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ORIGINAL ARTICLE

Electroacupuncture Improves Learning and Memory Abilities via Activating AMPK/mTOR-Induced Autophagy in APP/PS1 Mice

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Abstract

Alzheimer's disease (AD) has become a global public health problem characterized by memory and cognitive impairments. Electroacupuncture (EA) has been indicated to exert promising therapeutic effects on AD. This study aimed to further investigate the underlying mechanism of EA in AD treatment. APP/PS1 transgenic mice and wide-type mice underwent with or without EA treatment at GV20 and BL23 acupoints. Morris water maze test was utilized for examining the learning and memory of mice. Hematoxylin–eosin, Congo red, immunofluorescence, and TUNEL staining were employed for detecting the pathological changes of mouse brain hippocampus. Western blotting was implemented for measuring protein levels of autophagy- and AMPK/mTOR pathway-associated markers. APP/PS1 mice exhibited significant impairments in the spatial learning and memory. EA treatment improved the cognitive impairments, reduced amyloid-beta (Aβ) deposition, and alleviated neuronal apoptosis in the hippocampal tissues of APP/PS1 mice. EA promoted autophagy and activated the AMPK/mTOR signaling pathway in the hippocampus of APP/PS1 mice. EA improves the cognitive deficits, enhances Aβ clearance, and attenuates neuronal apoptosis in APP/PS1 mice in part by activating AMPK/mTOR-mediated autophagy.

Keywords Alzheimer's disease · Electroacupuncture · Autophagy · Cognition · AMPK/mTOR

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Abbreviations

AD	Alzheimer's disease
EA	Electroacupuncture
Aβ	Amyloid-beta
AMPK/mTOR	Adenosine monophosphate-activated protein kinase/mammalian target of rapamycin
CNS	Central nervous system
Wt	Wide-type
MWM	Morris water maze
H&E	Hematoxylin–eosin
IF	Immunofluorescence
TUNEL	Terminal-deoxynucleotidyl transferase-mediated nick end labeling
PBS	Phosphate-buffered saline
DAPI	4', 6-Diamidino-2-phenylindole
SD	Standard deviation
ANOVA	Analysis of variance

Introduction

Alzheimer's disease (AD), also called senile dementia, is a common neurodegenerative disease and has become a global public health issue (Lane et al. 2018). The disease mainly affects people over the age of 65, leading to progressive memory impairment, communication problems, personality disorders, and other cognitive dysfunctions (Mantzavinos and Alexiou 2017). Chronic deposition of amyloid-beta (Aβ) and neuronal loss in the brain are considered as the primary neuropathological hallmarks of AD (Peng et al. 2016; Fu et al. 2016). Evidence has suggested that reduction or prevention of Aβ accumulation can effectively improve cognitive functions in animal models of AD (Huang et al. 2020; Luo et al. 2020a). With aging of the population, the prevalence of AD is increasing rapidly which brings great burdens to the society and economy especially in developing countries (Zhang et al. 2021a). Nonetheless, there are currently no ideal treatment methods available for AD (Li et al. 2022). Thus, it is imperative to investigate the pathogenesis of AD and find effective approaches for treating or preventing this disease.

Autophagy is a highly conserved self-degradative process that removes dysfunctional components and provide nutrition and energy to cells (Glick et al. 2010). Emerging evidence has illustrated that dysfunction of autophagy is closely associated with AD. Dysfunctional autophagy leads to Aβ and tau deposition and contributes to AD progression (Chen et al. 2021). Immature autophagosome accumulation was observed in the brains of patients with AD (Nixon et al. 2005). Intriguingly, aberrant autophagosome accumulation is found to precede the formation of Aβ plaques (Li et al. 2017; Zhang et al. 2021b). Activation of autophagy can ameliorate cognitive deficits and amyloid pathology in mouse models of AD (Luo et al. 2020b). Many studies have illuminated that adenosine monophosphate-activated protein kinase/mammalian target of rapamycin (AMPK/mTOR) signaling pathway is crucial for autophagy activation (Wang and Hu 2021). Moreover, it was reported

that activated AMPK downregulates mTOR signaling to promote autophagy, consequently enhancing Aβ clearance and ameliorating cognitive impairments (Ding et al. 2022).

