

## Original Article

# Electroacupuncture promotes locomotor recovery by inhibiting the activation of JNK and p38 MAPK signaling pathways in spinal cord injury rats

Junfeng Zhang<sup>1</sup>, Yaochi Wu<sup>1</sup>, Rong Zhang<sup>2</sup>, Shisheng Li<sup>1</sup>, Yuanzhi Fan<sup>1</sup>

<sup>1</sup>Department of Acupuncture, Tuina and Traumatology, The Sixth People's Hospital Affiliated to Shanghai Jiaotong University, Shanghai 200233, China; <sup>2</sup>Department of Chinese Medicine, Jinqiao Community Health Service Center of Pudong New District, Shanghai 201206, China

Received December 22, 2015; Accepted October 20, 2016; Epub November 15, 2016; Published November 30, 2016

**Abstract:** This study is aimed to evaluate the locomotor recovery of spinal cord injury (SCI) rats who having been treated with electroacupuncture (EA) and the related molecular mechanism. SD male rats were constructed to SCI models and divided into 4 groups. SCI, SCI + EA: SCI models were treated with electrical acupuncture on Jiaji for 30 min; SCI + ANI group: SCI rats were injected intraperitoneally with Anisomycin (0.1 mg/kg IP), and SCI + EA + ANI group: rats were simultaneously treated with electrical acupuncture on Jiaji and injected intraperitoneally with Anisomycin. BBB score and inclined plane test were used to observe the promotion of EA on the locomotor recovery of rats with SCI. The activation of astrocyte and microglia was assayed through determination of the levels of GFAP and Iba-1 respectively using western blotting and immunohistochemistry. The activation of JNK and p38 MAPK, and apoptosis were detected using western blotting through measuring the levels of p-JNK, JNK, p-c-Jun, c-Jun, p-p38, p38 and activated caspase 3, 9. After surgery 28 days, BBB score and angle of inclination of rats in SCI + EA group were evidently higher than that in SCI, SCI + ANI and SCI + EA + ANI group. In addition, SCI induced increased production of GFAP, Iba-1 and activated caspase 3, 9 were significantly reduced by EA, and this improvement effect could be reversed by ANI, an agonist of JNK and p38 MAPK. In conclusion, EA can improve the locomotor recovery of spinal cord of SCI rats by inhibiting the activation of astrocytes and microglia, and apoptosis. JNK and p38 MAPK signaling pathways might play a vital in this process.

**Keywords:** Electroacupuncture, spinal cord injury, JNK, p38 MAPK, locomotor recovery

## Introduction

Spinal cord injury (SCI) is a high-cost, low-incidence neurological disability [1]. It occurs through various countries throughout the world with an annual incidence of 15 to 40 cases per million, with the causes of these injuries ranging from motor vehicle accidents and community violence to recreational activities [2]. It is damage to the thick bundle of nerves that runs from the brain to the lower back [3]. SCI resulting in permanent paralysis and loss of sensation may appear to many individuals as one of the most devastating experiences imaginable [4]. The evaluation and treatment of SCI continues to evolve, enhanced by new imaging modalities [5].

Electroacupuncture (EA) was reported promote differentiation of mesenchymal stem cells,

regeneration of nerve fibers and partial functional recovery after SCI [6]. It could improve the survival and migration of neural stem cells (NSCs) transplanted in injured spinal cord as well as the potential mechanisms [7]. EA has been proven to contribute towards neurologic and functional recoveries in SCI [8]. Ding et al. reported EA could efficiently promote bone marrow mesenchymal stem cells (MSCs) survival and differentiation, axonal regeneration and finally, functional recovery in the transected spinal cord [9]. Acupuncture (AP) could relieve neuropathic pain (NP) by inhibiting Jun-N-terminal kinase (JNK) activation in astrocytes after spinal cord injury (SCI) [10]. Bernateck et al. reported can relieve peripheral NP as well as acute or chronic inflammatory pain via inhibition of microglial activation and production of inflammatory mediators in animal models [11]. Therefore, we want to understand whether SCI,

## Electroacupuncture promotes locomotor recovery in SCI

which is similar to AP but not exactly the same with AP, promote locomotor recovery after SCI in rats by inhibiting the activation of JNK and p38 MAPK.

In this study, we prepared the SCI models and treated them with EA, Anisomycin (ANI) and EA + ANI, respectively. Then, the locomotor recovery of rats after treated for different times was determined. Moreover, the protein expression levels of GFAP, Iba-1, JNK, p-JNK, c-Jun, p-c-Jun, p38 MAPK, p-p38 MAPK and activated caspase 3, 9 in L4L5 spinal cord tissues were determined by western blot assay and/or immunohistochemistry.

### Materials and methods

#### *Rat models of spinal cord injury*

Adult male SD rats (230-270 g; Animal Center of Shandong University) with damage of T10 segment were used for all experiments. The rats were maintained in a climate-controlled vivarium with a 12 h light-dark cycle with free access to food. Then, SCI models were constructed: SD rats were injected intraperitoneally with 100 mg/mL chloral hydrate (300 mg/kg) in the left 1 cm of abdomen midline. Rats were prostrate lied, fixed on the operating table and back wool was cut. Then, the back of rats was sterilized with iodophor and cut along spine spines. Vertebral plate and spine on T10 parts were removed. Spinal cord on T10 section was striked by NYU Blow device (Curtiss-Wright, Grimethorpe) with 60 g cm (10 g × 6 cm) to result in the SCI of T10 segment.

#### *Treatment for SCI rats models*

SCI rat models were conducted Basso-Beattie-Bresnahan scale (BBB) score and inclined plane test before and after surgery for 1, 7, 14, 21 and 28 days. BBB is a 22-point scale (scores 0-21) that systematically and logically follows recovery of hindlimb function from a score of 0, indicative of no observed hindlimb movements, to a score of 21, representative of a normal ambulating rodent. Inclined plane test was performed by the method described previously [12]. In brief, animals were tested in two positions (right side or left side up) on the testing apparatus (i.e., a board covered with a rubber mat containing horizontal ridges spaced 3 mm apart). The maximum angle at which a rat could

