International Journal of Pharmacology

ISSN 1811-7775

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OPEN ACCESS International Journal of Pharmacology

ISSN 1811-7775
DOI: 10.3923/ijp.2018.164.178

Research Article

Electroacupuncture Rescued the Impairment of Hippocampal Neurons in Perimenopausal Depression Rats via Activating the CREB/BDNF Pathway

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Abstract

Background and Objective: Perimenopausal depression is caused by the impaired function of the ovarium prior to menopause and characterized by a persistent feeling of sadness and loss of interest. Electroacupuncture (EA) therapy has been demonstrated to be effective in patients with depression. The effects of EA in perimenopausal depression as well as the underlying mechanisms remain unclear. This study aimed to elucidate the therapeutic effect of EA on perimenopausal depression and its correlation with the CREB/BNDF signaling pathway to understand the mechanisms underlying the EA effect. **Materials and Methods:** Chronic Unpredictable Mild Stress (CUMS) combined with bilateral ovariectomy (OVX) were used to establish a rat model of perimenopausal depression. The Open Field Test (OFT) and sucrose preference tests were used to examine the anxiety level and anhedonia effect of OVX+CUMS rat model and the effects of EA. The MTT assay and flow cytometry were used to detect the cell viability and apoptosis of hippocampal neurons. The immunohistochemistry and ELISA assay were utilized for measuring the proteins expressions and RT-PCR for mRNA expressions, respectively. Comparisons among more than two groups were made using one-way analysis of variance (ANOVA) followed by the Least Significant Difference (LSD) test. **Results:** The EA at the "Shenshu", "Baihui" and "Sanyinjiao" points could reverse the increased and reduced sucrose preference induced by CUMS and OVX. Meanwhile, the reduced cell viability, increased cell apoptosis and impaired function of hippocampal neurons in the perimenopausal depression model rats were prevented by EA treatment. Moreover, EA increased p-CREB and BDNF expression in the hippocampal neurons and this effect was found to be suppressed by the CREB antagonist KG-501. **Conclusion:** The EA treatment could rescue the impairment induced by OVX+CUMS via CREB/BDNF pathway activation. Taken together, the results suggest that EA could be a potential therapy for perimenopausal depression.

Key words: Perimenopausal depression, electroacupuncture therapy, hippocampal neurons, functional impairment, CREB/BDNF signaling pathway

Received: June 19, 2017 Accepted: November 30, 2017 Published: January 15, 2018

Citation: Shao-Heng Li, Li-Tong Wang, Xue Deng, Ya-NanJiao, Liang Kong, Martin Fu, Lian-Qun Jia, Jing-Xian Yang and Lu Ren, 2018. Electroacupuncture rescued the impairment of hippocampal neurons in perimenopausal depression rats via activating the CREB/BDNF pathway. Int. J. Pharmacol., 14: 164-178.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Perimenopausal depression is caused by the impaired function of the ovarium prior to menopause and with a persistent feeling of sadness, loss of interest and sleep disorder[1]. It is estimated to affect 30% of women and to have a negative influence on the quality of life of patients[2]. Hormone replacement therapy and antidepressant treatment are widely used and have been demonstrated efficacy clinically but with limitations due to the side effects, which can induce endometrial cancer and breast cancer resulting from chronic hormone treatment[3]. Therefore, it is necessary and imperative to develop a novel therapy for treating perimenopausal depression.

Electroacupuncture (EA), a Chinese traditional therapy, has been demonstrated to be clinically effective in patients with psychiatric disorders including depression[4-6]. However, little is known about the effectiveness of EA therapy for perimenopausal depression, the potential mechanisms of which remain unclear.

The hippocampus is considered the critical brain region in memory and mood-related behaviors, exhibiting impairment in response to stress[7]. It has been demonstrated that chronic stress induces neuronal loss and dendritic atrophy in the hippocampus, contributing to the progression of depression[8,9]. The hippocampus is believed to be the major brain region affected in perimenopausal depression, with neuronal death and structural changes. Most antidepressants promote hippocampal neurogenesis, whereas blocking hippocampal neurogenesis could eliminate the antidepressant action[10,11]. Therefore, one way to promote hippocampal neurogenesis in depression patients may be to decrease the impairment in the brain induced by chronic stress.

Cyclic AMP Response Element Binding protein (CREB) is a nuclear transcription factor that is activated by phosphorylation of serine 133 in multiple signaling pathways[12]. Brain Derived Neurotrophic Factor (BDNF) is downstream of CREB, the pathway that promotes neurogenesis, neuronal survival and maintenance of neuron structure and function. The expression of phosphorylated CREB (p-CREB) and BDNF have been shown to be reduced in the hippocampus in depression rats induced by Chronic Unpredicted Mild Stress (CUMS). This represents that the impairments of neurogenesis and hippocampal damage occur in depression. Therefore, activation of CREB/BDNF pathway promotes neuronal survival and function in the hippocampus and thus, this pathway is potentially an efficient target for treating depression[13,14].

In this study, CUMS combined with bilateral ovariectomy (OVX) were used to create an animal model for perimenopausal depression in female rats and then the effects of EA treatment in hippocampal neurons were examined to further explore the molecular mechanisms of the effects of EA treatment in depression. The results suggested that EA treatment significantly reversed the increased anxiety-like behaviors and decreased sucrose preference in perimenopausal depression rats. EA treatment promotes neuronal activity and suppresses cell apoptosis in the hippocampus via activating the CREB/BDNF pathway. The data suggested that EA treatment could be a potentially effective therapy for treating perimenopausal depression.

MATERIALS AND METHODS

Animals: Female Sprague-Dawley (SD) rats aged with 56-62 days and weighing 200-250 g were purchased from Weitonglihua Co. (SCXK 2013-0001, Beijing, China) and used in this study. The rats were group-housed in a 22±2°C environment with 12 h/12 h light/darkness in a specific pathogen-free room with relative humidity 55-65% and given free access to water and food. A total of 30 rats were divided randomly into 5 groups (n = 6/group) including control, sham, model, Clomipramine Hydrochloride (CH) treatment and EA treatment groups. All experimental procedures were approved by the community of Liaoning University of Traditional Chinese Medicine according to the rules for animal ethics.

Bilateral OVX: Rats fasted for 24 h before surgery. Rats were anesthetized with 30 mL kg^{-1} of 10% chloral hydrate (Sigma Chemical Co., St Louis, MO, USA) via intraperitoneal injection and then the fur over the dorsal lumbar area was shaved. To avoid contamination, povidone-iodin (0.5%, Sigma) followed by an alcohol (Sigma) rinse was used to disinfect the skin. A longitudinal incision (1-2 cm) was made in the midline area of the lower abdomen and then the ovaries were removed. The skin incision was closed with stainless steel wound clips (2 or 3 each side). The control group received no treatment and the sham group underwent the same surgical procedure except the ovaries were not removed[15]. All chemicals used in the study were of analytical grade.

CUMS: All rats except those in the control and sham groups were subjected to a chronic unpredictable stress paradigm modified from that of Willner *et al.*[16]. The paradigm consisted of seven stressors including tail clamp for 1 min at a 1 cm distance from the tail end; swimming in 4°C cold water for

5 min; placement in an experimental room at 45°C for 5 min; 24 h of food deprivation and 24 h of water deprivation; restricted stress for 1 h; cage tilting and damp sawdust for 24 h (200 mL water per individual cage, which is enough to wet the sawdust bedding) and circadian disruption by inversing the light and dark cycle. Rats were individually housed and subjected to one of these stressors daily in a random order. The whole stress procedure lasted for 21 days.

EA treatment: The EA was performed at 3 points including bilateral Shenshu located on the back of rats (bilateral 5 mm next to the second lumbar vertebra, 6 mm depth), bilateral Sanyinjiao (10 mm upper in the malleolus tip of the hind region, 5 mm depth) and Baihui (the middle of the parietal region, 2 mm depth). An electrical current at 18 V with high/low frequencies of 4 and 20 Hz was added after the EA needle was inserted into the appropriate point. The EA treatment was given daily (20 min per treatment) for 28 days. Only the EA group received EA treatment[17]. The rats in the sham group were held for 20 min without any treatments, while the control rats were kept in their cages as usual. The CH was dissolved in saline and 20 mg kg^{-1} CH was administered intragastrically to rats in the CH group daily for 28 days.

Open Field Test (OFT): The center time or distance in the OFT was used to measure anxiety-like behaviors in rodents. A Plexiglas chamber divided into a central zone and a periphery zone was equipped with a camera on the top. Rats were acclimated to the room with open field equipment for 2 h before starting the experiment. Then they were placed in the chamber to freely explore for 3 min and their locomotor activity was monitored by the camera (Chengdu TME Technology Co., Ltd., Chengdu, China). The distance and time that the rats moved in the center were recorded and the test was performed once at weeks 0, 3 and 7.

