NEURAL REGENERATION RESEARCH
October 2016, Volume 11, Issue 10
www.nrronline.org

RESEARCH ARTICLE

Antidepressant effect of electroacupuncture regulates signal targeting in the brain and increases brain-derived neurotrophic factor levels

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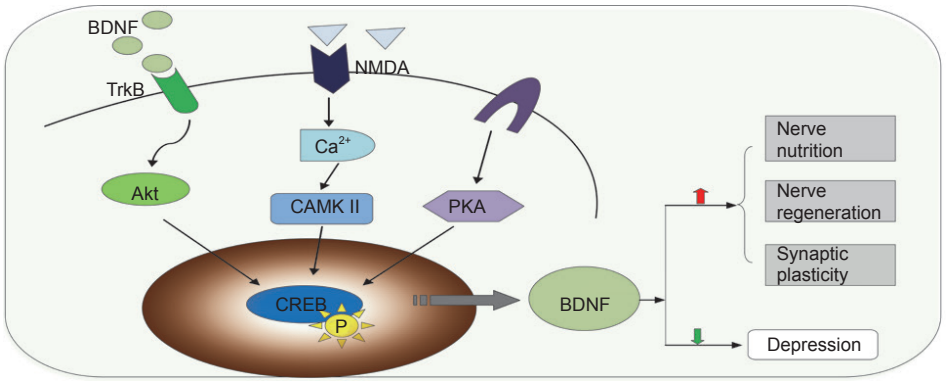
How to cite this article: *Duan DM, Tu Y, Liu P, Jiao S (2016) Antidepressant effect of electroacupuncture regulates signal targeting in the brain and increases brain-derived neurotrophic factor levels. Neural Regen Res 11(10):1595-1602.*

Funding: *This study was supported by the General Program of the National Natural Science Foundation of China, No. 81273847.*

Graphical Abstract

Antidepressive effect of electroacupuncture (EA) may be medicated by increased cyclic adenosine monophosphate response element binding protein (CREB) phosphorylation and brain-derived neurotrophic factor (BDNF) levels



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doi: *10.4103/1673-5374.193238*

Accepted: *2016-09-18*

Abstract

Electroacupuncture improves depressive behavior faster and with fewer adverse effects than antidepressant medication. However, the antidepressant mechanism of electroacupuncture remains poorly understood. Here, we established a rat model of chronic unpredicted mild stress, and then treated these rats with electroacupuncture at *Yintang* (EX-HN3) and *Baihui* (DU20) with sparse waves at 2 Hz and 0.6 mA for 30 minutes, once a day. We found increased horizontal and vertical activity, and decreased immobility time, at 2 and 4 weeks after treatment. Moreover, levels of neurotransmitters (5-hydroxytryptamine, glutamate, and γ-aminobutyric acid) and protein levels of brain-derived neurotrophic factor and brain-derived neurotrophic factor-related proteins (TrkB, protein kinase A, and phosphorylation of cyclic adenosine monophosphate response element binding protein) were increased in the hippocampus. Similarly, protein kinase A and TrkB mRNA levels were increased, and calcium-calmodulin-dependent protein kinase II levels decreased. These findings suggest that electroacupuncture increases phosphorylation of cyclic adenosine monophosphate response element binding protein and brain-derived neurotrophic factor levels by regulating multiple targets in the cyclic adenosine monophosphate response element binding protein signaling pathway, thereby promoting nerve regeneration, and exerting an antidepressive effect.

Key Words: *nerve regeneration; depression; chronic unexpected mild stress; electroacupuncture; brain-derived neurotrophic factor; neurotransmitter; cyclic adenosine monophosphate response element binding protein signal pathway; protein kinase A; TrKB; fluoxetine; neural regeneration*

Introduction

Depressive symptoms are strongly associated with the stress reaction, which is largely modulated by cranial nerves (Revollo et al., 2011). Stimulation can activate or upregulate adaptive modifications in certain cranial nerves, resulting in reversible changes in maintenance of nerve cells and their connections. In contrast, chronic stress or stimulation can cause nerve cell apoptosis (Rogóz et al., 2005; Rojas et al., 2011; Sterrenburg et al., 2011).

Electroacupuncture (EA) is a traditional therapy that has been widely used in China for thousands of years for the treatment of various conditions, including depression. Previously, we found that EA improves depressive behavior, possibly through N-acetylaspartate and choline in the hippocampus and frontal lobe (Duan et al., 2010). Further, we also demonstrated that depressive symptoms were alleviated faster and presented fewer adverse effects after EA treatment combined with antidepressant medication (*i.e.*, fluoxetine), compared with antidepressant medication alone (Duan et al., 2009). However, the underlying mechanisms for the rapid effect of EA on depression remain mostly unknown.

