Original Article



# Electroacupuncture alleviates neuropathic pain caused by spared nerve injury by promoting AMPK/mTOR-mediated autophagy in dorsal root ganglion macrophage

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**Background:** Dorsal root ganglia (DRG) plays an important role in mediating the peripheral sensation transduction through the primary afferent neurons in pain research. Neuropathic pain (NP) is a syndrome of hyperalgesia, spontaneous pain and allodynia caused by central or peripheral nerve injury. Recent trends of study are turning towards the development of therapies for the management of NP. Activation of autophagy in glial cells in the spinal cord has been reported to be associated with attenuation of NP, but the autophagic process in DRG is rarely studied.

**Methods:** The analgesic effect of electroacupuncture (EA) was evaluated in NP-induced rats developed using spared nerve injury (SNI). Acupuncture or EA was performed after 7 days of SNI at Zusanli (ST36) and Huantiao (GB30) acupoints. Then, the activation status of autophagy process in DRGs of rats treated with SNI and EA were investigated, and the possible mechanism of the analgesic effect of EA were explored.

**Results:** Application of EA has been found to reduce mechanical hyperalgesia. Autophagy indicator p62 was colocalized with the marker proteins for macrophages (CD11b), but not with NeuN (marker protein for neurons) or GFAP (marker protein for satellite glial cells), as shown by immunofluorescence. Western blots results indicate that the expression levels of p62, Beclin-1 and LC3-II in the L4-L6 DRG of rats in the SNI group were increased, compared with that in the control group. EA treatment resulted in decreased expression of p62 and increased expression of Beclin-1 and LC3-II/LC3-I. Furthermore, we explored the causal relationship between EA-induced suppression of NP and increased levels of autophagy in DRG using electron microscopy and the AMPK (AMP-activated protein kinase) inhibitor compound C.

**Conclusions:** SNI achieved a significant upregulation of autophagy levels in DRG macrophages. Furthermore, EA attenuated NP, which may contribute to the promotion of AMPK/mTOR (mammalian target of rapamycin)-mediated autophagy in DRG macrophages. Therefore, this strategy provides a new target for therapeutic intervention of NP.

**Keywords:** Neuropathic pain (NP); dorsal root ganglia (DRG); autophagy; electroacupuncture (EA)

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## Introduction

Dorsal root ganglia (DRG) include primary sensory neuronal bodies and glia cells, carrying somatosensory information to the central nervous system (CNS) through the soma (1), and thus play an import role in mediating neuronal network between the CNS and peripheral nervous system (2). DRG neurons have bipolar property, entering the spinal dorsal horn with the synaptic terminal, with the other terminal of undifferentiated morphology (3). Various receptors and ion channels are expressed in the membrane of DRG neurons, transforming to different patterns upon nerve injury, which are pivotal in numerous pathological and physiological conditions (4).

Neuropathic pain (NP) is a syndrome caused by central or peripheral nerve injury, mainly manifested as hyperalgesia, spontaneous pain and allodynia. NP has many features of neuroimmune mechanisms. Pain relief may be related to immunosuppression and inhibition of the reciprocal cellular relationship between neuronal and non-neuronal cells (5). It has been reported that after peripheral nerve injury, the cellular and molecular interactions among microglia cells and neurons in spinal dorsal horn, and also the resident macrophages in central nervous system, are involved in the induction and maintenance phase of NP (6,7). After peripheral nerve injury, along with the activation of the microglia in spinal dorsal horn, many researches discovered that ipsilateral DRG macrophages are also increased (8,9). Macrophages and satellite glial cells are normally present in the DRG. Nerve injury could induce reaction of these remote resident immune and glial cells in the DRG, which is enhanced by invading macrophages and T cells (5). Injury-induced macrophage invasion maybe due to chemokine (C-X3-C motif) ligand 1 (fractalkine) (10) and the chemokine CCL2 released by DRG neurons (11-13). DRG macrophages are pivotal in the initiation and maintenance phase of NP (14). There is a reciprocal cellular interaction between DRG sensory neurons and macrophages, which possibly contributes to the phenotype of NP (14). The immuno-intensity of macrophages in the DRG for major histocompatibility complex II increases 1 week after nerve injury, and persists for at least 3 months. By that moment, macrophages in the DRG shift from an initially rather scattered distribution to surround the cell bodies of neurons (5). Two months after nerve injury, a large proportion of macrophages in the DRG transform to active phagocytes, presumably contributing to removal of debris from injured sensory neurons (8), a substantial proportion of which degenerate after nerve transection. As a result, a predominant decrease of small unmyelinated neurons would be detected 2 months after nerve transection (15).

Electroacupuncture (EA), as a common form of acupuncture, involves electrical current and precisely controlled parameters (16), which makes this technique highly reproducible and better than manual acupuncture (17). Although EA is an adequate method of pain treatment, the exact mechanism of action for EA in NP needs further study (18).

Autophagy involves degrading damaged organelles and unneeded or aged proteins through autophagosome-lysosome pathway. It was first found under conditions of starvation (19). If nutrients are in short supply,

### Highlight box

**Key findings**

- Our work found the possible mechanism of EA induced analgesia in neuropathic pain is possibly through AMPK/mTOR pathway and autophagy induction of macrophages in dorsal root ganglia.

**What is known and what is new?**

- Electroacupuncture is an effective treatment for neuropathic pain. Impaired autophagy is involved in neuropathic pain and autophagy induction could lead to analgesia.
- Electroacupuncture induced analgesia is possibly through activation of AMPK/mTOR pathway and autophagy induction of macrophages in dorsal root ganglia.

**What is the implication, and what should change now?**

- Our research provides a fundamental basis for targeting autophagy pathway and application of EA in neuropathic pain therapy, and further elucidates the regulation and role of AMPK/mTOR signaling pathway in electroacupuncture mediated analgesia, offering new sights and targets for pain therapy.

autophagy process will be activated. A double membrane autophagosome will be formed around cellular content, which is then fused with a lysosome, and then these proteins and organelles are degraded to amino acids and fatty acids, and recycled back to the cells, which allow the cells to survive (20). Recent studies have suggested a role for autophagy in the nervous system, especially in relation to the pathogenesis of Alzheimer's disease (21), Parkinson's disease (22), amyotrophic lateral sclerosis (23,24), and spinal cord injury (25) in the peripheral nervous system. An altered expression of autophagy-associated proteins, indicating an impaired autophagy flux, has been well documented in rat models of NP (26). Other reports have demonstrated that intrathecal injection of an autophagy blocker in mice induced significant mechanical hypersensitivity, and treatment with the autophagy inducer rapamycin could ameliorate NP by activating autophagy in the spinal cord, suggesting that NP may have occurred due to impaired autophagy (27,28). In addition, studies have shown that peripheral nerve injury can cause changes in the autophagy activation of microglia (26,29), and electroacupuncture has been reported to reduce tactile allodynia and thermal hyperalgesia by inhibiting the activation of spinal cord microglia (30,31).