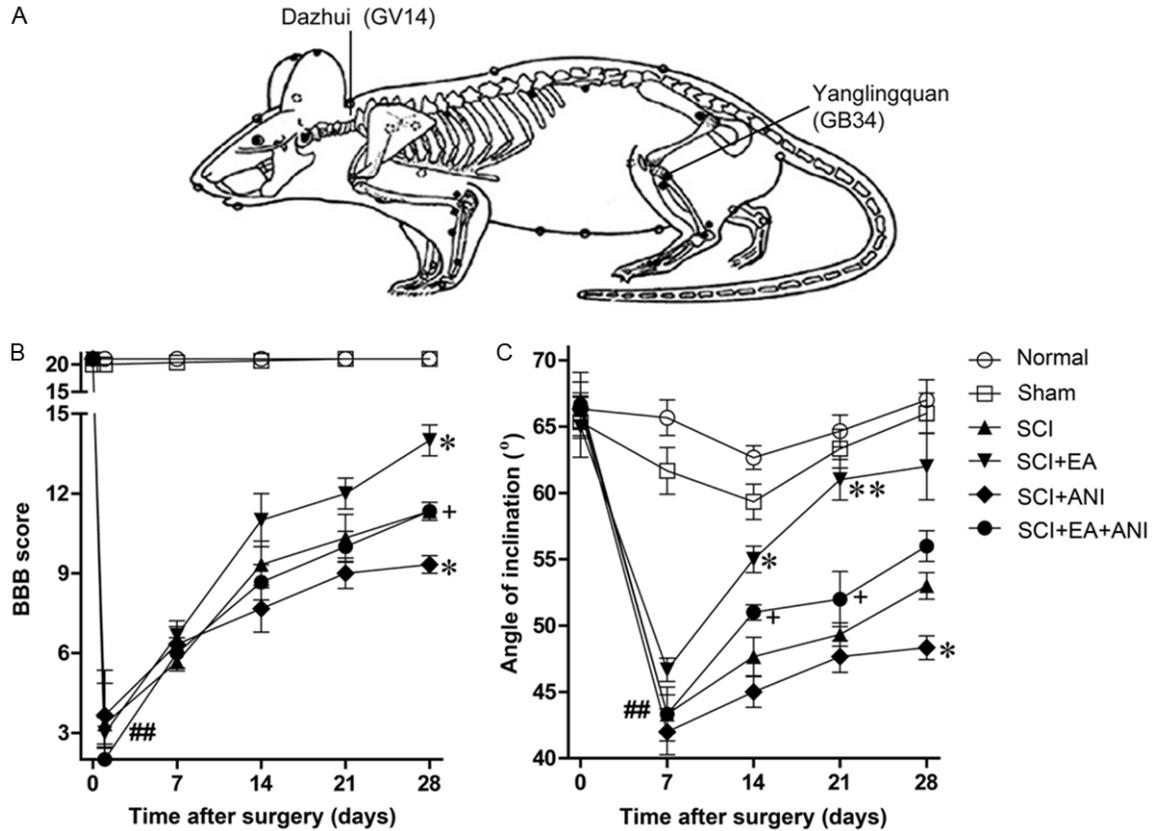
maintain its position for 5 s without falling was recorded for each position and averaged to obtain a single score for each animal. The ability to control and place the hindlimb precisely was tested on a horizontal grid as previously described [13]. Analysis was performed by counting the number of foot fall (mistake) in foot placing. Both animal's forepaws and hindpaw were dipped in red and blue dye (non toxic) and then walked across a narrow box (1 m long and 7 cm wide). The footprints were scanned, and digitized images were analyzed.

SCI rat models were randomly divided into 4 groups: SCI group, SCI + EA group, SCI + ANI and SCI + EA + ANI group. There were 20 Rats in each group and each group was divided into 3, 7, 14 and 28 d. For SCI + EA group, rats were treated with electrical acupuncture on Jiaji for 30 min (Frequency of electric acupuncture was 20 Hz) by G6805-1 multi-channel electric acupuncture therapeutic apparatus (Shanghai medical electronic instrument factory, China). For SCI + ANI group, SCI rat models were injected intraperitoneally with Anisomycin (0.1 mg/kg IP) after the models were constructed 4 days. For SCI + EA + ANI group, rats were simultaneously reacted with electrical acupuncture on Jiaji and injected intraperitoneally with Anisomycin (Sigma, St. Louis, MO, USA) after the models were constructed 4 days.

#### *Western blot analysis*

The rats were executed after anesthetized by intraperitoneal injection of ethyl carbamate (2.5%, 1.5 g/kg). The L4-L5 segments of spinal cord were isolated immediately in ice bath. Total proteins were prepared from spinal cord tissues using radioimmunoprecipitation assay (RIPA) lysis buffer (Sigma, USA) supplemented with protease inhibitors. An equal amount (50 µg) of cellular lysates was separated on 10% SDS-polyacrylamide gel electrophoresis (PAGE) minigels and transferred to nitrocellulose filter membranes (Hybond, Escondido, CA, USA). The membrane was blocked with Tris-buffered saline Tween-20 (TBST) containing 5% skimmed milk powder for 1 h at room temperature, followed by incubation in TBST containing 5% BSA (Sigma, St. Louis, MO, USA) and primary antibodies overnight at 4°C. Primary antibodies were detected using a peroxidase-coupled goat anti-rabbit secondary antibody (1:8000, ZSbio, Beijing, China) and EZ-ECL chemiluminescence

# Electroacupuncture promotes locomotor recovery in SCI



**Figure 1.** Electroacupuncture promoted the recovery of motor function in rats. A: Schematic diagram of EA on rats; B and C: Basso-Beaie-Bresnehan (BBB) score and angle of inclination of motor function in rats in SCI, SCI + EA, SCI + ANI and SCI + EA + ANI groups after surgery for 1, 7, 14, 21 and 28 days by BBB score method and inclined plane test, respectively. \* $P < 0.05$  and \*\* $P < 0.01$ , compared with SCI group, BBB score and angle of inclination in SCI + EA and SCI + ANI had significant difference; ## $P < 0.01$ , compared with sham group, BBB score and angle of inclination in SCI, SCI + EA, SCI + ANI and SCI + EA + ANI groups had significant difference; + $P < 0.05$ , compared with SCI + EA group, BBB score and angle of inclination in SCI + EA + ANI group had significant difference.

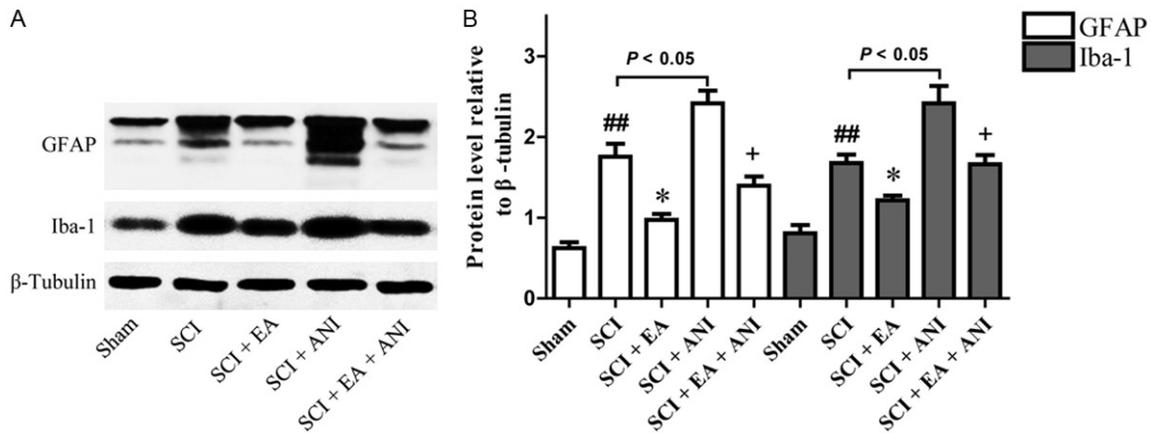
Detection kit for HRP (Biological Industries, Beit-Haemek, Israel). The following primary antibodies were used: rabbit GFAP (Cell Signaling Technology, Danvers, MA, USA), Iba-1, p-JNK, p-c-JUN, p-p38 MAPK, caspase 3, caspase 9 and rabbit pAb  $\beta$ -Tubulin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA).

