

# Ginsenoside Rg1 alleviates learning and memory impairments and A $\beta$ disposition through inhibiting NLRP1 inflammasome and autophagy dysfunction in APP/PS1 mice

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**Abstract.** Alzheimer's disease (AD) is a common neurodegenerative disorder. Amyloid  $\beta$  (A $\beta$ ) deposition is considered an important pathological feature of AD. Growing evidence has linked neuroinflammation and autophagy to A $\beta$  deposition in the progression of AD. However, there are few drug options for inhibiting neuroinflammation and autophagy to prevent AD. Ginsenoside Rg1 (Rg1), a steroidal saponin extracted from ginseng, has been reported to possess multiple neuroprotective effects. The present study aimed to evaluate whether Rg1 treatment could attenuate cognitive disorders and neuronal injuries by inhibiting NLRP1 inflammasome and autophagy dysfunction in an AD model of APP/PS1 mice. The results of behavioral tests indicated that Rg1 treatment for 12 weeks could significantly improve olfactory dysfunction as well as learning and memory impairments. The results of histopathological tests indicated that Rg1 treatment could reduce A $\beta$  deposition and neuronal damages in APP/PS1-9M mice. Additionally, the results of immunoblot, reverse transcription-quantitative PCR or immunohistochemistry demonstrated that Rg1 treatment significantly downregulated the expression levels of inflammation-related proteins of NLRP1, caspase1, IL-1 $\beta$  and TNF- $\alpha$ , as well as autophagy-related proteins of p-AMPK/AMPK, Beclin1 and LC3 II/LC3 I, and increased the expression levels of p-mTOR/mTOR and P62 in APP/PS1-9M mice. In addition,

the molecular docking analysis showed that there was favorable binding result between Rg1 and NLRP1. The present study suggested that Rg1 may alleviate learning and memory impairments and A $\beta$  disposition by inhibiting NLRP1 inflammasome and improving autophagy dysfunction, suggesting that Rg1 may be a potential therapeutic agent for delaying AD.

## Introduction

Alzheimer's disease (AD) is a common neurodegenerative disease worldwide in the elderly. The main neuropathological characteristic of AD is the deposition of amyloid  $\beta$  peptide (A $\beta$ ) in brain, which plays critical roles in promoting cognitive impairments and neuronal injuries in the onset and development of AD. A $\beta$  is mainly produced by the sequential cleavage of amyloid precursor protein (APP) through  $\beta$ - and  $\gamma$ -secretase (1). In progression of AD, the imbalance between A $\beta$  production and clearance contributes to elevation of A $\beta$  levels in the central nervous system (2), which leads to neuronal oxidative stress and inflammation, ultimately resulting in neuronal dysfunction and cognitive deficits (3). Although the specific mechanism of A $\beta$  accumulation remains unclear, increasing evidence suggested that alterations in autophagy and inflammation are closely associated with A $\beta$  generation and clearance in AD (4,5). However, there are still no effective drugs in improving neuronal injuries and cognitive dysfunction through modulation of autophagy and inflammation in AD.

Autophagy is a cell cycle process by which the cells hydrolyze macromolecules in response to various stress signals (6). Its primary function is to remove aged or damaged organelles and maintaining essential energy homeostasis (7). Additionally, autophagy plays an important role in the occurrence of numerous diseases, such as cancer, diabetes and neurodegenerative diseases (8). It has been reported that A $\beta$  peptides can be produced by cleavage of APP in autophagosomes during the autophagic renewal of APP-rich organelles (9,10). The autophagy function can affect A $\beta$  levels and thus modulates the pathological changes in AD (11). Moreover, autophagy also plays significant roles in metabolism of A $\beta$  and autophagy deficits may also cause the aggregation of A $\beta$  (12). Although

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how autophagy exactly affects AD pathology remains undefined, regulating autophagy may be an important target in developing new drugs for AD.

Neuroinflammation has long been considered to be associated with AD pathology. Neuroinflammation and A $\beta$  production are two critical early steps in the development of AD (13). Inflammasomes are large cytosolic multiprotein complexes that can activate caspase-1-mediated inflammatory responses (14). Caspase-1 is an important protease that causes the maturation and secretion of pro-inflammatory cytokines, such as IL-1 $\beta$  and IL-18 (15). The release of these cytokines contributes to synaptic dysfunction, neuronal death and inhibition of neurogenesis. Additionally, inflammatory cytokines (such as IL-1 $\beta$  and TNF- $\alpha$ ) are also reported to involve in the process of APP proteolytic cleavage to increase the production of A $\beta$ <sub>1-42</sub> peptides (16). Nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs) are important subfamilies of inflammasomes. Previously, the NLR-family pyrin domain-containing 1 (NLRP1) inflammasome, widely expressed in brain particularly in neurons, has received extensive attention. An ~25- to 30-fold increase of NLRP1 immunopositivity in neurons was observed in AD brains compared with non-AD brains (17). By contrast, inhibition of NLRP1 inflammasome could downregulate the A $\beta$  accumulation in AD model mice (18). A previous study by the authors indicated that NLRP1 activation is closely involved in aging-related cognitive impairments and neuronal injuries (19). However, the specific link between NLRP1 inflammasome and regulation of A $\beta$  production remains unclear.

Ginsenoside Rg1 (Rg1) is a saponin extracted from ginseng. Numerous experiments have shown that Rg1 has multiple neuroprotective effects both *in vivo* and *in vitro* (20,21). A recent study by the authors suggested that Rg1 can prevent H<sub>2</sub>O<sub>2</sub>-induced neuronal injury *in vitro* by inhibiting the activation of NLRP1 inflammasome in hippocampal neurons (22). A recent study by the authors also suggested that Rg1 treatment significantly attenuates neuronal injury and A $\beta$  generation and deposition in APP/PS1 mice (23). In recent years, it has been increasingly reported that autophagy can be regulated through the modulation of inflammatory pathways (24,25), suggesting that associating NLRP1 inflammasome with autophagy may provide new ideas for delaying AD. However, there is little study on whether Rg1 can improve AD pathology through regulating NLRP1 inflammasome and autophagic pathways. In the present study, it was hypothesized that Rg1 may reduce A $\beta$  deposition through inhibiting NLRP1 inflammasome and autophagy dysfunction, resulting in improvement of cognitive impairments and neuronal injuries. The effects of Rg1 treatment on olfactory detection ability, learning and memory function and A $\beta$  deposition in APP/PS1 mice were firstly explored. Meanwhile, the regulation of Rg1 treatment on NLRP1 inflammasome and autophagy dysfunction in APP/PS1 mice was further investigated. The present study may provide new targets and drug options for inhibiting the occurrence and development of AD.

## Materials and methods

**Animals and treatment.** The APP/PS1 transgenic AD model mice were provided by the Model Animal Research Center of

Nanjing University. The mice were bred in the animal center of Anhui Province. Male APP/PS1 mice were used in the present study, and age- and gender-matched WT littermates were utilized as control. Mice were housed in standard laboratory conditions with free access to standard food and water (temperature, 22-25°C; atmosphere, ~50-60%; 12-h light/dark cycle). The mice were raised in a standard laboratory with free food and water and were allowed to adapt to the laboratory conditions before testing. The experiment was performed (approval no. LLSC20211172) according to the protocol of Animal Ethics Committee of Anhui Medical University (Hefei, China).

To study the protective effects of Rg1 on APP/PS1 male mice, six-month old (WT) or APP/PS1 male mice were divided randomly into 6 groups (n=10 in each group; weight, 25-35 g): WT-9M group, APP/PS1-6M group, APP/PS1-9M model group, APP/PS1 + apocynin (50 mg/kg), APP/PS1 + Rg1 (5 mg/kg) and APP/PS1 + Rg1 (10 mg/kg) groups. Rg1 (Chengdu Desite Biotechnology Co.; Content >98%) and apocynin (Merck Millipore) were dissolved with water and were administered orally (0.1 ml/10 g) for 12 weeks. The doses of Rg1 and apocynin were reported in previous studies (26,27). The WT-9M and APP/PS1-9M groups were given equal volume of solvent for 12 weeks.

**Buried food test (BFT).** Previously, olfactory dysfunction has been reported in AD patients even in the early stages of AD (28). Therefore, the changes of olfactory dysfunction were examined using a previously described method (29). Briefly, mice were subjected to restricted diet for 24 h prior to the test. All mice were acclimated to the test chamber for 1 h prior to testing. A small piece of food was randomly placed in a corner of a clean test cage 1 cm below the bedding. The mouse was then placed in the test cage at a constant distance from the hidden food. ANY-maze behavioral tracking software was used to record the latency of the mouse to find food. If the mouse failed to find the buried food within 5 min, the latency period was recorded as 300 sec. A total of 9 h after the aforementioned experiment, surface pellet tests were performed using the same protocol, but with the pellets placed on the surface to exclude possible motor impairment.

