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High-frequency spinal cord stimulation produces long-lasting analgesic effects by restoring lysosomal function and autophagic flux in the spinal dorsal horn

Zhi-Bin Wang[1], Yong-Da Liu[1], Shuo Wang[2], Ping Zhao[1, *]

Graphical Abstract *High-frequency spinal cord stimulation (HF-SCS) maintains long-lasting analgesic effect by restoring lysosomal function and autophagy flux in the spinal dorsal neurons of neuropathic pain rats*
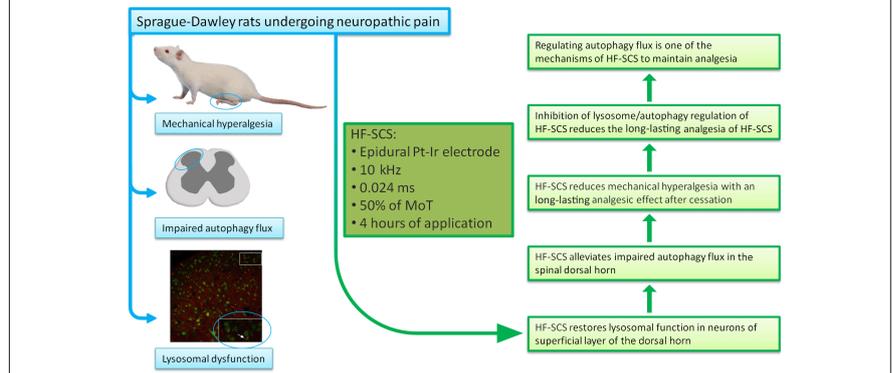
Abstract
High-frequency spinal cord stimulation (HF-SCS) has been established as an effective therapy for neuropathic pain. However, the analgesic mechanisms involved in HF-SCS remain to be clarified. In our study, adult rat neuropathic pain was induced by spinal nerve ligation. Two days after modeling, the rats were subjected to 4 hours of HF-SCS (motor threshold 50%, frequency 10,000 Hz, and pulse width 0.024 ms) in the dorsal horn of the spinal cord. The results revealed that the tactile allodynia of spinal nerve-injured rats was markedly alleviated by HF-SCS, and the effects were sustained for 3 hours after the stimulation had ceased. HF-SCS restored lysosomal function, increased the levels of lysosome-associated membrane protein 2 (LAMP2) and the mature form of cathepsin D (matu-CTSD), and alleviated the abnormally elevated levels of microtubule-associated protein 1A/B-light chain 3 (LC3)-II and sequestosome 1 (P62) in spinal nerve-injured rats. HF-SCS also mostly restored the immunoreactivity of LAMP2, which was localized in neurons in the superficial layers of the spinal dorsal horn in spinal nerve-injured rats. In addition, intraperitoneal administration of 15 mg/kg chloroquine for 60 minutes reversed the expression of the aforementioned proteins and shortened the timing of the analgesic effects of HF-SCS. These findings suggest that HF-SCS may exhibit long-lasting analgesic effects on neuropathic pain in rats through improving lysosomal dysfunction and alleviating autophagic flux. This study was approved by the Laboratory Animal Ethics Committee of China Medical University, Shenyang, China (approval No. 2017PS196K) on March 1, 2017.

Key Words: autolysosome; dorsal horn of spinal cord; dysfunction; electrical stimulation; high-frequency spinal cord stimulation; neuropathic pain; spinal nerve ligation

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Introduction

Neuropathic pain is caused by disease or injury of the nervous system. It is considered one of the most refractory pain syndromes, and it results in an enormously decreased quality of life and places an economic burden on individuals and society (Colloca et al., 2017; Liang et al., 2021). Because conventional pharmacological treatments are only moderately efficacious and have problematic side effects (Finnerup et al., 2005; Attal et al., 2010), spinal cord stimulation (SCS) has been considered an indispensable neuromodulatory treatment in these refractory conditions for decades (Papuć and Rejdak, 2013). Recently, 10 kHz high-frequency (HF)-SCS has been demonstrated to present superior long-term efficacy in the treatment of both back and leg pain (Kapural et al., 2015, 2016). However, our understanding of the underlying mechanisms of both the immediate and long-lasting analgesic effects of HF-SCS remains relatively limited.

