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Electroacupuncture inhibits excessive interferon-γ evoked up-regulation of P2X4 receptor in spinal microglia in a CCI rat model for neuropathic pain

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Editor's key points

- Electroacupuncture (EA) is effective in neuropathic pain but mechanisms are unclear.
- In a rat model of nerve injury, EA decreased allodynia and interferon-γ (IFN-γ) mediated microglial activation.
- The mechanism of the analgesic action of EA is via effects on IFN-γ and microglial cells.

Background. Although electroacupuncture (EA) is effective in the relief of neuropathic pain, the underlying mechanisms remain unclear. Previous studies have reported immunomodulatory effects of EA in rats. Since excessive release of interferon-γ (IFN-γ) after nerve injury transforms quiescent spinal microglia into an activated state with more neuropathic pain, associated with purinergic receptor P2X4 expression, it is possible that EA may mediate its analgesic effect by attenuating IFN-γ release and subsequent generation of P2X4R$^+$ microglia.

Methods. Male rats underwent chronic constriction injury (CCI) or IFN-γ intrathecal injection and von Frey tests were performed to evaluate the effect of EA on pain thresholds. Spinal IFN-γ and P2X4R expression levels were measured by immunohistochemistry, real-time PCR, enzyme immunoassay, and/or western blots. *In vitro* primary cultures of microglia were used to examine IFN-γ activation of P2X4R$^+$ cells.

Results. In CCI rats, EA treatment significantly increased paw withdrawal threshold relative to control. IFN-γ facilitated P2X4R$^+$ microglia activation both *in vitro* and *in vivo*. EA also down-regulated both P2X4R and IFN-γ expression in the spinal cord after CCI. However, EA did not exert the same analgesic effect after intrathecal IFN-γ injection.

Conclusions. EA ameliorated tactile allodynia after peripheral nerve injury by down-regulating excessive expression of IFN-γ in the spinal cord and subsequently reducing expression of P2X4R.

Keywords: acupuncture; interferon-γ; P2X4 receptor; neuropathic pain; spinal cord

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Electroacupuncture (EA) is a viable therapeutic intervention for chronic pain caused by nerve injury,[1–3] but the biological basis of its action remains unknown. In the last few decades, a great deal of research has focused on down-regulating neuronal activities by releasing neurotransmitters and modulators like opioids, serotonin, noradrenalin, adenosine, etc., and lately the importance of microglia in establishing and maintaining chronic neuropathic pain has emerged.[3–9] Indeed, inhibition of microglia activation attenuated nerve injury-induced pain hypersensitivity.[10 11] However, resent research showed that the P2X4 receptor (P2X4R), an ATP-gated purinergic receptor expressed on microglia, drove tactile allodynia after nerve injury, and pharmacologically blocking or genetically knocking out the P2X4R dramatically reduced pain hypersensitivity, even if microglia were highly activated.[12–14] The relationship between EA relief of mechanical allodynia and P2X4R positive (P2X4R$^+$) microglia is unclear.

Interferon-γ (IFN-γ) is a pro-inflammatory cytokine that has been implicated in the activation of microglia in neuropathic pain. Genetic knockout of the IFN-γ receptor impeded nerve injury-induced pain, and intrathecal injection of IFN-γ in naïve rats can lead to significant tactile allodynia.[15 16] IFN-γ also up-regulated spinal P2X4R, whereas such stimulation in P2X4R deleted mice blunted allodynia.[17 18]

Several studies have shown that EA has an immunomodulatory effect,[19–21] and we have found that EA attenuates release of pro-inflammatory cytokines in rats treated with endotoxin.[22] As an extension of this work, we hypothesized that EA would suppress spinal P2X4R by inhibiting spinal IFN-γ release and thereby relieving tactile allodynia induced by nerve injury.

Methods

Animals

Experiments were performed on adult male Sprague–Dawley rats weighing 200–250 g. Rats were housed in a temperature-(22–24°C) and light-controlled (12 h light–dark cycle) room with free access to food and water. Before experimental manipulation, rats were allowed to acclimatize to the housing facilities and were handled daily for at least 3 days. Anaesthetized

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animals were killed after experimentation by exposure to carbon dioxide under anaesthesia. All experimental protocols and animal-handling procedures were approved by the Animal Care Committee of Shanghai Jiao Tong University (Shanghai, China) and were in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health and complied with relevant sections of the ARRIVE guidelines.

