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# Molecular mechanisms underlying the effects of acupuncture on neuropathic pain

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## Research Highlights

(1) Previous studies of the mechanisms underlying acupuncture analgesia mainly focused on stimulating the release of endogenous opioid peptides, on adjusting the levels of pain-related neurotransmitter, and on neuropeptide levels. Chronic neuropathic pain not only refers to allodynia, but also the recovery of damaged nerves.

(2) Ephrin-B/EphB signaling participates in the modulation of neural development, synaptic plasticity or pain. However, few studies have addressed whether ephrin-Bs/EphBs are involved in the effects of acupuncture on neuropathic pain.

(3) This study investigated the expression of ephrin-Bs/EphBs in the spinal dorsal horn using rat models of neuropathic pain, and first verified that acupuncture for neuropathic pain and the recovery of neurological function are associated with the activation of ephrin-B/EphB signaling, indicating a potential mechanism of action for acupuncture in improving pain and repairing injured nerves.

## Abstract

Acupuncture has been used to treat neuropathic pain for a long time, but its mechanisms of action remain unknown. In this study, we observed the effects of electroacupuncture and manual acupuncture on neuropathic pain and on ephrin-B/EphB signaling in rats models of chronic constriction injury-induced neuropathic pain. The results showed that manual acupuncture and electroacupuncture significantly reduced mechanical hypersensitivity following chronic constriction injury, especially electroacupuncture treatment. Real-time PCR results revealed that ephrin-B1/B3 and EphB1/B2 mRNA expression levels were significantly increased in the spinal dorsal horns of chronic constriction injury rats. Electroacupuncture and manual acupuncture suppressed the high sion of ephrin-B1 mRNA, and elevated EphB3/B4 mRNA expression. Electroacupuncture significantly enhanced the mRNA expression of ephrin-B3 and EphB3/B6 in the dorsal horns of neuropathic pain rats. Western blot results revealed that electroacupuncture in particular, and manual acupuncture, significantly up-regulated ephrin-B3 protein levels in rat spinal dorsal horns. The results of this study suggest that acupuncture could activate ephrin-B/EphB signaling in neuropathic pain rats and improve neurological function.

## Key Words

neural regeneration; acupuncture; neuropathic pain; chronic constriction injury; electroacupuncture; spinal dorsal horn; ephrin-B/EphB signaling; nerve repair; grants-supported paper; neuroregeneration

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## INTRODUCTION

Peripheral nerve injury often leads to neuropathic pain, a chronic condition that can manifest behaviorally as spontaneous pain, hyperalgesia and allodynia<sup>[1-3]</sup>, and which also results in neurological dysfunction<sup>[4-6]</sup>. Management of neuropathic pain in patients still constitutes a remarkable therapeutic challenge owing to the modest and variable efficacy as well as the side effects of drugs<sup>[7-9]</sup>. Therefore, investigations into effective therapies for the treatment of neuropathic pain, with fewer adverse effects, are highly relevant. Acupuncture has been widely used to alleviate pain in human subjects and experimental animals with neuropathic pain<sup>[10-12]</sup>, which mainly involves spinal opioid, adrenergic, dopaminergic, serotonergic, and cholinergic receptors<sup>[13-17]</sup>. In addition, acupuncture has been used to relieve neurological dysfunction in neurodegenerative disorders and induces functional improvement following central nervous system injuries<sup>[18-20]</sup>. However, the molecular mechanisms underlying the effects of acupuncture treatment on peripheral nerve injury remain unknown.

Eph receptor tyrosine kinases and their membrane-bound ligands, ephrins, are involved in a variety of biological processes, such as tissue patterning, angiogenesis, and cardiovascular and skeletal development<sup>[21-23]</sup>. There are three ephrin-Bs (ephrin-B1–3) and five EphBs (EphB1–4 and EphB6). The interactions of ephrin-Bs/EphBs are well known to lead to bidirectional signals, forward and reverse signaling, and play a critical role in modulating multiple aspects of physiology and pathology in the central nervous system<sup>[24-26]</sup>. On the one hand, ephrin-B/EphB signaling can modulate synaptic efficacy in the spinal cord, contributing to sensory abnormalities in persistent pain conditions. Recent studies have demonstrated that activation of spinal ephrin-B1–2/ EphB1 signaling plays a critical role in the development and maintenance of chronic pain after peripheral nerve injury, cancer pain<sup>[27-29]</sup>. On the other hand, ephrin-B/EphB signaling actively participates in various aspects of neuronal

