Voluntary Exercise Ameliorates Neuropathic Pain by Suppressing Calcitonin Gene-Related Peptide and Ionized Calcium-Binding Adapter Molecule 1 Overexpression in the Lumbar Dorsal Horns in Response to Injury to the Cervical Spinal Cord

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Research

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Abstract

Background

Neuropathic pain (NP) is a frequent finding in patients diagnosed with spinal cord injuries (SCIs). We aimed to investigate the effects of voluntary exercise on NP after SCI and to elucidate its potential mechanisms.

Methods

A rat model of post-SCI NP induced by compression of the posterior or lateral cervical spinal cord was used to evaluate the effects of voluntary exercise by measuring the bilateral withdrawal of the hind paws using the Von Frey filament and Hargreaves tests. The place escape/avoid paradigm was used to evaluate supraspinal pain processing and somatosensory evoked potentials (SEPs) were used to examine disturbances in proprioception. Locomotor function was evaluated using Basso, Beattie, and Bresnahan (BBB) scoring. Pathologic findings in hematoxylin and eosin-stained tissue and magnetic resonance imaging were used to evaluate the morphological changes after SCI. The lesion size within the cervical spinal cord was evaluated by staining with Eriochrome cyanine R. Quantitative polymerase chain reaction and immunohistochemistry were used to assess the expression of calcitonin gene-related peptide (CGRP) and ionized calcium-binding adapter molecule 1 (Iba-1) in the lumbar dorsal horns.

Results

All injured rats developed mechanical hypersensitivity, hyposensitivity, and thermal hyperalgesia in the contralateral hind paws at one week post-injury. Rats that underwent lateral compression injury developed NP in the ipsilateral hind paws one week later than rats with a posterior compression injury. Our findings revealed that voluntary exercise ameliorated mechanical allodynia and thermal hyperalgesia, and significantly improved proprioception as measured by SEP, but had no impact on mechanical hypoalgesia or motor recovery and provided no significant neuroprotection after recovery from an acute SCI. SCI-induced NP was accompanied by increased expression of CGRP and Iba-1 in the lumbar dorsal horn. These responses were reduced in rats that underwent voluntary exercise.

Conclusions

Voluntary exercise ameliorates NP that develops in rats after compression injury. Increased expression of CGRP and Iba-1 in the lumbar dorsal horns of rats exhibiting symptoms of NP suggests that microglial activation might play a crucial role in its development. Collectively, voluntary exercise may be a promising therapeutic modality to treat NP that develops clinically in response to SCI.

Introduction

Spinal cord injury (SCI) is a devastating neurological event that is characterized by motor, sensory, and autonomic impairment of varying severity. Neuropathic pain (NP) is one of the most common

complications that develop in patients diagnosed with SCI. NP usually does not respond to pharmacological treatment because of the complex mechanisms involved. Therefore, it will be necessary to identify alternative options that can be used to prevent the development and/or treat the consequences of NP.

Bed rest and prolonged immobilization can result in abnormal sensory responses, for example, allodynia, in both animals and humans, even in the absence of apparent neurological trauma [1][2]. Physical activity is a non-invasive and clinically useful treatment that can promote the recovery of neurological function after an acute SCI. Exercise (swimming, treadmill training, and forced walking on an exercise wheel) initiated during the early or later phases post-injury can prevent or alleviate the development of allodynia in experimental animal models of SCI [3][4][5]. Voluntary exercise also plays a positive role in alleviating NP in these models [6][7]. However, the mechanisms underlying the impact of voluntary exercise and its capacity to ameliorate or relieve NP remain to be defined.

Results from various NP models point to abnormal changes in peripheral, spinal, subcortical, and cortical structures as among the factors associated with the development of NP. For example, results from several previous studies suggested that the loss of GABAergic interneurons, down-regulation of potassium-chloride transporter member 5, overexpression of glutamate, and activation of the ionotropic N-methyl-D-aspartate (NMDA) receptor in the spinal cord and brain are associated with the development of NP. Neuroinflammation, including activation of astrocytes and microglia, may also contribute to NP. Furthermore, SCI may not only lead to anatomical disruption and microenvironmental changes in the injured area, these injuries may also elicit NP via their impact on the structure and function of spinal cord segments below the lesion[8].

Previously, our group found that increased calbindin-D 28K staining density in L4-L6 spinal cord laminae I and II was associated with SCI-induced NP. Given that the dorsal horn functions as the integration center that links afferent information with sensory perception, information focused on irregular termination, distribution, and/or connection patterns of sensory fibers within this structure may be of significant importance toward our understanding of NP. Primary nociceptive afferent fibers exhibit dramatic maladaptive arborization into the deeper laminae of the lumbar dorsal horn after SCI that may be connected with the development of NP [9]. Likewise, calcitonin gene-related peptide (CGRP) is widely distributed within nociceptive pathways in the nervous system and may contribute to pain transmission and inflammation similar to our current understanding of its role in promoting migraine pain [10].

The relationship between physical exercise, maladaptive changes in the lumbar spinal cord, and the perception of pain has not been investigated extensively in the setting of compressive SCIs. In this study, we will evaluate the behavior of rats with posterior or lateral cervical spinal cord compression injuries. We will then test the effects of voluntary exercise on pain-associated behaviors and evaluate pain-related maladaptive changes in the distal lumbar spinal cord. Our findings reveal that voluntary exercise has a positive effect on SCI-induced mechanical allodynia and thermal hypersensitivity and that pain relief is

accompanied by reductions in CGRP and ionized calcium-binding adapter molecule 1 (Iba-1) expression in the lumbar dorsal horn.

Methods

1. Animal subjects

All experimental procedures were approved by the Research Ethics Committee of Sun Yat-sen University, Guangzhou, China, and conformed to all relevant regulatory standards. The ethic number is [2020]015. Eighty-six female Sprague-Dawley rats (initial weights, 250–300 g, average weight 287 g from the Laboratory Animal Center of Sun Yat-sen University, Guangzhou, China) were used to carry out the experiments. Rats were provided with access to food and water *ad libitum* and housed in groups of four per cage on a 12/12- hour light-dark cycle. Two rats were sacrificed before completion of the experimental protocol because of an anesthesia accident. The remaining 84 rats were divided into six groups: (1) no compression (sham, n = 12), (2) posterior spinal cord compression injury (P-SCI, n = 15), (3) lateral spinal cord compression injury (L-SCI, n = 15) groups, (4) sham with voluntary exercise (sham + V, n = 12), (5) P-SCI with voluntary exercise (P-SCI + V, n = 15), and (6) L-SCI with voluntary exercise (L-SCI + V, n = 15). Individual rats were assigned to voluntary exercise or no voluntary exercise groups based on paw withdrawal responses to the 2g von Frey hair filament at 1 week post-injury (1wpi) in order to create matched groups of exercised and not exercised animals. All rats were sacrificed 4 weeks after surgery.

2. Spinal cord injury

Rats in the sham, P-SCI, and L-SCI groups were anesthetized with a cocktail (3 mg/kg) containing ketamine (31.25 mg/kg), xylazine (1.58 mg/kg), and acepromazine (0.31 mg/kg) (Guangzhou FISCLAB Environ. Sci-Tech. Co., Ltd., Guangzhou, China). Briefly, following exposure of the C4–C6 spinal process and laminae via a posterior incision, a C5 laminectomy was performed to gain access to the epidural space. A sheet (1 × 3 × 5 mm^3) of water-absorbable polyurethane polymer was implanted into the C6 epidural space in the dorsal region of the spinal cord of the P-SCI group. For the L-SCI group, the sheet was implanted into the C6 epidural space in the lateral region of the spinal cord. For the sham group, only C5 laminectomy was performed. After surgery, all rats received a subcutaneous (s.c.) injection of Ringer's solution (200 μl) to prevent dehydration during the surgical procedure. Post-surgical care included close observation, manual bladder voiding twice per day, and treatment with antibiotics to prevent bladder infection (100 μl ampicillin via s.c. injection twice per day) for about 7 days after surgery until reflex bladder function was restored. All surgical procedures were performed by the same experienced investigator.

3. Voluntary exercise

Running wheels were placed in individual cages (Huaibei Jiubai Electronic Technology Co., Ltd. China) of the sham + V, P-SCI + V, and L-SCI + V groups at 1 wpi. Identical running wheels that were locked in place (i.e., immobile), were placed into the cages of the control groups (sham, P-SCI, and L-SCI). A counter was

connected to the wheel that recorded the frequency of running sessions during a 24 h period. The distance covered during each session was approximately 1 m. In all voluntary exercise groups, rats had free access to the running wheel freely over a 3-week training period. In the P-SCI + V and L-SCI + V groups, the average amount of exercise for 3 weeks was approximately 20,155 m between all rats and approximately 22,349 m in the sham + V group. Rats were acclimated to the running wheel for 2 days before the beginning of exercise protocol to avoid stress responses.

4. Magnetic Resonance Imaging (MRI)

A 3.0-T MR imager (Siemens Trio) was used to evaluate the location of the polyurethane polymer sheet used to generate the compression injury. Rats undergoing MRI evaluation were anesthetized with an intraperitoneal injection of 10% chloral hydrate (300 mg/kg) and placed in a prone position with a surface coil over the cervical spine regions to facilitate the acquisition of anatomical T2-weighted images (T2WIs). T2WIs were acquired with the following MRI parameters [11]: echo time/repetition time (TR) = 35/2500 ms (T2W) and 115/2500 ms (proton density-weighted [PDW]), slice thickness = 1 mm, interslice distance = 1.1 mm, and number of excitations (NEX) = 4. Fifteen axial scans were acquired from each rat that covered C3–C7 of the cervical spinal cord. Fifteen sagittal images of the cervical spinal cord (C3 to C7) were also acquired in an identical fashion.

