

Electroacupuncture Prevents the Depression-Like Behavior by Inhibiting the NF- κ B/NLRP3 Inflammatory Pathway in Hippocampus of Mice Subjected to Chronic Mild Stress

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Keywords

Electroacupuncture · Stress · Depression · Inflammation

Abstract

Background: The precise physiological mechanisms of acupuncture in the treatment of depression are still unknown. This study aimed to observe the effects of electroacupuncture (EA) on depression-like behavior of mouse in chronic mild stress (CMS) model and explore the underlying mechanism. **Methods:** The depression model was established by using CMS method for 6 weeks. After the third week of the CMS paradigm, EA treatment was performed daily for 15 min over a period of 3 weeks. The antidepressant-like effects of EA were evaluated using the sucrose preference test and the forced swimming test (FST). The protein levels of the nuclear factor-kappa B (NF- κ B), p-NF- κ B, inhibitor of NF- κ B, p-I κ B α , NOD-like receptor protein 3, interleukin (IL)-6, IL-1 β , IL-18, and tumor necrosis factor- α (TNF- α) in hippocampus of mice were detected. **Results:** Sucrose preference was decreased after 6 weeks of CMS and the effects of CMS was reversed by EA. CMS increased immobility time and decreased latency to the first immobility in the FST test, but these effects were reversed by EA. CMS-induced nuclear entry of NF- κ B (nuclear/

cytoplasmic ratio of NF- κ B) with an increase in protein levels of p-NF- κ B and p-I κ B α in the hippocampus. The CMS also increased NLRP3 levels in the hippocampus. However, these effects were reversed by EA. In addition, the levels of IL-6, IL-1 β , IL-18, and TNF- α in the hippocampus were increased by CMS, and these effects of stress were reversed by EA. **Conclusion:** EA prevented CMS-induced depressive-like behaviors by inhibiting NF- κ B/NLRP3 inflammatory pathway.

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Introduction

Major depressive disorder (MDD) is a common mental disorder associated with considerable morbidity, disability, and risk for suicide. MDD is estimated by the World Health Organization to affect more than 300 million people worldwide [1]. The pathogenesis of MDD is still not clear, even though there are various hypotheses. Robust evidence suggests that neuroinflammation is involved in the pathogenesis of MDD, patients show increased systemic inflammatory biomarkers [2, 3].

Selective serotonin reuptake inhibitors and selective norepinephrine and serotonin reuptake inhibitors are

first-line antidepressants for treating MDD, regrettably, only 40–60% of patients respond to these drugs [4]. Antidepressants have many side effects, such as nausea, insomnia, dryness, blurred vision, constipation, and sexual problems [5]. Clinically, depressive patients prefer complementary therapies to drug therapy [6]. Acupuncture is widely used in clinical practice and had been shown to have antidepressant effects in both clinical and animal studies [7, 8]. Combined antidepressants and acupuncture therapy are more effective than drugs alone [7].

The precise physiological mechanisms of acupuncture in the treatment of depression are still unknown. In recent years, the effect of acupuncture on central system inflammation and its underlying mechanism have attracted much attention [7]. Nuclear factor-kappa B (NF- κ B)/NOD-like receptor protein 3 (NLRP3) signaling pathway is the “star pathway” involved in inflammation. NF- κ B represents a central factor in inflammation, stress response, cell differentiation or proliferation as well as apoptosis [3]. NF- κ B/NLRP3 inflammatory pathway can be activated in the hippocampus by chronic mild stress (CMS), then increased the expression of inflammatory cytokines such as tumor necrosis factor- α (TNF- α), interleukin (IL)-6, IL-18, and IL-1 β [3, 9–11]. Whether acupuncture can improve depression through NF- κ B/NLRP3 inflammatory pathway is rarely reported.

Electroacupuncture (EA) is defined as the application of a small current through acupuncture needles; it is thought to deliver a greater dose of acupuncture compared with manual acupuncture, as a result of the intensity and duration of stimulation [8]. EA is widely used in clinical practice, as well as in experimental investigations into the physiological mechanisms of acupuncture. In present study, we investigated the antidepressant-like effects of EA in the CMS mouse model, which is a valid, reasonably reliable, and useful model of depression [12]. We also analyzed the effects of EA on the activation of NF- κ B/NLRP3 inflammatory pathway in the hippocampus. Fluoxetine, a classical antidepressant, was used in this study as a positive control. Administration of fluoxetine has been shown to significantly reverse the depressive-like behavior and inflammation in the hippocampus caused by CMS [3].