### Immunohistochemistry

The rats were perfused with 150 ml of 37°C normal saline (NS), and fixed with 400 ml of 4% paraformaldehyde in 100 mM phosphate buffer (4°C) under deep anesthesia by ethyl carbamate (2.5%, 1.5 g/kg, i.p.). After perfusion, the L4-L5 segments of spinal cord were removed immediately, post-fixed in 4% paraformaldehyde for 4 h at 4°C, and immersed from 10-30% sucrose solution for 24-48 h at 4°C for cryoprotection. Then, the tissues were sectioned at 40

$\mu\text{m}$  on a freezing microtome (Leica, Nussloch, Germany) under OTC (Miles Inc., Elkhart, IN) protection. After rinsed with 0.01 M PBS for three times, the sections were blocked with 10% normal donkey serum in 0.01 M PBS with 0.3% Triton-X-100 over night at 4°C, followed by incubation with mouse anti-Iba-1 (1:1000, ab15690, Abcam) and anti-GFAP (1:1000, ab10062, Abcam) primary antibody at 4°C for 24 h. After washed with 0.01 M PBS for 3 times, the sections were incubated with fluorescein isothiocyanate (FITC)-conjugated donkey anti-rat IgG (1:100, ab102181, Abcam) for 2 h at 4°C. After washed with PBS in dark and mounted with fluorescent mounting medium (0.01 M PBS: glycerinum, 1:1), the sections were examined under fluorescence microscope (Leica, Germany). Quantitative analysis of immunohistochemical staining was performed using Image-Pro Plus 6.0 software, and the results

## Electroacupuncture promotes locomotor recovery in SCI



**Figure 2.** Electricacupuncture restrained the high expression of GFAP and Iba-1 resulted by SCI. A: The protein expression of GFAP and Iba-1 in spinal cord tissue of lumbar vertebrae L4 and L5 by western blot assay. B: The densitometric analysis on western blot stripe of GFAP and Iba-1. <sup>##</sup> $P < 0.01$ , compared with sham group, GAFF level relative to  $\beta$ -Tubulin in SCI group had significant difference;  $*$  $P < 0.05$ , compared with SCI group, GAFF level relative to  $\beta$ -Tubulin in SCI + EA group had significant difference;  $*$  $P < 0.05$ , compared with SCI + ANI group, GAFF level relative to  $\beta$ -Tubulin in SCI + EA + ANI group had significant difference.

were expressed as  $IOD_{\text{treatment}}/IOD_{\text{normal}}$ . IOD: Integrated Optical Density.

### Statistical analysis

Data are presented as mean  $\pm$  SD values. Comparisons between vehicle and acupuncture-treated groups were made by unpaired Student's *t* test. Multiple comparisons between groups were performed one-way ANOVA. Behavioral scores from BBB analysis and inclined plane tests were analyzed by repeated measures ANOVA (time vs treatment). Tukey's multiple comparison was used as Post hoc analysis. Statistical significance was accepted with  $P < 0.05$ . All statistical analyses were performed by using SPSS 18.0 (SPSS Science, Chicago, IL).

### Results

#### Electroacupuncture promoted locomotor recovery of SCI rats

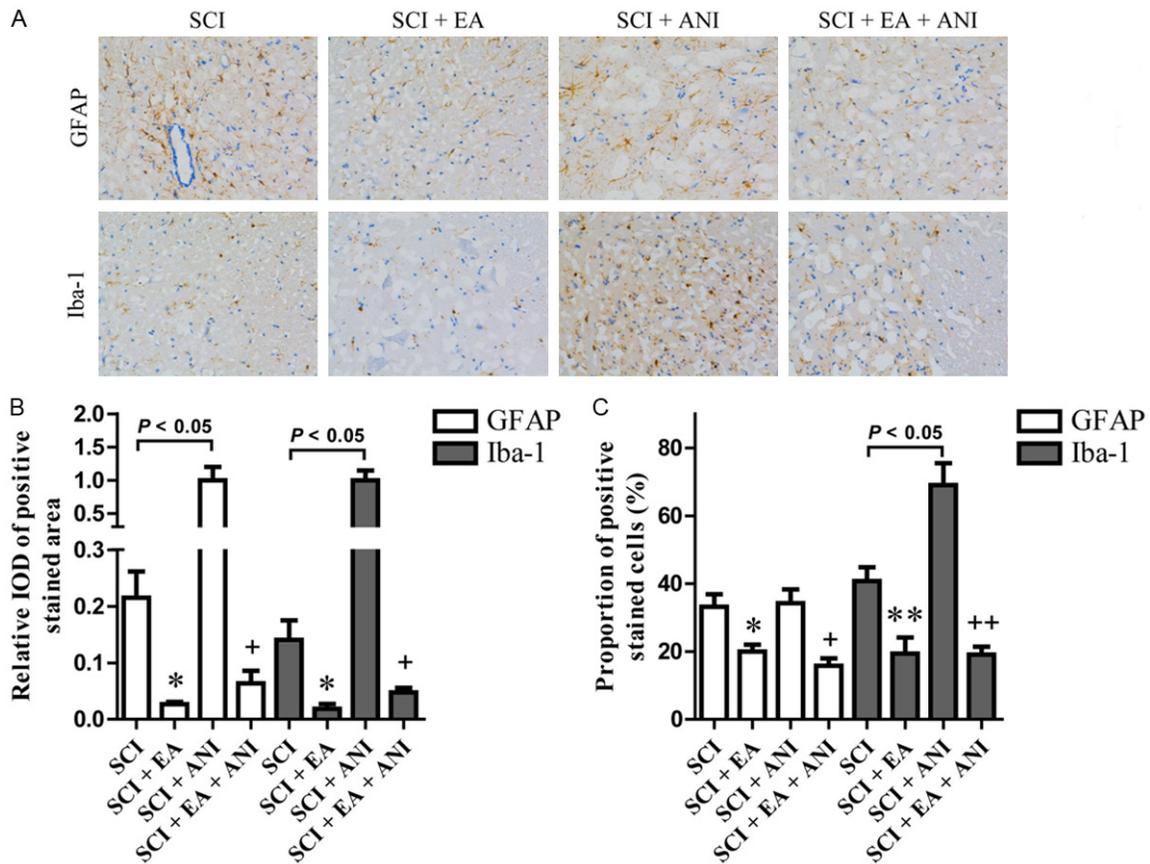
To explore the effect of EA on SCI, we constructed SCI rat models and evaluated the influence of EA on SCI rats. Schematic diagram of EA on rats was performed as shown in **Figure 1A**. BBB score was used to evaluate the behavior of rats in different groups before and after surgery 7, 14, 21 and 28 days, and results were shown in **Figure 1B**. As shown, BBB score in SCI, SCI + EA, SCI + ANI and SCI + EA + ANI groups were almost the same after surgery 7 days. BBB