Autophagy levels can be mainly regulated through the AMP-activated protein kinase (AMPK)/mammalian target of rapamycin (mTOR) pathway, where AMPK activation can alleviate chronic pain by inhibiting the mTOR signaling pathway (32). In addition, the expression of AMPK in the hypothalamus is related to the analgesic effect of electroacupuncture (33).

Herein, we hypothesized that EA might attenuate NP by promoting the AMPK/mTOR-mediated autophagy of macrophages in DRG. To test this hypothesis, the analgesic effects of EA were analyzed in the NP rat model, in which rats were subjected to spared nerve injury (SNI). We demonstrated that peripheral nerve injury could induce autophagy in DRG macrophages, and EA could mediate analgesia in SNI rats through inducing the autophagy process in DRG macrophages. Furthermore, we explored the causal relationship between EA-induced inhibition of NP and increased autophagic levels by using the AMPK inhibitor. We present the following article in accordance with the ARRIVE reporting checklist (available at https://atm.amegroups.com/article/view/10.21037/atm-22-5920/rc).

## Methods

### *Animal and surgery*

All animal experiments were conducted with the approval of the committee on animal experimentation at the Nanjing University of Chinese Medicine (NUCM, No. ACU210502), in compliance with national guidelines for the care and use of animals. A protocol was prepared before the study without registration. Adult male Sprague-Dawley rats (weighing 160–180 g) were housed at the NUCM with free access to food and water, in light-controlled rooms (12/12 h light/dark cycle) and maintained at a temperature of 23±2 ℃ for at least 3 days prior to experimentation. We created NP by SNI as described in Cichon *et al.* (34). The rats were anesthetized using isoflurane (2–3%), and then the left lateral sciatic nerve and its three terminal branches, namely the sural, tibial and common peroneal nerves, were exposed. A tight ligation was made around the left tibial and common peroneal nerve with silk sutures, and the part distal to the ligated point was sectioned, with at least 2–4 mm of distal nerve stump removed while keeping the sural nerve left intact. The muscle and skin tissues were closed under sterile condition. Similar surgical procedure was applied to the sham surgery rats, except for leaving intact both the common peroneal and tibial nerves.

### *Analysis of EA stimulation on rats*

For EA treatment, rats were restrained in opaque cloth bags with hind legs exposed. Insert two acupuncture needles (made of stainless steel) at least 6–7 mm into two acupuncture point on the same side as the injured side. We insert a needle into the ST36 5 mm lateral to the anterior tibial tubercle, marked with a notch. Another needle is at Huantiao point (GB30), which is at the intersection of the outermost third and middle third of the line connecting the most protruding point of the femur and the sacral hiatus. These two acupoints were selected as stimulation at "Zusanli" (ST36) and "Huantiao" (GB30) acupoints have already been reported to induce analgesia in various pain models (35-37). Han's Acupoint Nerve Stimulator (HANS, 200A, Nanjing Jisheng Medical Technology Co., Ltd, China) was used to generate the stimulation square waves of EA. One week after SNI, rats were stimulated with EA for 30 minutes once per day for seven consecutive days with

 

an EA stimulation frequency of 2 Hz while increasing the stimulation intensity stepwise from 1 mA to 2 mA to 3 mA after every 10 minutes. As previously reported, 2 Hz EA stimulation with intensity increasing in a stepwise manner for 30 min has better analgesic effect on NP than 100 Hz EA (38). For the SNI + A group, acupuncture needles were only superficially inserted into ST36 and GB30 without electrical stimulation.

### *Intrathecal drug administration*

Intrathecal administration by lumbar puncture was performed under isoflurane (2.5%) anesthesia as previously described, in which a 26G gauge needle has been inserted between the L5 and L6 vertebrae (39).

Dilute an approximately 10 μL the AMPK inhibitor compound C (0.2 μmol/kg, HY13418A, MedChemExpress) in DMSO once a day for 7 days 1 h before EA stimulation. DMSO alone was used as control. To ensure the quality of each injection, an injection-induced tail flick was observed.

### *Measurement of paw withdrawal threshold (PWT)*

Hypersensitivity intermittent mechanical stimulation of von-Frey filaments (0.38, 0.57, 1.23, 1.83, 3.66, 5.93, 9.13, and 13.1 g) was measured using up-down method (40). We consider sudden paws withdrawal, licking, and shaking as positive responses. Dixon's method and formula have been applied to measure PWT. Investigator performing behavioral experiments has been blinded in accordance to treatment conditions to minimize experimenter bias.

### *Immunofluorescence analysis*

Pentobarbital was used for anesthetizing rats, and 20 mL of 0.1 M PBS (phosphate-buffered saline) was used for perfusion, followed by 25 mL of fixative containing saturated picric acid (14%, v/v) and formaldehyde (4%) in PBS at 4 ℃. The L4-L6 DRG tissues were embedded, cut into sections (thickness 15 μm), and placed on slides. The slides were then blocked with 0.2% Triton X-100 and 5% normal donkey serum dissolved in PBS for 1 hour at room temperature followed by incubation with primary antibodies overnight at 4 ℃. A solution containing sodium azide (0.01%), bovine serum albumin (1%), and Triton X-100 (0.3%) in PBS was used to dilute the primary antibodies to their final working concentrations. FluoroShield histology mounting medium (Sigma-Aldrich) was applied to the slides following 45 minutes of incubation with secondary antibodies. The primary antibodies used were as follows: goat anti-glial fibrillary acidic protein (GFAP; 1:50, Abcam, ab53554), mouse anti-NeuN (1:20, Abcam, ab104224), mouse anti-CD11b (1:20, Abcam, ab1211), rabbit anti-p62 (1:50, Abcam, ab91526), and rabbit anti-goat (Alexa Fluor® 488) (1:200, Abcam, ab150192). The secondary antibodies used were the following: rhodamine Red-X-conjugated AffiniPure donkey anti-rabbit IgG (H + L) (1:200, Jackson ImmunoResearch, West Grove, PA, 711-295-152) and fluorescein-conjugated AffiniPure donkey anti-mouse IgG (H + L) (1:200, Jackson ImmunoResearch, 711-095-151). Images were visualized with a fluorescence microscope (Olympus BX51 microscope system, Melville, NY). Immunostaining results were analyzed with Image J software (NIH, Bethesda, MD). The areas with overlapping similarities have been evaluated among distinct animals. Any background signals arising from each subsequent slice were subtracted. The average IOD (integrated optical density) of sham-operated rats was determined and the IOD ratio among SNI rats was normalized to the average values of sham-operated rats. The data was analyzed by an investigator who was blinded to the experimental groups.