The hippocampus contains a high density and wide distribution of neurotransmitter receptors, and is known to be an important target region for hormones (Guo et al., 2015). Accordingly, the hippocampus plays a key role in the stress reaction and behavioral adaptation (Ruan et al., 2000). Functional and structural hippocampal changes are reported in individuals with major depressive symptoms or depression (Videbech and Petersen, 2001). Moreover, Franco et al. (2016) found that activation of the hypothalamic-pituitary-adrenal axis and increased cortisol content in response to chronic stress results in atrophy of hippocampal neurons. Cyclic adenosine monophosphate (cAMP) response element binding protein (CREB) and brain-derived neurotrophic factor (BDNF) are targets for diverse classes of antidepressants (Młyniec et al., 2015). Previous studies have shown that BDNF, CREB, and their upstream cAMP- and calcium-calmodulin-dependent protein kinase (CaMK)-pathways are involved in neural protection and regeneration (Blendy, 2006; Qi et al., 2008; Zubenko and Hughes, 2011; Shi et al., 2012).

Most current studies have focused on known mechanisms for the effect of EA in treating depression (*e.g.*, the neurotrophic hypothesis). In this study, we investigated the molecular mechanism of EA, which has a rapid antidepressant effect. Thus, we simultaneously compared EA and fluoxetine alone, as well as their combination, to identify the characteristics and molecular mechanisms of EA on the antidepressant effect.

Materials and Methods

Ethics statement

Animal studies were approved by the Animal Experimentation Ethics Committee of the General Hospital of Chinese PLA (archives No. X5-2014-03), and performed in accordance with the Principles of Laboratory Animal Care and China Legislation for the Use and Care of Laboratory Animals. Precautions were taken to minimize suffering and the number of animals used in each experiment.

Animals

In total, 110 male specific-pathogen-free Sprague-Dawley rats aged 6–8 weeks and weighing 150 ± 10 g were provided by Vital River (Beijing, China) (license No. SCXK (Jing) 2012-0003). All rats were maintained on a 12-hour light/dark cycle under controlled temperature conditions (22 ± 1°C), and with free access to standard food and water. One week after acclimation, the open field test was performed, as described previously (Dong et al., 2013).

Rats were placed in the center of an open-field box under quiet conditions, and their behavior recorded for 5 minutes. The open box was thoroughly cleaned before the next rat was observed. Rats with horizontal and vertical activity scores < 30 or > 120 were excluded. Finally, to establish a depression model, 102 rats were selected and randomly divided into two groups: non-chronic unexpected mild stress (CUMS) rats (CON, $n = 16$) and CUMS rats ($n = 86$). After 4 weeks of CUMS, 64 CUMS rats were successfully established according to behavior tests. From the 5th week, CUMS rats were randomly divided into four groups: CUMS rats (CUMS), EA only (EA), fluoxetine (Flu), and EA and fluoxetine combined (EA + Flu) ($n = 16$).

Establishment of a rat CUMS model

To establish a depression model, rats were subjected to isolation housing combined with CUMS (Tian et al., 2014). Chronic unexpected mild stress was performed for 28 days (7 days/cycle for four cycles), and included: swimming in cold water (14°C, 5 minutes), clipping tails (180 seconds), water deprivation (24 hours), fasting (24 hours), electric shock (electric shock of 30 V for 5 seconds, with 5-second intervals, for a total of 120 seconds), wet food (24 hours), and binding (3 hours). Each stimulus was randomly given for 4 weeks.

Electroacupuncture and fluoxetine administration

After CUMS was successfully established, rats were administrated EA and/or fluoxetine. A Hwato brand acupuncture needle (Hwato Brand, No. 30, 0.5 cun; Suzhou Medical Instrument Factory, Suzhou, China) was used for acupuncture at *Yintang* (EX-HN3) and *Baihui* (DU20) (depth, 0.2 cm, 15° angle to the skin), 1 hour before stimulus, once a day. The needle tip was placed individually on *Baihui* and *Yintang* towards the hind head and nasal tip. The needling location and depth was in accordance with the Atlas of Acupuncture Point for Experimental Animals, formulated by the Experimental Acupuncture Research Association of the National Acupuncture Society in China. Electroacupuncture was performed using sparse waves at 2 Hz and 0.6 mA for 30 minutes (Duan et al., 2014). Rats in the Flu group were intragastrically administered fluoxetine (20 mg/kg; Eli Lilly Company, Indianapolis, IN, USA) during the 4-week treatment period (from week 5 to week 8). EA and fluoxetine treatments lasted for 14 and 28 days, respectively.

Behavioral tests

Potential antidepressant behavioral effects were assessed in the open field test and forced swimming test using a self-made open field box with a black wall and bottom (40 cm high, 80 cm long, and 80 cm wide; the bottom contained an equal area of 25 blocks drawn with white lines), and transparent swimming tank (100 cm × 100 cm × 10 cm). The open field test was performed 2 and 4 weeks after EA treatment. Exploratory activity was determined by horizontal activity (crossing activity: frequency of crossing the square) and vertical activity (rearing activity: frequency of rearing). After the open field test, rats were forced to swim in water at 14°C for 5 minutes and immobility time (in seconds) recorded (Liu et al., 2016).