Autophagy is a lysosomal degradative process that can

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maintain the homeostasis of cells (Klionsky et al., 2016; Ray, 2020; Chen and Wang, 2021), facilitate nerve regeneration, and prevent pain chronification (Marinelli et al., 2014). Previous studies have reported that various abnormalities of autophagic status occur in different kinds of neuropathic pain models (Marinelli et al., 2014; Berliocchi et al., 2015; Guo et al., 2015). Moreover, the impaired autophagic flux of neuronal and glial cells affects the synthesis and release processes of neurotransmitters (e.g., glutamate or γ-aminobutyric acid) and neuroinflammatory mediators (Shi et al., 2013). This further constitutes an important component of its mechanisms in ectopic activity and peripheral and central sensitization in neuropathic pain conditions (Coull et al., 2005; Ji et al., 2006; Sun et al., 2007). Recent animal studies have confirmed that increasing autophagy through rapamycin, the mammalian target of rapamycin pathway inhibitor, can relieve neuropathic pain in rats with spinal nerve ligation (SNL) (Obara et al., 2011). In addition, blocking autophagy can aggravate neuropathic pain (Marinelli et al., 2014) or reduce the analgesic effect of hyperbaric oxygen (Liu et al., 2017). Moreover, 50 Hz SCS was reported to affect the activity of protein kinase B (AKT), which plays an important role in the autophagy process, in the superficial dorsal horn (Wu et al., 2008). Furthermore, our preliminary experiments demonstrated that HF-SCS can upregulate the autophagy markers microtubule-associated protein 1A/B-light chain 3 (LC3) and sequestosome 1 (P62, also known as SQSTM1) in the spinal cord of SNL rats. We therefore hypothesized that HF-SCS may attenuate hyperalgesia through increasing autophagic flux in the spinal cord of rats undergoing neuropathic pain. The aim of the study was to investigate this hypothesis.

Materials and Methods
Animals
All experimental procedures and protocols were approved by The Laboratory Animal Ethics Committee of China Medical University, Shenyang, China (approval No. 2017PS196K) on March 1, 2017, in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals to ensure minimal animal use and discomfort. One hundred and twenty-six healthy male Sprague-Dawley rats (4–5 weeks old, weighing 180–200 g at the beginning of the experiment) were provided by the Research and Development Center of Shengjing Hospital (license No. SCXK (Jing) 2014-0004) and were housed in separate cages under specific-pathogen-free conditions, with controlled temperature (24 ± 1°C) and humidity (55 ± 10%) and a 12-hour light/dark cycle. Food and water were freely available.

Experimental protocols
Experiment 1
Experiment 1 was performed to test for differences in mechanical withdrawal thresholds (MWTs) and the autophagy markers LC3 and P62 between sham-SNL rats, SNL rats who were undergoing HF-SCS treatment, and SNL rats who were not undergoing HF-SCS treatment. The protocol was as follows: 48 rats were randomly assigned to three experimental groups (n = 16 per group), and then received electrode implantation and SNL (or sham-SNL) surgery. Rats in the SNL + HF-SCS group then received 4 hours of HF-SCS. Next, 10 of the 16 rats were randomly chosen and sacrificed for western blot analysis (n = 5 per group) or immunofluorescence staining (n = 5 per group) immediately after stimulation cessation (at 240 minutes). These rats were not included in the behavior analysis because they lacked the MWT measurements from 360–480 minutes (**Table 1**).

