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Research report

Mitophagy activation by rapamycin enhances mitochondrial function and cognition in 5×FAD mice

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ABSTRACT

Alzheimer's disease (AD) is the most prevalent form of dementia, characterized by severe mitochondrial dysfunction, which is an intracellular process that is significantly compromised in the early stages of AD. Mitophagy, the selective removal of damaged mitochondria, is a potential therapeutic strategy for AD. Rapamycin, a mammalian target of rapamycin (mTOR) inhibitor, augmented autophagy and mitigated cognitive impairment. Our study revealed that rapamycin enhances cognitive function by activating mitophagy, alleviating neuronal loss, and improving mitochondrial dysfunction in 5 ×FAD mice. Interestingly, the neuroprotective effect of rapamycin in AD were negated by treatment with 3-MA, a mitophagy inhibitor. Overall, our findings suggest that rapamycin ameliorates cognitive impairment in 5 ×FAD mice via mitophagy activation and its downstream PINK1-Parkin pathway, which aids in the clearance of amyloid-β (Aβ) and damaged mitochondria. This study reveals a novel mechanism involving mitophagy regulation underlying the therapeutic effect of rapamycin in AD. This study provides new insights and therapeutic targets for rapamycin in the treatment of AD. However, there are still some shortcomings in this topic; if we can further knock out the PINK1/Parkin gene in animals or use siRNA technology, we can further confirm the experimental results.

1. Introduction

Alzheimer's disease (AD) is an age-related neurodegenerative disorder clinically characterized by decreased behavioral and cognitive abilities. The primary histopathological features of AD are extracellular amyloid-β (Aβ) deposition and intracellular tangles of phosphorylated tau proteins. Mitochondria is widely recognized to be crucial in the pathogenesis of AD, and mitochondrial dysfunction expedites the progression of AD. Mitochondria, one of the most vital cellular organelles and the primary site of energy production, are critical for maintaining the brain's energy balance and generating antioxidants that safeguard neurons. Mitochondrial dysfunction has been observed in the brain of patients with AD [1]. Mitochondria-mediated signaling pathways affect synaptic signaling and alter the structure and function of neurons; thus, mitochondria play an significant role in learning and memory [2].

The regulation of mitochondrial quality and preservation of mitochondrial homeostasis includes mitochondrial dynamics and mitophagy [3]. Mitophagy can selectively eliminate damaged or dysfunctional mitochondria and is a form of autophagy. When mitochondria sustain damage, cells initiate mitophagy, which subsequently removes the damaged mitochondria to maintain normal mitochondrial quality and function [4]. Conversely, if damaged mitochondria are not promptly eliminated, they lead to an increase in the production of reactive oxygen species (ROS), which subsequently triggers neuronal death. Therefore, the timely removal of damaged mitochondria is crucial for the preservation of mitochondrial function and structure. PINK1 and Parkin, the primary proteins involved in mitophagy, were found in increased amounts in the brains of APP/PS1 mice [5,6]. However, structural damage to the mitochondria was still observed in the brain regions of these mice [7], implying that the initiation of mitophagy was still insufficient to remove damaged mitochondria. The activation of autophagy reduces Aβ deposition and mitigates cognitive dysfunction in various animal models [8]. Therefore, enhanced mitophagy could be a novel therapeutic target in AD treatment.

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Rapamycin (RAPA) is a macrolide antibiotic primarily used as an immunosuppressant to prevent organ rejection following transplantation [9]. Interestingly, recent scientific findings suggest that in animal models [11–13], RAPA not only possesses anti-aging properties [10], but also plays a role in improving cognitive impairment and delaying the progression of age-related neurodegenerative diseases, including AD and Parkinson's disease.

A series of experiments have provided evidence that RAPA reduces Aβ fiber aggregation in neurons and the level of pathogenic $A\beta_{42}$ in the brain, thereby improving cognitive function in AD mice [14,15]. The mammalian target of RAPA, mTOR, is a RAPA target molecule belonging to the phosphatidylinositol kinase (PI3K) family of protein kinases. mTOR governs crucial cellular functions, such as protein synthesis, energy metabolism, lipid metabolism, autophagy, and mitochondrial and lysosomal biogenesis; hence, it is linked to numerous diseases, including AD [16]. RAPA is a specific mTOR inhibitor. Inhibiting of mTOR, can influence the cell cycle, cell growth and proliferation, cellular autophagy, and protein synthesis, and has shown promising neuroprotective effects in animal models of neurodegenerative diseases. Consequently, in recent years, researchers have examined the connection between RAPA and autophagy and discovered that RAPA is a mitophagy activator. Emerging evidence suggests that RAPA activates mitophagy in AD, thereby mitigating cognitive damage [17,18]. However, the underlying mechanism remains unclear.

In this study, we used 5 ×FAD mice to explored the effect of RAPA on cognitive impairment, and sought to understand the mechanism of mitophagy and the downstream pathways involved this process. We intraperitoneally administered RAPA in 2-month-old male 5 ×FAD mice for two months and examined spatial learning, Aβ production, and synaptic ultrastructure. Additionally, we evaluated mitochondrial morphology, energy metabolism, reactive oxygen species (ROS) production, dynamics, and mitophagy. Our results reveal that RAPA may be a promising agent for AD therapy via mitophagy and the downstream PINK1-Parkin pathway.

2. Materials and methods

2.1. Reagents

RAPA was synthesized with a purity of more than 99% by Professor Jie Huang and Professor Kua-liang Li of the Fujian Institute of Microbiology. 3-Methyladenine (3-MA) was obtained from MedChemExpress (Monmouth Junction, NJ, USA).

2.2. Animals and drug administration

Transgenic 5 ×FAD mice were sourced from The Jackson Laboratory and the generation of 5 ×FAD mice has been described in detail in a previous study [19]. The mice were housed in a well-ventilated, pathogen-free environment with a 12-hour light/dark cycle and unlimited access to food and water.

Two-month-old male 5 ×FAD mice were randomly allocated to the AD, AD+RAPA, AD+RAPA+ 3-MA, and AD+ 3-MA groups, while non-transgenic wild-type littermates were used as controls (WT group), with eight mice in each group. RAPA was dissolved in DMSO, conserved at − 20 °C, it was diluted in a carrier solution containing 5% Tween 80 immediately prior to administration. 3-MA was liquefied in normal saline by warming the solution to 60–70 °C directly before administration. Mice in the AD+RAPA group received intraperitoneal injections of RAPA at a dosage of 8 mg/kg every alternate day for 8 weeks [20]. The AD+RAPA+ 3-MA group was administered 3-MA at a dose of 15 mg/kg (i.p.) 1 h prior to RAPA injection [21], and the AD+ 3-MA group received an injection of 3-MA at a dose of 15 mg/kg (i.p.). Conversely, the mice in the WT and AD groups were administered an equivalent vehicle solution.

All procedures were performed in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Bethesda edition, revised 2011) and the Institutional Animal Care and Use Committee of Fujian Medical University (No. FJMU IACUC 2018–035, 2022–0438).

2.3. Morris water maze tests and open filed tests

Morris water maze (MWM) tests were conducted in accordance with a previously established protocol[22]. A circular pool with a diameter of 120 cm was used for the MWM test, with a transparent platform submerged 1 cm beneath the water at a fixed location. Non-toxic white paint was used to render the water opaque. During the test, curtains were used to encircle the pool to eliminate any potential influence from the observers, and spatial cues were positioned at fixed locations around the pool. In the acquisition phase, mice were introduced into the pool and allowed to swim for 60 s. Mice that were independently located on the platform were allowed to remain on the platform for 15 s, whereas those unable to find the platform were gently guided to it, allowed to stay for 15 s, and then returned to their cages. Each mouse underwent four trials per day for five consecutive days, with each trial involving placement in a pool from a different quadrant. On the sixth day, the platform was removed and the mice were introduced into the pool from the quadrant opposite the original platform location and allowed to search for 60 s. After each trial, the mice were carefully dried using a towel and kept warm using a heating system. The swimming trajectory, escape latency, swimming speed, and number of platform crossings of the mice over the course of six days were documented and analyzed using SAMRT2.5 (Pan Lab, Barcelona, Spain).

The open filed test (OFT) is a standard method used to evaluate rodent locomotion, exploration, and anxiety, and was conducted as previously described [23]. Mice were transported to the test room 1 h prior to the start of the test. Each mouse was placed in an open-field arena, constructed from opaque white plastic material measuring 50 cm × 50 cm with walls 50 cm high, and allowed to explore freely for 5 min. The total distance traveled (in meters), time spent in the central area (25 cm × 25 cm), and central entries were automatically recorded by the SMART 2.5 video tracking system.

2.4. Preparation of brain tissue

Following the behavioral tests, the mice were euthanized with pentobarbital sodium (50 mg/kg, intraperitoneally). For hematoxylin and eosin (H&E) staining, immunohistochemistry, transmission electron microscopy (TEM), and immunofluorescence (IF) staining, mice were perfused with 0.9% saline through the apex of the heart, followed by 4% paraformaldehyde. For enzyme-linked immunosorbent assays (ELISAs), western blotting, and the evaluation of malondialdehyde (MDA) level, superoxide dismutase (SOD) activity, and ATP content, the cortices were dissected on ice and subsequently stored at − 80 ℃.