Materials and Methods

Animals

Forty male C57BL/6J mice (6–8 weeks old and 20 ± 2 g of weight) were obtained from Hunan SJA Laboratory Animal Co., Ltd (Changsha, China). The mice were housed individually in plastic cage (290 mm $\times$ 178 mm $\times$ 150 mm) under standard condi-

tions ($23 \pm 1^\circ\text{C}$ and $55 \pm 2\%$ humidity) on a 12-h light/dark cycle and had ad libitum access to food and water. The mice were allowed to acclimatize to laboratory condition for 1 week prior to the experiments. This study was carried out in accordance with the Guidelines for the Care and Use of Laboratory Animals published by the US National Institutes of Health. Our study was approved by the Laboratory Animal Ethical Committee of Shenyang Medical College (SYYXY2018010601).

Experimental Design and Intervention

The CMS model was established according to our previous experiments as follows [13]: the mice were subjected to 7 unpredictable mild stressors for 6 weeks. The stress regime for each week consisted of 45° cage tilt (24 h), food and water deprivation (24 h), tail pinch (60 s, 1 cm from the end of the tail), reversal of day and night regimen (24 h), overnight stroboscopic light, restraint in a plastic tube (2 h), and moist bedding (12 h). The mice were subjected to one of these stressors on each day of the week, with the same stressor not applied for 2 consecutive days.

Forty mice were randomly divided into 4 groups:

1. Control group (CON group, $n = 10$): mice did not receive any intervention;
2. CMS group ($n = 10$): mice only received CMS for 6 weeks;
3. CMS + EA group ($n = 10$): after the third week of the CMS paradigm, EA treatment was performed daily for 15 min (min) over a period of 3 weeks. The acupuncture needles (30 mm in length and 0.3 mm in diameter) were inserted at a depth of 5 mm into the Baihui (GV 20), Shenshu (BL 23), and Taixi (KI 3) acupoints. The locations of these points were determined according to *Experimental Acupuncture Science* [14]. Baihui (GV 20) acupoint is at the midpoint between the auricular apices. Shenshu (BL 23) acupoint is located 1 cm lateral to the spine on each side at the height of the tip of the spinous process of the second lumbar vertebra. Taixi (KI 3) acupoint is located in the depression between the medial malleolus and the tendon (Fig. 1a). The needle handle was connected to G6805 EA apparatus (Suzhou Medical Appliance Factory, Shanghai, China), and the stimulation parameters were set as follows: sparse wave, 2 Hz, 2 V, and 0.6 mA current intensity;
4. CMS + fluoxetine group (CMS + FLU group, $n = 10$): after the third week of the CMS paradigm, mice received fluoxetine (10 mg/kg, Eli Lilly and Company, Indianapolis, IN, USA) [3].

After 6 weeks, all mice in the 4 groups underwent 4 consecutive days of behavioral testing (Fig. 1b).

Behavioral Procedures

Sucrose Preference Test

The sucrose preference test (SPT) was started 24 h after the last intervention. Forty-eight hours before the SPT, all mice were habituated to 2 drinking bottles containing 1% sucrose solution. After 12 h of deprivation of food and water, the mice were allowed free access to either of 2 bottles containing water or 1% sucrose solution for 12 h. In our experiment, the position of the bottles was switched every 4 h to prevent the influence of position preference on drinking. Sucrose preference was calculated as: sucrose intake (g)/total liquid intake (g) [3].

Forced Swimming Test

The FST was performed as previous described [3]. The mice were individually placed in a glass cylindrical container (21 cm in

