

The Molecular Mechanism of Scutellaria Barbata Flavonoids in Promoting Neurogenesis and Improving Memory Impairment Mediated by CREB Phosphorylation in Rats

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Research Article

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Abstract

Background

The present study was aimed to investigate the effect and molecular mechanism of *Scutellaria barbata* flavonoids (SBFs) in promoting neurogenesis and improving memory impairment mediated by CREB phosphorylation in rats.

Methods

Alzheimer's disease (AD) model was established by intracerebroventricular injection of amyloid beta-peptide 25–35 ($A\beta_{25-35}$) in combination with aluminum trichloride ($AlCl_3$) and recombinant human transforming growth factor- $\beta 1$ (RHTGF- β_1) (composited $A\beta$) in rats. The Morris water maze was used to screen the successful AD model of rats. The screened successful AD model rats were randomly divided into three groups including a model group and two drug groups of 140 mg/kg SBFs and 0.5 mg/kg Rolipram (Positive control drug). After administration for 38 days, the Morris water maze test was used to measure the learning and memory ability of the rats. HE staining was used to observe the morphology of neurons in the hippocampus and cerebral cortex regions of the rats' brains. Immunohistochemistry was used to detect the expression of NeuN in the hippocampal gyrus of rats. The mRNA expression of TrkB, RSK, CREB, and BDNF and the protein expression of NeuN, TrkB, RSK, P-CREB-Ser133, and BDNF in the hippocampus and cerebral cortex of the rats were assayed by quantitative real-time PCR (qPCR) and Western blotting methods.

Results

Intracerebroventricular injection of composited $A\beta$ induced memory impairment, impaired neurons, decreased the protein expression of NeuN in the hippocampal gyrus, increased the mRNA expression levels of TrkB, RSK and BDNF, decreased the mRNA expression level of CREB and the protein expression levels of NeuN, TrkB, RSK, P-CREB-Ser133 and BDNF in the hippocampus and cerebral cortex of rats. However, SBFs attenuated memory impairment which was induced by composited $A\beta$ in rats and ameliorated neuropathological changes in the brain, increased the expression of NeuN protein in the hippocampal gyrus, and regulated mRNA and protein expressions in composited $A\beta$ treated rats. Rolipram ameliorated learning and memory disorder induced by composited $A\beta$, increased the protein expression of P-CREB-Ser133, increased the expression of NeuN protein in the hippocampal gyrus, promoted neurogenesis, and improved the neuropathological changes in the brain. The effect of SBFs was also similar to Rolipram.

Conclusion

The effect of SBFs was consistent with the positive control drug Rolipram. SBFs could promote neurogenesis and improve learning and memory impairment in AD rats, and its mechanism was mediated by CREB phosphorylation.

Background

Alzheimer's disease (AD) is a common type of dementia. The main clinical symptoms of AD are manifested as progressive cognitive impairment and mental and behavioral abnormality [1]. The characteristic pathological changes in AD include neuronal loss, and the reduction of neurogenesis [2]. It is reported that CREB phosphorylation can promote neurogenesis and compensate damaged neurons, which is of great significance for improving cognitive impairment [3]. Several reports have suggested that BDNF-RSK-CREB signal pathway involved in neurogenesis, and plays a significant role. The molecules of BDNF, TRKB, and RSK, in this signaling pathway, activate CREB phosphorylation through a cascade reaction. Phosphorylated CREB induces the expression of downstream neurotrophic factors such as NGF, BDNF, and Egr-1, promotes neurogenesis, improves learning and memory disorders, and plays an anti-AD role [4]. In this study, intraventricular injection of composited A β to established AD model rat. A β_{25-35} produces neurotoxicity, aluminum inhibits the transformation of deposited A β into soluble A β , and RHTGF- β_1 promotes the deposition of A β and induces the occurrence of AD [5]. This multi-factor induced nerve damage and dysfunction highly mimic the pathophysiology and clinical manifestations of AD.

Many studies have shown that Chinese medicine has a potential value on the treatment of AD [6]. Scutellaria barbata flavonoids (SBFs), extracted from the aerial parts of Scutellaria barbata, in which scutellarin is the main component, have been proved to exert antipyretic, anti-inflammatory, antioxidant, and neuroprotective effects, and other pharmacological activities [7, 8]. However, it has not been reported whether the mechanism of SBFs in improving cognitive function impairment and neurogenesis reduction resulting from composited A β is related to CREB phosphorylation.

In this study, male SD rats were administered an intracerebroventricular injection of A β_{25-35} in combination with AlCl₃ and RHTGF- β_1 to establish the AD model. Rolipram, a CREB phosphorylation activator, was used as the positive control drug to explore whether the effect of SBFs is consistent with Rolipram in activating CREB phosphorylation and regulating the BDNF-RSK-CREB signaling pathway in promoting neurogenesis and ameliorating learning and memory disorder through CREB phosphorylation.

Materials

Animals

Fifty Specific pathogen free (SPF) male SD rat (weighing 280 $\pm$ 40g) were supplied by Beijing Huafukang Biotechnology Co., Ltd (SPF grade, Certification NO SCXK (Jing) 2019-0008). The rats were housed in a room with a warm room, under controlled a room temperature of 22-24°C, relative humidity of 55-60%, with 12 h of daily light and free access to food and water. The rats were allowed to acclimatize to the

laboratory environment for one week. Humanistic care for all rats was provided according to the guidelines of ethical review of animal welfare, and the protocol of experiments was supported by the Ethics Committee of Chengde Medical College, and the approval number CDMULAC-20190901-018.

Drug preparation and reagents

SBFs were prepared by the Institute of Chinese Medicine at Chengde Medical College. The aerial part of *Scutellaria barbata* was soaked in 80% ethanol for 12 h, and then heat reflux extraction was performed. Vacuum concentration was done until there was no alcohol smell, the pH was adjusted to 2 with concentrated hydrochloric acid, centrifuged and the supernatant was discarded to acquire SBFs, which was dried up at 60°C. Scutellarin was used as a reference substance. Ultraviolet spectrophotometry $\lambda=285$ nm was used to measure the purity of SBFs which was 81.65%. SBFs was dissolved with saturated sodium bicarbonate and the pH was adjusted to 7.2-7.4. Then, the mixture was diluted to the required concentration with sterilization by normal saline with 18% cyclodextrin containing 10% DMSO (Fig. 1). shows the original plant, dry herb, and SBFs extraction. Rolipram (Cat. R871830) was provided by Shanghai McLin Biochemical Technology Co., Ltd. of China. Rolipram was dissolved in normal saline with 18% cyclodextrin containing 10% DMSO and diluted to the desired concentration. $A\beta_{25-35}$ (Cat. MB10445-1) was supplied by Dalian Meilun Biotechnology Co. Ltd., China. RHTGF- β_1 (Cat. 100-21) was purchased from PEPROTECH Co. Ltd., China. $AlCl_3$ was provided by Tianjin Fuchen Chemical Reagent Co. Ltd., China. Antibodies BDNF and TRKB (Cat. BS90133, BS1431) were purchased from Bioword Technology, Inc., USA. Antibody P-CREB-Ser133 (Cat. #13323) was supplied by SABC Technology, Inc., USA. Antibody RSK (Cat. ab32114) was provided by Abcam Co. Ltd., China. Antibody NeuN (Cat. BM4354) was purchased from BOSTER Biotechnology Co. Ltd., China. BDNF, TRKB, RSK, and CREB Real-Time primer (Cat. NM_001270630.1-F/R, NM_001163168.2-F/R, CJAH172-01.02-F/R, NM_031107.1-F/R) were provided by Dalian Bao Bioengineering Co. Ltd., China. Rabbit IgG-Immunohistochemistry Kit (Cat. SA1022) was purchased from BOSTER Biotechnology Co., Ltd., China. TB GreenTM Premix Ex TaqTM and Prime Script RT reagent Kit were provided by TaKaRa Company.

Experimental design

Fifty healthy male SD rats were randomly divided into a sham operated group (10 rats) and a composited $A\beta$ -treated group (40 rats) and were accepted adaptive feeding for 1 week, then used to establish AD model. On day 45 of the operation, the Morris water maze test was used to screen successful AD models. On day 49 after the operation, the successful model rats were randomly divided into three groups, including the model group, 140 mg/kg SBFs group and 0.5 mg/kg Rolipram group. The rats were administrated of drugs for 38 days. On day 79 to 85 of operation, the Morris water maze test was used to detect learning and memory ability, and swimming speed in rats. On day 86 of the operation, all the rats were decapitated under anesthesia. The brain of three rats from each group was used for HE staining and immunohistochemistry detection. The brain tissues of other rats were stored in a -80°C refrigerator, which was used for testing of relevant indicators (Fig. 2).

Establishment of AD model rats

According to the surgical methods of Xiaoguang Wu [9], the rats were anesthetized with isoflurane inhalation and fixed on the brain stereotaxic apparatus. Taking the bregma as the point of origin, the first point [posterior: 2.0 mm to the bregma, lateral (L): 1.4 mm to the midline, depth: 4.6 mm], the second point [posterior (P): 0.8 mm to the bregma, lateral (L): 2.0 mm to the midline, depth: 4.6 mm], and the third point [Front (F): 2.0 mm to the bregma; lateral (L): 1.5 mm to the midline] were marked. Day 1 of the operation, RHTGF- β 1 (10 ng) was injected at the first point and fixed with screws, the catheter was inserted at the second point and the third hole was fixed with screws. The denture base material was used to cover the exposed skin incision. Day 2 of the operation, the operated group rats were microinjected with 1 μ l (4 μ g) A β ₂₅₋₃₅ in the morning for 14 days and 3 μ l AlCl₃ (1%) in the afternoon for 5 days. The rats of the sham group were subjected to the same operation and were microinjected with the same amount of saline. After the last microinjection of A β ₂₅₋₃₅, the mixture of a denture base, cannula, and miniscrew was removed from all rats and the skin of the head of rats was sutured.

Experimental equipment

Morris water maze is a circular pool of stainless steel 120 cm in diameter and 50 cm in depth. The pool was divided into four quadrants, and different markers were pasted on the inner wall of each quadrant, which provided a spatial reference for experimental animals. A platform was placed in the quadrants (Q3) as the target quadrant. In the experiment, water was injected into the maze, and edible melanin was added to make the water black. The platform was hidden 1.5 cm under the water surface, and the water temperature in the pool was maintained at 22 $\pm$ 1°C. All markings around the pool were maintained throughout the experiment. A camera and an acquisition card are connected with the computer, which was used to collect and analyze the data.