Surgery

The chronic constriction injury (CCI) model in rats was used as previously described by Bennett *et al.*[23] Briefly, male rats were anaesthetized with i.p. sodium pentobarbital (40–60 mg kg^{-1}) and adequate anaesthesia was ascertained by lack of response to a nociceptive stimulus. The right common sciatic nerve was loosely tied with four chromic catgut ties after exposure at the mid-thigh level (spaced ~1 mm apart). In this way, the nerve was slightly constricted, and the circulation through the epineural vasculature was not interrupted. Rats in the sham group underwent surgery, but without nerve constriction after sciatic nerve exposure.

For intrathecal administration in rats, under sodium pentobarbital anaesthesia, a 32-gauge intrathecal catheter (ReCathCo, Allison Park, PA, USA) was inserted through the atlanto-occipital membrane into the lumbar enlargement and externalized through the skin.[17 24] A single intrathecal dose of IFN-γ (1000 U; Millipore, Billerica, MA, USA) or artificial cerebrospinal fluid (ACSF, in mM: 126 NaCl, 2.5 KCl, 1.25 $NaH_2PO_4 \cdot H_2O$, 26 $NaHCO_3$, 25 dextrose, 2 $MgSO_4$, and 2 $CaCl_2$) was adminstered using a 25 μl Hamilton syringe.

EA treatment

The EA points used in this study were Huantiao (GB 30), which is located at the lateral 1/3 and medial 2/3 distance between the sacral hiatus and the greater trochanter of the femur. Stainless steel needles were inserted bilaterally to Huantiao point (GB 30) at a depth of 7 mm, and stimulation (current of 2 mA, 2 Hz) was delivered using an electrical stimulation device (HANS LH-202, Huawei Co., Beijing, China) for 30 min daily.[10] The treatment started on the day after CCI surgery or IFN-γ intrathecal injection and lasted for 14 days. Rats were awake and immobilized in a purpose built Perspex restrainer with the head, tail, and hind limbs protruding. The rats were habituated to the restrainer for 3 days before starting the treatment.

Behavioural tests

Paw withdrawal thresholds (PWT) were evaluated by the von Frey test using a calibrated series of von Frey 'hairs' ranging from 1 to 26 g. After habituation to the test chambers, the hairs were applied to the central region of the plantar surface of the right hind paw in ascending order (1, 1.4, 2, 4, 6, 8, 10, 15, and 26 g). Each hair was applied five times at 5 s intervals, and three withdrawals out of the five consecutive applications were considered responsive. The PWT was defined as the lowest hair force in grammes that produced a withdrawal response.

Microglia culture

Rat primary cultured microglia cells were prepared in accordance with a method described previously.[14] In brief, a mixed glial culture was prepared from neonatal Sprague–Dawley rats and maintained for 10–16 days in DMEM with 10% FBS. Microglia cells were collected immediately as the floating cells over the mixed glial culture by a gentle shake of the culture flasks, and transferred to coverslips for subsequent experiments. The purity of cultures reached about 95%, as determined by immunostaining of OX42.

Immunohistochemistry and immunocytochemistry

After defined survival times, rats were anaesthetized as described above and perfused transcardially with saline followed by 4% paraformaldehyde (PFA) with 4% sucrose in 0.1 M phosphate buffer (PB, pH 7.4). The L4–L5 segments of spinal cord were then removed, post-fixed in the same fixative for 4 h at 4°C, and immersed in 30% sucrose in PB for 24–48 h at 4°C. Transverse spinal sections (14 μm) were cut in a microtome cryostat and processed for immunofluorescence. Microglial cells were fixed with 4% PFA for 20 min at room temperature. All the sections or coverslips were blocked with 10% normal goat serum in 0.01 M phosphate-buffered saline (PBS, pH 7.4) with 0.3% Triton-X-100 for 1 h at room temperature and incubated over 48 h at 4°C with rabbit anti-P2X4R (1:500, Alomone, Jerusalem, Israel) or mouse anti-OX42 for microglia cells (1:100, Millipore, Billerica, MA, USA), mouse anti-GFAP for astrocyte cells (1:1000, Sigma, St Louis, MO, USA), and mouse anti-NeuN for neurones (1:200, Chemicon). The sections were then incubated for 2 h at room temperature with Alexa Fluor™ 488-conjugated goat anti-mouse IgG (1:500, Molecular Probes, Carlsbad, CA, USA), Alexa Fluor™ 594-conjugated goat anti-rabbit IgG (1:1000, Molecular Probes), and DAPI (1:1000, Sigma). Omission of the primary antibody served as a negative control. The stained sections were examined with a fluorescence microscope (BX-51, Leica, Wetzlar, Germany), and images were captured with a CCD spot camera.