### *Western blotting*

For Western blot assays, ipsilateral L4–L6 DRG were isolated from rats and homogenized in an ice-cold RIPA buffer. After centrifugation, the protein concentration of the homogenates was assayed using a detergent-compatible protein and bovine serum albumin standard. Proteins were separated by 10% or 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Membranes were blocked with skim milk for 1 hour, and then incubated with primary antibodies overnight at a temperature of 4 ℃. Tris buffered saline Tween (TBST) was used to wash the membranes, followed by incubation with the secondary antibodies for 2 hours at room temperature. An enhanced chemiluminescence kit (Amersham) was used to detect the immunoreactivity of membranes after washing with TBST three times. The antibodies used for this study were against p62 (1:1,000, Abcam, UK, ab91526), Beclin-1 (1:1,000, Abcam, UK, ab210498), LC3 (1:2,000, Abcam, UK, ab192890), AMPK (1:1,000, Cell Signaling Technology, USA, 5831S), phosphorylated (p)-AMPK (1:1,000, Cell Signaling Technology, USA, 2535S), mTOR (1:1,000, Cell Signaling Technology, USA, 2983S), and p-mTOR (1:1,000,

 

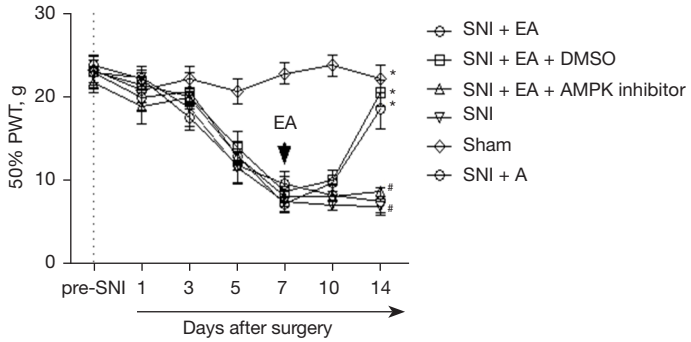


**Figure 1** Effects of repeated EA on mechanical hypersensitivity in SNI rats with or without AMPK inhibitor treatment. SNI (n=6) induced severe mechanical allodynia from day 7 after surgery, which persisted for 14 days. PWT in sham-operated rats (n=6) did not change significantly from baseline during the testing period. From day 7 to day 14 after surgery, electroacupuncture (n=6) significantly increased PWT. In the control group for electroacupuncture, acupuncture (n=6), did not significantly increase the PWT in SNI rats. DMSO was used as a solvent for AMPK inhibitor-compound C and administered as a control for compound C. EA-mediated analgesia effects in SNI rats were reversed by intrathecal injection of compound C 1 h before every electroacupuncture treatment. All the data were expressed as the mean ± SEM. *P<0.05 versus the SNI group; #P<0.05 versus the SNI + EA group, two-way mixed-model ANOVA. EA, electroacupuncture; PWT, paw withdrawal threshold; SNI, spared nerve injury; A, acupuncture; DMSO, dimethyl sulfoxide; AMPK, AMP-activated protein kinase; ANOVA, analysis of variance.

Cell Signaling Technology, USA, 2971S). The intensity of immunoreactive bands were quantified using Image J software. GAPDH was used as an internal protein loading control.

### *Transmission electron microscopy (TEM)*

Autophagosomes were detected using TEM. The ipsilateral L4–L6 DRG of the rats were harvested, 1 $mm^3$ tissue samples were obtained and fixed in glutaraldehyde (2.5%) at room temperature for 2 hours, and washed 3 times with PBS (10 minutes each time). After fixation for 1.5 hours in osmium acid solution (1%) at 4 ℃, the fixative solution was removed, and the samples were washed 3 times in PBS (15 minutes each time). The samples were dehydrated using a series of gradient alcohol, embedded, sectioned, and observed and photographed under electron microscopy (HITACHI, HT77000).

### *Statistical analysis*

Data shown are representative results of experiments with at the minimum three biological replicates of comparable outcomes. The data was analyzed via GraphPad Prism 5.0 software. The Bonferroni correction has been utilized in multiple comparisons in ANOVA. A Two-tailed test was carried out and data was presented as the mean ± SEM with P<0.05 is regarded as statistical significance.

## Results

### *Debilitation of mechanical hypersensitivity in SNI rat stimulated by EA*

Perform EA or acupuncture on SNI rats from day 7 to day 14 after SNI. Sham-operated rats without any treatment are considered controls. It was observed that during testing periods, PWT in sham-operated rats doesn't change much from the baseline. In addition, SNI was also observed to induce severe mechanical allodynia on day 7 after surgery, which persisted until day 14 (P<0.05, two-way mixed-model ANOVA). However, EA significantly increased PWT on day 14 after surgery (P<0.05, two-way mixed-model ANOVA). The PWT in SNI rats was not significantly increased by acupuncture in control group (*Figure 1*).