Measurement of neurotransmitters

Rats were sacrificed at 2 and 4 weeks after EA and fluoxetine treatment. The hippocampus was collected. Hippocampal content of serotonin (or 5-hydroxytryptamine, 5-HT), norepinephrine (NE), glutamate (Glu), and γ-aminobutyric acid (GABA) were determined by high performance liquid chromatography using the Agilent system (Santa Clara, CA, USA) (C18, 4.6 mm × 250 mm, 5 μm). Detection was performed using a fluorometer at an excitation wavelength of 340 nm and an emission wavelength of 450 nm. Briefly, the hippocampus was mixed with a precooled tissue extract (ethylenediaminetetraacetic acid-Na_2 0.0533 g, L-cysteine 0.1 g, and perchloric acid 0.787 mL; volume up to 100 mL) at 1:5 (weight (mg)/volume (μL)), and cooled to 0°C in an ice bath. After centrifugation at 3,000 r/min, 4°C for 15 minutes, 100 μL of supernatant was mixed with 100 μL of H_4ClO_3 (0.1 M). After the mixture was centrifuged at 15,000 r/min, 4°C for 20 minutes, the supernatant was filtered through a 0.45-μm filter membrane before analysis. The aqueous phase (A) consisted of a mixture of disodium hydrogen phosphate (35.814 g/L) and methanol (high performance liquid chromatography grade) at 7:3. The mobile phase was: 20 minutes, 0–9% methanol (B); 20–21 minutes, 9–16% B; 21–30 minutes, 16–16% B; 30–31 minutes, 16–30% B; 31–34 minutes, 30–30% B; 34–35 minutes, 30–0% B; 35–40 minutes, 0–0% B; with a gradient elution velocity of 1.0 mL/min (Liu et al., 2015).

Western blot assay

Rats were sacrificed at 2 (n = 8) and 4 weeks (n = 8) after EA and fluoxetine treatment. The hippocampus was collected and treated with precooled lysate containing protease inhibitors. The sample was triturated on ice for 10 minutes, shaken by a vortex for 30 seconds every 3 minutes, and centrifuged at 12,000 r/min, 4°C for 10 minutes. Total protein was measured using the bicinchoninic acid assay. Forty μg protein (20 μL) was electrophoresed on 8% and 10% separation gels at 120 V, and 4% stacking gels at 80 V. Protein was transferred to nitrocellulose membranes using the wet transfer method at 300 mA for 90 minutes. Membranes were blocked in 5% milk for 4 hours, washed with Tris-buffered saline Tween-20 (TBST) for 10 minutes three times, and incubated with primary antibody at 4°C overnight. The primary antibodies used were: rabbit anti-rat monoclonal antibodies against BDNF (1:5,000 dilution), CaMKII (1:3,000 dilution), protein kinase A (PKA) (1:2,500 dilution) (Abcam, Cambridge, MA, USA), CREB (1:1,000 dilution), phosphorylated-CREB (p-CREB) (1:1,000 dilution) (Cell Signaling, Danvers, MA, USA), TrkB (1:500; Biogot Biotechnology Co., Ltd., Nanjing, China), Akt (1:500; Bio-world, Dublin, OH, USA), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:2,000; Bioss, Beijing, China). After washing with TBST, membranes were incubated with goat anti-rabbit IgG (horseradish peroxidase conjugated, 1:5,000 dilution; Bioss) for 2 hours at room temperature. After a final washing with PBST three times each for 10 minutes, membranes were processed for chemiluminescence detection (0.125 mL/cm^2, room temperature for 2 minutes). Membranes were visualized using an ultraviolet photometry gel imager (Imaging System, Cambridge, MA, USA). Band intensities were quantified by optical density analysis (Dong et al., 2014).

Real-time polymerase chain reaction (PCR)

Total mRNA was extracted from the hippocampus using Trizol reagent (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's instructions (Gautam et al., 2016). Reverse transcription and real-time fluorescent quantitative PCR were performed using a cDNA Synthesis Kit and SYBR Green supermix (Bio-Rad, Hercules, CA, USA), according to the manufacturer's instructions. Primer sequences are shown in **Table 1**. Relative expression levels were analyzed using IQ5 software (Bio-Rad).

Statistical analysis

Data are presented as mean ± SD, and were analyzed with SPSS v.19.0 (IBM Corp., Armonk, NY, USA). One-way analysis of variance and Tukey's *post hoc* test were used to determine differences among groups. A two-tailed P value < 0.05 was considered statistically significant.

Results

Electroacupuncture improved behavior and body weight in a rat model of depression

No significant differences were observed in horizontal and vertical activity, immobility time, and body weight before and after stimulation. Compared with the CON group, horizontal activity, vertical activity, and body weight decreased, while immobility time increased significantly in the CUMS group (**Figure 1**). After 2 weeks of treatment with either EA or EA + Flu, horizontal activity and body weight significantly increased, and immobility time significantly decreased in the EA and combination groups compared with the CUMS group. However, there were no significant changes in the Flu group after 2 weeks of treatment. After 4 weeks of EA and EA + Flu treatment, horizontal activity, vertical activity, and body weight significantly increased, and immobility time significantly decreased in the EA and EA + Flu groups compared with the CUMS group. Except for vertical activity, similar effects were observed in the Flu group after 4 weeks of treatment.

