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Original Research

Electroacupuncture ameliorates post-traumatic stress disorder in rats via a mechanism involving the BDNF-TrkB signaling pathway

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Abstract: Post-traumatic stress disorder (PTSD) is a mental health condition triggered by a terrifying event, causing flashbacks, nightmares and severe anxiety. It develops in individuals who have experienced a shocking, scary, or dangerous event. Electroacupuncture is reported to be effective for the treatment of PTSD. The present study was carried out to investigate the protective effect of electroacupuncture in a rat model of PTSD, and the mechanism involved. Specific-pathogen-free male Sprague Dawley rats (n = 30) weighing 180 – 220 g (mean weight = 200 ± 20 g) were randomly assigned to three groups of ten rats each: control group, single-prolonged stress (SPS) group, and treatment group. The treatment group rats received electroacupuncture. Changes in PTSD-like behavior were assessed using locomotor activity, elevated plus-maze (EPM) and fear conditioning tests. The mRNA and protein expressions of brain-derived neurotrophic factor (BDNF) and tropomyosin receptor kinase B (TrkB) were determined using real-time quantitative polymerase chain reaction (qRT-PCR) and Western blotting, respectively. Co-immunoprecipitation (Co-IP) was used to measure BDNF and TrkB binding interaction, while chromatin immunoprecipitation (ChIP) was used to evaluate the binding between cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB) and its target genes. Electroacupuncture significantly increased locomotor activity and exploratory behavior, but significantly reduced general fear and anxiety in SPS rats ($p < 0.05$). It also significantly upregulated the mRNA and protein expressions of BDNF and TrkB, and increased the binding of BDNF to its receptor TrkB ($p < 0.05$). Electroacupuncture significantly increased the binding of CREB to BDNF promoter region ($p < 0.05$). Electroacupuncture ameliorates PTSD in rats via a mechanism involving the BDNF-TrkB signaling pathway.

Key words: Brain-derived neurotrophic factor; Electroacupuncture; Post-traumatic stress disorder; Single-prolonged stress; Tropomyosin receptor kinase B.

Introduction

Post-traumatic stress disorder (PTSD) is a mental health condition triggered by a terrifying event, causing flashbacks, nightmares and severe anxiety. It develops in individuals who have experienced a shocking, scary, or dangerous event. Post-traumatic stress disorder (PTSD) is characterized by lack of interest in social activities and heightened startle and arousal. The symptoms usually persist for more than 1 month and cause significant social or occupational dysfunction (1). In the U.S., the average lifetime probability of exposure to at least one severe trauma is estimated to be as high as 75 % (2). Experienced traumatic events account for 36.7 - 81.3 % of the total population, while the lifetime prevalence of PTSD in men and women are put at 5.0 and 10.4 %, respectively (3). After the Wenchuan earthquake in 2008, the prevalence of PTSD was 58.2 % two months after the earthquake, and 22.10 % eight months after the earthquake (4).

Acupuncture is a form of alternative medicine and a key component of Traditional Chinese Medicine (TCM) in which thin needles are inserted into the body. It is relatively safe, cost-effective, well-tolerated, and effective against mental disorders such as anxiety, dementia, and sleep/eating disorders) (5). The effectiveness of acupuncture on PTSD has been reported in literature (6, 7). Studies on different acupuncture treatments on patients with PTSD after the Great Earthquake showed significant improvement in symptoms such as fear and depression, and the curative effect was better than that of paroxetine. Studies have also shown that electroacupuncture is better, when compared with other acupuncture methods (8). Zoological study has also shown that electroacupuncture can effectively repair synaptic plasticity in the amygdala and hippocampi of rats with PTSD (9). However, the underlying molecular mechanisms remains unknown.