**Morris water maze (MWM) test.** MWM is an important method to evaluate learning and memory impairments (30). The MWM consists of a circular pool (120 cm in diameter and 60 cm in height) filled with water and a video tracking system on top. MWM test includes four training trials from first day (day 1) to day 4 and the exploration trials on day 5 as previously described (31). During the training trials, the animals were placed in the water from each of the 4 quadrants in turn and ensured that their heads were to the wall of the pool. The animals were given 60 sec to find a platform hidden underwater. If animals did not find the platform within 60 sec, they were guided to the platform and were allowed to stay on the platform for 30 sec to familiarize and memorize the location of the platform. The mean escape latency (MEL, sec) of the four trials each day was recorded to indicate the learning performance. On day 5, the platform was removed, and each mouse was detected a swimming probe trial for 60 sec from the first quadrant. The latency first to the platform (LFP, sec),

the swimming time in the platform quadrant (STP, sec), and the number crossing platform (NCP) were recorded to indicate the memory results.

**H&E staining.** After behavior test, the mouse was euthanized by cervical dislocation, and the brain tissues (n=4) were removed and fixed with 4% paraformaldehyde at 4°C for 24 h. The tissues were sectioned into 5- $\mu$ m after paraffin-embedded using a slicer (Leica Microsystems GmbH). The pathological changes were examined by using intelligent tissue slice imaging system (3DHISTECH) after the slice was stained with hematoxylin for 4 min and eosin for 40 sec at room temperature (RT).

**Thioflavin-S Staining.** The sections (n=4) were deparaffinized by xylene and were hydrated by graded alcohol. Then the slices were stained with Thioflavin-S (1%, MedChemExpress USA, HY-D0972) at 37°C for 5 min, then were hydrated with 70% ethanol. After being washed twice with PBS, the slices were stained with Hoechst 33258 at RT for 5 min. Then the slices were sealed and examined with an intelligent slice imaging system (3DHISTECH). The green density was double-blindly analyzed from 3 regions (magnification, x400) respectively in the cortex and hippocampal CA1 by using an Image-Pro Plus 6.0 analysis system (Media Cybernetics, Inc.) to evaluate the deposition of A $\beta$ .

**Immunofluorescence.** Briefly, the slides (n=4) were deparaffinized and hydrated as aforementioned. Then the slides were treated with 0.25% Triton X-100 (cat. no. ST797-100 ml; Beyotime Institute of Biotechnology) at RT for 30 min, and were blocked with 1%BSA (cat. no. ST2249-5g; Beyotime Institute of Biotechnology) at RT for 60 min. Next the slides were incubated with polyclonal A $\beta$ <sub>1-42</sub> (1:150; cat. no. bs-0076R; BIOSS) and post-synaptic density scaffolding protein 95 (PSD95) (1:100; cat. no. GB11277; Wuhan Servicebio Technology Co., Ltd.) antibodies overnight at 4°C. The second day, the slides were treated with a secondary antibody conjugated to Rhodamine (1:200; cat. no. ZF-0317; OriGene Technologies, Inc.) at RT for 60 min. Lastly, the slides were sealed with anti-fade medium and were imaged by an intelligent slice imaging system (3DHISTECH). The red fluorescence density was analyzed from 3 regions (magnification, x400) respectively in the cortex and hippocampal CA1 by using an Image-Pro Plus 6.0 analysis system to evaluate the changes of PSD95 and A $\beta$ <sub>1-42</sub> expression.

**Immunohistochemistry.** The slides (n=4) were deparaffinized and were hydrated as aforementioned. Then, the slides were treated with H<sub>2</sub>O<sub>2</sub> (3%) at RT for 10 min to eliminate endogenous peroxidase. Afterward, the slides were treated with boiling sodium citrate buffer (3.23 g/l, pH 6.0; OriGene Technologies, Inc.) to restore antigen. Next, the slides were blocked with 10% goat serum (cat. no. C0265; Beyotime Institute of Biotechnology) for 30 min at RT and were then incubated with specific primary antibodies against Beclin1 (1:100; cat. no. 11306-1-AP) and LC3 (1:100; cat. no. 14600-1-AP; both from ProteinTech Group, Inc.) overnight at 4°C. The next day, the slides were washed with PBS three times 5 min per time and were incubated with a rabbit-general secondary antibody

(1:500; cat. no. S0001; Affinity Biosciences) at RT for 1 h. The slides were stained by a DAB kit (OriGene Technologies, Inc.) to produce brownish staining. Then the slides were mounted and examined by an intelligent tissue slice imaging system (3DHISTECH). The quantification of Beclin1 and LC3 was detected blindly by using Image-Pro Plus 6.0 software from 3 fields (magnification, x400) respectively in the cortex and hippocampal CA1.

**Immunoblot analysis.** Total protein in the hippocampus and cortex tissues (n=4) were extracted with RIPA lysis buffer (Beyotime Institute of Biotechnology) containing protease and phosphatase inhibitors by using a cryogenic tissue grinder (Shanghai Jingxin Industrial Development Co., Ltd.) 60 Hz for 50 sec, at 4°C. The protein concentration was detected by using a BCA Protein Assay kit (Beyotime Institute of Biotechnology). The proteins (20  $\mu$ g) were separated by 12% SDS-PAGE and were transferred onto a PVDF membrane (MilliporeSigma). Then, the membrane was treated with 5% non-fat milk dissolved in TBS-0.05% Tween-20 (TBST) buffer at RT for 1 h and then incubated with corresponding primary antibodies overnight at 4°C: PSD95 (1:1,000), NLRP1 (1:2,000; cat. no. ab3683; Abcam), ASC (1:500; cat. no. bs-6741R; BIOSS), Caspase-1 (1:1,000; cat. no. ab1872; Abcam), IL-1 $\beta$  (1:1,000; cat. no. ab9722; Abcam), IL-6 (1:1,000; cat. no. 21865-1-AP; ProteinTech Group, Inc.), TNF- $\alpha$  (1:1,000; cat. no. WL01581; Wanleibio Co., Ltd.), AMPK (1:1,000; cat. no. AF6423; Affinity Biosciences), p-AMPK (1:1,000; cat. no. bs-4010; Bioworld Technology, Inc.), mTOR (1:1,000; cat. no. 66888-1-Ig; ProteinTech Group, Inc.), p-mTOR (1:1,000; cat. no. bs-4706; Bioworld Technology, Inc.), Beclin1 (1:1,000), LC3 (1:1,000), P62 (1:500; cat. no. WL02385; Wanleibio Co., Ltd.) and  $\beta$ -actin (1:2,000; cat. no. GB12001; Wuhan Servicebio Technology Co., Ltd.). The second day, the membrane was washed with TBST and was incubated in secondary antibody conjugated horseradish peroxidase (1:10,000; cat. no. ZF-2301; OriGene Technologies, Inc.) at RT for 1 h. After washed with TBST, the bands were visualized by an ECL reagent (Amersham; Cytiva) and were imaged with a Chemi mini imaging system (Q4600 Mini; Shanghai Bioshine Technology; URL, <http://bioshine.bioon.com.cn/>). The band density of protein was examined using an ImageJ 6.0 software (National Institutes of Health). The target band density was normalized to  $\beta$ -actin.

**Reverse transcription-quantitative (RT-q) PCR analysis.** The total RNAs were extracted from hippocampus and cortex tissues (n=4) by using a TRIzol<sup>®</sup> reagent (Invitrogen; Thermo Fisher Scientific, Inc.) as previously described (32). Firstly, the cDNA was synthesized with PrimeScript<sup>™</sup> Reverse Transcriptase buffers with dNTP (cat. no. RR037A; Takara Bio, Inc.) from total RNA. The thermocycling conditions were at 37°C for 15 min, then at 85°C for 5 sec followed by cooling to 4°C. The qPCR analyses for mRNAs of ASC, NLRP1, IL-1 $\beta$ , Caspase-1 and  $\beta$ -actin were examined with a SYBR<sup>®</sup>Premix Ex Taq II RT-PCR kit (cat. no. RR820A; Takara Bio). The level of  $\beta$ -actin mRNA was used as internal control. The primer sequences (TsingKe Biological Technology) were as follows: NLRP1 forward, 5'-TGGCACATCTAGGGAAATC-3' and reverse, 5'-TCCTCACGTGACAGCAGAAC-3'; ASC forward, 5'-GTCACAGAAAGTGGACGGAGTG-3' and reverse, 5'-CTC