Acupuncture is a widely practiced medicine technique that involves the insertion of sterile needles into acupoints on the body surface (Kaptchuk 2002). As a non-pharmacological treatment method, acupuncture has lower costs and higher safety in disease treatment (Jiang et al. 2023). Electroacupuncture (EA) is a modern acupuncture technique which has better repeatability and consistency as compared to the traditional acupuncture (Song et al. 2019). Studies have demonstrated that EA exhibits promising therapeutic effects on multiple central nervous system (CNS) diseases, such as stroke, Parkinson's disease, and vascular dementia (Zheng et al. 2020; Tamtaji et al. 2019; Ma et al. 2022). Importantly, previous evidence has indicated that EA treatment can ameliorate Aβ pathology and cognitive deficits in animal models of AD (Cai et al. 2019; Zheng et al. 2021). In addition, evidence suggests that EA treatment provides more beneficial effects on cognitive function in AD patients than western drugs (Yu et al. 2020a).

Despite the above findings, the mechanisms underlying the therapeutic effect of EA on AD remain to be further illustrated. It is unclear whether EA affects AMPK/mTOR signaling pathway-mediated autophagy in AD. Therefore, we aimed to verify the neuroprotective effect of EA on AD and explore the potential mechanism in APP/PS1 double-transgenic mice. Our results may provide a new perspective on the potential mechanisms by which EA affects AD and helps to develop new therapeutic ideas for the disease.

Materials and Methods

Mice

APP/PS1 transgenic mice (male, 9-month-old) on the C57BL/6 background and the wide-type (Wt) littermates were obtained from Cavens Laboratory Animal Co., Ltd. (Changzhou, China). All mice were housed in specific pathogen-free environments with free access to food and water. This study was approved by the Ethics Committee of The Central Hospital of Wuhan, Tongji Medical College, Huazhong University of Science and Technology and all animal experiments were conducted following the NIH Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize the sufferings of mice.

Animal Grouping and Intervention

Twenty Wt C57BL/6 mice and twenty APP/PS1 mice were randomly divided into four groups: Wt, Wt+EA, APP/PS1, and APP/PS1+EA ($n = 10$ mice per group). Based on traditional Chinese medicine theory and previous evidence, two acupuncture points were selected: *Baihui* (GV20, located on the bregma, midpoint between the auricular apices) and *Shenshu* (BL23, located adjacent to the second lumbar

vertebra). Mice in Wt+EA and APP/PS1+EA groups received EA stimulation for 20 min once a day for 15 days. Stainless steel filiform needles were inserted to a depth of 0.2 cm at GV20 and 0.5 cm at BL23. The needle handle was connected to an EA apparatus (Shanghai Medical Electronic Apparatus, Shanghai, China) and the parameters were set as follows: continuous wave, 2-Hz frequency, 1-mA current intensity, and 2-V voltage. Mice in the Wt and APP/PS1 groups did not receive EA stimulation but were handled and bound in the same conditions.

Morris Water Maze (MWM) Test

The MWM test was employed to assess the spatial learning and memory of mice according to previous reports (Li et al. 2014). The test was performed using a circular pool (diameter = 120 cm, height = 50 cm) filled with water (22–25 °C) with a platform (diameter = 10 cm) placed 1.5 cm below the opaque water in the midpoint of the target quadrant. The performances of mice were video recorded and analyzed using an automated analysis system (Daheng group, Beijing, China). In training trials (days 1–5), mice were allowed to swim freely to the hidden platform within 70 s. The time needed for finding the platform was recorded as escape latency. If the mouse did not find the platform within 70 s, it would be put on the platform for 15-s rest, and the escape latency was recorded as 70 s. In probe trial (day 6), the hidden platform was removed; mice were placed into the pool and allowed to swim freely for 70 s. The time that mice spent in the target quadrant and the times that mice crossed the original position of the platform were measured and recorded.