Experiment 2
Experiment 2 was conducted to explore the original influence of SNL surgery and HF-SCS treatment on autophagic flux in the spinal cord of rats. This was achieved by pre-applying chloroquine (CQ), which is an inhibitor of autophagosome degradation. CQ is widely used to neutralize lysosomal pH and prevent autophagosome degradation (Klionsky et al., 2016; Turksen, 2019). When animals receive an application of CQ, the additional increasing levels of LC3-II or P62 reflect the original amount of autophagosome degradation (Klionsky et al., 2012; Turksen, 2019). In the present study, 30 rats with electrode implantation were randomly assigned to six groups: sham + vehicle, sham + CQ, SNL + vehicle, SNL + CQ, SNL + HF-SCS + vehicle, and SNL + HF-SCS + CQ (n = 5 per group). The rats in each group received L5 nerve ligation (or sham ligation) and an intraperitoneal injection of either vehicle (saline, 3 mL/kg) or CQ (15 mg/kg, dissolved in 3 mL/kg saline; Sigma-Aldrich, St. Louis, MO, USA) 60 minutes before being tethered to the HF-SCS system. Only rats from the SNL + HF-SCS + vehicle and SNL + HF-SCS + CQ groups received valid electrical delivery. The ipsilateral L4–5 spinal cord was harvested immediately after the cessation of stimulation and was frozen at –80°C until its use in western blot assays (**Table 1**).

Table 1 | Number of animals, intervention and detection in each experiment

Experiment	Group	Number of rats	Electrode implantation	SNL surgery	HF-SCS treatment	Abdominal injection	Behavior tests[***]	Western blot[***]	ImmunofluoRescence[***]
1[*]	Sham	16	√	–	–	–	n=6	n=5	n=5
	SNL	16	√	√	–	–	n=6	n=5	n=5
	SNL+HF-SCS	16	√	√	√	–	n=6	n=5	n=5
2	Sham+vehivle	5	√	–	–	√ (vehivle)	–	n=5	–
	Sham+CQ	5	√	–	–	√ (CQ)	–	n=5	–
	SNL+vehivle	5	√	√	–	√ (vehivle)	–	n=5	–
	SNL+CQ	5	√	√	–	√ (CQ)	–	n=5	–
	SNL+HF-SCS+vehivle	5	√	√	√	√ (vehivle)	–	n=5	–
	SNL+HF-SCS+CQ	5	√	√	√	√ (CQ)	–	n=5	–
3[**]	Sham	/	√	–	–	–	–	n=5	√, n=5
	SNL	/	√	√	–	–	–	n=5	√, n=5
	SNL+HF-SCS	/	√	√	√	–	–	n=5	√, n=5
4	SNL+vehivle	11	√	√	–	√ (vehivle)	n=6	n=5	–
	SNL+HF-SCS+vehivle	11	√	√	√	√ (vehivle)	n=6	n=5	–
	SNL+HF-SCS+CQ	11	√	√	√	√ (CQ)	n=6	n=5	–

*Experiment 1 did not contain immunofluorescence analysis, but five rats of each group were sacrificed and the spinal cord were collected for further immunofluorescence staining. **Experiment 3 did not sacrifice additional rats, and the samples were collected from the animals in Experiment 1. ***The number of rats in each group was listed. CQ: Chloroquine; HF-SCS: high-frequency spinal cord stimulation; SNL: spinal nerve ligation.

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Experiment 3

Because normal lysosomal function is a key requirement for autophagosome degradation (Eskelinen, 2006; Coutinho et al., 2015), we investigated the differences in lysosomal function between the sham, SNL, and SNL + HF-SCS groups. No additional rats were sacrificed for this experiment, and the samples were collected from the animals described in Experiment 1. To evaluate lysosomal number and activity, which are crucial for lysosomal function, western blot analysis (n = 5 for each group) was performed for lysosome-associated membrane protein-2 (LAMP2) and cathepsin D (CTSD). We also assessed the colocalization of the lysosomal marker LAMP2 and the neuronal marker NeuN using confocal immunofluorescence microscopy (n = 5 per group) (**Table 1**).

