Effects of low- and high-frequency electroacupuncture on protein expression and distribution of TRPV1 and P2X3 in rats with peripheral nerve injury

Junying Du¹#, Junfan Fang¹#, Xuaner Xiang¹, Jie Yu¹, Xiaoqin Le¹, Yi Liang¹, Xiaoming Jin²*, Jiaoqiao Fang¹*

1. Department of Neurobiology and Acupuncture Research, the Third Clinical Medical College, Zhejiang Chinese Medical University. Key Laboratory of Acupuncture and Neurology of Zhejiang Province, Hangzhou 310053, China

2. Department of Anatomy and Cell Biology, Stark Neuroscience Research Institute, Indiana University School of Medicine (+1) 317-278-5766, USA

# Co-first author: The authors contributed equally to this study.

*Corresponding author. Xiao-Min Jin, PhD, Stark Neurosciences Research Institute & Department of Anatomy and Cell Biology, Indiana University School of Medicine, NB Building, 320w 15th St #141, Indianapolis, IN 46202. Email: xijin@iupui.edu.

Jian-Qiao Fang, M.D, PhD, Department of Neurobiology and Acupuncture Research, the Third Clinical Medical College, Zhejiang Chinese Medical University, 548 Binwen Road, Binjiang District, Hangzhou, Zhejiang Province, 310053, China. Tel.: (+86) 571-86673000, Fax: (+86) 571-86673000, E-mail: fangjianqiao7532@163.com.

Email addresses:

Junying Du: dujunying0706@163.com
Junfan Fang: fangjunfan0223@163.com
Xuaner Xiang: 530825902@qq.com
Jie Yu: 690474518@qq.com
Xiaoqin Le: 365703832@qq.com

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ABSTRACT

**Background:** Whether electroacupuncture (EA) stimulation at different frequencies has a similar effect on spared nerve injury (SNI) as on other neuropathic pain models, and how EA at different frequencies causes distinct analgesic effects on neuropathic pain is still not clear.

**Methods:** Adult male Sprague-Dawley rats were randomly divided into sham SNI, SNI, 2 Hz, 100 Hz and sham EA groups. Paw withdrawal threshold (PWT) and paw withdrawal latency (PWL) were measured. EA was performed once a day on days 1 to 14 after SNI. The expressions of transient receptor potential cation subfamily V member 1 (TRPV1) and peripheral purinergic P2X receptor 3 (P2X3) were determined by Western blotting and immunofluorescence. TRPV1 siRNA and P2X3 siRNA were administered by intrathecal injection. TRPV1 or P2X3 agonists were combined with EA.

**Results:** There were significant decreases in PWT but no changes in PWL in the 14 days after SNI. EA using 2 Hz or 100 Hz stimulation similarly increased PWT at every time point. The cytosol protein expression of P2X3 in the L4-6 DRG was increased, but the expression of TRPV1 was decreased in the SNI model. Both these effects were ameliorated by EA, with 2Hz stimulation having a stronger effect than 100 Hz stimulation. Blocking either TRPV1 or P2X3 specific siRNAs attenuated the decreased PWT induced by SNI. Administration of either a TRPV1 or P2X3 agonist inhibited EA analgesia.
Conclusions:

2 Hz and 100 Hz EA similarly induced analgesic effects in SNI. This effect was related to up-regulation and down-regulation, respectively, of cytosol protein expression of P2X3 and TRPV1 in L4-L6 DRG, with 2 Hz having a better effect than 100 Hz.

Key Words: electroacupuncture, TRPV1, P2X3, spared nerve injury
INTRODUCTION

Neuropathic pain is known to be one of the most difficult pain syndromes to manage and outcomes following treatment of neuropathic pain are often unsatisfactory. Results from clinical studies indicate that pharmacological and non-pharmacological therapies provide inadequate symptomatic relief for this type of pain.[1,2] New treatment strategies have been introduced in an attempt to better alleviate neuropathic pain.

Electroacupuncture (EA) is a promising complementary strategy for the treatment of neuropathic pain. Reviews have indicated that EA has few adverse events and could be considered a supplementary therapy to other non-pharmacological treatment options for neuropathic pain.[3-5] In the past, it was widely believed that EA alleviates various types of pain through the descending inhibitory system, especially via endogenous opioid peptides.[6-8] However, evidence suggests that opioids provide insufficient or equivocal relief from neuropathic pain.[1,9,10] Thus, some researchers believe that EA alleviates neuropathic pain not only by promoting endogenous opioid peptide release but also by regulating other mechanisms.[11,12] The mechanisms underlying the analgesic effect of EA on neuropathic pain remain unclear. Furthermore, according to established endogenous opioid theories, different parameters of EA (stimulation frequency, duration and location) produce variable therapeutic effects in terms of pain relief because of different levels of secretion of endogenous opioids.[13,14] If other mechanisms are involved in EA analgesia, it remains unknown how they contribute to the different effects of EA on neuropathic pain.

