

Electroacupuncture pretreatment exhibits anti-depressive effects by regulating hippocampal proteomics in rats with chronic restraint stress

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Abstract

The clinical effect of electroacupuncture on depression is widely recognized. However, the signal transduction pathways and target proteins involved remain unclear. In the present study, rat models of chronic restraint stress were used to explore the mechanism by which electroacupuncture alleviates depression. Rats were randomly divided into control, model, and electroacupuncture groups. Chronic restraint stress was induced in the model and electroacupuncture groups by restraining rats for 28 days. In the electroacupuncture group, electroacupuncture pretreatment at *Baihui* (GV20) and *Yintang* (GV29) acupoints was performed daily (1 mA, 2 Hz, discontinuous wave, 20 minutes) prior to restraint for 28 days. Open field tests and body weight measurements were carried out to evaluate the depressive symptoms at specific time points. On day 28, the crossing number, rearing number, and body weights of the model group were significantly lower than those in the control group. Behavior test results indicated that rat models of depressive-like symptoms were successfully established by chronic restraint stress combined with solitary raising. On day 28, an isobaric tag for a relative and absolute quantitation-based quantitative proteomic approach was performed to identify differentially expressed proteins in hippocampal samples obtained from the model and electroacupuncture groups. The potential function of these differential proteins was predicted through the use of the Cluster of Orthologous Groups of proteins (COG) database. Twenty-seven differential proteins (uncharacteristic proteins expected) were selected from the model and electroacupuncture groups. In addition to unknown protein functions, COG are mainly concentrated in general prediction function, mechanism of signal transduction, amino acid transport and metabolism groups. This suggests that electroacupuncture improved depressive-like symptoms by regulating differential proteins, and most of these related proteins exist in nerve cells.

Key Words: nerve regeneration; traditional Chinese medicine; electroacupuncture; depression; chronic restraint stress; iTRAQ; differential proteins; proteomics; multi-target effect; neural regeneration

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Introduction

Depression, characterized by negative moods, decreased physical activity, loss of interest in usual activities, feeling of helplessness and suicidal tendencies (Kessler et al., 2003), currently ranks as the fourth leading cause of disability worldwide (Mathers et al., 2006; Moussavi et al., 2007). Depression results from several biological factors, including inflammatory and immune processes, alterations in activation of the hypothalamic-pituitary-adrenal axis, variability in heart rate, increased activity of the sympathoadrenal and pituitary adrenal axis, reduction in circulating endothelial progenitor cells, increased levels of cortisol and catecholamine, altered activity of the autonomic nervous system, and oxida-

tion processes (Compare et al., 2011, 2013; Nemeroff et al., 2012). Depression is also associated with several unhealthy lifestyles, such as increased consumption of tobacco, alcohol and illicit substances, reduced physical activity, overeating and lack of medical adherence.

Various western medicine treatments including antidepressant medication and psychological therapy have a pivotal role in treating depression; however, almost one fourth of patients are unable to achieve favorable effects, especially the improvement of somatic symptoms (Bruce et al., 2005). Therefore, seeking alternative therapy for depression remains an urgent issue.

Electroacupuncture, functioning as a type of bi-directional

regulation of positive stress source, has been suggested to be effective in treating both physical and mental disorders (Cabioglu et al., 2007; Yang et al., 2014). However, there have been few basic experimental studies regarding the mechanism of electroacupuncture treatment in depression.

Hippocampal formation is involved in the stress response as a vital part of the negative feedback system regulating circulating glucocorticoids (McEwen, 2000; Liu et al., 2009). Moreover, the hippocampus is coupled to stress through fear and emotional processing *via* its connections to the amygdala (McIntyre et al., 2003; Roozendaal et al., 2004). Hippocampal formation is believed to be implicated in the etiology of depression, potentially through an important role in stress responses (Sheline, 2000; Nestler et al., 2002; Holm et al., 2010). Based on the role of hippocampal formation in stress and stress-related psychiatric disorders, it might be expected to find markers of stress-susceptibility/resilience in this region of the brain. Chronic psychological stress produces hippocampal-dependent cognitive deficits consistent with structural changes in the hippocampus. Stress-induced changes in hippocampal CA3 neurons are consistent with deficits in hippocampal function (McLaughlin et al., 2007).