### *The effect of EA on the expression of proteins related to autophagy in the L5 DRG*

On day 14 following SNI, the L4–L6 DRG ipsilateral to the injured side were collected to evaluate how EA affects the expression of autophagy-related proteins, namely, p62, Beclin-1, and LC3II (*Figure 2A*). The ubiquitin-binding protein p62 is integrated into autophagosomes by directly

 

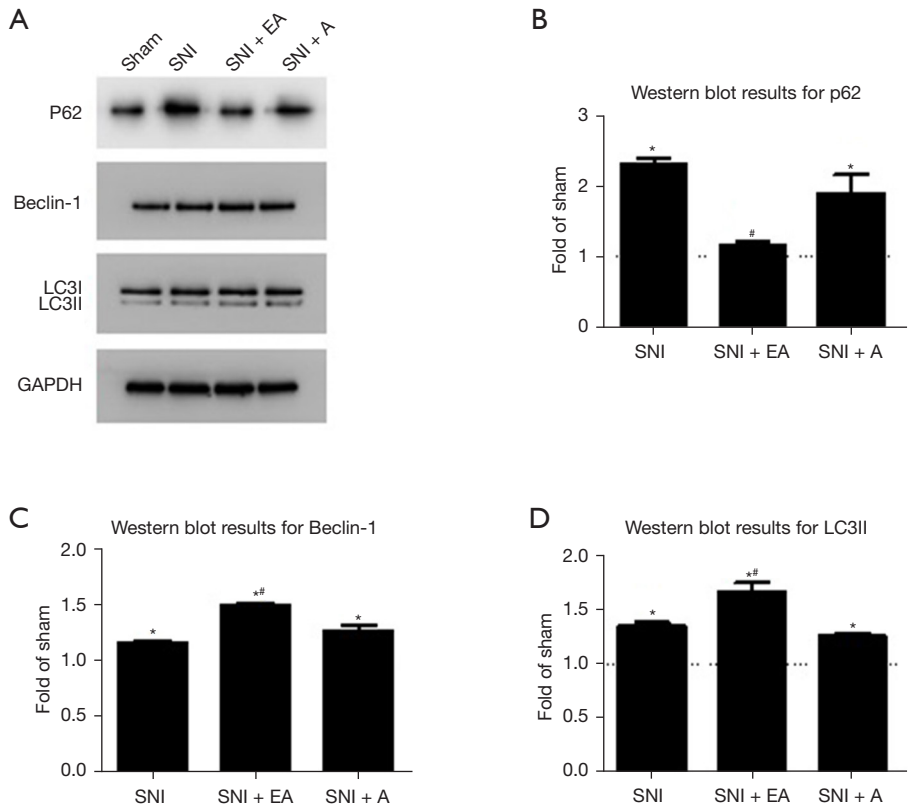


**Figure 2** Effects of repeated EA on the expression of autophagy-related proteins (p62, Beclin-1, and LC3II) in L4-L6 DRGs of rats after SNI. (A) Illustrative immunoblots showing p62, Beclin-1, and LC3II expression in the ipsilateral L4-L6 DRG of SNI rats (n=3), SNI rats administered electroacupuncture (n=3) or acupuncture (n=3), and sham-operated rats (n=3). (B-D) Quantification of protein levels for p62, Beclin-1, and LC3II from ipsilateral L4-L6 DRGs among distinct groups. All the data are expressed as the mean ± SEM. *P<0.05 versus the sham group; #P<0.05 versus the SNI group, two-way mixed-model ANOVA. EA, electroacupuncture; SNI, spared nerve injury; A, acupuncture; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DRG, dorsal root ganglion; ANOVA, analysis of variance.

binding to LC3 and is efficiently degraded in autolysosomes (41,42). Thus, autophagy is impaired when p62 accumulates. Beclin-1 protein mediates the initiation step and is crucial for autophagosome formation (43). LC3-I is the unbounded cytosolic form that is converted to LC3-II after being recruited to the autophagosomal membranes upon binding to phosphatidylethanolamine. LC3-II demonstrates greater electrophoretic mobility. The ratio of LC3-II to LC3-I is considered a reliable indicator of autophagy (43). We observed that the levels of p62 were significantly increased on day 14 after SNI compared to the control group (P<0.05, one-way ANOVA; *Figure 2B*). EA treatment reduced the increase in p62 expression. However, the level of p62 in the acupuncture group did not differ significantly from that in the SNI group (*Figure 2B*). A significant increase in Beclin-1 and LC3II expression levels was observed on day 14 in the SNI group (P<0.05, one-way ANOVA; *Figure 2C,2D*). However, Beclin-1 and LC3II expression levels in the SNI + EA group increased remarkably in comparison to the SNI group (P<0.05, one-way ANOVA; *Figure 2C,2D*). These results suggested that SNI enhanced the expression of p62 and blocked autophagy. The elevation of Beclin-1 and LC3II might be caused by impaired autophagosome clearance instead of autophagy induction.

### *SNI induced activation of DRG macrophages accompanied by impaired autophagy*

Immunofluorescence staining of the L4–L6 DRG was performed using antibodies against p62, the satellite glial cell marker GFAP, the neuronal marker NeuN, and the macrophage cell marker CD11b. As shown in *Figure 3*, p62,

 