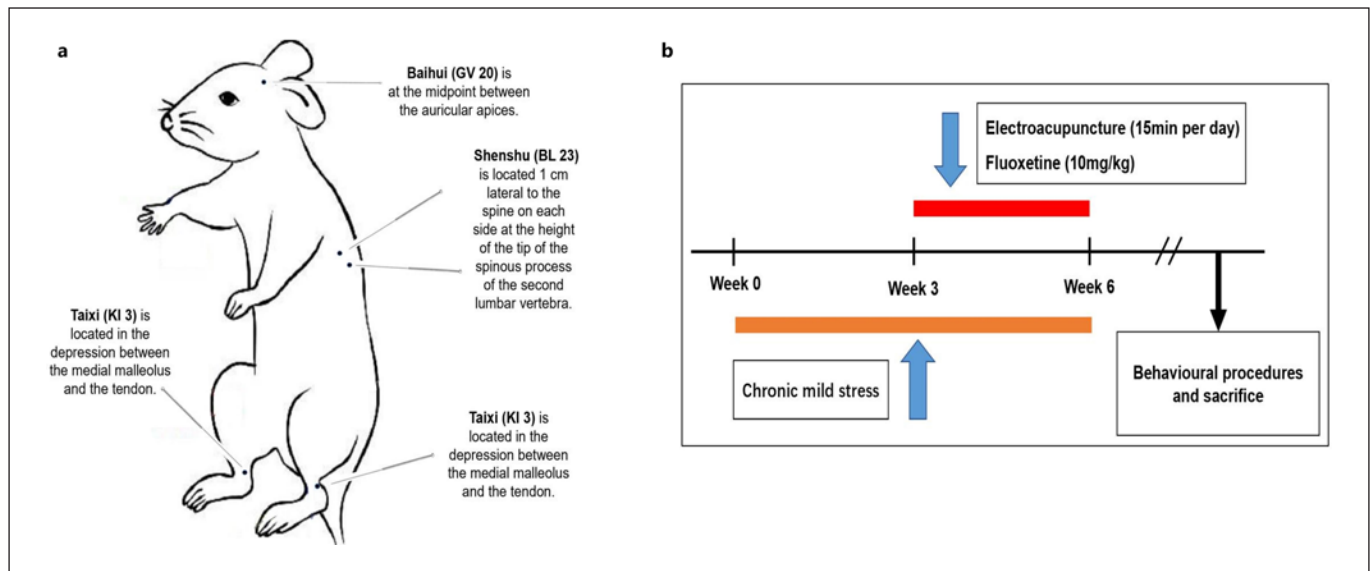


Fig. 1. Electroacupuncture points (a) and experimental design (b).

diameter $\times$ 46 cm in height) that was filled with water ($25 \pm 0.5^\circ\text{C}$) to a depth of 15 cm. Each mouse was exposed to a test session for 6 min. A mouse floating passively in the water without struggling is considered immobile. The immobility time during the last 4 min of the FST, and the latency to the first immobility, were analyzed using a video-tracking program (EthoVision, version 7.0; Noldus Information Technology, Wageningen, The Netherlands).

Brain Sample Collection and Biochemical Assays

After the behavior test, the mice were sacrificed by neck dislocation and the brains were rapidly removed, then the hippocampus were quickly dissected and stored at -70°C .

Enzyme-Linked Immunosorbent Assay

Enzyme-linked immunosorbent assay was used to detect IL-6, IL-1 β , IL-18, and TNF- α in the hippocampus [3]. The tissues were homogenized and centrifuged at 1,000 g for 30 min. Protein levels of cytokines in the supernatants were quantified using enzyme-linked immunosorbent assay kits (Enzyme research Biotechnology Co., Ltd., Shanghai, China), according to the manufacturer's protocol.

Western Blot

The protein levels of the NF- κB , p-NF- κB , p-I κB , I κB , and NLRP3 in the hippocampus of mice were detected by Western blot [3]. The tissues were suspended in lysis buffer, supplemented with a complete protease and phosphatase inhibitor cocktail set (Kanglang Biotechnology Co., Ltd., Shanghai, China), and centrifuged at 16,000 g for 30 min at 4°C . Bicinchoninic acid protein assay (Kanglang Biotechnology Co., Ltd., Shanghai, China) was used to detect protein concentration. The cytoplasmic and nuclear protein samples for NF- κB assay were extracted by a commercial kit purchased from Fushen Biotechnology Co., Ltd., Shanghai, China, according to the manufacturer's instructions. Samples were resolved using

7.5–10% criterion sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Puli Gene Technology Co., Ltd., Beijing, China), followed by transfer to 45- μm polyvinylidene fluoride membranes. Transfer was performed at 4°C in buffer containing 25 mM Tris, 192 mM glycine, and 20% methanol. Then the membranes were blocked with 5% skim milk and incubated with corresponding primary antibodies at 4°C for overnight. Membranes were washed with TBST, then incubated with horseradish peroxidase-conjugated secondary antibody for 1 h and thereafter washed with TBST for 1 h. Subsequently, the membranes were incubated with ECL reagent (Enzyme research Biotechnology Co., Ltd., Shanghai, China) for 2 min and exposed with an X-ray film. Densitometric analysis was performed using VisionWorks LS software (UVP, Upland, CA, USA). The ratio of individual proteins to lamin A/C (for nuclear protein) or GAPDH (for cytoplasmic and total protein) was then determined. The following primary antibodies were used: antibodies of anti-p-NF- κB (1:1,000; ImmunoWay, Plano, TX, USA), anti-NF- κB (1:1,000; ImmunoWay, Plano, TX, USA), anti-p-I κB (1:1,000; ImmunoWay, Plano, TX, USA), anti-I κB (1:1,000; ImmunoWay, Plano, TX, USA), anti-NLRP3 (1:1,000; Santa Cruz, Santa Cruz, CA, USA), anti-Lamin B (1:1,000; Santa Cruz, Santa Cruz, CA, USA), and anti-GAPDH (1:1,000; ImmunoWay, Plano, TX, USA). The following secondary antibodies were used: goat anti-rabbit IgG (1:8,000; Abbkine, Waltham, MA, USA) and goat anti-mouse IgG (1:8,000; Abbkine, Waltham, MA, USA).

