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ORIGINAL ARTICLE

Astaxanthin Improved the Cognitive Deficits in APP/PS1 Transgenic Mice Via Selective Activation of mTOR

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Abstract

Astaxanthin (Ast) is an effective neuroprotective and antioxidant compound used to treat Alzheimer's disease (AD); however, the underlying *in vivo* molecular mechanisms remain unknown. In this study, we report that Ast can activate the mammalian target of rapamycin (mTOR) pathway in the 8-month-old APP/PS1 transgenic mouse model of AD. Our results suggest that Ast could ameliorate the cognitive defects in APP/PS1 mice by activating the mTOR pathway. Moreover, mTOR activation perturbed the mitochondrial dynamics, increased the synaptic plasticity after 21 days of treatment with Ast (10 mg/kg/day), and increased the expression of Aβ-degrading enzymes, mitochondrial fusion, and synapse-associated proteins and decreased the expression of mitochondrial fission proteins. Intraperitoneal injection of the mTOR inhibitor, rapamycin, abolished the effects of Ast. In conclusion, Ast activates the mTOR pathway, which is necessary for mitochondrial dynamics and synaptic plasticity, leading to improved learning and memory. Our results support the use of Ast for the treatment of cognitive deficits.

Keywords Alzheimer's disease. astaxanthin. mitochondrial fusion. mitochondrial fission. synaptic plasticity

Introduction

Alzheimer's disease (AD), one of the most common neurodegenerative diseases, is characterized by the deposition of amyloid beta (Aβ) plaques, the formation of neurofibrillary tangles, and loss of neurons in the brain, leading to learning and memory disorders (Høgh 2017; Yilmaz 2015). Aβ (1–42) peptide, the major component of senile plaques, is toxic to neuronal cells and is considered to play a causative role in the development and progression of AD (Qu et al. 2011). Mammalian target of rapamycin (mTOR) is a serine-threonine kinase that regulates several important aspects of mammalian mitochondrial dynamics, protein synthesis, and cell function (Hung et al. 2012; Laplante and Sabatini 2012). The mTOR pathway is important for the acquisition and maintenance of memory paradigms, including spatial, social, and motor functions (Chen et al. 2019). Although the molecular mechanism underlying AD development and progression is unclear, several studies have confirmed the role of mTOR signaling in the pathophysiology of AD (Slipczuk et al. 2009; Zoncu et al. 2011).

Enhanced mitochondrial fission-fusion process impairs mitochondrial function and induces neuronal death in AD (Cadonic et al. 2016; Lu et al. 2017). Previous studies have reported that Aβ-induced Drp1 phosphorylation through Akt/mTOR activation promotes excessive mitochondrial fission, leading to neuronal apoptosis (Kim et al. 2016; Morita et al. 2017). The mTOR signaling pathway plays an important role in memory reconsolidation and maintaining synaptic plasticity by regulating protein synthesis in neurons (Morita et al. 2015; Parra et al. 2014; Parsons et al. 2006). Synaptic plasticity, which can be assessed by examining the altered dendrite morphology, is a prerequisite for learning and memory (Costa-Mattioli and Monteggia 2013; Hoeffer and Klann 2010; Townsend et al. 2006). mTOR activation mediates neural

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network remodeling, including spine plasticity, synaptic transmission, and axonal myelination, and is therefore important in neurodegenerative diseases (Lafay-Chebassier et al. 2005; Maiese et al. 2013; Pozueta et al. 2013). Therefore, regulating mitochondrial dynamics while maintaining synaptic plasticity, would be an effective therapeutic strategy for AD.

Astaxanthin (Ast), a keto-carotenoid, is abundantly present in the marine environment especially in the shells of crustaceans, salmon, trout, and starfish (Guerin et al. 2003). Ast can suppress age-related macular degeneration, prevent chemical-induced cancer, increase high-density lipoprotein levels, and enhance the immune function through its antioxidant and anti-inflammatory properties. It can cross the blood-brain barrier and affects multiple molecular targets within the central nervous system (Hussein et al. 2006; Ikeda et al. 2008; Lee et al. 2008). Previous studies have demonstrated the neuroprotective effects of Ast in animal models of neurodegenerative diseases (Choi et al. 2017; Lee et al. 2008). However, the molecular mechanisms underlying Ast-mediated neuroprotection remain poorly understood with little evidence *in vivo*.

Due to the aforementioned roles of mTOR in regulating memory function, it is a candidate target for treating cognitive dysfunctions in AD. This study aimed to investigate whether Ast ameliorates memory impairment by activating the mTOR pathway and whether the mechanism underlying the protective activity of Ast involves regulation of mitochondrial function and preventing synaptic plasticity damage. We hypothesized that Ast activates the mTOR pathway, thereby, enhancing mitochondrial function and synaptic plasticity and improving learning and memory.

Materials and Methods

Ethics Statement

This study complied with the Chinese Animal Care and Institutional Ethical Guidelines. All animal experiments were approved by the Institute of Laboratory Animal Science of Jinan University (approval number: 20,160,503,112,404).

Animals and Drug Administration

APPswe/PSEN1dE9 (APP/PS1, male, n = 48) double-transgenic (Tg) mice and the non-transgenic (non-Tg, male, n = 12) littermates were purchased from the Guangdong Medical Laboratory Animal Center. The APP/PS1 are double transgenic mice expressing a mutant human presenilin-1 with exon 9 deletion (PS1-dE9) and human APP bearing the Swedish mutation K594N/M595L (APPswe). This model is widely used in AD research (Xue et al. 2017). The animals were housed in standard plastic cages under conventional laboratory conditions (22–24 °C, 40–60% relative humidity) with a 12-h light/dark cycle. Food and water were available *ad libitum*.

Eight-month-old APP/PS1 transgenic mice were randomly divided into Tg (APP/PS1), Tg + Ast, Tg + Ast + RAPA, and Tg + RAPA groups (n = 12 in each group). The wild-type control group (WT; non-Tg) and the Tg group mice were fed corn oil (10 mL/kg body weight) as a vehicle in the same volume as Ast every day for 21 days. The Tg + Ast group received Ast (10 mg/kg/day, oral administration) for 21 days (Xue et al. 2017). The Tg + Ast + RAPA group mice were administered Ast orally every day for 21 days along with the mTOR inhibitor rapamycin (3 mg/kg body weight, intraperitoneal injection) every third day. The Tg + RAPA group mice also received rapamycin (3 mg/kg body weight, intraperitoneal injection) every third day for 21 days, but no Ast treatment. After the behavioral tests, the mice were euthanized, and tissues were collected.