To better understand the underlying mechanisms and develop novel therapeutic strategies to treat neuropathic pain, many animal models have been developed, including those induced by chronic constriction injury (CCI) of the sciatic nerve, spinal nerve ligation (SNL), partial SNL (pSNL), sciatic nerve transection (SNT) and spared
nerve injury (SNI). All these models are established by peripheral nerve injury, and SNI is a known animal model that can mimic several characteristics of clinical neuropathic pain. Results of studies using these animal models indicate that both peripheral purinergic P2X receptor 3 (P2X3) and transient receptor potential cation subfamily V member 1 (TRPV1) are important transducers of nociceptive stimulation and are expressed on small- and medium-diameter neurons of dorsal root ganglia (DRG). Many research studies have indicated that both P2X3 and TRPV1 are key components of the nociceptive pathway. Our previous studies and those by other researchers have indicated that EA may inhibit the upregulated expression of TRPV1 in the DRG and spinal cord in neuropathic pain and cancer pain. In addition, suppressing the expression of P2X3 through EA stimulation could reduce visceral pain and CCI-induced neuropathic pain. However, the role of TRPV1 and P2X3 in EA analgesia remains controversial.

Although it is considered that altered P2X3 and TRPV1 expression or function after nerve injury contribute to hypersensitivity in neuropathic pain, they have been thought to interact with each other in an inhibitory manner under physiological conditions. Another study demonstrated that P2X3 and TRPV1 interact in a facilitatory manner in temporomandibular disorders; this interaction could contribute to the peripheral sensitization known to underlie masseter hyperalgesia. Although peripheral TRPV1 and P2X3 seem to induce hyperalgesia, it remains unclear whether they play the same role in EA analgesia for neuropathic pain. Answering this question may provide a new perspective on EA analgesia.

In the present study, we established a SNI model and measured TRPV1 and P2X3 expression along with changes in pain threshold. Different EA stimulation frequencies were then applied to the SNI rats. The effect of EA on paw withdrawal threshold (PWT)
was quantified, and the regulatory effect of EA on P2X3 and TRPV1 was investigated. Finally, the effects of siRNA interference and specific agonists combined with EA on PWT were evaluated.

METHODS

Animals

Adult male Sprague-Dawley rats weighing 160–250 g (animal certificate no. SCXK(沪)2013-0016, Shanghai Laboratory Animal Center, Chinese Academy of Sciences) were used for this experiment. The animals were housed five per cage in a controlled environment (25±2 °C, 55±5 %, 12 h light/dark cycle) in the laboratory animal center of Zhejiang Chinese Medical University (SYKK(浙)2013-0184). The animals were provided with food and water ad libitum. During the research, we ensured that all animal experiments complied with institutional and governmental regulations regarding the ethical use of animals, and were approved by the Animal Ethics Committee of Zhejiang Chinese Medical University (ZSLL-2015-022).

Experimental Design

We designed five experiments within this study. In experiment 1, 24 rats were completely randomized into a sham SNI group (n=12) and an SNI group (n=12) to observe changes in pain threshold and protein expression of TRPV1 and P2X3 at different time points after surgery. In experiment 2, to compare the effects of different frequency and duration of EA on pain, 72 rats were randomized into sham SNI, SNI, 2Hz EA and 100 Hz EA groups (n=18 rats per group). Each group was further divided into three subgroups (n=6 each) receiving 20 min, 30 min or 45 min of EA stimulation. Experiment 3 was based on the earlier result to compare the effects of different frequencies of EA stimulation on protein expression of TRPV1 and P2X3. Sixty rats
were randomized into sham SNI, SNI, 2Hz EA, 100Hz EA and sham EA groups (n=12 rats per group). Experiment 4 aimed to determine the role of TRPV1 or P2X3 in SNI. Thirty rats were randomized into sham SNI, SNI, SNI+control siRNA, SNI+TRPV1 siRNA and SNI+P2X3 siRNA groups (n=6 rats per group). In experiment 5, to further study the mechanisms of EA analgesia in SNI, 24 rats were randomized into SNI+vehicle, SNI+2Hz EA+vehicle, SNI+2Hz EA+capsaicin and SNI+2Hz EA+αβ meATP groups (n=6 rats each).

