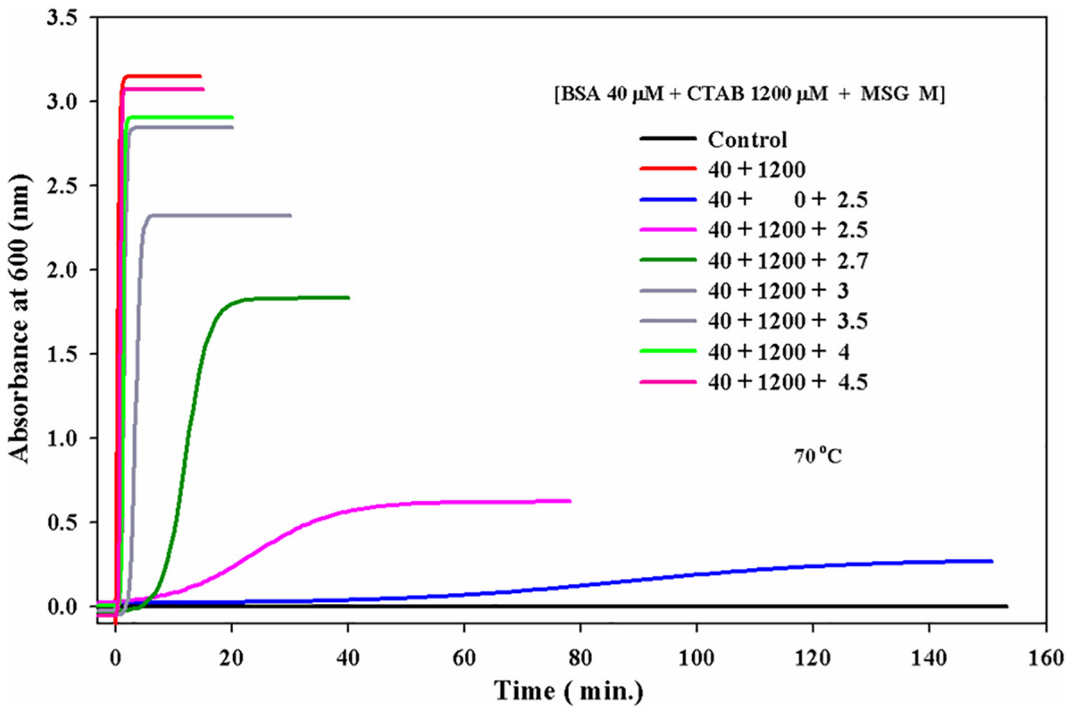

FIGURE 5 | Effect of monosodium glutamate on the kinetics of CTAB-induced aggregation of BSA at 70°C temperature 25 mM phosphate buffer, pH 7.0 and wavelength of 600 nm.

CTAB act as the homologous seeds in the nucleation phase of BSA aggregation.

Influence of Monosodium Glutamate on Reversibility of BSA

In this experiment, a UV-visible spectrum (in the range of 230 to 340 nm wavelength) of 5 µM of BSA was taken at the temperature of 25°C in the absence of MSG. At 280 nm, there was no scattering observed and the absorbance of this sample was 0.308. However, when the UV-Visible spectra were taken in the presence of MSG, at 280 nm, there was marked scattering observed and the absorbance of this sample was 0.727. BSA in the presence of MSG was subjected to thermal aggregation at a temperature of 80°C and subsequently the UV-Visible spectra were taken to this aggregated sample. The aggregated sample was extremely turbid, and there was no peak observed at all. Moreover, enormous scattering is observed and the absorbance of this sample at 208 nm is 1.188.

After the thermal aggregation, two UV-visible spectra of this aggregated sample were taken, once on cooling after half an hour at 25°C and another after overnight incubation at 25°C. In both the cases, all the peaks were removed and the spectra of thermally aggregated BSA in the presence of MSG were completely flattened. From this experiment, as shown in **Figure 6A**, it is clear that the BSA structure is perturbed greatly in the presence of MSG and at high temperature there is the formation of aggregates of BSA induced by MSG and these aggregates are completely irreversible in nature.

Effect of Monosodium Glutamate on the Measurement of the Hydrodynamic Size of BSA

Determining the size of a BSA protein in the absence or in the presence of MSG at room temperature, DLS measurements were performed. **Figure 6B** shows the results of DLS measurement, in which there is marked increase in the hydrodynamic diameter of BSA in the presence of MSG from 8.3 to 68.33 nm. From the results, it is clear that addition of MSG causes the aggregation of BSA with the increase in size.

Effect of Monosodium Glutamate on the Secondary Structure of Bovine Serum Albumin by Performing Far-UV CD Measurements

To know the effect of MSG on the secondary structure of protein BSA, far UV-CD was carried out, as depicted in **Figure 7A**. This experiment involves the titration of 5 µM BSA with the increasing concentration of MSG from 0.01 M to 0.1 M at the temperature of 25°C. **Figure 7B** shows the plot of mean residual ellipticity of BSA at 208 nm versus the concentration of MSG. Here, the experiment is performed at very low concentrations of MSG, since at 0.1 M MSG was involved in maximum denaturation of the secondary structure and the HT voltage value of the machine Jasco Spectropolarimeter crossed beyond 800. Beyond this HT voltage

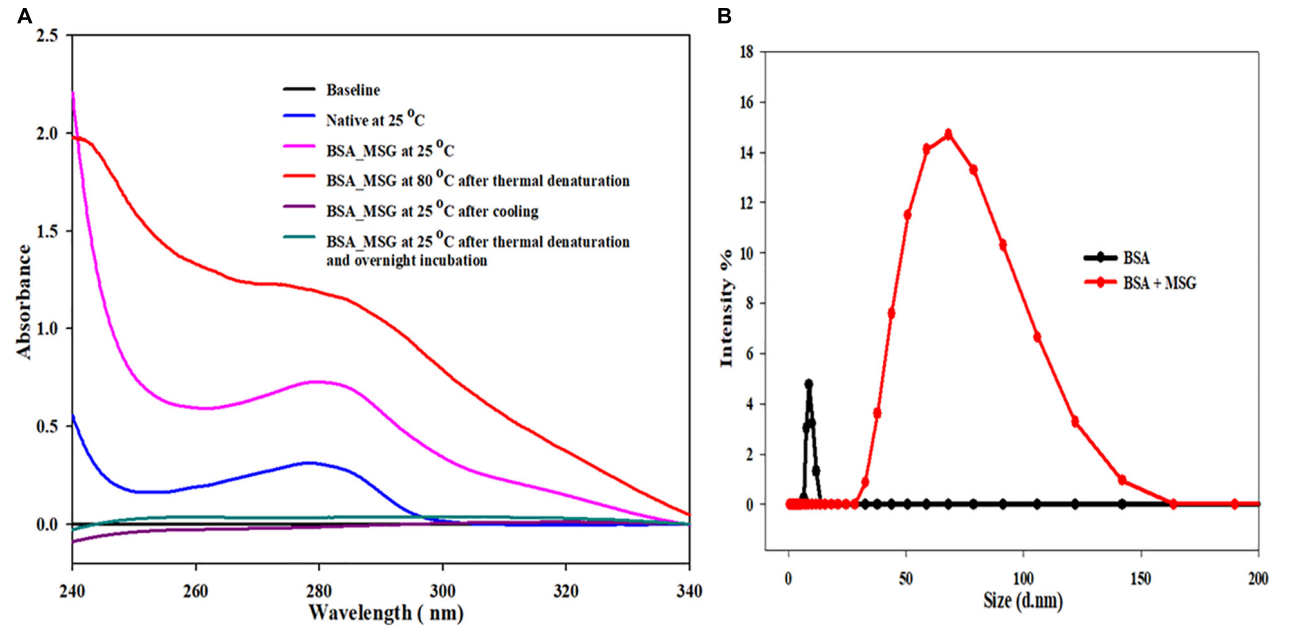

FIGURE 6 | (A) Determination of reversibility of BSA in the presence of monosodium glutamate. **(B)** Influence of MSG on the measurement of the hydrodynamic size of bovine serum albumin.

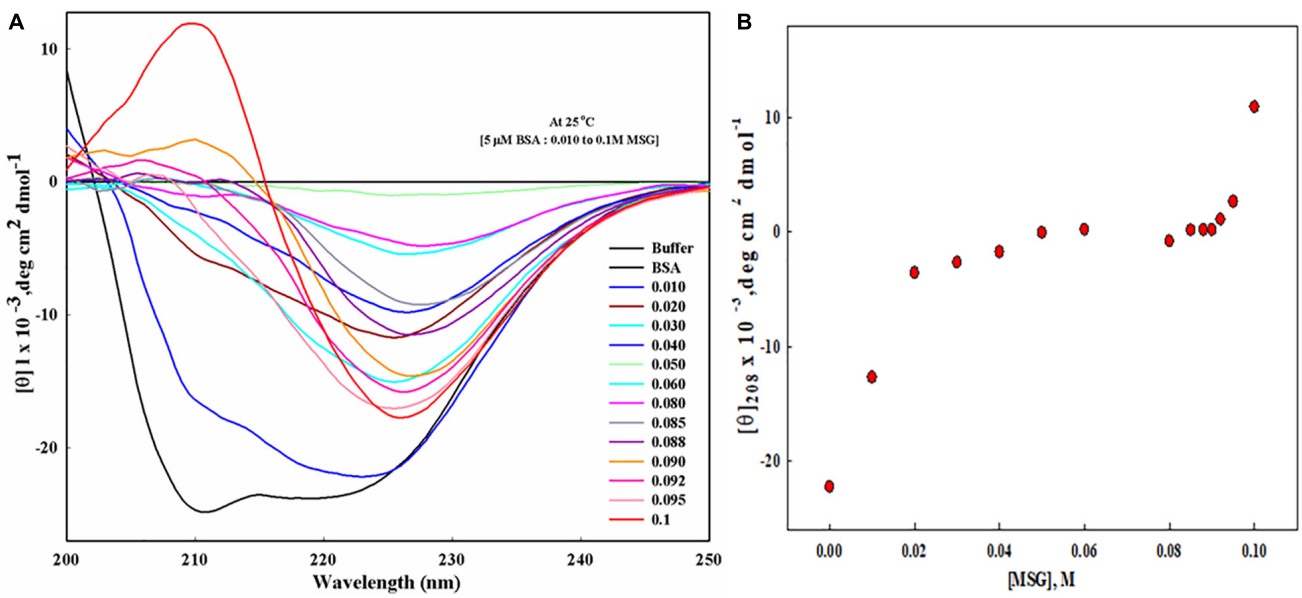

FIGURE 7 | (A) Far UV-CD spectra of BSA in presence of increasing concentration (from 0.01 M to 0.1 Molar) MSG at 25°C, 25 mM phosphate buffer, and pH. 7.0. **(B)** The plot of mean residual ellipticity of BSA at 208 nm versus the concentration of MSG.

value, the CD signal becomes disproportionate, data cannot be valued, and it can damage the machine. Thus, to prevent this issue, an experiment was performed at low concentrations of MSG. From **Figure 7A**, it is clear that in the presence of MSG there is a decrease in the secondary structure of BSA. Even at the low concentration of MSG, there is complete perturbation and denaturation of the secondary structure of BSA. The peak present at 210 nm is completely vanished even at a very low concentration, 0.001 M of MSG. Upon addition of further concentrations which includes 0.02 to 0.1 M of MSG, there is formation of a new peak at 226 nm. This is a very interesting observation as far as the effect of MSG on the secondary structure analysis by Far-UV CD measurement is considered.

Isothermal Titration Calorimetry Measurements

Figure 8 shows ITC measurement in which ligand MSG was titrated against the cell containing BSA. The concentration of BSA was 30 micromolars, and the ligand MSG was 900 micromolars. For determination of thermodynamic parameters, soft interaction, binding affinity, the determination of energetics of interaction of promoters and suppressors with the protein the pathway, and protein aggregation by quantifying the heat that is either liberated or consumed between MSG and BSA, ITC measurement was carried out.

The topmost side panel shows the raw data in power versus time (heat per unit of time liberated from every injection of the ligand with respect to the protein). This portion displays the data in 25 injections. The bottom side panel shows the raw data in the power standardization to the quantity of injections (kcal mol^{-1}) against its molar ratios with the addition of consecutive injections of ligand into the cell reaction comprising the protein solution. The amount of heat released as a function of the mole ratio of ligand to protein is shown in the bottom side panel. Further, in the bottom side panel, dots denote the experimental data and the line correlates the best-fitting model holding the number of identical and independent binding sites. The Origin software installed in the VP-ITC Calorimeter was used to analyze routine data and their fitting in the form of isotherm displaying the summaries about heat changes in the interaction. **Table 1** shows the various parameters, which include the association constant (K_a), binding enthalpy (ΔH), and equilibrium constant (K_d) accompanied with the ITC thermogram of BSA-MSG. Applying equation (2), (ΔG) free energy change was determined. The equilibrium dissociation constant was also evaluated (i.e., $K_d = 1/K_a$) from the value of binding affinity as given in **Table 1**.

DISCUSSION

Statistically, it has been estimated that the annual global demand for dietary MSG is near about 4 million metric tons (Markit, 2018). Asian countries are responsible for 88 percent of consumption, and consumption of China alone is 55 percent of the world's consumption, as shown in **Supplementary Figure 2A** as the pie chart (Handbook, 2015). China is globally the largest exporter, giving 44 percent of MSG to the world. Major countries which serve as export destinations for the Chinese MSG are as shown in **Supplementary Figure 2B** as the pie chart (Plateform, 2014). In recent years, consumption of dietary MSG has been observed to be increasing throughout the globe as the most extensively used flavoring agent in order to enhance and improve the taste in foods. It is predicted to witness a significant growth of USD $\sim$ 6,200 million of Global MSG in 2022 (Future, 2020). Therefore, despite such a huge global consumption of MSG, still MSG is undoubtedly the controversial focal point as MSG consumption in both human and animal studies has been linked with various neurological and physiological complications (Fuchsberger, 2019). The degeneration of the nervous system by the progressive loss of structure or function especially in the neurons of the brain is called neurodegeneration; a heterogeneous group of diseases occurs due to the process of neurodegeneration, including Alzheimer's diseases, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, multiple sclerosis, and Prion diseases, which are considered neurodegenerative diseases (Olney, 1960; Beas-Zarate et al., 1989; Samuels, 1999; Fraser, 2017; Shannon et al., 2017; Butnariu and Sarac, 2019; Fuchsberger, 2019). **Supplementary Figure 3** illustrates the possible impacts of excessive dietary MSG consumption on human health. The neurodegenerative diseases are associated with protein misfolding and aggregation. The association of MSG consumption with neurodegenerative diseases represents an important medical challenge to human beings. There are no studies about the role of MSG with protein folding, unfolding, misfolding, and aggregation. Thus, this is the first study that primarily involves the investigation of aggregation behaviors of bovine serum albumin in the presence of MSG.

The results from the kinetic measurements of BSA in the presence of increasing concentrations of MSG at 60°C temperature revealed that MSG was involved in the aggregation of BSA. The change in kinetic parameters such as A_{max}, b, y_o, $t^1/_2$, (t_{lag}), and Kapp min^{-1}, associated with aggregation of BSA in the presence of MSG at varying concentrations from 0.1 to 4.5 M at temperature 60°C, is shown in **Table 2**. From this table, it is clear that A_{max} from 0.00289 to 0.2110 and K_{app} from 0.21 to 0.48 increase whereas b from 4.70 to 2.12, $t^1/_2$ from 34.0 to 5.54, and t_{lag} 24.6 to 1.43 decrease. On the other hand, there was further increase in the aggregation of BSA by MSG at 70°C. The change in kinetic parameters such as A_{max}, b, y_o, $t^1/_2$, t_{lag}, and K_{app} min^{-1}, associated with aggregation of BSA in the presence of MSG at varying concentrations from 0.1 to 4.3 M at temperature 70°C, is shown in **Table 3**. From this table, it is clear that A_{max} from 0.04 to 0.88 and K_{app} from 0.04 to 0.43 increase whereas b from 24.7 to 2.21, $t^1/_2$ from 51.26 to 4.11, and t_{lag} from 1.78 to 0.59 decrease.

Furthermore, at temperature of 70°C, the aggregation kinetics of BSA in the presence of MSG was characterized by the presence of maximum turbidity. The pattern of MSG-induced aggregation of BSA occurs via a nucleation–polymerization mechanism and can be represented as the mathematical function having a characteristic of the sigmoid growth curve which entails a distinct lag or nucleation phase, tracked by a growth phase and subsequently by a saturation phase. In the nucleation phase, first the monomers of protein go through some modifications or conformational rearrangement and there are accumulation monomers which interact to form the nucleus of protein aggregate referred to as seeds or start aggregate or critical nucleus. The nucleation step is coupled with maximum activation energy or a transition state of maximum free energy in the pathway of aggregation and hence is thermodynamically unfavorable. There can be also the involvement of different protein intermediates such as partially denatured or mutated protein monomers and varied sizes of unstable oligomers. This step is reversible in nature and is the slowest or a rate-limiting step, which decides the rate of the whole aggregation kinetics. The start aggregates, which are small in size, can interact with the different cellular organelles and can turn toxic in nature. This interaction obstructs the functioning of proteasome

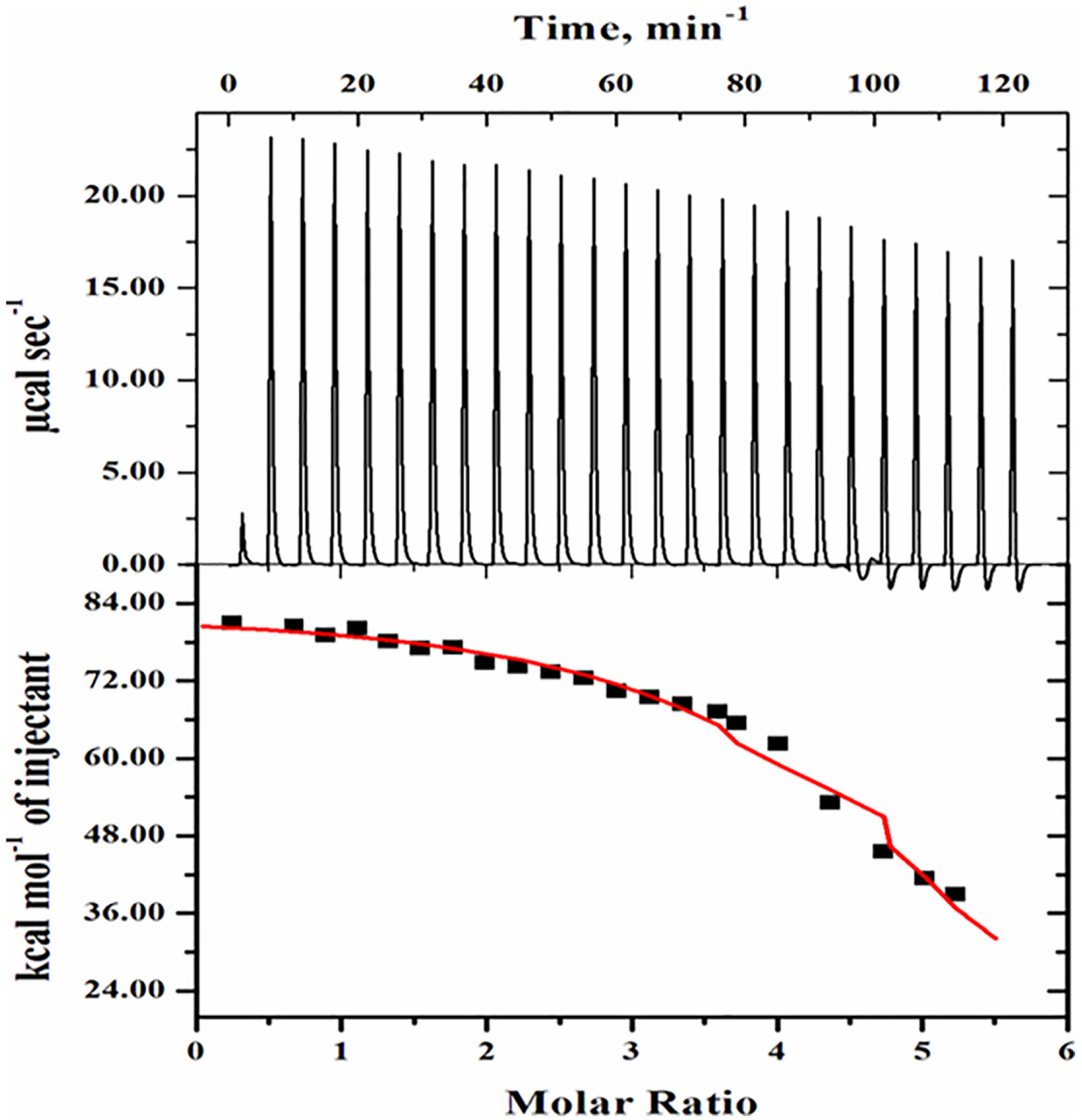

FIGURE 8 | Isothermal calorimetric titration of BSA in presence of monosodium glutamate. The top side panel shows the raw data in the power versus time (heat per unit of time released after each injection of the ligand into the protein). The bottom side panel shows the raw data in the power standardization to the amount of injections (kcal mol^{-1}) versus its molar ratios with the addition of consecutive injections of monosodium glutamate into the cell reaction containing BSA protein solution.

TABLE 1 | Binding parameters of BSA with monosodium glutamate evaluated from ITC measurements at pH 7.0 and 25°C.

Thermodynamic parameters (units)	N	K_a (M^{-1})	ΔH (cal mol^{-1})	ΔS (cal mol^1 deg^{-1})	ΔG (cal mol^{-1})
Step 1	5.18 (± 0.079)	1.82×10^5 ($\pm 3.31 \times 10^5$)	8.328×10^4 (± 1263)	303	−7014

and thus the aggregated protein cannot be degraded (Proctor et al., 2010). The occurrence of the lag phase comprehensively disturbs the speed of kinetics in protein aggregation and is frequently accompanied with increase in the concentration of nuclei during the course of the nucleation phase (Gelamo and Tabak, 2000; De et al., 2005; Librizzi and Rischel, 2005; Sharma et al., 2010b; Saha et al., 2016). At the temperature of 60°C, MSG-induced aggregation BSA is characterized by the absence of the lag phase; however, at the temperature of 70°C, MSG-induced aggregation BSA is outlined by means of the well-established lag phase. The lag time of the aggregation kinetics of 5 μM BSA was noticed to be 1.78, but in the presence of 0.1 molar MSG, the lag time of BSA aggregation was noticed to be increased abruptly about 54.00 per minute.

TABLE 2 | Change in kinetic parameters associated with aggregation of BSA in presence of monosodium glutamate at varying concentration from (0.1 to 4.5 M) at temperature 60°C.

S.No.	Co-solvents	a	y_o	b	t ½	t_{lag} (min)	K_{app} (min^{-1})
1	No Additives	0.00289	0.0029	4.70	34.0	24.6	0.21
2	0.1 M	0.0089	1.0152	4.30	9.66	1.06	0.23
3	0.2 M	0.0252	3.705	4.05	6.43	1.67	0.25
4	0.4 M	0.0420	0.111	2.97	9.98	4.04	0.33
5	0.6 M	0.0517	0.071	2.94	8.96	3.08	0.34
6	0.8 M	0.0669	0.1255	2.37	7.46	2.54	0.42
7	0.9 M	0.101	0.053	2.18	5.80	0.60	0.38
8	1.0 M	0.129	0.181	2.67	8.28	0.78	0.37
9	2.0 M	0.137	0.356	2.67	6.61	0.85	0.37
10	2.5 M	0.177	0.791	2.49	5.56	0.99	0.40
11	3.0 M	0.184	0.968	2.11	5.54	1.32	0.47
12	4.0 M	0.196	0.947	2.11	5.52	1.33	0.47
13	4.5 M	0.2110	1.1053	2.12	5.54	1.43	0.48

TABLE 3 | Change in kinetic parameters associated with aggregation of BSA in the presence of monosodium glutamate at varying concentrations from 0.1 to 4.5 M at temperature 70°C.

S. no.	Co-solvents	a	y_o	b	t ½	t_{lag} (min)	K_{app} (min^{-1})
1	No additives	0.04	0.0035	24.7	51.26	1.78	0.040
2	0.1 M	0.100	0.0086	16.92	86.01	54.00	0.06
3	0.5 M	0.15	0.0125	17.05	87.94	53.00	0.05
4	0.8 M	0.27	0.0169	19.57	87.57	48.32	0.05
5	1 M	0.45	−0.006	6.00	38.00	26.00	0.16
6	2 M	0.50	0.0176	5.67	32.87	23.39	0.17
7	2.5 M	0.62	0.0068	5.62	23.97	9.43	0.17
8	3 M	0.81	−0.0057	2.79	11.70	6.11	0.35
9	4 M	0.84	−0.0949	2.41	7.08	2.26	0.41
10	4.3 M	0.88	−0.2331	2.31	4.11	0.59	0.43

Further increasing the concentration of MSG from 0.1 to 4.3 molar subsequently reduces the lag time from 54.00 to 0.59, as shown in **Table 3**. The reduction of the lag phase may be due to the addition of new seeds in the nucleation step of BSA aggregation by MSG. There are reports that suggest that addition of new seeds reduces the lag phase. The rate of aggregation kinetics can be accelerated and reduced by the addition of performed fibrils through a process called seeding. It can be either through the addition of new seeds of the same protein (homologous seeding or self-seeding) or through the addition of new seeds of heterologous seeding or cross-seeding of different proteins. Thus, MSG may be involved in the induction of homologous seeds, which in turn reduces the lag phase of aggregation. In the growth phase, from the existing critical nucleus, aggregates further develop promptly in their size into the different types of aggregates like amyloid or amorphous aggregates, which are found to be associated with neurodegeneration. This step is irreversible in nature, and the concentration of nuclei remains constant (Dobson, 2006; Morris et al., 2009; Dasgupta and Kishore, 2017). Eventually, all the proteins are being incorporated into aggregates with no additional attachment of fibrils and the depletion of monomers marks the saturation phase.

To know whether MSG has any inhibitory effect or not on the aggregation kinetics, the increasing concentration of MSG was titrated against CTAB-induced BSA aggregation. The results revealed that CTAB-induced BSA aggregation in the presence of MSG was near the aggregation of CTAB-induced BSA. The change in kinetic parameters such as A_{max}, b, y_o, $t^1/_2$, t_{lag}, and K_{app} min^{-1}, associated with CTAB-induced BSA in the presence of MSG at varying concentrations from 0.1 to 4.5 M at temperature of 70°C is shown in **Table 4**. From this table, it is clear that A_{max} from 0.00076 to 3.08 and K_{app} from 0.21 to 9.34 increases significantly whereas b from 4.70 to 0.107, $t^1/_2$ from 34.43 to 0.84, and t_{lag} from 25.03 to 0.62 decrease. Thus, MSG was again involved in the induction of homologous seeds, which in turn reduces the lag phase of both BSA aggregation as shown in **Figure 2** and CTAB-induced BSA aggregation as shown in **Figure 5** and **Supplementary Figure 1B**. Furthermore, the influence of temperature on the kinetics of MSG-induced BSA aggregation was carried out in the presence of a fixed concentration, 1 molar, of MSG at 60°C to 85°C temperatures. The change in kinetic parameters such as A_{max}, b, y_o, $t^1/_2$, t_{lag}, and K_{app} min^{-1} was associated with BSA aggregation in the presence of a fixed concentration (1 molar) of MSG at variant temperatures starting from 60 to 85 as shown in **Table 5**. From this table, it is clear that A_{max} from 0.120 to 0.494 and K_{app} from 0.99 to 1.47 increase significantly whereas b from 1.01 to 0.68, $t^1/_2$ from 4.28 to 1.56 and (t_{lag}) 2.28 to 0.20 decrease. Here the lag phase was not prominent and even changing the temperature from 60°C to 85°C is not involved in inducing the seeding phenomenon in the nucleation phase of aggregation. However, there is a substantial increase in BSA-MSG aggregation, with the increase in absorbance. The plot between $\ln t_{lag}$ versus the reciprocal of temperature for MSG-induced aggregation of BSA at various temperatures depicts that this plot illustrates the nonlinear relationship with ln t_{lag} and there is a decrease in the lag time with the increase in temperature. All inset plots including A_{max} versus concentration at 60°C, A_{max} versus concentration at 70°C, and $\ln t_{ag}$ versus reciprocal of temperature show the significant nonlinear relationship in the turbidity measurements. Reports suggest that nonlinear relationships are associated with changes in the size of aggregates or changes in the morphology of aggregates in the turbidity measurements (Borgia et al., 2013). From 60 to 85°C temperatures, values of the rate constant acquired from time-dependent kinetic measurement were then used to evaluate the value of activation energy of the aggregation process as shown in **Table 6**. The results from the nonlinear fit of the plot of ln tlag vs. 1000/T associated with the nucleation phase of aggregation and the nonlinear fit of the plot of ln(k) vs. 1000/T was associated with nucleation growth of aggregation. Both plots depict a noticeable non-Arrhenius behavior when plotted over the full temperature range from 60 to 85°C. It has been reported that the protein aggregation which is promoted by temperature frequently shows the nonlinear behavior, and

TABLE 4 | Change in kinetic parameters associated with CTAB-induced BSA aggregation in the presence of monosodium glutamate at varying concentrations from 0.1 to 4.5 M at temperature 70°C.

S. no.	Co-solvents [BSA 40 μM + CTAB 1200 μM + MSG M]	a	y_o	b	t 1/2	t_{lag} (min)	K_{app} (min^{-1})
1	Control	0.00076	0.002	4.70	34.43	25.03	0.21
2	40 + 1200 + 2.5	3.15	1.40	0.21	0.30	0.37	4.7
2	40 + 0 + 2.5	0.297	0.018	19.57	87.46	48.32	0.05
3	40 + 1200 +2.5	0.629	0.006	7.018	23.79	9.76	0.14
4	40 + 1200 + 2.7	1.953	0.03	1.953	12.11	8.20	0.51
5	40 + 1200 + 3	2.32	0.05	0.442	3.47	2.59	2.27
6	40 + 1200 + 3.5	2.857	0.02	0.219	1.49	1.05	4.76
7	40 + 1200 + 4	2.91	0.005	0.131	1.33	1.06	7.63
8	40 + 1200 + 4.5	3.08	0.107	0.107	0.84	0.62	9.34

TABLE 5 | Change in kinetic parameters associated with BSA aggregation in the presence of fixed concentration (1 molar) of monosodium glutamate at different temperatures ranging from 60 to 85°C.

S. no.	Temperature (°C)	a	y_o	b	t 1/2	t_{lag} (min)	K_{app} (min^{-1})
1	60	0.120	0.015	1.5	5.80	2.28	0.67
2	63	0.195	0.023	1.31	4.56	1.94	0.76
3	65	0.228	0.013	1.13	4.12	1.86	0.88
4	70	0.248	0.012	1.00	3.93	1.92	1.00
5	75	0.265	0.007	0.57	3.14	1.99	1.75
6	78	0.353	0.010	0.59	3.17	1.99	1.69
7	80	0.406	0.010	0.59	3.17	1.99	1.69
8	81	0.431	0.013	0.59	2.17	0.99	1.69
9	82	0.490	0.023	0.48	1.44	0.48	2.08
10	84	0.488	0.023	0.55	1.69	0.59	1.81
11	85	0.494	0.020	0.73	1.56	0.20	1.47

TABLE 6 | Calculation of activation energies into two segments from nonlinear behavior shown by slopes by plotting ln tlag vs. 1000/T and ln(*k*) vs. 1000/T.

E_a	Lag phase	Growth phase
60 to 80°C	0.60 kcal/mol	9.90 kcal/mol
80 to 85°C	107.16 kcal/mol	4.44 kcal/mol

this type of aggregation is also referred to as non-Arrhenius protein aggregation (Wang and Roberts, 2013). Also, the report suggests that a change in temperature leads to a rate-limiting step of aggregation kinetics (Wang and Roberts, 2013), so the association of the non-Arrhenius behavior with the nucleation phase of MSG-BSA aggregation in the presence of temperature is well justified. Unfolding of protein is also accompanied with the increase in the activation energies (Lee and Timasheff, 1981; Sharma et al., 2010b), and here MSG was also observed with the increase in the activation energies.

The effect of MSG on the reversibility of bovine serum albumin was also studied. Our results suggest that at 25°C, UV-visible spectroscopic studies of native BSA depict no scattering but in the presence of MSG a noticeable scattering was witnessed. However, after thermal denaturation of BSA in the presence of MSG at 80°C, the solution turns exceedingly turbid and aggregation was visible with the naked eyes. It is reported that the native protein is stabilized by a covalent bond (disulfide bonds) and noncovalent interactions or electrostatic interactions such as ionic bonds, hydrogen bonds, van der Waals forces, and hydrophobic interactions (Ankarcrona et al., 1995). The process by which protein loses its native, well-defined folded structure (i.e., secondary, tertiary, and quaternary structure) which is formed under physiological conditions to an unfolded state or biologically inactive state (in which primary structure is retained) under non-physiological conditions or under some external stress is known as denaturation (Tanford, 1968). There is change in the physical, chemical, and biological properties of native protein by virtue of denaturation (Dill and Shortle, 1991). In denaturation, covalent peptide bonds are not disrupted; however, there is disruption of covalent interactions between disulfide bridges between cysteine groups, noncovalent dipole–dipole interactions between polar amino acid side chains, Van der Waals interactions between nonpolar amino acid side chains, alpha-helices and beta-pleated sheets, and random coil configuration (Bazoti et al., 2005; Sionkowska, 2005). It may not involve complete unfolding of proteins, but there can be the presence of a folded structure in random conformation. It may provide an unfolded state to the protein without any noncovalent interactions responsible for the folded protein and denatured state to protein with some noncovalent interactions responsible for the folded protein stability. The changes occurring in one part of protein induces the unfolding of the other part; thus, denaturation is cooperative in nature (Wang and Spector, 2000). Cooling the thermally denatured BSA in the presence of MSG at a temperature of 25°C or overnight incubation at 25°C, in both circumstances, UV-visible studies suggest that all peaks of the BSA spectra were absent, totally disproportionate, and flattened. Reports suggest that lowering the temperature of the solution, removal of denaturants, and readjusting the pH to the native state are some important processes through which most proteins may be refolded to their native state. The refolded proteins may restore their biological functions. Native and denatured states are in equilibrium with each other. Upon removal of denaturant, protein refolds back to native conformation from denatured state, which is called renaturation or refolding, thus denaturation may be reversible

(Roussel et al., 2013). The process by which unfolded (denatured) proteins are returned back to their native state partially or fully is known as renaturation (Rakic, 2012). **Figure 6A** clearly depicts that BSA forms aggregates in the presence of MSG and the aggregates are not reversible but are irreversible. Irreversible aggregates are the aggregates formed by misfolding of native monomers into stable or net-irreversible or higher-molecular-weight species which when formed cannot be dissociated without subjecting them to very higher concentrations of chemical denaturants, pressure, or temperature or by diluting the solution and is driven by hydrophobic interactions and hydrogen bonds (De Young et al., 1993; Tennent et al., 1995; Brummitt et al., 2012). The secondary and tertiary structures may be lost with the formation of irreversible aggregates. The concentrations of different aggregate species regulate the relative rates of the different processes. It has been reported that there is change in the viscosity of the solution on the formation of irreversible aggregates (Cromwell et al., 2006). The separation of irreversible aggregates can be also achieved by column chromatography (Wilson and Smith, 1959). The presence of irreversible aggregates cause degradation on the quality of products (Jiskoot et al., 2012).

In order to investigate the effect of MSG on the hydrodynamic size of BSA, a dynamic light scattering (DLS) experiment of BSA was performed in the absence and presence of MSG. DLS is a well-established technique that provides important structural information regarding biological macromolecules in solution especially to measure values of the hydrodynamic diameter, polydispersity, and existence of aggregates in the protein solution. Results depict that the value of the hydrodynamic diameter of BSA was observed to be about 8.3 nm (4.14 nm hydrodynamic radius) at pH 7.0, which is close to reported values. It has been reported that the value of the hydrodynamic diameter at pH 4.5 to 9.2 varies from 9.6 to 7.5 nm (Martinez-Landeira et al., 2002; Castelletto et al., 2007a,b). However, there was abrupt enhancement in the values of hydrodynamic diameter by the addition of MSG to the solution of BSA protein from 8.3 to 68. 33 nm (see **Figure 6B**). Thus, MSG causes the enhancement of the hydrodynamic diameter of BSA which could be due to unfolding of the BSA protein or due to the existence of aggregates in the solution. Also from the results of DLS measurements, structural deformation of protein by the MSG can be well justified.

The effect of MSG on the secondary structure of bovine serum albumin was studied from far-UV CD measurements. Far-UV CD ranging from 240 to 180 nm corresponds to the absorption of the peptide bond which is asymmetric in nature so molecules asymmetric in nature exhibit the phenomenon of CD. Far-UV CD provides information about the content of the secondary structure of protein which includes α-helix and β-sheet, turn, and random coil (Kelly and Price, 2000). The results shown in **Figure 7A** illustrate that a low concentration of MSG causes the substantial perturbation and denaturation of secondary of BSA. Moreover, even at the concentration of 0.001 M of MSG the peaks of BSA existing at 208 and 222 nm absolutely disappeared. The native spectrum of BSA illustrates strong negative signals at 208 and 222 nm and there was also the abrupt decrease in the value of mean residual ellipticity (MRE) or the negative signals of CD at 208 and 222 nm.

