Journal of Ethnopharmacology 321 (2024) 117462

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jethpharm

Ginsenoside RK3 promotes neurogenesis in Alzheimer's disease through activation of the CREB/BDNF pathway

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ARTICLE INFO

Keywords:
Ginsenoside RK3
AD
Neurogenesis
Cognitive function
Synaptogenesis

ABSTRACT

Ethnopharmacological relevance: In the ancient book "Shen Nong's Herbal Classic," *Panax ginseng* CA Mey was believed to have multiple benefits, including calming nerves, improving cognitive function, and promoting longevity. Ginsenosides are the main active ingredients of ginseng. Ginsenoside RK3 (RK3), a rare ginsenoside extracted from ginseng, displays strong pharmacological potential. However, its effect on neurogenesis remains insufficiently investigated.

Aim of the study: This study aims to investigate whether RK3 improves learning and memory by promoting neurogenesis, and to explore the mechanism of RK3 action.

Materials and methods: The therapeutic effect of RK3 on learning and memory was determined by the Morris water maze (MWM) and novel object recognition test (NORT). The pathogenesis and protective effect of RK3 on primary neurons and animal models were detected by immunofluorescence and western blotting. Protein expression of cAMP response element-binding protein (CREB)/brain-derived neurotrophic factor (BDNF) signaling pathway was detected by western blotting.

Results: Our results showed that RK3 treatment significantly improved cognitive function in APPswe/PSEN1dE9 (APP/PS1) mice and C57BL/6 (C57) mice. RK3 promotes neurogenesis and synaptogenesis in the mouse hippocampus. *In vitro*, RK3 prevents Aβ-induced injury in primary cultured neurons and promotes the proliferation of PC12 as well as the expression of synapse-associated proteins. Mechanically, the positve role of RK3 on neurogenesis was combined with the activation of CREB/BDNF pathway. Inhibition of CREB/BDNF pathway attenuated the effect of RK3.

Conclusion: In conclusion, this study demonstrated that RK3 promotes learning and cognition in APP/PS1 and C57 mice by promoting neurogenesis and synaptogenesis through the CREB/BDNF signaling pathway. Therefore, RK3 is expected to be further developed into a potential drug candidate for the treatment of Alzheimer's disease (AD).

1. Introduction

Cognitive impairments signify a breakdown in cerebral function. When individuals affected by these impairments lose their capacity for independent living, it places a substantial financial burden and emotional stress on both their families and caregivers (Hossain et al., 2020). Alzheimer's disease (AD) is a chronic neurodegenerative condition marked by gradual cognitive and behavioral deterioration ("2022

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https://doi.org/10.1016/j.jep.2023.117462
Received 19 September 2023; Received in revised form 12 November 2023; Accepted 14 November 2023
Available online 18 November 2023

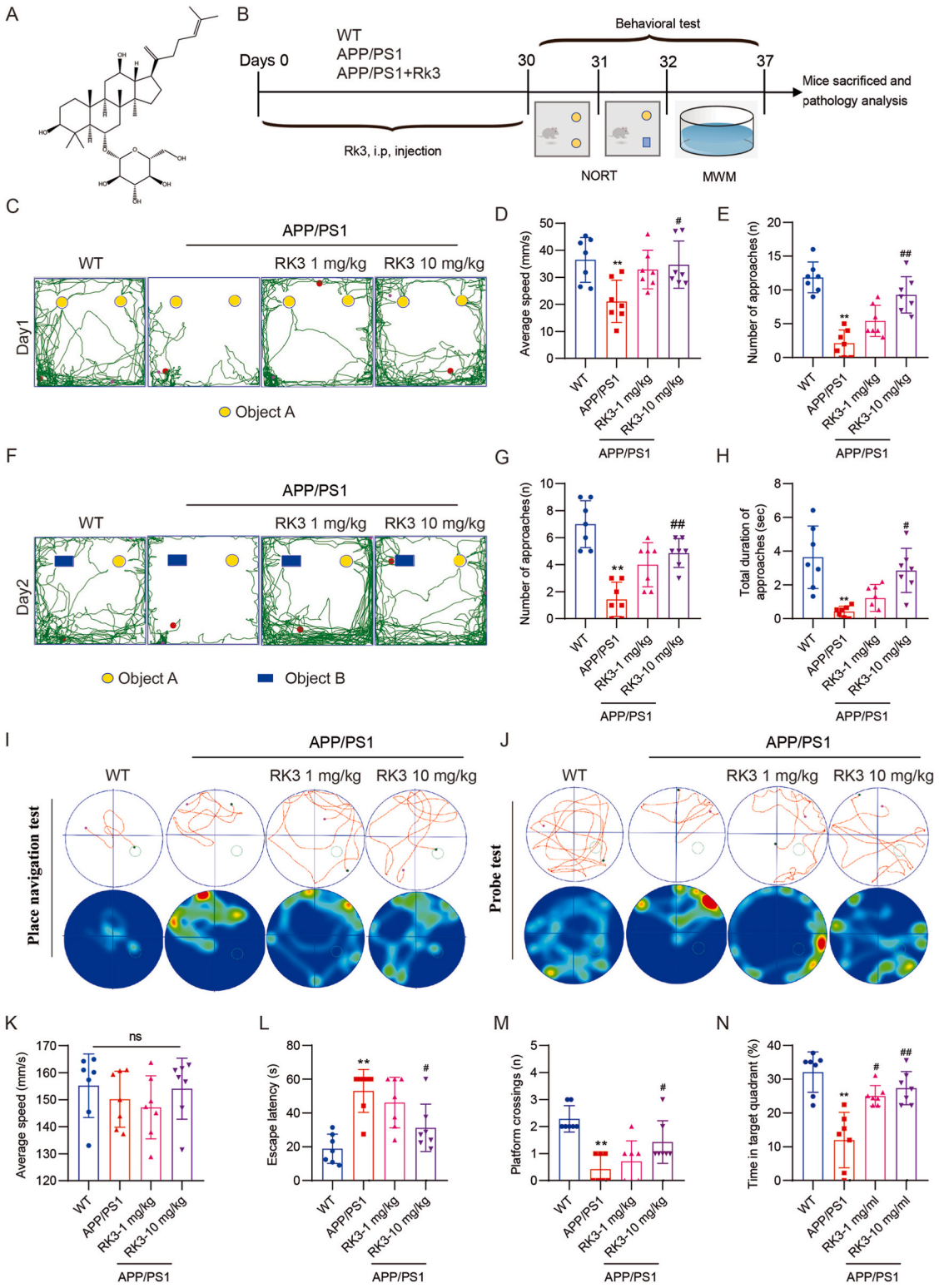

Fig. 1. Effects of Rk3 on behavioral impairments and cognitive deficits in APP/PS1 mice. (A) Chemical structure of Ginsenoside RK3. (B) Experimental design and treatment schedule. (C) Representative image of mice movement trajectories from the first day of NORT. (D) Average speed of the NORT on the first day. (E) The number of times mice touched objects on the first day of the NORT. (F) Representative images of mice movement trajectories for new object recognition experiments on the second day. (G) Number of mice touches on the new object on the second day of the NORT. (H) On the second day of the NORT the degree of liking of the mice to the novel object (time for the mice to touch the novel object). (I) On the fifth day of the MWM experiment, representative track map of mice swimming. (J) On the sixth day of the MWM experiment, after removing the hidden platform, the representative trajectory of the mice swimming. (K) Average speed of mice swimming in the MWM test. (L) On the fifth day of the MWM experiment, the time required for mice to find the hidden platform (escape latency). (M) On the 6th day of the MWM experiment, the number of mice cross-platform areas within 60s. (N) On the sixth day of the MWM experiment, the time the mice spent in the quadrant where the hidden platform was located. Data are presented as mean ± SEM of n = 7 mice per group. *P < 0.05 or **P < 0.01 versus the WT group; #P < 0.05 or ##P < 0.01 versus the APP/PS1 group.

