

Electroacupuncture effects on the P2X4R pathway in microglia regulating the excitability of neurons in the substantia gelatinosa region of rats with spinal nerve ligation

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Abstract. Electroacupuncture (EA) has been used to treat neuropathic pain induced by peripheral nerve injury (PNI) by applying an electrical current to acupoints with acupuncture needles. However, the mechanisms by which EA treats pain remain indistinct. High P2X4 receptor (P2X4R) expression levels demonstrate a notable increase in hyperactive microglia in the ipsilateral spinal dorsal horn following PNI. In order to demonstrate the possibility that EA analgesia is mediated in part by P2X4R in hyperactive microglia, the present study performed mechanical withdrawal threshold (MWT) and thermal withdrawal latency (TWL) tests in male Sprague-Dawley rats that had undergone spinal nerve ligation (SNL). The expression levels of spinal P2X4R were determined using reverse transcription-quantitative PCR, western blotting analysis and immunofluorescence staining. Furthermore, spontaneous excitatory postsynaptic currents (sEPSCs) were recorded using whole-cell patch clamp to demonstrate the effect of EA on synaptic transmission in rat

spinal substantia gelatinosa (SG) neurons. The results of the present study demonstrated that EA increased the MWT and TWL and decreased overexpression of P2X4R in hyperactive microglia in SNL rats. Moreover, EA attenuated the frequency of sEPSCs in SG neurons in SNL rats. The results of the present study indicate that EA may mediate P2X4R in hyperactive spinal microglia to inhibit nociceptive transmission of SG neurons, thus relieving pain in SNL rats.

Introduction

Electroacupuncture (EA) is used to treat neuropathic pain (NP) induced by peripheral nerve injury (PNI) (1,2). To the best of our knowledge, however, the mechanism has not yet been identified. Previous studies have noted that neuronal activities are associated with neurotransmitters and neuromodulators, such as opioids, interleukins, chemokines, serotonin and adenosine, and that the appearance and persistence of NP depends on hyperactive microglia (3-8). Suppression of microglia activation attenuates pain induced by nerve injury (9,10). Previous studies have demonstrated that the P2X4 receptor (P2X4R) triggers allodynia following PNI, and that relief of NP occurs both in mice injected intrathecally with a P2X4R antisense oligonucleotide and in mice lacking P2X4R (8,11). ATP is a transmitter that conveys sensory information between hyperactive microglia and nociceptive neurons. Hyperactive microglia induce or promote increased expression levels of P2X4R (8). In response to extracellular ATP, P2X4R can mediate a number of effects, such as the production and diffusion of bioactive factors, including cytokines and neurotrophic factors that can induce depolarization of dorsal horn sensory neurons (12). The physiological process of EA analgesia involves numerous transmitters and modulators, including acetylcholine opioid peptides, substance P, glutamate, γ -amino-butyric acid and other associated peptides (13). Previous studies have indicated

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that EA analgesia is associated with decreased purine and purinergic receptors, especially P2X4R (3,14). In light of this association, EA treatment may have a role in the relief of NP, which may be mediated in part by P2X4R in microglia in the spinal dorsal horn (SDH) (15,16). The present study demonstrated that the pain behavior and expression levels of P2X4R in microglia in the SDH were altered in spinal nerve ligation (SNL) rats. Whole-cell patch clamp techniques were used to investigate the variation in the frequency of spontaneous excitatory postsynaptic currents (sEPSC) in SNL rat spinal substantia gelatinosa (SG) neurons. Studies have demonstrated that EA treatment at 'Zusanli' (ST-36, at the posterolateral aspect of the knee joint, ~5 mm below the humeral head) and 'Kunlun' (BL-60, ~10 mm above the prominence of the lateral malleolus of the hind limb) points can relieve neuropathic pain, but its specific mechanism has not yet been elucidated (1,2). The aim of the present study was to investigate the potential mechanism by which EA treatment at 'Zusanli' and 'Kunlun' points relieves NP via the action on P2X4R.

Materials and methods

Experimental animals. The Institutional Animal Care and Use Committee of Wenzhou Medical University approved all experiments. A total of 72 male Sprague-Dawley rats (weight, 180-200 g; age, 6-8 weeks), bought from Wenzhou Medical University (Wenzhou, China), were kept on a standard laboratory diet at room temperature (20-22°C) and 12-h alternative light-dark cycle conditions in a pathogen-free room. The behavioral experiment was performed between 2:00 p.m. and 4:00 p.m. All experimental rats were randomly distributed into four groups (n=6): Control, sham, SNL and ipsilateral EA groups. All surgical procedures were performed using a microscope (Leica S8 APO; Leica Microsystems, Ltd; magnification, x4). The experimental rats were anesthetized with 5% chloral hydrate [350 mg/kg, intraperitoneal (i.p.)]. SNL surgery was performed as previously described (17-19). Briefly, an incision was made in the midline lumbar region of animals placed in a prone position. In order to expose the right L4-L5 spinal nerves completely, the right L5 vertebral transverse was cut. Following right L5 spinal nerve separation, it was ligated with 5-0 silk, and the incision was closed. In the sham group, the right L5 spinal nerve was exposed but not ligated. Pain thresholds were measured at days 0, 3, 5, 7, 10, 12 and 14 post-SNL.