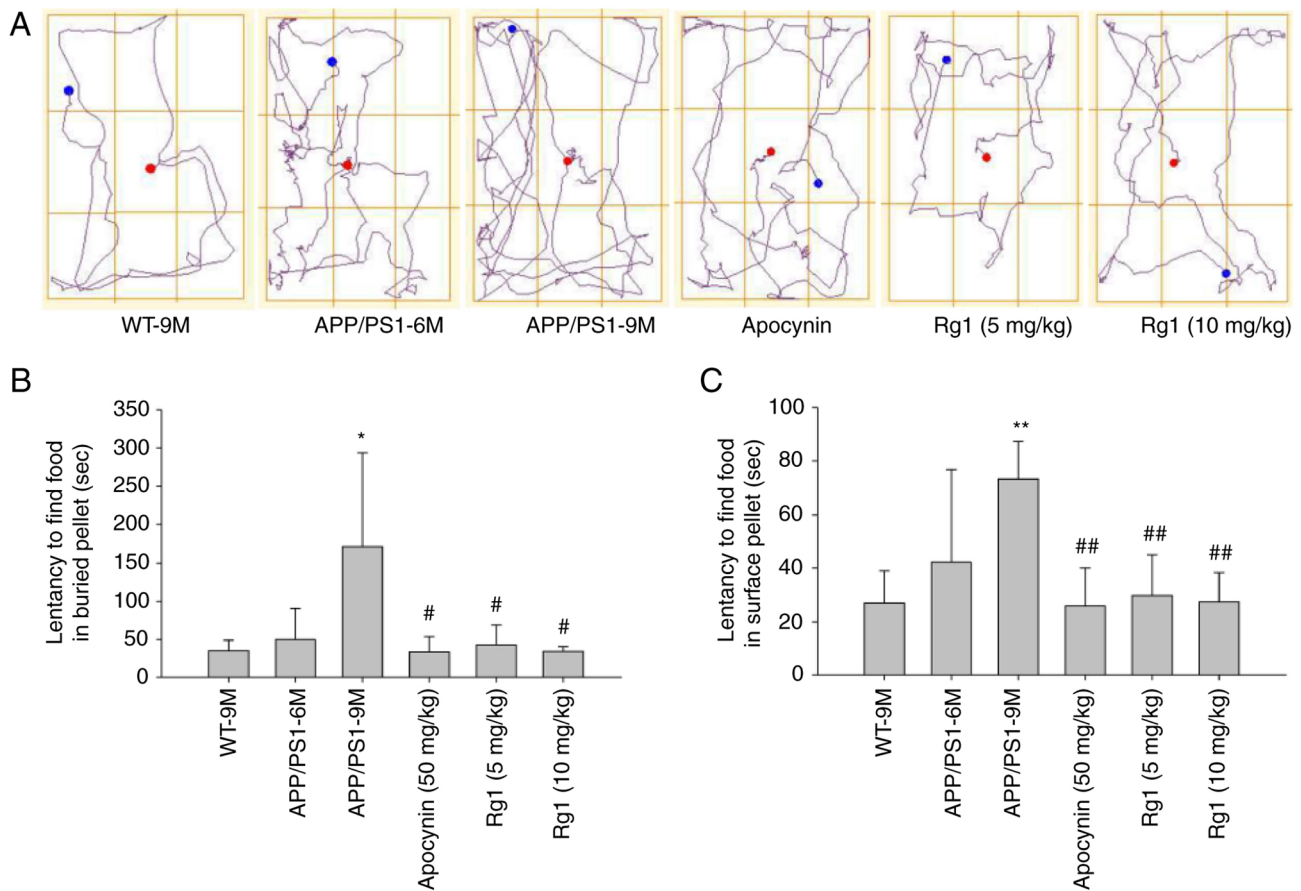


Figure 1. Rg1 treatment improves olfactory dysfunction in APP/PS1 mice. (A) The track of BFT. (B) Latency to find food in buried pellet (sec). (C) Latency to find food in surface pellet (sec). Results are expressed as the mean  $\pm$  SD (n=10). \* $P$ <0.05 and \*\* $P$ <0.01 vs. the WT-9M group; # $P$ <0.05 and ## $P$ <0.01 vs. the APP/PS1-9M group. Rg1, Ginsenoside Rg1; BFT, buried food test.

ATCTTGTCTTGGCTGGTG-3'; Caspase-1 forward, 5'-CGT GGAGAGAAACAAGGAGTG-3' and reverse, 5'-AATGAA AAGTGAGCCCCTGAC-3'; IL-1 $\beta$  forward, 5'-CTGCTTCCA AACCTTTGACC-3' and reverse, 5'-AGCTTCTCCACAGCC ACAAT-3'; and  $\beta$ -actin forward, 5'-GATTACTGCTCTGGC TCCTAGC-3' and reverse, 5'-GACTCATCGTAC TCCTGC TTGC-3'. The PCR protocol was at 95°C for 30 sec, followed by amplification at 95°C for 5 sec (40 cycles), then at 60°C for 30 sec. The qPCR was performed with a Real-time PCR System (cat. no. CFX96; Bio-Rad Laboratories, Inc.). The CT values of the samples were calculated, and the  $2^{-\Delta\Delta C_t}$  method was used to analyze transcript levels of the target mRNAs as previously described (33).

**Drug-target molecular docking.** To verify whether there is an association between Rg1 and target protein, the molecular docking between ginsenoside Rg1 and NLRP1 was monitored. Firstly, the 3D Protein Data Bank (PDB) format file of NLRP1 (PDB ID: 6XKK) was obtained from the RCSB PDB, the 2D SDF of Rg1 was downloaded (PubChem CID: 441923) from the PubChem database, and the 2D structure was imported into ChemBio3D14.0 software for structural optimization, revealing the 3D mol2 structure. Then, the drug molecules and proteins were imported into AutoDock Tool to perform hydrogenation, charge calculation, atom addition and ROOT docking. The lower binding energy indicates the improvement

of the docking result. Pymol 2.5.2 software (Schrödinger, Inc.) was used to analyze the docking results.

**Statistical analysis.** All results were showed as the mean  $\pm$  SD. The GraphPad Prism 8.0 software (GraphPad Software, Inc.) was used for statistical analysis. The results of four-day training in MWM were analyzed by repeated-measure two-way analysis of variance (ANOVA) and other data were analyzed by one-way ANOVA. Tukey's post hoc test was performed to compare the significance between groups.  $P$ <0.05 was considered to indicate a statistically significant difference.

## Results

**Rg1 treatment improves abnormal behavior function in APP/PS1 mice.** Firstly, BFT was used to observe the effects of Rg1 on olfactory function in APP/PS mice. The buried pellet results showed that there were no differences in the latency to find the food between the WT-9M and APP/PS1-6M group mice, while in the APP/PS1-9M group mice the latency to find the food was significantly increased compared with the WT-9M group mice. Meanwhile, it was revealed that Rg1 and apocynin treatment significantly reduced the latency to find the food compared with the APP/PS1-9M group mice (Fig. 1A and B,  $P$ <0.05). Additionally, the surface pellet test showed similar results to the buried pellet test (Fig. 1C,  $P$ <0.01), suggesting



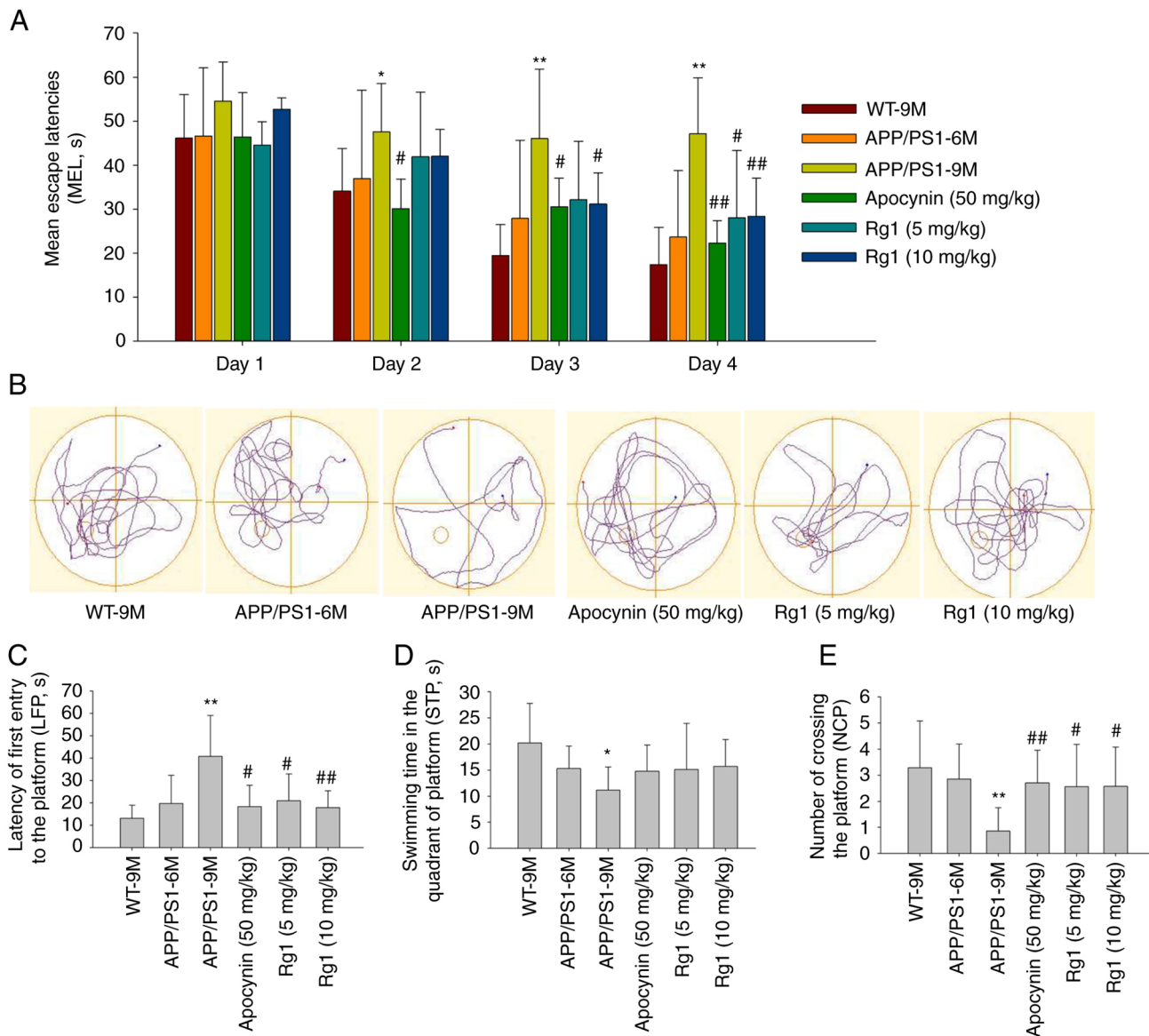


Figure 2. Rg1 treatment improves learning and memory ability in APP/PS1 mice. (A) The mean escape latencies. (B) The track of Morris water maze in the fifth day. (C) The latency of first entry platform. (D) Swimming time in platform quadrant. (E) Number of crossing the platform. Results are expressed as the mean  $\pm$  SD (n=10). \*P<0.05 and \*\*P<0.01 vs. the WT-9M group; #P<0.05 and ##P<0.01 vs. APP/PS1-9M group. Rg1, Ginsenoside Rg1.