Experiment 4

Experiment 4 was conducted to explore the relationship between the analgesic effects of HF-SCS treatment and the regulation of lysosomes/autophagy that was induced by HF-SCS treatment in the spinal cord of SNL rats. The MWT test was used for this analysis. We first used CQ to block the degradation of autophagosomes in SNL rats while they were receiving HF-SCS treatment, to observe whether a restoring effect of lysosomal function (or autophagic flux) participates in the analgesic mechanisms of HF-SCS. Thirty-three rats were randomly divided into three groups, as follows. First, to reveal whether the inhibition of autophagic flux affects the analgesic effects of HF-SCS treatment, we blocked autophagic flux in the spinal cord of SNL rats (SNL + HF-SCS + CQ group, n = 11), by abdominally injecting CQ (15 mg/kg, dissolved in 3 mL/kg saline) 60 minutes before HF-SCS treatment started. A vehicle (saline, 3 mL/kg) injection pretreatment was applied to the other two groups, in which rats received SNL (SNL + vehicle group, n = 11) or SNL and HF-SCS treatment (SNL + HF-SCS + vehicle group, n = 11). To confirm whether CQ exerted the appropriate effects, 5 of the 11 rats from each three groups were randomly chosen and sacrificed for western blot analysis when the HF-SCS (or sham-HF-SCS) treatment was stopped. These rats were excluded from the behavior analysis because they lacked the MWT measurements from t = 360–480 minutes (**Table 1**).

Electrode implantation

Briefly, the animals were anesthetized with pentobarbital (intraperitoneal, 40 mg/kg; Sigma-Aldrich), a small laminectomy was performed at the T13 lamina, and the sterilized micro-cathode, a rectangular platinum–iridium plate (dimensions 2.8 mm × 1 mm, thickness 0.1 mm), was inserted epidurally in the rostral direction. The insulated silver wire of the micro-cathode was secured to the spinous process of T12 to prevent movement of the lead. The anode, a circular platinum-iridium plate (diameter 6 mm, thickness 0.1 mm), was implanted and fixed in a subcutaneous pocket in the left chest wall. Both proximal ends of the electrodes were tunneled subcutaneously and connected to micro-plugs outside the neck of the animal for their later connection to an external neurostimulator. The surgical process was consistent with that of previous studies (Truin et al., 2011; Shechter et al., 2013). The electrodes and wires were manufactured by the Institute of Metal Research of the Chinese Academy of Sciences (Shenyang, China).

SNL

Rats that underwent electrode implantation were allowed to recuperate for 48 hours. If no motor dysfunction or mechanical hypersensitivity presented, they then received SNL or sham-SNL surgery. The SNL model was induced by performing a standard surgical procedure, as we have described previously (Liu et al., 2017). Under pentobarbital sodium anesthesia (intraperitoneal, 40 mg/kg), a midline incision was made in the skin at the L2–S2 levels, and the left paraspinal muscles (L3–S1 levels) were then bluntly dissected from the spinal processes. The left transverse process of the L6 vertebra was carefully removed to expose the left L5 spinal nerve. The left L5 spinal nerve was then tightly ligated with a 6-0 silk suture and transected 1 mm distal to the ligation. Complete hemostasis was confirmed, and the wound was then sutured in layers. In the sham-SNL groups, the SNL surgical procedure was identical except that the L5 nerve was left intact. Animals were then allowed to recuperate for 2 days before undergoing HF-SCS treatment.

HF-SCS treatment

Each rat was placed in an observation cage on a metal mesh floor that was surrounded by a transparent plexiglass chamber, and was allowed to move freely for at least 30 minutes to acclimatize. The electrode microcontacts were connected to a kilohertz-frequency stimulator (Model 2100, A-M Systems, Sequim, WA, USA) until the final behavior test was finished. When the exploratory activity had ceased, the motor threshold (frequency 2 Hz, pulse width 0.2 ms) was assessed in each rat before the HF-SCS treatment. Only the rats that were treated with HF-SCS then received 4 hours of SCS (50% of the animal's own motor threshold, frequency 10,000 Hz). The pulse width was set as 0.024 ms and monophasic pulses were used so that the results were comparable with earlier animal studies and the clinical application of HF-SCS (Shechter et al., 2013; Tiede et al., 2013; Song et al., 2014; Vallejo et al., 2017). Rats in the sham-SNL or SNL groups were only connected to the system; they did not receive HF-SCS treatment.