Therefore, the purpose of this study was to detect alterations of hippocampal protein expression prior to electroacupuncture pretreatment to elucidate the underlying mechanism by which electroacupuncture alleviates depression. According to traditional Chinese medicine, the function of two acupoints *Baihui* (GV20) and *Yintang* (GV29) is to replenish *qi* and boost *yang*, tranquilize the spirit and calm the mind (Shi, 2007). In the present study, we selected these two acupoints for electroacupuncture and identified differential proteins expressed during depression after electroacupuncture. These differential proteins might be potential targets of the anti-depressive effect of electroacupuncture pretreatment, which provides a reference for further research of depression.

Materials and Methods

Animals

Forty-five specific pathogen free (SPF) Sprague-Dawley male rats, aged 5 weeks, were supplied by Charles River Laboratories of Beijing, China (license No. SCXK (Jing) 2012-0001). Animals were housed at 18–26 °C, humidity 55%, in 12-hour light/dark cycles (light on at 8:00 a.m.), with free access to food and water. The study was performed in rats 3 days after environment acclimatization. All surgery was performed under chloral hydrate anesthesia, and all efforts were made to minimize animal suffering and to be consistent with the 3R principle of reduction, replacement, and refinement. All experimental procedures received approval from the Animal Ethics Committee of Beijing University of Chinese Medicine (permission No. Kj-dw-18-20131012).

Establishment of animal models of depression and acupuncture administration

All rats were randomly and equally divided into three groups with 15 rats per group. In the control group, herd manage-

ment, and 6-hour fasting for solids and liquids was performed from 9 a.m. to 3 p.m. per day, lasting for 28 days. In the model group, social isolation and chronic restraint stress were conducted daily for 28 days by the following method: rats were restrained in a self-made cylinder-shaped wire net (20-cm length and 5-cm diameter) from 9 a.m. to 3 p.m. After restraint, they were released with free access to water and food. In the electroacupuncture group, electroacupuncture pretreatment was conducted daily prior to restraint using the same method as for the model group.

During acupuncture administration, rats were maintained within a cloth bag. *Baihui* (GV20) and *Yintang* (GV29) acupoints were selected for electroacupuncture. GV20 is located above the apex auriculate, on the midline of the head, and GV29 at the midpoint between the two eyes (Liu et al., 2011). Sterilized disposable stainless steel needles (0.2 × 25.0 mm, Huan Qiu Brand, manufactured by Suzhou Medicine Co., Ltd., Suzhou, China) were inserted obliquely as deep as 5 mm for both points. Following the insertions, electrodes were added to the handle of needles (electroacupuncture apparatus used: SDZ-Velectronic needle therapy instrument, manufactured by Suzhou Medical Supplies Co., Ltd.). Electricity simulation parameters were 1 mA, 2 Hz, discontinuous wave, for 20 minutes.

Body weight measurement

Rat body weights were measured on days 0 and 28 of the study.

Open-field test

At days 0 and 28 of the study, an open-field test was conducted as previously described with modifications (Lu et al., 2011). A wood apparatus, composed of a square arena (80 × 80 cm²) with a 40-cm-high wall, was divided into 25 × 25-cm² equal squares drawn on the floor. A single rat was gently placed at the center of the floor and allowed to familiarize itself for 3 minutes. The activity of the rat was recorded by a camera installed on top of the lateral high wall. The crossing times (defined as at least three paws in a square) and the rearing times in 3 minutes (defined as the rat standing upright on its hind legs) were monitored by two observers who were blind to the experimental design through a monitor equipped by a camera which was installed 1 meter away from the apparatus. After one rat finished the test, alcohol was applied to clean the floor to exclude the intervention of odor signals.

Tissue collection

The hippocampus was carefully dissected out from 12 male Sprague-Dawley rats on day 28. The specific steps were as follows: (1) cut along the coronal suture and sagittal suture, then pull off both sides of parietal bone and inter parietal bone; (2) open the skull, remove the cerebral cortex covering it, and separate the rest of the hippocampus from the cortex covering it along the surface of the hippocampus towards the ventral part of the hippocampus; and (3) free the hippocampus from the surrounding tissue.