Morris Water Maze Test

The Morris water maze (MWM) was purchased from Chengdu Taimeng Technology Co., Ltd. The instrument was inspected, and the software was checked before use. The maze was filled with water and made opaque by adding titanium dioxide. The water level was maintained 1.5 cm above the platform, and the temperature was maintained at 23 °C. The position of all the reference objects was kept constant during the whole experiment, including the position of the experimenter. The surroundings were kept quiet and there was no direct light in the pool. The MWM experiment involves a spatial navigation test and spatial probe test. After continuous intragastric administration of Ast for 21 days, the water maze experiment was performed. In the spatial navigation test, the mouse was randomly placed in a quadrant such that the platform was not visible to the mouse, and was allowed to find the hidden platform. The computer system automatically recorded the trajectory of the mouse and the time (latency period) spent to find the hidden platform. If the mouse could not find the platform within 60 s, the mouse was guided to reach the platform and stay there for 10 s to consolidate its memory. At this time, the computer system automatically recorded the incubation period of 60 s. After the experiment, the mice were dried to reduce the effect of low-temperature stimulation. The probe trial was used to detect the memory retention ability in mice. The platform was removed on the 8th day of the water maze experiment, and the mouse was placed in the second quadrant and allowed to swim in the water for 60 s. The computer recorded the relevant data of the mouse movement within 60 s (mainly the number of times it crossed the platform and time spent in the target quadrant). After the experiment, the mice were wiped dry to reduce the effect of low-temperature stress.

Passive Avoidance Test

The Passive Avoidance Task (PAT) was used to evaluate the short-term memory in mice. The instrument was divided into six compartments, half of which were dark and the other half were illuminated. The compartments were connected by a circular hole with a gate in the middle. A device that can produce an electric shock was attached to the bottom of the dark compartment. When the mouse entered the dark compartment from the illuminated compartment, it received an electric shock (39 V for 3 s). The experiment was divided into the training session (day 1) and the test session (day 2).

On the 1st day of the experiment, the mice were trained for dark avoidance. With the gates closed, the mice were placed in the illuminated compartment for 5 min to adapt to the environment. When the mouse received the electric shock each time, the latency and the number of errors were measured. After 24 h of training, the mice were tested for dark avoidance, as described above. The latency and the number of errors were recorded.

Tissue Preparation

After the behavioral experiments, all animals were anesthetized using 1.25% tribromoethanol (Sigma, USA). For histology, four mice from each group were anesthetized and transcardially perfused with 4% paraformaldehyde in phosphate-buffered saline (PBS) at 4 °C. After perfusion, the brain was removed and immersion-fixed overnight at 4 °C. The brains of the remaining eight mice from each group were isolated and immediately snap-frozen in liquid nitrogen and stored at -80 °C until use for western blot and ELISA analysis.

Immunohistochemistry

The fixed brain tissue was embedded in paraffin and cut into 6-mm thick coronal sections. The sections were dewaxed and rehydrated. The endogenous peroxidase activity in the brain slices was quenched using 3% H_2O_2/methanol solution for 15 min. The sections were boiled in 0.01 m citrate buffer solution (pH 6.0) for 6 min, following which the citrate buffer was allowed to cool at room temperature. The sections were then washed with PBS, incubated in blocking solution (5% goat serum; Boster Bioengineering, Wuhan, China) for 30 min at 37 °C, followed by incubation in anti-Aβ primary antibody (1:100; Cell Signaling Technology, USA) at 4 °C overnight in a humidified chamber. The sections were washed with PBS and incubated with anti-rabbit IgG-horseradish peroxidase secondary antibody (1:200) at room temperature for 1 h. The sections were then developed using diaminobenzidine and counterstained with hematoxylin for 3 min. Subsequently, the sections were washed, placed on glass slides, and dried. Images were captured using a light microscope (Leica, Germany).

Aβ42 ELISA

Briefly, the cortex and the hippocampus were homogenized in Tris-buffered saline (TBS, pH 8.0) with protease inhibitors. The homogenates were sonicated and centrifuged. The supernatant was neutralization with 0.5 m Tris. The concentration of soluble Aβ42 was determined using the Aβ42 ELISA kit (CUSABIO, Wuhan, China) following the manufacturer's instructions.

Transmission Electron Microscopy (TEM)

The hippocampi of mouse from each group were immediately fixed in ice-cold glutaraldehyde (3%, pH = 7.4), then fixed with osmic acid (OsO4), embedded in EPON812 resin, and cut into 1 mm^3 chips for TEM (Hitachi HT7700). 1 mm thick fixed sections from the DG region of the hippocampus were analyzed to observe the structure of the synapse and mitochondria.

Western Blot Analysis

The tissue was lysed in 500 μl of lysis buffer (Beyotime, Nantong, Jiangsu, China) for 30 min at 4 °C. The lysate was centrifuged, and the supernatant was collected. The protein concentration in the supernatants was determined using the BCA kit (Beyotime, Jiangsu, China). The supernatants were mixed with loading buffer (Beyotime, Jiangsu, China) and boiled for 10 min. Equal amounts of proteins were loaded in each well of the gel. The proteins were resolved by gel electrophoresis (Beyotime, Jiangsu, China). We used 12% gels to resolve mitochondrial fission protein 1 (Fis1) and 8% gels to resolve β-tubulin, SYP, PSD95, S6, p-S6 (Ser240/244), Akt, p-Akt (Thr308), mTOR, p-mTOR (Ser2448), Drp1, Mfn1, Mfn2, Opa1, NEP and IDE. Once resolved, the proteins were electrophoretically transferred onto the PVDF membrane (Millipore, USA). The membranes were blocked using 5% skimmed milk powder (Beyotime, Jiangsu, China) overnight. The membranes were then washed thrice with TBS containing Tween 20 (TBST), and incubated with the primary antibody (table S1) overnight at 4 °C. Subsequently, the membranes were washed three times with TBST and incubated with the secondary antibody on a shaker at room temperature (table S1) for 1 h. The signal was measured on the gel imaging system using the enhanced chemiluminescence kit (ECL, Millipore, USA), and the results were analyzed using Quantity One software.