**Spared nerve injury**

SNI was performed to induce neuropathic pain, as previously described. During the operation, the rats were anesthetized by inhalation of isoflurane (3% for induction and 2% for maintenance) and the left sciatic nerve and its three terminal branches (namely the sural, common peroneal and tibial nerves) were exposed. The common peroneal and tibial nerves were tightly ligated with a 5.0 silk thread and sectioned distal to the ligation by removing 2 to 4 mm of the nerve stump. Adequate care was taken to avoid any contact with or stretching of the sural nerve. Both the muscle and skin were sutured. Only animals that developed mechanical allodynia were used. For sham-operated rats, the left sciatic nerve and its trifurcation were exposed but not manipulated. When the rats woke up from anesthesia, they were returned to their cages. To prevent bacterial infection, all rats received penicillin (80U intramuscularly) for 3 days.

**Behavioral testing**

**Paw withdrawal threshold.** Before PWT was measured, the rats were placed in transparent organic glass boxes (20 cm x 20 cm x 15 cm) on an elevated metal floor until they settled down. Von Frey filaments (Stoelting, IL, USA) weighing 0.4, 0.6, 1, 2, 4, 6, 8, 15 and 26 g were pressed onto the lateral plantar surface (sural nerve area) of the hind paw for approximately 6 to 8 s. The first filament applied was equal to a force...
of 2 g. If a negative response (no movement) was observed, expressed as X, a filament exerting a greater force was then applied; if a positive response (paw withdrawal) was observed, expressed as O, a filament of a lesser force was then applied. This process was repeated several times until the first “OX” or “XO” sequence was obtained, and then four additional “O” or “X” responses were measured. The PWT was calculated as described by Chaplan et al.[27]

**Paw withdrawal latency.** The radiant heat source of the test instrument (37370, UGO, Italy) was positioned on the lateral plantar surface of the hind paw after the rats settled in the test chamber, and the elapsed time was recorded as the paw withdrawal latency (PWL). The radiant heat was set to 35°C, and the cutoff time was 20 seconds. The PWL was repeated three times at each time point, at 2-minute intervals, and the mean of these values was used for further calculations.

**Electroacupuncture intervention**

The EA intervention procedure was performed as reported previously.[28] The intervention began after completion of the behavioral test on day 1 after SNI. The rats were gently immobilized with a special cotton retainer designed by our laboratory (patent no. ZL 2014 2 0473579.9, State Intellectual Property Office of the People’s Republic of China). Stainless-steel needles (0.18 mm x 13 mm) were inserted to a depth of 5 mm at bilateral ST36 (Zusanli) and BL60 (Kunlun). The needles were connected to a HANS Acupuncture Point Nerve Stimulator (LH-202H Huawei Co., Ltd., Beijing, China) with intensities of 0.5 mA, 1.0 mA or 1.5 mA, and frequencies of 2 Hz or 100 Hz, for 30 minutes, once per day, until the end of the experiment.

For rats in the sham EA group, needles were inserted subcutaneously at ST36 and BL60 (to 1 mm depth) and the needles were connected to the electrodes, however there was no electrical stimulation.
**Drug administration**

α,β-methyleneadenosine 5′-triphosphate lithium salt (αβ-meATP, P2X3 agonist) and capsaicin (TRPV1 agonist), both from Sigma-Aldrich (St. Louis, MO, USA), were used in current study. They were dissolved in dimethylsulfoxide (DMSO). The final concentration of DMSO was less than 2%. Before EA stimulation, α,β-meATP (600nmol, 25μl) and capsaicin (100μg, 25μl) were administered intradermally into the dorsum of the hind paw on 14 day.

**Tissue preparation**

After behavioral testing, the rats were anesthetized with 2% pentobarbital sodium (0.2 ml / 0.1 kg, intraperitoneally). For immunofluorescence, rats were quickly perfused under deep anesthesia with 150 ml of 0.9% NaCl (4°C) followed by 400 ml fresh 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS). The L4, L5 and L6 DRGs were removed and post-fixed in the same fixative for 3 hours at 4°C before serial transfer to 15% and 30% sucrose for dehydration. For Western blot analysis, the rats were quickly perfused with 150 ml cold sterilized saline after being deeply anesthetized. The L4, L5 and L6 DRGs were removed and stored at −80°C.

