Randomized Controlled Study

Exercise Facilitates the M1-to-M2 Polarization of Microglia by Enhancing Autophagy via the BDNF/AKT/mTOR Pathway in Neuropathic Pain

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Background: In neuropathic pain following peripheral nerve injury, microglia are rapidly activated and accumulated in the spinal cord. Physical exercise can alleviate neuropathic pain. However, the exact mechanism underlying this analgesic effect is not fully understood.

Objectives: We aimed to investigate the molecular mechanisms by which exercise alleviates neuropathic pain in relation to brain-derived neurotrophic factor (BDNF), microglia polarization, and autophagy.

Study Design: A randomized controlled animal study divided into 2 stages. The first stage comprised 4 groups each with 6 mice, and the second stage comprised 6 groups, 3 with 18 mice and 3 with 12 mice.

Setting: Department of Anesthesiology, Lanzhou University Second Hospital, Orthopaedics Key Laboratory of Gansu Province, Lanzhou University.

Methods: Von Frey filaments, Western blotting, immunofluorescence, and transmission electron microscopy analyses were conducted to detect relevant markers.

Results: After peripheral nerve injury, exercise training downregulated BDNF expression and reversed microglial activation, as indicated by the increased expression of the M2 marker CD206 and decreased expression of the M1 marker CD86 in the spinal dorsal horn of mice. Autophagy flux was enhanced after exercise training, as suggested by the increased expression of the autophagy markers LC3-II/LC3-I and Beclin1 and decreased expression of the autophagy adaptor protein p62. Furthermore, autophagy inhibition by 3-methyladenine aggravated M1 polarization and hyperalgesia, whereas autophagy induced by rapamycin promoted M2 polarization and reduced hyperalgesia. Intrathecal injection of BDNF significantly upregulated BDNF expression, inhibited autophagy, triggered M1 polarization of spinal microglia, and aggravated hyperalgesia. Furthermore, BDNF regulated autophagy through the AKT/mTOR pathway, thereby participating in exercise training-mediated polarization of microglia after nerve injury.

Limitations: The effect of exercise on autophagy and pain cannot be assessed in an in vitro model. The influence of intrathecal injection of BDNF on the metabolic changes in other neuronal cells and the subsequent effects on pain should be investigated. Further studies on how exercise training modulates microglial autophagy to alleviate neuropathic pain are needed.

Conclusions: Exercise training promoted the recovery of sciatic nerve injury in mice, possibly by regulating microglial polarization through BDNF/AKT/mTOR signaling-mediated autophagy flux. We confirmed the efficacy of exercise training in alleviating neuropathic pain and suggest a new therapeutic target for neuropathic pain.

Key words: Autophagy, brain-derived neurotrophic factor, exercise, microglial polarization, neuropathic pain

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Nonpharmacological approaches, such as treadmill exercise, attenuate chronic pain (1-4). However, how exercise reduces neuropathic pain (NP) remains unclear. Elucidating this mechanism will provide a theoretical basis for the application of exercise therapy and provide a new direction for the treatment of chronic pain.

The normalization of neurotrophic growth factors plays a significant role in promoting the recovery of nerve function (5-7). In NP pathogenesis, brain-derived neurotrophic factor (BDNF) expression in sensory neurons is upregulated, leading to microglia activation and abnormal tactile pain (8-12). The spinal cord signaling pathway mediated by BDNF is related to the occurrence and persistence of mechanical hyperalgesia (13). In a neuroinflammation model, exercise training can normalize the expression of inflammatory mediators and BDNF in the central nervous system by reducing the activation of glial cells (14-16). Therefore, exercise training is a trigger for microglial activation and NP induction. However, the molecular pathway by which BDNF drives microglia activation to induce NP remains unclear.

Microglial autophagy regulates the homeostasis of the nervous system. There are 2 main phenotypes of activated microglia: M1 and M2. The former promotes inflammation and neurotoxicity, whereas the latter exerts anti-inflammatory and neuroprotective effects (17-20). Increasing evidence indicates that autophagy is essential for inducing microglia polarization to the M2 phenotype and regulating their functions. Changing the polarization of microglia is a promising strategy for the treatment of NP (21-25). Therefore, we investigated whether BDNF is involved in exercise training-induced microglia polarization by regulating autophagy and explored the underlying molecular mechanisms. Treadmill exercise training may provide an effective treatment for NP .

Methods

Animal Experiments

C57BL/6 mice (weighing 18–20 g, male) were purchased from Lanzhou Veterinary Research Institute of the Chinese Academy of Agricultural Sciences (Animal Center License No. SCXK [Gan] 2015-0001). The mice were raised at a fixed room temperature of 23 ± 1°C, light/dark cycle of 12 h/12 h, and free access to food and water.

Mouse Model of NP

To establish an NP model, sciatic nerve injury (SNI) was generated in the left sciatic nerve of the mice (26). After anesthetization with an intraperitoneal injection of sodium pentobarbital (40 mg/kg), the outer skin of the left thigh was cut, and the biceps femoris was dissected to expose the terminal branches of the sciatic nerve. At the bifurcation point, the common peroneal nerve and tibial nerve were tightly ligated using 4-0 silk and cut distal to the junction. Touching and pulling of the sural nerve were avoided during the operation. In Group S, the sciatic nerve was exposed without ligation or cut.

Research Design

In the first stage, the mice ran at different speeds for 60 minutes a day, 5 days a week. This experiment involved 4 groups, each with 6 mice: 3 exercise groups and one control group. The mice in the exercise groups ran at 7 (SNT-7, n = 6), 10 (SNT-10, n = 6), and 15 (SNT-15, n = 6) m/min for 3 to 21 days after SNI surgery.

Group SNI (n = 6) mice did not undergo treadmill running after surgery. In the second stage, after the optimal speed was determined, mice were divided into 6 groups: 1) Sham (Group S) (n = 18); 2) SNI-detrained (Group SD) (n = 18), mice that did not run after SNI surgery; 3) SNI-trained (Group SNT) (n = 18), mice that ran after SNI surgery; 4) SNI-trained + rapamycin (Group SNR) (n = 12); 5) SNI-trained + 3-methyladenine (3-MA) (Group SNM) (n = 12); and 6) SNI-trained + BDNF (Group SNB) (n = 12).

Exercise Training

The protocol for treadmill exercise training (SA101, Jiangsu Sans Biotechnology Co. Ltd) was designed based on a previously reported method (Figs. 1A, 1C) (27-29). Except for Groups S and SD, all groups were subjected to adaptive running training for 2 weeks, 5 days a week. In the first week, the mice ran at a speed of 7 m/min for 10 minutes every day. Starting with the second week, the mice ran for 20 minutes a day at a speed of 7 m/min; the duration was increased by 10 minutes each day up to 60 minutes. The surgery was performed in the third week. Except for Groups S and SD, all groups ran for 60 minutes a day at a speed of 10 m/min starting from the third day postsurgery. Before running, all mice were adapted to the treadmill belt for 10 minutes. The incline of the treadmill was set to 0. During the running process, constant running on the treadmill was ensured by tapping the tail or hind limbs of the mouse. Various stimuli, such as electric shocks, were turned off to avoid deviations caused by stress stimuli.

Drug Administration

Rapamycin (Sigma-Aldrich) and 3-MA (Sigma-Aldrich) were dissolved in 2% dimethyl sulfoxide in saline and administered intraperitoneally every day starting at 30 minutes postsurgery. Recombinant human BDNF (R&D system) was reconstituted in 0.9% saline. This was administered intrathecally every 2 days starting at 30 minutes postsurgery. Group SNR was injected with 10 mg/kg rapamycin, and Group SNM was injected with 15 mg/kg 3-MA every day (30). Group SNB was injected with 50 ng/kg BDNF (31-33). On postsurgery day 14, all animals were euthanized by an overdose of sodium pentobarbital at 30 minutes following drug injection.

