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ORIGINAL RESEARCH

Electroacupuncture Inhibits NLRP3-Mediated Microglial Pyroptosis to Ameliorate Chronic Neuropathic Pain in Rats

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Background: Patients with neuropathic pain (NP), caused by injury or disease of the somatosensory nervous system, usually suffer from severe pain. Our previous studies revealed that electroacupuncture (EA) stimulation could effectively improve NP. However, the underlying mechanisms of EA have not been fully clarified. This study aimed to investigate the specific mechanisms of EA in alleviating NP, focusing on the pyroptosis.

Materials and Methods: Chronic Constriction Injury (CCI) model was established on the male Sprague-Dawley rats. CCI rats were treated with EA at acupoints GV20 and ST36 or/with the NOD-like receptor protein 3 (NLRP3) antagonist MCC950. EA treatment was administered for successive 14 days 7 days after the CCI surgery. The mechanical withdrawal threshold (MWT) and paw withdrawal latency (PWL) were performed during the experiment. At the end of the experiment, spinal cord segments and serum of rats were collected, ELISA detected the expression of inflammatory factors, immunofluorescence detected the microglia and neuron cells with pyroptosis biomarkers, and Western blot detected the NLRP3 pathway.

Results: EA treatment significantly alleviated pain hypersensitivity by increasing the MWT and PWL. Moreover, EA reduced levels of pro-inflammatory cytokines IL-1β and TNF-α in the spinal tissue. Mechanistically, the pyroptosis-related proteins, including NLRP3, N-GSDMD, Cleaved Caspase-1, IL-18 as well as IL-1β were downregulated by EA, indicating that EA attenuated the pyroptosis phenotype in NP rats. In particular, EA reduced the co-expression of NLRP3, Caspase-1 and N-GSDMD in microglia rather than in neuronal or astrocytic cells within the spinal cord of CCI rats. Pharmacological inhibition of NLRP3 inflammasome by MCC950 alleviates CCI-induced pain hypersensitivity while blocking EA's effect on anti-pyroptosis in CCI rats.

Conclusion: These findings demonstrate the EA ameliorates the neuroinflammation and pyroptosis to relieve chronic NP by suppressing NLRP3 inflammasome activation in microglia. EA may serve as a viable treatment therapy for chronic NP.

Keywords: NLRP3 inflammasome, electroacupuncture, neuropathic pain, pyroptosis, neuroinflammation

Introduction

Neuropathic pain (NP) is a persistent and irreversible condition caused by peripheral and/or central nerve injury, affecting an estimated 500 to 800 million individuals worldwide.[1] Patients with NP often experience symptoms such as allodynia and hyperalgesia, which significantly worsen the quality of life and impose a tremendous burden on public health.[2,3] NP remains a substantial clinical challenge due to its complex pathophysiology and limited effective treatments. In clinical, anti-depressants and anti-convulsants are recommended as the first-line treatments for NP.[4] However, pharmacological management of NP remains challenging because of limited efficacy and severe side effects associated with long-term medications. Therefore, it is necessary to identify a dependable technique to advance the therapy of NP.

Currently, a growing body of evidence suggests that the cause of NP is not restricted to changes in neuronal activity but may involve a network of interaction among neurons, glial, and immune cells.[5–7] Microglia, the resident immune

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cells, are considered crucial for the development of NP.[8–10] Upon peripheral nerve injury, microglia are swiftly activated in the dorsal horn of spinal cord, subsequently releasing inflammatory mediators that sensitize neurons, thereby exacerbating NP progression.[11,12] A significant increase in the level of inflammatory factors, such as tumor necrosis factor- α (TNF-α), interleukin-6 (IL-6), and interleukin-1β (IL-1β) contributes to neuroinflammation.[13] The inflammatory response caused by activated microglia is a protective response of the central nervous system after noxious stimulation. It helps to clear pathogens and cell debris, resist noxious stimulation, and promote tissue repair and regeneration.[14,15] If the inflammatory response is excessive and uncontrolled or persists for a long time, it will cause pathological neuroinflammation, resulting in cell death and tissue pathological damage.[16] Thus, controlling the ongoing inflammatory response in the spinal cord is beneficial for relieving NP.

Pyroptosis, a form of programmed cell death, contributes to the inflammatory response in NP pathogenesis by releasing pro-inflammatory cytokines.[17,18] During the biological process of pyroptosis, the NOD-like receptor protein 3 (NLRP3) inflammasome triggers the activation of caspase-1, which in turn processes gasdermin D (GSDMD) and the pro-inflammatory cytokines interleukin−1β (IL-1β) and interleukin−18 (IL-18). Furthermore, the N-terminal fragment of GSDMD inserts itself into the plasma membrane and forms pores, which facilitate the release of IL-1β and IL-18.[19,20] Furthermore, increasing evidence indicate that the NLRP3 inflammasome is activated following peripheral nerve injury, and suppressing the NLRP3 inflammasome in SNL and CCI model could alleviate NP.[21,22] Thus, inhibition of NLRP3 inflammasome may serve as a potential therapeutic approach for modulating NP.

Electroacupuncture (EA), a refined technique that integrates traditional manual acupuncture with modern electrotherapy, has been proven to effectively alleviate NP.[23,24] Both clinical and experimental studies have found that EA could successfully alleviate pain hypersensitivity by inhibiting neuroinflammation.[25–27] Moreover, previous research found that during the progression of NP, EA protected neuron damage in the spinal cord of NP rats to relieve pain hypersensitivity and exhibited rehabilitative implications in nerve repair.[28–31] However, whether inhibiting NLRP3 inflammasome-mediated pyroptosis in EA treatment against NP remains unknown.

Thus, in this study, we mimicked NP using the CCI rat model and investigated the potential role of EA in ameliorating NP through inhibition of pyroptosis in the spinal cord by regulating NLRP3 inflammasome activation. We explored the cellular distribution of pyroptosis in the dorsal horn of the spinal cord and examined whether EA affects NLRP3 inflammasome activation in specific spinal cells. Furthermore, pharmacological methods that specifically target against NLRP3 inflammasome were utilized to validate if EA alleviates NP via the NLRP3 inflammasome Our results will offer new insights into the therapeutic mechanism of EA, laying the foundation for a clinical application of EA therapy in NP.

Methods and Materials

Animals

Male Sprague Dawley rats weighed 250 g ± 50 g were purchased from Gempharmatech Co., Ltd. All rats were housed in the animal facility of the Animal Center of Shanghai Municipal Hospital of Traditional Chinese Medicine (TCM), where the room temperature maintained at 22 ± 0.5 °C. The lighting regimen followed a 12-hour day and night alternation, providing free access to food and water. All animal experiments were approved by the Animal Experiments Ethical Committee of Shanghai Municipal Hospital of TCM (SYXK 2024004) and conducted with the approval of the regulatory animal care guidelines of the National Institute of Health of the United States (Bethesda, MD, USA).

