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ORIGINAL RESEARCH

Electroacupuncture Regulates Macrophage Polarization to Alleviate the Neuropathic Pain Induced by Spared Nerve Injury

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Purpose: The current therapeutic strategies for neuropathic pain have limited efficacy. The activation of macrophages and pro-inflammatory responses following peripheral nerve injury can effectively prevent the progression of neuropathic pain. Macrophage polarization to the M2 or M1 (respectively anti- and pro- inflammatory) phenotypes frequently occurs during neuroinflammation. Electroacupuncture (EA) therapy has been shown to exert anti-inflammatory functions in several pain models, and has thus been applied to alleviate neuropathic pain. Therefore, the present study aimed to determine whether EA could reduce neuroinflammation and induce analgesia by regulating macrophage polarization.

Methods: Forty-five male rats were used to create a spared nerve injury (SNI) model of peripheral nerve injury. Subsequently, EA was applied to the ipsilateral *huantiao* (GB30) and *yanglingquan* (GB34), and the von Frey assay was conducted to monitor the effect of EA on the paw withdrawal threshold. Immunofluorescence analyses were further performed to detect the effects of EA on interleukin-1β (IL-1β) expression and peripheral macrophage polarization.

Results: EA attenuated pain behavior (P=0.002) and decreased inflammatory cytokines derived from macrophages (P=0.002 in the sciatic nerve; P=0.002 in the dorsal root ganglion, DRG) but not in Schwann (P>0.05) or mast (P>0.05) cells in SNI rats. In addition, EA increased M2 macrophage polarization (P<0.0001 in the sciatic nerve; P=0.001 in the DRG) and decreased M1 macrophage expression (P=0.036 in the sciatic nerve; P=0.022 in the DRG).

Conclusion: These data revealed that EA exerted analgesia by adjusting the polarization of macrophages and inhibiting the IL-1β expressing in macrophages in SNI rats.

Keywords: electroacupuncture, macrophage polarization, neuropathic pain, neuroinflammation, analgesia

Introduction

Neuropathic pain is caused by disease or primary lesion influencing the somatosensory system.[1] Clinical symptoms include paralgesic effects, such as hyperalgesia, spontaneous pain, and allodynia.[2] Neuropathic pain significantly affects sufferers' ability to work or participate in daily routines, seriously impacting quality of life.[3] The current therapies supply symptomatic mitigation to 1/4 of patients.[4] Dose-limiting and resistance side effects of available drugs make treating neuropathic pain more difficult. Therefore, the development of more effective therapies for neuropathic pain is essential.

Acupuncture is a non-pharmacological therapy with few side effects that has received increasing attention worldwide. The World Health Organization lists 43 different diseases which acupuncture treatment is suitable for, including asthma, addiction, stroke rehabilitation, and pain.[5] The analgesic effect is the most known advantage of acupuncture. Electroacupuncture (EA) is a treatment method that combines manual acupuncture with electrical stimulation to achieve similar or superior clinical effects to manual acupuncture.[6]

Cytokines released by immune cell activation are critical in the pathogenesis of neuropathic pain.[7] Macrophages, as a type of immune cell, display a series of reactions to injury, from resting to classical activation (M1 macrophages) or to

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alternative activation (M2 macrophages). During neuropathic pain, nerve injury promotes M1 macrophage phenotypic polarization by increasing the levels of pro-inflammatory factors. Increasing M2 macrophage polarization and suppressing M1 macrophage polarization have been confirmed to attenuate neuropathic pain.[8] Based on the above findings, we hypothesized that EA improves the neuroinflammatory response by regulating peripheral macrophage polarization to alleviate neuropathic pain.

Materials and Methods

Animals

Adult male Sprague-Dawley rats (180–200 g) were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. All procedures in this study were approved by the Animal Care Committee of Beijing University of Chinese Medicine (BUCM-4-2,020,112,302-4073), and performed in accordance with the ethical guidelines of the International Association for the Study of Pain and the Animal Research Reporting of In Vivo Experiments (ARRIVE) reporting guidelines.[9] The rats were housed in specific pathogen-free conditions with ad libitum access to standard food and water with a 12 h light-dark cycle.