Behavioral test

Mechanical allodynia was assessed as a behavioral measure of neuropathic pain (Shechter et al., 2013) by measuring MWTs using von Frey filaments (0.16, 0.4, 0.6, 0.8, 1.0, 1.4, 2.0, 4.0, 6.0, 8.0, 10.0, and 15.0 g; Stoelting Company, Wood Dale, IL, USA). The cutoff value was assigned as 15 g, as we have previously described (Liu et al., 2017). Behavior assessments were performed before electrode implantation (baseline), on the day of SNL (before surgery), before SCS, and every hour for 8 hours after the initiation of HF-SCS treatment (0, 60, 120, 180, 240, 300, 360, 420, and 480 minutes).

Western blot and immunofluorescence

The ipsilateral spinal dorsal horn (L4–5) was harvested for the determination of LC3, P62, LAMP2, CTSD, and glyceraldehyde-3-phosphate dehydrogenase expression by standard western blot analysis, as we have previously described (Liu et al., 2017). The images were visualized using an electrochemiluminescence western blotting detection kit (NC15079; Thermo Fisher Scientific, Waltham, MA, USA) and acquired on a C-300 system (Azure Biosystems, Dublin, CA, USA). Sections of the spinal dorsal horn (L4–5) were used for standard immunofluorescence analysis. The immunofluorescence images were taken under a Nikon C1Si confocal microscope, and the number of NeuN$^+$/LAMP2$^+$ cells was counted by Nikon NIS-Elements AR Analysis 4.50.00 software, as described previously (Liu et al., 2017). The western blot membranes or tissue sections were incubated with primary antibodies overnight at 4°C, and were then incubated with secondary antibodies for 2 hours at room temperature. The following antibodies were used: rabbit anti-LC3B antibody (1:2000, Cat# 48394, Abcam, Cambridge, MA, USA), rabbit anti-P62/SQSTM1 antibody (1:2000, Cat# P0067, Sigma-Aldrich), rabbit anti-CTSD antibody (1:2000, Cat# 75852, Abcam), rabbit anti-LAMP2 antibody (1:1000 for western blot, 1:50 for immunofluorescence, Cat# L0668, Sigma-Aldrich), anti-glyceraldehyde-3-phosphate dehydrogenase antibody (1:1000, Cat# 97166, Cell Signaling Technology, Danvers, MA, USA), mouse anti–NeuN antibody (1:200, Cat# 104224, Abcam), Alexa Fluor 594-conjugated goat anti-rabbit IgG

(1:200, Cat# 150080, Abcam), and Alexa Fluor 488-conjugated goat anti-mouse IgG (1:200, Cat# 150113, Abcam). In the sections stained by immunofluorescence, nuclei were visualized with 4′,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich).

Statistical analysis

All graphics and calculations were performed using GraphPad Prism version 8.0 (GraphPad Inc., La Jolla, CA, USA) and SPSS version 22.0 (IBM Corp., Armonk, NY, USA). Data were checked using Bartlett's test for equal variances and the Shapiro-Wilk test for normality. The MWT data from the behavioral test were analyzed using two-way analysis of variance (with SNL surgery or HF-SCS treatment as between-group factors and time points as repeated measure factors) followed by Tukey's *post hoc* test for comparisons between the three groups in Experiments 1 and 4. Parametric data from the western blot and immunofluorescence assays were compared between the three groups using one-way analysis of variance followed by Dunnett's multiple comparisons test in Experiments 1, 3, and 4. The data from the two groups in Experiment 2 were compared using the paired Student's *t*-test. All data are presented as the mean ± standard error of the mean (SEM). A *P*-value < 0.05 was considered statistically significant.

Results

General observations of all rats

In the present study, 111 out of 126 rats completed the experiment and were analyzed. Nine animals were excluded for at least one of the following reasons: impaired motor function, displacement of electrodes, undetectable motor threshold because of damage to the electrodes, significant reduction of body weight, or deteriorating health conditions. Another six rats did not develop mechanical hypersensitivity (> 50% reduction of MWT from the pre-injury baseline) on day 2 post-SNL; these rats were excluded from the subsequent studies. The general experimental procedure is shown in **Figure 1**.