5. Behavioral testing

Rats were acclimated to the individual testing set-up for two days for at least 1 h per day before behavioral testing and 30–60 min in each set-up on the days in which the tests were performed. All behavioral testing was performed on awake, unrestrained rats by the same investigator who was blinded to group identity.

Mechanical sensitivity determined by von Frey Filament testing

To evaluate changes in mechanical sensitivity after SCI with or without voluntary exercise, von Frey hair filaments with force calibrated to 2, 6, and 15g (Bioseb, USA) were applied to the hind paws of each rat. The filament sizes were chosen based on findings reported in previously published research on SCI in rats [12][13]. Each filament was applied five times to both the contralateral and ipsilateral hind paws. The frequency of positive responses to stimulation in the form of a percentage (i.e., the number of paw withdrawals divided by the number of applied stimuli) was used to document mechanical sensitivity. Testing with these filaments was performed on two consecutive days before surgery to obtain baseline levels of mechanical sensitivity of each rat. After surgery, mechanical allodynia, and hyposensitivity were examined at 1-week post-injury (7 dpi) as well as at 2, 3, and 4 weeks (14 dpi, 21 dpi, and 28 dpi, respectively) after the opportunity for voluntary exercise was (or was not) provided (Fig. 1).

Mechanical sensitivity using Place Escape/Avoidance Paradigm

Paw withdrawal in response to stimulation with a von Frey hair filament is a measure of stimulus-evoked behavior. Therefore, paw withdrawal in response to the different filaments might also be the result of evoked hyperreflexia. Given this possibility, supraspinal processing of nociceptive stimuli was evaluated by testing based on a place escape/avoidance paradigm (PEAP). Briefly, rats were placed in a chamber that included a dark and a light side. A removable partition that was laminated with black (facing the dark side) and white (facing the light side) adhesive foil was positioned in the middle of the chamber. The partition separated the dark from the light side and created a passage between them. The rats were free to move from the dark to the light side and vice versa. PEAP testing is based on the assumption that the rats have the active choice between the naturally preferred dark side *versus* pain relief in the more aversive light side. Each rat underwent 25 min of PEAP testing. To begin the test, rats were placed in the middle of the chamber and the time they spent on the dark side was measured. The first 10 min phase that involved no external stimuli was defined as the exploration phase (i.e., baseline). This was followed by a 15 min testing phase (3 x 5 min blocks) during which the hind paws were stimulated with a 2g von Frey filament every 15 s when a rat was present on the dark side of the chamber. But no nociceptive stimuli were administered to the rats while they remained in the light side of the chamber. The hind paws that were contralateral and ipsilateral to the spinal cord lesion were stimulated alternatively during this period. The PEAP was used to evaluate changes in mechanical sensitivity changes after injury at 4 wpi.

Thermal hypersensitivity using the Hargreaves test

The Hargreaves test (Hargreaves Apparatus; Ugo Basile) was used to examine thermal sensitivity after SCI with or without voluntary exercise. In brief, we used an infrared heat beam to stimulate the plantar surface of the hind paws of each rat five times on each side. The withdrawal latency to this heat stimulation was measured. Before testing, the baseline latencies to the heat stimulus were adjusted to approximately 13–14 s. A delay of at least 2 min between the two trials that test the same paw on the same rat, as well as a cut-off time of 15 s was set to avoid tissue damage. Similar to the von Frey testing, the baseline withdrawal latency of each rat was examined on two consecutive testing days before surgery. After surgery, thermal sensitivity was assessed at one week (7 dpi) post-injury, as well as at 2, 3, and 4 weeks (14 dpi, 21 dpi, and 28 dpi, respectively) after the opportunity for voluntary exercise was or was not provided.

Motor recovery using BBB scoring

The motor function of rats after SCI with or without voluntary exercise was assessed using Basso, Beattie, and Bresnahan (BBB) scoring. A single rat was placed randomly in an open field for at least 5 min and observed by two experienced investigators who were blinded to group identity. A maximum score of 21 points indicates normal function or complete recovery, whereas a score of 0 reflects complete paralysis of the hind limb. Lower scores (0–7) were used to document isolated joint movements with little to no hindlimb movement. Intermediate scores (8–13) reflected intervals of uncoordinated gait, while higher scores (14–21) were used to document appropriate coordination between the forelimb and the hindlimb.

6. Somatosensory evoked potentials (SEPs)

The somatosensory conductive function of the spinal cord was evaluated using an electrophysiological monitoring system (Nicolet Endeavor CR) at 4 weeks post-injury (wpi). The animals were evaluated under general anesthesia that was induced by intraperitoneal injection with 10% chloral hydrate at a dose of 300 mg/kg. The scalp and posterior neck skin of each rat was shaved and sterilized with topical iodine. The two SEP recording electrodes were inserted subcutaneously at the midline of the skull, including one at 2 mm anterior to the bregma (Fz) and another at the midpoint of the ears (Cz). Two SEP stimulation electrodes were placed on each side of the sciatic nerve. Constant current with a magnitude of 6 mA, duration of 0.02 ms, and frequency of 3.43 Hz was used to elicit SEPs. Cortical SEPs were recorded from the skull at Cz–Fz. SEP signals were filtered using a bandpass filter of 10 Hz to 250 Hz. We averaged the results from 256 SEP trials to improve the signal-to-noise ratio. A sensitivity of 20 lV/div and a time base of 5 ms/div were used to display the SEP responses.

Onset latency and peak-to-peak amplitude were measured at the endpoint of the study. The onset latency was measured as the time interval between the delivery of the stimulus to the first N wave from the baseline. Peak-to-peak amplitude is defined as the vertical distance between the P and N points. Each SEP test was repeated 3 times, and average values were recorded. We define an immeasurable SEP (i.e., below detectable limits) as a waveform that could not be identified by averaging over 500 sweeps. SEP responses recorded from each limb were classified individually. For the final analysis, the lowest SEP amplitudes detected for each of the four limbs were identified and used as the definitive data point.

7. Quantitative polymerase chain reaction (qPCR)

Briefly, rats were deeply anesthetized with pentobarbital and perfused transcardially with phosphate-buffered saline (PBS). The L4-L6 segment of the spinal cord was removed immediately thereafter. Total RNAs were isolated with RNA Isolator Total RNA Extraction Reagent (Vazyme, R401-01) according to the manufacturer's protocol and PrimeScipt RT Master Mix (Takara, RR036A) was used to synthesize cDNA. QPCR reactions (10 μL each) were prepared from 5 μl Premix Ex Taq II (Takara, RR820A), 0.5 μl primer (final concentration 10 nM), 2 μl DEPC water, and 2 μl cDNA. Reactions were run in a LightCycler 480 qPCR instrument (Roche) using the standard conditions, including 95°C for 5 min, followed by 40 cycles (95°C for 15 s, 56°C for 30 s, and 72°C for 30 s) and a melting curve. We documented relative levels of gene expression using the $2^{-\Delta\Delta Ct}$ method with data normalized to GAPDH [14]. Sequences for all RT-qPCR primers are shown in Table S1.

8. Eriochrome cyanine R (EC) staining of myelin

Eriochrome cyanine R (EC) staining was used to identify myelinated regions in the cervical spinal cord and to quantify spared tissue at the epicenter of each lesion. Spinal cord sections (25 μm thickness) were subjected to EC staining as previously described [15]. The digital images of EC-stained sections were obtained at 20× magnification using an Olympus BX53 microscope equipped with an XC camera. The percentage of the spared matter was determined by dividing the area representing the white matter area by the total cross-sectional area. Imaging and results were analyzed by the same investigator who was

blinded to the group identity of each sample using ImageJ (U. S. National Institutes of Health, Bethesda, MD, USA).

9. Hematoxylin and eosin staining and Immunohistochemistry

At the endpoint of the study, rats were euthanized with an overdose of intravenous sodium pentobarbital and perfused transcardially with 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PFA). Spinal cord segments C5-C7 and L4-L6 were collected, fixed overnight with 4% formaldehyde in PBS at 4°C, and embedded in paraffin. A series of 25-μm thick spinal cord sections were prepared for Hematoxylin and eosin (H&E) and immunohistochemical (IHC) staining. Microwave-mediated epitope retrieval was performed before staining. Sections were incubated overnight at 4◦C with rabbit anti-CGRP (1:150; Abcam, Cambridge, UK) and rabbit anti-Iba-1 (1:200) antibodies. The sections were then incubated with reagents provided in a ready-to-use DAKO ChemMate EnVisionTM kit (K500711; Dako; Agilent Technologies, Inc., Santa Clara, CA, USA) for 30 min at room temperature. Images of each section of the perilesional spinal cord were acquired at 100× or 200× magnification using an XC30 camera mounted on an Olympus microscope (Olympus Corporation, Tokyo, Japan).

The labeling densities of both CGRP and Iba-1 were quantified in the lumbar dorsal horn using ImageJ software (U. S. National Institutes of Health). Images were converted to grayscale and subjected to threshold evaluation. Labeling densities of CGRP and Iba-1 were quantified as the fraction of the area that exceeded the threshold level within the region of interest.