score in SCI + EA group increased continuously with time increased from 7 to 28 days and the value was much higher than that in SCI, SCI + ANI and SCI + EA + ANI group. BBB score in SCI and SCI + EA + ANI group kept almost the same with each other with the increase of time. While BBB score in SCI + ANI group was always lower than that in SCI and SCI + EA + ANI group. To further verify the influence of EA on SCI, inclined plane test was also performed. The angle of inclination in SCI, SCI + EA, SCI + ANI and SCI + EA + ANI groups was determined after surgery 7, 14, 21 and 28 days, respectively. The growth trend was similar to that of BBB score. Both BBB score and angle of inclination in SCI + ANI group were significantly lower than that in SCI group after surgery 28 days which indicated ANI increased the degree of SCI in rats (**Figure 1C**). Compared with SCI + EA group, the resilience of rat locomotor recovery in SCI + EA + ANI group was weakened.

#### Electroacupuncture inhibited the expression of GFAP and Iba-1

GFAP is an intermediate-filament protein expressed abundantly and almost exclusively in astrocytes of the CNS [14]. Iba-1 is specifically expressed in microglia and plays an important role in the regulation of the function of microglia [15]. In this study, the protein expression levels of GFAP and Iba-1 in spinal cord tissue of lumbar vertebrae L4 and L5 were determined by western blotting and immunohistochemistry.

## Electroacupuncture promotes locomotor recovery in SCI



**Figure 3.** Electricacupuncture suppressed the activation of spinal cord microglia and astrocytes resulted by SCI. A: Immunohistochemistry of GFAP and Iba-1 in spinal cord tissue of lumbar vertebrae L4 and L5. B: Relative integrated optical density (IOD) value of positive staining region. C: Percentage of GFAP or Iba-1 positive stained cells (positive cell number/number of nuclei, nuclear staining for blue). ## $P < 0.01$ , compared with sham group, GAFFP level relative to  $\beta$ -Tubulin in SCI group had significant difference; \* $P < 0.05$ , compared with SCI group, GAFFP level relative to  $\beta$ -Tubulin in SCI + EA group had significant difference; \* $P < 0.05$ , compared with SCI + ANI group, GAFFP level relative to  $\beta$ -Tubulin in SCI + EA + ANI group had significant difference.

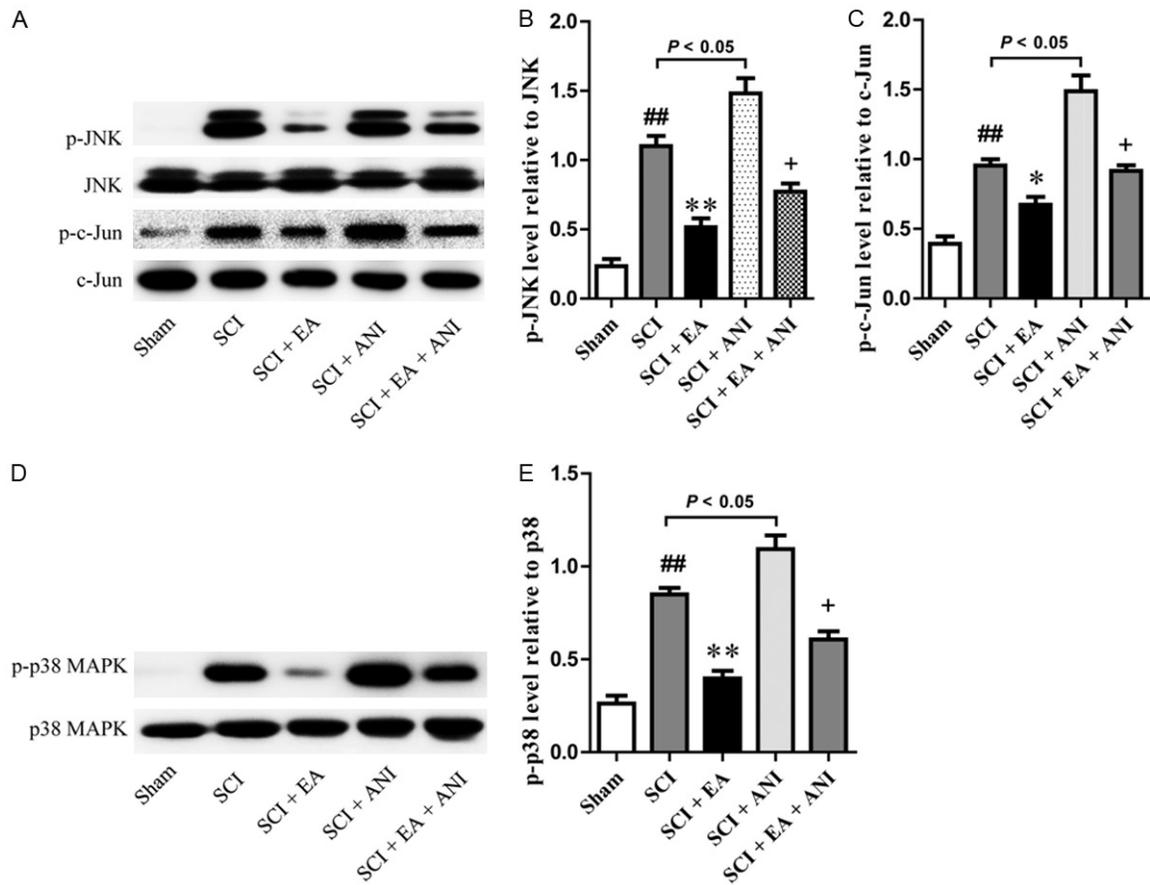
Western blot results showed GFAP level relative to  $\beta$ -Tubulin in SCI group increased significantly compared with that in sham group ( $P < 0.01$ ). The value in SCI + EA group was evidently lower than that in SCI group. GFAP level relative to  $\beta$ -Tubulin in SCI + ANI group increased markedly compared with that in SCI + EA group and also significantly higher than SCI group. Compared with GFAP level relative to  $\beta$ -Tubulin in SCI + ANI group, the value in SCI + EA + ANI group decreased markedly and was slightly higher than that in SCI + EA group. The protein expression level of Iba-1 in different groups and the comparison results kept almost the same with that of GFAP (**Figure 2A** and **2B**). The result of immunohistochemistry matched the western-blot. Compared with the SCI group, the relative IOD of positive stained area and proportion of positive stained cells for both GARP

and Iba-1 were significantly reduced in EA group (**Figure 3**), which was reversed by the administration of ANI, an agonist of JNK/p38 MAPK. ANI alone further promoted the increased positive stained area and cells for both GARP and Iba-1 in spinal cord of rats with SCI. These data suggesting that EA treatment could suppress SCI induced activation of astrocytes and microglia, which might be related to the inhibition of JNK and p38 MAPK signaling pathways.