**Immunofluorescence staining**

Sections of DRG (14 μm) were blocked with 5% normal donkey serum in tris-buffered saline tween (TBST; 0.1% Tween-20) for 1 hour at 37°C. DRG sections were then incubated with rabbit anti-P2X3 and rabbit anti-TRPV1 (both 1:1000 in 5% normal donkey serum, Abcam, Cambridge, MA, USA) antibodies for one night at 4°C, followed by incubation in fluorescein (FITC) AffiniPure donkey anti-rat IgG (H+L, Jackson, PA, West Grove, USA) for 1 hour at 37°C. An A1R confocal microscope (Nikon, Tokyo, Japan) was used to acquire images of the expression of P2X3 and TRPV1 in the L4, L5 and L6 DRG.
Western blot analysis

We determined the cytosol protein and total protein of the L4, L5 and L6 DRG according to the respective procedures. To extract cytosol protein, we used a nuclear and cytoplasmic protein extraction kit (Beyotime, China) containing 1% phenylmethylsulfonyl fluoride (PMSF, Beyotime, China) and a protease/phosphatase inhibitor cocktail (Applygen, China). To extract total protein, we used radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime) containing 1% PMSF (Beyotime) and a protease/phosphatase inhibitor cocktail (Applygen). Protein concentration was estimated using a BCA protein assay kit. Sodium dodecylsulfate-polyacrilamide gel electrophoresis (SDS-PAGE) gels (5% to 10%) were used to separate protein samples (20 μg), and the separated proteins were electrophoretically transferred to polyvinyl difluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA) and blocked with 5% low-fat milk in TBST for 1 hour at room temperature. Next, the samples were treated with rabbit anti-P2X3 and anti-TRPV1 (both 1:1000 in 5% normal donkey serum, Abcam) for one night at 4°C. The samples were then incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:10000, Abcam) for 1 hour at room temperature. Rabbit anti-GAPDH (HRP conjugate; 1:1000, CST, Danvers, MA, USA) was used as the internal control. The membranes were developed using an ECL kit (Pierce, USA) and the signals were then captured with an ImageQuant LAS 4000 system (EG, USA). The density of each band was measured using ImageQuant TL 7.0 analysis software (GE, USA). The mean expression level of the target protein in the animals in the sham SNI group was considered as 1, and the relative expression level of the target protein in all animals was adjusted as a ratio to the level of the sham SNI group.

Intrathecal catheter implantation and siRNA administration
siRNA administration was performed as previously described.[29] TRPV1 siRNA sequences (5’-CCAUUGAACGGCGGAACAUTT and 5’-AUGUUCCGCCGUUCA AUGGTT), P2X3 siRNA sequences (5’-CCGUCCAGCUGCUGAUUAUTT and 5’-AUAAUCAGCAGCUGGACGTTT) and control siRNA sequences (5’-UUCUCCGAACGUGUCACGUTT and 5’-ACGUGACACGUUCGGAGAATT) were dissolved in double distilled RNAse-free water to obtain a 100 μM stock solution and stored at -20°C. On the day of the experiment, the stock solution was mixed with the transfection reagent (i-fect) to achieve a final concentration of 2 μg / 10 μl, and then intrathecally administered to the appropriate groups of rats once daily for three days. After SNI, siRNA treatment was administered on alternate days for the whole experimental protocol.

**Statistical analysis**

All data were expressed as means ± standard error of the mean (SEM). The paw withdrawal threshold and paw withdrawal latency data were normally distributed and were therefore analyzed using repeated-measures analysis of variance (ANOVA) with between-subject factors. A one-way ANOVA for independent samples was used to compare differences between the groups at each time point. We used the independent sample t-test to compare the differences in TRPV1 and P2X3 expression between the two groups (figure 1A). For immunofluorescence and Western blot analysis, comparisons between groups were performed using one-way ANOVA followed by the Fisher protected least significant difference post hoc test. $P<0.05$ was considered statistically significant.

**RESULTS**

SNI increased expression of P2X3 and TRPV1 in the L4-6 DRG and decreased
As shown in Figure 1A, peripheral nerve injury significantly increased P2X3 and TRPV1 protein expression in the L4-6 DRG, similar to previous results. Along with the changes in P2X3 and TRPV1 expression, the PWT of the SNI group markedly decreased 1, 3, 7 and 14 days after SNI ($P<0.05$, Figure 1B). Unlike the change in PWT, the PWL of the SNI group did not decrease significantly within the 14 days after SNI compared with that of the sham SNI group ($P>0.05$, Figure 1C).

Effects of EA on PWT in SNI rats

Next, the effect of different frequencies of EA on PWT in SNI rats was determined. As shown in Figure 2, the PWT of the SNI group was decreased at 24 hours and remained at a lower level until 14 days after SNI. Both 2 Hz and 100 Hz EA stimulation significantly increased PWT at every time point after SNI ($P<0.05$ or $P<0.01$), and the analgesic effect of 2 Hz stimulation was not significantly different to that of 100 Hz stimulation ($P>0.05$). Furthermore, sham EA stimulation did not affect the decreased PWT induced by SNI at any time point after the operation ($P>0.05$).