Behavioral tests. The EA treatment time was fixed at 9:00-10:00 a.m. MWT and TWL tests time were fixed at 2:00-4:00 p.m. The Electronic von Frey anesthesiometer (IITC Life Science Inc.) was used to measure MWT to judge mechanical hyperalgesia. The experimental rats were allowed to acclimatize in the wire mesh-bottom cages (20x14x16 cm) for 30 min. The test probe was positioned at the base of third and fourth toes, and the pressure of the Electronic von Frey anesthesiometer was set to 0.1-70.0 g. Both lifting and licking the paw were considered to be positive responses. The maximum pressure was also recorded. Each hind paw was tested alternately six times at 5 min intervals. The average value was used for statistical analysis. Following the MWT test, rats were placed in a square, transparent, bottomless

acrylic box (16.0x12.5x14.0 cm) and allowed to acclimatize for 15 min before being subjected to a TWL test. The TWL was used to test thermal hyperalgesia using Plantar Test apparatus (Ugo-Basile S.R.L.) (13). The infrared source was set at 60°C under a glass plate and directed towards the plantar surface of the hind paw. Withdrawal of the paw led to the infrared source breaking off, at which point latency was measured. The hind paw of the experimental rats was tested five times at 15-min intervals and the TWL was expressed as the mean value.

EA treatment. EA treatment was started on day 7 post-SNL in the EA groups. The rats were maintained in fixation equipment (patent no. 201110021482.5; State Intellectual Property Office) (13). Acupuncture needles were percutaneously inserted 2-3 mm at the Zusanli and Kunlun points. EA stimulus (2/100 Hz; 1.5 mA) was delivered using an electrical stimulation device (HANS-200E; Nanjing Jisheng Medical Technology, Ltd.) for 30 min daily. The intensity was set at 1 mA, and the total stimulation period was 30 min for 7 days, which ensured the best curative effect of EA (Fig. 1).

Reverse transcription-quantitative PCR. Real-time amplification using SYBR-Green Supermix (Toyobo Life Science) and a Light Cycler 480 system (Roche Diagnostics GmbH) was performed using 4 ng cDNA extracted from L4-L5 segments with TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. RNA preparation and cDNA synthesis were performed as previously described (20). The PCR conditions consisted of an initial melting cycle at 95°C for 15 min, followed by 40 cycles of amplification at 95°C for 15 sec (denaturation), 60°C for 30 sec (annealing) and 72°C for 30 sec (extension). Primers were procured from Invitrogen (Thermo Fisher Scientific, Inc.): P2X4R forward, 5'-GGGTGAAGTTTATTCCAGC-3'; P2X4R reverse, 5'-GGGTGAAGTTTCTGCAGCC-3'; GAPDH forward, 5'-CTTCACCACCATGGAGAAGGC-3'; and GAPDH reverse, 5'-GGCATGGACTGTGGTCATGAG-3'. The quantification values were obtained from the quantification cycle (Cq) number at which the increase in the signal was associated with exponential growth of the PCR products. All samples were run in triplicate and repeated three times. RPS16 quantification was used as an internal control for normalization. Fold differences in mRNA levels over vehicle control were calculated using the $2^{-\Delta\Delta Cq}$ method (21).

Western blotting. Western blotting was performed as previously described (22) with minor modifications. The rats were deeply anesthetized with i.p. injection of 30 mg/kg pentobarbital sodium. The Rat Anesthesia Guidelines of University Minnesota (<https://www.researchservices.umn.edu/services-name/research-animal-resources/research-support/guidelines/anesthesia-rats>) were used to determine when the rat had entered deep anesthesia. Corneal reflexes were observed to disappear in the rat eye. Rats were observed to have no response after lightly clamping the fourth toe with tweezers, thereby confirming that rats had entered a state of deep anesthesia. The rats were sacrificed by decapitation. The proteins extracted from L4-L5 segments were quantified using a bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology). The proteins (42 kDa actin and