The plot of mean residual ellipticity of BSA at 208 nm versus the concentration of MSG shown in **Figure 7B** depicts the decrease in the negative ellipticity of protein from −22236 to 10880 in the presence of low concentrations of MSG. At concentrations from 0.02 to 0.1 M, MSG results in the disappearance of original peaks but results in the formation of a new peak at 226 nm, which is an attention-grabbing observation as far as the influence of MSG on the secondary structure analysis by far-UV CD measurement is considered. The formation of a new peak at 226 nm is an indication of aggregated or disordered proteins. These disordered proteins are unstructured or partially structured, rich in random coils, and or pre-molten globules (Clements et al., 1992), and also these proteins are deprived of an ordered secondary structure and three-dimensional structure referred to as intrinsically disordered proteins (IDPs) and is noticed by CD as "random coil," "unordered," or "disordered." This observation can provide some evidence to diseases triggered by protein misfolding (Anand et al., 2011; Habchi et al., 2014; Knowles et al., 2014; Lopes et al., 2014; Li et al., 2015). Reports also suggest that from the far-UV CD measurement there is the presence of a secondary structure mainly with α-helix and random coil by titration of SDS with acid-induced denatured cyt c (Keiderling and Xu, 2002; Xu and Keiderling, 2004). Thus, from the far-UV CD measurements, our results suggest that a low concentration of MSG is involved in the unfolding of a secondary structure of protein with the disappearance of original peaks and the formation of a unique peak.

The protein folding under optimum physiological conditions in which unfolded proteins fold into a three-dimensional native state and functional state. These native proteins are stabilized by intermolecular interactions, which are thermodynamically favorable and regulated process (Fersht and Daggett, 2002; Daggett and Fersht, 2003; Baldwin, 2007; Nickson and Clarke, 2010). **Figure 8** represents the ITC profile acquired after titration of BSA with MSG at pH 7.0 specifies with the positive heat pulse in the upper panel describing the binding of MSG with BSA. The interaction is endothermic in nature with a stoichiometry of n = 5.18 (± 0.079), K_a (M^{-1}) = 1.82 × 10^5 (± 2.6 x 10^4), ΔH = {8.328 × 10^4 ± 1263} cal mol^{-1}, and ΔS = 303 cal mol^{-1} deg^{-1}. From these binding parameters, using equation (2), change in Gibbs free energy was also calculated (ΔG = -7014 cal mol^{-1}). Different binding and thermodynamic parameters associated between BSA-MSG interactions estimated from ITC measurements at pH 7.0 and 25°C are shown in **Table 1**. ITC thermogram data reveals the best-fitting with one site-binding model. The enthalpy change calculated is mostly positive which also suggests that the binding of interaction is endothermic in nature, entropy change (ΔS) calculated is positive, and Gibbs free energy change (ΔG) is largely negative which means the reaction is spontaneous in nature (Perozzo et al., 2004; Jia et al., 2014). There is noticeable heat of interaction or a specific heat pattern that mentions the binding affinity of MSG with BSA which is very much appreciable. Evaluation of properties of heat reactions including sign, magnitude, pattern, and shape on the thermogram is very much significant in determining the formation of different types of aggregates and has been accompanied with aggregation kinetics of proteins (Ikenoue et al., 2014). The ionic

interaction, hydrogen bonding, hydrophobic interaction, and van der Walls forces have a vital role in binding affinity of protein with ligand (Sharma et al., 2010a; Ikenoue et al., 2014), and the endothermic heat of interaction between BSA and MSG indicates involvement of a hydrophobic interaction (Perozzo et al., 2004; Song et al., 2016; Patel and Bummer, 2017). A higher value of endothermic heat of interaction $\Delta H = \{832.8 \times 10^3 (\pm 1263 \times 10^3)\}$ is suggestive of protein–MSG interaction or the formation of a protein–MSG complex. We speculate that this coupling of MSG with BSA causes aggregation in the protein (Ghosh et al., 2018). As there are reports suggesting that calcium causes the aggregation of α-synuclein with endothermic heat of interaction (Jain and Bhat, 2014), SDS-induced hydroxypropyl methylcellulose (HPMC) aggregation was also observed to be endothermic in nature (Patel and Bummer, 2017) and the polymer-induced surfactant aggregation is also coupled with endothermic heat of interaction (Loh et al., 2016).

CONCLUSION

In conclusion of this work, we present the first report in support of the association of dietary MSG with protein misfolding and aggregation. This study demonstrates that MSG is involved in the promotion of protein aggregation via the mechanism of nucleation-dependent polymerization. CTAB-BSA aggregation was also promoted considerably in the presence of MSG. There is temperature dependence, non-Arrhenius nature, and endothermic heat of interaction revealed by MSG-induced BSA aggregation. MSG is involved in a marked increase in hydrodynamic diameter of the native structure which is the mark of unfolding and aggregation. Irreversible aggregate formation is caused by MSG which was also observed to be associated with the unfolding of the secondary structure of protein with the vanishing of native protein peaks and the formation of an exclusive peak, which is an important finding. This is the first study which justifies the role of MSG in protein misfolding and aggregation and provides the commencement for further investigations of the role of MSG in protein misfolding and aggregation, their exact mechanism, and their relationship with human physiological and neurodegenerative diseases.

AUTHOR CONTRIBUTIONS

IA performed all the experiments of biophysical and aggregation studies and prepared the first draft of the manuscript. ZP and SB assisted in the ITC experiment. AS, FA, MIH, MFA, AH, and AI designed the experiments, monitored the experimental work, and prepared the final draft of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2021.636454/full#supplementary-material

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Triadin Decrease Impairs the Expression of E-C Coupling Related Proteins in Muscles of MPTP-Induced Parkinson's Disease Mice

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Parkinson's disease (PD), caused by destruction of dopaminergic neurons in the brain, leads to motor symptoms like bradykinesia, tremor, and walking impairments. While most research effort focuses on changes in neuronal pathology we examined how muscle proteins were altered in a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of PD. A Ca^{2+} release channel complex, consisting of ryanodine receptors (RYR), triadin (TRDN), and calsequestrin (CSQ1), is important for excitation-contraction coupling in the sarcoplasmic reticulum membrane in muscles. Thus, we investigated changes in the RYR Ca^{2+} release channel components in PD mice model. Based on a report that TRDN deletion impairs skeletal muscle function, we also investigated how the knock-down of TRDN affects other components of the RYR channel in the PD model. In this study, the expression levels of the components of RYR channels decreased in the quadriceps femoris muscle of MPTP-induced PD mice and in C2C12 cells treated with 1-methyl-4-phenylpyridinium. We show that decreased TRDN levels decrease RYR and CSQ1 levels. These results suggest that the levels of proteins related to Ca^{2+} channel function decreased in this model, which could impair muscle function. We conclude that muscle function alterations could add to the bradykinesia and tremor in this model of PD.

Keywords: TRDN, Parkinson's disease, Ca^{2+} channel, MPTP, MPP+, skeletal muscle, C2C12

INTRODUCTION

Parkinson's disease (PD) is a brain disorder that is caused by the degeneration of dopaminergic cells in the substantia nigra (SN). The symptoms of PD include bradykinesia (slow movement), tremor, muscle rigidity, postural instability, and walking impairments. The symptoms related to the neuromuscular function are mainly observed in patients with PD. PD usually occurs in elderly people and causes dangerous situations like falling which can cause secondary injury. Alleviating the symptoms related to muscles will improve the patients' quality of life. Furthermore, it was shown that bradykinesia could be due to insufficient recruitment of muscle force for the initiation

of movement (Berardelli et al., 2001). Therefore, in this study, we focused on muscle function in PD and investigated factors related to Ca^{2+} channels in muscles.

Motor neurons in contact with skeletal muscles signal the muscle to contract by releasing acetylcholine into the synaptic cleft. Acetylcholine depolarizes the muscle fiber and triggers an action potential in the muscle fiber membrane, which travels to the transverse tubule (T-tubule). This action potential moves to the sarcoplasmic reticulum (SR), which has Ca^{2+} channels that release Ca^{2+} into the cytoplasm to enable muscle contraction. When the level of Ca^{2+} in the cytoplasm is high, the muscle contracts by binding Ca^{2+} to the troponin complex (Campbell et al., 2010; Hudspeth et al., 2013).

Muscle contraction is regulated by Ca^{2+} levels in the cytoplasm, which in turn are related to Ca^{2+} channels. Ryanodine receptors (RYRs), calsequestrin (CSQ1), triadin (TRDN), junctin, and dihydropyridine receptors are proteins involved in Ca^{2+} homeostasis (Oddoux et al., 2009) and excitation contraction (E-C) coupling (Sutko and Airey, 1996). In this study, we focused on the role of TRDN in muscles. Furthermore, proteins such as RYRs and CSQ1s, which are related to TRDN in the SR membrane Ca^{2+} channel, were studied.

Triadin has been mostly studied in the cardiac and skeletal muscles. TRDN regulates Ca^{2+} release from the SR. In the cardiac muscle, *TRDN* mutations trigger the leakage of Ca^{2+} ions from the SR lumen because CSQ1 is unable to inhibit the release of Ca^{2+} ions by RYR (Roux-Buisson et al., 2012). TRDN deletion impairs muscle function (Oddoux et al., 2009). In addition, although CSQ1 has a role as a luminal calcium sensor for RYR (Beard et al., 2005), TRDN is also able to sense the Ca^{2+} level in the SR lumen by mediating interactions between RYR and CSQ1 (Zhang et al., 1997).

In this study, TRDN-related factors were studied in a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced PD mouse model (Brady et al., 2005). The quadriceps femoris (QF) muscle was used because the QF is the most important muscle for walking and jumping. The mouse model was a semi-chronic model induced by 4 weeks of MPTP administration. The C2C12 cell line, which is an immortalized mouse myoblast cell line, was used to determine the change in TRDN-related factors after treatment with 1-methyl-4-phenylpyridinium (MPP+) and TRDN siRNA.

We hypothesized that Ca^{2+} channels related to muscle contraction may be impaired in PD. The purpose of this study was to determine the association between the Ca^{2+} channel components, especially TRDN, and PD. Furthermore, this study could contribute to advancing the research on the mechanism of bradykinesia, one of the symptoms of patients with PD.

MATERIALS AND METHODS

MPTP-Induced PD Mouse Model

Six-week-old male C57BL/6 mice (20–22 *g*; DBL, Korea) were divided into two groups: the control group and MPTP-treated group (MPTP). In the control group, mice were injected intraperitoneally with 100 μL phosphate-buffered saline (PBS) once a day for 4 weeks, while in the MPTP group, mice were injected intraperitoneally with MPTP-HCL (20 mg/kg of free base; Sigma, United States) in PBS (100 μL) every 24 h for 4 weeks to produce a semi-chronic model of PD. On the day after the final MPTP treatment, mice were anesthetized using Alfaxan and perfused transcardially with cold PBS for western blotting. The MPTP model of PD shows dopaminergic cell death in the SN (Choi and Lim, 2010; Yeo et al., 2013). All animal experiments conducted for this study were approved by the Sang Ji University Animal Experimentation Committee.

Rotarod Test

Rotarod tests were performed to evaluate the motor ability of MPTP mice before the last MPTP injection. Training was performed in the second week for 2 days at 30 rpm for 15 min, once a day. The rotarod treadmill diameter was 280 mm, and the test was performed in an accelerated mode for 4 min from 10 rpm to 50 rpm in 5-min running time. After 4 min of accelerated mode, 50 rpm was maintained for 1 min until completion. The time until the first fall or the first drop was measured. The test was repeated two times and the measurement of last test was used as results for statistical analysis ($n = 5$/group).

Pole Test

The pole test was conducted to evaluate the motor ability before the last MPTP injection. In the pole test, a wooden vertical pole (length 548 mm, diameter 8 mm) was used. The time the mouse took while moving from the top to the bottom of the pole was measured. Training was performed in the second week for 2 days, once a day. The test was performed two times and the measurement of last test was used as results for statistical analysis ($n = 5$/group).

Immunohistochemistry

The brains of MPTP-induced PD mice were perfused with 4% paraformaldehyde and fixed in 4% paraformaldehyde for 1 day at 4°C. After fixation, the brains were dehydrated with 30% sucrose buffer for 2 days at 4°C. Coronal sections (40 μm) were cut using a cryomicrotome. Immunohistochemical analysis was performed using an ABC kit, a Mouse on Mouse (M.O.M) immunodetection kit (Vector Laboratories, CA, United States), and a modification of the avidin-biotin-peroxidase method. Sections encompassing the striatal and SN regions were incubated in 3% H_2O_2 with PBS (pH 7.4), and then incubated in blocking buffer [1% bovine serum albumin (BSA), 10% horse serum in PBS]. When the mouse anti-tyrosine hydroxylase (TH) antibody (1:200; Santa Cruz Biotechnology, United States) was used, tissues were treated with an M.O.M mouse Ig-blocking reagent (Vector Laboratories, CA, United States) at room temperature for 1 h before incubation with the primary antibody overnight at 4°C. Thereafter, the sections were treated with a biotinylated anti-mouse IgG and an avidin-biotin-peroxidase complex, which reacted with diaminobenzidine-hydrogen peroxide. Dopaminergic neuronal cells were analyzed using a Nikon X-cite series 120Q microscope (Nikon, Japan).

Western Blotting

The QF muscle tissue was homogenized in 20 mM radioimmunoprecipitation assay buffer using a sonicator (Qsonica Q55, United States) on ice for 20 min.

C2C12 cells were incubated and homogenized in Tris–Triton cell lysis buffer (GenDEPOT, United States) for 20 min on ice.

After the tissues and the C2C12 cells were centrifuged at 12,000 rpm at 4°C for 15 min, supernatant samples were separated using 4–15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride membranes (Pall Life Science, United States). The membranes were blocked with 3% BSA at room temperature and then incubated with primary antibody overnight and washed with 0.1% Tris–buffered saline with Tween 20. The membrane was also incubated with a secondary antibody for 1 h and washed with 0.1% Tris–buffered saline with Tween 20.

Rabbit anti-TRDN (1:2000, Cloud-Clone Corp., United States), mouse anti-CSQ1 (1:500, Santa Cruz Biotechnology, United States), mouse anti-RYR (1:500, Santa Cruz Biotechnology, United States), and mouse anti-β actin (1:5000; Santa Cruz Biotechnology) antibodies were used as primary antibodies.

Immunofluorescence

Longitudinal QF muscle cryosections were used for immunofluorescence in the control and MPTP groups. The sections were fixed in 4% paraformaldehyde and methanol. After fixation, the sections were incubated in blocking buffer (1% BSA, 5% goat serum in PBS) for 1 h. The samples of both groups were incubated with the primary antibodies, mouse anti-RYR (1:200, Invitrogen, United States) or mouse anti-CSQ1 (1:200, Invitrogen, United States) and rabbit anti-TRDN (1:200, Cloud-Clone Corp., United States), followed by secondary antibodies, goat anti-mouse IgG (H + L) fluorescein isothiocyanate (FITC)-conjugated (CUSABIO, United States), and goat anti-rabbit IgG (H + L) tetramethylrhodamine (TRITC)-conjugated (Novex, United States). Finally, DAPI (1 μg/mL) was used to label the cell nuclei. Photographic documentation was performed using a Nikon X-cite series 120Q microscope (Nikon, Japan). The exposure parameters were the same for the control and MPTP groups.

C2C12 cells were fixed in 4% paraformaldehyde and blocked for an hour. Rabbit anti-TRDN (1:200, Cloud-Clone Corp., United States) was used as the primary antibody, followed by a secondary antibody, goat anti-rabbit IgG (H + L) TRITC-conjugated (Novex, United States). Thereafter, DAPI (1 μg/mL) was used to label the cell nuclei. The control and MPP + group staining procedures were performed concurrently.

Cell Lines and Cultures

C2C12 cells from the mouse-engineered myoblast cell line were cultured under standard culture conditions (5% CO_2, 37°C). Dulbecco's modified Eagle's medium (BioWest, United States) containing 10% fetal bovine serum (GenDEPOT, United States) and 100 U/mL of penicillin-streptomycin (Gibco, United States).

MPP + Treatment

C2C12 cells were treated with 1, 2.5, or 5 mM MPP + iodide (Sigma) for 18 h. MPP + was administered at the same time in each experiment.

Short Interfering RNA Knockdown

C2C12 cells were incubated in Opti-MEM medium (Gibco, United States) at least 1 day before siRNA (*short interfering RNA*) transfection. The transfection reagent and TRDN siRNA were applied (3.5:1) when the density of C2C12 cells was 30%. Transfection was continued for 48 h. siRNA against TRDN (5-UC AUG UGG GUA GAC UCA GU-3) and negative control duplexes (5-UUC UCC GAA CGU GUC ACG UTT-3) were used (Bioneer Inc., South Korea).

Imaging Software

ImageJ software developed at the National Institutes of Health and the Laboratory of Optical and Computational Instrumentation (University of Wisconsin) was used to analyze the images.

Statistical Analysis

Statistical analyses were carried out with Student's *t*-test and analysis of variance in SPSS 25 (SPSS Inc. Released 2017, PASW Statistics for Windows, Version 25.0, United States). All values are expressed as mean ± standard error.

RESULTS

The MPTP-induced PD mouse model was created by injecting MPTP-HCL (20 mg/kg) every 24 h for 4 weeks. To check the status and motor ability of mice in the control and MPTP groups, rotarod and pole tests were performed. The results are shown in **Figure 1**. In the rotarod test, the mice in the MPTP group fell earlier than the mice of the control group did, with an average difference of 44 s. In the pole test, the mice in the MPTP group arrived on average earlier at the bottom than control mice did. The mice in the MPTP group did not grab the pole by their hind leg but slid down, which seemed to be caused by a lack of strength. This indicates that the MPTP mice showed decreased motor ability compared to that of control mice.

To ensure that the PD mouse model met the PD characteristics, TH expression was analyzed in the SN and the striatum using immunohistochemistry. The results showed that TH expression was reduced in both areas in the MPTP group. Furthermore, dopaminergic cell numbers decreased in the SN in the MPTP group (**Figure 2**). This decrease was caused by the destruction of dopaminergic cells by MPTP. This result indicates that the MPTP-induced PD model was well established.

Western blot analysis of QF muscle tissue (**Figure 3A**) showed that the expression levels of TRDN, RYR, and CSQ1 were reduced in the MPTP group (**Figures 3B,C**). RYR and CSQ1 levels were more than 50% lower compared to those in the control group. Considering that TRDN, RYR, and CSQ1 are components of a Ca^{2+} channel in the SR membrane of T-tubules, this could indicate reduced levels of Ca^{2+} channels in the SR membrane.

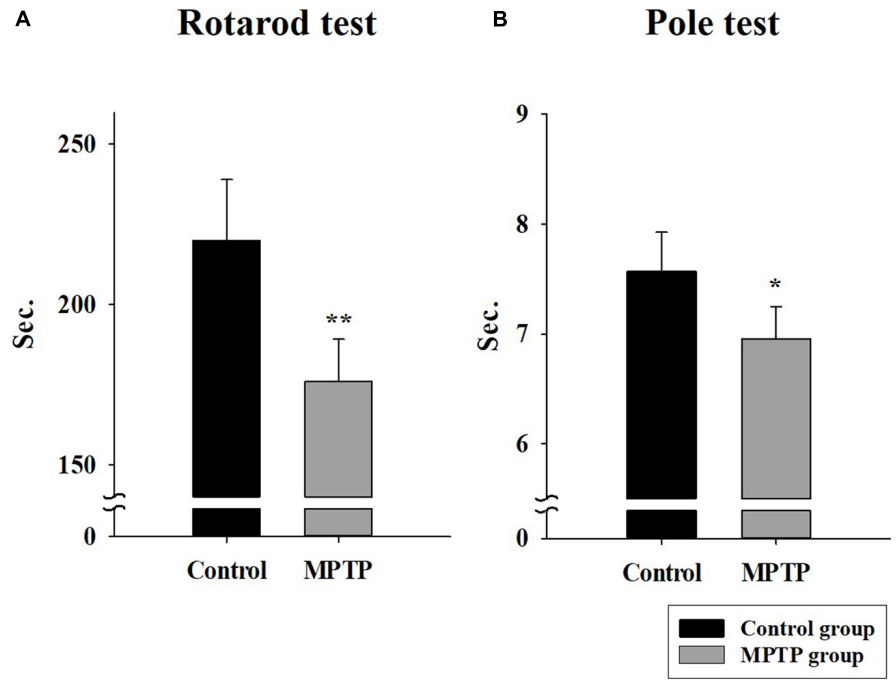

FIGURE 1 | Rotarod and pole tests in the control group and the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced Parkinson's disease group. **(A)** The rotarod (28 mm diameter) test was conducted in Accel Forward mode for 4 min from 10 to 50 rpm during 5 min of running time. The final minute was carried out at constant 50 rpm. ($n = 5$, $^{**}P < 0.005$) **(B)** Pole test. The length and diameter of the pole were 548 mm and 8 mm, respectively. The mouse ran from the top to the bottom of the vertical wooden pole. ($n = 5$, $^{*}P < 0.05$) Statistical analyses were carried out with Student's *t*-test.

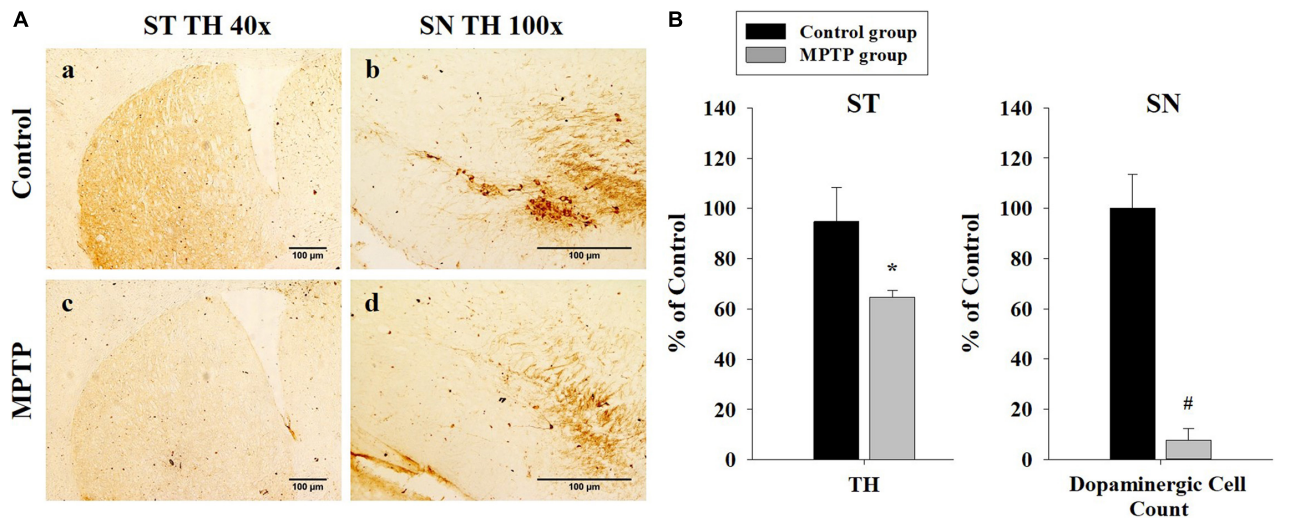

FIGURE 2 | Immunohistochemistry analysis of tyrosine hydroxylase expression in the striatum and the substantia nigra (SN) in the control and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated (MPTP) groups. **(A)** Tyrosine hydroxylase staining is much stronger and darker in the striatum and the SN of the control (a, b) than that of the MPTP (c, d) mice because dopaminergic cells are destroyed in the SN of the MPTP mouse model. (scale bar, 100 μm). **(B)** The left graph shows the amount of tyrosine hydroxylase in the striatum, comparing panel a with panel c. The right graph shows the dopaminergic cell count in the SN, comparing the dopaminergic cell number in panel b with panel d. ($n = 3$, $^{*}P < 0.05$, and $^{\#}P < 0.0005$) Statistical analyses were carried out with Student's *t*-test.

Immunofluorescence staining was carried out in longitudinal cryosections of the QF muscle to observe changes in RYR, CSQ1, and TRDN levels between the control group and the MPTP group. All processes were performed simultaneously under the same conditions. The results showed that the fluorescence intensity of RYR and TRDN decreased in the MPTP group (**Figure 4**), and the intensity of CSQ1 and TRDN also decreased in the MPTP group (**Figure 5**), which is consistent with the

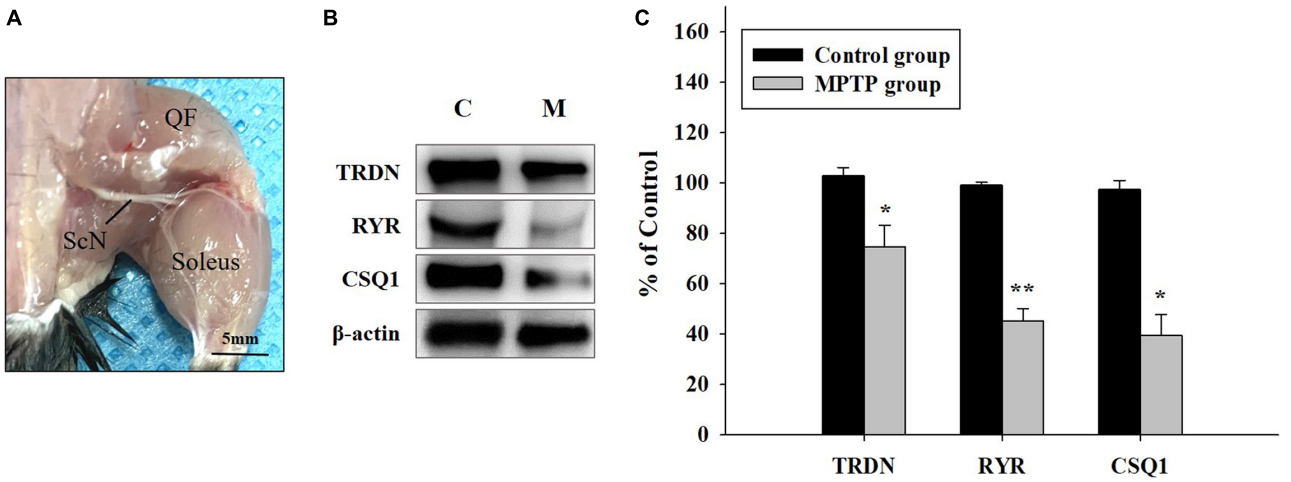

FIGURE 3 | Western blot analysis of proteins linked to skeletal muscle contraction. **(A)** Tissue of the quadriceps femoris muscle, which is important for walking and jumping, was used in western blot analysis, as shown in **B**. (ScN, sciatic nerve). **(B)** Western blot analysis of triadin (TRDN), ryanodine receptor (RYR), and Calsequestrin 1 (CSQ1) which are factors of the Ca^{2+} channel in sarcoplasmic reticulum membrane from the control and 1-methyl-4-phenyl-1,2,3,6-tetrahydro pyridine treated (MPTP) groups. **(C)** Protein levels are shown as bar graphs from western blot results from **B**. ($n = 3$, $^{*}P < 0.05$, and $^{**}P < 0.005$) Statistical analyses were carried out with Student's *t*-test.

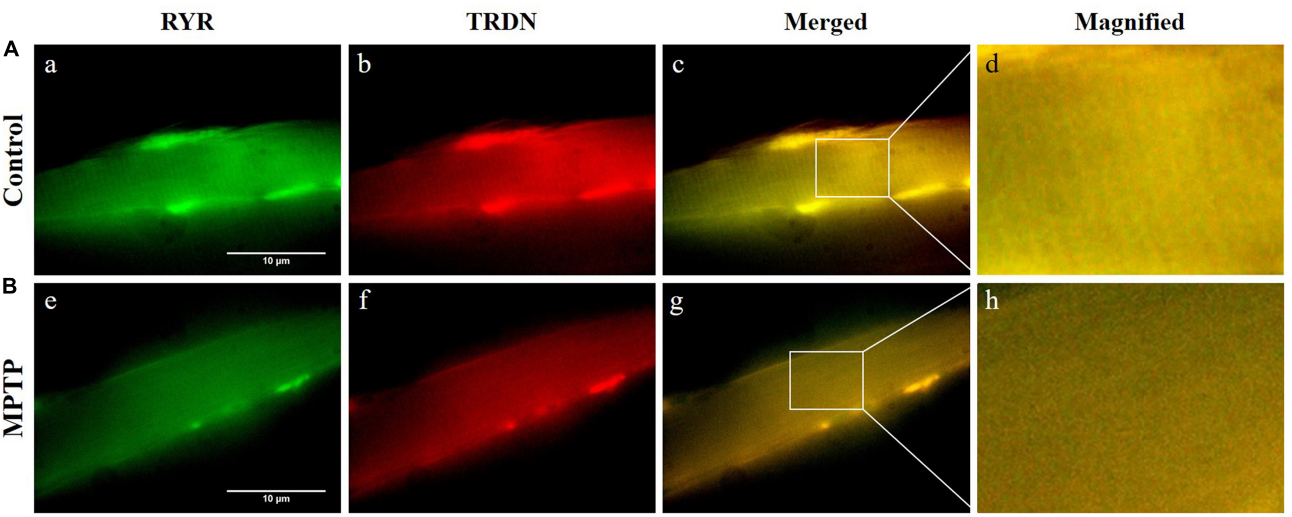

FIGURE 4 | Immunofluorescence staining of ryanodine receptor (RYR) and triadin (TRDN) in skeletal muscle tissue (quadriceps femoris region). **(A)** Control group. a, RYR; b, TRDN; c, merge of a and b; and d, a 4 times magnified figure of a white rectangular box in c panel. **(B)** 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine treated (MPTP) group. e, RYR; f, TRDN; g, merge of e and f; and h, a 4 times magnified figure of a white rectangular box in g panel. (scale bar, 10 μm).

results shown in **Figure 3**. We hypothesized that the decrease in TRDN, RYR, and CSQ1 expression levels in skeletal muscle tissue would match the results at the cellular level.

To investigate the results at the cellular level, we used C2C12 mouse myoblast cells. After inducing the PD model with the neurotoxin MPP+, we examined the effect of MPP+ on C2C12 cells at increasing MPP + concentrations (Nicklas et al., 1987). Higher MPP + concentrations induced a larger decrease in the expression levels of the TRDN, RYR, and CSQ1 components of the Ca^{2+} channel in C2C12 cells (**Figure 6**).

We also analyzed the changes in the components of the Ca^{2+} channel in C2C12 cells treated with MPP + when TRDN levels were decreased via TRDN siRNA (**Figure 7**). Because the protein levels of TRDN, RYR, and CSQ1 decreased in C2C12 cells treated with 1 mM MPP+ (**Figure 6**), MPP + treatment was performed equally with 1 mM in **Figure 7**. When 10 or 100 nM TRDN siRNA was added to MPP+ -treated C2C12 cells, the TRDN expression

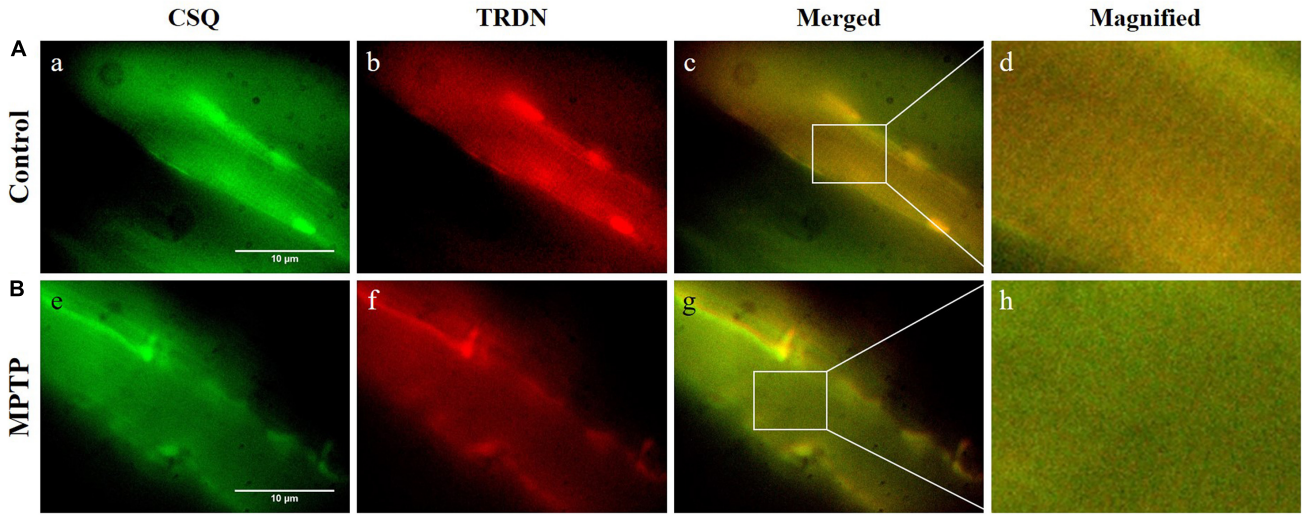

FIGURE 5 | Immunofluorescence staining of Calsequestrin 1 (CSQ1) and triadin (TRDN) in skeletal muscle tissue (quadriceps femoris region). **(A)** Control group. a, CSQ1; b, TRDN; c, merge of a and b; and d, a 4 times magnified figure of a white rectangular box in c panel. **(B)** 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine treated (MPTP) group. e, CSQ1; f, TRDN; g, merge of e and f; and h, a 4 times magnified figure of a white rectangular box in g panel. (scale bar, 10 μm).

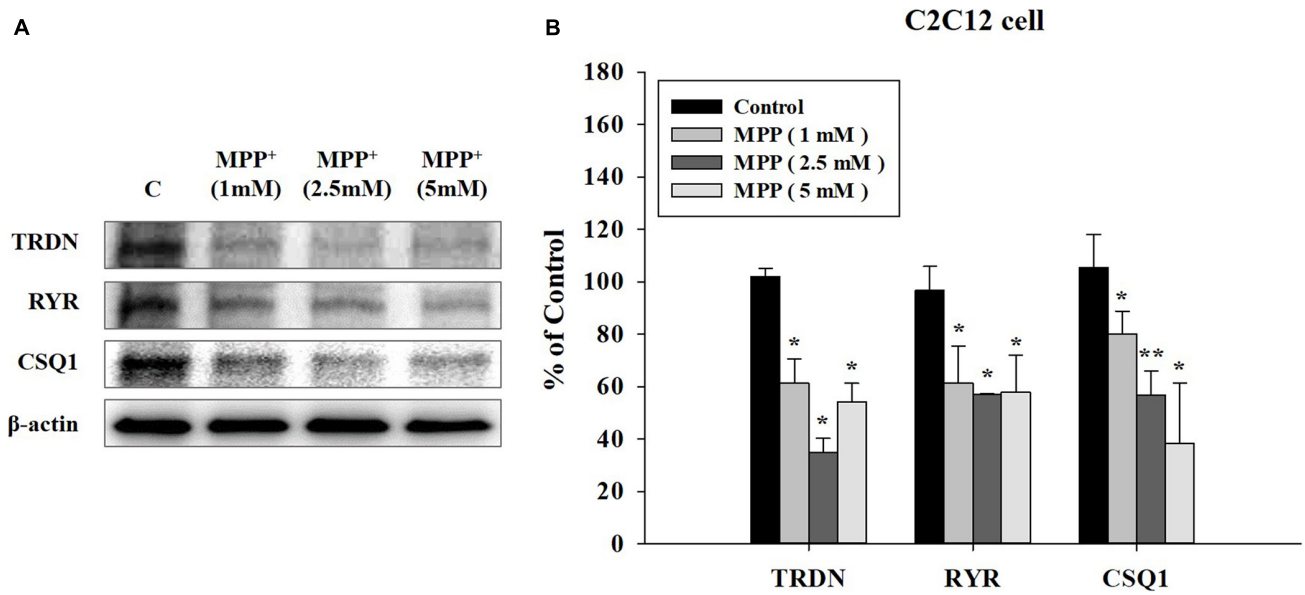

FIGURE 6 | Western blot analysis of components linked to Ca^{2+} channels in sarcoplasmic reticulum at different 1-methyl-4-phenylpyridinium (MPP+) concentrations in C2C12 cells. **(A)** Western blot analysis of triadin (TRDN), ryanodine receptors (RYR), and Calsequestrin 1 (CSQ1), which are components of the Ca^{2+} channel in the sarcoplasmic reticulum membrane, at different MPP + treated concentrations: 1, 2.5, and 5 mM. MPP+ was applied for 18 h. **(B)** Quantified protein levels of the western blot results in **A**. ($n = 3$, $^{*}P < 0.05$, and $^{**}P < 0.005$) Statistical analyses were carried out with analysis of variance in SPSS 25.

level also decreased. Furthermore, the expression levels of RYR and CSQ1 also decreased (**Figure 7**). This indicates that in this PD model, decreasing TRDN levels also decreased RYR and CSQ1 expression.

To observe the change in TRDN expression at the cellular level according to MPP + treatment, immunofluorescence staining was performed in C2C12 cells in the control and MPP+ (1 mM)-treated group (**Figure 8** and **Supplementary Figure 1**). As indicated by the arrows in **Figures 8A**, b, TRDN was clearly observable in the control group. However, TRDN was not observed in the MPP + treated group (**Figures 8B**, e). Thus, the decreased TRDN level in the PD model was verified once again.