The neurotrophic hypothesis of depression postulates that neuronal plasticity is a key factor in the development of depression and in the clinical response to antidepressants. Brain-derived neurotrophic factor (BDNF), an important protein in this process, is ubiquitously expressed in the central nervous system (CNS). It plays an important role in learning, memory, neuroprotection and pathological processes of neurodegenerative diseases (10). The normal function of BDNF and its specific receptor tropomyosin receptor kinase B (TrkB) is closely related to cognition, learning and memory functions of the brain (11). The involvement of

BDNF-TrkB signaling pathway in psychiatric disorders has been reported. This pathway has been implicated in the onset of inflammation-related depression and is a potential therapeutic target (12). Antidepressants act to improve the function of key brain regions via a mechanism involving the BDNF-TrkB signaling pathway (12, 13). The concentration of BDNF in peripheral blood of patients with PTSD is significantly reduced relative to normal healthy patients, and its upregulation leads to improvement in symptoms such as anxiety, depression, and fear (14). The BDNF-TrkB signaling pathway and resultant changes in the structure and function of relevant brain regions are the pathological bases for the clinical manifestations of PTSD (15). The aim of this study was to investigate the protective effect of electroacupuncture in rat model of PTSD and the mechanism involved.

Materials and Methods

Materials

Acupuncture needles were purchased from Suzhou Medical Appliance Factory (China) and HANS-200 acupoint nerve stimulator was produced by Nanjing Jisheng Medical Treatment Technology Co. Ltd. (China). Nuclear/cytoplasmic protein extraction kit and protein A/G beads were obtained from Beyotime Biotechnology (China). Bicinchoninic acid (BCA) protein kit, SYBR qPCR mix and Nanodrop 2000 spectrophotometer were products of Thermo-Fisher Scientific (USA). Rabbit polyclonal anti-BDNF, TrkB, MEK1 (phospho S298), MEK1, p-ERK1/2, ERK1/2, phosphoinositide 3-kinase (PI3K), p-Akt, Akt, p-CREB, CREB, lamin A and β-actin antibodies were obtained from Abcam (UK). Enhanced chemiluminescence (ECL) was a product of GE Healthcare Life Science (USA), while PrimeScript reverse transcription reagent kit with gDNA eraser was purchased from Takara Bio Inc. (Japan).

Experimental rats

Specific-pathogen-free male Sprague Dawley rats (n = 30) weighing 180 – 220 g (mean weight = 200 ± 20 g) were obtained from the Model Animal Research Center of Nanjing University, Jiangsu, China. They were housed in metal cages under standard conditions and had free access to standard feed and water. The rats were exposed to 12-h light/12-h dark cycle and maintained at 25 ˚C and 65 % humidity. They were acclimatized to the laboratory conditions for seven days prior to commencement of the study. The study protocol was approved by the Institutional Animal Ethics Committee of Chengdu University of Traditional Chinese Medicine, Sichuan, China and the study procedures were carried out according to the guidelines of the National Institute of Health (NIH) for the use and care of experimental animals.

The rats were randomly assigned to three groups of ten rats each: control group, single-prolonged stress (SPS) group, and treatment group. The treatment group rats received electroacupuncture.

Preparation of rat model of PTSD

Rat model of PTSD was prepared according to the guidelines of the Advances in Basic and Clinical Research International Conference of the Japanese Ministry of Education (2005). Each rat was first restrained via placement inside a plastic tube for 2 h and thereafter subjected to forced swim test for 20 min in a rectangular sink filled to 30 cm with water maintained at 24 ^{o}C. Then, the rats were anesthetized with ether in a cylinder until they became unconscious and were finally returned to their cages.

Electroacupuncture treatment

The rats were immobilized on a 6 × 12 cm wooden pedal, 50 cm above the ground. With the rat abdomens facing down, the rat limbs were fixed on the pedal with medical tape, and their necks, chests and waists were fixed with adjustable nylon Velcro. The stimulation points of were *Baihui* (GV20), *Shenting* (GV24), and *Shenshu* (BL23, bilateral). The three points were accurately located by referring to "Experimental Acupuncture" textbook and using the method of comparative anatomy. Instruments used in the electroacupuncture consisted of 32G Hwato disposable acupuncture needles (0.25 mm diameter × 25 mm length, and HANS-200 acupoint nerve stimulator. Two sets of electrical stimulation were used (GV24 and GV20) and were connected to the anode, and the bilateral BL23 was connected separately to the cathode. Parameters of electroacupuncture included a 2/100 Hz dilatational frequency wave with an automatic shift between 2 and 15 Hz of stimulation (3 sec duration for each shift), and a current intensity of 1 mA. Electroacupuncture of all regions was performed each day for 20 min consecutively for 3 weeks.

