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Original Research

``Olfactory three-needle" acupuncture enhances synaptic function in Aβ_{1-42}-induced Alzheimer's disease via activating PI3K/AKT/GSK-3β signaling pathway

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Synaptic dysfunction and neuronal loss are related to cognitive impairment of Alzheimer's disease. Recent evidence indicates that regulating the phosphatidylinositol 3-Kinase (PI3K)/AKT/GSK-3β pathway is a therapeutic strategy for improving synaptic plasticity in Alzheimer's disease. Here, we investigated "olfactory three-needle" effects on synaptic function and the PI3K/AKT/GSK-3β signaling pathway in β-amyloid$_{1-42}$(Aβ_{1-42})-induced Alzheimer's disease rats. A three-needle olfactory bulb insertion for 28 days alleviated Aβ_{1-42}-induced Alzheimer's disease rats' cognitive impairment as assessed by performance in the Morris water maze test. Furthermore, the three-needle electrode inhibited neuro-apoptosis and neuro-inflammation. It significantly upregulated the protein expression of postsynaptic density protein 95, synaptophysin, and GAP43, indicating a protective effect on hippocampal synaptic plasticity. Additionally, the activation level of PI3K/AKT signaling and the phosphorylation inactivation of GSK-3β were significantly enhanced by the "olfactory three-needle". Our findings suggested that the three-needle acupuncture is a potential alternative to improve synaptic plasticity and neuronal survival of Alzheimer's disease brain in rodents.

Keywords

Alzheimer's disease; Olfactory bulb; Three-needle; Synaptic plasticity; PI3K/AKT/GSK-3β signaling; Acupuncture

1. Introduction

Alzheimer's disease (AD) is one of the most prevalent neurodegenerative disease characterized by the abnormal accumulation of amyloid-beta (Aβ) peptide and intracellular tau-containing neurofibrillary tangles, which leads to progressively marked deficits in memory and other behavior disorders [1, 2]. Synapse loss occurs at the early stage of AD, deteriorates progressively, and closely correlates with the cognitive decline [3]. As memory is stored in the brain synapses, synaptic plasticity is thought to underlie learning and memory [4]. As a primary site of adult neurogenesis, Hippocampus plays a pivotal role in acquiring, consolidating, and recognizing the declarative and spatial memory [5]. The loss of hippocampal neurons and synapses in AD causes severe impairment of hippocampal-dependent behaviors, including spatial learning and memory [6, 7]. Therefore, improving synaptic plasticity is proposed as the potential therapeutic strategy for rescuing cognitive dysfunction in AD.

PI3K/AKT/GSK-3β signaling pathway is implicated in neuronal network maintenance, cell survival and longevity [8]. Disturbance of this signaling pathway is the vital mechanism of inducing tau hyperphosphorylation to form neurofibrillary tangles in AD brain [9]. The phosphorylation activation of AKT can inhibit the glycogen synthase kinase-3β (GSK-3β) through inducing phosphorylation inactivation of GSK-3β. The "GSK-3β hypothesis of AD" [10] proposed that the aberrant GSK-3β activity contributes to several features of this pathology such as tau phosphorylation, memory impairment, microglia-mediated inflammation neuronal apoptosis.

Excessive production of the insoluble 42-amino acid-long Aβ_{42} peptides was almost invariably found in the neocortex and hippocampus of early-onset autosomal dominant AD the presence of gene mutations [11]. Accumulating evidence has shown that the activation of GSK-3β is directly disturbed by Aβ exposure in AD brains. Generally, soluble or diffusible oligomeric aggregates of Aβ [12, 13] have been implicated in tau hyperphosphorylation [14], loss of dendritic spine density [15], disruption of memory, neuronal death [16], and neurotoxic inflammatory response [17]. As an insulin-suppressing agent, Aβ oligomers have been shown to inhibit insulin signal transduction by antagonizing insulin receptors, ultimately causing a dysfunction in the down-

stream PI3K/AKT/GSK-3β signaling pathway and a series of characteristic damage changes in AD, including tau hyperphosphorylation, neuronal apoptosis, and synaptic loss [18]. Therefore, Aβ accumulation plays a vital role in the pathological process related to the PI3K/AKT/GSK-3β signaling pathway.

Both animal and clinical studies have approved that electroacupuncture is a potential therapy for AD [19–21]. Our previous studies report an electroacupuncture protocol with GV29 and IL20 named as "olfactory three-needle" to improve olfactory dysfunction and cognitive deficits in AD patients in clinical applications, which has been confirmed an improvement of cognitive deficits in SAMP8 mice through inhibiting Aβ deposition, tau phosphorylation, and oxidative stress damage in hippocampal region [22, 23]. Unlike the acupoint of other electroacupuncture, GV29 in the nasal root and IL20 in the bilateral nasal ala were the main three acupoints used in "olfactory three-needle" therapy, which closely related to the olfactory system through stimulating the olfactory nerve in front of the olfactory bulb. Furthermore, we have further confirmed that olfactory stimulation with "olfactory three-needle" or eugenol enhances spatial learning and memory abilities of SAMP8 mice [22, 23]. The olfactory system has complex neural connections and can connect to the hippocampus and prefrontal regions of the brain, involved in learning and memory abilities [24]. In the present study, we investigate the effects of electroacupuncture with "olfactory three-needle" on synaptic function and PI3K/AKT/GSK-3β pathway. Our study may provide a novel finding of electroacupuncture's olfactory nerve mechanisms, improving AD's synaptic function.

2. Method

2.1 Preparation of Aβ_{1-42}-induced AD rat model

Male six-month-old Sprague-Dawley (SD) rats (body weight 220 $\pm$ 20 g, SPF grade) were purchased from the Chengdu Dashuo Experimental Animal Co. Ltd, Chengdu, People's Republic of China (certificate No. SCXK201302). Rats were housed in normal plastic cages with free access to commercial water and diet under a 12 h light/dark cycle at an invariable room temperature (25 °C). In this study, AD model rats were established, referring to the previously described method [25]. In brief, rats were first injected stereotaxically with 10 μg aggregated Aβ_{1-42} (Sigma-Aldrich, USA) in the lateral ventricle at accurate positions based on the mediolateral (ML), dorsoventral (DV), and anteroposterior (AP) coordinates (2.8 mm, 2.2 mm and 3.0 mm, respectively), to locate the CA1 region of the hippocampus.