Statistical Analysis

Statistical analysis was performed using SPSS Statistics 20.0 (SPSS Inc., Chicago, IL, USA). Data were expressed as mean $\pm$ S.E.M. Potential differences between the mean values were analyzed using one-way analysis of variance (ANOVA) followed by the LSD post hoc test. Values of $p < 0.05$ were considered statistically significant.

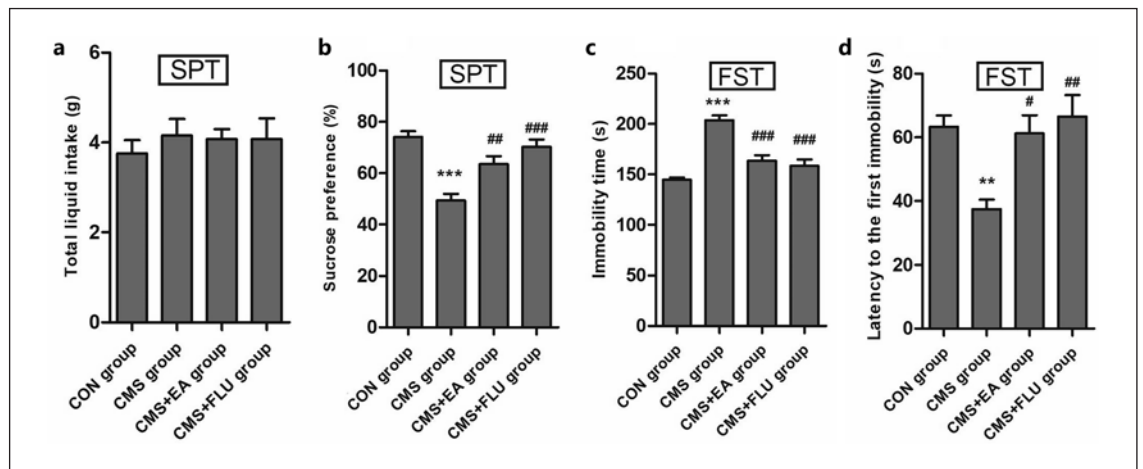


Fig. 2. Effect of EA and fluoxetine on SPT (a, b) and FST (c, d). The data are presented as mean $\pm$ S.E.M. ($n = 10$ mice per group). ** $p < 0.01$, *** $p < 0.001$ versus CON group. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ versus CMS group.

Results

Depressive-Like Behavior in SPT and FST

The results of one-way ANOVA test analysis showed that there were no significant differences in total liquid intake in the SPT among the 4 groups [$F(1, 36) = 0.764$, $p = 0.317$], and there were significant differences in sucrose preference in the SPT among the 4 groups [$F(1, 36) = 16.270$, $p < 0.001$]. Sucrose preference was decreased after 6 weeks of CMS ($p < 0.001$) and the effects of CMS were reversed by EA ($p = 0.008$) or fluoxetine ($p < 0.001$) (Fig. 2a, b).

There were significant differences in the immobility time [$F(1, 36) = 25.793$, $p < 0.001$] and the latency to the first immobility [$F(1, 36) = 7.042$, $p = 0.001$] among the 4 groups. CMS increased immobility time in the FST test ($p < 0.001$) and decreased latency to the first immobility ($p = 0.009$), but these effects were reversed by EA ($p < 0.001$, $p = 0.012$, respectively) or fluoxetine ($p < 0.001$, $p = 0.004$, respectively) (Fig. 2c, d). These findings demonstrated that EA could reduce depressive-like behavior induced by CMS.

