

Electroacupuncture ameliorated neuropathic pain induced by chronic constriction injury via inactivation of PI3K/AKT pathway

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Abstract

Objective: To explore the analgesic effect of electroacupuncture on neuropathic pain and the role of PI3K/AKT pathway in microglial activation in spinal cord.

Methods: Chronic constriction injury model was established using Sprague-Dawley rats to mimic neuropathic pain. The pain intensity was then detected by paw withdrawal threshold and thermal withdrawal latency. Real-time quantitative PCR (qRT-PCR), Western blot were used to evaluate the activation of PI3K/AKT pathway as well as microglia in L4-5 of all rats. Proinflammatory cytokines TNF- α and IL-6 were analyzed by Enzyme-linked immunosorbent assay (ELISA). **Results:** It was demonstrated that chronic constriction injury-induced hyperalgesia was significantly improved by LY294002 intrathecal administration and electroacupuncture stimulation at “Zusanli” 足三里 (ST36) and “Yanglingquan” 阳陵泉 (GB34). Also, treatment with electroacupuncture significantly reduced the activation of microglia and downregulated the levels of TNF- α and IL-6, which was similar to the outcomes of LY294002 intrathecal administration. Furthermore, the expression and phosphorylation of PI3K/AKT signaling was markedly suppressed by electroacupuncture treatment.

Conclusions: These findings indicated that electroacupuncture exhibited the analgesic effect on CCI rats by inhibiting the activation of microglia and production of proinflammatory cytokines in spinal cord through blocking the microglial PI3K/AKT signaling activation.

Keywords: Neuropathic pain, electroacupuncture, PI3K/AKT pathway, microglia

INTRODUCTION

Neuropathic pain (NP) occurred in 6% to 8% of patients experiencing a disorder or injury of nervous system¹, which is characterized by spontaneous pain, hyperalgesia and allodynia.² The current analgesics not only exert a few troublesome side effects, but also can't satisfactorily solve the problem.³ Thus it's of vital urgency to explore the pathogenesis and therapeutic strategies of NP. One key component facilitating the maintenance of NP has considered to be persistent existence of neuroinflammation in spinal dorsal horn following nerve injury.^{4,5} Also, polarization and proliferation of microglia, especially subsequent over-release of inflammatory cytokines such as IFN- γ , IL-6 and TNF- α are causative factors resulting in spinal neuroinflammation which is responsible for the central sensitization.⁶⁻⁸ Thus, the disruption of microglial activation and proinflammatory cytokine action in spinal cord

might be a potentially novel treatment strategy for NP. However, the potential mechanism by which initiates the activation of microglia in NP has yet to be explored.

It is well established that phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) pathway are involved in modulating nociceptive information. Accordingly, the close relationship between PI3K/AKT pathway and NP has been elucidated by recent research. Liu W *et al.* have reported that the expression of PI3K/AKT increased in the L4-6 spinal cord of NP model rats, and importantly, inhibiting the activation of PI3K/AKT pathway efficiently attenuated the development of spinal central sensitization.⁹ Moreover, PI3K/AKT pathway plays a crucial role in the activation of spinal microglia in NP model.¹⁰ Therefore, blocking the activation of microglial PI3K/AKT signaling might be promising therapeutic strategy of NP.

Electroacupuncture (EA) treatment, clinically effective in relieving NP¹¹⁻¹³, has been widely used due to easy practice and little side-effects. Accumulating evidence revealed that spinal glial cells might participate in the process of EA analgesia in NP models.¹⁴⁻¹⁶ It has been demonstrated that the analgesic effect of EA may be partly mediated by inhibition of inflammation and glial activation.¹³ Li *et al.* reported that EA stimulation at “Zusanli” 足三里 (ST36) and “Yanglingquan” 阳陵泉 (GB34) reduced the portion of the activated microglia and behavioral hypersensitivity.¹⁷ However, little is known about the innate action of microglia in spinal cord in the process of EA analgesia.

In this study, we hypothesized that the underlying mechanism of EA analgesia correlated with its inhibitory effect on microglial via inactivation of PI3K/AKT pathway. Hence, we first examined whether EA promoted recovery of hyperalgesia in rats after chronic constriction injury (CCI). Then the activation of spinal microglia and release of proinflammatory cytokines in CCI rats were detected under EA treatment and LY294002 (PI3K/AKT inhibitor) administration. We also evaluated the effect of EA on inactivation of PI3K/AKT pathway.