Antibodies

Polyclonal rabbit anti-IL-1β antibody was purchased from GeneTex (Irvine, CA, USA; 1:1000). Polyclonal goat anti-Iba-1 and monoclonal mouse anti-mast cell tryptase antibodies were purchased from Abcam (Cambridge, MA, USA; 1:100 and 1:1000, respectively). Monoclonal mouse anti-CD206 antibody was purchased from Proteintech (USA; 1:800 dilution). Polyclonal rabbit anti-iNOS antibody was purchased from Thermo Fisher Scientific (USA; 1:800 dilution). The monoclonal mouse anti-S100 beta antibody was obtained from Proteintech (USA; 1:1000 dilutions). Secondary antibodies corresponding to the primary antibodies–donkey anti-rabbit IgG conjugated with Dylight 594 (1:400), donkey anti-goat IgG conjugated with Dylight 488 (1:400), and donkey anti-mouse IgG conjugated with Dylight 488 (1:400)–were purchased from Jackson ImmunoResearch.

Spared Nerve Injury (SNI) Surgery

The SNI model is a known animal model that mimics several characteristics of clinical neuropathic pain relevant to peripheral nerve injury,[10] and is easy to operate and replicate compared with other neuropathic pain models, such as the chronic constriction injury (CCI), partial sciatic ligation (PSL) and spinal nerve ligation (SNL) models. It is further possible to study injured and non-injured tissues separately using SNI.

The rats were assigned to four groups: Sham, SNI, SNI+ EA, SNI+ Sham EA. Rats were randomly chosen to undergo SNI or Sham surgeries by a technician who did not conduct any of the pain-related behavior, histology, or molecular pathway experiments, or statistical analysis. Some rats died due to anesthesia and restraint, leaving only 12 rats in the Sham group, 10 in the SNI group, 12 in the SNI+ EA group, and 11 in the SNI+ Sham EA group.

Rats were anaesthetized with 1% sodium pentobarbital (0.4 mL/100 g, i.p)., and the SNI procedure was executed on the left posterior limb, as described previously.[11] In brief, the common peroneal, tibial, and sural nerves were separated, after which the tibial and common peroneal nerves were ligated with 5–0 sutures and severed far from the ligation site, disconnecting the 2 to 4 mm stump from the terminal nerve. The sural nerve was retained intact. Sham animals underwent an equivalent operation to expose the nerves, without any severance. One day later, the SNI rats were randomized into three groups using the random number table method to receive no treatment, EA or Sham EA. The rats with motor dysfunction or paralgesia were excluded from the study. The rats were observed daily for self-harm.

EA Treatment

The most commonly used acupoints for relieving neuropathic pain in humans and rodents are *huantiao* (GB30) and *yanglingquan* (GB34).[12,13] Anatomically, GB 30 is located on the sciatic nerve and GB34 is situated on the common peroneal nerve. SNI is induced by injury to the tibial and common peroneal nerves. EA stimulation with 2 Hz at 1-2-3mA for 30 min has a better analgesic effect on neuropathic pain than 100 Hz EA.[14] Han et al[15] previously reported that repeated

or prolonged EA resulted in EA tolerance. To avoid tolerance, 2 Hz EA was applied once every alternate day. Overall, in the present study, rats in the SNI+EA group were subjected to EA on the ipsilateral GB30 and GB34 starting from days 1 to 21 post-SNI surgery, once every other day, 11 times. EA (2 Hz) was administered at 1-2-3 mA for 30 min. The current was transferred using a Han's Acupoint Nerve Stimulator (LH202; China). In the SNI+ Sham EA group, the needles were shallowly inserted at both the GB30 and GB34 acupoints, without manual manipulation or electrical stimulation.