2.2 Animal grouping and treatments

One week after the operation, rats treated with Aβ_{1-42} were randomly divided into three groups (n = 10 rats per group) and severally treated with NS (Aβ_{1-42} group), donepezil 4 mg/kg (Aβ_{1-42} + Don group) and "Olfactory three-needle" (Aβ_{1-42} + Ae group) once per day for 28 consecutive days. While rats in the sham group and control group were without any treatment.

For the "olfactory three-needle" treatment group, Aβ_{1-42}-induced AD rats were immobilized by mouse bags. The disposable sterile electroacupuncture needles (0.3 mm $\times$ 13 mm) (Huatuo Medical Instrument Company, Suzhou, PR China) were used to puncture the GV29 with transverse puncturing to the nasal root direction at a depth of 10 mm and the bilateral LI20 with shallowly puncturing towards the interior and superior at a depth of 2 mm (shown in Fig. 1), which was connected to an electric stimulator (Han Shi, Nanjing, PR China) with electrical stimulation (1.5 mA, 15 Hz) for 10 minutes daily within 28 consecutive days, as description as our previous study [23]. For the donepezil treatment group, Aβ_{1-42}-induced AD rats were treated with donepezil hydrochloride tablets (H20050978, Eisai, Co. Ltd, PR China), which were crushed dissolved in distilled water, with the dose at 4 mg/kg by oral administration once per day for 28 consecutive days.

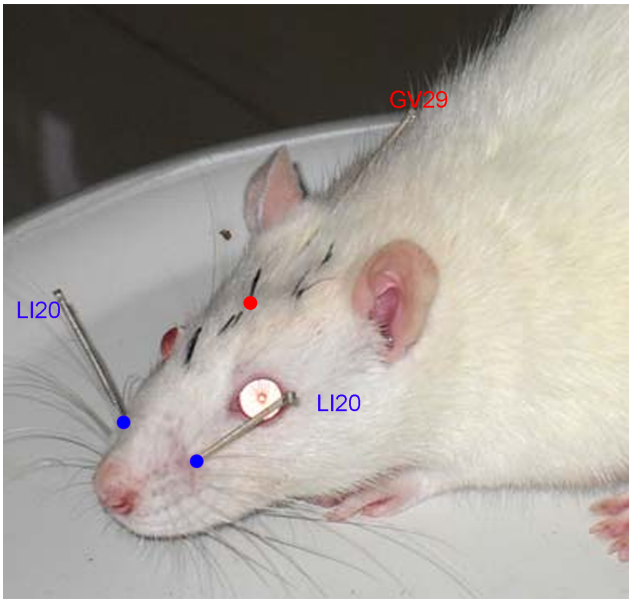

Fig. 1. The location of acupoints for "olfactory three-needle" applied in this study. The red point indicated the locations of GV29 on the head of mice; Blue points indicated the locations of bilateral IL20 above the nostril of mice.

2.3 Morris water maze test

Morris water maze tests assessed the rats' spatial learning and memory abilities according to a previous study [22]. At 24 h after "olfactory three-needle" treatment for 24 days, rats in all the group were trained for four trials per day and received place navigation trial for 5 consecutive days in a circular pool (Intex Recreation Corporation, Long Beach, CA, USA; 91 cm diameter and 40 cm height) containing a 7 cm-diameter hidden platform submerged 1 cm below the water surface. Every rat was placed in the water facing the pool wall to locate the hidden platform within 60 s. The rats that

failed to find the platform were guided to stay there for 10 s. The time and distance to find the platform (escape latency) were recorded to reflect the spatial learning ability. On the sixth day, the quadrant-target duration within the 60s and the number of times the rats crossed the platform area in a spatial probe trial were recorded to assess the memory ability.

2.4 Immunohistochemical and histopathological analyses

At 24 h after the behavioral test, five rats selected randomly from each group were sacrificed and perfused transcardially with 20 mL of ice-cold sterilized saline followed by 40 mL of 4% paraformaldehyde. The brain was taken out, and the hippocampus tissue was stripped and cut into 5 μm sections using a cryostat (Leica, CM3050S, Tokyo, Japan). Immunohistochemistry of Aβ and p-tau was performed on 5 μm paraformaldehyde-fixed brain sections. After incubation with the primary antibody at 4 °C overnight (anti-beta-Amyloid, Abcam, 1 : 1000; anti-p-tau, Abcam, 1 : 1000), the brain sections were incubated with gota anti-rabbit IgG secondary antibody (Boster Biological Technology co., Ltd., PR China) at 37 °C for 30 minutes. All procedures were performed according to the protocols of the immunoassay kit. The brain slices were concurrently stained with H&E before assessing the histopathology. Images were captured using a light microscope (Nikon Eclipse 80i, Nikon, Japan).

2.5 TUNEL staining

TUNEL staining was used to label apoptotic cells by a fluorescent-TdT FragEL^{TM} DNA Fragmentation Detection Kit (Calbiochem, Darmstadt, Germany). Briefly, the frozen coronal brain sections of the same brain region from each rat were incubated with proteinase K for 10 min at room temperature and washed three times with TBS. These sections were then incubated with TdT equilibration buffer for 30 min and followed with the TUNEL reaction mixture for 90 min at 37 °C in a dark cassette. After washed three times by TBS, these sections were covered by mounting media to observe apoptotic cells, which emitted yellow-green fluorescence in the nucleus under an exciting light of 488 nm. The average number of apoptotic neurons in six random visual fields per section in penumbra was used for statistical analysis.

2.6 Nissl staining

For Nissl staining, the brain sections were fixed on the polylysine-coated slides, dried overnight, rehydrated in distilled water, and immersed in 1% cresyl violet for 20 min. After being rinsed by distilled water and dehydrated by graded serried ethanol, these sections were submerged in xylene and then coverslipped. Nissl-positive cells in the pyramidal layer of the CA1 area were observed to assess neuronal loss under a microscope (Leica, DM6000 B, Tokyo, Japan) by three pathologists blind to this study. The average number of Nissl-positive cells in six random visual fields per section in penumbra was used for statistical analysis.