2.5. Histological analysis

Brain tissues were fixed with 4% paraformaldehyde for 48 h, embedded in paraffin, sectioned into 4-μm thick samples, then dewaxed with xylene, dehydrated with gradient alcohol, rinsed with water, stained, and finally sealed with neutral resin. H&E staining was performed to count the neuronal pyramidal cells. Immunohistochemistry was used to observe the Aβ deposition. Stained sections were examined under a microscope, and the resulting images were processed using Image J analysis software (National Institutes of Health, USA) to quantify pyramidal neuronal cells and Aβ deposits.

2.6. Transmission electron microscopy

The CA1 region of the mouse hippocampus was harvested and fixed with 3% buffered glutaraldehyde and 1% osmium tetroxide. The

samples were dehydrated in a graded ethanol series and embedded in EPON 618. Five sections were viewed under a transmission electron microscope (Philips, Amsterdam, Netherlands), providing at least 50–100 synapses and mitochondria per mouse for morphometric analysis using Image-Pro Plus 6.0. Synaptic features such as synaptic cleft width, synaptic interface curvature, and post-synaptic density (PSD) were measured using previously established methods[24]. Mitochondrial characteristics, including specific surface area, numerical density, and volume density, were quantified using stereological methods[25,26].

2.7. Immunofluorescence staining

Brain tissues were fixed with 4% paraformaldehyde for 48 h, embedded in paraffin, sectioned into 4 μm samples, then dewaxed with xylene, dehydrated with gradient alcohol, rinsed with water, stained, and finally sealed with neutral resin. The sections were immersed in 3% hydrogen peroxide solution, incubated for 25 min at room temperature in the dark, and then blocked with goat serum for 30 min. The cells were then incubated with the primary antibody (LC3B, cat. 18725–1-AP, Proteintech; COVIX, cat. GB11250, Servicebio) overnight at 4 °C and with the secondary antibody conjugated to FITC (cat. GB22301, Servicebio) or Cy-3 (cat. GB21301, Servicebio) for 60 min at 25 °C. Cell nuclei were labeled with 40–6-diamino-2-phenylindole (DAPI, Molecular probe) (DAPI, cat. G1012, Servicebio). Statistical evaluations and analyses were performed using a Nikon fluorescence microscopy (Nikon ECLIPSE Ni) and Image-Pro Plus 6.0 software.

2.8. Oxidative stress assessment

MDA levels and SOD activity were measured using a Lipid Peroxidation MDA Assay Kit (cat. S0131S, Beyotime Biotechnology), and a Total Superoxide Dismutase Assay Kit with WST-8 (cat. S0101S, Beyotime Biotechnology), according to the manufacturer's guidelines.

2.9. ELISA

The cortical tissue was rinsed with ice-cold phosphate buffered saline (PBS) to remove residual blood, and subsequently homogenized on ice using a glass homogenizer with PBS (9 mL PBS to 1 mg tissue). After centrifugation at 5000 × *g* for 5 min, the supernatants were processed using a mouse Complex I ELISA kit (cat. RX202220M, Quanzhou Ruixin Biological Technology) according to the manufacturer's instructions.

2.10. ATP assay

ATP generation in cortical tissue was quantified using an Enhanced ATP Assay Kit (cat. S0027, Beyotime Biotechnology), according to the manufacturer's guidelines. Tissue homogenates were gathered by centrifugation at 12,000 × *g* and 4 ℃ for 5 min, followed by the addition of 20 μL supernatant and 100 μL ATP assay working solution to a 96-well opaque plate. Luminescence was measured using a SpectraMax iD3 microplate reader and the total ATP concentration was deduced from a standard curve.

2.11. Assessment of mitochondrial membrane potential

Pure mitochondria were procured using a Tissue Mitochondrial Isolation Kit (#C3606, Beyotime Biotechnology) according to the manufacturer's guidelines. In brief, the cortical tissue was homogenized with a glass homogenizer, centrifuged at 600 × *g* for 5 min at 4℃, the supernatant was collected and centrifuged at 11,000 g for 10 min at 4℃ to obtain the mitochondrial precipitate. The mitochondrial membrane potential was gauged using an Enhanced mitochondrial membrane potential assay kit with JC-1 (Δψm).

2.12. Western blot analysis

Proteins from the cortical tissue were extracted utilizing RIPA buffer (cat. P0013C, Beyotime Biotechnology), supplemented with protease and phosphatase inhibitors (TaKaRa Biological Technology Co., Ltd.). The proteins (20 μg) were initially segregated on 8–12% (w/v) sodium dodecyl sulfate polyacrylamide gels, followed by their transfer onto polyvinylidene difluoride membranes using a Trans-Blot Turbo (Bio-Rad, Singapore). The membranes were blocked with QuickblockTM Western blocking buffer (cat. P0252, Beyotime Biotechnology) for 30 min, then subjected to an overnight incubation at 4 °C with various primary antibodies at a 1:1000 dilution: β-actin (cat. A2103, Abcam), anti-synapsin (cat. 2312 S, CST) and PSD95 (cat. GB11277, Servicebio), OPA1 (cat. 80741 S, CST) and MFN2 (cat. 11925 S, CST) and DRP1 (cat. 8570 S, CST), Phospho-DRP1(ser637) (cat. 4867 S, CST) and PINK1 (cat. 23274–1-AP, Proteintech) and Parkin (cat. ab77924, Abcam), LC3 (cat. SAB5701328, Sigma), Beclin-1 (cat. 207612, Abcam) and mTOR (cat. 2972 S, CST), and SQSTM1/p62 (cat. ab109012, Abcam). The membranes were then washed 3 times in 0.1% TBS/Tween-20 and incubated with an anti-rabbit secondary antibody (cat. 7074 S, CST) at room temperature for 2 h. Finally, the bands were visualized using a Chem-iDoc imaging system (Bio-Rad), and quantitative analysis was conducted using ImageJ software.

2.13. Statistical analysis

All data were presented as mean±standard error of the mean (SEM). For the MWM test, escape latency data during the acquisition trials were analyzed using two-way analysis of variance (two-way ANOVA) of repeated measures, whereas one-way ANOVA was conducted on the data obtained from the probe trial. Comparisons among different groups were performed using one-way ANOVA followed by Tukey's post-hoc test for multiple group comparisons. IBM SPSS Statistics (version 24.0; IBM Corp., Armonk, NY, USA) and GraphPad Prism software (version 7.0; San Diego, CA, USA) were used for the data analysis. $P < 0.05$ was deemed significant.

3. Results

3.1. Rapamycin augments learning and memory capabilities and ameliorates synaptic damage in 5 ×FAD mice

The impact of RAPA on cognitive impairment in 5 ×FAD mice was evaluated. In the MWM test, there was a notable increase in escape latency within the AD group from days 4 to 5 ($P < 0.05$, Fig. 1A), which was significantly counteracted by RAPA administration ($P < 0.05$, Fig. 1A). Concurrently, the number of target platform crossings in the AD group ($P = 0.029$, Fig. 1B) decreased on day 6, which was reversed by RAPA administration ($P = 0.005$, Fig. 1B). We recorded the total distance, central times, and central entries in the OFT to further assess cognitive dysfunction. We found that the total distance, central time, and central entries were significantly decreased in the AD group compared to those in the WT group ($P = 0.032$, $P = 0.041$, $P = 0.014$, Fig. 1D-F), which was significantly reversed by the administration of RAPA ($P = 0.019$, Figs. 1C and 1F). However, there were no significant differences in the central time.

Synapses are the fundamental biological basis off learning and memory in the brain. Postsynaptic Density Protein 95 (PSD95) is a crucial protein for maintaining synaptic stability and plasticity and is a key component of the learning mechanism [27]. Therefore, we evaluated the expression of synapse-associated proteins in the cortex using western blotting. The results indicated that the expression of PSD95 and SYN in mice treated with RAPA was considerably higher than that in 5 ×FAD mice ($P = 0.050$, $P = 0.042$, Fig. 1G-I). These findings suggest that RAPA can ameliorate cognitive impairments in 5 ×FAD mice.