### *Electroacupuncture inhibited the activation of JNK and p38 MAPK*

To test the speculation, the phosphorylation of c-Jun, c-Jun NH2-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) was determined using western blot assay. JNK belongs to the mitogen-activated protein kinase

## Electroacupuncture promotes locomotor recovery in SCI



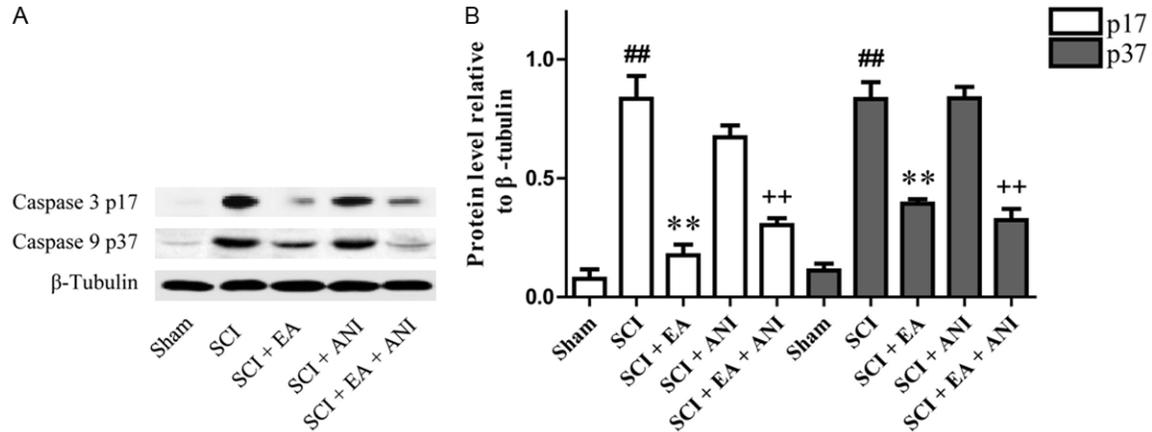
(MAPK) family [16], and plays important roles in cellular processes and stress [17]. P38 MAPK family member function in a cell context-specific and cell type-specific manner to integrate signals that affect proliferation, differentiation, survival and migration [18]. The phosphorylation levels of c-Jun, JNK and p38 were remarkably increased in SCI rats, which were alleviated by EA stimulation (**Figure 4**). ANI alone effectively promoted the activation of JNK and p38, and could attenuate the inhibition effect of EA on JNK and p38 activation.

### *Electroacupuncture inhibited the activation of caspase 3 and caspase 9*

It was well documented that activated spinal glial cells release a variety of proinflammatory factors, and play an important role in apoptosis

of neurons after SCI. After confirmation of the inhibition effect of EA on the activation of glial cells, we tried to investigate the effect of electric acupuncture on apoptosis in SCI. It is well known that three important pathways are involved in cell apoptosis, namely death receptor pathway, mitochondrial pathway, and endoplasmic reticulum pathway. Among them, the mitochondrial pathway is the most important intracellular apoptotic signal cascade system, which is divided into caspase dependent and caspase independent pathways. Caspase protein, also known as apoptosis enzyme, is a class of aspartate dependent cysteine protease, which plays an important role in the regulation of apoptosis. The irreversible open of mitochondrial permeability transition pore (MPTP), located between the outer and inner membranes of mitochondria induced by vari-

## Electroacupuncture promotes locomotor recovery in SCI



**Figure 5.** Electricacupuncture restrained the high production of cleaved caspase 3 and cleaved caspase 3 resulted by SCI. A: The protein expression of cleaved caspase 3, 9 in spinal cord tissue of lumbar vertebrae L4 and L5 by western blot assay. B: The densitometric analysis on western blot stripe of cleaved caspase 3, 9. ##P < 0.01, compared with sham group, p-p38 level relative to p38 in SCI group had significant difference; \*\*P < 0.01, compared with SCI group, p-p38 level relative to p38 in SCI + EA group had significant difference; \*P < 0.05, compared with SCI + ANI group, p-JNK level relative to JNK in SCI + EA + ANI group had significant difference.

ous unfavorable factors, leads to the release of cytochrome C (Cyt C) and apoptosis inducing factor from mitochondria into the cytoplasm. The binding of Cyt C and apoptotic protease activating factor-1 (APAF 1) leads to the activation of caspase-9. Activated caspase-9 activates other caspase, such as caspase-3, 7. Activation of caspase family proteins irreversibly initiates protein degradation and apoptosis. In this work, we found the activated caspase-3 and caspase-9 levels in SCI group were markedly higher than that in normal group, indicating that SCI induced apoptosis in spinal cord (Figure 5). EA treatment significantly reduced the increased levels of activated caspase-3, 9, suggesting EA could suppress SCI induced apoptosis. In addition, ANI treatment could effectively reverse the reducing effect of EA on activated caspase-3, 9 levels, suggesting JNK and p38 pathways might play a vital role in regulating apoptosis after SCI. Considering the increased number of glial cells, showed by immunohistochemistry, we speculated that SCI mainly induced neurons apoptosis in spinal cord.

### Discussion

SCI is a high-cost, low-incidence neurological disability. This condition has a significant impact on the individual, the family, health care service delivery systems, and society in general [19]. The spinal cord is the major conduit

through which motor and sensory information travels between brain and body [20]. SCI can lead to paraplegia or quadriplegia. Although there are no fully restorative treatments for SCI, various rehabilitative, cellular and molecular therapies have been tested in animal models [21]. Anisomycin (ANI) is a protein synthesis inhibitor and could inhibit long-term memory formation. It produced decrements in long-term memory by raising free tyrosine levels and by the accumulation of catecholamines (CAs) [22]. Electroacupuncture have been shown is effective in relieving human pain sensation. It can promote the differentiation of mesenchymal stem cells, regeneration of nerve fibers and partial functional recovery after SCI [23]. EA was reported can improve the survival and migration of neural stem cells (NSCs) transplanted in injured spinal cord as well as the potential mechanisms [8]. However, the molecular mechanism about the promotion of EA on the restoration of function in SCI is still unknown.