2.7 Western blotting

Isolated hippocampus tissues were removed from the remaining five rats of every group and rapidly homogenized. The protein extracts were mixed with sample buffer, heated for 10 min and then centrifuged for 10 min at 12,000 × g. After estimating the protein concentration with a bicinchoninic acid (BCA) kit (Sigma-Aldrich), 50 μg total proteins were transferred to the polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA) by 10% SDS-PAGE in Tris-glycine transfer buffer. After blocked in PBST within 5% milk for 1 h, PVDF membranes were incubated at 4 °C overnight with the following primary antibodies: rabbit anti-Synaptophysin (ab14692, 1 : 1000, Abcam), rabbit anti-PSD95 (ab18258, 1 : 1000, Abcam), rabbit anti-GAP43 (ab134075, 1 : 1000, Abcam), rabbit anti-PI3K (#4249, 1 : 1000, Cell Signaling Technology), rabbit anti-AKT (sc-8312, 1 : 1000, Santa Cruz, CA, USA), rabbit anti-phospho-AKT (sc-271964, 1 : 1000, Santa Cruz), mouse anti-GSK-3β (#9832, 1 : 1000, Cell Signaling Technology), rabbit anti-phospho-GSK-3β (#5558, 1 : 1000, Cell Signaling Technology), and mouse anti-β-actin (A1978, 1 : 10000, Sigma), conjugated to horseradish peroxidase were used as secondary antibodies. After washing three times by PBST, PVDF membranes were incubated with an anti-mouse or anti-goat IgG antibody (PerkinElmer Life Sciences, Waltham, MA, USA) for 1h at room temperature. Protein bands were visualized by enhanced chemiluminescence (Millipore), and images were captured under a Bio-Image system (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

2.8 Statistical analysis

Statistical analysis was performed with the SPSS 23.0, and the data were exhibited as the mean ± SD. Two-tailed Student's *t*-test and two-way *ANOVA* with repeated measures were used to analyze the orientation navigation test data in different groups simultaneously. Also, data of spatial probe test, WB and other data were analyzed by One-Way *ANOVA*.

3. Results

3.1 "Olfactory three-needle" ameliorated the spatial learning and memory impairment of Aβ_{1-42}-induced AD rats

To investigate the effect of "olfactory three-needle" on AD rats' behavior deficits, the Morris water maze test was employed to measure the ability of spatial learning and memory after the treatments on day 31 to 35 and day 36, respectively (Fig. 2A). In an orientation navigation test of five consecutive days (Fig. 2B), Aβ_{1-42}-treated rats showed an apparent deficit in spatial memory with longer escape latency than control or sham rats. Still, it could be significantly rescued by "olfactory three-needle" treatment. Despite apparent improvements in days 1, 2, and 3 in the orientation navigation test, donepezil could not shorten the escape latency of Aβ_{1-42}-treated rats on days 4 and 5.

In the spatial probe test, Aβ_{1-42}-treated rats performed impaired spatial memory with fewer cross-platform (Fig. 2C) and more time spent in crossing platforms than control or sham rats (Fig. 2D). After 28 days of treatments, "olfactory three-needle" and donepezil both significantly improved the spatial memory of Aβ_{1-42}-treated rats by increasing