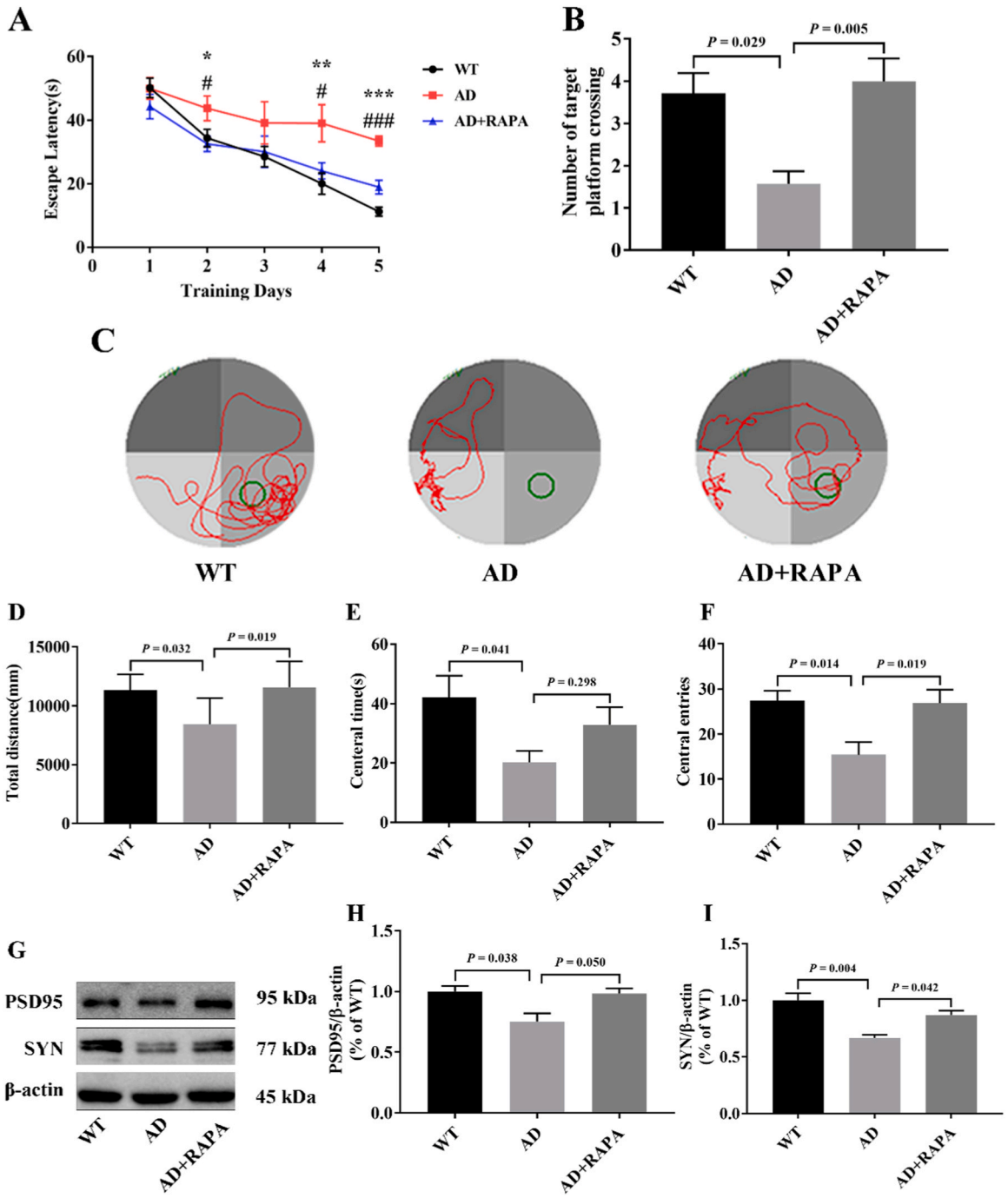

Fig. 1. Rapamycin improves learning memory and synaptic damage in 5 ×FAD mice. (A) Escape latency during 5 days of hidden platform tests. (B) Representative traces of swimming paths from each group in the probe test. (C) Crossings of the target quadrant during the probe trial. (D) The total distance. (E) Time in the central area. (F) Number of the central area entries. n = 8 per group for MWM tests and OFTs. (G-I) Protein expression of PSD95 and SYN in the cortices of the mice by western blotting. n = 3 per group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. the WT group. $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ vs. the AD group.

3.2. Rapamycin improves the histological features of neuronal pyramidal cells and diminishes Aβ deposition in 5 ×FAD mice

Histological alterations using HE staining. Our observations revealed that, in contrast to the WT group, pyramidal cells in the hippocampal CA1 region of the AD group were loosely arranged and disordered, and RAPA enhanced these histological features (Fig. 2A). The data demonstrated that the number of pyramidal cells in the hippocampal CA1 region of the AD+RAPA group was significantly higher than that in the AD group ($P = 0.001$, Fig. 2B). Aβ deposition is a significant pathological characteristic of AD. Similarly, immunohistochemical findings indicated that the Aβ deposition in the hippocampal CA1 of AD+RAPA mice significantly decreased compared to the AD group ($P < 0.001$, Fig. 2D). These findings suggest that RAPA enhances histological features and diminishes Aβ deposition in 5 ×FAD mice.

3.3. Rapamycin induces mitophagy and enhances mitochondrial structure and function in 5 ×FAD mice

Mitochondria are essential organelles for the normal functioning and survival of neurons, with functional changes arising from structural alterations [28]. Therefore, we examined the effects of RAPA on the

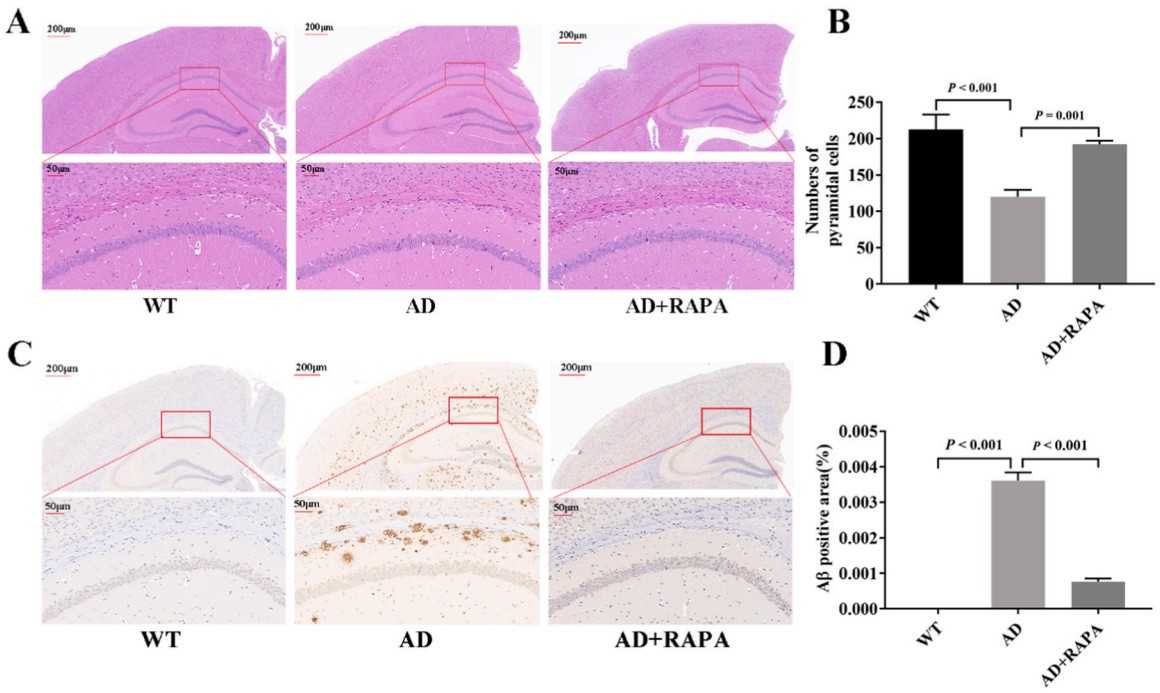

Fig. 2. Rapamycin improves the histological characteristics of neuronal pyramidal cells and diminishes Aβ deposition in 5 ×FAD mice. (A) Representative images of the histological alterations in the hippocampal CA1 region. Scale bar = 200 μm. Magnified images are displayed in the lower column. Scale bar = 50 μm. (B) The pyramidal cells numbers in the hippocampal CA1 region. (C) Representative images of immunohistochemical photographs for Aβ in the hippocampal CA1 region. Scale bar = 200 μm; magnified images are displayed in the lower column. scale bar = 50 μm. (D) Quantitative analysis of Aβ deposition. n = 3 per group.

mitochondrial structure and function. We observed relatively unscathed mitochondrial structures and clearly delineated mitochondrial cristae in the hippocampus using TEM in the AD+RAPA group compared to the AD group in 5 ×FAD mice (Fig. 3A). Concurrently, quantitative analysis revealed that RAPA significantly augmented the specific surface area and volume density of mitochondria ($P = 0.038$ and $P = 0.004$, respectively; Figs. 3B and 3D). No variation was detected in the numeric density across all the groups. Furthermore, we investigated whether RAPA could mitigate the imbalance of cortical mitochondrial dynamics in 5 ×FAD mice. Western blot analysis of the protein extracts demonstrated that the levels of phosphorylation of dynamin-related protein 1 at the s637 site, mitofusin 2 (MFN2), and optic atrophy 1 (OPA1) were elevated in the AD+RAPA group ($P = 0.041$, $P = 0.002$, $P = 0.071$, Fig. 3E-H). These findings suggest that RAPA significantly enhances mitochondrial structure and equilibrats mitochondrial dynamics.

Mitochondria are the primary loci of oxidative stress. Mitochondrial dysfunction can result in the leakage of reactive oxygen species[29] and increased levels of oxidative stress in tissues. We also detected a reduction in the oxidative stress response in the cortex of AD+RAPA mice, which included a decrease in Malondialdehyde (MDA) levels and an increase in Superoxide Dismutase (SOD) activity compared to the AD group ($P = 0.02$, $P = 0.044$; Fig. 3I and J).

The mitochondria are the principal sites of energy production. In line with these results, compared with the AD group, ATP levels and respiratory chain complex I activity in the cortex were increased in the AD+RAPA group ($P = 0.068$, $P = 0.007$, Fig. 3K and L). Normal mitochondrial membrane potential aids in the preservation of the normal physiological functions of the mitochondria, and a decline in mitochondrial membrane potential often signifies impaired mitochondrial function. As shown in Fig. 3**M**, the mitochondrial membrane potential was significantly lower in the AD group than that in the WT group ($P = 0.02$). Conversely, the mitochondrial membrane potential in the AD + RAPA group was notably higher than that in the AD group ($P = 0.016$).