Mechanical Allodynia

Mechanical allodynia was estimated by calculation of the 50% paw withdrawal threshold (PWT), which responded to a range of von Frey filaments (Ugo Basile, Ita), and were measured using the "up-and-down" method.[16] A total of 8 von Frey filaments were selected (0.4 g, 0.6 g, 1.0 g, 2.0 g, 4.0 g, 6.0 g, 8.0 g and 15.0 g). Every test began with 2.0 g vertically imposed on the ipsilateral plantar for roughly 2–3 s. The sudden withdrawal of stimulation was recorded as a significant reaction. When a negative or positive reaction occurred, larger or smaller filaments were used, respectively. Processing was repeated six times after the first difference was observed. If a rat did not react to the 15.0 g filament, 15.0 g was recorded. If a rat reacted to the 0.4 g filament, 0.25 g was noted.

Immunofluorescence

Immunofluorescence was performed as previously described.[17] Twenty-one days after the SNI procedure, rats were anesthetized with 1% sodium pentobarbital (0.4 mL/100 g, i.p.) and subjected to intracardiac perfusion with 0.9% saline followed by 4% paraformaldehyde. The left sciatic nerve and L4-L6 dorsal root ganglia (DRG) were post-fixed in 4% paraformaldehyde for 6–8 h. Tissues were subsequently dehydrated in 30% sucrose. Tissues were sections into 20μm slices on a cryostat (CM1950, Leica). The slices were blocked with phosphate buffer containing 5% donkey serum for 1h at 37°C, and incubated with primary antibodies at 4°C overnight. Subsequently, sections were incubated with secondary antibodies. The slices were counterstained with the nuclear marker DAPI (100 ng/mL, Sigma-Aldrich) carrying blue fluorescence for 10 min at room temperature, mounted on slides, coverslipped with glycerin-gelatin, and then cover-slipped onto a confocal imaging system (FV1200, Olympus, Tokyo, Japan).

Statistical Analysis

IBM SPSS Statistics 21.0 and GraphPad Prism 9 software were applied for statistical analyses, and all results were displayed as the mean ±SEM. Parametric or non-parametric statistical analysis of the data were applied depending on the pass of the normal distribution testing. For data comparison between different groups at a certain time point, immuno-fluorescence was analyzed by one-way ANOVA with Dunnett's test (equal variance) or Dunnett'sT3 multiple comparison test (unequal variance). Statistical significance was set at $P < 0.05$.

Results

To assess the analgesic effect of EA on SNI, the von Frey test was conducted before (baseline, BL) and on days 1, 3, 5, 7, 14, and 21 after surgery (Figure 1A). In the SNI group, the rats showed a lower PWT one day after the surgical procedure compared to the Sham group and were retained for not less than 21 days (Figure 1B). EA enhanced PWT from day 3 compared to SNI rats. Sham EA did not relieve mechanical allodynia in rats with SNI.

Increasing evidence has indicated that pro-inflammatory cytokine enhancement-induced neuroinflammation may contribute to the progression and maintenance of neuropathic pain. In order to investigate the analgesic mechanism of EA, the levels of IL-1β expressed in the sciatic nerve and DRG was tested. The expression of IL-1β in the sciatic nerve and DRG was enhanced in SNI rats. EA treatment inhibited the expression of IL-1β, but not Sham EA (Figure 1C-F).

EA Inhibited Immune Cells Expansion Induced by SNI

The first cells to respond to peripheral nerve injury are the innate immune cells, including macrophages, mast cells, and Schwann cells. The number of macrophages and mast cells and the fluorescence intensity of Schwann cells in the ipsilateral sciatic nerve and DRG of the SNI group were significantly increased compared to the Sham group. Treatment with EA, but not Sham EA, alleviated the expansion of immune and Schwann cells (Figure 2).