Intrathecal Injection

Mice were subjected to 3% isoflurane anesthesia and maintained at 1.5–2%. For intrathecal injection, a half-inch 30G needle attached to a 25 μL Hamilton syringe was inserted diagonally upward between the spinous and transverse processes of the L5 and L6 vertebrae of the spinal column (34). A reflective flick of the tail was considered a sign of the accuracy of each injection. The volume of a single intrathecal injection was 4 μL.

Assessment of Mechanical Hyperalgesia

We assessed mechanical hyperalgesia in mice according to the paw withdrawal threshold described by Chaplan, et al (35). In the first stage, mechanical hyperalgesia was assessed at 2–3 days before SNI surgery as well as at 3, 7, 14, and 21 days post SNI surgery to evaluate functional recovery. The second stage was conducted before SNI surgery as well as at 3, 7, and 14 days post SNI surgery. Von Frey filaments of different forces (0.6, 1.0, 1.4, 2.0, and 4.0 g, North Coast Medical) were used to stimulate the lateral edge of the left hind paw of the mice. The mechanical stimulus threshold was determined using the up-and-down method (35).

Western Blotting Analysis

Total protein was extracted using radioimmunoprecipitation assay (RIPA) lysis buffer, fractionated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to polyvinylidene difluoride (PVDF) membranes. Subsequently, the blots were blocked with 5% nonfat dried milk for one hour and then co-incubated with primary antibodies separately at 4°C overnight.

The antibodies used included anti-BDNF antibody (1:1000, ab108319; Abcam), anti-CD86 antibody (1:1000, ab112490; Abcam); anti-CD206 antibody (1:1000, ab64693; Abcam); anti-Beclin1 antibody (1:1000, ab62557; Abcam); anti-LC3II antibody (1:2000, ab192890; Abcam); anti-p62 antibody (1:10000, ab109012; Abcam); anti-TrkB antibody (1:1000, ab181560; Abcam); anti-mTOR antibody (1:1000, ab134903; Abcam); anti-p-mTOR antibody (1:1000, ab109268; Abcam); anti-AKT antibody (1:1000, ab8805; Abcam); anti-p-AKT antibody (1:1000, ab38449; Abcam); and anti-GAPDH antibody (1:1000, AF7021).

Following 3 washes with tris buffered saline-tween (TBST), the membranes were reacted with the appropriate horseradish peroxidase- (HRP) conjugated secondary antibody (ZB-2301; ZSGB-BIO) at room temperature for 2 hours. Finally, the protein bands were visualized using Pierce™ enhanced chemiluminescence (ECL) (Thermo Scientific), and detected using the Bio-Rad ChemiDoc MP imaging system (UVP Inc.) and analyzed by the ImageJ software (public domain).

Immunofluorescence

The spinal cord was dissected, followed by fixation in 4% paraformaldehyde for 2 hours and in 30% sucrose solution at 4°C for 24 hours. The specimens were sectioned on a cryostat and then incubated with anti-Beclin1 (1:10000), anti-p62, anti-LC3II, anti-CD86, anti-CD206, anti-BDNF, and anti-Iba-1 primary antibodies at 4°C overnight. Next, the samples were incubated with fluorescein isothiocyanate (FITC) (1:500; Servicebio) or Cy-3 (1:500; Servicebio) goat anti-rabbit secondary antibody for one hour, in which the nuclei were counterstained with DAPI (Servicebio). Finally, pictures of the glass slides were taken using a confocal fluorescence microscope (Nikon Eclipse C1).

Transmission Electron Microscopy Analysis

L4–L6 spinal cord tissue samples were fixed in 2.5% glutaraldehyde for 2 hours, cut into small pieces (one mm3), and then fixed in 2.5% glutaraldehyde at 4°C for 24 hours. Subsequently, the samples were postfixed in 1% phosphate-buffered osmium tetroxide. After dehydration in acetone, the samples were cut into ultra-thin sections and then stained with uranyl acetate and lead citrate. A transmission electron microscope (T7780, Hitachi) was used to observe the ultrastructure.

Statistical Analyses

Statistical analyses were performed using GraphPad Prism (GraphPad Software). Data are presented as

the means ± standard deviations, and the normality of data distribution was determined by the Shapiro–Wilk test ($P > 0.05$). Significant differences were assessed using repeated measures analysis of variance (ANOVA) or Student's t test. $P < 0.05$ was considered statistically significant.

Results

Treadmill Exercise Improves Pain-related Behaviors Post SNI Surgery

From 3 to 21 days post SNI surgery, mice exhibited mechanical allodynia-like behaviors (Fig. 1B). However, allodynia was progressively reversed from 7 to 21 days postsurgery in Group SNT, compared with Group SNI (7 days, $*P < 0.05$; 14 days, $**P < 0.01$; 21 days, $**P < 0.01$). These results suggest that the treadmill running protocol effectively alleviated pain-related behaviors; there was no difference in the improvement in mechanical withdrawal between the different exercise programs. Hence, exercise at 10 m/min (SNT-10) was used to investigate the mechanism of action of treadmill exercise in alleviating hyperalgesia within 14 days postsurgery.

In the second stage, the withdrawal threshold decreased from 3 days post SNI surgery compared with Group S ($***P < 0.001$) (Fig. 1D). In the SNT groups,