Studies have suggested that the fusion of autophagosomes and lysosomes is disrupted in AD, leading to the significant accumulation of damaged mitochondria in neurons [30,31], which subsequently accelerates AD progression. Mitophagy, a selective type of autophagy, that eliminates damaged mitochondria. The PINK1-Parkin pathway is a classical mitophagy pathway that plays a pivotal role in maintaining mitochondrial quality. To explore whether mitophagy plays a part in RAPA's enhancement of cognitive function in 5 ×FAD mice, this study examined the impact of RAPA on the expression levels of mitophagy and downstream PINK1-Parkin pathway marker proteins via western blotting. We found a substantial increase in LC3II expression. Western blot analysis revealed a substantial increase in the expression of PINK1, Parkin, Beclin1, LC3II, and mTOR in the cortex of the AD group compared to that in the WT group ($P = 0.01$, $P = 0.005$, $P = 0.008$, $P < 0.001$, respectively, Fig. 4B-E), and mTOR was also increased ($P = 0.056$; Fig. 4G). Concurrently, the expression of p62 was significantly lower, indicating activation of mitophagy in the cortex of 5 ×FAD mice. However, the introduction of RAPA further increased the expression of PINK1, Parkin, Beclin1, and LC3II ($P = 0.09$, $P = 0.014$, $P = 0.030$, $P < 0.001$, respectively, Fig. 4B-E), whereas it reduced the expression of p62 and mTOR in the cortex of 5 × FAD mice compared to the AD group ($P = 0.003$, $P = 0.033$; Fig. 4F and G). These findings suggested that RAPA enhanced mitophagy in 5 ×FAD mice.

3.4. Mitophagy suppression reverses the alleviated cognitive dysfunction and neuronal damage by rapamycin in 5 ×FAD mice

Mitochondrial abnormalities are recognized as significant pathological factors in AD. The regulation of mitochondrial quality and maintenance of mitochondrial homeostasis involves two pathways: mitochondrial dynamics and mitophagy [3], with mitophagy receiving special attention. Mitophagy, a selective form of autophagy, selectively eliminates malfunctioning mitochondria and plays a vital role in maintaining mitochondrial quality [32]. To further investigate whether the upregulation of mitophagy induced by RAPA is essential for its effects on AD, 3-MA was used to pharmacologically inhibit mitophagy.

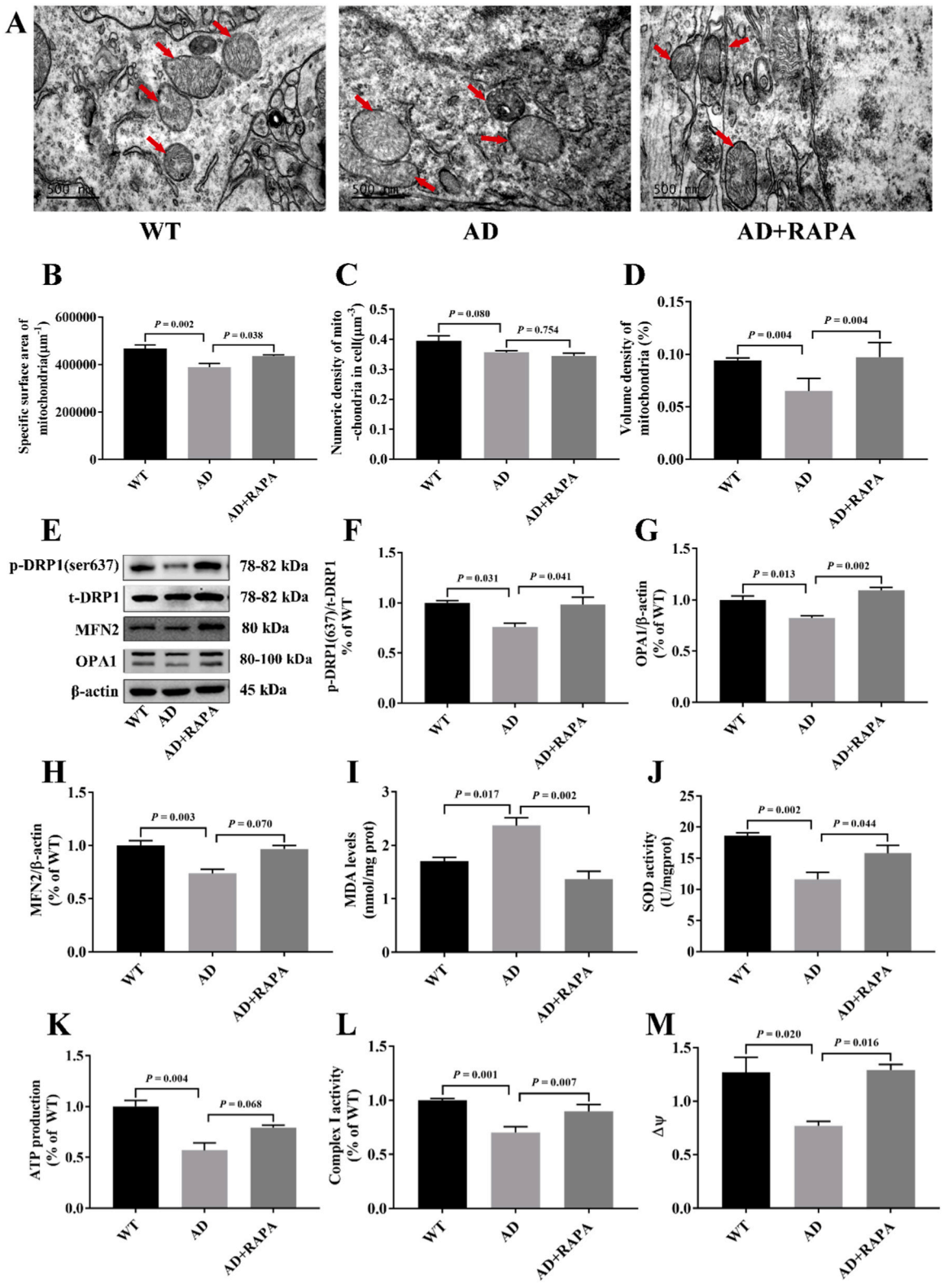

Fig. 3. RAPA alleviates mitochondrial morphology and functional impairments in 5 ×FAD mice. (A) Representative images of mitochondria morphology in the hippocampal CA1 region under TEM. The red arrowheads indicate mitochondria. Scale bar = 500 nm; (B-D) Quantification of specific surface area (B), numeric density (C), and volume densities of mitochondria (D). n = 3 per group. (E) Relative expressions of proteins related to mitochondrial dynamics determined by western blotting in the cortices homogenate. (F)-(H) Relative quantification analysis of p-Drp1(ser637) (F), MFN2 (G), and OPA1 (H). Oxidative stress injury was evaluated based on the MDA level (I) and SOD activity (J) in the cortex. Energy metabolism was determined based on the ATP level (K) and respiratory chain complex I activity (L) in the cortex. (M) The mitochondrial membrane in the cortex. n = 3 per group.

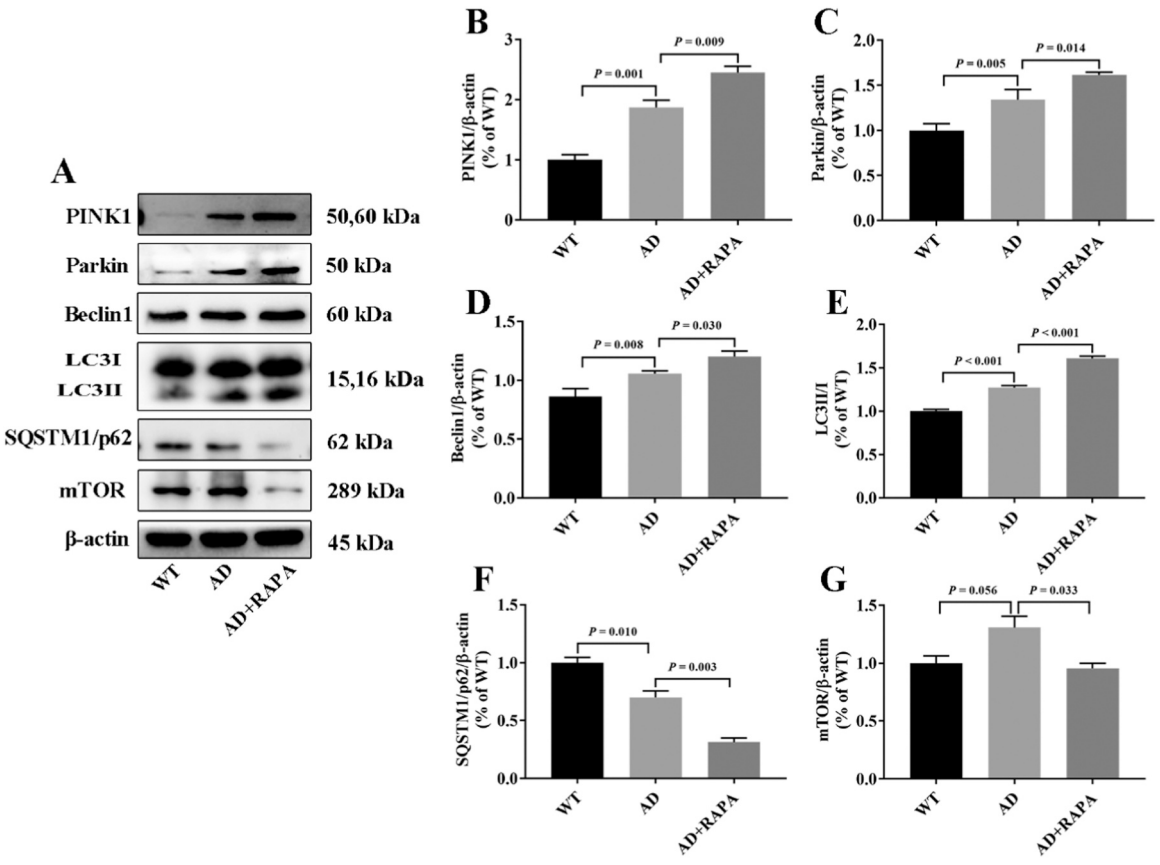

Fig. 4. RAPA induces mitophagy in the cortices of 5 ×FAD mice. (A) Relative expressions of proteins related to mitophagy as determined by western blotting in the cortical homogenate. (B)-(G) Relative quantification analysis of PINK1 (B), Parkin (C), Beclin1 (D), LC3 (E), SQSTM1/p62 (F), and mTOR (G). n = 3 per group.