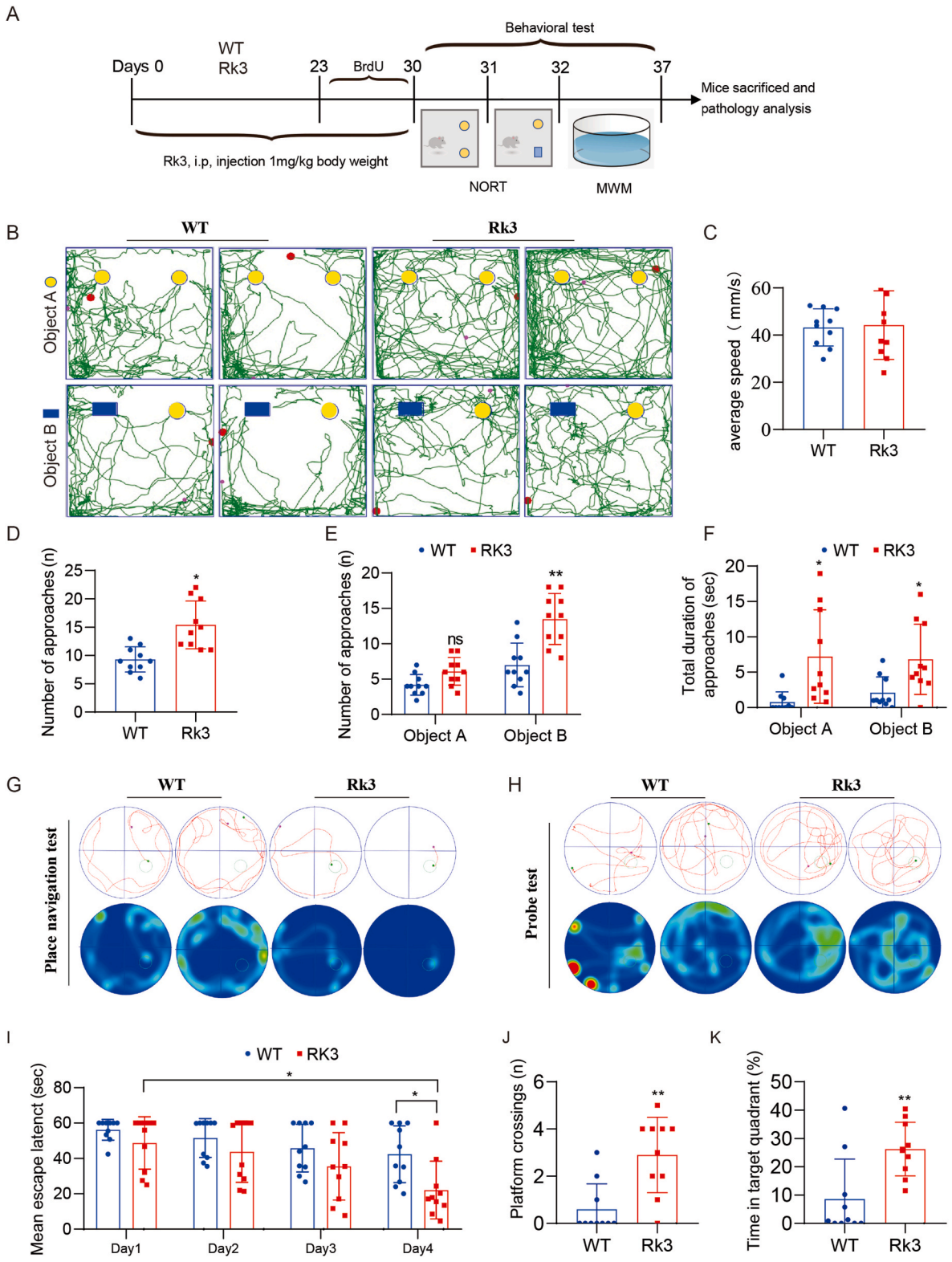

Fig. 2. Rk3 improved the learning and memory ability of 6-month aged mice. (A) Experimental design and treatment schedule. (B) Representative image of mice movement trajectories from the first day of NORT. (C) Average speed of the NORT on the first day. (D) The number of times mice touched objects on the first day of the NORT. (E) Number of touches of old object A and new object B by mice on the second day of the NORT. (F) The exposure time of mice to the novel object and the old object on the second day of NORT. (G) On the fourth day of the MWM experiment, representative track map of mice swimming. (H) On the fifth day of the MWM experiment, after removing the hidden platform, the representative trajectory of the mice swimming. (I) Time required for mice to find the hidden platform on the fourth day of the MWM experiment. (J) On the fifth day of the MWM experiment, after the hidden platform was removed, the number of times mice in each group passed through the platform area within 60 s. (K) On the fifth day of the MWM experiment, the time the mice spent in the target quadrant where the platform was located after the hidden platform was removed. Data are presented as mean ± SEM of n = 10 mice per group. *P < 0.05 or **P < 0.01 versus the WT group.

Alzheimer's disease facts and figures," 2022; Xu et al., 2023). The hippocampus, closely associated with cognitive functions, plays a pivotal role in learning and memory processes (Mu and Gage, 2011). It is considered the primary neurogenic niche within the adult brain (Kempermann et al., 2015). Hippocampal neurogenesis involves the stimulation of neural stem cell (NSC) proliferation within the subgranular zone (SGZ), followed by their differentiation into mature neurons within the dentate gyrus (DG). β-amyloid (Aβ) is produced by cleavage of amyloid precursor protein (APP) by β-secretase and γ-secretase (Wang et al., 2019). Studies have shown that Aβ oligomers may be the most neurotoxic and pathological form in AD, impair synaptic function and cognitive function (Behar et al., 2021). In AD, the formation of Aβ plaques disrupts multiple pathways, resulting in the suppression of neurotrophic signaling and reduced neurogenesis, ultimately impacting memory and cognitive functions (Bahlakeh et al., 2022). This implies the pivotal role of hippocampal neurogenesis in cognition and learning (Y. Liu et al., 2023b). Furthermore, another significant hallmark of the hippocampus related to memory and learning is the structural and functional disruption of hippocampal synapses, synaptic dysfunction, and alterations in neurogenesis patterns. These changes are regarded as early pathological indicators that contribute to the progression of AD (Wei et al., 2022; Zhang et al., 2017).

AD still lacks effective treatments, necessitating innovative therapeutic approaches for AD patients. In recent years, there has been a growing use of natural remedies known for their multifaceted actions, reduced side effects, and enhanced safety profiles in the management of various diseases, including AD (Newman and Cragg, 2016; Xu et al., 2023). *Panax ginseng* Meyer, a traditional Chinese medicine, has been employed medicinally for centuries across Asian countries (Sun et al., 2022; Xie et al., 2018). This revered herb, known for its enduring health benefits and anti-aging properties, finds its earliest endorsement in "*Shen Nong's Herbal Classic*", a text with over 5000 years of history (M. Liu et al., 2019a; Zhang et al., 2021). This ancient record extols ginseng's ability to nurture vital organs and, with prolonged use, to pacify the nervous system, mitigate convulsions, enhance cognitive function, and extend life. Subsequent generations of traditional Chinese medicine embraced this wisdom, incorporating ginseng into health regimens for insomnia, memory lapses, dementia, and other ailments (Feng et al., 2022). Originally harnessed as a sustenance and energy source, ginseng's extensive pharmacological effects, particularly in neurological disorders, have gradually come to light (J. Liu et al., 2023a; Tan et al., 2022). Comprising diverse chemical compounds, ginsenosides, the principal bioactive constituents of ginseng, stand out (Kim et al., 2013). Among these, RK3, a rare ginsenoside derived from ginseng, exhibits remarkable pharmacological efficacy (Bai et al., 2021; Han et al., 2019). While previous investigations have highlighted RK3's protective role in conditions such as kidney injury (Baek et al., 2017), lung cancer (Duan et al., 2017), alcoholic liver disease (Qu et al., 2019), diabetes (Y. Liu et al., 2019), and colitis (Tian et al., 2020), its involvement in neurogenesis - a domain marked by multifaceted pharmacological activities - remains poorly understood.