Test method

Before the formal test of the water maze, all rats were allowed to swim in the Morris water maze for 180 s to acclimatize to the water environment. In the formal test, the rats were tested at the two test points twice a day, and the average latency of the test during the two-point trial in the morning and two-point trial in the afternoon per day was taken as the intraday learning score. In the two test points, the rats respectively faced the pool wall of the platform and were placed into the water once from the distal end and once from the proximal end. The rats were allowed to have a 10 s rest between the two trials. Every rat was allowed to swim in the water maze within 60 s, and the latency was defined as the time taken from entering the water to finding the platform.

Screening of successful AD model rats

In this test, the platform was placed in quadrant IV, which was the target quadrant. The test was conducted for 4 consecutive days, and the performance of day 4 in Morris water maze test were used as index of the successful model screening. The screening rate (SR) was defined as (A-B)/B $\times$ 100%, the

latency of each rat with an intracerebroventricular injection of composited A β to find the hidden platform was regarded as "A", and B is the latency for the rats in the sham group to find the hidden platform. When the SR was greater than 0.2 for a composited A β rat, that rat was considered a successful AD model rat.

Detection of the learning and memory ability of rats

The Morris water maze test was divided into four parts, including positioning navigation trial, probe trial, opposite platform trial, and visible platform trial, which were used to measure memory acquisition, memory retention, memory re-learning ability, and swimming speed of the rats, respectively. The administration of the drug was continuously performed during the test period.

Determination of the rat's memory acquisition ability

The trial of day 1 and 2 was used to measure the memory acquisition ability by positioning navigation trial. The platform was placed in the same position as at model screening (quadrant IV), the latency of rats to find the platform was recorded, and the average latency of the test during the two-point trial in the morning and two-point trial in the afternoon per day was recorded as the intraday memory acquisition score.

Determination of the rat's memory retention ability

The trial of day 3 was used to determine the memory retention ability by probe trial. The platform was taken out of the pool and a probe trial was performed. The rat was placed in the water from a point (proximal or distal), the swimming time and swimming distance in quadrant IV (target quadrant) were recorded and the number of cross target quadrants of the rat in 60 s was recorded as the intraday memory retention score.

Determination of the rat's memory re-learning ability

The trial of day 4 to day 6 was used to measure the memory re-learning ability by opposite platform trial. The platform was placed in quadrant II. It recorded the latency of rats. The average latency of the test during the two-point trial in the morning and two-point trial in the afternoon per day was recorded as the intraday memory re-learning score.

Determination of the rats' swimming speed

The trial of day 7 was used to measure the swimming speed of the rats by visible platform trial. The platform was set up to about 2 cm above the water surface.

Preparation of sample

On day 38 of the administration of drugs to rats, rats were euthanized under anesthesia. Three rats were randomly selected from each group, the brain tissues were taken out, and the left and right brains were

separated and fixed with 4% paraformaldehyde for HE staining and NeuroD1 indicator detection. The brain tissues of other rats were stored in a -80°C refrigerator for testing of relevant indicators.

Detection of morphological changes in hippocampal and cortical neural cells by HE staining

The rats' brain tissues embedded in paraffin, and cut into 5-μm thick sections, then operated according to the routine steps of HE staining and photographed under a 400-fold microscope.

NeuN protein expression by immunohistochemistry detection

Paraffin sections were routinely dewaxed with water, blocked with 5% BSA, NeuN primary antibody was added, and left overnight at 4°C. Second antibody was added, DAB chromogenic reaction, seal slices, and placed under a microscope and photographed.

Measurement of the mRNA expressions of BDNF, TRKB, RSK, and CREB by qPCR

Total RNA was extracted from the hippocampus and cerebral cortex by the Tri-zol method, and the concentration of RNA and OD 260 / 280 values were detected (1.8-2.1 were qualified). RNA was reverse transcribed into cDNA. β-actin (Forward primer: 5'-GGAGATTACTGCCCTGGCTCCTA-3', Reverse primer: 5'-GACTCATCGTACTCCTGC TTGCTG-3'). BDNF (Forward primer: 5'-GGAGATTACTGCCCTGGCTCCTA-3', Reverse primer: 5'-GACTCATCGTACTCCTGCTTGCTG-3'). TRKB (Forward primer: 5'-TCCGGTTAAAGTTTGTGGCTTAC-3', Reverse primer: 5'-CAAGTCAAGGTGGCGGAAATG-3'). RSK (Forward primer: 5'-CAGACCGAGGGCAAGCTCTA-3', Reverse primer: 5'-GTGCCAGCTCAGC CAGGTAA-3'). CREB (Forward primer: 5'-ACAGTTCAAGCCCAGCCACAG-3', Reverse primer: 5'-GCACTAAGGTTACAGTGGGAGCAGA-3'). The reverse transcription product cDNA was amplified, and the dissolution curve of the amplified product was obtained. The $2^{-\Delta\Delta CT}$ method was used for relative quantitative calculation of the data.

Measurement of the protein expression of NeuN, BDNF, TRKB, RSK, and P-CREB-Ser133 by western blotting

Western blotting was performed according to standard methods. The first antibody of NeuN, BDNF, TRKB, RSK, and P-CREB-Ser133 was added separately and overnight at 4°C, then for 1 hour at room temperature with the second antibody. The protein bands were analyzed by Image J software after exposed on ultra-sensitive ECL chemiluminescence agent, GAPDH protein was used as an internal control.

Statistical analysis

Results were expressed as mean ± SD. All statistical analyses were analyzed by SPSS 24.0 software and were analyzed by one-way ANOVA. $P < 0.05$ means statistically significant.

Results

Screening Of Successful Ad Model Rats

The rats were screened for successful AD model from day 46 to 49 of the experiment after the operation. As depicted in Fig. 3, the latency of all rats to find the hidden platform was gradually reduced with the prolongation of training. According to the $SR \geq 0.2$ of each rat in the composited A β -treated group on the day 4 of training, in which the number of rats with $SR \geq 0.2$ was 24, and the number of rats with $SR < 0.2$ was 10, the model success rate in the present study was 70.58%.

The effects of SBFs and Rolipram on memory acquisition of AD rats

The results showed that the latencies of rats in the model group increased by 84.35% (day 1, $P < 0.01$) and 177.14% (day 2, $P < 0.01$), compared to sham group. The latencies decreased by 48.89% (day 1, $P < 0.01$) and 54.36% (day 2, $P < 0.01$) for 140 mg/kg SBFs rats, and 30.15% (day 1, $P < 0.05$) and 49.97% (day 2, $P < 0.01$) for 0.5 mg/kg Rolipram rats, compared to model group. There was no significant difference between the SBFs 140 mg/kg group and the Rolipram 0.5 mg/kg group (Fig. 4).

The effects of SBFs and Rolipram on memory retention of AD rats

(Fig. 5A, 5B, 5C, 5D) represent the swimming time, swimming distance crossing number, and swimming tracks, respectively in the Morris water maze test of rats. The swimming time, the swimming distance and the number of crossed platforms in the target quadrant decreased by 17.36% ($P < 0.05$), 15.51% ($P < 0.05$), and 24.89% ($P < 0.05$) in the model group, respectively. However, SBFs and Rolipram could regulate the above abnormal change of rats induced by composited A β to varying degrees. increased by 12.58% (swimming time), 14.62% (swimming distance) and 24.68% (number of crossed platforms) for 140 mg/kg SBFs rats (All $P < 0.05$), 22.30% (swimming time), 24.65% (swimming distance) and 54.27% (number of crossed platforms) for 0.5 mg/kg Rolipram rats (All $P < 0.05$), respectively. There was no obvious difference between the effects of the SBFs 140 mg/kg group and the Rolipram 0.5 mg/kg group.

The effects of SBFs and Rolipram on re-learning ability of AD rats

The latencies of rats in the model group decreased by 2.80% ($P > 0.05$), 74.00% ($P < 0.01$), and 102.14% ($P < 0.01$), compared to sham group. However, compared to model group, the latencies reduced by 5.61% (day 4, $P > 0.05$), 28.33% (day 5, $P > 0.05$), and 38.40% (day 6, $P < 0.01$) for 140 mg/kg SBFs rats, and decreased by 10.41% (day 4, $P > 0.05$), increased by 30.47% (day 5, $P > 0.05$), and decreased by 36.58% (day 6, $P < 0.01$) for 0.5 mg/kg Rolipram rats. There was no remarkable difference between the SBFs 140 mg/kg group and the Rolipram 0.5 mg/kg group (Fig. 4).

The effects of SBFs and Rolipram on swimming speed of AD rats

The results showed that there was no significant difference in the time for rats in each group to find a visual platform ($P > 0.05$).

The effect of SBFs and Rolipram on the morphology of hippocampal and cortical neurons in AD rats

The results of HE staining showed that the cells in the hippocampal CA1 region of the sham group were complete in morphology with round nuclei and numerous in number. The structure of the cerebral cortex was clear, neurons were normal, and there were no abnormal morphological cells or ectopic phenomena. In the model group, the number of cells in the hippocampal CA1 region was decreased, and the number of glial cells was increased. Cells in the cerebral cortex were round with shortened protrusions, stained nuclei, and neuronophagia were present. Compared to model group, the number of nerve cells in the hippocampal CA1 region and cerebral cortex of rats in the SBFs group and the Rolipram group was increased, and the pathological characteristics, such as nuclear staining, edge shift, and neuronophagia, were improved to different degrees. The effects of the SBFs 140 mg/kg group and the Rolipram 0.5 mg/kg group were similar (Fig. 6).

The Effect of SBFs and Rolipram on the protein expression of NeuN in the hippocampal gyrus of the rats

The results showed that NeuN protein was expressed in the cell nucleus, which was brownish-yellow. The color of nerve cells in the hippocampal gyrus of rats in the model group was lighter, the intercellular space was widened, the cell body was swollen, and the nucleus was deeply stained. In the SBFs group and Rolipram group, the damage to hippocampal neurons was significantly improved, and the neurons were arranged neatly, and the morphology was normal. The color of brown-yellow particles was deepened. The number of NeuN positive expression cells in the model group decreased by 64.00% ($P < 0.01$), compared to sham group. Compared to model group, the number of NeuN positive expression cells in the SBFs 140 mg/kg group and the Rolipram 0.5 mg/kg group increased by 81.33% ($P < 0.01$) and 104.00% ($P < 0.01$), respectively. There was no significant difference in the effects of the SBFs 140 mg/kg group and the Rolipram 0.5 mg/kg group (Fig. 7).