Statistical Analysis

All data are presented as mean ± standard deviation (SD). One-way analysis of variance was used to analyze the significant differences among the groups. A p-value of less than 0.05 was considered statistically significant. Significance was indicated by $*p < 0.05$, $**p < 0.010$, and $***p < 0.001$.

Results

Ast Alleviated Cognitive Impairment via mTOR Activation

In AD animal models, mTOR regulates a series of physiological processes involved in learning and memory (Bhattacharya et al. 2012). To elucidate the effect of Ast on learning and memory in APP/PS1 mouse, the MWM test and PAT were performed. In the MWM test, APP/PS1 mice showed a longer escape latency in the spatial navigation test as compared to the WT group. Ast treatment significantly reduced the escape latency in APP/PS1 mice ($p < 0.05$, Fig. 1A). Representative tracks of each mice group on day 7 of the navigation test and day 8 of the spatial probe test are shown in Fig. 1B. In the exploratory experiment, compared with the WT group, APP/PS1 mice stayed in the target quadrant for a significantly shorter duration and crossed the target quadrant fewer times ($p < 0.05$, Fig. 1C and D). Spatial learning was improved in the Ast-treated APP/PS1 mice ($p < 0.05$). After 24 h of training, we performed the PAT to assess the conditional fear and memory in mice, and observed that memory retention was significantly improved after Ast treatment ($p < 0.05$, Fig. 1E and F). These findings suggest that Ast treatment significantly alleviates the cognitive impairments in APP/PS1 transgenic mice. Moreover, the observed effects of Ast treatment were significantly attenuated by intraperitoneal injection of the mTOR inhibitor rapamycin ($p < 0.05$, Fig. 1A and F) suggesting that Ast improves learning and memory via mTOR activation in APP/PS1 transgenic mice.

Ast Treatment Activated the mTOR Pathway

Ast treatment significantly enhanced both working memory and memory recall in APP/PS1 mice as determined by Morris water maze; these effects of Ast were attenuated by co-administration of rapamycin. Next, we investigated whether the neuroprotective properties of Ast are mediated via the activation of the mTOR pathway in APP/PS1 transgenic mice. Ast treatment markedly increased the levels of phosphorylated (activated) mTOR and phosphorylated S6 in the cerebral cortex of APP/PS1 transgenic mice ($p < 0.05$; Fig. 2A and D).

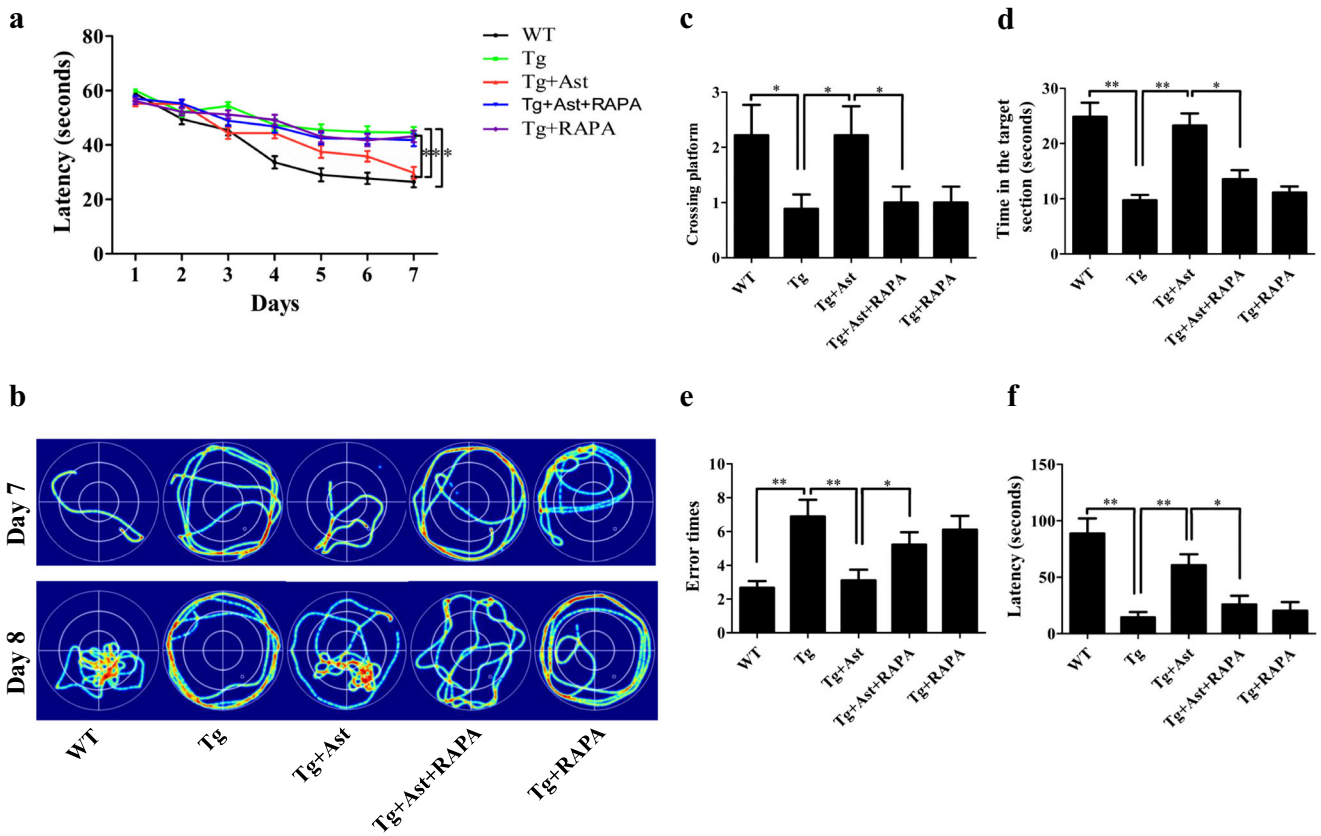

Fig. 1 Astaxanthin alleviates cognitive impairment in APP/PS1 transgenic mice via mTOR activation. **(A)** Escape latency of each group in the spatial navigation test; **(B)** Representative tracks of each group of mice on day 7 of the spatial navigation test and on day 8 of the spatial probe test; **(C)** Number of target platform crossings in the spatial probe test; **(D)** Time spent in the target quadrant in the spatial probe test; **(E)** Number of errors in each group in the step-through PAT; **(F)** Latency of each group in the step-through PAT. Data are presented as the mean ± SD; n = 12 per group; $^{*}p < 0.05$, $^{**}p < 0.01$. Analyzed by a one-way ANOVA followed by Bonferroni post hoc test