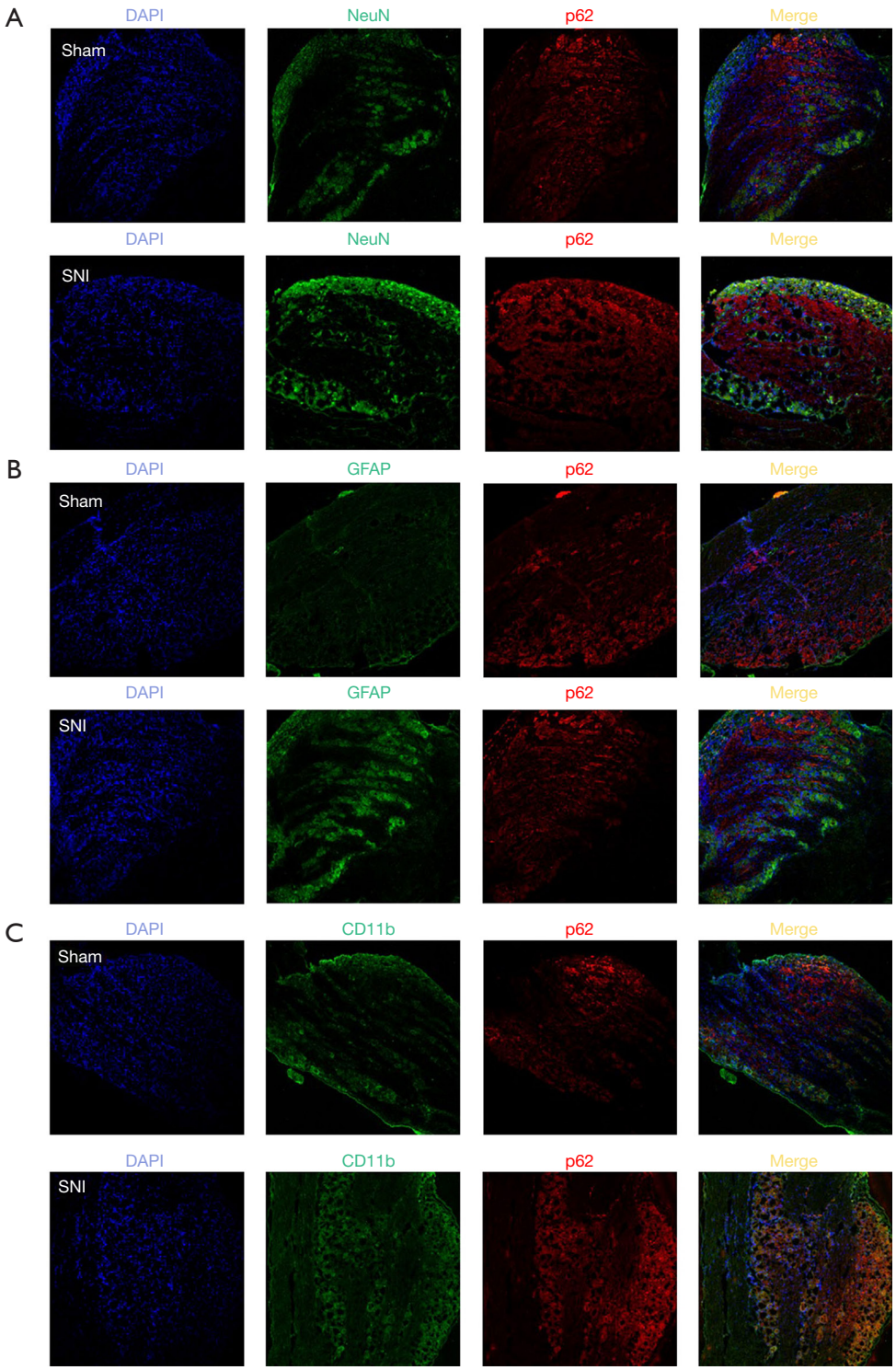


**Figure 3** Immunofluorescence analysis of the L4-L6 DRGs for the colocalization of p62 (representing for autophagy activation), NeuN (marker for neurons), GFAP (marker for satellite glial cells), and CD11b (marker for macrophages) at 14 days in sham and SNI rats. After SNI, p62, NeuN, GFAP, and CD11b showed significant stronger immunoreactivities in DRG compared with that in the sham group. P62 (red) was colocalized with CD11b (green), but not with NeuN (green) nor GFAP (green) in the SNI group. Red, p62; Green, NeuN, GFAP and CD11b; Blue, DAPI; magnification, ×40. SNI, spared nerve injury; DRG, dorsal root ganglion; GFAP, glial fibrillary acidic protein.

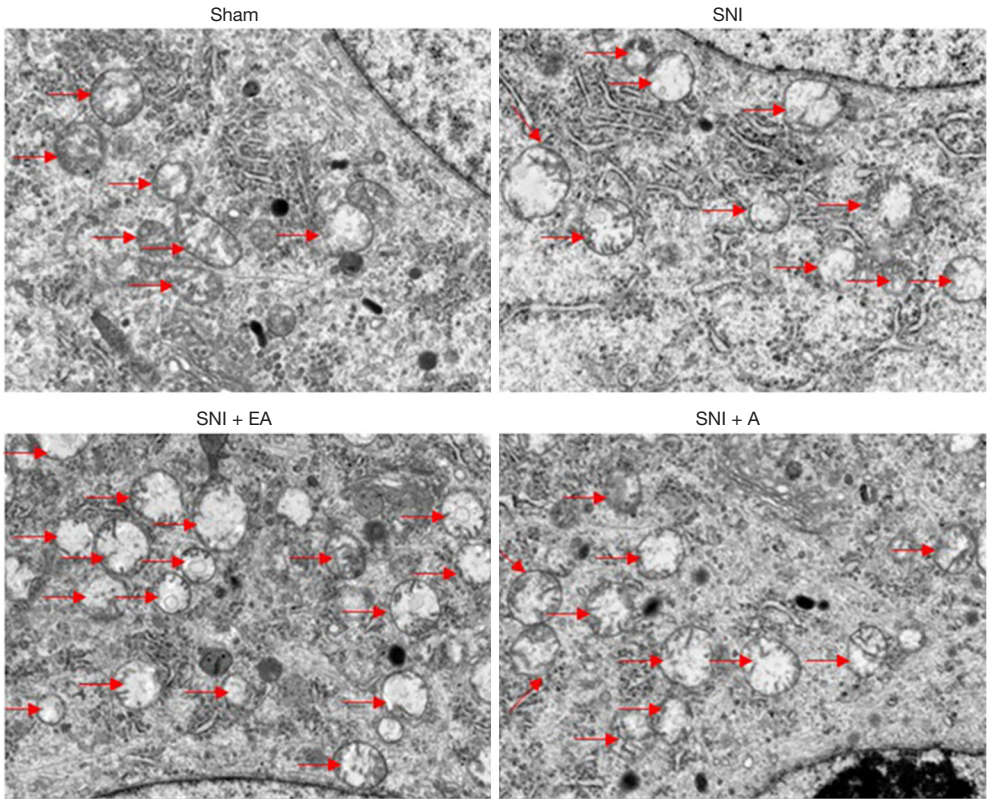


**Figure 4** Autophagy in macrophages of DRG was identified by electron microscopy. Rat samples from four groups (SNI, SNI + EA, SNI + A, Sham) were collected on day 14 post-surgery after the paw withdrawal threshold was quantified. Red arrows indicate autophagosomes. Electroacupuncture in SNI rats induced autophagosome formation in macrophages of DRG. Scale bar, 2 μm. SNI, spared nerve injury; EA, electroacupuncture; A, acupuncture; DRG, dorsal root ganglion.