10. Statistical analysis

Two-way analysis of variance (ANOVA) with Fisher's least significant difference (LSD) post hoc test was used to analyze behavioral test data and to reveal overall group differences and significant changes over time. One-way ANOVA with Fisher's LSD post hoc test was used to analyze lesion size and expression levels of CGRP and Iba-1. All data are presented as mean ± standard error of the mean (SEM). Prism 7 software (GraphPad Software Inc., La Jolla, CA, USA) was used to perform statistical analysis. The threshold for significance was set at $p < 0.05$.

Results

1. Assessment of SCI-associated damage by MRI and H&E staining

MRI and H&E staining were performed to determine whether the compression protocol used in this study resulted in damage to the posterior and lateral spinal cord. MRI analysis identified the compression materials at sites that were either directly posterior or lateral to the spinal cord where they elicited P-SCI or L-SCI, respectively (Fig. 2A). H&E-stained sections of spinal cord tissue from rats in the sham group revealed normal histology. By contrast, H&E-stained spinal cord tissue from rats subjected to

compression protocols exhibited vesicular degeneration and cavitation within the compressed regions (Fig. 2B).

2. Voluntary exercise has no impact on the recovery of motor function after SCI

Motor recovery was evaluated weekly beginning at 1 wpi using BBB scoring. Rats with SCIs exhibited significant deficits in hindlimb motor function compared with rats that underwent sham procedures at all time-points evaluated in this study (Fig. 2C, two-way ANOVA, $p < 0.0001$). Voluntary exercise had no impact on motor recovery (Fig. 2C). These results are consistent with the patterns of tissue sparing observed in the spinal cord lesions of rats that did or did not participate in voluntary exercise (see below). Although the sizes of the spared epicenters of these lesions were significantly different in rats with P-SCI *versus* L-SCI, no differences in the recovery of locomotor function were observed.

3. Voluntary exercise suppresses mechanical hypersensitivity that develops in response to SCI

To determine whether rats develop NP after compressive SCI and whether voluntary exercise results in reduced pain behaviors, rats were tested for mechanical and thermal sensitivity both before the injury and weekly thereafter.

Our findings revealed that rats with compressive SCIs developed both mechanical hypersensitivity and hyposensitivity in the contralateral hind paw at 1 wpi (Fig. 3A and 3B). Rats with P-SCI developed mechanical hypersensitivity and hyposensitivity in the ipsilateral hind paw at 1 wpi, while those with L-SCI developed these responses at 2 wpi.

Stimulation of the contralateral hind paw using 2g and 4g von Frey filaments revealed that rats with P-SCI or L-SCI exhibited a significantly higher overall response rate compared to their sham-treated counterparts (two-way ANOVA, $p < 0.0001$). No differences were detected between the responses of rats with P-SCI *versus* L-SCI. However, the response rate of rats with P-SCI or L-SCI that participated in voluntary exercise was lower than that observed in rats with SCI that did not exercise from 2 through 4 wpi (Fig. 3A and 3B, $^{oooo}p < 0.0001$, $^{vvv}p < 0.001$, $^{vvvv}p < 0.0001$). ipsilateral hind paw revealed that rats with L-SCI required an additional week to reach the same response rate to 2g and 4g von Frey filaments when compared to the response rate of rats with P-SCI rats (Fig. 3D and 3E). Rats that participated in voluntary exercise after P-SCI exhibited lower levels of mechanical hypersensitivity of the ipsilateral hind paw. Voluntary exercise also suppressed the mechanical hypersensitivity that developed in rats with L-SCI.

Stimulation of the contralateral hind paw of rats with P-SCI or L-SCI using a 15g von Frey filament revealed an overall significantly lower response rate than that observed in sham-treated animals (two-way

ANOVA, $^{****}p < 0.0001$, $^{\#\#\#\#}p < 0.0001$). Voluntary exercise had no impact on the response rate to this larger diameter filament in either of these groups (Fig. 3C). Testing of the ipsilateral hind paw revealed that rats with L-SCI had a lower response rate to 15g von Frey filament compared with sham-treated animals at 2 wpi, while rats with P-SCI rats exhibited a reduced response rate after 1 wpi (Fig. 3D).

4. Mechanical hypersensitivity that develops in response to SCI is associated with aversive supraspinal responses

PEAP testing was performed at the final time point (4 wpi) to evaluate the supraspinal processing of nociception. Briefly, rats that are allowed to move freely between dark and light chambers will naturally spend more time in the dark. However, when the dark chamber is paired with painful stimuli (von Frey 2g filament), the rats may ultimately choose to spend more time on the light side. Analysis of the time spent on the dark *versus* light side revealed that rats with P-SCI and L-SCI spent less time in the dark chamber at 4 wpi (Fig. 3G). However, after three weeks of voluntary exercise, all rats with SCIs spent less time on the dark side compared with those that did not participate in voluntary exercise (Fig. 3G).

5. Voluntary exercise suppresses thermal hypersensitivity that develops in response to SCI

The Plantar Test (Hargreaves method) was used to examine the thermal sensitivity of the rat hind paws. Significant differences were detected between various groups (two-way ANOVA: $^{****}p < 0.0001$, $^{\#\#\#\#}p < 0.0001$). Rats with P-SCI and L-SCI developed significant thermal hypersensitivity at 1 wpi when compared with their baseline values and to the responses of sham-treated rats. Thermal sensitivity persisted through the end of the experiment (4 wpi). All rats with SCI exhibited reductions in the withdrawal latency at the contralateral hind paw of ~8–9 s from baseline values at 1 wpi. By contrast, the withdrawal latency decreased only by ~3–4 s in the ipsilateral hind paw of rats with L-SCI. Interestingly, voluntary exercise had a positive impact on the thermal hypersensitivity that developed in response to SCI (Fig. 4). The withdrawal latency increased by ~3–4 s in the contralateral hind paws of rats with SCI that participated in voluntary exercise compared to the sham-treated group and rats with SCI that did not participate in voluntary exercise. However, none of the response rates of rats with SCI ultimately returned to baseline levels (Fig. 4A). Voluntary exercise increased the withdrawal latency in the ipsilateral hind paw by 3–4 s in rats with P-SCI rats and suppressed the more severe thermal hypersensitivity exhibited by L-SCI rats (Fig. 4B).

6. Voluntary exercise resulted in improved somatosensory evoked potentials in rats with SCI

Somatosensory evoked potentials (SEPs) were examined in order to evaluate the functionality of ascending sensory and proprioception pathways after SCI (Fig. 5). As shown in Fig. 5A, categories of SEP responses that have been modeled in rats include N and P points, which refer to the upward (N; negative) and downward (P; positive) peaks of the far-field cortical potential. Onset latency was measured as the time delay before N and the amplitude was measured as the voltage differences between N and P. Rats with L-SCI exhibited significant decreases in amplitude and prolonged latency at the side that was ipsilateral to the compression compared to the sham-treated controls (two-way ANOVA, $^{**}p < 0.01$, $^{****}p < 0.0001$). Greater deterioration of SEPs was observed on the ipsilateral *versus* the contralateral side in response to the compression lesion in rats with L-SCI. By contrast, similar deterioration was observed on both sides of the lesion in rats with P-SCI.

Rats with L-SCI showed no significant differences in onset latency on the side that was contralateral to the lesion when compared to the responses of sham rats. Participation in voluntary exercise resulted in significant improvements in SEP amplitudes on both sides of the lesion in rats with L-SCI (two-way ANOVA, $^{\#}p < 0.05$, $^{\#\#\#\#}p < 0.0001$). After voluntary exercise, the differences between the ipsilateral and contralateral SEP amplitudes were not significant, although the SEP latency detected on the side that was ipsilateral to the lesion remained significantly longer than responses detected on the contralateral side.

Rats with P-SCI exhibited similar amplitude decreases and prolongation of onset latency on both sides of the compression lesion. Participation in voluntary exercise resulted in improvements in the amplitude but had minimal impact on SEP latency of rats with P-SCI.

7. Voluntary exercise does not result in modification of the structural changes that result from a compressive SCI

To exclude the possibility that differences in lesion size might influence the changes in pain sensitivity secondary to SCI and examine the impact of voluntary exercise on processes that promote neuroprotection, white matter sparing was evaluated in Eriochrome cyanine R (EC)-stained coronal sections of the rat spinal cord (Fig. 6A) The extent of white matter sparing was quantified at the lesion epicenter (Fig. 6B). We found no significant differences in white matter sparing at the lesion epicenters when comparing spinal cord tissue from rats with SCI (either P-SCI or L-SCI) that have or have not participated in voluntary exercise (Fig. 6B). Interestingly, spinal cord tissues from injured rats exhibit a comparatively large percentage of white matter and complete loss of gray matter at the lesion epicenters compared to sham-treated controls (one-way ANOVA, $^{***}p < 0.001$). Tissues from rats with L-SCI exhibit a smaller fraction of white matter loss at the lesion epicenter compared with tissues from rats with P-SCI (one-way ANOVA, $^{***}p < 0.001$).

8. SCI-induced increases in calcitonin gene-related peptide (CGRP) expression are suppressed by voluntary exercise

Calcitonin gene-related peptide (CGRP) is a neuropeptide that may be expressed in response to peptidergic C and Aδ innervation and that has been detected at high levels in the skin, blood, cerebrospinal fluid, and spinal cord tissue in association with a variety of chronic pain conditions. Therefore, we quantified CGRP expression bilaterally in lumbar laminae I and II by using qPCR and IHC (Fig.7).