that there was no motor dysfunction in the experimental mice. These results suggested that Rg1 treatment can improve olfactory dysfunction in APP/PS1 mice.

Then, MWM was conducted to examine the learning and memory ability of mice. On the first day (day 1), there was no significant difference in escape latency among the groups. With the progress of the experiment, the escape latency became increasingly shorter in the WT-9M and administration groups. While in APP/PS1-9M group, the escape latency was significantly longer than that of the WT-9M mice in days 2-4 (Fig. 2A, P<0.05 or P<0.01). Meanwhile, it was identified that the escape latency was significantly reduced by apocynin treatment in days 2-4, by Rg1 (5 mg/kg) in day 4 and Rg1 (10 mg/kg) in days 3-4 compared with APP/PS1-9M group (Fig. 2A, P<0.05 or P<0.01). On the fifth day of the probe test, the results showed that the LFP was significantly increased, the NCP and the STP were significantly decreased in APP/PS1-9M mice compared with the WT-9M group (Fig. 2B-E, P<0.05 or P<0.01). While

compared with the APP/PS1-9M group, treatment with apocynin and Rg1 (5 and 10 mg/kg) could significantly reduce the LFP and increase the NCP (Fig. 2B-E, P<0.05 or P<0.01). The results indicated that Rg1 treatment can significantly improve the learning and memory impairments in APP/PS1 mice.

*Rg1 treatment alleviates neuronal damages and A $\beta$  depositions in APP/PS1 mice.* The H&E staining results revealed that, compared with WT-9M, there were few pathological changes in the cortex and hippocampal CA1 areas in APP/PS1-6M mice, while in APP/PS1-9M mice, neuronal damages, such as pyknosis and nucleoli inconspicuous (black arrows) and A $\beta$  plaques (yellow arrows), were increased in the cortex and hippocampal CA1 (Fig. 3). Compared with APP/PS1-9M, treatment with apocynin and Rg1 (5 and 10 mg/kg) could alleviate the neuronal damages in the cortex and hippocampal CA1 (Fig. 3). The results indicated that Rg1 treatment could improve neuronal damages in APP/PS1 mice.

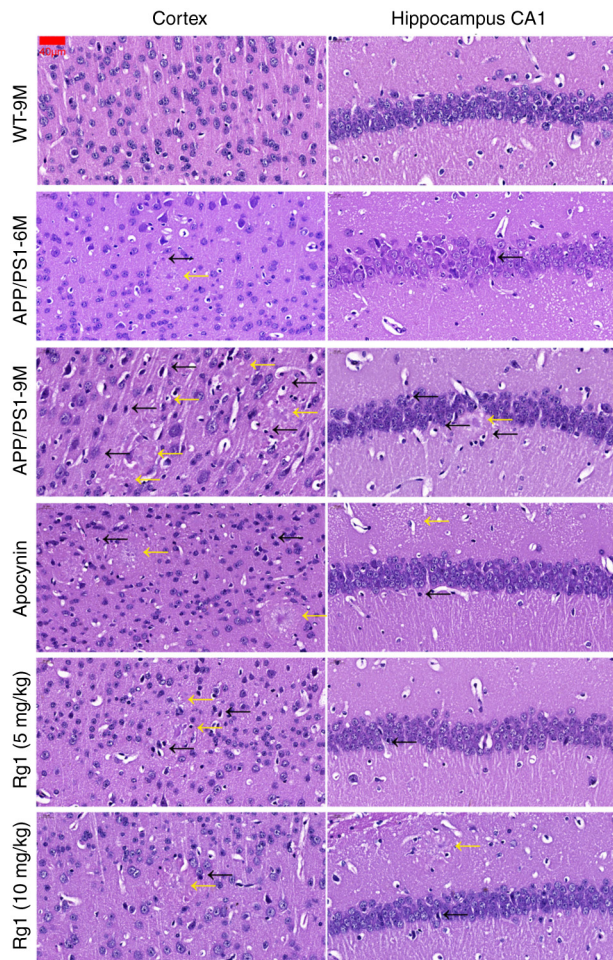


Figure 3. Rg1 treatment alleviates neuronal damage in APP/PS1 mice. The results of H&E staining in the cortex and hippocampus CA1 (magnification, x400; scale bar, 40  $\mu$ m; n=4). Rg1, Ginsenoside Rg1.

To confirm the effect of A $\beta$  deposition, the Thioflavin-S staining and immunofluorescence were conducted to detect the A $\beta$  deposition and A $\beta_{1-42}$  level. The Thioflavin-S staining results demonstrated that there was a small amount of A $\beta$  deposition in the cortex and hippocampal CA1 in APP/PS1-6M mice, while in APP/PS1-9M mice, the A $\beta$  depositions (yellow arrows) were significantly increased compared with WT-9M (Fig. 4A-C,  $P < 0.05$  or  $P < 0.01$ ). Compared with APP/PS1-9M, treatment with apocynin and Rg1 could significantly reduce A $\beta$  depositions in the cortex and hippocampal CA1 (Fig. 4A-C,  $P < 0.05$  or  $P < 0.01$ ). The immunofluorescence results of A $\beta_{1-42}$  were similar to that of Thioflavin-S staining, suggesting that A $\beta_{1-42}$  expression was significantly increased in APP/PS1-9M mice, and was significantly downregulated by apocynin and Rg1 treatment in the cortex and hippocampal CA1 (Fig. 4D-F,  $P < 0.05$  or  $P < 0.01$ ). The results indicated that Rg1 treatment decreases A $\beta$  generation and deposition in APP/PS1 mice.

*Rg1 treatment increases PSD95 expression in APP/PS1 mice.* AD models not only have A $\beta$  deposition, but also are accompanied by synaptic dysfunction (34). Therefore, the expression of PSD95 was detected by immunofluorescence and immunoblot analysis. The immunofluorescence results showed that the expression of PSD95 was significantly

decreased in the cortex and hippocampal CA1 in both APP/PS-6M and APP/PS1-9M mice compared with WT-9M mice, particularly in APP/PS1-9M mice (Fig. 5A-C,  $P < 0.01$ ). After Apocynin and Rg1 treatment, the expression of PSD95 was significantly increased in the cortex and hippocampal CA1 (Fig. 5A-C,  $P < 0.01$ ). The immunoblot results of PSD95 were similar to that of immunofluorescence results, suggesting that the expression of PSD95 was significantly decreased in APP/PS-6M and APP/PS1-9M mice, and was significantly increased after Apocynin and Rg1 treatment (Fig. 5D,  $P < 0.05$  or  $P < 0.01$ ). These results indicated that Rg1 treatment can improve synaptic dysfunction in APP/PS1 mice.

*Rg1 treatment decreases the expression of NLRP1 inflammasome in APP/PS1 mice.* It has been reported that NLRP1 inflammasome is involved in learning and memory impairments and neuronal damages during the aging process in mice (19). Therefore, the effect of Rg1 treatment on the expression of NLRP1 inflammasome proteins was further examined through immunoblot and RT-qPCR. The results showed that, compared with WT-9M, there was no significant increase in the expression of NLRP1 inflammasome-related proteins in the APP/PS1-6M group. While in the APP/PS1-9M group, the expression levels of NLRP1, ASC, caspase-1, IL-1 $\beta$ , IL-6 and TNF- $\alpha$  were significantly increased. Compared with the APP/PS1-9M group, after apocynin and Rg1 treatment, the expression levels of these proteins were significantly downregulated (Fig. 6A-H,  $P < 0.05$  or  $P < 0.01$ ). Similar results were observed using qPCR; the levels of NLRP1, ASC, caspase-1 and IL-1 $\beta$  mRNA were significantly increased in the APP/PS1-9M mice and were significantly decreased after apocynin and Rg1 treatment (Fig. 7A-D,  $P < 0.05$  or  $P < 0.01$ ). These results suggested that Rg1 treatment can inhibit NLRP1 inflammasome in APP/PS1 mice.