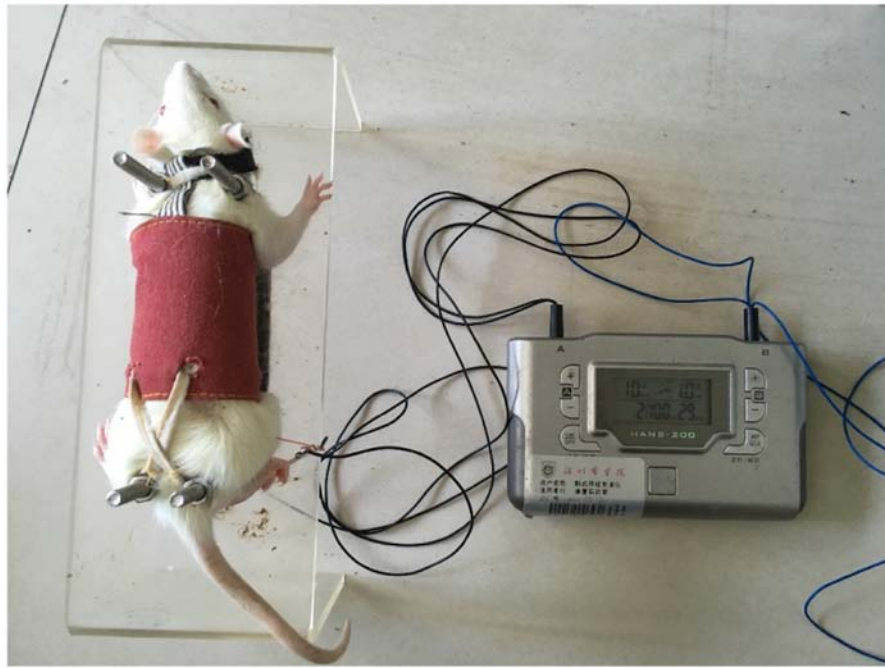


Figure 1. Use of comfortable fixation equipment can decrease stress and enhance convenience in acupuncture research.

62 kDa P2X4R) were subjected to 8% SDS-PAGE and transferred to a PVDF membrane. The membranes were blocked with 5% non-fat milk for 2 h at 4°C and incubated overnight at 4°C with anti-rat P2X4R polyclonal antibody (1:1,000; Alomone Labs) or GAPDH antibody (1:4,000; Sigma-Aldrich, Merck). Membranes were subsequently incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (goat anti-rabbit IgG; 1:3,000; cat. no. 7074; Cell Signaling Technology, Inc.) for 2 h at 4°C. These membranes were washed 3 times with TBST (0.1% Tween-20) (5 min/time) after incubating with HRP-conjugated antibody. The bands were detected using the ECL method (BeyoECL Plus; cat. no. P0018S; Beyotime Institute of Biotechnology) and exposed to radiography films.

Immunofluorescence staining. Immunofluorescence staining was performed as previously described with minor modifications (23). On day 14 post-SNL, the rats were anesthetized with 10% chloral hydrate (350 mg chloral hydrate/kg). The rats exhibited no signs of peritonitis following administration of chloral hydrate. The rats were perfused through the ascending aorta with physiological saline. Subsequently, rats were fixed with 4% paraformaldehyde for 4 h in 0.1 M phosphate buffer at pH 7.2-7.4, 4°C. Following fixation, the heartbeat disappeared and the body was stiff. The lumbosacral section was dehydrated, cleared and embedded in paraffin for transverse paraffin sections. Transverse spinal cord sections (5 µm) were excised and mounted on poly-L-lysine-coated slides. Sections were deparaffinized and rehydrated in descending alcohol series. Then, sections were immersed in antigen repair buffer (sodium citrate; pH 6.0) and heated in a microwave oven at 100°C for 20 min and allowed to cool naturally. The slides were blocked with 3% H₂O₂ for 10 min at room temperature and 10% normal goat serum (Gibco; Thermo Fisher Scientific, Inc.) with 0.3% Triton X-100 in PBS for 1 h at 4°C.

The sections were incubated with rabbit anti-P2X4 (1:200; cat. no. 13534-1-AP; ProteinTech Group Inc.) and mouse anti-ionized calcium-binding adapter molecule 1 (Iba-1; 1:400; cat. no. ab15690; Abcam) antibodies for 16 h at 4°C. The secondary antibodies were tetraethyl rhodamine isothiocyanate (1:1,000; cat. no. AP31444TC-N; OriGene Technologies, Inc.) conjugated to rabbit anti-P2X4 IgG and fluorescein conjugated to goat anti-mouse IgG (1:5,000; cat. no. BL003A; Biosharp Life Sciences), and the incubation was performed for 1 h at 37°C. Slides were washed three times (5 min/time) with PBS and incubated with DAPI staining solution (1:1,000; cat. no. C1005; Beyotime Institute of Biotechnology) for 10 min at 25°C, then washed a further three times with PBS (5 min/time). Images were captured using a BX41 fluorescence microscope (Olympus Corporation; magnification, x10 and x40). Image-Pro Plus software (version 5.1; Media Cybernetics, Inc.) was used to determine the staining intensity.

Section preparation. The spinal cord sections from rats were prepared as previously described (13,24). Briefly, rats were anesthetized as aforementioned, then transcardially perfused with ~70 ml of ice-cold, oxygenated (95% O₂, 5% CO₂) cutting solution containing: 105.0 N-methyl-D-glucamine, 105.0 HCl, 2.5.0 KCl, 1.2 NaH₂PO₄, 26.0 NaHCO₃, 25.0 glucose, 10.0 MgSO₄, 0.5 CaCl₂, 5.0 L-ascorbic acid, 3.0 sodium pyruvate and 2.0 mM thiourea (pH 7.4, 295-305 mOsm). The lumbosacral section was removed in the cutting solution. All ventral and dorsal roots were cut and the pia-arachnoid membrane was removed. Transverse spinal sections (300 µm) were cut using a vibratome (VT1200S; Leica Microsystems, Ltd.) and placed in an incubator filled with normal oxygenated (95% O₂, 5% CO₂) Krebs solution for at least 30 min at 32°C. The normal Krebs solution contained: 117.0 NaCl, 3.6 KCl, 2.5 CaCl₂, 1.2 MgCl₂, 1.2 NaH₂PO₄, 25.0 NaHCO₃, 11.0 D-glucose, 0.4 ascorbic acid and 2.0 mM pyruvate.