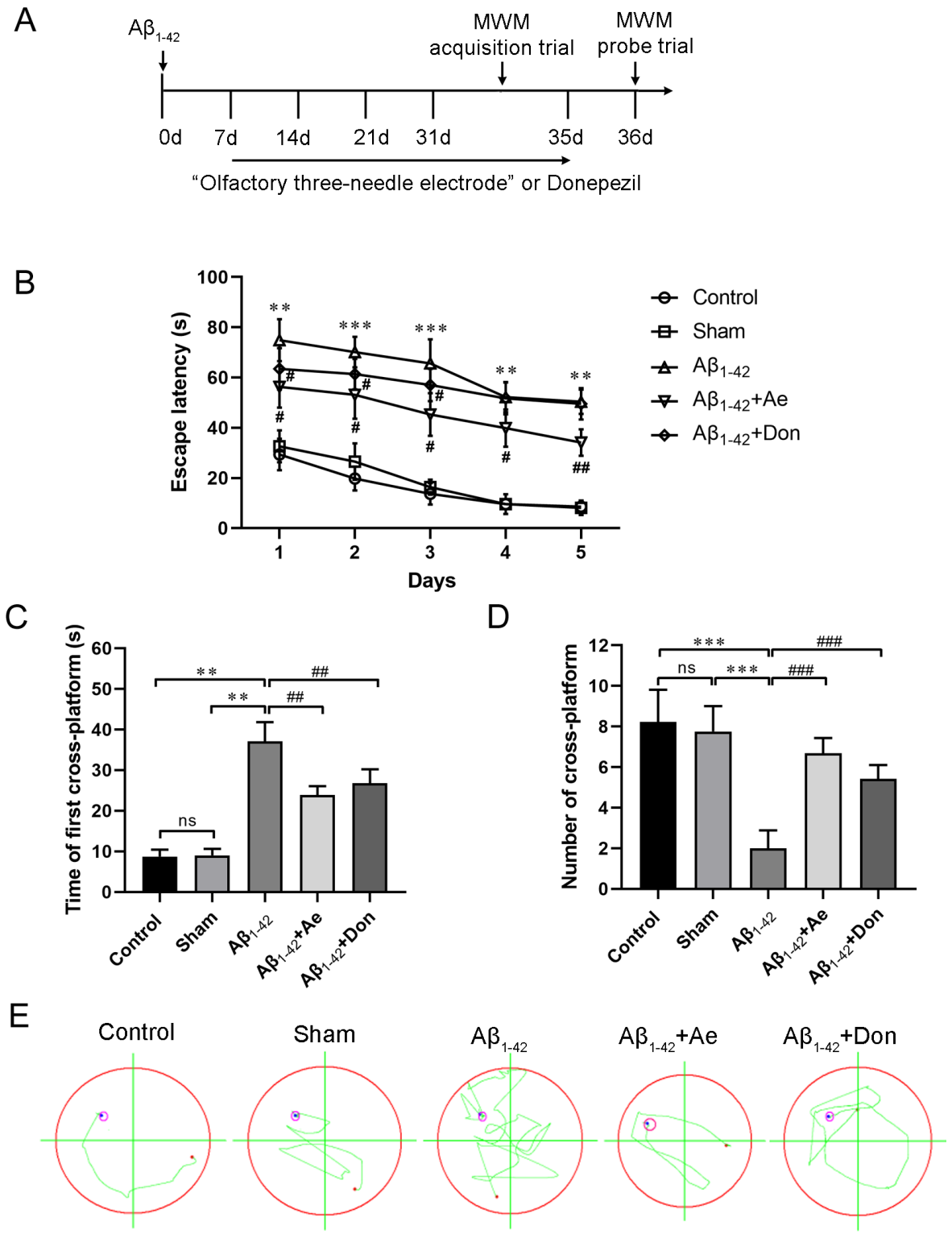

Fig. 2. "Olfactory three-needle" ameliorated the spatial learning and memory impairment of Aβ_{1-42}-induced AD rats. (A) Experimental design for the animal study. (B) Escape latency time during the orientation navigation test of five consecutive days after "olfactory three-needle" treatment. (C) Time was spent in the target quadrant during the spatial probe test. (D) Platform crossing numbers in the spatial probe test. (E) Swimming trajectories of groups. Data are expressed as means ± SD, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. Aβ_{1-42} treated rats.

the cross-platform numbers and shortening the time cross-platform (Fig. 2C, D, E). Overall, the defect of spatial learning and memory in Aβ_{1-42}-treated rats was ameliorated by "olfactory three-needle" treatment by improving spatial learning and memory abilities.

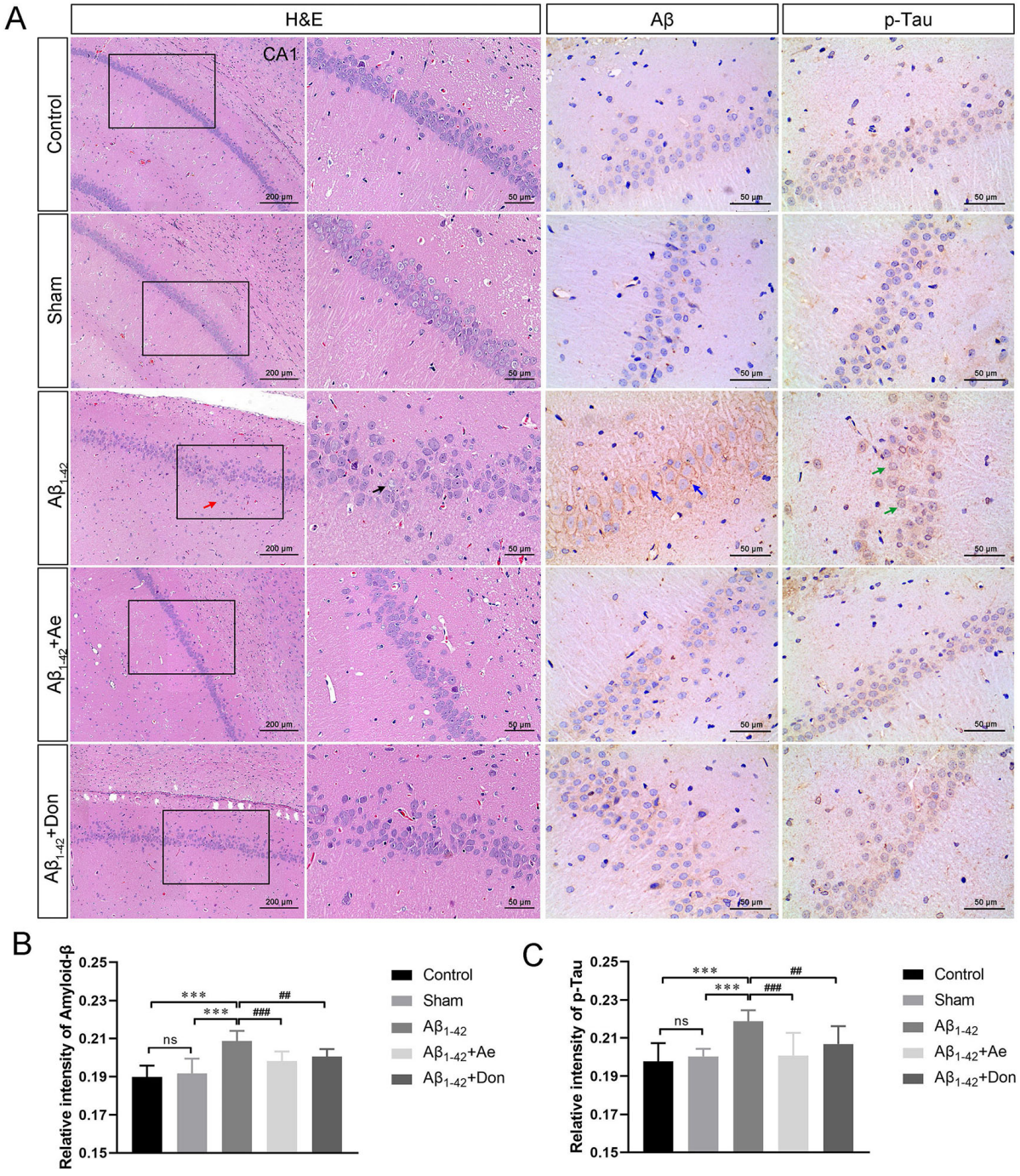

Fig. 3. "Olfactory three-needle" reduced Aβ deposition and tau hyperphosphorylation in the hippocampus of Aβ_{1-42}-treated rats. (A) Representative HE and immunohistochemical staining for Aβ and p-tau positive areas in the hippocampal CA1 region. The red arrow indicated that cells scattered outside the pyramidal cell layer. The black arrow indicated the necrosis of pyramidal cells. (B, C) Quantification of the integrated optical density of Aβ and p-tau by immunoreactivity. Blue arrow indicated that Aβ deposition on the intercellular substance between cells. The green arrow indicated that p-tau accumulation in neuron cells. Data are expressed as means ± SD, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. Aβ_{1-42} treated rats.