Chronic Constriction Injury (CCI)-Induced NP Model

Following a week of adaptive feeding, rats were assigned to receive sciatic nerve ligation to construct a CCI rat model.[32–34] Briefly, rats were deeply anesthetized with isoflurane, and the right sciatic nerve was exposed. Subsequently, 4 ligatures, each spaced 1 mm apart, were carefully applied to the nerve (Figure 1A). The degree of constriction was determined by observing slight muscular twitching of the ipsilateral limb. For the Sham group, similar procedures were performed on the right sciatic nerve without the application of any ligatures.

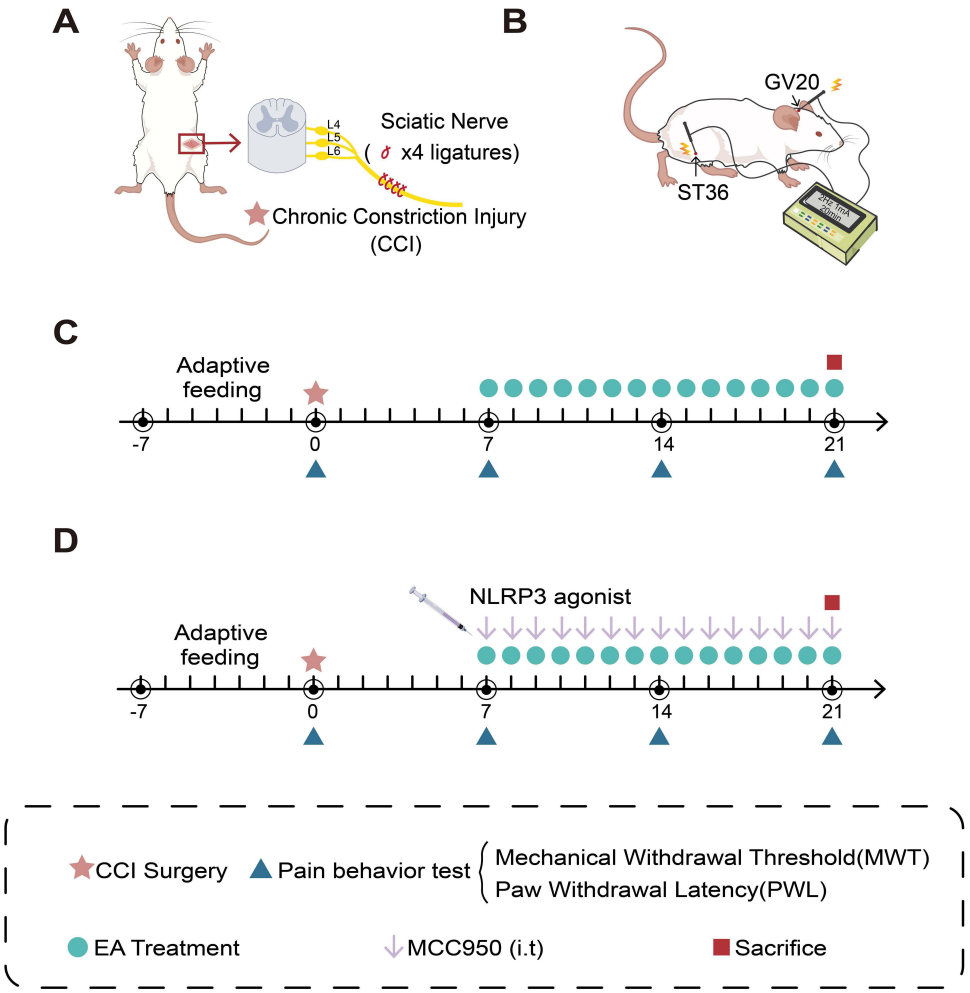

Figure 1 The establishment of the CCI induced NP modeling and EA's intervention CCI rats. (**A**) Demonstration of the CCI surgery. (**B**) The intervention protocol of EA treatment. (**C**) The study protocol of EA treatment in CCI rats in experiment 1. (**D**) The study protocol of EA and MCC950 treatment in CCI rats in experiment 2.

EA Treatment

EA therapy started on day 7 after the CCI operation as in our previous work.[29] After administering isoflurane anesthesia, rats were securely immobilized on a plate table The EA group received therapy at Baihui (GV20, in the midline of the head) and Zusanli (ST36, 5 mm beneath the capitulum fibulae and located laterally and posterior to the knee-joint) on the injured side (Figure 1B). EA stimulation was delivered using an electronic acupuncture instrument (SDZ-II, Hwato) for a total of 20 minutes per day. Acupuncture needles were inserted for 2–3 mm and stimulated (2 hz, 1 mA) for 20 min with an electric stimulation device. Meanwhile, the Sham group and CCI group only received isoflurane anesthesia without EA treatment.

Intrathecal Injection

This method of intrathecal injection has been described in detail by Hylden et al.[35] In brief, the microsyringe was inserted into the L5 spinous space and advanced carefully until the tail flicking was observed. Specific NLRP3 inflammasome inhibitor MCC950 (#S8930, Selleck, Houston, USA) was prepared in DMSO and diluted in PBS before use.[36] It was applied via an intrathecal catheter (30 μg/rat/day, 20 μL injection volume).[37] During the process, if the rats demonstrated tail movement, the intrathecal injection was successful.

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Experimental Design and Grouping

In experiment 1 (Figure 1C), to explore the therapeutic role of EA on NP, rats were randomly divided into three groups (n = 8 per group): the Sham group, the CCI group (performed CCI operation), and the CCI + EA group (CCI rats treated with EA). On day 7 after the CCI operation, rats in the CCI + EA group were administrated once a day by EA, while the Sham and CCI group rats received anesthesia only for control. Behavioral assessments were conducted at baseline and on days 7, 14, and 21 post-CCI surgery, followed by euthanasia of the rats and collection of spinal cord tissues for further analyses.

In experiment 2 (Figure 1D), to further understand the molecular mechanism of EA in CCI rats, we used the NLRP3 inhibitor MCC950 respectively. Rats were randomly assigned to five groups (n = 8/group): Sham, CCI, CCI + EA, CCI + MCC950, CCI + MCC950 + EA. The L4 - L6 spinal cord of each group was collected for Western blot analysis (n = 3/group) 21 days after CCI.