The PC12 cell line is derived from a transplantable mouse pheochromocytoma. PC12 cells have typical neuronal characteristics and are a commonly used neural cell line for drugs and nervous system-related diseases. They are used to evaluate the neuroprotective effects of drugs (Chua and Lim, 2021; Zhong et al., 2023). Our preliminary experimental results showed that RK3 has the ability to enhance PC12 cell proliferation. Notably, our previous studies have shown that RK3 has the potential to alleviate neuronal apoptosis and oxidative stress (She et al., 2023). We therefore hypothesized: RK3 also stimulate hippocampal neuronal proliferation, thereby improving cognitive performance and learning in mice. To confirm this, we used the Tg-APPswe/PS1dE9 (APP/PS1) model mouse and normal C57 mice to explore the role and mechanism of RK3 on memory impairment and neurogenesis.

2. Methods

2.1. Reagents

Aβ 1–42 was purchased from Ontores Biotechnologies, specific details were listed in Supplementary Table 1. DMEM medium was procured from Sigma. RK3 (95% purity, CAS: 364,779-15-7) was ordered from Chengdu DeSiTe Biological Technology (Chengdu, China). ™Neurobasal Medium (21103049), Gibco™ B-27™ Additive (17504044). FD Rapid Golgi Stain Kit (FD Neuro Technologies, PK401). Fetal bovine serum (FBS) and 0.25% Trypsin were purchased from Life Technologies (Grand Island, NY, USA) were ordered from Calbiochem (San Diego, CA, USA). HifairIII 1st Strand cDNA Synthesis SuperMix for qPCR (gDNA digester plus) kit (Cat. No. 11141ES60, Yeasen). Phosphatase inhibitor cocktail (MCE, HY-K0010/HY-K0021), Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, 23,227). Quantitative polymerase chain reaction (PCR) was performed using the SYBR Green kit (Cat. No.11201ES08, Yeasen). Penicillin/Streptomycin (Carlsbad, CA, USA). MTT (Sigma; M2128). PVDF was bought from Bio-Rad. CREB inhibitor 666–15 (MCE, HY-101120). Bovine Serum Albumin (FD0030). LipofectamineTM 2000 (MACKLIN, C804359). Hydrolytic chloride (Thermo Fisher Scientific, Carlsbad, CA, USA). Antibodies used in this study was presented in Table S2.

2.2. Animal model and drug administration

APPswe/PS1dE9 (APP/PS1) mice (30–32g, female, 8-month-old) were bred at the Animal Laboratory of Hangzhou Medical College and procured from the Jackson Laboratory. They were kept in a controlled environment at a temperature of 24–26 °C with a 12-h light-dark cycle and had unrestricted access to food and water.

To assess the effect of RK3 on the cognitive abilities, the APP/PS1 mice were divided into four groups, each group consisting of seven individuals: the WT group, the APP/PS1 group, the APP/PS1 + RK3 (1 mg/kg) group and the APP/PS1 + RK3 (10 mg/kg) group (Han et al., 2019). The WT and APP/PS1 groups received daily intraperitoneal injections of 1xPBS, while the APP/PS1 + RK3 group received daily intraperitoneal injections of RK3 dissolved in PBS. After one month, when the drug treatment concluded, we conducted two behavioral tests, the novel object recognition test (NORT) and the Morris water maze (MWM), to evaluate the mice's learning and memory capacities. The experimental workflow for Experiment 1 is depicted in Fig. 1B.

To further explore the influence of RK3 on learning and cognition, the six-week-old WT mice were divided into two groups, each comprising ten individuals: the WT group and the RK3 (1 mg/kg) group. The RK3 group received RK3 dissolved in 1xPBS, while the WT group was administered an equivalent volume of 1xPBS. Mice received intraperitoneal injections once daily for one month. During the last seven days of the treatment period, BrdU (a thymidine analogue) was co-administered at a dose of 50 mg/kg. Behavioral assessments were conducted after the dosing regimen was completed. The experimental schedule for Experiment 2 is outlined in Fig. 2A.

2.3. MWM test

In MWM experiments conducted on days 1–4, a hidden platform approximately 1 cm below the water surface was located in the center of a quadrant within the pool. During this phase, the time to find the hidden platform was recorded. On the fifth day of the experiment, the hidden platform was removed, affording the mice a 60 s period of free exploration within the pool, constituting the spatial exploration test. Data acquisition and subsequent analysis were performed utilizing the MWM automated imaging and processing system (VisuTrack, Shanghai, China).

2.4. NORT

Briefly, we utilized the NORT to evaluate the recognition and memory functions of the mice. The experiment commenced with each mouse having 5 min to explore two identical circular objects on the first day. On the subsequent day, one of the circular objects was replaced with a square-shaped one, and each mouse was again given a 5 min exploration period. We recorded both the duration and frequency of each mouse's interactions with these objects during these sessions for analysis.

2.5. Tissue samples preparation

Following the conclusion of the behavioral tests, all mice from different group were euthanized with an intraperitoneal injection of 1% pentobarbital (40 mg/kg). Subsequently, some of the mice underwent brain extraction, which was followed by a 24 h fixation period in 4% paraformaldehyde (PFA). After fixation, specific samples were subjected to a dehydration process, embedded in OCT compound, and stored at −80 °C for future analysis. Brain tissue from another group of mice was also securely stored at −80 °C for later use.

2.6. Golgi staining

After the mouse was euthanized, the hippocampus was meticulously dissected from the brain tissue. Subsequently, Golgi staining was conducted using the FD Fast Golgi Staining Kit following the manufacturer's guidelines. High-resolution images were captured utilizing a laser-scanning confocal microscope.

2.7. Immunofluorescence (IF)

Brain tissue samples were sectioned into 20 μm slices. These sections underwent permeabilization with 0.3% Triton X-100 for 10 min, followed by a 1 h incubation in a 10% BSA solution. Subsequently, they were exposed to specific primary antibodies and left to incubate overnight at 4 °C. The following day, they were treated with the corresponding secondary antibodies. Finally, the sections were mounted using an anti-fading agent that included DAPI.

2.8. Cell culture and treatments

Newborn C57BL/6 (C57) mice brains were used to isolate primary neurons within 24 h. The brain was then meticulously dissected and rinsed with cold 1xHBSS. After precise removal of blood vessels and meninges under a microscope, the brain tissue was finely minced. Subsequently, enzymatic digestion was performed using 0.1% trypsin at 37 °C for 20 min. Upon termination of the digestion process, the cell suspension obtained was filtered through a 0.45 μm pore-sized device and then centrifuged at 1000 g for 5 min to eliminate the supernatant. The cells were subsequently resuspended and seeded on poly-D-lysine-coated plates in Neurobasal Medium with the addition of 1% B27.

PC12 cells were maintained in 10% DMEM medium (comprising 10% FBS and 1% penicillin/streptomycin) in a cell incubator set at 37 °C with 5% CO2. The culture medium was refreshed every two to three days, and cell passages were performed using 0.25% trypsin when they reached 90% confluency.

2.9. Gene knockdown

Specific siRNA is transfected into cells through Lipofectamine 2000 to silence specific genes. CREB siRNA was purchased from Gene Pharmaceutical Co., Ltd. The sequences of CREB siRNA and scrambled siRNA are shown in Table S1. Mix CREB siRNA at a final concentration of 50 nM with 2 μL Lipofectamine2000 in 200 μL serum-free Opti-MEM. After incubation at room temperature for 20 min, the mixture was added to primary neurons in DMEM without FBS, and after 6 h of incubation, it was changed to DMEM containing 10% FBS.