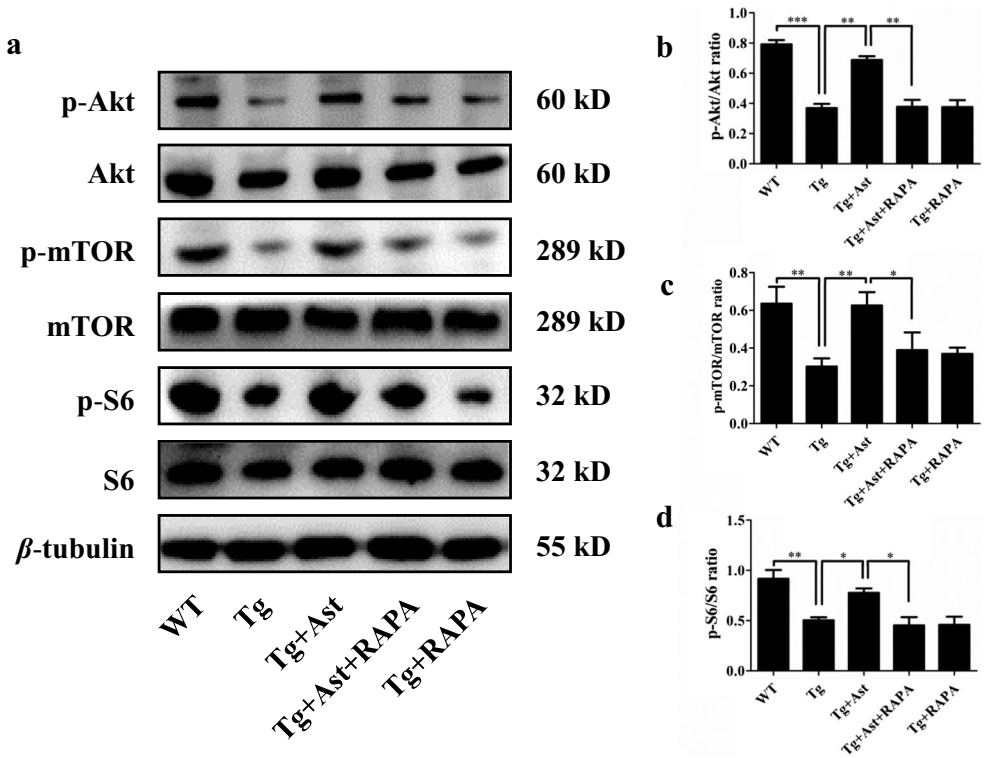

Fig. 2 Astaxanthin activates mTOR signaling in APP/PS1 transgenic mice. Twenty-one days after Ast treatment, western blotting was performed to analyze the levels of Akt/mTOR/S6 pathway-related proteins in the cerebral cortex of all groups. Total Akt, mTOR, and S6 levels were used as controls. β-tubulin was used as the loading control. Representative immunoblot (A) and protein expression quantification (B-D) of each group. Data are expressed as mean ± SD; n = 08 per group; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Analyzed by a one-way ANOVA followed by Bonferroni post hoc test

This increase in the levels of p-mTOR and p-S6 in the cerebral cortex was attenuated by intraperitoneal injection of the mTOR inhibitor rapamycin ($p < 0.05$, Fig. 2A and D). These findings suggest that Ast could effectively activate the mTOR pathway in APP/PS1 transgenic mice.

Ast Attenuated Synaptic Damage via mTOR Activation

The mTOR signaling pathway plays an important role in the consolidation of memory and the maintenance of synaptic plasticity during memory formation by regulating the neuronal protein synthesis (Morita et al. 2015). The mTOR-S6 pathway facilitates the synthesis of synaptic proteins involved in potentiating synaptic plasticity. To determine the effect of Ast on the expression of synaptic functional proteins in the cerebral cortex of APP/PS1 transgenic mice, we analyzed the synapse-related markers SYP, GAP43, and PSD95 by western blot. Compared with the Tg group, the expression of SYP, GAP43, and PSD95 proteins in the Tg + Ast group was significantly increased ($p < 0.05$; Fig. 3A and D). These results suggest that Ast can effectively increase the expression of synapse-related proteins, thereby exerting a neuroprotective effect. This effect of Ast on the expression of synapse-related markers was substantially reduced by rapamycin treatment.

Ast Regulated Mitochondrial Dynamics via mTOR Activation

Because mTOR activation is also involved in mitochondrial fusion/fission dynamics (Townsend et al. 2006), we further investigated whether Ast affects mitochondrial dynamics via mTOR activation. To address this, we analyzed the expression of mitochondrial fusion markers Mfn1, Mfn2, and Opa1 by western blot. Results revealed that compared to wild-type mice, the expression of Mfn1, Mfn2, and Opal proteins was significantly reduced in APP/PS1 mice. Ast treatment significantly increased the level of these proteins in APP/PS1 transgenic mice ($p < 0.05$; Fig. 3A and E-G), and rapamycin administration abolished this effect.

Next, we evaluated the effects of Ast on mitochondrial fission protein expression in APP/PS1 transgenic mice. Our results showed that compared to WT mice, the levels of Drp1 and Fis1 were significantly increased in APP/PS1 mice. Ast treatment significantly decreased the level of the mitochondrial fusion protein in APP/PS1 transgenic mice ($p < 0.05$; Fig. 3A, H, I). These findings suggest that Ast could effectively increase mitochondrial fusion and inhibit mitochondrial fission in APP/PS1 transgenic mice. This effect of Ast on mitochondrial dynamics was significantly reduced by rapamycin treatment ($p < 0.05$; Fig. 3A, H, I), suggesting that Ast regulates mitochondrial fusion/fission dynamics via mTOR activation.