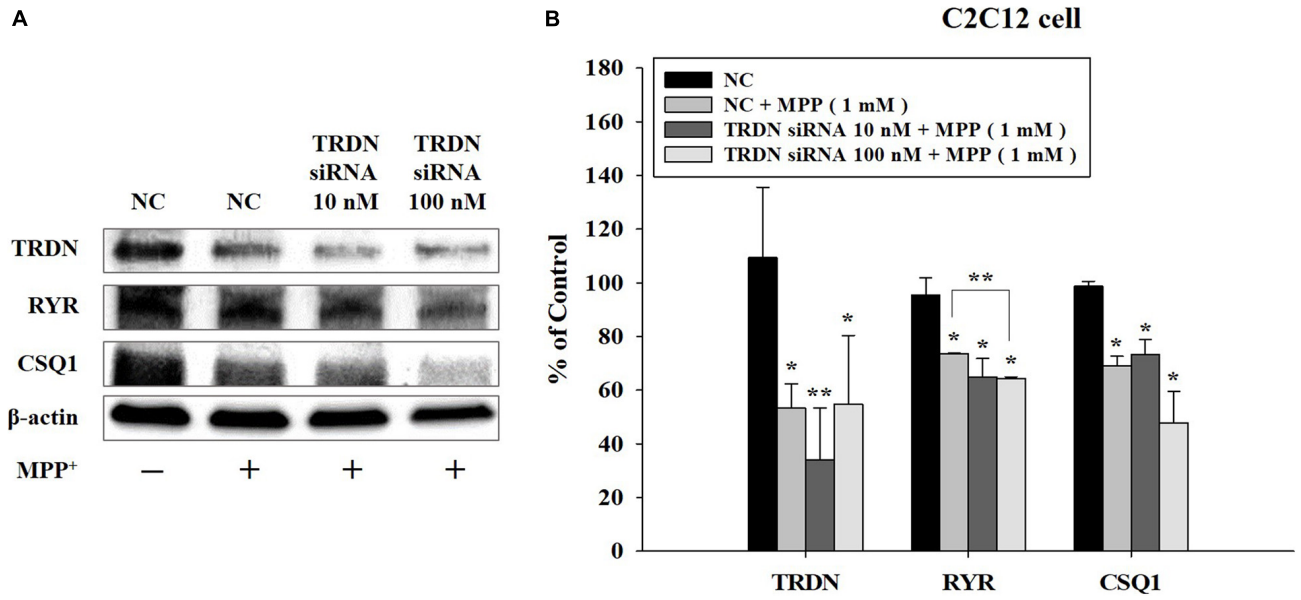

FIGURE 7 | Western blot analysis of the components linked to Ca^{2+} channels in sarcoplasmic reticulum after treatment of C2C12 cells with 1-methyl-4-phenylpyridinium (MPP +) and triadin (TRDN) siRNA. **(A)** Western blot analysis of TRDN, ryanodine receptors, and Calsequestrin 1, which are proteins in the sarcoplasmic reticulum membrane, after MPP+ and TRDN siRNA application. Negative control cells were treated with negative control siRNA (100 nM) in the same condition as TRDN siRNA treatment. MPP+ was applied at a concentration of 1 mM for 18 h. The incubation time of TRDN and negative control siRNA was 48 h. **(B)** Quantified protein levels from the western blot results shown in **A**. Negative control (NC), negative control siRNA treatment (100 nM for 2 days); TRDN siRNA 10 nM, TRDN siRNA treatment (10 nM for 2 days); TRDN siRNA 100 nM, TRDN siRNA treatment (100 nM for 2 days). ($n = 3$, $^{*}P < 0.05$, and $^{**}P < 0.005$).

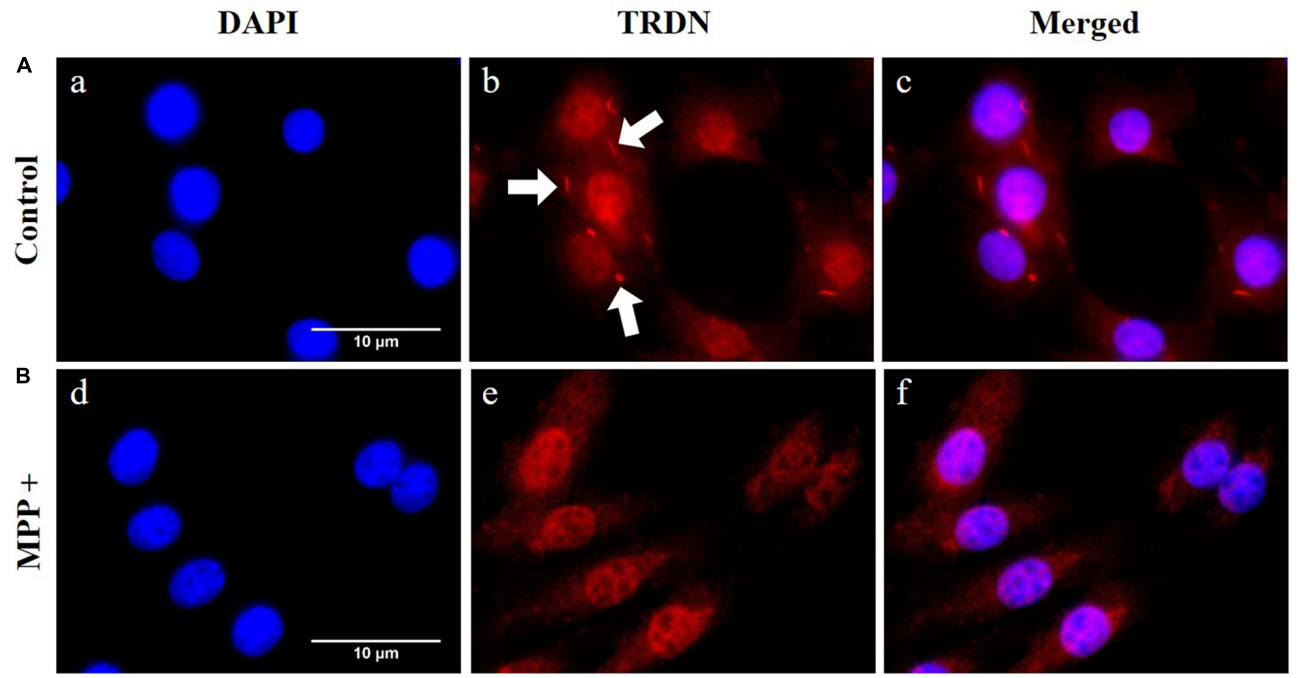

FIGURE 8 | Immunofluorescence staining of triadin (TRDN) in C2C12 cells. **(A)** Control group. a, nuclei; b, TRDN; and c, merge of a and b. **(B)** 1-methyl-4-phenylpyridinium (MPP+) group. d, nuclei; e, TRDN; and f, merge of d and e. (scale bar, 10 μm).

DISCUSSION

In this study, we developed an MPTP-induced PD mouse model and used it to verify that the expression levels of Ca^{2+} channel components decreased in the leg QF muscle. In addition, we showed that the expression levels of TRDN, RYR, and CSQ1 were decreased by MPP + treatment in C2C12 cells at the cellular level. These results indicate that expression of the Ca^{2+} channel components TRDN, RYR, and CSQ1 were reduced in the PD model. Furthermore, the results showed that Ca^{2+} channel expression was decreased in the SR membrane of skeletal muscle. Because the Ca^{2+} channel related to RYR in the T-tubules is important for E-C coupling in muscle (Sutko and Airey, 1996), it is considered that the results of this study showing decreased RYR Ca^{2+} channel expression would be important in PD. As Ca^{2+} release from the SR lumen in the muscle is important for contraction, insufficient Ca^{2+} release because of decreased Ca^{2+} channel expression could impair the adjustment of muscle contraction. This might be linked to bradykinesia (slow movement), one of the symptoms seen in patients with PD. However, it is necessary to further investigate the factors related to Ca^{2+} homeostasis regulation.

In addition, CSQ1 is known as a luminal regulator of RYR activity and inhibits the Ca^{2+} release channels (Beard et al., 2004). The lower expression of CSQ1 in our PD model (**Figures 3**, **5**) may induce Ca^{2+} leakage from the SR that could delay the restoration of the Ca^{2+} concentration in the SR lumen during contraction. TRDN expression was also decreased in the PD model (**Figures 3**, **6**), resulting in poor Ca^{2+} regulation in the SR because one of the functions of TRDN is the regulation of Ca^{2+} release from the SR, and the loss of TRDN leads to RYR Ca^{2+} leakage. These changes demonstrate that the function of the Ca^{2+} release channel is impaired or that the number of Ca^{2+} release channels decreases, which impairs Ca^{2+} release from the SR lumen and leads to symptoms related to muscle function in PD, such as bradykinesia or tremor.

To test whether TRDN is able to regulate CSQ1 expression (Oddoux et al., 2009), we used TRDN siRNA knockdown in our PD model (**Figure 7**). This reduction in TRDN induced a decrease in RYR and a tendency to decrease CSQ1 (**Figure 7**). Therefore, TRDN might be an important factor in the regulation of Ca^{2+} release from the SR in PD. It is anticipated that restoring decreased TRDN levels could alleviate the symptoms of PD, such as bradykinesia, tremor, or walking impairments. This is meaningful for improving the quality of life of patients with PD. However, other Ca^{2+} homeostasis regulation factors and the precise regulation mechanisms will need to be investigated in future studies. Furthermore, it is reported that dysregulated Ca^{2+} homeostasis in dopaminergic neuron leads to impaired mitochondria (Cali et al., 2014) and alterations of Ca^{2+} homeostasis including depletion of endoplasmic reticulum Ca2 + storage are implicated with neurodegenerative process (Mattson, 2012; Schapira, 2013; Zaichick et al., 2017). In this regard, the alteration of triadic proteins in neurons related to Ca^{2+} channel in endoplasmic reticulum of dopaminergic neuron would be prospective research direction and the changes of mitochondria in muscle related to Ca^{2+} homeostasis would be also interesting research in a forward study.

In conclusion, the expression levels of RYR, TRDN, and CSQ1, which are components of the Ca^{2+} channel related to E-C coupling, decreased in the QF muscle of MPTP-induced PD mice and in C2C12 cells treated with MPP+. Decreasing TRDN levels reduced RYR and CSQ1 levels and this might cause a Ca^{2+} leak via RYRs and a decrease in Ca^{2+} release channel levels. In addition, this would delay Ca^{2+} uptake in the SR lumen and muscle contraction until the necessary Ca^{2+} concentration is reached in the cytoplasm, potentially linking this mechanism to PD symptoms. These results suggest that the levels of proteins related to Ca^{2+} channel function decreased in this model, which could impair muscle function. We conclude that muscle function alterations could add to the bradykinesia and tremor in this model of PD. Restoring the components of the Ca^{2+} channel investigated in this study might relieve the symptoms related to muscle function in PD.

DATA AVAILABILITY STATEMENT

The data will be available upon request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee (IACUC) of Sangji University.

AUTHOR CONTRIBUTIONS

SY and MS: conceptualization. SY and MS: methodology. MS: software. SY and MS: validation. SY and MS: formal analysis. MS: investigation. SY: resources. MS: data curation. MS: writing—original draft preparation. SY: writing—review and editing. MS: visualization. SY: supervision. SY: funding acquisition. Both authors have read and agreed to the published version of the 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/fnins.2021.649688/full#supplementary-material

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REVIEW
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Role of the Peripheral Nervous System in PD Pathology, Diagnosis, and Treatment

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Studies on Parkinson disease (PD) have mostly focused on the central nervous system—specifically, on the loss of mesencephalic dopaminergic neurons and associated motor dysfunction. However, the peripheral nervous system (PNS) is gaining prominence in PD research, with increasing clinical attention being paid to non-motor symptoms. Researchers found abnormal deposition of α-synuclein and neuroinflammation in the PNS. Attempts have been made to use these pathological changes during the clinical diagnosis of PD. Animal studies demonstrated that combined transplantation of autologous peripheral nerves and cells with tyrosine hydroxylase activity can reduce dopaminergic neuronal damage, and similar effects were observed in some clinical trials. In this review, we will systematically explain PNS performance in PD pathology and its clinical diagnostic research, describe PNS experimental results [especially Schwann cell (SC) transplantation in the treatment of PD animal models] and the results of clinical trials, and discuss future directions. The mechanism by which SCs produce such a therapeutic effect and the safety of transplantation therapy are briefly described.

Keywords: Parkinson's disease, dopaminergic neuron, peripheral nervous, Schwann cell, graft

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INTRODUCTION

Parkinson disease (PD) is a typical neurodegenerative disorder of the nervous system. A pathologic hallmark of PD is the presence of intracytoplasmic inclusions known as Lewy bodies (LBs) composed of α-synuclein aggregates in neurons in the pars compacta of the substantia nigra (SN). The 6-stage theory proposed by Braak to explain the pathology and clinical development of PD postulates that neurodegeneration begins in the peripheral nervous system (PNS), with progressive involvement of the central nervous system (CNS) from caudal to rostral brain areas and corresponding clinical manifestations (Braak et al., 2003; Braak and Del Tredici, 2017). This hypothesis has been substantiated by studies demonstrating α-synuclein deposition in the gastrointestinal nervous system (Qualman et al., 1984; Kupsky et al., 1987; Wakabayashi et al., 1988; Iranzo et al., 2014) and cardiac denervation detected by imaging preceding the loss of dopaminergic neurons in the SN (Salsone et al., 2012). Although it cannot fully explain the clinical course of PD, this theory provides a framework for studying of PD and has yielded important insights into the disease.

The clinical diagnosis of PD is mostly based on motor symptoms, which may not appear until there is damage to 30–50% of dopaminergic neurons in the brain (Fearnley and Lees, 1991; Ma et al., 1997; Lang and Lozano, 1998). As such, early diagnosis of PD is critical for preserving the integrity of motor neurons and related motor functions. Although there are no reliable blood biomarkers for PD, imaging of dopaminergic neurons is a reliable method for detecting early-stage PD. Positron emission tomography (PET) and single-photon emission computed tomography (SPECT) can reveal the density of presynaptic terminals of SN dopaminergic neurons projecting into the striatum for early and accurate assessment of disease progression (Brooks and Pavese, 2011). Meanwhile, new treatment methods such as transplantation of induced pluripotent stem cells are being investigated (Kikuchi et al., 2017; Kikuchi et al., 2018), although their safety and efficacy require further assessment, and ethical concerns must be addressed (Yasuhara et al., 2017; Sonntag et al., 2018). The diagnosis and treatment of PD based on CNS manifestations does not take into account all aspects of the disease, and increasing attention has been paid to PNS involvement (Wakabayashi et al., 2010; Comi et al., 2014). Specifically, Schwann cells (SCs) have been studied for their potential to support injured neurons and promote neuroregeneration (Kim et al., 2013; Brosius Lutz and Barres, 2014; Jessen et al., 2015). SCs have also been linked to pathologic changes in PD and may be important for its clinical diagnosis as well as treatment (Tu et al., 1998; Timmer et al., 2004; Zhang et al., 2019). This review discusses PNS manifestations in PD, with a focus on the role of SCs in the pathologic changes associated with the disease and their potential application to PD diagnosis and treatment (**Figure 1**).

PATHOLOGIC CHANGES IN THE PNS IN PD

Parkinson disease is a multisystem disease with numerous clinical manifestations. In addition to the typical motor symptoms, there are many non-motor symptoms such as skin sensory and olfactory abnormalities and autonomic dysfunction, which are accompanied by aberrant protein deposition in the PNS. In PD patients with dysphagia, α-synuclein aggregates have been detected in the pharyngeal motor and sensory branches of the vagus nerve, as well as in the glossopharyngeal and internal superior laryngeal nerves (Mu et al., 2013a,b). LBs have also been found in the dorsal vagus ganglion (Jackson et al., 1995; Kövari et al., 2007), parasympathetic sacral nuclei (Bloch et al., 2006), cardiac plexus (Orimo et al., 2008; Salsone et al., 2012), and esophageal and intestinal nerves (Qualman et al., 1984; Kupsky et al., 1987; Wakabayashi et al., 1988; Iranzo et al., 2014). Bilateral vagus nerve transection was shown to block the spread of α-synuclein from the gut to the brain and prevent neurodegeneration (Kim et al., 2019), supporting Braak's theory that PD spreads from the PNS to the CNS. LBs have also been found in the skin of PD patients (Dabby et al., 2006; Ikemura et al., 2008; Miki et al., 2010; Doppler et al., 2014; Sharma, 2014); in 279 postmortem specimens of the abdominal wall and upper arm skin, immunopositivity for phosphorylated α-synuclein was detected in unmyelinated fibers of the dermis in 20 of 85 patients with LB pathology in the CNS and adrenal glands, while the remaining 194 patients without LB pathology had negative immunoreactivity. A retrospective analysis of 142 patients with PD found that the sensitivity of this skin test was ∼70% (Ikemura et al., 2008); however, in a clinical trial, the detection rate of cutaneous α-synuclein was just 10%. In 2 out of 20 patients with confirmed PD, a skin test revealed abnormal α-synuclein accumulation in non-medullated fibers of the anterior chest skin. The loss of peripheral nerve fibers is an inherent feature of PD that reflects two aspects of abnormal α-synuclein aggregation in the CNS: axonal degeneration and neuronal death. Thus, detection of phosphorylated α-synuclein in dermal nerve fibers may be a highly specific method for PD diagnosis, albeit with low sensitivity (Doppler et al., 2014).

Schwann cells are abundant in the PNS, and their density around myelinated axons is markedly reduced in PD patients (Kanda et al., 1996). α-Synuclein immunoreactivity was observed in SCs near spinal nerve roots but not in the myelin sheath or axons; moreover, astrocytes and oligodendrocytes were positive for α-synuclein expression but oligodendrocyte progenitor cells were negative (Papadopoulos et al., 2006). A recent clinical study reported that phosphorylated α-synuclein deposits were present in SCs of the sural nerve in PD patients but almost absent in axons (Zhang et al., 2019). SCs with cytoplasmic structures containing phosphorylated α-synuclein have also been observed in patients with multiple system atrophy (MSA) (Nakamura et al., 2015). Thus, SCs are implicated in neurodegenerative diseases characterized by abnormal α-synuclein accumulation and may serve as a pathologic marker for disease diagnosis.

Neuroinflammation is an important pathological manifestation of PD, and reactive microglia were first found in the brains of PD autopsy cases over 30 years ago (McGeer et al., 1988). Microglia can scavenge abnormal α-synuclein produced by neurons and produce a variety of pro-inflammatory factors, such as interleukin (IL)-1β, IL-6, and tumor necrosis factor-α (TNF-α) in the brain and cerebrospinal fluid (CSF) of patients (Harms et al., 2021). The researchers also found large numbers of activated microglia in areas outside the SN, and this activation was not directly linked to the presence of LBs, suggesting that the role of microglia in PD neuroinflammation goes beyond the scavenging of abnormal proteins and dead neurons (Imamura et al., 2003). Axonal degeneration of peripheral nerves and activation of SCs with secretion of multiple inflammatory factors are also observed in PD patients, but these lesions are not necessarily associated with sensory abnormalities in patients (Zhang et al., 2020). Welleford et al. performed RNA sequencing on the sural nerve of six patients undergoing DBS surgery. They first intercepted a section of the sural nerve. Two weeks after the initial injury, another section of the nerve fascicles of the distal and pre-degenerated stump of the nerve was dissected and processed for RNA-sequencing studies. The results showed that SCs upregulated the expression of genes related to dedifferentiation, immunity, and growth, such as IL-6, IL-10, leukemia inhibitory factor (LIF), and glial cell-derived neurotrophic factor (GDNF) significantly increased

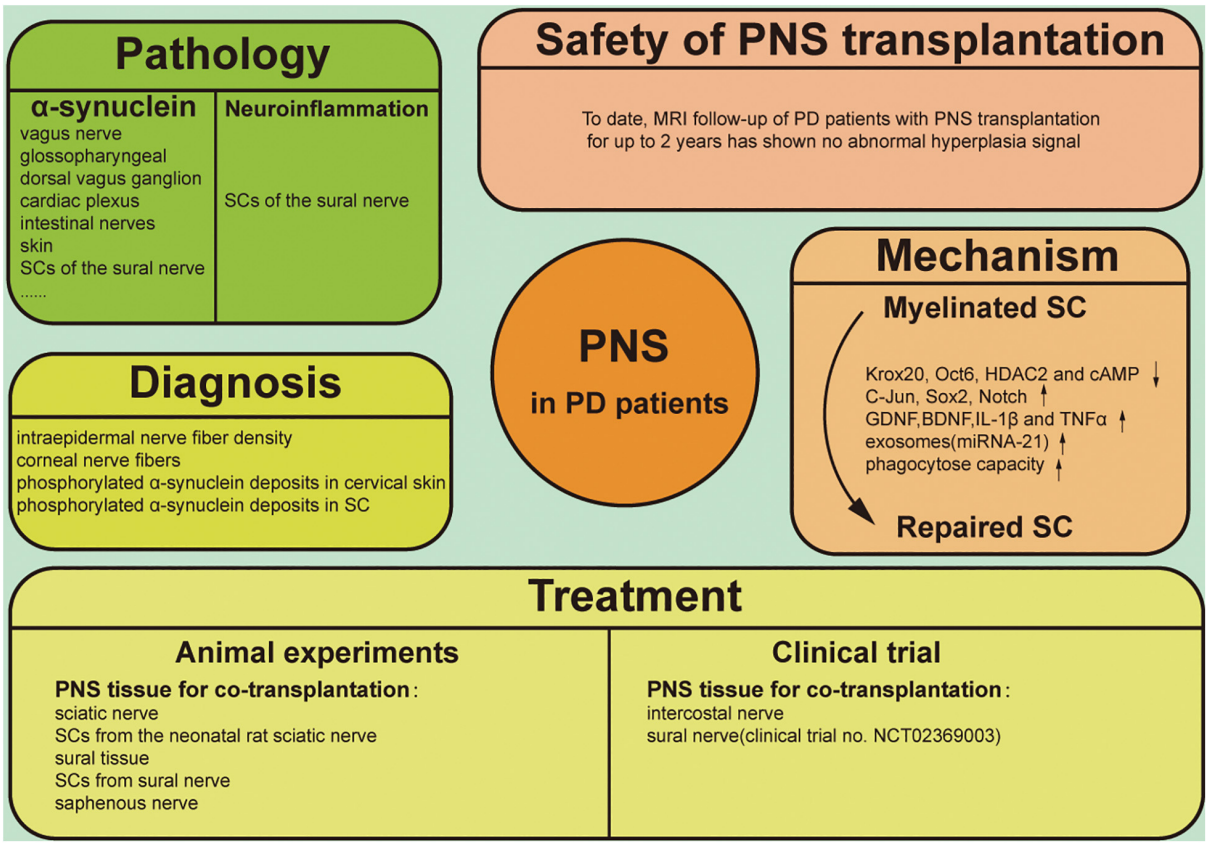

FIGURE 1 | Graphical illustration of PNS involving the pathology, diagnosis, treatment, therapeutic mechanism and safety of clinical application in PD.

(Welleford et al., 2020). Collectively, the evidence indicates that SCs are involved in PD neuroinflammation.

PNS IN THE DIAGNOSIS OF PD

Peripheral nervous system manifestations of PD are useful for adjunctive diagnostic screening, and skin biopsies are widely used for diagnosis (**Table 1**). Intraepidermal nerve fiber density was lower in PD patients than in healthy subjects, with a greater reduction on the more severely affected side that was accompanied by localized skin sensory and tactile abnormalities (Lin et al., 2016; Nolano et al., 2017; Jeziorska et al., 2019). Another study confirmed that the densities of intraepidermal and corneal nerve fibers were significantly reduced in PD patients compared to normal subjects (Kass-Iliyya et al., 2015). Notably, epidermal nerve fiber density and autonomic innervation were significantly reduced in idiopathic (I) PD patients, while skin biopsies in patients with parkinsonism were normal (Giannoccaro et al., 2015). IPD patients also had small nerve fiber lesions in the skin of the legs and phosphorylated α-synuclein deposits in cervical skin, whereas patients with parkinsonism of different etiologies were presumed to lack synuclein deposits and had normal nerve fibers, with no pathologic protein deposits (Donadio et al., 2014). Phosphorylated α-synuclein was mostly deposited in SCs and largely absent in the axons (Zhang et al., 2019). It should be noted that α-synuclein deposition is not an exclusive feature of PD; it is also observed in patients with dementia with LBs and MSA (Tu et al., 1998; Giasson et al., 2000; Wakabayashi et al., 2002; Nishie et al., 2004; Foulds et al., 2012) and therefore cannot be used on its own to diagnose PD.

APPLICATIONS OF PNS IN PD TREATMENT

Evidence for SC Involvement in PD From Animal Models

Although the relationship between pathologic changes and PD progression is not fully understood, the therapeutic potential of SCs for PD has been widely investigated in animal experiments based on their neuroregenerative capacity in the PNS (**Table 2**). In the 1980s, Aguayo and colleagues demonstrated that PNS grafts could support CNS neuron survival following injury and guide axonal regeneration in mice. Transplanted dopaminergic neurons survived and extended fibers into a peripheral nerve bridge formed by homotopic sciatic nerve covering the skull that connected the midbrain tissue graft

TABLE 1 | Application of PNS in clinical diagnosis and trials.

Authors	Patient	Diagnosis method	Outcomes
Clinical diagnosis			
Lin et al., 2016	Twenty-eight PD patients	Skin biopsy, contact heat-evoked potential (CHEP)	PD patients had reduced intraepidermal nerve fiber density and CHEP amplitude
Jeziorska et al., 2019	Twenty-three PD patients	Skin biopsy	Intraepidermal nerve fiber density and subepidermal nerve fiber length were lower in more affected versus less affected side
Nolano et al., 2017	Fifty-four PD patients	Skin biopsy	Intraepidermal nerve fiber density was lower in patients and the loss of it was higher in the more affected side
Kass-Iliyya et al., 2015	Twenty-six PD patients	Corneal confocal microscopy (26/26), skin biopsy (24/26)	PD patients had significantly reduced in corneal nerve fiber density and intraepidermal nerve fiber density
Giannoccaro et al., 2015	Twenty-two idiopathic Parkinson disease (IPD), and eleven parkinsonism patients	^{123}I-MIBG myocardial scintigraphy and Skin biopsy	In the IPD group, both ^{123}I-MIBG scintigraphy and skin biopsy results were abnormal in 91% of patients. In parkinsonism, results of both tests were normal in all patients
Donadio et al., 2014	Twenty-one IPD and twenty parkinsonism patients	Skin biopsy	IPD patients showed a small nerve fiber neuropathy prevalent in the leg with phosphorylated a-synuclein deposited in the cervical skin. Parkinsonism patients did not show these signs
Zhang et al., 2019	Sixteen PD patients	Sural nerve biopsy	Deposition of phosphorylated α-synuclein was found in 16/16 PD patients
Authors	**Patient**	**Implantation method**	**Outcomes**
Clinical trials			
Date et al., 1995	A 55-year-old woman and a 43-year-old patient with advanced Parkinson's disease	Co-grafts of adrenal medulla and peripheral nerve into the bilateral caudate nuclei	Both patients showed improvement in PD symptoms after transplantation
Watts et al., 1997	Five patients with advanced Parkinson's disease	Patients received unilateral intrastriatal adrenal medulla-intercostal nerve co-grafts	The clinical improvement from this procedure is sustained for 24 months
López-Lozano et al., 1999	Four patients PD	The adrenal medulla and intercostal nerve were implanted into right caudate nucleus	Improved symptoms in on and off phases persist in all four cases
Nakao et al., 2004	Four patients with PD	Thoracic sympathetic ganglia into the brain	Two fold increase in the duration of the "on" phase induced by a single dose of levodopa
van Horne et al., 2018	Eight patients with a diagnosis of idiopathic PD	Patients receive bilateral DBS and unilateral segments of the sural nerve of the STN	The lateralized UPDRS scores showed a more significant overall reduction in scores on the side contralateral to the graft

over the superior colliculus and the caudate-putamen nucleus (Aguayo et al., 1984).

To investigate the influence of the CNS environment on peripheral neuron grafts, one study compared the therapeutic efficacy of adrenal medullary cells transplanted alone or with sciatic nerve fragments into the striatum of mice with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced PD (Date et al., 1990). The results showed that the number of tyrosine hydroxylase (TH)-positive chromaffin cells, density of TH fibers, and concentration of dopamine in the brain were higher in the sciatic nerve group. Similarly, grafting of the sciatic nerve along with embryonic midbrain tissue in a rat model of 6-hydroxydopamine (6-OHDA)-induced PD resulted in greater improvement in an apomorphine-induced rotation test compared to the monograft group (van Horne et al., 1991). Immunofluorescence analysis revealed the secretion of basement membrane components by SCs, but an immune response was rarely observed. Collectively, these results suggest that PNS grafts can survive in the CNS and can promote the orderly growth of new nerve fibers, with consequent functional improvements.

In animal experiments where PNS fragments were transplanted into the brain for therapeutic purposes, SCs were identified as the functional component. Direct grafting of SCs is another possible approach for neuronal regeneration in

TABLE 2 | Application of PNS in animal experiments.

Authors	Animal model	Graft	Outcomes
Animal test			
Aguayo et al., 1984	6-OHDA lesioned right nigrostriatal of Female Sprague-Dawley rats	Fetal mesencephalic and heterologous sciatic nerve	Monoaminergic neurons within the implant extended axons along the entire length of the nerve bridges and some of these fibers extended into the striatum
Date et al., 1990	MPTP-treated male C57BL/6 mice	Adrenal medullae and sciatic nerve	The co-grafted mice showed a better survival of adrenal medullary chromaffin cells and longer fibers of host DA neurons
van Horne et al., 1991	6-OHDA lesioned male Fisher-344 rats	Fetal ventral mesencephalic and sciatic nerve	The co-graft group revealed a significantly more significant decrease in rotation than the VM group
Wilby et al., 1999	6-OHDA lesioned female rats	SCTM41 ± GDNF and nigra graft	Co-grafts improved the survival of intrastriatal embryonic dopaminergic neuronal grafts. Bridge grafts promoted the growth of axons through the grafts to the striatum
Timmer et al., 2004	6-OHDA lesioned medial forebrain bundle (MFB) of female Sprague–Dawley rats	Ventral mesencephalic tissue and Schwann cells	21/23 kDa FGF-2-secreting SCs promoted the survival of dopaminergic neurons
Kordower et al., 1990	MPTP lesioned aged female rhesus monkeys	Autologous adrenal chromaffin cells and sural nerve	Co-grafted chromaffin cells exhibited multipolar neuritic processes and numerous chromaffin granules
Watts et al., 1995	Right hemi parkinsonian by left intracarotid injection of MPTP	Autologous adrenal chromaffin cells and sural nerve	Animals undergoing autologous co-grafts demonstrated improved motor performance than the control animal
Xia et al., 2012	6-OHDA lesioned right caput nuclei caudate and right dorsal caudate putamen	SCs and NSCs	Co-transplantation of SCs and NSCs could effectively cure PD in macaques
Collier et al., 1994	MPTP lesioned adult male St Kitts African Green monkeys	Monkey saphenous nerve and embryonic ventral mesencephalic tissue	Morphological observations indicated that no evident augmentation of the morphology of grafted dopamine neurons
Howel et al., 2000	MPTP-induced hemi parkinsonian model in rhesus monkeys	Adrenal chromaffin cells and sural nerve	Recovery of behavioral function after surgical treatment, with adrenal co-grafted monkeys showing the highest degree of improvement

the brain. To this end, GDNF-secreting SCs were engineered by lentiviral transduction from neonatal rat sciatic nerve cultures purified from SCs (SCTM41) (Wilby et al., 1999). Transplantation of either SCTM41 or SCTM41-GDNF improved the survival of intrastriatal embryonic dopaminergic neuron grafts and promoted neurite outgrowth into the host neuropil, although SCTM41-GDNF had a more potent effect. In a bridging experiment, both types of SCs induced axonal growth of grafted cells into the striatum, with SCTM41-GDNF being more effective both in terms of the density and total number of TH-positive axons. In another study, modification of SCs to secrete basic fibroblast growth factor-2—a cytokine with mitogenic and pro-survival activities—promoted graft survival and neuronal growth in the CNS (Timmer et al., 2004).

Primate studies are necessary to verify the clinical applicability of SCs to treat PD. When equal amounts of autologous adrenal medulla and sural tissue were transplanted into the brains of rhesus monkeys, the graft survival rate was 4–8 times higher than with adrenal medulla transplantation alone (Kordower et al., 1990). Moreover, medullary cells in the co-transplant group had more chromaffin granules and neurites that formed synaptic connections with surrounding axons. These findings were substantiated by a similar study (Watts et al., 1995). Rhesus monkeys with 6-OHDA-induced injury transplanted with neural stem cells from aborted fetal mesencephalic tissue in combination with SCs showed improved motor function 1 month later, with recovery of fine motor control after 4 months; PET scanning revealed accumulation of ^{18}F-FP-β-CIT (a radioligand used for dopamine transporter quantification) in the injured striatum (Xia et al., 2012). However, cell-based therapy in primates has not been as effective as anticipated. For instance, there was no significant functional improvement when a hollow tube with a semi-permeable polymer as a carrier was used to insert a saphenous nerve segment into the lateral ventricle, with transplantation of monkey embryo-derived mesencephalon tissue into the caudate nucleus 2 mm rostral and 2 mm caudal to the polymer implant (Collier et al., 1994). Although the authors claimed that this experimental strategy avoided the brain tissue damage associated with conventional transplantation, spatial separation between the transplanted and native tissues may have prevented successful graft integration. Monkeys treated by co-transplantation of sural nerve and an adrenal graft showed greater behavioral improvements than the surgical control; however, the 3,4-dihydroxyphenylacetic acid and homovanillic acid levels in CSF were not different between

the two groups after 10–12 months (Howel et al., 2000). Moreover, there was significant interindividual variability in fine hand movement recovery, which precluded group analysis. This problem is rarely encountered in behavioral studies using rodents and highlights the complexity of primate behavior. Given the importance of primate experiments for developing new therapies, more rational designs for behavioral experiments and refinement of pathophysiologic tests are needed.

Personalized PD Therapy Using SCs

Schwann cells have been used to treat spinal cord injury (Saberi et al., 2008, 2011; Yazdani et al., 2013; Oraee-Yazdani et al., 2016; Anderson et al., 2017), but few trials have investigated their use in PD (**Table 1**). Several trials conducted in the 1990s showed that adrenal medulla transplantation combined with peripheral nerve grafting alleviated motor impairment (Date et al., 1995, 1996; Watts et al., 1997; López-Lozano et al., 1999). These studies used autologous sympathetic nerves as grafts and reported a significantly longer "on" period in four patients during follow-up. At the 1-year follow-up of eight PD patients who underwent deep brain stimulation (DBS) of the subthalamic nucleus with concurrent autologous sural nerve grafting in the SN, the Unified Parkinson's Disease Rating Scale III (UPDRs-III) scores decreased from 32.5 ± 9.7 at baseline to 25.1 ± 15.9, with a more prominent reduction for the contralateral limb. The complications associated with obtaining the autologous sural nerve were that three participants described a patch of numbness on the lateral aspect of the foot. This phenomenon was also manifested in some patients who took biopsy of the sural nerve, and one patient developed local superficial cellulitis and recovered after antibiotic treatment. Postoperative magnetic resonance imaging (MRI) showed there was no significant edema or hemorrhage in the graft target zone (van Horne et al., 2018). The number of patients in these trials was low, but a phase 1 clinical trial has been initiated with an estimated enrollment of 72 patients (clinical trial no. NCT02369003). The study started in February 2015 and plans to complete the primary outcome in September 2020. Adverse events will be collected to measure the safety and tolerability of the grafting procedure. Dopamine neurodegeneration at 12 or 24 months will be assessed using SPECT imaging and compared to scans obtained taken before DBS surgery. This trial will investigate whether autologous SCs are effective in repairing lesioned dopaminergic neurons in the CNS. If successful, it would herald the development of a new treatment for PD in which patients can provide their own tissue as a source of growth factors that could arrest or reverse the ongoing cellular loss underlying their devastating dysfunction.

There are some outstanding issues that must be addressed regarding the therapeutic application of autologous SCs. First, the intercellular interactions and molecular mechanisms of SCs in promoting neuronal regeneration remain to be elucidated. Secondly, LBs have been detected in the SCs of PD patients, calling into question the safety of using autologous SCs for cell-based therapy. On the other hand, clinical trials have shown that such pathologic alterations do not influence the therapeutic effects of SCs in CNS; in fact, α-synuclein accumulation may activate SCs, thereby enhancing nerve repair.

SC MECHANISMS FOR NEURONAL REPAIR AND REGENERATION

Schwann cells are a type of peripheral glial cell originating from the neural crest that initially differentiate into SC precursor cells, then immature SCs, and finally into myelinating and non-myelinating cells that retain the ability to dedifferentiate to an immature SC state (Bhatheja and Field, 2006; Griffin and Thompson, 2008; Jessen et al., 2015). SCs secrete a basal lamina composed of growth-promoting laminin, type IV collagen, and heparin sulfate proteoglycans (Bunge et al., 1990), which are critical for the SC myelinating function. More importantly, SCs proliferating after nerve injury may form a channel that promotes axonal regeneration along residual SC structures known as Büngner bands (Chen et al., 2007; Faroni et al., 2015). In contrast, oligodendrocytes in the CNS do not secrete basal lamina, so the healthy CNS is free of these basal lamina components except at the pial surface and sites of contact between astrocytes and blood vessels (Squire et al., 2009; Martini et al., 2010). SC maturation is accompanied by the establishment of autocrine circuits involving platelet-derived growth factor, insulin-like growth factor-1, and neurotrophin-3 that allow SCs to survive after nerve injury and promote peripheral nerve regeneration (Jessen and Mirsky, 1999). Up to 30 or 40% of oligodendrocytes may be lost following CNS injury, and those that survive may be unable to support neuroregeneration (Ludwin, 1990). SC autocrine and cytokine secretion functions are not limited to the PNS, as demonstrated in animal models and clinical trials of SC transplantation into the brain.

Exosomes are secretory vesicles containing mRNA and microRNA secreted by cells, and they have a variety of biological effects. It is thought that supplementing their natural function could enable targeted delivery of drug molecules (Mehryab et al., 2020). Multiple studies have confirmed that SC can secrete exosomes and that they have a powerful promotion effect on neuronal axon regeneration and improve neuronal viability (Hu et al., 2019; Yu et al., 2021). It was found that miRNA-21 expression was increased in secreted exosomes after SC upregulated the expression of c-jun and Sox2, which are key in promoting neurite growth by SC-derived exosomes (López-Leal et al., 2020). Glutamate and calcium ions may also play a role in this process (Hu et al., 2019).

