Inhibition of spinal microglia and astrocytes contributes to the anti-allodynic effect of electroacupuncture in neuropathic pain induced by spinal nerve ligation

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ABSTRACT
Objective Besides neurons, activated microglia and astrocytes in the spinal cord dorsal horn (SCDH) contribute to the pathogenesis of chronic pain. Electroacupuncture (EA) has been used widely to treat various chronic pain diseases, however, the underlying mechanisms of EA are still not fully understood.

Methods Male Sprague-Dawley rats were randomly divided into four groups, including an untreated healthy Control group (n=14), a True-spinal nerve ligation (SNL) group that underwent SNL and remained untreated (n=25), a True-SNL +EA group that underwent SNL followed by EA treatment (n=25), and a Sham-SNL group that underwent sham surgery and remained untreated (n=15). SNL was performed unilaterally at L5 and EA was applied to ST36 and BL60 bilaterally once per day. Paw withdrawal thresholds (PWTs) were measured ipsilaterally at baseline and 1, 3, 7, and 14 days after ligation. Activation of microglia and astrocytes in the SCDH were examined bilaterally by immunofluorescence staining, and concentrations of interleukin-1β (IL-1β) and interleukin-6 (IL-6) were measured in the ipsilateral SCDH by ELISA.

Results SNL significantly decreased PWTs and activated glial cells in the superficial laminae of the ipsilateral SCDH. In rats with SNL, glial fibrillary acidic protein (GFAP) immunoreactivity peaked at 7 days and was maintained until 14 days post-ligation. EA significantly alleviated SNL-induced mechanical allodynia. Furthermore, EA reduced microglial activation (OX-42 positive ratios) in the lumbar SCDH at 3 days post-ligation and suppressed astrocyte activation (GFAP positive ratios) at all time points observed.

Conclusions EA stimulation alleviates SNL-induced neuropathic pain, at least in part through inhibition of spinal glial activation. Moreover, inhibition of spinal microglia and astrocyte activation may contribute to the immediate effects and maintenance of EA analgesia, respectively.

INTRODUCTION
Neuropathic pain, which is characterised by spontaneous pain, allodynia (pain evoked by non-noxious stimuli) and hyperalgesia (increased pain intensity in response to noxious stimuli), has recently been defined by expert consensus as “pain arising as a direct consequence of a lesion or disease affecting the somatosensory system”.1 Epidemiological data reveal a high prevalence of neuropathic pain in pain clinics: among 2173 surveyed patients, definite, probable and possible neuropathic pain was cited in 639 (29.4%), 304 (14.0%) and 97 (4.5%) of cases, respectively.2

Efforts to determine the underlying mechanisms of chronic pain have generally focused on the neuron. However, accumulating evidence suggests that glial cells also play an important role in the initiation and maintenance of chronic pain.1-3 Robust activation of spinal glia, especially microglia and astrocytes, has been observed in various models of neuropathic pain.1,2-5 Activated glia release proinflammatory cytokines, such as interleukin-1β (IL-1β) and interleukin-6 (IL-6), which facilitate pain transmission.3 It has been reported that inhibition of spinal glial activation significantly attenuated mechanical pain by

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intrathecal injection of minocycline (a microglial activation inhibitor), fluorocitrate (an astrocytic activation inhibitor), and propentofylline (a glial activation inhibitor). Moreover, activation of spinal microglia and astrocytes had different time courses. Minocycline could only prevent mechanical allodynia in the early stages whereas fluorocitrate could reverse existing mechanical allodynia. Moreover, pretreatment of intrathecal minocycline suppressed glial fibrillary acidic protein (GFAP) expression in the spinal dorsal horn in a rat model of arthritis induced by complete Freund’s adjuvant. These results demonstrate that activation of microglia and astrocytes contributes to the initiation and maintenance of neuropathic pain, respectively.