Specimen Collection

After MWM test, all mice were sacrificed by cervical dislocation under anesthesia and the hippocampal tissues were harvested. A portion of the tissues was fixed in 4% paraformaldehyde and paraffin embedded for histologic analysis, and the remainder was stored at – 80 °C for subsequent protein isolation.

Hematoxylin–Eosin (H&E) Staining

The paraffin-embedded hippocampal tissues were cut into sections (5 μm thick). After dewaxing and rehydration, the sections were stained with hematoxylin (Solarbio, Beijing, China) for 15 min, treated with 5% acetic acid, and then rinsed twice with tap water, followed by staining with eosin (Solarbio) for 2 min. Then, the sections were dehydrated in graded ethanol, made transparent by xylene, and sealed with neutral resin (Sigma-Aldrich, St. Louis, MO). The stained tissues were observed by a light microscope (Nikon, Tokyo, Japan).

Congo Red Staining

After dewaxing and rehydration, the hippocampal tissue sections were soaked in Highman Congo red staining solution (G1535, Solarbio) for 10 min, followed by

treating with alkaline solution for 5 s and washing with tap water. The sections were then stained with Lillie–Mayer hematoxylin staining solution for 2 min and washed with distilled water. After dehydration in graded alcohol, transparency by xylene, and sealing with neutral resin, the sections were observed under a light microscope (Nikon).

Immunofluorescence (IF) Staining

The hippocampal tissue sections were deparaffinized, rehydrated, and subjected to heat-mediated antigen retrieval. After blocking with 0.1% gelatin, the sections were incubated with anti-Aβ primary antibody (sc-28365, 1:200, Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4 °C. After that, the sections were incubated with the secondary antibody (ab150113, 1:500, Abcam, Shanghai, China) at room temperature for 1 h, counterstained with 4',6-diamidino-2-phenylindole (DAPI, Solarbio), mounted, and then sealed with nail polish. Images were captured using a fluorescence microscope (Nikon) and relative IF intensity was evaluated by ImageJ software (NIH, Bethesda, MD).

Terminal Deoxynucleotidyl Transferase-Mediated Nick End Labeling (TUNEL) Staining

Cell apoptosis in the hippocampal CA1 region was detected using TUNEL Apoptosis Assay Kit (Beyotime, Shanghai, China) following the manufacturer's guidelines. In brief, deparaffinized tissue sections were permeabilized with 20-μg/ml DNase-free proteinase K, rinsed three times with phosphate-buffered saline (PBS), and then incubated with 3% hydrogen peroxide in PBS for 20 min. Subsequently, the sections were incubated with biotin labeling solution (50 μl) for 1 h at 37 °C without light. After three PBS washes, the sections were treated with 50-μl Streptavidin-HRP solution for 30 min at room temperature. The sections were counterstained with DAPI. TUNEL-positive cells were observed under a microscope (Nikon) and analyzed using Image-Pro Plus 6.0 software (Media Cybernetics, Silver Spring, MD).

Western Blotting

Protein extraction from murine hippocampal tissues was performed using Radio Immunoprecipitation Assay lysis buffer (Solarbio). A bicinchoninic acid assay kit (Solarbio) was employed for protein concentration evaluation. Protein samples (20 μg) were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, blotted onto polyvinylidene fluoride membranes (Beyotime) and blocked with 5% defatted milk. The membranes were incubated at 4 °C overnight with primary antibodies (Table S1), followed by incubation with the secondary antibody (ab97080, 1:5000, Abcam) for 1 h. Blot signaling was visualized using an enhanced chemiluminescence detection kit (Solarbio) and ImageJ software (NIH) was utilized for relative protein level quantitation.