3.2 "Olfactory three-needle" reduced Aβ deposition and tau hyperphosphorylation in the hippocampus of Aβ$_{1-42}$-induced AD rats

The arrangement of vertebral neuron cells in the CA1 area of Aβ_{1-42}-treated rats was disordered, with a small number of vertebral cell necrosis. 28 days after treatments, vertebral neuron cells' arrangement in the hippocampus was improved by "olfactory three-needle" and donepezil (Fig. 3A). Immunohistochemistry revealed the presence of Aβ and p-tau immunoreactivity in the hippocampus CA1 area of Aβ_{1-42}-treated rats. Aβ deposition on the intercellular substance be-

tween neurons and p-tau accumulation in neurons is the hallmark characteristic in AD and AD-like brain aging, which is also the leading cause of neurodegeneration. However, the integrated optical density of Aβ and p-tau immunostaining was significantly decreased in Ae and donepezil groups (Fig. 3B, C), which suggested that both "olfactory three-needle" and donepezil could decrease the production of Aβ and p-tau in the hippocampus.

3.3 "Olfactory three-needle" inhibited neuro-apoptosis and neuroinflammation in the hippocampus of Aβ_{1-42}-induced AD rats

Aβ deposition is neurotoxic to neurons and will lead to neuronal apoptosis and neuro-inflammation. Compared to control or Sham rats, the apoptotic neurons were significantly increased in Aβ_{1-42}-treated rats. The apoptotic neurons' level was decreased by the treatments of "olfactory three-needle" and donepezil (Fig. 4A, B). Moreover, the level of apoptotic neurons in the "olfactory three-needle"-treated group was lower than the donepezil-treated group. The mRNA expression of IL-1β, TNF-α, and IL-6 in the hippocampus was significantly increased in Aβ_{1-42}-treated rats. Still, there was no difference in the expression of those inflammatory cytokines between Aβ_{1-42}-treated rats and donepezil-treated rats. Moreover, "olfactory three-needle" treatment decreased the expression of IL-1β and TNF-α but not included IL-6 (Fig. 4C, D, E).

3.4 "Olfactory three-needle" improved neuronal activity and synaptic plasticity in the hippocampus of Aβ_{1-42}-induced AD rats

The ratio of viability neurons in the CA1 area of the hippocampus was decreased in Aβ_{1-42}-treated rats compared to control or sham rats, and "olfactory three-needle" and donepezil treatments improved the ratio of viability neurons (Fig. 5A, B). Moreover, Aβ_{1-42}-treated rats showed a pronounced decrease in hippocampal synaptic protein expression, including SYN, PSD95, and GAP43, compared with control or sham rats. Remarkably, the levels of SYN, PSD95, and GAP43 were significantly increased by "olfactory three-needle" treatment compared with the Aβ_{1-42}-treated group. In contrast, there were no significant changes between the donepezil treated group and the Aβ_{1-42}-treated group (Fig. 5C, D). Taken together, these results demonstrated that "olfactory three-needle" treatment substantially increased the expression of SYN, PSD95, and GAP43 in the hippocampus of Aβ_{1-42}-treated rats, suggesting that "olfactory three-needle" likely alleviates the decline in MWM performance in these rats by improving hippocampal synaptic plasticity.

3.5 "Olfactory three-needle" enhanced PI3K/AKT/GSK-3β signaling in the hippocampus of Aβ_{1-42}-induced AD rats

Western blot analysis showed a significant decrease in PI3K expression and the activity levels of p-AKT/AKT and p-GSK-3β/GSK-3β in Aβ_{1-42}-treated rats compared with those in control or sham rats, which suggested that the PI3K/AKT/GSK-3β signaling in the hippocampus was inhibited by Aβ_{1-42} (Fig. 6). However, the protein expression of PI3K (Fig. 6A, B), as well as the activity levels of p-AKT/AKT and p-GSK-3β/GSK-3β (Fig. 6A, C, D), were both enhanced by "olfactory three-needle" treatment. In contrast, the donepezil-treated rats showed no significant changes compared with Aβ_{1-42}-treated rats. Those data indicated that the "olfactory three-needle" greatly enhanced PI3K/AKT/GSK-3β signaling in the hippocampus of Aβ_{1-42}-treated rats, a potential mechanism the "olfactory three-needle" to improve synaptic plasticity.

4. Discussion

Synaptic dysfunction and neuronal loss are the leading causes of the early development of AD [26]; recovering synaptic dysfunction has been proposed as a promising therapeutic approach for AD. The present study demonstrates that "olfactory three-needle" rescued the spatial learning and memory dysfunction through improving neuro-apoptosis and neuro-inflammation, and synaptic plasticity in the hippocampus of Aβ_{1-42}-induced AD rats. Also, we show that the "olfactory three-needle" treatment enhanced the PI3K/AKT/GSK-3β signaling pathway, which is involved in AD-related synaptic pathophysiology [10]. In general, these results suggest that "olfactory three-needle" could improve synaptic function probably via activating the PI3K/AKT/GSK-3β signaling pathway.

Electroacupuncture has been suggested as an effective therapeutic intervention for AD in many clinical and animal studies [20, 27, 28]. Our previous studies have reported that "olfactory three-needle" improved cognitive deficits of SAMP8 mice through inhibiting Aβ deposition, tau phosphorylation, and oxidative stress damage in the hippocampal region [23]. Unlike the acupoint of other electroacupuncture, GV29 in the nasal root and IL20 in the bilateral nasal ala were the main three acupoints used in "olfactory three-needle" therapy, which is implicated in the olfactory system. In the multiple steps of adult neurogenesis, the survival and integration of newborn neurons are strongly affected by the olfactory system [29, 30]. The generation of newborn neurons from the olfactory bulb after adulthood is a significant learning-induced remodeling of structural and functional plasticity. Olfactory learning has been shown to selectively promote remodeling of both inhibitory and excitatory inputs in the deep dendritic domain of adult-born neurons [31]. Cognitive deficits of AD are attributable to the disruptions of synaptic functions and neuronal loss, correlated to the memory deficit's severity in AD [32]. Hence, we believe that the olfactory system's stimulation through electroacupuncture may be a valid alternative to improve synaptic plasticity and neuronal survival of neurodegenerative diseases.