The Effects of SBFs and Rolipram on the mRNA expression of BDNF, TRKB, RSK, and CREB, and the protein expression of NeuN, BDNF, TRKB, RSK, and P-CREB-Ser133 of rats

The effects of SBFs and Rolipram on the protein expression of NeuN of rats

Compared with sham group, the content of NeuN protein reduced by 19.60% (hippocampus, $P < 0.01$), and increased by 17.48% (cerebral cortex, $P < 0.05$) in the model group. Compared with model group, the content of NeuN protein increased by 22.57% (hippocampus, 140 mg/kg SBFs, $P < 0.01$) and 24.97% (cerebral cortex, 140 mg/kg SBFs, $P < 0.01$), respectively. The expression of NeuN protein reduced by 63.08% (hippocampus, 0.5 mg/kg Rolipram, $P < 0.01$) and increased by 18.24% (cerebral cortex, 0.5 mg/kg Rolipram, $P < 0.05$), respectively. The effect of SBFs 140 mg/kg was stronger than that of Rolipram 0.5 mg/kg (Fig. 8).

The effects of SBFs and Rolipram on the mRNA and protein expression of TRKB of rats

Compared to sham group, the expression of TRKB mRNA of rats in the model group increased by 120.98% (hippocampus, $P < 0.01$) and 15.04% (cerebral cortex, $P < 0.01$), respectively. However, SBFs and Rolipram could regulate the expression levels of TRKB mRNA of rats induced by composited A β to varying degrees. Compared with model group, the expression levels of TRKB mRNA of rats with composited A β decreased by 49.82% (hippocampus, SBFs 140mg/kg, $P < 0.01$) and 38.52% (hippocampus, Rolipram 0.5mg/kg, $P < 0.01$), respectively. The expression of TRKB mRNA was reduced by 15.26% (cerebral cortex, SBFs 140 mg/kg, $P < 0.01$) and 12.43% (cerebral cortex, Rolipram group 0.5 mg/kg, $P < 0.05$), respectively (Fig. 9A).

The expression of TRKB protein reduced by 28.02% (hippocampus, $P < 0.01$) and 36.64% (cerebral cortex, $P < 0.05$) in the model group, compared with sham group. While, after administration of 140 mg/kg SBFs and 0.5 mg/kg Rolipram for 38 days, compared to model group, the protein expression of TRKB increased by 23.96% (hippocampus, $P < 0.05$) and 3.33% (hippocampus, $P > 0.05$), respectively. The expression of TRKB protein increased by 25.64% (cerebral cortex, 140 mg/kg SBFs, $P < 0.05$) and 19.14% (cerebral cortex, 0.5 mg/kg Rolipram, $P < 0.05$), respectively. The effect of SBFs 140 mg/kg was stronger than Rolipram 0.5 mg/kg (Fig. 9B).

The effects of SBFs and Rolipram on the mRNA and protein expression of RSK of rats

The expression of RSK mRNA of composited A β rats increased by 160.88% (hippocampus, $P < 0.01$) and 208.97% (cerebral cortex, $P < 0.01$), respectively, compared to sham group. However, after treated with SBFs and Rolipram for 38 days, compared with model group, the RSK mRNA expression levels were attenuated by 6.58% (hippocampus, SBFs 140 mg/kg, $P > 0.05$) and 38.73% (hippocampus, Rolipram 0.5 mg/kg, $P < 0.01$), respectively. The mRNA expression of RSK reduced by 59.82% (cerebral cortex, SBFs 140 mg/kg, $P < 0.01$) and 64.30% (cerebral cortex, Rolipram 0.5 mg/kg, $P < 0.01$), respectively. The effect of Rolipram 0.5 mg/kg was stronger than that of SBFs 140 mg/kg (Fig. 10A).

Compared with sham group, the expression of RSK protein in the model group was attenuated by 16.69% (hippocampus, $P < 0.01$) and 13.29% (cerebral cortex, $P > 0.05$). Compared with model group, after administration of 140 mg/kg SBFs and 0.5 mg/kg Rolipram for 38 days, the RSK protein expression increased by 42.23% (hippocampus, SBFs 140 mg/kg, $P < 0.01$) and 43.32% (hippocampus, Rolipram 0.5 mg/kg, $P < 0.01$), respectively. However, there was no significant change in the protein expression of RSK in the cerebral cortex. The effects of SBFs 140 mg/kg and Rolipram 0.5 mg/kg were similar (Fig. 10B).

Effects of SBFs and Rolipram on the expression of CREB mRNA and the expression of P-CREB-Ser133 protein of rats

The mRNA expression levels of CREB decreased by 24.77% (hippocampus, $P < 0.01$) and increased by 70.00% (cerebral cortex, $P < 0.01$) of the model group, respectively, compared to sham group. However, SBFs and Rolipram could regulate the expression of CREB mRNA of rats induced by composited A β to

varying degrees. The mRNA expression levels of CREB increased by 3.30% (hippocampus, SBFs 140 mg/kg, $P > 0.05$) and 33.30% (hippocampus, Rolipram 140 mg/kg, $P < 0.01$), and decreased by 14.98% (cerebral cortex, SBFs 140 mg/kg, $P < 0.01$) and 6.13% (cerebral cortex, Rolipram 140 mg/kg, $P > 0.05$). In the hippocampus, the effect of Rolipram 0.5 mg/kg was stronger than that of SBFs 140 mg/kg. In the cerebral cortex, the effect of SBFs 140 mg/kg was stronger than that of Rolipram 0.5 mg/kg (Fig. 11A).

The expression of P-CREB-Ser133 protein was attenuated by 55.05% (hippocampus, $P < 0.01$) and 28.23% (cerebral cortex, $P < 0.01$), respectively, in the model group, compared to sham group. However, SBFs and Rolipram could regulate the expression of P-CREB-Ser133 protein of rats induced by composited A β to varying degrees, the expression of P-CREB-Ser133 protein increased by 55.97% (hippocampus, SBFs 140 mg/kg, $P < 0.01$) and 59.85% (hippocampus, Rolipram 0.5 mg/kg, $P < 0.01$), and by 42.18% (cerebral cortex, SBFs 140 mg/kg, $P < 0.01$), and 11.20% (cerebral cortex, Rolipram 0.5 mg/kg, $P < 0.05$). In the hippocampus, the effect of SBFs 140 mg/kg was similar to that of Rolipram 0.5 mg/kg. In the cerebral cortex, the effect of SBFs 140 mg/kg was stronger than that of Rolipram 0.5 mg/kg (Fig. 11B).

Effect of SBFs and Rolipram on the BDNF mRNA and protein expression of rats

The expression of BDNF mRNA in the model group increased by 84.18% (Hippocampus, $P < 0.01$) and 53.95% (Cerebral cortex, $P < 0.01$), respectively, compared to sham group. However, SBFs and Rolipram could regulate the expression of BDNF mRNA of rats induced by composited A β to varying degrees. The mRNA expression levels of BDNF decreased by 33.35% (hippocampus, SBFs 140 mg/kg, $P < 0.01$) and 30.87% (hippocampus, Rolipram 0.5 mg/kg, $P < 0.01$), and decreased by 34.37% (cerebral cortex, SBFs 140 mg/kg, $P < 0.01$) and 36.07% (cerebral cortex, Rolipram 0.5 mg/kg, $P < 0.01$). The effects of SBFs 140 mg/kg and Rolipram 0.5 mg/kg were similar (Fig. 12A).

The protein expression of BDNF decreased 36.23% (hippocampus, $P < 0.01$) and 54.16% (cerebral cortex, $P < 0.01$) in the model group. However, SBFs and Rolipram could regulate the expression of BDNF protein of rats induced by composited A β to varying degrees. Compared to model group, the expression of BDNF protein increased by 31.67% (hippocampus, SBFs 140 mg/kg, $P < 0.05$) and 99.93% (hippocampus, Rolipram 0.5 mg/kg, $P < 0.01$), respectively. The expression of BDNF protein increased by 56.07% (cerebral cortex, SBFs 140 mg/kg, $P < 0.01$), and 11.73% (cerebral cortex, Rolipram 0.5 mg/kg, $P < 0.05$), respectively. The effect of Rolipram 0.5 mg/kg was stronger than SBFs 140 mg/kg in the hippocampus, and the effect of SBFs 140 mg/kg was stronger than Rolipram 0.5 mg/kg in the cerebral cortex (Fig. 12B).

Discussion

AD is a neurodegenerative disease, and its main clinical manifestation is cognitive dysfunction [10]. The Morris water maze test can reflect the changes in animals' cognitive ability, and it is a common method for AD behavior evaluation [11]. It is known that cognitive function is an advanced function of the brain.

The cerebral cortex is the advanced nerve center of the body, which has the functions of language, learning, memory, and thinking, and the hippocampus is responsible for the storage and conversion of short-term and long-term memory. The structure and functional state of these parts have great significance for the formation and maintenance of learning and memory, which are the most prominent areas of AD pathological changes [12]. In AD patients or AD animal models, the loss of neurons in the hippocampus and cerebral cortex of the brain and the neural structure damage result in learning and memory disorders [13]. Neurogenesis can improve learning and memory impairment, a process in which neural precursor cells proliferate and differentiate into new mature neurons. Normally, the newly-born mature neurons can migrate to the brain injury site, compensate for the damaged neurons, and restore the normal function of neurons [14]. However, in the pathological condition of AD, neural precursor cells in the dentate gyrus of the hippocampus cannot develop into mature neurons, thus impacting neurological function [15]. Neuronal nuclei (NeuN) is a neuron-specific nuclear protein, which is the protein marker of mature neurons and an important target of neural structure and function. In pathological states, the number of mature neurons in the brain decreases, and the expression of NeuN decrease accordingly [16]. In this study, it was found that intracerebroventricular injection of composited A β markedly induced learning and memory impairment, histopathological changes, and the decrease of neurogenesis in rats. SBFs improved learning and memory dysfunction, neuropathological changes, and neurogenesis in rats treated with composited A β . Rolipram can also reverse the learning and memory impairment, and neuropathological changes, and decreases neurogenesis caused by composited A β in rats. It was observed that the effect of SBFs is similar to Rolipram, and their effects on improving learning and memory impairment are related to their neuroprotective effects and increasing the number of mature neurons in the brain.

