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Electroacupuncture relieves chronic pain by promoting microglia M2 polarization in lumbar disc herniation rats

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Electroacupuncture has an effective analgesia on chronic pain caused by lumbar disc herniation (LDH) clinically, however, the underlying mechanism is unclear. In this study, we investigated whether electroacupuncture alleviated pain in LDH model rats by inducing spinal microglia M2 polarization. We established a noncompression LDH rat model by implanting autologous caudal nucleus pulposus into L5/L6 nerve root. Electroacupuncture (30 min/day) treatment on the ipsilateral side was started on the 8th postoperative day, once a day for consecutive 7 days. Paw withdrawal threshold (PWT) and paw withdrawal latency (PWL) were tested for pain behavior. Western blotting was used to detect the protein expression in lumbar enlargement (L5/L6). Immunofluorescence was used to detect iNOS$^+$/Iba-1$^+$ and Arg-1$^+$/Iba-1$^+$ and CB2R$^+$/Iba-1$^+$ in lumbar enlargement (L5/L6). We show that PWT and PWL decreased in the LDH group while Iba-1, iNOS, and TNF-α expression increased significantly in lumbar spinal dorsal horn (SDH) after LDH surgery, and revealing that microglia were activated and polarized towards proinflammatory M1 phenotype. Electroacupuncture treatment significantly increased PWT and PWL while reducing Iba-1, iNOS, and TNF-α expression, interestingly, Arg-1 and IL-10 expression were significantly increased. Moreover, electroacupuncture treatment led to CB2 receptors on microglia upregulation, while NF-κB and p-NF-κB expression in lumbar SDH downregulation. Our study indicated that electroacupuncture may reduce nociceptive hyperalgesia by inhibiting microglia activation and microglia M1 polarization and promoting microglia M2 polarization in lumbar SDH of LDH rats, which may be caused by the activation of CB2 receptors on microglia and inhibition of NF-κB pathway in lumbar SDH. *NeuroReport* **34: 638–648 Copyright © 2023 The Author(s). Published by Wolters Kluwer Health, Inc.**

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Keywords: electroacupuncture, lumbar disc herniation, microglia, polarization, spinal dorsal horn

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Introduction

Lumbar disc herniation (LDH) is a common chronic disease, which seriously affects the life of patients and is one of the most important causes of disability in the world [1], and it brings a heavy burden to medical security and social support [2]. Electroacupuncture originated from traditional acupuncture and is modified by pulsed electricity instead of manipulation [3]. Electroacupuncture is effective in the treatment of chronic pain caused by LDH clinically [4], however, the underlying mechanism remains unclear. The analgesic mechanism of electroacupuncture includes activation of a variety of bioactive chemicals through peripheral, spinal, and supraspinal [5].

Microglia are the major immune effector cells in the central nervous system (CNS), and they are similar to peripheral macrophages in function. Under physiological conditions, microglia maintain a ramified cell shape, which was called M0 phenotype [6]. Microglial polarization is related to various neurological disorders such as CNS infection, nerve injury, nerve inflammation, chronic pain, and neurodegeneration [7–10]. Some studies have shown that neuropathic pain could be reduced by promoting microglia M2 polarization [11]. Recent studies have shown that electroacupuncture promotes the brain microglia M2 polarization to reduce neuroinflammation, which has been reported in many neurological diseases, such as cerebral ischemia [12] and Alzheimer's disease [13]. Electroacupuncture may reduce neuroinflammation by inhibiting the nuclear factor kappa B (NF-κB)

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Highlights

(1) Electroacupuncture relieved chronic pain in lumbar disc herniation rats.
(2) Electroacupuncture reduced inducible nitric oxide synthase and proinflammatory cytokine expression.
(3) Electroacupuncture promoted arginase-1 (Arg-1) and anti-inflammatory cytokine expression.
(4) Electroacupuncture upregulated CB2 receptors and suppressed nuclear factor kappa B (NF-κB) and *p*-NF-κB expression.
(5) Electroacupuncture inhibited microglia activation and promoted microglia polarization toward the M2 phenotype.

(p65) pathway to promote the brain microglia polarization toward the M2 phenotype [14–16]; however, how electroacupuncture regulates the spinal microglia polarization to alleviate pain is not well understood.

Our lab previously reported that activation of the cannabinoid 2 receptor (CB2R) had anti-inflammatory and analgesic effects, and intrathecal injection of CB2 receptor agonists inhibited microglia activity, reduced the expression of proinflammatory cytokines, and alleviated pain [17,18]. CB2R is a type of cannabinoid receptor, which is found mainly on peripheral immune cells, but is mainly expressed in microglia in CNS [19]. Previous studies have shown that electroacupuncture activates CB2 receptors on peripheral tissue to produce anti-inflammatory and analgesic effects [20], consistently, the treatment of selective CB2 receptor agonists on spinal cord injury models or microglia *in vitro* also produces anti-inflammatory and analgesic effects [21]; however, whether the anti-inflammatory and analgesic effects of electroacupuncture is through activation of CB2 receptors on spinal microglia remains unclear.

Materials and methods

Animals

In this study adult male Sprague–Dawley rats (220 ± 20 g) were used. Animals were raised under controlled laboratory conditions (22 ± 2 °C, 06 : 00 ~ 18 : 00 lighting) with free access to fresh water and laboratory diet. Care and handling of rats were provided to ameliorate suffering in accordance with the guidelines of the ethical guidelines for the use of experimental animals [22].

Lumbar disc herniation model

A rat model of lumber disc herniation was performed as described in detail previously [23]. In brief, the rats were deeply anesthetized with sodium pentobarbital (50 mg/kg body weight, intraperitoneally). The hair on the lower back of rat was shaved, and the skin was sterilized with 0.5% chlorhexidine and covered with clean paper. A midline incision from L3 to S1 was made over the lumbar spine, and then the left L5 and L6 upper facet and half-lamina were resected to expose the lumbar 5 and lumbar 6 nerve roots. The autologous caudal nucleus pulposus was obtained from the second and third coccygeal intervertebral discs. The obtained nucleus pulposus (about 5 mg) was implanted next to the left L5 and L6 nerve roots, just near the corresponding dorsal root ganglion. The amount of nucleus pulposus implanted was approximately equal between rats that were regarded as LDH group. The Sham group took nucleus pulposus but was not implanted into the nerve roots, with other procedures remaining the same as those in the LDH Group. Special care was taken to minimize mechanical compression during surgery and prevent infection and minimize the influence of inflammation. After surgery, the rats were raised in individual cages in the animal room until they fully recovered.