development, such as neuronal migration, axon guidance and synaptic plasticity<sup>[26, 30-31]</sup>. Furthermore, ephrin-B/EphB signaling also plays significant roles in key aspects of the nervous system's response to damage, and is involved in axon regeneration, cellular remodeling and scar formation<sup>[32-34]</sup>. These studies indicated that ephrin-B/EphB signaling may be involved in pain modulation and functional recovery at the spinal cord level after peripheral nerve injury. We hypothesized that ephrin-B/EphB signaling in the spinal cord involved in acupuncture effectively attenuates pain and ameliorates functional outcomes after peripheral nerve injury.

Manual acupuncture and electroacupuncture are frequently used in clinical practice, and both have shown therapeutic effects on neuropathic pain and functional improvement after neural injuries in pre-clinical and clinical studies<sup>[13, 35-38]</sup>. The acupoints *Zusanli* (ST36) and *Sanyinjiao* (SP6) are often used to modulate neural functions and to enhance the immune system, resulting in alleviation of pain and improvement of neurological disorders<sup>[39-41]</sup>. The present study aimed to explore whether neuropathic pain affects the expression of ephrin-Bs/EphBs in the spinal dorsal horn of chronic constriction injury rat models of neuropathic pain, and then examined whether manual acupuncture and electroacupuncture could interfere with the neuropathic pain by modulating ephrin-B/EphB expression.

## RESULTS

### Quantitative analysis of experimental animals

A total of 37 male Sprague-Dawley rats were used in the experiment to establish chronic constriction injury models. Chronic constriction injury-operated rats were divided into three groups: (1) chronic constriction injury ( $n = 9$ ), (2) chronic constriction injury + electroacupuncture treatment ( $n = 9$ ), and (3) chronic constriction injury + manual acupuncture treatment ( $n = 10$ ). Sham-operated rats were used as controls

( $n = 9$ ). All rats were involved in the final analysis.

**Effects of electroacupuncture and manual acupuncture on mechanical hypersensitivity of chronic constriction injury rats**

As shown in Figure 1A, the basal paw withdrawal threshold was similar for all groups before surgery. Compared with control rats, chronic constriction injury rats displayed a profound decrease in ipsilateral paw withdrawal threshold in a time-dependent manner, reaching the lowest level at day 9 after chronic constriction injury of the sciatic nerve. No significant change was observed among chronic constriction injury, electroacupuncture and manual acupuncture groups at day 7 post-injury. The paw withdrawal threshold in the contralateral hind paw of all the rats was not significantly different from the pre-lesion baseline value at all time points (data not shown). The paw withdrawal threshold in the ipsilateral hind paw of chronic constriction injury group without treatments remained significantly lower than that in the control group at various time points measured after chronic constriction injury of the sciatic nerve ( $P < 0.01$ ).

Electroacupuncture and manual acupuncture stimulations were performed every other day from day 8. One day after the first treatments (day 9), hypersensitivity remained unchanged. However, the paw withdrawal threshold in the ipsilateral hind paw increased gradually following the electroacupuncture and manual acupuncture treatments. At day 17, mechanical allodynia was significantly alleviated in the electroacupuncture and manual acupuncture groups compared with the chronic constriction injury group ( $P < 0.01$ ), and this trend lasted until the end of the experiment (Figure 1A). On the other hand, the electroacupuncture stimulation showed a better tendency to improve the paw withdrawal threshold than manual acupuncture stimulation (Figure 1A). The area under the curve for the paw withdrawal threshold changes over time (Figure 1B) also indicated that electroacupuncture treatment had a better overall effect than manual acupuncture treatment ( $P < 0.05$ ).