It is generally suspected that duration of stimulation, another important parameter of EA, could also affect the analgesic effect of EA. Therefore, we further investigated whether different durations of EA stimulation would induce different analgesic effects in SNI rats. EA stimulation was administered for 20, 30 or 45 minutes each time. The results are shown in Figure 2A-C. Similar to the results shown in Figure 2D, EA significantly improved the PWT of SNI rats at 25 and 73 hours after the surgery, and 2 Hz EA did not differ from 100 Hz EA ($P>0.05$). No effect of different durations of stimulation was observed in this study. The effect of EA on PWL was not investigated, given SNI did not induce any significant change in this parameter (Figure 1C).
Effects of EA on expression of P2X3 and TRPV1 in the L4-6 DRG

Because there was no change in PWL in the SNI rats and different durations of EA stimulation did not produce different analgesic effects, only the PWT in SNI rats and the effect of EA administered for 30 minutes were investigated in the following experiments.

Western blotting and immunofluorescence were used to observe cytosol and total P2X3 protein expression and the cells and areas positive in the L4, L5 and L6 DRG. Compared with that of the sham SNI group, the SNI group showed a significant decrease in P2X3 cytosol protein in the L4, L5 and L6 DRG ($P<0.01$), and total protein was not obviously changed ($P>0.05$), as shown in Figure 3A-D. 2 Hz EA stimulation markedly increased the expression of P2X3 cytosol protein in the L4, L5 and L6 DRG ($P<0.01$), while 100 Hz and sham EA had no effect ($P>0.05$).

As shown in Figure 3E-H, compared with that of the sham SNI group, TRPV1 cytosol protein expression in the L4, L5 and L6 DRG of the SNI group was significantly higher, and total protein expression was not obviously changed. In addition, both 2 Hz and 100 Hz EA stimulation markedly decreased the expression of TRPV1 cytosol protein in the L4, L5 and L6 DRG ($P<0.01$), and the effect of 2 Hz EA on the expression of TRPV1 cytosol protein was greater than that of 100 Hz EA ($P<0.01$). Sham EA stimulation did not affect the expression of TRPV1 cytosol protein in the L4-6 DRG ($P>0.05$) and neither EA (at 2 Hz or 100 Hz) nor sham EA stimulation had any effect on the expression of TRPV1 total protein in the L4-6 DRG ($P>0.05$).

Effects of EA on the number of P2X3- and TRPV1 positive cells in the L4-6 DRG

As shown in Figure 4A-C, the SNI group showed significantly more P2X3-positive cells in the L4 and L6 DRG than in the sham SNI group ($P<0.05$), and the
P2X3-positive area in the L5 and L6 DRG of the SNI group was also greater \((P<0.05)\). Furthermore, 2 Hz EA stimulation decreased the number of P2X3-positive cells in the L4 and L6 DRG and the P2X3-positive area in the L4, L5 and L6 DRG, while 100 Hz EA stimulation decreased the P2X3-positive area in the L6 DRG alone. Sham EA stimulation did not affect the P2X3-positive area or number of positive cells in the L4, L5 and L6 DRG.

As shown in Figure 4D-F, the SNI group had more TRPV1-positive cells in the L5 and L6 DRG than the sham SNI group. Furthermore, 100 Hz EA stimulation reduced the number of TRPV1-positive cells in the L5 DRG, and 2 Hz EA stimulation reduced the number of TRPV1-positive cells in the L6 DRG and the TRPV1-positive area in the L4 DRG.

**Effect of siRNA interference on PWT and protein expression**

Intrathecal catheter implantation and injection of control siRNA caused no significant change in TRPV1 and P2X3 expression (Figure 5A-B). There were no differences in TRPV1 or P2X3 expression between the SNI group and the SNI+control siRNA group; TRPV1 and P2X3 levels in L4-6 DRG in both groups were higher than those of the sham SNI group. Intrathecal TRPV1 siRNA treatment decreased protein expression of TRPV1 in the L4-6 DRG. As shown in Figure 5A, protein expression of TRPV1 in the L4-6 DRG of the SNI+TRPV1 siRNA group was significantly lower than that of the sham SNI group, SNI group and SNI+control siRNA group \((P<0.01)\). Further intrathecal P2X3 siRNA treatment also decreased protein expression of P2X3 in the L4-6 DRG \((P<0.01;\) figure 5B).

Regardless of whether an intrathecal catheter was implanted or not, there were no differences in PWT at baselines among the sham SNI group, the SNI group, the SNI+control siRNA, the SNI+TRPV1 siRNA and the SNI+P2X3 siRNA group \((P>0.05,\)
Both TRPV1 siRNA and P2X3 siRNA pretreatment improved the PWT of SNI rats \( (P<0.01) \), while control siRNA treatment had no effect on PWT \( (P>0.05) \); Figure 5C).