Sucrose preference test: Stress-induced anhedonia in mice is associated with a strong decrease in sucrose preference, which is a putative indicator of anhedonia in rodents[18]. On the day before the test, subjects were individually housed and fasted from food and water. Subsequently, they were presented with food and 2 bottles filled with 1% (w/v) sucrose (Sigma-Aldrich, St. Louis, MO, USA) in tap water and the rats were acclimated to the bottles and sucrose for 24 h. The test began with 1 bottle changed to tap water while 1% sucrose was kept in the other bottle and the rats were allowed to freely choose which bottle to drink from for 24 h. Bottles were weighed before and after they were presented to the rats. Sucrose preference was calculated according to the percentage of sucrose intake out of the total liquid intake.

Primary hippocampal neuron culture: The hippocampus was isolated from the brain and removed immediately from rats sacrificed bycervical dislocation. The tissue was homogenized and then digested in 4 mL of 0.25% Trypsin (Gibco, Invitrogen Corporation, Grand Island, NY, USA) for 15 min at 37°C. Digestion was ended by addition of neurobasal Dulbecco's Modified Eagle Medium (DMEM) containing 10% Fetal Bovine Serum (FBS) and 1% penicillin/streptomycin (Gibco, Invitrogen Corporation) and then the number of neurons in each sample was recorded. The hippocampal neurons were seeded at a density of 1×10^6 cells mL^{-1} on a culture plate coated by poly-lysine and incubated in a 37°C incubator with 5% CO_2 and 95% air. The medium was replaced with another medium containing 4 μg mL^{-1} cytarabine after 3 days in culture and then replaced with the normal medium after 48 h. After 9 days in culture, the cells were used in the following experiments[19].

MTT assay: The MTT assay was used to measure the viability of hippocampal neurons. First, 10 μL MTT buffer (Invitrogen) was added to each well of a 96 well plate containing hippocampal neurons and plates were incubated at 37°C for 4 h. Subsequently, the MTT buffer was exchanged with 150 μL dimethyl sulfoxide (DMSO) and the plates were incubated for 15 min. The absorbance value at 492 nm was read by a microplate spectrophotometer (MR-96, Mindray, Shenzhen, China)[20].

Flow cytometry: Using a cell apoptosis measurement kit for flow cytometry (KeyGen, Nanjing, China), we detected cell apoptosis among cultured hippocampal neurons[21]. The cultured neurons were placed in 6-well plates, digested with EDTA-free trypsin solution for 3 min and washed with Phosphate Buffered Saline (PBS) twice (centrifugation for 5 min at a speed of 2000 rpm). Then we added 0.5 mL Binding Buffer, 5 μL Annexin V-Light 650 and 5 μL propidiumiodide before incubation for 15 min at room temperature in darkness. Cell apoptosis in these samples was assessed by flow cytometry.

Immunohistochemistry: The cultured cells were post-fixed with 4% paraformaldehyde at room temperature for 30 min and then incubated with 1% Triton X-100 for 15 min followed by 1 h of blocking in 5% goat serum. Subsequently, the cells from different groups were incubated with specific primary antibodies including mouse anti-NF-M (1:150), anti-CREB

(1:150), anti-p-CREB (1:150) and anti-BDNF (1:150, all antibodies were purchased from Abcam, Cambridge, MA, USA) at 4°C overnight, followed by incubation with the appropriate Cy3-conjugated secondary anti-mouse antibody (1:200, Jackson, West Grove, PA, USA) for 1 h at room temperature. The cells were mounted on glass slides with a mounting buffer containing DAPI. Images were captured using a Nikon epifluorescence microscope (Nikon Eclipse E800; Nikon, Tokyo, Japan)[22]. The morphological analysis was performed for 30-50 cells from each animal. A total number of 150-500 cells for each group was statistically analyzed. The neurite number and length were determined using ImageJ software (US National Institutes of Health, Bethesda, MD, USA)[23].

Enzyme-linked immunosorbent assay (ELISA): The ELISAs were used to detect the contents of SYP, CREB, p-CREB and BDNF in lysis buffer, according to the instructions of the ELISA kit (Shanghai Lengton Bioscience Co., Ltd, Shanghai, China).

Reverse Transcription (RT)-PCR: The hippocampal neurons of the rats were immediately isolated and placed on ice after administration of diethyl ether anesthesia to rats. Total RNA was extracted from hippocampal tissues using Trizol (Invitrogen). The cDNA was synthesized using a First Strand cDNA Synthesis Kit (Thermo Scientific, Lafayette, CO, USA), according to the manufacturer's instructions. Then 2 µL cDNA was used in the following PCR according to the instructions for the PCR Master Mix Kit (Thermo). The images were visualized on a 1% agarose gel with Ethidium Bromide (EB) and captured by the 4100 Gel imaging system (Tanon Science and Technology Co., Shanghai, China). The background density was subtracted from the target gene band density and normalized to that of β-actin, which was used as the loading control gene. The specific primers sequences are listed in Table 1.

Western blot analysis: Proteins were extracted with a ReadyPrep protein extraction kit according to the manufacturer's instructions (R and D Systems, MN, USA) from the cells. Equal amounts of proteins (50 µg) were loaded on 10% SDS-PAGE gels and transferred onto polyvinylidenedifluoride membranes. The membranes were blocked for 1 h with a 5% BSA in Tris-buffered saline solution and Tween 20 (10 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20; TBS-T). Membranes were then incubated overnight at 4°C with primary antibodies. After 3 washes, incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies was performed for 1 h at room temperature. Quantitative chemiluminescence was achieved using ECL western blotting detection reagents (Millipore). Signal intensity was quanti ed using Image J software. The following antibody was used: rabbit anti-synaptophysin (1:1000; Abcam). Forcontrol loading, blots were stripped and reprobed with mouse anti-beta actin (1:2000; Abcam).

Statistical analysis: All data are expressed as the Mean±Standard Deviation (SD). All statistical analyses were carried out using SPSS 13.0 (SPSS Inc., Chicago, IL, USA). Comparisons among more than two groups were made using one-way analysis of variance (ANOVA) followed by the Least Significant Difference (LSD) test. The differences were considered statistically significant if $p<0.05$[24].

RESULTS

EA treatment prevented the increased anxiety-like behaviors and reduced sucrose preference induced by OVX and CUMS: The time and distance in the OFT central zone were assessed to examine the effect of EA on anxiety-like behaviors in the perimenopausal depression model induced by OVX and CUMS. The results showed that there was no difference among the 5 groups before OVX and CUMS with respect to the duration of time spent in the central zone of the OFT ($p>0.05$). However, OVX and CUMS treatment in the model, CH and EA groups significantly decreased the central time compared to that of the control or sham group, which suggested the anxiety level was increased after OVX and CUMS induction in week 3 ($p<0.01$). Moreover, the CH and EA treatment prevented the reduction incentral time compared to the model group, indicating the rescue effects of CH or EA treatment in increased anxiety-like behaviors induced by OVX and CUMS (Fig. 1a, $p<0.01$). Similarly, the increased distance

Table 1: Primer sequences for CREB, BDNF and β-actin

Gene name		Primer sequence	Product size (bp)
CREB	Forward	5′-GACATCGGCTCCACTCGGTT-3'	162
	Reverse	5'-CCGAGTGCTCCGAGATGAAT-3'	
BDNF	Forward	5'-CCCTGGCTGACACTTTTGAG-3'	160
	Reverse	5'-TCCAGCAGAAAGAGCAGAGG-3'	
β-actin	Forward	5'-TACCCACGGCAAGTTCAACG-3'	122
	Reverse	5'-CACCAGCATCACCCCATTTG-3'	