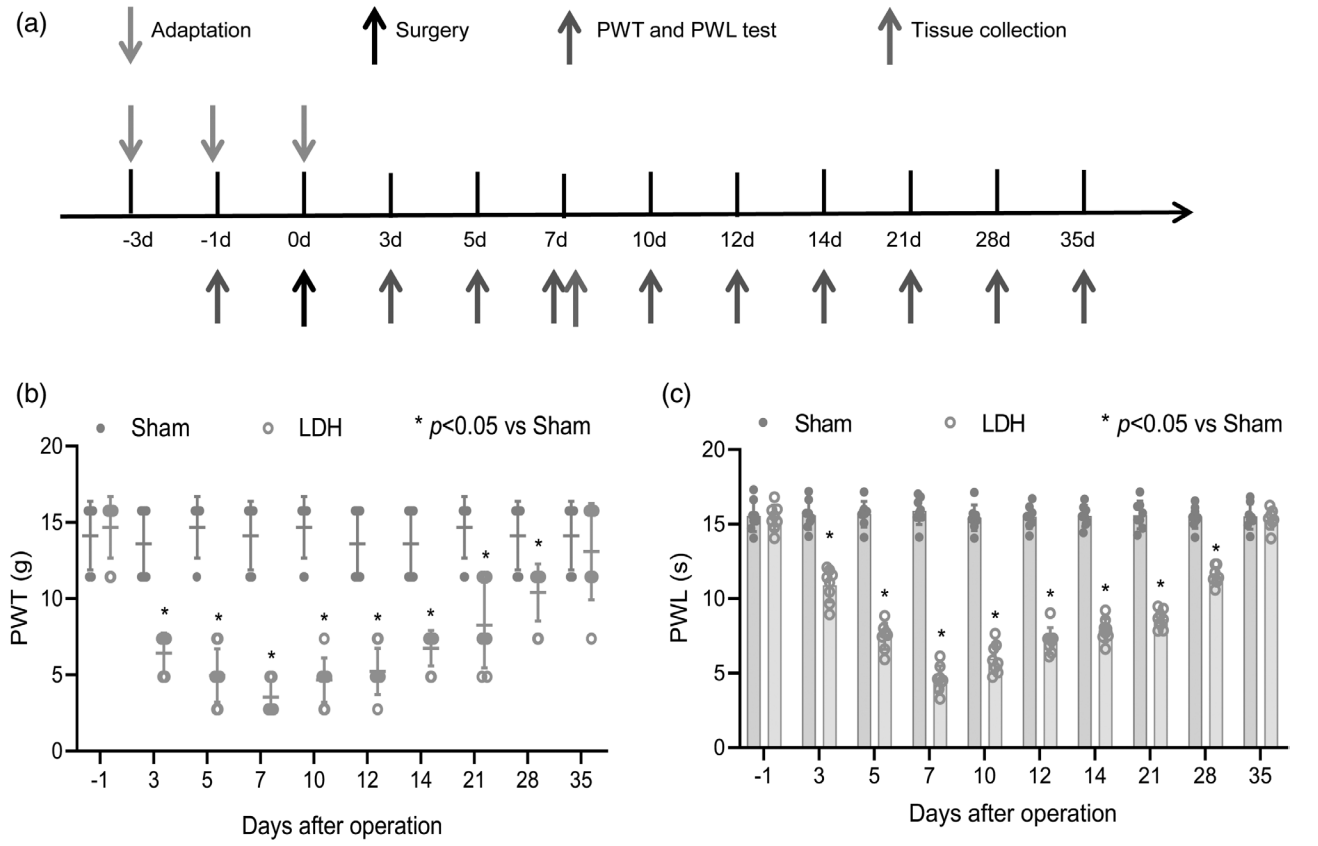
Electroacupuncture stimulation

Rats in the Sham-electroacupuncture and electroacupuncture groups received treatment from the 8th day after surgery for 7 consecutive days, 30 min once a day. The rats were placed in a self-made immobilization apparatus, with the left hindlimb and low back exposed and the rest of the body fixed, and the rats could receive electroacupuncture treatment comfortably without anesthesia [24]. 'Huantiao' (GB-30 acupoint, located at the junction of the lateral 1/3 and medial 2/3 of the distance between the sacral fissure and the greater trochanter of the femur) and 'Yanglingquan' (GB-34 acupoint, located in the depression below the anterior aspect of the fibular tuberosity) were chosen in this study. Acupuncture needles (0.25 × 13 mm, Suzhou Medical Appliance Factory, Suzhou, China) were inserted acupoint at a depth of 3 and 5 mm unilaterally on the ipsilateral side. Conscious rats were electrically stimulated for 30 min with 2 Hz in frequency and 2–3 mA in intensity by an SDZ-IIB electronic acupuncture instrument (Suzhou Medical Appliance Factory). The Sham-electroacupuncture group rats went through the same procedure except without electrical stimulation.

Nociceptive behavior tests

All behavior tests were performed by the experimenter who was blind to all groups. The rats were adapted to acclimatize for at least 30 min before tests. The timing of the behavior tests is shown in Figs. 1a and 3a. Paw withdrawal threshold (PWT) was used to assess the mechanical allodynia using von Frey filaments (Aesthesio, California, USA) as previously described [25]. The rats were placed into individual transparent plexiglass compartments (10 × 10 × 20 cm) onto a metal mesh floor (graticule: 0.5 cm × 0.5 cm). Von Frey filaments (1.98, 2.74, 4.87, 7.37, 11.42, 15.76, and 20.3 g) were applied perpendicularly to the plantar surface of the rat's left hind paw with sufficient force to bend the filaments for 6–8 s or until the rat withdrew. The test was conducted every 10 min for a total of three rounds