2.10. Aβ oligomer preparation

In this research, we prepared stock solutions of Aβ1-42 in sterile DMSO at a concentration of 10 mM. It's noteworthy that, as previously mentioned, Aβ1-42 tends to aggregate when incubated at 37 °C for 7 days prior to utilization (Friedlander, 2003; M. Liu et al., 2019b; Zhao et al., 2022). Consequently, we subsequently diluted it to a concentration of 10 μM in the culture medium to facilitate its application in the study.

2.11. MTT assay

Briefly, PC12 cells or primary neurons were initially plated in 96-well plates. Once the cells adhered to the well surfaces, they underwent sequential drug treatments. Following drug exposure, MTT (0.5 mg/ml) was added to each well and allowed to incubate for 3–4 h. Subsequently, formazan crystals were solubilized with DMSO, and absorbance was quantified at 570 nm utilizing a microplate reader. Cell viability was determined as a percentage relative to the control group.

2.12. Network pharmacology

Initially, we compiled relevant AD-associated targets by consolidating data from the Human Gene Database (GeneCards). Subsequently, we gathered all pharmacological targets of RK3 from Swiss Target Prediction and SuperPred sources. Following this, we identified the overlapping candidate genes by employing the reviewed (Swiss-Prot) and Human settings within the UniProt database, ensuring data accuracy (R. Li et al., 2021b). To explore pivotal biological processes and pathways, we conducted Genome (KEGG) pathway enrichment analysis (Li et al., 2024).

2.13. Western blot analysis

Cultured cells or brain tissue samples under various treatments were lysed in ice- RIPA lysis buffer supplemented with protease phosphatase inhibitor cocktail. After denaturation at 95 °C, samples were loaded onto an SDS-PAGE gel and transferred to a 0.22 μm PVDF membrane at 300 mA for 1.5 h. The PVDF membrane containing protein bands was blocked with 3% BSA for 1 h at room temperature and incubated with selective primary antibody overnight at 4 °C. Incubate with horseradish peroxidase (HRP)-conjugated secondary antibody for an additional 2 h at room temperature. After ECL exposure, band intensities were quantified using Image J software.

2.14. Statistical analysis

Statistical analyses were performed using GraphPad Prism 8 (GraphPad Software, San Diego, CA). Error bars were presented as mean ± SEM. The statistical significance between multiple groups was determined using one or two-way ANOVA followed by Tukey's post-hoc test. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Effects of RK3 on cognition and learning in mice

To assess whether RK3 (Fig. 1A) administration could treat cognitive deficits in APP/PS1 mice. The administration method and experimental procedure are shown in Fig. 1B. On the first day of NORT, the movement trajectory is shown in Fig. 1C, and APP/PS1 showed obvious slow movement, RK3 treatment increased the movement speed of APP/PS1 mice (Fig. 1D), and significantly improved the exploration of things in

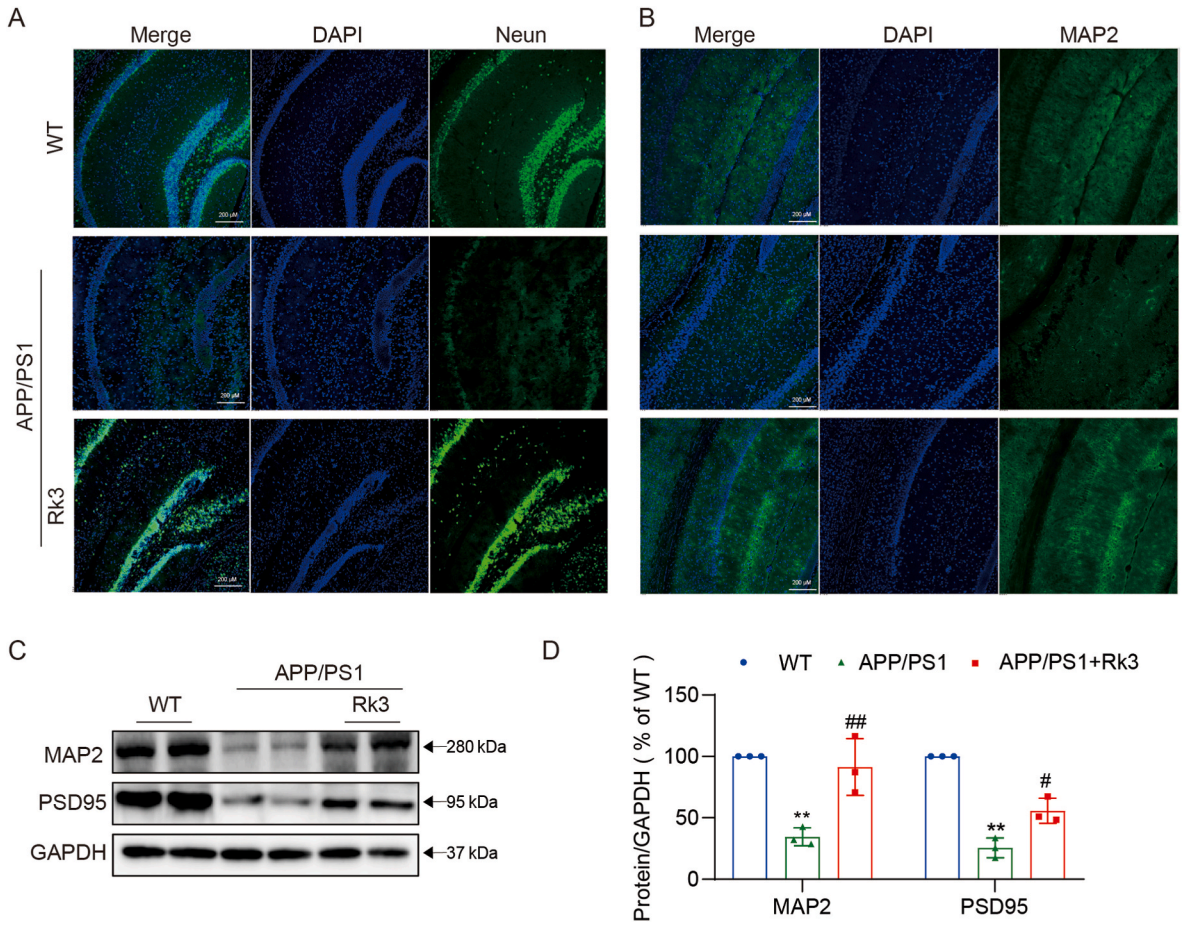

Fig. 3. Rk3 treatment prevents synaptic proteins and neurodegeneration in APP/PS1 mice. (A) Representative images of immunofluorescence staining for Neun (green), DAPI (blue) in the hippocampus of mouse brain tissue [Scale bar = 200 μm]. (B) Representative images of immunofluorescence staining for MAP2 (green), DAPI (blue) in the hippocampus of mouse brain tissue [Scale bar = 200 μm]. (C) Protein expression of MAP2 and PSD95 in mouse brain tissue hippocampus. (D) Quantification of corresponding Western blot intensities. *P < 0.05 or **P < 0.01 versus the WT group; #P < 0.05 or ##P < 0.01 versus the APP/PS1 group. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

APP/PS1 mice (Fig. 1E). On the second day of NORT, the movement trajectory is shown in Fig. 1F. It can be seen from the number of explorations and exploration time of new objects that RK3 treatment improved the recognition and exploration ability of APP/PS1 mice (Fig. 1G and H). MWM for assessing spatial learning and memory in mice. During the training period (from day 1 to day 5), APP/PS1 mice spent longer time than WT group looking for the hidden platform, while compared with APP/PS1 mice, RK3-treated mice had shorter escape latencies to detect hidden platforms during training (Fig. 1I, L). And the change was not due to swimming ability (Fig. 1K). In the probe test (day 6), the number of target quadrant crossings and target quadrant dwell time were significantly reduced in the APP/PS1 group compared with the WT group (Fig. 1J, 1M-N), demonstrating that APP/PS1 mice have cognitive and memory impairment. After RK3 drug treatment, the number of target quadrant crossings and target quadrant residence time were effectively increased. These experimental results indicated that RK3 could improve learning and memory impairment in APP/PS1 mice.