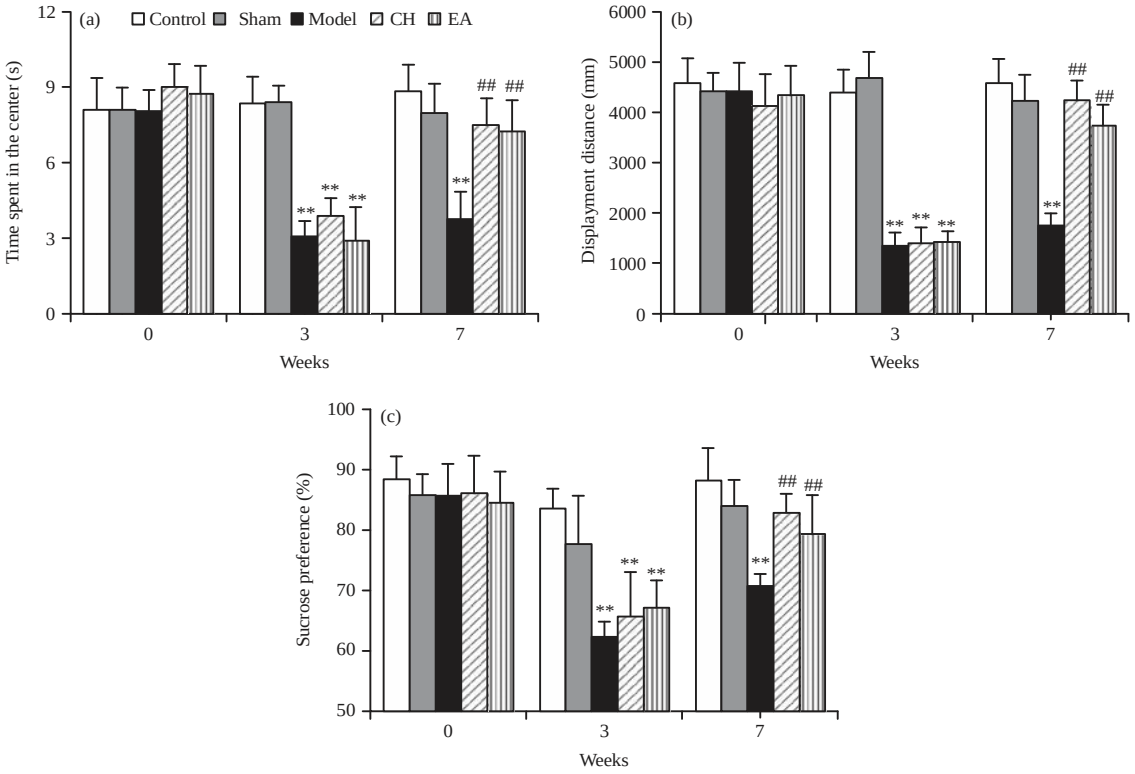

Fig. 1(a-c): EA treatment reverses the increased anxiety-like behaviors and reduced sucrose preference induced by OVX and CUMS, (a)Time spent in the centerat weeks 0, 3 and 7, (b) Displayment distanceat weeks 0, 3 and 7 and (c) Sucrose preference test at weeks 0, 3 and 7

Data are expressed as Mean±SD, **p<0.01 compared with the control or sham group, ##p<0.01 compared with the Model group

moved in the central zone induced by OVX and CUMS was prevented by CH or EA treatment (Fig. 1b, p<0.01), which was consistent with our findings in Fig. 1a.

Furthermore, in order to investigate the anhedonia effect of EA treatment in the perimenopausal depression animal model, we tested sucrose preference in the 5 groups. The results showed no differences among the 5 groups before OVX and CUMS treatment. The OVX and 3 weeks CUMS treatment significantly decreased (p<0.05) the sucrose preference in the model, CH and EA groups compared to that in the control or sham group. However, 4 weeks CH or EA treatment reversed the decreased sucrose preference at the 3rd week, whereas the model group, which did not receive any treatments, did not exhibit any rescued effect in sucrose preference (Fig. 1c, p<0.01). Taken together, these results demonstrated that the EA treatment can rescue the increased anxiety-like behaviors and reduced sucrose preference in this perimenopausal depression animal model.

EA treatment rescued the decreased cell viabilityand increased cell apoptosis induced by OVX and CUMS: Neurofilament Medium (NF-M) was used as a neuronal marker to identify neurons in the hippocampus of the control groupand DAPI to label cell nuclei to confirm the quality of our cell cultures. We found that the approximately 90% of the cells were NF-M-positive neurons from four different slides (Fig. 2a, b, p>0.05). Next, the MTT assay showed that the cell viability was significantly decreased in the model group compared with the control group (p<0.01), whereas increased cell viability was observed in the CH and EA treatment groups (Fig. 3a, p<0.01).

Meanwhile, we found that cell apoptosis measured by flow cytometry was increased in the model group compared with the controlor shamgroups, whereas CH and EA treatment significantly suppressed the increased cell apoptosis rates (Fig. 3b, c, p<0.01).

EA treatment rescued the decreased neurite number and length as well asthe reduced SYP protein expression in hippocampal neurons induced by OVX and CUMS: Furthermore, the morphological changes of hippocampal neurons among the five groups were observed. Figure 4 shows a decreased number (p<0.01) and length (p<0.01) in

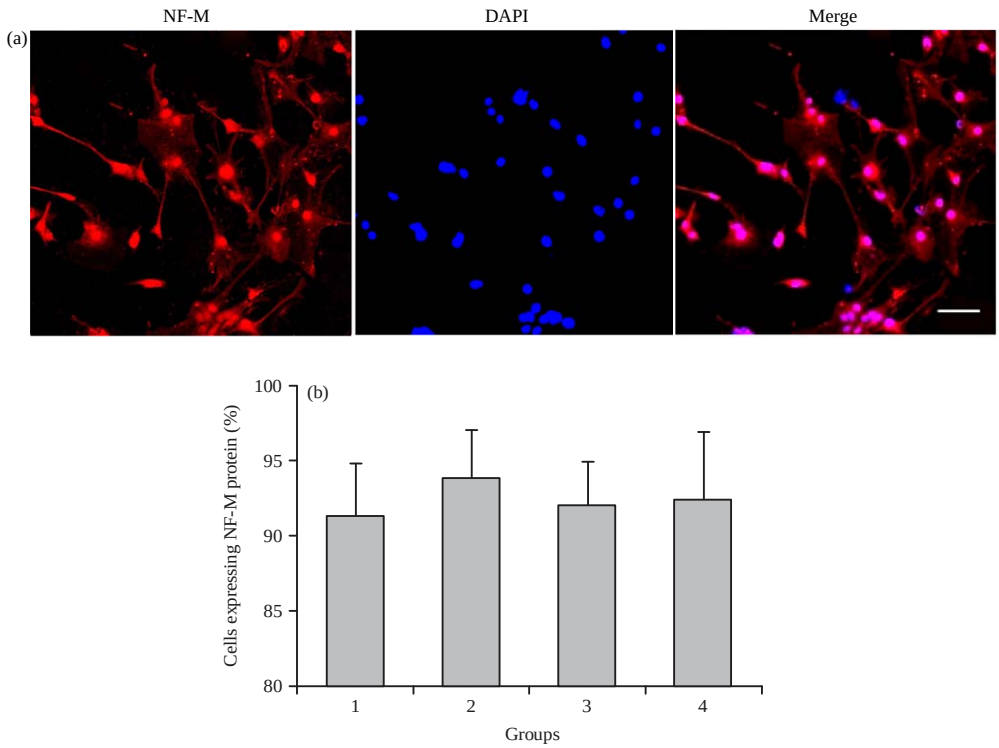

Fig. 2(a-b): Verification of primary culture of hippocampal neurons, (a) Representative images of neurofilamentmedium (NF-M)-positive cells (red signal) in hippocampal neurons cultures. DAPI staining (blue) shows the cell nuclei, Scale bar =50 μm and (b) Quantification of the percentages of NF-M-positive cells
Data are expressed as Mean±SD

the model group, where as the CH ($p<0.01$) or EA ($p<0.05$, $p<0.01$) treatment prevented the decrease in neurite number and length, which demonstrated that the EA treatment rescued the morphological changes in hippocampal neurons in the perimenopausal depression model.

The synapse is the critical connection between neurons that is responsible for the information transmission. Synaptophysin (SYP) is a synaptic protein specifically located in the presynaptic membrane regulating neurotransmitter release[25]. Therefore, we detected SYP protein expression in the hippocampus by Western blotting showed that the SYP protein level was significantly decreased by CUMS and OVX treatment, compared to levels in the control or sham groups ($p<0.01$, Fig. 5a, b). However, CH or EA treatment increased SYP protein expression compared to that in the model group ($p<0.01$, Fig. 5a, b). These results suggested the EA treatment can reverse the decreased SYP protein expression in the depressive condition, which may promote the functional recovery of synapses. In addition, the ELISA data showed results similar to those in the Western blotting experiment with respect to SYP concentration (Fig. 5c, $p<0.01$), which further confirmed that EA treatment rescued the reduced synaptic function in the perimenopausal depression animal model.

EA treatment promoted hippocampal neuron recovery by activating CREB/BDNF pathway-related genes and proteins: In order to study the molecular mechanism underlying the protective effects of EA treatment on hippocampal neuron function, we detected CREB and BDNF expression, which are known to be involved in regulating hippocampal function. The RT-PCR analysis showed that the model group had lower CREB and BDNF mRNA levels compared with the control and sham groups (Fig. 6a-b, $p<0.01$) but EA treatment suppressed the reduced mRNA expression in the model group (Fig. 6a, b, $p<0.01$). Moreover, the CREB suppressor KG-501[26] could block the rescue effect of EA treatment, suggesting it was indeed regulated by CREB/BDNF signaling. Consistently, by using immunofluorescence staining, we observed that although the total CREB amount did not differ among all the groups (Fig. 7a, b, $p>0.05$). Both p-CREB expression and BDNF expression were decreased in the model group (Fig. 7a, b,