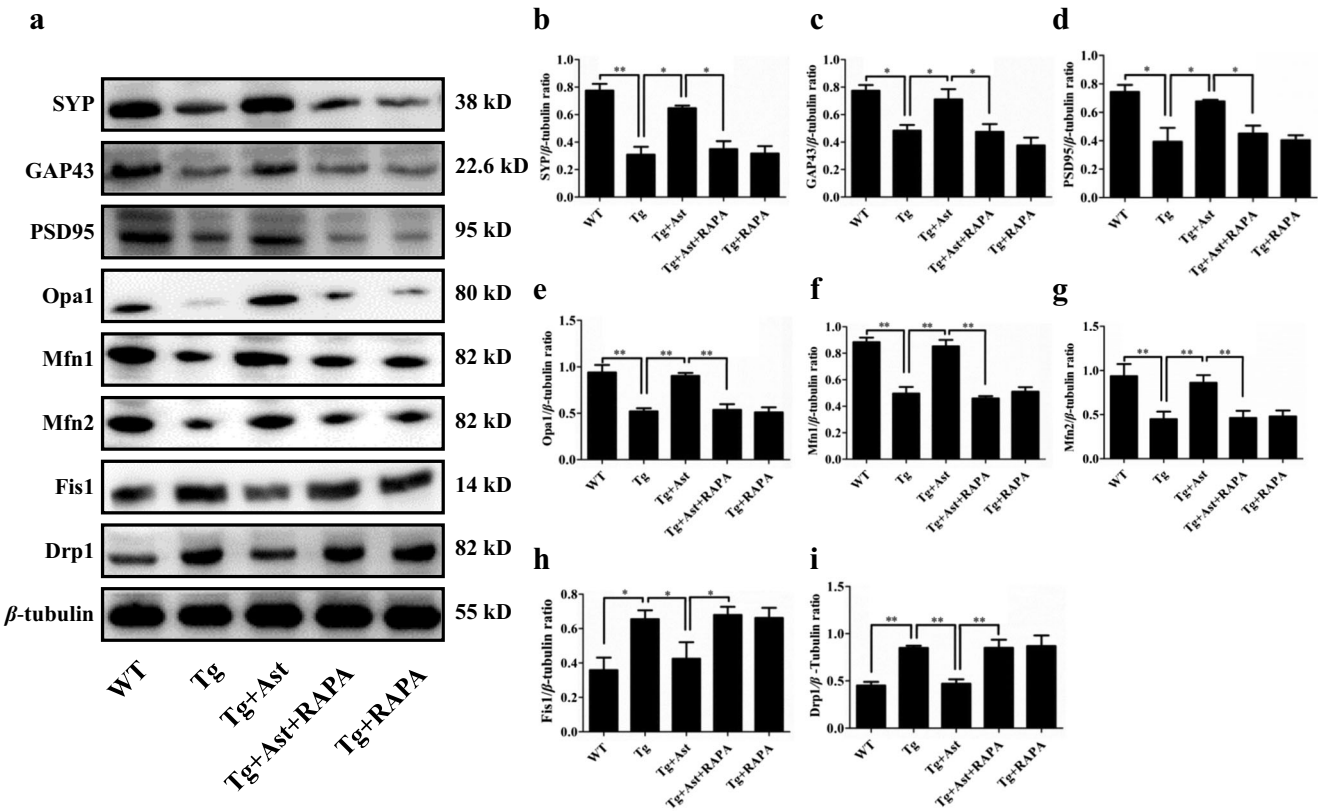

Fig. 3 Astaxanthin affects synaptic plasticity and mitochondrial fusion and fission via mTOR activation in APP/PS1 transgenic mice. Representative western blot of total protein extracted from the cerebral cortex **(A)** and quantification of the synapse-related proteins, including SYP **(B)**; GAP43 **(C)** and PSD95 **(D)**, and mitochondrial proteins, including **(E)** Opa1; **(F)** Mfn1; **(G)** Mfn2; **(H)** Fis1; and **(I)** Drp1. Data are presented as mean ± SD; n = 08 per group; $^{*}p < 0.05$, $^{**}p < 0.01$. Analyzed by a one-way ANOVA followed by Bonferroni post hoc test

Western blot analysis revealed that the expression of proteins involved in regulating synaptic plasticity and mitochondrial dynamics was increased in Ast treated APP/PS1 mice brain. Next, we tried to visualize the synaptic junctions and mitochondria in the hippocampus of Ast treated APP/PS1 mice by TEM. As shown in Fig. 4, the synaptic structures in the hippocampus can be clearly observed through TEM. Compared with the Tg group, the Tg + Ast group showed a significantly reduced synaptic damage and the synaptic gaps were more evident. These results suggest that Ast can effectively improve synaptic plasticity in the hippocampus of APP/PS1 mice.

We also investigated the effect of Ast on mitochondrial fusion and fission in the hippocampus of APP/PS1 mice through TEM. Compared with the Tg group, the number of mitochondria in the fusion state (oval, long rod-shaped) within the hippocampal region was significantly increased in the Tg + Ast group, whereas, the number of mitochondria in the fission state (spherical) was significantly decreased. The above results suggest that Ast can effectively promote mitochondria fusion and reduce mitochondrial fission in the hippocampus of APP/PS1 mice to maintain normal mitochondrial dynamic balance. Thus, consistent with the western blot results, our TEM results showed that Ast treatment significantly improved both synaptic plasticity and mitochondrial dynamics in the hippocampus of APP/PS1 mice, and this effect of Ast was attenuated by co-administration of rapamycin.