**Effects of electroacupuncture and manual acupuncture on mRNA expression of ephrin-Bs and EphBs in the spinal dorsal horns of chronic constriction injury rats**

Real-time PCR analyses were used to investigate whether the mRNA expression levels of ephrin-Bs (ephrin-B1, ephrin-B2, and ephrin-B3) and EphBs (EphB1, EphB2, EphB3, EphB4, and EphB6) in the spinal dorsal horn changed under chronic constriction injury-induced

neuropathic injury and after electroacupuncture and manual acupuncture treatments. Ephrin-B1, ephrin-B3, EphB1, and EphB2 mRNA levels in the spinal dorsal horn were significantly enhanced after chronic constriction injury-induced neuropathic injury ( $P < 0.05$  to  $P < 0.01$ ) (Figure 2). Furthermore, this upward trend of these genes was not inhibited by electroacupuncture and manual acupuncture treatments except for ephrin-B1, whose mRNA levels were not different between the electroacupuncture and control groups. In contrast, the mRNA levels of EphB3 and EphB4 were significantly up-regulated after electroacupuncture and manual acupuncture treatments ( $P < 0.01$ ; Figure 2). Specifically, the mRNA levels for EphB3, EphB6 and ephrin-B3 were dramatically increased in the spinal dorsal horn after electroacupuncture stimulation compared with the control, chronic constriction injury and manual acupuncture groups.

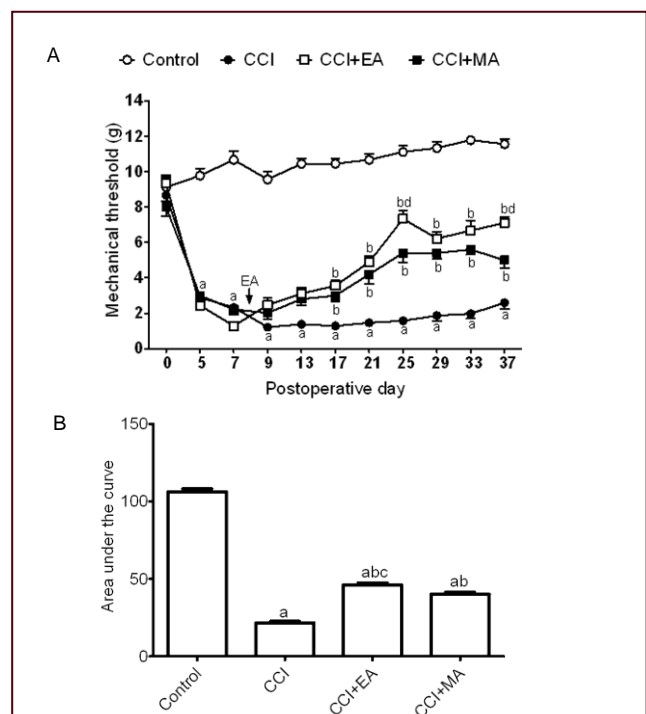


Figure 1 Effect of electroacupuncture (EA) and manual acupuncture (MA) stimulation on mechanical hypersensitivity induced by chronic constriction injury (CCI) in rats.

(A) Mechanical threshold of rats at different time points measured by the hind paw withdrawal response to von Frey hair stimulation.

(B) Area under the curve of graph A (from day 0 to day 37).

The data are expressed as mean  $\pm$  SEM. There are nine rats in each group, except for the CCI + MA treatment group, which contains 10 rats. <sup>a</sup> $P < 0.01$ , vs. control group; <sup>b</sup> $P < 0.01$ , vs. CCI group; <sup>c</sup> $P < 0.05$ , <sup>d</sup> $P < 0.01$ , vs. CCI + MA group. Two-way analysis of variance followed by Bonferroni's *post hoc* test (A) or one-way analysis of variance followed by Bonferroni's *post hoc* test (B) was used.