Locomotor activity assay

Each rat was placed in an open-field arena (40 × 40 × 50 cm) to allow free exploration within 30 min. Video tracking was used to record horizontal movement of each rat over 6 consecutive blocks of 5 min each.

Elevated plus-maze test

Elevated plus-maze (EPM) was used to assess anxiety-like behavior in the rats by comparing their inclination to explore the open arms of the maze. Each rat was placed on the center of the plus-maze (10.0 cm wide, 50.0 cm long, and 55.0 cm off the floor) with wal-

Table 1. Primer sequences used for qRT-PCR.

Gene	Sequence
BDNF	Forward: 5'-AATAATGTCTGACCCCAGTGCC-3'
	Reverse: 5'- CTGAGGGAACCCGGTCTCAT-3'
TrkB	Forward: 5'- TGCTCAAGTTGGCGAGACAT-3'
	Reverse: 5'-GTCCCAGGAGTTCAGCTCAC-3'

ls (40.0 cm high) on the two opposing arms (16). During a 5-min test, the number of entries into, and time spent in the opened and closed arms were recorded. and the percentages of open arm entries and time spent in the open arms were calculated. Rat behavior was recorded and scored. The EPM test was used as an indicator of general anxiety-like behavior, with lower percentage indicative of higher levels of anxiety.

Fear conditioning test

Fear conditioning behavioral training was performed in 2 chambers of similar sizes (A and B) on the day after EPM test. Contextual fear conditioning was performed in chamber A only, while cued fear conditioning was performed in chambers A and B. Both chambers provided different context stimuli. Chamber A provided auditory stimulus and mild electric foot shock, while chamber B provided only auditory stimulus. The duration of proportional freezing induced by auditory (cue) or context stimulus (chamber environment) was used as an index of conditional fear. In addition to acquisition of fear conditioning, the rats were tested in extinction and recall paradigms. Extinction was measured by placing rats in chamber A without foot shocks. Each training session involved 10 placements (blocks). Four consecutive sessions were performed for contextual fear memory and 3 consecutive sessions for cued fear memory. At the end of extinction paradigm, a recall paradigm was performed, whereby rats were exposed a second time to foot shocks, followed by extinction-like training sessions.

Protein extraction

After successful completion of electroacupuncture and behavioral tests, the rats were anaesthetized via intraperitoneal injection of sodium pentobarbital at a dose of 80 mg/kg bwt and euthanized. Their brains were then excised for analysis.

Nuclear/cytoplasmic proteins were extracted from the brain tissues using protein extraction kit. Approximately 100 mg of amygdala tissue was homogenized with ice-cold phosphate buffer (0.05 M) (60 mg tissue/200 µL buffer) containing extraction reagents A and B in 20:1 ratio (for cytoplasmic protein extraction) and phenylmethylsulfonyl fluoride (PMSF, 1 mM final concentration) using a glass tissue homogenizer. Cytoplasmic and nuclear proteins were extracted using their respective kits according to the manufacturer's instructions. The extracted proteins were quantified using bicinchoninic acid (BCA) protein kit.

Western blotting

A portion of total cell protein (50 µg) from each sample was separated on 10 % sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis and transferred to a fixed polyvinylidene fluoride membrane at 110 V and 90 °C for 120 min. Subsequently, non-fat milk powder (5 %) in Tris-buffered saline containing 0.2 % Tween-20 (TBS-T) was added with gentle shaking at 37 °C and incubated to block non-specific binding of the blot. Incubation of the blots was performed overnight at 4 °C with primary antibodies of rabbit polyclonal anti-BDNF, TrkB, MEK1, MEK1, p-ERK1/2, ERK1/2, PI3K, p-Akt, Akt, p-CREB, CREB, lamin A and β-actin, each at a dilution of 1 to 1000. Then, the membrane was washed thrice with PBS and further incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody for 1h at room temperature. The blot was developed using an X-ray film. Grayscale analysis of the bands was performed using Enhanced Chemiluminescence (ECL). The respective protein expression levels were normalized to that of β-actin which was used as a standard.