After confirming that RK3 could treat cognitive deficits in APP/PS1 mice, we further explored whether RK3 could improve cognition and learning ability in C57 mice. The experimental procedure is shown in Fig. 2A. On the first day of NORT, RK3 administration significantly increased the number of explorations of objects in C57 mice, while the average speed of the mice remained unchanged (Fig. 2B–D). After the new object was replaced on the second day, it can be seen from the number of touches and the exploration time of the new object that the recognition and short-term memory ability of the mice after RK3 administration was improved (Fig. 2E and F). During the MWM training period (from day 1 to day 4), RK3-administered mice spent less time searching for hidden platforms than WT mice (Fig. 2G and I). In the detection test (day 5), compared with the WT group, the number of target quadrant crossings and the target quadrant residence time in the RK3-administered group were significantly increased (Fig. 2H, 1J-K). It was proved that after administration of RK3, mice had improved cognitive and memory abilities. Taken together, the results of this study suggest that RK3 can improve learning and memory in mice.

3.2. *RK3 promotes neurogenesis and synaptogenesis in the hippocampus of mice*

Neurogenesis and synaptic structure function contribute to hippocampus-dependent learning and memory. To further explore the mechanism by which RK3 improves cognitive deficits in mice, we evaluated the expression of neurons and synapse-related proteins closely related to learning and memory. As assessed by IF staining in brain tissue samples (Fig. 3A), a marked reduction in neuronal expression occurred in the hippocampus of APP/PS1 mice, and RK3 administration significantly ameliorated the reduction in neuronal expression in the APP/PS1 hippocampus (Supplementary Fig. 2 A and 2 B). Expression of the dendritic marker microtubule-associated protein 2 (MAP2) and the postsynaptic marker postsynapticdensityprotein95 (PSD95) were further examined to explore changes in excitatory synapses associated with dendritic and spine alterations. Both MAP2 and PSD95 are

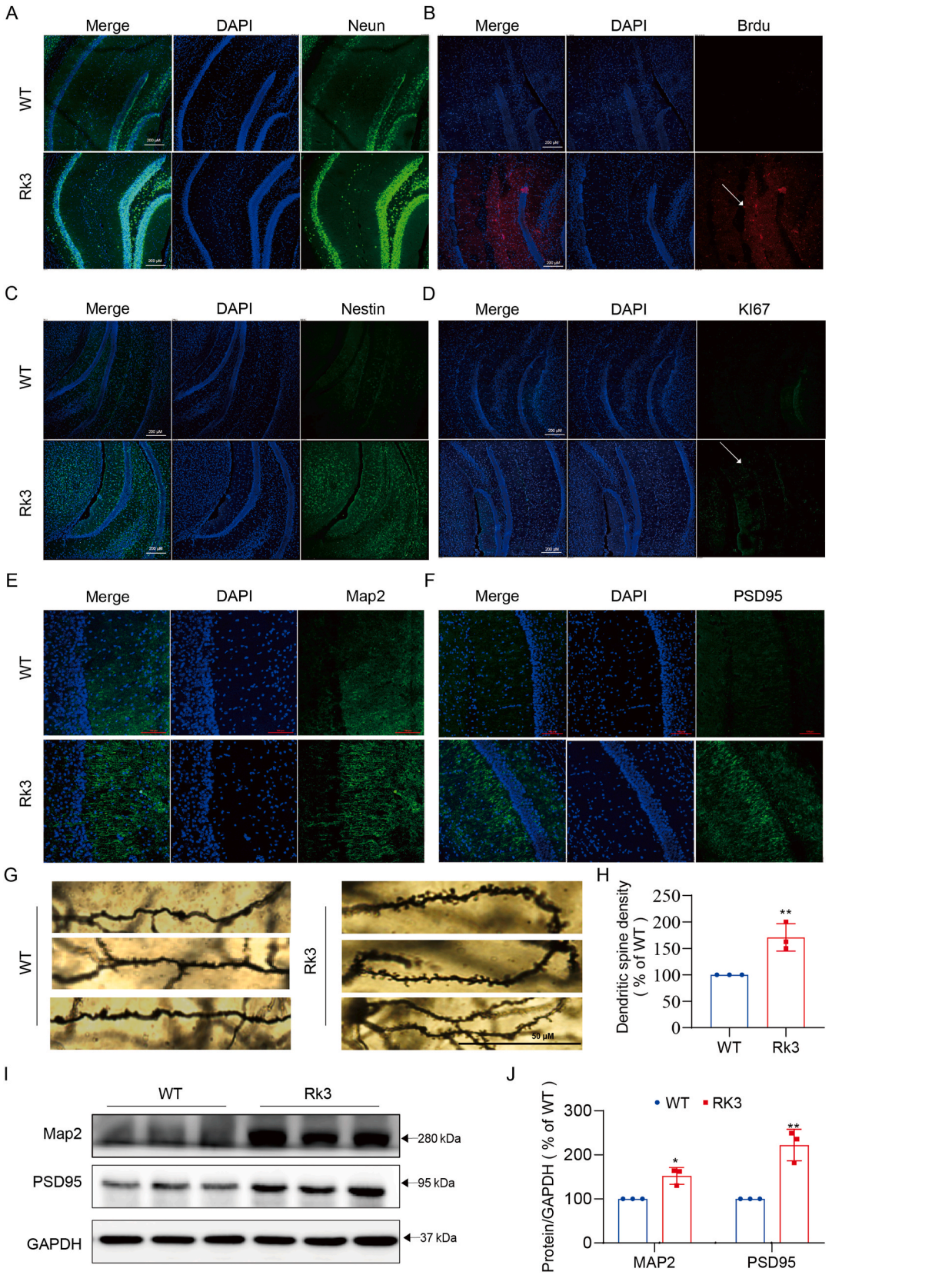

Fig. 4. Rk3 treatment promotes synaptogenesis and neurogenesis in mice. (A–F) Representative images of immunofluorescence staining of mouse brain tissue hippocampus [scale bar = 200 μm]. (G–H) Golgi staining results. (G) Representative photomicrographs of Golgi apparatus staining experiments in the hippocampus of mice brain tissue. (H) Total spines number per 50 μm area. (I) Protein expression of MAP2 and PSD95 in mouse brain tissue hippocampus. (J) Quantification of corresponding Western blot intensities. *P < 0.05 or **P < 0.01 versus the WT group.