As depicted in Fig. 5, the administration of 3-MA reversed the effects of RAPA on the MWM and OFT. In the MWM, the AD+RAPA+ 3-MA group demonstrated a significant increase in escape latency ($P < 0.05$, Fig. 5 A) and a reduced the number of platform crossings ($P = 0.018$, Fig. 5B) compared to the AD + RAPA group. Conversely, in the OFT, 3-MA reduced the total distance, central time, and central entries in 5 ×FAD mice. The AD+ 3-MA group showed no significant difference, compared with the AD group, suggesting that 3-MA alone was not neurotoxic to 5 ×FAD mice.

In this study, we examined the synapses in the hippocampal CA1 region using TEM. The findings revealed that RAPA treatment increased the synaptic cleft width and postsynaptic density (PSD) thickness ($P < 0.001$, respectively, Fig. 5H and J), and this group displayed a more regular synapse structure (Fig. 5 G). However, 3-MA counteracted the effect of RAPA on synapses, and the western blot results also showed that 3-MA reduced the expression of the synapse-associated (synapse related) proteins PSD95 and SYN ($P = 0.027$, $P = 0.031$, Fig. 5K-M). These results suggest that RAPA enhances learning and spatial memory and alleviates synaptic damage by up-regulating mitophagy.

Furthermore, the administration of 3-MA undermined the beneficial effects of RAPA on the enhancement of histological features and Aβ clearance. Relative to the AD + RAPA group, neuronal pyramidal cells in the CA1 region of the hippocampus in the AD + RAPA + 3-MA group were arranged more loosely, and the number of pyramidal cells was notably diminished ($P = 0.01$, Fig. 6 A and B). Concurrently, Aβ deposition was significantly escalated in the AD + RAPA + 3-MA group in comparison to the AD + RAPA group ($P = 0.020$, Fig. 6 C and D).

3.5. Mitophagy suppression reverses the alleviated mitochondrial dysfunction by rapamycin in 5 ×FAD mice

The equilibrium of mitochondrial dynamics is contingent upon the regulation of the mitochondrial division protein DRP1, and the fusion proteins MFN2 and OPA1, which contribute to normal mitochondrial functionality [33]. 3-MA reversed the effects of RAPA on mitochondrial morphology. We observed that the specific surface area and volume density of the mitochondria were significantly diminished ($P < 0.001$ and $P = 0.045$, respectively; Fig. 7B and D), whereas the numerical density of the mitochondria remained unchanged. In addition, our immunoblot analysis results indicated a decrease in the expression of p-DRP1(ser637), MFN2, and OPA1 in the AD + RAPA + 3-MA group compared to the AD + RAPA group ($P = 0.056$, $P = 0.017$, $P = 0.09$, Fig. 7E-H).

Furthermore, administration of 3-MA significantly counteracted the effect of RAPA on mitochondrial function. Specifically, 3-MA increased the MDA levels ($P = 0.031$; Fig. 7I) and decreased the SOD activity ($P = 0.008$; Fig. 7 J). In terms of energy metabolism, 3-MA reduced ATP production and respiratory chain complex I activity ($P = 0.744$ and $P = 0.014$, respectively; Fig. 7 K and L). Additionally, 3-MA led to a decrease in mitochondrial membrane potential ($P = 0.038$; Fig. 7 M). These results suggested that RAPA mitigated mitochondrial dysfunction by enhancing mitophagy.

To determine the relationship between the effects of RAPA on improved cognitive function and mitophagy as well as its downstream pathways, we examined the expression levels of mitophagy and downstream PINK1/Parkin pathway-related proteins in the cortex. Western blot analysis demonstrated that 3-MA counteracted the RAPA-induced upregulation of PINK1, Parkin, and LC3 expression ($P < 0.001$, $P < 0.001$, $P = 0.001$, Fig. 8B-C and E), and Beclin1 expression was

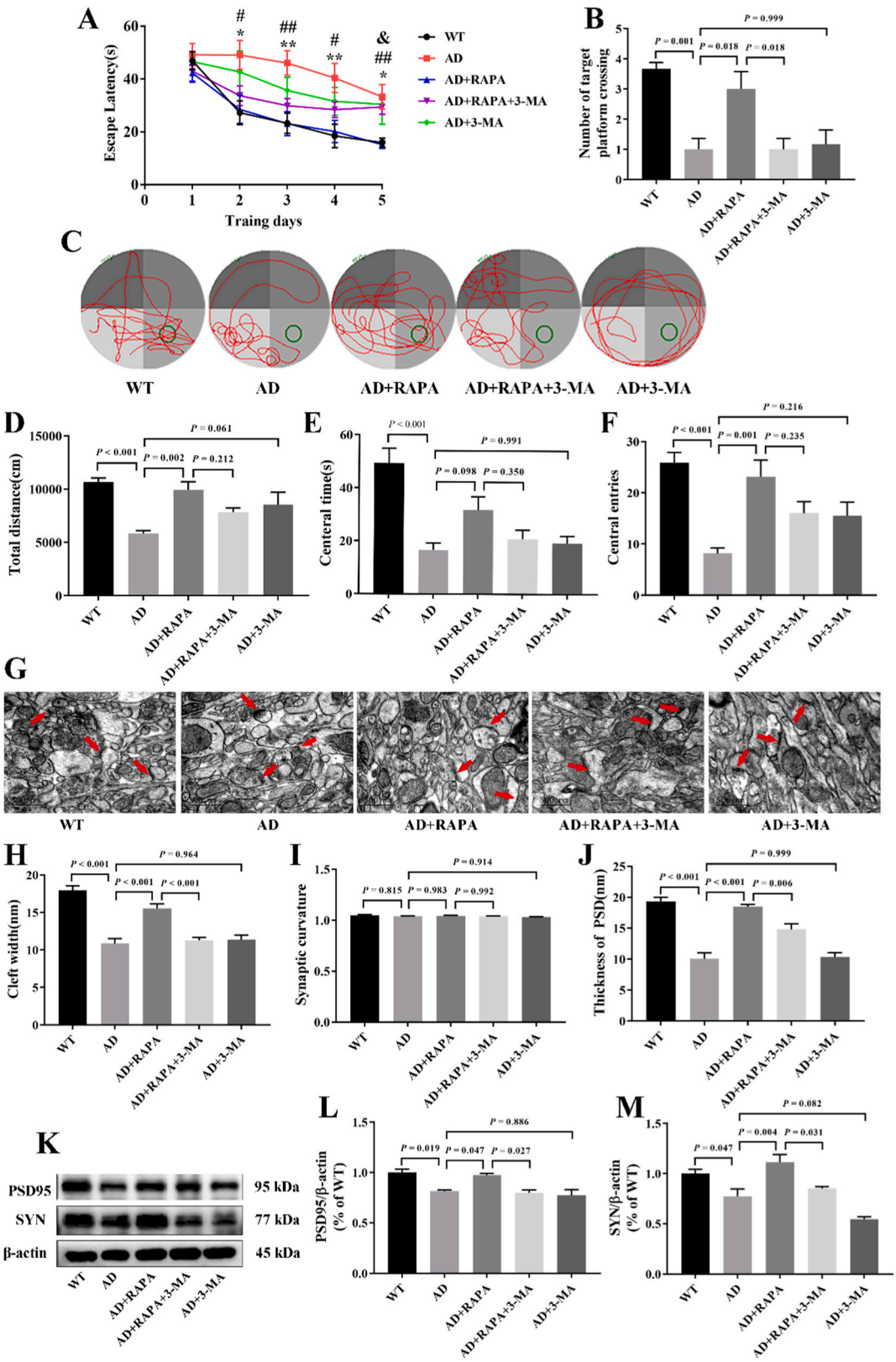

Fig. 5. 3-MA reversed the effect of rapamycin on learning and spatial memory and alleviated synaptic damage in 5 ×FAD mice. (A) Escape latency during the 5 days of hidden platform tests. (B) Representative traces of swimming paths in each group during the probe test. (C) Crossing of the target quadrant during the probe trial. (D) Total distance. (E) Time spent in the central area. (F) Number of entries into the central area. n = 6 per group. (G) Representative TEM images of synaptic morphology in the hippocampal CA1 region. Red arrowheads indicate the synapses. Scale bar = 500 nm; (H-J) Quantification of synaptic cleft width (H), synaptic curvature (I), and PSD thickness(J). (K) Relative expression of synapse-related proteins determined by western blotting of cortical homogenates. (L-M) Relative quantification of PSD95 (L) and SYN (M). n = 3 per group.