Real-time quantitative PCR (qRT-PCR)

Bilateral amygdala was isolated from the brains, and 0.25 g of tissue was used for qRT-PCR. Trizol RNA extraction reagent was used to extract total RNA from cells of each group and RNA concentration and purity were assessed spectrophotometrically.

Complementary DNA (cDNA) synthesis kit (PrimeScript RT reagent kit) was used to perform cDNA synthesis reaction according to the instructions of the manufacturer. Light Cycler 1536 RT-PCR detection system was used for the estimation of the mRNA expressions of BDNF and TrkB. Variation in the cDNA content was normalized using glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The PCR reaction mixture (20 µl) consisted of 6.4 µl of dH_2O, 1.6 µl of gene-specific primer (10 µM), 2 µl of synthesized cDNA and 10 µl of SYBR Premix Ex Taq™ II. The Ct value of U6 was taken as the internal parameter and $2^{-\Delta\Delta Ct}$ was used to calculate the relative expression levels of the proteins**.** Primers used for the qRT-PCR are as shown in Table 1.

Co-IP assay

Whole brains of rats were washed twice with phosphate-buffered saline (PBS) and lysed with 250 µL of ice-cold radio-immunoprecipitation assay (RIPA) buffer containing protease and phosphatase inhibitors. The tissue lysate was transferred to 1.5 mL Eppendorf tubes and placed on a low-speed shaker at 4 °C for 15 min, and then centrifuged at 14,000 g for 15 min at 4°C. The resultant supernatant was collected and cleared with protein A/G-agarose microbeads to remove non-specific binding proteins. The mixture was thereafter centrifuged at 14,000 g for 15 min at 4°C to obtain a supernatant. Total protein concentration in the supernatant was determined using BCA assay kit. The total protein solution was diluted to 1 µg/µL with PBS, and then made up to 500 µL with primary antibody. The antigen-antibody mixture was gently mixed on a shaker at 4°C overnight and centrifuged at 14,000 g for 5 sec to obtain pellet, which was then washed thrice with 800 µL of ice cold PBS. After the addition of loading buffer, the supernatant was subjected to SDS-PAGE and Western blotting.

ChIP assay

Cell suspension resulting from the trypsinization of rat brains was used for ChIP assay. The cells were cross-linked and sonicated according to standard procedures (17). Immunoprecipitation was performed in four replicates and carried out at 4°C overnight with 1 µg of rabbit anti-rat CREB antibody or an irrelevant IgG antibody as a negative control. Immunocomplexes formed were recovered with 50 µL protein A/G beads. Input DNA and purified immunoprecipitated DNA were analyzed using qRT-PCR. Quantification of the ChIP-DNA

was carried using standard methods (18).

Statistical analysis

Data are expressed as mean ± SEM. Statistical analysis was performed using SPSS (20.0). Groups were compared using Student's *t*-test. Statistical significance was assumed at $p < 0.05$.

Results

Effect of electroacupuncture on contextual fear conditioning

There were no significant differences in proportional time of freezing among the groups 3 min before the acquisition of contextual fear conditioning and training ($p > 0.05$; Figure 1A). During the training, freezing became more pronounced in all the groups as rats moved from block 1 to 5 (Figure 1A). In the extinction paradigm, freezing time was significantly reduced in the three groups within each training session (10 blocks), as well as during the entire course of the training (Figures 1B-1E),and electroacupuncture treatment significantly reduced the incidence of freezing and promoted the extinction of contextual fear memory during the entire course of training ($p < 0.05$). In the recall paradigm, it resulted in a disproportionate and statistically signifificant increase in freezing time in the SPS group when compared with the SPS+EA, and Ctrl groups, the percentage of freezing time in the SPS+EA group was signifificantly less than the SPS group (Figure 1F, $p < 0.05$).

Effect of electroacupuncture on cued fear conditioning

There were no significant differences in proportional freezing time among the groups 3 min before the acquisition of cued fear conditioning ($p > 0.05$; Figure 2A). Freezing time was significantly longer in SPS group than in the control and treatment groups ($p < 0.05$, Figure 2A). Electroacupuncture treatment significantly reduced the incidence of freezing and promoted the extinction of cued fear memory during the course of training ($p < 0.05$, Figures 2B-2E). However, towards the end of the recall paradigm (blocks 8 and 10), there were no significant differences in freezing time among the groups ($p > 0.05$).