Quantitative real-time PCR

Real-time amplification, using an iCycler (Bio-Rad, Hercules, CA, USA) and iQSYBR Green supermix (Bio-Rad), was performed on 4 ng cDNA derived from spinal cord segments L4–L5 ipsilateral to the injured nerve. RNA preparation and cDNA synthesis was performed as described.[25] Primers used for PCR were obtained from Invitrogen (Carlsbad, CA, USA): P2X4R, sense GGGTGAAGTTTTATTCCAGC, antisense GGGTGAAGTTTTCTGCAGCC; IFN-γ, sense ATCTGGAGGAACTGGCAAAAGGACG, antisense CCTTA GGCTAGATTCTGGTGACAGC; GAPDH, sense CTTCACCACCATGGA GAAGGC, antisense GGCATGGACTGTGGTCATGAG.

Western blotting

The protein extracted from spinal cord or cultured microglia was quantified using the DC protein assay (Bio-Rad). Equivalent protein were subjected to 8% SDS–PAGE, and transferred electrophoretically to nitrocellulose membranes. After blocking with 1% BSA, membranes were incubated with anti-rat P2X4R polyclonal antibody (1:1000; Alomone) or β-actin polyclonal

antibody (1:4000; Sigma) and then were incubated with horseradish peroxidase-conjugated secondary antibody. The blots were detected using a chemiluminescence method (ECL system, GE Healthcare, Chalfont St Giles, UK) and exposed to radiography films.

Enzyme immunoassay

The protocol used in this study was a variation of that described by Lin *et al.*[26] Rats were anaesthetized and perfused with ice-cold PBS, and the L4–L5 spinal cords ipsilateral to the nerve injury were removed, then immediately homogenized in 250 μl of PBS with complete protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland) using a motorized homogenizer. The extracts were incubated on ice for 5 min, and centrifuged at 10 000 *g* for 10 min. The protein content of each extract was determined by the DC protein assay. IFN-γ was determined using a murine commercially available enzyme immunoassay kit (R&D Systems, Minneapolis, MN, USA) following the manufacturer's instructions.

Statistical analysis

Data are expressed as mean and SD, and the Lilliefors test was used to assess distribution of the data. Both pre-EA and pre-IFN-γ treatment baseline measures for von Frey test were analysed by one-way analysis of variance (ANOVA). Post-treatment time course measures for PWT were analysed by two-way ANOVA (treatment × time) followed by Newman–Keuls *post-hoc* test. For groups where $n \leq 5$ data were assumed to be non-parametric and were analysed using appropriate tests.

Results

EA-induced relief of tactile allodynia in CCI rats is accompanied by inhibition of up-regulated P2X4R on spinal microglia

EA treatment increased PWT in CCI rats

Baseline measures of PWT to von Frey hairs on both hind paws did not differ between EA and CCI groups. Following unilateral sciatic nerve constriction, evident tactile allodynia developed within 1 day and persisted over 14 days in the ipsilateral hind paw. PWT in contralateral hind paws and bilateral hind paws of the sham group rats remained stable for 14 days. EA on bilateral Huantiao acupoints (GB 30) resulted in increased PWT on Day 2 and showed significant improvement by Day 5. On Day 14, PWT of the EA group was greatly improved and there was no statistical difference between the EA and sham groups. Two-way ANOVA analysis revealed a significant effect of EA treatment ($P<0.01$) and interaction between EA treatment and time ($P<0.01$) (Fig. 1).

EA treatment reduced spinal P2X4R expression in CCI rats

There was a prominent increase in $P2X4R^+$ microglia spinal cords of CCI rats. In addition, the number of $P2X4R^+$ microglia (identified by P2X4R and OX-42) was significantly suppressed after EA treatment (Fig. 2A). We also found that P2X4R was specifically expressed on microglia, rather than spinal astrocytes (identified by glial fibrillary acidic protein, GFAP) or neurones (identified by neuronal nuclei, NeuN) (Supplementary material Fig. S1). Data from real-time PCR indicated that elevated P2X4R expression in CCI rats was suppressed by EA on Day 3, and this difference between the EA group and CCI group was amplified on Days 7 and 14 ($P<0.01$). Western blot showed similar results at the same protein level ($P<0.01$) (Fig. 2B and C).

EA attenuates overexpression of IFN-γ in spinal cord of CCI rats

A marked decrease in IFN-γ mRNA and protein was seen in the ipsilateral spinal cord after EA relative to CCI rats (Fig. 3). A decrease in mRNA expression was seen on Day 3 and continued to decline; at Days 7 and 14 both mRNA and protein expression were decreased (EA compared with CCI $P<0.01$; CCI compared with Sham, $P<0.01$; EA compared with Sham, $P<0.05$).