Behavioral Tests

Mechanical Withdrawal Threshold (MWT)

The assessment of the MWT was conducted using Von Frey fibers (North Coast, USA). Individual rats were accommodated in a plexiglass observation chamber with multiple compartments, allowing them freedom of movement, and providing suitable conditions for a duration of 30 to 60 minutes on a suspended metal mesh. The MWT, in response to the application of a von Frey probe to the foot, was determined by employing the up-down method. A progressive series of Von Frey fibers with logarithmically increasing force was administered to specific sites on the central region of the plantar surface of the affected hind paw. Each Von Frey fiber was delicately applied to the designated area for approximately 2–3 seconds, with an ensuing interval of 20 minutes between stimuli.

Paw Withdrawal Latency (PWL)

To assess nociceptive responses to thermal stimuli, PWL was determined by the radiant thermal apparatus (IITC, USA, II-390). Rats were placed in a suitable plastic chamber for approximately 30 minutes to allow them to adapt to the new environment. Once the rats have settled down and ceased exploring, position the radiant heat source directly beneath the mid-plantar surface of the right hind paw. The intensity of the light source was set to 22% ~ 32% and the cut-off time was 20 seconds, to avoid excessive heating injury. The latency time was recorded, and the evaluation was repeated three times, with intervals of 5 to 10 minutes between each trial, subsequently calculating the mean value. All behavioral testing measurements were conducted by investigators in a blinded manner. All behavioral testing measurements were conducted by investigators blindly.

Enzyme-Linked Immunosorbent Assay (ELISA)

Neuroinflammation levels were assessed by measuring the inflammatory cytokines tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6) and interleukin-1β (IL-1β) in the spinal cord utilizing an ELISA kit. TNF-α (EK382), IL-6 (EK306) and IL-1β (EK301BHS) ELISA kits were supplied by Multisciences Biotech Co., Ltd. (Hangzhou, China). For endogenous opioids or neurotransmitters, serum substance P (D751030, Sangon Biotech) and β-endorphin (D731172, Sangon Biotech) were detected, respectively. The concentrations (in pg/mL) were determined through the utilization of linear regression curves derived from the dataset corresponding to a range of protein standards supplied in the kit, which were subsequently averaged across replicate readings.

Immunofluorescence (IF) Staining

Prior research indicates that in conditions of peripheral nerve injury, both spinal dorsal horn microglia and neuronal cells experience pyroptosis, but astrocytes show negligible pyroptosis.[38,39] Thus, to identify specific cell type in the spinal cord undergoing pyroptosis, co-staining of the microglial biomarker (Iba-1), or neuronal marker (NeuN) with NLRP3 or Caspase-1 or N-GSDMD was performed. The lumbar enlargement L4 - L6 of the spinal cord was perfused with a 10% neutral buffered formalin solution, employed for pathological scrutiny. Paraffin sections were processed sequentially by dewaxing to water, antigen repairing, incubating with 0.1% Triton X-100, and blocking with 5% BSA. Then the sample sections of the spinal cord were treated with primary antibodies anti-rabbit NLRP3 (1:20, DF7438, Affinity) or anti-rabbit Caspase-1 (1:100, 22,915-1-AP, Proteintech) or N-GSDMD (1:1000, DF13758, Affinity) and anti-mouse Iba-1

(1:50, ab283319, Abcam) or anti-mouse antibodies NeuN (1:1000, ab279296, Abcam) overnight at 4°C, then incubated with fluorescent-labeled secondary antibodies for 1 hour, and counterstained with DAPI (P0131, Beyotime, Shanghai, China). Finally, the sections were scrutinized through a confocal microscope (Leica, DM6). Image J software was employed for the enumeration of co-labeled cells.

RNA Extraction and Real-Time PCR

Spinal cord tissue was collected for examination into the expression of pyroptosis-related genes using RT-PCR. RNA was extracted using Trizol reagent (Thermo Fisher Scientific, Waltham, MA). The cDNA was synthesized using a reverse transcription kit (R223-01, Vazyme, China) according to the manufacturer's guide. Real-time PCR was performed on ABI 7500 (Foster City, CA, America) using SYBR Green PCR master mix (TaKaRa, RR420A) by 2 -ΔΔCT formula. Primers were listed in Table S1.

Western Blot

For the detection of proteins, spinal cord lysates were prepared using RIPA buffer (P0013B, Beyotime, Shanghai, China) containing protease and phosphatase inhibitors. Protein concentrations were determined utilizing a BCA protein assay kit (P0010, Beyotime, Shanghai, China). Equal quantities of protein (20 μg) were resolved via SDS-PAGE and subsequently transferred onto PVDF membranes (Millipore, Billerica, City, State, USA). The membranes were incubated overnight at 4 °C with primary antibodies, followed by a 1-hour incubation with secondary antibodies for band detection using the ChemiDOC Western blot technique (BIO-RAD). After the first primary antibody incubation, the PVDF membranes were subjected to a stripping buffer (PS107, EpiZyme) to remove the first antibody at room temperature for 20 minutes with gentle agitation. Following stripping, the membranes were rinsed three times with Tris-buffered saline containing 0.1% Tween-20 for 5 minutes each to ensure complete removal of the stripping buffer and reactivation of the membrane. The membranes were then blocked again for 1 hour at room temperature and then incubated with antibodies overnight at 4°C. The experiments were repeated 3 times. The Western blot analysis employed the following primary antibodies: NLRP3 (1:1000, ab283819, Abcam), GSDMD N-Terminal (1:1000, DF13758, Affinity), Cleaved Caspase-1 (1:1000, AF4005, Affinity), IL-1β (1:1000, ab9722, Abcam) and IL-18 (1:1000, DF6252, Affinity). β-actin (1:1000, #4970, CST) served as an internal control.

Statistical Analysis

All data are expressed as mean ± standard deviation (SD) of at least three independent experiments. Statistical analyses were tested by GraphPad Prism 9.0 (GraphPad Software, Inc., USA). Data comparison at different time points were analyzed by repeated measures analysis. Group comparisons were assessed with one-way *ANOVA* or two-way *ANOVA* with Bonferroni's post hoc test for the comparison of multiple columns. Pairwise comparisons between groups were analyzed by the Student's *t*-test. P values < 0.05 were considered to indicate statistical significance.