Protein preparation

Hippocampal samples were ground to a powder in liquid nitrogen and lysed with lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 40 mM Tris-HCl, pH 8.5) containing 1 mM phenylmethyl sulfonylfluoride and 2 mM phenylmethyl sulfonylfluoride (final concentration). After 5 minutes, 10 mM dithiothreitol (final concentration) was added to the samples. The resulting suspension was sonicated at 200 W for 15 minutes, and then centrifuged at 4°C, 30,000 × g for 15 minutes. The supernatant was mixed well with a 5× volume of chilled acetone containing 10% (v/v) trichloroacetic acid and incubated at -20°C overnight. After centrifugation at 4°C, 30,000 × g, the supernatant was discarded. The precipitate was washed with chilled acetone three times. The pellets were air-dried and dissolved in lysis buffer (7 M urea, 2 M thiourea, 4% NP40, 20 mM Tris-HCl, pH 8.0–8.5). The suspension was sonicated at 200 W for 15 minutes, and centrifuged at 4°C, 30,000 × g for 15 minutes. The supernatant was transferred to another tube to reduce disulfide bonds in proteins of the supernatant, 10 mM dithiothreitol (final concentration) was added and incubated at 56°C for 1 hour. Subsequently, 55 mM iodoacetamide (final concentration) was added to block cysteines, and incubated for 1 hour in a dark room. The supernatant was mixed well with a 5× volume of chilled acetone for 2 hours at -20°C to precipitate proteins. After centrifugation at 4°C, 30,000 × g, the supernatant was discarded, the pellets were air-dried for 5 minutes, dissolved in 500 μL of 0.5 M triethylammoniumbicarbonate (Applied Biosystems, Milan, Italy), and sonicated at 200 W for 15 minutes. Finally, samples were centrifuged at 4°C, 30,000 × g for 15 minutes, and the supernatant was transferred to a new tube and quantified. The proteins in the supernatant were kept at -80°C for further analysis.

iTRAQ™ labeling and SCX fractionation

Total protein (100 μg) was extracted from each sample solution and then digested with Trypsin Gold (Promega, Madison, WI, USA) with a ratio of protein:trypsin of 30:1 at 37°C for 16 hours. After trypsin digestion, peptides were dried by vacuum centrifugation. Peptides were reconstituted in 0.5 M triethylammoniumbicarbonate and processed according to the manufacturer's protocol for 8-plex iTRAQ reagent (Applied Biosystems). Briefly, one unit of iTRAQ reagent was thawed and reconstituted in 24-μL isopropanol. Samples were labeled with the iTRAQ tags as follows: Sample H45 (No.117), and Sample H50 (No.116). After incubation at room temperature for 2 hours, the isobaric-tag-labeled peptides were pooled and dried by vacuum centrifugation.

SCX chromatography was carried out using a LC-20AB HPLC Pump system (Shimadzu, Kyoto, Japan). The iTRAQ labeled peptides were reconstituted with 4-mL buffer A (25 mM NaH₂PO₄ in 25% ACN, pH 2.7) and loaded onto a 4.6 × 250-mm² Ultremex SCX column containing 5-μm-sized particles (Phenomenex, Torrance, CA, USA). The peptides were eluted at a flow rate of 1mL/min with a gradient of buffer A for 10 minutes, 5–60% buffer B (25 mM NaH₂PO₄, 1 M KCl in 25% ACN, pH 2.7) for 27 minutes, and 60–100%

buffer B for 1 minute. The system was then maintained with 100% buffer B for 1 minute before equilibrating with buffer A for 10 minutes prior to the next injection. Absorbance at 214 nm was measured, and fractions were collected every minute. The eluted peptides were pooled into 20 fractions, desalted with a Strata X C18 column (Phenomenex) and vacuum-dried.