*Rg1 treatment improves autophagy dysfunction in APP/PS1 mice.* Autophagy also plays important roles in A $\beta$  generation and metabolism, and its malfunction is involved in the progress of AD (35). Therefore, the effect of Rg1 on autophagy function in APP/PS mice was further studied. The results showed that there were no significant differences in the expression levels of p-AMPK/AMPK, p-mTOR/mTOR, Beclin1, LC3 II/LC3 I and P62 in APP/PS1-6M mice compared with WT-9M. While in APP/PS1-9M mice, the levels of p-AMPK/AMPK, Beclin1 and LC3 II/LC3 I were significantly increased, and the levels of p-mTOR/mTOR and P62 were significantly decreased (Fig. 8A-G,  $P < 0.05$  or  $P < 0.01$ ). Moreover, apocynin and Rg1 treatment could significantly reverse the changes of these proteins in APP/PS1 mice (Fig. 8A-G,  $P < 0.05$  or  $P < 0.01$ ). Furthermore, the expression levels of Beclin1 and LC3 were detected by immunohistochemistry. There were similar results that the expression levels of Beclin1 and LC3 were increased in APP/PS1-9M mice and were significantly decreased after Rg1 treatment in cortex and hippocampus CA1 (Figs. S1 and S2,  $P < 0.05$  or  $P < 0.01$ ). These results indicated that Rg1 treatment can improve autophagy dysfunction in APP/PS1 mice.

*Molecular docking shows potential interaction of Rg1 with NLRP1.* Molecular docking was used to further verify the



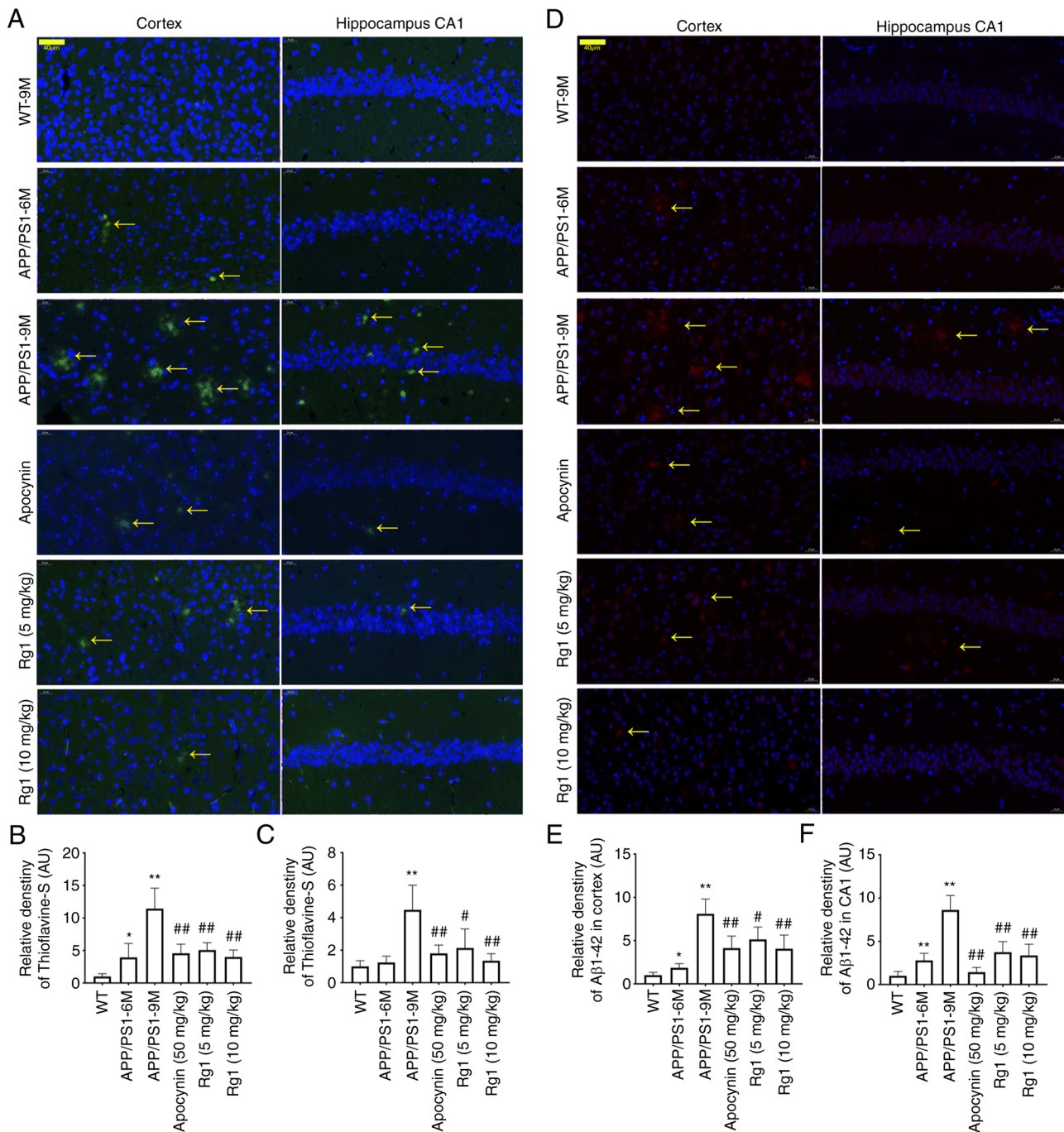


Figure 4. Rg1 treatment alleviates A $\beta$  deposition in APP/PS1 mice. (A) The results of Thioflavin-S staining in the cortex and hippocampus CA1 (magnification, x400; scale bar, 40  $\mu$ m). (B) Relative density of Thioflavin-S staining in cortex over WT. (C) Relative density of Thioflavin-S staining in hippocampus CA1 over WT. (D) The results of A $\beta_{1-42}$  in the cortex and hippocampus CA1 (immunofluorescence, x400; scale bar, 40  $\mu$ m). (E) Relative density of A $\beta_{1-42}$  in cortex over WT. (F) Relative density of A $\beta_{1-42}$  in hippocampus CA1 over WT. Results are expressed as the mean  $\pm$  SD (n=4). \*P<0.05 and \*\*P<0.01 vs. the WT-9M group; #P<0.05 and ##P<0.01 vs. the APP/PS1-9M group. Rg1, Ginsenoside Rg1.

interaction between Rg1 and NLRP1. The results showed that several hydrogen bonds may form between Rg1 and NLRP1 (Fig. S3A and B). Meanwhile, the binding energy (affinity: -5.31 kcal/mol) in the docking analysis showed favorable binding results between Rg1 and NLRP1 (Fig. S3C).

## Discussion

Although it is well known that the pathological features of AD are obvious, the pathogenesis remains not completely

clear and there is currently little effective treatment for AD. The A $\beta$  cascade hypothesis is dominated in the field of AD research and provides an intellectual framework for therapeutic interventions (36). Based on this, various mechanisms, and pathways of A $\beta$  generation and deposition have been established, such as ROS oxidative stress, mitochondrial dysfunction and inflammatory process (37). In recent years, increasing evidence has focused on the changes in neuroinflammation and autophagy, which has brought new ideas to explore the pathogenesis of AD. Ginsenoside Rg1 has been