Results

EA Attenuated Pain Hypersensitivity and Spinal Inflammatory Response in CCI Rats

The successful induction of NP-like hypersensitivity was confirmed 7 days after CCI by a marked reduction in the MWT and PWL. During the NP progression, CCI rats exhibited significant decreases in MWT and PWL compared with the Sham group. Conversely, 14 days of EA treatment showed substantial improvements in MWT and PWL in NP rats in the time-dependent manner, restoring them to 77.6% and 74.0% of the Sham group on day 21, respectively (Figure 2A and B). These results suggest that EA could significantly attenuate CCI-induced pain sensitization in the NP model, and its analgesic effect intensifies over time. In addition, ELISA assays showed a significant substantial reduction in analgesic β- endorphin levels and an elevation in pain inducing substance P in the serum of CCI rats. EA partially recovered the aberrant levels of them to the normalcy (Figure 2C and D). Moreover, we test the level of pro-inflammatory cytokines, including IL-1β, IL-6 and TNF-α in the spinal tissue. The content of IL-1β, IL-6 and TNF-α in the spinal cord of CCI rats was obviously elevated in the spinal cord in comparison to the Sham group, suggesting that the CCI operation induced local inflammation. EA therapy

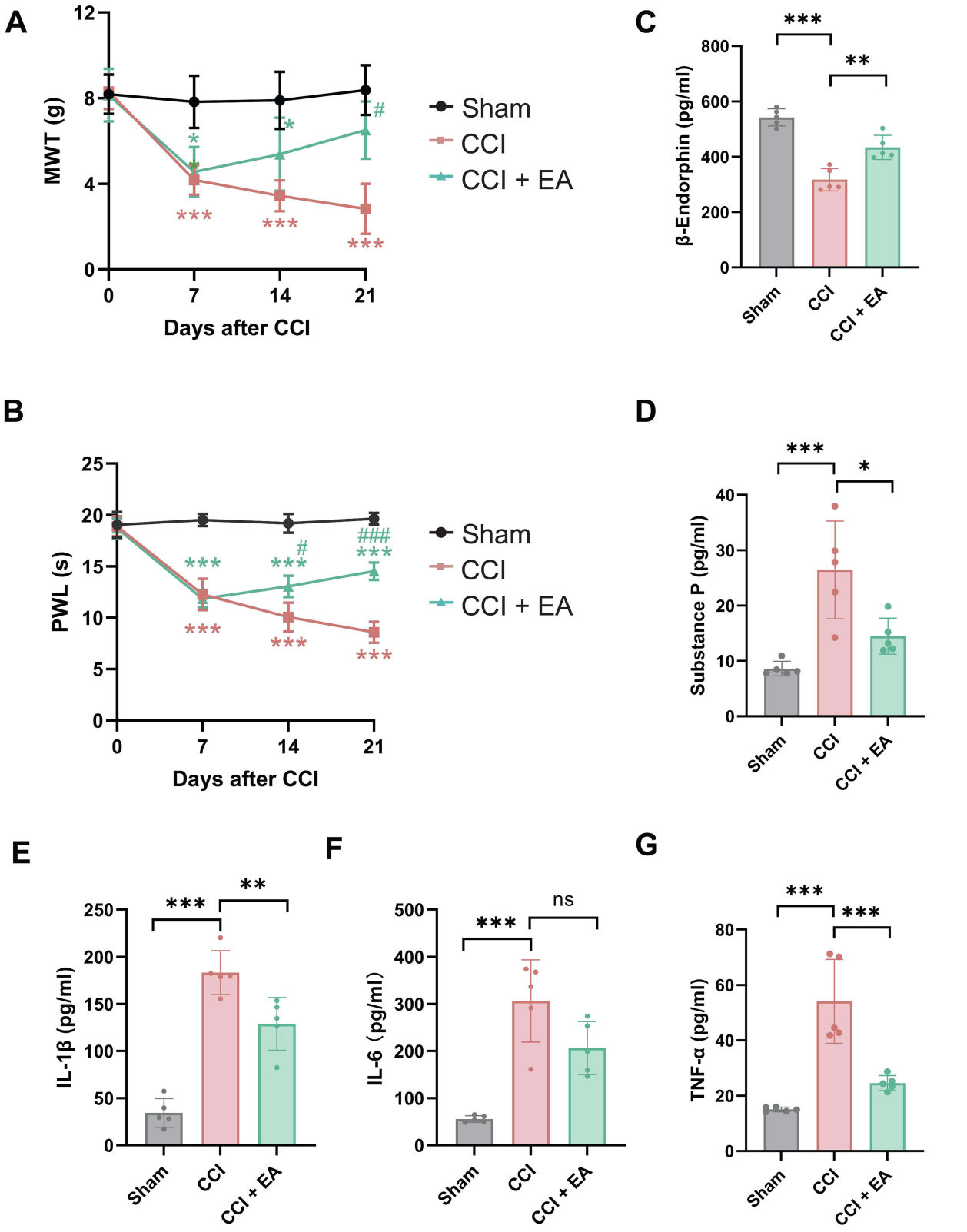

Figure 2 EA reduced pain hypersensitivity and suppressed the spinal inflammatory responses in CCI-induced NP rats. (**A**) Effects of EA on MWT induced by CCI surgery. (**B**) Effects of EA on PWL in CCI-induced NP models. **P* < 0.05, ****P* < 0.001. vs Sham group. #*P* < 0.05, ###*P* < 0.001 vs CCI group. n = 8. (**C**) The level of β-Endorphin in the spinal cord tissue of each group. (**D**) The level of SP in the serum of each group. (**E**–**G**) The level of IL-1β, IL-6 and TNF-α in the spinal cord tissue of each group. **P* < 0.05, ***P* < 0.01, ****P* < 0.001. ns, no significant difference. n = 5.

could reduce the production of IL-1β and TNF-α, although IL-6 levels remained unchanged (Figure 2E–G). These results showed that EA could attenuate pain hypersensitivity and anti-inflammatory responses in the spinal cord of CCI rats.

EA Mitigated CCI-Induced Pyroptosis Levels in the Spinal Cord

Pyroptosis is a pro-inflammatory type of programmed cell death that relies on the activity of GSDMD driven by the NLRP3 inflammasome. We then examined the expression of pyroptosis-associated biological markers better to understand EA's anti-inflammatory effects on the CCI rats. First, the protein levels of key pyroptosis-related proteins, such as NLRP3, N-GSDMD, Cleaved Caspase-1, IL-18 and IL-1β were detected by Western blot analysis (Figure 3A). These proteins exhibited considerable up-regulation in the spinal cord of CCI rats, while EA treatment decreased their expression (Figure 3B–F). To further confirm the effect of EA against pyroptosis, we further tested the mRNA level of *NLRP3, IL-18* and *IL-1β*. Expectedly, EA treatment markedly decreased the overexpression of *NLRP3, IL-18* and *IL-1β* in the spinal cord tissue (Figure 3G–I). These results demonstrated that pyroptosis is involved in the NP progression, and EA exhibits a notable anti-pyroptotic effect in the spinal cord of NP rats.