LC-ESI-MS/MS analysis based on Triple TOF 5600

Each fraction was re-suspended in buffer A (5% ACN, 0.1%FA) and then centrifuged at 20,000 × g for 10 minutes, and the mean peptide concentration was 0.5 μg/μL. A 10-μL volume of supernatant was loaded onto a 2-cm C18 trap column on a LC-20AD nanoHPLC (Shimadzu, Kyoto, Japan) through the use of autosampler. Then, the peptide samples were eluted onto a homemade 10-cm analytical C18 column (inner diameter 75 μm) and loaded at 8 μL/min for 4 minutes. Thereafter, a 35-minute gradient was run at 300 nL/min starting from 2–35% buffer B (95%ACN, 0.1%FA), followed by a 5-minute linear gradient to 60%, 2-minute linear gradient to 80%, maintenance at 80% buffer B for 4 minutes, and finally returning to 5% in 1 minute. Data acquisition was performed with a Triple TOF 5600 System (AB SCIEX, Concord, ON, USA) fitted with a Nanospray IIIsource (AB SCIEX) and a pulled quartz tip as the emitter (New Objectives, Woburn, MA, USA). Data were acquired using the following parameters: ion spray voltage 2.5 kV, curtain gas 30 psi, nebulizer gas 15 psi, and interface heater temperature 150°C. The MS was operated with a reflection pattern of greater than or equal to 30,000 FWHM for TOF MS scans. For information associated with data collection (IDA), survey scans were acquired in 250 ms and as many as 30 product ion scans were collected if exceeding a threshold of 120 counts per second (counts/s) and with a 2+ to 5+ charge-state. Total cycle time was fixed to 3.3 seconds. The Q2 transmission window was 100 Da for 100%. Four time bins were summed for each scan at a pulse frequency value of 11 kHz through monitoring of the 40-GHz multichannel TDC detector with four-anode channel detection. A sweeping collision energy setting of 35 ± 5 eV coupled with iTRAQ adjust rolling collision energy was applied to all precursor ions for collision-induced dissociation. Dynamic exclusion was set for 1/2 of peak width (15 seconds) and then the precursor was refreshed off the exclusion list.

Data analysis

The raw files were converted to MGF files with Proteome Discoverer 1.2 software (PD 1.2; Thermo; 5600-msconverter) and the MGF files were searched. Protein identification was conducted using Mascot search engine (Matrix Science, London, UK; version 2.3.02) against a database containing 39,925 sequences. For protein identification, a mass tolerance of 0.1 Da (ppm) was permitted for intact peptide masses and 0.05 Da for fragmented ions, with allowance for one missed cleavage in the trypsin digests. Gln-> pyro-Glu (N-term Q), Oxidation (M), and Deamidated (NQ) were the potential variable modifications, and Carbamidomethyl (C), iTRAQ8 plex (N-term), and iTRAQ8plex (K) were the

Table 1 Differential (downregulated) proteins identified by iTRAQ coupled with LC-MS/MS

Accession IPI rat-v3.87	Description	COG function	EA vs.control (P value)	Model vs. EA(P value)
IPI00327925	Adenylate cyclase type 5	Adenylate cyclase, family 3 (some proteins contain HAMP domain)	> 0.05	< 0.05
IPI00193259	Tyrosine 3-monooxygenase	Phenylalanine-4-hydroxylase	> 0.05	< 0.05
IPI00187593	Sodium-dependent dopamine transporter	Na ⁺ -dependent transporters of the SNF family	< 0.05	< 0.05
IPI00199077	Calcium/calmodulin-dependent 3',5'-cyclic nucleotide phosphodiesterase1B		< 0.05	< 0.05
IPI00324300	Angiotensin-converting enzyme	Not found	> 0.05	< 0.05
IPI00780260	Protein Ankrd63	FOG: Ankyrin repeat	> 0.05	< 0.05
IPI00382226	Neurabin-1	Not found	> 0.05	< 0.05
IPI00230972	Microtubule-associated protein	Not found	> 0.05	< 0.05
IPI00202366	Protein Diras1	GTPase SAR1 and related small G proteins	> 0.05	< 0.05
IPI00204316	ATP synthase-coupling factor 6, mitochondrial	Not found	> 0.05	< 0.05
IPI00208200	Phosphatide cytidyltransferase	CDP-diglyceride synthetase	> 0.05	< 0.05
IPI00369414	Sodium channel subunit beta-4	Not found	> 0.05	< 0.05
IPI00331856	Carbonyl reductase [NADPH] 1	Dehydrogenases with different specificities (related to short-chain alcohol dehydrogenases)	> 0.05	< 0.05

All data conform to normal distribution and variance (one-way analysis of variance followed by the least significance difference test). COG: Cluster of Orthologous Groups of proteins; LC-MS/MS: liquid chromatography-tandem mass spectrometry/mass spectrometry; EA: electroacupuncture.