**Effect of P2X3 or TRPV1 agonist combined with EA on PWT**

We further used specific P2X3 or TRPV1 agonist combined with EA to determine their role in mediating the effect of EA on PWT. As shown in Figure 5D, compared with the SNI+vehicle group, the PWTs of the SNI+2Hz EA+vehicle group, SNI+2Hz EA+capsaicin group and SNI+2Hz EA+αβ meATP group were significantly increased 14 days after SNI \( (P<0.01) \). Compared with the SNI+2Hz EA+vehicle group, PWTs in the SNI+2Hz EA+capsaicin group and SNI+2Hz EA+αβ meATP group were markedly decreased.

**DISCUSSION**

In the present study, we observed and compared the regulatory effect of different frequencies and durations of EA stimulation on the PWT of SNI rats, and investigated why EA administered by different frequencies produced different analgesic effects on SNI rats. We found that SNI rats exhibited a reduction in PWT but no change in PWL at every time point after operation. EA, regardless of the parameters (duration and frequency), alleviated the peripheral nerve injury-induced pain, and the analgesic effect of 2 Hz stimulation was not significantly different to that of 100 Hz stimulation. SNI induced a decrease in the cytosol protein expression of P2X3 in the L4, L5 and L6 DRG, whereas the cytosol protein expression of TRPV1 in the L4, L5 and L6 DRG was increased. However, the total protein expression of neither P2X3 nor TRPV1 was significantly different. Furthermore, 2 Hz EA stimulation significantly increased the cytosol protein expression of P2X3 and decreased that of TRPV1 in the L4-6 DRG, and
the regulatory effects were stronger than that of 100 Hz. In addition, both TRPV1 siRNA and P2X3 siRNA mitigated the decreased PWT induced by SNI. Either TRPV1 agonist or P2X3 agonist could inhibit the EA analgesia.

Animal models have been developed to determine the mechanisms of neuropathic pain and explore novel treatment strategies. By its dependency on sympathetic activity, neuropathic pain can be divided into two categories: sympathetically maintained pain (SMP) and sympathetically independent pain (SIP).[30] Several animal models have been used to represent SMP. For example, chemical or surgical sympathectomy has been shown to inhibit neuropathic signs such as mechanical allodynia and thermal hyperalgesia in both CCI and SNL models.[31, 32] However, chemical or surgical sympathectomy has been shown to be ineffective at reducing neuropathic pain.[33] SNI is a well-established animal model of neuropathic pain that is very simple to produce and can result in profound and reliable pain behaviors. In addition, because intraperitoneal application of guanethidine does not inhibit any neuropathic signs, the SNI model may represent SIP and thus could be a useful tool for elucidating the mechanisms of neuropathic pain.[33] A major characteristic of the SNI model is the enhanced mechanical sensitivity without any change in heat thermal thresholds.[26] We observed similar behavioral outcomes; a decrease in PWT occurred as early as day 1 after SNI surgery and maintained on day 14. However, PWL was not changed in SNI rats at any of the experimental time points.

TRPV1 in the DRG might participate in the production of nociceptive responses after nerve injury. However, the changes in PRV1 expression levels in the DRG varies in different neuropathic pain models. The number of TRPV1-immunoreactive cells in the DRG has been shown to be reduced after CCI,[34] but increased in undamaged DRG neurons after partial nerve injury.[35] mRNA expression of TRPV1 receptors in the DRG
was decreased following SNI,[36] but increased in the spared L4 DRG in rats with L5 spinal nerve ligation.[37] In the present study, we showed that SNI induced an increase in the total protein expression of TRPV1, and separately upregulated the cytosol protein expression of TRPV1 in the L4-L6 DRG. The total protein expression of TRPV1 did not change when the protein was extracted separately from the L4-L6 DRG. However, the trend towards higher TRPV1 expression in the L4 and L5 DRG was similar to the results of total protein. Consistently, our quantification of TRPV1-immunoreactive cells in the L4-L6 DRG showed more TRPV1-positive cells and areas in the L5-L6 DRG after SNI.