We detected a significant increase in CGRP expression was significantly increased in laminae I and II of the lumbar spinal cord in rats with P-SCI or L-SCI. Both groups of rats with SCI exhibited significant 1.7-fold increases in CGRP expression compared to sham-treated controls (one-way ANOVA, $^{***}p < 0.001$). No significant differences were detected between rats with P-SCI *vs.* L-SCI. Interestingly, voluntary exercise resulted in significant reductions in CGRP expression in lumbar laminae I and II in rats with SCI. By contrast, no differences in CGRP expression were detected in sham *vs.* sham+V controls.

9. SCI-induced increases in microglial activation are suppressed by voluntary exercise

CGRP is a mediator of microglial activation and may promote nociceptive signaling in the dorsal horn [16]. Based on our detection of CGRP overexpression in the lumbar spinal cords of rats with P-SCI and L-SCI, we proceeded to evaluate microglial activation in the lumbar dorsal horn in experiments designed to detect and quantify expression of ionized calcium-binding adapter molecule 1 (Iba-1).

We found significantly increased expression of Iba-1 bilaterally in the lumbar spinal cord of rats with P-SCI compared to sham-treated controls (Fig. 8). Participation in voluntary exercise suppressed Iba-1 overexpression in these rats (one-way ANOVA, $^{***}p < 0.001$). By contrast, Iba-1 expression was significantly increased in the ipsilateral lumbar dorsal horn, but not in the contralateral lumbar dorsal horn of rats with L-SCI. Voluntary exercise also suppressed Iba-1 overexpression in the ipsilateral lumbar dorsal horn in rats with L-SCI. Similar to our findings for CGRP expression, we detected no statistically significant differences in Iba-1 expression in the lumbar spinal cord when comparing results from the sham vs. the sham+V groups..

Discussion

The findings presented in our study demonstrate clearly that voluntary exercise initiated during the early phase (1 wpi) of recovery from a compressive SCI results in reductions in mechanical allodynia and thermal hyperalgesia. We also found that compressive SCI-induced NP is associated with increased expression of both CGRP and Iba-1 in the lumbar dorsal horn, which are reduced in rats that participated in voluntary exercise. Thus, we conclude that voluntary exercise selectively influences disturbed mechanical and thermal sensation that develops after posterior and lateral compressive SCIs and reduces the extent of injury-induced maladaptive changes in the lumbar dorsal horn.

An understanding of how neuropathic pain arises after SCI and what might be done to it will require ongoing mechanistic studies performed in animal models [17]. Rats represent the most common and clinically relevant animal model used to study the development of NP [18]. Most of the recent studies focused on NP secondary to SCI include contusion, clip compression, or hemisection procedures. However, many patients experience pain symptoms in response to spinal cord compression in clinical settings. Spinal cord compression can result from ossification of the posterior longitudinal ligament (OPLL), cervical spondylotic myelopathy (CSM), and spinal cord tumors. Furthermore, given the structural differences between the spinal cords of rodents and those of larger animals (e.g., medial *vs.* lateral positioning of the corticospinal tract), it will be particularly important to understand the behavioral performance of animals with compressive lesions generated at different anatomical locations. Therefore, we performed an extensive characterization of rats subjected to both posterior and lateral cervical compression injuries with experiments that addressed mechanical and thermal sensitivity as well as supraspinal pain processing. Injured rats developed significant mechanical allodynia, mechanical hypoalgesia, and thermal hyperalgesia. The behavioral results from our cervical compression model are consistent with published reports of mechanical allodynia, mechanical hypoalgesia, and thermal hyperalgesia that develop in response to peripheral and central nerve injury[19][20][21]. Furthermore, we found that rats subjected to a lateral cervical compression injury rats developed mechanical allodynia, hypoalgesia, and thermal hyperalgesia in the ipsilateral hind paw after a delay of two weeks. By contrast, the contralateral hind paws of rats with both P-SCI and L-SCI developed to NP within 1 wpi. Hatashita et al. [22] reported that contralateral mechanical allodynia could be detected as soon as two days after a hemilateral spinal nerve injury. Similarly, Kang et al. [23] demonstrated that spinal cord hemisection resulted in prominent mechanical allodynia and thermal hyperalgesia at both contralateral and ipsilateral hind paws after only one day post-injury and that the administration of diluted bee venom could suppress pain-related behavior at the ipsilateral hind paw. The reasons why rats with P-SCI *vs.* L-SCI rats presented with contralateral and ipsilateral NP at different timepoints required further exploration.

Interestingly, participation in voluntary exercise ameliorated the mechanical allodynia and thermal hypersensitivity but not loss of sensory function in both contralateral and ipsilateral hind paws of rats with compressive cervical SCIs. These results suggest that loss of sensory function (i.e., mechanical hyposensitivity) that develops in response to a compressive cannot be recovered via these actions. It is not clear why voluntary exercise has a selective influence on mechanical allodynia and thermal hyperalgesia but not mechanical hypoalgesia. This observation might be explained by the fact that sensory neurons are unable to renew themselves. In a previous study that featured a clip compression SCI rat model, early initiation of intensive treadmill training attenuated the development of thermal hypersensitivity after 2 weeks and reduced mechanical allodynia after 7 weeks [24]. Clip compression models typically result in more severe neurological dysfunction than the model featured in our current study. Thus, it may take longer to detect exercise-associated reversals of NP. Similarly, findings reported by Li et al. [25] revealed that treadmill training could significantly mitigate both mechanical allodynia and thermal hyperalgesia at 1 wpi in a rat spinal cord contusion model. Furthermore, our results are consistent with those of Dugan et al. [24] who found that intensive treadmill training had no impact on

overall functional locomotor recovery. A more precise study of locomotor responses, including an analysis of gait with Catwalk, may reveal differences between the groups that could not be detected using BBB scoring. However, this is beyond the scope of our current research.

In addition to reductions in both mechanical and thermal hypersensitivity, we also found that participation in voluntary exercise significantly improved SEP at 4 wpi. These results suggested that voluntary exercise results can ameliorate SCI-induced disturbances in proprioception. This is the first study to evaluate the effects of exercise on disturbances in proprioception that developed in response to SCI in rats. Of note, Qaiser et al. [26] demonstrated that it was possible to alter proprioception in patients diagnosed with incomplete SCI using a passive training protocol that was combined with feedback. More studies will be needed to confirm and extend these observations.

To the best of our knowledge, this is the first research study that focused on the impact of voluntary exercise on NP that develops in response to posterior and lateral cervical SCIs in rats. Our findings can serve as a basis for future studies designed to explore the molecular and cellular mechanisms underlying the development and persistence of NP in patients with OPLL, CSM, or spinal tumors and the role of voluntary exercise in promoting effective rehabilitation.

Because the lumbar dorsal horn plays an important role in the development and persistence of NP that develops in response to SCI, we also evaluated maladaptive changes of the dorsal horn at this site, which is below the level of the injury. There is increasing evidence that CGRP plays a role in the development of NP [27]. Results from a mouse model of spinal cord contusion injury suggested that early treadmill training can suppress NP by reducing CGRP expression, detected in this study by its labeling density in laminae III–IV of lumbar dorsal horn [28]. In this study, we also detected increased CGRP expression in laminae I–II, but not in laminae III–IV after a cervical spinal cord compression injury. Furthermore, we found that participation in voluntary exercise suppressed injury-induced CGRP overexpression in both ipsilateral and contralateral lumbar dorsal horns. Future studies will be needed to explore whether SCI induces aberrant plasticity of the neural circuitry involving peptidergic nociceptive fibers in laminae I–II that may contribute to the development of NP [29].

CGRP has been implicated in the development of neurogenic inflammation and is upregulated in association with inflammation and neuropathic pain [30]. Activation of spinal microglia also contributes to the development of NP following peripheral and central nerve injury [31][32]. Therefore, we explored the impact of spinal cord compression and voluntary exercise on microglial activation in the lumbar dorsal horn. The primary phase of microglial activation reportedly peaks at 7 dpi. Microglia undergo reactivation after 14 dpi followed by a peak at 60 dpi that persists through 180 dpi [33]. Similar to the results presented in a previous study, Iba-1 expression was increased in the lumbar spinal cord at 4 wpi; voluntary exercise resulted in a significant decrease in Iba-1 overexpression in the lumbar dorsal horn. Qi et al. [34]demonstrated that an increase in Iba-1 activation in the dorsal horn may account for the occurrence of stress-induced hyperalgesia in a model of chronic inflammatory pain. Many studies have demonstrated that Aβ-fibers can produce and release CGRP in inflammatory states and that this response

might contribute to NP that can develop in response to nerve injury and in inflammatory pain models [35] [36]. CGRP is localized within the primary afferent terminals and neurons in laminae I and II that mainly receive signals from peptidergic nociceptive fibers. Therefore, CGRP overexpression might be the result of a phenotypic switch of Aβ-fibers to a nociceptor phenotype. The relationship between CGRP and Iba-1 expression in response to SCI requires confirmation and further study.