Table 2 Differential (upregulated) proteins identified by iTRAQ coupled with LC-MS/MS

Accession IPI rat-v3.87	Description	COG function	EA vs. control (P value)	Model vs. EA (P value)
IPI00203440	ATPase, Na ⁺ /K ⁺ transporting, beta 2 polypeptide	Not found	> 0.05	< 0.05
IPI00195109	Serine hydroxymethyltransferase	Glycine/serine hydroxymethyltransferase	> 0.05	< 0.05
IPI00212816	Protein kinase C delta type	Serine/threonine protein kinase	> 0.05	< 0.05
IPI00869863	Carboxypeptidase M (predicted)	Predicted carboxypeptidase	> 0.05	< 0.05
IPI00231350	Parvalbumin alpha	Ca ²⁺ -binding protein (EF-hand superfamily)	> 0.05	< 0.05
IPI00231900	Fatty acid-binding protein, brain	Not found	> 0.05	< 0.05
IPI00382202	Isoform 2 of haptoglobin	Not found	> 0.05	< 0.05
IPI00765347	Adenylate kinase isoenzyme 5	Adenylate kinase and related kinases	> 0.05	< 0.05
IPI00208117	Choline transporter-like protein 1 (fragment)	Not found	> 0.05	< 0.05
IPI00207426	Voltage-dependent calcium channel gamma-8 subunit (fragment)	Not found	> 0.05	< 0.05
IPI00371167	Glucose phosphate mutase protein Pgm2l1	Phosphomannomutase	> 0.05	< 0.05
IPI00363718	Enolase-phosphatase 1	Predicted enolase-phosphatase	> 0.05	< 0.05
IPI00389828	Isoform 1 of ermin	Not found	> 0.05	< 0.05
IPI00230941	Vim vimentin	Not found	> 0.05	< 0.05

All data conform to normal distribution and variance (one-way analysis of variance followed by the least significance difference test). COG: Cluster of Orthologous Groups of proteins; LC-MS/MS: liquid chromatography-tandem mass spectrometry/mass spectrometry; EA: electroacupuncture.

fixed modifications. The charge states of peptides were set to +2 and +3. Specifically, an automatic decoy database search was performed in Mascot by choosing the decoy checkbox in which a random sequence of database was generated and tested for raw spectra as well as the real database. To reduce the probability of false peptide identification, only peptides with significance scores (≥ 20) at the 99% confidence interval by Mascot probability analysis greater than "identity" were counted as "identified". The identification of each confident protein involved at least one unique peptide. For protein quantitation, a protein should contain at least two unique peptides. The quantitative protein ratios were weighted and

normalized by the median ratio in Mascot. Only ratios with P values < 0.05 were used, and only fold changes > 1.2 were considered statistically significant.

The differential proteins were further analyzed by pathway enrichment to determine which main biochemical pathways and signaling transduction pathways they were part of.

Statistical analysis

The behavior test results are expressed as the mean \pm SD and were analyzed statistically using SPSS 17.0 software (SPSS, Chicago, IL, USA). All data conformed to normal distribution and variance. One-way analysis of variance, followed by