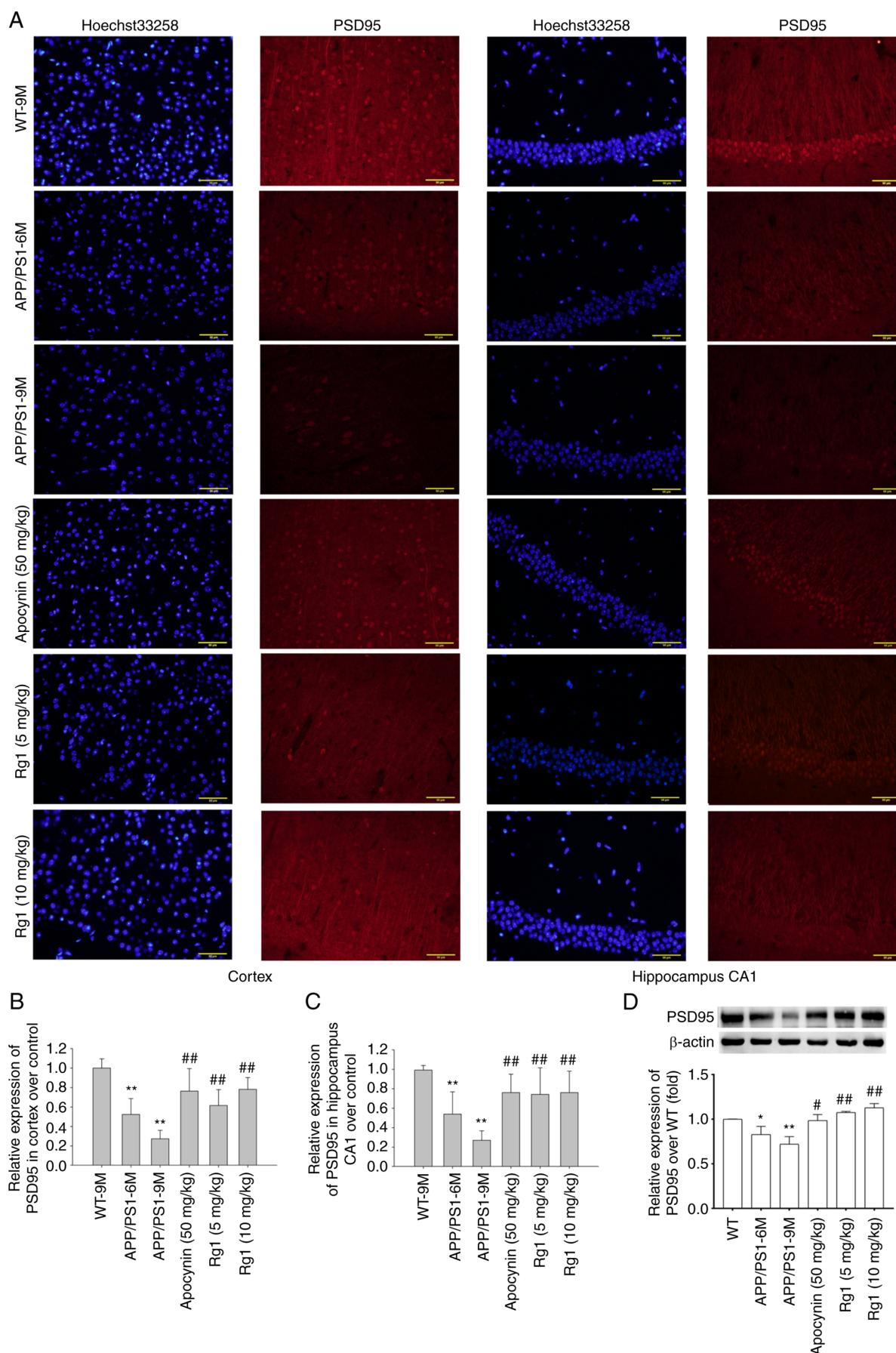


Figure 5. Rg1 treatment increases the expression of PSD95 in APP/PS1 mice. (A) The expression of PSD95 in the cortex and hippocampus CA1 (magnification, x400; scale bar, 50  $\mu$ m; n=4). (B) Relative expression of PSD95 in cortex over WT. (C) Relative expression of PSD95 in hippocampus CA1 over WT. (D) Relative expression of PSD95 (western blot analysis, n=4). Results are expressed as the mean  $\pm$  SD. \* $P$ <0.05 and \*\* $P$ <0.01 vs. the WT-9M group; # $P$ <0.05 and ## $P$ <0.01 vs. the APP/PS1-9M group. PSD95, post-synaptic density scaffolding protein 95; Rg1, Ginsenoside Rg1.



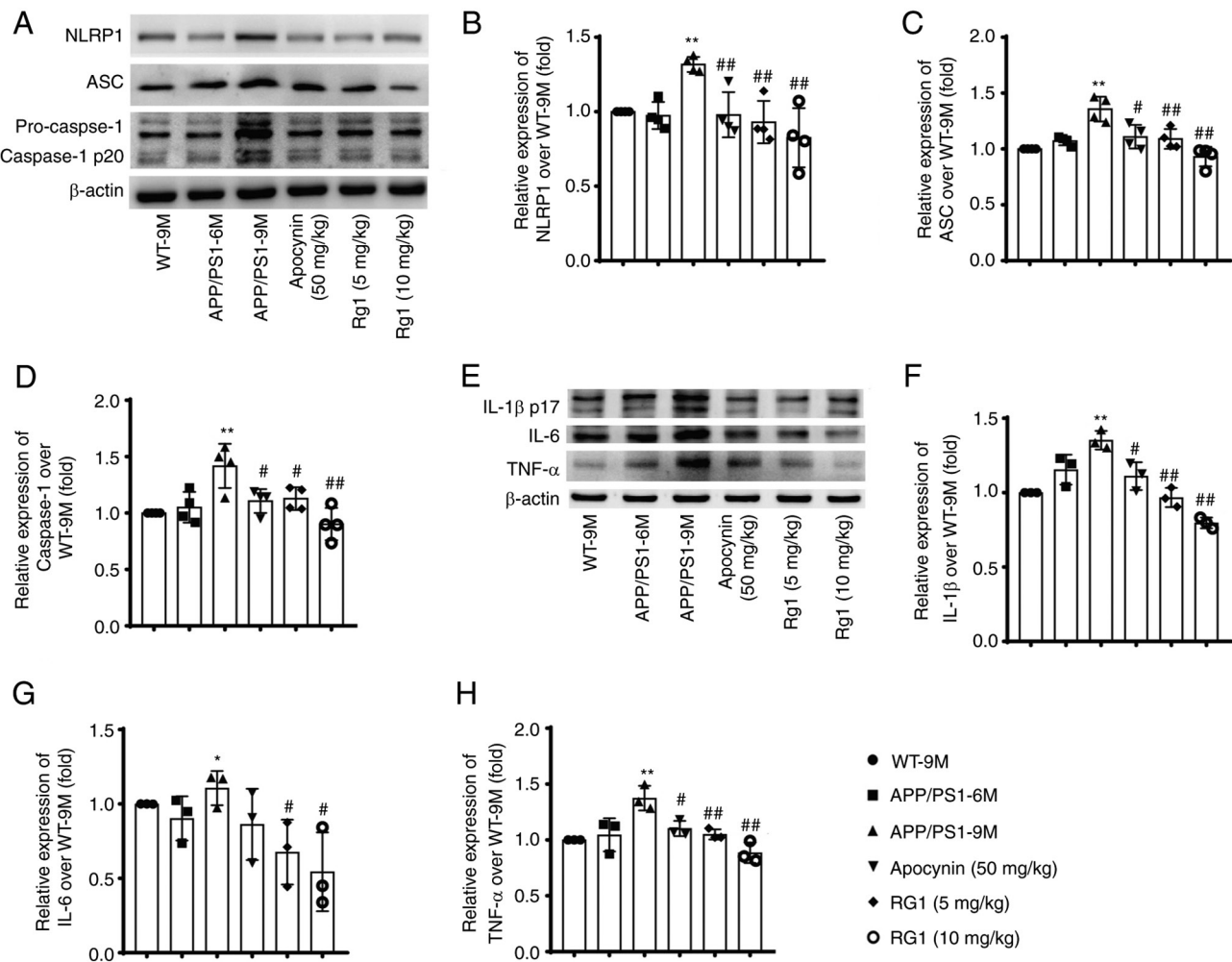


Figure 6. Rg1 treatment decreases the expression of NLRP1 inflammasome in APP/PS1 mice (western blot analysis). (A) The bands of NLRP1, ASC, Caspase-1 and β-actin. (B-D) Relative expression of (B) NLRP1, (C) ASC and (D) Caspase-1 over WT (n=4). (E) The bands of IL-1β, IL-6, TNF-α and β-actin (n=3). (F-H) Relative expression of (F) IL-1β, (G) IL-6 and (H) TNF-α over WT. Results are expressed as the mean ± SD. \*P<0.05 and \*\*P<0.01 vs. the WT-9M group; #P<0.05 and ##P<0.01 vs. the APP/PS1-9M group. Rg1, Ginsenoside Rg1.

reported to possess neuroprotective effects in numerous neurodegenerative diseases (20,21). Particularly, Rg1 treatment could protect against lipopolysaccharide (LPS)-induced neuroinflammation and neuronal apoptosis (38,39). A recent study by the authors indicated that Rg1 treatment could significantly ameliorate LPS-induced cognitive impairments and neuroinflammation through inhibiting NLRP1 inflammasome in mice (40). Additionally, another recent study indicated that Rg1 treatment could promote autophagy, resulting in the inhibition of NLRP3 inflammasome in acute liver injuries (41). However, whether Rg1 protects against AD through regulating autophagy remains unknown. Therefore, it was hypothesized that Rg1 treatment may alleviate neuronal damages and Aβ depositions by inhibiting NLRP1 inflammasome and autophagy dysfunction in APP/PS1 mice, which may provide a new therapeutic strategy for AD. In the present study, the results showed that Rg1 treatment significantly alleviated the olfactory dysfunction, learning and memory impairments, Aβ deposition and neuronal damage in APP/PS1 mice. The results also demonstrated that Rg1 treatment significantly reduced the expression levels of NLRP1 inflammasome, as well as reversed the AMPK/mTOR pathway and inhibited

the autophagy function in APP/PS1 mice. The present results suggested that Rg1 ameliorates Aβ depositions and neuronal damages by regulating NLRP1 inflammasome as well as the AMPK/mTOR-mediated autophagic pathway.