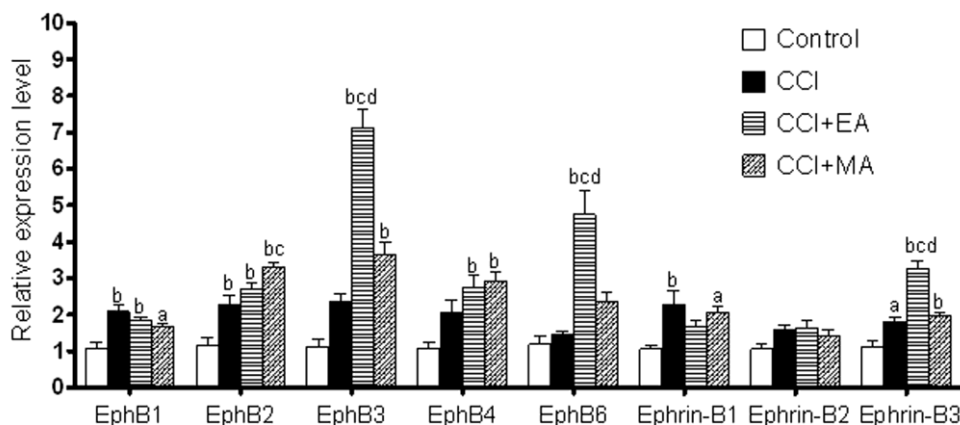


Figure 2 Effects of electroacupuncture (EA) and manual acupuncture (MA) on ephrin-B and EphB gene expression in the spinal dorsal horns of chronic constriction injury (CCI) rats.

The mRNA levels were detected by real-time PCR, and the data (fold change in target gene relative to control) are expressed as mean ± SEM of eight rats in each group. <sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.01, vs. control group; <sup>c</sup>*P* < 0.01, vs. CCI group; <sup>d</sup>*P* < 0.01, vs. CCI + MA group. One-way analysis of variance, followed by Bonferroni's *post hoc* test was performed.

### Effects of electroacupuncture and manual acupuncture on the expression of ephrin-B3 protein in the spinal dorsal horn of chronic constriction injury rats

Because ephrin-B3 was the only ephrin-B family member that was significantly up-regulated at the mRNA level in the spinal dorsal horn region after electroacupuncture treatment (Figure 2), and because electroacupuncture treatment had a better therapeutic effect in chronic constriction injury rats than manual acupuncture treatment (Figure 1), we focused on the expression of this protein after electroacupuncture and manual acupuncture treatments. Inconsistent with the mRNA results (Figure 2), chronic constriction injury-induced neuropathic injury did not significantly increase ephrin-B3 protein levels in the spinal dorsal horn (Figure 3). However, the absorbance of the bands for ephrin-B3 protein on western blots of samples from the electroacupuncture and manual acupuncture groups was significantly higher than that in western blots of samples from the control and chronic constriction injury groups (*P* < 0.05 or *P* < 0.01; Figure 3). Furthermore, the ephrin-B3 protein level was more significantly increased in the electroacupuncture group than in the manual acupuncture group (*P* < 0.05; Figure 3).

## DISCUSSION

In the present study, we investigated whether repeated long-term electroacupuncture and manual acupuncture treatments could improve the chronic constriction in-

jury-induced neuropathic injury in rats. We also tested whether the electroacupuncture- and manual acupuncture-induced therapeutic effect involved spinal ephrin-B/EphB signaling modulation.

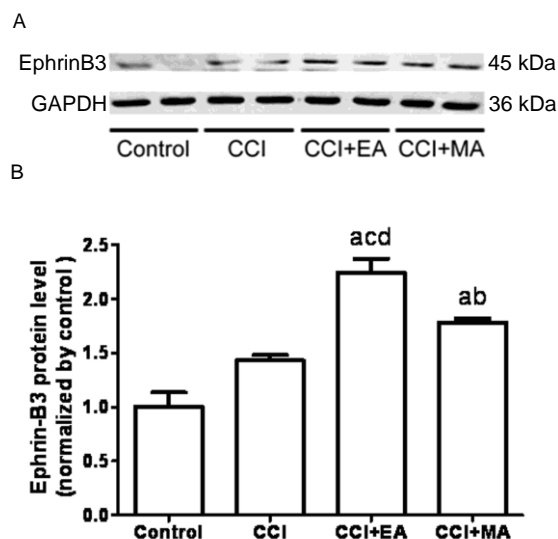


Figure 3 Effects of electroacupuncture (EA) and manual acupuncture (MA) on the expression of ephrin-B3 protein in the spinal dorsal horns of chronic constriction injury (CCI) rats.

(A) Electrophoresis results.

(B) Quantitative analysis of the expression of ephrin-B3 protein.