EA Inhibits Microglia Pyroptosis in the Dorsal Spinal Cord of CCI Rats

Previous studies using the CCI-induced NP model found that the expression of NLRP3 and its downstream effectors were increased in neurons and glia of the spinal cord dorsal horn.[40] Therefore, to better understand the effect of EA on the cellular distribution of pyroptosis after CCI, we performed the double IF staining of NLRP3, Caspase-1 and N-GSDMD with cellular biomarkers of microglia (Iba-1), neurons (NeuN) and astrocytes (GFAP) in the spinal cord among groups. Compared to the Sham group, the CCI rats exhibited co-staining of NLRP3, Caspase-1, and N-GSDMD in the majority of activated Iba-1^{+} microglia in the spinal cord. EA could significantly downregulated NLRP3, Caspase-1 and N-GSDMD expression in microglia cells compared to the CCI group (Figure 4A–C). Furthermore, the quantitative analysis showed that EA treatment reduced 12.87% of the NLRP3/Iba-1, 18.74% of the Caspase-1/Iba-1 and 7.85% of the N-GSDMD/Iba-1 co-expression compared with the CCI group respectively (Figure 4D–F). However, EA therapy failed to inhibit the co-expression of NLRP3, Caspase-1 and N-GSDMD with NeuN or GFAP (Figure S1). These findings indicate that EA predominantly inhibits pyroptosis in microglia rather than neurons or astrocytes.

EA Alleviated Pyroptosis by Inhibiting NLRP3 Inflammasome Activation

Subsequently, to further ascertain the role of NLRP3 inflammasome in the effects of EA on microglia pyroptosis and NP progression, we pharmacologically blocked NLRP3 inflammasome activation by MCC950 (an NLRP3 inhibitor) alongside EA intervention 7 days after CCI surgery (as depicted in Figure 1D). Behavioral analysis results indicated that, both EA treatment and MCC950 treatment significantly ameliorated mechanical and thermal pain of CCI rats (Figure 5A and B). However, the treatment that combined EA with MCC950 did not further relieve the threshold. These results revealed that the inhibition of NLRP3 inflammasome contributes the NP recession, highlighting the critical contribution of EA in the NP of CCI rats. Then the crucial proteins of NLRP3-mediated pyroptosis were detected by Western blot (Figure 5C). Notably, both EA and MCC950 administration reduced the expression of NLRP3, N-GSDMD, Cleaved Caspase-1 and its downstream IL-18 and IL-1β in the spinal cord compared to the CCI rats (Figure 5D–H). Furthermore, the combined intervention of MCC950 with EA did not further reverse EA-induced downregulation of pyroptosis proteins compared to the CCI rats treated with EA alone. Finally, we investigated the role of NLRP3 in the inflammatory cascade and after CCI by analyzing the expression of TNF-α, IL-6 and IL-1β. Compared to the CCI group, the content of pro-inflammatory cytokines TNF-αand IL-1βin CCI rats were also lowered while the expression of IL-6 was unaffected (Figure 5I–K). The above results indicated that EA could target on spinal NLRP3 inflammasome to alleviate microglial pyroptosis.

Discussion

NP poses a great challenge in clinical treatment due to its complex and incomprehensible underlying mechanism. In this study, we applied rats induced by CCI surgery, a well-established and commonly utilized animal model of peripheral NP, which elicits comparable clinical behavioral manifestations to those observed in NP patients, such as allodynia and hyperalgesia.[41–44]