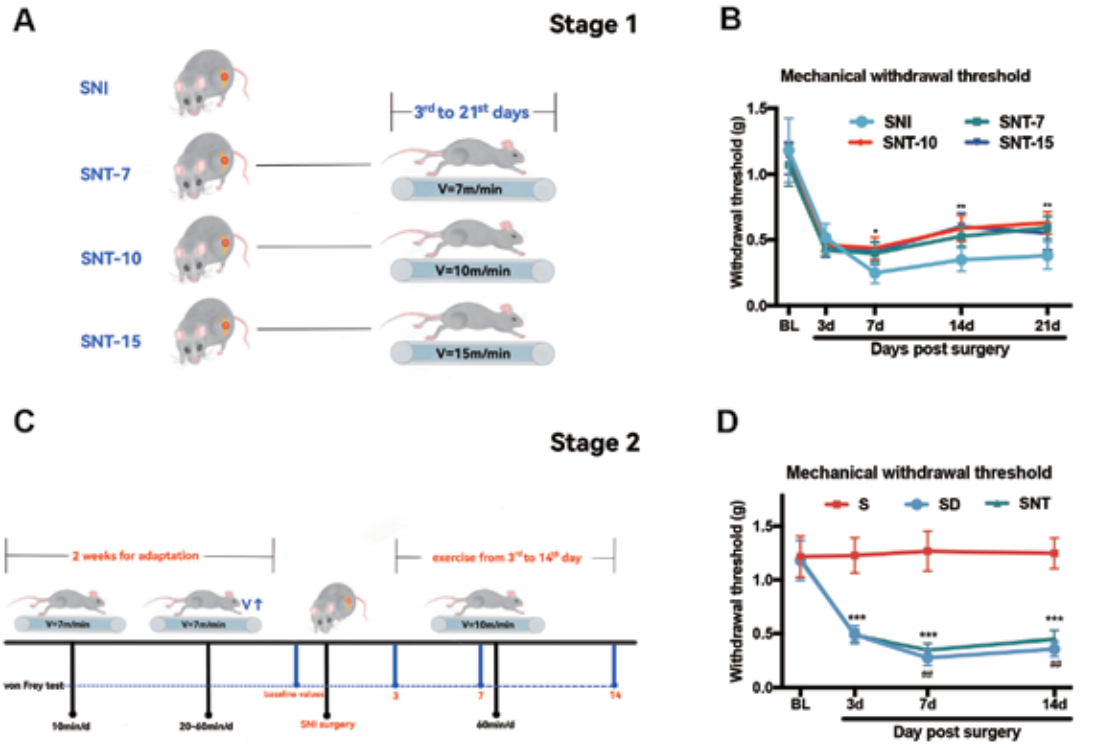

Fig. 1. *Protocol for treadmill exercise training and time-course of mechanical paw withdrawal threshold measured by von Frey hair stimulation. Representation of the two stages of the treadmill running protocols. (A) Stage 1: definition of intensity and duration of continuous running. The mice were divided into four groups: (1) SNI group (n=6), (2) SNI-trained-7 (SNT-7, n=6) group, (3) SNI-trained-10 (SNT-10, n=6) group, (4) SNI-trained-15 (SNT-15, n=6) group. (B) Mechanical hyperalgesia was evaluated before SNI surgery and on days 3, 7, 14, 21 after SNI surgery. Data points represent mean ± standard deviation from experiments. $*P < 0.05$, $**P < 0.01$ versus the SNI group, two-way repeated-measures ANOVA for each time point. (C) Stage 2: The mice were divided into six groups: (1) Sham (S) group, (2) SNI-detrained (SD) group: mice without running after SNI surgery, (3) SNI-trained (SNT) group: mice with running after SNI surgery, (4) SNI-trained + rapamycin (SNR) group, (5) SNI-trained + 3-MA (SNM) group, and (6) SNI-trained + BDNF (SNB) group. (D) The withdrawal threshold values of the S (n = 6), SD (n = 6), and SNT (n = 6) groups were determined at baseline and at 3, 7, and 14 days after SNI or sham surgery. Data points represent mean ± standard deviation from experiments. $***P < 0.001$ versus the S group; $##P < 0.01$ versus the SD group, two-way repeated-measures ANOVA for each time point.*

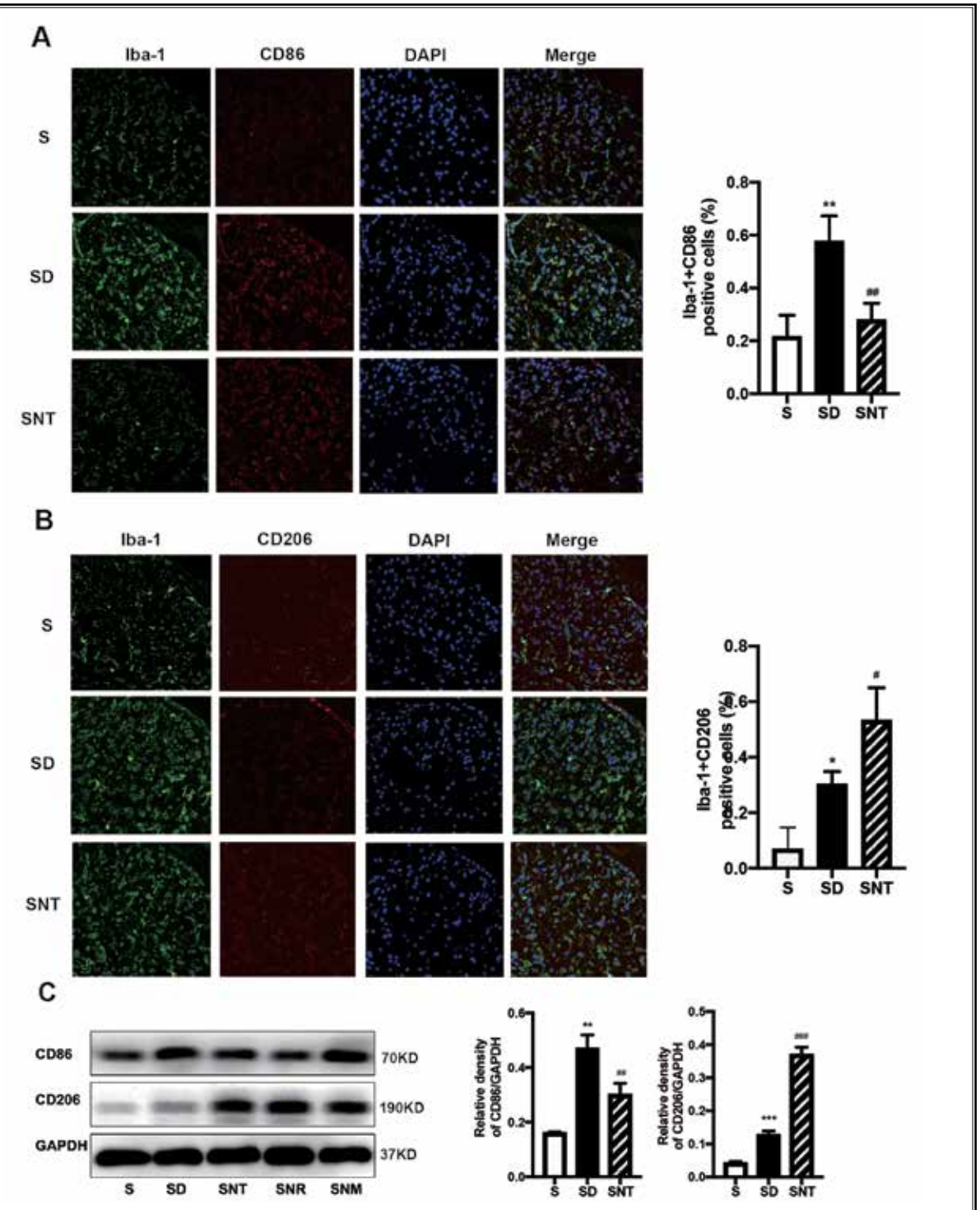

Fig. 2. *Treadmill exercise training promotes M1-to-M2 polarization of microglia in mice after SNI surgery. (A, B) Samples of the spinal cord of mice were collected to detect the expression of CD86 and CD206 by immunofluorescence, CD86 and CD206 expression by western blotting analysis (C) at 14th day after SNI surgery (Bar = 20 μm). Data were expressed as mean ± standard deviation. *P < 0.05, **P < 0.01, and ***P < 0.001 versus the S group; #P < 0.05, ##P < 0.01, and ###P < 0.001 versus the SD group, one-way ANOVA followed by Tukey's test of multiple comparisons.*

allodynia was gradually reversed from 7 to 14 days after surgery (##$P < 0.01$). Taken together, these results indicated that treadmill training effectively alleviated pain-related behaviors in NP model mice.

Exercise Promotes M1-to-M2 Polarization of Microglia in Mice Post SNI Surgery

In order to examine whether treadmill exercise can change the polarization of microglia, immunofluorescent staining and western blotting were conducted to analyze the expression of M1 (CD86) and M2 (CD206) markers. L4–L6 spinal cord samples of mice were stained for Iba-1, which is a marker of microglia activation.

Immunofluorescence analysis results showed that the number of Iba-1+ CD86+ and Iba-1+ CD206+ cells in the dorsal horn of the ipsilateral spinal cord increased post SNI surgery (Figs. 2A-C, *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$). After treadmill exercise training, the expression of the CD206 protein increased significantly, whereas that of CD86 decreased significantly (CD206, #$P < 0.05$; CD86, ##$P < 0.01$). The number of Iba-1+ CD206+ cells increased and Iba-1+ CD86+ cells decreased in the dorsal horn of the ipsilateral spinal cord (CD206, ###$P < 0.001$; CD86, ##$P < 0.01$). These findings suggest that exercise promotes the polarization of microglia from the M1 to M2 phenotype.

Exercise Promotes Autophagy of Microglia in the Spinal Dorsal Horn

Compared with Group S, the expression of spinal autophagy-related proteins (LC3-II/LC3-I and Beclin1) significantly increased in Group SD (#$P < 0.05$, ***$P < 0.001$; Fig. 3A); after exercise training, it was further increased to a higher level than that in Group SD (#$P < 0.05$). Double immunofluorescence staining further proved that the accumulation of immunostained LC3 spots (red) was increased, showing co-localization with Iba-1 (green) (#$P < 0.05$; Fig. 2C). The SNI model showed significantly increased expression of p62 in microglia, which suggests impaired autophagy. Our results indicate that exercise training significantly reduces the SNI-induced expression of p62 in microglia (#$P < 0.05$).