Fig. 1



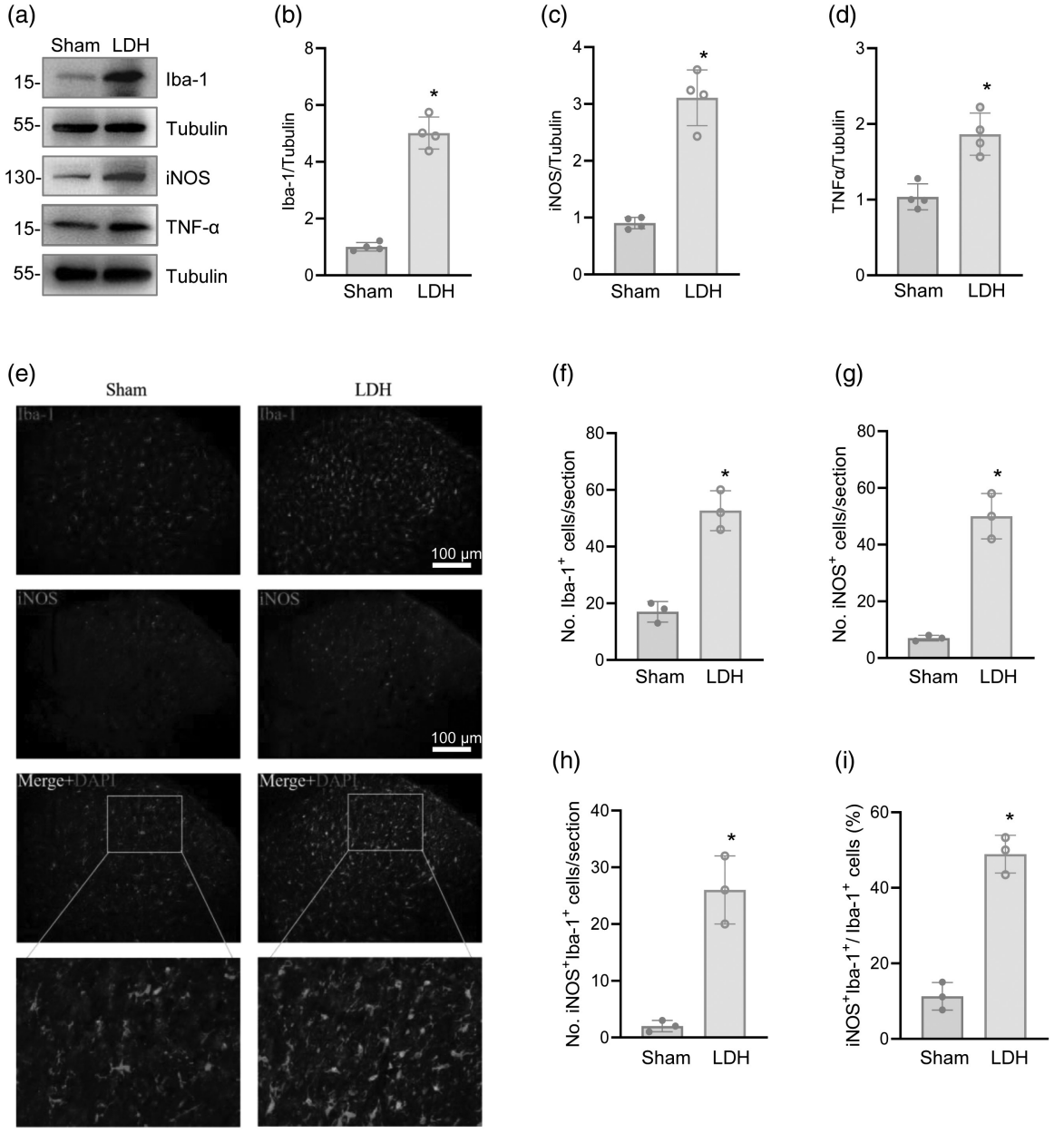
Mechanical and thermal pain thresholds were significantly decreased in LDH rats. (a) Schematic diagram of the experimental procedure. (b) Paw withdrawal threshold, two-way ANOVA, $n = 8$, $^*P < 0.05$. (c) Paw withdrawal latency, two-way ANOVA, $n = 8$, $^*P < 0.05$. ANOVA, analysis of variance; LDH, lumbar disc herniation.

of testing. A stimulus-related withdrawal of the paw was considered a withdrawal response. Finally, the lowest von Frey filament stimulus strength that produced three positive responses was considered the PWT threshold. The tactile stimulus producing a 50% likelihood of withdrawal was determined by the 'up-down' calculating method. The cutoff strength of von Frey filaments was set at 20.3 g to avoid potential injury. Paw withdrawal latency (PWL) was used to assess the thermal hyperalgesia determined in a manner similar to that described previously [26]. After acclimation for 30 min, a radiant heat source setting at 60 °C was focused on the middle of the left hind paw from underneath the glass. The time required to cause the withdrawal of the paw from the stimulus was measured to the nearest 0.01 s (cutoff time 20 s). The test was conducted every 10 min for a total of three rounds of testing. The average of these values in each group was considered the PWL threshold.

Western blotting

The protocols for western blotting were described previously [27]. In brief, after being deeply anesthetized with pentobarbital sodium (50 mg/kg body weight, intraperitoneally), the lumbar spinal dorsal horn (SDH) (L5-6) from the ipsilateral side was quickly extracted and stored in liquid nitrogen [28]. Tissue samples were homogenized in lysis buffer. The homogenate was centrifuged at 12 000 r/min for 15 min at 4 °C, and then the supernatant was collected. The protein concentration was determined by the BCA Protein Assay Kit, following the manufacturer's instructions. Protein samples were separated on SDS-PAGE (10%) and transferred onto polyvinyl difluoride (PVDF) membranes (BIO-RAD, California, USA) at 200 mA for 2 h at 4 °C. Subsequently, the membranes were blocked with 3% BSA for 2 h at room temperature (RT) and incubated overnight at 4 °C with the following primary antibodies: mouse anti-Iba-1 (1 : 500, Santa Cruz, California, USA, sc-32725), rabbit anti-iNOS (1 : 500, proteintech, Chicago, USA, 18985-1-AP), rabbit anti-arginase I (1 : 500, GeneTex, GTX109242), rabbit anti-TNF-α (1 : 500, Affinity, Georgia, USA, Ab-AF7014), mouse anti-IL-10 (1 : 500, Santa Cruz, sc-365858), rabbit anti-CB2R (1 : 400, Thermo Fisher, Massachusetts, USA, PA1-746A), rabbit anti-NF-κB (1 : 1000, Genetex, Texas, USA, GTX102090) and rabbit anti-*p*-NF-κB (1 : 1000,