HF-SCS treatment reduces mechanical hyperalgesia with long-lasting analgesic effects after cessation and affects the levels of autophagy markers in the spinal dorsal horn

The results from Experiment 1 demonstrated that electrode implantation did not significantly affect the MWTs of rats in any groups compared with their pre-implantation values (**Figure 2A**, pre-SNL *vs.* baseline, respectively). Tactile hypersensitivity was observed in the SNL and SNL + HF-SCS groups at the pre-HF-SCS time point (both *P* < 0.05, *vs.* pre-SNL, respectively), which was 2 days after SNL surgery. This finding suggests that tactile hypersensitivity was successfully induced by SNL, as has been described in previous studies (Kim and Chung, 1992). HF-SCS treatment significantly increased MWTs to maximal levels at 60 minutes after the onset of stimulation; this increase was maintained for 3 hours after stimulation cessation (*P* < 0.05, *vs.* SNL group; **Figure 2A**). The SNL group had significantly increased expression of P62 and LC3-II (*P* < 0.05, *vs.* sham group; **Figure 2B–D**), which was partly reversed by HF-SCS treatment (*P* < 0.05, *vs.* SNL group; **Figure 2B–D**).

HF-SCS treatment alleviates the blockage of late autophagic flux that is caused by nerve injury

The results of Experiment 2 showed that, after CQ application, the levels of both LC3-II and P62 were markedly increased in the sham-SNL rats (*P* < 0.05, *vs.* sham + vehicle group; **Figure 3A–C**). This finding reflects the normal status of autophagosomal degradation in the spinal cord of rats under physiological conditions. However, there were no significant differences in the expression of LC3-II or P62 in the SNL rats undergoing sham-HF-SCS between those that received CQ and those that did not (*P* > 0.05; **Figure 3D–F**), suggesting

that spinal autophagosome degradation was already defective in the SNL rats. In contrast, the application of CQ caused a marked accumulation of LC3-II and P62 in the SNL rats receiving HF-SCS treatment (*P* < 0.05, *vs.* SNL + HF-SCS + vehicle group; **Figure 3G–I**). This finding indicates that HF-SCS treatment can restore autophagosome degradation and alleviate the impairment of autophagic flux in SNL rats.

HF-SCS treatment can reverse the lysosomal dysfunction of spinal cord neurons in SNL rats

The western blot results of Experiment 3 revealed a significant decrease in LAMP2 and the mature form of CTSD (matu-CTSD) in the SNL group (*P* < 0.05, *vs.* sham group; **Figure 4A–C**), indicating that both lysosomal quantity and activity were decreased in the SNL group. However, HF-SCS treatment increased the levels of LAMP2 and matu-CTSD in the spinal horn dorsal cord of never-injured rats (*P* < 0.05, *vs.* SNL group; **Figure 4A–C**). The confocal immunofluorescence results demonstrated that spinal LAMP2 was primarily co-localized with the neural marker NeuN (Jongen-Rêlo and Feldon, 2002). Furthermore, HF-SCS treatment significantly attenuated the SNL-induced downregulation of LAMP2 immunoreactivity in spinal neurons, especially in neurons in the superficial layers of the ipsilateral dorsal horn (*P* < 0.05, *vs.* SNL group; **Figure 5A–D**).

HF-SCS-induced regulation of autophagic flux participates in the mechanism of analgesia maintenance when stimulation is ceased

The results of Experiment 4 revealed that the HF-SCS-induced decrease of the abnormally elevated levels of P62 in SNL rats was significantly reversed when CQ was applied (*P* < 0.05, SNL + HF-SCS + CQ group *vs.* SNL + HF-SCS + vehicle group; **Figure 6A** and **B**), indicating that inhibition by CQ had an impact on autophagy in the spinal cord of rats of SNL + HF-SCS + CQ group, as expected. Furthermore, during HF-SCS treatment, the MWTs of rats who had received pretreatment with CQ were not significantly different from those without CQ treatment at 60–240 minutes after stimulation cessation (*P* > 0.05; **Figure 6C**). However, once the HF-SCS instrument was turned off, the CQ-induced inhibition of autophagy was able to rapidly reduce the restored MWTs of nerve-damaged rats, at 300–420 minutes after stimulation cessation (*P* < 0.05, SNL + HF-SCS + CQ group vs. SNL + HF-SCS + vehicle group; **Figure 6C**). In addition, the MWTs of SNL + HF-SCS + CQ group were not significantly different compared with the SNL group at 360–480 minutes after stimulation cessation (*P* > 0.05; **Figure 6C**).