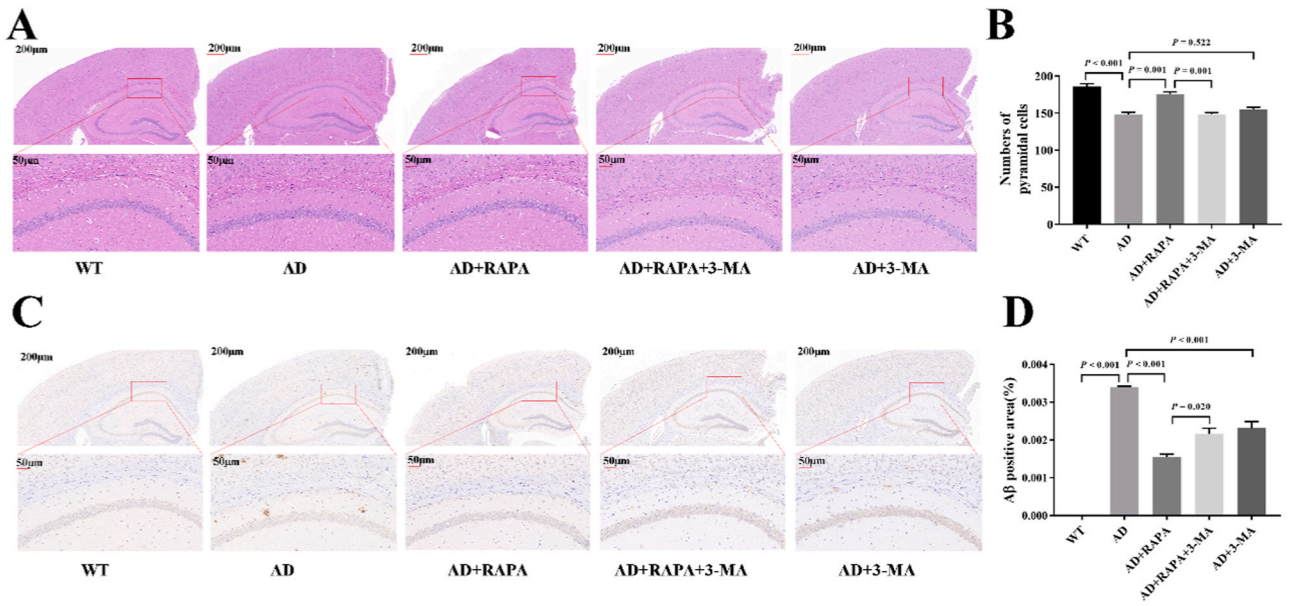

Fig. 6. 3-MA reversed the beneficial effects of rapamycin on the histological characteristics of neuronal pyramidal cells and the reduction of Aβ deposition in the hippocampal CA1 region of 5 ×FAD mice. (A) Representative images of the histological alterations in the CA1 region of the hippocampus. Scale bar = 200 μm. Magnified images are shown in the lower column. Scale bar = 50 μm. (B) The number of pyramidal cells in the hippocampal CA1 region. (C) Representative images of immunohistochemical photographs for Aβ in the hippocampal CA1 region. Scale bar = 200 μm. Magnified images are shown in the lower column. scale bar = 50 μm. (D) Quantitative analysis of Aβ deposition. n = 3 per group.

decreased ($P = 0.143$, Fig. 8D). 3-MA also counteracted RAPA-induced downregulation of p62 and mTOR ($P = 0.004$, $P < 0.001$, Fig. 8F-G). Furthermore, we identified the co-localization of autophagic microsomes (LC3) and mitochondria (COXIV) using immunofluorescence. The results indicated that RAPA increased the LC3-COXIV co-localization area ($P = 0.02$, Fig. 9 A and B), while 3-MA counteracted the RAPA-induced increase in the LC3-COXIV co-localization area ($P = 0.01$, Fig. 9 A and B). These findings suggested that RAPA enhanced cognitive function and mitigated mitochondrial dysfunction by upregulating mitophagy via the downstream PINK1/Parkin pathway.

4. Discussion

In the current study, we used RAPA to elucidate the role of mitophagy in alleviating cognitive dysfunction in AD mice. RAPA may enhance AD by promoting mitophagy, laying the foundation for further research on RAPA treatment of AD.

Aβ deposition is the main pathological feature of AD, and has neurotoxicity. The action mechanism of Aβ in AD is very complex. Although recent studies have proposed a variety of mechanisms for the pathogenesis of AD, Aβ still believed to play an important role in the occurrence and development of AD. Some studies have shown that the *TREM2* gene, expressed in microglia, is closely related to Aβ [34], while some studies have demonstrated that the WNT/β-Catenin pathway is related to the formation of Aβ [35]. Excessive Aβ deposition mediates cognitive dysfunction and synaptic damage in AD, which worsens with disease progression [36]. Previous studies have found that individuals with AD exhibit Aβ deposition and loss of synapses, as well as reduced expression of synaptic-related proteins [37,38]. In this study, 5 ×FAD mice expressing the five familial AD mutations were used as the experimental animals. These mutations can increase the formation of Aβ, which makes these mice an ideal model for simulating amyloidosis in AD cases. Through the water maze and open field experiments, we demonstrated that RAPA can improve the learning and memory abilities of 5 ×FAD mice, reduce Aβ deposition, and improve the morphological damage of synapses in the hippocampal CA1 region. These results are consistent with those of prior reports indicating that RAPA enhances synaptic plasticity in APP/PS1 mice and improves cognitive function [17]. Shi et al. also showed that RAPA improves cognitive impairment and reduces Aβ deposition in AD model mice [39].

Mitochondrial dysfunction is one of the pathogenic mechanisms of AD, and its structural changes are the standard pathological features, manifested by alterations in mitochondrial morphology, reduced energy generation, impaired electron transport chain, and increased oxidative stress [11,40]. Reducing the production of oxidative stress can improve the cognitive dysfunction of mice [41,42]. It has been found that mitochondrial-mediated signal pathways affect synaptic signal transduction and changes in the structure and function of neurons, thus playing an important role in learning and memory [2,43]. The main factor driving the changes in mitochondrial morphology is mitochondrial dynamics, which play a vital role in maintaining mitochondrial form and function [44]. We observed significant mitochondrial swelling, decreased expression of the mitochondrial fission protein p-DRP1 (ser637), increased oxidative stress, and decreased ATP levels in 5 ×FAD mice, indicating mitochondrial dysfunction in the AD state. Zheng et al. have demonstrated that RAPA improves mitochondrial dysfunction in streptozotocin-induced diabetic mice and balances mitochondrial dynamics [45]. Similarly, RAPA exerted neuroprotective effects against mitochondrial dysfunction induced by vascular dementia in rats [18], suggesting its potential to improve mitochondrial dysfunction. In our study, rapamycin improved mitochondrial dysfunction in 5 ×FAD mice. Therefore, we speculated that RAPA may improve cognitive dysfunction in 5 ×FAD mice by alleviating mitochondrial dysfunction.

RAPA, as a specific inhibitor of mTOR, has been shown to have neuroprotective effects against neurodegenerative diseases in animal models by blocking mTOR. Moreover, studies have found that the activity of the mTOR pathway in damaged areas of the brain in patients with AD is significantly higher than that in normal brain tissue [46]. Several studies have shown that RAPA reduces tau protein levels, alleviates cognitive impairment, and improves learning and memory abilities in dementia animal models via affecting the mTOR/p70S6K, AMPK/mTOR, and PI3K/AKT/mTOR signaling pathways [15,18,47]. Civiletto et al. demonstrated that RAPA promotes mitophagy by