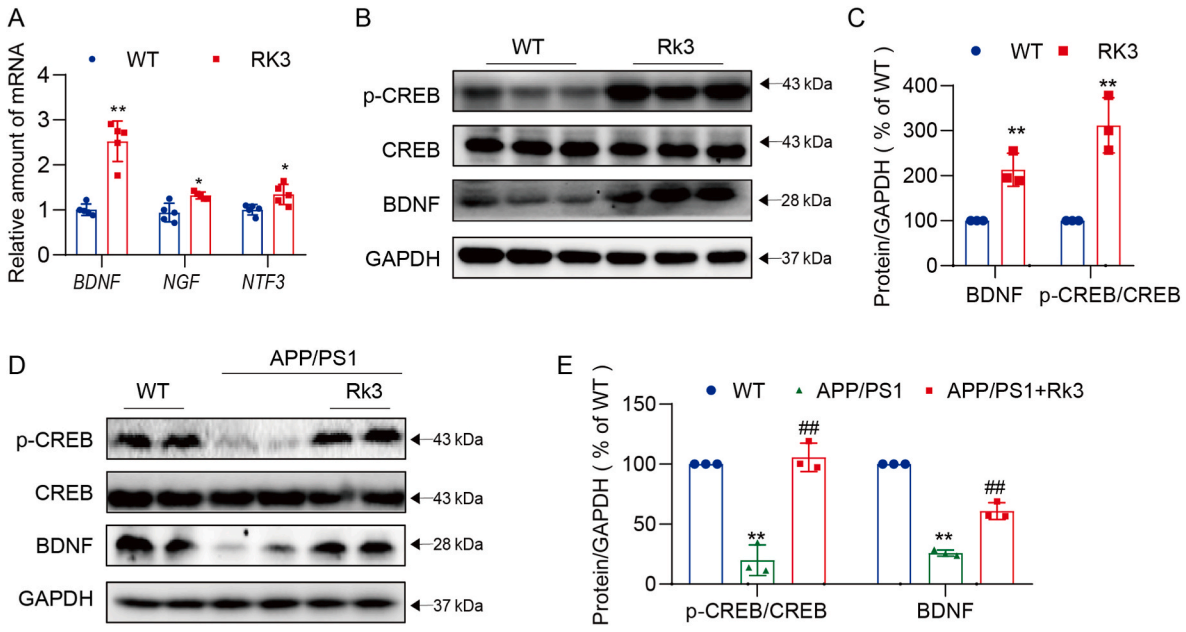

Fig. 5. Neuroprotection of RK3 in mice through activation of the CREB/BDNF pathway. (A) The mRNA levels of BDNF, NGF and NTF3 in the hippocampus of mouse brain tissue. (B) Protein expression of p-CREB, CREB, and BDNF in mouse brain tissue hippocampus. (C) Quantification of corresponding Western blot intensities. (D) Protein expression of p-CREB, CREB, and BDNF in mouse brain tissue hippocampus. (E) Quantification of corresponding Western blot intensities. *P < 0.05 or **P < 0.01 versus the WT group; #P < 0.05 or ##P < 0.01 versus the APP/PS1 group.

important markers of synaptogenesis and play a crucial role in synaptic plasticity. RK3 administration also significantly reversed synaptic damage in APP/PS1 mice (Fig. 3B). It was further confirmed by WB experiment that compared with the WT group, the expression of MAP2 and PSD95 in APP/PS1 mice was significantly reduced, and the expression of MAP2 and PSD95 in APP/PS1 mice was significantly increased after RK3 treatment (Fig. 3C and D). These results demonstrate that RK3 treatment improves neuronal and synaptic damage in AD models.

The role of RK3 on neurogenesis and synaptogenesis in C57 mice was next explored. RK3 administration significantly increased expression in neurons in the hippocampus (Fig. 4A and Supplementary Fig. 2 C). As reduced neurogenesis could be due to effects on proliferation, differentiation or survival that underlie neurogenesis, we labeled dividing cells by using: 5-bromodeoxyuridine (Brdu). RK3 increases the number of Brdu cells in the mouse hippocampus (Fig. 4B and Supplementary Fig. 2 D). To validate the Brdu result, the levels of endogenous markers Nestin and Ki 67 in proliferating cells were examined. The result was similar to the Brdu result (Fig. 4C and D and Supplementary Fig. 2E and F). These data suggest that RK3 administration promotes hippocampal neurogenesis in adult mice.

Next, we further explored whether the phenotypic improvement after RK3 administration was related to synaptic changes. Golgi staining was performed in the CA1 region of the hippocampus to examine dendritic spine density in mice. RK3 administration significantly increased dendritic spine density in mice compared with control mice (Fig. 4G and H). IF experiment confirmed that RK3 administration significantly promoted synaptic growth (Fig. 4E and F and Supplementary Fig. 2G and H), and WB experiment further confirmed that RK3 administration promoted the expression of MAP2 and PSD95. These results indicated that RK3 administration promoted the improvement of synapse-associated protein expression and synaptic density. Taken together, RK3 promotes learning and memory in mice by promoting neurogenesis and synaptogenesis.

3.3. RK3 activates the CREB/BDNF pathway

We investigated the mechanism behind RK3 treatment of AD neurogenesis. First, we collected 14,386 AD-related genes from the Genecard database and identified the pharmacological targets of RK3 using the Swiss Target Prediction software and the SuperPred database. After biocorrection and duplicate gene removal using the UniProt database, we obtained 246 RK3-related targets (Supplementary Fig. 3A). We then identified 118 crossover genes shared by AD and RK3-related targets, and KEGG enrichment analysis of the 118 crossover genes showed that the revealed pathways included neurotrophin signaling pathway and cAMP signaling pathway (Supplementary Fig. 3B). Neurotrophic factors (NTFs) are involved in the regulation of neuronal function, including several key aspects of neurogenesis, synaptic plasticity, and neuroprotection. NTFs include nerve growth factor (NGF), BDNF, and neurotrophin-3 (NT-3) (Nordvall et al., 2022). Therefore, we further determined whether the expression levels of BDNF, NGF and NTF3 changed after RK3 administration. Compared with the WT group, RK3 administration significantly promoted the expression of neurotrophic factors, but the most significant effect was on BDNF (Fig. 5A). In the cAMP signaling pathway, phosphorylation of CREB promotes the transcription of BDNF neurotrophin, which in turn promotes neurogenesis (Huang et al., 2021; Weng et al., 2023). Therefore, we explored whether RK3 promoted the phosphorylation level of CREB, thereby promoting the expression of BDNF. RK3 administration activated p-CREB and promoted BDNF expression, both in WT and APP/PS1 mice (Fig. 5B–E).

3.4. RK3 protects primary neurons through the CREB/BDNF pathway

To further verify the protective effect of RK3 in vitro, we extracted primary neurons, presented neurons are shown in Fig. 6A. As expected, RK3 protected neurons from Aβ damage (Fig. 6B). To verify that RK3 is related to neuronal growth, we proceeded to stain the synapses of primary neurons with PSD95. Synapse outgrowth in primary neurons is protected after RK3 pretreatment (Fig. 6C). These results indicate that RK3 has a protective effect on the survival and synapse growth of primary neurons. Next, we further explored the mechanism of this protective effect, detected the expression of p-CREB, CREB, and BDNF, and found that RK3 promoted the expression of BDNF by activating the phosphorylation of CREB (Fig. 6D–F). 666–15, as an inhibitor of CREB,