Data (absorbance ratio to GAPDH) are expressed as mean ± SEM of four rats in each group. <sup>a</sup>*P* < 0.01, vs. control group; <sup>b</sup>*P* < 0.05, <sup>c</sup>*P* < 0.01, vs. CCI group; <sup>d</sup>*P* < 0.01, vs. CCI + MA group. One-way analysis of variance, followed by Bonferroni's *post hoc* test was performed.

Although repeated electroacupuncture and manual acupuncture treatments did not totally resolve the hypersensitivity caused by chronic constriction injury within the time frame of 37 days for this study, they significantly improved mechanical thresholds compared with chronic constriction injury groups at day 6 after the first treatment. Repeated electroacupuncture and manual acupuncture up-regulated mRNA levels for EphB3, EphB6 and ephrin-B3 in the spinal dorsal horn. Furthermore, ephrin-B3 protein levels were significantly increased after electroacupuncture and manual acupuncture, and positively correlated with the therapeutic effects of acupuncture treatments. This result revealed that long-term acupuncture treatment at bilateral *Zusanli* and *Sanyinjiao* can activate spinal ephrin-B/EphB signaling to alleviate mechanical hypersensitivity and improve neural function.

Nowadays, electroacupuncture and manual acupuncture treatments are extensively used to alleviate neuropathic pain and improve nervous function in the clinic<sup>[42-45]</sup>. In manual acupuncture treatment, the acupuncture needle is inserted into the acupoint and twisted up and down by hand until a feeling of “*Deqi*”, which is described as a sensation of numbness, soreness or heaviness, reflecting the activation of afferent nerve fibers, is experienced<sup>[46]</sup>. Traditional acupuncturists adjust the acupuncture manipulation to achieve therapeutic purposes according to the conditions of patients<sup>[47]</sup>. In electroacupuncture treatment, a stimulating current is delivered to acupoints *via* the needles using an electrical stimulator. Compared with manual acupuncture stimulation, electroacupuncture stimulation is easy to control and accurately reproduce. Little is known about the different therapeutic benefits of electroacupuncture and manual acupuncture treatments. Several studies have found that electroacupuncture stimulation produces greater pressure pain detection threshold elevations and is more effective in some aspects in regulating disturbed estrous cyclicity compared with manual acupuncture stimulation<sup>[48-49]</sup>. However, another study showed that there was no difference in alleviating headache, trigeminal neuralgia, and retro-auricular pain between electroacupuncture and manual acupuncture<sup>[44]</sup>. The present study demonstrated that electroacupuncture treatment was superior to manual acupuncture treatment in terms of improvements after chronic constriction injury and an overall analgesic effect. However, we have to point out that this result does not suggest that electroacupuncture had a better therapeutic effect than manual acupuncture. In

electroacupuncture stimulation, acupoints were stimulated by a continued stimulus and the current intensity was gradually increased during treatment. Based on a previously described method<sup>[36]</sup>, the needles were only operated for 15 seconds every 10 minutes in the manual acupuncture stimulation used in this study. Obviously, the intensity of stimulus in the electroacupuncture treatment was stronger than the intensity in the manual acupuncture treatment. Previous studies have shown that acupuncture with optimal high-intensity stimulation is more effective than treatments with less stimulation, such as minimal acupuncture<sup>[50-51]</sup>. The results from this study confirmed that the intensity of acupuncture plays an important role in improving neuropathic injury and pain.