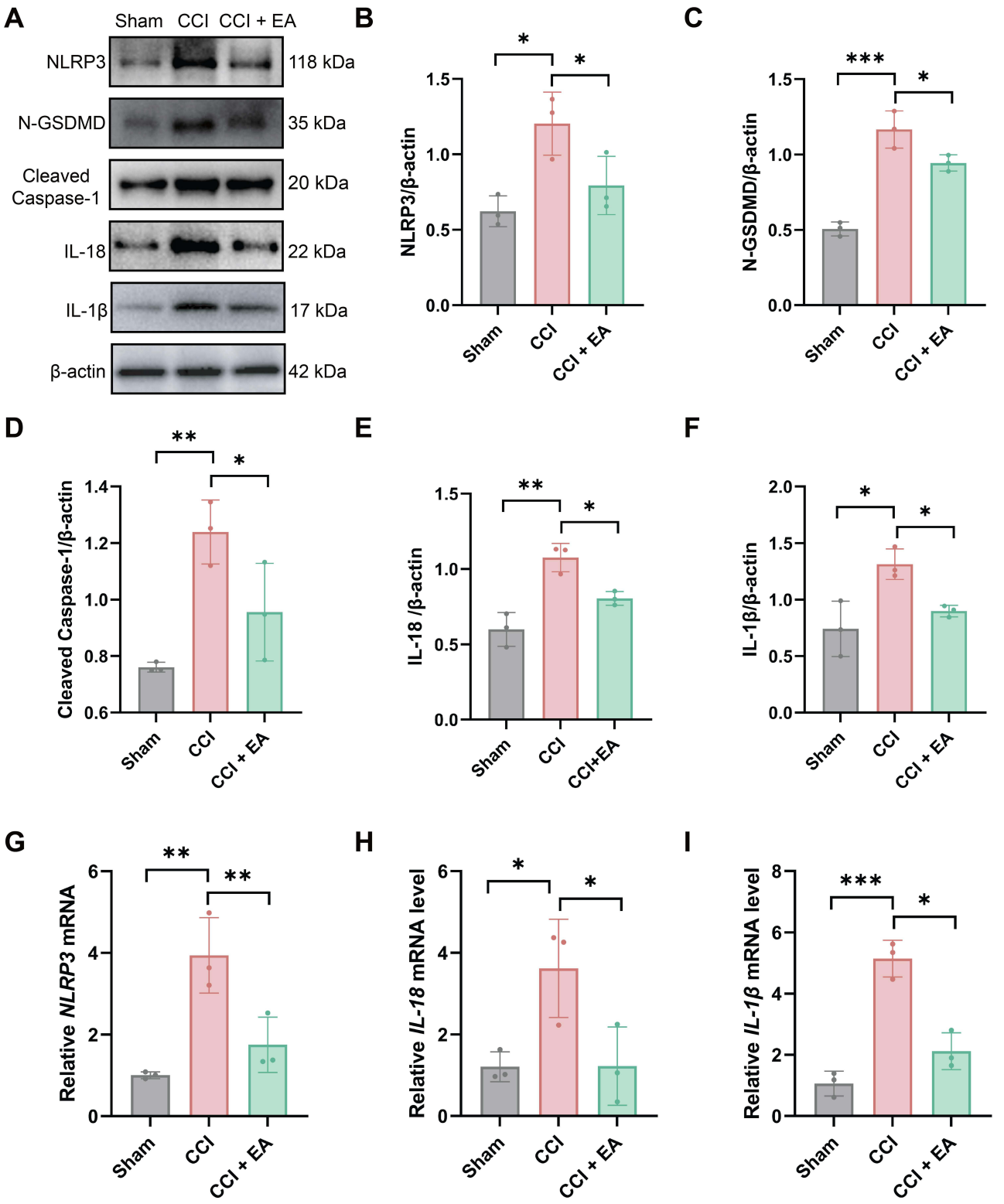

Figure 3 EA attenuated CCI-induced pyroptosis phenotype in the spinal cord. (**A**) The protein expression of NLRP3, N-GSDMD, Cleaved Caspase-1, IL-18 and IL-1β in the spinal cord of each group. β-actin was used as an internal control. (**B** and **F**) The relative expression of NLRP3, N-GSDMD, Cleaved Caspase-1 and IL-18 protein in each group. Data presented as means ± s.d. **P* < 0.05, ***P* < 0.01, ****P* < 0.001. n = 3. (**G** and **I**) The mRNA expression levels of the pyroptosis-related gene of *NLRP3, IL-18, IL-1β*, respectively. Data presented as means ± s.d. **P* < 0.05, ***P* < 0.01, ****P* < 0.001. n = 3.

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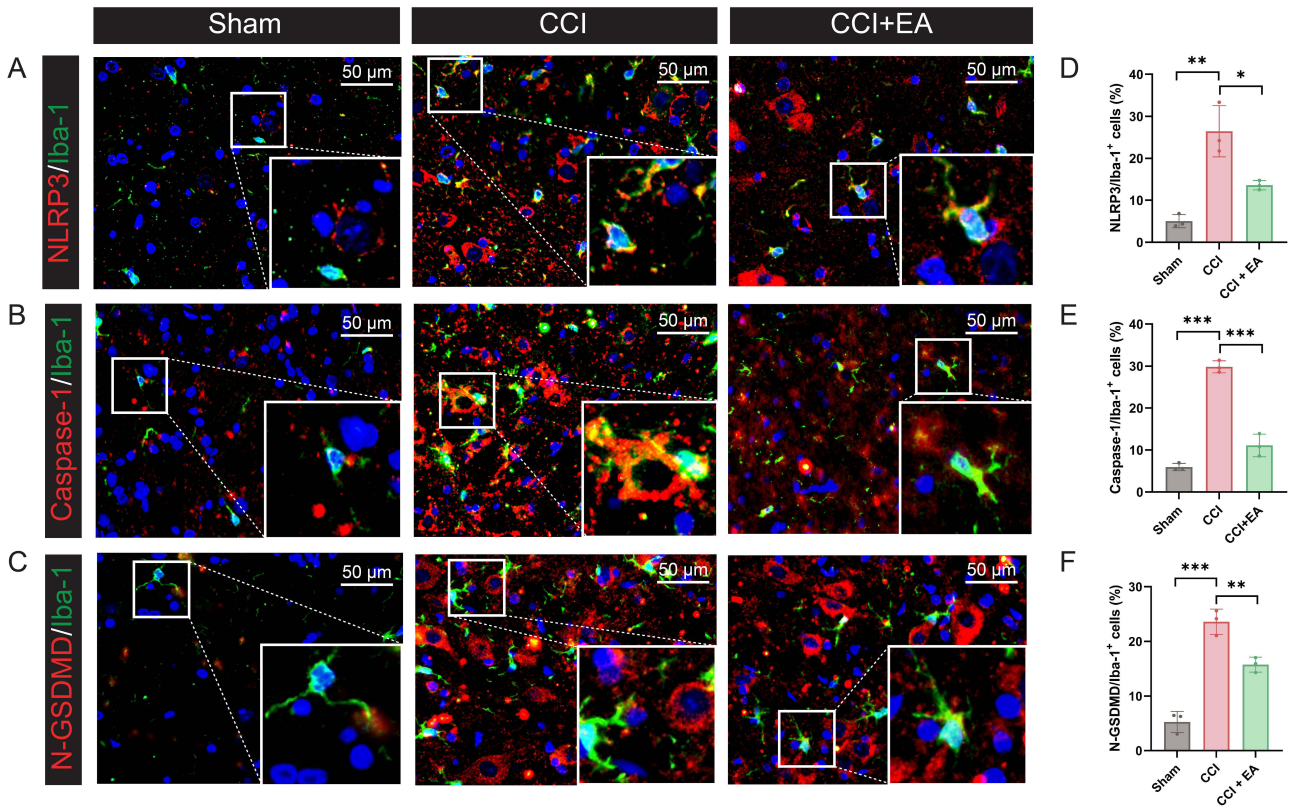

Figure 4 EA inhibited the pyroptosis in microglia following CCI operation. (**A**) Representative images of immunofluorescent analysis of Iba-1$^+$ (green), NLRP3$^+$ (red) maker, and DAPI (blue) staining of nuclei in the spinal cord dorsal horn among groups. Scale bars, 50 μm. (**B**) Representative images of immunofluorescent analysis of Iba-1$^+$ (green), Caspase-1$^+$ (red) maker, and DAPI (blue) staining of nuclei in the spinal cord dorsal horn among groups. Scale bars, 50 μm. (**C**) Representative images of immunofluorescent analysis of Iba-1$^+$ (green), N-GSDMD$^+$ (red) maker, and DAPI (blue) staining of nuclei in the spinal cord dorsal horn among groups. Scale bars, 50 μm. Quantification analysis the number of NLRP3/Iba-1$^+$ (**D**), Caspase-1/Iba-1$^+$ (**E**), N-GSDMD/Iba-1$^+$ (**F**) in the spinal cord dorsal horn. Data presented as means ± s.d. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, n = 3.

Subsequently, we assessed the therapeutic efficacy of EA on the pain hypersensitivities exhibited by CCI rats. Our findings revealed that EA significantly mitigated both mechanical and thermal hypersensitivities induced by CCI surgery. Furthermore, EA suppressed microglial pyroptosis and neuroinflammation. This evidence hinted that EA treatment at acupoints ST36 and GV20 has a certain antinociceptive effect on NP by inhibiting microglial pyroptosis in the spinal cord.