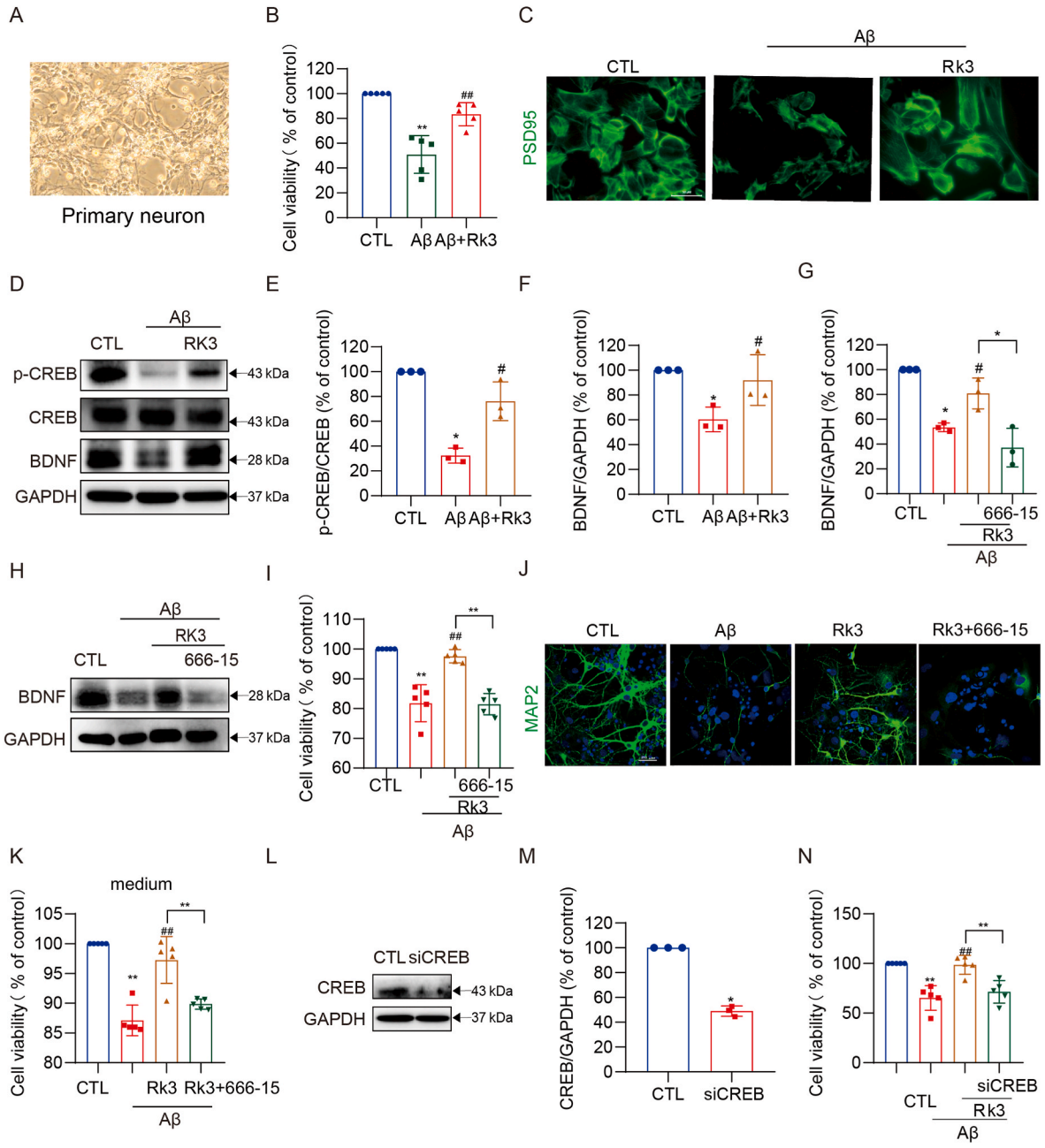

Fig. 6. RK3 protects synaptogenesis in primary neurons by activating CREB/BDNF. (A) Flow chart of primary neuron extraction. (B) Primary neuron. (C–G) Primary neurons were pretreated with 1.25 μM RK3 for 24h and then induced with or without 10 μM Aβ1-42 for another 24h. (C) Cell viability was measured using the MTT assay after drug treatment. (D) Primary neurons were stained with PSD 95 antibody. Scale bar = 50 μm. (E) Protein expression of p-CREB, CREB, and BDNF in mouse brain tissue hippocampus. (F–G) Quantification of corresponding Western blot intensities. (G–N) Primary neurons were pretreated with 5 μM 666–15 for 1 h. Afterwards, 1.25 μM RK3 was used to treat for 24 h. Finally, primary neurons were exposed to 10 μM Aβ for 24 h. (A) Primary neurons were stained with MAP2 antibody. (G) Protein expression of BDNF in primary neuron. (H) Quantification of corresponding Western blot intensities. (I) Cell viability was measured using the MTT assay after drug treatment. Scale bar = 20 μm (J) Primary neurons were stained with MAP2 antibody. (K) The drug-treated primary neuron medium in Fig. 7(D) was added to another primary neuron. Cell viability was measured using the MTT assay. (L–M) Protein expression of CREB in primary neuron and quantification of corresponding Western blot intensities. (N) Cell viability was measured using the MTT assay. *P < 0.05 or **P < 0.01 versus the CTL group; #P < 0.05 or ##P < 0.01 versus the Aβ group.

was selected by us to verify the protective effect of RK3 through CREB/BDNF. As expected, RK3 reversed the Aβ-induced decrease in BDNF expression, while 666–15 pretreatments also inhibited BDNF expression (Fig. 6G and H). 666-15 blocked the protective effect of RK3 on viability and synapses in primary neurons (Fig. 6I and J). To assess the effects of mediators released by neurons on neurons. Add the medium treated in Fig. 6G to another primary neuron to determine whether RK3 has a protective effect. It was found that RK3 pretreated medium could protect primary neurons from Aβ-induced damage, while 666–15 pretreatment blocked the protective effect of RK3 (Fig. 6K). For further verification, we selected CREB siRNA to knock out CREB, and the knockout efficiency is shown in Fig. 6L-M. Consistent with the results of 666–15 pretreatment, the knockdown of CREB reversed the protective effect of RK3 against Aβ induction (Fig. 6N). It shows that RK3 plays a role through the CREB/BDNF pathway.

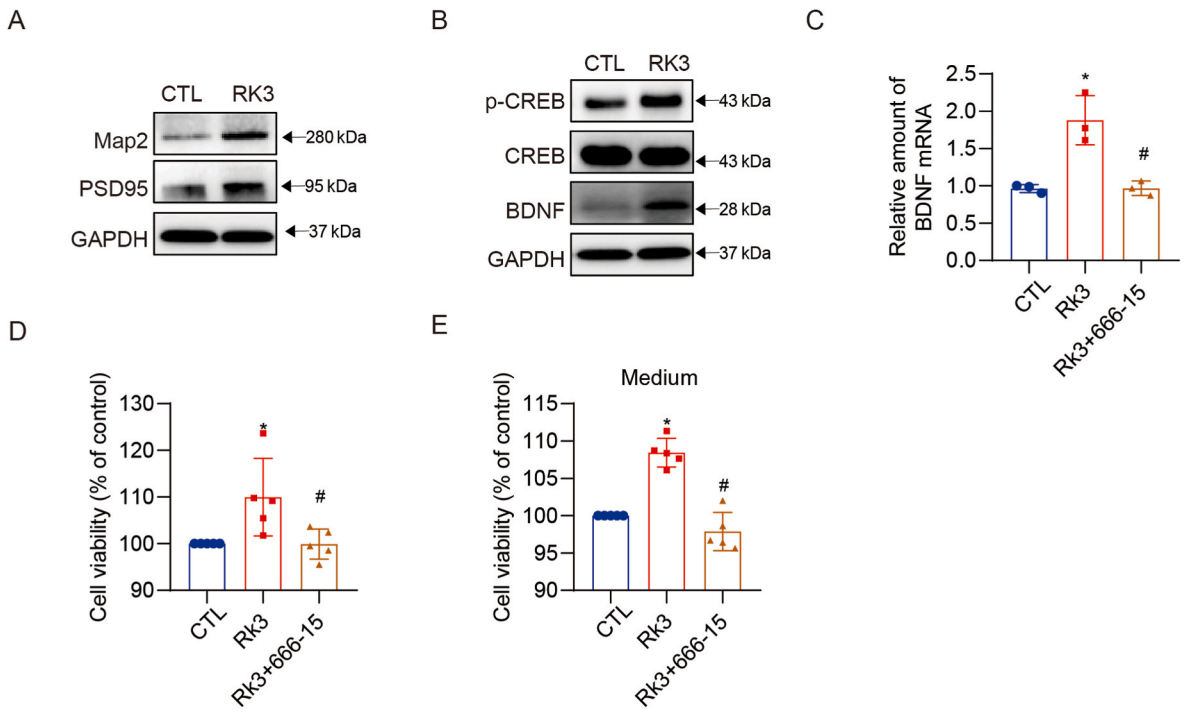

Fig. 7. The protective effect of RK3 on PC12 cell is reversed by inhibition of CREB. (A) Representative Western blot analysis of MAP2 and PSD95 in PC12 cell. GAPDH was used as the loading control. (B) Representative Western blot analysis of p-CREB, CREB and BDNF in PC12 cell. GAPDH was used as the loading control. (C) The mRNA levels of BDNF in the PC12 cell. (D) Cell viability was measured using the MTT assay. (E) Add the PC12 medium in Fig. 7 (D) to another PC12 cell. Results are presented as mean ± SEM (n = 3). *P < 0.05 or **P < 0.01 versus the CTL group; #P < 0.05 or ##P < 0.01 versus the RK3 group.