EA Inhibits the Activation of NF- κ B/NLRP3 Pathway in the Hippocampus of CMS Mice

As is shown in Figure 3, the results of one-way ANOVA test analysis showed that there were significant differences in nuclear/cytoplasmic ratio of NF- κ B [$F(1, 16) = 10.460$, $p < 0.001$], p-NF- κ B protein levels [$F(1, 16) = 9.711$, $p < 0.001$], p-I κ B α protein levels [$F(1, 16) = 6.739$, $p = 0.004$], and NLRP3 protein levels [$F(1, 16) = 15.970$,

$p < 0.001$] among the 4 groups. The CMS-induced nuclear entry of NF- κ B (nuclear/cytoplasmic ratio of NF- κ B, $p < 0.01$) with an increase in protein levels of p-NF- κ B ($p < 0.001$) and p-I κ B α ($p = 0.031$). Both of EA and fluoxetine reduced the nuclear entry of NF- κ B ($p = 0.004$, $p < 0.001$, respectively) and decreased the protein levels of p-NF- κ B ($p = 0.019$, $p < 0.01$, respectively) and p-I κ B α ($p = 0.027$, $p = 0.030$, respectively). The CMS also increased NLRP3 levels in the hippocampus ($p < 0.001$), but the effects were reversed by EA ($p = 0.004$) and fluoxetine ($p = 0.004$). These findings demonstrated that EA and fluoxetine can inhibit NF- κ B/NLRP3 pathway activation in the hippocampus induced by CMS.

EA Decreases the Levels of Inflammatory Cytokines in the Hippocampus of CMS Mice

As is shown in Figure 4, the results of one-way ANOVA test analysis showed that there were significant differences in IL-6 levels [$F(1, 16) = 11.321$, $p < 0.001$], IL-1 β levels [$F(1, 16) = 7.630$, $p = 0.004$], IL-18 levels [$F(1, 16) = 35.032$, $p < 0.001$], and TNF- α levels [$F(1, 16) = 6.910$, $p = 0.006$] among the 4 groups. The levels of IL-6, IL-1 β , IL-18, and TNF- α in the hippocampus were increased by CMS ($p = 0.020$, $p = 0.005$, $p < 0.001$, $p = 0.025$, respectively), and these effects of stress were reversed by EA ($p = 0.031$, $p = 0.018$, $p < 0.001$, $p = 0.022$, respectively) and fluoxetine ($p < 0.001$, $p = 0.004$, $p < 0.001$, $p = 0.039$, respectively). These findings demonstrated that EA and fluoxetine can decrease the inflammatory cytokines levels in the hippocampus induced by CMS.