Our previous study has substantiated that applying EA at acupoints ST36 and GV20 exerts a notable effect on NP. EA emerges as a potent therapeutic modality with relatively minimal adverse effects on NP management in clinical settings.[27] A multicenter randomized controlled trial showed that EA treatment was beneficial for reducing pain and improving quality of life in patients with painful diabetic peripheral neuropathy, while also being well tolerated and safe during this study.[45] The main mechanisms of the analgesic effect of EA are attributed to robust anti-inflammation and neuroimmune regulation.[46] Consistently, our findings have demonstrated that EA therapy exerts a beneficial impact on the inflammatory response during the NP process decreasing the level of TNF-α and IL-1β and IL-6 in the spinal cord. In addition, EA increased the expression of the neuron marker NeuN, suggesting that EA mitigates the neuron loss induced by CCI. According to these findings, EA stimulation of peripheral acupoints might have an effective anti-neuroinflammatory effect.

Pyroptosis, a form of programmed cell death characterized by activation It has been ascertained that neuroinflammation is markedly modulated by the NLRP3 inflammasome.[47] NLRP3, ASC and Caspase-1, as a proinflammatory complex in microglia, can promote maturation and secretion of inflammatory cytokine IL-1β and 18 which induce pyroptosis.[48,49] An expanding body of research has elucidated the involvement of the NLRP3 inflammasome in the pathological manifestations of NP,[50] and the deletion of NLRP3 prevents mechanical hypersensitivity in mice.[51] A similar study using a CCI-induced NP model also found that the expression of NLRP3 and its downstream effectors were increased in neurons and glial cells of the spinal cord dorsal horn.[52] Previous studies have proved that NLRP3 was primarily

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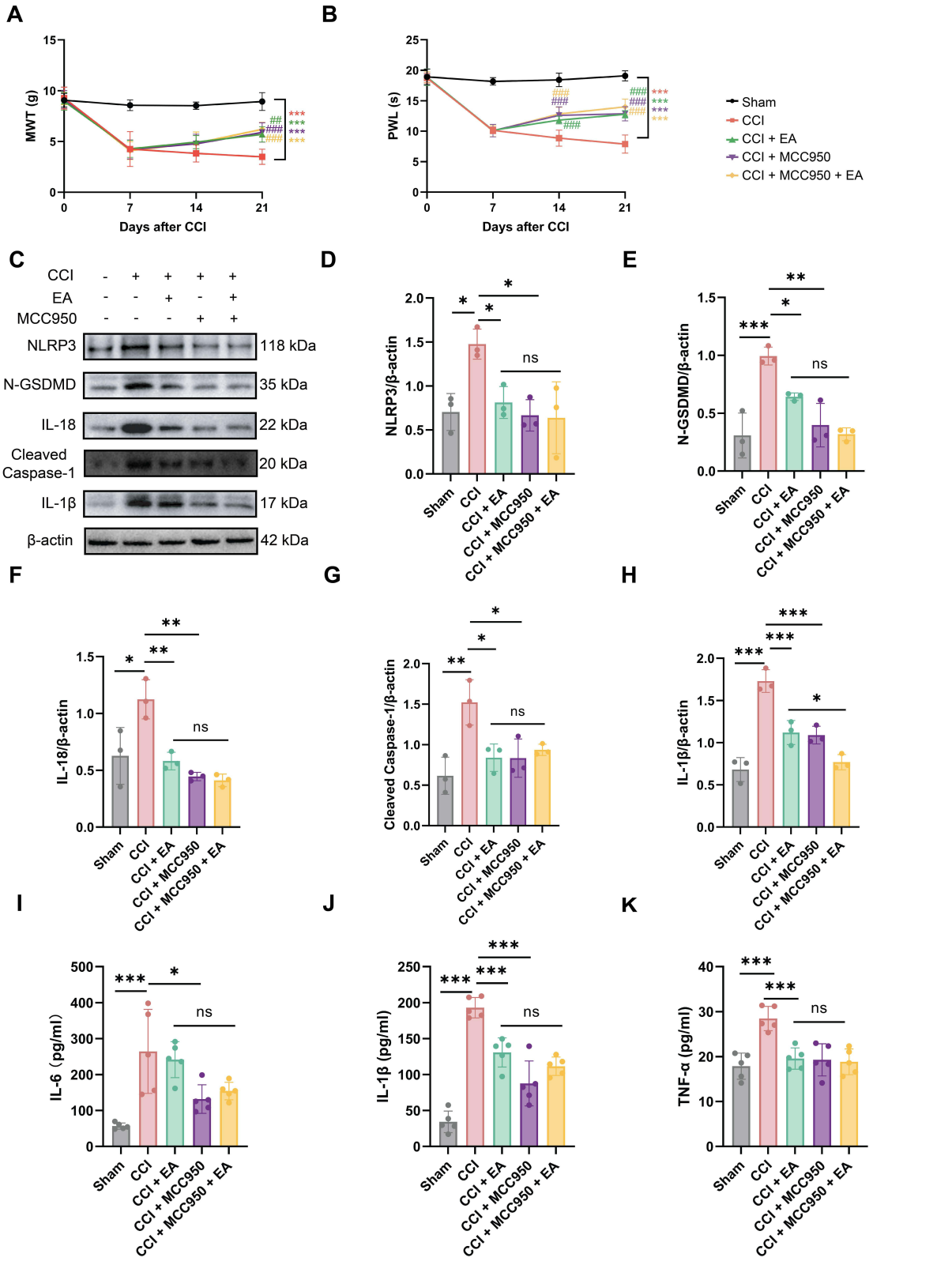

Figure 5 Inhibition of NLRP3 inflammasome activation relieved CCI-induced pain behaviour and abolished the anti-pyroptosis effect of EA. (**A** and **B**) The effects of EA on MWT and PWL in CCI-induced NP models. n = 8. Data presented as means ± s.d. ***$P < 0.001$. vs Sham group. ##$P < 0.01$, ###$P < 0.001$. vs CCI group. (**C**) The protein expression of NLRP3, N-GSDMD, Cleaved Caspase-1, IL-18 and IL-1β in the spinal cord of each group. β-actin was used as an internal control. (**D** and **H**) The relative expression of NLRP3, N-GSDMD, Cleaved Caspase-1, IL-18 and IL-1β protein in each group. Data presented as means ± s.d. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. n = 3. (**I** and **K**) The level of IL-6, IL-1β and TNF-α in the spinal cord of each group. Data presented as means ± s.d. *$P < 0.05$, ***$P < 0.001$. ns, no significant difference.n = 5.