Next, double immunofluorescence staining showed that post SNI surgery, the percentage of beclin1 and p62-positive microglia increased significantly (*$P < 0.05$; Figs. 2B, 2D); furthermore, after exercise, compared with Group SD, the percentage of beclin1-positive microglia increased, whereas that of p62-positive microglia decreased in Group SNT (#$P < 0.05$). Exercise training not only enhanced the expression of autophagy markers but also reduced the burden of autophagy substrates.

Microglia in the dorsal horn of the spinal cord were assessed via transmission electron microscopy. Microglial morphology in Group S was normal and complete. A few mitochondria were scattered in the cytoplasm and were regular in shape. No autophagosomes were detected. In contrast, Group SD displayed swelled and vacuolated mitochondria. A few autophagosomes were observed in microglia. Group SNT showed a lower degree of mitochondrial swelling and significantly more autophagosomes than Group SD group (Fig. 3E). Taken together, these findings suggest that the number of autophagosomes in the spinal cord microglia of SNI mice increased after treadmill exercise, thereby increasing the level of autophagy.

Exercise Mediates Microglial Activation by regulating Autophagy

Rapamycin and 3-MA were used to activate or inhibit autophagy, respectively, in NP mice. Our results showed that rapamycin further promoted autophagy and elevated the mechanical withdrawal threshold after SNI surgery, whereas 3-MA reduced it ($F_{6,117} = 3.156$, $P < 0.01$; Fig. 4A). Thus, we hypothesized that activation of autophagy can alleviate noxious behavior and play a protective role against peripheral nerve injury.

The autophagy inducer rapamycin downregulated Iba-1 expression in mice post SNI surgery, whereas the autophagy inhibitor 3-MA upregulated Iba-1 expression (Figs. 4C, 4D). Compared with Group SNT, p62 expression post SNI surgery significantly increased, and the LC3-II/LC3-1 ratio significantly decreased following treadmill exercise and 3-MA treatment (###$P < 0.001$ and #$P < 0.05$; Figs. 3A and 4B). These findings indicate that 3-MA partially abolished the exercise-induced upregulation of autophagy.

To evaluate autophagy induction at the molecular level, we performed double immunofluorescence staining for LC3 and p62 post SNI surgery. The results show that compared with Group SNT, the number of LC3-positive microglia was significantly decreased, whereas that of p62-positive microglia was significantly increased in Group SNM (##$P < 0.01$ and #$P < 0.05$; Figs. 4C, 4D). In contrast, compared with Group SNT, after a combination of treadmill exercise and rapamycin treatment, Group SNR showed a significantly increased LC3-II/LC3-I ratio (***$P < 0.001$; Figs. 3A, 4B). This result

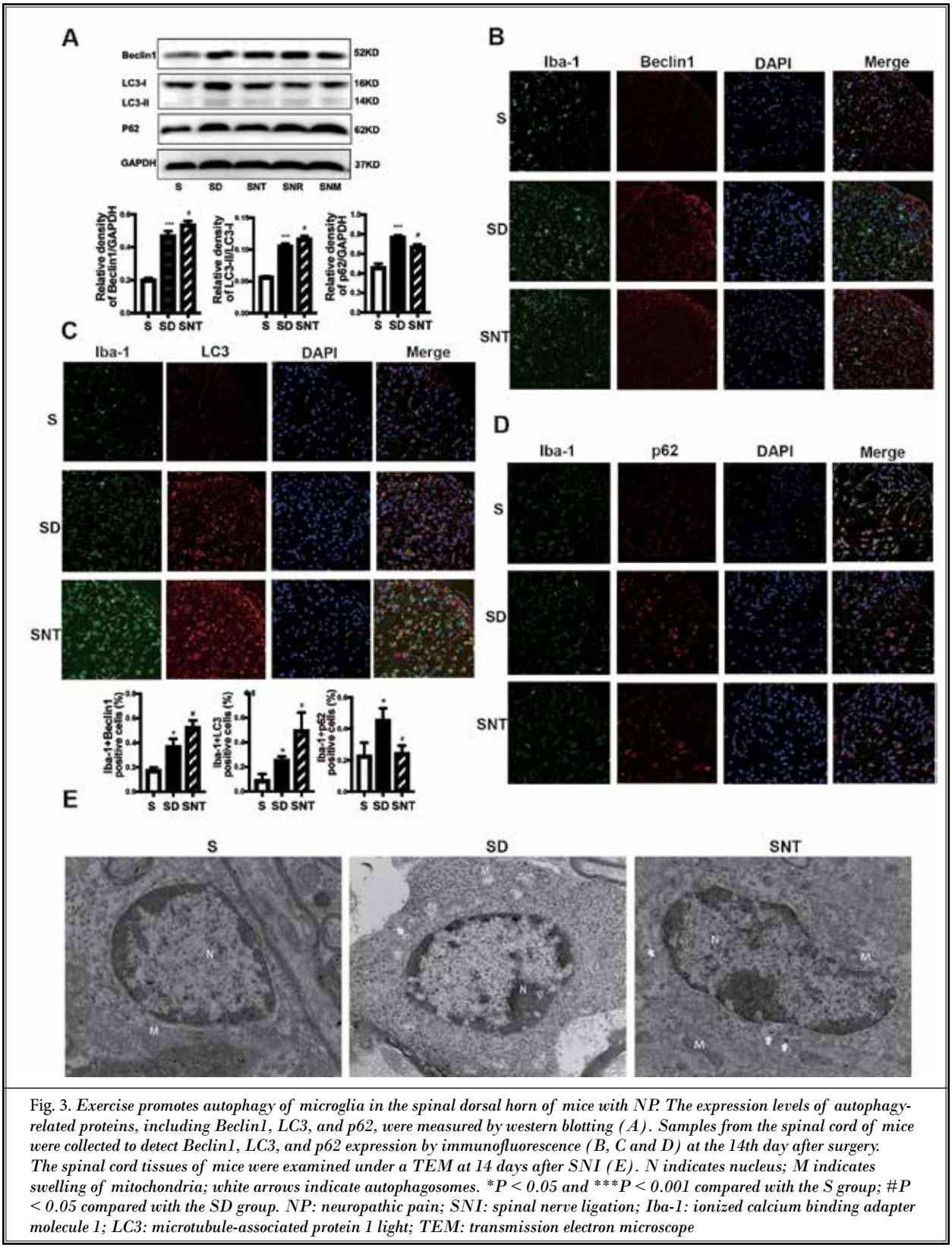

Fig. 3. *Exercise promotes autophagy of microglia in the spinal dorsal horn of mice with NP. The expression levels of autophagy-related proteins, including Beclin1, LC3, and p62, were measured by western blotting (A). Samples from the spinal cord of mice were collected to detect Beclin1, LC3, and p62 expression by immunofluorescence (B, C and D) at the 14th day after surgery. The spinal cord tissues of mice were examined under a TEM at 14 days after SNI (E). N indicates nucleus; M indicates swelling of mitochondria; white arrows indicate autophagosomes. *P < 0.05 and ***P < 0.001 compared with the S group; #P < 0.05 compared with the SD group. NP: neuropathic pain; SNI: spinal nerve ligation; Iba-1: ionized calcium binding adapter molecule 1; LC3: microtubule-associated protein 1 light; TEM: transmission electron microscope*