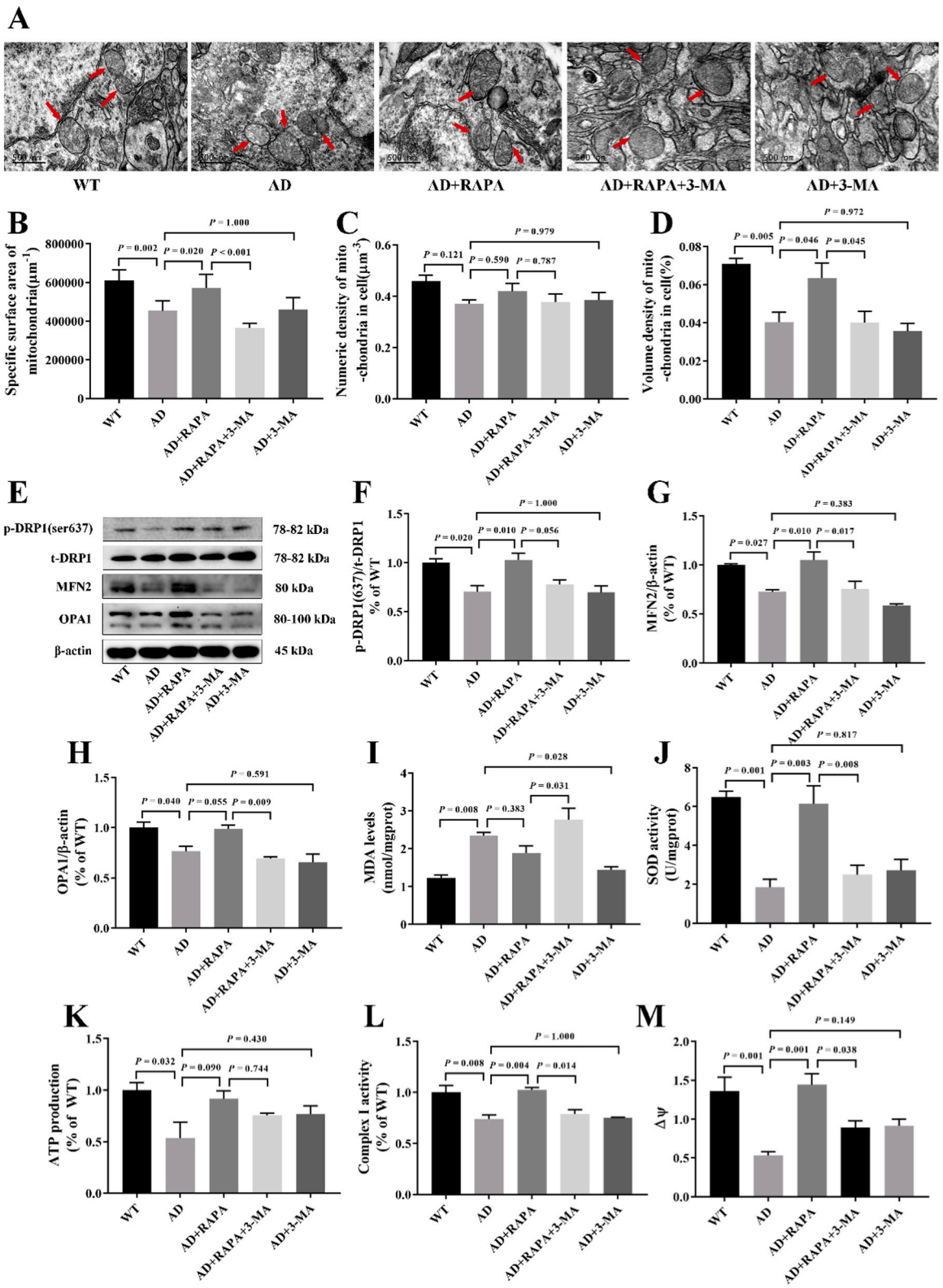

Fig. 7. 3-MA counteracts the effect of rapamycin in improving mitochondrial structure and function in 5 ×FAD mice. (A) Representative TEM images of the mitochondrial morphology in the CA1 region. Red arrowheads indicate mitochondria. Scale bar = 500 nm. (B-D) Quantification of the specific surface area (B), numeric density (C), and volume density of mitochondria (D). n = 3 per group. (E) Relative expression of proteins related to mitochondrial dynamics in the cortical homogenate as determined by western blotting. (F)-(H) Relative quantification of p-Drp1(ser637) (F), MFN2 (G), and OPA1 (H). Oxidative stress injury was determined based on MDA level (I) and SOD activity (J), and energy metabolism was determined based on ATP level (K), respiratory chain complex I activity (L), and mitochondrial membrane (M) in the cortices.

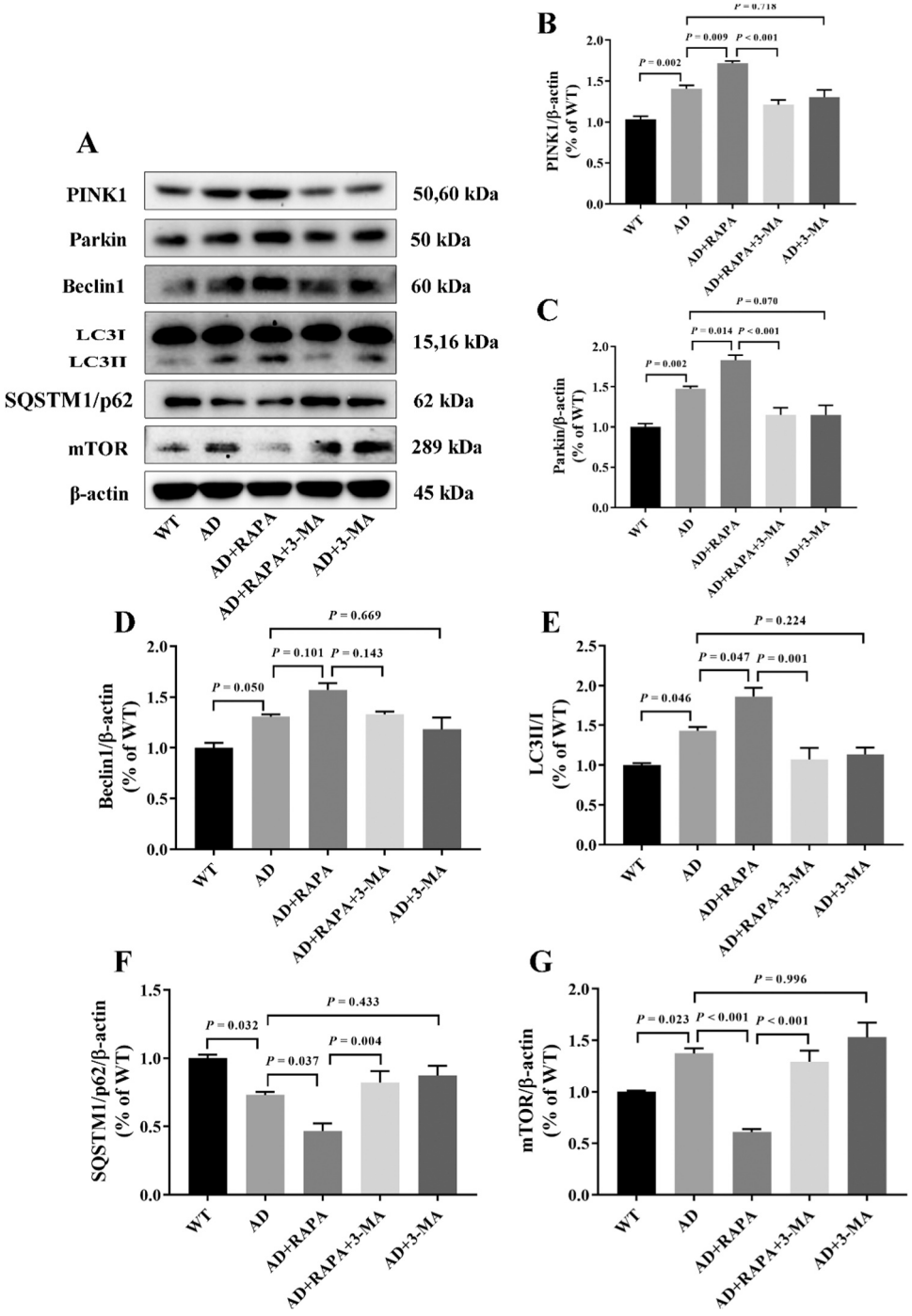

Fig. 8. 3-MA suppresses the effect of rapamycin on mitophagy in the cortices of 5 ×FAD mice. (A) Relative expression of proteins associated with mitophagy in the cortical homogenate as determined by western blotting. (B)-(G) Relative quantification of PINK1 (B), Parkin (C), Beclin1 (D), LC3 (E), SQSTM1/p62 (F), and mTOR (G). n = 3 per group.

inducing autophagosome formation and enhancing lysosomal biogenesis [48]. The up-regulation of lysosome biogenesis may help to alleviate the pathogenesis of Aβ in AD [49]. Studies have shown that activating autophagy can improve cognitive impairment, reduce Aβ deposition in AD, and delay the progression of AD [50,51]. However, both domestic and international studies on improving AD using RAPA have mainly focused on its effects after binding to mTOR. In recent years, scholars have focused on the relationship between RAPA and autophagy, and have found that RAPA is an activator of mitophagy. In muscle-specific *Cox15* knockout mice, RAPA can promote mitophagy by inducing the formation of autophagosomes and enhancing the occurrence of lysosomes in the muscle tissue [48]. A few number studies have shown that RAPA can activate mitophagy and improve cognitive function in rats with vascular dementia and in APP/PS1 mice [17,18]; however, the specific mechanism remains unclear. Zhang et al. showed that the effects of rapamycin on AD-induced cognitive and synaptic plasticity defects, as well as mitochondria-dependent apoptosis, may be related to the enhancement of mitophagy [17]. However, this study did not provide conclusive evidence that rapamycin-induced mitophagy is directly related to the improvement of cognitive and synaptic functions, and there is no evidence supporting whether rapamycin clears damaged mitochondria. This is only a preliminary mention that the improvement