Ephrin-B/EphB signaling is an important signaling pathway for pain modulation and is involved in neuronal development and the response to injury<sup>[26, 28, 32]</sup>. After peripheral nerve injury, the levels of ephrin-B1 and EphB1 proteins were significantly up-regulated in the spinal dorsal horn<sup>[52-53]</sup>. Furthermore, ephrin-B1-Fc could produce a dose- and time-dependent thermal and mechanical hyperalgesia *via* intrathecal injection, accompanied by an increase in the levels of spinal phosphorylated mitogen-activated protein kinases and c-Fos<sup>[28]</sup>. The mitogen-activated protein kinases, including p38, extracellular signal-regulated kinase, and c-Jun N-terminal kinase, are a family of serine/threonine protein kinases that transduce extracellular stimuli into intracellular post-translational and transcriptional responses. Their activation is involved in the modulation of nociceptive information and peripheral and central sensitization produced by intense noxious stimuli<sup>[54-58]</sup>. Activation of ephrin-B2/EphB1 signaling in the spinal dorsal horn and primary sensory neurons activates astrocytes and microglial cells and up-regulates the level of phosphorylation of NR1 and NR2B receptors, and up-regulates the phosphorylation level of Src within the N-methyl-D-aspartate receptor complex, and increases the activity of matrix metalloproteinase-2/9 and subsequent Ca<sup>2+</sup>-dependent signals, all of which result in pain behaviors<sup>[27]</sup>. Intrathecal application of blocking reagents, including EphB1-Fc, EphB2-Fc and ephrin-B1-Fc, could relieve nerve injury-induced pain<sup>[52, 59]</sup>. In the current study, real-time PCR data also demonstrated significant overexpression of EphB1, EphB2 and ephrin-B1 mRNA levels after chronic constriction injury in the spinal dorsal horn. However, previous studies have shown that acupuncture treatment modulates the expression and phosphorylation of spinal N-methyl-D-aspartate receptor subunits and inhibits p38MAPK and extracellular sig-

nal-regulated kinase activation in the spinal dorsal horn to attenuate mechanical allodynia in a neuropathic pain model<sup>[60-64]</sup>. Although the up-regulation of these three genes seemed not to be inhibited by acupuncture stimulations, this does not show that acupuncture for the treatment of neuralgia does not exert effects on ephrin-B1–EphB1/B2 signaling, which should be determined in further neuropharmacological and behavioral tests.

Ephrin-B3, a member of the ephrin-B gene family, plays an important role in brain development and repair after nervous system injury<sup>[65-66]</sup>. Ephrin-B3 and EphB1 cooperatively regulate the proliferation and migration of neural progenitors in the hippocampus, suggesting that they may act as candidate targets for modulating the production and integration of new neurons to treat neurodegenerative diseases or brain injury<sup>[67]</sup>. A trans-synaptic interaction between ephrin-B3 and EphB2 regulates synapse density and the formation of dendritic spines by inhibition of postsynaptic Ras/mitogen-activated protein kinase signaling<sup>[68]</sup>. Ephrin-B3 is expressed in the injured mouse optic nerve where it recruits macrophages, which express EphB3, to promote sprouting of damaged retinal axons<sup>[69]</sup>. EphA4 can initiate cell death in the absence of ephrin-B3. However, infusion of soluble ephrin-B3 reduced cell death in the germinal zones<sup>[70]</sup>. The present study showed that only the ephrin-B3 mRNA level was significantly increased in the spinal dorsal horns of neuropathic injury rats after electroacupuncture treatment among three ephrin-Bs. Moreover, electroacupuncture and manual acupuncture increased the ephrin-B3 protein level in the spinal dorsal horn, especially after electroacupuncture stimulation, consistent with the alleviation of neuropathic hypersensitivity after acupuncture in the present study. Taken together, our findings provide new information that acupuncture increases the expression and synthesis of ephrin-B3 in the spinal dorsal horn, which subsequently promotes the restoration of injured neural function and finally results in neuropathic pain alleviation. Nevertheless, the underlying mechanism requires further investigation.

In summary, the present study shows for the first time that the effects of acupuncture on neuropathic injury and nociception are associated with an increase in the levels of ephrin-B3 in the spinal dorsal horns of chronic constriction injury rats. This new information may improve our understanding of the mechanisms underlying the effects of acupuncture on neuropathic injury and pain.

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## MATERIALS AND METHODS

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### Design

A randomized, controlled animal experiment.

### Time and setting

This study was performed at the Department of Acupuncture, Shanghai University of Traditional Chinese Medicine and at the Research & Development Department, National Engineering Research Center for Biochip Technology, Shanghai, China, from March 2011 to September 2012.

### Materials

A total of 37 male Sprague-Dawley rats were obtained from the Experimental Animal Center, Shanghai University of Traditional Chinese Medicine in China (License No. SCXK (Hu) 2007-0005), aged 3–4 months, weighing 180–220 g. Animals were housed under a 12-hour light/dark cycle, and allowed free access to food and water. The room temperature was maintained at  $23 \pm 1^\circ\text{C}$  and relative humidity at 40–60%. Rats were acclimatized to the environment for 7 days prior to experiment. All procedures were in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China<sup>[71]</sup>.