It is thought that P2X3 in the DRG also contributes to the nociceptive responses after nerve injury. Like the expression of TRPV1, changes in the expression of P2X3 receptors vary among injury models. The number of P2X3-immunoreactive cells in the DRG were shown to be higher after CCI,[38] but lower in the injured L5 and L6 DRG when the sciatic or spinal nerves were ligated.[39, 40] mRNA expression of P2X3 receptors may be decreased[41] or unchanged after nerve ligation.[37] The expression of P2X3 receptors on the cell membrane was significantly enhanced, whereas the total expression of P2X3 receptors remained unchanged after SNI.[42] Here, we showed that SNI induced a reduction in the cytosol protein expression of P2X3, whereas the total protein expression of P2X3 remained unchanged. However, a trend of higher total protein expression of P2X3 in the L4 and L5 DRG was also noted. In addition, there were more P2X3-positive cells in the L4 and L6 DRG after SNI. All these results showed that P2X3 and TRPV1 may have different functions in SNI rats.

The interference of siRNA was then used to investigate the role of TRPV1 and P2X3 in neuropathic pain. The results indicated that inhibition of TRPV1 and P2X3 expression in the L4-L6 DRG could alleviate the pain induced by peripheral nerve
injury. The function of TRPV1 was consistent with previous results. However, the reason why lowering cytosol protein expression of P2X3 in the L4-L6 DRG would alleviate pain is still unclear. One hypothesis is that lower cytosol protein expression of P2X3, accompanied by higher unchanged total protein expression, may indicate a higher member protein expression. Further study of this hypothesis is therefore necessary.

EA is known to have a positive effect on pain relief that is associated with its stimulation parameters (e.g., acupoint, frequency, duration). Our previous studies have demonstrated that stimulation at ST36 and BL60 can produce a good analgesic effect. Hao et al. observed that the acupuncture parameters (stimulation mode, retention time, frequency) affected the outcomes for tension-type headaches. Our previous research revealed that EA has a parameter-specific effect on chronic inflammatory pain relief, which primarily depends on the stimulation frequency but not on the duration of stimulation. In this study, we compared and observed that both low-frequency and high-frequency EA induced analgesic effects in SNI rats, with no statistically significant differences between them. These results are inconsistent with those of several other animal studies of EA for neuropathic pain, which demonstrated that low-frequency EA had a better analgesic effect on neuropathic pain than high-frequency EA. Yu et al. showed that EA stimulation exhibits significant antinociceptive effects on CCI-induced neuropathic pain, and 2 Hz EA has a better analgesic effect than 15 Hz EA. In another study, EA at a high frequency of 100 Hz was ineffective for the treatment of SNL-induced neuropathic pain, whereas EA at a low frequency of 2 Hz was adequate. We speculate that these differences may be related to variations in sample size, needling location and animal models between studies. Furthermore, in our previous research we observed that the parameter-specific analgesic effect of EA
was at least partially related to the mediation of the protein level of TRPV1 and P2X3 expression in the DRG of complete Freund’s adjuvant (CFA)-exposed rats.\textsuperscript{[44]} In the present study, we showed that different frequencies of EA had different regulatory effects on the protein expression of P2X3 and TRPV1—attenuating the decrease in cytosol protein expression of P2X3 and suppressing the increase in cytosol protein expression of TRPV1—and 2 Hz stimulation had a stronger regulatory effect than 100 Hz stimulation. EA did not affect the total expression of P2X3 and TRPV1. We could speculate that EA may influence the membrane protein expression by modulating the cytosol protein expression of P2X3 and TRPV1. Furthermore, specific agonists of P2X3 and TRPV1 could inhibit the analgesic effect of EA. These results strongly support our hypothesis that EA produces an analgesic effect by regulating expression of P2X3 and TRPV1 in primary sensory neurons. Our future research will further investigate how EA regulates P2X3 and TRPV1.

Conclusions

EA using 2 Hz or 100 Hz frequency for 20, 30 or 45 minutes had similar and significant analgesic effects in a SNI model of neuropathic pain. This effect was associated with up-regulation of cytosol protein expression of P2X3 and down-regulation of cytosol protein expression of TRPV1 in the L4-L6 DRG, with 2 Hz EA being more effective than 100 Hz EA.

Data availability

The data supporting the findings of this study are available from the corresponding author upon request.

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Conflicts of Interest
None declared

Author contributions
JD wrote the initial draft of the manuscript. JD and JF performed the immunohistochemistry, Western blotting, tissue fractionation and associated analyses. JY and XL supervised the data analysis, study direction, image acquisition and manuscript design and revisions. XX and YL performed experiments and contributed to the design, data analysis and writing of the manuscript. XJ and JF designed and performed the experimental protocols described. All authors read and approved the final version of the manuscript accepted for publication.