Discussion

The present study demonstrated that HF-SCS application alleviated the blockage of autophagic flux by restoring impaired lysosomal function in the spinal neurons, especially in the superficial layers of the ipsilateral dorsal horn, of animals with neuropathic pain. Furthermore, the regulation of autophagic flux was one of the molecular cytological mechanisms of HF-SCS treatment, and it played an especially important role in the "carryover" maintenance of the analgesic effects.

Previous investigations (Linderoth and Foreman, 2017; Vallejo et al., 2017; Chakravarthy et al., 2018) have indicated that the fundamental basis of HF-SCS treatment might involve several mechanisms, including blockade of depolarization by inactivating sodium channels, desynchronization of neural signals from clusters of neurons firing in synchrony, and membrane integration. In the present research, we revealed that HF-SCS treatment indeed relieved hyperalgesia in SNL rats, and maintained its analgesic effect until 3 hours after stimulation cessation. These findings do not support the depolarization blockade of input from the periphery, but

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Figure 1 │ Timeline of the experimental procedures.
Timeframe for the behavior tests (B), electrode implantation (EI), spinal nerve ligation or sham surgery (S), abdominal injection (Inj), western blot assays (WB), immunofluorescence experiments (IF), and high-frequency spinal cord stimulation treatment (HF-SCS).
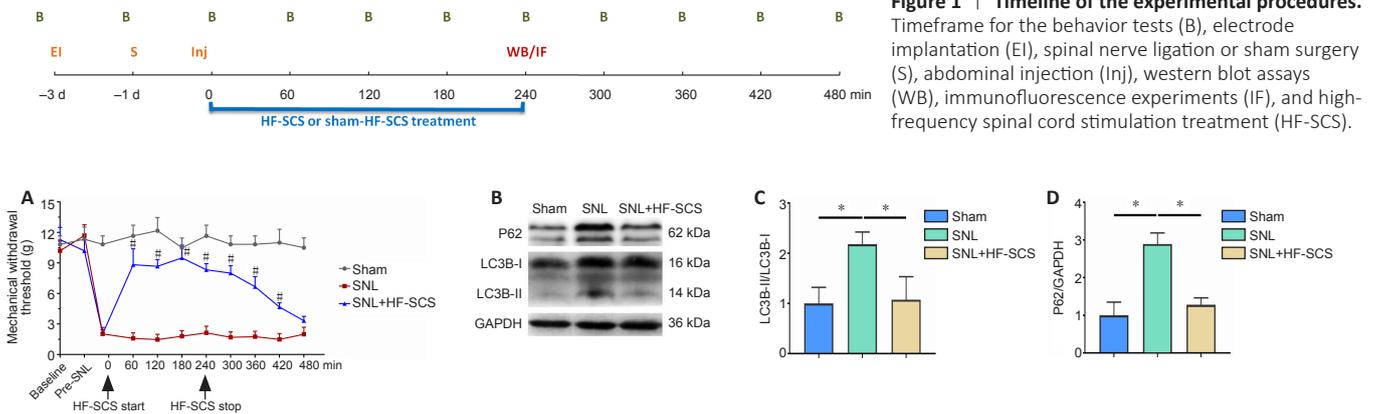
Figure 2 │ Effects of HF-SCS on the ipsilateral mechanical withdrawal thresholds and expression of autophagy markers in the ipsilateral spinal cord in neuropathic pain rats.
(A) The mechanical withdrawal threshold was measured using von Frey monofilaments. Data are presented as the mean ± SEM (n = 6 per group). #$P < 0.05$ vs. SNL group (two-way analysis of variance followed by Tukey's *post hoc* test). (B–D) Western blot analysis of LC3 and P62 expression. Data are presented as the mean ± SEM (n = 5 per group). *$P < 0.05$ (one-way analysis of variance with Dunnett's multiple comparisons test). GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; HF-SCS: high-frequency spinal cord stimulation; LC3: microtubule-associated protein 1A/B-light chain; P62: sequestosome 1; SNL: spinal nerve ligation.