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expressed in neurons and microglia in the rat spinal cord, and only a few astrocytes were found to express NLRP3.[53] Our results showed that NLRP3 was predominantly expressed in microglia and neurons in the spinal cord, and only a few astrocytes were found to express NLRP3. Moreover, we found that Caspase-1 and N-GSDMD were colocalized in both microglia and neurons in the spinal cord. These investigations revealed a significant role for NLRP3-mediated neuroinflammation in peripheral nerve injury-induced NP models. Our findings indicate that CCI surgery increased NLRP3, N-GSDMD, Cleaved Caspase-1, IL-18 and IL-1β. EA treatment suppresses pyroptosis in the spinal cord of CCI-induced NP rats, as evidenced by reduced expression of key pyroptosis-related proteins. This suggests that EA may exert its analgesic effects by inhibiting microglial pyroptosis in the spinal cord.

To answer if EA alleviated pyroptosis in CCI rats by inhibiting NLRP3 inflammasome, we pharmacologically inhibited the NLRP3 inflammasome through MCC950. As expected, the inhibition of NLRP3 inflammasome using MCC950 alleviated allodynia, consistent with the previous reports, demonstrating that NLRP3 inflammasome is involved in the progression of NP. Interestingly, MCC950 combined with EA did not further inhibit NLRP3 activation or improve pain hypersensitivity, suggesting that EA itself is strong enough to inhibit NLRP3 activation and neuroinflammation, confirming that EA works through NLRP3 inflammasome inhibition.

Previous studies have demonstrated that EA possesses extensive anti-inflammatory and neuroprotective effects.[54,55] Notably, EA can regulate the activation of glia,[56,57] decrease cytokines[58,59] and modulate pathways such as the NF-kappaB,[60] mitogen-activated protein kinase[61] and Janus kinase 2/signal transducer and activator of transcription 3 pathways.[62] These multi-faceted mechanisms provide EA a significant advantage in NP management and these activities may be indirectly or directly involved in the inhibition of NLRP3 inflammasome activation[63] and alleviate neuropathic pain.[64] The above findings lead us to speculate that EA treatment relieves NP through multiple mechanisms, except the inhibition of NLRP3 inflammasome.

While this study effectively investigates molecular changes in the spinal cord and the treatment of CCI-induced NP, its limitation lies in its limited exploration of the mechanisms. First, pyroptosis is an inflammatory death pathway, which can be divided into Caspase-1 dependent classical pathway and Caspase-4, 5, 11 dependent non-classical pathway according to its activation mechanism[65] However, in this study, we did not involve the pyroptosis process of other non-

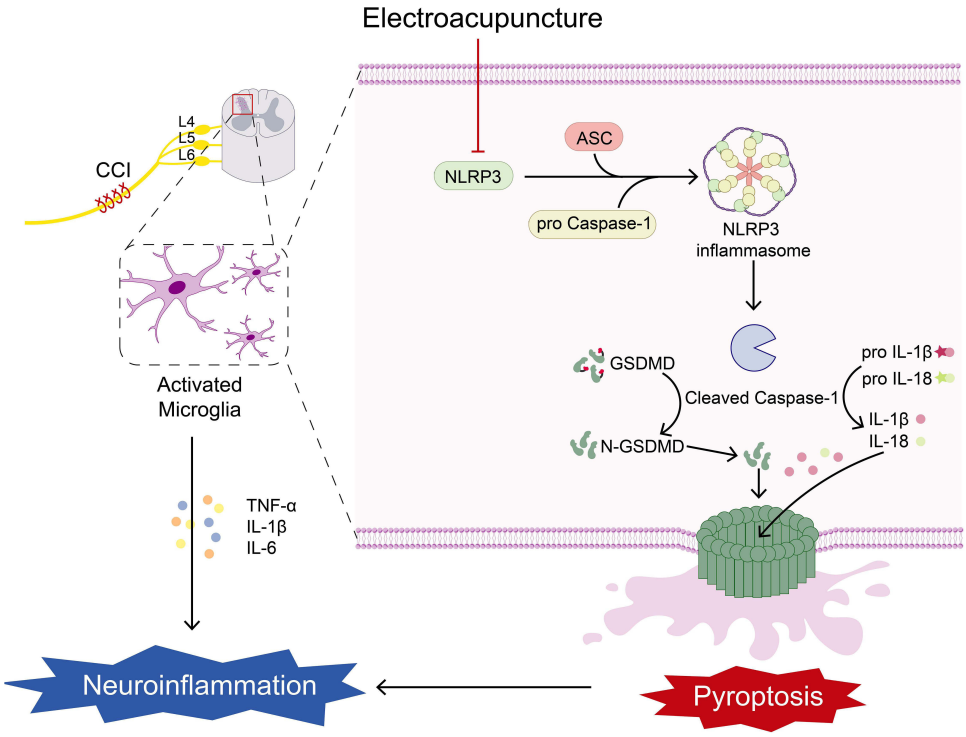

Figure 6 A schematic diagram illustrating the mechanism of EA treatment in peripheral nerve injury-induced NP.

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classical pathways, which requires further experimental verification. Second, we solely described the analgesic effect of EA mediated by regulating pyroptosis-specific indicators in NP rats. GV20 is a key acupoint for regulating brain function and low-intensity electroacupuncture stimulation at ST36 has been confirmed to activate vagal-adrenal anti-inflammatory pathway.[66] However, whether the combination of other acupoints can achieve the same effect and the mechanism of how EA treatment on ST36 and GV20 remotely inhibits pyroptosis activity remains unclear. Thus, further studies can employ chemogenetic-DREADD techniques to further elucidate the molecular mechanisms by which EA inhibits pyroptosis activity in the spinal cord.

This study illustrated that EA therapy may suppress microglial pyroptosis of the spinal cord and neuroinflammation to alleviate pain hypersensitivity. As a non-pharmacological treatment with fewer side effects, EA shows a significant effect on NP management. These discoveries offer new avenues for treating NP and improving the quality of life for NP patients.

Conclusion

Collectively, EA at acupoints ST36 and GV20 emerges as a promising therapeutic modality for NP. Our findings reveal that EA inhibits neuroinflammation and microglia pyroptosis by suppression NLRP3 inflammasome activation (Figure 6). However, further studies are still warranted to elucidate the precise molecular mechanisms underlying the effects of EA and to explore its clinical applicability in NP patients.

Abbreviations

NP, neuropathic pain; EA, electroacupuncture; CCI, chronic constriction injury; NLRP3, NOD-like receptor protein 3; MWT, mechanical withdrawal threshold; PWL, paw withdrawal latency; TNF-α, tumor necrosis factor- α; IL-6, interleukin-6; IL-1β, interleukin-1β; GSDMD, gasdermin D; TCM, Traditional Chinese Medicine; ELISA, Enzyme-Linked Immunosorbent Assay; IF, Immunofluorescence.

Data Sharing Statement

The original contributions presented in the study are included in the article/Supplementary Materials, further inquiries can be directed to the corresponding authors.

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Disclosure

The authors report no conflicts of interest in this work.

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