Electroacupuncture (EA), a modified acupuncture technique, has been used for several decades in the treatment of chronic pain. Spinal nerve ligation (SNL) is a widely used model of neuropathic pain, which can be alleviated by repeated EA. In rats with SNL, spinal astrocytes and microglia on the side ipsilateral to the lesion were heavily stained and appeared to show an activated state, and intrathecal injection of minocycline significantly attenuated mechanical allodynia in this model. It has been reported that repetitive EA stimulation significantly suppresses spinal glial activation in other pain models, and EA analgesia has been potentiated by inhibition of spinal astrocyte or microglial activation. However, whether the analgesic effect of EA in SNL-induced pain is related to the inhibition of spinal glial activation remains unclear. Moreover, the potential role of spinal microglia and astrocytes in EA analgesia has not been clarified. Therefore, in our study, we used rats with SNL at L5 to model neuropathic pain and investigated the effect of EA on paw withdrawal thresholds (a marker of pain) and activation of spinal glial cells.

METHODS
Experimental rats
Experiments were performed on male Sprague-Dawley rats weighing 220–260 g. All experimental animals were obtained from the animal experiment centre attached to Zhejiang Chinese Medical University (No. SYXXK(沪) 2008-0115). They were housed five per cage with free access to food and water and maintained in temperature- and light-controlled rooms (24±2°C, 12 h/12 h light/dark cycle with lights on at 07:00). Before experimental manipulation, rats were allowed to acclimatise to the housing facilities for 1 week. All experimental procedures were approved by the Animal Ethics Committee of Zhejiang Chinese Medical University.

Establishment of neuropathic pain model
SNL surgery was performed according to the method described by Kim and Chung. Briefly, rats were initially anaesthetised with intraperitoneal (ip) 10% chloral hydrate (0.35 mL/100 g bodyweight). An incision was made on the back between L4 and S2, followed by retraction of the paravertebral musculature from the vertebral transverse processes. The L6 transverse process was partially removed with the help of bone rongeurs. A 3/0 silk suture was tied tightly to ligate the L5 spinal nerve and the wound was closed in two layers (fascia and skin). Sodium penicillin (20 IU/day per rat) was given intramuscularly for three consecutive days to prevent postoperative infection. Sham surgery consisted of exposing the nerve in the same manner, but without ligation. Any animals with disability of movement after surgery were excluded due to presumed damage to the L4 spinal nerve.

Experimental groups and EA treatment
Seventy-nine Sprague-Dawley rats were divided randomly into the following four groups: (1) the Control group (n=14) without any treatment; (2) the True-SNL group (n=25) that received SNL surgery followed by immobilisation but no EA stimulation; (3) the True-SNL+EA group (n=25) that received SNL surgery, immobilisation and EA stimulation; and (4) the Sham-SNL group (n=15) that received sham surgery in place of SNL.

Rats in the True-SNL+EA group were gently immobilised using a special cotton retainer designed by our laboratory (Patent No. ZL 2014 2 0473579.9, State Intellectual Property Office of the People’s Republic of China). Stainless steel acupuncture needles (Φ 0.25 mm×13 mm length, Suzhou Medical Appliance Factory) were inserted to a depth of 5 mm at ST36 (Zusanli) bilaterally (5 mm lateral to and below the anterior tubercle of the tibia) and BL60 (Kunlun) bilaterally (at the level of the ankle joint, between the tip of the lateral malleolus and Achilles tendon). The homolateral needles were connected to the output terminals of a HANS Acupuncture point Nerve Stimulator (Hans-100, Huawei Co, Ltd, Beijing, China). The EA parameters were set as follows: constant square wave current output (pulse width: 0.6 ms at 2 Hz, 0.2 ms at 100 Hz); intensities ranging from 1 to 2 mA (15 min each, total 30 min); 2 and 100 Hz alternating frequencies (automatically shifting between 2 and 100 Hz stimulation for 3 s each). EA stimulation was administered for 30 min, once daily, and started after the completion of the first behavioural test on day 1 post-surgery. Treatment continued until day of euthanasia between 3 and 14 days.