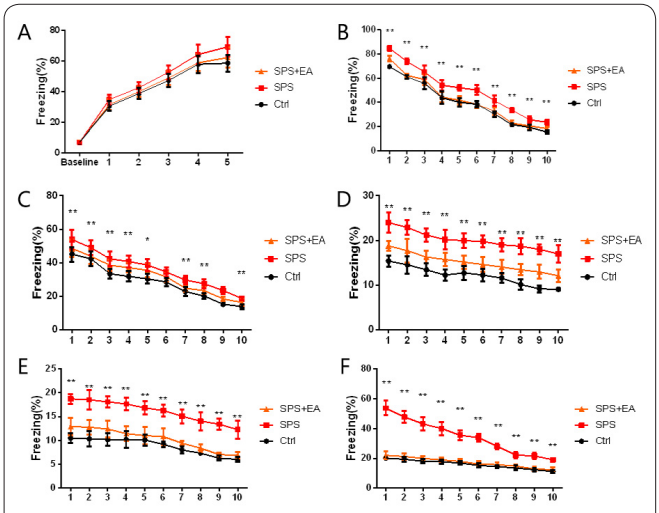

Figure 1. Extinction of contextual fear memory. (A): Percentage of freezing during acquisition; (B, C, D, & E): percentage of freezing during extinction; and (F): percentage of freezing during recall of contextual fear conditioning. *$p < 0.05$, compared with control group; **$p < 0.05$, compared with SPS group.

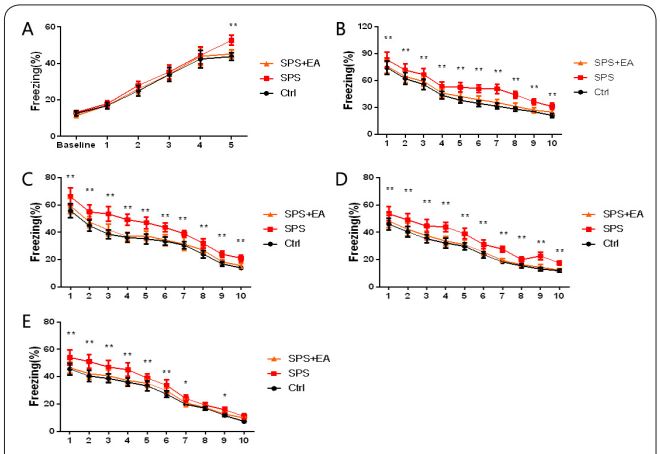

Figure 2. Extinction of cued fear memory. (A): Percentage of freezing time during acquisition; (B, C, D): percentage of freezing time during extinction; and (C): percentage of freezing time during recall of cued fear conditioning. *$p < 0.05$, when compared with control group; **$p < 0.05$, when compared with SPS group.

Locomotor activity assessment

There were no significant differences in locomotor activity among the groups at the beginning of testing block (block 1) ($p > 0.05$; Figure 3A). In blocks 2-6 of free exploration, rats in the treatment group moved significantly faster and covered longer distances, when compared with those of SPS group ($p < 0.05$). Electroacupuncture significantly increased locomotor activity and exploratory behavior, but significantly reduced general fear in SPS rats ($p < 0.05$). There were no significant differences in current levels that elicited notice reactions, withdrawal reactions (flinch), and/or vocalization (vocalize) among the groups ($p > 0.05$, Figure 3 B). The percentage of time spent in the open arms and the number of entries to the open arms were significantly lower in SPS group than in treatment group ($p < 0.05$; Figures 3C and 3D).