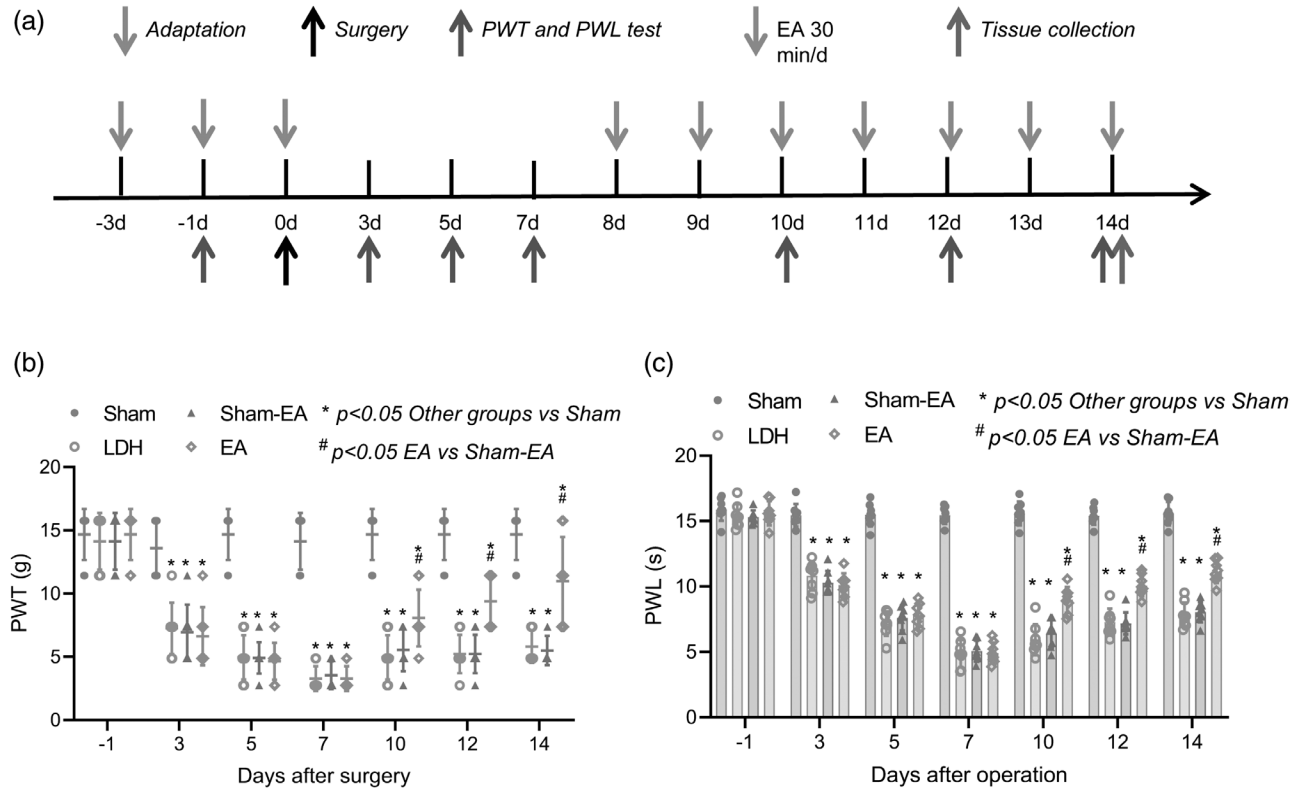
Fig. 2



Microglia in lumbar SDH of LDH rats were activated and polarized toward M1 phenotype, and released proinflammatory cytokines. (a) Representative western blots. (b) Quantification of Iba-1, (c) iNOS, and (d) TNF-α in lumbar SDH on postoperative day 7 in each group, two-tailed *t*-test, $n = 4$, $^*P < 0.05$. (e) Colabeling staining of Iba-1 (microglia marker, red) with iNOS (M1 polarization marker, green) in lumbar SDH on 7 days postoperation in each group. The enlarged images revealed that these two markers were colabeled. Quantitative analyses of the (f) number of Iba-1^+ cells, (g) number of $iNOS^+$ cells, (h) number of Iba-1^+ and $iNOS^+$ double-positive cells, (i) ratio of $iNOS^+$ and Iba-1^+ double-positive cells to Iba-1^+ cells, two-tailed *t*-test, $n = 3$, $^*P < 0.05$. LDH, lumbar disc herniation; SDH, spinal dorsal horn.

cell signaling, Boston, USA, #3033). After washing in TBST (0.5% Tween-20 in TBS), the PVDF membranes were incubated with horseradish peroxidase (HRP) conjugated secondary antibodies polyclonal Goat Anti-Mouse-HRP (Jackson ImmunoResearch Laboratories; Pennsylvania, USA, 1 : 5000) and polyclonal Goat Anti-Rabbit-HRP (Jackson ImmunoResearch Laboratories; 1 : 5000) in TBS and 1% BSA for 1 h at RT. Then, the membranes were visualized in an automated chemiluminescence system (Tanon, Shanghai, China). Anti-α-tubulin (1 : 5000, Cell Signaling, #3873) was used as the loading control, and the ImageJ software (NIH, Bethesda, Maryland, USA) was used to measure the gray value of each band.

Fig. 3



Electroacupuncture treatment increased mechanical and thermal pain threshold in LDH rats. (a) Schematic diagram of the experimental procedure. (b) Paw withdrawal threshold, two-way ANOVA, $n = 8$, $^{*}P < 0.05$, other groups versus sham; $^{\#}P < 0.05$, electroacupuncture versus sham-electroacupuncture. (c) Paw withdrawal latency, two-way ANOVA, $n = 8$, $^{*}P < 0.05$, other groups versus sham; $^{\#}P < 0.05$ electroacupuncture versus sham-electroacupuncture. ANOVA, analysis of variance; LDH, lumbar disc herniation.