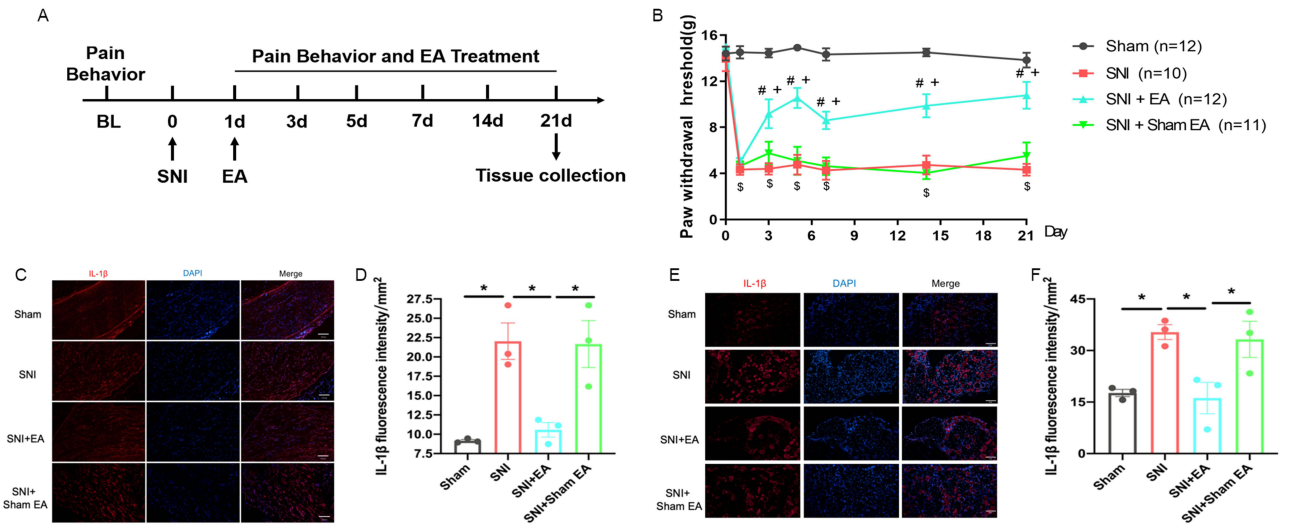

Figure 1 The effect of EA on persistent mechanical hypersensitivity and IL-1β expression. Timeline of the experimental program (**A**) and the time course of paw withdrawal threshold testing (**B**). Immunofluorescence images of sciatic nerve (**C**) and DRG (**E**) sections showing IL-1β (red) staining with DAPI (blue)-labeled cell nuclei. Merged panels show double-labeled cells. Summary results show the fluorescence intensity of IL-1β at sciatic nerve (**D**) and DRG (**F**). Results are displayed as the mean ± SEM. The numbers of rats are indicated by points or numbers in the figure. $P<0.05, compared with the Sham group; #P < 0.05, compared with the SNI group; +P< 0.05, compared with the SNI+ Sham EA group; *P<0.05, compared between two groups.