METHODS

Animal grouping

Sprague-Dawley rats (males, 6-8 weeks old, 200-250 g) were purchased from Southwest University Animal Center (LuZhou, SiChuan, China). All animals were housed at standard laboratory conditions (12-hlight/dark cycle) and given free access to food and water. All rats were randomly divided into five groups (n=20 in each group): Sham group (sciatic nerve of rats were exposed without ligation), CCI group (rats were subjected to CCI), CCI+EA group (rats were subjected to CCI receiving EA treatment), CCI+LY294002 group (rats were subjected to CCI receiving LY294002 intrathecal injection), CCI+NS group (rats were subjected to CCI receiving the same volume of 0.9% NaCl injection). The investigators were blinded about the group belonging. All experiments conformed to National Institutes of Health Guide for the Care and Use of Laboratory Animals and the Chinese Council's Guide for the Care and Use of Laboratory Animals.

Chronic constriction injury model establishment

CCI surgery was performed using the standard

protocol as described by Bennett and Xie.¹⁸ Briefly, the rats were firstly deeply anaesthetized and the right sciatic nerve was exposed from skin and muscle. Four ligatures (4-0 silk) were subsequently made around the nerve with a 1.0mm interval between each. Rats in the sham group were not ligated after exposure of the nerve. To avoid infection, penicillin was intraperitoneally administrated at a dose of 80000IU. PWT and TWL were employed in all CCI rats to confirm the neuropathic pain development one week after surgery.

Intrathecal catheter and intrathecal administration

After anesthesia, the ligamentum flavum was exposed at the site of L5-6. Then, 32-gauge polyethylene intrathecal catheter (CR3212 Cth RSR 32G, Recath Co, LLC, Allison Park, PA, USA) was inserted into blow arachnoid membrane. When the rats woke up from anesthesia, 20 μ L 2% lidocaine was intrathecally injected to confirm the catheter was placed in proper location. If two hind legs were paralysed within 30s and recovered in 30mins, it could be regarded as successful operation. Finally, LY294002 (Akt inhibitor; 10 μ g, 20 μ L) or 20ul of 0.9% NaCl were administrated intrathecally for consecutive 14 days after CCI.

Electroacupuncture treatment

According to classic theory of traditional Chinese medicine, “Zusanli” (ST36) and “Yanglingquan” (GB34) were selected as acupoints for their efficacy on treating low back pain. The self-made cloth bag was used for rats immobilization. Filiform needles (Huatuo, Suzhou Medical Appliance Manufactory, Jiangsu, China) were inserted into two acupoints with a depth of 5 mm which corresponded to ST36 and GB34 in humans. ST36 is located 5 mm beneath the capitulum fibulae and posterior-lateral to the knee joint, and GB34 is about 5 mm superior-lateral to ST36. EA was carried out with a HANS Apparatus (Hans-200A, Jisheng Medical Technology, Co., Ltd., Nanjing, China) at 1 mA, 2HZ for 30 min a day.

Behavioral tests

Paw withdrawal threshold (PWT) and Thermal withdrawal latency (TWL) tests were measured on 1d before surgery and on 3 d, 7 d and 14 d after surgery. Rats were habituated in Plexiglas chambers 1 to 2 h daily for three days prior to behavioral testing. PWT was assessed by

stimulating the plantar surface of ipsilateral hind paw with von Frey filaments (North Coast Medical, San Jose, CA) and following the up-down method.¹⁹ Briefly, after 30 min of acclimation to plastic cage, the ipsilateral mid-plantar surface of right hind paw was perpendicularly pressed with a series of 7 von Frey filaments (2, 4, 6, 8, 10, 15, and 26 g, respectively). The latency from stimulus beginning to withdrawal of hind paw was recorded (30 s was the maximal latency). Each rat received ten tests by single filament before being pricked by the next larger filament. For the measurement of TWL, rats were placed in a plastic cage with the transparent glass plate for at least 30 min in order to adapt to the test environment. A plantar algesimeter (Tes7370, Ugo Basile, Comerio, Italy) was placed underneath the glass and aimed at plantar surface of ipsilateral hind paw. TWL was determined as the time from starting heat stimulation to paw withdrawal. PWT and TWL tests were repeated three times with 5 min intervals consecutively and the average duration of three measurements were determined as the final data.

Western blot

Briefly, rats were deeply anaesthetized with peritoneal injection of 10% chloral hydrate (300 mg/kg). The exposed right sciatic nerve was tracked to locate L4-5 spinal cord which was then exposed by rongeur and harvested using tissue scissors. Proteins were extracted by homogenizing spinal tissues in cold immunoprecipitation assay buffer. After centrifugation, supernatants were collected and protein concentration was measured using bicinchoninic acid (BCA) method (Thermo Scientific). Next, protein samples were loaded onto SDS-polyacrylamide gels (Beyotime, Shanghai, China), separated and electrically transferred to polyvinylidene difluoride (PVDF) membrane. After being blocked with 5% evaporated milk, membranes were incubated with primary antibodies overnight at 4°C, including anti-Iba1 antibodies (rabbit, 1:500, Bio-Rad), anti-p-PI3K antibodies (rabbit, 1:1000, Bio-Rad), anti-p-AKT antibodies (rabbit, 1:1000, Bio-Rad), anti-β-actin

antibodies (rabbit, 1:2000, Sigma) as a loading control. The membranes were subsequently washed 3 times in TBST for 5 min each and further incubated with horseradish peroxidase-conjugated secondary antibody (1:2000, ZSGB-BIO, ORIGENE, Beijing, China) for 2 h. Images were revealed by ECL solution and exposed by Hyperfilm (Millipore). ImageJ was used to analyze the densitometry.