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Spared nerve injury (SNI) induced changes in transient receptor potential cation subfamily V member 1 (TRPV1) and peripheral purinergic P2X receptor 3 (P2X3) total protein expression level in the L4-6 dorsal root ganglion (A, n=5-7 per group) and paw withdrawal threshold (B, n=6 per group) but not paw withdrawal latency (C, n=6 per group). Data are mean±SEM. *P<0.05, compared to sham SNI group.
Figure 2

Effects of electroacupuncture (EA) with 2 Hz and 100 Hz stimulation lasting 20 min (A), 30 min (B and D) or 45 min (C) on the paw withdrawal threshold of spared nerve
injury (SNI) rats over 3 days (A-C) and 14 days (D), and schematic of experimental protocol (EA). Data are mean±SEM (n=6 per group). *P<0.05, **P<0.01, compared with sham SNI group; △P<0.05, △△P<0.01, compared with SNI group; # P<0.05, ## P<0.01, compared with 2 Hz group; ○P<0.05, ○○P<0.01, compared with 100 Hz group.
Figure 3

Effect of electroacupuncture (EA) with 2 Hz and 100 Hz stimulation on peripheral purinergic P2X receptor 3 (P2X3) and transient receptor potential cation subfamily V member 1 (TRPV1) protein expression in the L4-6 dorsal root ganglion of spared nerve injury (SNI) rats, measured by Western blotting. Panels show Western blotting bands.
and optical band density analysis, respectively, for P2X3 cytosol protein (A and B),
P2X3 total protein (C and D), TRPV1 cytosol protein (E and F) and TRPV1 total protein
(G and H). Data are mean±SEM (n=6-7 per group). ** P<0.01, compared with sham
SNI group; △△ P<0.01, compared with SNI group; # P<0.05, ## P<0.01, compared
with 2 Hz group; ○ P<0.05, compared with 100 Hz group.
Figure 4

Effect of electroacupuncture (EA) with 2 Hz and 100 Hz stimulation on peripheral purinergic P2X receptor 3 (P2X3)-positive and transient receptor potential cation subfamily V member 1 (TRPV1)-positive neurons in the L4-6 dorsal root ganglion (DRG) of spared nerve injury (SNI) rats, measured by immunofluorescence. Panels A and D show immunofluorescence confocal micrographs of the L4, L5 and L6 DRG in sham SNI, SNI, 2 Hz, 100 Hz and sham EA groups. Sections show immunohistochemical green labeling for P2X3-positive neurons (A) and TRPV1-positive neurons (D). Scale bars = 100 μm. Remaining panels show quantitative analysis of P2X3-positive cell counts (B) and area (C), and TRPV1-positive cell counts (E) and area (F). Data are mean±SEM (n=3-5 per group). *P<0.05, compared with sham SNI group; △ P<0.05, △△ P<0.01, compared with SNI group; # P<0.05, ## P<0.01, compared with 2 Hz group; ○ P<0.05, ○○ P<0.01, compared with 100 Hz group.
Figure 5

Involvement of peripheral purinergic P2X receptor 3 (P2X3) and transient receptor potential cation subfamily V member 1 (TRPV1) in the spared nerve injury (SNI) rat model of neuropathic pain and analgesic effect of electroacupuncture (EA). All data are mean±SEM (n=6 per group). [A] Optical band density analysis and Western blotting bands of TRPV1 total protein expression in the dorsal root ganglion (DRG). ** P<0.01, compared with sham SNI group; △△ P<0.01, compared with SNI group; && P<0.01, compared with control siRNA group. [B] Optical band density analysis and Western blotting bands of P2X3 total protein expression in DRG. [C] Changes in paw withdrawal threshold (PWT) in sham SNI, SNI, SNI+control siRNA, SNI+TRPV1 siRNA, SNI+P2X3 siRNA, SNI+2Hz EA+vehicle, SNI+2Hz EA+capsaicin, SNI+2Hz EA+αβ meATP groups. ** P<0.01, compared with sham SNI group; △△△ P<0.01, compared with SNI group; &&& P<0.01, compared with control siRNA group. [D] Paw withdrawal threshold (PWT) in SNI+vehicle, SNI+2Hz EA+vehicle, SNI+2Hz EA+capsaicin, SNI+2Hz EA+αβ meATP groups. ** P<0.01, compared with sham SNI group; △△△△ P<0.01, compared with SNI group; &&&& P<0.01, compared with control siRNA group.
siRNA and SNI+P2X3 siRNA groups. ** $P<0.01$, compared with sham SNI group; 
$\Delta \Delta P<0.01$, compared with SNI group; && $P<0.01$, compared with control siRNA group. [D] Effects of P2X3 or TRPV1 agonist combined with EA on PWT. $\Delta \Delta P<0.01$, compared with SNI+vehicle group; ## $P<0.01$, compared with SNI+2Hz EA+vehicle group.