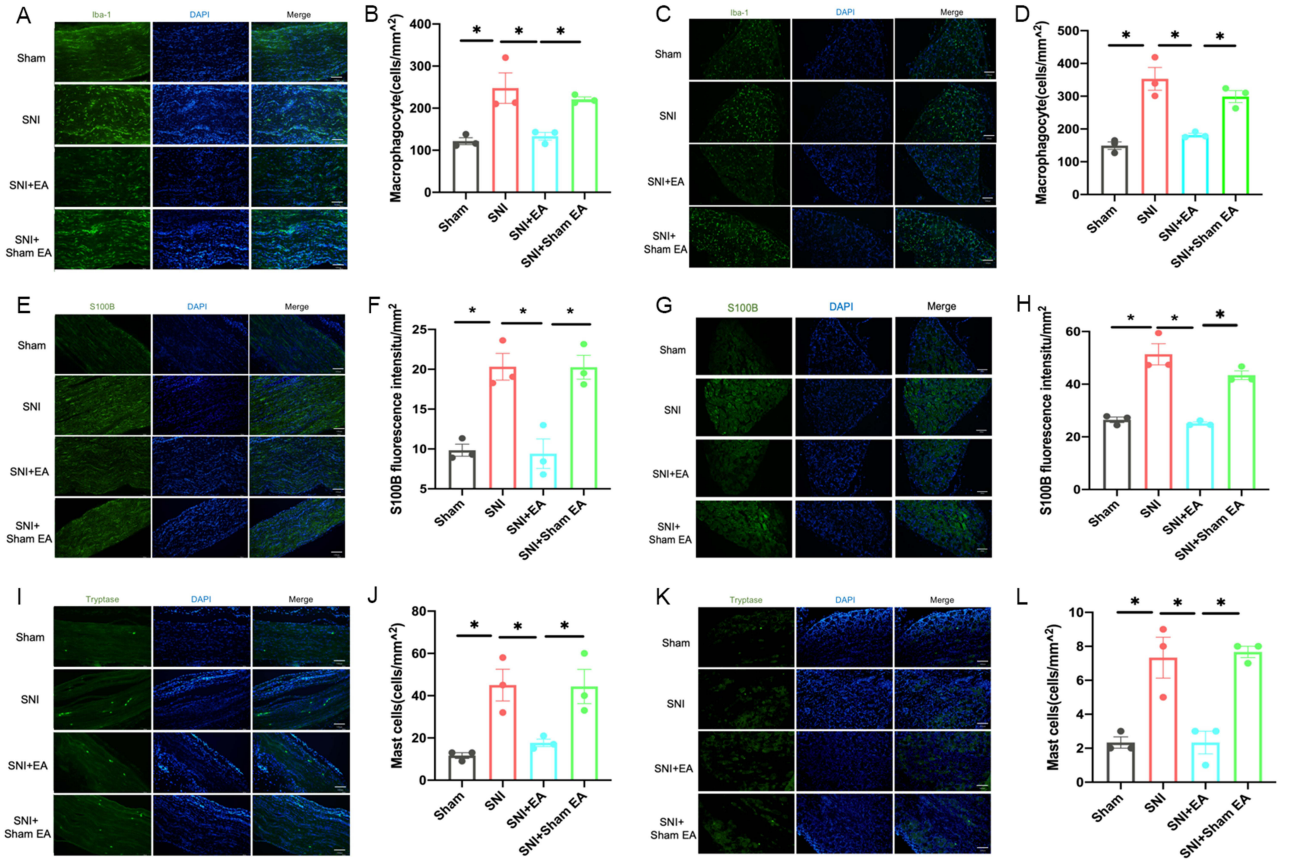

Figure 2 The effect of EA on immune cells expansion induced by SNI. Immunofluorescence images of sciatic nerve (**A, E** and **I**) and DRG (**C, G** and **K**) sections showing Iba-1 positive-macrophages (green, **A**, **C**), S100B positive-Schwann cells (green, **E**, **G**), and tryptase-positive mast cells (green, **I**, **K**), with DAPI (blue)-labeled cell nuclei. Merged panels show double-labeled cells. Summary results show the number of macrophages (**B** and **D**) and mast cells (**J** and **L**), and the fluorescence intensity of Schwann cells (**F** and **H**) in the tissue sections. Results are displayed as the mean ± SEM. The numbers of rats are indicated by points in the figure. *P < 0.05, compared between the two groups.

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EA Decreased IL-1β Derived From Macrophages

To determine which cells expressed IL-1β in the SNI rats, we performed immunofluorescence with double-labeling. IL-1β was primarily co-expressed with macrophages labeled with Iba-1, not with tryptase (mast cell marker) and S100B (Schwann cell marker) (Figure 3). Consistently, EA, but not Sham EA, rats showed decreased IL-1β expression in macrophages.

EA Regulated Macrophage Polarization

We further investigated the percentage of M2 and M1 macrophages in the sciatic nerve and DRG. Immunofluorescence double-labeling experiments reveled that SNI rats had higher cell numbers of the marker of M1 macrophage polarization, iNOS, but not the marker of M2 macrophages, CD206. EA, but not Sham EA, decreased the number of M1 macrophages and increased the number of M2 macrophages in the sciatic nerve and DRG (Figure 4).