Real time quantitative PCR

L4-5 spinal cords were taken out of -80°C refrigerator. Trizol solution (TaKaRa Kyoto, JPN) was used to lyse the samples and extract total RNA in accordance with the manufacturer's protocol. PCR reaction was run in a Real-Time PCR machine (Takara, Kyoto, JPN) with the Platinum SYBR Green qPCR SuperMix-UDG kit (Life Technologies Corporation, Carlsbad, CA, USA) according to the manufacturer's instructions. The cDNA was synthesized by reverse transcription with the following program: 40 cycles of 55°C for 10s and 75°C for 20s. The primer sequences of PI3K/AKT and GAPDH were listed in Table 1. The thermocycling conditions in the amplification were as follows: 95°C for 5min, then 40 cycles of 95°C for 10s, 55°C for 10 s, 75°C for 20 s. Relative expression was calculated using the $2^{-(Ct_{\text{experimental sample}} - Ct_{\text{internal control sample (GAPDH)})}$ method.

Enzyme-linked immunosorbent assay

After deep anaesthesia, rats were transcardially perfused with PBS. L4-L5 spinal cords of each rat were rapidly isolated and homogenized with RIPA lysis buffer containing phenylmethylsulfonyl fluoride at 4°C. Samples were centrifuged for 15 min at 4°C for supernatants collection. Then, the measurement of IL-6 and TNF-α were determined through ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's protocol. The standard curve was included in each experiment.

Statistical analysis

SPSS 17.0 software (SPSS Inc., Chicago, IL, USA) was used for the statistical analysis. The

Table 1: The primer sequences of PI3K/AKT and GAPDH

Gene	Forward	Reverse
PI3K	5'-TGCTATGCCTGCTCTGTAGTGGT-3'	5'-GTGTGACATTGAGGGAGTCGTTG-3'
AKT	5'-GTGCTGGAGGACAATGACTACGG-3'	5'-AGCAGCCCTGAAAGCAAGGA-3'
GAPDH	5'-GCAAGTTCAACGGCACAG-3'	5'-CTCAACAGTATAAAGAGC-3'

quantitative data are presented as Mean \pm SEM. Iba1 expression among four groups were analyzed with the one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls post hoc test. Other data were analyzed using two-way analysis of variance (ANOVA) followed by Holm-Sidak post-hoc analysis. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Electroacupuncture and intrathecal LY294002 alleviated neuropathic pain induced by chronic constriction injury in rats.

To evaluate the mechanical allodynia and thermal hyperalgesia, paw withdrawal threshold (PWT) and thermal withdrawal latency (TWL) were employed. As the results revealed, the PWT in CCI group were significantly decreased as time prolonged compared to sham group, but that in CCI+EA and CCI+LY294002 groups were markedly higher than CCI and CCI+NS groups (Figure 1A, $P < 0.05$). Correspondingly, the TWL in these four groups exhibited a marked reduction on 3 d. CCI and CCI+NS groups maintained a downward trend over time while the other two groups showed slight upward trend from 3 d to 14 d (Figure 1B, $P < 0.05$). These results revealed that the CCI rats developed mechanical allodynia and thermal hyperalgesia after operation. EA increased the PWT and TWL of CCI rats, and similar effects were displayed by intrathecal injection of LY294002 (AKT inhibitor).

Electroacupuncture treatment and intrathecal LY294002 administration suppressed microglial activation in chronic constriction injury rats.

Microglial activation is key element of the maintenance of NP following CCI operation. In order to test the effect of EA treatment and PI3K/AKT inhibition on microglia after CCI, western blot was performed to evaluate the expression of Iba-1 (microglial biomarker) at the L4-5 of spinal cord. As is shown in Figure 2, the integrated optical density (IOD) of Iba-1/ β -actin in CCI group significantly increased on postoperative 14d compared with sham group. Besides, western blot analysis showed that Iba-1 expression in rats L4-5 of CCI+EA group and CCI+LY294002 group were markedly decreased compared with CCI and CCI+NS groups ($P < 0.05$). Our data revealed that persistent treatment with EA and LY294002 intrathecal administration for 14 days after CCI

surgery could inhibit the activation of microglia.