We were greatly intrigued by the results of the immunohistochemical studies performed with anti-Iba-1 that revealed microglial activation in both ipsilateral and contralateral lumbar dorsal horns of rats with P-SCI, but only in the contralateral dorsal horn of rats with L-SCI. These findings may explain why the pain on the ipsilateral hind paw emerged one week later compared with the contralateral hind paw. Additional studies will be needed to confirm this finding. The mechanisms underlying differential Iba-1 expression remain unclear and may be related to changes in the microenvironment that develop at levels below initial spinal cord injury. These responses and associated mechanisms are studied only rarely. Experiments performed in transgenic reporter mice will be needed to pursue this issue in our future work. Moreover, Iba-1 overexpression and thus microglial activation in the lumbar dorsal horn that develops after SCI was suppressed in response to voluntary exercise. These results are consistent with those reported in many previous studies that examined the impact of exercise and its role in suppressing microglia activation in a variety of neurological diseases. For example, Xiong et al. [37] reported that long-term treadmill exercise could improve spatial memory by regulation of microglial activation in a mouse model of Alzheimer's disease. Similarly, treadmill exercise could reduce neuronal damage via suppressing microglial activation in a mouse model of Parkinson's disease [38]. Treadmill running was found to reduce Purkinje cell apoptosis via inhibition of microglial activation and suppression of reactive astrocytes [39].

Collectively, our findings suggest that voluntary exercise suppresses mechanical allodynia, thermal hyperalgesia, and disturbances in proprioception that develop in response to SCIs in rats. Increased expression of CGRP and Iba-1 in rats with NP suggests that microglial activation might play one or more critical roles in the development of this condition. This hypothesis is further supported by our findings that revealed reduced levels of CGRP and Iba-1 expression in association with voluntary exercise-mediated suppression of NP. Thus, our research provides evidence that voluntary exercise is a therapeutic modality that might be used clinically to prevent the development of NP in patients diagnosed with SCIs.

Abbreviations

NP: Neuropathic pain; SCI: spinal cord injury; NMDA: the ionotropic N-methyl-D-aspartate; CGRP: calcitonin gene-related peptide; Iba-1: ionized calcium-binding adapter molecule 1; wpi: week post-injury; s.c.: subcutaneous; T2WIs: T2-weighted images; PEAP: place escape/avoidance paradigm; BBB: Basso, Beattie, and Bresnahan; SEPs: Somatosensory evoked potentials; qPCR: Quantitative polymerase chain reaction; PBS: phosphate-buffered saline; EC: Eriochrome cyanine R; PFA: phosphate buffer; IHC: immunohistochemical; H&E: Hematoxylin and eosin; ANOVA: analysis of variance; SEM: mean ± standard error of the mean; OPLL: the posterior longitudinal ligament; CSM: cervical spondylotic myelopathy

Declarations

Ethics approval and consent to participate

All experimental procedures were approved by the Research Ethics Committee of Sun Yat-sen University, Guangzhou, China, and conformed to all relevant regulatory standards. The ethic number is [2020]015.

Consent for publication

Not applicable.

Availability of data and materials

The data that support the findings of this study are available from corresponding author on reasonable request.

Competing interests

The authors declare they have no competing interests.

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Authors’ contributions

WLC and YW contributed to the conception and design of the study. XC, ZRY, WJH, JCC, WC, LW, XL, JWC and WWZ contributed to the experiments and analysis of data. XC, ZRY and WJH contributed to drafting the text and preparing the figures. The authors read and approved the final manuscript.

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Figures

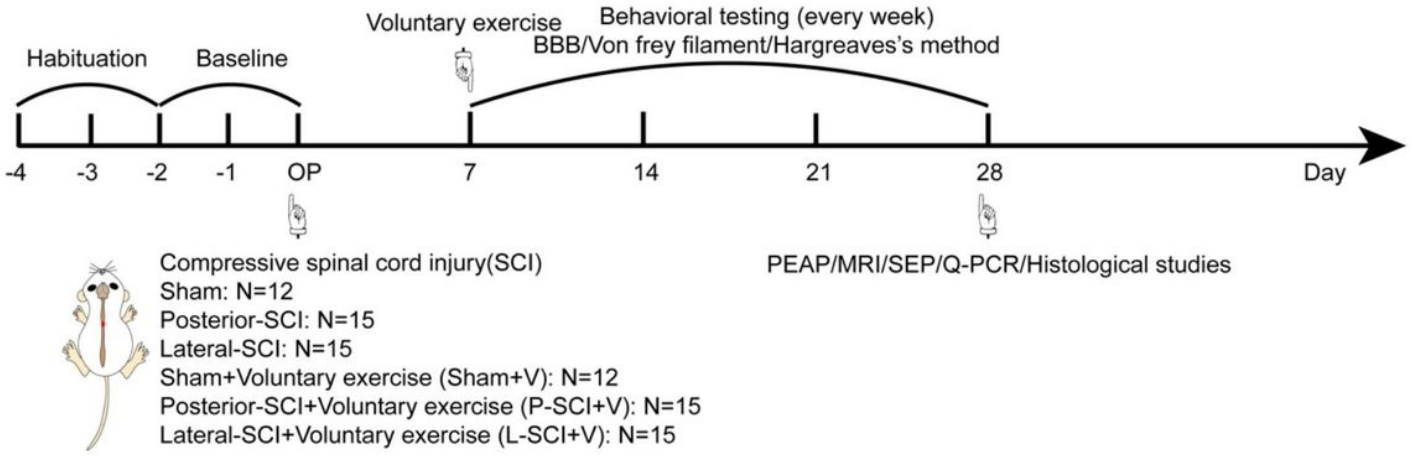

Figure 1

Experimental design.

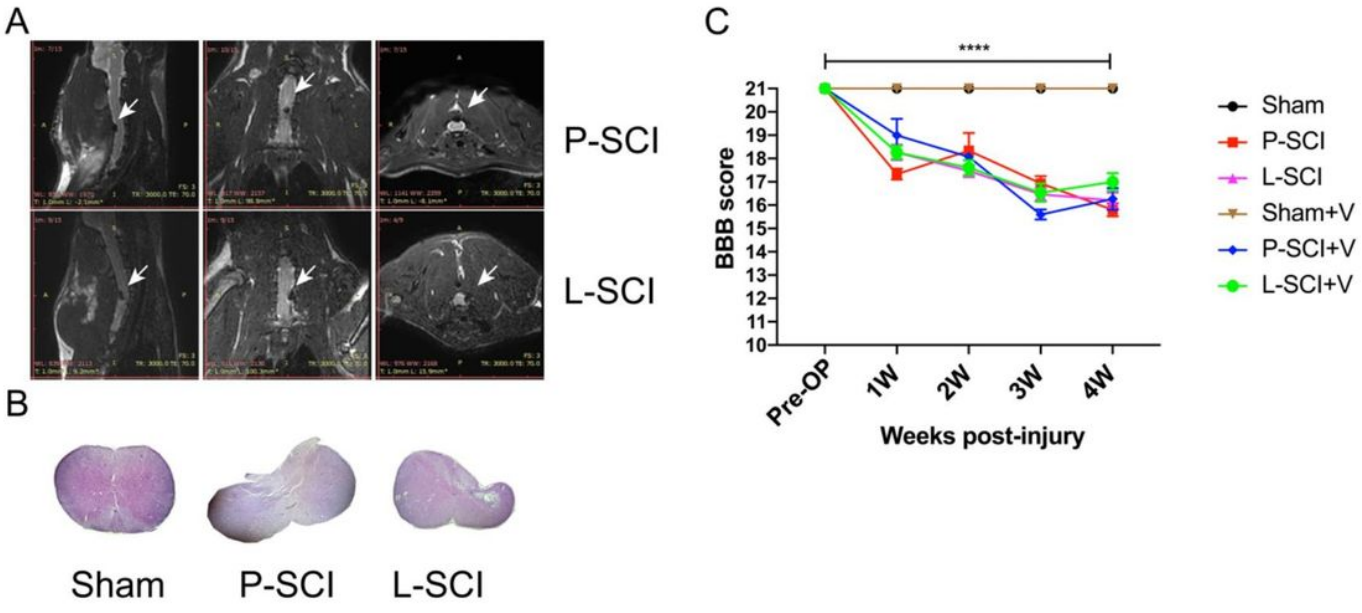

Figure 2

Voluntary exercise has no impact on motor recovery. (A) Location of the compressive material (white arrow) revealed by T2-weighted magnetic resonance imaging (T2-MRI). (B) H&E-stained spinal cord tissues exhibit vesicular degeneration in the compressed regions. (C) Rats were tested weekly beginning at 1 wpi in an open field. Motor recovery was evaluated using Basso, Beattie, and Bresnahan (BBB)

scoring. Posterior and lateral spinal cord injuries (P-SCI and L-SCI, respectively) in the rats in the voluntary exercise and no voluntary exercise groups both showed significant motor deficits compared to the two sham groups (two-way ANOVA, ****p < 0.0001) although there were no significant differences between the two groups.