Statistical Analysis

Data are expressed as the mean ± standard deviation (SD). Statistical analysis was implemented using SPSS 25.0 software (IBM, Armonk, NY). Difference comparisons in difference treatment groups were performed by one-way analysis of variance (ANOVA) with Tukey's post hoc analysis. Two-way ANOVA was utilized for escape latency analysis in the MWM test. $p < 0.05$ indicated statistical significance.

Results

EA Improves the Spatial Learning and Memory of APP/PS1 Mice

The MWM test was performed to evaluate the spatial learning and memory of mice in each group. In comparison to the Wt group, the APP/PS1 group displayed significantly enhanced escape latency from day 2 onward, while EA stimulation markedly decreased the escape latency of APP/PS1 mice (Fig. 1A). In addition, no significant difference was observed in the escape latency between the Wt group and the Wt+EA group (Fig. 1A). Similarly, the results of the probe trial demonstrated that mice in the APP/PS1 group spent less time in the target quadrant (Fig. 1B) and had less frequency crossing the original position of the platform than those in the Wt group (Fig. 1C), indicating that the APP/PS1 mice had obvious memory impairment. However, the time spent in the target quadrant and the number of times crossing the original platform location were prominently increased in the APP/PS1+EA group (Fig. 1B, C). These data suggest that EA stimulation ameliorates cognitive deficits in APP/PS1 mice.

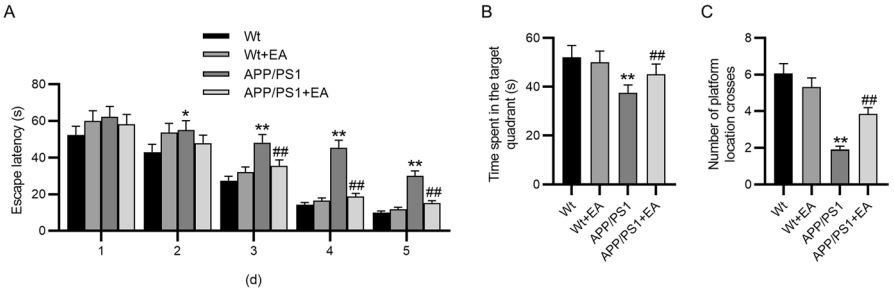

Fig. 1 EA improves the spatial learning and memory of APP/PS1 mice. The MWM test was implemented to evaluate the spatial learning and memory of mice in each group. **A** The escape latency of each group assessed by the training trails (days 1–5). **B**, **C** Time spent in the target quadrant (**B**) and the number of times crossing the platform location (**C**) measured by the probe trail (day 6). $^{*}p < 0.05$, $^{**}p < 0.01$ vs. the Wt group, and $^{\#\#}p < 0.01$ vs. the APP/PS1 group

EA Alleviates the Pathological Changes in the Hippocampus of APP/PS1 Mice

Subsequently, we examined the pathological changes in mouse brain hippocampus. As depicted by H&E staining, the Wt and Wt+EA groups did not show any lesion of pathological significance. Relative to those in the Wt group, the hippocampal neurons in the APP/PS1 group were shrunken and disorderly arranged, with prominent cellular loss. Nonetheless, the above pathological changes in the hippocampus of APP/PS1 mice were alleviated by EA treatment (Fig. 2A). We measured Aβ expression in mouse hippocampus by Congo red staining and IF staining. Congo red staining showed significant Aβ deposition in the hippocampus of the APP/PS1 mice compared with that of the Wt mice, whereas EA treatment markedly decreased Aβ deposition in the hippocampal tissues of APP/PS1 mice (Fig. 2B). Similar trends were observed in the results of IF staining (Fig. 2C, E). Moreover, the number of apoptotic neuronal cells was significantly increased in the APP/PS1 group in comparison to the Wt group, as demonstrated by TUNEL staining (Fig. 2D, F). EA stimulation markedly attenuated neuronal apoptosis in the hippocampal CA1 region of APP/PS1 mice (Fig. 2D, F). These results indicate that EA reduces brain Aβ deposition and neuronal apoptosis in APP/PS1 mice.