Electroacupuncture treatment and intrathecal LY294002 administration downregulated levels of TNF- α and IL-6.

Since the microglia were activated after spinal cord injury, the release of proinflammatory cytokines such as TNF- α and IL-6 were up-regulated to facilitate the neuroinflammation which was responsible for central sensitization.⁸ As shown, TNF- α and IL-6 levels in CCI and CCI+NS groups increased significantly from 3 d to 14 d after surgery compared with sham rats ($P < 0.05$). EA and LY294002 both depleted the up-regulation of TNF- α and IL-6 in L4-5 spinal cord of CCI rats (Figure 3A, B, $P < 0.05$). This tendency corresponded to microglial activation.

Electroacupuncture and intrathecal LY294002 administration inactivated PI3K/AKT signaling

In order to validate the involvement of PI3K/AKT pathway in NP development, we tested the phosphorylation levels of PI3K/AKT by western blot (Figure 4A) and detected the mRNA levels of PI3K/AKT by qRT-PCR (Figure 4B). And the current data showed that both protein and mRNA expression of PI3K/AKT was significantly increased in CCI and CCI+NS groups from 3 d to 14 d post operation ($P < 0.05$). After intervention of EA and intrathecal injection of LY294002 in CCI rats, the expression and phosphorylation levels of PI3K/AKT signaling demonstrated a significant decrease ($\#P < 0.05$). Our data revealed that EA and intrathecal LY294002 administration could inhibit the activation of PI3K/AKT signaling pathway.

DISCUSSION

In this study, we found that intrathecal injection of LY294002 and EA treatment applied to ST36 and GB34 markedly increased the PWT and TWL of rats following CCI. Microglia activation was depressed and levels of TNF- α and IL-6 were descended in CCI rats with EA treatment and PI3K/AKT inhibition. Further, EA was revealed to be effective in inactivating PI3K/AKT signaling pathway. These results suggested that EA stimulation alleviated neuroinflammation induced by CCI through inhibiting PI3K/AKT signaling.

The pain threshold of CCI rats was significantly decreased on 3 d and sustained to 14 d after CCI, which is similar to the outcomes of a previous study.²⁰ Following EA stimulation of ST36 and GB34, the PWT and TWL were markedly

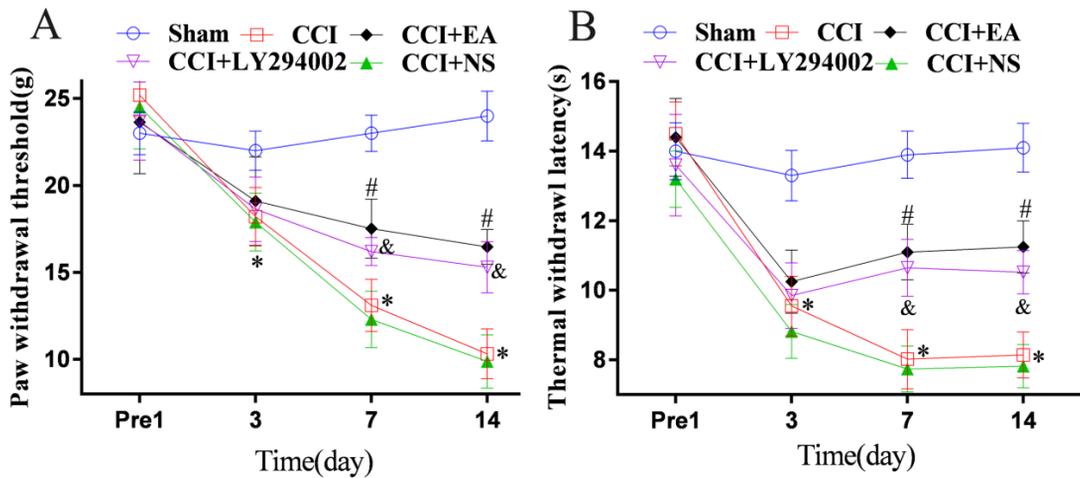


Figure 1. Effects of EA and LY294002 on hyperalgesia in CCI-induced neuropathic pain. (A) Mechanical allodynia and (B) thermal hyperalgesia were examined at 1 d before CCI and 3 d, 7 d, 14 d post CCI. PWT, paw withdrawal threshold; TWL, thermal withdrawal latency; CCI, Chronic constriction injury; EA, Electroacupuncture; NS, 0.9% NaCl. Data are presented as the mean \pm SEM. Significance of PWT and TWL were analyzed with two-way analysis of variance (ANOVA) followed by Holm-Sidak post-hoc analysis. * $P < 0.05$ vs. sham group, # $P < 0.05$ vs. CCI group, & $P < 0.05$ vs. CCI +NS group (n=5/group).