Aβ peptide deposition and the subsequent formation of amyloid plaques in the brain have been recognized as an important event in the neuro-pathogenesis of AD [33]. The neurotoxicity of Aβ has been proven to involve intracellular hyperphosphorylated tau neurofibrillary tangles. The ac-

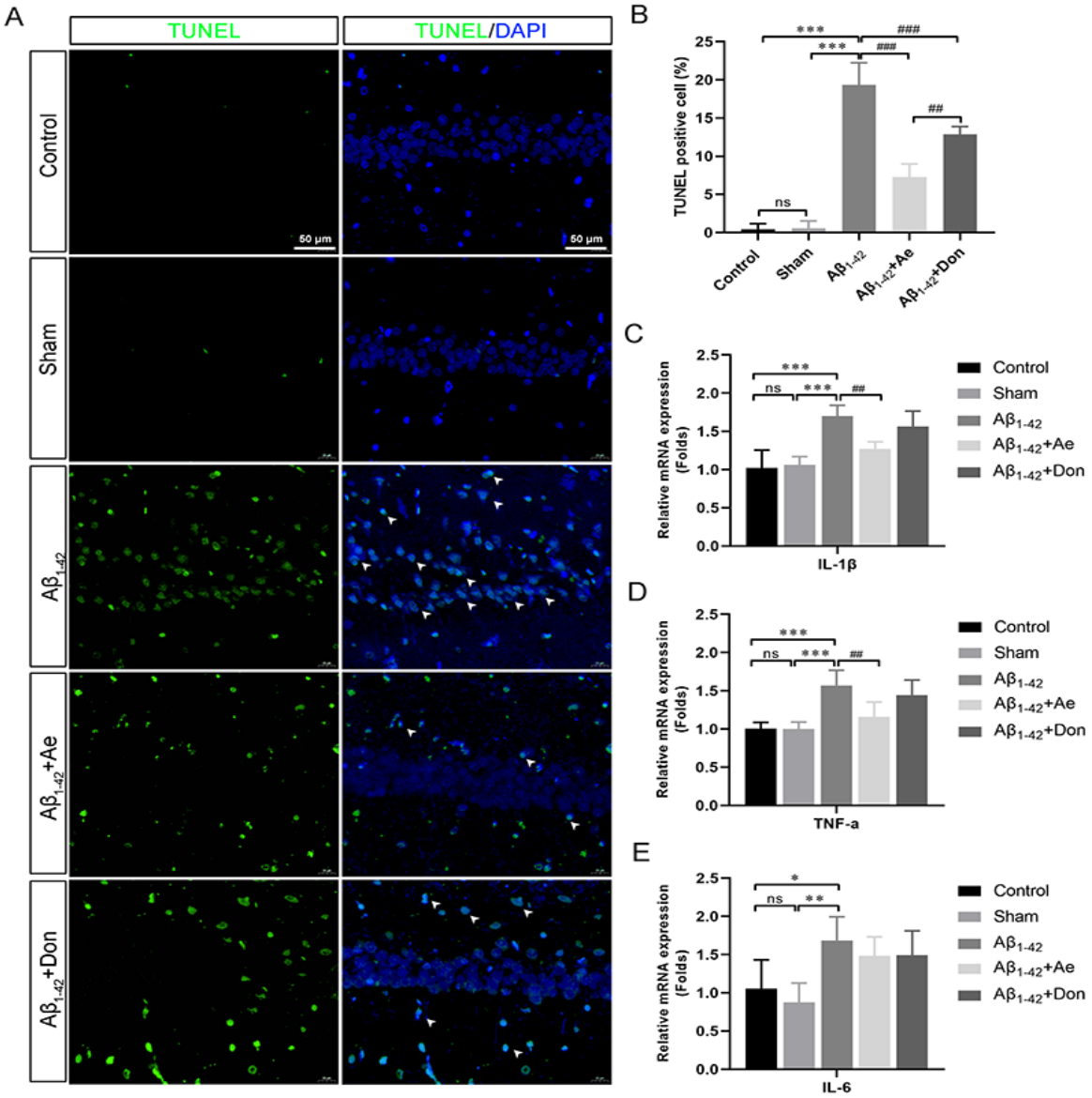

Fig. 4. "Olfactory three-needle" inhibited neuro-apoptosis and neuroinflammation in the hippocampus of Aβ_{1-42}-induced AD rats. (A) Representative pictures of TUNEL staining in the hippocampus, the green signal represented TUNEL positive nucleus, and the blue signal represented a normal nucleus. The white arrows point to apoptotic cells. (B) Percent of apoptotic cells in the hippocampus. (C, D, E) The mRNA expression of IL-1β, TNF-α, and IL-6. Data are expressed as means ± SD, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. Aβ_{1-42} treated rats.

tivation of microglia-mediated neuroinflammation response resulted in neuronal damage, apoptosis, and, eventually, spatial memory impairment [34, 35]. In this study, we found that "olfactory three-needle" not only decreased Aβ deposition in the hippocampus of Aβ_{1-42}-induced AD rat but also inhibit the expression of tau hyperphosphorylation and pro-inflammatory cytokines IL-1β and TNF-α, as well as the damage and apoptosis of hippocampal neurons. Therefore, this clearance of Aβ may be one possible mechanism of "olfactory three-needle", stimulating the olfactory system in ameliorating cognition degeneration in rats.