Table 1 Primer sequences for real-time PCR

Primer	Forward sequence	Reverse sequence
TrkB	5′-CTG GGG CTT ATG CTT GCT GGT-3′	5′-TGA TGT TCT CTG GGT CAA TGC TGT T-3′
Akt	5′-TCT ACG GTG CGG AGA TTG TG-3′	5′-TCC CGG TAC ACC ACG TTC TT-3′
BDNF	5′-GGC GGC AGA CAA AAA GAC TG-3′	5′-ATG CCT TTT GTC TAT GCC CC-3′
CaMKII	5′-AGA ATG GGA CAC AGT GAC AC-3′	5′-ATG GAG GCA ACA GTA GAA CG-3′
CREB	5′-CCA GCA GGA ATA ACA AC-3′	5′-AGG CTT CCA ATG CTA CCA GA-3′
PKA	5′-GGA GCA GTT GCG AGA TTT CA-3′	5′-TGG GAA GTC TTG GAT CTG TG-3′
GAPDH	5′-TAC CCA CGG CAA GTT CAA CG-3′	5′-CAC CAG CAT CAC CCC ATT TG-3′

BDNF: Brain-derived neurotrophic factor; CaMKII: calcium-calmodulin-dependent protein kinase II; CREB: cyclic adenosine monophosphate response element binding protein: PKA: protein kinase A; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

Effect of EA on neurotransmitters in the hippocampus in a rat model of depression

Neurotransmitter levels in the hippocampus are shown (**Figure 2**). Levels of 5-HT, NE, Glu, and GABA decreased significantly in the CUMS group compared with the CON group. Further, compared with the CUMS group, 5-HT levels were significantly higher in the EA, Flu, and EA + Flu groups after 2 and 4 weeks of treatment, while GABA and Glu levels were significantly higher in the EA and EA + Flu groups. There were no significant differences in GABA and Glu levels between the Flu and CUMS groups after 2 weeks of treatment. Compared with the CUMS group, no significant differences were observed in NE levels among the groups.

Effect of EA on protein expression of BDNF and BDNF-related factors in the hippocampus of a rat depression model

Protein levels of BNDF and BDNF-related factors in the hippocampus are shown (**Figure 3**). Compared with the CON group, levels of TrkB, PKA, phosphorylated-CREB (pCREB), and BDNF protein were lower in the CUMS group, while CaMKII levels were higher. Additionally, TrkB, pCREB, and BDNF protein levels were higher in the EA group compared with the CUMS group after 2 and 4 weeks of EA. These changes were only observed in the Flu group after 4 weeks of Flu treatment. Further, PKA levels increased, while CaMKII levels decreased after 4 weeks of EA, Flu, and EA + Flu. Protein levels were comparable in the EA + Flu and EA groups.

Effect of EA on BDNF- and CREB-related gene expression levels in the hippocampus of a rat model of depression

Levels of six BDNF and CREB pathway genes are shown (**Figure 4**). Compared with the CUMS group, hippocampal levels of TrkB mRNA (after 2 and 4 weeks of EA) and PKA mRNA (after 4 weeks of EA) were increased. In contrast, CaMKII mRNA levels were decreased after 4 weeks of EA. Flu treatment increased TrkB mRNA levels and decreased CaMKII mRNA levels after 4 weeks of intragastric administration. Hippocampal levels of TrkB, PKA, and CaMKII mRNA were comparable in the EA + Flu and EA groups. No significant differences were observed in BDNF, CREB, and Akt mRNA levels among all groups compared with the CUMS group after 2 or 4 weeks of treatment.

Discussion

Using a CUMS rat model of depression, we found that EA treatment increased horizontal and vertical activity in the open field test, and decreased immobility time in the forced swim test, suggesting improved behavior. Furthermore, neurotransmitter levels (5-HT, Glu, and GABA) and protein levels of BDNF and BDNF-related factors (TrkB, PKA, pCREB) were higher in the hippocampus of EA-treated rats. Additionally, we found increased PKA and TrkB mRNA levels and decreased CaMKII levels in EA-treated rats. Taken together, our findings suggest that the CREB and BDNF pathways may be involved in the antidepressive effect of EA in this CUMS rat model.

CREB, a stimulus-inducible transcription factor, as well as its upstream pathways are a possible mechanism linking BDNF with development of depression. CREB promotes transcription of downstream genes, increases BDNF expression, and accelerates neuronal growth and formation of synaptic connections (Reichardt, 2006). CREB-mediated transcriptional activity is dependent on the phosphorylation status of CREB, which can be induced by kinases such as CaMK II, PKA, ERK, and AKT (Li et al., 2011). Recently, the CREB signaling pathway was shown to be involved in neuronal plasticity as a key intersection between stress and response (Dunham et al., 2009). Lu et al. (2013) found that EA may induce neuronal regeneration and alter neural plasticity, possibly through regulation of the CREB signaling transduction pathway.