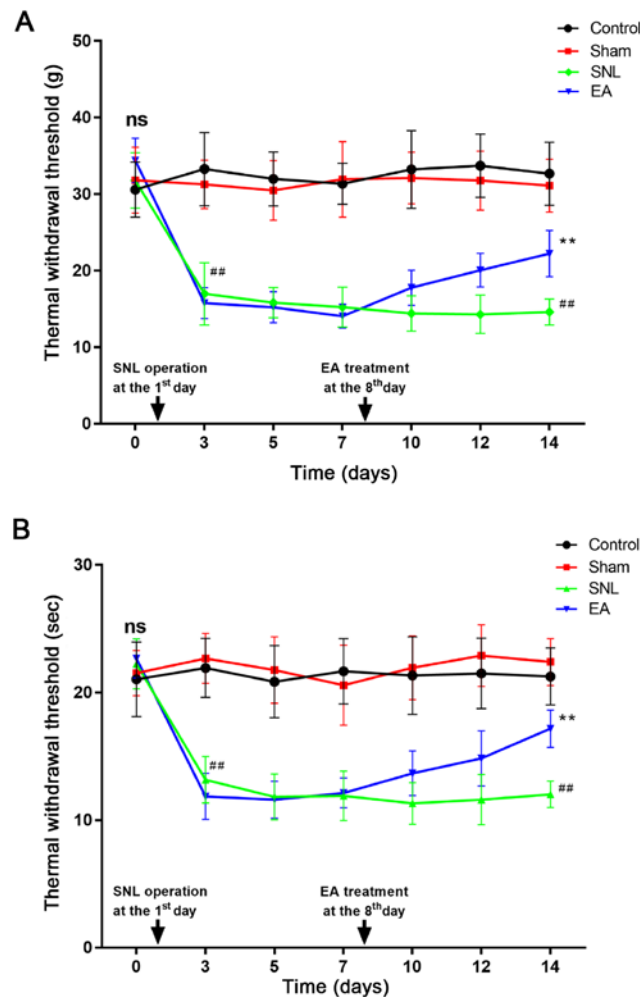


Figure 2. Treatment with EA results in a significant analgesic effect. (A) Mechanical allodynia. (B) Thermal hyperalgesia. The graph demonstrates the changes in withdrawal threshold. The threshold was observed pre- and post-EA. The values of MWT and TWL were notably decreased on day 3 post-SNL. The values of MWT and TWL of rats treated with EA were significantly higher than those in the SNL group. ** $P < 0.01$ vs. SNL group; # $P < 0.01$ vs. sham group. EA, electroacupuncture; MWT, mechanical withdrawal threshold; TWL, thermal withdrawal latency; SNL, L-5 spinal nerve ligation; ns, not significant.

Patch-clamp recordings. The patch-clamp recording procedures were performed as previously described (24,25). Specifically, the section was placed in a recording chamber beneath a BX51WI upright light microscope (Olympus Corporation; magnification, $\times 20$). The procedure of tight-seal, whole-cell patch-clamp and the recordings were performed at room temperature ($22\text{--}24^{\circ}\text{C}$) with artificial cerebrospinal fluid perfusion. SG neurons were identified using an infrared and differential interference contrast camera (cat. no. BX51WI; Olympus Corporation; magnification, $\times 20$ and $\times 100$). The recording pipettes were made from borosilicate glass capillaries (optical density, 1.5 mm; inner diameter, 1.12 mm; Sutter Instrument Company) with a micropipette puller (P-97; Sutter Instrument Company) and had a resistance of 4–6 M Ω when filled with a solution containing: 130.0 K-gluconate, 5.0 KCl, 4.0 Mg-ATP, 10.0 phosphocreatine, 0.3 Li-GTP and 10.0 mM HEPES (pH 7.4 adjusted with KOH, 300 mOsm). The frequency of spontaneous excitatory postsynaptic currents (sEPSC) was recorded using an EPC-10 amplifier with a lowpass filter at 5 kHz using Patchmaster software (version UI325; HEKA Elektronik GmbH; Harvard Bioscience, Inc.).