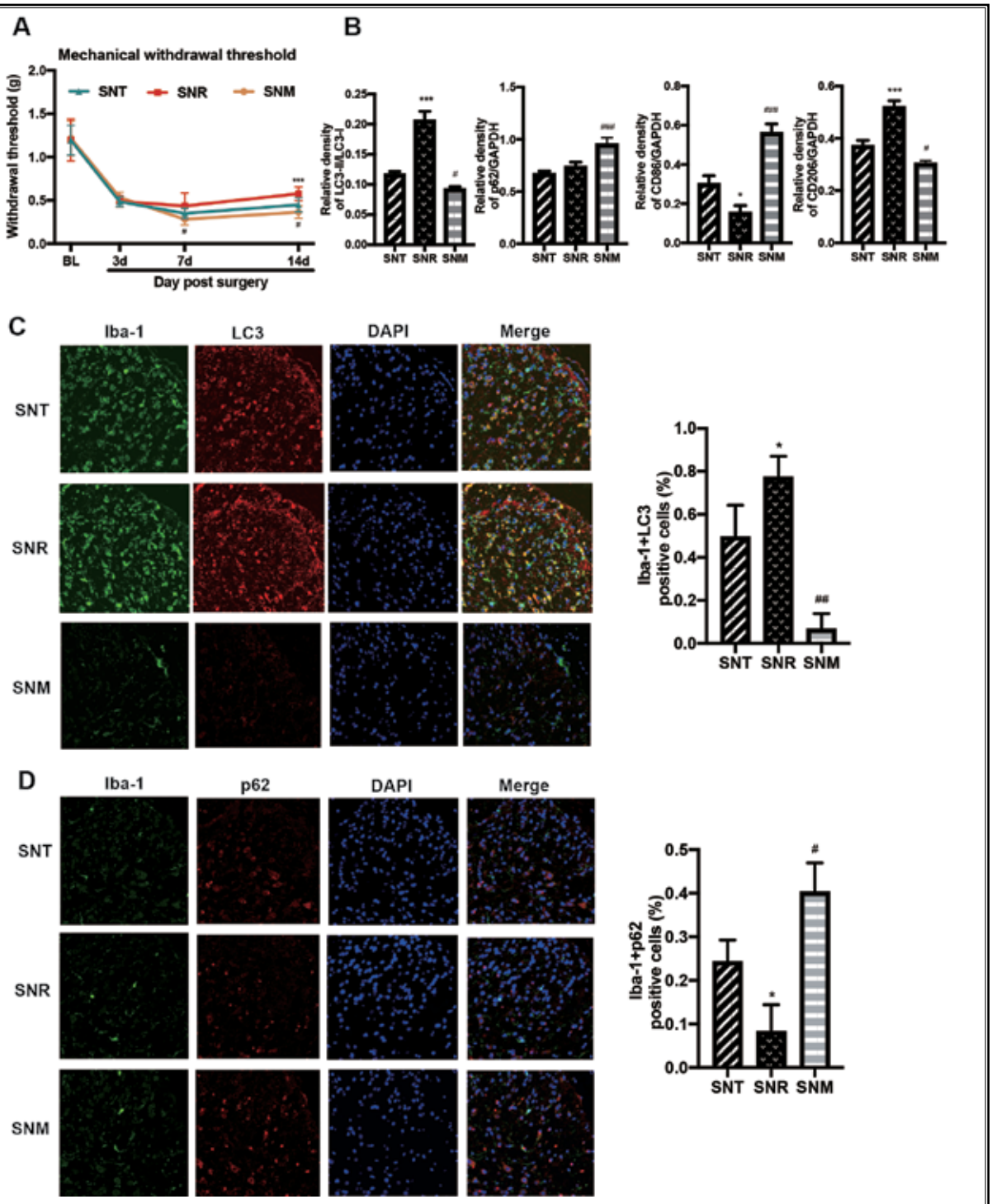

Fig. 4. *Exercise mediates microglial activation by regulating autophagy. Rap and 3-MA were administered intraperitoneally to mice. Mechanical withdrawal threshold (A) was detected before and at the 3rd, 7th, and 14th days after surgery. The expression levels of autophagy-related proteins, including Beclin1, LC3, and p62, were measured by western blotting (B) and immunofluorescence (C), respectively. Data are expressed as mean ± standard deviation in each group. *P < 0.05, ***P < 0.001, #P<0.05, ##P<0.01, and ###P<0.05 compared with the SNT group. RAP: Rapamycin; 3-MA: 3-methyl adenine; Iba-1: ionized calcium binding adapter molecule 1; LC3: microtubule-associated protein 1 light*

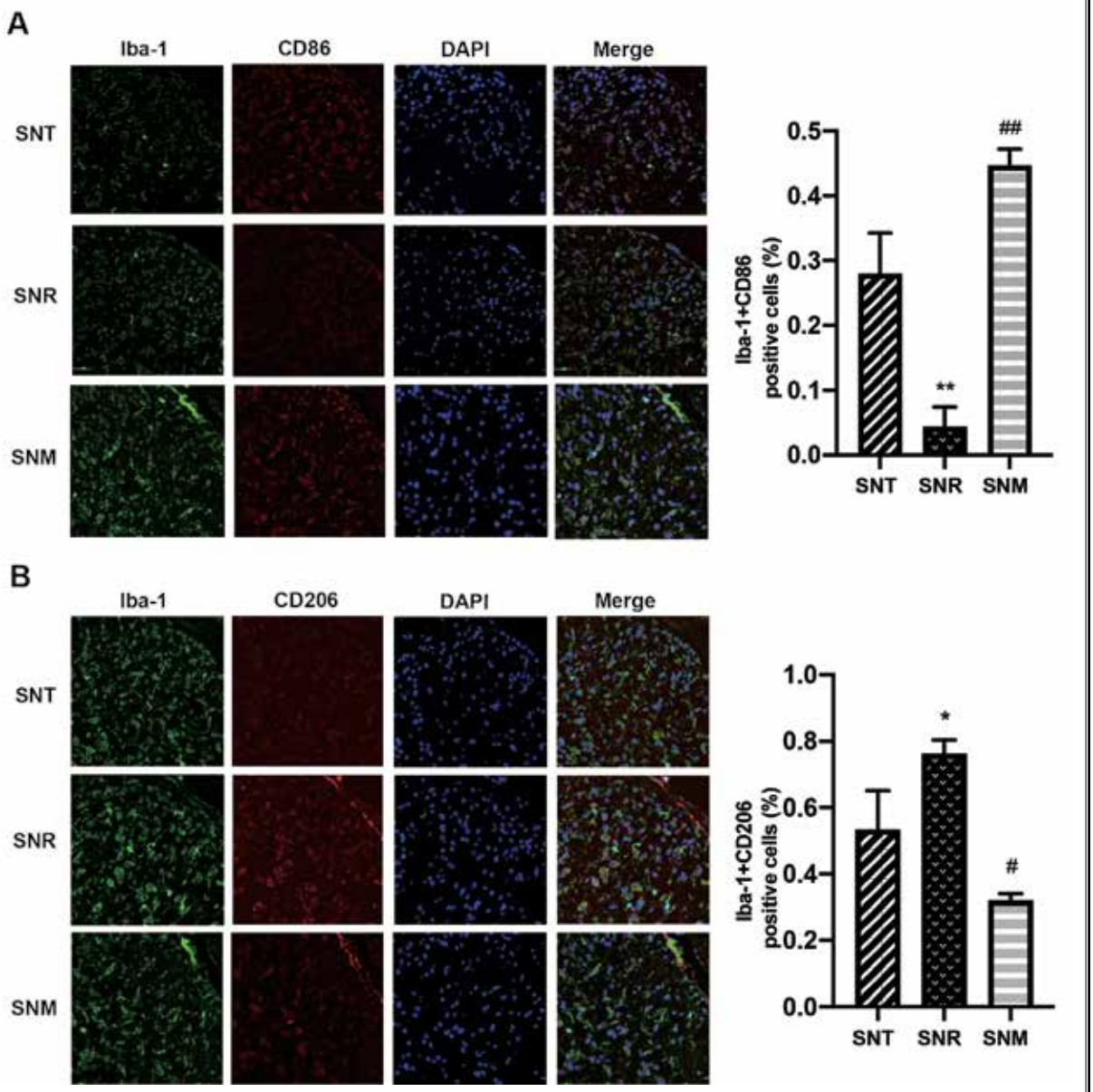

Fig. 5. *Exercise mediates microglial activation by regulating autophagy. Rap and 3-MA were administered intraperitoneally to mice. The expression levels of microglia polarization-related proteins CD86 (A) and CD206 (B) were measured by immunofluorescence. Data are expressed as mean ± standard deviation in each group. *P < 0.05, **P < 0.01, #P < 0.05 and ##P < 0.01 compared with the SNT group. RAP: Rapamycin; 3-MA: 3-methyl adenine; Iba-1: ionized calcium binding adapter molecule 1*