In this study, SCI rat models were constructed and divided into 4 groups: SCI, SCI + EA, SCI + ANI and SCI + EA + ANI group. BBB score assay and inclined plane test were performed to observe the influence of EA on the restoration of locomotor recovery in rats before and after surgery for 1, 7, 14, 21 and 28 days. Results showed the BBB score after blown by the weight was decreased significantly compared with the

## Electroacupuncture promotes locomotor recovery in SCI

sham group, which illustrated modeling was successful. During the recovery process of SCI, results of BBB score after surgery 28 days and inclined plane test after surgery 14 and 21 days showed EA significantly ( $P < 0.05$ ) promoted the recovery of SCI in rats ( $P < 0.05$ ). After surgery 28 days, BBB score and angle of inclination in SCI + ANI group were significantly lower than those in SCI group, which indicated ANI ANI increased degree of spinal cord injury in rats increase the degree of SCI in rats. Compared with SCI + EA group, the recovery capability of locomotor function in rats in SCI + AE + ANI group was significantly weakened, which might indicate the activation of JNK and p38 MAPK would significantly hinder the promotion of EA on the recovery of SCI.

Following SCI, various cellular and molecular events occur that induce glial activation in the spinal dorsal horn, such as the ionic imbalances, neuroinflammation, alterations of cell cycle proteins, and the release of glutamate, proinflammatory cytokines, ATP, reactive oxygen species (ROS) and neurotrophic factors [24]. It is well documented that activated microglia and astrocytes in the spinal cord contribute to inflammatory injury and neuron apoptosis through releasing a large amount of pro-inflammatory cytokines, and inhibition of the activation of spinal cord glial cells could effectively promote the functional recovery and relieve neuropathic pain after SCI [25, 26]. GFAP is the major protein constituent of glial intermediate filaments in differentiated fibrous and protoplasmic astrocytes of the central nervous system [27]. The mutation of GFAP could result in the dysfunction of astrocytes [28]. In the vertebrate central nervous system (CNS), astrocytes are the most abundant and functionally diverse glial cell population [29]. Astrocytes play very dynamic and interactive roles that are important for the normal functioning of the central nervous system [30]. The decrease of GFAP after SCI rats treating with EA indicated EA could effectively restrain the excessive proliferation of astrocytes. Ionized calcium-binding adapter molecule 1 (Iba-1) is a novel calcium-binding protein, and is specifically expressed in microglia in the brain [15]. It is involved in the Rac signalling pathway and the key molecule in microglial activation [31]. The decrease of Iba-1 in SCI rats after treating with EA indicated EA could effectively restrain the activation of microglial.

The inhibition effect of EA on the activation of microglia and astrocytes in spinal cord has been widely reported [32, 33], however the underlying mechanisms remain largely unknown. EA with different frequencies promotes the release of different kinds of neuropeptides [34, 35], which contribute to the presynaptic activation of opioid receptors on the primary afferent terminals. Presynaptic activation of opioid receptors leads to a direct reduction of  $Ca^{2+}$  conductance or indirect in  $K^{+}$  conductance, and in turn decreases release of transmitters such as excitatory amino acids and SP [36, 37]. All the substance P (SP), ATP, CGRP and glutamate can activate microglia and astrocytes through binding to receptors, such as NMDA, AMPA/KA, NK-1, ATP, and CGRP receptors. Thus, one of mechanisms of EA suppressing spinal glial activation might be to attenuate the release of some neurotransmitters/neuromodulators from primary afferent terminals and spinal nociceptive neurons via inactivation of endogenous opioid systems [33]. Another important mechanism for the inhibitory effect of EA on spinal glia activation might be related to the opioid receptors on glial cells. EA mediated activation of glial opioid receptors may suppress the activation of glial cells via modulating intracellular signaling pathway such as ERK/p38 MAPK cascades [38, 39]. Doo C. Choi et al. demonstrated that acupuncture inhibited microglia activation after SCI in rats through suppressing ROS-induced p38MAPK and ERK activation in microglia [40]. Whereas, Jee Y. Lee et al. reported acupuncture at Shuigou (GV26) and Yanglingquan (GB34) acupoints inhibited astrocyte activation through inactivation the JNK pathway in astrocytes at L4-L5 level of spinal cord after SCI in rats [26]. EA is a modified technique of acupuncture that utilizes electrical stimulation. The effect of EA on the activation of JNK and p38 MAPK has not been determined. In this work, we found during the recovery process of SCI, the activated JNK and p38 MAPK signaling pathways were significantly inhibited by EA. Moreover, simulation of the activation of JNK/p38 MAPK using JNK/p38 MAPK activator ANI significantly promoted the protein expression of GFAP and Iba-1, indicating that the JNK and p38 MAPK signaling pathways play an important role in the activation of astrocytes and microglia. However, double labeling with Abs for p-p38MAPK or p-JNK and for cell-type specific markers (NeuN for Neurons, CC1 for oligoden-

drocytes, Iba-1 for microglia, and GFAP for astrocytes) needed to be performed to identify the cell types expressing p-p38MAPK or p-JNK.

p38 MAPKs are a conserved subfamily of MAPKs involved in the response to stress found in eukaryotic cells from yeast to mammals [41]. They played a central role in mediating cellular response to environmental stress, growth factors and cytokines [42]. p38 MAPK pathway signaling is known to participate in cell proliferation, apoptosis, and differentiation, in a manner dependent on the cellular context [43]. Increases in the activated forms of ERK 1/2, p38 MAPK, and CREB are correlated with the expression of at-level mechanical allodynia following spinal cord injury [44]. JNKs are critical regulators of transcription [43]. The relative extent of JNK, p38, or ERK activation has been proposed to determine cell fate after injury [45]. p38 MAPK and JNK are preferentially activated by environmental stresses and are actively involved in various stress responses [46].

SCI is currently divided into primary and secondary injuries according to the disease progression and the occurrence mechanism. Primary injury is the instantaneous, irreversible physical damage injury of spinal cord caused by continuity destruction, fracture or dislocation of the spinal canal. The secondary injury is induced by a series of malignant biochemical reactions, including bleeding, edema, ischemia reperfusion, inflammatory reaction and active oxygen radicals, and is the main factor leading to neuronal death and loss of nerve function. The mechanism of secondary injury of SCI involves local microcirculation disturbance, nerve inflammation injury, reactive oxygen free radical damage, toxic action of excitatory amino acid, electrolyte imbalance and apoptosis of nerve cells. Among them, a series of immune inflammatory response induced nerve cell apoptosis is considered to be the key reason for secondary injury of spinal cord [47, 48]. Thus, inhibition of the apoptosis of spinal cord neurons may be the key to the successful treatment for SCI. In this work, we evaluated the nerve cell apoptosis after SCI through determination the protein levels of cleaved caspase-3 and 9. We found, the production of cleaved caspase-3 and 9 was significantly increased after SCI, suggesting SCI promoted the spinal nerve cell apoptosis in rats. EA treatments remark-

ably attenuated the SCI induced apoptosis, which was reversed by the ANI administration. These data demonstrated that JNK and p38 MAPK might be involved in the adverse effect of EA on SCI induced spinal cell apoptosis. However, more detailed researches need to be done to investigate the types of apoptotic cells, and the mechanisms underlying the inhibition effect of EA on apoptosis.