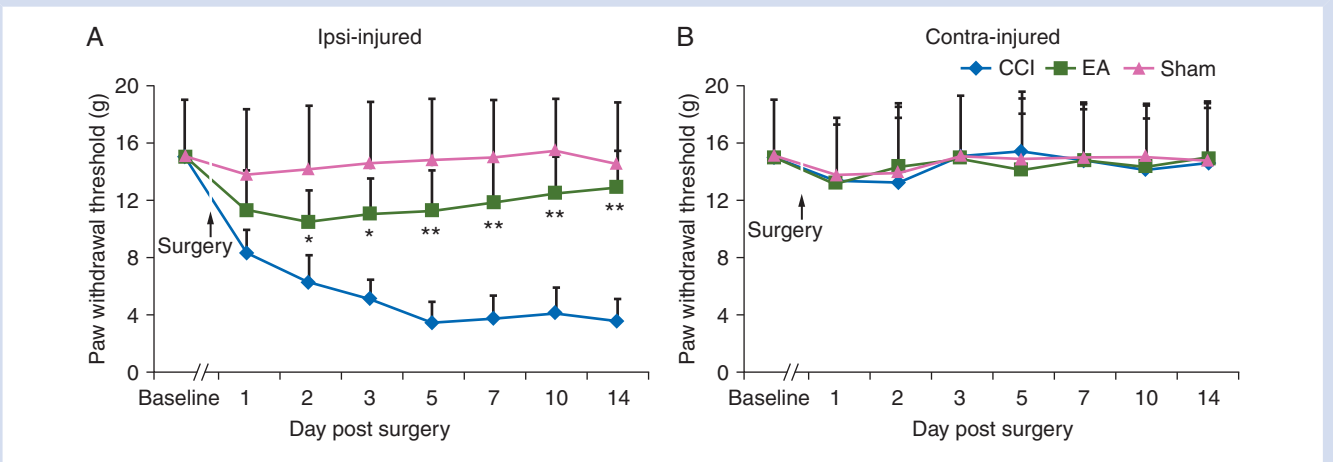

Fig 1 PWTs for von Frey test in rats with chronic sciatic nerve constriction. (A) ipsilateral; (B) contralateral paw. EA significantly reduced allodynia shown by raised ipsilateral PWT. Mean (SD), $n=12$.