Western blotting

Protein expressions of BDNF and TrkB, and phos-

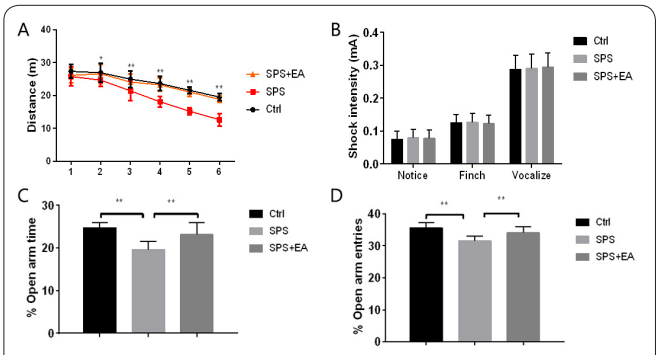

Figure 3. Effect of electroacupuncture on locomotor activity. (A): Locomotor activity; (B): foot shock sensitivity; (C): percentage of time spent in the open arms; and (D): percentage of time spent in EPM. *$p < 0.05$, compared with control group; **$p < 0.05$, compared with SPS group.

phorylation of key proteins in the BDNF-TrkB signaling pathway (p-MEK, p-ERK1/2, PI3K, p-Akt, and p-CREB) were significantly down-regulated in SPS group, relative to control group, but were significantly upregulated by electroacupuncture treatment ($p < 0.05$). These results are shown in Figures 4A-4I.

qRT-PCR , Co-IP and CHIP

Messenger RNA (mRNA) expressions of BDNF and TrkB were significantly down-regulated in SPS group, when compared with control group, but were significantly upregulated by electroacupuncture ($p < 0.05$; Figures 5A and 5B). The results of Co-IP indicated that electroacupuncture treatment significantly increased the binding of BDNF to TrkB ($p < 0.05$; Figure 5C). The results of CHIP showed tha electroacupuncture significantly increased the binding of CREB to the BDNF promoter region ($p < 0.05$; Figure 5D).

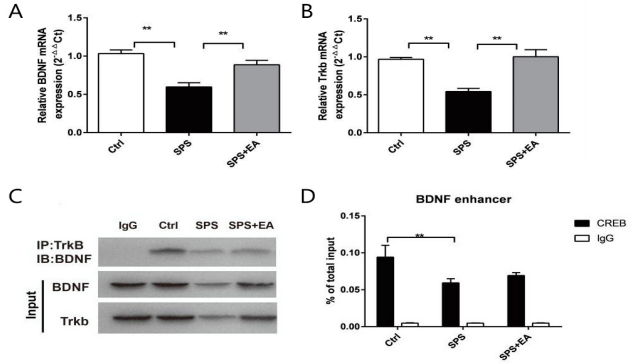

Figure 5. Effect of electroacupuncture on mRNA expressions of BDNF and TrkB. (A and B): mRNA expressions of BDNF and TrkB as measured using qRT-PCR; (C): The binding of BDNF to TrkB as determined via immunoprecipitation. (D): the binding of CREB to BDNF promoter region as determined via Chromatin Immunoprecipitation; *$p < 0.05$, compared with control group; **$p < 0.05$, compared with SPS group.