Immunofluorescence

After being deeply anesthetized with pentobarbital sodium (50 mg/kg body weight, intraperitoneally), perfused with normal saline via the ascending aorta, and then followed by 4% paraformaldehyde. Then the spinal cord lumbar segments were extracted quickly, postfixed in 4% paraformaldehyde, and then dehydrated in 30% sucrose at 4 °C. Serial frozen sections were cut into 20 μm thick slides in a freezing microtome, washed in PBS, and permeabilized with 0.5% Triton X-100 for 30 min, and then blocked with 7% donkey serum for 1 h at RT. Subsequently, the sections were incubated overnight at 4 °C with the following primary antibodies: goat anti-Iba-1 (1 : 1000, Abcam, Cambridge, UK, ab5076), rabbit anti-iNOS (1 : 500, proteintech, 18985-1-AP), rabbit anti-arginase I (1 : 500, cell signaling, #93668), rabbit anti-CB2R (1 : 40, Thermo Fisher, PA1-746A). After washing, they were with secondary antibodies conjugated with Alexa Fluor 488 and 555 (Invitrogen; California, USA, 1 : 500) for 45 min at RT. Finally, the sections were covered with DAPI (4',6-Diamidino-2-phenylindole; Sigma-Aldrich; Darmstadt, Germany, 200 ng/ml). Images were acquired using Olympus Front mounted fluorescent microscope (Tokyo, Japan).

Statistical analysis

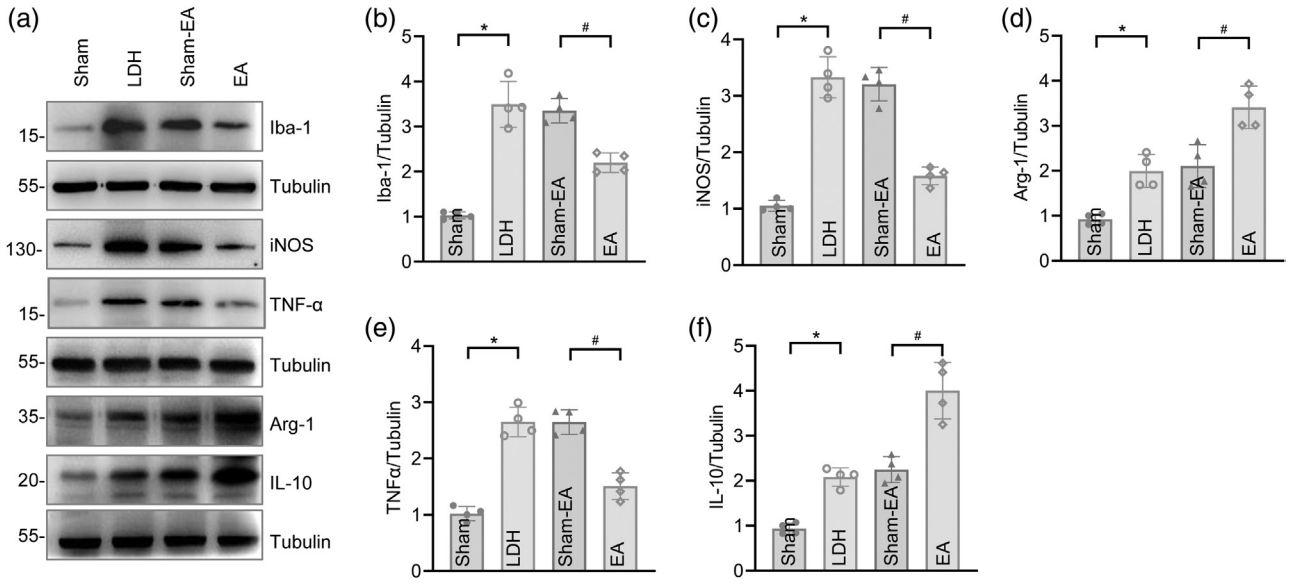
Statistical analyses were performed using Prism 8 (Graphpad Software, San Diego, California, USA). The measurement data conforming to normal distribution are expressed as mean ± SD. Comparisons between two groups were analyzed with the two-sided *t*-test. Comparisons between three or more groups were analyzed with one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Data from the mechanical hyperalgesia and thermal hyperalgesia tests were analyzed with two-way ANOVA followed by Dunnett's multiple comparisons test. Normality was checked before analysis. $P < 0.05$ was considered statistically significant.

Results

Mechanical and thermal pain thresholds were significantly decreased in lumbar disc herniation rats

The PWT and PWL were measured on preoperative day 3 and postoperative day 1, 3, 5, 7, 10, 12, 14, 21, 28, and 35 (Fig. 1a). In the LDH group, PWT and PWL on the ipsilateral side decreased significantly after LDH surgery. Minimum value was reached on postoperative day 7, and

Fig. 4



Electroacupuncture treatment inhibited microglia activation and microglia M1 polarization, and promoted microglia M2 polarization while reducing proinflammatory cytokines, and increasing anti-inflammatory cytokines in lumbar SDH of LDH rats. (a) Representative western blots. (b) Quantification of Iba-1, (c) iNOS, (d) Arg-1, (e) TNF-α and (f) IL-10 in lumbar SDH on postoperative day 14 in each group, one-way ANOVA, $n = 4$, $^{*}P < 0.05$, LDH versus sham; $^{\#}P < 0.05$, electroacupuncture versus sham-electroacupuncture. ANOVA, analysis of variance; Arg-1, arginase-1; LDH, lumbar disc herniation; SDH, spinal dorsal horn.

this change persisted through postoperative day 28, and then returned to baseline level on day 35 after LDH surgery (Fig. 1b and c).

Microglia were activated, polarized toward M1, and released proinflammatory cytokines in lumbar spinal dorsal horn of lumbar disc herniation rats

On postoperative day 7, western blotting and immunofluorescence showed a significant increase in Iba-1 in lumbar SDH of the LDH group compared to the sham group (Fig. 2a, b, e, f). Microglia in lumbar SDH were significantly activated. Further results showed that M1 marker iNOS in ipsilateral lumbar SDH increased compared to the sham group after surgery (Fig. 2a, c, e, g). Similar changes in proinflammatory cytokines TNF-α also increased (Fig. 2a and d). Double immunofluorescence staining showed that the iNOS increased significantly compared to the sham group, and was colabeling with Iba-1 in lumbar SDH (Fig. 2e, g, h, i).

Electroacupuncture treatment attenuated the mechanical and thermal pain in lumbar disc herniation rats

The PWT and PWL were measured on preoperative day 3 and postoperative days 1, 3, 5, 7, 10, 12, and 14 (Fig. 3a). Except in the sham group, the PWT and PWL of the other three groups decreased significantly after surgery (Fig. 3b and c). Electroacupuncture treatment started from postoperative day 8, and the PWT and PWL increased significantly in the electroacupuncture group from postoperative day 10–14 compared to the sham-electroacupuncture group (Fig. 3b and c).