Paw withdrawal threshold measurement and immunofluorescenec staining
In each experimental group, eight to nine rats were tested for mechanical allodynia of the plantar surface of the hindpaw before surgery and 24 h, 3 days, 7 days, and 14 days afterwards. Paw withdrawal thresholds (PWTs) were measured according to the
method described by Liang et al.9 At the end of the 14-day period, four to five rats from each group of eight to nine were euthanased and underwent immunohistochemical analysis of their spinal cord tissues. In the True-SNL and True-SNL+EA groups an additional 10 animals per group were euthanised in the same manner at day 3 (n=5 each) and day 7 (n=5 each). In keeping with the 3Rs principle of reduction, no additional animals were used in the Control and Sham-SNL groups (reflected in their lower baseline numbers). Accordingly, the 14-day immunohistochemical assessments for these two groups were also used as negative controls for the two True-SNL groups at both 3 and 7 days. Rats were anaesthetised by ip administration of 10% chloral hydrate before being transcardially perfused with sterilised saline, followed by 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS). The L4–L6 segments were removed, post-fixed for 6 h, then placed in 15–30% sucrose until equilibration. The tissue was cryosectioned at 30 μm, and the sections were collected in 0.1 M PBS (containing 30% sucrose, 30% ethylene glycol) and stored at −20°C. After blocking with 2% normal goat serum in PBS containing 0.3% Triton X-100 for 1 h at 37°C, sections were incubated with mouse anti-integrin αX-100 for 1 h at 37°C, sections were incubated with mouse anti-integrin α (OX-42, a microglial marker; 1:400, MCA275GA, Serotec) or mouse anti-GFAP (an astrocyte marker; 1:1000, MAB3402, Millipore) primary antibody overnight at 4°C, followed by fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (1:400, 115-096-072, Jackson) for 1 h at 37°C. Sections were then washed and coverslipped with mounting medium. Sections from different groups were processed together in the same batches to minimise staining variability.

Sections were analysed with a fluorescence microscope (MD2500, Leica), and images were captured with a charge-couple device (CCD) camera. Quantification of glial immunoreactivity was performed using Image-Pro Plus 6.0 software (Thermo) under blinded conditions. The total area of the superficial laminae of the spinal cord dorsal horn (SCDH) was traced and measured, and the number of positive pixels within the traced area was determined and expressed as the percentage of pixels positive for immunoreactivity. Five non-consecutive sections determined the average for each rat, and four to five rats were included in each group.

ELISA
The remaining six rats per experimental group (that had not undergone behavioural testing and/or immunohistochemistry) were euthanased at 7 days postsurgery. Under terminal anaesthesia (ip injection of 10% chloral hydrate followed by cervical dislocation), samples of lumbar spinal cord were removed and the quarter containing the ipsilateral SCDH was dissected on an ice-chilled plate, then immediately frozen in liquid nitrogen. Tissues were homogenised in radio-immunoprecipitation assay (RIPA) buffer (Beyotime Biotechnology) and centrifuged at 17968 × g (14000 rpm) for 15 min. The supernatant was collected. IL-1β (#RLB00) and IL-6 (#R6000B) levels were assessed by ELISA (R&D Systems) according to the manufacturer’s instructions. Each sample was examined in duplicate and averaged.

Statistical analysis
The Statistical Package for the Social Sciences (SPSS) V20.0 (Chicago, Illinois, USA) was used for statistical analysis. Data are expressed as mean±SEM. Differences between groups and time points were analysed using one-way analysis of variance (ANOVA) with post hoc test of least significance difference. A value of p<0.05 was considered to be statistically significant.