Following neuron injury (**Figure 2**), SCs phagocytose cell fragments and secrete inflammatory factors such as TNF-α, LIF, IL-1, and IL-6 that recruit other phagocytes (Kass-Iliyya et al., 2015; Kikuchi et al., 2018). Fatty acids released during myelin breakdown also regulate the inflammatory response by producing prostaglandins and leukotrienes that facilitate immune cell penetration into damaged nerve tissue (Martini et al., 2008). Phagocytosed SC-derived myelin fragments induce macrophage differentiation toward an anti-inflammatory phenotype (Boven et al., 2006). SCs induce the egress of macrophages out of Büngner bands during subsequent myelination by interacting

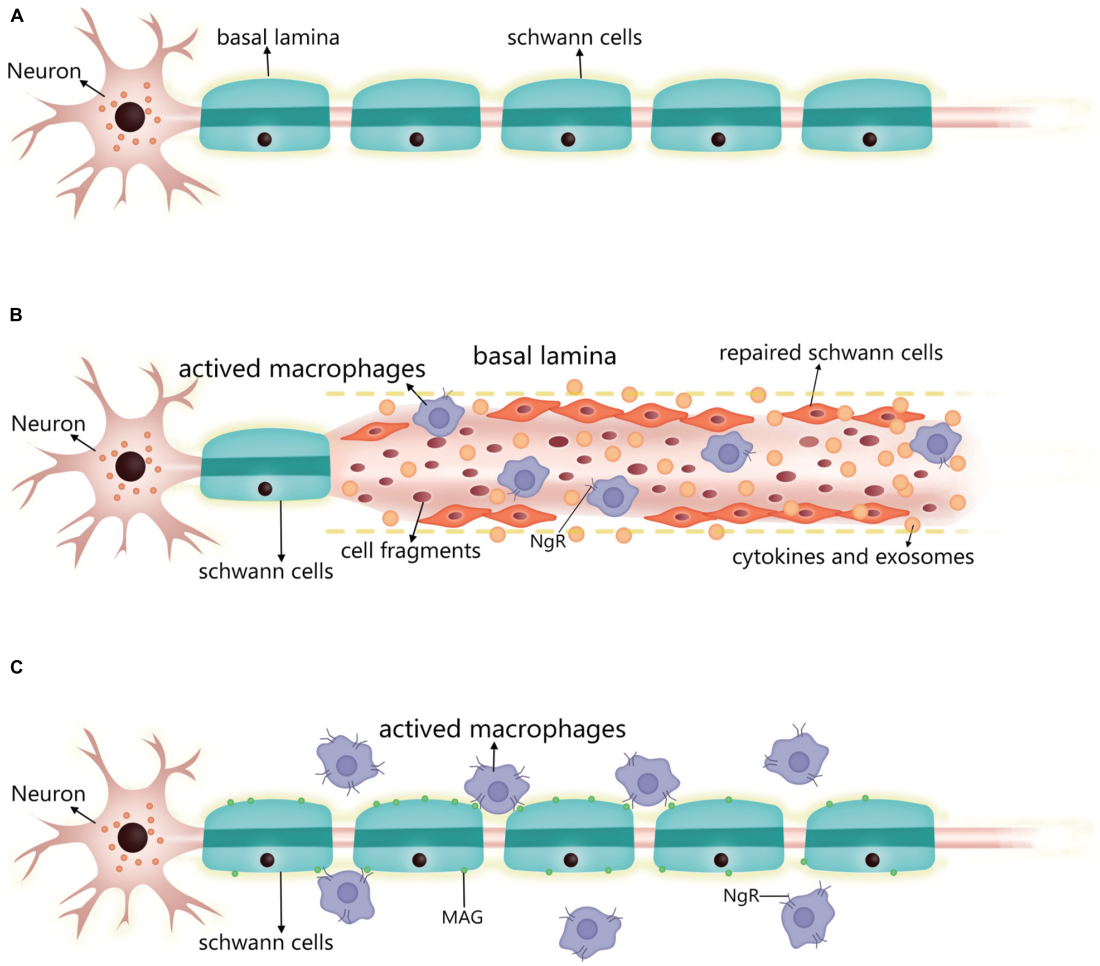

FIGURE 2 | Changes in SC after PNS injury and the performance of the nerve repair process. **(A)** Normal myelinated SC and basal lamina formation. **(B)** Post-injury, residual SCs upregulate repair-related signals into repaired SCs, which can align along the residual basal lamina to guide neuronal axon growth, while secreting cytokines and exosomes to promote neuronal survival and neurite growth. The repaired SCs also enhance phagocytosis to scavenge cell fragments, secreting inflammatory factors to regulate surrounding inflammation and recruiting macrophages into the injury to enhance cell fragment clearance. At this stage, the macrophage surface expresses a small amount of NgR. **(C)** After the repair is complete, the SC re-formed myelin sheath wraps around the axon and produces a basal lamina. The SC expresses MAG, which interacts with the macrophage surface NgR to induce the macrophage to migrate out of the nerve. At this stage, macrophage expression of NgR is significantly increased. SC, Schwann cell; PNS, peripheral nervous system; NgR, Nogo receptor; MAG, myelin-associated glycoprotein.

with the macrophage Nogo receptor (NgR) and myelin-associated glycoprotein (Fry et al., 2007; David et al., 2008). If these macrophages are not cleared, their prolonged residence in the nerve can lead to chronic inflammation and nerve damage. Thus, following axonal injury, surviving SCs secrete cytokines and chemokines that prevent further neuronal damage, participate in postinjury debris removal, recruit macrophages that participate in debris clearance, and modulate the local inflammatory response.

The myelinated SC is transformed into a repair state after injury in two steps. The expression of myelination-related signaling molecules such as Krox20, Oct6, HDAC2, and cAMP is downregulated, while that of repair-related signaling molecules such as c-jun, Sox2, and Notch is promoted (Jessen and Arthur-Farraj, 2019). c-Jun is a key regulator in the SC injury response. After injury, it is rapidly upregulated and negatively regulates the myelin program, and promotes expression of the repair program (Arthur-Farraj et al., 2012; Fazal et al., 2017). Additionally, zinc finger E-box binding homeobox (Zeb) 2 has been identified as an essential regulator of SC differentiation, myelination, and nerve repair (Quintes et al., 2016). The timing of the expression of these reparative molecules differed after injury, with cytokine expression (e.g., IL-1β and TNFα) showing upregulation 1 day after injury, GDNF expression peaking at about 1 week, brain-derived neurotrophic factor expression peaking at 2–3 weeks, and c-jun also being rapidly expressed after injury and lasting at least 7–10 days (Rotshenker, 2011; Jessen and Mirsky, 2019). Moreover, SCs were also found to upregulate some inflammation-related signaling molecules and secrete pro-inflammatory factors

to regulate the inflammatory response at the affected site 2 weeks after the injury (Kikuchi et al., 2018; Welleford et al., 2020). In conclusion, the transition process of SCs to the repair form after injury is very complex and regulated by multiple factors.

SAFETY OF CELL-BASED THERAPY

There are strict ethical and safety requirements for human cell transplantation therapy. Animal-derived mesencephalic tissue is commonly used in animal experiments but has little possibility of being used in clinical settings. Even the use of human embryonic stem cells from aborted fetuses for clinical purposes is hampered by tremendous ethical obstacles. Therefore, donor tissue is usually derived from patients themselves, which has the advantage of a reduced risk of graft rejection.

A major concern in cell-based therapy is the possibility that autologous cells can grow and differentiate into neoplasms in a non-native environment. In one case study of a 43-year-old female patient, cranial MRI at 1, 12, and 24 months following autologous peripheral nerve transplantation showed signal enhancement at the graft site but there were no abnormal signals in other brain regions (Date et al., 1995). In a similar study, no abnormal hyperplasia, cerebral infarction, or cerebral hemorrhage was detected on MRI of the graft site 1 year after transplantation surgery (van Horne et al., 2018). Although these results are encouraging, they do not provide sufficient evidence for the safety of peripheral neuron grafting in the treatment of PD, given the paucity of cases. However, relevant insight can be garnered from studies in which peripheral neurons were used for the treatment of injured spinal cord, which is part of the CNS. In eight patients with chronic spinal cord injury who were transplanted with mesenchymal stem cells combined with SCs, MRI examination at 6, 12, and 18 months postsurgery showed no tumor-like tissue growth (Yazdani et al., 2013). Similarly, in six patients with subacute spinal cord injury treated with autologous SCs of sural nerve origin, there was no MRI evidence of hyperplasia up to 12 months later (Anderson et al., 2017).

CONCLUSION

The PNS is relevant to the diagnosis and treatment of PD, but its clinical application has not been fully exploited. For instance, it may be possible to develop a compound that can transiently chelate α-synuclein *in vivo* with no harm or low toxicity to humans, and detection of this complex by radiographic or other imaging methods could provide a diagnostic tool for PD and other neurodegenerative diseases characterized by abnormal protein aggregation. PD treatment should also be differentiated according to the disease stage, in accordance with Braak's six-stage theory. There are no incompatibilities between drugs, DBS, and cell-based therapy in the treatment of PD, but the timing of each intervention may be critical for maximizing efficacy. Intracerebral therapy by transplantation of autologous SCs derived from the PNS is a promising therapeutic strategy that can potentially prevent or slow PD progression. For this approach to be successful, basic questions such as the mechanisms underlying the interaction between a patient's own SCs and brain dopaminergic neurons must be answered.

AUTHOR CONTRIBUTIONS

MC designed the study. CM and WZ reviewed the literature. CM wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Monoamine Levels and Parkinson's Disease Progression: Evidence From a High-Performance Liquid Chromatography Study

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Parkinson's disease (PD) is associated with dysfunction of monoamine neurotransmitter systems. We investigated changes in the levels of monoamine and their metabolites in PD patients, together with their association to clinical profiles. PD patients and age-matched control subjects (n = 40 per group) were enrolled. Using high-performance liquid chromatography (HPLC) with an electrochemical detector, levels of monoamines (dopamine, DA; norepinephrine, NE; epinephrine, EPI; and serotonin, 5-HT) were measured in plasma, while the metabolites (homovanillic acid, HVA; vanillylmandelic acid, VMA; and 5-hydroxyindoleacetic acid, 5-HIAA) were measured in urine. Plasma DA level was not significantly different between PD and control groups. PD patients had significantly higher plasma NE but lower EPI and 5-HT levels. PD patients had a significantly higher HVA/DA ratio and lower VMA/NE ratio than control subjects, while the 5-HIAA/5-HT ratio was not different between the groups. Regarding the association between monoamine levels and clinical profiles, the DA level had a negative relationship with disease duration and the 5-HT level had a negative relationship with severity of motor impairment. These findings emphasized the involvements of several neurotransmission systems and their association with clinical profiles in PD patients, demonstrated by quantification of monoamine levels in peripheral body fluids. This could benefit appropriate pharmacological treatment planning in respect of monoamine changes and might also help predict subsequent clinical symptoms.

Keywords: Parkinson's disease, monoamine, dopamine, norepinephrine, epinephrine, serotonin

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INTRODUCTION

Parkinson's disease (PD) is a neurodegenerative disorder characterized by motor impairments including bradykinesia, rigidity, rest tremor, and postural instability, combined with a variety of non-motor symptoms (Przedborski, 2017). Hallmark pathologies of PD are Lewy body deposition and progressive deterioration of dopaminergic neurons in substantia nigra, leading to depletion of central dopamine (DA) level (Braak et al., 2003). There is no specific test for PD diagnosis

nowadays. Neurologists usually diagnose PD based on clinical assessment and dopaminergic medication responsiveness.

Apart from abnormality of DA, neuroimaging and post-mortem brain tissue studies have demonstrated imbalances of other monoamine neurotransmitters in PD including norepinephrine (NE), epinephrine (EPI), and serotonin (5-HT) (Barone, 2010). Thus, management of PD has focused on several affected neurotransmitter systems rather than the dopaminergic system alone (Barone, 2010). However, there is lack of information on the alteration of monoamine levels measuring in peripheral body fluids in different stages of PD. In addition, many reports showed contradictory information regarding the association between monoamine dysfunctions and clinical profiles of PD such as age, gender, medications, disease duration, and severity (Lunardi et al., 2009; Olivola et al., 2014; Kaasinen and Vahlberg, 2017).

The objective of this study was to investigate the alteration of DA, NE, EPI, and 5-HT levels in plasma along with levels of their metabolites in the urine of PD patients. Additionally, we aimed to compare the levels of monoamines in early and advanced stages and also evaluate their associations with clinical profiles including age, gender, L-DOPA equivalent daily dose (LEDD), disease duration, and motor severity.

MATERIALS AND METHODS

Participants

Participants in this study were male and female PD patients aged between 30 and 80 years and healthy gender- and age-matched control groups. Based on a previous report (Tong et al., 2015), the sample size estimation for our primary objective (PD versus control groups) was 16 participants per group ($\alpha = 0.05$, 80% power). However, our secondary objective was to compare the monoamine levels between early- and advanced-stage patients in the PD group. We thus recruited the PD patients until there were at least 16 participants categorized in either early or advanced stages. Eventually, there were 40 participants in the PD group (24 and 16 participants categorized in early and in advanced stages, respectively) and 40 participants in the control group. Patients were diagnosed with PD according to the UK Parkinson's Disease Society Brain Bank Clinical Diagnostic Criteria and recruited between February 2018 and November 2019 from the outpatient clinic at Chulalongkorn Centre of Excellence on Parkinson's Disease and Related Disorders, King Chulalongkorn Memorial Hospital, Thailand.[1] Demographic information and clinical data including disease duration, comorbidities, current medications, and LEDD were recorded. Disease severity was assessed based on the modified Hoehn and Yahr (HY) scale and was classified as early and advanced stages when scales were 1–2.5 and 3–5, respectively. Cognitive function was evaluated using the Thai Version of Mini-Mental State Examination (MMSE-Thai 2002). Patients were excluded when (1) they had other identified central nervous system abnormalities such as cognitive disorders, cerebrovascular disease, and traumatic brain injury; (2) they had psychiatric comorbidities; and (3) they were taking medications which possibly interfere the monoamine concentration including selective 5-HT reuptake inhibitors, 5-HT-NE reuptake inhibitors, tricyclic antidepressants, neuroleptics, and β-adrenergic antagonists. Participants had discontinued parkinsonian drugs or other medications which could disturb monoamine levels at least 12 h prior to collection of the specimens, as stated in a previous study (Lian et al., 2018). Foods and beverages including coffee, tea, banana, chocolate, vanilla, and citrus fruits were also restricted during this period.

[1] www.chulapd.org

Neurotransmitter and Metabolite Levels Determination

Specimen Collection

Plasma and urine were obtained to determine the neurotransmitter and metabolite concentrations, respectively. Blood samples (3 ml) were collected from a cubital vein, drawn into an EDTA tube, immediately centrifuged to separate plasma at 3,000 × *g* (4°C) for 10 min, and stored at −80°C until analyzed. Plasma DA, NE, EPI, and 5-HT were measured by HPLC with an electrochemical detector. At the same time, a single urine sample was collected in a container with 10 ml (32%) hydrochloric acid per liter of urine and pH was adjusted to 1–2. In these urine samples, the levels of homovanillic acid (HVA), vanillylmandelic acid (VMA), and 5-hydroxyindoleacetic acid (5-HIAA), metabolites of DA, NE/EPI, and 5-HT, respectively, were quantified.

HPLC Analysis

Levels of the neurotransmitter and metabolite were determined by the *ClinRep*® Complete Kits for catecholamines, 5-HT, and VMA/HVA/5-HIAA (*München, Germany*). Analysis was achieved with HPLC *Chrome* systems (*Gräfelfing, Germany*), consisting of isocratic pump CLC 300, programmable autosampler injection CLC 200, and electrochemical detector model CLC 100. The chromatographic peaks were separated by the *Recipe*'s special reversed-phase columns (*München, Germany*) and peaks identified by the *Easyline* analysis software program (*München, Germany*). Using an internal standard technique, monoamine concentrations were calculated by comparing the retention time and peak area with a calibration curve. Sample preparations and monoamine concentration measurements were performed according to the manufacturer's instructions as previously described (Andersen et al., 2017). Briefly, for determination of DA, NE, and EPI, plasma (1 ml) and an internal standard (50 μl) were centrifuged with a washing solution and eluting reagent. The eluted sample (40 μl) was then injected to an electrochemical detector (500 mV, 1 nA) at a flow rate of 1 ml/min. For analysis of 5-HT, plasma (200 μl) was mixed and centrifuged with internal standard (10 μl) and precipitant solution (200 μl). Supernatant (20 μl) was injected at a flow rate of 1 ml/min to the electrochemical detector (450 mV, 20 nA). For HVA/VMA/5-HIAA analyses, the urine sample (50 ml) and internal standard (1 ml) were mixed, washed, and added with the eluting reagent. The eluted solution (20 μl) was then injected to the electrochemical detector (800 mV, 50 nA) at a flow rate of 0.9 ml/min.

Statistical Analysis

We performed statistical analysis with *SPSS Statistics 22* (*IBM Corporation, New York, NY, United States*). All data were tested for normal distribution using Kolmogorov–Smirnov. The demographic data, monoamine levels, metabolite levels, and ratios between PD and control groups were compared using the independent *t*-test (two-tailed) for parametric data or Mann–Whitney *U*-test for non-parametric data. Comparisons of plasma monoamine levels between early and advanced stages of PD patients were also performed by the Mann–Whitney *U*-test. The relationships between plasma monoamine levels and clinical profiles were tested by using multiple linear regression models with the stepwise method. Plasma monoamine levels were log-transformed to fit the linear model. Age, gender, LEDD, disease duration, and motor severity were set as the covariates. The standardized coefficients (β), 95% confidence interval (CI), and coefficient of multiple determination (R^2) were presented. Statistical significance was defined as p-value < 0.05.

RESULTS

Demographic Data and Clinical Profiles

The mean ages of control subjects and PD patients were 55.5 ± 6.3 and 57.6 ± 8.5 years, respectively ($p = 0.22$). The majority of participants were male in both groups. In the PD group, the average disease duration was 13.2 ± 7.1 years and the mean LEDD was 1055.3 ± 656.9 mg/day (**Table 1**). Other PD-related medications including trihexyphenidyl 1–2 mg/day and clonazepam 0.25–2 mg/day had been taken by 6 (15.0%) and 15 (37.5%), respectively. In the PD group, histories of essential hypertension, type 2 diabetes mellitus, and hypercholesterolemia were documented in four (10.0%), two (5.0%), and three (7.5%) patients, respectively. Medications taken for their underlying diseases were amlodipine 5–10 mg/day in three (7.5%), enalapril 10 mg/day in one (2.5%), losartan 50 mg/day in one (2.5%), metformin 500–1,000 mg/day in two (5.0%), and statins in three (7.5%) patients.

PD patients were classified into two disease severity subgroups; 24 (60%) patients were in the early stage (modified HY stages 1–2.5), and 16 (40%) patients were in the advanced stage (modified HY stages 3–5). Early-stage patients were younger, had a shorter disease duration, and had a lower LEDD than the advanced-stage ones (**Table 2**). There were no significant differences in the proportion of patients taking trihexyphenidyl (20.8 and 6.2%, $p = 0.37$) and clonazepam (37.5 and 37.5%, $p = 1.00$) between the early and advanced subgroups, respectively.

TABLE 1 | Demographic data and clinical characteristics of control and PD groups.

Characteristics	Control($n = 40$)	Parkinson($n = 40$)	p-value
Age [years, mean ± SD]	55.50 ± 6.33	57.55 ± 8.48	0.22[a]
Males: females	22:18	27:13	0.30[b]
MMSE score [mean ± SD]	28.08 ± 1.94	28.50 ± 1.52	0.46[a]
Disease duration [years, mean ± SD]	NA	13.18 ± 7.11	–
Modified H&Y stages of PD			
- Average modified H&Y stage [mean ± SD]	NA	2.76 ± 1.06	–
- Frequency of patients [N (%)]			
Stage 1	NA	3 (7.5%)	–
Stage 1.5	NA	2 (5%)	–
Stage 2	NA	6 (15%)	–
Stage 2.5	NA	13 (32%)	–
Stage 3	NA	8 (20%)	–
Stage 4	NA	4 (10%)	–
Stage 5	NA	4 (10%)	–
LEDD [mg/day, mean ± SD]	NA	1055.3 ± 656.9	–

MMSE = Mini-Mental State Examination; LEDD = L-DOPA-equivalent daily dose; SD = standard deviation.
[a]*Independent t-test.*
[b]*Chi-square test.*

Comparisons of Plasma Monoamine Levels Between PD and Control Groups

Figures 1A–D show the levels of plasma monoamine and represent their HPLC chromatograms of the control and PD groups. The plasma DA level was not significantly different between PD patients and control subjects (389.85 ± 48.06 versus 346.45 ± 37.37 ng/l, $p = 0.864$). The plasma NE level was significantly higher in PD patients than in control subjects (1,336.72 ± 235.87 versus 295.48 ± 31.14 ng/l, $p < 0.001$). Compared to control subjects, PD patients had a significantly lower plasma EPI (584.70 ± 66.84 versus 676.73 ± 66.81 ng/l, $p = 0.027$) and 5-HT levels (14.81 ± 3.11 versus 31.20 ± 6.15 μg/l, $p = 0.014$).

Comparisons of Urinary Metabolite Levels Between PD and Control Groups

Figures 2A–C show the levels of urinary HVA, VMA, and 5-HIAA and the HPLC chromatograms of the control and PD groups, respectively. The urinary HVA level was significantly higher in PD patients than in control subjects (12.94 ± 1.78 versus 4.43 ± 0.45 mg/l, $p < 0.001$). The urinary VMA level was not significantly different between the PD and control groups (14.26 ± 2.94 versus 9.36 ± 1.10 mg/l, $p = 0.917$). On the other hand, the urinary 5-HIAA level was significantly lower in PD patients than in control subjects (1.54 ± 0.27 versus 4.14 ± 0.63 mg/l, $p < 0.001$).

Comparisons of the Metabolite/Monoamine Ratio Between PD and Control Groups

Figures 3A–D exhibit the ratio of HVA/DA, VMA/NE, VMA/EPI, and 5-HIAA/5-HT, respectively. The findings showed that PD patients had a significantly higher HVA/DA ratio than control subjects (0.054 ± 0.009 versus 0.021 ± 0.003, $p < 0.001$).

TABLE 2 | Demographic and clinical characteristics of early and advanced stage PD patients.

Characteristics	Early stage(n = 24)	Advanced stage(n = 16)	p-value
Age [years, mean ± SD]	54.3 ± 8.5	62.5 ± 5.7	0.001**[a]
Males: females	18:6	9:7	0.30[b]
Disease duration [years, mean ± SD]	10.8 ± 5.7	16.8 ± 7.7	0.01*[a]
LEDD [mg/day, mean ± SD]	886.6 ± 472.1	1,338.3 ± 798.3	0.04*[a]

LEDD = L-DOPA-equivalent daily dose; SD = standard deviation.
[a]*Independent t-test.*
[b]*Chi-square test.*
**p<0.05; **p<0.01.*

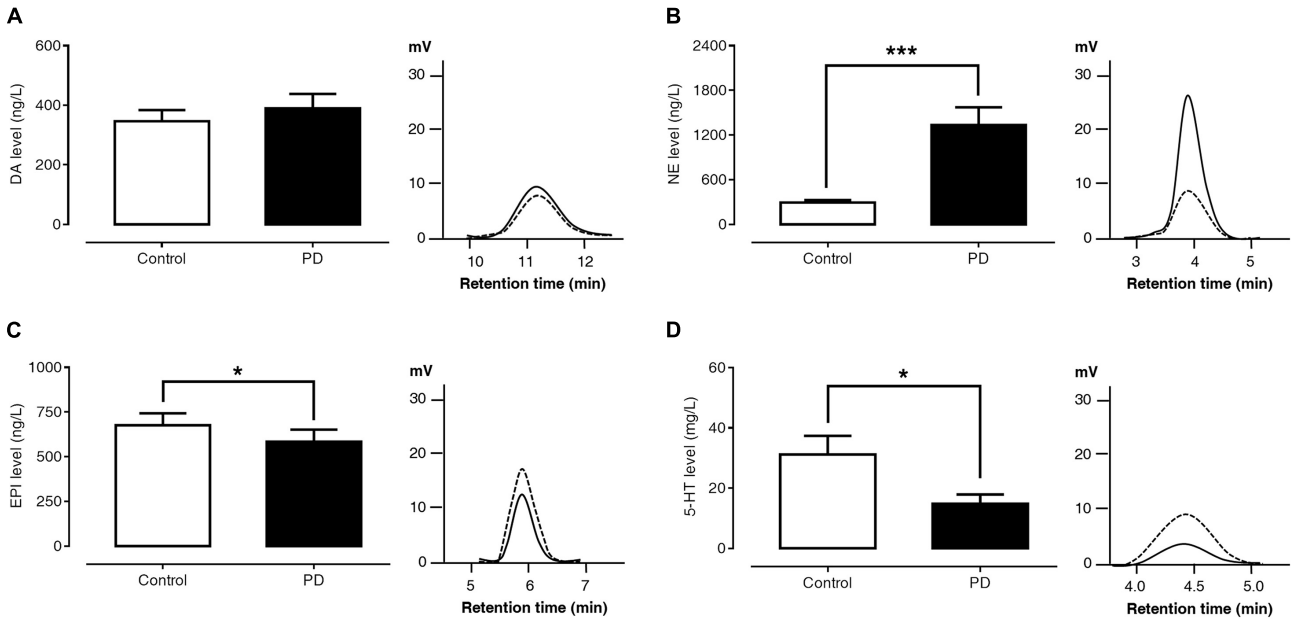

FIGURE 1 | Comparisons of plasma DA **(A)**, NE **(B)**, EPI **(C)**, and 5-HT **(D)** levels and HPLC chromatograms between control subjects (dash lines) and PD patients (solid lines). Data are presented as mean ± SEM (*$p < 0.05$, ***$p < 0.001$).

In contrast, the VMA/NE ratio of PD patients was significantly lower than that of control subjects (0.021 ± 0.004 versus 0.045 ± 0.007, $p < 0.001$). The ratios of VMA/EPI (0.039 ± 0.009 versus 0.016 ± 0.002, $p = 0.29$) and 5-HIAA/5-HT (0.804 ± 0.315 versus 1.171 ± 0.514, $p = 0.74$) were not significantly different between the PD and control groups.

Association Between Plasma Monoamine Levels and Clinical Profiles of PD Patients

Figures 4A–D present the levels of plasma DA, NE, EPI, and 5-HT in early-stage and advanced-stage PD patients. Between these two subgroups, there were no significant differences in plasma levels of DA (393.12 ± 64.12 versus 384.94 ± 74.52 ng/l, $p = 0.984$), NE (1,317.84 ± 298.83 versus 1,365.05 ± 395.14 ng/l, $p = 0.624$), and EPI (634.66 ± 100.75 versus 509.77 ± 71.18 ng/l, $p = 0.935$). However, plasma 5-HT levels in advanced PD patients were significantly lower than in early-stage patients (7.44 ± 2.11 versus 19.72 ± 4.77 μg/l, $p = 0.024$).

The contributions of clinical profiles, including age, gender, LEDD, disease duration, and motor severity, to plasma monoamine levels were evaluated by multiple linear regression analyses (**Table 3**). From these analyses, we found that disease duration had a negative relationship with plasma DA level (β = −0.328, 95% CI [−0.033, −0.002], $p = 0.025$) and motor severity had a negative relationship with plasma 5-HT level (β = −0.351, 95% CI [−0.910, −0.060], $p = 0.026$). LEDD was also the significant determinant of both plasma DA (β = 0.379, 95% CI [0.118, 0.835], $p = 0.011$) and NE levels (β = 0.394, 95% CI [0.141, 1.059], $p = 0.012$).

DISCUSSION

This study demonstrated the alteration in the levels of DA and other monoamine neurotransmitters in peripheral body fluids by quantifying plasma monoamine levels and their urinary metabolites in PD patients. As there have been few reports determining these chemicals in plasma and urine of PD patients,

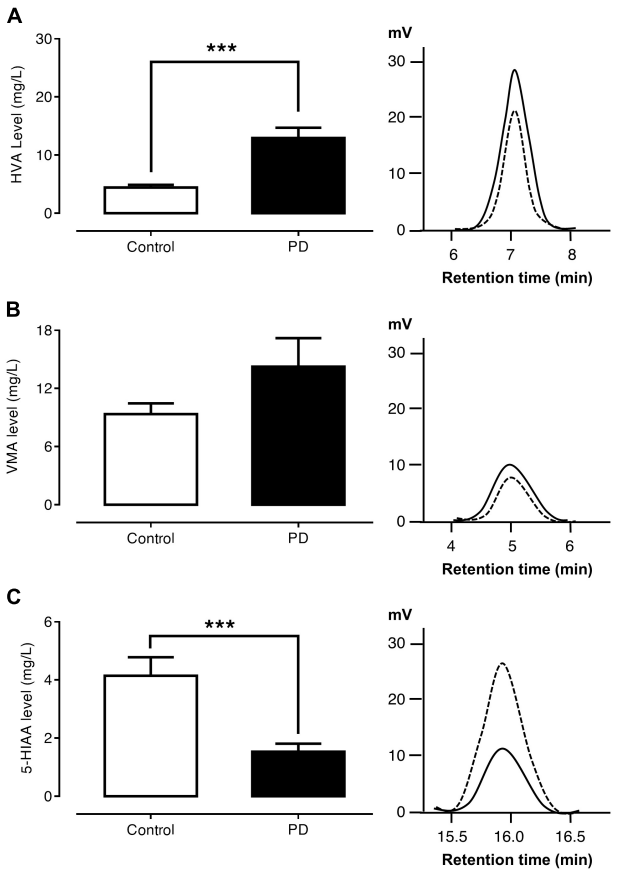

FIGURE 2 | Comparisons of urinary HVA **(A)**, VMA **(B)**, and 5-HIAA **(C)** levels and HPLC chromatograms between control subjects (dash lines) and PD patients (solid lines). Data are presented as mean ± SEM (***$p < 0.001$).

we would discuss by comparing our results to the previous ones in central body fluid, imaging, or post-mortem brain tissue studies instead.

For the dopaminergic system, our analyses showed that PD patients have significantly higher urinary HVA levels and HVA/DA ratio than control subjects. Additionally, in PD patients, the plasma DA level increased in parallel with the higher LEDD. These findings are consistent with a previous study by Andersen et al. (2017) reporting increased DA and HVA levels in the cerebrospinal fluid of PD patients treated with L-DOPA, and a decrease in untreated PD patients. When nigrostriatal degeneration progresses in PD, the surviving dopaminergic neurons compensate the loss by increasing DA synthesis, storage, release, and turnover through upregulation of aromatic amino acid decarboxylase (AADC) and vesicular monoamine transporter type 2 (VMAT2) (Lee et al., 2000). These compensatory responses may explain why DA and HVA were increased in PD patients. The activity of monoamine oxidase (MAO) in PD patients is also increased (Lee et al., 2000). The rise in HVA thus appears to be greater than the rise in DA.

L-DOPA administration may be an additional factor causing abnormal increases in DA and its metabolite. L-DOPA can be taken up by non-dopaminergic neurons, particularly serotonergic and noradrenergic neurons and astrocytes, leading to the increase in production of DA as these neurons possess plentiful AADC and VMAT2 which are essential for DA synthesis and storage (Carta et al., 2008a,b; Pavese et al., 2011). Furthermore, long-term use of L-DOPA also stimulates angiogenesis and changes the permeability of the blood–brain barrier to increase its diffusion into the brain (Ohlin et al., 2011). All the above factors may be implicated in the rise of DA levels in the synaptic cleft, extracellular fluid, and peripheral circulation (Sossi et al., 2007). In this study, we attempted to minimize the effect of L-DOPA by having the patients discontinue their medications for 12 h prior to specimen collection, which is much longer than the half-life of L-DOPA.

Among PD patients, we also found that the plasma DA level decreased in parallel with the longer disease duration. Similarly, Lunardi et al. (2009) reported a negative correlation between DA level in CSF and disease duration in PD patients. Additionally, they found that the HVA/DA ratio was higher in the patients with a longer disease duration (Lunardi et al., 2009). Therefore, the negative relationship between DA level and disease duration may be explained by both the progressive degeneration of dopaminergic neurons and the abnormal increase in DA degradation.

Regarding NE and EPI, this study demonstrated significantly increased plasma NE with a decreased VMA/NE ratio in PD patients, indicating that they have a high rate of NE synthesis with a low rate of its degradation. The plasma EPI level was significantly lower in PD than in control groups, while the VMA/EPI ratio was not different between the groups. Information regarding the alterations of NE and EPI remains controversial. A study by Chia et al. (1993) showed that PD patients had a higher plasma NE without a difference in plasma EPI level compared to control subjects. Similarly, Andersen et al. (2017) revealed that the NE level in CSF was significantly increased, whereas the ratio of methoxy-4-hydroxyphenylglycol (MHPG)/NE was significantly decreased in PD patients treated with L-DOPA. On the other hand, a study by Eldrup revealed no differences in plasma DA, NE, and EPI between PD patients and control subjects (Eldrup et al., 1995). In an electrophysiology study, it was reported that the firing rate of noradrenergic neurons in the locus coeruleus was increased in PD rats compared to normal ones (Wang et al., 2009). Normally, DA is converted into NE and EPI by the catalyzed activities of dopamine beta-hydroxylase (DBH) and phenyl-ethanolamine-N-methyltransferase enzyme (PNMT), respectively. The study by Kopp et al. (1982) suggested that PD patients had enhanced activity of DBH without change in PNMT activity in the brainstem. This may be the reason why PD patients in our study had increased NE, but not EPI, levels.

For the serotonergic system, we found that PD patients had remarkably decreased plasma 5-HT and urinary 5-HIAA. However, the ratio of 5-HIAA/5-HT in PD was not different from the control group. From these results, it can be assumed that PD patients have a reduced 5-HT synthetic rate but unchanged turnover rate. These results agree with several previous studies which reported that 5-HT and 5-HIAA levels were significantly

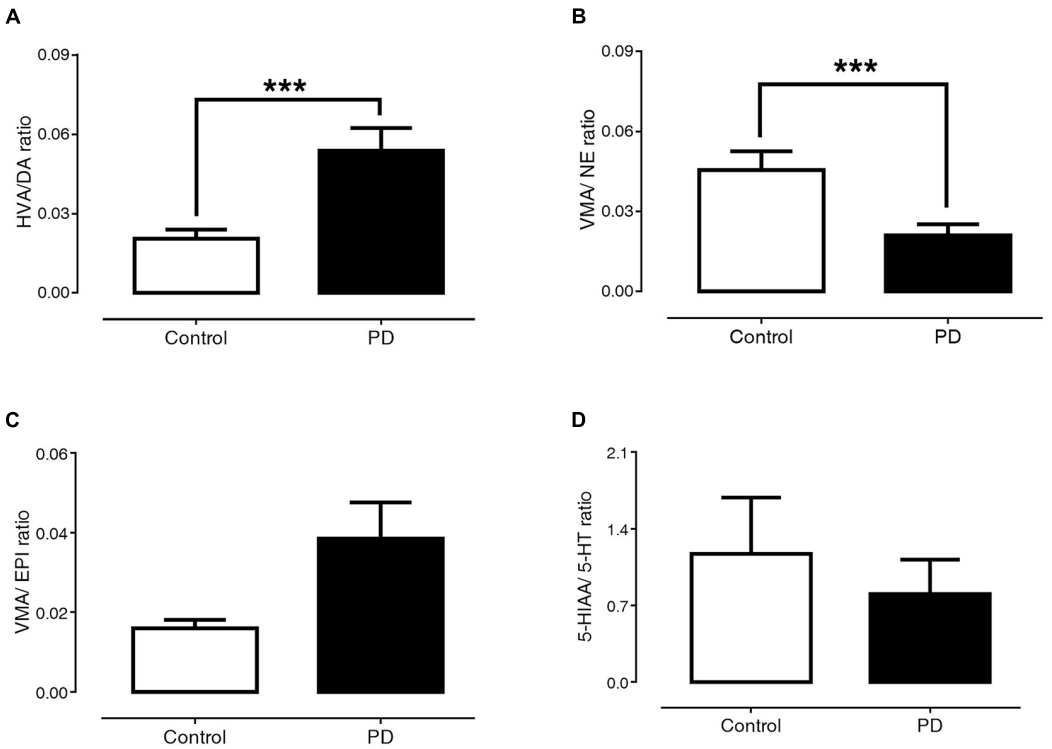

FIGURE 3 | Comparisons of HVA/DA **(A)**, VMA/NE **(B)**, VMA/EPI **(C)**, and 5-HIAA/5-HT **(D)** ratio between control subjects and PD patients. Data are presented as mean ± SEM (***$p < 0.001$).