EA Activates Autophagy in the Hippocampus of APP/PS1 Mice

To explore the effect of EA on autophagy in the hippocampus, we measured the expression levels of autophagy-associated markers in each group. There were no significant differences in the expression levels of these markers between the Wt and Wt+EA groups, indicating that EA did not impact autophagy in the brain of normal mice. Notably, relative to those in the Wt mice, the ratio of LC3-II/LC3-I and protein levels of Beclin1, autophagy-related 5 (ATG5), and ATG7 were enhanced in APP/PS1 mice (Fig. 3A–C, E, F), while p62 protein level was significantly reduced (Fig. 3A, D), indicating that the autophagy was repressed in the hippocampus of APP/PS1 mice. However, the above effects were prominently counteracted by EA intervention in APP/PS1 mice (Fig. 3A–F), suggesting that EA promoted the autophagy activation in APP/PS1 mouse brain hippocampus.

EA Regulates the AMPK/mTOR Signaling Pathway

The AMPK/mTOR signaling pathway plays a critical role in the regulation of autophagy (He and Klionsky 2009). To investigate whether EA activated autophagy by regulating AMPK/mTOR signaling pathway, we examined the effect of EA on this pathway in the hippocampus of APP/PS1 mice by assessing the protein levels of related markers, including AMPKα, AMPKβ1, mTOR, and its downstream target p70S6K. As displayed by the results, the APP/PS1 group exhibited significantly decreased protein levels of p-AMPKα and p-AMPKβ1 and elevated levels of p-mTOR and p-p70S6K as compared to the Wt group (Fig. 4A–E), suggesting the inactivation of AMPK signaling and activation of mTOR signaling in APP/PS1