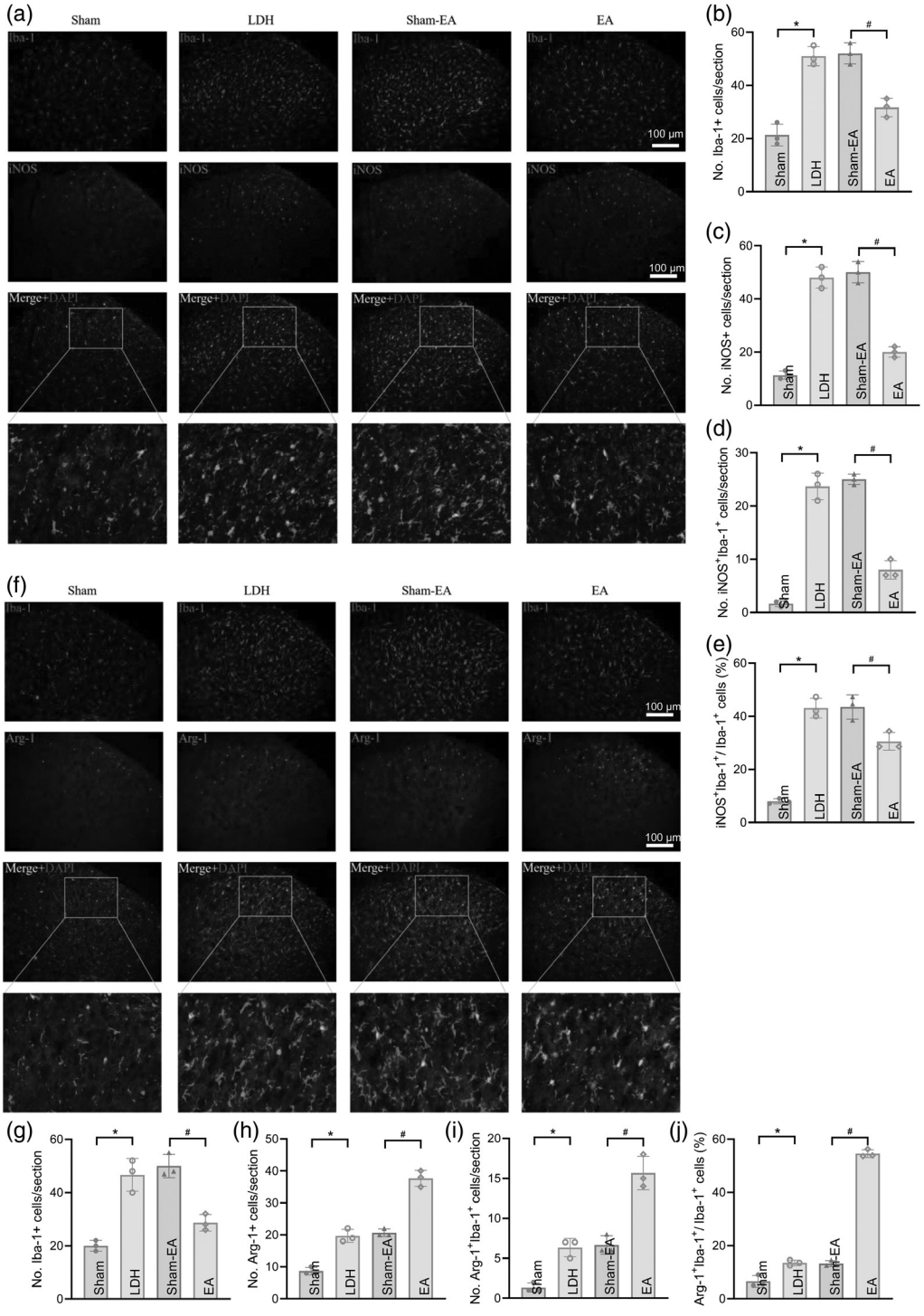
Electroacupuncture treatment inhibited microglia activation in lumbar spinal dorsal horn of lumbar disc herniation rats

After 1 week of electroacupuncture treatment from postoperative day 8, western blotting and immunofluorescence on postoperative day 14 showed a significant increase in Iba-1 in lumbar SDH of the LDH group compared to the sham group, but a significant decrease in the electroacupuncture group compared to the sham-electroacupuncture group (Fig. 4a and b and Fig. 5a and b). These results showed that electroacupuncture treatment inhibits microglia activation in lumbar SDH of LDH rats.

Electroacupuncture treatment inhibited microglia M1 polarization and promoted microglia M2 polarization in lumbar spinal dorsal horn of lumbar disc herniation rats