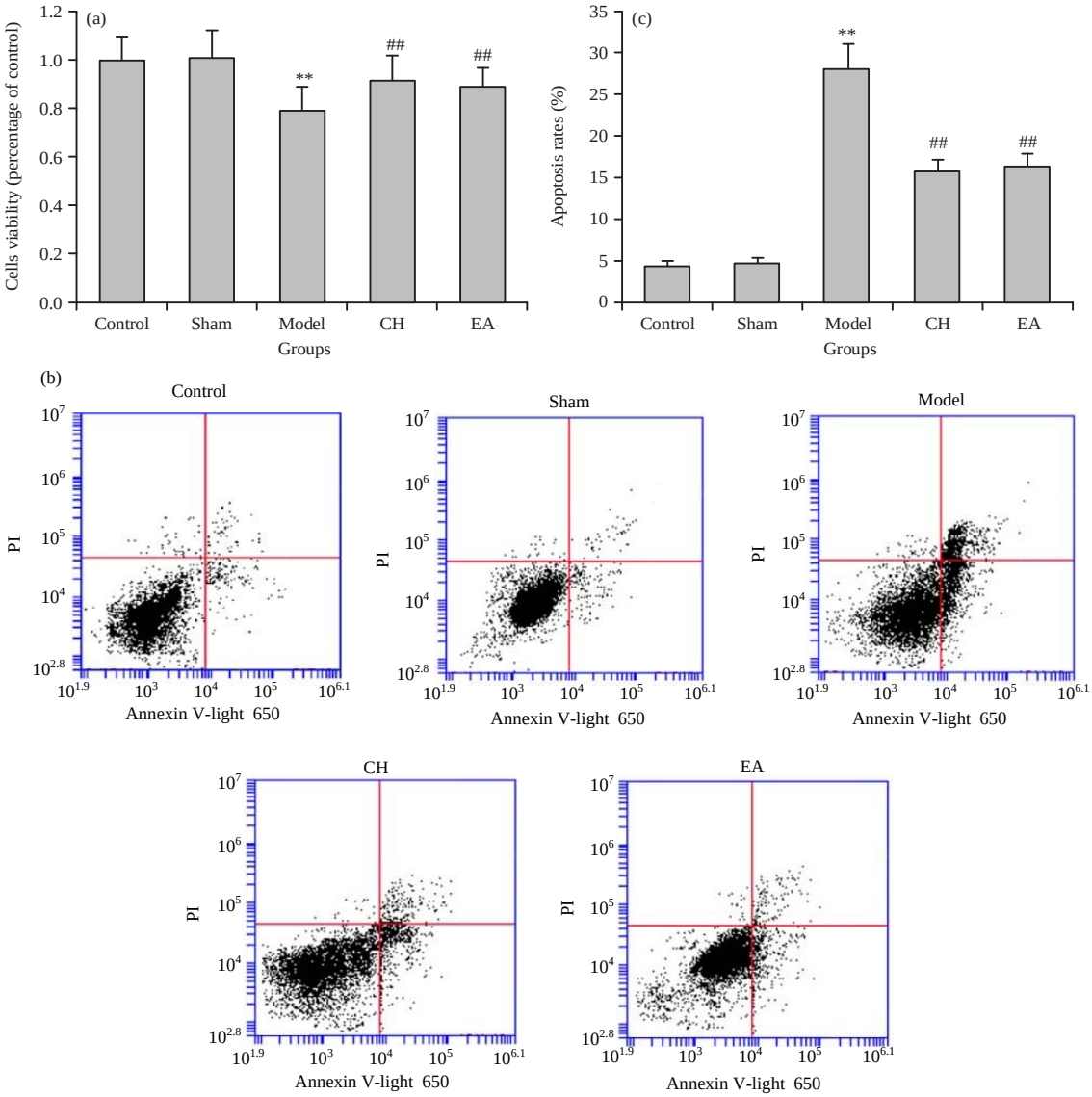

Fig. 3(a-c): EA treatment rescues the decreased cell viability and increases cell apoptosis induced by OVX and CUMS, (a) Cell viability was assessed by MTT assay, (b) Cell apoptosis was detected by flow cytometric analysis and (c) Quantification of the percentages of apoptotic cells

Data are expressed as Mean±SD, **p<0.01 compared with the control or sham group, ##p<0.01 compared with the Model group

p<0.01) but these decreases were prevented by EA treatment. However, the protective effect of EA treatment was blocked by treatment with a CREB suppressor KG-501 (Fig. 7a, b, p<0.01). Meanwhile, the ELISA further demonstrated that the concentrations of total CREB (Fig. 7c, p>0.05), p-CREB and BDNF showed similar alterations as seen in the immunofluorescence results (Fig. 7d, e, p<0.05). Taken together, these results suggested that EA treatment may promote hippocampal neuron recovery by activating CREB/BDNF signaling pathway-related genes and proteins.

DISCUSSION

Clomipramine Hydrochloride (CH) is a tricyclic antidepressant that is widely used in clinical treatment of depression[27,28], thus, CH was used as a positive control treatment in the current study to exclude the possibility of a false positive effect. This study demonstrated that EA treatment exerted a similar protective effect compared with CH. Our experiment also illustrated that EA treatment could suppress the increased anxiety, reduced sucrose preference and impairment of hippocampal neuronsvia the CREB/BDNF

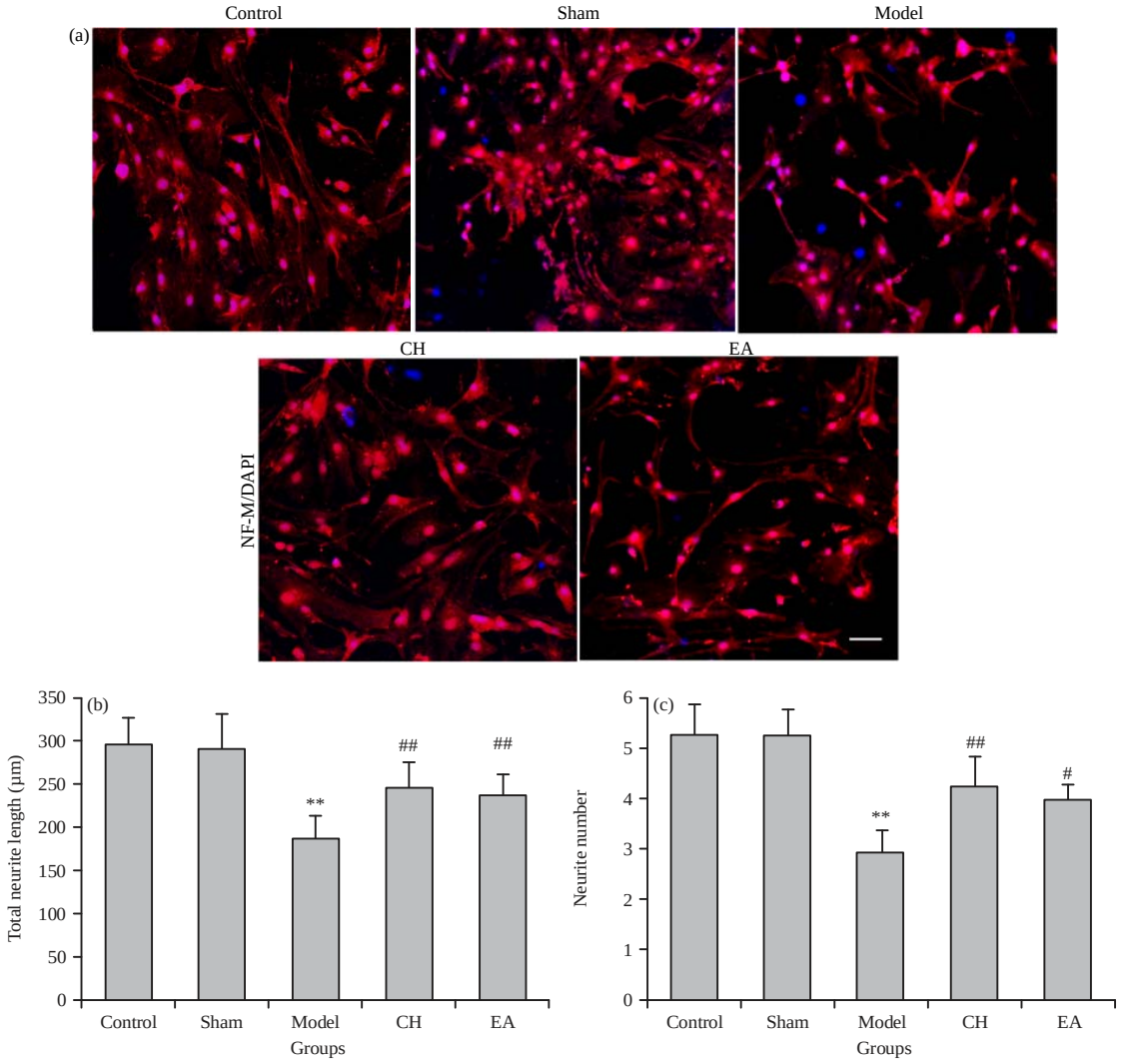

Fig. 4(a-c): EA treatment rescues the decreased number and length of hippocampal neuron neurites induced by OVX and CUMS, (a) Representative images from the individual groups, Scale bar = 50 μm, (b) Totalneurite length and (c) Neurite number

Data are expressed as Mean±SD, **p<0.01 compared with the control or sham group, #p<0.05, ##p<0.01 compared with the model group

signaling pathway in the OVX plus CUMS-induced perimenopausal depression model. Therefore, EA treatment could be a potentially effective therapy for perimenopausal depression.