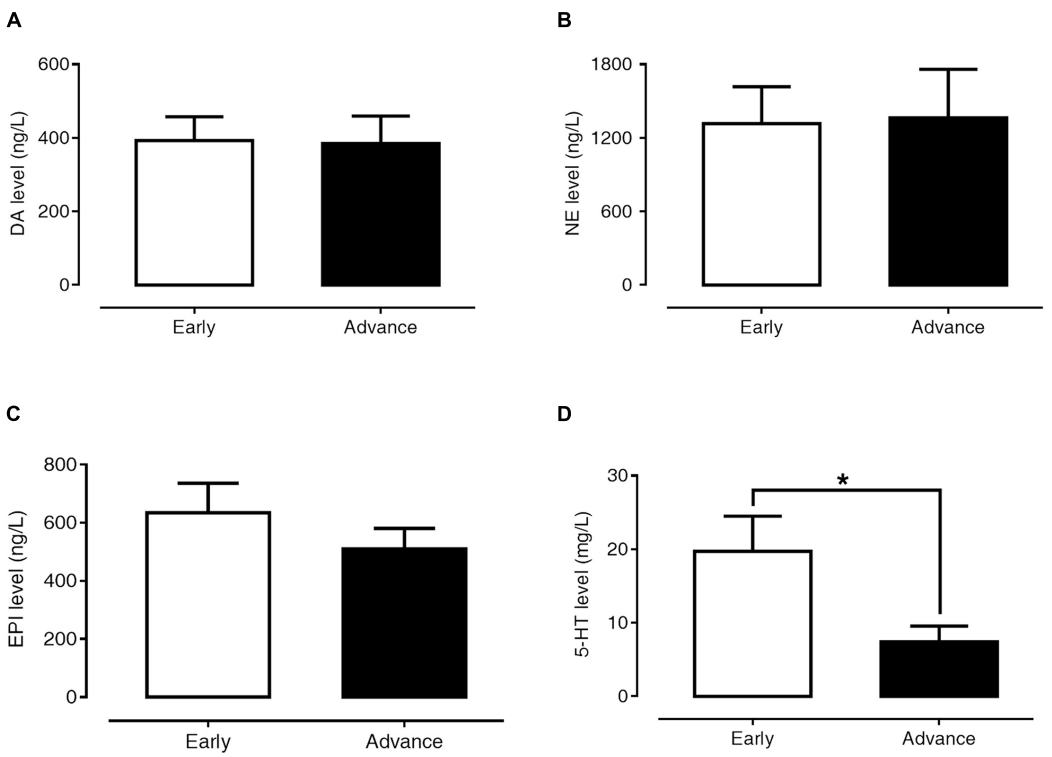

FIGURE 4 | Levels of plasma DA **(A)**, NE **(B)**, EPI **(C)**, and 5-HT **(D)** in early- and advanced-stage PD patients. Data are presented as mean ± SEM (*$p < 0.05$).

TABLE 3 | Multiple linear regression model for the association between plasma monoamine levels and clinical profiles of PD patients.

Dependent variables	Independent variables	Standardized coefficients (β)	95% CI	*p*-value
Plasma DA level ($R^2 = 0.274$)	LEDD	0.379	0.118, 0.835	0.011*
	Disease duration	−0.328	−0.033, −0.002	0.025*
Plasma NE level ($R^2 = 0.155$)	LEDD	0.394	0.141, 1.059	0.012*
Plasma 5-HT level ($R^2 = 0.123$)	Motor severity (0 = early, 1 = advanced)	−0.351	−0.910, −0.060	0.026*

CI = confidence interval; R^2 = coefficient of multiple determination.
$^*p < 0.05$.

lower in PD patients compared to healthy controls, in both CSF and peripheral circulation (Olivola et al., 2014; Tong et al., 2015). The decrease of 5-HT may result from Lewy body deposition and subsequent destruction of raphe nuclei (Braak et al., 2003). Moreover, many studies suggest that L-DOPA could inhibit 5-HT production. As mentioned earlier, serotonergic neurons are susceptible to uptake of L-DOPA, which might act as a competitive inhibitor of 5-HT synthesis (Goldstein and Frenkel, 1971).

This study also revealed that PD patients in the advanced stage (i.e., high motor severity) had significantly lower plasma 5-HT levels than patients in the early stage (i.e., low motor severity). This association is similar to a previous study regarding 5-HT dysfunction, and positron emission tomography (PET) study, which revealed a greater loss of serotonergic terminals at the raphe nuclei and striatum in advanced PD compared to early PD patients (Politis et al., 2010). Another two PET studies also reported that 5-HT transporter-binding markers and 5-HT_{1A} receptors in the raphe nuclei, caudate, and putamen negatively correlated with tremor severity in PD patients (Doder et al., 2003; Loane et al., 2013). Moreover, Coppen et al. (1972) showed that tryptophan co-treatment in PD patients was more effective in improving motor symptoms. Thus, 5-HT depletion may contribute to the severity of motor impairment in PD.

This study is limited by the small number of participants. In addition, a concurrent study on the activities of enzymes related to monoamine metabolism may lead to a better understanding of the monoamine system changes in PD. Furthermore, the measurement of monoamines and metabolites in peripheral body fluid may not precisely reflect their levels or activities in the central nervous system. However, our findings are in the same direction with several studies measuring monoamine levels in CSF or determining their activities by the neuroimaging technique. Considering this, the potential use of monoamine level measurement in peripheral body fluid as PD biomarkers should be investigated in future studies.

In conclusion, our study demonstrated the alteration of monoamine neurotransmitter in peripheral body fluids of PD patients. Correlations between disease severity and plasma 5-HT level, as well as disease duration and plasma DA level, were also demonstrated. This information contributes to our wider knowledge of multi-neurotransmitter dysfunction in PD, thus enhancing the evaluation of neurotransmitter status, prediction of subsequent symptoms, planning of appropriate disease management, and monitoring of the effectiveness of treatments.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Internal Review Board, Faculty of Medicine, Chulalongkorn University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

PW acquired the data and drafted the manuscript. PW, ST, and SB-p analyzed and interpreted the data. ST, SB-p, RB, and OP revised the manuscript. ST and SB-p obtained the funds. All authors designed the study and approved the final version of the manuscript.

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Electroacupuncture Improves M2 Microglia Polarization and Glia Anti-inflammation of Hippocampus in Alzheimer's Disease

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Background: Alzheimer's disease (AD) is a neurodegenerative disease characterized by loss of recognition and memory. Neuroinflammation plays pivotal roles in the pathology of AD and affects the progression of the disease. Astrocyte and microglia, as main immune executors in the central nervous system (CNS), participate into the inflammatory response in AD. Glia polarize into different phenotypes during neurodegeneration. Pro-inflammatory glia produce cytokines (IL-1β, TNF-α, and IL-6) resulting into debris aggregates and neurotoxicity. Anti-inflammatory phenotypes produce cytokines (IL-4 and IL-10) to release the inflammation. Electroacupuncture is a useful treatment that has been found to slow the neurodegeneration in animals through experimentation and in humans through clinical trials. The aim of this study was to uncover the mechanisms of glia activation, microglia polarization, and cytokine secretion regulated by electroacupuncture as a treatment for AD.

Methods: Twenty male Sprague–Dawley (SD) rats were randomly divided into four groups: Control group (Control), Normal saline group (NS), AD group (AD), and Electroacupuncture group (Acupuncture). The AD and Acupuncture groups were bilaterally injected with $A\beta_{1-42}$ into the CA1 field of the hippocampus. The Acupuncture group received electroacupuncture stimulation on the acupoint "Baihui" (GV20) for 6 days per week for a total of 3 weeks. The Morris Water Maze (MWM) was used to evaluate learning and memory capacity. Immunofluorescence was used to stain GFAP and Iba1 of the DG and CA1 in the hippocampus, which, respectively, expressed the activation of astrocyte and microglia. The M1 microglia marker, inducible nitric oxide synthase (iNOS), and M2 marker Arginase 1 (Arg1) were used to analyze the polarization of microglia. The pro-inflammatory cytokines (IL-1β, TNF-α, and IL-6), anti-inflammatory cytokines (IL-4 and IL-10), and pathway-molecules (p65 and Stat6)

were tested to analyze the glia inflammatory response by immunofluorescence and polymerase chain reaction (PCR).

Results: The MWM results showed that electroacupuncture improves the escape latency time and the swimming distance of AD rats. The number of GFAP and Iba1 cells significantly increased in AD rats, but electroacupuncture decreased the cells. The iNOS-positive cells were significantly increased in AD, and electroacupuncture decreased the positive cells. Electroacupuncture elevated Arg1-positive cells in AD rats. Electroacupuncture decreased the glia pro-inflammatory cytokine expression and increased the anti-inflammatory cytokine expression in AD rats. Furthermore, electroacupuncture inhibited the NF-κB pathway molecule (p65) while raising the Stat6 pathway molecule (Stat6).

Conclusion: These results provide evidence that electroacupuncture improves the recognition abilities and memory of AD rats. Electroacupuncture inhibits the activation of glia and polarizes microglia toward the M2 phenotype. Electroacupuncture decreased the pro-inflammatory cytokines (IL-1β, TNF-α, and IL-6) and increased the anti-inflammatory cytokines (IL-4 and IL-10). Furthermore, electroacupuncture affects the immune responses through inhibition of NF-κB pathway but activation of Stat6 pathway.

Keywords: acupuncture, Alzheimer's disease, microglia, astrocyte, cytokines

INTRODUCTION

The most prevalent characteristic of Alzheimer's disease (AD) is failure of recognition and memory that is accompanied with other clinical features, such as poor judgment or hallucinations. It is extremely difficult to treat the disease and relieve the symptoms (Knopman et al., 2021). Acupuncture, a very useful treatment of traditional Chinese medicine (TCM), has been reported to bring symptom relief or increase the effects of drugs for AD. Acupuncture could improve the scores of Mini Mental State Examination (MMSE). For example, donepezil and acupuncture increased MMSE scores than donepezil alone (MD 2.37, 95% CI 1.53–3.21) (Zhou et al., 2015). We have also shown that electroacupuncture improved Morris Water Maze (MWM) scores in AD model rats (Xie et al., 2018). By using resting-state functional magnetic resonance imaging (rs-fMRI), it was found that acupuncture altered amplitude of low-frequency fluctuations (ALFFs) in some brain areas of AD patients (Zheng et al., 2018). However, the cellular and molecular mechanisms are still unclear in regard to the process of how exactly electroacupuncture treats AD.

The main neuropathologic occurrences of AD are external neuronal β-amyloid plaques (Aβ) and intra-neuronal neurofibrillary tangles (Tau protein) accumulation. The disturbance of immune response has been recognized as core etiology for AD debris aggregation. Immune cells (microglia, macrophage) and non-immune cells (astrocyte, endothelial cell) extremely activate and pro-inflammatory cytokines are secreted during the progression of AD (Knopman et al., 2021). The pathological debris and inflammatory substances activate microglia and astrocytes. The activated microglia transformed into two phenotypes, M1 and M2, in which M1 mainly promotes pro-inflammation factors and neurotoxicity while M2 promotes anti-inflammation factors which lead to protective function (Tang and Le, 2016). It seemed that microglia tended to polarize toward M1 phenotype in AD. $A\beta_{1-42}$ triggered the NF-κB pathway that resulted in an increase of M1 microglial markers and a decrease of M2 markers (Xie et al., 2020). The M1 microglia and pro-inflammatory astrocytes produce pro-inflammatory cytokines, such as TNF-α and IL-1β, which enhance the accumulation of debris, oxidative stress, and neurotoxicity (Minter et al., 2016; Rodriguez-Arellano et al., 2016). Some cytokines (IL-1β, IL-6, and TNF-α) increase the risk of AD; however, deletion of these cytokines treats the neurotoxic effects of Aβ plaques and rescues recognition impairments (Minter et al., 2016). The pro-inflammatory factors cause an inflammatory response and neurotoxicity, but anti-inflammatory factors, such as IL-4 and IL-10, extricate the damage and promote tissue repair (Minter et al., 2016; Tang and Le, 2016).

Acupuncture is one of the widely used Chinese traditional therapies and is commonly utilized in other countries. Previous clinical reports and animal experiments supported that Acupuncture improved the symptoms of AD. Acupuncture improves the astrocytic ultrastructure in AD rats, in which the swelling mitochondrial and endoplasmic reticulum had been released (Tang et al., 2019). Manual acupuncture inhibited inflammasome IL-1β production and neuronal apoptosis in SAMP8 mice (Ding et al., 2017). The regulation of the immune response and its constituents are very important mechanisms of acupuncture. We previously found that electroacupuncture balanced the Treg/Th17 ratio through increasing IL-10 and decreasing IL-6 (Sun et al., 2017). Recently, we reported that acupuncture not only activated astrocytes and microglia in the hippocampus of DSS-induced colitis but also treated

the mitochondrial dysfunction (Zhang et al., 2020). We also found that electroacupuncture polarized microglia into the M2 phenotype which improved the anti-inflammatory effects in AD rat models (Xie et al., 2018), but it is still not clear if electroacupuncture activates and polarizes glia or affects the cytokine production in different hippocampal areas.

RESULTS

The Effect of Electroacupuncture in Memory and Learning of Alzheimer's Disease

In order to assess the effects of electroacupuncture on memory and learning, we performed the MWM experiment with rats. Learning and memory have been analyzed *via* the escape latency time and swimming distance. The probe trial has been used to evaluate the memory capacity. The results showed that the AD group (average 39.15 s) was significantly longer compared with Control (average 18.45 s) and NS (average 18.25 s) groups in the escape latency after the 6 days of training, but the mean escape latency of the Acupuncture group (average 21.35 s) was shorter than that of the AD group (**Figure 1A**). The AD group (average 26.78 mm/s) was significantly lower compared with the Control (average 37.37 mm/s) and NS (average 41.93 mm/s) groups of the mean swimming speed at the sixth day. However, electroacupuncture improved the mean swimming speed upon comparing all groups (**Figure 1B**). The swimming distance of the Control group (average 72.85 cm) and NS group (average 72.05 cm) was shorter than that of the AD group (average 213.00 cm) on the sixth day, but electroacupuncture decreased the total swimming distance in the Acupuncture group (average 105.60 cm) (**Figure 1C**). The probe times of the AD group (average 5.0) were less than the Control (average 7.6) and NS (average 7.8). The probe times was improved by electroacupuncture (average 7.2) (**Figure 1D**).

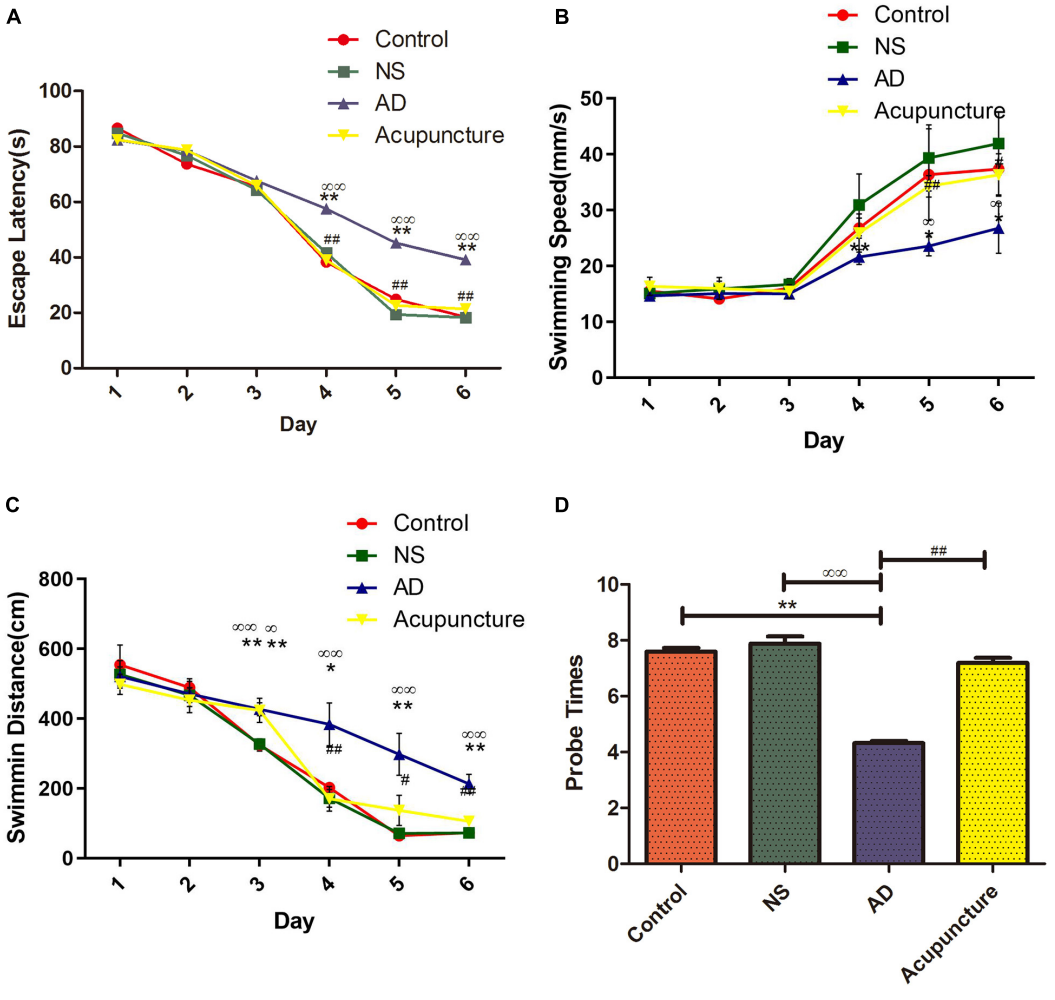

FIGURE 1 | Evaluation of learning and memory capacity in the Morris Water Maze. **(A)** The mean escape latency from star point to the platform. **(B)** The mean swimming speed from the star point to the platform. **(C)** The mean swimming distance. **(D)** Probe times of trails. Compared with the Control group, $^{**}p < 0.01$; compared with the NS group, $^{\circ\circ\circ}p < 0.01$; compared with the AD group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ ($n = 5$).

Electroacupuncture Released the Glia Activation in Alzheimer's Disease

The Iba1 is a cytoplasmic helix–loop–helix protein with F-actin binding and actin-cross-linkage. The up-regulation of Iba1 expresses the activation of microglia (Hendrickx et al., 2017). The Iba1$^+$ cell levels significantly elevated in the AD group (average 109.8 of CA1 and 104.2 of DG) compared with Control (average 88.4 of CA1 and 47.2 of DG) and NS (average 88.2 of CA1 and 47.4 of DG) groups ($p < 0.01$), but there is no significant difference between the AD and Acupuncture groups ($p > 0.05$) (**Figures 2A1–5, B1–5**). We used GFAP staining to observe the activation of astrocytes. The GFAP$^+$ cells of the Control group (average 159.6 of CA1 and 127.4 of DG) and NS group (average 161.4 of CA1 and 129.4 of DG) are less than the AD group (average 232.6 of CA1 and 239.0 of DG) ($p < 0.01$). The number of positive cells decreased in the Acupuncture group (average 160 of CA1 and 130.6 of DG) ($p < 0.01$) (**Figures 2C1–5, D1–5**). These results indicate that electroacupuncture inhibited the activation of glia both in the DG and CA1 of the hippocampus.

Electroacupuncture Improving the M2 Microglia Polarization

To prove that microglia undergo polarization, iNOS was used to mark M1, and Arg1 was used to mark M2. The iNOS is a special enzyme which catalyzes the arginine into citrulline and NO and is usually selected as a functional marker to express the M1 microglia. Arg1 metabolizes arginine into urea and ornithine, then these substances metabolize into hydroxyproline, proline, and polyamine. The Arg1 often is used to identify the M2 microglia (Tang and Le, 2016). The iNOS- and Arg1-positive cells were expressed both in pyramidal cell layer and white matter in the hippocampus (**Figure 3**). We measured the expression of Iba1$^+$iNOS$^+$ and Iba1$^+$Arg1$^+$ cells in CA1 and DG. The double staining results revealed that the number of Iba1$^+$iNOS$^+$ in AD mice (average 39.0 of CA1 and 26.8 of DG) increased, compared with Control (average 19.8 of CA1 and 13.2 of DG) and NS (average 19.2 of CA1 and 13.0 of DG) ($p < 0.05$) (**Figures 3A1–3,5, B1–3,5**). Electroacupuncture decreased the Iba1$^+$iNOS$^+$ cells both in CA1 (average 23.0) and DG (average 16.8) areas ($p < 0.05$) (**Figures 3A4–5, B4–5**). Electroacupuncture alone

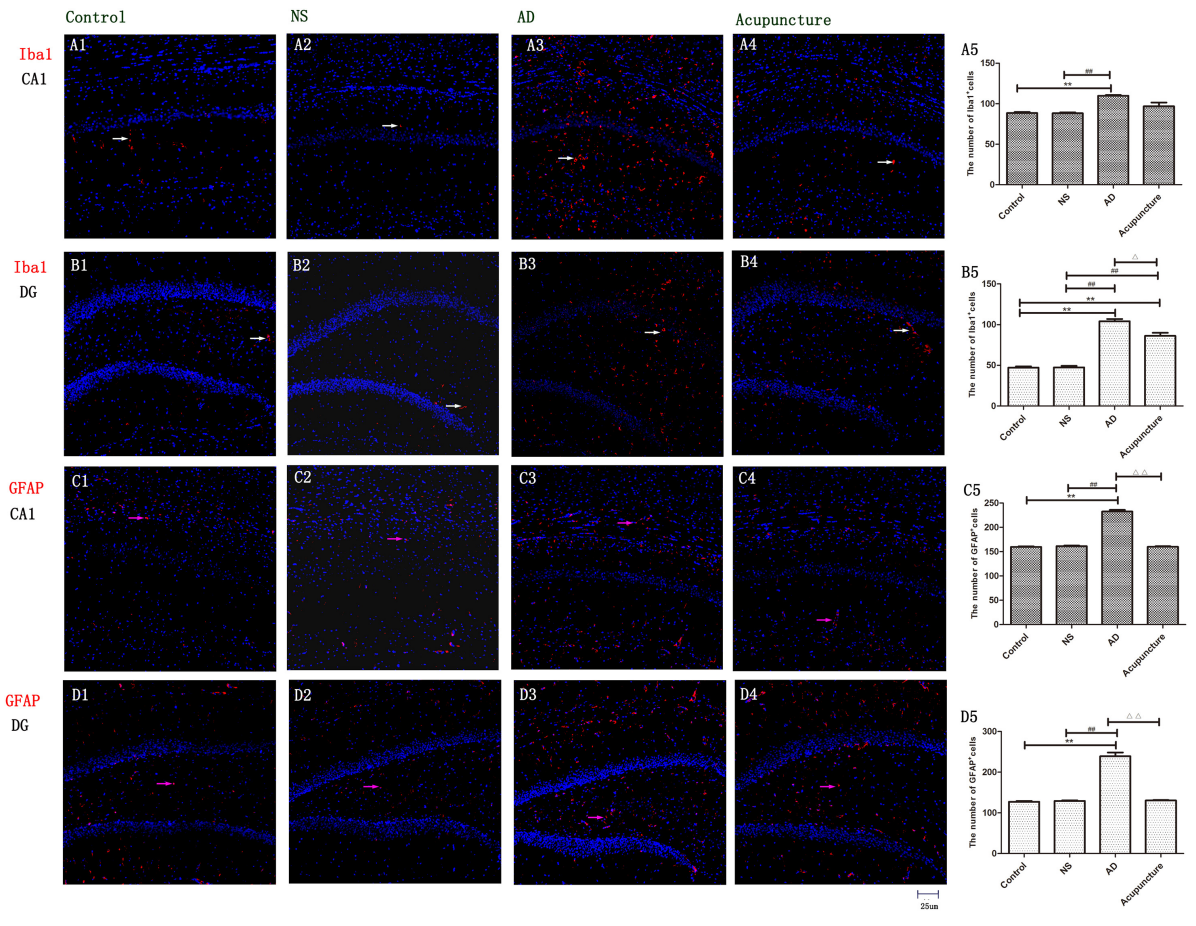

FIGURE 2 | The activation of glia in DG and CA1. **(A1–4)** Microglia of CA1 were stained with anti-Iba1 in four groups. **(B1–4)** Microglia of DG were stained with anti-Iba1. (The white arrow express the red Iba1-positive cells.) **(C1–4)** Astrocyte of CA1 was stained with anti-GFAP. **(D1–4)** Astrocyte of DG was stained with anti-GFAP (the pink arrow express the red GFAP-positive cells). **(A5,B5,C5,D5)** The number of positive stained cells in four groups. Error bars: SE. Compared with Control group, $^{**}p < 0.01$; compared with NS group, $^{\#\#}p < 0.01$; compared with AD group, $^{\triangle}p < 0.05$, $^{\triangle\triangle}p < 0.01$ ($n = 5$).

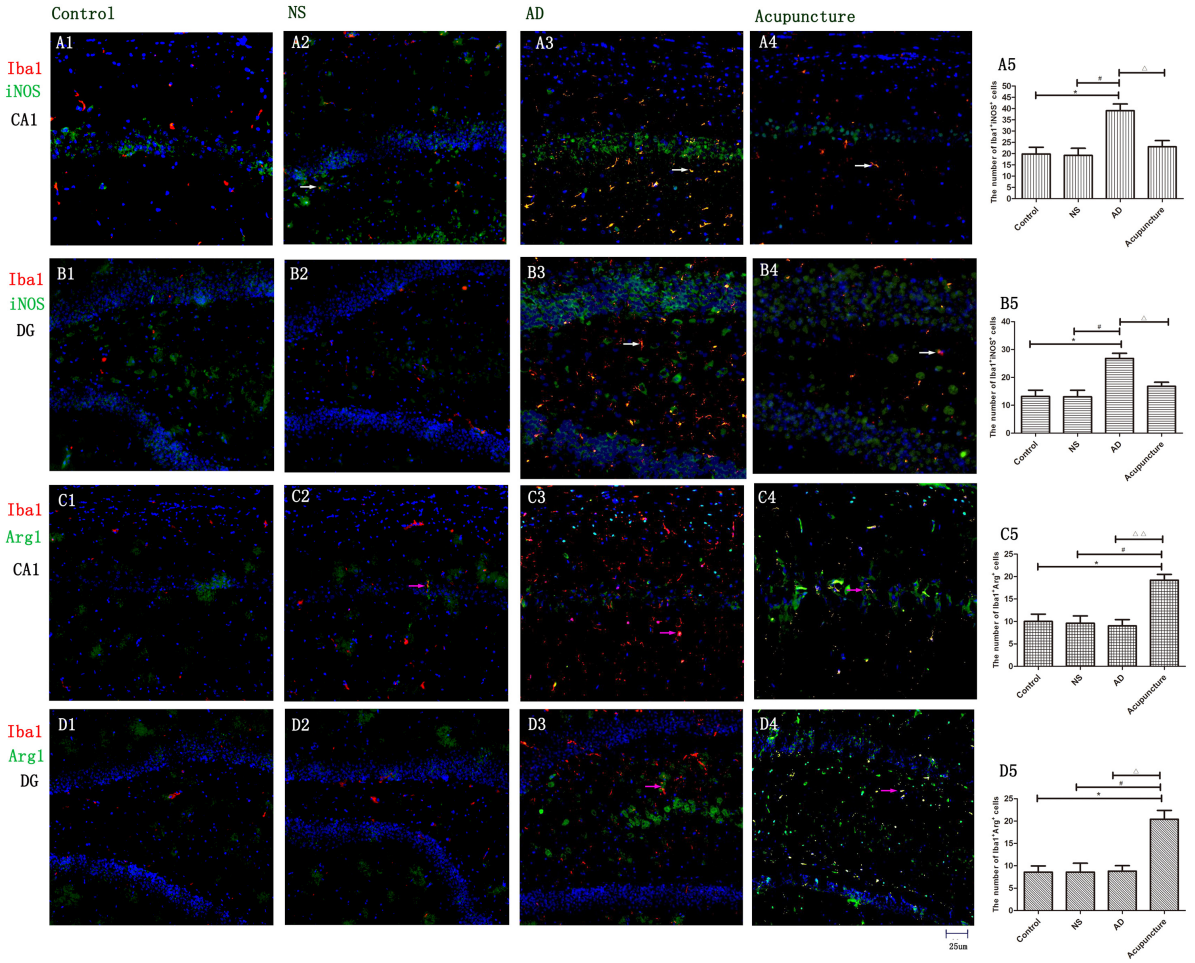

FIGURE 3 | The microglia polarization in different groups. **(A1–4)** Microglia of CA1 was double-stained with anti-iNOS and anti-Iba1 antibody and was observed on fluorescent image. (Red expresses Iba1-positive cells, green expresses iNOS-positive cells, and blue expresses DAPI. The white arrow expresses $iNOS^{+}Iba1^{+}$ cells.) **(B1–4)** Microglia of DG was double-stained with anti-iNOS and anti-Iba1 antibody and was observed on fluorescent image (Red expresses Iba1-positive cells, green expresses iNOS-positive cells, and blue expresses DAPI. The white arrow expresses $iNOS^{+}Iba1^{+}$ cells.) **(C1–4)** Microglia of CA1 was double-stained with anti-Arg1 and anti-Iba1 antibody and was observed on fluorescent image. (Red expresses Iba1-positive cells, green expresses Arg1-positive cells, and blue expresses DAPI. The pink arrow expresses $iNOS^{+}Iba1^{+}$ cells.) **(D1–4)** Microglia of DG was double-stained with anti-Arg1 and anti-Iba1 antibody and was observed on fluorescent image (Red expresses Iba1-positive cells, green expresses Arg1-positive cells, and blue expresses DAPI. The pink arrow expresses $iNOS^{+}Iba1^{+}$ cells.) **(A5,B5,C5,D5)** The number of positive stained cells in four groups. Error bars: SE. Compared with Control group, $^{*}p < 0.05$; compared with NS group, $^{\#}p < 0.05$; compared with AD group, $^{\triangle}p < 0.05$, $^{\triangle\triangle}p < 0.01$ ($n = 5$).

increased the $Iba1^{+}Arg1^{+}$ cell concentration about twofold in the Acupuncture group (average 15.4 of CA1 and 20.4 of DG), compared with the Control (average 6.4 of CA1 and 8.6 of DG), NS (average 7.2 of CA1 and 8.6 of DG), and AD (average 8.2 of CA1 and 8.8 of DG) groups ($p < 0.05$) (**Figures 3C1–5, D1–5**). However, there is no significant difference of $Iba1^{+}Arg1^{+}$ cells among Control, NS, and model groups ($p > 0.05$).

Electroacupuncture Reduces the Pro-inflammatory Cytokines of Glia in Alzheimer's Disease

Glia produce pro-inflammatory cytokines, such as IL-1β and TNF-α, which not only enhances the inflammatory response but also elevates neurotoxicity in AD (Grimaldi et al., 2019). Here, we double-stained the different cytokines (IL-1β, TNF-α, and IL-6) and glia markers (GFAP and Iba1). We found that $Iba1^{+}IL\text{-}1\beta^{+}$ or $Iba1^{+}TNF\text{-}\alpha^{+}$ cells were increased in AD (average 30.0 of CA1 and 30.8 of DG for IL-1β, and 24.8 of CA1 and 21.6 of DG for TNF-α), compared to the Control (average 10.4 of CA1 and 10.4 of DG for IL-1β, and 12.2 of CA1 and 9.2 of DG for TNF-α), and NS (average 12.2 of CA1 and 12.2 of DG for IL-1β, and 11.4 of CA1 and 9.2 of DG for TNF-α) groups ($p < 0.01$) (**Figures 4A1–3,5–8,10, B1–3,5–8,10**). Electroacupuncture decreased the pro-inflammatory factor-positive cells in the DG (average 12.4 for IL-1β and 9.0 for TNF-α) ($p < 0.01$) (**Figures 4A9–10, B9–10**). But there is no difference between the Acupuncture (average 22.0 for IL-1β and 15.4 for TNF-α) and AD groups in the CA1 ($p > 0.05$) (**Figures 4A4–5, B4–5**). There

is no significant difference of Iba1$^+$IL-6$^+$ cells among the four groups ($p > 0.05$) (**Figures 4D1–10**).

The GFAP$^+$IL-1β$^+$ or GFAP$^+$TNF-α$^+$ or GFAP$^+$IL-6$^+$ expression in cells was increased in the AD group (average 29.4 of CA1 and 34.6 of DG for IL-1β, 48.6 of CA1 and 35.0 of DG for TNF-α, and 35.2 of CA1 and 28.2 of DG for IL-6), compared with Control (average 15.2 of CA1 and 20.2 of DG for IL-1β, 29.2 of CA1 and 22.0 of DG for TNF-α, and 12.4 of CA1 and 10.6 of DG for IL-6), and NS (average 15.6 of CA1 and 20.8 of DG for IL-1β, 32.4 of CA1 and 21.0 of DG for TNF-α, and 12.2 of CA1 and 12.8 of DG for IL-6) groups ($p < 0.05$). The number of positive cells decreased due to electroacupuncture (average 17.4 of CA1 and 21.2 of DG for IL-1β, 33.4 of CA1 and 20.2 of DG for TNF-α, and 12.4 of CA1 and 13.2 of DG for IL-6) ($p < 0.05$, $p < 0.01$) (**Figures 5A1–10, B1–10, D1–6**). The mRNA of IL-6 was elevated in AD compared to Control and NS groups. Electroacupuncture reduced it (**Figure 6A**).

Electroacupuncture Elevates the Glia Anti-inflammatory Cytokines in Alzheimer's Disease

IL-4 and IL-10 are very essential anti-inflammatory cytokines with a crucial role in inhibiting the inflammatory pathological process (da Silva et al., 2015; Bhattarai et al., 2016; Kinney et al., 2018). To quantify the anti-inflammatory cytokines affected by electroacupuncture in AD, we double-stained glia markers and cytokines (IL-4 and IL-10). We found that the expression of Iba1$^+$IL-4$^+$ or Iba1$^+$IL-10$^+$ cells in the AD group (average 5.4 for IL-4 and 4.8 for IL-10 in CA1) was two times less than the Control (average 14.0 for IL-4 and 11.2 for IL-10) and NS (average 12.4 for IL-4 and 10.2 for IL-10) groups in CA1 ($p < 0.05$). The positive cells of the Acupuncture group (average 24.2 for IL-4 and 20.4 for IL-10) were more than the other three groups in CA1 ($p < 0.01$) (**Figures 4C1–5, E1–5**). The expression of Iba1$^+$IL-4$^+$ or Iba1$^+$IL-10$^+$ cells significantly increased in the Acupuncture group (average 20.8 for IL-4 and 21.0 for IL-10), compared to the Control (average 8.6 for IL-4 and 8.2 for IL-10), NS (average 8.8 for IL-4 and 8.8 for IL-10), and AD (average 10.6 for IL-4 and 9.2 for IL-10) groups in the DG area of the hippocampus ($p < 0.01$) (**Figures 4C6–10, E6–10**). The GFAP$^+$IL-4$^+$ or GFAP$^+$IL-10 cells in the AD group (average 11.0 of CA1 and 7.6 of DG for IL-4, and 10.8 of DG and 11.0 of CA1 for IL-10) were about two times less than the Control (average 20.0 of CA1 and 20.2 of DG for IL-4, and 20.8 of DG and 22.4 of CA1 for IL-10), NS (average 20.0 of CA1 and 20.4 of DG for IL-4, and 23.8 of CA1 and 21.0 of DG for IL-10), and Acupuncture (average 20.6 of CA1 and 20.6 of DG for IL-4, and 26.6 of CA1 and 21.0 of DG for IL-10) groups ($p < 0.05$, $p < 0.01$) (**Figures 5C1–10, E1–10**). The mRNA results reveal that IL-4 and IL-10 decreased in AD mice models compared to the Control and NS mice models (**Figures 6B, C**). So we can conclude that electroacupuncture increased these anti-inflammatory cytokines expression.

Electroacupuncture Inhibits the Glia p65 but Increases the Stat6 in Alzheimer's Disease

The p65 is the core molecule that contains the activated transcriptional domain. Some pro-inflammatory proteins (such

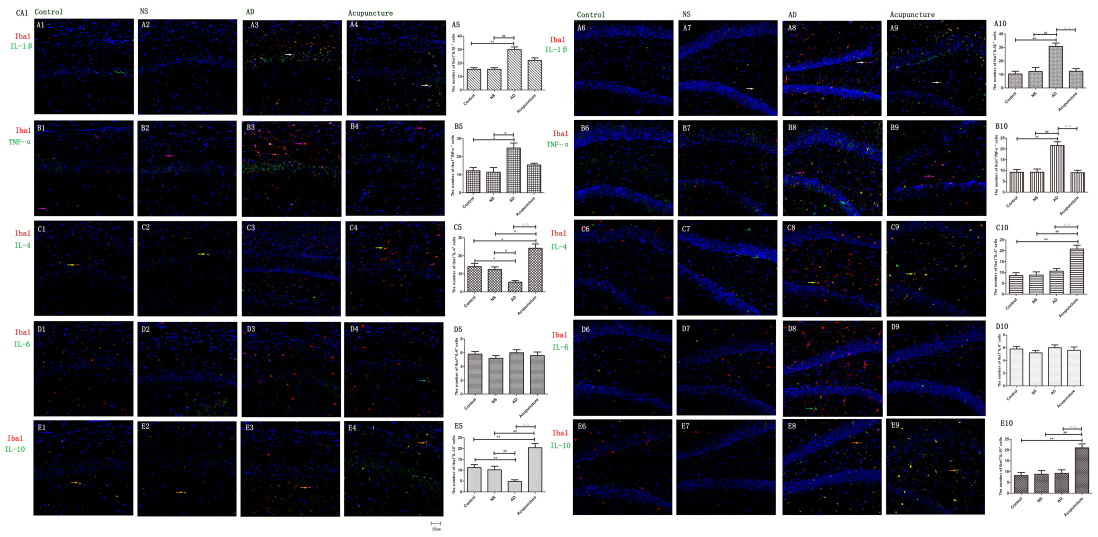

FIGURE 4 | The microglial cytokines in different groups. The microglial cytokines were double-stained with cytokine antibodies and anti-Iba1 antibody. **(A1–4)** Double staining with IL-1β and Iba1 (CA1 area). **(A6–9)** Double staining with IL-1β and Iba1 (DG area). **(B1–4)** TNF-α and Iba1 (CA1 area). **(B6–9)** TNF-α and Iba1 (DG area). **(C1–4)** IL-4 and Iba1 (CA1 area). **(C6–9)** IL-4 and Iba1 (DG area). **(D1–4)** IL-6 and Iba1 (CA1 area). **(D6–9)** IL-6 and Iba1 (DG area). **(E1–4)** IL-10 and Iba1 (CA1 area). **(E6–9)** IL-10 and Iba1 (DG area). (Red expresses Iba1-positive cells, green expresses cytokine-positive cells, and blue expresses DAPI. The white arrow expresses IL-1β$^+$Iba1$^+$ cells. The pink arrow expresses TNF-α$^+$Iba1$^+$ cells. The yellow arrow expresses IL-4$^+$Iba1$^+$ cells. The green arrow expresses IL-6$^+$Iba1$^+$ cells. The beige arrow expresses IL-10$^+$Iba1$^+$ cells.) **(A5,B5,C5,D5,E5,A10,B10,C10,D10,E10)** The number of positive double-stained cells in different groups. Error bars: SE. Compared with Control group, $^*p < 0.05$, $^{**}p < 0.01$; compared with NS group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$; compared with AD group, $^{\Delta\Delta}p < 0.01$ ($n = 5$).