There is a consensus that the BDNF-RSK-CREB signaling pathway plays an important role in synaptic plasticity and learning and memory functions of the brain [17]. In the BDNF-RSK-CREB signaling pathway, the interaction between BDNF and RSK can regulate CREB phosphorylation, in which RSK can activate CREB phosphorylation and mediate BDNF expression. At the same time, the combination of BDNF and TRKB can activate RSK molecules, thus forming a positive feedback regulation mechanism of BDNF-TRKB-RSK-CREB-BDNF [18] (Fig. 13)

CREB is a nuclear transcription factor that plays a role in the phosphorylation of the Ser-133 site. Phosphorylation through CREB can regulate mRNA and protein expressions of BDNF, NGF, and other neurotrophic factors, promote neurogenesis and improve learning and memory impairment [19]. In the present study, it was found that intracerebroventricular injection of composited A β decreased CREB mRNA and P-CREB-Ser133 protein expressions in the hippocampus and cerebral cortex of rats. However, SBFs increased the expressions of CREB mRNA and P-CREB-Ser133 protein of composited A β rats, and Rolipram reversed the decreased expression of CREB mRNA and P-CREB-Ser133 protein induced by composited A β . It is suggested that SBFs has the same effect as Rolipram, which can activate CREB phosphorylation, thus playing a role in neurogenesis and memory formation. BDNF, an important member of the neurotrophic factor family, is the upstream and downstream signal molecule of CREB, which can regulate brain development, synapse formation, and learning and memory [20]. BDNF combined with

TRKB activates the intracellular signal transduction pathway, reverses neuronal loss, and promotes neurogenesis [21]. It was discovered that TRKB is related to the pathogenesis of AD. The expression of TRKB is decreased in the hippocampus and cerebral cortex of AD patients [22]. RSK, a member of the serine/threonine-protein kinase factor family, is a downstream signal molecule of TRKB. The active RSK molecule can activate CREB phosphorylation, it is beneficial in promoting neurogenesis and improving learning and memory disorders [23]. In this study, treatment with composited A β differently increased the mRNA expression of BDNF, TRKB, and RSK of composited A β rats, and differently decreased the protein expression of BDNF, TRKB, and RSK of composited A β rats, thus inhibiting the phosphorylation of CREB. However, SBFs could regulate the mRNA expression of BDNF, TRKB, and RSK to different extents, and increase the protein expression of BDNF, TRKB, and RSK of composited A β rats, thereby promoting phosphorylation of CREB. These results indicate that the effect of SBFs is consistent with Rolipram, which can activate the BDNF-RSK-CREB signaling pathway, partially increase the mRNA expression of BDNF, TRKB, and RSK, up-regulate the protein expression of BDNF, TRKB, and RSK in the hippocampus and cerebral cortex to a different degree, promote the phosphorylation of CREB, which in turn promote neurogenesis and improve learning and memory impairment.

Studies have proved that the nervous system has the ability of functional compensation [24, 25]. In the present study, in the BDNF-RSK-CREB signal pathway, due to the effect of CREB phosphorylation, at the mRNA level, compensatory mRNA expression occurred in nerve cells. The composited A β rats showed markedly increased mRNA expression levels of BDNF, TRKB, and RSK in the brain, compared with sham group, but the protein expression in the brain was significantly lower than that in the sham group. It was observed that intracerebroventricular injection combined with A β significantly reduced the effective mRNA translation of BDNF, TRKB, and RSK in the brain of rats, thus significantly reducing the protein expression of BDNF, TRKB, and RSK. After the administration of SBFs and Rolipram, the mRNA expression levels of BDNF, TRKB, and RSK in the brain were significantly decreased, and the protein expression levels of BDNF, TRKB, and RSK in the brain were significantly increased without significant difference from the sham group. The results show that SBFs and Rolipram could effectively regulate the signal molecules of the BDNF-RSK-CREB signaling pathway, and tend to a normal level, which is beneficial to promoting neurogenesis and improving learning and memory disorders.

The above-mentioned results indicate that SBFs and Rolipram could improve learning and memory impairment, ameliorate neuropathological changes, promote neurogenesis and up-regulate the BDNF-RSK-CREB signaling pathway based on the fact that Rolipram is the activator of CREB phosphorylation. These results suggest that the effect of SBFs is the same as Rolipram, which might also be an activator of CREB phosphorylation. The results indicate that SBFs has the function of promoting neurogenesis and ameliorating learning and memory disorder, which may be due to its promotion of CREB phosphorylation.

In summary, SBFs promotes neurogenesis and improves memory impairment, which is related to the mediation of CREB phosphorylation by SBFs. This study confirms that SBFs could be beneficial in the treatment of neurodegenerative diseases such as AD.

Declarations

Authors' contributions

Shang Yazhen, Zhao Chunying and Li Caixia conceived and designed the experiment. Liu Xinyang, Liu Yuling and Ma Shuai performed the test and wrote the manuscript. Cheng Jianjun and Gao Yang provided the technique assistance. Shang Yazhen, Zhao Chunying and Li Caixia revised the manuscript. All authors read and approved the final manuscript.

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None declared.

Competing interests

None.

Availability of data and materials

Not applicable.

Ethics approval

All procedures for the animal study were reviewed and approved by the Animal Ethics Committee of Chengde Medical College (No. CDMULAC-20190901-018). All animal procedures were carried out in accordance with the Regulations of Experimental Animal Administration issued by the State Committee of Science and Technology of China on Oct. 31, 1988.

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Figures



Figure 1

Scutellaria barbata flavonoids. *A* The aerial part of *Scutellaria barbata*. *B* The dried aerial part of *Scutellaria barbata*. *C* The dried powder aerial part of *Scutellaria barbata*.

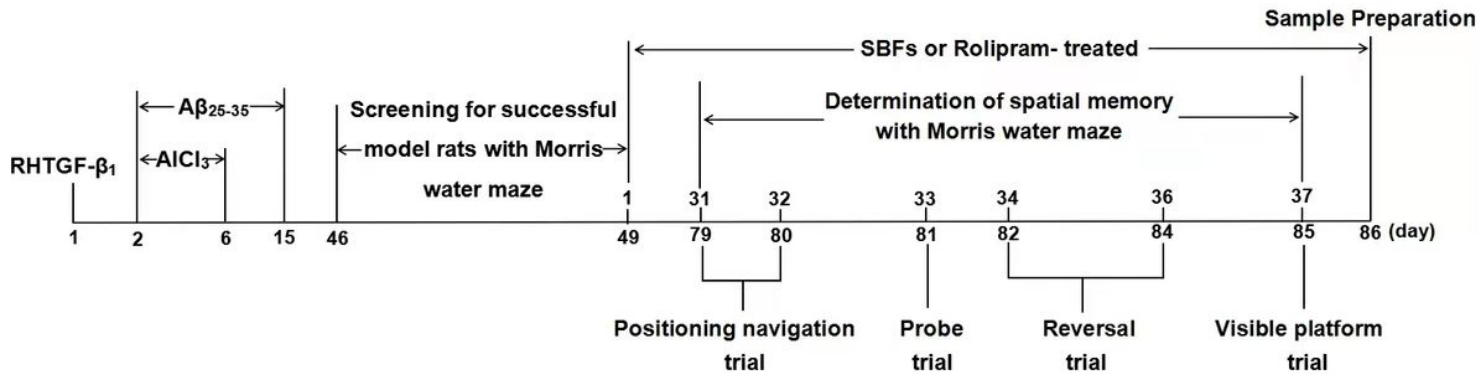


Figure 2

Timeline of experimental design.

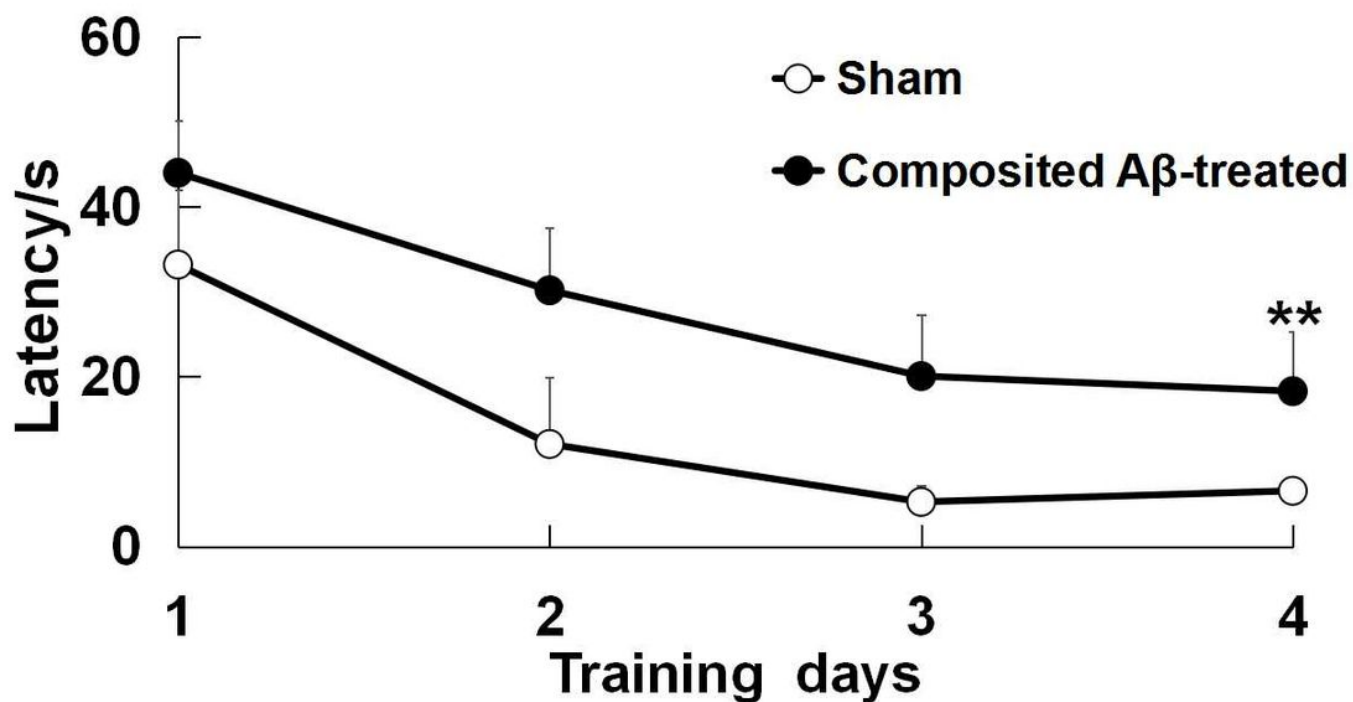


Figure 3

Screening for successful AD model of rats. Mean±SD. ** $P < 0.01$ versus the sham group.