RESULTS
Ipsilateral PWTs at different time points
Following SNL, rats frequently exhibited sudden licking of the operated hindpaw while resting, and limped on the ipsilateral side while walking. Differences in baseline PWTs, reflecting the pain threshold of the rats, did not differ significantly among the four groups (table 1). Data were unavailable for three rats (one in the True-SNL group and two in the True-SNL+EA group) that were excluded due to movement disability. Compared with the Control group, ipsilateral PWTs of the rats with true SNL began to decline at 24 h post-ligation (p<0.01) and were maintained at a lower level for 14 days (p<0.01). By contrast, those of the rats in the Sham-SNL group reduced slightly at the 24 h time point, then quickly returned to normal. Following EA treatment, compared with day 1, PWTs of rats in the True-SNL+EA group improved significantly (p<0.01). Accordingly, they were higher than those in the True-SNL group at 3 and 7 days post-SNL (p<0.001), but not significantly so at 14 days.

Activation of astrocytes (GFAP immunohistochemistry)
Figure 1 illustrates the fluorescent ratio of GFAP in lumbar SCDH as a marker of astrocyte activation. Data were unavailable from one rat in the True-SNL group on day 3 because the spinal cord tissue sample was inadvertently damaged. The GFAP positive ratio in laminae I–II of the ipsilateral SCDH of rats with SNL was greater at 3 and 7 days compared to 14 days post-ligation (21.29±0.81% and 21.84±0.71% vs 15.91±1.05%, respectively, p<0.01 each). After EA, the ipsilateral GFAP positive ratio of the True-SNL+EA group was lower than that in the True-SNL group at 3 days (14.94±1.02% vs 21.29±0.81%, p<0.01), 7 days (14.06±0.90% vs 21.84±0.71%, p<0.01), and 14 days (13.25±0.32 vs 15.91±1.05%, p<0.05). At 14 days, astrocyte activation in the
Table 1

<table>
<thead>
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<th>Group</th>
<th>n</th>
<th>Baseline</th>
<th>Day 1 (24 h)</th>
<th>Day 3</th>
<th>Day 7</th>
<th>Day 14</th>
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<td>Control</td>
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<td>22.42±0.79</td>
<td>22.44±1.22†</td>
<td>22.17±1.70</td>
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<tr>
<td>True-SNL</td>
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<td>25.96±1.85</td>
<td>19.14±0.90**§</td>
<td>15.58±1.70***§§</td>
<td>16.18±0.94**§§</td>
<td>16.96±1.32*§§</td>
</tr>
<tr>
<td>True-SNL+EA</td>
<td>7</td>
<td>23.56±1.61</td>
<td>15.61±1.20**§§</td>
<td>22.44±0.89▲##</td>
<td>23.73±1.37▲###</td>
<td>21.14±1.33#</td>
</tr>
</tbody>
</table>

Values are mean±SEM.
† p=0.015, ‡ p=0.026, * p<0.001 and ** p<0.001 compared with the Control group.
▲ p<0.001 compared with the Sham-SNL group.
§ p=0.001 and §§ p<0.001 compared with baseline.
# p=0.005, ## p=0.001 and ### p<0.001 compared with Day 1.
EA, electroacupuncture; SNL, spinal nerve ligation.

Figure 1
Astrocyte activation (glial fibrillary acidic protein (GFAP) immunohistochemistry). (A) Representative sections of GFAP positive cells (bars=100 μm) in the ipsilateral spinal cord dorsal horn (SCDH) at three different time points (3, 7 and 14 days post-surgery) in rats undergoing spinal nerve ligation (SNL surgery) and receiving electroacupuncture (True-SNL+EA group, n=5 each) or no treatment (True-SNL group, n=4 at D3 and n=5 at D7 and D14) and at a single time point (14 days post-surgery) in healthy rats (Control group, n=4) and rats undergoing sham surgery (Sham-SNL group, n=4). Enlargements in the insets demonstrate the changes in the expression of GFAP. (B) Quantification of the positive ratio of GFAP in superficial laminae of the ipsilateral SCDH. ** p<0.01 compared with the Control group. ▲ p<0.05 and ▲▲ p<0.01 compared with the True-SNL group. •• p<0.01 compared with the Sham-SNL group. ••• p<0.01 compared with day 1.

true-SNL group was increased compared to both the Control and Sham-SNL groups (15.91±1.05% vs 10.17±0.61% and 11.31±0.97, respectively, p<0.01 each) in the ipsilateral SCDH. By contrast, there were no significant differences between groups at 14 days post-SNL in the contralateral GFAP positive ratio.