NeuN, GFAP, and CD11b showed significantly stronger immunoreactivities in SNI rats, compared with that in the sham group, which indicated that DRG neurons, satellite glial cells, and macrophages were all activated after SNI. Increased p62 immunoreactivity among SNI rats compared to sham-operated rats indicated impaired autophagy. P62 (red) was colocalized with CD11b (green), but not with NeuN (green) nor GFAP (green) in the SNI group, suggesting that p62 was expressed mostly in macrophages, but not in neurons nor satellite glial cells.

### *EA in SNI rats induced autophagosome formation in DRG macrophages*

After quantification of PWT, L4-L6 DRG samples from four groups (SNI, SNI + EA, SNI + A and control) were collected on postoperative day 14. TEM showed that autophagosome formation enhanced significantly in DRG macrophages of SNI rats compared with sham-operated rats. EA in SNI rats significantly increased autophagosome formation compared to the SNI group (*Figure 4*). SNI rats were not affected by acupuncture in terms of autophagosome formation. SNI rats might have increased autophagosomes due to impaired clearance rather than autophagy induction. Whereas increased autophagosome formation in EA treated SNI rats might be due to autophagy activation by EA. The results elucidated that EA could induce autophagy progression in DRG macrophages among rats with SNI.

### *EA could reduce NP by promoting AMPK/mTOR-mediated autophagy in DRG macrophages*

To determine the causal relationship between EA-induced NP decay and enhanced autophagy, compound C, an AMPK inhibitor, was injected intrathecally. As control, DMSO, a solvent for compound C, was used. From postoperative day 5, PWT decreased with SNI, reached

 

nadir on postoperative day 7 and remained at a low level until postoperative day 14 (P<0.05, two-way mixed-model ANOVA; *Figure 1*). After surgery, EA administered between days 7 and 14 significantly increased PWT on day 14 after surgery, which was reversed by the intrathecal injection of compound C 1 hour before every EA treatment (P<0.05, two-way mixed-model ANOVA; *Figure 1*). As a control, DMSO did not significantly influence the analgesic effect of EA on SNI rats.

We examined the role of AMPK/mTOR signaling in EA-induced autophagy by measuring autophagy-related proteins, including p62, Beclin-1, LC3II, and p-AMPK/AMPK, p-mTOR/mTOR expression in the ipsilateral L4–L6 DRG of SNI rats with or without EA or compound C treatment on day 14 post-surgery (*Figure 5*). Compared to the SNI group, EA considerably reduced the expression of p62, as well as p-mTOR/mTOR, and thus, led to a significant increase in the expression of Beclin-1, LC3II, and p-AMPK/AMPK. After administration of compound C, the effects of EA were reversed, which indicated that EA induced autophagy via increasing the activity of AMPK, thus inhibiting the activity of mTOR, that is, the AMPK/mTOR signaling pathway was affected.

The function of the AMPK/mTOR signaling pathway in EA-induced autophagy was further evaluated by observing the autophagosomes in the L4–L6 DRG macrophages using electron microscopy. We observed that compared to the sham group, autophagosome formation was significantly enhanced in EA-treated SNI rats, which was subsequently reversed after the administration of compound C (*Figure 6*). These results suggested that EA induces autophagy in DRG macrophages of rats with SNI via the AMPK/mTOR signaling pathway.

## Discussion

In this study, the antinociceptive impact and its mechanism of EA on NP was examined. In ipsilateral L4-L6 DRGs, SNI-induced activation of macrophages was accompanied by reduced autophagy. EA alleviated SNI-induced NP and activated the autophagy process in DRG macrophages. Furthermore, intrathecal administration of AMPK inhibitor C reversed EA-induced NP inhibition and autophagy activation. These results indicate that EA treatment activates macrophage autophagy and alleviates NP via AMPK/mTOR pathway.

Complex mechanisms are involved in NP. Abundant molecular and cellular changes take place at the peripheral nerve system and CNS after peripheral nerve injury, which lead to peripheral and central pain sensitivity (44,45). Consistent with previous studies (14,46), this research found that neurons, satellite glial cells, and macrophages in L4–L6 DRG all showed stronger immunoreactivity after SNI.

EA is effective in treating various kinds of pain. Both peripheral and central mechanisms are involved in EA induced analgesic effect. Numerous bioactive chemicals from peripheral, spinal, and supraspinal systems are reported to be related to the mechanism of EA mediated analgesia (18). When combined with low-dose conventional analgesics, EA could provide more effective pain management both in animal and clinical studies (47-50), maximizing the effect of integrative medicine and minimizing the risk of debilitating side effects, which suggests a synergistic mechanism of this combination (47). Possible explanations of this synergic effect are maybe due to the influence of the spinal COX-2, thus inhibiting the production of PGE2, and alleviating the central hyperalgesia (49,50). There are already many clinical case studies about EA treatments of NP. The purpose of our study is to explore the deep mechanism of EA induced analgesia, and related clinical case studies are also being undertaken by our teams.

Autophagy could be stimulated by a variety of cellular stresses, with common cytoprotective characteristics (51). LC3 is an autophagic protein in mammals that is mainly present in the membrane of autophagosomes in the cytosol. Upon induction of autophagy, LC3I from the cytosol is converted into LC3II. This conversion happens in the autophagosome membrane (52). The plasma membrane, cytoplasm, and nucleus contain Beclin-1, which is essential for the localization of autophagic proteins to a pre-autophagosomal structure (53). P62 is considered to be selective autophagy receptor that is primarily degraded by autophagy; thus, an increase in the p62 levels correspond to a reduction in autophagy (54). P62 was found to have a connection with LC3 and ubiquitinated substrates. In additions, it is incorporated into autophagosomes and subjected to degradation in autophagosomes (55). Recent studies have uncovered that there is an impaired autophagy process in spinal astrocytes, microglia and GABAergic interneurons following peripheral nerve injury, which may account for the induction and maintenance phase of NP (26,56). Activation of autophagy is involved in acute spinal cord injury (25). Herein, we showed that SNI rats exhibited significant mechanical allodynia; the expression level of p62 in ipsilateral DRG was elevated to a significant level; and colocalization of macrophages and p62 were

 