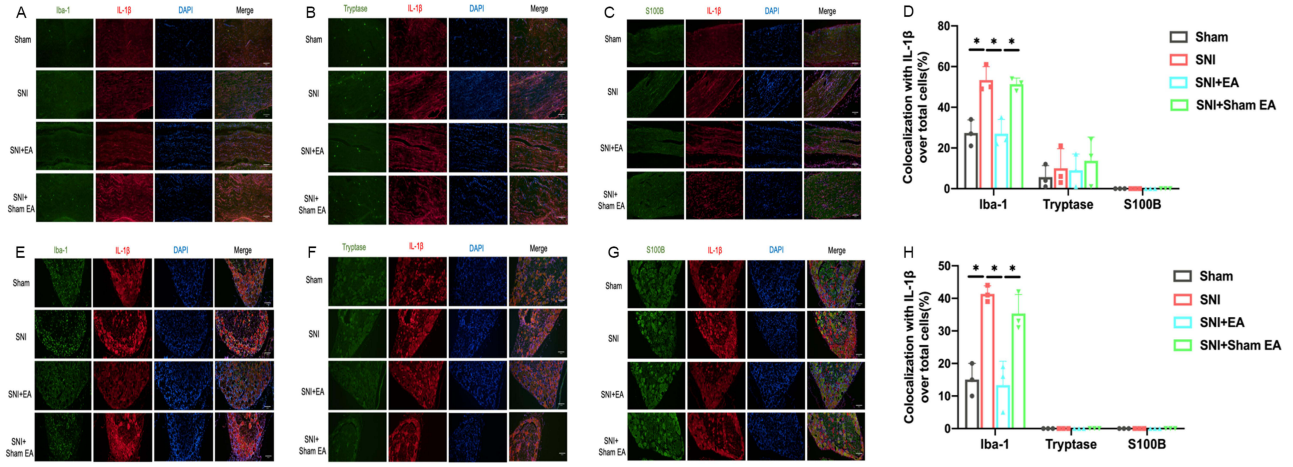

Figure 3 Effect of EA on IL-1β derived from macrophages. Immunofluorescence images of sections showing Iba-1 positive-macrophages (green, (**A** and **E**), Tryptase positive-mast cells (green, (**B** and **F**), S100B positive-Schwann cells (green, (**C** and **G**), and IL-1β cells (red). DAPI (blue)-labeled cell nuclei. Merged panels show double-labeled cells. Summary results displaying the proportion of double-labeled cells in the sciatic nerve and DRG (**D** and **H**). Results are displayed as mean ± SEM. The numbers of rats are indicated by points in the figure. *P < 0.05, compared between the two groups.