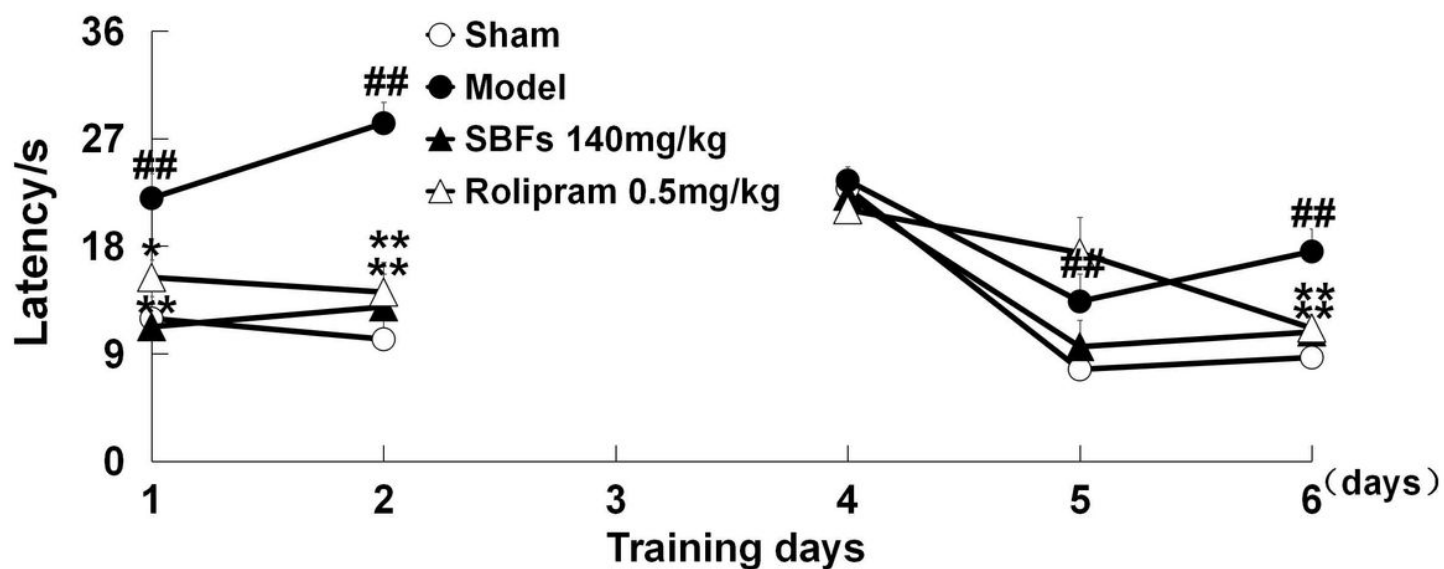


Figure 4

Effect of SBFs and Rolipram on memory acquisition and memory re-learning impairment. Mean±SD. ## $P < 0.01$ versus the sham group. * $P < 0.05$, ** $P < 0.01$ versus the model group.

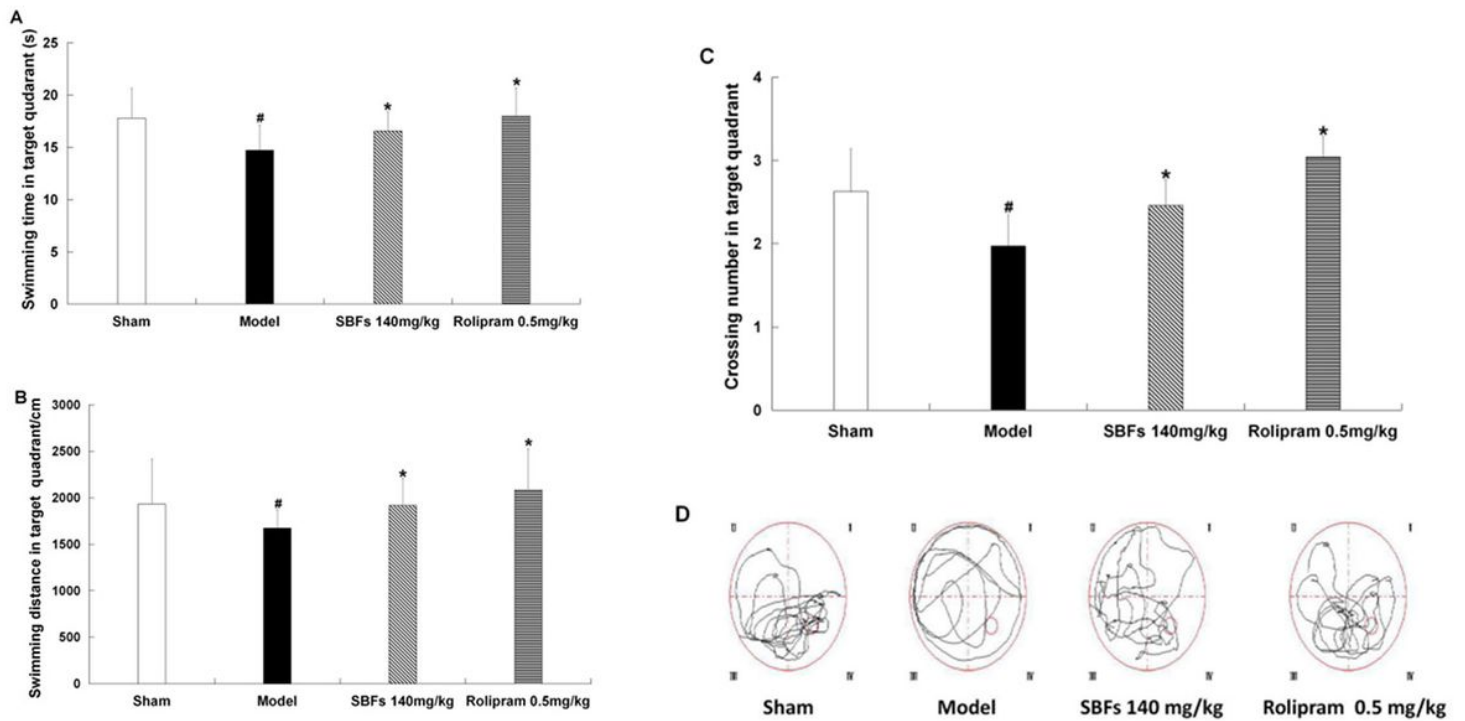


Figure 5

Effect of SBFs and Rolipram on memory retention. **A** Swimming time. **B** Swimming distance. **C** Crossing number in the target quadrant within 60 s in the probe trial (no platform). **D** Representative swimming tracks of different experimental groups in the probe trial. Mean±SD. [#] $P < 0.05$ versus the sham group. ^{*} $P < 0.05$ versus the model group.