To explore the potential molecular mechanism of "olfactory three-needle" to improve Aβ_{1-42}-induced learning and memory impairment, the expression of synapse-related molecules forming the structural basis of synaptic plasticity of learning and memory were investigated. SYN and PSD95, which are the markers of pre-and post-synaptic terminals, respectively, are considered to represent the structural bases of plasticity underlying learning and memory. SYN expression reflects the synaptic density and distribution, which is implicated in the formation and reconstruction of synapsis [36, 37]. SYN may directly affect the synaptic structure and influence synaptic plasticity by regulating neurotransmitter release [38]. PSD95, as the structural basis of post-synaptic plasticity, is also vital for information transmission and memory formation [39]. It is reported that the reduced level of PSD95 in the hippocampus results in the impairment of learning and memory function [40]. GAP43 is a growth-

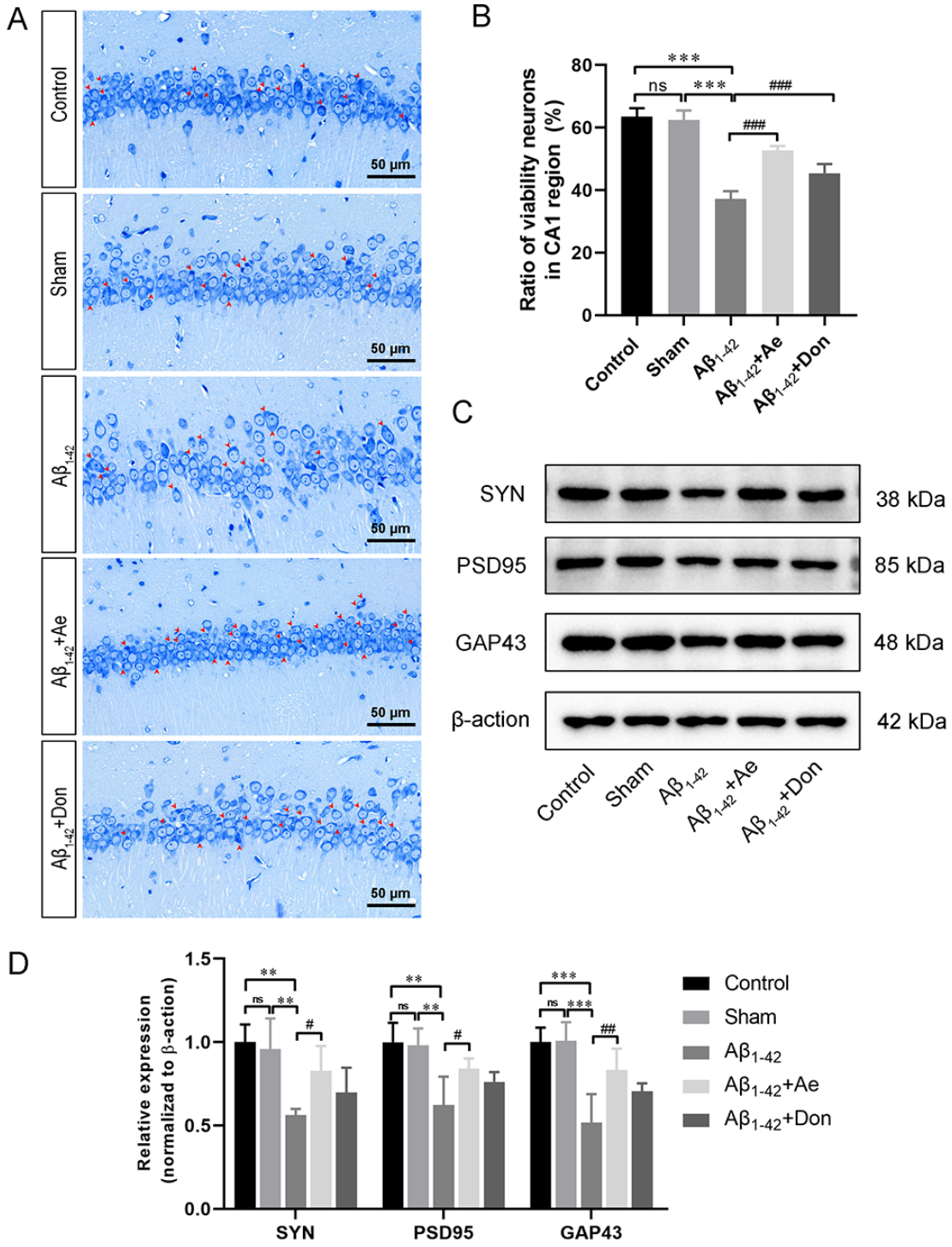

Fig. 5. "Olfactory three-needle" improved neuronal activity and synaptic plasticity in the hippocampus of Aβ_{1-42}-induced AD rats. (A) Nissl staining for the hippocampal CA1 region of rats. The red arrows point to viability neurons with Nissl's body. (B) The ratio of viability neurons in the CA1 area of rats. (C) Representative western blot bands of SYN, PSD95, and GAP43 in the hippocampus. (D) Relative protein expression of SYN, PSD95, and GAP43. Data are expressed as means ± SD, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. Aβ_{1-42} treated rats.

associated phosphoprotein with high expression levels during neuronic development, axonal regeneration, and neuronic sprouting [41]. Therefore, stimulating the olfactory system to increase the expression of synapse-related SYN, PSD95, and GAP43 of hippocampal CA1 was the molecular basis for "olfactory three-needle" to improve synaptic plasticity and cognitive deficits in Aβ_{1-42}-induced rats.

As the most commonly used anticholinesterase inhibitor for AD clinical treatment, Donepezil has been proven to enhance neurogenesis, improve cognition, learning, and