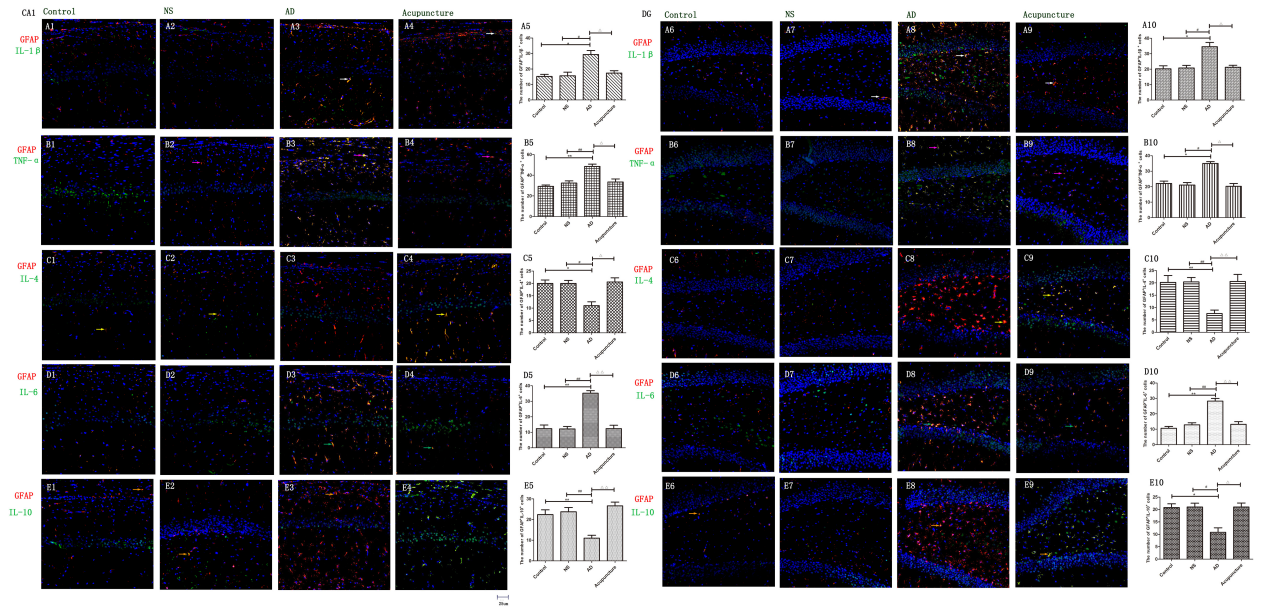

FIGURE 5 | The astrocytic cytokines in different groups. The astrocytic cytokines were double-stained with cytokine antibodies and anti-GFAP antibody. **(A1–4)** Double staining with IL-1β and GFAP (CA1 area). **(A6–9)** Double staining with IL-1β and GFAP (DG area). **(B1–4)** TNF-α and GFAP (CA1 area). **(B6–9)** TNF-α and GFAP (DG area). **(C1–4)** IL-4 and GFAP (CA1 area). **(C6–9)** IL-4 and GFAP (DG area). **(D1–4)** IL-6 and GFAP (CA1 area). **(D6–9)** IL-6 and GFAP (DG area). **(E1–4)** IL-10 and GFAP (CA1 area). **(E6–9)** IL-10 and GFAP (DG area). (Red expresses GFAP-positive cells, green expresses cytokine-positive cells, and blue expresses DAPI. The white arrow expresses IL-1β^{+}GFAP^{+} cells. The pink arrow expresses TNF-α^{+}GFAP^{+} cells. The yellow arrow expresses IL-4^{+}GFAP^{+} cells. The green arrow expresses IL-6^{+} GFAP^{+} cells. The Beige arrow expresses IL-10^{+}GFAP^{+} cells.) **(A5,B5,C5,D5,E5,A10,B10,C10,D10,E10)** The number of positive double-stained cells in different groups. Error bars: SE. Compared with Control group, $^{*}p < 0.05$, $^{**}p < 0.01$; compared with NS group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$; compared with AD group, $^{\triangle}p < 0.05$, $^{\triangle\triangle}p < 0.01$ ($n = 5$).

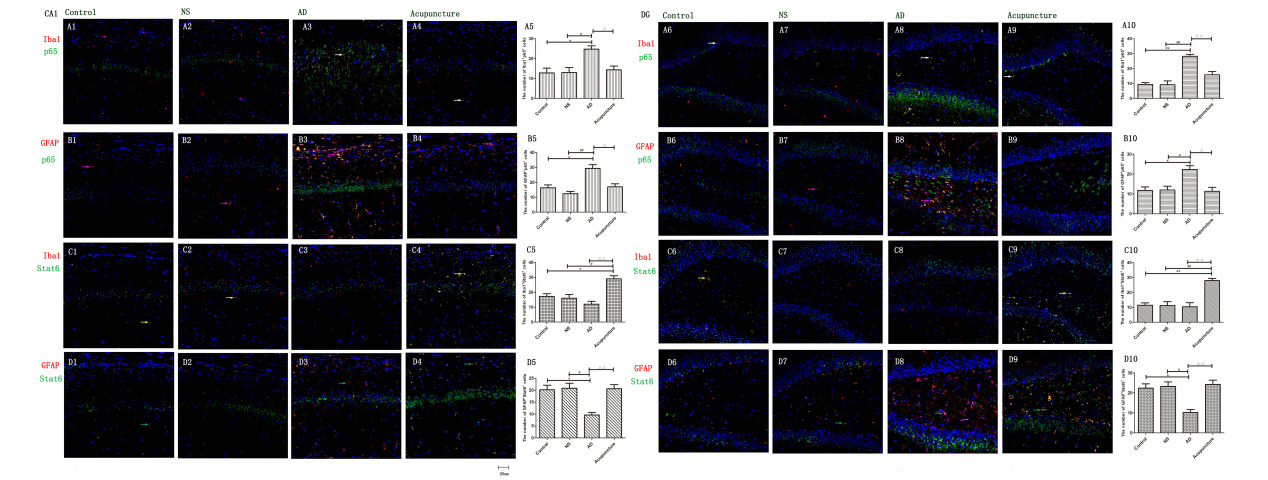

FIGURE 6 | The mRNA expression of cytokines and pathway molecules in hippocampus. **(A–C)** Quantitative data on the relative expression of cytokines mRNA. **(A)** IL-6, **(B)** IL-4, **(C)** IL-10. **(D,E)** Quantitative data on the relative expression of pathway molecules mRNA. **(D)** p65 and **(E)** Stat6 ($n = 5$). Compared with Control group, $^{*}p < 0.05$, $^{**}p < 0.01$; compared with NS group, $^{\#\#}p < 0.01$; compared with AD group, $^{\triangle\triangle}p < 0.01$.

as IL-1β and LPS) induce phosphorylation of IκBs, which are recognized as inhibitory proteins. The p65 translocates into the nucleus and combines its domain after IκBs degradation (Zhong et al., 1998). The number of Iba1^{+}p65^{+} cells in the AD group (average 24.8 of CA1 and 28.2 of DG) were twofold more than the Control (average 12.8 of CA1 and 9.4 of DG), NS (average 13.0 of CA1 and 9.2 of DG), and Acupuncture (average 14.4 groups of CA1 and 15.8 of DG) groups ($p < 0.05$, $p < 0.01$) (**Figures 7A1–10**). The cells expressing GFAP^{+}p65^{+} in the AD group (average 29.4 of CA1 and 22.4 of DG) were less than the Control (average 16.4 of CA1 and 11.8 of DG), NS (average 12.4 of CA1 and 12.0 of DG), and Acupuncture groups (average 17.0

of CA1 and 11.4 of DG) ($p < 0.05$) (**Figures 7B1–10**). The p65 mRNA expression increased in AD groups, compared with the other three groups ($p < 0.01$) (**Figure 6D**).

Stat6 is the main downstream molecule of IL-4 pathways (Bhattarai et al., 2016). Electroacupuncture increased the $Iba1^+Stat6^+$-positive cells in Acupuncture groups (average 28.0 in DG and 29.0 in CA1), compared to the Control (average 17.2 of CA1 and 11.4 of DG), NS (average 16.0 of CA1 and 11.2 of DG), and AD (average 12.0 of CA1 and 10.4 of DG) groups ($p < 0.01$) (**Figures 7C1–10**). The $GFAP^+Stat6^+$ cells significantly decreased in the AD groups (average 9.6 in CA1 and 10.2 in DG), compared to the Control (average 20.2 in CA1 and 22.4 in DG), and NS (average 20.8 of CA1 and 23.2 of DG) groups ($p < 0.05$). Surprisingly, electroacupuncture restored the loss of positive cells in the Acupuncture group (average 20.6 in CA1 and 24.2 in DG) ($p < 0.01$) (**Figures 7D1–10**). The Stat6 mRNA expression decreased in the AD group when compared with the Control and NS groups, but the electroacupuncture increased Stat6 mRNA expression ($p < 0.01$) (**Figure 6E**).

DISCUSSION

The activation of microglia and astrocytes are featured in the neurodegeneration, which play very important roles in several functions of the central nervous system (CNS), including inflammation, plasticity, and repair (Knopman et al., 2021). Recently, it was discovered that different types of activated microglia contributed to many physiologies and pathology in neurodegeneration (Tang and Le, 2016). In the present study, we observed that microglia tend to polarize toward the M1 phenotype, that accompanied pro-inflammation in AD rats (Xie et al., 2018). Here, we investigated the microglial polarization in different areas of the hippocampus. Results showed that microglia polarized into an M1 phenotype both in the DG and CA1 areas in the AD group; however, electroacupuncture reversed this effect, which decreased M1 microglia and increased M2 microglia. These results indicated that rebalancing microglia polarization in the hippocampus would be one of the mechanisms of electroacupuncture in AD. The basic function of the DG has been recognized as neurogenesis because it contains neural progenitor cells in adults (Nakashiba et al., 2012). The microglia have a large effect on neurogenesis *via* inflammatory mediation and phagocyted ability (Sierra et al., 2010; Gebara et al., 2013). In the absence of the immune response, the loss of microglia-induced progenitor cells decreased (Gebara et al., 2013), and activated microglia maintain a balance of neurogenesis *via* phagocytosis, the ability of which is affected by the polarization of microglia (Sierra et al., 2010; Tang and Le, 2016). In this study, we found that electroacupuncture tended to improve the microglia polarization into the M2 phenotype and enhance the anti-inflammatory ability in DG area of hippocampus. These results indicated that electroacupuncture would help neurogenesis *via* increasing microglia phagocytosis and inhibiting the pro-inflammation process. The other area of hippocampus, CA1, is usually recognized as an important region that is involved in cognitive processes, especially memory and learning (Rodriguez et al., 2010). The increase of activated microglia was related with memory impairment. It was reported that the number of activated microglia showed a positive relationship with the aggregation of Aβ deposits and neurofibrillary tangles in CA1 (McGuiness et al., 2017). This study supported that electroacupuncture inhibited excessive microglia activation and restored the microglia pro-inflammatory process. This alteration would be one of mechanism of electroacupuncture that is beneficial to neuroprotection and tissue repair. Like microglia, astrocytes are known to have a crucial role in physiological aging and AD progression. The fundamental functions of astrocytes include inflammation, homeostasis, and regeneration (Rodriguez-Arellano et al., 2016; Knopman et al., 2021). Activated astrocytes surround the Aβ plaques and abnormally release neurotransmitters to disturb the normal neuronal activity (Knopman et al., 2021). It was previously recognized that the astrocyte activity of AD patients increased in some areas, especially in the hippocampus, but not in the whole brain (Rodriguez-Arellano et al., 2016). The high density of astrocytes in AD patients mainly accumulated in the CA1/2 regions of the hippocampus (Marlatt et al., 2014). Contrarily, we observed that the activation of astrocytes increased both in CA1 and DG areas. Lian et al. (2016) reported that astrocytic C3 release enhanced Aβ aggregation *via* trigging the microglia C3R, which has been known to be an important crosstalk between glia in the pathology of AD. Thus, inhibiting the excessive activation of astrocytes is one of effective way to slow or delay the progression of the disease. Maintaining homeostasis has widely been recognized as a fundamental mechanism of electroacupuncture (Sun et al., 2017; Xie et al., 2018). We previously reported that acupuncture restores the altercations of Treg and Th17 that triggered the pro-inflammatory response in ulcerative colitis (UC) (Sun et al., 2017). Liang et al. (2016) observed that acupuncture inhibited glia inflammatory response which was the mechanism that relieved neuropathic pain. According our results, we approved that electroacupuncture could inhibit the activation of both microglia and astrocytes to improve the memory loss in AD, while improving the M2 microglia polarization to support the neuroprotection.

The regulation of cytokine is one of fundamental roles of acupuncture, specifically by maintaining the balance of different immune cells, such as T cells and microglia (da Silva et al., 2015; Sun et al., 2017; Xie et al., 2018). In a previous study, we discovered that electroacupuncture decreased the pro-inflammatory factor concentration (IL-1β and TNF-α) in AD animal models (Xie et al., 2018). We further supported that electroacupuncture increased some Treg cytokines (TGF-β, IL-10, and IL-2) while decreasing some Th17 cytokines (IL-6 and IL-17A) *via* the TLR pathway and manipulating the TL17/Treg ratio (Sun et al., 2017). The M2 microglia secreted protective proteins which inhibit neurodegeneration (Tang and Le, 2016). Here, we observed that electroacupuncture expressed anti-inflammatory function in hippocampus. Electroacupuncture increased the concentration of anti-inflammatory factors (IL-4 and IL-10) and decreased the concentration of pro-inflammatory factors (IL-1β, TNF-α, and IL-6) of microglia and astrocytes

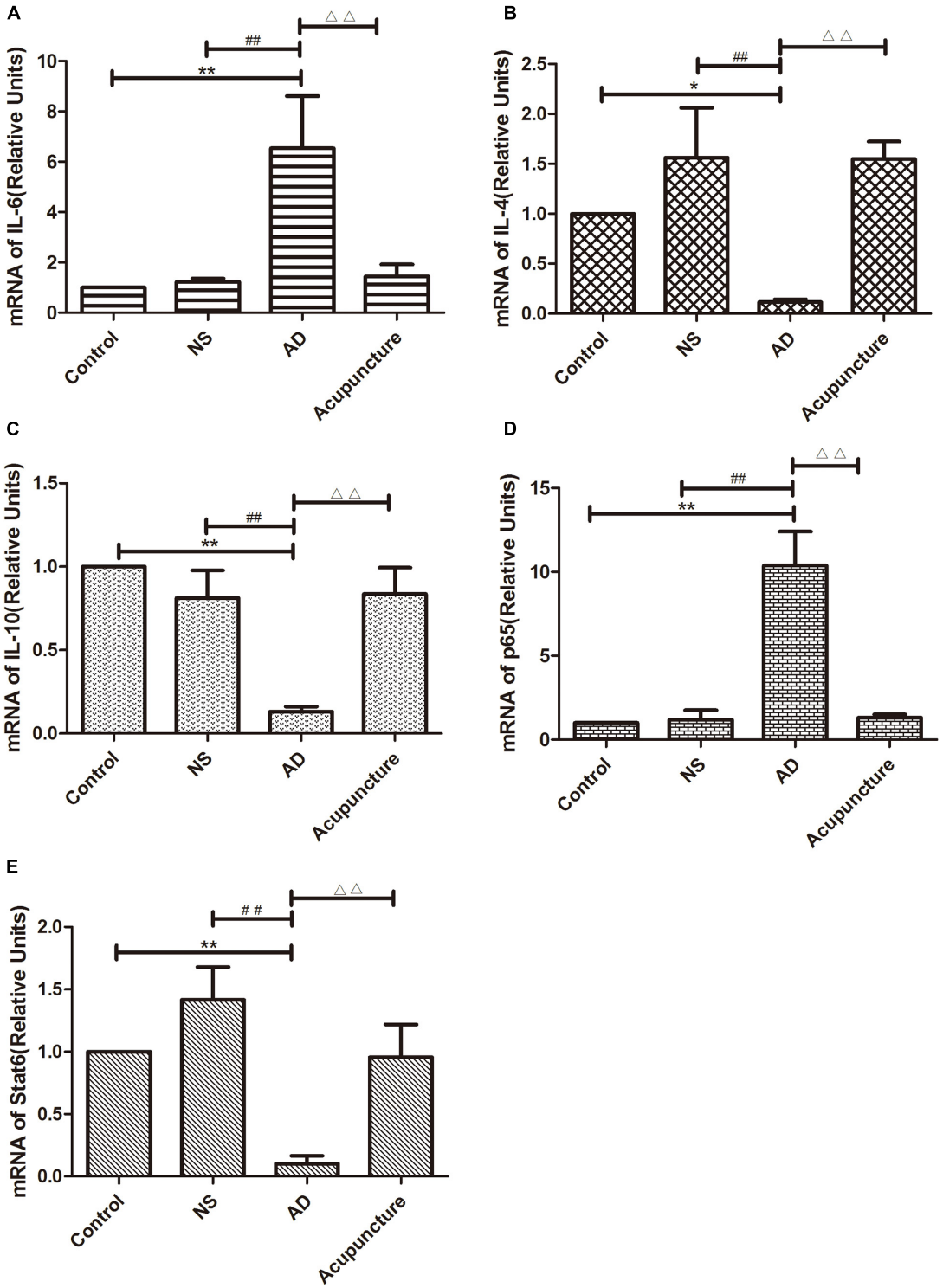

FIGURE 7 | Glia p65 and Stat6 expressed in different groups. **(A, B)** p65 was stained in glia. **(A1–4)** Double staining with p65 and Iba1 (CA1 area). **(A6–9)** p65 and Iba1 (DG area). **(B1–4)** p65 and GFAP (CA1 area). **(B6–9)** p65 and GFAP (DG area). p65 and GFAP (CA1 area). **(C,D)** Stat6 was stained in glia. **(C1–4)** Stat6 and Iba1 (CA1 area). **(C6–9)** Stat6 and Iba1 (DG area). **(D1–4)** Stat6 and GFAP (CA1 area). **(D6–9)** Stat6 and GFAP (DG area). (Red expresses Iba1 or GFAP-positive cells, green expresses p65- or Stat6-positive cells, and blue expresses DAPI. The white arrow expresses $p65^{+}Iba1^{+}$ cells. The pink arrow expresses $p65^{+}GFAP^{+}$ cells. The yellow arrow expresses $Stat6^{+}Iba1^{+}$ cells. The green arrow expresses $Stat6^{+}GFAP^{+}$ cells.) **(A5,B5,C5,D5,E5,A10,B10,C10,D10,E10)** The number of positive cells in different groups ($n = 3$). Error bars: SE. Compared with Control group, $^{*}p < 0.05$, $^{**}p < 0.01$; compared with NS group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$; compared with AD group, $^{\triangle}p < 0.05$, $^{\triangle\triangle}p < 0.01$ ($n = 5$).

in the hippocampus, which was associated with the inhibition of glial activation and M2 microglial polarization. IL-4 and IL-10 are the major anti-inflammatory factors that antagonize the inflammatory damage and promoted recovery responses (da Silva et al., 2015; Tang and Le, 2016). The dimers of IL-4 and IL-13 activate the Stat6 pathway *via* combing with IL-4 receptors (Bhattarai et al., 2016). IL-4 and IL-13 are widely accepted as M2 macrophage/microglia polarization stimulators (Bhattarai et al., 2016; Tang and Le, 2016). The IL-4/Stat6 pathway withstands the pro-inflammatory response and improves neurogenesis (Bhattarai et al., 2016). Here, we found electroacupuncture could increase the levels of Stat6 and the anti-inflammatory factors IL-4, both in the astrocyte and the microglia. These results indicated acupuncture would affect IL-4/Stat6 pathway to control the polarization of microglia and inflammatory process of glia in AD. Unlike protective M2 microglia, M1 microglia and activated astrocytes produce some pro-inflammatory cytokines (IL-1β and TNF-α), which enhance the tissue damage and neurotoxicity (Rodriguez-Arellano et al., 2016; Tang and Le, 2016). The NF-κB pathway has been widely recognized as a main inflammatory signal involving into the progression of AD, which was related with cognitive decline (Zhao et al., 2018). Pro-inflammatory cytokines activate the NF-κB pathway, and initiation of the pathway enhances some toxic cytokines. Finally, the inflammatory chain causes neurodegeneration (Tang and Le, 2016). Electroacupuncture inhibited glia activation, decreased pro-inflammatory cytokine, and down-regulated the NF-κB pathway in the hippocampus. These results indicated that NF-κB should be the signaling mechanism that electroacupuncture acts on to control the pro-inflammatory response in AD.

CONCLUSION

Electroacupuncture inhibited the activation of glia and improved the M2 microglia polarization in both the CA1 and DG areas of the hippocampus in AD. The anti-inflammatory effects are associated with increasing levels of anti-inflammatory cytokines (IL-4 and IL-10) and decreasing levels of pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) of glia. The NF-κB and Stat6 pathways might be mechanisms in which the treatment works through.

MATERIALS AND METHODS

Morris Water Maze

The MWM was used to evaluate the learning and memory capacity of the animals on day 5 after $A\beta_{1-42}$ injection. The water maze consisted of a flat black metal cylindrical tank (150 cm in diameter and 60 cm in height) that was equipped with a fixed platform (9 cm diameter and 30 cm height) below the surface of the water. A camera recording system was used to record the motion of the animals, accompanied with the MWM analysis software. The swimming pool was divided into four quadrants (I, II, III, and IV) and the platform was located in the four quadrants. The temperature of water was 22 ± 1℃ and the indoor environment was room temperature. Before the learning and memory trail, animals were faced with adaptive training for 3 days, in which animals were put on the platform for 10 s to familiarize themselves with the task. For the spatial memory trail, animals were placed in different quadrants of the same pool, and the trail was ended once the animal reached the platform. If the animals did not reach to the platform within 90 s, animals were helped to the platform and the latency data were recorded as 90 s. The spatial memory of the animals was analyzed *via* some data, including the time, swimming distance, and speed from when the animal was put in the water to the time it took for the rat to reach the platform. The time of spatial memory trail was 6 days. On Day 7, a spatial probe trial was conducted *via* the same tank without the platform. The times of crossing the original platform location was recorded within 120 s.

Animal

Three-month-old Sprague–Dawley (SD) rats were bought from the Sichuan Dashuo Experimental Animal Co., Ltd. [License number: SCXK (chuan) 2015-030]. All experiments were performed in accordance with the guidance of the Institutional Animal Care and Use Committee of Chengdu University of Traditional Chinese Medicine. Rats were individually housed with free access to water and food at a room temperature in ventilated conditions. These rats were performed in adaptive feeding for 1 week. After that, rats were randomly divided into four groups: the Control group, the Normal saline group (NS), the AD group (AD), and the Electroacupuncture group (Acupuncture).

$A\beta_{1-42}$ Injection Induced the Alzheimer's Disease Rat Model

The surgery was performed as previously described (Li et al., 2014). The AD and electroacupuncture rats were bilaterally injected with $A\beta_{1-42}$ into the CA1 field of hippocampus. In brief, rats were anesthetized with intraperitoneal injection of 10% chloral hydrate and fixed on stereotaxic frame. Bilateral injection locations were chosen with the guidance of the rat brain in stereotaxic coordinates (George Paxinos, 2006). The implanted location: AP = 3 mm; L = 2 mm; and V = 4 mm. Each injection contained 2.5 μl $A\beta_{1-42}$ (0.01 mg/L) and the NS group had the same volume (0.9%) injected into them.

Electroacupuncture Treatment

The Acupuncture group got electroacupuncture treatments. The treatments began 3 days after surgery and the days of total treatment was 18. Each treated period was 6 days and 1 no-treatment day between each period. Stainless steel needles (0.24 mm in diameter and 13 mm in length) were inserted into the acupoint "Baihui" (GV20). The "Baihui" locates at the intersection between the sagittal midline of the head and the coronary midpoint of the two ears. The location of GV20 was selected according to Government Channel and Points Standard GB12346-90 of China and "The Veterinary Electroacupuncture of China." The depth of injection was 2 mm.

Needle handles were connected with an electroacupuncture apparatus (Hans-200, China). Stimulating parameters: frequency 20 Hz, current strength 20 mA, voltage 2–4 V, and the treatment duration was 30 min.

Immunofluorescence Analysis

The rats were killed by intraperitoneal injection (10% chloral hydrated, 1 ml/100 g) and 4% paraformaldehyde (PFA) was used to fix *via* cardiac perfusion. Coronal brain slices (15 μm thick) were prepared and used for staining. Slices were washed in PBS for 5 min, three times, then blotted in 5% goat serum for 1 h at room temperature. Slices were incubated with the primary antibodies overnight at 4°C. Primary antibodies used: mouse anti-Iba1 (1:200 Sigma American), mouse anti-GFAP (1:200 Sigma American), rabbit anti-iNOS, anti-Arg1, anti-IL-1β, anti-TNF-α, anti-IL-4, anti-IL-6, and anti-IL-10 (1:200; Bioss, Beijing, China). After having been washed in PBS three times, these slices were incubated with secondary antibodies at 37°C for 2 h. Secondary antibodies: Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:200; Bioss, Beijing, China), Cy3-conjugated goat ant-mouse IgG (1:200; Bioss, Beijing, China). Then these slices were washed three times again and stained with DAPI (Biyuntian, China) for 5 min. Results were imaged using a 7266-fluorescence microscope (Leica, Japan).

Quantitative Polymerase Chain Reaction Analysis

These rats were sacrificed by cervical dislocation. The hippocampus tissues were extracted and ground into pieces. The RNA of the whole sample was isolated and extracted using TRIzol (Abcame). The cDNA was extracted by using cDNA Synthesis Kit (Biyuntian, China). Quantitative RT-PCR (Q-PCR) was performed on a polymerase chain reaction (PCR) cycler (Bio-Rad CFX96) utilizing the synthetic primers and SYBR Green (Sangon Biotech, China). Samples were subjected to the following reaction systems: 95°C for 3 min, 95°C for 10 s, renaturation for at 60°C for 30 s, and repeated for 45 cycles. $^{-\Delta\Delta}$Ct was used to calculate the relative expression of mRNA. The sequences of the primers were as follows: IL-6, forward, 5′-AGAAGACCAGAGCAGATTTT-3′ and reverse, 5′-GAGAAAAGAGTTGTGCAATG-3′; IL-4, forward, 5′-CTTTGAACCAGGTCACAG-3′ and reverse, 5′-CTCGTTCTCCGTGGTGTT-3′; IL-10, forward, 5′-CAGAAATCAAGGAGCATTTG-3′ and reverse, 5′-CTGCTCCACTGCCTTGCTTT-3′; p65, forward, 5′-CTGTTTCCCCTCATCTTTCCCT-3′ and reverse, 5′-CTGGTCCTGTGTAGCCATTGA-3′; and Stat6, forward, 5′-ATGCTTCCATGCAACTCAGC-3′ and reverse, 5′-GCTCCTGAAAAGATGGCAGT-3′.

Statistical Assay

Data were expressed as means ± SEM. One-way ANOVA Multiple was used to value the comparisons between different groups. Differences were considered significant at $p < 0.05$.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Review Board of Chengdu University of Traditional Chinese Medicine.

AUTHOR CONTRIBUTIONS

SY and QW conceived and designed the experiments. LX, YL, NZ, CL, AS, GW, YS, and HL performed the experiments. LX, YL, NZ, CL, and QW analyzed the images and data. SY, QW, LX, AS, and GW wrote the manuscript. LX and YL contributed equally to this work. All authors contributed to the article and approved the submitted version.

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Neuroprotective Effects of Ceftriaxone Involve the Reduction of Aβ Burden and Neuroinflammatory Response in a Mouse Model of Alzheimer's Disease

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Ceftriaxone (CEF) is a safe and multipotent antimicrobial agent that possesses neuroprotective properties. Earlier, we revealed the restoration of cognitive function in OXYS rats with signs of Alzheimer's disease (AD)-like pathology by CEF along with its modulating the expression of genes related to the system of amyloid beta (Aβ) metabolism in the brain. The aim of this study was to determine the effects of CEF on behavior, Aβ deposition, and associated neuroinflammation using another model of an early AD-like pathology induced by Aβ. Mice were injected bilaterally i.c.v. with Aβ fragment 25–35 to produce the AD model, while the CEF treatment (100 mg/kg/day, i.p., 36 days) started the next day after the surgery. The open field test, *T*-maze, Barnes test, IntelliCage, and passive avoidance test were used for behavioral phenotyping. Neuronal density, amyloid accumulation, and the expression of neuroinflammatory markers were measured in the frontal cortex and hippocampus. CEF exhibited beneficial effects on some cognitive features impaired by Aβ neurotoxicity including complete restoration of the fear-induced memory and learning in the passive avoidance test and improved place learning in the IntelliCage. CEF significantly attenuated amyloid deposition and neuroinflammatory response. Thus, CEF could be positioned as a potent multipurpose drug as it simultaneously targets proteostasis network and neuroinflammation, as well as glutamate excitotoxicity, oxidative pathways, and neurotrophic function as reported earlier. Together with previous reports on the positive effects of CEF in AD models, the results confirm the potential of CEF as a promising treatment against cognitive decline from the early stages of AD progression.

Keywords: neurodegeneration, drug repurposing, amyloid, microglia, cognition, behavior

INTRODUCTION

Drug repurposing (also called drug repositioning or drug reprofiling) is a process of redeveloping a compound for application in a different pathology and finding new therapeutic indications for the existing drugs. The premise of repositioning is that the drugs that have previously passed clinical trials will minimize the risk of failure in future late-stage clinical trials due to toxicity and thus lead to faster drug approvals (Li and Jones, 2012). It has been growing in importance in the last few years and becoming mainstream in the drug research area and industry. This strategy appeared to be quite an effective approach in psychopharmacology as well. For instance, the antibiotic minocycline was proposed as an effective adjuvant treatment of schizophrenia to improve its negative symptoms (Zhang and Zhao, 2014). The present study was focused on another antimicrobial drug with neuroprotective properties, ceftriaxone (CEF).

CEF is a cephalosporin antibiotic drug of the 3rd generation. It is highly soluble in water and penetrates the blood–brain barrier (Nau et al., 1993). Pathological accumulation of the glutamate, the main excitatory neurotransmitter, in synapses leads to excitotoxic death of neurons in a number of neurological diseases. Glutamate is eliminated from the synaptic cleft mainly by means of glutamate transporter-1 (GLT-1). In 2005, it was hypothesized that CEF might be effective for the treatment of several neurodegenerative disorders associated with elevated glutamate levels, including cerebral ischemia, amyotrophic lateral sclerosis, and epilepsy. This assumption was based on the ability of CEF to increase the activity of GLT-1 in astrocytes with subsequent normalization of glutamate levels. This property of CEF was revealed during a wide screening of more than 1,000 drugs on sections of organotypic cultures of rat spinal cord cells (Rothstein et al., 2005). In a number of further works, its anti-excitotoxic effect was confirmed (Chu et al., 2007; Lipski et al., 2007; Hota et al., 2008).

The beneficial effect of CEF has been demonstrated on motor deficits in rats in an experimental model of Parkinson's disease (Leung et al., 2012). The drug is actively studied in preclinical studies on models of neurological disorders (ischemia, myotonic dystrophy, alcohol, and drug addiction, etc.) in animals (Hakami and Sari, 2017; Hammad et al., 2017; Krzyzanowska et al., 2017; LaCrosse et al., 2017; Sicot et al., 2017; Stennett et al., 2017). Materials appeared on clinical trials of the CEF as a neuroprotective agent in amyotrophic lateral sclerosis (Cudkowicz et al., 2014) or Parkinson's disease dementia.[1] As glutamate-induced excitotoxicity is a prominent event in AD brains, the effects of the CEF in AD models were examined as well. The studies revealed positive effects of the CEF on AD-like pathology (Zumkehr et al., 2015; Hefendehl et al., 2016; Tikhonova et al., 2017). However, the additional mechanisms of its neuroprotective effects such as an activity targeted at proteostasis network or pathological aggregation of proteins were suspected.

[1]https://clinicaltrials.gov/ct2/show/NCT03413384

Pathological aggregation and accumulation of Aβ and associated neuroinflammation in the brain tissue is considered to play a core role in the pathogenesis of AD (Selkoe and Hardy, 2016). Early stages of AD are associated with disturbances in amyloid metabolism and accumulation of amyloid oligomers that are the most toxic forms of amyloid that lead to synaptic and neuronal dysfunctions and initiate the pathological cascade (Haass and Selkoe, 2007; Mroczko et al., 2018). However, the potential impact of the CEF on this mechanism is scantily studied.

In recent years, when studying the mechanisms of the neuroprotective action of the CEF in models of neurodegenerative diseases (Alexander's disease, Parkinson's disease) *in vitro*, its ability to directly influence the expression and pathological aggregation of proteins that cause neurotoxicity and neurodegeneration was found (Bachetti et al., 2010; Ruzza et al., 2014). Our group revealed the CEF modulating the expression of genes related to the system of Aβ metabolism in the brain of 5-month-old OXYS rats in a model of an early stage of AD-like progression (Tikhonova et al., 2018). Here we checked whether CEF might influence Aβ burden and associated neuroinflammatory response in the brain at early stages of AD-like pathology. The aim of this study was to determine the effects of the CEF on behavior, Aβ deposition, and neuroinflammation using another model of an early AD-like pathology induced by Aβ neurotoxicity in mice.

MATERIALS AND METHODS

Reagents

The following main reagents were used: Aβ25–35 fragment (Sigma, United States), CEF (Roche, Switzerland), a mouse monoclonal antibody to Aβ (cat. # NBP2-13075, 1:1,000 dilution; Novus Biologicals, United States), a rat monoclonal antibody to CD54/ICAM-1 (cat. # 16-0542-81, 1:300 dilution; eBioscience, Thermo Fisher Scientific, United States), a goat polyclonal antibody to microglial marker AIF-1/IBA1 (cat. # NB100-1028, 1:200 dilution; Novus Biologicals, United States), an Alexa Fluor 568-conjugated goat anti-mouse IgG polyclonal antibody (cat. # ab175473, 1:400 dilution, Abcam, United Kingdom), an Alexa Fluor 594-conjugated goat anti-rat IgG polyclonal antibody (cat. # ab150160, 1:500 dilution; Abcam, United Kingdom), and an Alexa Fluor 488-conjugated donkey anti-goat IgG polyclonal antibody (cat. # ab150129, 1:200 dilution; Abcam, United Kingdom).

Experimental Procedures Involving Animals

Male C57Bl/6J mice (2 months old, 20–25 g) from the Federal State Budgetary Scientific Institution "Scientific Research Institute of Neurosciences and Medicine" (SRINM) (Novosibirsk, Russia) were used. Animals were kept on a standard laboratory diet and under standard conditions (light–dark cycle: 14 h light and 10 h dark; temperature: 20–22°C; relative humidity: 50–60%). All the experimental procedures were carried out in accordance with the guidelines of the NIH Guide for the Care

and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the SRINM. Every effort was made to minimize the number of animals used and their suffering.

Experiments were conducted on a pharmacological model of neurodegeneration caused by central injection of an amyloid beta (Aβ) fragment 25–35. Mice were subdivided into four groups (15–20 animals each): (1) bilateral injections of sterile water into the lateral ventricles of the brain (i.c.v.) and intraperitoneal (i.p.) administration of saline (0.9% NaCl solution, 100 μl/10 g) for 36 days, (2) bilateral i.c.v. injections of sterile water and i.p. administration of CEF (100 mg/kg/day for 36 days), (3) bilateral i.c.v. injections of an Aβ fragment (Aβ25–35) and i.p. administration of saline for 36 days, and (4) bilateral i.c.v. injection of Aβ25–35 and i.p. administration of CEF for 36 days. All animals underwent stereotaxic surgery on day 0. The treatment with CEF was started on day 1. During the 2nd–5th weeks after the introduction of Aβ or vehicle into cerebral ventricles, behavioral testing was performed, after which biological samples were collected. On day 37, four randomly selected mice per group were sacrificed by exposure to CO_2 and transcardially perfused with phosphate-buffered saline (PBS) and followed by 4% paraformaldehyde in PBS; then, their brains were rapidly removed and post-fixed in PBS containing 30% sucrose at 4°C. After being immersed in the embedding Tissue-Tek O.C.T. compound (Sakura Finetek, United States), the brains were frozen and stored at −70°C until sectioning into 30-μm-thick slices with a cryostat HistoSafe MicroCut-SADV (China).