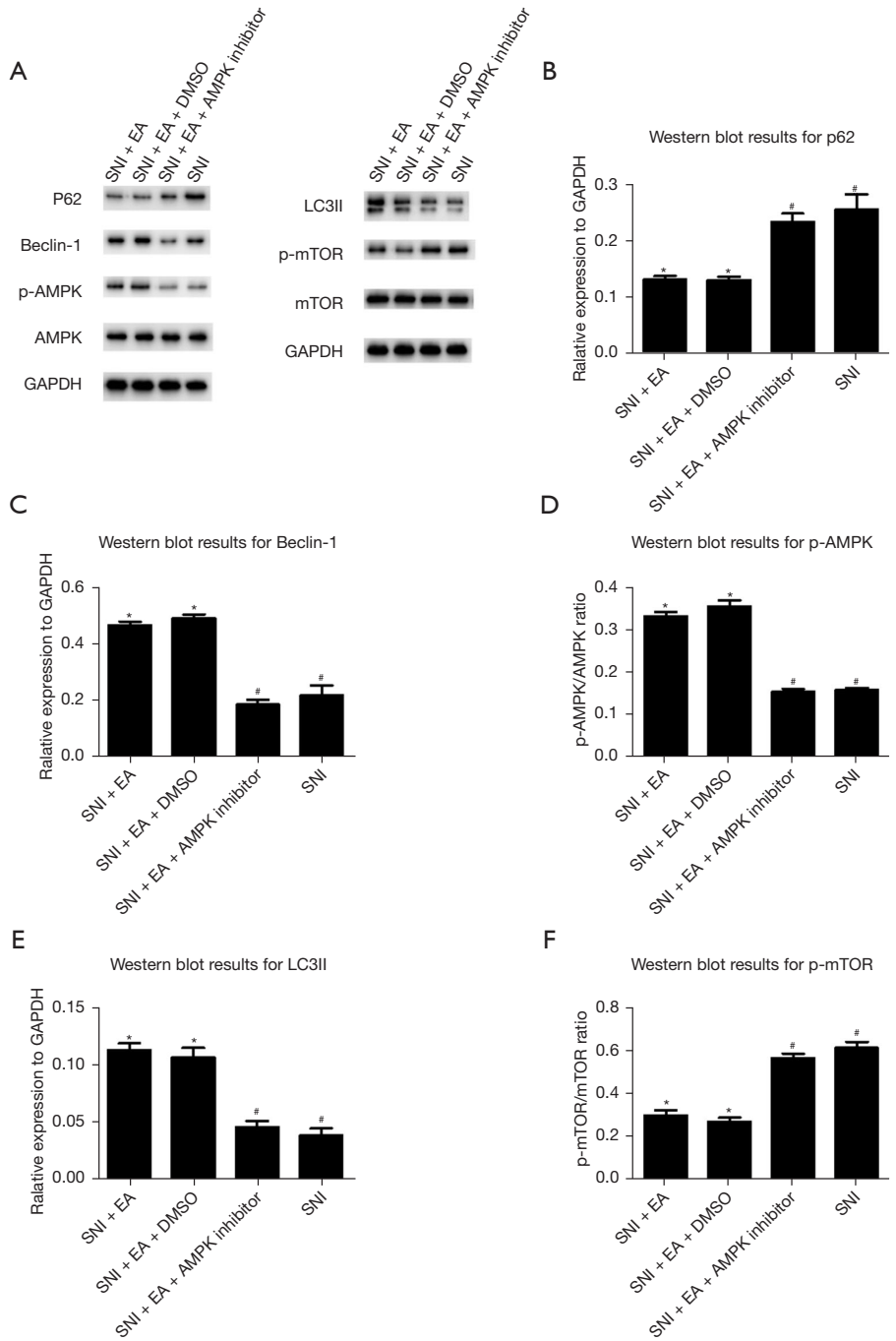


**Figure 5** Western blot results for p62, Beclin-1, AMPK, LC3II, and mTOR protein expression in the ipsilateral L4–L6 DRG of SNI rats with or without electroacupuncture or compound C treatment on day 14 after surgery. (A) Representative immunoblots in the SNI + EA group (n=3), SNI + EA + DMSO group (n=3), SNI + EA + AMPK inhibitor group (n=3), and SNI group (n=3). (B-F) Quantification of p62, Beclin-1, p-AMPK, LC3II, and p-mTOR protein levels in the ipsilateral lumbar spinal cord across different groups. All data are expressed as the mean ± SEM. *, P<0.05 versus the SNI group; #, P<0.05 versus the SNI + EA group, one-way ANOVA. SNI, spared nerve injury; EA, electroacupuncture; DMSO, dimethyl sulfoxide; AMPK, AMP-activated protein kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; mTOR, mammalian target of rapamycin; DRG, dorsal root ganglion; SEM, standard error of mean; ANOVA, analysis of variance.

 