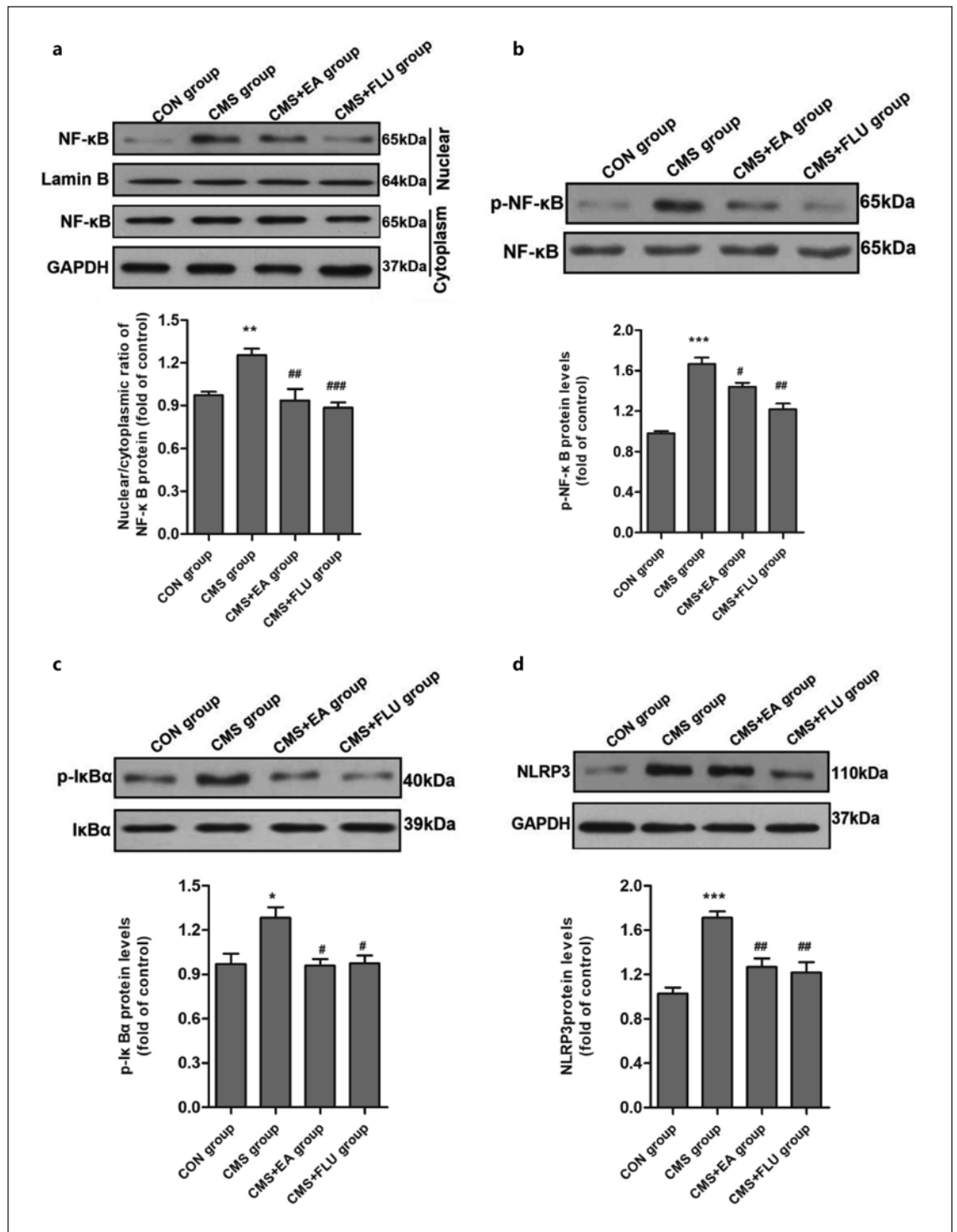


Fig. 3. Effects of EA and fluoxetine on NF-κB/NLRP3 pathway in the hippocampus. **a** The nuclear entry of NF-κB was quantitated by the nuclear/cytoplasmic ratio of NF-κB protein levels normalized by lamin B or GAPDH, respectively. The image placed above is representative images of the Western blot. **b** The relative protein levels of p-NF-κB were normalized to NF-κB. The image placed above is representative images of the Western blot. **c** The relative

protein levels of p-IκBα were normalized to IκBα. The image placed above is representative images of the Western blot. **d** The relative protein levels of NLRP3 were normalized to GAPDH. The data were expressed as fold of control values ($n = 5$ mice per group). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, versus CON group. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ versus CMS group.