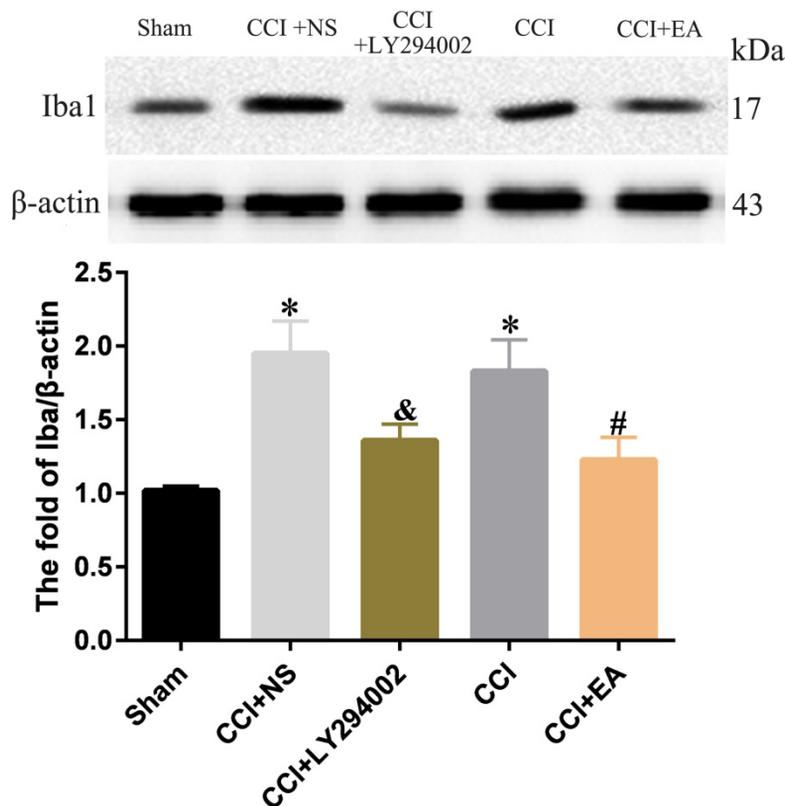


Figure 2. Effects of EA treatment and intrathecal LY294002 administration on microglial activation. The protein level of the microglial marker Iba1 in sham, CCI+NS, CCI+LY294002, CCI and CCI+EA groups. CCI, Chronic constriction injury; EA, Electroacupuncture; NS, 0.9% NaCl. Data are presented as the mean \pm SEM. Significance of Iba1 levels were analyzed with one-way ANOVA followed by Student-Newman-Keuls post hoc test. * $P < 0.05$ vs. sham group, # $P < 0.05$ vs. CCI group, & $P < 0.05$ vs. CCI +NS group (n=4/group).

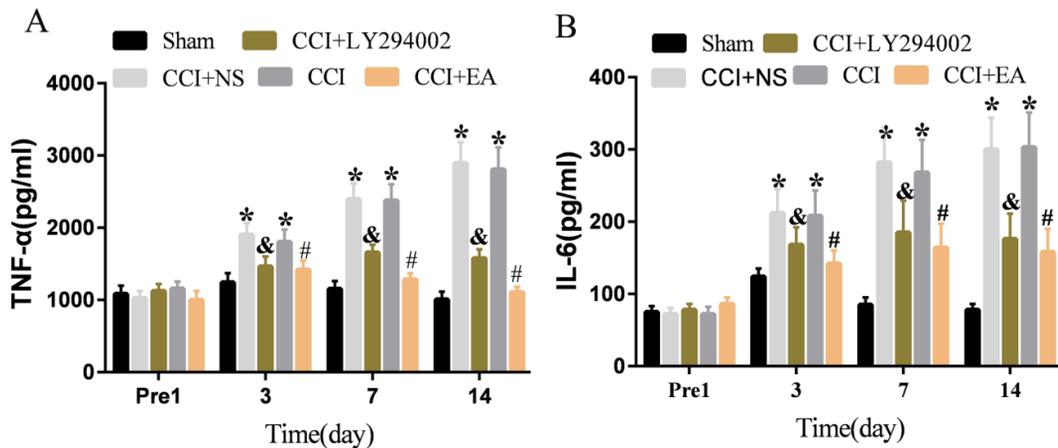


Figure 3. The anti-inflammatory effect of EA and intrathecal LY294002 treatment. The protein levels of (A) TNF- α and (B) IL-6 in L4-5 spinal cord were examined by ELISA on 1 d before CCI and 3 d, 7 d, 14 d after CCI. CCI, Chronic constriction injury; EA, Electroacupuncture; NS, 0.9% NaCl; ELISA, Enzyme-linked immunosorbent assay; d, day. Data are presented as the mean \pm SEM. Significance of TNF- and IL-6 levels were analyzed with two-way ANOVA followed by Holm-Sidak post-hoc analysis. * $P < 0.05$ vs. sham group, # $P < 0.05$ vs. CCI group, & $P < 0.05$ vs. CCI + NS group (n=4/group).