Fluoxetine, a selective 5-HT reuptake inhibitor, is the most commonly prescribed antidepressant (Arroll et al., 2005). This selective 5-HT reuptake inhibitor binds selectively to 5-HT transporters on serotonergic neurons in the central nervous system. Consequently, fluoxetine inhibits 5-HT reuptake and increases 5-HT concentration in the synaptic cleft within several hours after administration. However, serotonergic neuronal discharge may be reduced because of a negative feedback mechanism involving 5-HT1A receptors. Therefore, increased 5-HT is inhibited with long-term treatment of selective 5-HT reuptake inhibitors, leading to the delayed effect of antidepressants (Wolak et al., 2015). In clinical settings, it takes 2–3 weeks for 5-HT1A receptor

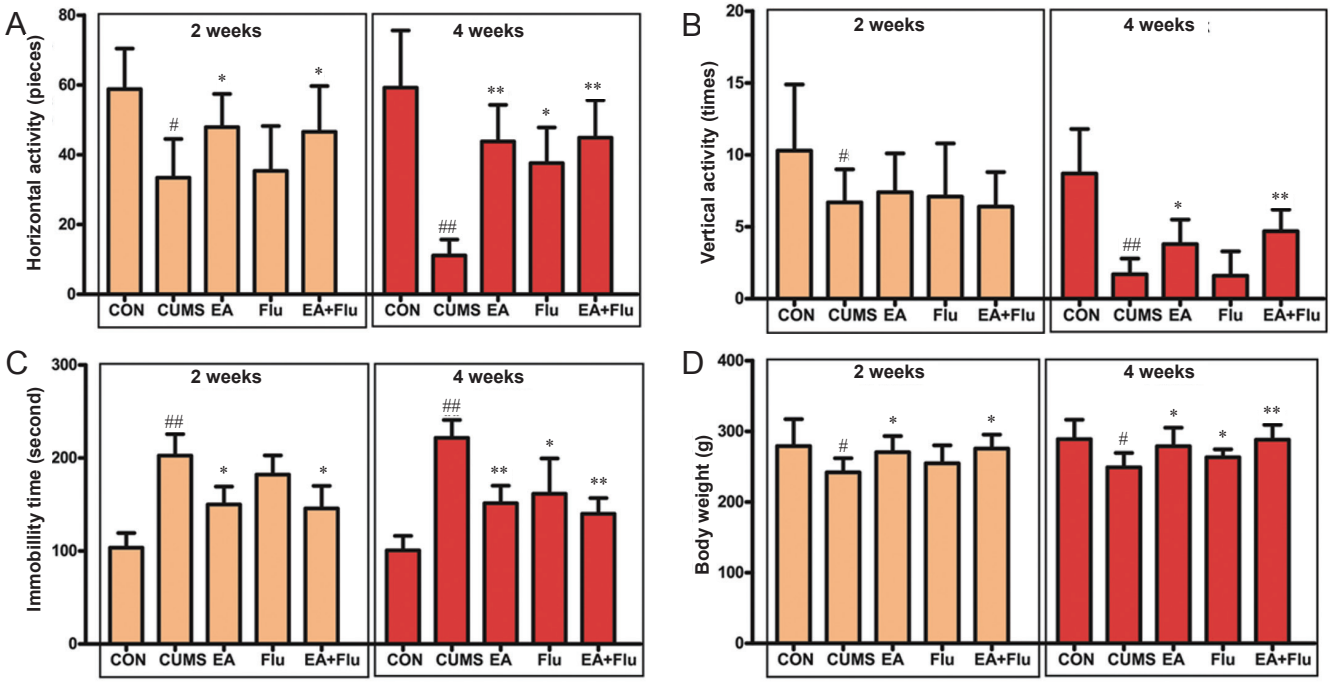

Figure 1 Effect of EA and Flu on behavior and body weight in CUMS rats.
(A) Horizontal activity (crossing activity: frequency of crossing the square) in the open field test; (B) vertical activity (rearing activity: frequency of rearing) in the open field test; (C) immobility time (5 minutes) in the forced swimming test; and (D) body weight. Data are presented as the mean ± SD. One-way analysis of variance and Tukey's *post hoc* test were used to examine differences among groups. #$P < 0.05$, ##$P < 0.01$, *vs*. control group; *$P < 0.05$, **$P < 0.01$, *vs*. CUMS group. CON: Control; CUMS: chronic unexpected mild stress; EA: electroacupuncture; Flu: fluoxetine.