suggests that rapamycin promotes exercise-induced autophagy. Furthermore, immunofluorescence staining results showed that compared with Group SNT, Group SNR had a significantly increased number of LC3-positive microglia and a significantly decreased number of p62-positive microglia (*$P < 0.05$; Figs. 4C, 4D), suggesting that exercise stimulated autophagy post SNI, which was further enhanced by rapamycin and was partially abolished by 3-MA.

In addition, compared with those in Group SNT, the numbers of Iba-1+ CD86+ and Iba-1+ CD206+ cells in the spinal dorsal horn after 3-MA treatment increased and

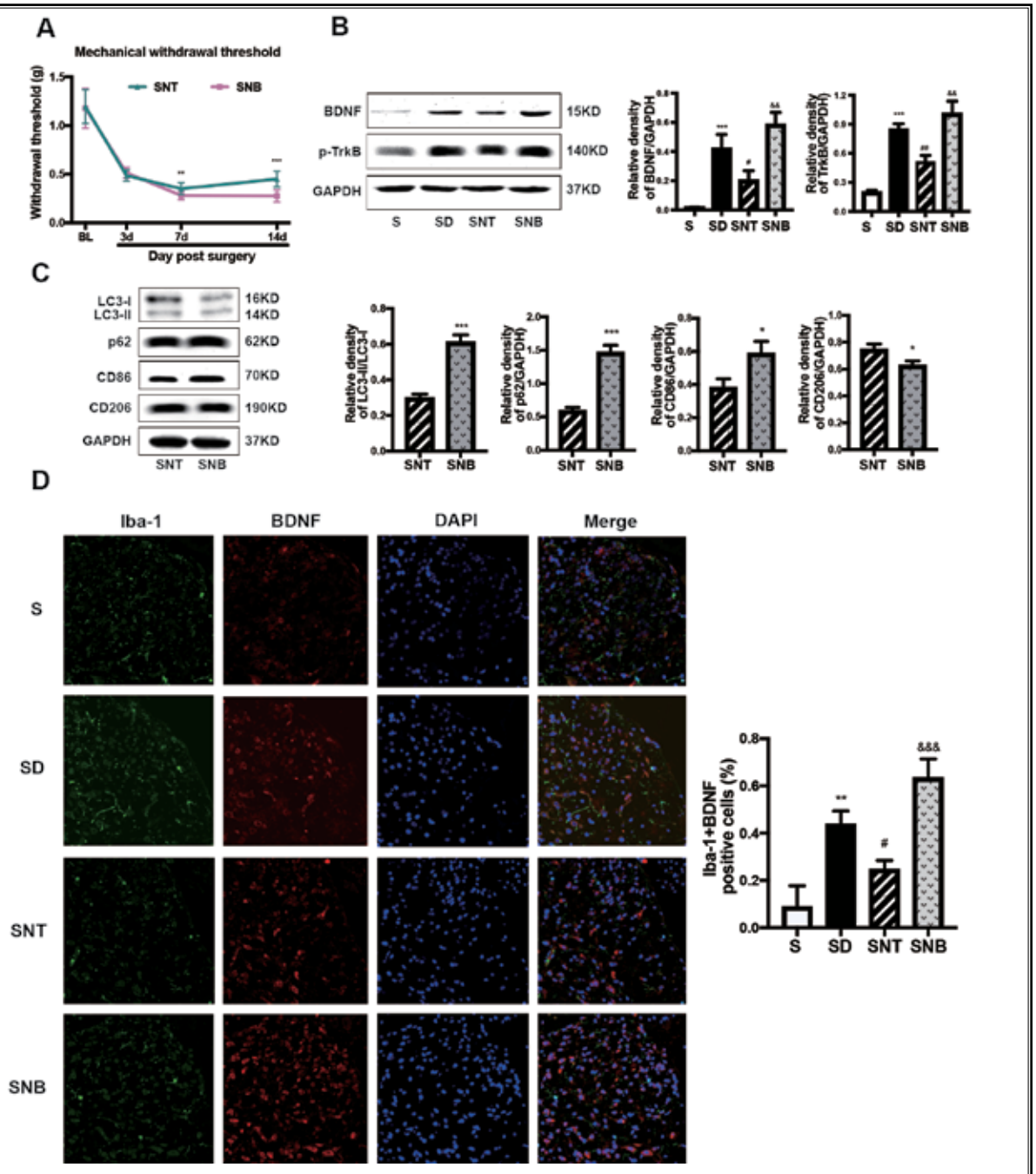

Fig. 6. *BDNF participates in exercise-regulated microglial autophagy and affects microglia activation. Mechanical withdrawal threshold (A) was detected before and at the 3rd, 7th, and 14th days after surgery. Data are expressed as mean ± standard deviation in each group. **P < 0.01, and ***P < 0.001 compared with the SNT group. The expression of BDNF proteins and TrkB phosphorylation levels were measured by western blotting (B). Data are expressed as mean ± standard deviation in each group. ***P < 0.001 compared with the S group; #P < 0.05 and ##P < 0.01 compared with the SD group; $$P < 0.01 compared with the SNT group. The expression of autophagy-related and microglia polarization-related proteins, including LC3, p62, CD86 and CD206 were measured by western blotting (C). Data are expressed as mean ± standard deviation in each group. *P < 0.05 and ***P < 0.001 compared with the SNT group. The expression of BDNF proteins was measured by immunofluorescence (D). Data are expressed as mean ± standard deviation in each group. **P < 0.01 compared with the S group; #P < 0.05 compared with the SD group; $$$P < 0.001 compared with the SNT group. Iba-1: ionized calcium binding adapter molecule 1; LC3: microtubule-associated protein 1 light; BDNF:Brain-derived neurotrophic factor; p-TrkB: tropomysin-related kinase B phosphorylation*

decreased, respectively, in Group SNM (##$P < 0.01$ and #$P < 0.05$; Figs. 5A, 5B). In contrast, rapamycin showed the opposite effect. Compared with Group SNT, Group SNM showed a decreased number of Iba-1+ CD86+ cells and an increased number of Iba-1+ CD206+ cells in the spinal dorsal horn following rapamycin treatment (**$P < 0.01$ and *$P < 0.05$). Western blotting analysis further confirmed these results(#$P < 0.05$ and ###$P < 0.001$; Figs. 2C and 4B).

Furthermore, after treadmill exercise and rapamycin administration, CD206 protein expression increased (***$P < 0.001$), whereas CD86 protein expression decreased (*$P < 0.05$). Taken together, these results confirm that exercise training mediated the polarization of mouse spinal cord microglia by regulating autophagy in NP.

BDNF Participates in Exercise-regulated Microglial Autophagy and Affects Microglia Activation

BDNF-Trk (tropomyosin receptor kinase)B signaling in the spinal cord has been well established in NP (36-39). Post SNI surgery, the mechanical retraction threshold of the ipsilateral side began to decrease at postsurgery day 3 (Fig. 6A). Compared with Group SNT, allodynia post BDNF injection continued to aggravate in Group SNB group, even at postsurgery day 7 (7 days, **$P < 0.01$; 14 days, ***$P < 0.001$). These results indicate that intrathecal BDNF administration reversed the efficacy of treadmill training in reducing pain-related behaviors in NP model mice.