increased compared with CCI group, suggesting that repeated EA stimulation exhibited an analgesic effect, which is identical to the results of recent researches.^{17,21} Correspondingly, the expression levels of PI3K/AKT mRNA and p-PI3K/p-AKT were obviously up-regulated in L4-5 spinal cord of CCI rats, suggesting an activation of spinal PI3K/AKT signaling after CCI. These changes of spinal molecules are in agreement with a previous report⁹ which revealed increased PI3K/AKT expression and phosphorylation in the L4-6 spinal cord of CCI rats facilitated pain development. As is mentioned, PI3K/AKT signal pathway plays an important role in process of cell proliferation in many diseases.²²⁻²⁴ Thus we certainly believed that PI3K/AKT activation might be a mediator for NP progression. In addition, there was an obvious up-regulation of Iba-1 in L4-5 spinal cord of CCI rats in the present study. It is well known that Iba1 is a microglial marker^{25,26}, implying that microglia were activated in CCI rats. Taken together, it is reasonable to say that microglial activation is closely associated with PI3K/AKT signaling activation in NP.

The indispensable role of PI3K/AKT in the activation of microglia was confirmed in the present study by intrathecal injection of PI3K/AKT signaling inhibitor LY294002. A study had reinforced the dose-dependent inhibitory effect of intrathecal LY294002 on the pain hypersensitivity.²⁷ Although no obvious side effect such as motor inhibition and dysesthesia

were mentioned by intrathecal LY294002 in the previous study^{28,29}, we decided to choose a low dose of 10ug for the purpose of safety. Intrathecal delivery LY294002 or 0.9% NaCl vehicle did not display any motor disturbance in this experiment, indicating the feasibility of intrathecal device. After intrathecal injection of LY294002, the mechanical allodynia and thermal hyperalgesia of CCI rats were significantly relieved, which suggested the PI3K/AKT inhibition was a potential strategy for the treatment of NP. Additionally, the microglial marker Iba-1 of CCI rats also exhibited an obvious down-regulation by intrathecal LY294002, which revealed a close relationship between PI3K/AKT signaling and microglia activation. Previous studies reported that PI3K/AKT pathway was a key element for the release of proinflammatory mediators in lipopolysaccharide-induced microglial activation.^{30,31} Consistently, we found that proinflammatory cytokines such as TNF- α and IL-6 were down-regulated in the treatment of intrathecal LY294002 compared with CCI rats. These results indicated that the neuroinflammation induced by microglial activation was attenuated by inhibition of PI3K/AKT pathway. Most importantly, our data demonstrated that PI3K/AKT was involved in the pathogenesis of NP by participating in the activation of spinal microglia.

The relevant current intensity, stimulation frequency and duration, even location of acupuncture all affect the EA analgesic effects.³²⁻³⁵