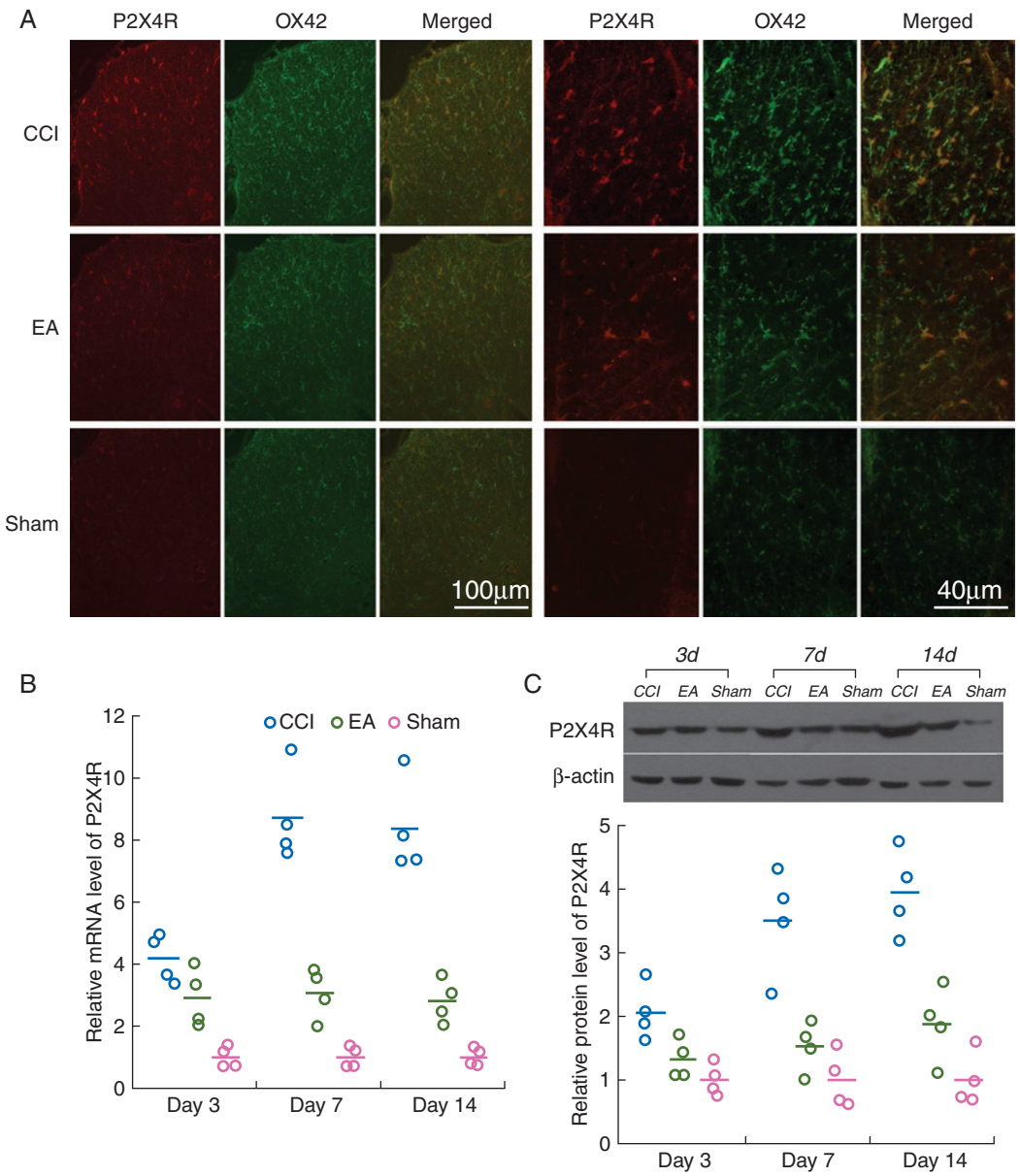

Fig 2 P2X4R expression in spinal dorsal horn from rats undergoing chronic sciatic nerve constriction. (A) Representative photomicrographs of immunofluorescence of P2X4R (red) and OX42 (green) on the ipsilateral spinal dorsal horn on Day 14 post-nerve injury. (B) Real-time PCR showing relative mRNA level of P2X4R in spinal dorsal horn ipsilateral to injured nerve post-surgery (individual data points shown, $n=4$ for each group). (C) Representative western blot of P2X4R protein detected by anti-P2X4R antibody and relative level in the spinal cord ipsilateral to the nerve injury post surgery (individual data points shown, $n=4$ for each group).

IFN-γ signalling is responsible for P2X4R$^+$ microglia activation

In culture microglia show classic ramified morphology, antler-like fine processes, and slender cytoplasm. When treated with 50 U ml^{-1} of IFN-γ for 24 h, P2X4R$^+$ microglia displayed an activated appearance with large cell bodies and short and thick processes, and the percentage of P2X4R$^+$ cells rose from 16.7% to 77.2% (Fig. 4A). Western blotting showed that P2X4R protein expression was markedly elevated after treatment with IFN-γ at three different doses at all time points ($P<0.01$) (Fig. 4B).

EA has no effect on analgesia or P2X4R$^+$ microglia in naive rats after intrathecal injection of IFN-γ

In line with the previous work, intrathecal injection of IFN-γ produced marked and long-lasting tactile allodynia.[17] PWT applied to the hind paw progressively decreased within 1 day, peaked on Day 3, and persisted for at least 10 days after the

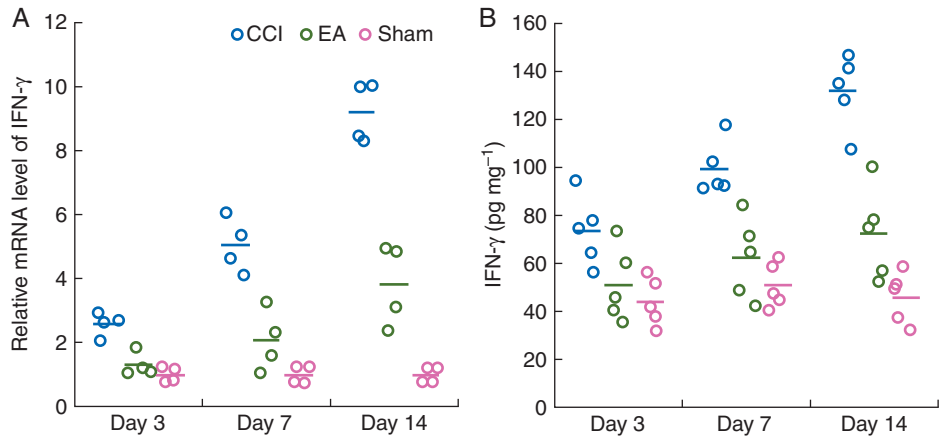

Fig 3 IFN-γ expression in spinal dorsal horn from rats undergoing chronic sciatic nerve constriction. (A) Real-time PCR (n=4) and (B). IFN-γ measured by enzyme immunoassay (n=5) on different days post-surgery (individual data points shown).