The present study showed that EA could effectively promote the recovery of SCI by inhibiting the activation of astrocytes and microglia in spinal cord through suppressing the activation of JNK and p38 MAPK signaling pathways. Moreover, EA also restrained SCI induced spinal cell apoptosis. However, immunohistochemical staining and immunofluorescence double labeling on spinal cord tissue were needed to further verify the results.

### Acknowledgements

This study was supported by Shanghai Cultivation Plan of New Stars in Xinglin (Grant No.: ZYSNXD011-RC-XLXX-20130046), Lu's Acupuncture Inheritance Study of Shanghai Schools of Traditional Chinese Medicine (Grant No.: ZYSNXD-CC-HPGC-JD-004) and Scientific Research Project of Chinese Medicine of Shanghai Health and Family Planning Committee (Grant No.: ZJ2016008 and 2014LP026B); Plan of action of Traditional Chinese Medicine of Shanghai for three years (Grant No.: ZY3-JSFC-1-1008) and Project of Shanghai Commission of Science and Technology (Grant No.: 16401933900).

### Disclosure of conflict of interest

None.

**Address correspondence to:** Dr. Yaochi Wu, Department of Acupuncture, The Sixth People's Hospital Affiliated to Shanghai Jiaotong University, 600# Yishan Road, Shanghai 200233, China. Tel: +86-21-24058567; Fax: +86-21-24058567; E-mail: yochi-wu@126.com

### References

- [1] Elliott TR and Rivera P. Spinal cord injury. Handbook of Psychology 2003.
- [2] Sekhon LH and Fehlings MG. Epidemiology, demographics, and pathophysiology of acute spinal cord injury. Spine 2001; 26: S2-S12.

## Electroacupuncture promotes locomotor recovery in SCI

- [3] Richards JS, Kewman DG and Pierce CA. Spinal cord injury. *Handbook of Rehabilitation Psychology* 2000; 11-27.
- [4] Sadowsky C, Volshteyn O, Schultz L and McDonald J. Spinal cord injury. *Disabil Rehabil* 2002; 24: 680-687.
- [5] McDonald JW and Sadowsky C. Spinal-cord injury. *Lancet* 2002; 359: 417-425.
- [6] Yan Q, Ruan JW, Ding Y, Li WJ, Li Y, Zeng YS. Electro-acupuncture promotes differentiation of mesenchymal stem cells, regeneration of nerve fibers and partial functional recovery after spinal cord injury. *Exp Toxicol Pathol* 2011; 63: 151-156.
- [7] Chen YY, Zhang W, Chen YL, Chen SJ, Dong H and Zeng YS. Electro-acupuncture improves survival and migration of transplanted neural stem cells in injured spinal cord in rats. *Acupunct Electrother Res* 2008; 33: 19-31.
- [8] Li WJ, Pan SQ, Zeng YS, Su BG, Li SM, Ding Y, Li Y and Ruan JW. Identification of acupuncture-specific proteins in the process of electro-acupuncture after spinal cord injury. *Neurosci Res* 2010; 67: 307-316.
- [9] Ding Y, Yan Q, Ruan JW, Zhang YQ, Li WJ, Zhang YJ, Li Y, Dong H and Zeng YS. Electro-acupuncture promotes survival, differentiation of the bone marrow mesenchymal stem cells as well as functional recovery in the spinal cord-transected rats. *BMC Neurosci* 2009; 10: 35.
- [10] Lee JY, Choi DC, Oh TH and Yune TY. Analgesic effect of acupuncture is mediated via inhibition of JNK activation in astrocytes after spinal cord injury. *PLoS One* 2013; 8: e73948.
- [11] Bernateck M, Becker M, Schwake C, Hoy L, Passie T, Parlesak A, Fischer MJ, Fink M and Karst M. Adjuvant auricular electroacupuncture and autogenic training in rheumatoid arthritis: a randomized controlled trial. *Forsch Komplementmed* 2008; 15: 187-193.
- [12] Rivlin AS and Tator CH. Objective clinical assessment of motor function after experimental spinal cord injury in the rat. *J Neurosurg* 1977; 47: 577-581.
- [13] Merkler D, Metz GA, Raineteau O, Dietz V, Schwab ME and Fouad K. Locomotor recovery in spinal cord-injured rats treated with an antibody neutralizing the myelin-associated neurite growth inhibitor Nogo-A. *J Neurosci* 2001; 21: 3665-3673.
- [14] Eng LF, Ghirnikar RS and Lee YL. Glial fibrillary acidic protein: GFAP-thirty-one years (1969-2000). *Neurochem Res* 2000; 25: 1439-1451.
- [15] Hwang IK, Yoo KY, Kim DW, Choi SY, Kang TC, Kim YS and Won MH. Ionized calcium-binding adapter molecule 1 immunoreactive cells change in the gerbil hippocampal CA1 region after ischemia/reperfusion. *Neurochem Res* 2006; 31: 957-965.
- [16] Chen N, Nomura M, She QB, Ma WY, Bode AM, Wang L, Flavell RA and Dong Z. Suppression of skin tumorigenesis in c-Jun NH2-terminal kinase-2-deficient mice. *Cancer Res* 2001; 61: 3908-3912.
- [17] Tong C, Yin Z, Song Z, Dockendorff A, Huang C, Mariadason J, Flavell RA, Davis RJ, Augenlicht LH and Yang W. c-Jun NH 2-terminal kinase 1 plays a critical role in intestinal homeostasis and tumor suppression. *Am J Pathol* 2007; 171: 297-303.
- [18] Wagner EF and Nebreda ÁR. Signal integration by JNK and p38 MAPK pathways in cancer development. *Nat Rev Cancer* 2009; 9: 537-549.
- [19] Proctor MR. Spinal cord injury. *Crit Care Med* 2002; 30 Suppl: S489-S499.
- [20] Bradbury EJ, Moon LD, Popat RJ, King VR, Bennett GS, Patel PN, Fawcett JW and McMahon SB. Chondroitinase ABC promotes functional recovery after spinal cord injury. *Nature* 2002; 416: 636-640.
- [21] Thuret S, Moon LD and Gage FH. Therapeutic interventions after spinal cord injury. *Nat Rev Neurosci* 2006; 7: 628-643.
- [22] Flood JF, Smith GE, Bennett EL, Alberti MH, Orme AE and Jarvik ME. Neurochemical and behavioral effects of catecholamine and protein synthesis inhibitors in mice. *Pharmacol Biochem Behav* 1986; 24: 631-645.
- [23] Wong AM, Leong CP, Su TY, Yu SW, Tsai WC and Chen CP. Clinical trial of acupuncture for patients with spinal cord injuries. *Am J Phys Med Rehabil* 2003; 82: 21-27.
- [24] Gwak YS, Kang J, Unabia GC and Hulsebosch CE. Spatial and temporal activation of spinal glial cells: role of gliopathy in central neuropathic pain following spinal cord injury in rats. *Exp Neurol* 2012; 234: 362-372.
- [25] Lee SM, Yune TY, Kim SJ, Park DW, Lee YK, Kim YC, Oh YJ, Markelonis GJ and Oh TH. Minocycline reduces cell death and improves functional recovery after traumatic spinal cord injury in the rat. *J Neurotrauma* 2003; 20: 1017-1027.
- [26] Lee JY, Choi DC, Oh TH and Yune TY. Analgesic effect of acupuncture is mediated via inhibition of JNK activation in astrocytes after spinal cord injury. *PLoS One* 2013; 8: e73948.
- [27] Anděřová M, Kubinová Š, Mazeř T, Chvátal A, Eliasson C, Pekny M and Syková E. Effect of elevated K<sup>+</sup>, hypotonic stress, and cortical spreading depression on astrocyte swelling in GFAP-deficient mice. *Glia* 2001; 35: 189-203.
- [28] Wang Y, Cheng X, He Q, Zheng Y, Kim DH, Whitemore SR and Cao QL. Astrocytes from the contused spinal cord inhibit oligodendrocyte differentiation of adult oligodendrocyte precursor cells by increasing the expression of bone morphogenetic proteins. *J Neurosci* 2011; 31: 6053-6058.