Discussion

Post-traumatic stress disorder (PTSD) is a mental health condition that is triggered by a terrifying event either experienced or witnessed. The symptoms may include flashbacks, nightmares and severe anxiety, as well as uncontrollable thoughts about the event. This study investigated the protective effect of electroacupuncture in a rat model of PTSD, and the mechanism involved. The results indicate that SPS rats showed longer retention of learned fear memories in the extinction paradigm of fear conditioning test. This finding is in agreement with the hypothesis that PTSD develops as a result of fear learning and reinforcement (19). Electroacupuncture significantly reduced fear memory retention in the rats. The results of elevated plus-maze (EPM) test showed that SPS also significantly increased the level of general anxiety-like behavior in the rats, which was ameliorated by electroacupuncture. Similarly, in the locomotor activity test, SPS rats were less exploratory (more hesitant or fearful), a behavior that was significantly reversed by electroacupuncture. Threshold tests provided supportive evidence that the effects of electroacupuncture in the fear conditioning paradigm were not caused by reduced sensation towards foot shocks. These results suggest that the effects of electroacupuncture may be mediated preferentially by the fear system in the CNS. It is likely that electroacupuncture specifically modulates fear memory in the absence of other factors that negatively affect learning, memory, sensation, or motor activity. The results of qRT-PCR and Western blotting showed that SPS significantly reduced BDNF and TrkB protein and mRNA expressions in the amygdala. However, electroacupuncture treatment significantly reversed the effects of SPS on the levels of expression of these proteins. Co-immunoprecipitation (Co-IP) results suggest that the reduced expressions of BDNF and TrkB could be due to reduced BDNF binding to TrkB. In this study, SPS inhibited the BDNF-TrkB signaling pathway. The phosphorylations of key proteins in this signaling pathway such as ERK, Akt, and CREB were significantly reduced by SPS, but were significantly increased by electroacupuncture. The results of ChIP indicated that the reduced activation of BDNF-TrkB signaling pathway also reduced CREB binding to the BDNF promoter, an indication that SPS may have exerted a feedforward loop inhibition on BDNF-TrkB signaling pathway. These results are in agreement with those of previous reports (20). It is important to note that electroacupuncture significantly reversed the effects of SPS at the different stages of the signaling pathway: this provides a sound mechanistic explanation at the molecular level for its protective effect on fear learning and behavior. These results are in agreement with those of previous studies involving TrkB agonists (21, 22). Reports that support the involvement of BDNF-TrkB signaling pathway in the amelioration of fear learning and reinforcement in PTSD abound in literature (15, 23, 24). In a previous study, it was reported that

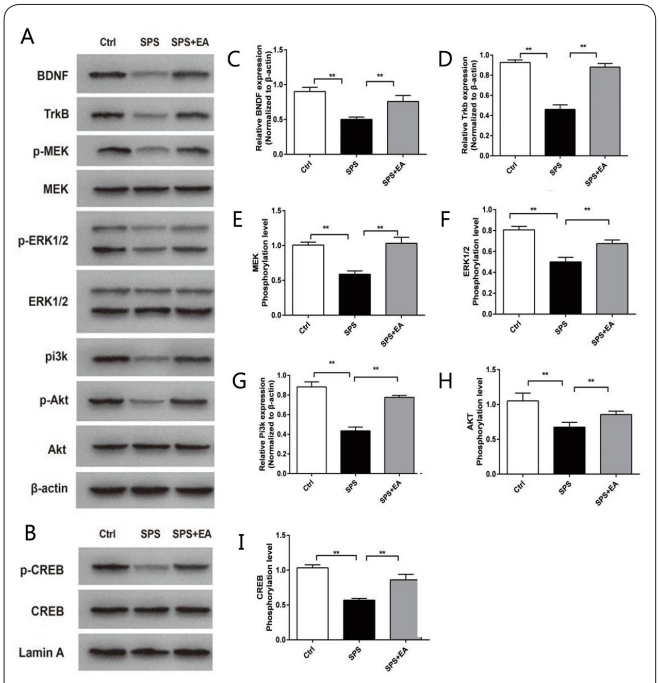

Figure 4. Effect of electroacupuncture on the expressions of BDNF-TrkB signaling pathway proteins in amygdala tissue of SPS rats. (A-I): Protein expressions of BDNF, TrkB,p-MEK, p-ERK1/2,p-PI3k, p-Akt, and p-CREB as measured using Western blotting. *$p < 0.05$, compared with control group; **$p < 0.05$, compared with SPS group.

the binding of CREB to the synaptic key protein PSD95 was significantly reduced by SPS, but was increased significantly by electroacupuncture treatment. This may be the point of interaction between synaptic plasticity and BDNF-TrkB signaling pathway, or it may be the key mechanism engaged by acupuncture in ameliorating PTSD.

Electroacupuncture ameliorates PTSD in rats via a mechanism involving the BDNF-TrkB signaling pathway.

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Conflicts of interest
There are no conflicts of interest in this study.

Author's contribution
All work was done by the author s named in this article and the authors accept all liability resulting from claims which relate to this article and its contents. The study was conceived and designed by Mi Li , Yiqiang Xie and Kai Li; Mi Li, Kai Li and Kun Niu collected and analysed the data; Mi Li wrote the text and all authors have read and approved the text prior to publication.
Kai Li and Yiqiang Xie are co-corresponding authors. Mi Li and Yiqiang are co-first authors.

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