**Activation of microglia (OX-42 immunohistochemistry)**

Figure 2 illustrates the fluorescence ratio of OX-42 in lumbar SCDH as a marker of microglial activation. After SNL, the OX-42 positive ratio in the superficial laminae of the ipsilateral SCDH was greater at 3 days compared to both 7 and 14 days post-ligation (6.75±0.54% vs 5.07±0.61% and 3.56±0.52%; p<0.05 and p<0.01, respectively). The ratios in the True-SNL+EA group was significantly lower than the True-SNL groups at 3 days post-ligation only (3.66±0.34% vs 6.75±0.54%, p<0.01). At this stage, ratios in the True-SNL group were significantly greater than the Sham-SNL group and Control group in the ipsilateral SCDH (6.75±0.54% vs 1.96±0.59% and 1.71±0.32%, respectively, p<0.01 each) and contralateral SCDH (3.42±0.18% vs 1.78±0.13% and 1.16±0.25%, respectively, p<0.01 each). Moreover, OX-42 positive ratios on the contralateral side were significantly lower in the True-SNL+EA versus True-SNL groups (3.21±0.26 vs 3.42±0.18%, p<0.01).

**IL-1β and IL-6 in ipsilateral SCDH**

Table 2 shows the IL-1β and IL-6 concentrations in the ipsilateral SCDH as measured by ELISA on day 7. Data were unavailable for one rat in the Sham-SNL that died following an anaesthetic overdose and one rat in each of the True-SNL and True-SNL+EA groups that were excluded due to movement disability. The IL-1β concentrations of rats with SNL were significantly higher than those of the Control group (p<0.01), whereas those of the Sham-SNL group were marginally increased relative to healthy controls (p<0.05). Similarly, the spinal IL-6 concentrations after SNL surgery increased significantly compared with the Control group (p<0.01) but were not significantly different following sham surgery. There were no significant differences between the True-SNL+EA and True-SNL groups in either IL-1β or IL-6.

**DISCUSSION**

Our results indicate that repeated EA effectively alleviated the mechanical allodynia induced by L5 SNL at 3 and 7 days post-SNL. Activation of spinal microglia and astrocytes reached their peak at an early phase (D3) and later phase (D7) of neuropathic pain modeling, respectively. Astrocyte activation was significantly inhibited by repetitive EA at all time points examined, whereas microglial activation was suppressed significantly only at 3 days post-SNL. These findings suggest that inhibition of spinal microglial activation may participate in the anti-allodynic effect of EA in the early phase of neuropathic pain; inhibition of astrocyte activation appears to last slightly longer.

The SNL model, which was first reported by Kim and Chung, is a classic neuropathic pain model that is used widely. Jinmo Chung, the founder of the SNL model, pointed out that a successful surgery would result in obvious signs of mechanical allodynia. In parallel with Chung’s reports, the present results showed that mechanical allodynia was successfully induced by L5 SNL, as reflected by the rapid and obvious decrease in paw withdrawal thresholds, and maintained until 14 days post-ligation. In our present study, repetitive EA mitigated the decrease in mechanical pain threshold at day 3 and 7 after surgery, in keeping with previous reports.