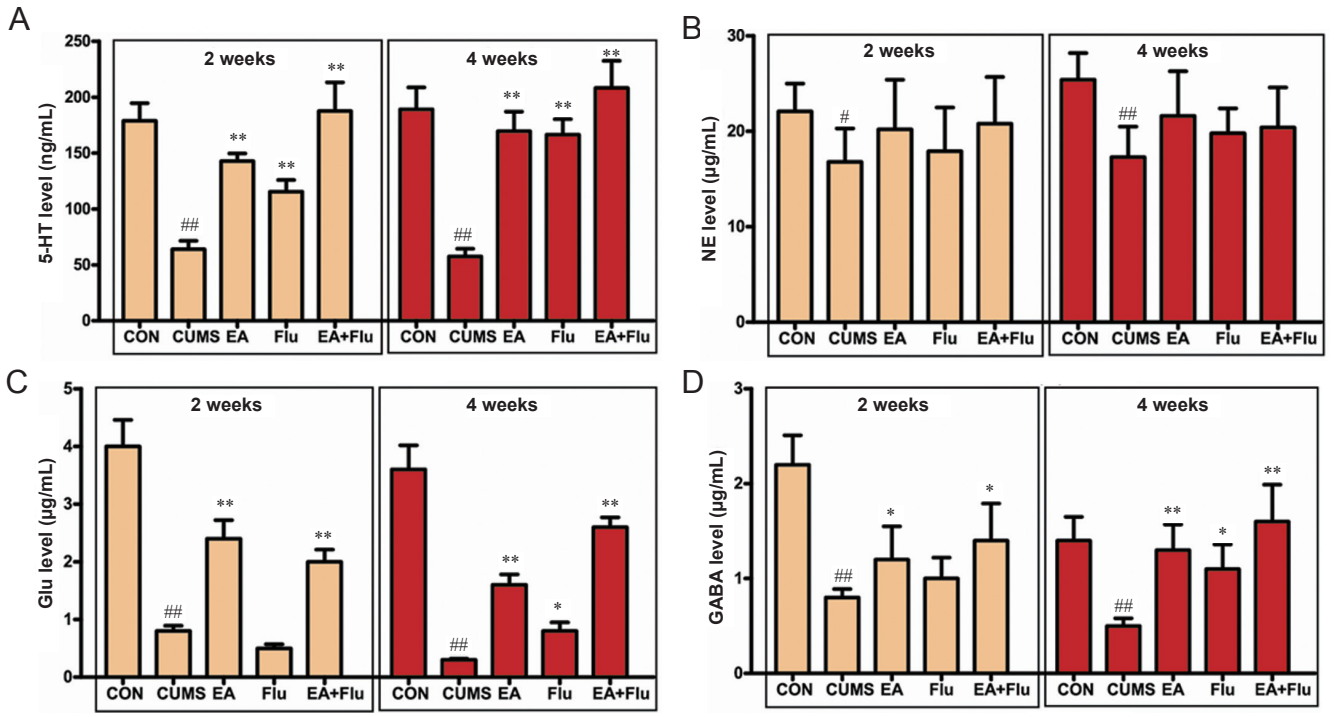

Figure 2 Effect of EA and Flu on neurotransmitters in the hippocampus of CUMS rats (detected by high performance liquid chromatography).
(A) 5-HT in blood; (B) NE in blood; (C) Glu in the hippocampus; and (D) GABA in the hippocampus. Data are presented as the mean ± SD. Experiments were performed five times. One-way analysis of variance and Tukey's *post hoc* test were used to examine differences among groups. #$P < 0.05$, ##$P < 0.01$, *vs*. control group; *$P < 0.05$, **$P < 0.01$, *vs*. CUMS group. CON: Control; CUMS: chronic unexpected mild stress; EA: electroacupuncture; Flu: fluoxetine; 5-HT: 5-hydroxytryptamine; NE: norepinephrine; Glu: glutamate; GABA: γ-aminobutyric acid.