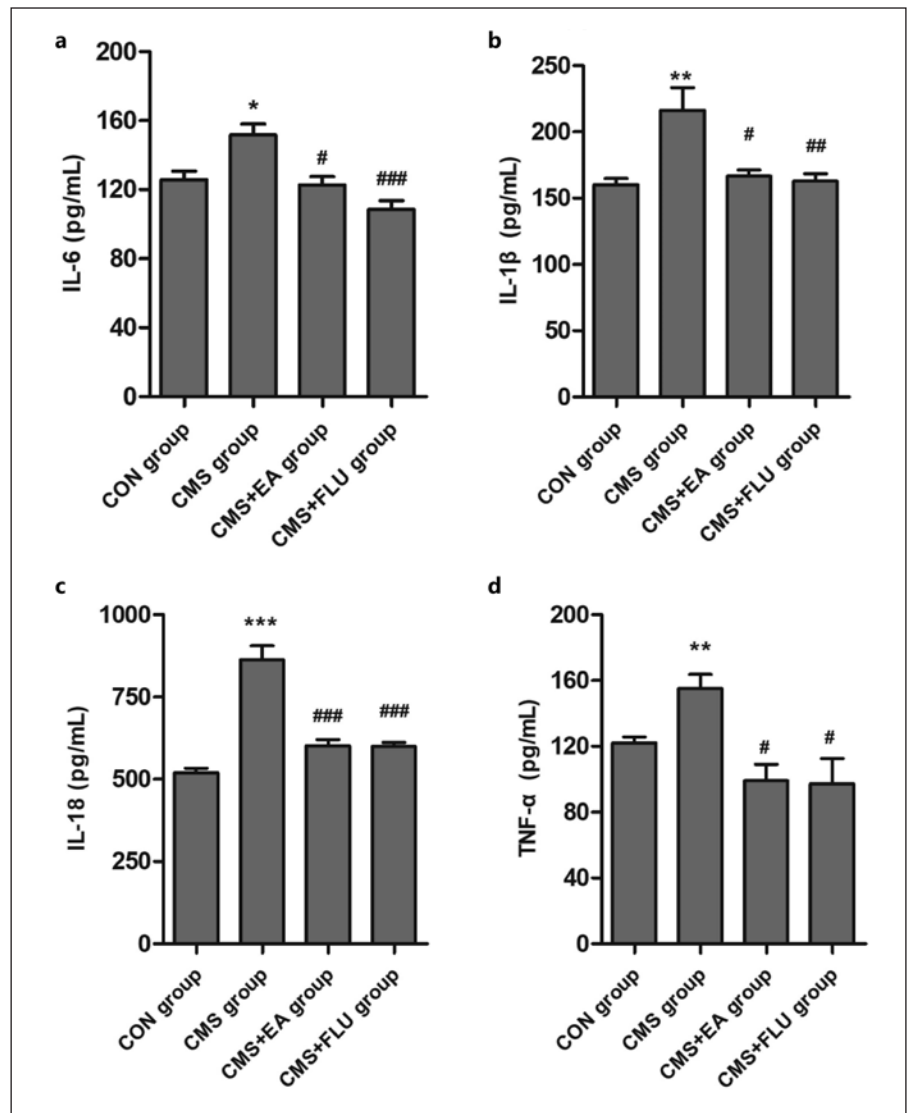


Fig. 4. Effects of EA and fluoxetine on the expression of IL-6 (a), IL-1β (b), IL-18 (c), and TNF-α (d) in the hippocampus of CMS mice. The data were expressed as fold of control values ($n = 5$ mice per group). * $p < 0.05$, ** $p < 0.01$; *** $p < 0.001$, versus CON group. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ versus CMS group.

Discussion

The biological mechanism of acupuncture in the treatment of depression is still unclear. In present study, we found EA prevented the depression-like behavior by inhibiting the NF-κB/NLRP3 inflammatory pathway in hippocampus of mice subjected to CMS.

Based on Chinese medicine theory, “Mutual Assistance of Kidney and Brain (MAKB),” the pathological basis of depression is considered as consumption and deficiency of kidney-essence and incoordination between the brain and kidney. Kidney-tonifying herbs, such as “Bushen Huoxue Herbs,” “Bushen Tiaogan formula,” “Bushen-Shugan granule,” which can improve the de-

pressive symptoms in the patients with Parkinson’s disease [15, 16], and the women during peri-menopause [17, 18]. In our study, GV 20, BL 23, and TKI 3 were selected as EA points according to the MAKb theory. GV20 is an acupoint of the Du meridian (the government vessel), which is located on the highest place of the head where all the yang meridians meet. Acupuncture at GV 20 acupoint could clear the mind, lift the spirits, tonify yang, strengthen the ascending function of the spleen, eliminate interior wind, and promote resuscitation [19]. Thus, the acupoint GV 20 acupoint is specifically used in the treatment of neurological and psychiatric disease such as traumatic brain injury, headache, stroke, Alzheimer’s disease, depression, and anxiety [19–21]. Previ-

ous studies showed that EA at GV 20 acupoint had a protective effect on depressive-like behavior, which may be through regulating brain-derived neurotrophic factor/tyrosine kinase receptor B protein expression, as well as p-ERK1/2 and p-p38 protein expression in the hippocampus [21, 22]. BL 23 is a shu acupoint in the bladder channel of the Foot Tai Yin meridian and affects nourishment of the kidney, strengthening of the lumbar vertebrae, and clearing of suseup [23]. Acupuncture at BL 23 acupoint can be used for treating weak kidney, fatigue, and aches of the waist and knees [23]. EA at BL 23 acupoint, which directly communicate with or nourish the brain, and then enhance endogenous brain-derived neurotrophic factor expression and improve the survival environment for intracerebral neurons, as well as suppress apoptosis of hippocampal neurons [24, 25]. KI 3 is the Shu Stream point and Yuan Primary point of kidney meridian. Acupuncture at KI3 is usually used in the treatment of kidney disease, cognitive impairment, hearing loss, and insomnia, which has been proved by numerous studies [26–29]. An early study demonstrated that manual stimulation of the KI 3 acupoint influenced significantly peripheral vascular circulation, by means of sympathetic stimulation causing vasoconstriction [30]. Acupuncture is widely used in clinical practice and had been shown to have antidepressant effects in both clinical and animal studies [7, 8, 31–33].