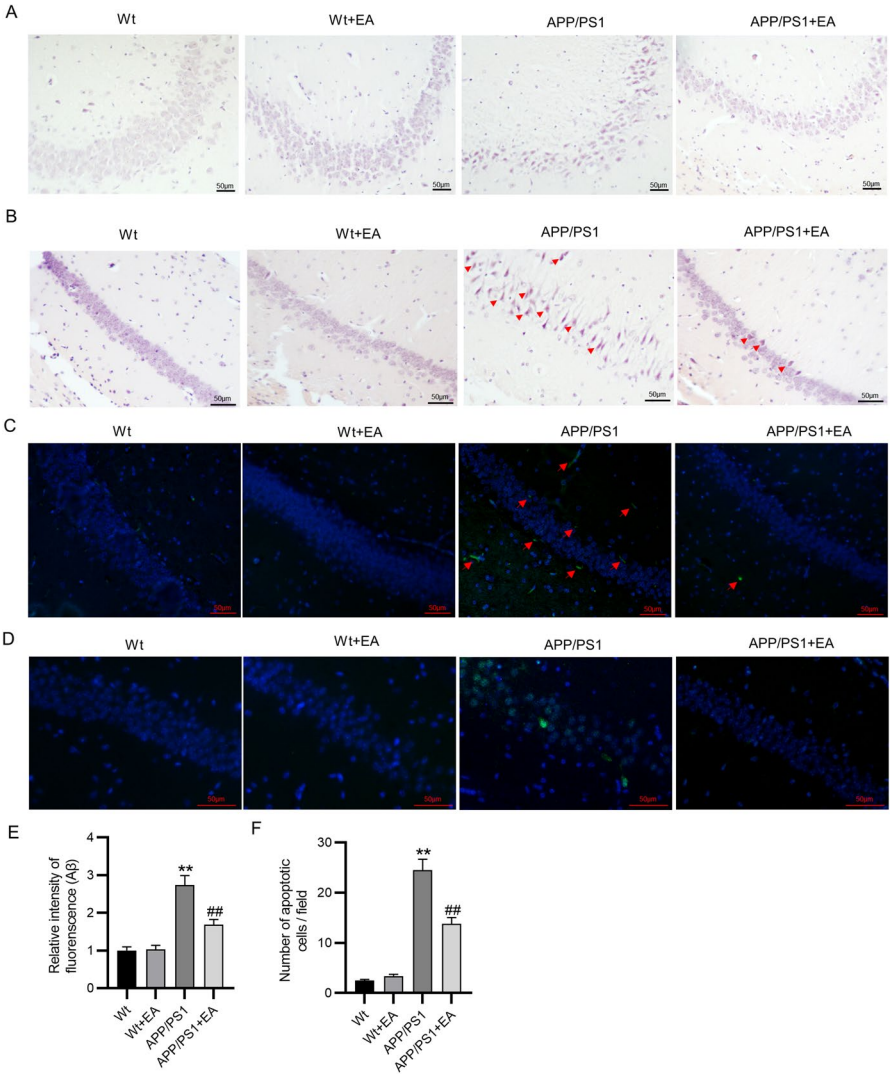

Fig. 2 EA alleviates the pathological changes in the hippocampus of APP/PS1 mice. **A** Representative images of H&E staining for morphological observation of mouse brain hippocampus in each group. **B** Congo red staining for detecting Aβ plaque formation in the hippocampus region. **C** IF staining for detecting Aβ deposition (red arrows) in the hippocampal tissues. **D** TUNEL staining for evaluating neuronal apoptosis (red arrows indicate the apoptotic cells) in the hippocampal CA1 region. **E** Relative fluorescence intensity. **F** Quantification of apoptotic cells. $^{**}p < 0.01$ vs. the Wt group; $^{\#\#}p < 0.01$ vs. the APP/PS1 group (Color figure online)

mice. However, EA treatment facilitated AMPKα and AMPKβ1 phosphorylation and suppressed mTOR and p70S6K phosphorylation in APP/PS1 mice (Fig. 4A–E). These reveal that EA activates AMPK signaling and inactivates mTOR signaling in the hippocampus of APP/PS1 mice.

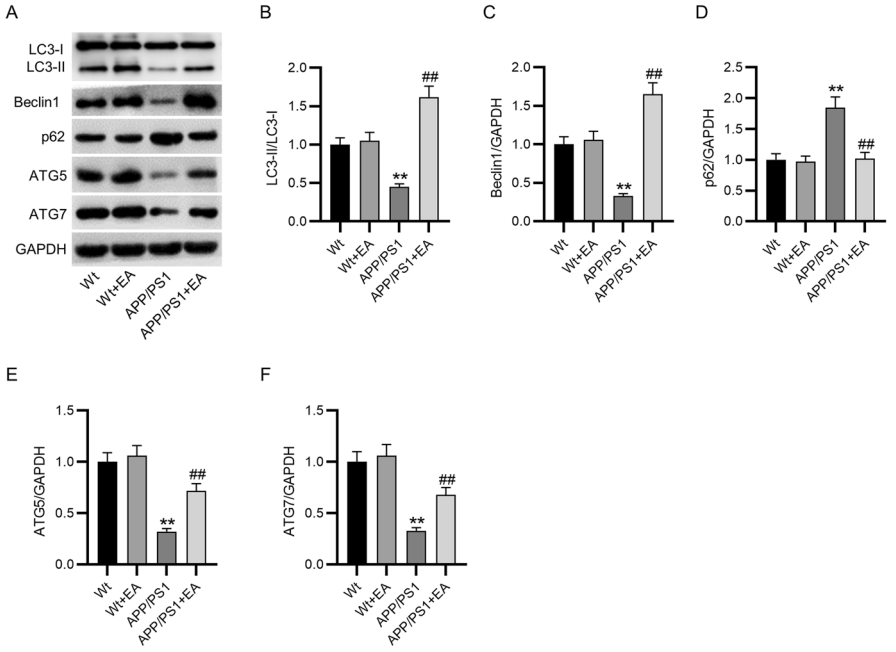

Fig. 3 EA activates autophagy in the hippocampus of APP/PS1 mice. **A** Western blotting for detecting protein levels of autophagy-associated markers in the hippocampus of each group. **B–F** The quantitative results of western blotting. $^{**}p < 0.01$ vs. the Wt group; $^{\#\#}p < 0.01$ vs. the APP/PS1 group

Discussion

The present study revealed that EA stimulation improved the spatial learning and memory of APP/PS1 mice, reduced Aβ deposition, and alleviated neuronal apoptosis in mouse brain hippocampus. Moreover, EA enhanced autophagy and activated AMPK/mTOR signaling pathway in the hippocampal tissues of APP/PS1 mice.