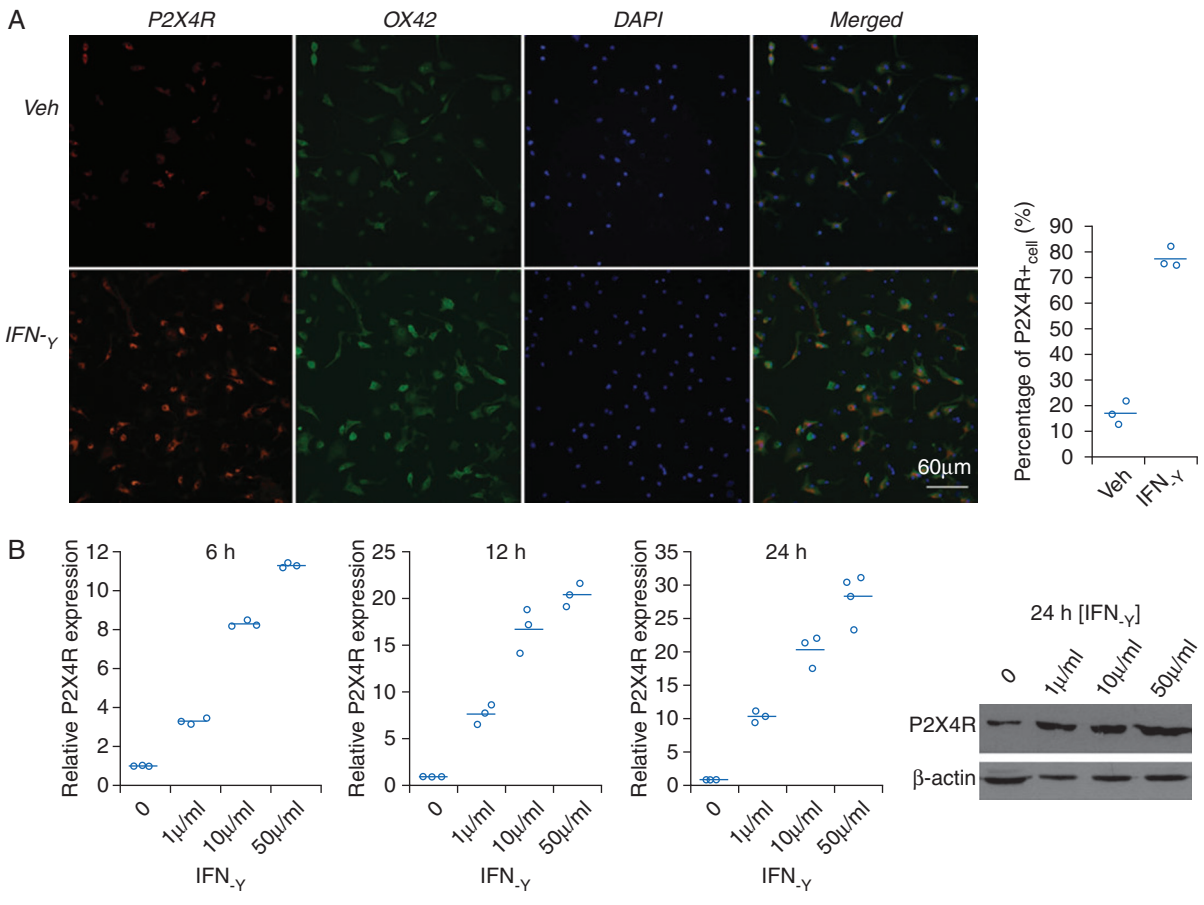

Fig 4 P2X4R expression in cultured microglia. (A) Representative photomicrographs of immunofluorescence in microglia (P2X4R, red; OX42, green) with or without IFN-γ treatment for 24 h. Percentage of P2X4R$^+$ cells are shown on the right. (B) Representative western blot (right) and relative expression (left) of P2X4R in cultured cells with or without IFN-γ treatment. Individual data points shown, n=3 for each group. **P<0.01.