Statistical analysis. Statistical significance was determined using SPSS Statistics software (version 16.0; SPSS, Inc.). Data are presented as the mean \pm standard error of the mean of three experimental repeats. Behavioral results with multiple comparisons were statistically analyzed by a mixed analysis of variance (ANOVA) for repeated measures, followed by Sidak's test. The other data were carried out using one-way analysis of variance, followed by Tukey's test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

EA reverses SNL-induced mechanical allodynia and thermal hyperalgesia. Baseline measures of MWT and TWL did not differ between groups (Fig. 2). MWT and TWL were recorded on day 3 post-SNL to avoid measuring the effects of postoperative pain, as previously described (13). Mechanical allodynia and thermal allodynia developed at day 3 post-SNL and were sustained until day 14. In the EA groups, all rats were tested 30 min post-EA treatment. As presented in Fig. 2A and B, the values of MWT and TWL in SNL rats notably decreased from day 3 to day 14 post-SNL compared with the control and sham

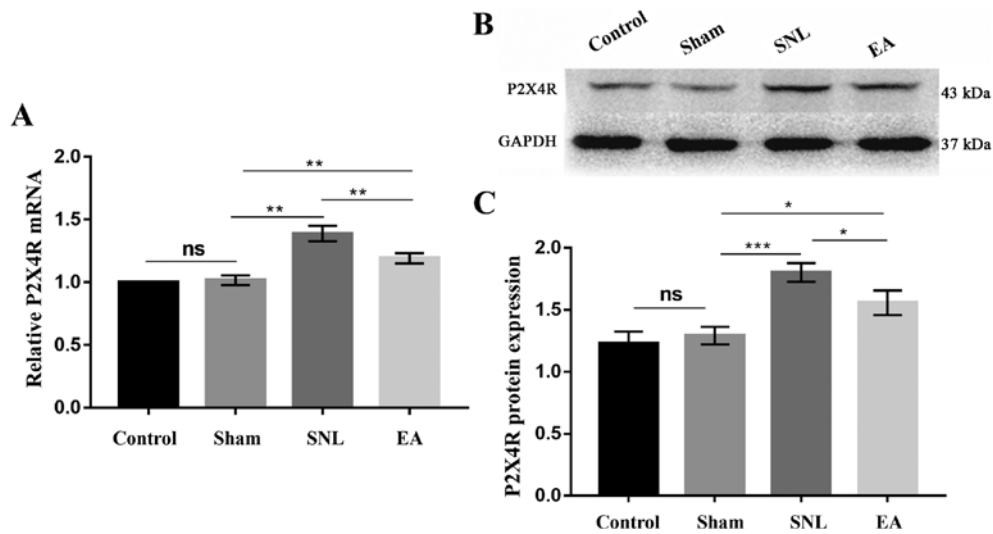


Figure 3. EA prevents P2X4R upregulation in SNL rats. (A) Quantification of relative P2X4R mRNA levels in SNL rats. (B) Western blotting analysis of total P2X4R protein in L4-L6 demonstrated notable upregulation in SNL rats. GAPDH was used as the loading control. (C) Summary data from western blotting experiments. * $P < 0.05$. ** $P < 0.01$. *** $P < 0.001$. EA, electroacupuncture; P2X4R, P2X4 receptor; SNL, spinal nerve ligation; ns, not significant.