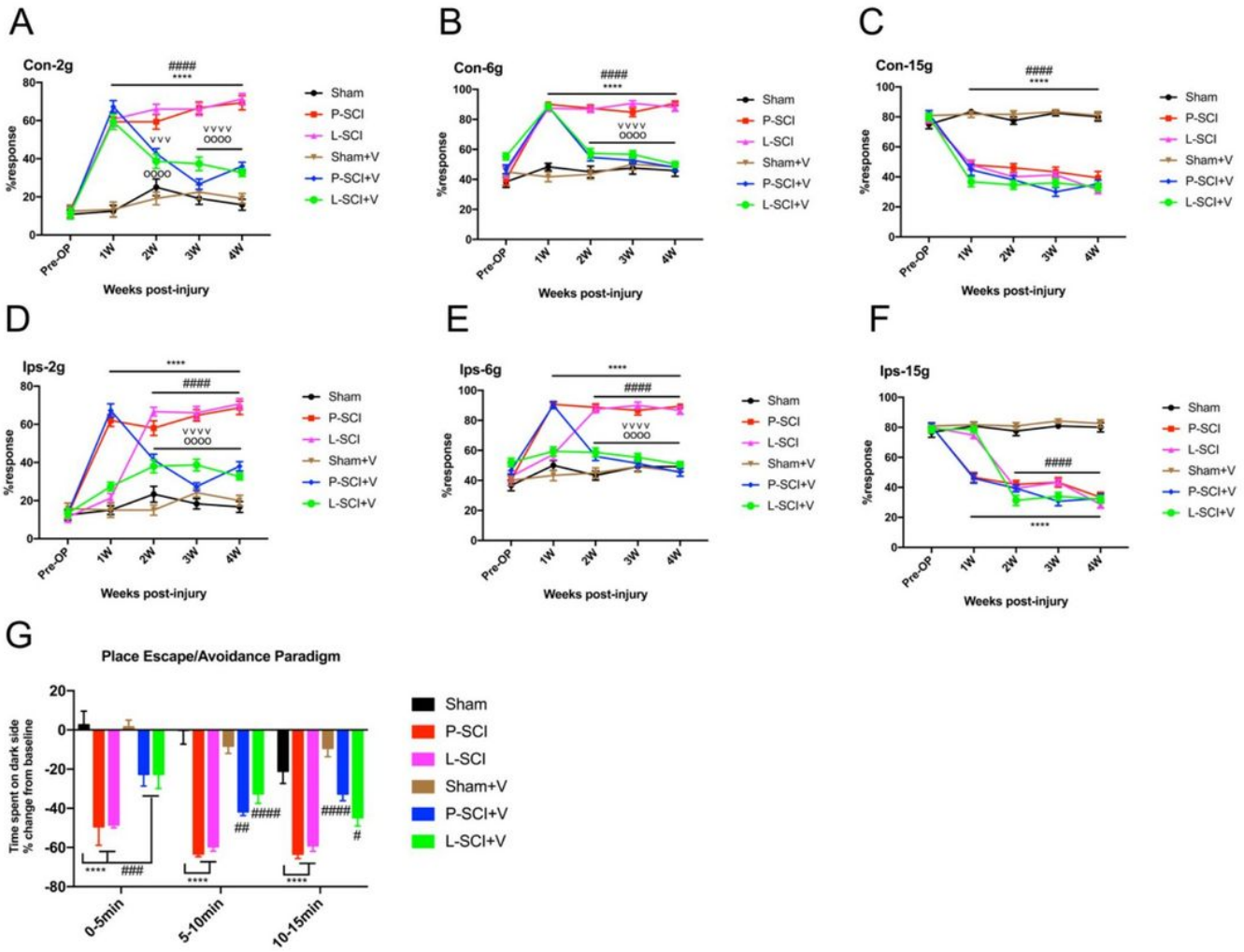

Figure 3

Effects of a compressive spinal cord injury (SCI) and voluntary exercise on mechanical sensitivity as assessed by stimulation with von Frey filaments linked to a place escape/avoidance paradigm. (A–B) L-SCI-induced mechanical hypersensitivity detected in the ipsilateral hind paws in response to light filaments (2g and 6g) developed one week later compared with the analogous responses observed in rats with P-SCI. SCI- induced mechanical hypersensitivity was suppressed in rats that participated in voluntary exercise (two-way ANOVA ####p < 0.001 for L-SCI vs. sham; ****p < 0.0001 for P-SCI vs. sham; oooop < 0.0001 for L-SCI+V vs. L-SCI; vvvvp < 0.0001 for P-SCI+V vs. P-SCI). (C) Stronger filaments (15g) revealed significant hyporesponsiveness of the ipsilateral hind paws in experiments with rats with SCI compared to those that were sham-treated regardless of voluntary exercise (two-way ANOVA, ****p < 0.0001 and ####p < 0.0001 for P-SCI and L-SCI vs. sham, respectively). (D–E) P-SCI- and L-SCI-induced mechanical

hypersensitivity detected in the contralateral hind paws in response to light filaments (2g and 6g) appears at 1 wpi. SCI-induced mechanical hypersensitivity was suppressed in rats that participated in voluntary exercise (two-way ANOVA, ####p < 0.0001 for L-SCI vs. sham; ****p < 0.0001 for P-SCI vs. sham; oooop < 0.0001 for L-SCI+V vs. L-SCI; vvvp < 0.001 and vvvvp < 0.0001 for P-SCI+V vs. P-SCI). (F) Voluntary exercise had no impact on SCI-induced mechanical hyposensitivity detected in the contralateral hind paws. (G) In the PEAP study, rats with P-SCI and L-SCI that did not participate in voluntary exercise spent significantly less time in the dark chamber with light mechanical stimuli compared with rats that were sham-treated and those with SCI that participated in voluntary exercise (P-SCI+V and L-SCI+V; two-way ANOVA, **p < 0.01 and ****p < 0.0001 for SCI vs. sham; #p < 0.05, ##p < 0.01, ###p < 0.001, and ####p < 0.0001 for all SCI+V vs. SCI; sham, n = 12; P-SCI, n = 15; L-SCI, n = 15; sham+V, n = 12; P-SCI+V, n = 15; L-SCI+V, n = 15).

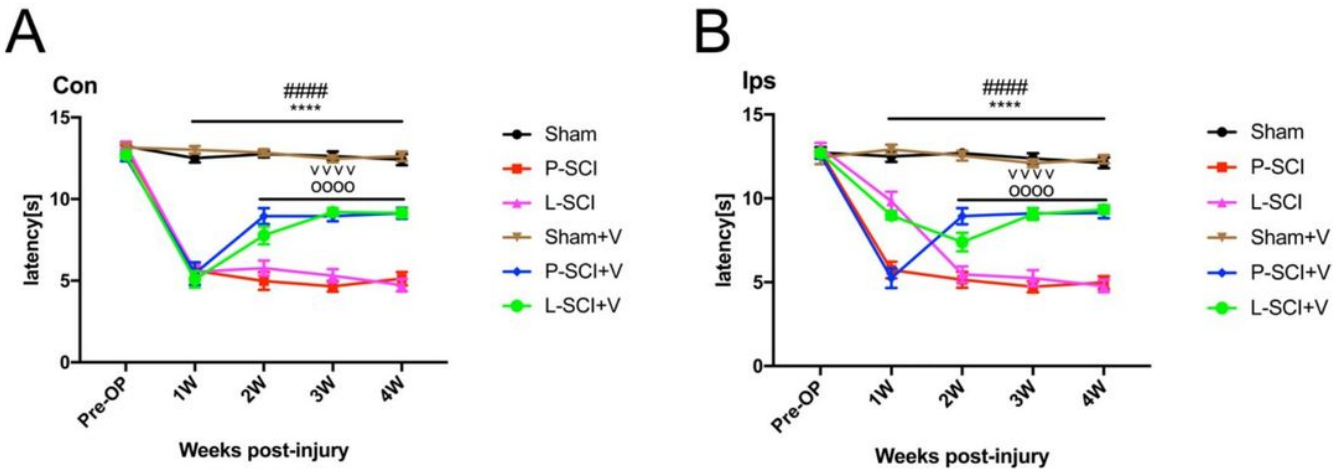

Figure 4

Voluntary exercise suppresses compressive spinal cord injury (SCI)-induced thermal hypersensitivity responses Rats with both P-SCI and L-SCI rats developed significant thermal hypersensitivity and decreased response latencies to heat stimuli in both the (A) ipsilateral and (B) contralateral hind paws that were reversed by voluntary exercise. Thermal hypersensitivity of the ipsilateral hind paws develops in response to L-SCI one week later than to P-SCI (two-way ANOVA, ****p < 0.0001 for P-SCI vs. sham; ####p < 0.001 for L-SCI vs. sham; vvvvp < 0.0001 for P-SCI+V vs. P-SCI; oooop < 0.0001 for L-SCI+V vs. L-SCI; sham, n = 12; P-SCI, n = 15; L-SCI, n = 15; sham+V, n = 12; P-SCI+V, n = 15; L-SCI+V, n = 15).