The APP/PS1 double transgenic mouse is a common AD model, which shows significant memory dysfunction, Aβ deposition and neuronal damages similarity to pathological features of AD at 8 months of age or even earlier (42). Therefore, the protective effects of Rg1 in APP/PS1 mice from 6-9 months old were studied. Additionally, Apocynin, as a NOX inhibitor by reducing ROS generation, has been reported to possess protective effect against neurodegenerative diseases (43,44) and has anti-inflammatory effects (45). Apocynin has also been reported to ameliorate neuronal damage by regulating autophagy (46,47). Therefore, apocynin was chosen as a positive drug in the current study. It was found that the expression levels of NLRP1 inflammasome and autophagy-related proteins were significantly decreased in the apocynin-treated mice, suggesting that apocynin has a regulatory effect on inflammation and autophagy in AD. Furthermore, previous studies have suggested that olfactory dysfunctions (OD) may be an early biomarker

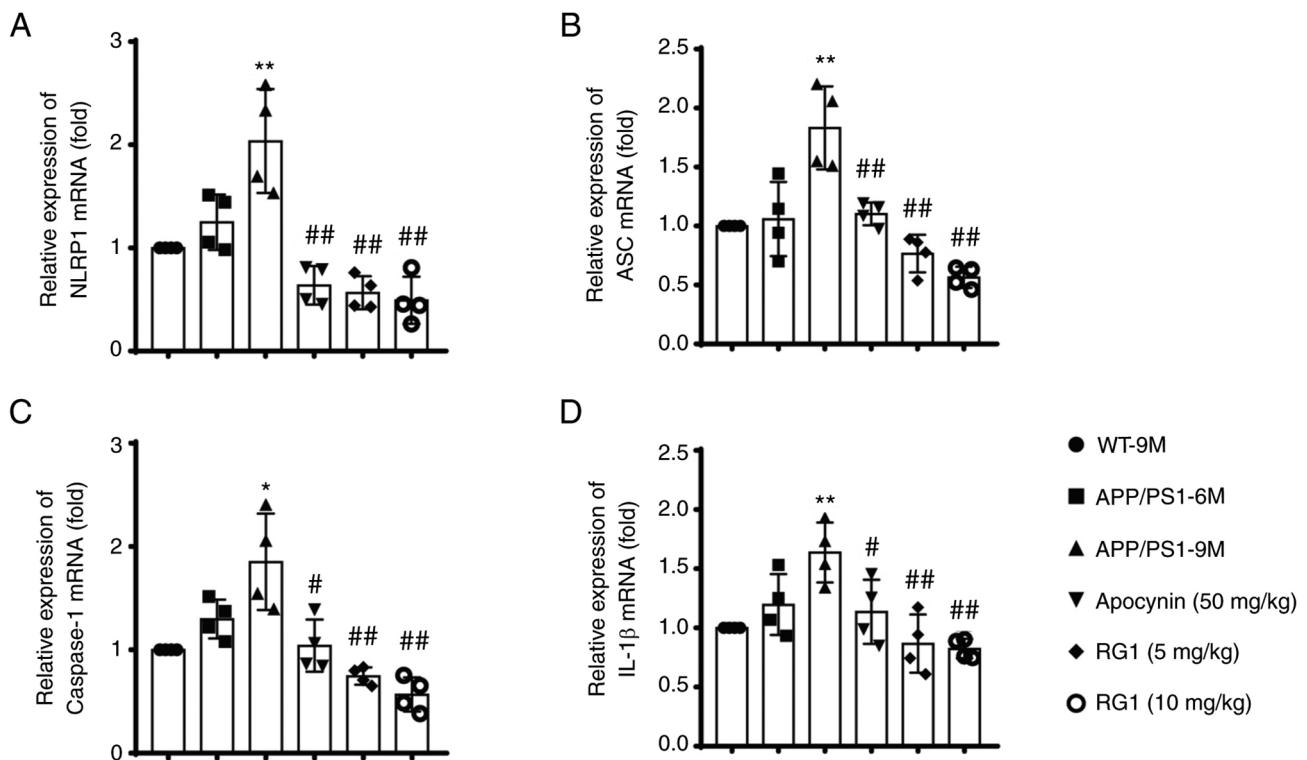


Figure 7. Rg1 treatment decreases the mRNA expression levels of NLRP1 inflammasome in APP/PS1 mice (reverse transcription-quantitative PCR). (A-D) Relative expression of (A) NLRP1, (B) ASC, (C) Caspase-1 and (D) IL-1 $\beta$  mRNA over WT. Results are expressed as the mean  $\pm$  SD (n=4). \*P<0.05 and \*\*P<0.01 vs. the WT-9M group; #P<0.05 and ##P<0.01 vs. the APP/PS1-9M group. Rg1, Ginsenoside Rg1.

for neurodegeneration (48). It has been reported that OD is observed in 85% of early-stage AD patients (48). The present study confirmed that there was no significant OD in APP/PS1-6M mice, but the APP/PS1-9M mice had a significant OD. The result of MWM was similar to the BFT that the APP/PS1-9M mice showed significant learning and memory impairments but not in APP/PS1-6M mice. Meanwhile, it was found that Rg1 and apocynin treatment could significantly improve the OD and cognitive impairments in APP/PS1-9M mice, suggesting that Rg1 could attenuate the progression of AD. The structural and functional dysfunctions in cortex and hippocampus, which are also vulnerable to A $\beta$  accumulation and are closely correlated to AD progression (49). The results of the present study indicated that the APP/PS1-9M mice exhibited significant neuronal injuries and A $\beta$  depositions in the cortex and hippocampus CA1 regions, and Rg1 treatment significantly alleviated the neuronal damages and A $\beta$  accumulation. Synapse loss also correlates with cognitive impairments in AD. PSD95, an important protein regulating synapse function, is significantly reduced in the cortex and hippocampus regions of patients with AD and is correlated negatively with cognitive dysfunction of AD (50). The present results revealed that the expression of PSD95 was significantly reduced in APP/PS1-9M mice but was increased by Rg1 treatment. These data suggested that Rg1 treatment could ameliorate neuronal injuries and A $\beta$  deposition in APP/PS1 mice.

Inflammation is considerably reported as an essential factor of neuronal damage in the progression of AD (51). It has been reported that a number of proinflammatory cytokines are elevated in brain tissues of AD, such as TNF- $\alpha$ , IL-6 and

IL-1 $\beta$  (52,53). The TNF- $\alpha$  secreted by activated microglia is involved in cognitive impairment (54). The IL-1 $\beta$  suppresses the long-term potentiation (LTP), and induces learning and memory impairments by activating p38-MAPK and GSK3 signaling (55). Furthermore, overexpression of these cytokines contributes to synaptic plasticity dysfunction and neuronal damages in 5xFAD mice (56). Furthermore, a handful of studies suggested that the proinflammatory cytokines, such as TNF- $\alpha$  and IL-1 $\beta$ , also play crucial roles in A $\beta$  accumulation of AD. IL-1 $\beta$  and TNF- $\alpha$  overexpression could increase A $\beta$  generation from APP through enhancement of  $\beta$ - and  $\gamma$ -secretase (57). In the present study, it was identified that the expression levels of IL-1 $\beta$ , IL-6 and TNF- $\alpha$  were significantly increased accompanied significant elevation of A $\beta$ <sub>1-42</sub> generation and A $\beta$  deposition in APP/PS1-9M mice. Rg1 treatment could significantly inhibit the expression levels of IL-1 $\beta$ , IL-6 and TNF- $\alpha$  and attenuate learning and memory impairments and A $\beta$  deposition. These data demonstrated that Rg1 may alleviate A $\beta$  deposition and learning and memory impairments through inhibiting neuroinflammation. A recent study by the authors suggested that Rg1 treatment could improve LPS-induced neuronal injuries by downregulating the expression levels of NLRP1 inflammasomes in HT22 cells (58). Therefore, it was hypothesized that Rg1 may delay the progression of AD by inhibiting NLRP1 inflammasome in APP/PS1 mice. The NLRP1 inflammasome is extensively expressed in central nervous system, particularly in neurons and serves as a platform for the recruitment of the apoptosis-associated speck-like protein containing a CARD (ASC) and procaspase-1 protease. Once activated, ASC activates caspase-1 and then induces the processing and maturation of