Ast Decreased Aβ Accumulation via mTOR Activation

We next investigated whether Ast treatment could reduce Aβ accumulation in the brain of APP/PS1 mice. We quantified the levels of Aβ42 in the cortical and hippocampal tissues using a specific Aβ42 ELISA kit. We also performed histochemical staining to detect amyloid plaques in the cortex and the hippocampus. Our results revealed that Ast reduces Aβ levels in APP/PS1 transgenic mice brain ($p < 0.05$; Fig. 5A and E). This effect of Ast was

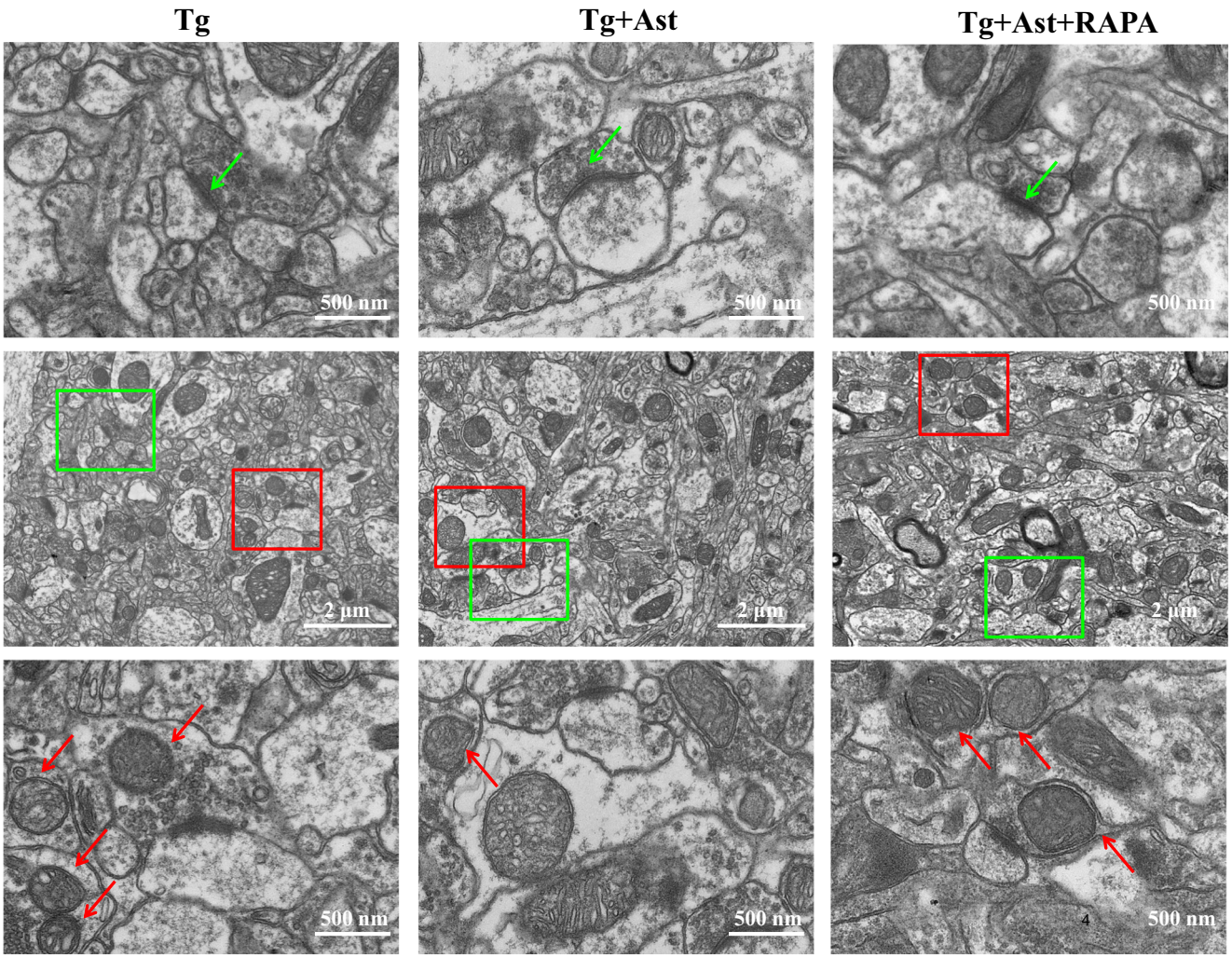

Fig. 4 Ast attenuated synaptic damage and mitochondrial dynamics via mTOR activation in the hippocampus of APP/PS1 transgenic mice. Green arrows/boxes indicate synapse; red arrows/boxes indicate mitochondria in the fission state (spherical)

significantly attenuated by rapamycin treatment ($p < 0.05$; Fig. 5A and E). These findings suggest that Ast-mediated reduction in Aβ accumulation in APP/PS1 transgenic mice brain is mTOR dependent.

Ast Increased the Levels of Aβ-degrading Enzymes via mTOR Activation

Ast treatment significantly decreased the levels of Aβ in the cortex and the hippocampus of APP/PS1 mice as determined by histochemical staining and ELISA, and this effect of Ast was attenuated by co-administration of rapamycin. Next, we investigated whether Ast could elevate the expression of Aβ-degrading enzymes including neprilysin (NEP) and insulin-degrading enzyme (IDE) via activation of the mTOR pathway in APP/PS1 transgenic mice. Results showed that compared to the Tg group, the expression of IDE and NEP was significantly increased in the Tg + Ast group ($p < 0.05$; Fig. 6A and C). Thus, Ast treatment significantly increased the levels of Aβ-degrading enzymes in APP/PS1 mice brain, and this effect of Ast was attenuated by co-administration of rapamycin. These results are consistent with our findings of histochemical staining and ELISA for Aβ.

Discussion

Our study demonstrated that Ast ameliorates cognitive deficits in APP/PS1 mice model of AD. Moreover, our results identified the key intracellular pathway involved in Ast-mediated improvement of cognitive functions in AD mice and solved the long-standing problem of the functional adaptation of mTOR signaling in mitochondrial dynamics and synaptic plasticity.

Herein, we demonstrated that Ast could improve the learning and memory abilities of APP/PS1 transgenic mice by activating the mTOR pathway. It has been previously reported