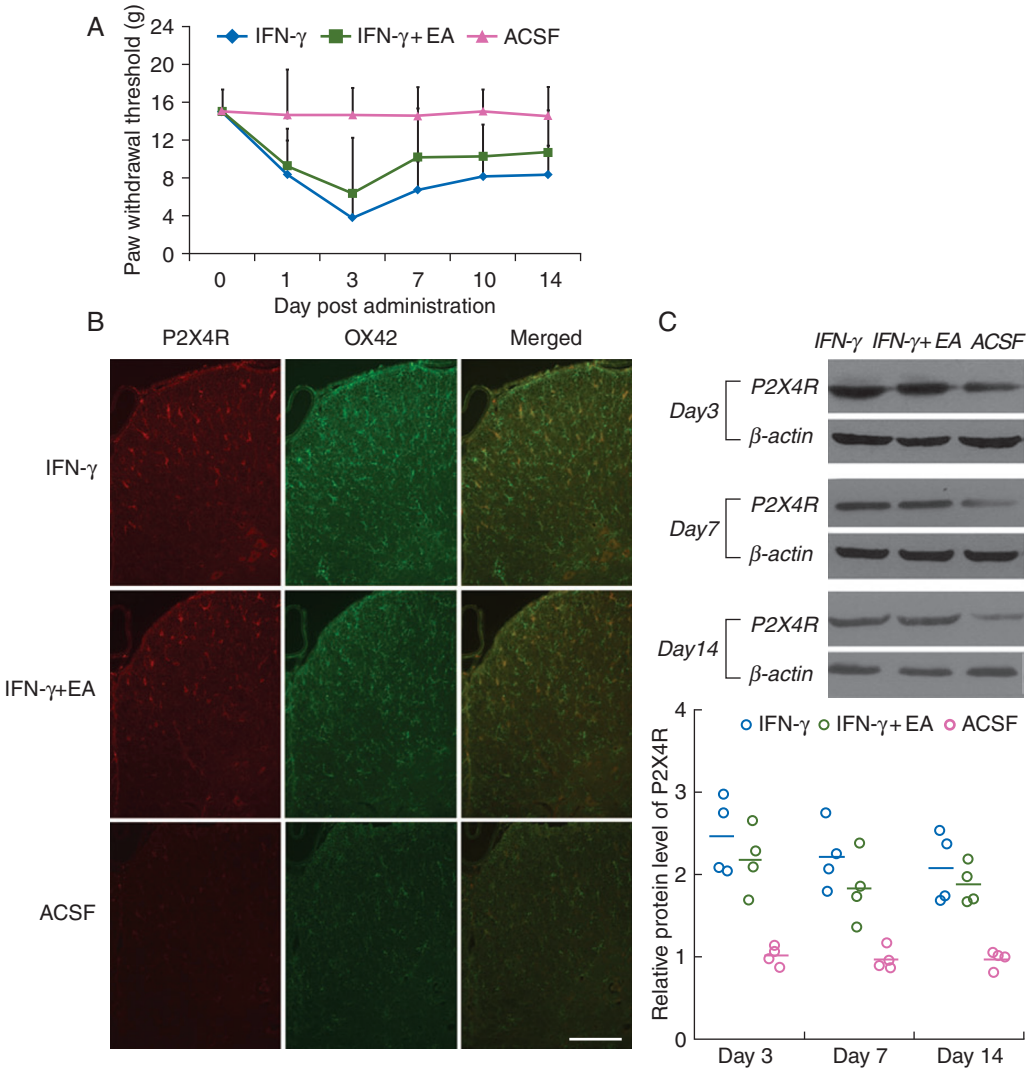

Fig 5 Effect of EA on rats after spinal administration of IFN-γ. (A) von Frey PWT. Mean (SD), $n=10$ for each group. (B) Representative photomicrographs of immunofluorescence in spinal microglia in rats on Day 7 after intrathecal injection of IFN-γ. P2X4R (red) and OX42 (green); scale bar, 100 μm. (C) Representative western blot (top) and relative P2X4R expression (bottom, $n=4$) in spinal dorsal horn on different days after IFN-γ administration. Individual data points shown.

administration (IFN-γ or IFN-γ+EA compared with ACSF, $P<0.01$). Daily EA treatment did not significantly affect IFN-γ-induced PWT (Fig. 5A). Immunofluorescence showed that intrathecal injection of IFN-γ also promoted P2X4R$^+$ microglia, and these changes were not reversed by EA (Fig. 5B). Western blots indicated that P2X4R protein expression was also not affected by EA for up to 14 days after IFN-γ administration (Fig. 5C).

Discussion

The current study demonstrated that EA treatment significantly inhibited spinal P2X4R$^+$ microglia in CCI rats, and this occurred concomitantly with a marked decrease in mechanical allodynia. Importantly, reducing IFN-γ expression in the spinal cord of CCI rats, but not disrupting IFN-γ function and/or IFN-γ receptor signalling cascades in microglia, down-regulated P2X4R-induced by EA. Taken together, these results suggest that the microglia-to-neurone interactions mediated by spinal microglia P2X4Rs seem to be attenuated by EA via its immunomodulatory effects in the spinal cord.