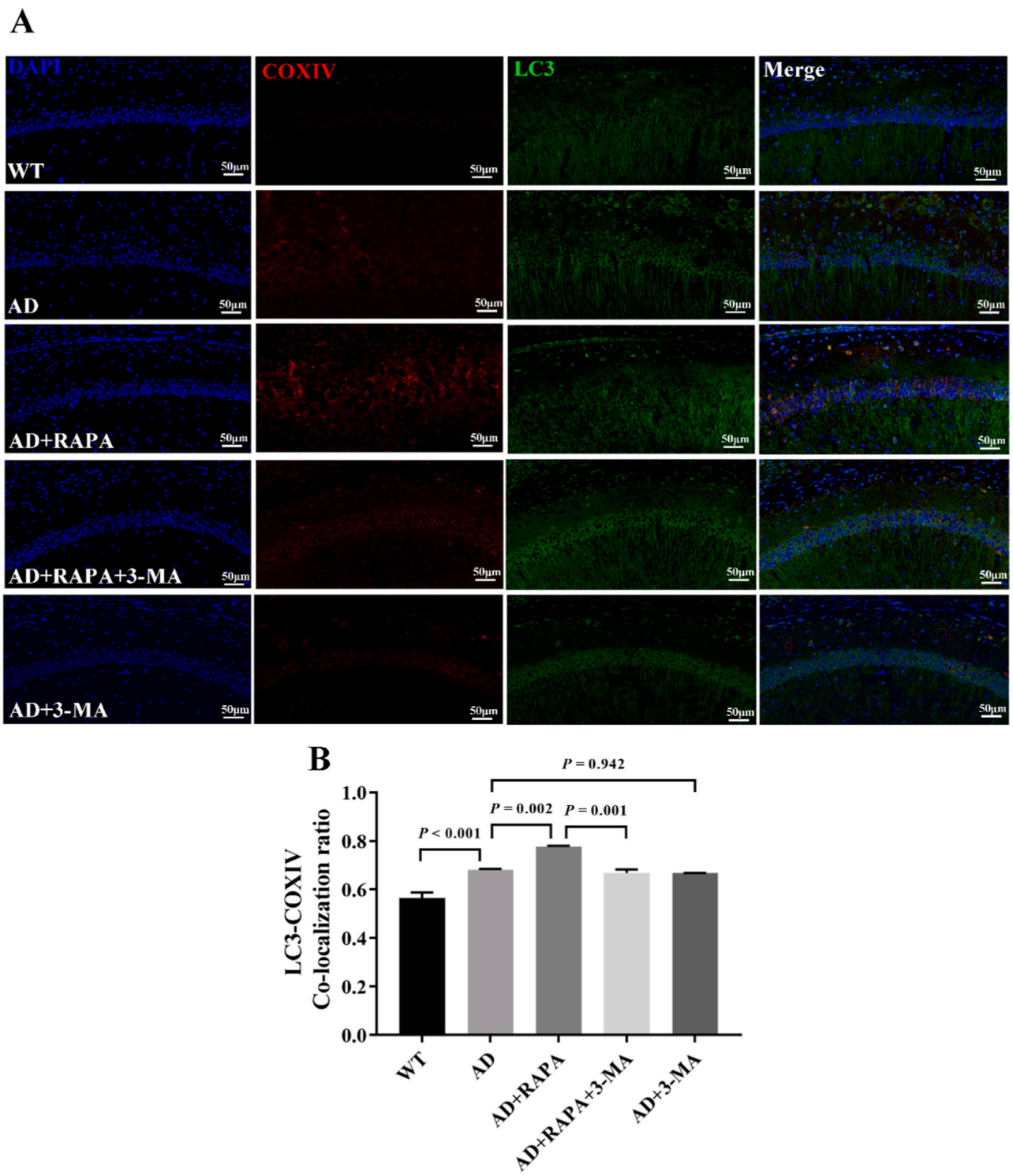

Fig. 9. 3-MA suppresses the effect of rapamycin on mitophagy in the hippocampal of 5 ×FAD mice. (A) Representative images of LC3-COXIV co-localization in the hippocampal region. Scale bar = 50 μm. (B) Quantification of the LC3-COXIV co-localization ratio in the hippocampal region. n = 3 per group.

in cognitive function induced by rapamycin may be related to mitophagy. Therefore, whether rapamycin-induced improvement in cognitive impairment and synaptic plasticity is related to the enhancement of mitophagy is not yet well established. We sought to investigate the precise role of mitophagy in improving cognitive dysfunction in AD mice through RAPA. The PINK1-Parkin pathway is an essential regulatory pathway of mitophagy, which, when activated, promotes the clearance and repair of mitochondria. PINK1 is a serine/threonine kinase that is located in the inner mitochondrial membrane. When mitochondrial function is impaired, the mitochondrial membrane potential decreases, causing PINK1 to accumulate in the mitochondrial membrane. It recruits Parkin protein from the cytoplasm to interact with microtubule-associated protein light chain 3 (LC3) on the mitochondria, thereby promoting mitophagy [52,53]. LC3 as a mitophagy marker. Moreover, p62 binds to ubiquitinated mitochondria to engulf cells through its LC3 binding domain, and can also serve as a marker of mitophagy [54]. Beclin1 is involved in autophagosomes formation during autophagy [55]. Additionally, previous studies have shown that

autophagy is activated in 3–6-month-old APP/PS1 transgenic mice, especially in 6-month-old mice [5]. Furthermore, Li et al. demonstrated increased expression of PINK1 and Parkin in APP/PS1 transgenic mice, indicating the activation of mitophagy in mice [6]. In line with these findings, our study identified mitophagy activation in the cortex of 5 ×FAD mice. However, although mitophagy is initiated in the AD brain, mitochondrial structural damage is still observed [7], indicating that mitophagy initiation is insufficient to clear damaged mitochondria. To address this issue, we investigated whether the expression of mitophagy-related proteins (PINK1, Parkin, LC3 and Beclin1) and the downstream PINK1-Parkin pathway were upregulated in 5 ×FAD mice after treatment with RAPA, suggesting that RAPA further activates mitophagy and the downstream PINK1-Parkin pathway. We also conducted immunofluorescence co-localization experiments, which showed an increased colocalization area of LC3 and COXIV with RAPA, further confirming the enhancement of mitophagy by RAPA. We hypothesized that RAPA improves cognitive function in AD mice by activating mitophagy and the downstream PINK1-Parkin pathway.

To verify this hypothesis, we conducted further research using the mitophagy inhibitor 3-MA. 3-MA inhibits autophagosome formation during the initiation phase of autophagy and has been successfully used to inhibit mitophagy. Studies both domestically and internationally have shown that 3-MA can inhibit autophagy in electric acupuncture-modulated AD animal models, thereby reversing improvements in learning and memory abilities [56]. Additionally, Deng et al. demonstrated that 3-MA can inhibit autophagy activity in APP/PS1 mice and increase Aβ deposition [57]. When 3-MA was used to inhibit mitophagy, we found that 3-MA reduced the expression levels of mitophagy-related proteins in the AD+RAPA+ 3-MA group, such as PINK1, Parkin, LC3. Additionally, decreased the co-localization area of LC3-COXIV, thereby reversing the effect of RAPA-induced mitophagy and its downstream PINK1-Parkin pathway. Therefore, 3-MA intervention in mice 1 h before RAPA treatment altered the effect of RAPA on mitophagy. Meanwhile, 3-MA reversed the improvement of RAPA on cognitive function in 5 ×FAD mice, leading to a decline in learning and memory abilities, increased Aβ deposition, and reduced hippocampal pyramidal neurons, thus reversing the neuroprotective effect of RAPA on synaptic injury. 3-MA also reversed the effects of RAPA on mitochondrial structure and function, as it counteracted mitochondrial morphological damage, decreased the expression of p-DRP1(ser637) protein, reduced energy production, and increased oxidative stress levels. These results demonstrate that 3-MA reverses the effect of RAPA in activating mitophagy and the downstream PINK1-Parkin pathway, as well as the neuroprotective effect of RAPA in 5 ×FAD mice. Therefore, RAPA activates mitophagy and the downstream PINK1-Parkin pathway, alleviating mitochondrial dysfunction and improving brain injury and cognitive impairment. These findings provide new insights into the treatment of AD. However, rapamycin and 3-MA used in this study are not specific mitophagy activators or inhibitors, have a wide range of effects on the autophagy pathway, and cannot be accurately located in the mitochondria, which is a limitation of this study.

In addition to PINK1-Parkin pathway, previous studies have proposed that RAPA affects autophagy through the PI3K/AKT/mTOR signaling pathway [18], and that inhibiting the PI3K/AKT/mTOR signaling pathway can activate autophagy [58]. mTOR is a vital nutrient sensor that plays a crucial role in cellular metabolism, growth, proliferation, apoptosis, and oxidative stress [14] and is therefore associated with many diseases. Due to the involvement of the mTOR pathway in numerous physiological functions, RAPA may affect the progression of AD through multiple mechanisms and pathways. Previous studies have primarily focused on the interaction between RAPA and mTOR; however, there is relatively limited research on the role of mitophagy in the effects of RAPA on AD. Therefore, further investigation is required to explore the underlying mechanisms. In this context, understanding the interplay between RAPA, mTOR, and mitophagy is pivotal for understanding the full scope of RAPA's impact of RAPA on AD. RAPA is primarily used for clinical immunosuppression, and reports suggest that mTOR inhibitors at doses lower than the clinical dose can enhance immune function in the elderly and reduce infection rates as immune function declines [59]. The imbalance between inflammation and the immune system plays a vital role in the occurrence and development of AD [60]. Moderate endoplasmic reticulum stress can reduce neuroinflammation and cognitive impairment, suspending or delaying the progression of neurodegenerative diseases [61]. Abnormal immune cell activity can lead to increased inflammatory responses that trigger oxidative stress and mitochondrial dysfunction. Therefore, the effects of RAPA on AD may be associated with immune function and inflammation. Further investigation of the regulatory mechanisms of mitophagy and related signaling pathways, and exploring their impact on immune function and inflammation, can help deepen our understanding of the pathogenesis of AD and provide a theoretical basis for the development of new treatment strategies.

5. Conclusion

We demonstrated that RAPA may improve mitochondrial function in 5 ×FAD mice by activating mitophagy and the downstream PINK1-Parkin pathway, thereby exerting its neuroprotective effects and improving cognitive impairment. This study provides new insights and approaches for RAPA treatment of AD.

CRediT authorship contribution statement

Wenrong Zheng: Conceptualization, Writing – original draft. **Kuaiang Li:** Writing – review & editing. **Meihua Zhong:** Resources. **Kejun Wu:** Data Curation. **Lele Zhou:** Resources. **Jie Huang:** Data Curation. **Libin Liu:** Project administration. **Zhou Chen:** Supervision.

Declaration of Competing Interest

The authors have no conflict of interests to declare.

Data Availability

Data will be made available on request.

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