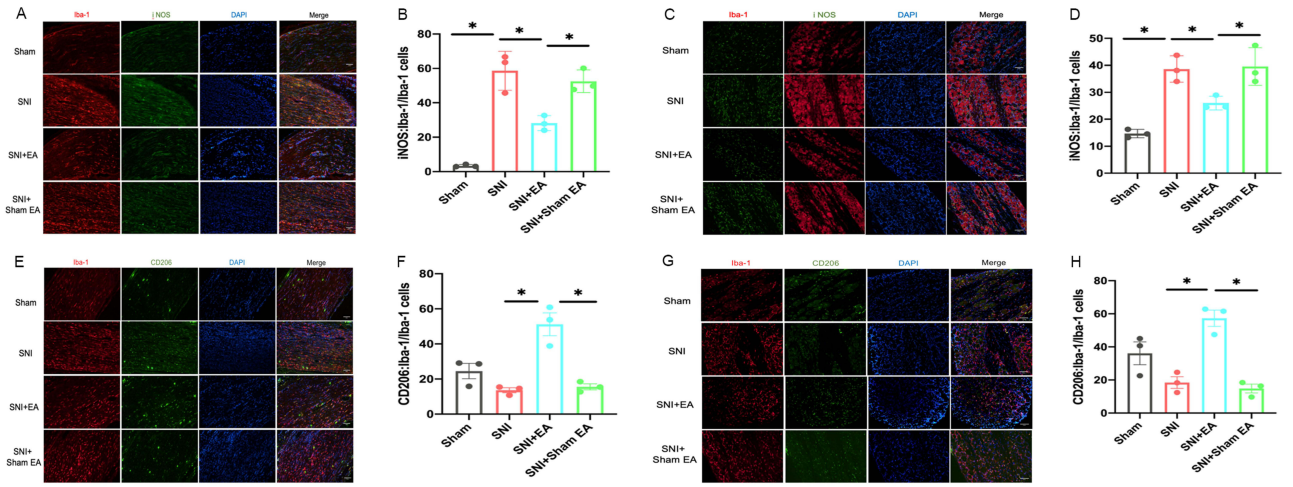

Figure 4 Effect of EA on macrophage polarization. Immunofluorescence images of sciatic nerve and DRG sections showing iNOS immunopositive macrophages (green, (**A** and **C**), CD206 immunopositive macrophages (green, **E** and **G**), and Iba-1 positive-macrophages (red). DAPI (blue)-labeled cell nuclei. Merged panels show double-labeled cells. Summary results display the proportion of double-labeled cells in sciatic nerve (**B** and **F**) and DRG (**D** and **H**) sections. Results are displayed as mean ± SEM. The numbers of rats are indicated by points in the figure. *P < 0.05, compared between the two groups.

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Discussion

In the present study, we investigated the anti-inflammatory effects of EA on neuropathic pain in an SNI rat model used to mimic mechanical pain. Overall, our results demonstrated that EA could relieve mechanical nociception and reduce IL-1β expression derived from macrophages in the sciatic nerve and DRG of SNI rats and that these roles of EA may be accomplished by modulating the macrophage polarization paradigm.

Neuropathic pain, derived from the pathology of the nervous system, induces hyperalgesia and allodynia. Four animal models of nerve injury have thus far been applied to simulate neuropathic pain in humans: CCI,[18] PSL,[19] SNL[20] and SNI model.[11] These models are not particularly consistent with human symptoms, but all exhibit allodynia and hyperalgesia in PWT experiments. In SNI model rats, the tibial and common peroneal nerves are sheared remotely from the sciatic nerve trunk without damaging the sural nerve in SNI rats. The uninjured sural nerve ensures hyperalgesia and allodynia. Because of the additional material left in situ, we observed no excessive inflammation. Cellular and biochemical studies were conducted on injured and noninjured tissues at the central (spinal cord) and peripheral (nerve and DRG) levels of the SNI. The animals used for SNI have been expanded from rats to mice and juvenile rats.[21,22] In the present study, we explored the effects of EA on neuropathic pain using an SNI rat model. Neuropathic pain involves events that promote pain sensitivity in the sensory nervous system. Pain hypersensitivity is primarily characterized by both thermal (heat and/or cold) and mechanical allodynia. The withdrawal response latency induced by nociceptive heat stimulation was not modified in SNI rats.[11]

Prior research has shown that EA modified a feedback regulation function of multiple afferent pathways through multiple pathways, including the stimulation-frequency, -intensity, and -intervals of EA stimulation. Several studies have shown that different types of neuropeptides are released by EA at different frequencies,[23] with low frequency EA being most effective for neuropathic pain.[24] Indeed, one study showed that 2 Hz EA induced a longer lasting inhibitory effect on mechanical allodynia than 100 Hz, as assessed using von Frey filament assay.[25] As previously reported, 2 Hz EA stimulation, with intensity increasing in a stepwise manner over 30 min, exerts better analgesic effect on neuropathic pain than 100 Hz EA.[14] Some results have further demonstrated that prolonged or repeated EA stimulation would lead to EA tolerance.[15,26] Accordingly, we selected 2 Hz, 1-2-3mA EA treatment of SNI-induced neuropathic pain rats with once every other day. To examine the analgesic effects of EA in neuropathic pain induced by SNI, we evaluated mechanical allodynia in SNI model rats. Consistent with a previous report,[27,28] model rats showed mechanical allodynia that lasted for at least 21 days after SNI. EA treatment attenuated the mechanical allodynia induced by SNI, demonstrating that it may have a beneficial effect on neuropathic pain, but the underlying mechanism needs to be explored.