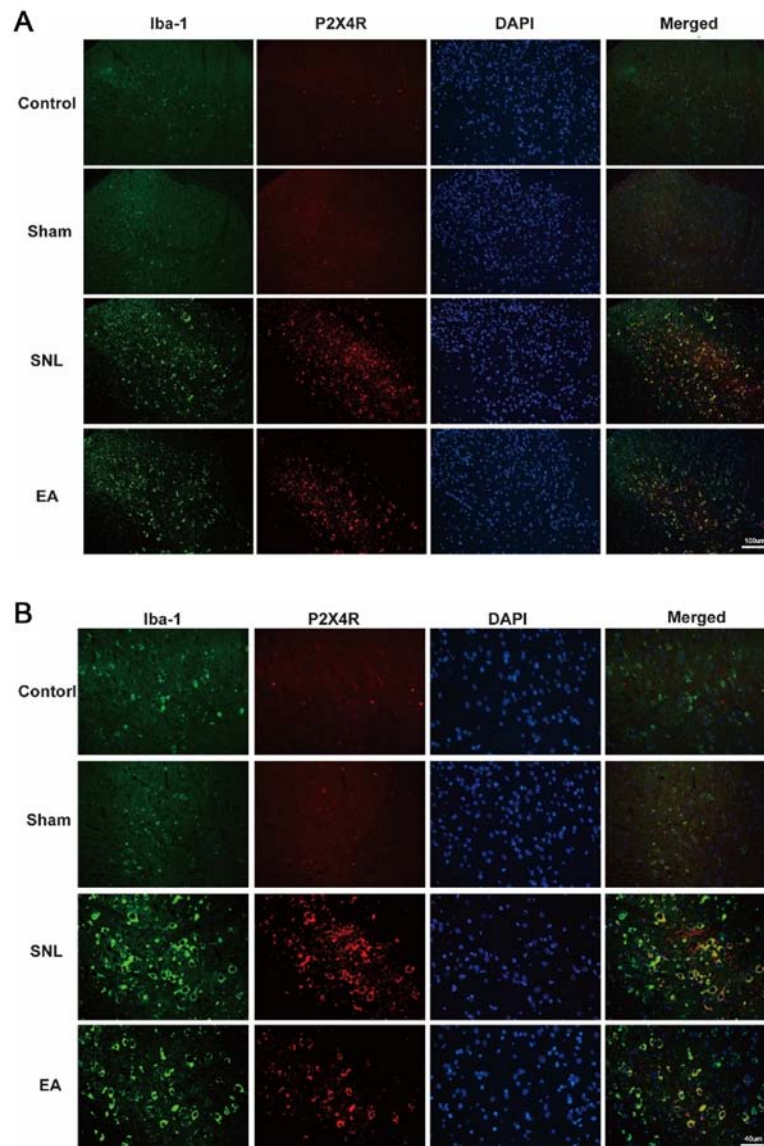


Figure 4. Representative photomicrographs of immunofluorescence of P2X4R (red) and Iba-1 (green) on the ipsilateral spinal dorsal horn on day 14 post-SNL (n=6). Scale bars, (A) 100 and (B) 40 μm . P2X4R, P2X4 receptor; SNL, spinal nerve ligation; Iba-1, ionized calcium-binding adapter molecule 1.

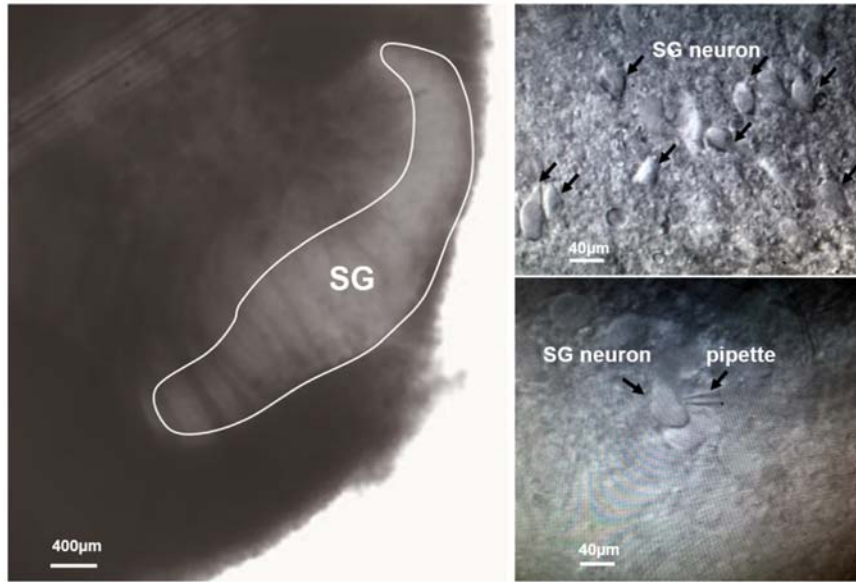


Figure 5. Representative images of the SG region in a transverse spinal cord section from a rat at low (left) and high magnification (right). SG, substantia gelatinosa.