Bilateral OVX combined with CUMS or individual housing of rats are widely used to establish a perimenopausal depression model in rats and the combined use of CUMS is reported to be more reliable than an individually housed combination[29,30]. OVX is used to simulate the occurrence of natural perimenopausal[31-33]. The CUMS model is a long-term and efficient model that has been widely used in many studies[34-41]. The long-term unpredicted mild stress stimuli can mimic the stressful simulations happening in the daily life of humans, which may cause psychological stress to ultimately induce depression. Thus, the major factors for a successful CUMS model are variable and unpredictable stimulation. According to the CUMS model of Willner *et al.*[16], we chose 7 different stressors to mimic the stressors happening in humans and the order of exposure to the stressors in each day is shown in Table S1. This study also demonstrated that OVX combined with CUMS is a reliable way to mimic perimenopausal depression since the increased anxiety-like behaviors and reduced sucrose preference performance were observed in the behavioral results.

Hippocampal atrophy and neuronal loss have been widely reported in patients with perimenopausal depression[9]. Chronic stress can induce alterations in the function and morphology in the hippocampus, thus impair learning and

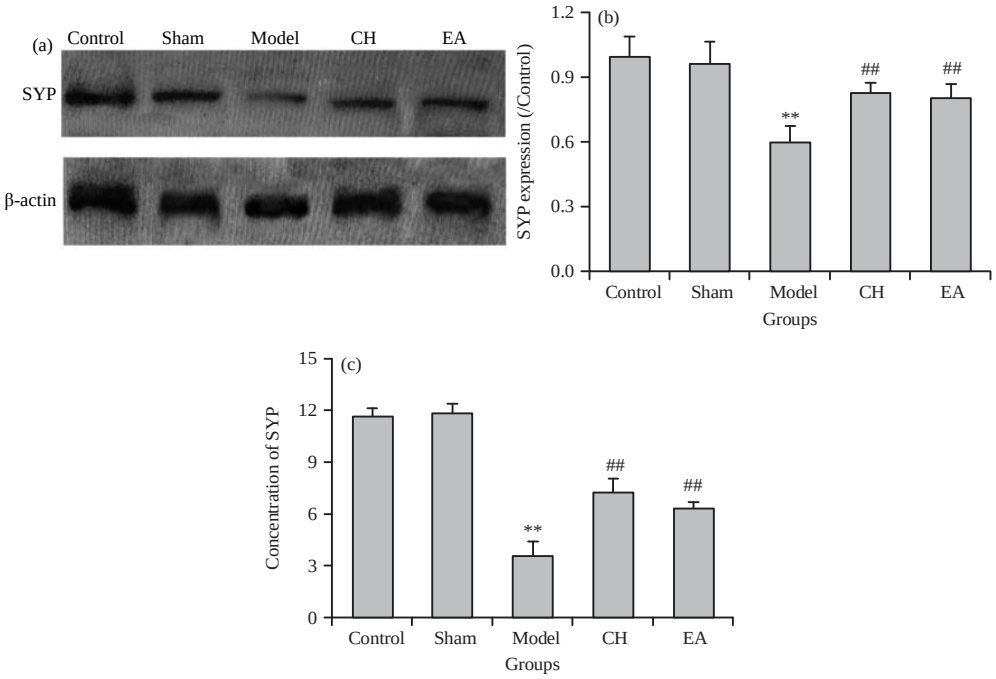

Fig. 5(a-c): EA treatment rescued the reduced expression of SYP protein in hippocampal neurons induced by OVX and CUMS, (a) Protein level of SYP detected by Western blot, (b) Quantitative analysis of results shown in (a) and (c) SYP concentration measured using ELISA

Data are expressed as Mean±SD, **p<0.01 compared with the control or sham group, ##p<0.01 compared with the model group

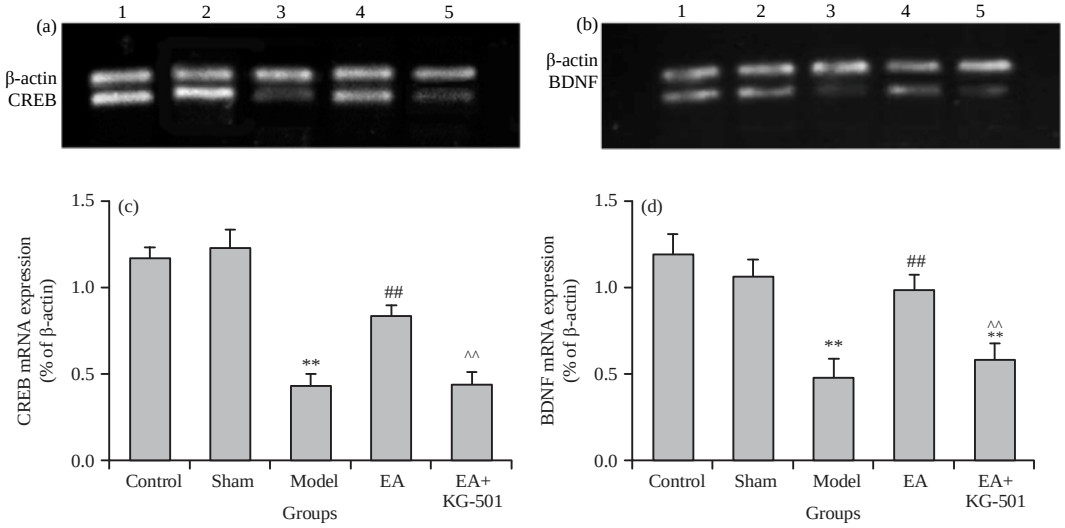

Fig. 6(a-d): EA treatment promoted hippocampal neuron recovery by activating the CREB/BDNF pathway-related genes, (a) Expression of CREB and (b) BDNF were detected by RT-PCR in neurons after EA treatment. β-actin was included as a loading control, (c) Relative optical density of CREB and (d) BDNF mRNA was acquired by Image J

Data are expressed as Mean±SD, **p<0.01 compared with the control or sham group, ##p<0.01 compared with the model group, ^^p<0.01 compared with the EA group

memory and reduce excitability, cognitive ability and sensitivity in response to reward stimuli, which are the major symptoms of depression. Also, stress can damage the hippocampal neuronal function and suppress neurogenesis in the adult hippocampus[42]. Previously, reduced numbers of neurons and atrophy of glia and nerve fiber networks were

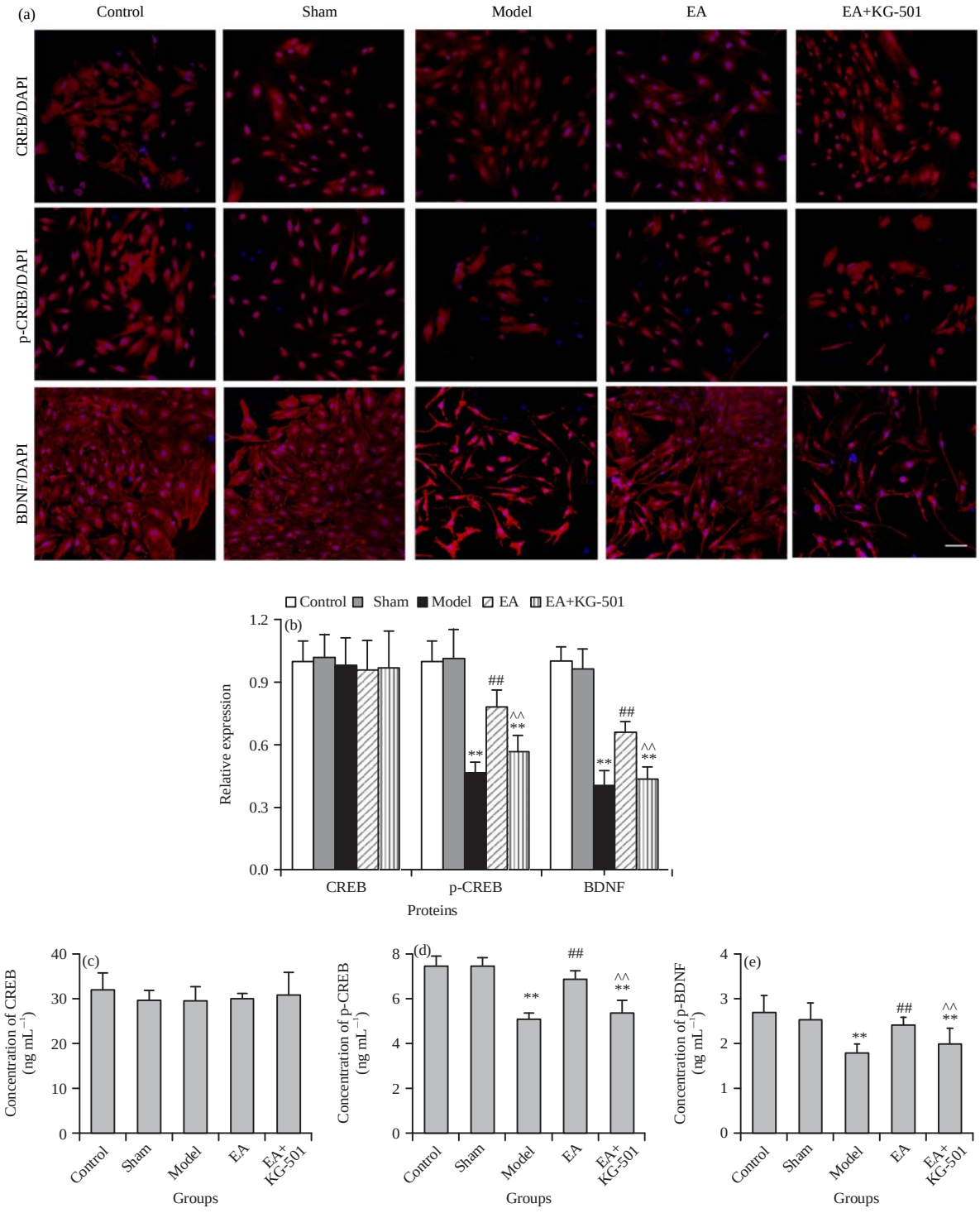

Fig. 7(a-e): EA treatment promoted hippocampal neuron recovery by activating the CREB/BDNF pathway-related proteins, (a) Representative images from immunofluorescence experiments. Red color indicates a positive signal for CREB, p-CREB and BDNF and blue color indicates a positive signal for DAPI, Scale bar =25 μm, (b) Quantification of the CREB, p-CREB and BDNF immunoflurescence intensity, (c) CERB, (d) p-CREB and (e) BDNF concentrations measured by ELISA assay