The Model of Alzheimer's Disease and Drug Administration

Aβ25–35 was dissolved in sterile water at a concentration of 1 mg/ml and stored at −20°C until use. Before administration to the animals, the prepared Aβ solution was thawed and incubated for 4 days at 37°C to form aggregates. Injections into cerebral ventricles were performed as described earlier (Tikhonova et al., 2020). The mice were anesthetized by administration of a 2.5% solution of avertin (2,2,2-tribromoethanol and 2-methyl-2-butanol, 100 μl/10 g, i.p.; Sigma-Aldrich Co.). The Aβ solution or sterile water was injected bilaterally with a Hamilton syringe (25 μl, model 1702 RN SYR, with a 22s ga needle, 2 in.), using a micropump (injection rate 0.8 μl/min). The needle was left at the injection site for 2 min after the injection. A total of 10 μl of the solution (9.4 nmol) were injected. The following coordinates adapted from the mouse brain atlas were used (Paxinos and Franklin, 2013): AP: -0.5 mm, ML: ±1 mm, and DV: −3 mm from the bregma, midline, and skull surface, respectively.

The rationale behind the CEF dosage (100 mg/kg/day) adopted in the current study was based on our recent studies showing neuroprotective effects of CEF in correcting behavioral and neuronal deficits (Tikhonova et al., 2017) and modifying the expression of genes related to the system of Aβ metabolism in the brain (Tikhonova et al., 2018) in another AD model (OXYS rats). Mice were weighed weekly during the experiment to adjust the drug dosage. The drug administration that preceded the testing of behavior or the collection of bio-samples was performed in 1 day prior to the corresponding manipulation in order to avoid the acute effects of CEF.

Behavioral Tests

Each animal was handled for 5 min/day on three consecutive days, before taking into the experiment.

IntelliCage

7–8 mice of each group were tested in an observer-independent setting using the IntelliCage apparatuses (TSE systems, Germany). The IntelliCage for mice with minor modifications has been described in sufficient details in a number of studies (Galsworthy et al., 2005; Barlind et al., 2010; Benner et al., 2015; Fischer et al., 2017). Briefly, it consists of a transparent cage (20.5 × 55 × 37.5 cm; Tecniplast, 2000P) equipped with four operant learning chambers (15 × 15 × 21 cm), which fit in to the corners of the housing cage. Each corner chamber holds two bottles of water (eight bottles per cage in total) that are separated from the living part of the cage by a circular automatically closable door (13 mm in diameter) with sensors for controlling access to bottles and an RFID antenna for identifying mice. Individual identification of a mouse was provided by subcutaneous implantation of a microchip into its interscapular region under light anesthesia 1 week before the experiment. To study the advanced conditioned responses (i.e., patrolling behavior), there are three colored LEDs above each door. Doors open when a sensor is activated with a mouse's nosepoke. Having opened the door, the mouse receives positive reinforcement (drinking water) or negative stimulation (trigger an air puff). IntelliCage Plus software controls an experimental protocol and registers automatically the number and duration of visits to corners, nosepokes, and licks. As a social group containing up to 16 mice could be tested at a time in the IntelliCage, we used two IntelliCage devices, one for Aβ-treated mice and another for vehicle (H_2O)-injected mice. A protocol in the experiment included the tests for place learning, place learning reversal, àvoidance conditioning, avoidance extinction, and patrolling behavior. The protocol details are presented as **Supplementary Table 1**.

The rest of the mice were tested in the *T*-maze, Barnes, open field, and passive avoidance tests. They were housed in groups of four to five in acrylic cages (25 × 40 × 20 cm) in an animal room. In 2 weeks after surgery (i.c.v. Aβ administration, day 0), the mice were subjected to tests for behavioral phenotyping: the *T*-maze test on days 15–17, Barnes test on days 20–25, open field on day 27, and passive avoidance test on days 34–36. All observations were performed during the light phase between 12:00 and 20:00 h. For behavioral testing, the animals were placed individually in a clean cage (25 × 40 × 20 cm) and transported to a dim observation room (28 lx of the red light) with sound isolation reinforced by a masking white noise of 70 dB. Performance in the behavioral tests was monitored using a video camera (Sony, China) positioned above an apparatus and processed with original EthoVision XT software (Noldus, Netherlands). The test equipment was cleaned using 20% ethanol and thoroughly dried before each test trial.

The *T*-Maze Test

The test was conducted according to the spontaneous alteration protocol at red lighting of 28 lx (Deacon and Rawlins, 2006). *T*-shaped apparatus consists of a start arm (30 × 7 cm) and two side arms (37 × 7 cm) with plastic walls of 20 cm high. Start zone in the start arm is 18 × 7 cm, while central zone between the side arms is 7 × 7 cm. All compartments are separated by automatic slide doors controlled remotely by the EthoVision XT software (Noldus, Netherlands). The test consisted of three trials per day during three consecutive days for each mouse. Each trial included two choice runs. At the beginning of each run, a mouse was placed in the start zone. During each run, the mouse made a choice of a side arm by entering into it. In the first run, right after the choice was made, a slide door separating the side arm with the mouse shut down, and the mouse had stayed in the selected arm for 30 s until the second run. In the second run, a mouse should choose a side arm opposite to that chosen in the first run (correct choice). Correct responses in the nine trials were recorded. The percentage of correct choices was regarded as an index of working memory (Deacon and Rawlins, 2006; Paul et al., 2009). The duration of each run was restricted by 90 s.

Barnes Maze Test

The test assesses spatial learning and memory. A mouse was placed on an elevated open circular arena (d = 120 cm, height from the floor = 90 cm) with 40 holes (d = 5 cm, distance between holes = 8 cm). An escape box was placed beneath one of the holes, and its location was randomly assigned to four positions for each mouse. Aversive bright lighting (1,000 lx) and the stress of being in the open space motivated an animal to search for the escape box to hide. Visual cues placed in the testing room provided spatial orientation. Testing was conducted according to the standard protocol (Dudchenko, 2004; Paul et al., 2009) and consisted of three phases: habituation (1 day, two sessions of 3 min), acquisition (4 days, four sessions of 3 min/day), and testing trial (1 day, one session of 60 s). *Habituation*: a mouse was placed near the hole with the escape box attached ("goal hole"); if the animal did not find the goal hole within 3 min, it was gently guided to the escape box and left there for 60 s. *Acquisition*: the animal was placed in the center of a platform and was free to explore the platform and search for the goal hole and escape box; if the animal did not find the goal hole within 3 min, it was gently guided to the escape box and left there for 60 s. The latency of finding the goal hole was recorded. Episodic memory was assessed as the dynamics of the latency in the four consecutive sessions on the first training day. During the *Testing trial*, the escape box was removed, and mice moved freely for 60 s. Exploratory activity (by the total number of nosepokes) and long-term memory and learning (by the percentage of the nosepokes to the goal hole) were evaluated.

The Open Field Test

This test was carried out in an apparatus with a square arena (40 × 40 cm) and plastic walls 37.5 cm high brightly lit from above (1,000 lx). A mouse was placed near the wall, and its movements were recorded for 10 min. The following parameters were determined: general locomotion (the distance traveled in cm), vertical locomotor and exploratory activity (rearing number), anxiety (time spent in the central part of the arena), and emotionality (defecation number).

The Passive Avoidance Test

Training on the passive avoidance reaction was performed by a standard single-session method in an experimental chamber with dark and light compartments and an automated Gemini Avoidance System apparatus (San Diego Instruments, CA, United States) as described in detail earlier (Tikhonova et al., 2020). The Gemini software automatically recorded the latency of the transfer to the dark compartment, and the data of testing served as a measure of acquisition of the conditioned passive avoidance reaction.

Nissl Staining and Immunohistochemical Analysis

Nissl Staining

Coronal slices along the frontal cortex (AP = 2.93–2.57 mm) or hippocampus (AP = -1.91 to −2.45 mm) of each mouse brain were made. Unstained brain sections were identified according to the mouse brain atlas (Paxinos and Franklin, 2013). Nissl staining, used to measure the neuronal density, was performed as described in our previous reports (Weng et al., 2016; Tikhonova et al., 2017). The image was captured and analyzed using a microscope Nikon Eclipse Ci (Nikon, China) coupled to a Nikon DS-Fi2 camera (Nikon, China) and Image Pro Plus Software 6.0 (Media Cybernetics, CA, United States). The neuronal density was measured by a semi-quantitative method as described earlier (Ho et al., 2014; Tikhonova et al., 2017) since it is difficult to directly count the number of neurons in a 30-μm thick brain section because the neurons are tightly packed. We calculated the percentage of an area of interest (AOI) in the 3rd layer of the frontal cortex (AOI size: 93,023 μm^2) and CA1 or CA3 area of the hippocampus (AOI size: 88,502 μm^2) occupied by Nissl-stained cells. The analyzer was blind to the treatment.

Immunohistochemical Analysis

Brain sections for the Immunohistochemical (IHC) analysis were randomly taken from the same animals that were used for the histological assay counting the density of neurons with Nissl staining. The IHC analysis was performed according to a protocol described in detail previously (Tikhonova et al., 2017, 2020). Antibodies used are listed in the Reagents section. The fluorescence images were finally obtained by an Axioplan 2 (Carl Zeiss) imaging microscope and then analyzed in Image Pro Plus Software 6.0 (Media Cybernetics, MD, United States). Fluorescence intensity associated with the expression of specific proteins (Aβ, CD54, or IBA1) was measured as background-corrected optical density (OD) with subtraction of staining signals of the non-immunoreactive regions in the images converted to grayscale. The AOI size was 18,208 μm^2 in the 3rd layer of the frontal cortex or in the dentate gyrus of the hippocampus, 19,353 μm^2 in the CA1 area, or 26,100 μm^2 in the CA3 area of the hippocampus. The analyzer was blind to the treatment.

Data Analysis

The results were presented as mean ± SEM and compared using a two-way ANOVA followed by *post hoc* Fisher's least significant difference (LSD) test. The independent variables for the two-way ANOVA were Aβ administration [control (mice administered i.c.v. with H_2O) or Aβ-treated mice] and CEF treatment (saline- or CEF-treated mice). Repeated-measures ANOVA followed by Fisher LSD *post hoc* comparison was applied to analyze the data of the passive avoidance test/Barnes test with Aβ administration and CEF treatment as between-subject variables and time (training or test/number of a session on the first day of training) as a repeated measure. The level of significance was defined as $p < 0.05$. STATISTICA 10.0 software (StatSoft, Tulsa, OK, United States) was used to perform all statistical analyses.

RESULTS

Analysis of Behavioral Effects of Ceftriaxone in the Aβ-Induced Mouse Alzheimer's Disease Model

The efficacy of CEF in recovering cognitive function was evaluated using behavioral phenotyping: the test of passive avoidance learning (**Figure 1**), Barnes test (**Figure 2**), *T*-maze test (**Figure 3F**), and IntelliCage paradigm (**Figures 3A–E**). To assess the CEF effects on general locomotion and exploratory activity, the open field test was applied (**Table 1**).

The Passive Avoidance Test

There was a significant influence of the CEF treatment [$F(1,17) = 5.2$, $p < 0.05$], learning (repeated measures) [$F(1,17) = 33.0$, $p < 0.001$], and of the interaction between these factors [$F(1,17) = 6.2$, $p < 0.05$] on the step-through latency. Latency to enter a dark compartment during training (before the foot shock) did not differ significantly among the experimental groups (**Figure 1**). As evidence of learning on testing day, 24 h after receiving the foot shock, control mice of the H_2O + saline group showed increased step-through latencies, often ∼10-fold greater than latencies on training day, so did the mice of the H_2O + CEF and Aβ25–35 + CEF groups. In contrast to those groups, the step-through latencies of Aβ25–35 + saline-treated mice (AD model) were sharply reduced and did not differ from the latencies during training ($p > 0.05$), indicating memory impairment. Thus, CEF treatment prominently improved the learning deficit in the mouse Aβ25–35-induced AD model as evidenced by a significantly longer retention latencies in comparison with Aβ25–35 + saline-treated mice ($p < 0.01$). Noteworthy, CEF treatment augmented step-through latencies in the Aβ25–35 + CEF group up to values observed in control mice of the H_2O + saline group.

Barnes Test

Episodic spatial memory was estimated in the Barnes test. Latencies of finding the goal box at four trials on the first day of training were assessed. The dynamics of learning is summarized in **Figure 2A**. There was a significant influence of the learning (repeated measures) [$F(3,117) = 17.0$, $p < 0.001$] and of the interaction between the factors of learning and Aβ25–35 administration [$F(3,117) = 3.01$, $p < 0.05$]. Control mice of the H_2O + saline group ($p < 0.01$), as well as mice of the H_2O + CEF ($p < 0.05$) group, showed a significant decrease in the latency of finding the goal hole by the third trial, while mice of both groups administered with Aβ25–35 demonstrated the significant latency reduction by the fourth trial. However, animals of the Aβ25–35 + CEF group had shorter latency in the fourth trial than those of the Aβ25–35-induced AD model without CEF treatment ($p < 0.05$). On the test day, no significant effects of the factors on the index of exploration (the total number of nosepokes to holes) were found (**Figure 2B**), while the parameter of spatial memory and learning (% of the goal hole nosepokes) was significantly influenced by the interaction between the factors of Aβ25–35 administration and CEF treatment [$F(1,38) = 4.9$, $p < 0.05$; **Figure 2C**]. The percentage of the goal hole nosepokes was markedly augmented in the Aβ25–35 + CEF group as compared with the Aβ25–35 + saline group ($p < 0.05$).

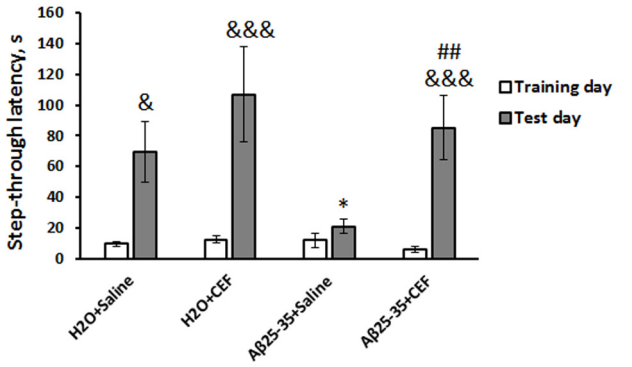

FIGURE 1 | Effects of the CEF and Aβ25–35 administration (AD model) on memory retrieval in mice in the passive avoidance test. The data are expressed as mean ± SEM of the values obtained in an independent group of animals (n = 5–6 per group). Statistically significant differences: $^{\&}p < 0.05$, $^{\&\&\&}p < 0.001$ compared with values of the same group on the training day; $^{*}p < 0.05$ vs. the "H_2O + saline" group on the test day; $^{\#\#}p < 0.01$ vs. the "Aβ + saline" group on the test day.

IntelliCage

In the place learning test, there was a significant influence of learning (repeated measures) [$F(4,104) = 76.03$, $p < 0.001$] and of its interaction with the factor of Aβ25–35 administration [$F(4,104) = 7.29$, $p < 0.001$] on the percentage of correct visits (**Figure 3A**). All experimental groups demonstrated a significant increase in the percentage of correct visits on days 2–5 of place learning testing compared with the first day (learning period) of this phase. LSD *post hoc* test revealed that on the first day of place learning testing, the percentage of correct visits was substantially reduced in the Aβ25–35 + saline group in comparison with the control mice of the H_2O + saline group ($p < 0.01$) indicating episodic memory and learning disturbances in mice exposed to the neurotoxic effects of Aβ25–35, while mice of the Aβ25–35 + CEF group did not show a significant decrease in the parameter as compared with the groups that did not receive Aβ25–35 injections. However, on the next days, the percentage of

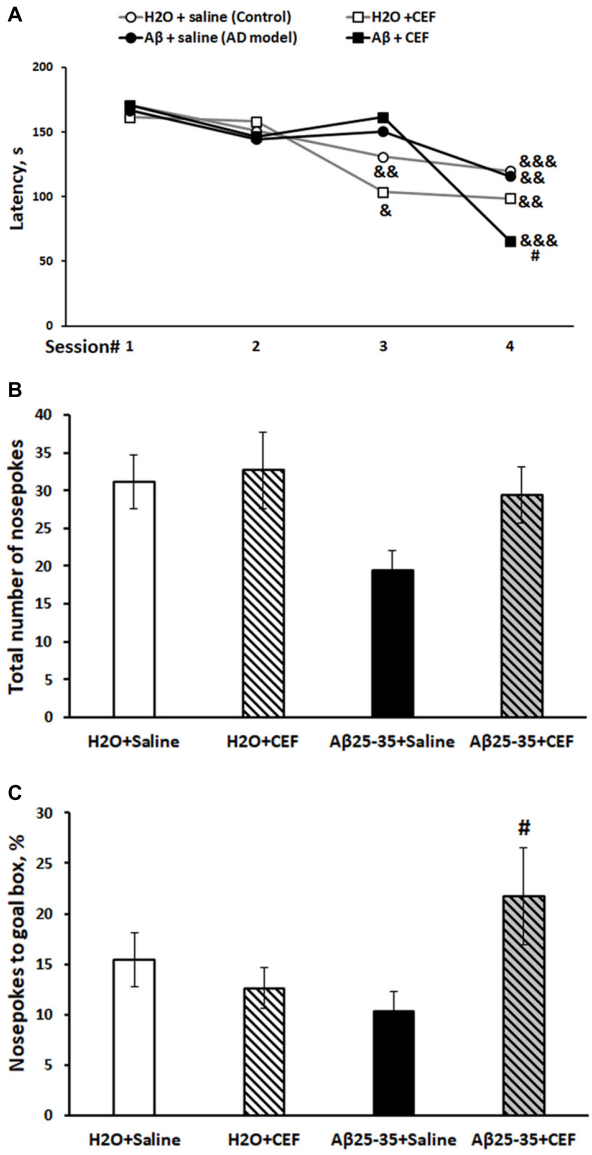

FIGURE 2 | Effects of the CEF and Aβ25–35 administration (AD model) on spatial memory and learning in mice in the Barnes test. **(A)** Episodic memory and learning were evaluated by the latency (s) to find an escape box during the first day of training. **(B)** General exploratory activity was estimated by the total number of nosepokes on the test day. **(C)** Long-term spatial memory was evaluated by the percentage of nosepokes to the target hole on the test day. The data are expressed as mean ± SEM of the values obtained in an independent group of animals (n = 7–15 per group). Statistically significant differences: $^{\&}p < 0.05$, $^{\&\&}p < 0.01$, $^{\&\&\&}p < 0.001$ compared with values of the same group on the first training session; $^{\#}p < 0.05$ compared with respective values of the "Aβ + saline" group.

correct visits increased in mice of the Aβ25–35 + saline group to the level of mice treated with H_2O instead of Aβ25–35. The most profound difference in the percentage of correct visits between the mice treated with Aβ25–35 of the Aβ25–35 + saline group and those of the H_2O + saline group was noted during the period of 3–6 h on the first day, while mice of the Aβ25–35 + CEF group did not show a significant decrease in the parameter as compared with the groups that did not receive Aβ25–35 injections (learning (repeated measures) factor [$F(3,75) = 7.29$, $p < 0.001$], Aβ25–35 administration [$F(1,25) = 9.2$, $p < 0.01$]; **Figure 3B**).

In the place learning reversal test, there was a significant influence of learning (repeated measures) [$F(4,104) = 33.83$, $p < 0.001$] on the percentage of correct visits, as well as on the percentage of incorrect visits [$F(4,104) = 33.0$, $p < 0.001$], while the effects of other factors or their interaction were insignificant. All experimental groups demonstrated a significant increase in the percentage of correct visits (**Figure 3C**) and simultaneous significant decrease in the percentage of incorrect visits (data not shown) on days 2–5 of place learning reversal testing compared with the first day (learning period) of this phase. No significant intergroup differences were found. Neither intergroup differences were observed on the first day [learning (repeated measures) factor [$F(3,75) = 10.71$, $p < 0.001$] for the percentage of correct visits and learning (repeated measures) factor [$F(3,75) = 15.79$, $p < 0.001$] for the percentage of incorrect visits; data not shown]. Thus, the reversal learning ability was observed in all the groups studied. Aβ25–35 administration or CEF treatment did not affect this feature significantly.

Similarly, in the avoidance conditioning test, there was a significant influence of learning (repeated measures) [$F(3,78) = 624.2$, $p < 0.001$] on the percentage of incorrect visits, while the effects of other factors or their interaction were insignificant. All experimental groups demonstrated a significant decrease in the percentage of incorrect visits (**Figure 3D**) since the first day of training compared with the percentage of the corner at previous phase (the last day of the place learning reversal test when the corner was assigned as correct and mice were not punished for its visiting). On days 2–3 of the avoidance conditioning test, further decrease in the percentage of incorrect visits was observed in all groups as compared with the first day of training. Thus, the learning ability at avoidance conditioning was observed in all the groups studied. Aβ25–35 administration or CEF treatment did not affect this feature significantly.

The avoidance extinction test revealed a significant influence of learning (repeated measures) [$F(5,130) = 26.1$, $p < 0.001$] and Aβ25–35 administration [$F(1,26) = 10.55$, $p < 0.01$] on the percentage of visits to the corner that was assigned as incorrect during the avoidance conditioning test, while the effects of other factors or their interaction were insignificant. All experimental groups demonstrated a gradual increase in the percentage of the visits during the avoidance extinction test (**Figure 3D**). Mice of the H_2O + saline or H_2O + CEF group had shown the increased percentage of the visits since the second day of the avoidance extinction phase compared with the last day of the avoidance conditioning test. However, mice treated with Aβ25–35 revealed a retarded extinction of avoidance learning. Mice of the Aβ25–35 + saline group had demonstrated a significant increase in the percentage of the visits since the fourth day, while mice of the Aβ25–35 + CEF group since the fifth day of the avoidance extinction test. Noteworthy, values of the parameter in the Aβ25–35 + CEF group were significantly lower than those in the H_2O + saline or H_2O + CEF group on all days of the avoidance extinction test (**Figure 3D**).

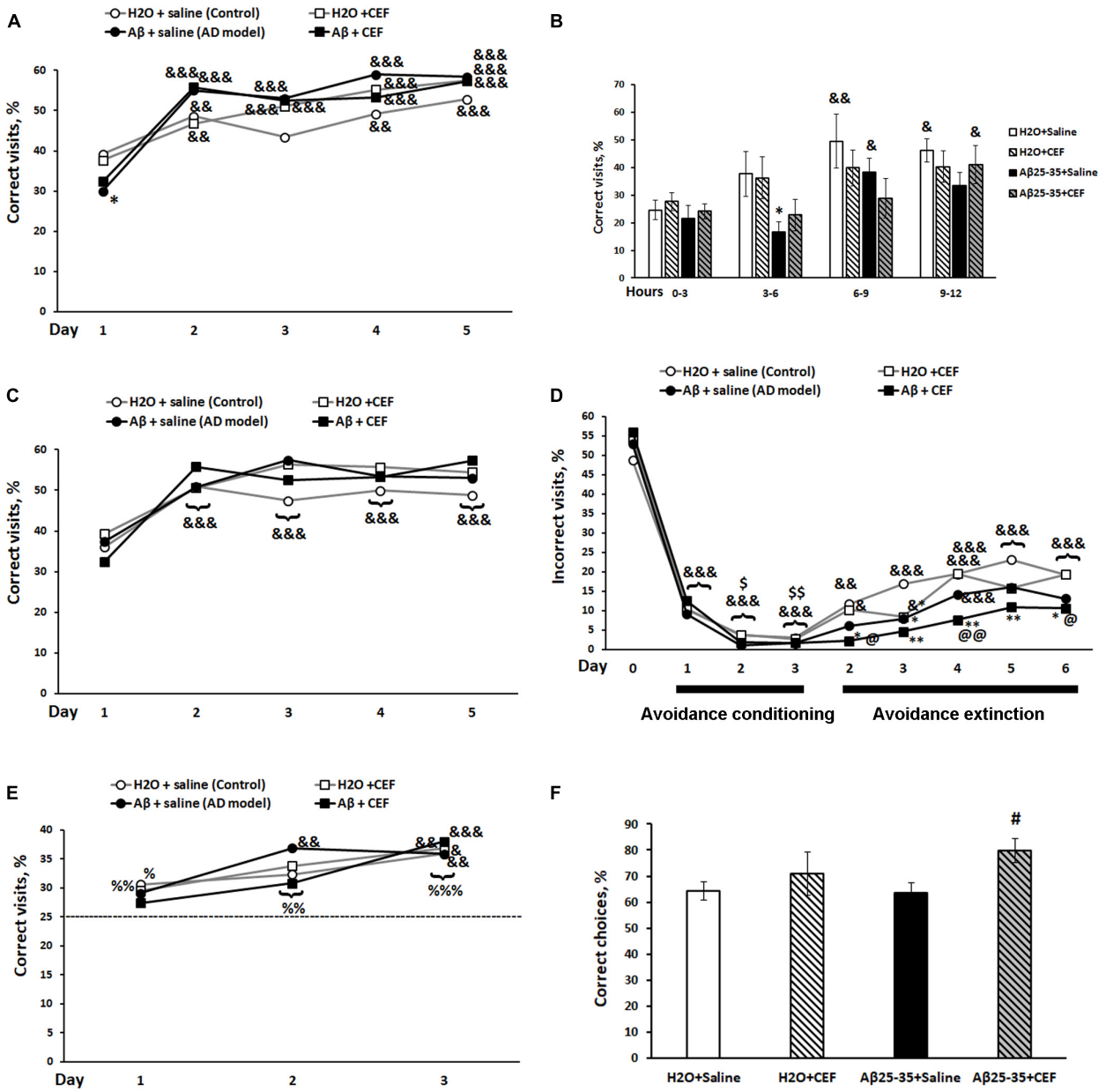

FIGURE 3 | Effects of the CEF and Aβ25–35 administration (AD model) on the place learning **(A,B)**, place learning reversal **(C)**, avoidance conditioning and extinction **(D)**, patrolling behavior **(E)** in the IntelliCage, or on the working spatial memory in the *T*-maze test **(F)** in mice. The dynamics of place learning in the IntelliCage was evaluated during the whole period of the test with the values pooled daily **(A)** or during the first day of this phase of the test with the values pooled for 3 h **(B)**. The data are expressed as means **(A,C–E)** or mean ± SEM **(B,F)** of the values obtained in an independent group of animals (n = 7–8 per group). Statistically significant differences: $^{\&}p < 0.05$, $^{\&\&}p < 0.01$, $^{\&\&\&}p < 0.001$ compared with values of the same group at the first timepoint of the phase, for avoidance conditioning—compared with values of the same group on the last day of the previous phase (day 0 in the figure), for avoidance extinction—compared with values of the same group on the last day of the previous phase (day 3 of the avoidance conditioning test); $^{\$}p < 0.05$, $^{\$\$}p < 0.01$ compared with values of the same group on the first day of the avoidance conditioning test; $^{\%}p < 0.05$, $^{\%\%}p < 0.01$, $^{\%\%\%}p < 0.001$ compared with the chance level (25%); $^{*}p < 0.05$, $^{**}p < 0.01$ compared with respective values of the "H_2O + saline" group; $^{\#}p < 0.05$ compared with respective values of the "Aβ + saline" group; $^{@}p < 0.05$, $^{@@}p < 0.01$ compared with respective values of the "H_2O + CEF" group.

The test for patrolling behavior also revealed a significant effect of learning (repeated measures) on the percentage of correct visits [$F(2,52) = 21.1$, $p < 0.001$], while the effects of other factors or their interaction were insignificant. According to LSD *post hoc* test, all experimental groups demonstrated a significant increase in the percentage of correct visits on the third day of testing compared with the first day of training. All groups had shown a significantly increased level of correct visits as compared with the chance level (25%) since the second day of the test for patrolling behavior (**Figure 3E**). Thus, the working

TABLE 1 | Effects of the CEF and Aβ25–35 administration (AD model) on the behavior of mice in the open field test.

Index	Group				Effects (*F*, *p*)
	H_2O + saline	H_2O + CEF	Aβ25–35 + saline	Aβ25–35 + CEF	
Locomotor activity (path length, cm)	2728.0 ± 106.2	3224.7 ± 223.2*	2668.2 ± 157.9	2580.5 ± 184.9$	**Aβ:** $F(1,37) = 4.4$, $p < 0.05$; **CEF:** $F(1,37) = 1.5$, $p > 0.05$; **Aβ × CEF:** $F(1,37) = 3.0$, $p > 0.05$
Exploratory activity (no. of rearings)	65.9 ± 3.9	63.5 ± 5.5	60.1 ± 5.3	58.4 ± 7.0	**Aβ:** $F(1,37) < 1$; **CEF:** $F(1,37) < 1$; **Aβ × CEF:** $F(1,37) < 1$
Anxiety (time in the center, s)	31.8 ± 4.1	34.1 ± 7.7	26.9 ± 3.7	21.9 ± 2.7	**Aβ:** $F(1,37) = 3.1$, $p > 0.05$; **CEF:** $F(1,37) < 1$; **Aβ × CEF:** $F(1,37) < 1$
Emotionality (no. of fecal boluses)	1.40 ± 0.48	1.17 ± 0.65	2.08 ± 0.59	2.14 ± 0.51	**Aβ:** $F(1,37) = 1.8$, $p > 0.05$; **CEF:** $F(1,37) < 1$; **Aβ × CEF:** $F(1,37) < 1$

*Data are presented as mean ± SEM of the values obtained in an independent group of animals (n = 7–15 per group). Statistically significant differences: *p < 0.05 vs. the "H_2O + saline" group; $ p < 0.05 vs. the "H_2O + CEF" group.*

memory was not disturbed in all the groups studied. Aβ25–35 administration or CEF treatment did not affect this feature significantly. However, it should be noted that the Aβ25–35-treated groups did not differ significantly in the percentage of correct visits in comparison with the chance level on the first day of the test for patrolling behavior, while the H_2O-treated groups had significantly augmented level of correct responses compared with the chance level on the first day of the test. That may indicate to the retarded learning of a new rule in the Aβ25–35-treated groups.

T-Maze Test

When comparing the indices of the working spatial memory in the *T*-maze test using the spontaneous alteration protocol, a significant influence of CEF treatment [$F(1,34) = 5.2$, $p < 0.05$] but not the Aβ25–35 injection factor [$F(1,34) < 1$] or interaction between the factors [$F(1,34) < 1$] on the percentage of correct choices was revealed. The percentage of correct choices in the Aβ25–35 + CEF group was higher than that in the Aβ25–35 + saline group ($p < 0.05$) (**Figure 3F**).

Open Field Test

Evaluation of general locomotor and exploratory activity and some other parameters was carried out by an open field test. The results are summarized in **Table 1**. There was a significant influence of Aβ25–35 administration [$F(1,37) = 4.4$, $p < 0.05$] on the locomotion (distance traveled), while the effects of CEF treatment or interaction between the factors were insignificant. However, mice of the Aβ25–35 + saline group did not differ significantly from those of the H_2O + saline group or the Aβ25–35 + CEF group in the distance traveled. Moreover, the groups studied did not differ significantly in the indices of exploratory activity (number of rearings), anxiety (time spent in the center of the arena), or emotionality (number of fecal boluses) as well.

Analysis of Ceftriaxone Effects on Neuronal Density, Aβ Accumulation, and Neuroinflammation in the Aβ-Induced Mouse Alzheimer's Disease Model

Nissl Staining

We have not found significant differences in the neuronal density in the frontal cortex or hippocampal CA1 and CA3 regions between the groups. Neither Aβ25–35 administration nor CEF treatment affected this feature in C57Bl6/J mice significantly. The detailed results are presented as **Supplementary Table 2**.

At the same time, a pronounced influence of Aβ25–35 administration or CEF treatment on Aβ accumulation and neuroinflammatory features was revealed.

Aβ Staining

In mice subjected to central administration of the Aβ25–35, Aβ burden was significantly reduced after CEF therapy in the frontal cortex and hippocampus (**Figure 4**). Significant effects of the factors of Aβ25–35 administration [$F(1,8) = 62.2$, $p < 0.001$], CEF treatment [$F(1,8) = 30.1$, $p < 0.001$], and their interaction [$F(1,8) = 46.3$, $p < 0.001$] on the levels of Aβ in the frontal cortex in mice were found. Similarly, the content of Aβ was significantly augmented in the CA1 and CA3 regions or the dentate gyrus of the hippocampus in mice of the Aβ25–35 + saline group given Aβ25–35 injections compared with control mice of the H_2O + saline group, and it decreased to the level of the control group after CEF therapy (**Figures 4C,D,E**). In the CA1 area of the hippocampus, significant effects of the Aβ25–35 administration [$F(1,8) = 19.1$, $p < 0.01$] and the interaction between the factors of Aβ25–35 and CEF treatment [$F(1,8) = 7.8$, $p < 0.05$] on the Aβ levels were observed. In the CA3 area of the hippocampus, significant effects of the Aβ25–35 administration [$F(1,8) = 23.2$, $p < 0.01$], CEF treatment [$F(1,8) = 9.5$, $p < 0.05$], and the

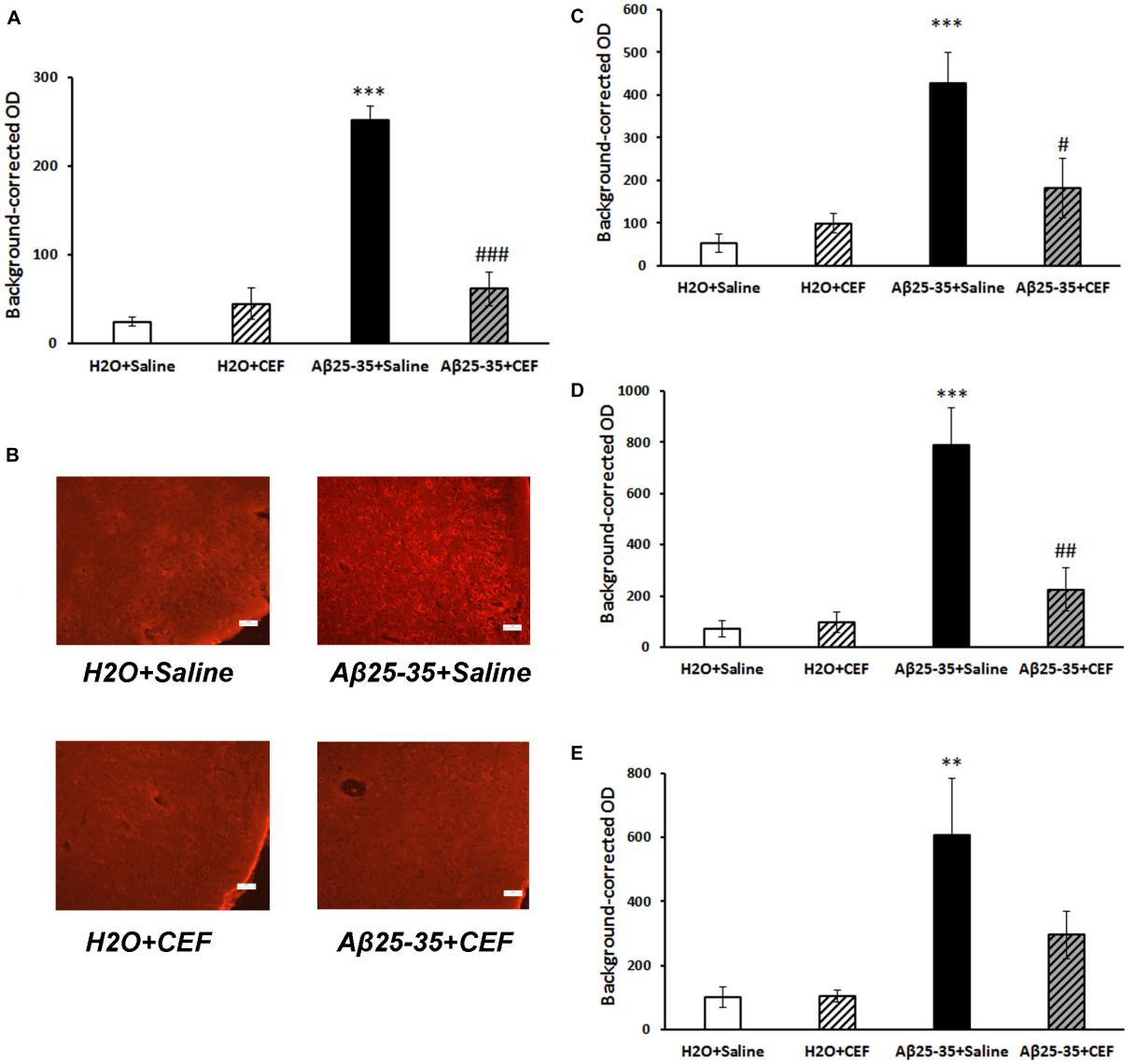

FIGURE 4 | Effects of the CEF and Aβ25–35 administration (AD model) on the Aβ accumulation in the frontal cortex **(A,B)** or hippocampus (**C** in the CA1 area; **D** in the CA3 area; **E** in the dentate gyrus) in mice. **(A,C–E)** Quantitative results. The data are expressed as mean ± SEM of the values obtained in an independent group of animals (n = 3–4 per group). Statistically significant differences: $^{**}p < 0.01$, $^{***}p < 0.001$ vs. the "H_2O + saline" group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. the "Aβ + saline" group. **(B)** Aβ immunoreactivity in the frontal cortex. Magnification, ×200; bar, 50 μm.

interaction between the factors [$F(1,8) = 11.3$, $p < 0.01$] on the Aβ levels were found. Aβ25–35 administration [$F(1,8) = 12.3$, $p < 0.01$] but not CEF treatment or interaction of the factors influenced significantly the content of Aβ in the dentate gyrus of the hippocampus.

For neurodegenerative disorders and AD in particular, neuroinflammation is one of the key pathogenetic features. Hence, we evaluated the effects of CEF treatment on neuroinflammatory indices.

CD54 Expression

The expression of inflammatory marker CD54 was significantly increased in the frontal cortex and hippocampus in mice of the Aβ25–35 + saline group given Aβ25–35 injections compared with control mice of the H_2O + saline group, while it decreased substantially to the level of the control group after CEF therapy (**Figure 5**).