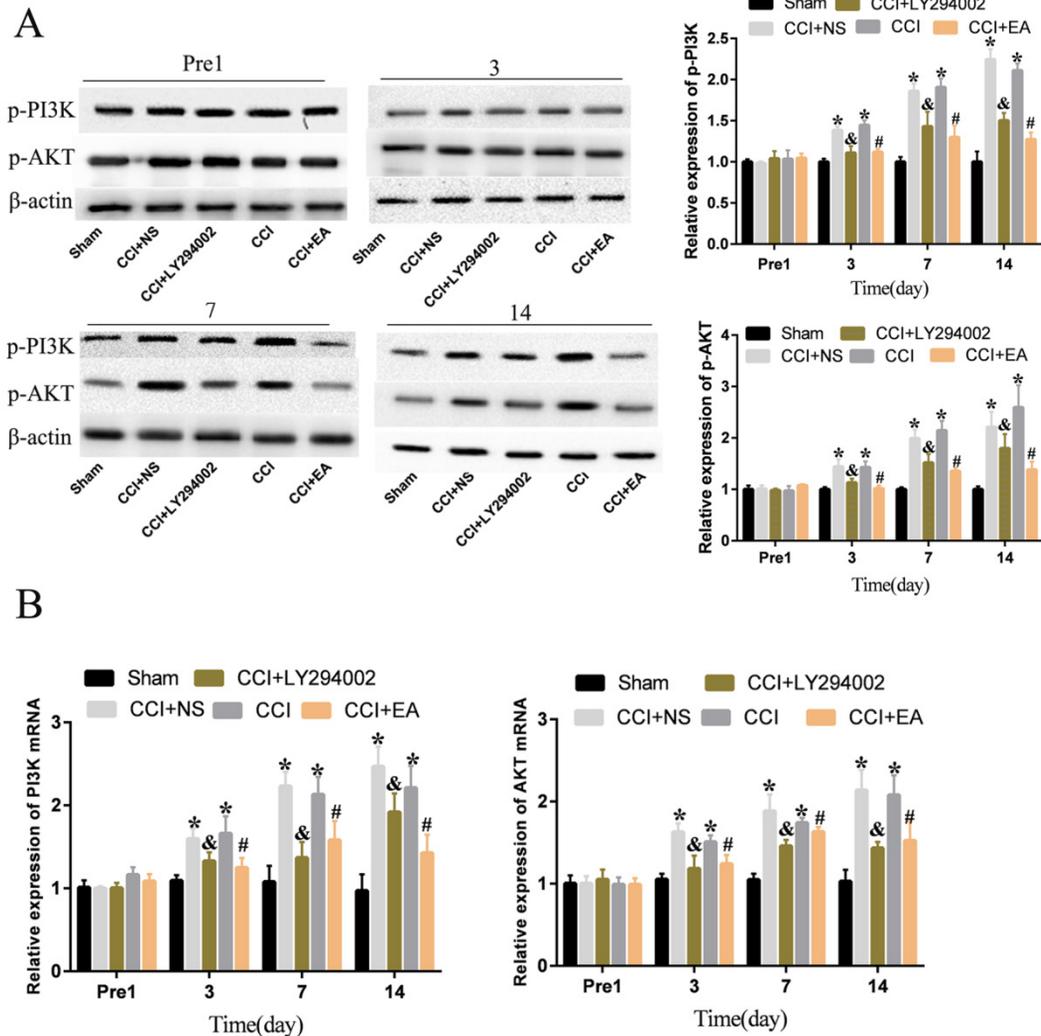


Figure 4. EA and intrathecal LY294002 administration inhibited the activation of PI3K/AKT pathway in CCI rats. (A) The western-blot gel bands of p-PI3K/AKT and the (Mean \pm SEM) of IOD of p-PI3K/ β -actin and p-AKT/ β -actin in all rats, $n=5$ /group. (B) The (Mean \pm SEM) of mRNA level of PI3K and AKT in L4-5 spinal cord of all rats, $n=5$ /group. CCI, Chronic constriction injury; EA, Electroacupuncture; NS, 0.9% NaCl. Significance of data were analyzed with two-way ANOVA followed by Holm-Sidak post-hoc analysis. * $P<0.05$ vs. sham group, # $P<0.05$ vs. CCI group, & $P<0.05$ vs. CCI +NS group.

2 Hz EA was chosen in the this study, because the previous study reported that 2 Hz EA brought better outcomes in relieving pain compared to 100 Hz.^{36,37} Although the analgesic effect of 1 mA applied in our study slightly weaker than 3.0 mA, it's enough to relieve NP with 30 min daily.^{38,39} To ensure the animal immobilization during EA stimulation, the rats in this study were restrained by the self-made cloth bag with the hind limbs extending through two holes. And EA was carried out in the dark and quiet condition for keeping the rats calm. Under the treatment of EA, the pain hypersensitive behaviors were well

attenuated in the experiment. The mechanism of EA analgesia might be attributed to inhibiting the activation central neuroglia and modulating the release of endorphins.^{14,35} EA also was regarded as an alternative non-invasive strategy for nerve stimulation, especially the sensory afferent nerve.⁴⁰ Not like mere basal tissue stimulation, this kind of mechanical stimulation abrogated pain nociception in sensory nerves by inhibiting adenylyl cyclase and blocking calcium channels.⁴¹ In the present study, the up-regulation of Iba-1 and TNF- α , IL-6 was greatly suppressed by EA, which suggested that EA inhibited the activation of