### Methods

#### ***Animal model of chronic constriction injury of the sciatic nerve***

Rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (40 mg/kg). Chronic constriction injury of the sciatic nerve was induced as previously described<sup>[72]</sup>. The biceps femoris and the gluteus superficialis were separated by blunt dissection, and the left sciatic nerve was exposed at the mid-thigh level. Proximal to the trifurcation, 10 mm of nerve was carefully freed from the adhering tissue. The ligatures (4-0 silk) were tied loosely around the nerve with 1 mm spacing, until they elicited a brief twitch in the respective hindlimb, but did not interrupt the epineurial circulation. The skin was sewed up with silk sutures (4-0) and the rats were left to recover in heated cages. Sham-operated rats underwent exposure of the left sciatic nerve without ligation.

#### ***Application of acupuncture***

Electroacupuncture or manual acupuncture was given to the respective groups of rats once every other day from days 8 to 36 after nerve injury. The rats were placed in well-ventilated Plexiglas restraint barrels with the hind limb exposed for needling. Stainless-steel needles (Huatuo acupuncture needle, Suzhou Medical Appliance Factory, Suzhou, Jiangsu Province, China), 0.3 mm in

diameter and 3 mm in length, were bilaterally inserted into the hind legs, one at *Zusanli* and the other at *Sanyinjiao*. For electroacupuncture stimulation<sup>[39]</sup>, constant-current square-wave electrical stimulation generated by a programmed pulse generator (LH200, Beijing Huawei Industrial Developing Company, Beijing, China) was given *via* the two needles for 30 minutes. The frequency was set at 15 Hz and the intensity of stimulation was increased stepwise from 0.5 to 1.0 and then 1.5 mA, with each step lasting for 10 minutes. For manual acupuncture stimulation<sup>[36]</sup>, a stainless-steel needle was inserted to a depth of 0.3 mm and rotated at a rate of two spins per second for 15 seconds in each acupoint's direction, for a total of 30 seconds of needle rotation. Subsequently, the animals were allowed to rest with the needles still inserted for an additional 30 minutes.

**Behavioral test for assessment of mechanical thresholds**

Evaluations were conducted the day after acupuncture. Mechanical allodynia was measured based on the hind paw withdrawal response to von Frey hair stimulation as previously described<sup>[73-74]</sup>. A series of nine calibrated von Frey hairs were applied to the central region of the plantar surface of one hind paw in ascending order (1, 1.4, 2, 4, 6, 8, 10, 15, and 26 g). The hair was applied only when the rat was stationary and standing on all four paws. The stimulation was sustained for 2 seconds. A withdrawal response was considered valid only if the hind paw was completely removed from the customized platform. Each hair was applied five times at 5-second intervals. If a withdrawal response was not induced more than twice during five applications of a hair, the next ascending hair in the series was applied in a similar manner. Once the hind paw was withdrawn from a particular hair in three out of the five consecutive applications, the rat was considered responsive to that hair. The next descending hair was applied until the hind paw was withdrawn less than three out of the five times. The paw withdrawal threshold was defined as the lowest hair force in grams that produced at least three withdrawal responses in five tests.

**Quantitative real-time PCR**

Rats were deeply anesthetized with sodium pentobarbital (60 mg/kg, intraperitoneally) and the lumbar enlargements of the spinal dorsal horns of rats were quickly removed, frozen immediately in liquid nitrogen, and stored at -80°C for further use.

Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA). The RNA concentration and purity were analyzed using a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA), with the spectral absorption at 260 and 280 nm. RNA quality was assessed using a Lab-on-chip Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA, USA). For cDNA synthesis, oligo(dT) primers, 1 µg of each total RNA sample, and the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Burlington, CA, USA) were used, following the guidelines of the manufacturer. Real-time PCR was conducted in duplicate with GAPDH as an internal control using the Prism 7900 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) and SYBR® Premix Ex Taq™ II (Takara, Dalian, China). The primer sequences are listed in Table 1. Cycling conditions were as follows: 95°C for 10 minutes, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. After cycling, a melting protocol was performed for 15 seconds at 95°C, 1 minute at 60°C, and 15 seconds at 95°C, to control for product specificity. The fold change in target gene cDNA level relative to GAPDH was determined as follows: fold change = 2<sup>-ΔΔCt</sup>, where ΔΔCt = (Ct<sub>Target</sub> - Ct<sub>Control</sub>) test - (Ct<sub>Target</sub> - Ct<sub>Control</sub>) control. Ct values were defined as the number of the PCR cycles at which fluorescence signals were detected.