## Electroacupuncture promotes locomotor recovery in SCI

- [29] Agius E, Decker Y, Soukkaieh C, Soula C and Cochard P. Role of BMPs in controlling the spatial and temporal origin of GFAP astrocytes in the embryonic spinal cord. *Dev Biol* 2010; 344: 611-620.
- [30] Porter JT and McCarthy KD. GFAP-positive hippocampal astrocytes in situ respond to glutamatergic neuroligands with increases in [Ca<sup>2+</sup>]<sub>i</sub>. *Glia* 1995; 13: 101-112.
- [31] Ehninger D and Kempermann G. Regional effects of wheel running and environmental enrichment on cell genesis and microglia proliferation in the adult murine neocortex. *Cereb Cortex* 2003; 13: 845-851.
- [32] Sun S, Qi-Liang MY, Cao H, Li T, Han M, Pan H, Wang YQ, Zhao ZQ and Zhang YQ. Is functional state of spinal microglia involved in the anti-allodynic and anti-hyperalgesic effects of electroacupuncture in rat model of monoarthritis? *Neurobiol Dis* 2007; 26: 558-568.
- [33] Sun S, Cao H, Han M, Li TT, Zhao ZQ and Zhang YQ. Evidence for suppression of electroacupuncture on spinal glial activation and behavioral hypersensitivity in a rat model of monoarthritis. *Brain Res Bull* 2008; 75: 83-93.
- [34] Sluka KA, Deacon M, Stibal A, Strissel S and Terpstra A. Spinal Blockade of Opioid Receptors Prevents the Analgesia Produced by TENS in Arthritic Rats. *J Pharmacol Exp Ther* 1999; 289: 840-846.
- [35] Zhang SP, Zhang JS, Yung KK and Zhang HQ. Non-opioid-dependent anti-inflammatory effects of low frequency electroacupuncture. *Brain Res Bull* 2004; 62: 327-334.
- [36] Dickenson AH and Sullivan AF. Electrophysiological studies on the effects of intrathecal morphine on nociceptive neurones in the rat dorsal horn. *Pain* 1986; 24: 211-222.
- [37] Malmberg AB and Yaksh TL. The effect of morphine on formalin-evoked behaviour and spinal release of excitatory amino acids and prostaglandin E. *Br J Pharmacol* 1995; 114: 1069-1075.
- [38] Schulz R, Eisinger DA and Wehmeyer A. Opioid control of MAP kinase cascade. *Eur J Pharmacol* 2004; 500: 487-497.
- [39] Belcheva MM, Clark AL, Haas PD, Serna JS, Hahn JW, Kiss A and Coscia CJ. Mu and kappa opioid receptors activate ERK/MAPK via different protein kinase C isoforms and secondary messengers in astrocytes. *J Biol Chem* 2005; 280: 27662-27669.
- [40] Choi DC, Lee JY, Lim EJ, Baik HH, Oh TH and Yune TY. Inhibition of ROS-induced p38MAPK and ERK activation in microglia by acupuncture relieves neuropathic pain after spinal cord injury in rats. *Exp Neurol* 2012; 236: 268-282.
- [41] Martín-Blanco E. p38 MAPK signalling cascades: ancient roles and new functions. *Bioessays* 2000; 22: 637-645.
- [42] Recio JA and Merlino G. Hepatocyte growth factor/scatter factor activates proliferation in melanoma cells through p38 MAPK, ATF-2 and cyclin D1. *Oncogene* 2002; 21: 1000-1008.
- [43] Pramanik R, Qi X, Borowicz S, Choubey D, Schultz RM, Han J and Chen G. p38 Isoforms have opposite effects on AP-1-dependent transcription through regulation of c-Jun the determinant role of the isoforms in the p38 MAPK signal specificity. *J Biol Chem* 2003; 278: 4831-4839.
- [44] Crown ED, Ye Z, Johnson KM, Xu GY, McAdoo DJ and Hulsebosch CE. Increases in the activated forms of ERK 1/2, p38 MAPK, and CREB are correlated with the expression of at-level mechanical allodynia following spinal cord injury. *Exp Neurol* 2006; 199: 397-407.
- [45] Park KM, Chen A and Bonventre JV. Prevention of kidney ischemia/reperfusion-induced functional injury and JNK, p38, and MAPK kinase activation by remote ischemic pretreatment. *J Biol Chem* 2001; 276: 11870-11876.
- [46] Ito K, Hirao A, Arai F, Takubo K, Matsuoka S, Miyamoto K, Ohmura M, Naka K, Hosokawa K and Ikeda Y. Reactive oxygen species act through p38 MAPK to limit the lifespan of hematopoietic stem cells. *Nat Med* 2006; 12: 446-451.
- [47] Byrnes KR, Stoica BA, Fricke S, Di GS and Faden AI. Cell cycle activation contributes to post-mitotic cell death and secondary damage after spinal cord injury. *Brain* 2007; 130: 2977-2992.
- [48] Esposito E, Mazzon E, Paterniti I, Impellizzeri D, Bramanti P, Cuzzocrea S. Olprinone attenuates the acute inflammatory response and apoptosis after spinal cord trauma in mice. *PLoS One* 2010; 5: e12170.