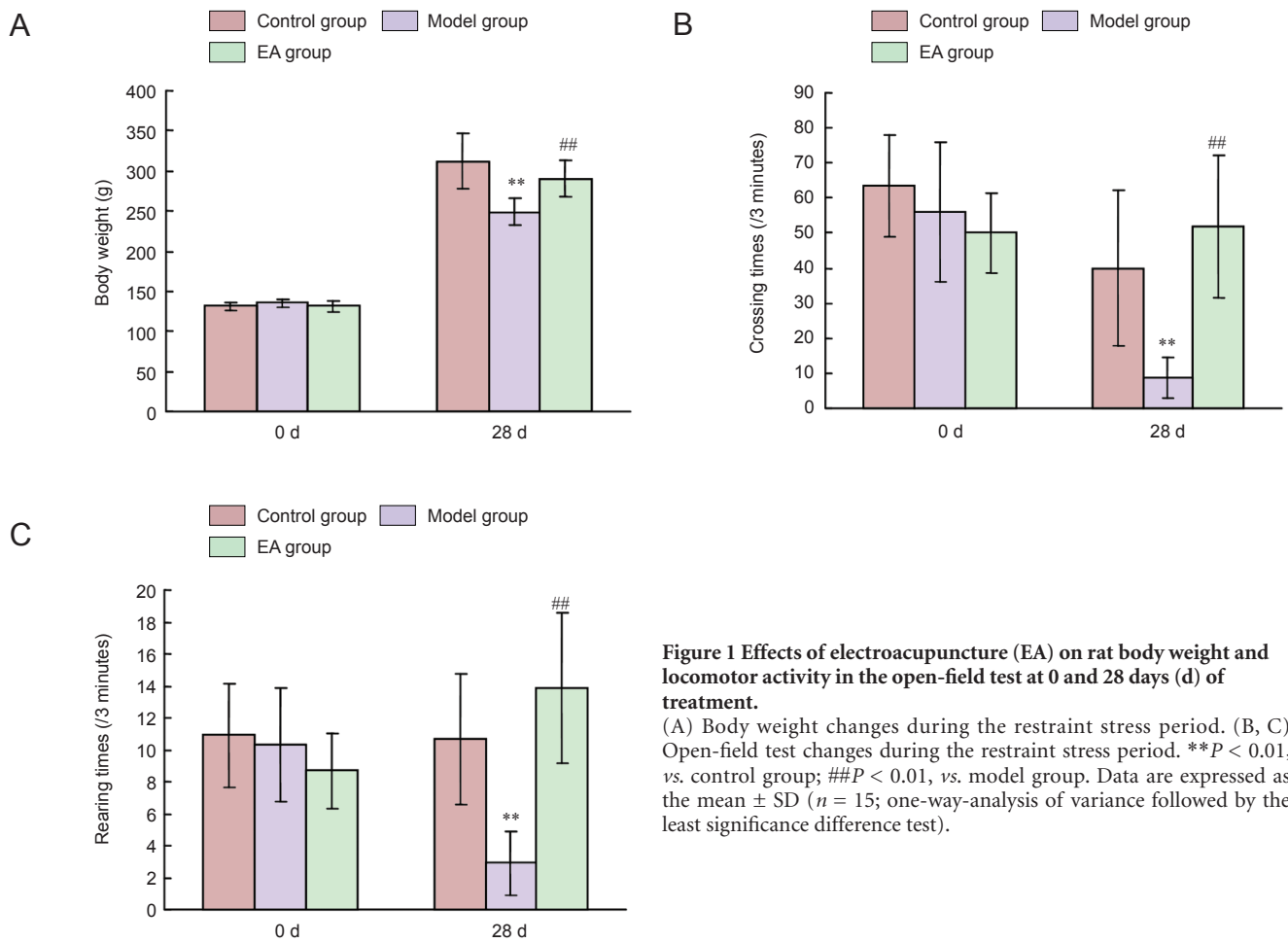


Figure 1 Effects of electroacupuncture (EA) on rat body weight and locomotor activity in the open-field test at 0 and 28 days (d) of treatment.

(A) Body weight changes during the restraint stress period. (B, C) Open-field test changes during the restraint stress period. ** $P < 0.01$, vs. control group; ## $P < 0.01$, vs. model group. Data are expressed as the mean \pm SD ($n = 15$; one-way-analysis of variance followed by the least significance difference test).

the least significance difference test, was performed to compare the differences between groups. A level of $P < 0.05$ was considered statistically significant.

Results

Body weight changes during restraint stress period

As shown in **Figure 1A**, there was no significant difference in bone weight among the three groups at the beginning of the study. After 28 days of induction, rat body weights were significantly decreased in the model group compared with the control group ($P < 0.01$), and rat body weight was significantly increased in the electroacupuncture group compared with the model group ($P < 0.01$).

Open-field test changes during the restraint stress period

The exploratory and locomotor activities of rats were determined using the open-field test. As shown in **Figure 1B**, no difference in crossing numbers was observed among the three groups on day 0. After 28 days of the restraint stress procedure, the crossing numbers in the model group were significantly lower compared with the control group ($P < 0.01$). The crossing numbers in the electroacupuncture group were significantly higher compared with the model group ($P < 0.01$). On day 0, there was no significant difference in rearing numbers among the three groups ($P > 0.05$).

Figure 1C shows that restraint stress reduced the rearing numbers of rats in the model group compared with the control group ($P < 0.01$). The rearing numbers were significantly increased in the electroacupuncture group compared with the model group ($P < 0.01$). According to the behavior comparison between control and model groups, rats in the model group exhibited a depressive-like state.

Differential proteins

Immunodepleted protein fractions were then profiled using iTRAQ coupled to a liquid chromatography-tandem mass spectrometry/mass spectrometry (LC-MS/MS)-based approach. The identified proteins in the depressed animals were used for both chronic restraint stress and electroacupuncture pretreatment comparisons. A total of 4,240 and 4,352 proteins were identified separately by two computers using the Mascot software. Differentially regulated proteins (fold change > 1.2 with $P < 0.05$) were selected. These cut-offs were selected based on literature investigating the reproducibility of iTRAQ quantification (Moulder et al., 2010). The potential function of these differential proteins was predicted using the COG database. Thirteen proteins were downregulated in the model group compared with the electroacupuncture group, and two differential proteins were downregulated in the electroacupuncture group compared

with the control group (Table 1). Fourteen proteins were up-regulated in the model group compared with the electroacupuncture group, and there were no differential proteins between electroacupuncture and control groups (Table 2).