Data are expressed as Mean±SD, **p<0.01 compared with the control or sham group, ##p<0.01 compared with the model group, ^^p<0.01 compared with the EA group

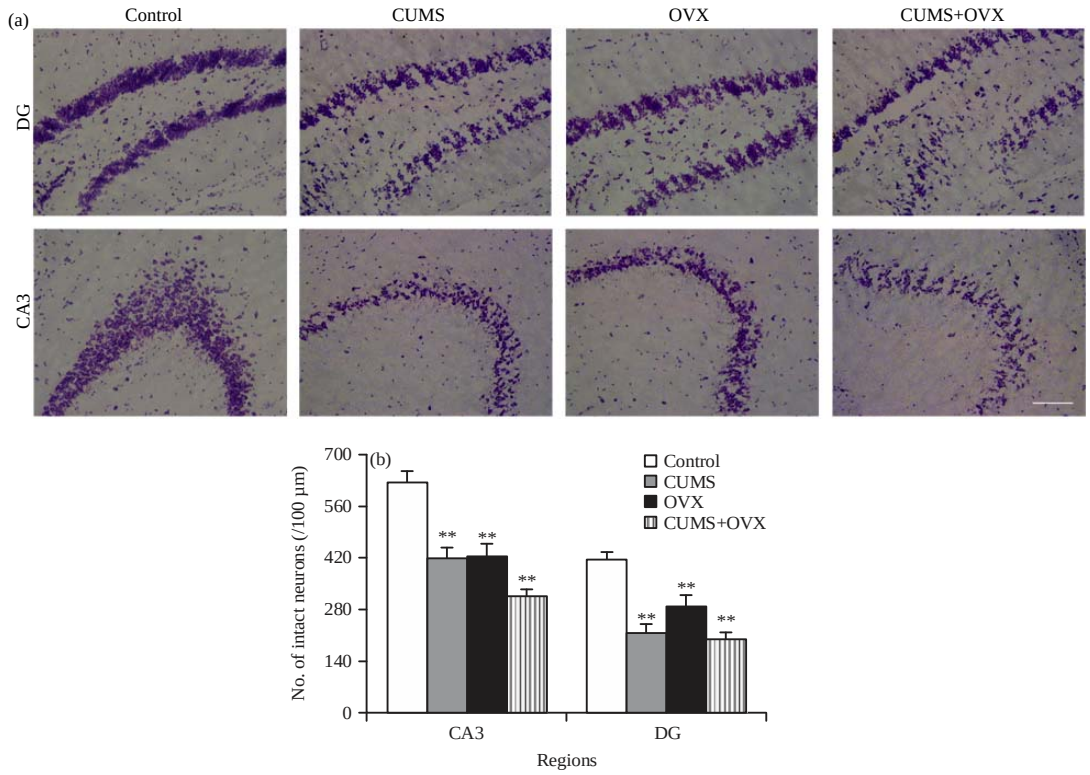

Fig. S1(a-b): Comparison of neuronal survival in hippocampal DG and CA3 from brain sections of rats of the three models, (a) Representative photographs of Nissl-stained hippocampal DG and CA3 sections. Scale bar = 100 μm and (b) Numbers of neurons in the hippocampal area (DG and CA3)

**p<0.01 vs. control group

observed in the hippocampus of patients with depression based on autopsy reports[43]. In order to validate the influence of CUMS+OVX on hippocampal neurons, we designed a CUMS only group and an OVX only group as the controls and utilized Nissl staining to study the effects on hippocampal neurons in the DG and CA3 sub-regions. The results further confirmed our perimenopausal depression model established by using a combination of CUMS and OVX (Fig. S1 and S2).Therefore, enhancement of hippocampal neuron viability, improvement of the morphology and inhibition of cell apoptosis in the hippocampus are necessary to help cure perimenopausal depression.

In the EA therapy, after insertion of the needle into the specific points, the stimulation effect is increased at the points by using different frequency currents. Much attention has been paid to the hippocampal functional recovery with EA therapy in recent years[5,17,44]. The EA treatment at the points "Baihui" and "Yintang" has been demonstrated to improve the depression-like behaviors induced by chronic stress possibly via alteration of the expression of depression-related genes and proteins[45,46]. Yang *et al.*[47] also showed that EA treatment could improve the depression-like behaviors via activating the ERK signaling pathway and increase the number of neuronal precursors in the hippocampal dentate gyrus area. In this study were treated the depressed rats at 3 points including "Shenshu", "Baihui" and "Sanyinjiao" at the same time using EA needles. This treatment could significantly improve ($p<0.05$) the behavioral performances by reducing their anxiety level, increasing their sucrose preference and improving function of the hippocampal neurons.

The NF is a cytoskeletal protein primarilyexpressed in the myelin sheath of axons and is important in the stability of mature axon[48]. This study showed that the NF-M-positive signal was reduced in depressed rats, accompanied by decreases in neurite number and length; however, these effects were all abolished in the EA treatment group, which together indicated the beneficial effect of EA treatment in neurite recovery. SYP is specifically expressed in the presynaptic membrane, which closely associates with the synaptic vesicles to thereby regulate neurotransmitter release. The SYP has been widely applied as a marker of presynaptic terminals to assess the number of synapses and the density change of pre-synapses[49]. Here immunofluorescence and ELISA were employed to detect the SYP expression in the

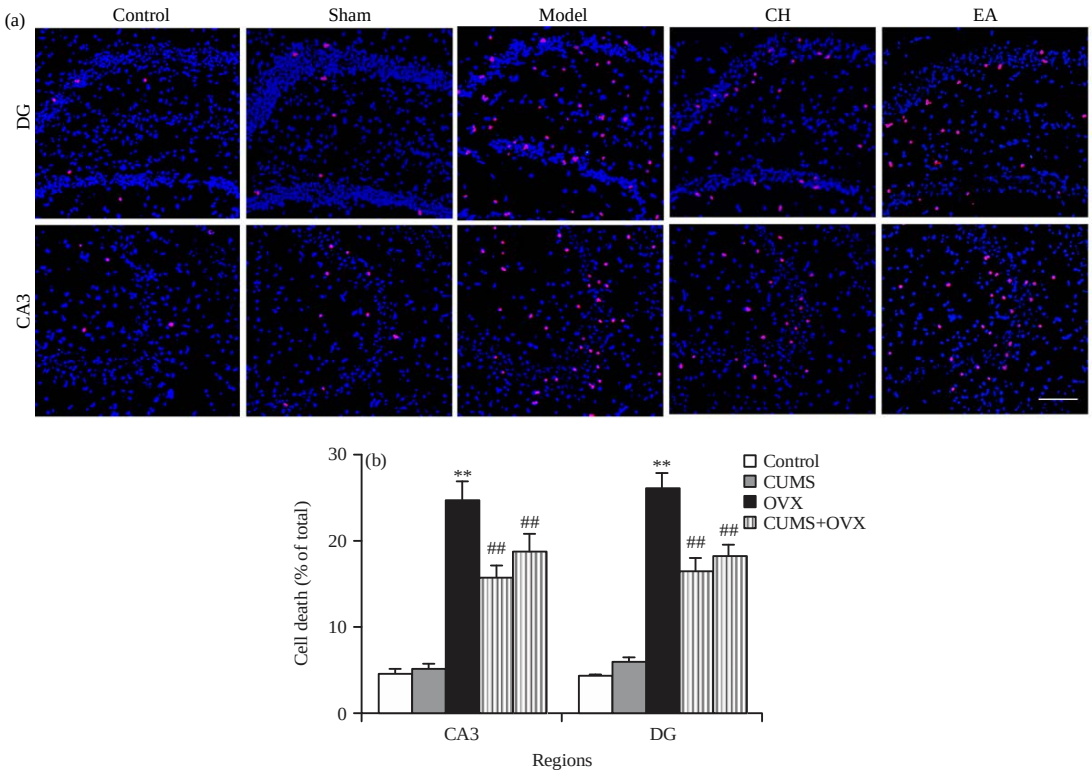

Fig. S2(a-b): EA treatment rescued the increased cell apoptosis induced by OVX and CUMS in the rats, (a) On immunocytochemical staining, apoptotic cells in the DG area (upper) and CA3 area (lower) were stained by TUNEL (red) and nuclei were stained by DAPI (blue). Scale bar = 75 μm and (b) Quantification of the percentages of apoptotic cells