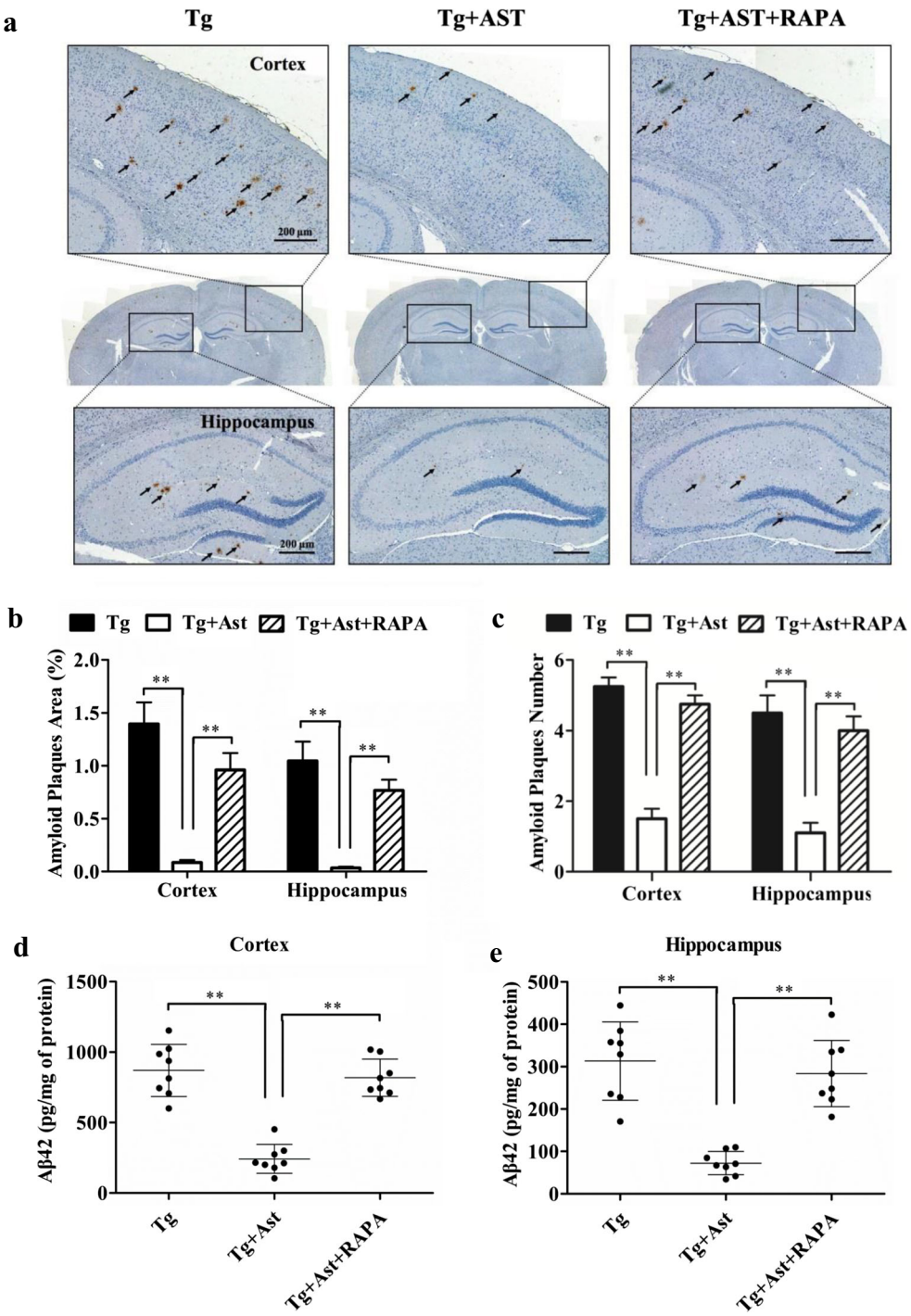

Fig. 5 Astaxanthin decreases Aβ accumulation via mTOR activation in APP/PS1 transgenic mice. **(A)** Histochemical staining of amyloid plaques in the brain; the black arrows indicate Aβ42 accumulation; **(B)** Amyloid plaques area (%) in the brain sections of each group (n = 03) **(C)** Number of amyloid plaques in each group (n = 03); **(D-E)** Concentration of Aβ42 in the cortex and hippocampus of each group determined using a specific Aβ42 ELISA kit (n = 08); Data are presented as mean ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Analyzed by a one-way ANOVA followed by Bonferroni post hoc test

that mTOR activation is essential for hippocampal-dependent memory functions and mTOR inhibition has been shown to impair memory consolidation (Lafay-Chebassier et al. 2005; Slipczuk et al. 2009). Suppressing mTOR signaling in AD animal models reduces AD pathology and improves cognition, while a decreased mTOR activity in peripheral lymphocytes appears to correlate with AD progression (Caccamo et al. 2018; Vartak et al. 2019). mTOR is localized to the synaptic region, where it regulates the synthesis of locally translated proteins and is upregulated in an activity-dependent manner, which is essential for synaptic plasticity including long term potentiation (LTP) (Chen et al. 2019). Our findings are consistent with those of the previous studies showing that Ast prevents ischemia-reperfusion injury via mTOR activation in mice and decreases cognitive decline in type-2 diabetic mice model by activating Akt (the upstream regulator of mTOR) and attenuating oxidative stress (Li et al. 2017, 2016).

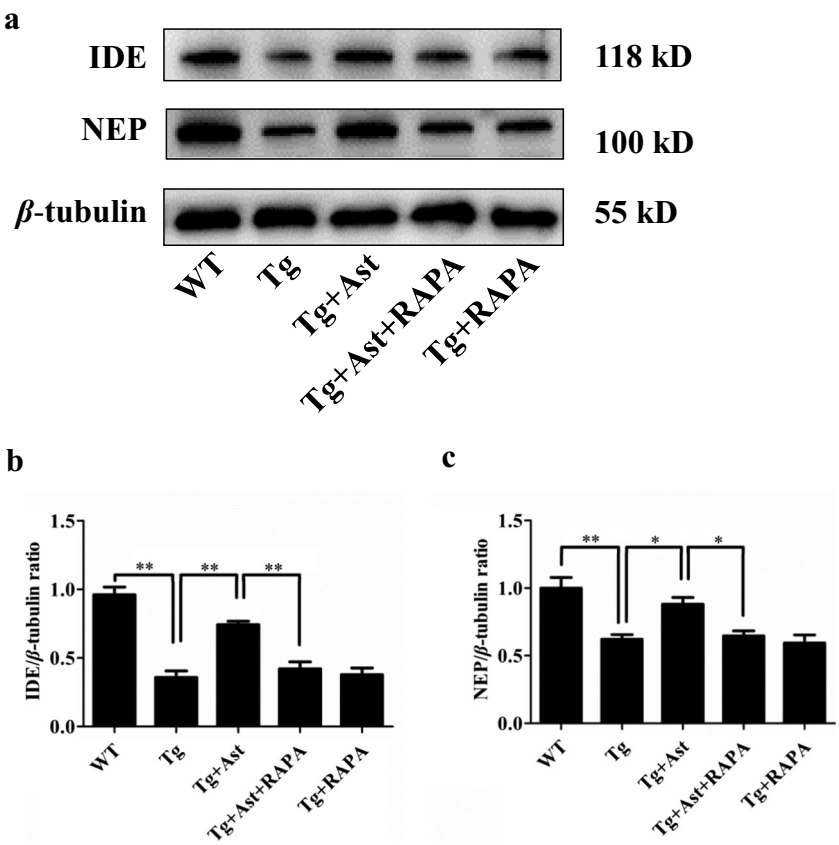

Fig. 6 Astaxanthin affects Aβ-degrading enzymes expression via mTOR activation in APP/PS1 transgenic mice. Representative western blot of total protein extracted from the cerebral cortex **(A)** and quantification of the proteins including IDE **(B)** and NEP **(C)**. Data are presented as mean ± SD; n = 08 per group; $^{*}p < 0.05$, $^{**}p < 0.01$. Analyzed by a one-way ANOVA followed by Bonferroni post hoc test