Discussion

iTRAQ technology was used in this study to quantitatively analyze proteomics of the hippocampus of chronic restrained rats and electroacupuncture pretreated rats. iTRAQ can be used for parallel comparison of protein abundance by determining the peak intensity of reporter ions released by iTRAQ-tagged peptides and is an important tool in the area of quantitative proteomic study.

iTRAQ has been used in a study to determine the mechanism involved in the anti-depressive effects of electroacupuncture pretreatment. Using this method, 27 proteins related to emotional diseases were successfully identified. To identify potential curative mechanisms of early intervention depressive state, the current study preliminarily explored proteins that were associated with depression by comparing chronic restraint stress rats with electroacupuncture pretreated rats.

Intriguingly, a recent study suggested that dopamine played a critical role in the anti-inflammatory potential of electroacupuncture (Torres-Rosas et al., 2014). These findings offer novel evidence for the involvement of electroacupuncture in modulating immune responses *via* the neural-immune network. The present study identified proteins related to depression or stress, such as dopamine transporter (also dopamine active transporter, DAT, SLC6A3) (Meyer et al., 2001; Yang et al., 2008) and protein kinase C (Coull et al., 2000). A large body of literature showed that dopamine insufficiency might be a susceptible factor for depression and treatment targeting dopamine had favorable effects (Liang et al., 2011). This indicates that neurotransmitters such as dopamine might mediate the antidepressant-like effects of electroacupuncture by regulating immune responses; meanwhile, they serve as a direct effector *per se* in treating depression. These results are consistent with the present findings.

The neurobiological mechanisms underlying the pathophysiology and therapeutics of bipolar disorders are still unknown. In recent years, protein kinase C has emerged as a potentially important factor in mania (Abrial et al., 2012). Protein kinase C inhibitors were recently reported to ease moods (Einat, 2014). In the present study, protein kinase C δ showed a tendency to be increased after chronic restraint stress, which was consistent with the results of a previous study (Coull et al., 2000). Protein kinase C can activate the cascade system, leading to mitogen-activated protein kinase (MAPK) phosphorylation, induction of the protein phosphorylation of the MAPK gene and regulation of the phosphorylation of the Elk-1 protein. Protein kinase C activates Elk-1 protein and binds with serum reaction components, followed by serum response factors, to regulate gene expression. In the present study, protein kinase C δ was shown to be related to the neurotrophin signaling pathway, vascular

smooth muscle contraction and tight junction pathway after chronic restraint stress. A genetic polymorphism in the DAT gene (DAT1), known as a Variable Number of Tandem Repeats (VNTR), is associated with dopamine-related disorders, and influences the amount of proteins expressed. In addition, protein kinase C and MAPK can adjust the DAT transit speed of dopamine or cause DAT internal conformational changes (Vandenbergh et al., 1992).

This study initially investigated delineating primary comparative protein profiles in the model and electroacupuncture groups using iTRAQ, then confirmed interesting candidate biomarkers on a larger sample of depression (caused by chronic stress). Some of the “traditional” methods such as western blot or ELISA using samples of hippocampus in addition to serum samples/supernatant should be performed for further verification.

In the present study, electroacupuncture pretreatment was implemented in rats under slightly restrained conditions. A previous study (Liang, 2012) demonstrated no significant difference in serum adrenocorticotrophic hormone and corticosterone between normal rats with or without acupuncture. This finding suggests that acupuncture administration does induce a stress response.

Depression induced by chronic restraint stress is an ideal model to mimic human depression symptoms. Chronic restraint stress affects multiple related proteins and through the modulation of these proteins, electroacupuncture can improve depressive symptoms. The specific targets identified in this study warrant further investigation.

Author contributions: ZG and YT conceived the study; ZG, XQY, YCW and LS conducted experimental procedures; ZG and TWG wrote the paper; XJY, WYZ, YW and XHZ were responsible for data analysis and literature search. All authors approved the final version of this paper.

Conflicts of interest: None declared.

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