**p<0.01 vs. control group, ##p<0.01 vs. model group

Table S1: Stressors exposure was randomly treated in rats every day

Stressor	Fast food and water (24 h)	Tail clamp (1 min)	Restraint stress (1 h)	Forced swim in cold water (5 min, 4°C)	Forced swim in high temperature water (5 min, 45°C)	Cage tilting without bedding (24 h)	Reversal of day and night (24 h)
day	1	2	3	4	5	6	7
1		*					
2				*			
3	*						
4							*
5					*		
6						*	
7			*				
8	*						
9							*
10				*			
11		*					
12			*				
13	*						
14					*		
15						*	
16			*				
17				*			
18		*					
19					*		
20							*
21						*	

*Represents that rats were subjected the stressor daily

hippocampal neurons and revealed reduced expression in the depressed rat hippocampus, suggesting a synaptic damage or loss. However, EA treatment significantly enhanced ($p<0.05$) the SYP expression compared with that in the group that did not receive EA treatment. Thus, EA therapy was again demonstrated to be effective for the recovery of hippocampal neuronal function.

The CREB as a selective binding CEB nucleoprotein was isolated from PC12 cell lines and rat brain tissue by Yamamoto *et al.*[50] and their study showed that CREB can enhance gene transcription and thus increase the CREB downstream gene activity. Serine 133 of CREB is phosphorylated by protein kinase A (PKA) to regulate the transcriptional function[51]. Activation of CREB further activates downstream signaling molecular BDNF, which binds to its receptor Trk B to activate intracellular signaling cascades involved in neurogenesis, synaptic plasticity and recovery from neuronal damage[51]. In the present study, the mRNA expression of CREB and BDNF along with the protein expression of p-CREB and BDNF were decreased in depressed rats, EA therapy rescued the reduced expression of CREB/BDNF signal pathway-related genes and proteins. By employing the CREB suppressor KG-501 to block the effect of CREB, It was demonstrated that the improvement induced by EA was indeed regulated by the CREB/BDNF pathway. KG-501 is a small molecule compound that acts by blocking the interaction between CREB and CRE, which in turn inhibits the phosphorylation of CREB,and this compound is widely used in animal studies[26]. The use of KG-501 indeed demonstrated that the effects of EA treatment were regulated by the CREB/BDNF signaling pathway.

CONCLUSION

This study clearly shows that EA can improve the anxiety/depression-like behaviors, enhance the viability of hippocampal neurons, reduce neuronal apoptosis and promote neuronal morphology and function in perimenopausal depression. These effects may be attributed to the activation of the CREB/BDNF signaling pathway. Together the evidence presented in this study indicates that EA therapy is a potential effective treatment for perimenopausal depression.

SIGNIFICANCE STATEMENT

This study discovers the electroacupuncture rescued the impairment of hippocampal neurons in perimenopausal depression rats via activating the CREB/BDNF pathway that can be beneficial for patients with perimenopausal depression. This study will help the researcher to uncover the critical area of Chronic Unpredictable Mild Stress (CUMS) combined with bilateral ovariectomy (OVX) that many researchers were not able to explore. Thus, a new theory on potential therapy for perimenopausal depression may be arrived at targeting the CREB/BDNF pathway.

ACKNOWLEDGMENTS

This study was supported by the National Natural Science Foundation of China and financially supported with Grant No. 81373718.

REFERENCES

1. Miller, L.J., C. Girgis and R. Gupta, 2009. Depression and related disorders during the female reproductive cycle. Women's Health, 5: 577-587.
2. Bromberger, J.T., K.A. Matthews, L.L. Schott, S. Brockwell and N.E. Avis *et al.*, 2007. Depressive symptoms during the menopausal transition: The Study of Women's Health Across the Nation (SWAN). J. Affective Disord., 103: 267-272.
3. Farquhar, C., J. Marjoribanks, A. Lethaby, J.A. Suckling and Q. Lamberts, 2009. Long term hormone therapy for perimenopausal and postmenopausal women. Cochrane Database Syst Rev. 10.1002/14651858.CD004143.pub3.
4. Aung, S.K.H., H. Fay and R.F. Hobbs III, 2013. Traditional Chinese medicine as a basis for treating psychiatric disorders: A review of theory with illustrative cases. Med. Acupunct., 25: 398-406.
5. Le, J.J., T. Yi, L. Qi, J. Li, L. Shao and J.C. Dong, 2016. Electroacupuncture regulate hypothalamic-pituitary-adrenal axis and enhance hippocampal serotonin system in a rat model of depression. Neurosci. Lett., 615: 66-71.
6. Xu, J., Y. She, N. Su, R. Zhang, L. Lao and S. Xu, 2015. Effects of electroacupuncture on chronic unpredictable mild stress rats depression-like behavior and expression of p-ERK/ERK and p-P38/P38. Evidence-Based Complement. Altern. Med. 10.1155/2015/650729.
7. Luo, D., R. Ma, Y. Wu, X. Zhang, Y. Liu, L. Wang and W. Fu, 2017. Mechanism underlying acupuncture-ameliorated depressive behaviors by enhancing glial glutamate transporter in Chronic Unpredictable Mild Stress (CUMS) rats. Med. Sci. Monit., 23: 3080-3087.
8. Lupien, S.J., B.S. McEwen, M.R. Gunnar and C. Heim, 2009. Effects of stress throughout the lifespan on the brain, behaviour and cognition. Nat. Rev. Neurosci., 10: 434-445.
9. Yau, S.Y., B.W.M. Lau, J.B. Tong, R. Wong and Y.P. Ching *et al.*, 2011. Hippocampal neurogenesis and dendritic plasticity support running-improved spatial learning and depression-like behaviour in stressed rats. PLoS ONE, Vol. 6. 10.1371/journal.pone.0024263.