Acupuncture originated in China, and is now an accepted alternative therapy for various diseases in both Eastern and Western countries. Acupuncture-induced analgesia has been used widely to alleviate various types of pains, particularly chronic pain.[1 4] Recent studies have evaluated the mechanisms underlying the beneficial effects of acupuncture or EA in neuropathic pain.[27–29] Various neurotransmitters and neuromodulators, including primarily opioid peptides, serotonin, norepinephrine, glutamate, γ-amino-butyric acid and adenosine,

are believed to be responsible for the beneficial effects of EA on neuropathic pain.[3–7] We believe this is the first study to identify P2X4R-positive cells, a specific phenotype of microglia, as an important target of EA in analgesia. Microglia are known as resident macrophages in the central nervous system, and they switch immediately from a 'resting' to 'activated' state after nerve injury. This switch is characterized by proliferation and morphological changes identified by microglial markers like OX-42 or Iba1. Previous work has already verified that EA can suppress spinal microglia activation in a rat model of monoarthritis,[10 11] as evidenced by reduced OX-42 expression and stereotypic microglial changes. Microglial cells expressing P2X4R are pivotal cellular intermediaries in the pathogenesis of nerve injury-induced pain hypersensitivity.[12] Proliferation and morphological changes of microglia *per se* are insufficient to produce pain hypersensitivity, since pharmacological blockade or genetic knockout of P2X4R in nerve-injured mice resulted in a robust increase in cell number and evident morphological change, but no pain hypersensitivity.[12] We demonstrated in this current study that EA treatment attenuated progression of mechanical allodynia by suppressing spinal P2X4R expression in CCI rats.

IFN-γ is unique to other inflammatory cytokines since it promotes spinal P2X4R expression and induces allodynia.[17 18 30] We demonstrated here that the anti-allodynic effect of EA is via down-regulation of IFN-γ. IFN-γ is predominantly produced by Th1 lymphocytes (one of the two major subtypes of T helper lymphocytes) in peripheral blood and may be the initiator of spinal inflammation after nerve injury.[15] Blood T lymphocytes can infiltrate into the spinal cord and interact with microglia through releasing pro-inflammatory cytokines and contribute to the maintenance of neuropathic pain.[31–33] In accordance with these findings, others have reported that allodynia induced by nerve injury was attenuated in rodents using immunosuppressants or elimination of T lymphocytes.[15 34–36] The present work showed that EA treatment greatly reduced spinal IFN-γ expression in CCI rat, which suggested EA may mediate its effect through modulation of IFN-γ signalling.

Here, we provide evidence linking EA-induced analgesia and P2X4R$^+$ microglia immunomodulation. We demonstrated previously that EA produced an immune-modulatory effect with increased survival in rats with endotoxaemia.[22] Similarly, Torres-Rosas and colleagues recently reported that EA at the sciatic nerve regulated systemic inflammation and down-regulated serum IFN-γ levels in a mouse model of sepsis.[37] Perhaps similar experimental approaches will provide further insight into the mechanism of EA modulation of neuropathic pain.

We also addressed whether EA treatment reduced spinal expression of IFN-γ or modulated IFN-γ function and/or signalling cascades in microglia. Therefore, IFN-γ was administered intrathecally to naïve rats to mimic the allodynia of neuropathic pain. In this experiment, EA treatment was therapeutically ineffective. Thus, EA modified spinal P2X4R$^+$ microglia, at least in part, by reducing IFN-γ release in the spinal cord. Increased fibronectin, Lyn tyrosine kinase and CCL2 were also reported to up-regulate microglial P2X4R or promote its trafficking to the cell surface after peripheral nerve injury.[38–40] Whether EA suppresses this up-regulation of P2X4R is currently under investigation, but our present results show that IFN-γ is crucial in the molecular processes mediating the down-regulation of P2X4R in spine.

In addition, P2X4R has only been shown to contribute to tactile hyperalgesia induced by peripheral nerve injury.[14] The analgesic effect of EA on thermal hyperalgesia and inflammatory pain indicates the existence of other modulated pathways. Indeed, a more thorough understanding of the mechanisms underlying these phenomena will contribute to the refinement of therapeutic strategies for a variety of neurological disorders.

In summary, we found that EA produced an analgesic effect on neuropathic pain via an immunomodulation process. Excessive spinal IFN-γ release induced by nerve injury was greatly inhibited by EA, which reduced P2X4R$^+$ microglia and reversed the pathophysiological process of neuropathic pain.

Supplementary material

Supplementary material is available at *British Journal of Anaesthesia* online.

Authors' contributions

X.-M. C., J. X. and B.-J. Z. were responsible for conduct of the study. J.G. S. undertook statistical analysis. X.-R. W. designed the study and prepared the first draft of the manuscript. All authors approved the final version of the paper.

Declaration of interest

None declared.

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