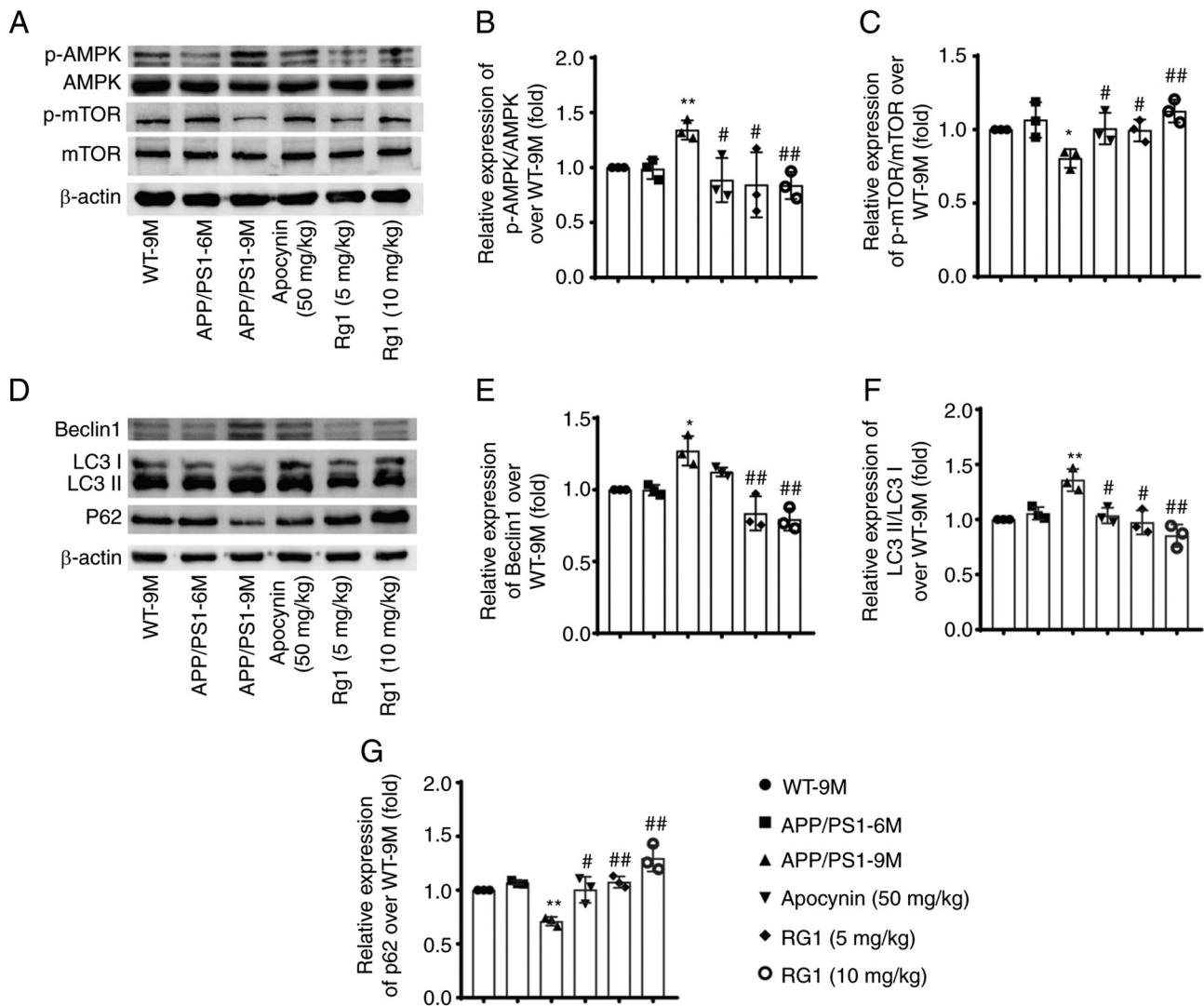


Figure 8. Rg1 treatment improves autophagy dysfunction in APP/PS1 mice (Western blot analysis). (A) The bands of p-AMPK, AMPK, p-mTOR, mTOR and  $\beta$ -actin. (B and C) Relative expression of (B) p-AMPK/AMPK and (C) p-mTOR/mTOR over WT. (D) The bands of Beclin1, LC3, P62 and  $\beta$ -actin. (E-G) Relative expression of (E) Beclin1, (F) LC3 II/LC3 I and (G) P62 over WT. Results are expressed as the mean  $\pm$  SD (n=4). \*P<0.05 and \*\*P<0.01 vs. the WT-9M group; #P<0.05 and ##P<0.01 vs. the APP/PS1-9M group. Rg1, Ginsenoside Rg1.

the IL-1 $\beta$ , IL-6 and IL-18 (59). Previous studies have shown that NLRP1 inflammasome is upregulated and accompanied by neuronal damage and cognitive decline in mice model of AD (15). The present results showed that the expression levels of NLRP1, ASC, Caspase-1, IL-1 $\beta$ , IL-6 and TNF- $\alpha$  were all significantly increased in APP/PS1-9M mice, and Rg1 treatment significantly decreased their expression levels. In addition, the molecular docking analysis showed that there was favorable binding result between Rg1 and NLRP1. These results confirmed that activation of NLRP1 inflammasome is involved in AD progression and Rg1 may alleviate learning and memory impairments and A $\beta$  disposition through inhibiting the NLRP1 inflammasome.

Autophagy is a lysosomal degradation pathway essential for survival, differentiation, development and homeostasis in cells (60). There are three stages in autophagy biogenesis, including phagosome membrane separation, phagosome elongation and phagocytosis of random cytoplasmic contents, and autophagosome maturation and fusion with lysosomes (61). Increasing evidence has implicated autophagy in numerous

major neurodegenerative disorders, such as frontotemporal dementia, AD, Parkinson's and Huntington's disease (62-65). Under normal conditions, autophagy can remove accumulated intracellular toxicants or damaged organelles from neuronal cells (66). For example, autophagy can facilitate the degradation and clearance of APP as well as APP cleavage products including A $\beta$  (67,68). However, autophagy dysfunction is also involved in the accretion of noxious proteins in the AD brain (35). Therefore, autophagy may be a double-edged sword in the progression of AD, and its function is controversial (11,69). On the one hand, autophagy can protect cells against apoptosis and necrosis by degrading harmful substances (70). On the other hand, its excessive activation can lead to autophagic stress, which is defined as a relatively sustained imbalance in which rates of autophagosome vacuoles formation exceed rates of autophagosome vacuoles degradation (71). The accumulated autophagic vacuoles were found to be increased in AD brains and in APP/PS1 mice, suggesting a source of A $\beta$  generation (72). Therefore, excessive autophagy may contribute to autophagic stress,

and its normal function will be blocked, thus aggravating the severity of AD (73). Beclin1 is an autophagy-associated protein that regulates the formation of autophagosomes. During the process of autophagy, a cytosolic form of LC3 (LC3 I) is conjugated to phosphatidylethanolamine to form LC3-phosphatidylethanolamine (LC3 II), which is recruited to autophagosome membranes (74). P62 is an autophagic receptor that recognizes ubiquitinated proteins and interacts with LC3-II in the autophagosomes (75). Furthermore, the lysosome is an essential organelle for A $\beta$  generation in autophagic vacuoles after autophagy activation (76). The results of the present study indicated that the expression levels of Beclin1 and LC3-II/LC3-I were decreased and p62 expression was increased in parallel with A $\beta$  deposition in APP/PS1-9M mice. Meanwhile, it was found that treatment with Rg1 can significantly reverse these changes in APP/PS1 mice. These data suggested that there may be autophagic stress in APP/PS1-9M mice, and Rg1 may alleviate A $\beta$  deposition by inhibiting autophagy dysfunction in APP/PS1 mice. To confirm the effect of Rg1 on regulation of autophagy in AD, the change of the AMPK/mTOR pathway, which plays an important role in the modulation of autophagy, was further explored (77). The AMP-activated protein kinase (AMPK) and mTOR are two main nutrient-sensing pathways in response to stress. The AMPK/mTOR pathway can activate autophagy by decreasing the activity of mTOR. In the present study, it was found that the p-AMPK/AMPK was significantly upregulated and the p-mTOR/mTOR was significantly down-regulated in APP/PS1-9M mice. In addition, Rg1 significantly decreased p-AMPK/AMPK and increased mTOR/mTOR in APP/PS1-9M mice. The results further revealed that Rg1 may regulate autophagy function through regulation of AMPK/mTOR pathway in APP/PS1 mice.

Additionally, it has been reported that excessive IL-1 $\beta$  can induce autophagy dysfunction in primary cultures of neurons, astrocytes and microglia (78). Moreover, the Beclin1 assembly rate is positively correlated with IL-1 $\beta$  and TNF- $\alpha$  levels, and conversely, TNF- $\alpha$  levels were negatively correlated with mTOR levels in APP/PS1 mouse brain tissue (79). These data suggested that interaction of inflammation and autophagy may be closely correlated with AD progression. The present study suggested that both NLRP1 inflammasome and autophagy dysfunction coexist in APP/PS mice. Rg1 treatment may delay the progression of AD by inhibiting NLRP1 inflammasome and autophagy dysfunction in APP/PS1 mice.

In general, the present study indicated that Rg1 treatment improves olfactory dysfunction, learning and memory impairments, neuronal damages and A $\beta$  depositions in APP/PS1 mice. Meanwhile, the present results revealed that Rg1 can regulate NLRP1 inflammasome as well as AMPK/mTOR-mediated autophagic function in APP/PS1 mice. These data suggested that Rg1 may alleviate A $\beta$  deposition and AD progression by inhibiting NLRP1 inflammasome and autophagy dysfunction. However, the present study only provided the basic experimental results that Rg1 treatment ameliorated cognitive dysfunction due to the inhibition of NLRP1 inflammasome and autophagy in APP/PS1 mice and its exact mechanism needs to be confirmed *in vitro*. Additionally, as the interrelationship between NLRP1 inflammasome and autophagy are complex, more research

is required to fully elucidate the possible mechanisms in progression of AD.

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## Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

## Authors' contributions

WZL and WPL conceived and designed the study and critically revised the manuscript for intellectually important content. LK, LH and XL performed the experiments and statistical analysis and wrote the manuscript. YS collated the data. HZ and PJ were mainly responsible for the histological experiments. YS and HZ confirm the authenticity of all the raw data. PJ, RS and CW helped to perform the experiments and wrote part of the manuscript. All authors read and approved the final version of the manuscript.

## Ethics approval and consent to participate

All experiments involving animals were approved (approval no. LLSC20211172) by the Ethics Committee of Laboratory Animals of Anhui Medical University (Hefei, China).

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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