The CMS model is a valid, reasonably reliable, and useful model of depression [12]. The model includes a variety of mild unpredictable stressors; the total stress time generally lasts for 3–6 weeks. For mice, the application of a longer exposure protocol resulted in an approximately 190% stronger behavioral effect (6 weeks in comparison to 4 and 5 weeks) [34]. After 6 weeks of stress, mice showed obvious depression-like behavior and neuroinflammation [35]. In the present study, mice were subjected to a 6-week CMS program and were treated with drugs or EA for the last 3 weeks. It is pity that we did not assess the behavior and biochemical indicators of mice at week 3. However, in our previous studies we found the sucrose preference was significantly decreased starting at 3 week in CMS model [3]. Our results from present study showed that EA at GV 20, BL 23, and TKI 3 acupoints increased sucrose preference and latency to the first immobility, as well as decreased immobility time, indicating EA was able to prevent depressive-like behavior induced by CMS.

The precise physiological mechanisms of acupuncture in the treatment of depression are still unknown. Previous studies have shown that acupuncture can modulate neurogenesis and synaptogenesis [36], regulate neuroen-

docrine function [7], attenuate neuroinflammation and neuronal apoptosis [37, 38], and regulate the expression of neurotrophic factors and their receptors [39, 40] in the central nervous system. In recent years, the effect of acupuncture on central system inflammation and its underlying mechanism have attracted much attention. NF- κ B/NLRP3 signaling pathway is the “star pathway” involved in inflammation. The phosphorylation levels of I κ B α and NF- κ B, and the number of NF- κ B into the nucleus are key to determine whether the classical NF- κ B signaling pathway is activated or not [41]. NLRP3 is the most widely studied inflammasome. NLRP3 activation can be divided into 2 necessary phases. The first stage is induced by NF- κ B pathway activation initiating IL-1 β , IL-18, and NLRP3 transcription and translation. The second stage is related to the assembly of the NLRP3 inflammasome [42]. Activation of NF- κ B/NLRP3 pathway amplifies inflammatory damage, and then the inflammatory cytokines levels were increased, such as IL-6, IL-1 β , IL-18, and TNF- α [3, 10]. Inhibiting NF- κ B/NLRP3 pathway may be a therapeutic target for depression [43]. With reference to previous studies [44, 45], the mice were sacrificed by neck dislocation and the hippocampus were rapidly removed. The hippocampus is the important structure involved in stress and emotion [46, 47]. CMS caused significant damage to the hippocampal neurons, increased inflammation, and oxidative stress, activated the NF- κ B/NLRP3 pathway [48, 49]. Consistent with previous finding [3, 10, 11], we found NF- κ B/NLRP3 inflammatory pathway was activated and the levels of IL-6, IL-1 β , IL-18, and TNF- α were increased by CMS in the hippocampus, and these effects were reversed by EA. The results indicating that EA exerts antidepressant effect by inhibiting central inflammatory injury.

Several limitations of our study should also be acknowledged. First, we did not detect the mRNA expression level of the target molecule. Anyway, we detect nuclear entry of NF- κ B (nuclear/cytoplasmic ratio of NF- κ B) and p-NF- κ B protein; it indicates that NF- κ B inflammatory signaling pathway is activated. Second, only male mice were used in this study. We did not explore the effect of EA on female depressed mice. Whether estrogen interfere with the therapeutic effect of acupuncture needs to be analyzed in the future. Third, we did not detect the effect of EA on systemic inflammation of mice. Fourth, the effect of EA on inflammation in other brain regions should also be detected, which is conducive to a deeper understanding of the therapeutic mechanism of EA.

Conclusions

The present study demonstrated that EA at GV 20, BL 23, and TKI 3 acupoints prevented CMS-induced depressive-like behaviors by inhibiting NF- κ B/NLRP3 inflammatory pathway. Additional preclinical studies and clinical trials are needed to better elucidate the effects of this therapeutic strategy.

Statement of Ethics

This study was carried out in accordance with the Guidelines for the Care and Use of Laboratory Animals published by the US National Institutes of Health. Our study was approved by the Laboratory Animal Ethical Committee of Shenyang Medical College (SYYXY2018010601).

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

All authors contributed extensively to this work. T.X. and B.S. designed the study, supervised the conductance of the experiments, data collection, and analysis, and wrote up the draft of the manuscript. Q.W., H.B., H.H., L.G., N.Q., and X.J. performed experiments, analyzed data, and helped in manuscript preparation. Q.W. and H.H. helped in study design, data analysis and interpretation, and writing up the manuscript. Y.W. polished the language of the manuscript.

Data Availability Statement

All data included in this study are available upon request by contact with the corresponding author.

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