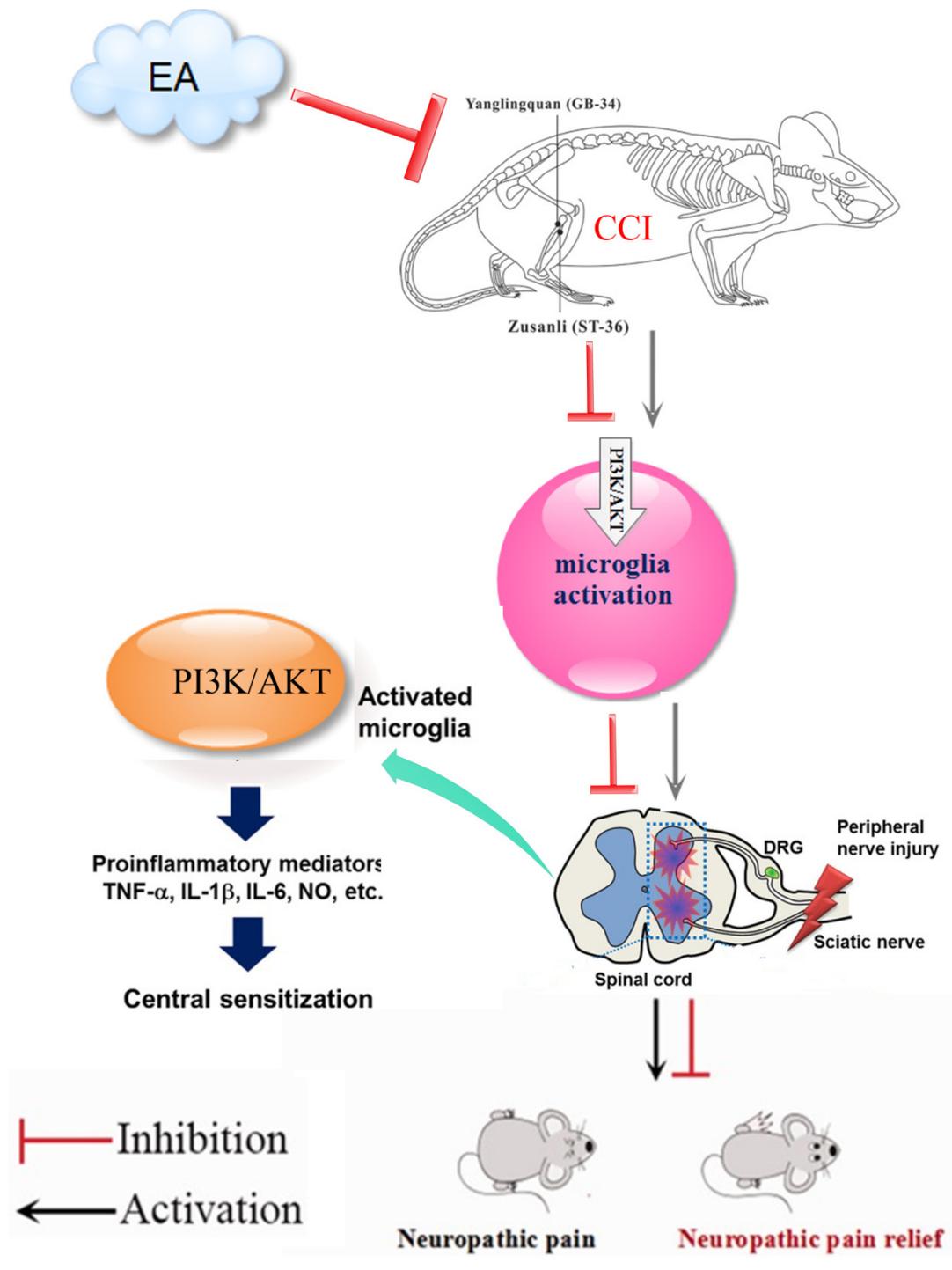


Figure 5. EA suppressed the microglial PI3K/AKT pathway thus inhibited the activation of microglia after CCI. Moreover, EA depleted the microglial proinflammatory cytokines which mediated central sensitization. CCI, Chronic constriction injury; EA, Electroacupuncture.

microglia and microglial proinflammatory release in spinal cord. Accumulating researches revealed that the EA analgesia was closely associated with microglia, which acted as an inhibitor for limiting the further activation of spinal microglia in peripheral nerve injury, thereby restraining the microglia-induced neuroinflammation.^{15,42,43} Unfortunately, the exact mechanism by which EA worked on microglia still remains largely unknown. However, we also found that EA therapy reversed both the expression and phosphorylation of PI3K/AKT in the L4-5 spinal cord of CCI rats. Kim et al had suggested that EA could significantly down-regulate the activation of PI3K/AKT pathway in L4-5 spinal cord of carrageenan-induced inflammatory pain rat, believing PI3K/AKT pathway played an important role in EA analgesia effect.⁴⁴ Consistent with the above-mentioned, results in the current study indicated that EA might block the microglial PI3K/AKT pathway activation and reduce the production of proinflammatory mediators in the development of neuropathic pain (Figure 5).

Taken together, this study demonstrate that the critical role of PI3K/AKT signaling in the effect of EA on alleviating neuropathic pain in CCI rats. EA inhibited the activation of microglia and therefore suppressed the production of proinflammatory cytokines in spinal, which may be attributed to its inhibitory effect on microglial PI3K/AKT signaling activation.

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DISCLOSURE

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Conflict of interest: None

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