In addition, SNI induced a marked increase in BDNF and TrkB phosphorylation levels postsurgery, compared with those in Group S, while treadmill exercise markedly reduced the levels (***$P < 0.001$, #$P < 0.05$ and ##$P < 0.01$; Fig. 6B). Thus, compared with those in Group SNT group, BDNF and TrkB phosphorylation levels increased significantly in Group SNB ($$$P < 0.01$).

The central nervous system maintains its functional capacity through signaling and synaptic transduction (40,41). Compared with Group SD, BDNF expression in microglia was reduced in Group SNT (#$P < 0.05$; Fig. 6D). In contrast, compared with Group SNT, BDNF expression in microglia was significantly increased in Group SNB (&&&$P < 0.001$).

The results also show that BDNF inhibits exercise-induced autophagic flux post SNI. Compared with Group SNT, the expression of LC3-II/LC3-I was reduced in Group SNB, whereas p62 was significantly elevated (***$P < 0.001$; Fig. 6C). Double immunofluorescence staining showed that the accumulation of LC3 (red) was reduced and that of p62 was increased, showing co-localization with Iba-1 (green) (*$P < 0.05$ and **$P < 0.01$; Figs. 7A, 7B). Thus, intrathecal injection of BDNF inhibits treadmill exercise-induced autophagy activation in microglia in the NP model.

Further, immunofluorescence staining revealed that post BDNF treatment, the number of Iba-1+ CD86+ cells in the ipsilateral spinal dorsal horn increased, whereas that of Iba-1+ CD206+ cells decreased (**$P < 0.01$ and *$P < 0.05$; Figs. 7C, 7D). Western blotting results were consistent with these findings. Compared with Group SNT, CD86 protein expression after intrathecal injection of BDNF increased significantly, whereas that of CD206 decreased in Group SNB (*$P < 0.05$; Fig. 6C). Collectively, these findings reveal that the inhibition of BDNF expression can promote autophagy flux and enhance microglial polarization to the M2 phenotype.

Exercise Activates Autophagy by Enhancing BDNF/Akt/mTOR Signaling

Our results show that NP induced a significant increase in p-AKT and p-mTOR levels in Group SD (***$P < 0.001$; Fig. 7E). After treadmill exercise, the phosphorylation levels of AKT and mTOR in Group SNT were lower than those in Group SD (###$P < 0.001$ and #$P < 0.05$). To elucidate the underlying mechanism, we examined the relationship between exercise-induced autophagic flux and the BDNF/AKT/mTOR pathway. Intrathecal injection of BDNF induced a significant increase in p-mTOR expression (&&&$P < 0.001$) and suppressed autophagy. However, exercise training inhibited the AKT/mTOR pathway, thereby promoting the M2 polarization of microglia. Autophagy triggered by AKT/mTOR may be a downstream target of exercise training in inducing BDNF to regulate pain hypersensitivity. Overall, these findings indicate that exercise-activated autophagic flux through the BDNF/AKT/mTOR pathway regulates microglia polarization, thereby reducing NP-induced hyperalgesia.

Discussion

After determining the optimum treadmill running protocol, we investigated the mechanism by which exercise training relieves hyperalgesia. We found that treadmill training effectively alleviated pain-related behaviors in NP model mice. Moreover, exercise training reduced the expression of BDNF in the spinal dorsal horn after nerve injury and reversed hyperalgesia. Thus, we speculated that the normalization of BDNF

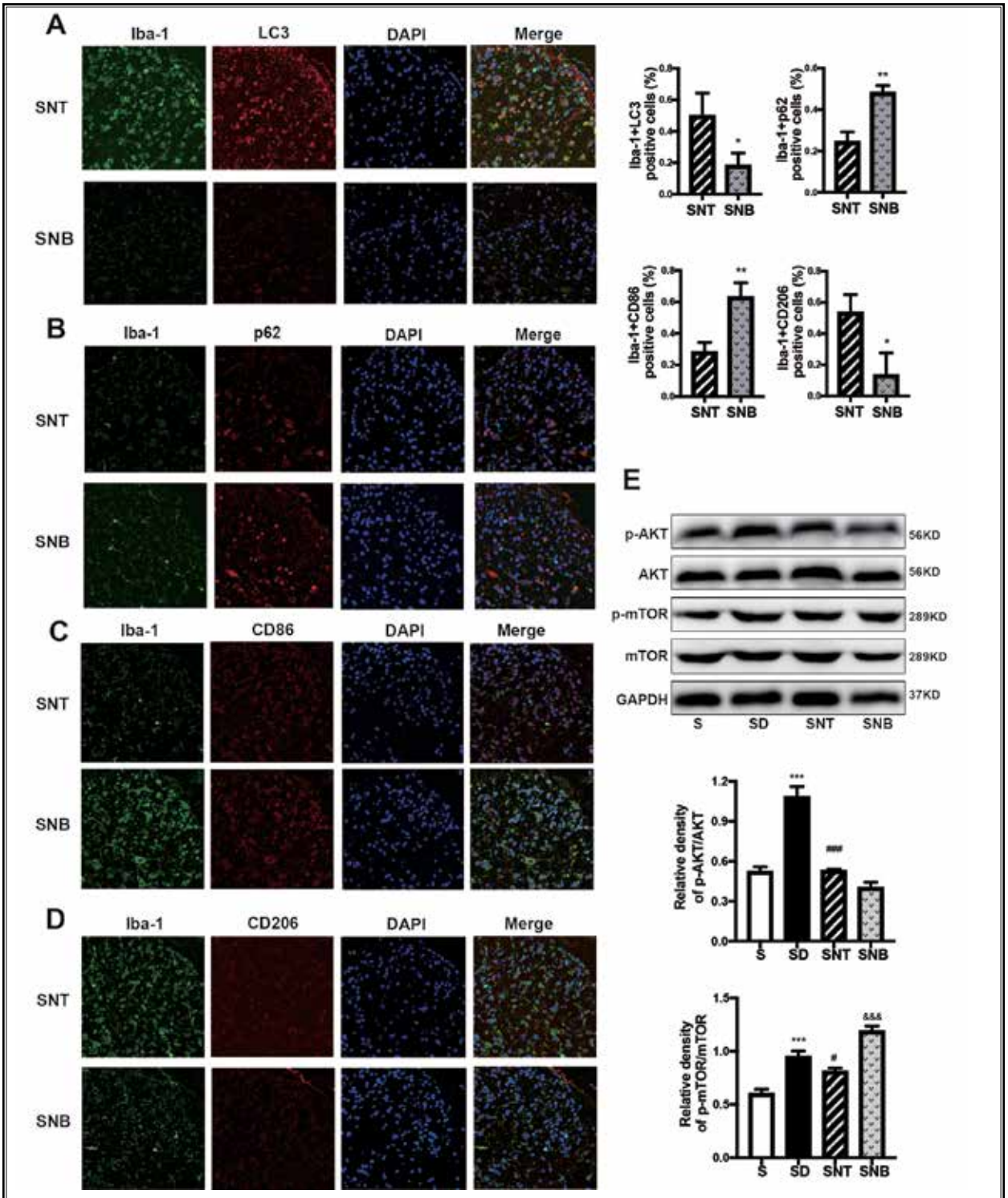

Fig. 7. *Exercise activates autophagy by enhancing BDNF/Akt/mTOR signaling. The protein expression of LC3 (A), p62 (B), CD86 (C) and CD206 (D) were measured by immunofluorescence. Data were expressed as mean ± standard deviation in each group. *P < 0.05 and **P < 0.01 compared with the SNT group; The expression of AKT/mTOR pathway were measured by western blotting (E). ***P < 0.001 compared with the S group; #P < 0.05 and ###P < 0.001 compared with the SD group; &&&P < 0.001 compared with the SNT group. Iba-1: ionized calcium binding adapter molecule 1*

expression might be involved in the attenuation of mechanical hypersensitivity and contribute to the recovery of pain-related behaviors.