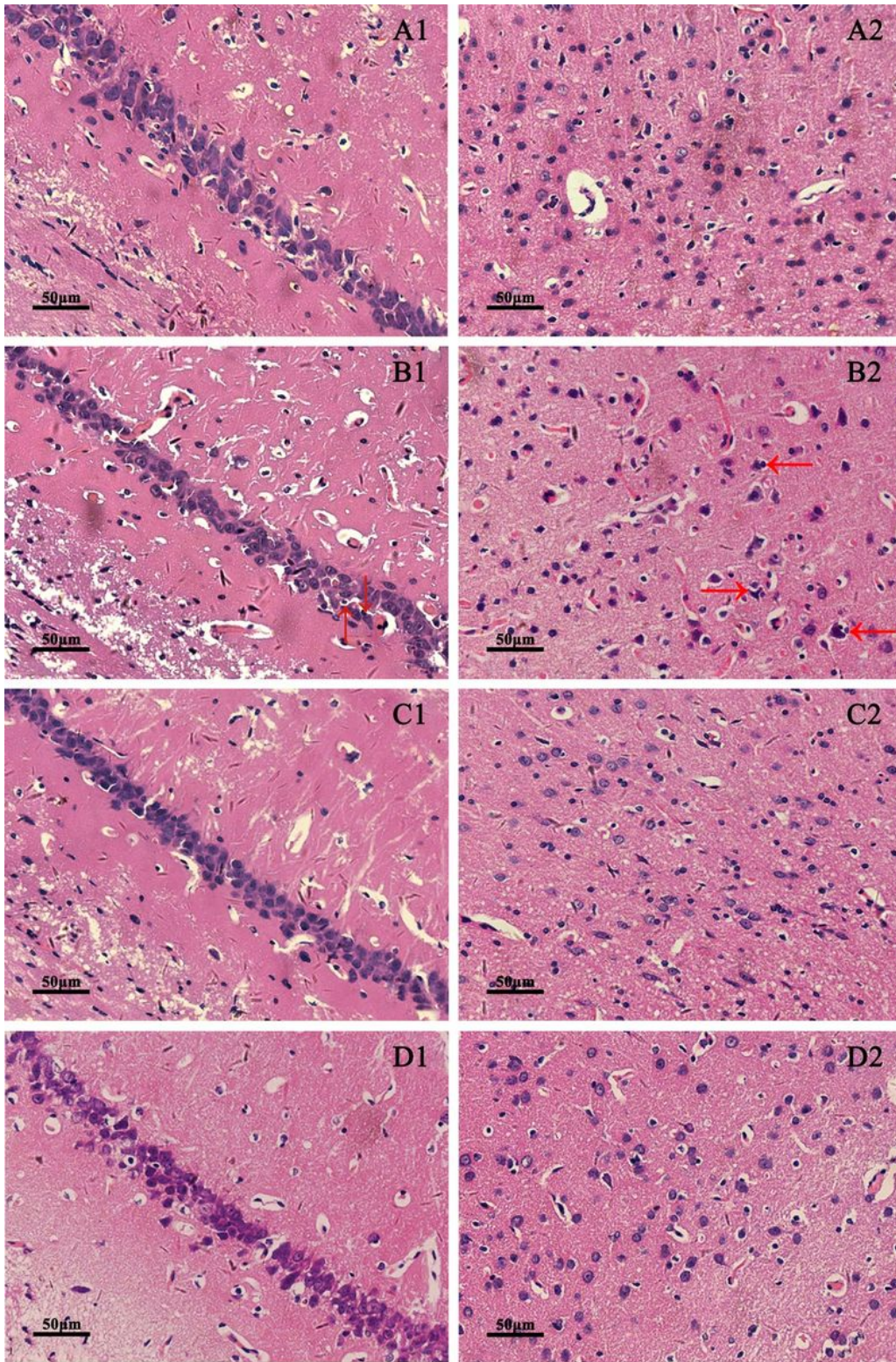


Figure 6

Effect of SBFs and Rolipram on neuropathological change of rats. *A1-D1* Hippocampal CA1 region. *A2-D2* Cerebral cortex. *A1-A2* Sham group. *B1-B2* Model group. *C1-C2* SBFs 140 mg/kg group. *D1-D2* Rolipram 0.5 mg/kg group $\times 400$. The vertical arrow points to the neuronophagia in the hippocampal CA1 region (red arrows). The transverse arrow points to the neuronophagia in the cerebral cortex (red arrows). Scale bar = 50 μm .

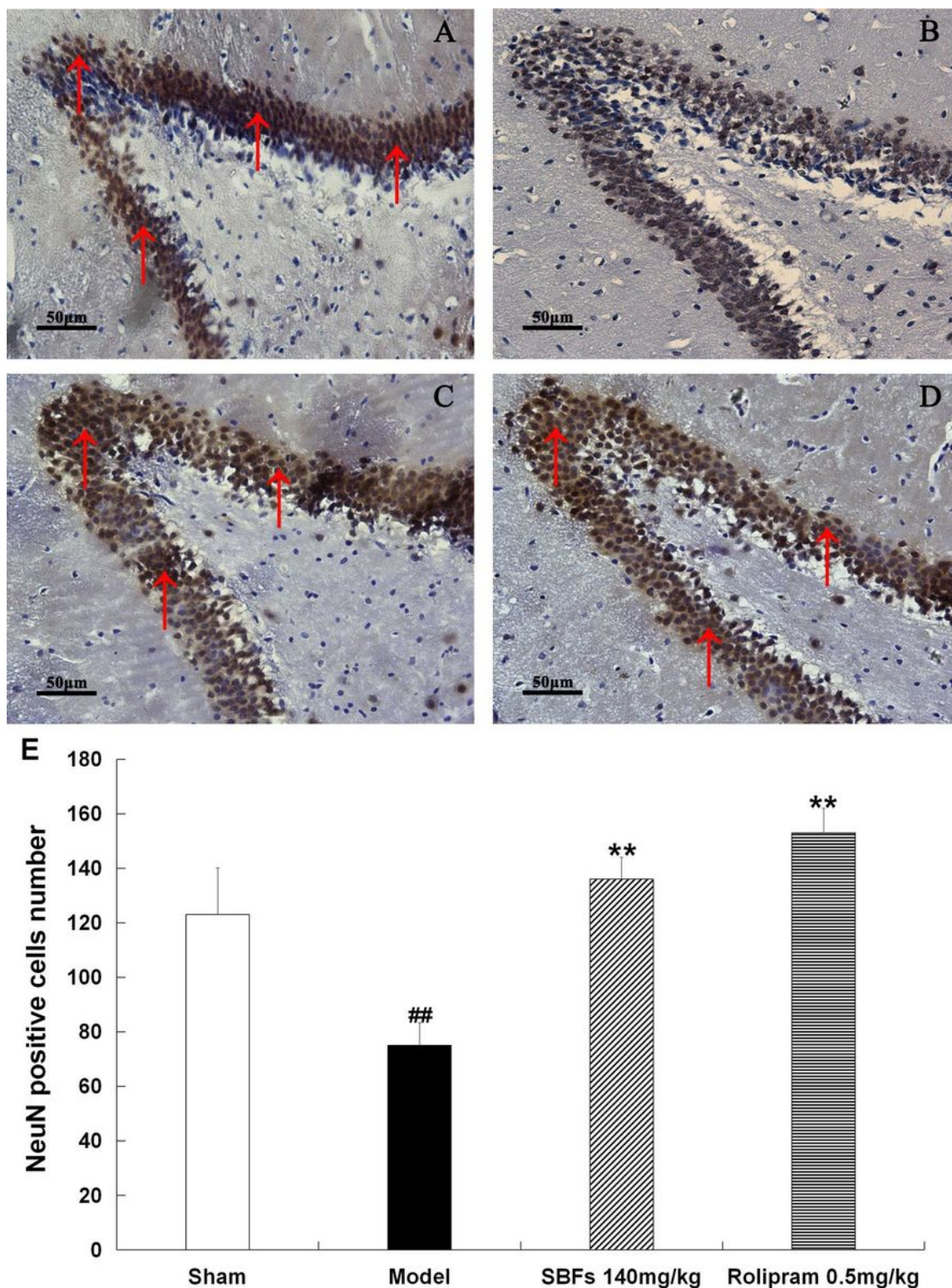


Figure 7

Effect of SBFs and Rolipram on the expression of NeuN protein in the hippocampus gyrus of the rats. *A* sham group. *B* model group. *C* SBFs 140 mg/kg group. *D* Rolipram 0.5 mg/kg group. Brown staining represents the protein expression of NeuN protein (red arrows). *E* The number of cells in the NeuN positive protein expression, $\times 400$. Scale bar = 50 μ m. ## $P < 0.01$ versus the sham group. ** $P < 0.01$ versus the model group.

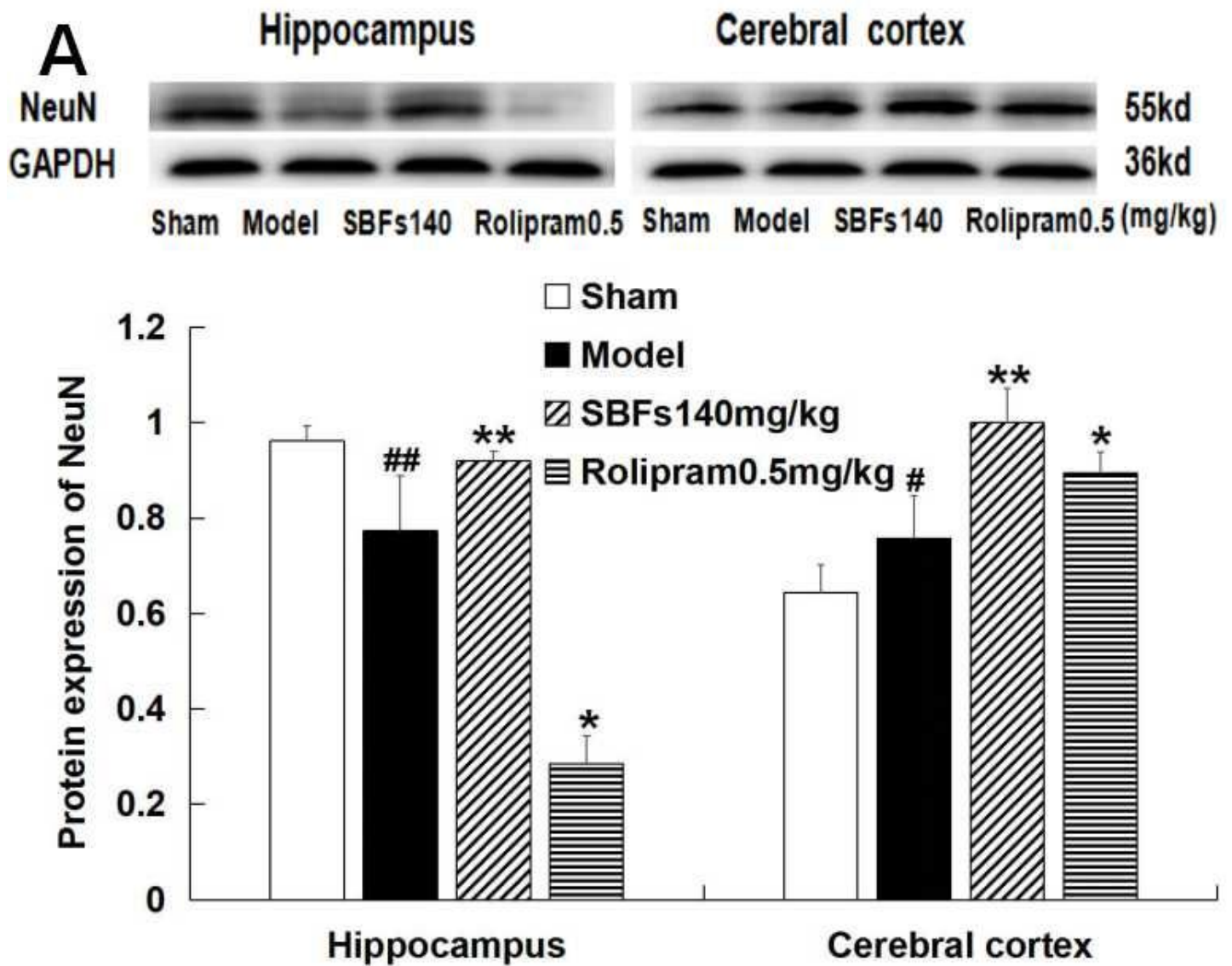


Figure 8

Effect of SBFs and Rolipram on the NeuN protein expression. Mean±SD. n = 5. [#]*P* < 0.05, ^{##}*P* < 0.01 versus the sham group. ^{*}*P* < 0.05, ^{**}*P* < 0.01 versus the model group.