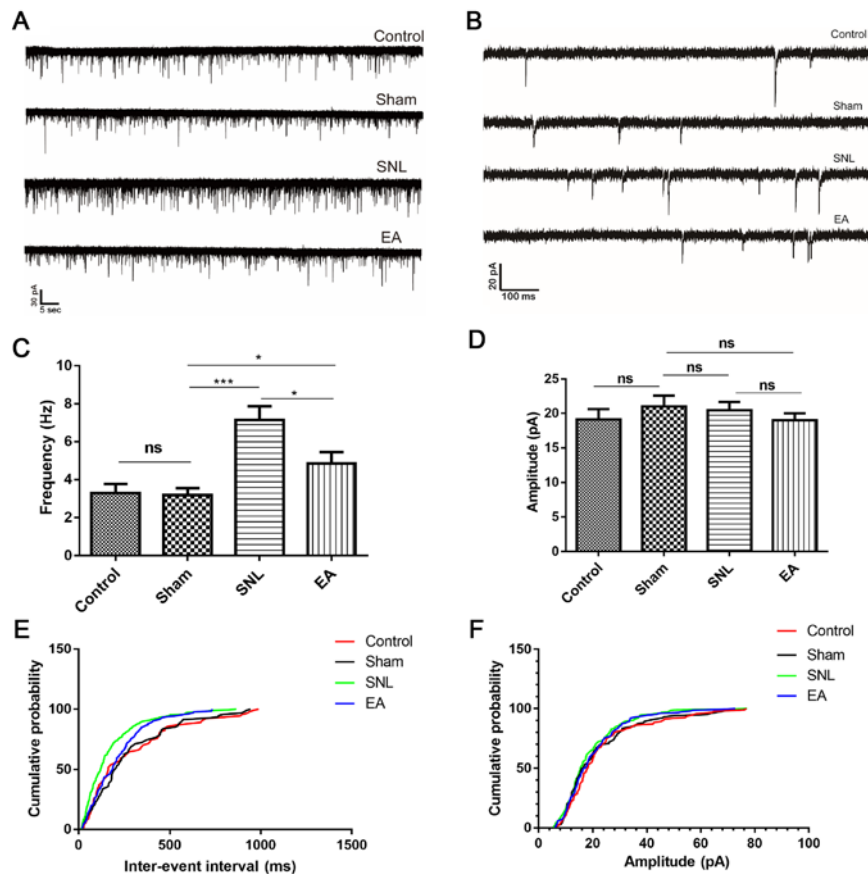


Figure 6. SNL dose-dependently increases the frequency but not the amplitude of sEPSCs in SG neurons. EA treatment decreased the frequency but not the amplitude of sEPSCs in SG neurons. (A) Representative recordings of sEPSCs obtained 2 min at a holding potential of 70 mV after the current stabilized. (B) Enlarged view of representative recordings of sEPSCs. (C and D) Summary bar graphs of sEPSC frequency and amplitude in the four groups. SNL group exhibited an enhanced frequency of sEPSCs in SG neurons. EA treatment lowered the frequency of sEPSCs without altering the amplitude of sEPSCs. (E and F) Cumulative probability of the sEPSCs inter-event interval and amplitude plots from the same neuron. * $P < 0.05$. *** $P < 0.001$. SNL, spinal nerve ligation; sEPSC, spontaneous excitatory postsynaptic currents; SG, substantia gelatinosa; EA, electroacupuncture; ns, not significant.

groups ($P < 0.01$). In the EA groups, the values of MWT and TWL notably increased from day 7 to day 14 compared with

those of the SNL groups ($P < 0.01$). The results indicate that EA relieved pain behavior in SNL rats.

EA decreases expression levels of P2X4R mRNA and protein in the spinal cord of SNL rats. In order to detect the effect of EA and to demonstrate the role of P2X4R in maintaining NP, the mRNA and protein levels of P2X4R in the spinal cord were investigated. As presented in Fig. 3A, P2X4R mRNA expression levels in the SNL group were higher than those in the control and sham groups ($P<0.01$). However, the relative expression levels of P2X4R mRNA in the EA group decreased in comparison with those in the SNL group ($P<0.01$) following 7 days of EA treatment. The results of P2X4R protein expression level analysis are presented in Fig. 3B and C. The relative expression levels of P2X4R protein in the control, sham, SNL and EA groups were 1.229 ± 0.043 , 1.291 ± 0.031 , 1.800 ± 0.034 and 1.557 ± 0.044 , respectively. The SNL group exhibited upregulated P2X4R protein levels compared with those in the control and sham groups ($P<0.001$). The P2X4R protein levels in the EA group were significantly lower than those in the SNL group ($P<0.05$). The results indicate that EA inhibited the upregulation of P2X4R protein expression levels in SNL rats.

EA decreases immunofluorescence staining of P2X4R and Iba-1. Upregulation of ionized calcium-binding adapter molecule 1 (Iba-1) is a marker of microglia activation (26). The results of double immunofluorescence staining are presented in Fig. 4A (magnification, $\times 20$) B (magnification, $\times 40$). The co-expression of P2X4R and Iba-1 was notable in the SNL group in contrast with the control group. The number of P2X4R⁺ microglia in the EA group was significantly lower following EA treatment. These results indicate that EA inhibited microglia activation and suppressed the expression levels of the P2X4R receptor.

EA decreases the frequency of sEPSCs in spinal cord SG neurons in the SNL group. Spinal cord SG neurons are predominantly excitatory neurons (24) and form a nociceptive circuit, which receives input from afferent C-fibers and sends output to lamina I projection neurons (27). It was hypothesized that EA may modulate neurotransmitter release and synaptic transmission by increasing expression levels of P2X4R in the spinal cord. The present study recorded sEPSCs in SG neurons in spinal cord sections from rats (Figs. 5 and 6A and B). The SNL group exhibited an enhanced frequency of sEPSCs in SG neurons ($P<0.001$; Fig. 6C and E) compared with the control and sham groups. In the EA group the frequency of sEPSCs was significantly decreased ($P<0.05$; Fig. 6C and E) but the amplitude of sEPSCs was not significantly altered ($P>0.05$; Fig. 6D and F) compared with the SNL group.

Glutamate AMPA/kainate receptors mediate sEPSCs, and excitatory synaptic transmission causes frequency changes in sEPSCs (28-30). Therefore, EA may inhibit excitatory synaptic transmission by decreasing glutamate release from presynaptic terminals, which may result in EA-induced suppression of P2X4R expression levels in microglia.

Discussion

The results of the present study demonstrated that EA treatment alleviated nerve injury-induced tactile allodynia and thermal hyperalgesia by inhibiting activation of spinal microglia-mediated P2X4R and by regulating the excitability

of neurons in the SG region of SNL rats. The findings of the present study demonstrated the underlying mechanisms of the therapeutic effect of EA on NP in regards to purinergic receptor family modulation.