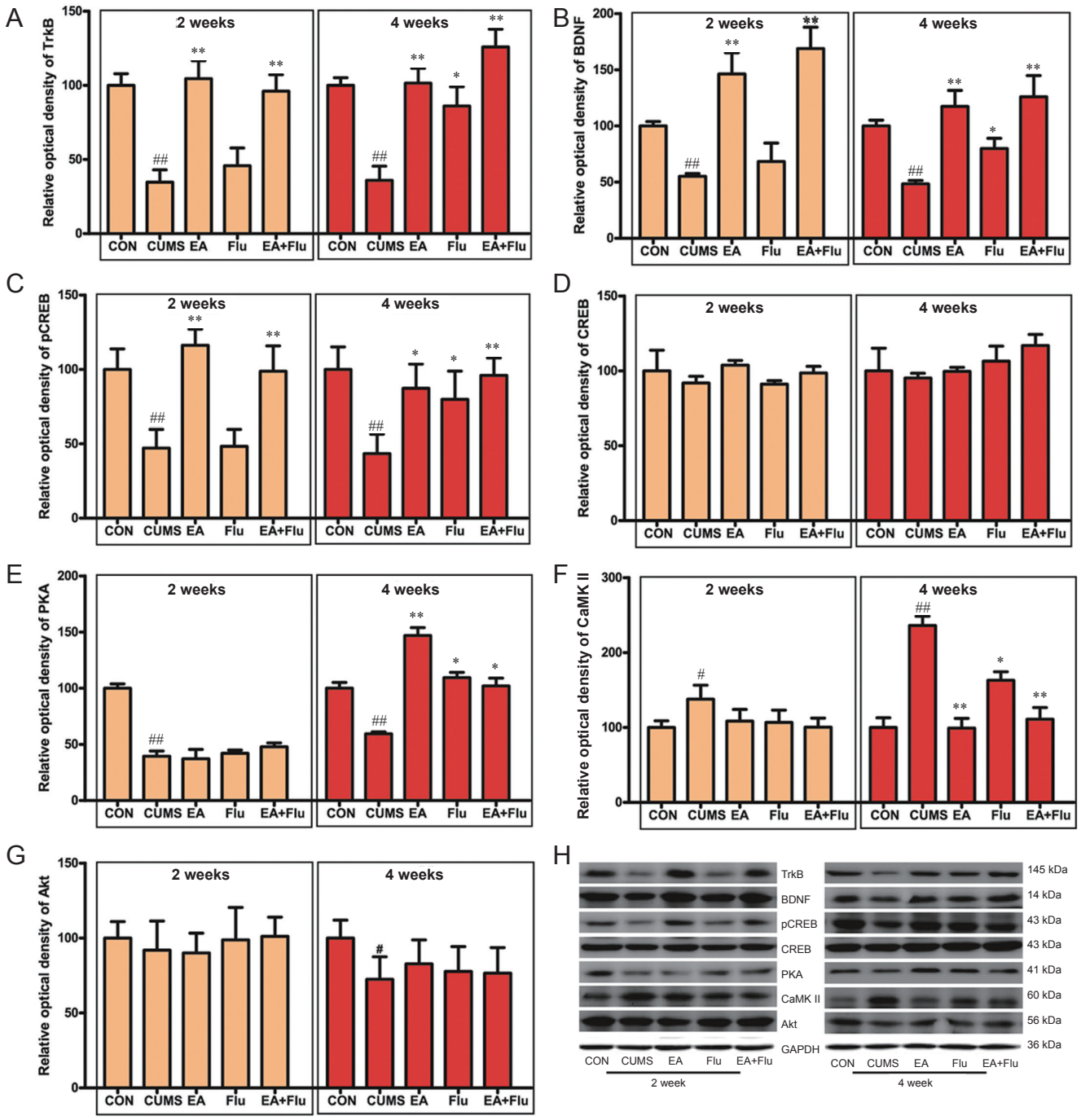

Figure 3 Effect of EA and Flu on protein levels in the hippocampus of CUMS rats (western blot assay).
Immunoreactivity of TrkB (A), BDNF (B), pCREB (C), CREB (D), PKA (E), CaMKII (F), and Akt (G) in the hippocampus. Protein bands of BNDF and BDNF-related factors in the hippocampus (H). Data are presented as the mean ± SD. Experiments were performed four times. One-way analysis of variance and Tukey's *post hoc* test were used to examine differences among groups. #$P < 0.05$, ##$P < 0.01$, *vs*. control group; *$P < 0.05$, **$P < 0.01$, *vs*. CUMS group. CON: Control; CUMS: chronic unexpected mild stress; EA: electroacupuncture; Flu: fluoxetine; BDNF: brain-derived neurotrophic factor; pCREB: phosphorylated-CREB; CREB: cyclic adenosine monophosphate response element binding protein; PKA: protein kinase A; CaMKII: calcium-calmodulin-dependent protein kinase.

desensitization to exhibit an antidepressant effect after administration of fluoxetine (Czachura and Rasmussen, 2000). This delayed clinical effect is strongly associated with decreased compliance and increased risk of suicide. Although treatment with multiple selective 5-HT reuptake inhibitors or 5-HT1A receptor antagonists may counteract this delayed clinical effect, such treatment can produce more side effects. Therefore, there is an urgent clinical need for multi-target, fast-acting antidepressant therapies to overcome the delayed clinical effect.

In this study on depression, EA has been shown to improve depressed behavior after 2 weeks, which is faster than Flu. Further, EA also increases protein levels of CREB and related factors, and improves regeneration of damaged