Significant influence of Aβ25–35 administration [$F(1,11) = 5.42$, $p < 0.05$] was revealed on the levels of CD54 in the frontal cortex. In the CA1 area of the hippocampus, the interaction between the factors of Aβ25–35 and CEF treatment influenced significantly the expression of CD54 [$F(1,10) = 5.5$, $p < 0.05$]. In the CA3 area of the hippocampus, a significant effect of CEF treatment [$F(1,9) = 9.5$, $p < 0.05$] on the CD54 levels was found. Aβ25–35 administration [$F(1,9) = 6.95$, $p < 0.05$], as well as the interaction between the factors of Aβ25–35 and CEF

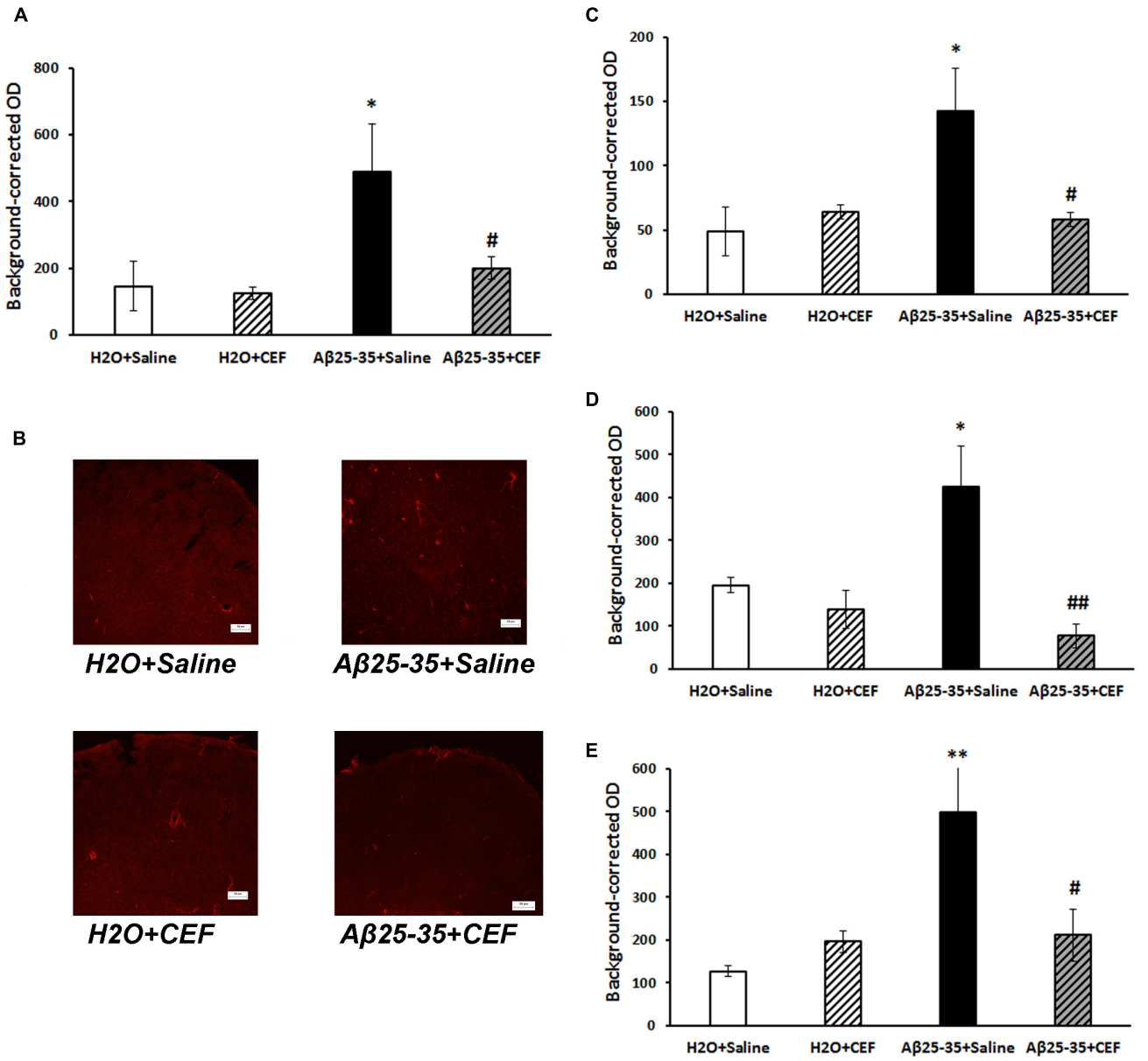

FIGURE 5 | Effects of the CEF and Aβ25–35 administration (AD model) on the expression of inflammatory marker CD54 in the frontal cortex **(A,B)** or hippocampus (**C** in the CA1 area; **D** in the CA3 area; **E** in the dentate gyrus) in mice. **(A,C–E)** Quantitative results. The data are expressed as mean ± SEM of the values obtained in an independent group of animals (n = 3–4 per group). Statistically significant differences: $^{*}p < 0.05$, $^{**}p < 0.01$ vs. the "H_2O + saline" group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. the "Aβ + saline" group. **(B)** CD54 immunoreactivity in the frontal cortex. Magnification, ×200; bar, 50 μm.

treatment [$F(1,9) = 5.85$, $p < 0.05$], had a significant effect on the expression of CD54 in the dentate gyrus of the hippocampus.

Microglia Activation

Microglia activation was assessed by the expression of IBA1 marker. Its expression was significantly increased in the frontal cortex in mice of the Aβ25–35 + saline group given Aβ25–35 injections compared with control mice of the H_2O + saline group ($p < 0.001$), while it decreased substantially to the level of the control group after CEF therapy (**Figures 6A,B**). Significant effects of the factors of Aβ25–35 administration [$F(1,10) = 37.4$, $p < 0.001$], CEF treatment [$F(1,10) = 17.5$, $p < 0.01$], and their interaction [$F(1,10) = 21.0$, $p < 0.01$] on the levels of IBA1 in the frontal cortex in mice were found.

At the same time, in the hippocampus, IBA1 expression was significantly influenced only by Aβ25–35 administration in the CA1 area [$F(1,8) = 26.3$, $p < 0.001$] or dentate gyrus [$F(1,8) = 94.4$, $p < 0.001$] but not in the CA3 region [$F(1,8) = 2.8$, $p > 0.05$], while the effects of CEF or interaction of the factors were insignificant (**Figures 6C–E**).

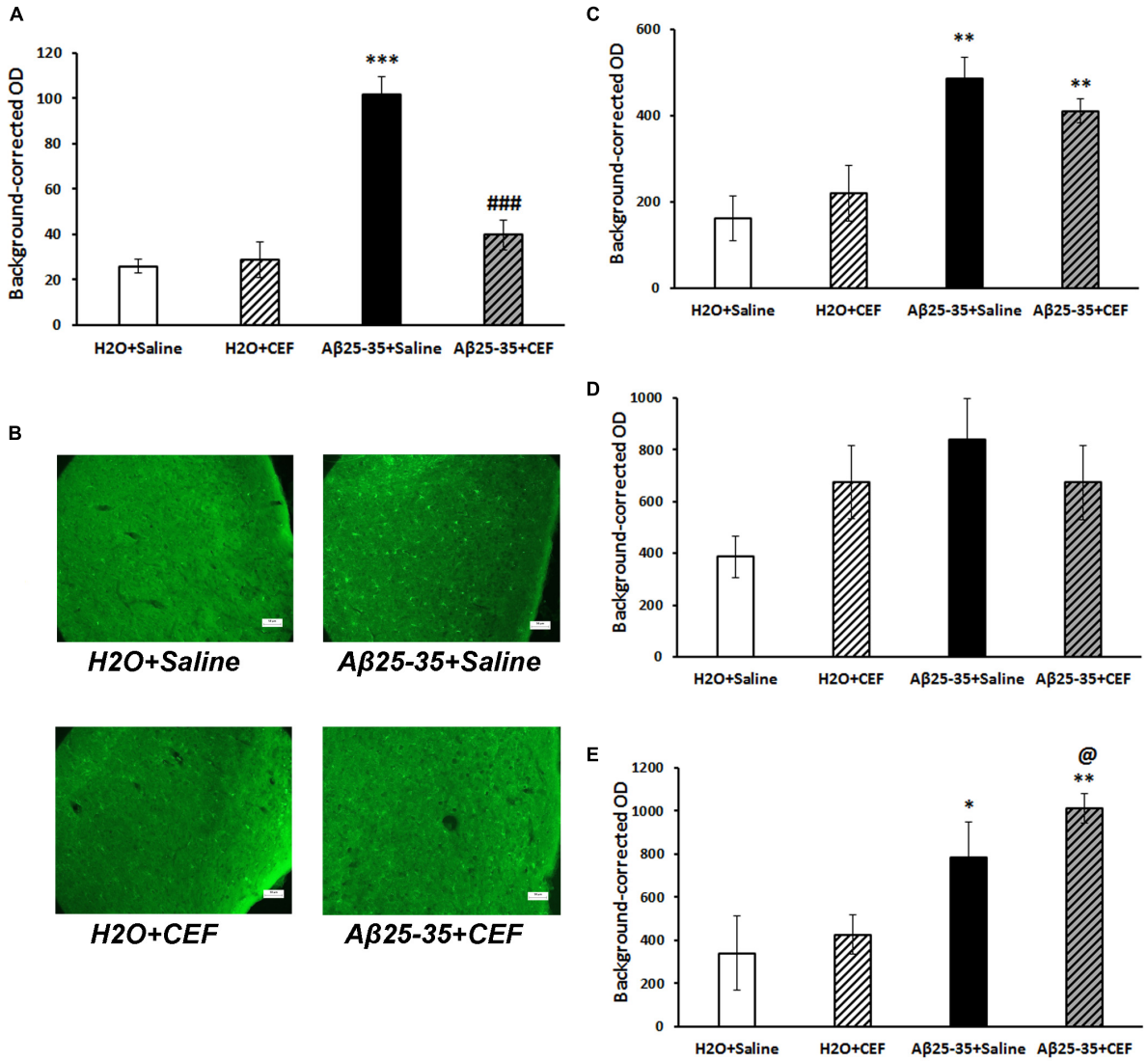

FIGURE 6 | Effects of the CEF and Aβ25–35 administration (AD model) on the expression of microglial marker IBA1 in the frontal cortex **(A,B)** or hippocampus (**C** in the CA1 area; **D** in the CA3 area; **E** in the dentate gyrus) in mice. **(A,C–E)** Quantitative results. The data are expressed as mean ± SEM of the values obtained in an independent group of animals (n = 3–4 per group). Statistically significant differences: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. the "H_2O + saline" group; $^{###}p < 0.001$ vs. the "Aβ + saline" group; $^{@}p < 0.05$ vs. the "H_2O + CEF" group. **(B)** IBA1 immunoreactivity in the frontal cortex. Magnification, ×200; bar, 50 μm.

It should be noted that in the frontal cortex, Aβ burden had strong positive correlation with both CD54 expression ($r_{12} = 0.74$, $p < 0.01$) and microglia activation ($r_{12} = 0.72$, $p < 0.01$). Aβ accumulation also correlated positively with microglia activation in the hippocampal CA1 area ($r_{12} = 0.82$, $p < 0.01$) or dentate gyrus ($r_{12} = 0.59$, $p < 0.05$) but not in the CA3 region. Positive correlation between Aβ burden and CD54 expression was found in the hippocampal CA3 area ($r_{11} = 0.61$, $p < 0.05$) but not in CA1 or dentate gyrus.

DISCUSSION

Beta-lactam antibiotics including CEF have been considered an optimistic group of drugs for treating neurodegenerative disorders (Kumari and Deshmukh, 2021). Through modulating the transcription and expression of the GLT-1, CEF protects neurons from excitotoxic neuronal damage (Rothstein et al., 2005). Moreover, CEF ameliorates symptoms across multiple rodent models of neurological diseases and substance use disorders associated with glutamate excitotoxicity-induced neuronal dysfunction (Tai et al., 2019; Yimer et al., 2019; Smaga et al., 2020). Since the glutamate mediated excitotoxicity is one of the essential pathogenic factors involved in various neurodegenerative pathologies including AD (Goncalves-Ribeiro et al., 2019), neuroprotective effects of CEF were studied in AD models as well (Smaga et al., 2020; Kumari and Deshmukh, 2021).

The beneficial effects of CEF on AD-related pathology were revealed earlier using transgenic mouse AD models [3xTg-AD (Zumkehr et al., 2015) and APPPS1 (Hefendehl et al., 2016) strains] or a genetic rat model of spontaneous AD (OXYS

strain) (Tikhonova et al., 2017). In transgenic murine models of advanced stages of AD-like pathology with highly expressed Aβ plaques (Zumkehr et al., 2015; Hefendehl et al., 2016), CEF neuroprotective effects were attributed to the attenuation of glutamatergic excitotoxicity induced by Aβ deposits, while no pronounced effect on APP processing, overall Aβ species levels (except for the increase in Aβ40 levels in the CEF-treated mice), or plaque pathology was observed (Zumkehr et al., 2015). In 5-month-old OXYS rats that correspond to an early stage of AD-like progression, our group revealed novel targets of CEF as it modulated the expression of genes related to the system of Aβ metabolism in the brain, namely, it affected mRNA levels of *Bace1*, *Ace2*, *Mme*, *Ide*, *Ece1*, and *Epo* (Tikhonova et al., 2018). Here, we checked whether CEF might influence Aβ burden at early stages of AD-like pathology. Indeed, in mice subjected to central administration of the Aβ25–35, Aβ deposition was significantly reduced after CEF therapy in the frontal cortex and hippocampus. Thus, we confirmed the CEF effects on Aβ-related hub of AD-like pathology. One may suggest that in the models of advanced stages of AD-like pathology with highly expressed Aβ deposits, those mechanisms activating enzymes of Aβ degradation are insufficient for considerable clearance from Aβ aggregates. In these cases, activation of other mechanisms such as macroautophagy that are responsible for segregation and eradication of pathological protein aggregates appears to be of benefit (Xin et al., 2018). It should be noted that CEF does not induce autophagy but rather has an inhibitory effect (Cui et al., 2014; Korolenko et al., 2020). In the mouse Aβ-induced AD model, CEF treatment reduced the augmented autophagy level in the brain (Korolenko et al., 2019).

Another process contributing much to the AD pathology is neuroinflammation. It is considered to be tightly involved into the amyloid cascade (Selkoe and Hardy, 2016). Hence, one may expect attenuation of Aβ-induced neuroinflammation due to the reduction of Aβ burden after CEF treatment. Indeed, the expression of a proinflammatory marker CD54 was substantially reduced by CEF in both the frontal cortex and hippocampus. However, the expression of a marker of microglia activation IBA1 was decreased in the frontal cortex after CEF treatment, but it remained augmented in the hippocampal regions. Moreover, no significant correlation was found between Aβ accumulation and the neuroinflammatory markers in the certain hippocampal areas. We consider that more complicated mechanism of the CEF anti-inflammatory effect takes place. Besides Aβ-related effect, effects of the CEF on other pathways regulating and modulating microglia function might be proposed. The suggestion is in a good agreement with recent findings on the CEF effects on microglial phagocytosis of glutamatergic synapses in the hippocampus of rats microinjected with Aβ1–40 through the reduction of synaptic production of the complement C1q (Wu et al., 2020).

AD has a multifactorial etiology and involves various pathological processes (e.g., neurotoxicity of protein aggregates, oxidative stress, neuroinflammatory response, disturbed neurotrophic function and neurogenesis, synaptic and neurotransmission dysfunction, ion disbalance, etc.) that often closely interact and overlap. Hence, multipurpose or multi-target therapy aimed at various important pathogenetic hubs in the course of AD is regarded currently as a relevant and promising approach (Sahoo et al., 2018). CEF appears to be a prospective drug of that kind as it potently and simultaneously targets glutamate excitotoxicity (Rothstein et al., 2005), oxidative pathways (Lewerenz et al., 2009; Stennett et al., 2017), neurotrophic function (Kaur and Prakash, 2017), neurogenesis (Ho et al., 2019), Aβ accumulation, and neuroinflammatory response as shown here.

Along with the beneficial effects on Aβ burden and neuroinflammatory response in the brain, CEF effectively prevented cognitive deficits in Aβ-treated mice. Aβ25–35 fragment used in the work is characterized by high neurotoxicity due to the high aggregative properties (Haass and Selkoe, 2007; Walsh and Selkoe, 2007). Although pharmacological Aβ-induced model of AD corresponds to early stages of AD-like pathology progression, mice or rats with Aβ-induced neurotoxicity demonstrate certain alterations in cognitive function including deficits in working memory, learning, or spatial memory (Park et al., 2011; Choi et al., 2013; El Bitar et al., 2014; Wu et al., 2020). In the present study, fear-associated memory and learning was considerably disturbed in Aβ-treated mice according to the passive avoidance test that is in a good agreement with a previous finding (Maurice et al., 1996). The behavioral response in the passive avoidance test was completely recovered by the CEF treatment. At the same time, the indices of working memory in the *T*-maze test or IntelliCage (patrolling behavior) or long-term spatial memory in the Barnes test were not significantly affected by Aβ administration. However, learning was slightly retarded in the Aβ25–35-treated groups on the first day of learning in the Barnes test or on the first day of the test for patrolling behavior in the IntelliCage; mice treated with Aβ25–35 revealed a retarded extinction of avoidance learning. Mice given CEF gained better scores when performing in the *T*-maze test (the working memory estimated) or in the Barnes test (long-term spatial memory and learning estimated) than the Aβ25–35 + saline group. In the IntelliCage, mice demonstrated different disturbances depending on a model of AD applied (Codita et al., 2010; Platt et al., 2011; Sekiguchi et al., 2011; Masuda et al., 2016). In the present study, we revealed a deficit of place learning on the first day of testing in the Aβ-treated mice that was reversed by the CEF treatment. It should be mentioned that the open field test revealed no significant differences in the indices of locomotion, exploratory activity, or anxiety between the controls and Aβ-treated mice or between the Aβ25–35 + saline and Aβ25–35 + CEF groups. Hence, the observed effect of CEF on cognitive function was specific and did not depend on general changes in locomotor or exploratory behavior. The beneficial effect of CEF on cognitive functions agrees well with the previous findings on the restoration of impaired cognition in the animal models of neurodegenerative disorders (Zumkehr et al., 2015; Weng et al., 2016; Tikhonova et al., 2017; Ho et al., 2019).

We may conclude that the CEF recovered Aβ-induced pathology and related cognitive impairment. Its neuroprotective activity involved the effects on Aβ burden and neuroinflammatory response in the brain. Hence, the CEF could be positioned as a potent multipurpose drug as it simultaneously

targets proteostasis network and neuroinflammation, as well as glutamate excitotoxicity, oxidative pathways, neurotrophic function, and neurogenesis as reported earlier. Together with previous reports on the beneficial effects of the CEF in AD models (Zumkehr et al., 2015; Hefendehl et al., 2016; Tikhonova et al., 2017), the results of the study confirm the potential of the CEF as a promising treatment against cognitive decline from the early stages of AD progression.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Local Ethics Committee of the Scientific Research Institute of Neurosciences and Medicine.

AUTHOR CONTRIBUTIONS

MAT, TA, Y-JH, and LA: conceptualization. MVT, AA, MO, AB, and ND: methodology and formal analysis. MVT, AA, MO, and ND: investigation. MAT: data curation and writing—original draft preparation. MAT, MO, and AA: visualization. TA: writing—review and editing. MAT, TA, and LA: supervision, project administration, and funding acquisition. All authors have read and agreed to the published version of the manuscript.

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SUPPLEMENTARY MATERIAL

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CASE REPORT
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Bilateral Globus Pallidus Interna Combined With Subthalamic Nucleus Variable Frequency Deep Brain Stimulation in the Treatment of Young-Onset Parkinson's Disease With Refractory Dyskinesia: A Case Report

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Background: Main motor characteristics in Parkinson's disease (PD) include bradykinesia, rigidity, and tremors. With the development of neuromodulation techniques, it has become possible to use deep brain stimulation (DBS) to control the symptoms of PD. However, since the subthalamic nucleus(STN) and globus pallidus interna (GPi) DBS have their own advantages and disadvantages, it is difficult to control symptoms of the patients. It is essential to find new stimulation methods.

Case Presentation: A 33-year-old male PD patient with onset at the age of 12 years. The onset of the disease is presented with bradykinesia and progressively developed severe choreic dyskinesia with the use of medications. We then performed a thorough evaluation of the patient and decided to perform bilateral globus pallidus interna combined with subthalamic nucleus variable frequency DBS (bSGC-DBS) implantation, and after 2 years of follow-up the patient's bradykinesia and dyskinesia symptoms and quality of life improved significantly.

Conclusions: This is the first case of bSCG-DBS in a PD patient with refractory dyskinesia, and the first report of encouraging results from this clinical condition. This important finding explores multi-electrode and multi-target stimulation for the treatment of dystonia disorders.

Keywords: Parkinson's disease, dyskinesia, deep brain stimulation (DBS), treatment, subthalamic nucleus

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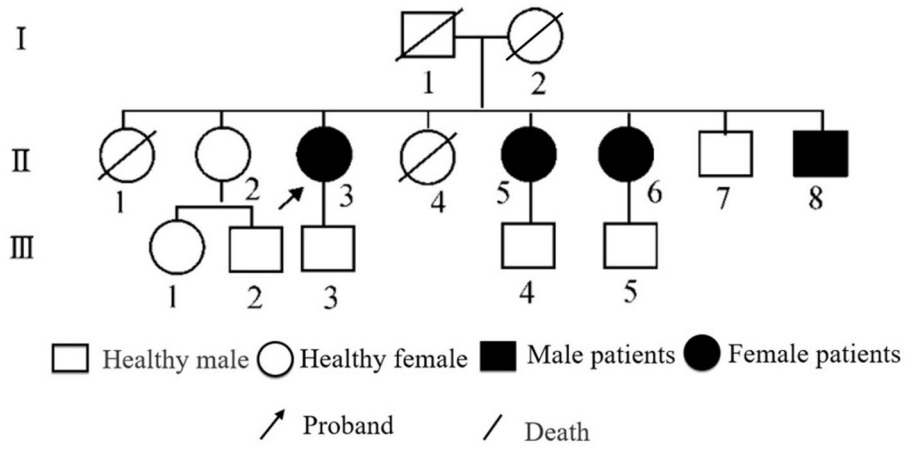

FIGURE 1 | Genetic family diagram of the patient.

BACKGROUND

Main motor characteristics in Parkinson's disease (PD) include bradykinesia, rigidity, and tremors. These characteristics, with the exception of variable tremors, can be significantly improved through levodopa treatment. This is especially beneficial early in the disease, wherein improvements can be retained through intermittent dosing in waking hours. In contrast, dopamine replacement therapy in PD has been associated with several medication-induced complications, of which dyskinesia is considered to be the most baneful influence. As such, a decreased levodopa dose with shortened dosage time intervals has been the most commonly used method for dyskinesia treatment, although some cases can still be difficult to manage (Benabid et al., 2000). Therefore, the development of new therapeutic interventions to reduce the impact of dyskinesia in PD is an important need.

CASE PRESENTATION

The patient was a 33-year-old man who presented with unsteady gait, limb tremors, and bradykinesia at the age of 12 years. Initially, the patient was treated with oral levodopa and benserazide, amantadine, and benzhexol, resulting in an improvement in his symptoms. However, these symptoms gradually worsened, and the dosage was gradually increased. Despite this, the symptoms could not be controlled, and the patient gradually developed severe chorea-like dyskinesia of the extremities. Furthermore, 5 years after taking the drug, during the drug-off period, the patient had significant bradykinesia and was unable to take care of himself. On assessment of the patient's family history, it was found that the patient's two sisters had similar symptoms (**Figure 1**). As a result, the patient and his two sisters underwent genetic testing for a single-gene genetic disorder. We found heterozygous mutations in the PRKN gene in the patient and his two sisters, leading to a diagnosis of "familial hereditary young-onset PD." (**Supplementary Material**).

A series of examinations was performed on the patient before surgery. Cranial magnetic resonance imaging (MRI) revealed no significant abnormalities other than mild brain atrophy. Cranial PET-CT showed decreased 18F-Dopa concentration in both the posterior putamen and decreased FDG metabolism in the left frontal temporal caudate nucleus head, left inferior parietal gyrus, and right posterior middle lobe gyrus. Electroencephalography showed no abnormalities.

The patients were assessed with a detailed scale to assess the severity of their symptoms, psychological status, and quality of life. The UPDRS-III, UPDRS-IV scale is used to assess the severity of the patient's symptoms. The NMSS (Parkinson's Non-motor Symptom Scale) is an assessment of Parkinson's non-motor symptoms. The PDQ-39 scale was used to evaluate the quality of life and psychological state of the patients. We found that the patient had severe bradykinesia and dyskinesia.

Previous randomized controlled trials (RCTs) comparing STN-DBS with GPi-DBS have demonstrated the beneficial role of STN-DBS in medication reduction and the role of GPi-DBS in the reduction of dyskinesia severity. However, high frequency stimulation of the STN in PD patients can induce intense dyskinesias that are similar to those induced by levodopa. The patient was unable to lower his medication because of intractable bradykinesia and had severe choreic dyskinesia. After discussion, our team concluded that neither the single use of bilateral STN-DBS nor the single use of GPi-DBS could improve all symptoms of the patient.

We then decided to perform bSGC-DBS implantation. The DBS electrode (model PINS G102R-new; PINS Medical Co., Ltd, Beijing, China) was implanted into the bilateral STN(L301) and GPi(L302).

We conducted pre-operative head placement of the patient with a Leksell-G orientation instrument, followed by an MRI scan. According to the scanning results, the frame coordinates of GPi were determined as (left: $X = 126.5$, $Y = 107$, $z = 116.5$; right: $x = 78$, $y = 107$, $z = 116.5$) and the frame coordinates of STN (left: $x = 114.5$, $y = 104$, $z = 118.5$; right:

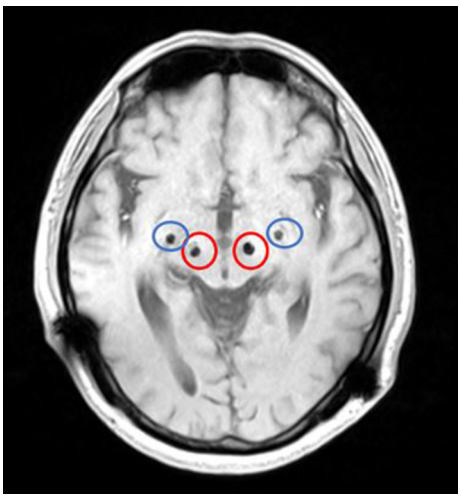

FIGURE 2 | MRI (magnetic resonance imaging) after bSCG-DBS implantation (the red circle is STN, and the blue circle is GPi).

$x = 88.5$, $y = 104$, $z = 118.5$). The implantation point of the GPi was posterior, 28 mm from the implantation point of the STN (**Figure 2**). Microelectrode monitoring was performed to determine the location of the target (**Figure 3**). After electrode implantation, temporary intraoperative test stimulation was applied to the patient, and the stimulation parameters were adjusted to 3.5 V voltage, 90 us pulse width, and 150 Hz frequency, when the patient reached a comfortable state. During the temporary stimulation, there was no visual tract or internal capsule stimulation and no nausea or adverse speech reaction.

In the 2nd week after surgery, we performed the first parameter adjustment for the patient's DBS. First, we activated the bilateral electrodes of the GPi and started with the following parameters: 90 μs for pulse width, 125 Hz for frequency, and 3.0 V for voltage. When we tried to adjust the parameters upwards, the patient's bilateral lower extremity dyskinesia became apparent. When we reduced the GPi pulse width to 70 μs, the patient's lower extremity dyskinesia disappeared, but the bradykinesia was still present. Electrodes in the STN were not activated when the parameters were first adjusted. In the 3rd month after surgery, we performed a second parameter adjustment. This time we made a slight adjustment in the GPi parameters, and the patient showed good control of dyskinesia without fluctuating symptoms. However, since the patient's bradykinesia did not improve with GPi-DBS alone, we subsequently activated the electrodes in the bilateral STN, with the following adjusted parameters: 70 μs for pulse width, 130 Hz for frequency, and 2.0 V for voltage. As a result, the patient's limb dyskinesia improved significantly, and bradykinesia also improved. In the 6th month after surgery, we performed the third parameter adjustment. At this time, the overall condition of the patient improved, as compared to the previous one. Because previous studies have shown that STN-DBS variable frequency stimulation (VFS) can increase gait speed and reduce the number of freezing episodes (Follett et al., 2010). For this adjustment, we adjusted the stimulation mode of the STN to VFS with parameters of 90, 105, 125, and 105 Hz, alternating every 0.1 s. In the 12th month after surgery, the patient showed further improvement in walking and limb dyskinesia, when compared to that in the previous period, and dyskinesia had been largely controlled. We performed the fourth parameter adjustment by adjusting the VFS mode parameters of STN to the following: 90 Hz-2 s; 130 Hz-7 s; 160 Hz-7 s; and 170 Hz-2 s. In the following 12 months, no parameter adjustment was performed, since the patient's symptoms were stable. Specific adjustment parameters are presented in **Supplementary Material**. At 24 months after surgery, the patient's dyskinesia symptoms had largely disappeared, and the bradykinesia was significantly improved. The dosage has also dropped dramatically (**Figures 4A–F**). Furthermore, the patient noted that he was able to take care of himself and was very satisfied with the overall outcome. We attempted to switch STN and GPi on and off alternately, subsequently evaluating patients' UPDRS-III and UPDRS-IV scores (**Figures 4G,H**). In the UPDRS-IV scale, the score of dyskinesia part was 6 before surgery, 2 in 3 months after surgery, 1 in 6 months after surgery, 1 in 12 months after surgery, and 2 in 24 months after surgery.

DISCUSSION AND CONCLUSIONS

With the development of neuromodulation techniques over the past 30 years, deep brain stimulation (DBS) has been found to improve levodopa-responsive symptoms, dyskinesia, and tremors. Subthalamic nucleus DBS (STN-DBS) and globus pallidus interna DBS (GPi-DBS) have now been assessed by new studies to be valid in dyskinesia treatment, showing clinical significance in practice (Fox et al., 2018). However, regarding treatment outcomes, numerous studies have verified the absence of remarkable discrepancies between these two targets, although controversies still exist about their respective treatment outcomes (Jia et al., 2018). Moreover, previous RCTs comparing STN-DBS with GPi-DBS have demonstrated the conducive role of STN-DBS in medication reduction and GPi-DBS in the reduction of dyskinesia severity (Sharma et al., 2010; Mansouri et al., 2018). However, High-frequency stimulation of the STN in PD patients can induce intense dyskinesias that are similar to those induced by levodopa (Vincent et al., 2016). Hence, the goals of DBS could be of great importance for target selection.

Based on the respective advantages of STN-DBS and GPi-DBS, this study developed bilateral STN-DBS in combination with GPi-DBS (bSCG-DBS) for the treatment of PD with severe dyskinesia. Furthermore, it was found that this new stimulation mode had better efficacy than STN-DBS or GPI-DBS alone. Herein, we report the first case of bSCG-DBS treatment in a patient with familial hereditary young-onset Parkinson's disease with refractory dyskinesia.

In this case report, STN stimulation was more effective for reducing bradykinesia, while GPi stimulation was more effective for reducing dyskinesia. Although in RCTs that STN DBS and GPI DBS are equally effective for the treatment of parkinsonism, in this case report we found that STN DBS was more effective

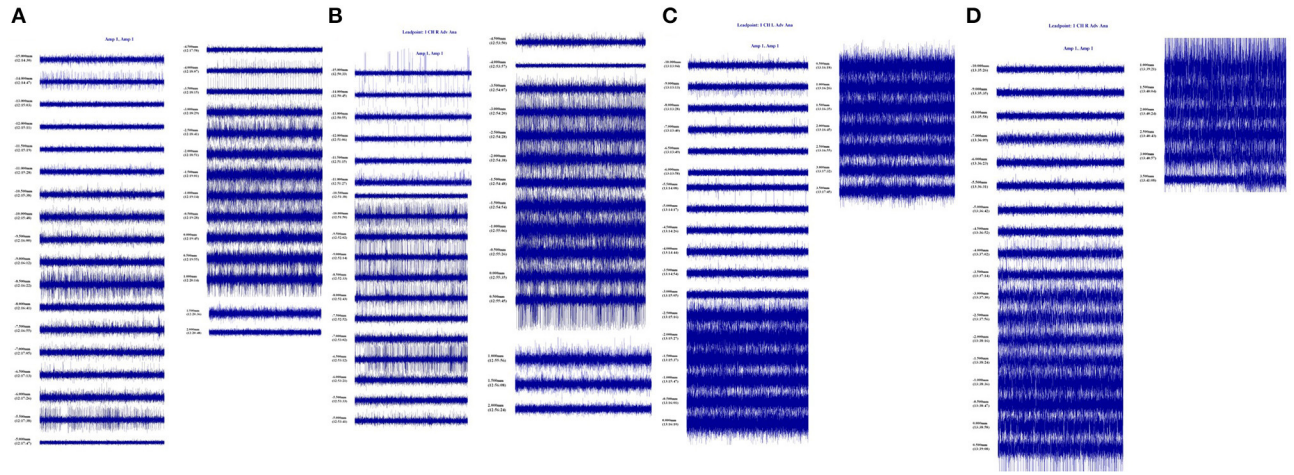

FIGURE 3 | Intraoperative microelectrode monitoring was recorded. **(A)** GPi on the left: during the monitoring process, GPe electrical activity was recorded from 11.0 mm above the target to 5.5 mm above the target, GPi discharge was recorded from 4.0 mm above the target to 1.5 mm below the target, and the electrode was placed at 1.0 mm below the target. **(B)** GPi on the right side: during the monitoring process, GPe electrical activity was recorded from 11.0 to 4.5 mm on the target, and GPi discharge was recorded from 3.5 to 1.5 mm below the target; The electrode was placed 1.0 mm below the target. **(C)** left STN: electrical activity of STN is recorded from 2.5 mm above the target to 3.5 mm below the target during monitoring: electrode is placed at 3.0 mm below the target. **(D)** Right STN: electrical activity of STN was recorded from 4.0 mm above target to 3.0 mm below target during monitoring; The electrode was placed 2.5 mm below the target.

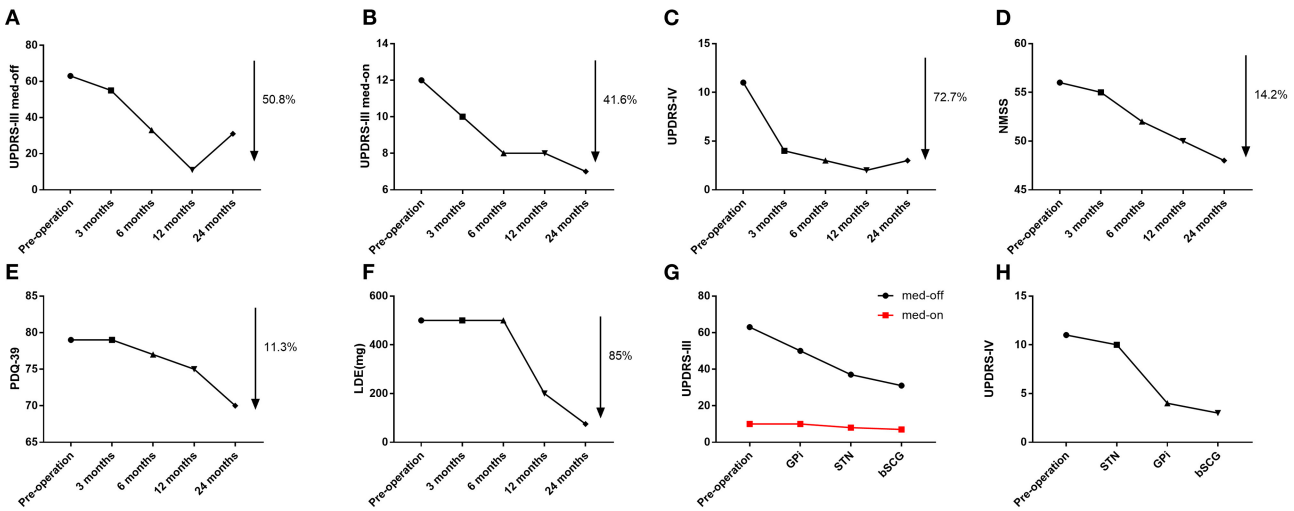

FIGURE 4 | (A) Trends in UPDRS-III off-period scores. **(B)** Trends in UPDRS-III on-period scores. **(C)** Trends in UPDRS-IV scores. **(D)** Trends in NMSS scores. **(E)** Trend in PDQ-39 scores. **(F)** Trends in Levodopa Equivalents (LDE). **(G)** Comparison of UPDRS-III score in 4 conditions (pre-surgery, GPi stimulation, STN stimulation, and combined GPi and STN stimulation). **(H)** Comparison of UPDRS-IV score in 4 conditions (pre-surgery, GPi stimulation, STN stimulation, and combined GPi and STN stimulation).

for the reduction of bradykinesia, while GPI DBS was more effective for the reduction of dyskinesia (Wong et al., 2019). In conclusion, STN stimulation appears to be insufficient to control refractory choreiform dyskinesia; therefore, the combination of GPi and STN stimulation provided some moderate advantage over STN/GPi stimulation alone.

To our knowledge, this is a case of bSCG-DBS in a PD patient with refractory dyskinesia, and the report of satisfactory results from this clinical condition. However, further studies are needed to confirm this important finding, which explores multi-electrode and multi-target stimulation for the treatment of PD.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the First Affiliated Hospital of USTC, Division of Life Sciences and Medicine. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

BC and JM jointly completed the experiment and the writing. CX, PC, and MJ are responsible for post-operative parameter regulation. CN took overall control of the whole study. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2021.782046/full#supplementary-material

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