10. Arantes-Goncalves, F. and R. Coelho, 2006. [Depression and treatment. Apoptosis, neuroplasticity and antidepressants]. Acta Medica Portuguesa, 19: 9-20, (In Portuguese).
11. Schloesser, R.J., S. Orvoen, D.V. Jimenez, N.F. Hardy and K.R. Maynard *et al*., 2015. Antidepressant-like effects of electroconvulsive seizures require adult neurogenesis in a neuroendocrine model of depression. Brain Stimul., 8: 862-867.
12. Hu, Y., Q. Wen, W. Liang, T. Kang and L. Ren *et al*., 2013. Osthole reverses beta-amyloid peptide cytotoxicity on neural cells by enhancing cyclic AMP response element-binding protein phosphorylation. Biol. Pharmaceut. Bull., 36: 1950-1958.
13. Liu, Z., Y. Qi, Z. Cheng, X. Zhu, C. Fan and S.Y. Yu, 2016. The effects of ginsenoside Rg1 on chronic stress induced depression-like behaviors, BDNF expression and the phosphorylation of PKA and CREB in rats. Neuroscience, 322: 358-369.
14. Zhang, X., Y. Song, T. Bao, M. Yu and M. Xu *et al*., 2016. Antidepressant-like effects of acupuncture involved the ERK signaling pathway in rats. BMC Complement. Altern. Med., Vol. 16. 10.1186/s12906-016-1356-x
15. Li, L.H., Z.C. Wang, J. Yu and Y.Q. Zhang, 2014. Ovariectomy results in variable changes in nociception, mood and depression in adult female rats. PLoS ONE, Vol. 9. 10.1371/journal.pone.0094312.
16. Willner, P., A. Towell, D. Sampson, S. Sophokleous and R. Muscat, 1987. Reduction of sucrose preference by chronic unpredictable mild stress and its restoration by a tricyclic antidepressant. Psychopharmacology, 93: 358-364.
17. Liu, Q., J. Yu, W.L. Mi, Q.L. Mao-Ying, R. Yang, Y.Q. Wang and G.C. Wu, 2007. Electroacupuncture attenuates the decrease of hippocampal progenitor cell proliferation in the adult rats exposed to chronic unpredictable stress. Life Sci., 81: 1489-1495.
18. Russo, E., R. Citraro, A. Davoli, L. Gallelli, E.D. di Paola and G. de Sarro, 2013. Ameliorating effects of aripiprazole on cognitive functions and depressive-like behavior in a genetic rat model of absence epilepsy and mild-depression comorbidity. Neuropharmacology, 64: 371-379.
19. Li, S., Y. Yan, Y. Jiao, Z. Gao and Y. Xia *et al*., 2016. Neuroprotective effect of osthole on neuron synapses in an Alzheimer's disease cell model via upregulation of MicroRNA-9. J. Mol. Neurosci., 60: 71-81.
20. Mi, Y.J., B. Hou, Q.M. Liao, Y. Ma and Q. Luo *et al*., 2012. Amino-Nogo-A antagonizes reactive oxygen species generation and protects immature primary cortical neurons from oxidative toxicity. Cell Death Differ., 19: 1175-1186.
21. Zou, W.W., H.P. Xiao, M.N. Gu, K.X. Liu and Z.Q. Liu, 2013. Propofol induces rat embryonic neural stem cell apoptosis by activating both extrinsic and intrinsic pathways. Mol. Med. Rep., 7: 1123-1128.
22. Zhang, N., Q. Wen, L. Ren, W. Liang and Y. Xia *et al*., 2013. Neuroprotective effect of arctigenin via upregulation of P-CREB in mouse primary neurons and human SH-SY5Y neuroblastoma cells. Int. J. Mol. Sci., 14: 18657-18669.
23. Hannan, M.A., J.Y. Kang, M. Mohibbullah, Y.K. Hong and H. Lee *et al*., 2014. *Moringa oleifera* with promising neuronal survival and neurite outgrowth promoting potentials. J. Ethnopharmacol., 152: 142-150.
24. Li, S.H., P. Gao, L.T. Wang, Y.H. Yan and Y. Xia *et al*., 2017. Osthole stimulated neural stem cells differentiation into neurons in an Alzheimer's disease cell model via upregulation of microRNA-9 and rescued the functional impairment of hippocampal neurons in APP/PS1 transgenic mice. Front. Neurosci., Vol. 11. 10.3389/fnins.2017.00340
25. Sheng, M. and C.C. Hoogenraad, 2007. The postsynaptic architecture of excitatory synapses: A more quantitative view. Annu. Rev. Biochem., 76: 823-847.
26. Pistollato, F., J. Louisse, B. Scelfo, M. Mennecozzi and B. Accordi *et al*., 2014. Development of a pluripotent stem cell derived neuronal model to identify chemically induced pathway perturbations in relation to neurotoxicity: Effects of CREB pathway inhibition. Toxicol. Applied Pharmacol., 280: 378-388.
27. Micale, V., A. Tamburella, G.M. Leggio, C. Mazzola, V.L. Volsi and F. Drago, 2008. Behavioral effects of saredutant, a tachykinin NK2 receptor antagonist, in experimental models of mood disorders under basal and stress-related conditions. Pharmacol. Biochem. Behav., 90: 463-469.
28. Rossi, M., B. Rotblat, K. Ansell, I. Amelio and M. Caraglia *et al*., 2014. High throughput screening for inhibitors of the HECT ubiquitin E3 ligase ITCH identifies antidepressant drugs as regulators of autophagy. Cell Death Dis., Vol. 5. 10.1038/cddis.2014.113.
29. Ma, S., J. Wu, Y. Feng and B. Chen, 2011. Elevated estrogen receptor expression in hypothalamic preoptic area decreased by electroacupuncture in ovariectomized rats. Neurosci. Lett., 494: 109-113.
30. Wang, Y., Y. Xu, H. Sheng, X. Ni and J. Lu, 2016. Exercise amelioration of depression-like behavior in OVX mice is associated with suppression of NLRP3 inflammasome activation in hippocampus. Behav. Brain Res., 307: 18-24.
31. Go, H., J.A. Ryuk, H.W. Lee and B.S. Ko, 2015. Palmiwon attenuates hepatic lipid accumulation and hyperlipidemia in a menopausal rat model. Menopause, 22: 872-884.
32. Vieira-Potter, V.J., J. Padilla, Y.M. Park, R.J. Welly and R.J. Scroggins *et al*., 2015. Female rats selectively bred for high intrinsic aerobic fitness are protected from ovariectomy-associated metabolic dysfunction. Am. J. Physiol.-Regul. Integr. Comp. Physiol., 308: R530-R542.
33. Liu, J., H. Lin, Y. Huang, Y. Liu, B. Wang and F. Su, 2015. Cognitive effects of long-term dydrogesterone treatment used alone or with estrogen on rat menopausal models of different ages. Neuroscience, 290: 103-114.

34. Tang, M., P. Jiang, H. Li, H. Cai, Y. Liu, H. Gong and L. Zhang, 2015. Antidepressant-like effect of n-3 PUFAs in CUMS rats: Role of tPA/PAI-1 system. Physiol. Behav., 139: 210-215.
35. Li, J., Y. Li, B. Zhang, X. Shen and H. Zhao, 2016. Why depression and pain often coexist and mutually reinforce: Role of the lateral habenula. Exp. Neurol., 284: 106-113.
36. Qin, T., F. Fang, M. Song, R. Li, Z. Ma and S. Ma, 2017. Umbelliferone reverses depression-like behavior in chronic unpredictable mild stress-induced rats by attenuating neuronal apoptosis via regulating ROCK/Akt pathway. Behav. Brain Res., 317: 147-156.
37. Luo, Y., S. Kuang, L. Xue and J. Yang, 2016. The mechanism of 5-lipoxygenase in the impairment of learning and memory in rats subjected to chronic unpredictable mild stress. Physiol. Behav., 167: 145-153.
38. Zhu, X., G. Ye, Z. Wang, J. Luo and X. Hao, 2017. Sub-anesthetic doses of ketamine exert antidepressant-like effects and upregulate the expression of glutamate transporters in the hippocampus of rats. Neurosci. Lett., 639: 132-137.
39. Liu, S., T. Du, Z. Liu, Y. Shen, J. Xiu and Q. Xu, 2016. Inverse changes in L1 retrotransposons between blood and brain in major depressive disorder. Scient. Rep., Vol. 6. 10.1038/srep37530.
40. Gao, X., F.Z. Zhuang, S.J. Qin, L. Zhou and Y. Wang *et al*., 2016. Dexmedetomidine protects against learning and memory impairments caused by electroconvulsive shock in depressed rats: Involvement of the NMDA Receptor Subunit 2B (NR2B)-ERK signaling pathway. Psychiatry Res., 243: 446-452.
41. Xu, P., K.Z. Wang, C. Lu, L.M. Dong and J.L. Zhai *et al*., 2016. Antidepressant-like effects and cognitive enhancement of the total phenols extract of *Hemerocallis citrina* Baroni in chronic unpredictable mild stress rats and its related mechanism. J. Ethnopharmacol., 194: 819-826.
42. Liang, J.H., L. Yang, S. Wu, S.S. Liu and M. Cushman *et al*., 2017. Discovery of efficient stimulators for adult hippocampal neurogenesis based on scaffolds in dragon's blood. Eur. J. Med. Chem., 136: 382-392.
43. Lopim, G.M., D.V. Campos, S.G. da Silva, A.A. de Almeida, R. Lent, E.A. Cavalheiro and R.M. Arida, 2016. Relationship between seizure frequency and number of neuronal and non-neuronal cells in the hippocampus throughout the life of rats with epilepsy. Brain Res., 1634: 179-186.
44. Yang, L., N. Yue, X. Zhu, Q. Han and B. Li *et al*., 2014. Electroacupuncture promotes proliferation of amplifying neural progenitors and preserves quiescent neural progenitors from apoptosis to alleviate depressive-like and anxiety-like behaviours. Evidence-Based Complement. Altern. Med. 10.1155/2014/872568
45. Guo, Z., Y. Tu, T.W. Guo, Y.C. Wu and X.Q. Yang *et al*., 2015. Electroacupuncture pretreatment exhibits anti-depressive effects by regulating hippocampal proteomics in rats with chronic restraint stress. Neural Regener. Res., 10: 1298-1304.
46. Duan, D., X. Yang, T. Ya and L. Chen, 2014. Hippocampal gene expression in a rat model of depression after electroacupuncture at the Baihui and Yintang acupoints. Neural Regener. Res., 9: 76-83.
47. Yang, L., N. Yue, X. Zhu, Q. Han, Q. Liu, J. Yu and G. Wu, 2013. Electroacupuncture upregulates ERK signaling pathways and promotes adult hippocampal neural progenitors proliferation in a rat model of depression. BMC Complement. Altern. Med., Vol. 13. 10.1186/1472-6882-13-288
48. Perrot, R., R. Berges, A. Bocquet and J. Eyer, 2008. Review of the multiple aspects of neurofilament functions and their possible contribution to neurodegeneration. Mol. Neurobiol., 38: 27-65.
49. Zhou, H. and G. Liu, 2015. Regulation of density of functional presynaptic terminals by local energy supply. Mol. Brain, Vol. 8. 10.1186/s13041-015-0132-z
50. Yamamoto, K.K., G.A. Gonzalez, W.H. Biggs III and M.R. Montminy, 1988. Phosphorylation-induced binding and transcriptional efficacy of nuclear factor CREB. Nature, 334: 494-498.
51. Platenik, J., Z. Fisar, R. Buchal, R. Jirak, E. Kitzlerova, M. Zverova and J. Raboch, 2014. GSK3β, CREB and BDNF in peripheral blood of patients with Alzheimer's disease and depression. Progr. Neuro-Psychopharmacol. Biol. Psychiatry, 50: 83-93.