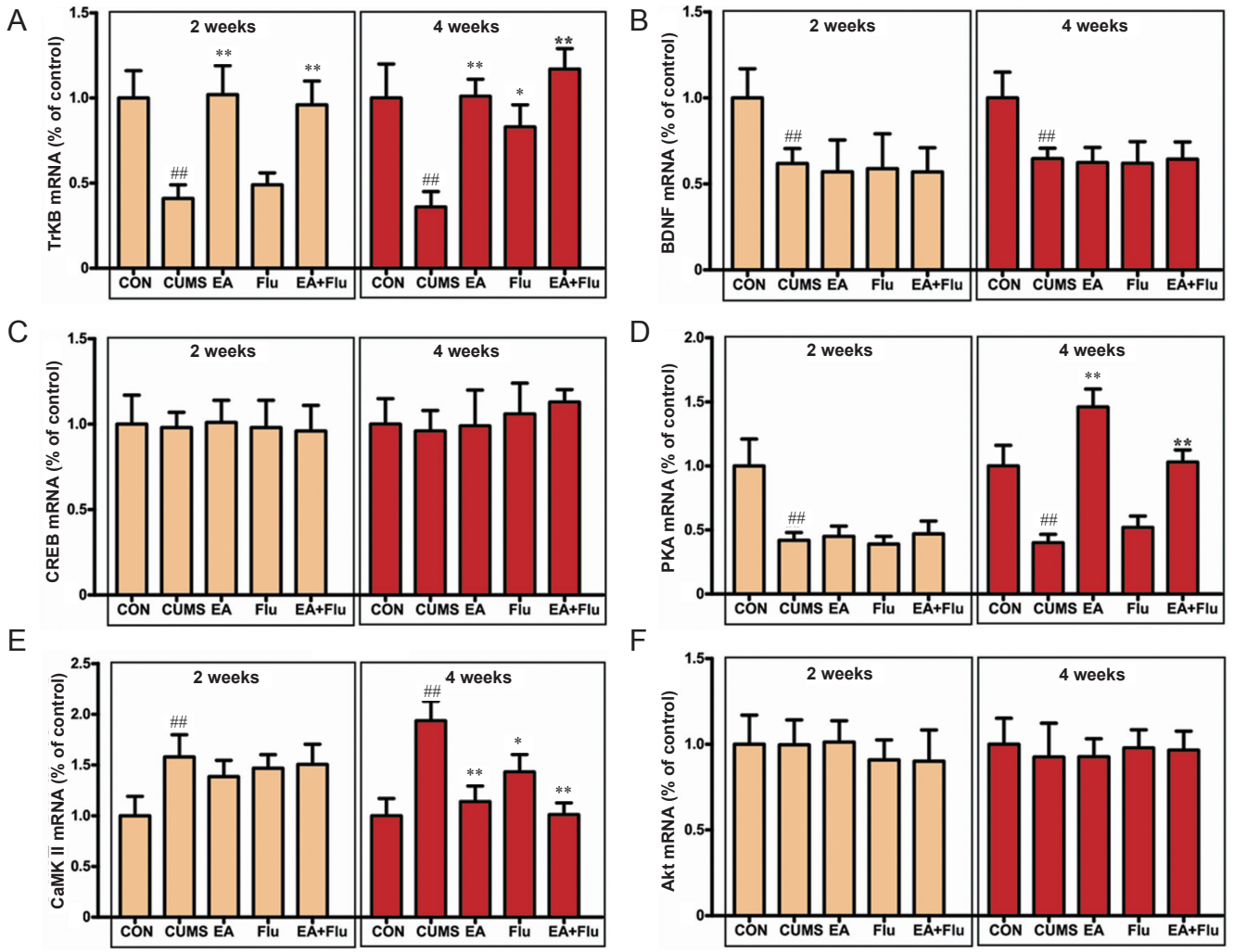

Figure 4 Effect of EA and Flu on mRNA levels of BDNF- and CREB-related genes in the hippocampus of CUMS rats (real-time PCR).
mRNA levels of (A) TrkB, (B) BDNF, (C) CREB, (D) PKA, (E) CaMKII, and (F) Akt in the hippocampus. Data are presented as the mean ± SD. Experiments were performed five times. One-way analysis of variance and Tukey's *post hoc* test were used to examine differences among groups. #P < 0.05, ##P < 0.01, *vs*. control group; *P < 0.05, **P < 0.01, *vs*. CUMS group. CON: Control; CUMS: chronic unexpected mild stress; EA: electroacupuncture; Flu: fluoxetine; BDNF: brain-derived neurotrophic factor; CREB: cyclic adenosine monophosphate response element binding protein; PKA: protein kinase A.

nerves after only 2 weeks of treatment, which together may contribute to its fast action. We also found changes in CREB and related-factors in EA-treated rats, further suggesting that EA may exhibit an antidepressant effect partially through the CREB pathway. We observed increased BDNF protein levels, which is consistent with a previous finding that the CREB-BDNF pathway is a key link in promoting neuronal regeneration and neurotrophy (Larsen et al., 2010). Other reported mechanisms underlying the antidepressant effect of EA include suppression of apoptosis-inducing factors and protection of nerve cells against stimulation.

Based on our findings using the CUMS rat model, EA exhibits a more rapid effect than fluoxetine, possibly by increased CREB phosphorylation and BDNF expression *via* regulation of the CREB pathway (TrkB-CREB-BDNF, PKA-CREB-BDNF, and CaMKII-CREB-BDNF). Our results will help further understanding of the molecular mechanisms and characteristics of EA treatment on depression, and also provide an experimental basis for a treatment strategy for depression with traditional Chinese medicine alone or in combination with western medicine. Nevertheless, as caveats to our study, we only used the CUMS rat model and did not examine all proteins within the CREB signaling pathway. Thus, our conclusions must be validated in other animal models in future studies.

Author contributions: *DMD served as a guarantor of integrity of the entire study, participated in study concept, study design, and paper preparation. YT was in charge of literature research, data analysis/interpretation, and statistical analysis. PL was responsible for experimental studies, data acquisition, and data analysis/interpretation. SJ participated in paper editing, statistical analysis, and paper revision/review. All authors approved the final version of the paper.*
Conflicts of interest: *None declared.*
Plagiarism check: *This paper was screened twice using CrossCheck to verify originality before publication.*
Peer review: *This paper was double-blinded and stringently reviewed by international expert reviewers.*

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Copyedited by James R, Yajima W, Yu J, Qiu Y, Li CH, Song LP, Zhao M