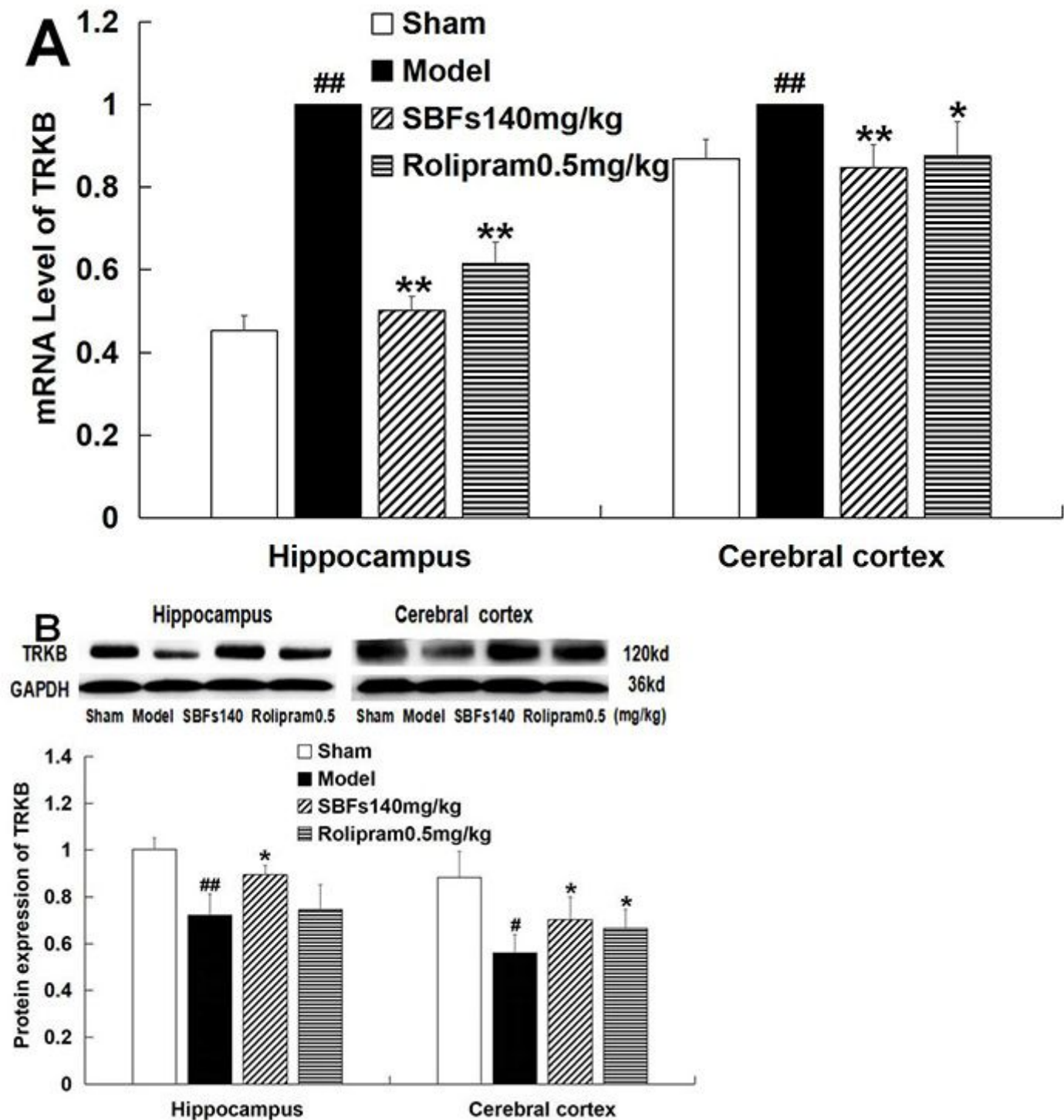


Figure 9

A Effect of SBFs and Rolipram on the TRKB mRNA expression. Mean $\pm$ SD. n = 5. ## P < 0.01 versus the sham group. * P < 0.05, ** P < 0.01 versus the model group.

B Effect of SBFs and Rolipram on the expression of TRKB protein. Mean $\pm$ SD. n = 5. # P < 0.05, ## P < 0.01 versus the sham group, * P < 0.05 versus the model group.

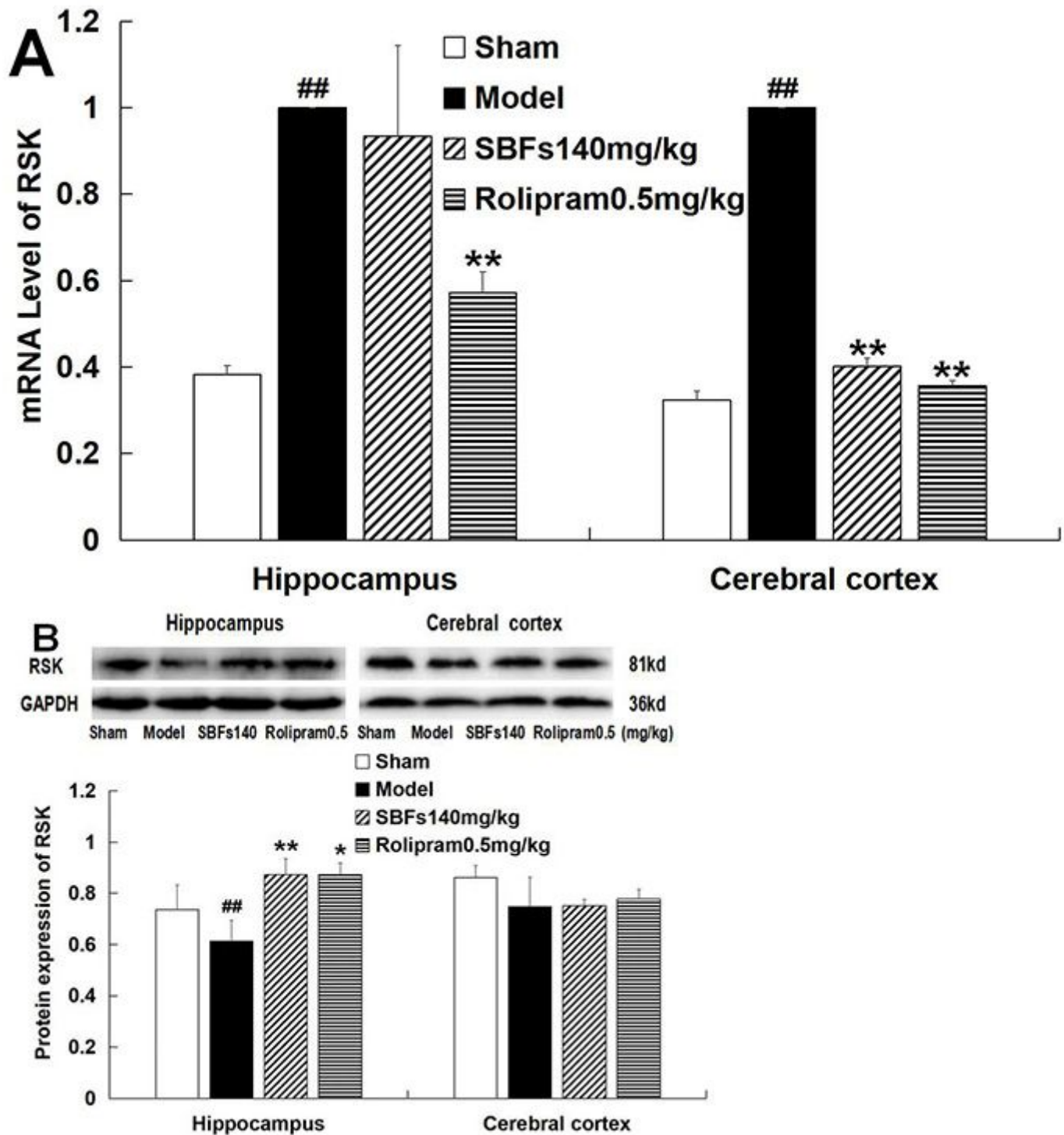


Figure 10

A Effect of SBFs and Rolipram on the expression levels of RSK mRNA. Mean±SD. n = 5. ## $P < 0.01$ versus the sham group. ** $P < 0.01$ versus the model group.

B Effect of SBFs and Rolipram on the expression of RSK protein. Mean±SD. n = 5. ## $P < 0.01$ versus the sham group. * $P < 0.05$, ** $P < 0.01$ versus the model group.