Acupuncture is used worldwide as a treatment for a number of conditions, particularly for pain (4,31). Zusanli and Kunlun points, first described in 'HuangDiNeiJing-LingShu-BenShu' (an ancient Chinese book, recorded in 200 BC), are commonly used acupoints to treat a number of symptoms (including pain relief) both in clinical practice and in research. It has been reported that EA stimulates the Zusanli point to relieve NP via inhibition of COX2 expression levels, activation of opioid receptors M1 mAChR, β_2 nAChR and endothelin-B receptors, and secretion of neuroactive mediators (32-34). Studies have also demonstrated that acupuncture at the Kunlun point can alleviate NP by inhibiting the p38 MAPK pathway and the expression levels of prostaglandin E2 and G protein-coupled kinase 2 (35,36). In the present study, increased sensitivity to thermal and mechanical stimulation was observed in SNL rats. SNL rats exhibit abnormal hyperalgesia and mechanical irritation, which is similar to human NP symptoms and behavior induced by injury and dysfunction of the peripheral nervous system (37). In the present study, the MWT and TWL in the EA group were significantly increased compared with the SNL group, indicating that EA may relieve mechanical pain and thermal pain in SNL rats. These findings indicated that EA treatment at the Zusanli and Kunlun points may be beneficial in the treatment of NP.

Spinal microglia have been demonstrated to be immediately activated following nerve injury and are necessary for the initiation and maintenance of pain hypersensitivity (38). The ATP receptors P2X4R and P2X7R have been demonstrated to be predominately expressed in the microglia of the spinal cord (11,39,40). Furthermore, a previous study has demonstrated that DRG P2X3R is involved in the analgesic effect of EA in rat models of chronic constriction injury (13). Following binding of ATP, microglial ionotropic P2X4R leads to increased microglia activation, which exaggerates pain states (41). Inhibition of spinal P2X4R⁺ microglia significantly alleviates tactile allodynia induced by nerve injury but not that induced by thermal hyperalgesia (11,42). Moreover, P2X4R knockout has been demonstrated to increase sensitivity to thermal hyperalgesia in an inflammatory mouse model (43). In the present study, the expression levels of P2X4R protein and mRNA were notably decreased compared with those in SNL rats following EA treatment. Therefore, the effects of EA on NP may be associated with the expression levels of P2X4R. Moreover, the results of the present study indicate that P2X4R was co-expressed with Iba-1 in the SDH. These results are consistent with the results of previous studies (11,44). The present study also demonstrated that co-expression of P2X4R and Iba-1 in the SDH of SNL rats was increased compared with that in control rats. Upregulated Iba-1 was associated with the activation of microglia. The results of the present study indicated that microglia were activated following PNI. Following EA treatment, the co-expression of P2X4R with Iba-1 in the SDH was decreased compared with that in the SNL group. EA may attenuate the transmission of nociceptive information by inhibiting the expression levels of P2X4R in SDH microglia, thus relieving pain behaviors in SNL rats.

sEPSCs were the most important indicator reflecting the excitatory transmission of neurons recorded. In general, the frequency of sEPSC changes was associated with presynaptic mechanisms. Increased presynaptic transmitter release led to increased sEPSC frequency. The amplitude of sEPSC was associated with pre- and post-synaptic mechanisms. In the present study, whole-cell patch clamp results demonstrated no significant difference in the amplitude of sEPSCs in spinal SG neurons in the four groups, but the frequency of sEPSCs was significantly different. Compared with the control group, the SNL group exhibited higher sEPSC frequency in SG nociceptive neurons. Additionally, the present study demonstrated that EA significantly decreased the frequency of sEPSCs but did not affect the amplitude of sEPSCs. These results indicated that EA may attenuate the transmission efficiency between synapses in the SG region of the SDH during NP by decreasing the excitability of neurons that transmit pain signals from the peripheral nerves to the spinal cord. In addition, previous studies have also demonstrated that EA has anti-inflammatory effects (45-48) and that microglia activation is associated with inflammation (49). The SNL rat model can induce both NP and inflammatory pain (50-52). The present study indicates that the mechanism underlying EA treatment of NP involves the transmission efficiency between synapses.

In light of the downregulated P2X4R mRNA and protein expression levels in the EA group, the present study demonstrated that the analgesic effects of EA analgesia may be mediated in part by P2X4R. In future, pharmacological, chemogenic and optogenetic methods may be used to further characterize the analgesic effects of EA mediated by P2X4R.

In conclusion, the present study demonstrated that mechanical allodynia and thermal hyperalgesia may be attenuated by the analgesic effects of EA. EA may exert analgesic effects by inhibiting P2X4R-mediated activation of spinal microglia and decreasing the excitability of neurons in the SG region of SNL rats. However, further research is required in order to verify these effects and to identify the underlying molecular mechanism of EA in animals with PNI.

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Availability of data and materials

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YZ, CJ, XJ, JC, XC and XY performed the laboratory experiments, collected and analyzed the data and interpreted the results. KZ wrote the manuscript. JW, MJ and GY analyzed the data. MJ and GY revised the manuscript. KZ, WT and SJ designed the experiments, supervised the study and revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All experiments were approved by the Institutional Animal Care and Use Committee of Wenzhou Medical University (approval no. WMU 174890).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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