We also observed that Ast exerts neuroprotective effects by significantly increasing the expression of SYP, GAP43, and PSD95 proteins via mTOR activation. Ast treatment also significantly reduced synaptic damage in APP/PS1 mice brain as determined by TEM, and this effect of Ast was attenuated by co-administration of rapamycin. Our TEM results are consistent with our findings on protein expression. Previous studies in various animal models also showed that mTOR is the key signaling pathway involved in regulating synaptic plasticity (Cammalleri et al. 2003; Kou et al. 2019). Loss of mTOR signaling has been shown to impair long-term potentiation and synaptic plasticity in AD models. Synaptic plasticity can be detected by evaluating changes in dendritic morphology, which is a prerequisite for learning and memory (Lafay-Chebassier et al. 2005; Townsend et al. 2006). A verity of synaptic proteins, such as SYP and PSD95 are the key regulators of the structure and functions of dendritic spines (Huang et al. 2018; Ikeda et al. 2008). mTOR-S6 pathway promotes synaptic protein synthesis and enhances synaptic transmission (Luft et al. 2004).

Interestingly, Ast treatment increased the expression of Mfn1, Mfn2, and Opa1 in APP/PS1 transgenic mice brain, and decreased the Drp1 and Fis1 levels, suggesting that Ast promotes mitochondrial fusion by activating mTOR and inhibit mitochondrial fission. Ast treatment also significantly improved mitochondrial dynamics in APP/PS1 mice brain as determined by TEM and western blot analysis, and this effect was attenuated by co-administration of rapamycin. A previous study showed that mTOR regulates mitochondrial dynamics and cell survival (Morita et al. 2017). Mitochondrial dynamics are influenced by the fusion and fission processes (Chen and Chan 2009). Regulatory factors such as Opal, Mfn1, and Mfn2 are involved in regulating mitochondrial fusion. Opa1 regulates the inner membrane fusion and cristae formation (Detmer and Chan 2007), while Mfn1 and Mfn2 regulate outer membrane fusion (Detmer and Chan 2007). Several features of mitochondrial dynamics are dysfunctional in early-stage AD, including a disruption in the balance between mitochondrial fusion and fission. A large GTPase, Drp1, mediates mitochondrial division and is recruited from the cytoplasm to the mitochondrial outer membrane through several mitochondrial outer membrane protein adaptors (including Mff) (Cadonic et al. 2016; Losón et al. 2013).

Further, our study demonstrated that Ast significantly decreased the Aβ plaque load in the cortex and hippocampus through mTOR activation. We also observed that Ast treatment significantly enhanced the levels of Aβ-degrading enzymes in APP/PS1 mice brain, which was attenuated by co-administration of rapamycin. This is consistent with our findings on histochemical staining and ELISA of Aβ. It has been

shown that the activation of mTOR and p70S6K could prevent cell death caused by Aβ exposure and ameliorate the toxic effect of Aβ on microglia *in vitro* (Lafay-Chebassier et al. 2005; Shang et al. 2012; Song et al. 2020). Moreover, an increase in the levels of phosphorylated/activated mTOR and p70S6 kinase influences neurofibrillary pathology in AD (An et al. 2003; Bhattacharya et al. 2012). Other groups also show beneficial effect of RAPA on APP/PS1 mice for their spatial learning and Abeta clearance (Lin et al. 2013; Wang et al. 2019). But our findings are consistent with some recent studies showing that activation of the mTOR pathway could promote neuronal cell survival, neuroprotection, and protein aggregate clearance in animal models of neurodegenerative diseases, which was attenuated by co-administration of rapamycin (Cammalleri et al. 2003; Chen et al. 2019; Heras-Sandoval et al. 2014).

The mTOR signaling pathway is a key regulator of cell differentiation, proliferation, senescence, and apoptosis. mTOR signaling activation is associated with both mitochondrial dynamics and synaptic plasticity in AD (Ma et al. 2010; Morita et al. 2015). mTOR can undergo autophosphorylation on its serine/threonine residues and regulates the synthesis of other proteins by phosphorylating and activating S6 kinase (Burnett et al. 1998). In our study, the mTOR phosphorylation was significantly increased after Ast treatment. However, the effects of Ast on APP/PS1 mice were significantly attenuated by the mTOR inhibitor rapamycin. Taken together, these findings demonstrate the role of Ast in activating the mTOR signaling pathway, promoting mitochondrial fusion and fission dynamics, and maintaining synaptic plasticity.

Our study has a few limitations. First, although we have evaluated the neuroprotective effects of Ast, we did not evaluate its role in regulating autophagy and tau phosphorylation. Second, we did not evaluate the redox signaling and have not utilized the conditional knockout mice to explore the underlying molecular mechanisms. Finally, the study was limited to *in vivo* experiments with no *in vitro* data. In future studies, we will try to focus on the *in vitro* neuroprotective properties of Ast. Nevertheless, our findings provide a potential new therapeutic option for the treatment of AD.

Conclusions

Ast improves learning and memory in AD mice by activating the mTOR signaling pathway. Ast-treatment affects mitochondrial fusion/fission dynamics and enhances synaptic plasticity in AD mice. Taken together, our results establish the central role of the mTOR signaling pathway in Ast-induced mitochondrial dynamics and synaptic plasticity, providing more supporting evidence for the use of Ast in patients with mental disorders and cognitive impairment.

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Compliance with Ethical Standards

Conflict of Interest No conflicting relationship exists for any author.

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