The activation of microglia in the spinal cord is regarded as an essential step in the pathogenesis of NP caused by neurotrophic factors (42-45). We found that the microglial marker Iba-1 and the M1 marker CD86 in the ipsilateral spinal dorsal horn were elevated, which induced the activation of microglia to the M1 phenotype, resulting in mechanical allodynia-like behaviors. Post treadmill exercise, the expression of CD86 and Iba-1 decreased, the expression of M2 microglia marker CD206 increased, the paw withdrawal threshold measured by von Frey filaments significantly increased, and the hyperalgesia caused by nerve injury was significantly reduced. These results suggest that the activation of microglia is inhibited further to relieve neural pain. In addition, the expression of BDNF and p-TrkB proteins was upregulated and downregulated after exercise. Although it has been known that signal transmission between microglia and neurons is an integral part of NP transmission, how this signal occurs remains unclear. We hypothesize that blocking this microglia-neuron signaling pathway may be a promising treatment for NP.

In this study, exercise training enhanced the expression level of autophagy markers as well as promoted autophagosome formation. Autophagy is the process of removing metabolites and repairing damaged organelles during cellular metabolism, which plays a key role in the occurrence and development of neurological diseases. Rapamycin is an autophagy activator that mainly acts on mTOR kinase and Beclin-1. 3-MA is an autophagy inhibitor, which plays an inhibitory role in the formation and development of autophagosome (46-47). Therefore, rapamycin and 3-MA were selected to evaluate the mechanisms underlying the exercise-induced promotion of autophagy.

Rapamycin promoted the activity of microglia induced by BDNF, further elevating the expression of LC3-II, beclin1, and p62 proteins. 3-MA exerted the opposite effect. These findings suggest that exercise training suppressed microglia action by enhancing autophagy. Meanwhile, our results indicate that exercise training stimulated autophagy, contributing to the M2 polarization of microglia. However, the molecular mechanism underlying microglial autophagy in NP remains unclear. We speculate that exercise training inhibited the activation of microglia through BDNF-mediated autophagy

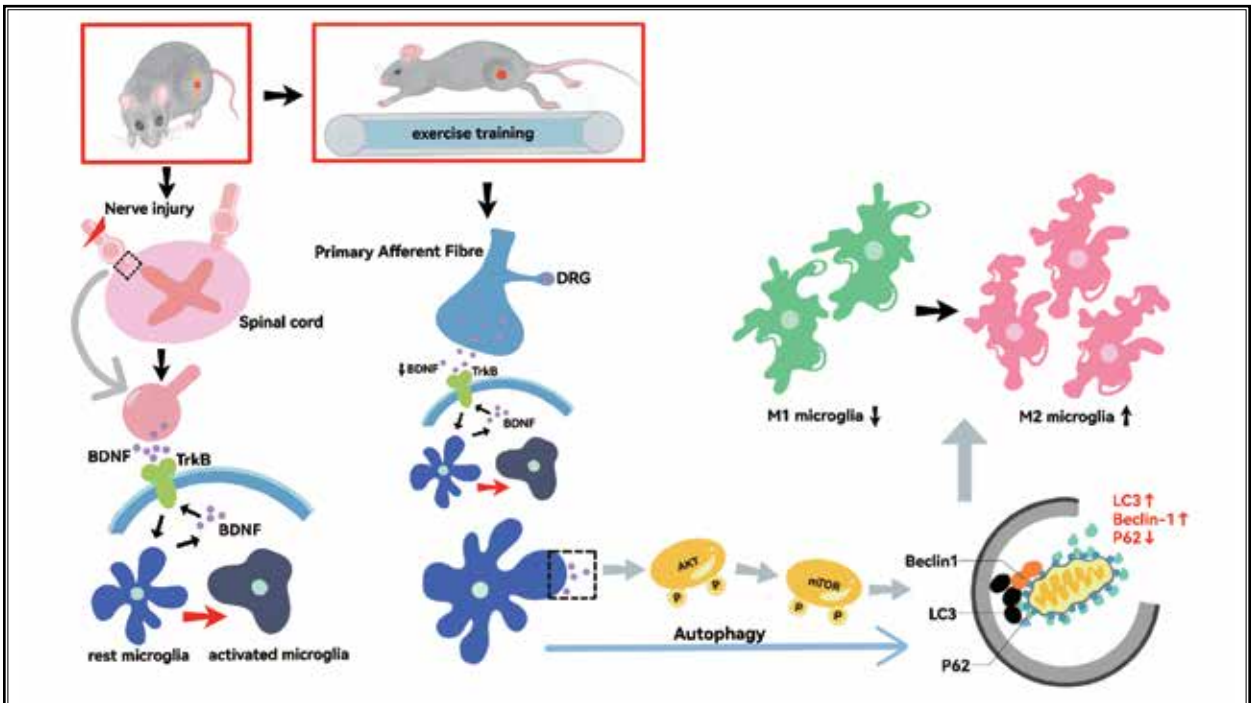

Fig. 8. *A simplified summary of the activation of autophagic flux through the effect of exercise on the BDNF/AKT/mTOR pathway in microglia. Exercise inhibits the expression of BDNF in the spinal horns, which can promote autophagy flux and microglia transition to the M2 phenotype, thereby further reducing the hyperalgesia caused by NP.*

activation, thus leading to reduced hyperalgesia. Nevertheless, the specific molecular mechanism remains to be explored.

Next, we studied the effect of autophagy flux on phenotypic changes in spinal microglia. Our results reveal that AKT triggers autophagy after BDNF-induced microglia activation. To verify that the BDNF-AKT-mTOR pathway is involved in the regulatory effect of exercise training on autophagy in vivo, we used drug intervention. Group SNB showed increased expression of p-Akt/Akt and p-mTOR/mTOR, showing that intrathecal injection of BDNF upregulated the AKT/mTOR pathway, thus suppressing autophagy. Taken together, these results show that autophagy triggered by AKT/mTOR and BDNF may be a downstream target of exercise training in regulating hypersensitivity to pain. The promotion of microglia autophagy plays an essential role in regulating inflammation and BDNF (Fig. 8).

Nevertheless, our study mainly focused on the relationship between treadmill exercise and neuropathic pain-related central responses. The effect of exercise on autophagy and pain cannot be assessed in an in vitro model. Further studies are needed to provide direct evidence for a causal relationship between exercise-induced neuropathic pain relief and cellular mechanisms. Our study also did not investigate whether intrathecal injection of BDNF induces metabolic changes in other neuronal cells that affect pain perception. Further research is needed on how exercise training modulates microglial autophagy to alleviate NP. At the same time, the number of animals used was small, and there were no clinical data or samples to validate our conclusions. We plan to carry out future studies to overcome these limitations.

In conclusion, the present study reveals, for the first time, that the inhibition of BDNF expression promoted autophagy flux and microglia polarization to the M2 phenotype by inhibiting AKT/mTOR signaling. Moreover, the activation of microglial autophagy stimulates M2 polarization, thus, attenuating BDNF-induced neurotoxicity in microglia; in contrast, the inhibition of microglial autophagy has the opposite effect.

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Author Contributions

J.B. and B.G. conceived and designed the study. X.W. and S.W. performed the experiment. Q.Y. collected the data and performed the statistical analyses. Y.T. revised the statistical analyses and figures. J.B. wrote the manuscript.

Ethics Approval

All animal protocols were approved by the Animal Ethics Committee of the Second Hospital of Lanzhou University (D2019-003).

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