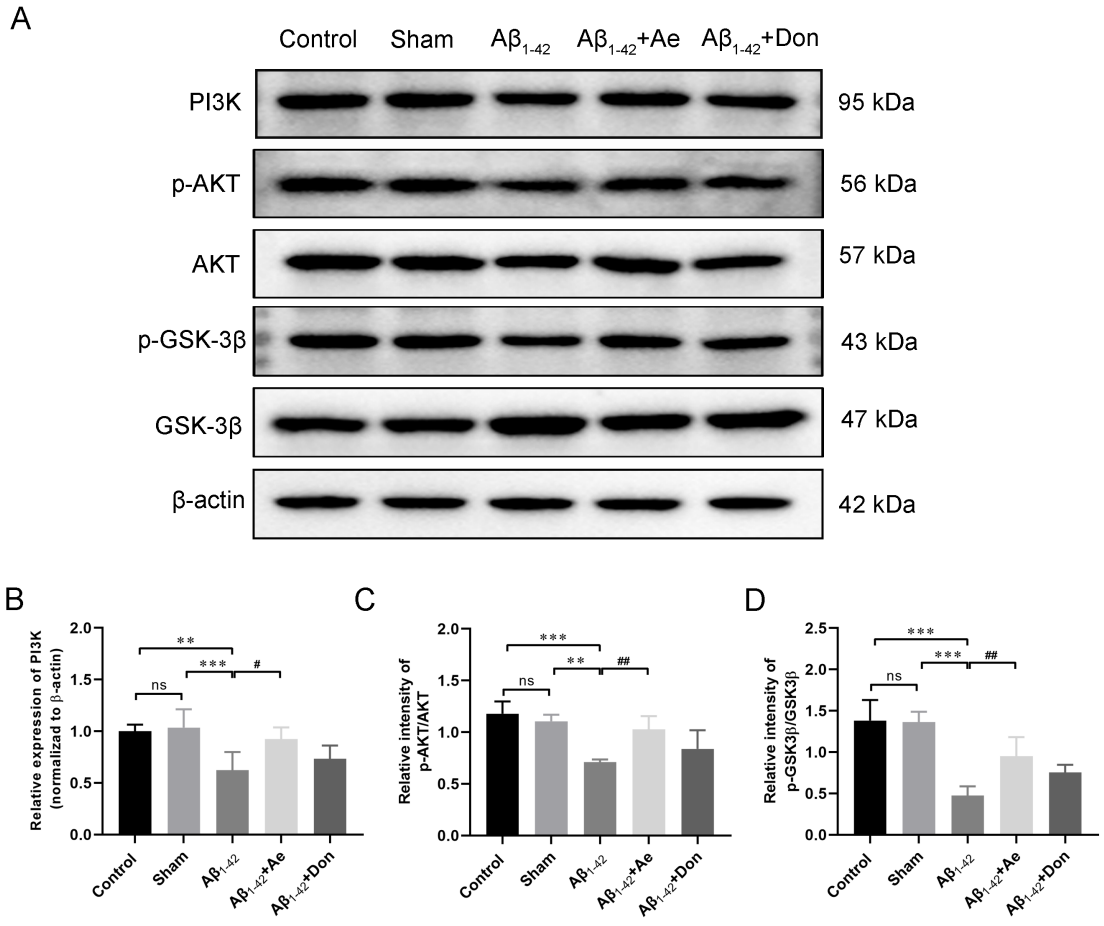

Fig. 6. "Olfactory three-needle" enhanced PI3K/AKT/GSK-3β signaling in the hippocampus of Aβ_{1-42}-induced AD rats. (A) Representative western blot bands of PI3K, p-AKT, AKT, p-GSK-3β and GSK-3β in the hippocampus. (B) Relative protein expression of PI3K. (C) The relative expression of p-AKT/AKT. (D) The relative expression of p-GSK-3β/GSK-3β. Data are expressed as means $\pm$ SD, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. Aβ_{1-42} treated rats.

memory and prevent aging progress by decreasing cholinergic loss neurons [42]. Recent studies have revealed that donepezil can inhibit the Aβ deposition by inhibiting acetylcholinesterase and its receptors, which plays an essential role in promoting Aβ peptides in AD neuro-pathogenesis [43]. Many studies have shown that donepezil possesses neuroprotective activity. Still, it could not improve the structure and density of neural synapses [44, 45], consistent with our results. The synapse-related protein expressions of hippocampal CA1 were not significantly improved by donepezil in Aβ_{1-42}-induced AD rats. However, "olfactory three-needle" could improve synaptic function by promoting the expression of synapse-related proteins. As a potential therapy for AD, the combination of electroacupuncture and donepezil applied in AD is worthy of expectation and attention.

It has been proposed that GSK-3β activity might exert a central role in Aβ production, tau phosphorylation, and neurodegeneration during the development of AD [10]. Overexpression of GSK-3β in the conditional transgenic mice engendered tau hyperphosphorylation pathology and neuronal death [46, 47]. Mainly, GSK-3β plays a vital role in the stress response for neurons through compromising the transcriptional activity of the cAMP response element-binding (CREB) protein to downregulate the expression levels of brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF) and other neurotrophic factors, which are important for the regulation of long-term memory and the maintenance of synaptic plasticity, thereby contributing to the neuronal degeneration of AD [48, 49]. The GSK-3β activity also has a critical role in inducing the differentiation and migration of inflammatory cells. Thus, the secretion of pro-inflammatory cytokines [50] could potentially deteriorate microglia-induced inflammatory responses in the vicinity of Aβ plaques.

Furthermore, GSK-3β is involved in modulating critical steps of apoptotic signaling pathways [51]. PI3K/AKT signaling is the upstream regulator of GSK-3β, which phosphorylates and inhibits GSK-3β [8]. Our current results showed that "olfactory three-needle" treatment enhanced the activation level of PI3K/AKT signaling and promoted the phosphorylation inactivation of GSK-3β. Thus, "olfactory three-needle" enhanced PI3K/AKT signaling to inhibit GSK-

3β, thereby increasing synapse-related molecule expression, inhibiting inflammatory cytokines (IL-1β and TNF-α) and neuronal apoptosis, which may be a potential mechanism of the "olfactory three-needle"-mediated protection of synaptic plasticity.

In conclusion, we provided evidence that "olfactory three-needle" could rescue the cognitive deficits of Aβ_{1-42}-induced AD rats by improving synaptic plasticity, neuro-apoptosis and neuro-inflammation through enhancing PI3K/AKT/GSK-3β signaling pathway.

Author contributions

YW and AZ conceived and designed the experiments. YW, HY, QW, BR, TG, TG and HC performed the experiments. YG, LX and ZL analyzed the data. ZL and HL contributed to the reagents and materials. YW and AZ wrote the manuscript.

Ethics approval and consent to participate

The experiment was carried out in the Shaanxi Key Laboratory of Acupuncture and Medicine of the Shaanxi University of Chinese Medicine. All the animal experiments were in accordance with the guidelines of the Shaanxi Province Experimental Animal Management Committee with a permit number (AM2019-0161).

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Conflict of interest

The authors declare no competing interests.

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