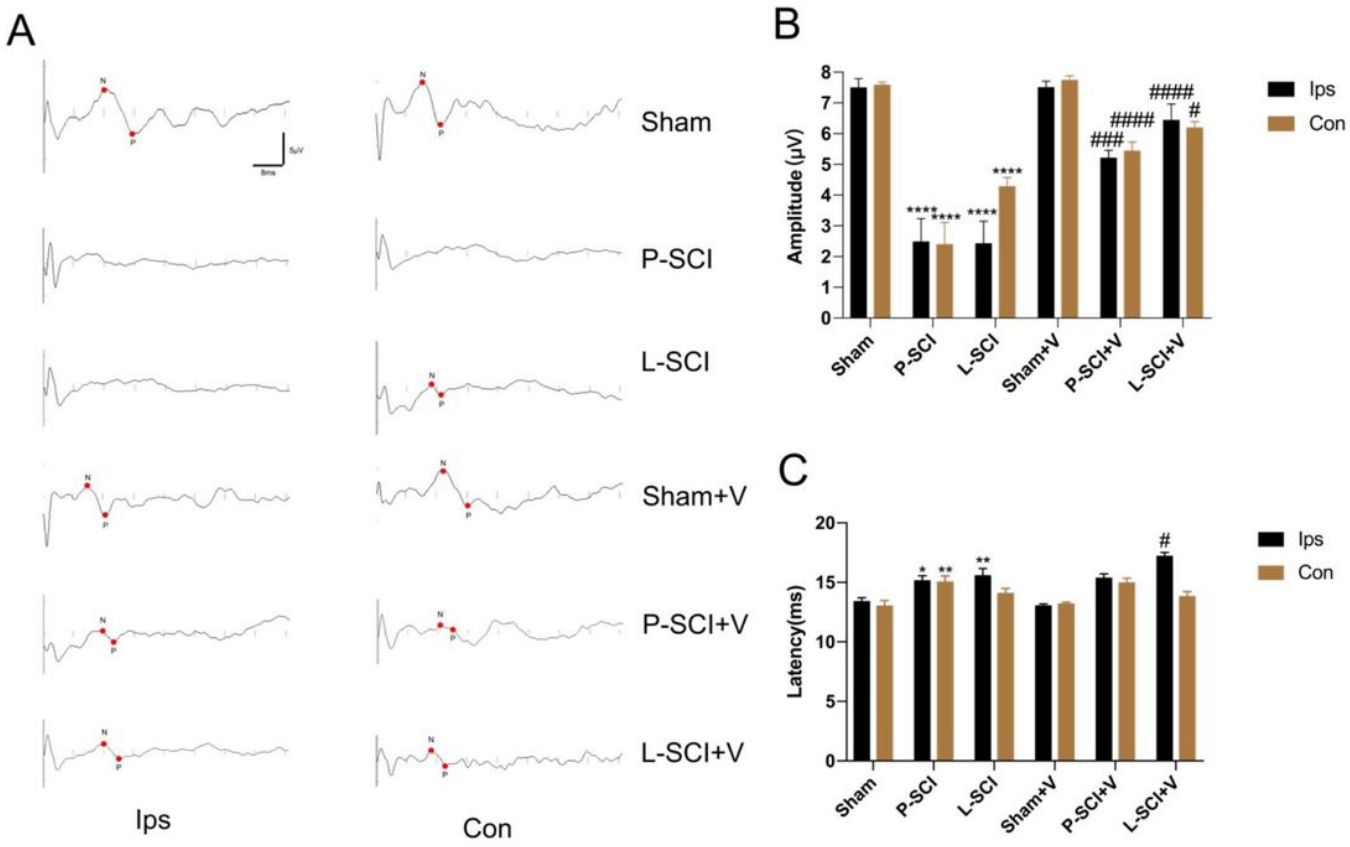

Figure 5

Participation in voluntary exercise resulted in improved SEPs after spinal cord compression injury (A) Somatosensory evoked potentials (SEPs) elicited by stimulation of the sciatic nerve at contralateral and ipsilateral sides of the lesions in all groups. (B) Quantification of peak-to-peak amplitude at both contralateral and ipsilateral sides in all groups. (C) Quantification of oneset latency at both contralateral and ipsilateral sides in all groups. (two-way ANOVA, *p < 0.05, **p < 0.01, and ****p < 0.0001 for SCI vs. sham; #p < 0.05, ###p < 0.001, and ####p < 0.0001 for SCI+V vs. and SCI; n = 8 rats per group)

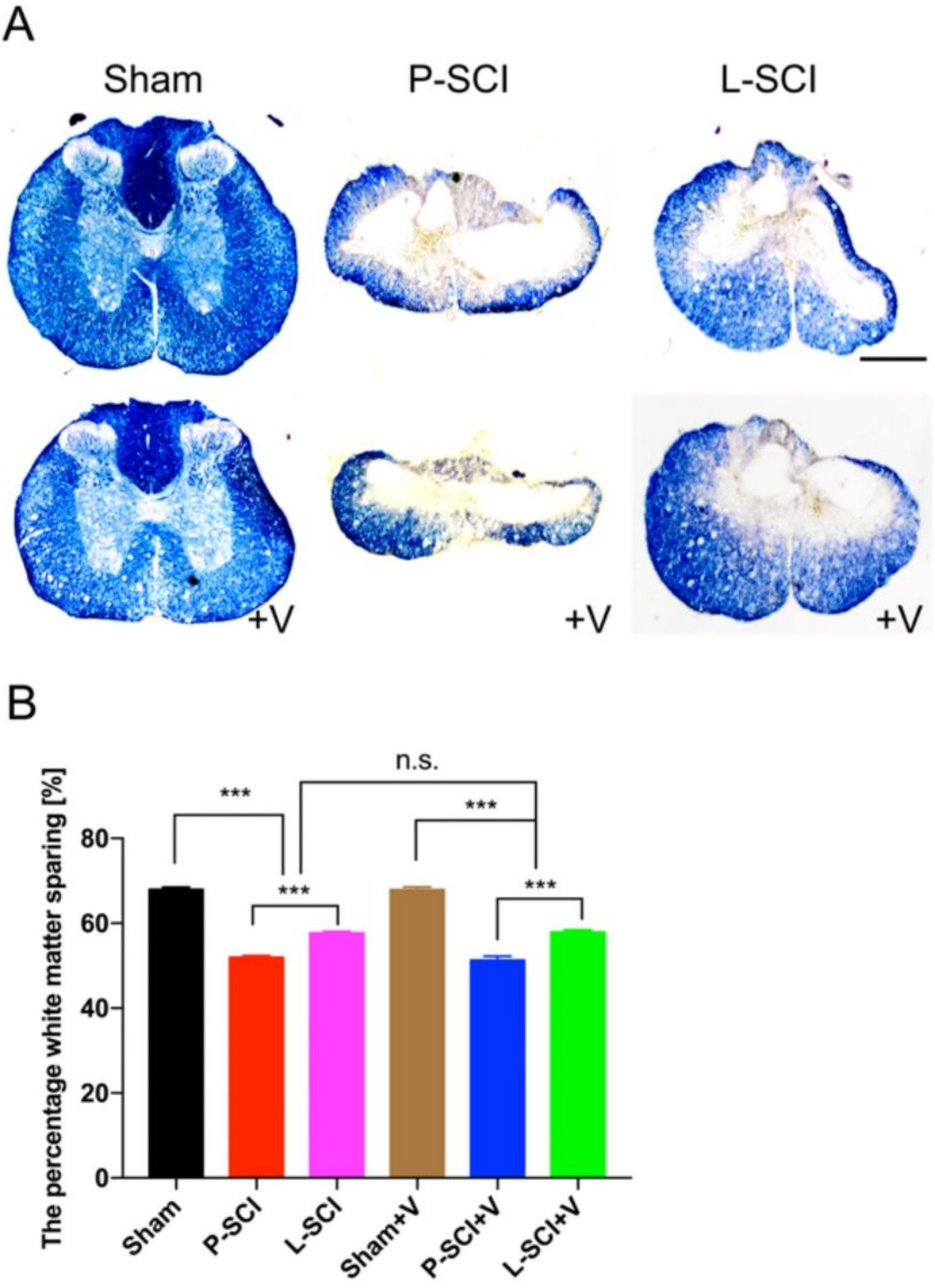

Figure 6

Voluntary exercise does not result in tissue sparing. (A) Representative Eriochrome cyanine R (EC)-stained coronal sections of spinal cord tissue from rats from each experimental group. The lesion epicenters exhibit loss of gray matter and a comparatively large percentage of white matter in rats with P-SCI and L-SCI that did or did not participate in voluntary exercise (scale bar, 500 μm). (B) Quantification of the percentage of white matter in each section reveals significantly more loss of gray matter in rats with P-

SCI rats compared to those with L-SCI (one-way ANOVA, ***p < 0.001, n.s. no significance). Participation in voluntary exercise had no impact on white matter sparing (sham, n = 12; P-SCI, n = 15; L-SCI, n = 15; sham+V, n = 12; P-SCI+V, n = 15; L-SCI+V, n = 15).