AD is characterized by progressive loss of memory and cognitive functions (Maity et al. 2021). Guo et al. demonstrated that acupuncture at GV20 and BL23 acupoints improves memory and ameliorates neuronal loss in a rat AD model (Guo et al. 2016). Consistently, Yu et al. indicated that EA stimulation at the two acupoints alleviates D-galactose-triggered memory impairment and AD-like pathology by inhibiting GSK3 β/mTOR pathway (Yu et al. 2020b). Based these studies, we selected GV20 and BL23 acupoints for EA stimulation in this study. In accord with the above evidence, our study revealed that EA treatment at GV20 and BL23 acupoints significantly improved the spatial learning and memory of APP/PS1 mice, as measured by MWM test, a well-established method for testing hippocampal-dependent learning (Bromley-Brits et al. 2011).

The hippocampus is a brain area crucial for learning and memory and is particularly vulnerable to injury in the early stages of AD (Mu and Gage 2011). Additionally, neurons in the hippocampus are the dominant cell responsible for learning and memory (Lin et al. 2018). Our study revealed that EA intervention

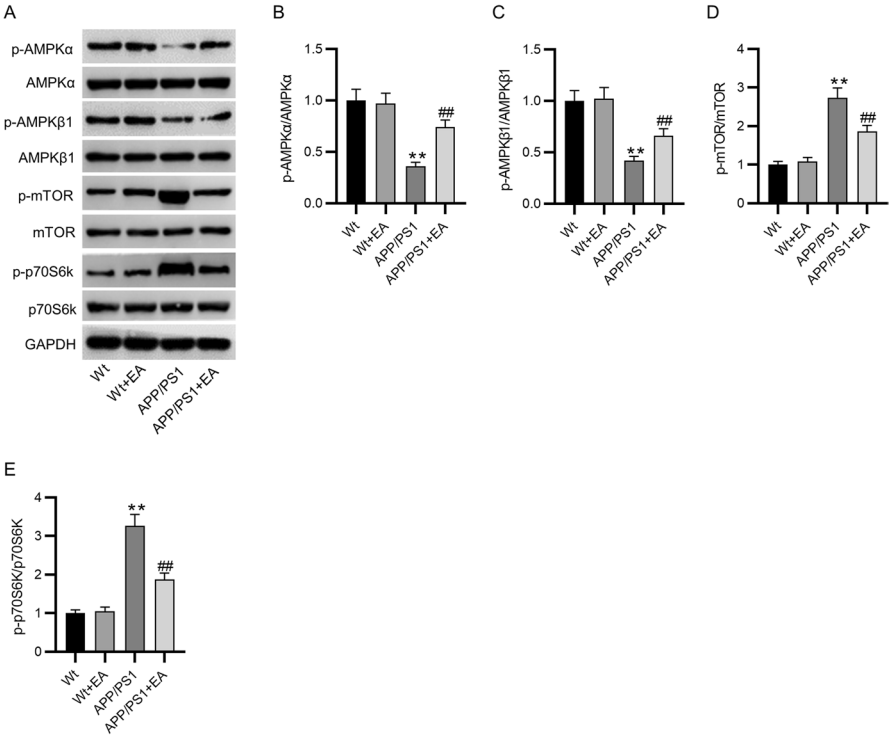

Fig. 4 EA regulates the AMPK/mTOR signaling pathway. **A** Western blotting for evaluating protein levels of AMPK/mTOR pathway-related markers in the hippocampal tissues of each group. **B**–**E** Quantitative results of western blotting. $^{**}p < 0.01$ vs. the Wt group; $^{\#\#}p < 0.01$ vs. the APP/PS1 group