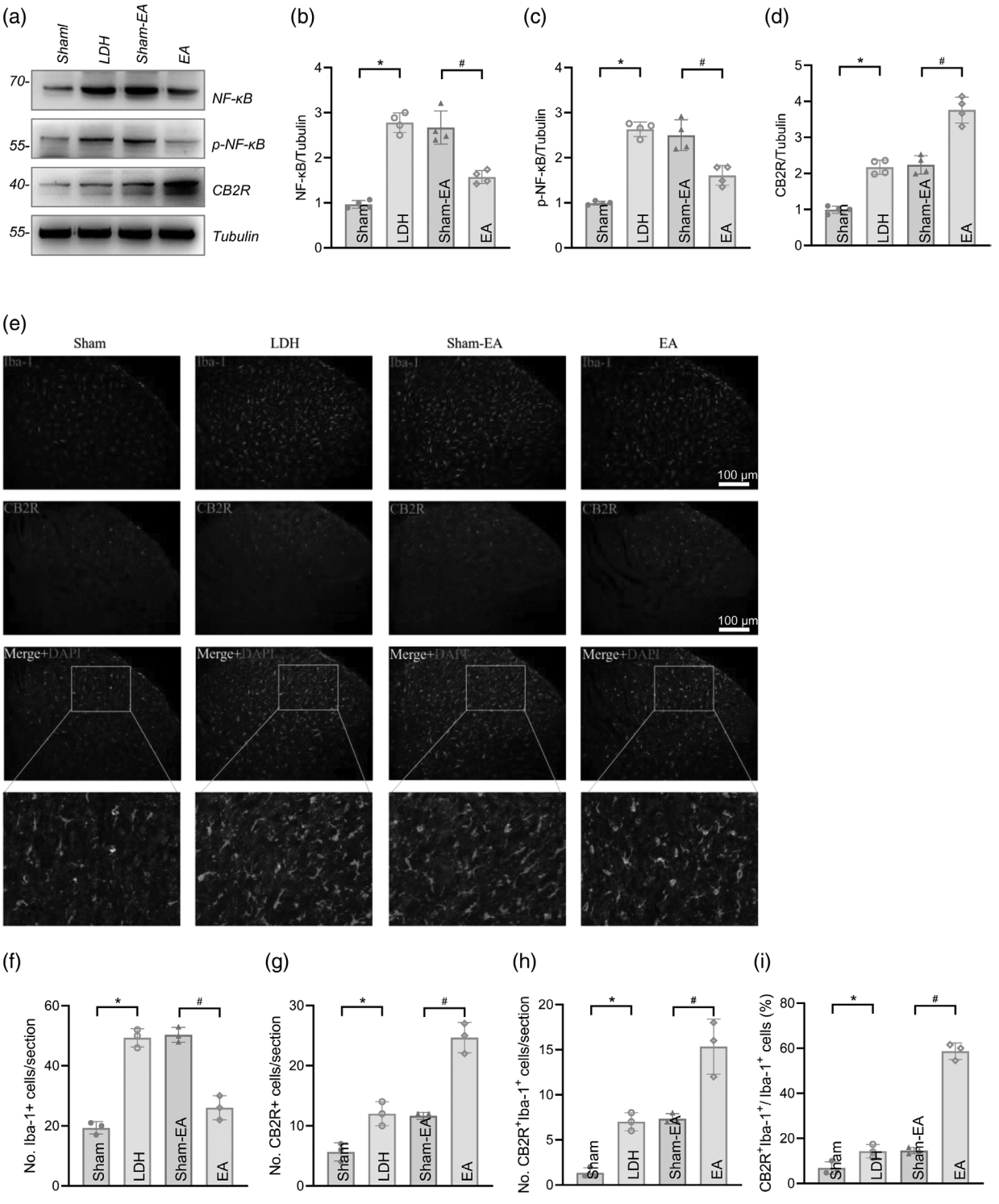
Western blotting and immunofluorescence showed that M1 marker iNOS was increased in the LDH group compared to the sham group, while significantly reduced in the electroacupuncture group compared to the sham-electroacupuncture group (Fig. 4a and c and Fig. 5a and c), and compared with the Sham-electroacupuncture group the M2 marker arginase-1

Fig. 5



Electroacupuncture treatment inhibited microglia activation and microglia M1 polarization and promoted microglia M2 polarization in lumbar SDH of LDH rats. (a) Colabeling staining of Iba-1 (red) with iNOS (green) in lumbar SDH on postoperative day 14 in each group. The enlarged images revealed that these two markers were colabeled. Quantitative analyses of the (b) number of Iba-1$^+$ cells, (c) number of iNOS$^+$ cells, (d) number of Iba-1$^+$ and iNOS$^+$ double-positive cells, (e) ratio of iNOS$^+$ and Iba-1$^+$ double-positive cells to Iba-1$^+$ cells, one-way ANOVA, $n = 3$, $^*P < 0.05$, LDH versus Sham; $^{\#}P < 0.05$, electroacupuncture versus sham-electroacupuncture. (f) Colabeling staining of Iba-1 (red) with Arg-1 (green) in lumbar SDH on postoperative day 14 in each group. The enlarged images revealed that these two markers were colabeled. (g) Quantitative analyses of the number of Iba-1$^+$ cells, (i) number of Arg-1$^+$ cells, (h) number of Iba-1$^+$ and Arg-1$^+$ double-positive cells, (j) ratio of Arg-1$^+$ and Iba-1$^+$ double-positive cells to Iba-1$^+$ cells, one-way ANOVA, $n = 3$, $^*P < 0.05$, LDH versus Sham; $^{\#}P < 0.05$, electroacupuncture versus sham-electroacupuncture. ANOVA, analysis of variance; Arg-1, arginase-1; iNOS, inducible nitric oxide synthase; LDH, lumbar disc herniation; SDH, spinal dorsal horn.

Fig. 6



Electroacupuncture treatment upregulated CB2 receptors on microglia and inhibited NF-κB and *p*-NF-κB expression in lumbar SDH of LDH rats. (a) Representative western blots. Quantification of (b) NFκB-p65, (c) *p*-NFκB-p65 and (d) CB2R in lumbar SDH on postoperative day 14 in each group, one-way ANOVA, $n = 4$, $^{*}P < 0.05$, LDH versus sham; $^{\#}P < 0.05$, electroacupuncture versus sham-electroacupuncture. (e) Colabeling staining of Iba-1 (red) with CB2R (green) in lumbar SDH on postoperative day 14 in each group. The enlarged images revealed that these two markers were colabeled. Quantitative analyses of the (f) number of Iba-1^{+} cells, (g) number of $CB2R^{+}$ cells, (h) number of Iba-1^{+} and $CB2R^{+}$ double-positive cells, (i) ratio of $CB2R^{+}$ and Iba-1^{+} double-positive cells to Iba-1^{+} cells. one-way ANOVA, $n = 3$, $^{*}P < 0.05$, LDH versus sham; $^{\#}P < 0.05$, electroacupuncture versus sham-electroacupuncture. ANOVA, analysis of variance; CB2R, cannabinoid 2 receptor; LDH, lumbar disc herniation; NF-κB, nuclear factor kappa B; SDH, spinal dorsal horn.

(Arg-1) in the electroacupuncture group increased significantly (Fig. 4a and d; Fig. 5f and h). Double immunofluorescence staining showed that the iNOS or Arg-1 were colabeling with Iba-1 in lumbar SDH (Fig. 5a and f). These results demonstrated electroacupuncture treatment inhibited microglia M1 polarization and promoted microglia M2 polarization in lumbar SDH of LDH rats.

Electroacupuncture treatment reduced proinflammatory factors and increased anti-inflammatory factors in lumbar spinal dorsal horn of lumbar disc herniation rats

Western blotting showed that the LDH group exhibited increased TNF-α expression compared to the sham group, whereas electroacupuncture treatment reduced TNF-α and increased IL-10 expression compared to the sham-electroacupuncture group (Fig. 4a, e, f). These results displayed that electroacupuncture treatment reduced proinflammatory cytokines while increasing anti-inflammatory factors.