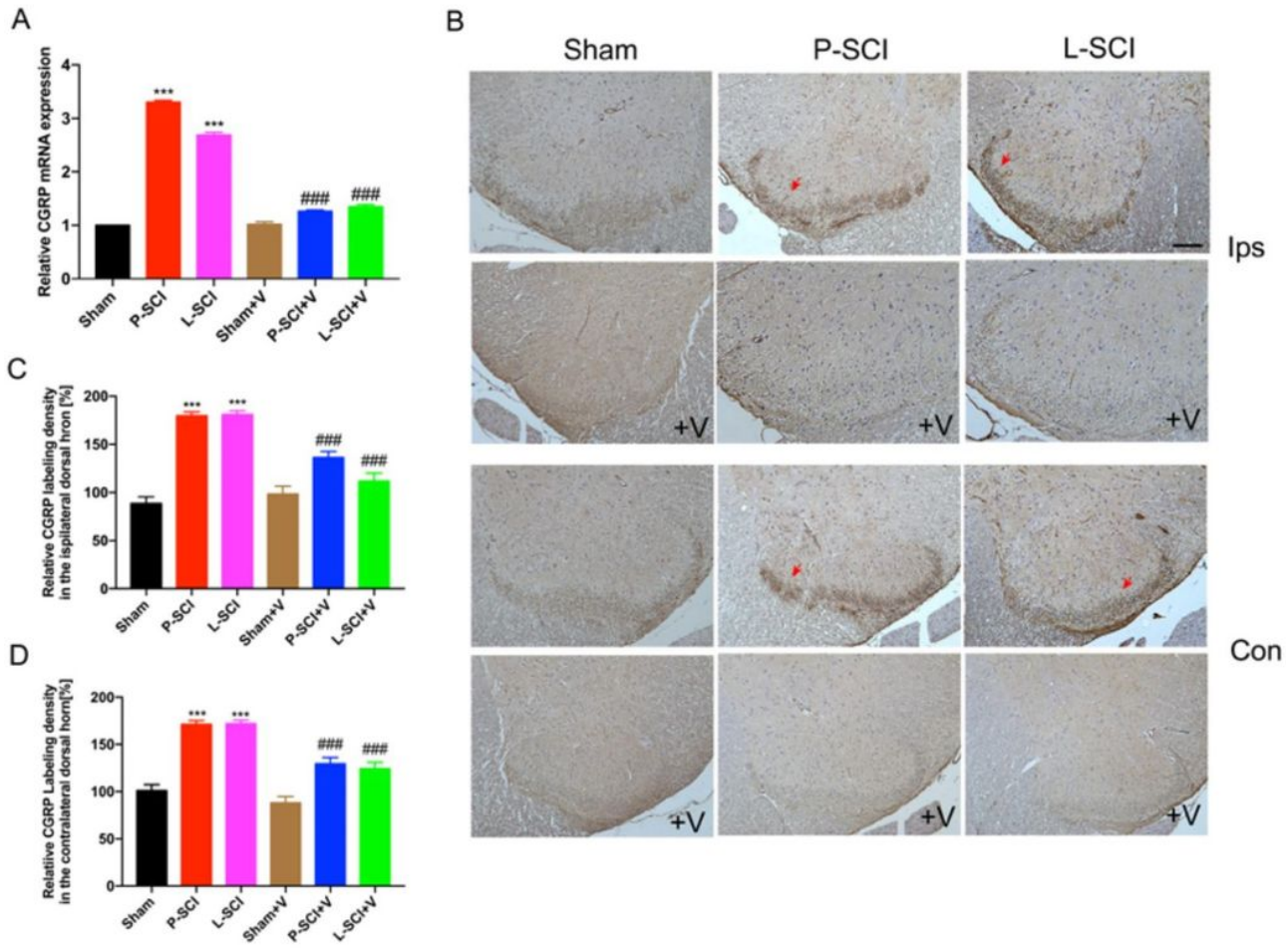

Figure 7

Compressive cervical SCI-induced expression of CGRP in the lumbar dorsal horn (L4-L6) was suppressed by voluntary exercise. (A) QPCR analysis demonstrated significantly increased levels of CGRP mRNA in the lumbar spinal cord (L4-L6) in rats with compressive SCIs. Participation in voluntary exercise suppressed the observed increase in CGRP mRNA levels at this site (n=3 rats per group). (B) Representative images documenting CGRP expression in coronal sections of the ipsilateral and contralateral L4-L6 dorsal horns of rats in each of the experimental groups (red arrow; scale bar = 100μm). (C–D) Quantification of the CGRP expression in lumbar laminae I and II of the ipsilateral and contralateral spinal dorsal horn. CGRP expression was significantly reduced in rats with SCI that participated in voluntary exercise (P-SCI+V and L-SCI+V) compared to those with SCI that did not participate in voluntary exercise (P-SCI and L-SCI; one-way ANOVA, ***p < 0.001 for SCI vs. sham; ###p < 0.001 for SCI vs. SCI+V). Voluntary exercise had no impact on CGRP expression in the spinal cords of

sham-treated rats; (sham, n = 9; P-SCI, n = 12; L-SCI, n = 12; sham+V, n = 9; P-SCI+V, n = 12; L-SCI+V, n = 12).

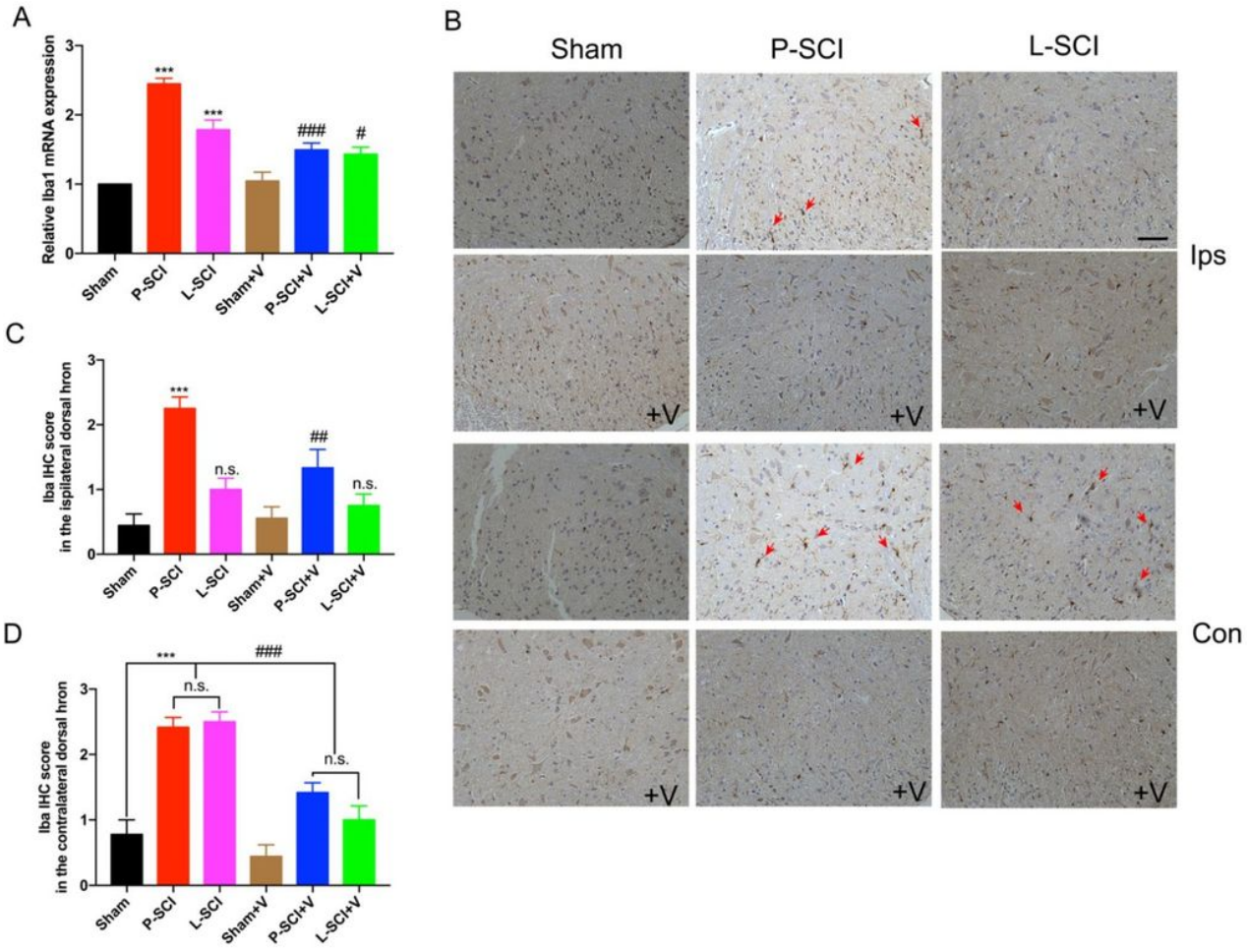

Figure 8

Compressive cervical SCI-induced expression of Iba-1 in the lumbar dorsal horn (L4-L6) was suppressed by voluntary exercise. . (A) QPCR analysis demonstrated significantly increased levels of Iba-1 mRNA level in the lumbar spinal cord (L4–L6) in rats with compressive cervical SCIs. Participation in voluntary exercise suppressed the observed increases in Iba-1 mRNA levels at this site (n=3 rats per group). (B) Representative images documenting Iba-1 expression in coronal sections of the ipsilateral and contralateral L4-L6 dorsal horns of rats in each of the experimental groups (red arrow, scale bar = 50 μm). (C–D) Quantification of the Iba-1 expression in the ipsilateral and contralateral lumbar dorsal horns. Iba-1 expression was significantly increased in the ipsilateral and contralateral spinal dorsal horn of rats with P-SCI compared to sham-treated controls; increased expression of Iba-1 was suppressed in the rats with P-SCI that participated in voluntary exercise. Increased expression of Iba-1 was detected in the contralateral, but not in the ipsilateral lumbar dorsal horn in rats with L-SCI. Participation in voluntary exercise suppressed the increased Iba-1 expression detected in the contralateral spinal dorsal horn of rats

with L-SCI (one-way ANOVA, ***p < 0.001 for SCI vs. sham; ###p < 0.001 for SCI vs. SCI+V). Participation in voluntary exercise had no impact on the levels of Iba-1 detected in sham-treated rats; (sham, n = 9; P-SCI, n = 12; L-SCI, n = 12; sham+V, n = 9; P-SCI+V, n = 12; L-SCI+V, n = 12).

Supplementary Files

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- TableS1.docx