3.5. RK3 promotes the proliferation of PC12 cells

PC12 cells were used to further validate the results in primary neurons. RK3 also promotes the expression of MAP2 and PSD95 in PC12 cells through the CREB/BDNF pathway (Fig. 7A and B). After adding 666–15, RK3 promoted the mRNA expression level of BDNF in PC12 cells, and 666-15 blocked this effect (Fig. 7C). RK3 administration significantly promoted cell proliferation, while 666–15 treatment inhibited RK3-induced cell proliferation (Fig. 7D). We obtained similar results when exploring the effect of mediators released by PC12 on additional PC12 cells (Fig. 7E). In summary, RK3 promotes PC12 cell proliferation through the CREB/BDNF pathway.

4. Discussion

In this study, we found that RK3 promotes neurogenesis, thereby enhancing learning and memory abilities in APP/PS1 mice and normal WT mice. *In vitro*, RK3 can promote cell proliferation of primary neurons and PC12 cells while exerting a protective effect against Aβ-induced neurotoxicity. RK3 administration can promote gene expression in the CREB/BDNF pathway, and the effect of RK3 is reduced after silencing CREB. Our findings support further exploration of RK3 as a potential drug candidate for AD prevention or treatment.

In AD, patients typically experience progressive memory loss and severe cognitive impairment, ultimately leading to inevitable mortality ("2022 Alzheimer's disease facts and figures," 2022). Despite extensive research efforts aimed at discovering strategies for AD prevention and cure, there is still a critical unmet need for therapies that can effectively decelerate the progression of neurodegeneration in AD. Ginseng, a traditional Chinese herbal remedy with a dual role in medicine and cuisine, exhibits neuroprotective effects within the complex pathological framework of AD (Kim et al., 2018). The primary bioactive components in Panax ginseng, ginsenosides, demonstrate the ability to safeguard neurons both in controlled laboratory conditions and living organisms. They achieve this by orchestrating vital biological processes, encompassing the mitigation of oxidative stress, counteracting excitotoxicity, preventing apoptotic neuronal demise, and regulating kinase and ubiquitin-proteasome signaling pathways (Cho, 2012).

The principal ginsenosides found in ginseng, namely Rb1, Rb2, Re, Rd, and Rc, function to inhibit the activity of β-secretase, leading to a subsequent reduction in Aβ production (Li et al., 2023; Tan et al., 2022; Yang et al., 2020). Additionally, ginsenosides Rg1 and Rh2 play integral roles in the regulation of the APP pathway (Sun et al., 2022). Notably, ginsenoside Rd has been reported to indirectly regulate tau hyperphosphorylation (J. Li et al., 2021a). Furthermore, ginsenoside Rb1 exhibits the capability to enhance BDNF expression, stimulate the proliferation, and facilitate the differentiation of endogenous neural stem cells. This multifaceted action ultimately leads to the amelioration of cognitive function in mice with AD (Zhao et al., 2018). Despite the structural diversity among numerous ginsenosides, they exert distinct and varying protective effects across a spectrum of disease models. It is imperative to conduct further investigations into the molecular targets and mechanisms of action associated with individual ginsenosides and even ginseng extracts in order to comprehensively comprehend their biological activities.

The hippocampus, a brain region pivotal for learning and memory, exhibits heightened susceptibility to early-stage damage in AD (Zhang et al., 2023). Neurogenesis within the brain's hippocampus holds a pivotal role in cognitive processes, with its extent diminishing as age progresses (Ren et al., 2023). In the course of neuronal development, the establishment of synaptic connections forms the bedrock of central nervous system function, representing a pivotal stride in memory development. Hence, augmenting both neurogenesis and synaptogenesis emerges as a promising therapeutic strategy for mitigating cognitive impairment linked to AD (Y. Liu et al., 2023b). In this study, we consequently investigated the potential of RK3 in promoting neurogenesis.

Neurotrophins comprise a diverse family of proteins that contribute to a variety of cellular processes. Transduction of these factors into the NSCs niche activates regulatory mechanisms for neurogenesis, synaptic

plasticity, and learning and memory improvement (Bahlakeh et al., 2022; Ribeiro and Xapelli, 2021). We tested three neurotrophic factors BDNF, NTF3, and NGF, among which RK3 had the most significant effect on BDNF (Fig. 5A). BDNF also plays a crucial role in neuroprotection and synaptic plasticity (Komander, 2010; Pak et al., 2022). Phosphorylation of CREB regulates the expression of BDNF to affect the survival of neurons. In this study, we also found that RK3 activates the CREB/BDNF pathway in vitro and in vivo. However, whether RK3 directly activates CREB needs to be further explored. Moreover, the study of a single mechanism of action is not enough to fully understand the pharmacodynamics and mechanism of RK3 on AD. Therefore, proper multi-mechanism studies are needed to thoroughly explore the mechanism of action of RK3 in AD.

5. Conclusion

In conclusion, this study demonstrated that RK3 promotes learning and cognition in APP/PS1 and C57 mice by promoting neurogenesis and synaptogenesis, and we also showed that RK3 mainly plays a role based on CREB/BDNF. In summary, the above results prove that RK3 has a protective ability in nerves, so RK3 is expected to be further developed into a potential drug candidate.

Ethics statement

Institutional Review Board Statement: The animal study protocol was approved by Hangzhou Medical College (2022–001).

CRediT authorship contribution statement

Lingyu She: participated in the drafting of the article, carried out the experiments. **Hao Tang:** carried out the experiments. **Yuqing Zeng:** carried out the experiments. **Liwei Li:** carried out the experiments. **Li Xiong:** carried out the experiments. **Jinfeng Sun:** carried out the experiments. **Fan Chen:** carried out the experiments. **Juan Ren:** carried out the experiments. **Jing Zhang:** carried out the experiments. **Wei Wang:** contributes to methodology. **Xia Zhao:** contributed to the literature search and study design, contributes to manuscript modification. **Guang Liang:** contributed to the literature search and study design, contributes to manuscript modification.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

The study was supported by a grant from Zhejiang Provincial Key Scientific Project (2021C03041 to G.L.), Natural Science Foundation of Zhejiang province (LQ23H090018 to X.Z. and LTGY23H090001 to W. W.) and Hangzhou medical college (00004F1RCYJ2109 to X.Z.).

Abbreviations

Alzheimer's disease AD
APPswe/PSEN1dE9 APP/PS1
β-amyloid Aβ
Brain-derived neurotrophic factor BDNF
C57BL/6 C57
cAMP-response element binging protein CREB
4′,6-Diamidino-2′-phenylindole DAPI
Dentate gyrus DG
Dulbecco's modified Eagle's medium DMEM
Microtubule-associated protein 2 MAP2
Immunofluorescence IF
Horseradish peroxidase HRP
3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide MTT
Morris water maze MWM
Small interfering RNA siRNA
Namely nerve growth factor NGF
New Object Recognition Test NORT
Neural stem cells NSCs
Neurotrophin-3 NT-3
Neurotrophic factors NTFs
Paraformaldehyde PFA
Poly vinylidene fluoride PVDF
Ginsenoside RK3 RK3
Subgranular zone SGZ
Neuronal Nuclei NeuN

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jep.2023.117462.

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