Multitudinous evidence has indicated that the immune responses induced by non-neuronal cells, such as immune cells and neuroinflammation may participate in neuropathic pain. Localized inflammation induced by peripheral nerve injury mediates mechanical hypersensitivity. Schwann cells weaken the medullary sheath at the damaged position, which is an essential precondition for nerve regeneration.[29] Furthermore, in the context of neuropathic pain, mast cells degranulate, inducing cytokines to sensitize nociceptors and promote mechanical and thermal hypersensitivity, highlighting the significance of the immune reaction in neuropathic pain.[30,31] Infiltrating macrophages are recruited to the location of resident macrophages and participate in the phagocytosis of the medullary sheath. In addition to their phagocytic function, macrophages release cytokines.[32] The exhaustion of macrophage circulation ameliorates hypersensitivity and allodynia in models of neuropathic pain.[33] Consistently, in the current study, the quantity of mast cells and macrophages and the fluorescence intensity of Schwann cells at the ipsilateral sciatic nerve and DRG is increased in SNI rats. Previous study has reported that EA stimulation was significantly reduced the number of mast cells, together with the alleviation of pain hypersensitivity.[34] EA alleviated SNI-induced neuropathic pain and macrophages expression by activated the autophagy process in DRG.[35] Similarly, we currently found that treatment with EA significantly decreased the quantity of mast cells and macrophages and the fluorescence intensity of Schwann cells. Furthermore, we demonstrated that EA regulated IL-1β expression, which was derived from macrophages in the sciatic nerve and DRG after SNI.

Neuroinflammation has been confirmed to participate in the etiopathogenesis of neuropathic pain.[36–38] Tissue-resident macrophages play a vital role in homeostasis. Resident and recruited macrophages proliferate and undergo functional and phenotypic changes in response to factors released by neurons or immune cells in the local tissue microenvironment. Many

studies have shown that macrophages can be sensitized into M1 or M2 macrophages in a polarizing manner.[39] Under physiological conditions, homeostasis of the inflammatory microenvironment is sustained by the balance between M2 and M1 macrophages. Upon tissue injury, macrophage can shortly respond triggering classical activation.[40] Macrophage polarization to the pro-inflammatory M1 macrophage has been shown to be involved in the advancement of neuropathic pain.[41,42] In accordance with previous studies, we found that SNI facilitated polarization of macrophages to the M1 phenotype. The analgesic effect of acupuncture act through phenotypic modulation involving the inhibition M1 macrophage polarization.[43] Meanwhile, prior research has shown that EA may alleviate pain by regulating the number of macrophages, as well as promoting the M2 polarization of local macrophages, and inhibiting the release of pro-inflammatory cytokines.[44] Consistently, in this study, the level of M1 macrophages in SNI rats treated with EA stimulation was decreased, but the M2 macrophages was obviously increased, indicating that EA balances the polarization of M1 and M2 macrophages to ameliorate neuropathic pain.

The present study has some limitations. First, we primarily focused on the regulation of macrophage polarization by EA. However, we did not investigate the underlying mechanisms. Second, a previous study has implicated DRG-resident macrophages in both the genesis and maintenance of neuropathic pain; however, we did not explore which type of macrophages in the sciatic nerve or DRG are more associated with the anti-inflammatory and analgesic effects of EA. Further studies are required to address the limitations of the present study.

Conclusions

Taken together, EA can effectively relieve neuropathic pain by adjusting the ratio of M1- and M2-polarized macrophages, which may be one of the mechanisms underlying its analgesic and anti-inflammatory effects. In addition, we will explore how EA influences other immune cells or cytokine pathways in neuropathic pain in different animal models or in human studies in the future research.

Abbreviations

Animal Research: Reporting of In Vivo Experiments (ARRIVE); chronic constriction injury (CCI); dorsal root ganglia (DRG); electroacupuncture (EA); interleukin-1β (IL-1β); partial sciatic ligation (PSL); paw withdrawal threshold (PWT); spared nerve injury (SNI); spinal nerve ligation (SNL).

Data Statement

All data in the current findings are attainable from the corresponding author.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

All authors declare no competing interests.

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