Electroacupuncture treatment upregulated CB2 receptors on microglia and inhibited NF-κB pathway in lumbar spinal dorsal horn of lumbar disc herniation rats

CB2 receptors, NF-κB, and *p*-NF-κB protein expression in lumbar SDH were examined by western blotting. The results showed that the expression of NF-κB and *p*- NF-κB in lumbar SDH was increased in the LDH group compared to the sham group, and decreased in the electroacupuncture group compared to the sham-electroacupuncture group (Fig. 6a–c). Compared to the sham-electroacupuncture group, CB2 receptors expression in lumbar SDH was significantly increased in the electroacupuncture group (Fig. 6a and d). Immunofluorescence indicated the CB2 receptors were mainly colabeling with Iba-1 in lumbar SDH, and the number of $CB2R^+$ and $Iba\text{-}1^+$ microglia in the electroacupuncture group was increased significantly compared to those in the sham-electroacupuncture group (Fig. 6e, g, h, i). These results suggested that electroacupuncture treatment activated CB2 receptors on microglia and inhibited NF-κB pathway in lumbar SDH of LDH rats.

In summary, electroacupuncture treatment inhibits microglia activation and microglia M1 polarization and promoted microglia M2 polarization, which reduces neuroinflammation and relieves chronic pain. The analgesic effect may be caused by the activation of CB2 receptors on microglia and inhibition of NF-κB pathway in lumbar SDH.

Discussion

The study showed that when lumbar SDH led to nociceptive hyperalgesia, microglia were activated and polarized toward M1 phenotype and released proinflammatory cytokines; however, electroacupuncture treatment which reduced nociceptive hyperalgesia in the LDH rats inhibited microglia activation and microglia M1 polarization, and promoted microglia M2 polarization, while reducing proinflammatory cytokines and increasing anti-inflammatory factors in lumbar SDH.

LDH is a common chronic disease, which is the cause of lower back pain and radicular pain [29]. Although mechanical compression of the nerve root is traditionally thought to be the cause of chronic pain, some studies have shown that the release of proinflammatory cytokines caused by the nucleus pulposus is an important pathological mechanism in causing radicular pain and that the release of proinflammatory cytokines by the nucleus pulposus caused severe pain even in the absence of mechanical compression [30]. Therefore, this study established a noncompression LDH by using rat autologous caudal nucleus pulposus implantation to induce chronic pain.

The lumbar SDH is an important part of the body for the transmission or integration of injurious information. This site shows a large distribution of neuroactive substances and receptors [31]. The role of microglia, as a first line of defense, is particularly crucial at this site; however, how electroacupuncture regulates spinal microglia polarization in lumbar SDH to alleviate pain is not well understood. In this study, the left side (ipsilateral to the surgery) equivalent of human acupuncture points 'Huantiao' (GB-30) and 'Yanglingquan' (GB-34) were used, which were used for the treatment of chronic pain caused by LDH clinically [32]. Previous studies have shown that acupuncture needles inserted into acupoints will not produce analgesic effect without electric stimulation (sham-electroacupuncture) or manual stimulation [33,34]. Sham-electroacupuncture and electroacupuncture groups of rats were placed in a self-made immobilization apparatus, and the electroacupuncture groups of rats could receive electroacupuncture treatment comfortably without anesthesia [35].

Under normal physiological conditions, microglia maintain a ramified cell shape which is known as the M0 phenotype [36]. When the microenvironment is abnormally altered, such as nerve injury, inflammation, or ischemia, microglia rapidly proliferate, migrate to abnormal areas, and polarize towards the classical M1 phenotype, which is geared toward killing pathogens or infected cells, and trigger the antigen presentation response [37]. M1 phenotype microglia produce a large number of proinflammatory cytokines, including nitric oxide (NO), reactive oxidative species, TNF-α, IL-1β, IL-6, IFN-γ [38], during which the expression of the M1 marker CD16 and iNOS are upregulated. M2 phenotype microglia produce anti-inflammatory factors such as IL-4 and IL-10, during which the expression of the M2 marker CD206, TGF-β, and the metabolic or Arg-1 are upregulated [39–42].

In this study, the microglia marker Iba-1 and the M1 marker iNOS were colabelled in lumbar SDH, and their expression was significantly increased in LDH rats, while expression of the proinflammatory cytokines TNF-α was also increased but significantly decreased after electroacupuncture treatment. In contrast, the M2 markers Arg-1 and Iba-1 were colabeled in lumbar SDH and increased in expression after electroacupuncture, while expression of the anti-inflammatory cytokine IL-10 was also increased. Notably, Microglial research has advanced yet has been constrained by a series of dichotomies, such as resting versus activated and M1 versus M2. This dichotomous classification of good or bad microglia is inconsistent with the broad range of microglia states and functions in development, plasticity, aging, and disease. When defined by transcriptomics and proteomics, macrophage responses are more complex than simply M1 and M2. We know that nomenclatures and categories are artificial constructs and that biology is seldom black and white but rather an extended continuum of greys [7].

Previous studies have shown that electroacupuncture stimulation of acupoints elevates endogenous cannabinoids to activate CB2 receptors and reduces inflammation [43]. In CNS, CB2 receptors are mainly expressed in microglia [44]. Especially in the presence of inflammation, CB2 receptors on microglia are significantly increased [30]. In this study, we showed that compared to the sham-electroacupuncture group CB2 receptors on microglia in lumbar SDH were significantly increased in the electroacupuncture group. Moreover, $CB2R^+$ and $Iba\text{-}1^+$ cells colabeled and increased in the electroacupuncture group. In addition, NF-κB and *p*-NF-κB in lumbar SDH were increased in the LDH group, compared to the sham group, while decreased in the electroacupuncture group compared to the sham-electroacupuncture group. These results suggested that electroacupuncture treatment activated CB2 receptors on microglia and inhibited NF-κB pathway in lumbar SDH.

In general, our results provided mechanistic insight into analgesic effect of electroacupuncture treatment; however, the mechanism underlying electroacupuncture upregulating CB2 receptors on microglia in LDH rats is not fully understood. Further studies are required to address how electroacupuncture upregulates CB2 receptors on microglia in LDH rats. Our study also had several limitations, including limited samples and focus on only LDH rat model. Further studies would use more animal models, advanced methods, and examine additional LDH-related chronic pain.

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Conflicts of interest

There are no conflicts of interest.

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