**Western blot assay**

The spinal dorsal horn was homogenized in cold lysis buffer (Beyotime, Haimen, Jiangsu Province, China) and centrifuged at 13 200 × g for 15 minutes at 4°C. The amount of total protein in the supernatant was quantified using an Enhanced BCA Protein Assay Kit (Beyotime). Samples (20 µg total protein per loading) were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel and electrotransferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA).

Table 1 Sequences of primers used for quantitative real-time PCR

Symbol	GenBank	Forward primer (5'-3')	Reverse primer (5'-3')	Product length (bp)
EphB1	NM_001104528	CGT GCT TGT TCG TGA CAG AT	CAT ATT CCA GCC CCT TGA GA	84
EphB2	NM_001127319	GTT CCC TGG ACT CCT TCC TC	GTT CAT GTC CGC CAG GTA CT	110
EphB3	NM_001105868	CCT ACC CTT GGT GCT GTC AT	CCC GTC TTT CAC ACA CAC AC	144
EphB4	XM_001069453	ACC AAC CCA GCA GTT CTG AC	AAG TGC AAT CCA ATG GGA AG	122
EphB6	NM_001107857	GTT GTG TGC AAG GAA TGT GG	AAG GAA CTT CAT GGC TGG TG	247
ephrin-B1	NM_017089	GGC AAG CAT GAG ACT GTG AA	GCT GTG TAT GCT TGC GAT GT	205
ephrin-B2	NM_001107328	GCT CAA GTA CCG CAG GAG AC	CGT AGT GTG GGC AGA AGA CA	167
ephrin-B3	NM_001100980	ACG GTC TGT CTT TTG GTT GG	GCA CAG GAA GGA CAA GAA GC	169
GAPDH	NM_017008	TCC TGC ACC ACC AAC TGC TTA G	AGT GGC AGT GAT GGC ATG GAC T	102

The membranes were blocked with 5% non-fat milk overnight at 4°C, and then incubated with primary antibodies recognizing GAPDH (mouse anti-rat monoclonal, 1:5 000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or ephrin-B3 (rabbit polyclonal, 1:1 000; Abcam, Cambridge, UK) for 2 hours at 22°C. The membranes were incubated with a horseradish peroxidase-conjugated goat anti-mouse IgG (1:5 000; Santa Cruz Biotechnology) or goat anti-rabbit IgG (1:5 000; Santa Cruz Biotechnology) for 2 hours at 22°C. The signal was visualized using Enhanced Chemiluminescence Plus reagent (GE Healthcare, Buckinghamshire, UK) and detected using a Clinx Chemiscope Mini Series Western Blot Imaging System (CLiNX Science Instruments, Shanghai, China). For semiquantitative analysis of ephrin-B3, the integrated absorbance of each band was measured using Image-Pro Plus (Meyer Instruments, Houston, TX, USA). The integrated absorbance was normalized to that of GAPDH and expressed as arbitrary units.

### Statistical analysis

Data are presented as mean  $\pm$  SEM. Differences in paw withdrawal latency were assessed using two-way analysis of variance with repeated measures in GraphPad Prism Version 5.0 (GraphPad, La Jolla, CA, USA). The Bonferroni's *post hoc* test was used to compare data between two groups. For the area under the curve of the paw withdrawal threshold changes over time, comparisons between groups were performed using one-way analysis of variance followed by the Bonferroni's *post hoc* test in GraphPad Prism Version 5.0. For real-time PCR and western blot assays, comparisons between groups were performed using one-way analysis of variance with Bonferroni's *post hoc* test using SPSS 16.0 software (SPSS, Chicago, IL, USA). A value of  $P < 0.05$  was considered statistically significant.

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