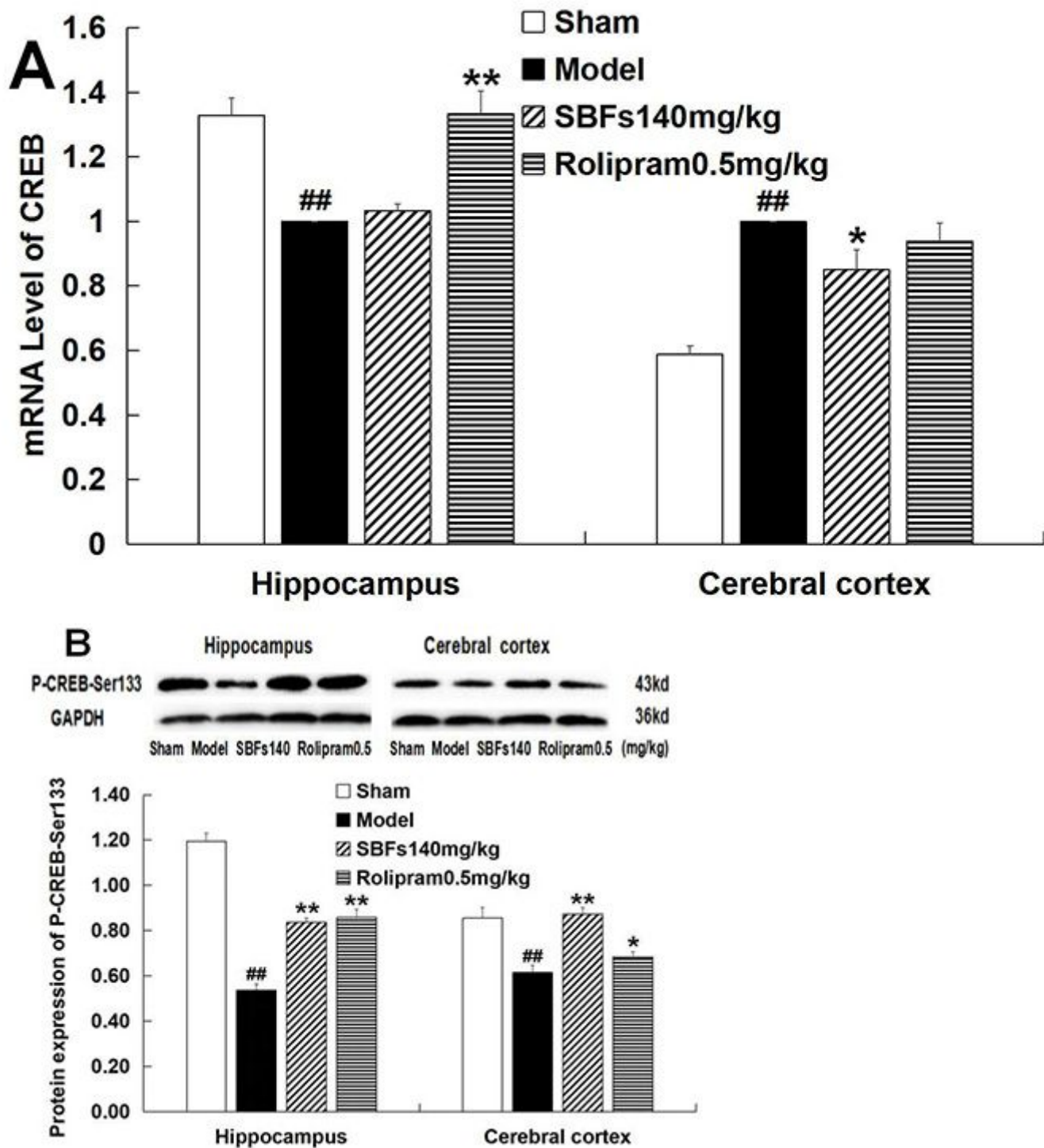


Figure 11

A Effect of SBFs and Rolipram on the expression of CREB mRNA. Mean±SD. n = 5. ^{##}*P* < 0.01 versus the sham group. ^{*}*P* < 0.05, ^{**}*P* < 0.01 versus the model group.

B Effect of SBFs and Rolipram on the expression P-CREB-Ser133 protein. Mean±SD. n = 5. ^{##}*P* < 0.01 versus the sham group. ^{*}*P* < 0.05, ^{**}*P* < 0.01 versus the model group.

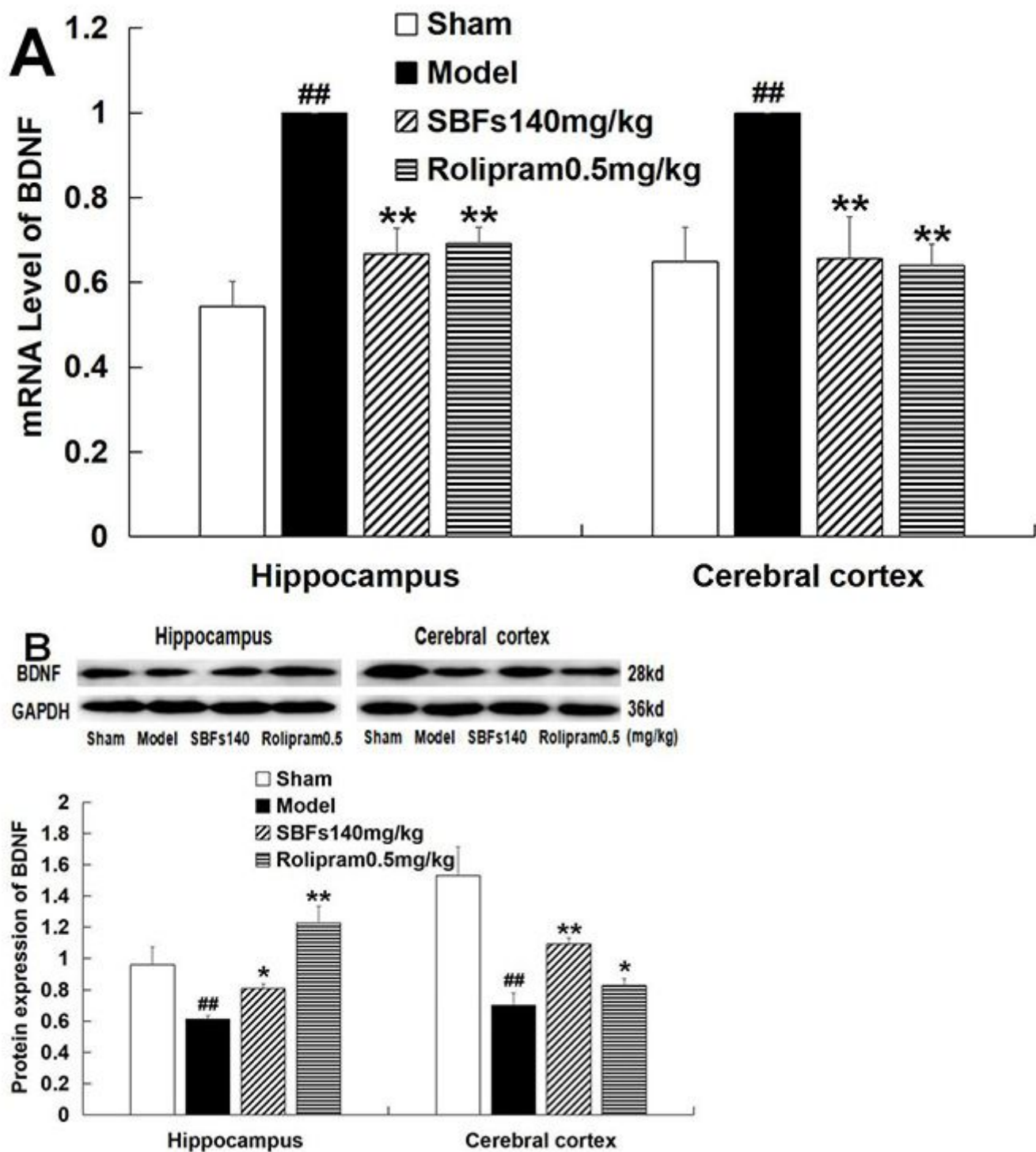


Figure 12

A Effect of SBFs and Rolipram on the expression of BDNF mRNA. Mean±SD. n = 5. ## $P < 0.01$ versus the sham group. ** $P < 0.01$ versus the model group.

B Effect of SBFs and Rolipram on the expression of BDNF protein. Mean±SD. n = 5. ## $P < 0.01$ versus the sham group. * $P < 0.05$, ** $P < 0.01$ versus the model group.

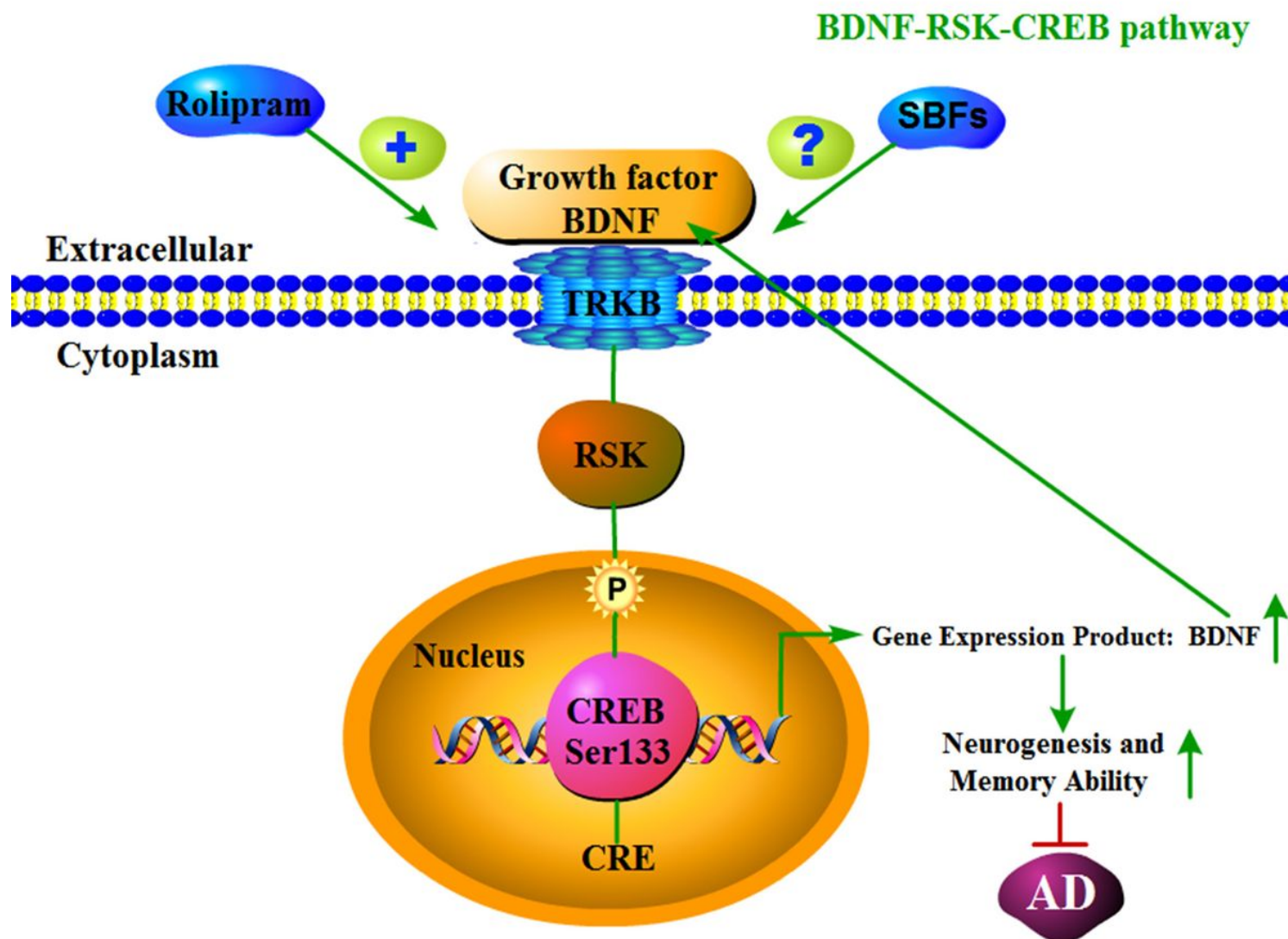


Figure 13

BDNF-RSK-CREB signal pathway.