prominently ameliorated the morphological changes of mouse hippocampus and alleviated neuronal loss and apoptosis in the hippocampus of APP/PS1 mice, further elucidating that EA could improve the impairments in learning and memory of mice with AD. Furthermore, we verified that EA stimulation reduced Aβ deposition in the hippocampus of APP/PS1 mice, which is a crucial hallmark of AD pathology. These results were consistent with those in previous reports which showed that EA could enhance Aβ clearance in animal models of AD (Zheng et al. 2021; Liang et al. 2021).

Mounting evidence has illuminated that autophagy is beneficial to Aβ and impaired autophagy contributes to the pathogenesis of AD (Zhang et al. 2021b). LC3 (microtubule-associated protein 1 light chain 3) is a soluble protein ubiquitously distributed in mammalian tissues and cultured cells (Tanida et al. 2008). LC3-I to LC3-II conversion increased levels of Beclin1 and ATGs are common hallmarks of autophagy, while accumulation of p62 is considered an indicator of impaired autophagy flux and its level is decreased when autophagy is induced (Ramadan et al. 2017; Bjørkøy et al. 2009). Previous reports have suggested that EA activates autophagy lysosomal pathway in the brains of 5XFAD mice (Zheng

et al. 2021). Consistently, we found that EA stimulation enhanced the ratio of LC3-II/LC3-I protein levels of Beclin1 and ATG5/7 but decreased p62 protein expression in the hippocampus of APP/PS1 mice, indicating the promoting effect of EA on autophagy. Furthermore, a recent study has demonstrated that EA activates AMPK/mTOR-mediated autophagy, thereby alleviating the neuropathic pain caused by spared nerve injury (Cui et al. 2022). The AMPK/mTOR signaling pathway is a pivotal regulatory pathway of cellular autophagy. Activated AMPK phosphorylates and represses mTOR, a negative regulator of autophagy, consequently enhancing autophagy (Zhu et al. 2021). Nonetheless, it is unclear whether EA activates autophagy in APP/PS1 mice by mediating AMPK/mTOR signaling pathway. Here, our results depicted that EA stimulation facilitated phosphorylation of AMPKα and AMPKβ1 but hindered that of mTOR and its effector p70S6K in the hippocampus of APP/PS1 mice. These indicated that EA activated AMPK signaling and blocked mTOR signaling in the mouse AD model.

It is worth noting that our study has some limitations. First, although EA treatment affected the expression levels of AMPK/mTOR signaling-related proteins in APP/PS1 mice, the results did not directly show that EA ameliorated cognitive deficits in an AMPK/mTOR signaling-dependent manner. Thus, future studies are necessary to verify the involvement of AMPK/mTOR signaling pathway in EA-mediated protective role in AD. Moreover, we only performed histopathological and biochemical analyses of the hippocampus. Further studies are needed to elucidate the beneficial effects of EA and its mechanism of action in AD by analyzing its effects on histopathological and biochemical changes in the cortex region of the brain.

In conclusion, this study reveals that EA treatment improves cognitive deficits, enhances Aβ clearance, and attenuates neuronal loss in the hippocampus of APP/PS1 mice probably by enhancing autophagy and activating AMPK/mTOR signaling pathway. These findings might help to deepen the understanding of the potential mechanism underlying EA therapeutic effects on AD.

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Author Contributions WW, LL, and YW conceived and designed the experiments. CM, YW, FY, WW, LL, and YW carried out the experiments. YW, FY, WW, LL, and YW analyzed the data. YW, FY, WW, LL, and YW drafted the manuscript. All authors agreed to be accountable for all aspects of the work. All authors have read and approved the final manuscript.

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Declarations

Conflict of interest The authors declare no competing interests.

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