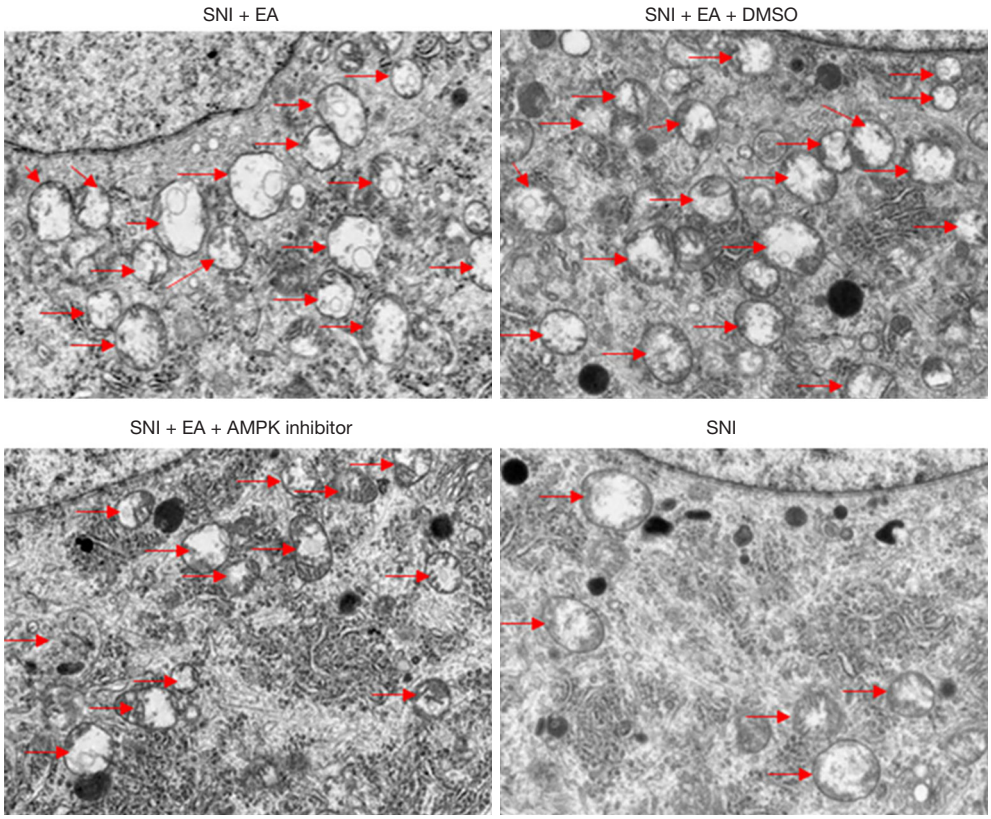


**Figure 6** Autophagosomes were observed with electron microscopy. Ipsilateral L4–L6 DRG samples were collected from the four groups (SNI + EA, SNI + EA + DMSO, SNI + EA + AMPK inhibitor, and SNI) on day 14 post-surgery after the paw withdrawal threshold was quantified. EA-induced autophagosome formation was shown in DRG macrophages, which was inhibited by intrathecal injection of the AMPK inhibitor. Autophagosomes are indicated by red arrows. Scale bar, 2 μm. SNI, spared nerve injury; EA, electroacupuncture; DMSO, dimethyl sulfoxide; AMPK, AMP-activated protein kinase; DRG, dorsal root ganglion.

detected after SNI, indicating that autophagic flux was blocked in macrophage autophagy after peripheral nerve injury. In addition, EA administration significantly reversed mechanical allodynia, which was accompanied by a decrease in p62 levels, indicating that EA enhanced autophagy. The expression of LC3-II and Beclin-1 was enhanced after EA stimulation, suggesting enhanced autophagy. Significant increased expression of Beclin-1 and LC3II was observed in the SNI group compared to sham-operated rats (*Figure 2C*,*2D*). Our results suggest that autophagy was alleviated after SNI based on the increased expression levels of p62. Elevated Beclin-1 and LC3II levels following nerve injury may be due to impaired autophagosome clearance rather than enhanced autophagy. Our study showed that activation of macrophage autophagy process may be involved in the mechanism of EA mediated analgesia.

It has been shown that the interactions between satellite glial cells and sensory neurons may contribute to the transmission of immune cell activation between peripheral and CNS, which lead to spinal sensitization of NP (46). Following neutrophil or macrophage invasion, there is satellite glia cell proliferation and increased coupling. Leukocyte elastase is then released from T cells, which leads to excitation of DRG neurons (46). DRG macrophages are involved in the maintenance of mechanical allodynia induced by nerve injury. The activation of microglial in the ipsilateral spinal dorsal horn is along with the pattern of mechanical allodynia induced by SNI, both of which are long lasting. DRG macrophages are also pivotal in the maintenance of hypersensitivity induced by nerve injury, in which the mechanisms may be related to the cellular communication between sensory neurons and DRG macrophages (14).

Our results are in line with previous reports (14,46),

 

which have indicated that neurons, satellite glia cells, and macrophages in L4–L6 DRG were all activated following SNI. Our double-labeled immunofluorescence results showed that after SNI, p62 is expressed in only in DRG macrophages but not in satellite glia cells or neurons. We also observed that EA induced autophagy in DRG macrophages in rats with SNI, as evidenced by the results obtained from TEM. Activation of macrophages in the presence of NP may be associated to impaired autophagy, and the enhancement of autophagy in macrophages may be responsible for EA's positive effects on NP. We examined the correlation between autophagy and the analgesic effects of EA on NP based on the changes in the expression of LC3, Beclin-1, and p62 found in DRGs of SNI rats after EA treatment.

The activation of the AMPK/mTOR pathway is critical for reducing chronic pain and controlling autophagic flux (32). AMPK expression in the hypothalamus can be linked to the analgesic effect of EA, and the expressed levels of AMPK in responding rats were higher than those in non-responding rats (33). To determine the role of the AMPK/mTOR pathway in EA-mediated autophagy in macrophages, we adopted a pharmacological inhibitor that has the potential to inhibit AMPK activity. Via TEM and autophagy-related proteins, we demonstrated that inhibiting AMPK activity attenuated autophagy induction and inhibits EA-induced autophagy in macrophages. Based on the results, AMPK/mTOR is involved in triggering autophagy in DRG macrophages upon EA stimulation in SNI rats. However, due to the high expense of gene knock-out experiment, we did not do research on target genes, which would be our future direction of study.

Our research provides further insight into the underlying mechanism of EA induced analgesic effect in NP caused by peripheral nerve injury and provides evidence for the AMPK/mTOR signaling pathway in modulating autophagy of DRG macrophages induced by EA stimulation for NP. There have been autophagy-targeting drugs emerging as targets in NP researches. This study provides a fundamental basis for targeting autophagy pathway and application of EA in NP therapy, and further elucidates the regulation and role of AMPK/mTOR signaling pathway in EA mediated analgesia, offering new sights and targets for pain therapy.

## Conclusions

Our study elaborated EA's analgesic impact is partly related to AMPK/mTOR pathway activation and autophagy induction in DRG macrophages, providing a novel therapeutic target for NP.

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## Footnote

*Reporting Checklist:* The authors have completed the ARRIVE reporting checklist. Available at https://atm.amegroups.com/article/view/10.21037/atm-22-5920/rc

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*Conflicts of Interest:* All authors have completed the ICMJE uniform disclosure form (available at https://atm.amegroups.com/article/view/10.21037/atm-22-5920/coif). The authors have no conflicts of interest to declare.

*Ethical Statement:* The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All animal experiments were conducted with the approval of the committee on animal experimentation at the Nanjing University of Chinese Medicine (NUCM, No. ACU210502), in compliance with national guidelines for the care and use of animals.

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