Accumulating evidence suggests that activated microglia and astrocytes in the spinal cord contribute to the pathogenesis of neuropathic pain. Blockade of spinal glial activation can prevent, and possibly even reverse, various aberrant pain states. Further studies have revealed that the activation of spinal glia provoked by peripheral nerve injuries is dynamic rather than static. For example, spinal GFAP mRNA measured by real-time reverse transcription polymerase chain reaction (RT-PCR) did not significantly increase until postoperative day 4 and continued to increase until 28 days after L5 nerve transection. After L5 nerve transection, immunofluorescence staining of ionised calcium binding adaptor molecule 1 (Iba-1, a microglial marker) increased in the entire L5 dorsal horn on days 1–4 followed by the deep laminae on day 7 after nerve injury. Conversely, GFAP expression increased at later time points from day 4 in the deep laminae) to day 7 in the entire dorsal horn. In the current study, notable increases in both GFAP and OX42 immunoreactivity were observed in the superficial laminae of the ipsilateral dorsal horn. Moreover, the peak intensity of GFAP and OX42 staining occurred on day 7 and day 3 post-SNL respectively, which are comparable with previous findings. However, it must be noted that this was a serial slaughter study (animals were euthanased at different time points: 3, 7 and 14 days post-SNL), therefore the results at each time point are derived from a different group of rats.

A slight increase in contralateral OX42 at day 3, but not GFAP staining at day 14, was observed. It had been reported that rats may develop mechanical and cold allodynia in the contralateral paw after SNL. Chronic intraperitoneal injection of minocycline significantly attenuated OX42 immunoreactivity in the contralateral spinal cord of rats with SNL. This phenomenon may reflect contralateralisation, which has previously been reported and may involve microglial activation. Thus, the differential roles of contralateral microglial and astrocyte activation at different stages of SNL-induced neuropathic pain need further study.
Previous reports suggest that microglial cells and astrocytes play different parts in the development of neuropathic pain. For example, the activation of microglial cells in the ipsilateral SCDH increased within 1 day of nerve injury; however, astrocytes were activated later than microglial cells and were implicated in the maintenance of mechanical allodynia after spinal nerve injury. Results of pharmacological studies confirm that intrathecal administration of minocycline only prevents mechanical allodynia in the early stages, whereas fluorocitrate may reverse existing mechanical allodynia. Neuropathic pain induced by a variety of methods (including chronic constriction injury, tetanic stimulation of the sciatic nerve, and S1/S2 spinal nerve transection) can be alleviated by EA treatment in association with the inhibition of spinal glial activation. However, there is a
lack of understanding of the potential role that spinal glial cells play in EA analgesia for SNL-induced neuropathic pain. The present study showed that, although SNL-induced microglial activation appeared in the early phase of neuropathic pain, the activation of spinal astrocytes was maintained for a longer period (2 weeks). Moreover, both microglial and astrocytic activation were inhibited by repetitive EA treatment in the early and later phases of neuropathic pain, respectively. This suggests that inhibition of spinal microglial activation contributes to the early phase of EA-induced analgesia while suppression of astrocyte activation participates in the maintenance of EA analgesia.

Similar to other immune cells, activated microglia and astrocytes in the SCDH release pro-inflammatory cytokines that are thought to facilitate pain transmission. IL-1β and IL-6 are the main pro-inflammatory cytokines and are implicated in a variety of painful conditions. We found that the content of IL-1β and IL-6 in ipsilateral SCDH increased markedly after SNL surgery. There was no statistically significant impact of EA on either cytokine, although this study may have been underpowered to detect any differences.

In conclusion, this study has demonstrated that EA stimulation exerts an analgesic effect in SNL-induced neuropathic pain, at least in part through inhibition of spinal glial activation. Moreover, inhibition of spinal microglial and astrocyte activation might differentially contribute to the immediate effects and maintenance of EA analgesia, respectively.

Contributors YL designed the experimental protocols and wrote the initial draft of the manuscript. YQ contributed to the immunofluorescence staining and image acquisition. JD performed the ELISAs and behavioural measurements. JL performed the animal experiments and EA treatments. JF and JZ assisted in the preparation of the immunofluorescence staining. JF modified the design of this study and revised the manuscript.

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Competing interests None declared.

Ethics approval This study was prospectively approved by the Animal Ethics Committee of Zhejiang Chinese Medical University (reference number ZSLL-2012-020) and the Moral Committee on Research Animals of the People’s Republic of China, and was conducted in accordance with guidelines for animal welfare equivalent to the National Research Council’s ‘Guide for the Care and Use of Laboratory Animals’.

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