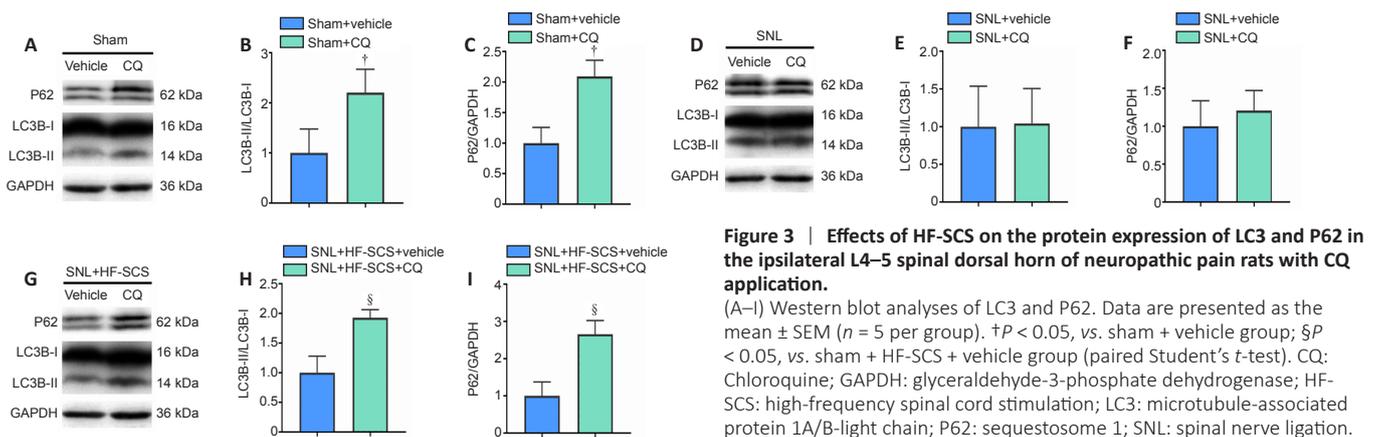
Figure 3 │ Effects of HF-SCS on the protein expression of LC3 and P62 in the ipsilateral L4–5 spinal dorsal horn of neuropathic pain rats with CQ application.
(A–I) Western blot analyses of LC3 and P62. Data are presented as the mean ± SEM (n = 5 per group). †$P < 0.05$, vs. sham + vehicle group; §$P < 0.05$, vs. sham + HF-SCS + vehicle group (paired Student's *t*-test). CQ: Chloroquine; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; HF-SCS: high-frequency spinal cord stimulation; LC3: microtubule-associated protein 1A/B-light chain; P62: sequestosome 1; SNL: spinal nerve ligation.

Figure 4 │ Effects of HF-SCS on lysosomal dysfunction in the spinal dorsal horn of neuropathic pain rats.
(A) Bands of LAMP2, pro-CTSD, and matu-CTSD. (B, C) Quantitative results of LAMP2 expression and matu-CTSD/pro-CTSD. Data are presented as the mean ± SEM (n = 5 per group). *$P < 0.05$ (one-way analysis of variance followed by Dunnett's multiple comparisons test). GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; HF-SCS: high-frequency spinal cord stimulation; LAMP2: lysosome-associated membrane protein-2; matu-CTSD: mature form of cathepsin D; pro-CTSD: pro-mature form of cathepsin D; SNL: spinal nerve ligation.

rather suggest the existence of other, as-yet-undiscovered pain-relieving mechanisms in HF-SCS treatment, which might contribute to re-establishing cellular homeostasis and rehabilitating injured nerve tissue. Thus, we investigated the protein expression of LC3 and P62, and demonstrated that HF-SCS treatment affected the abnormal status of autophagy in the spinal dorsal horn of SNL rats. LC3-II is a specific protein that associates with autophagosome membranes through degradation (Klionsky et al., 2016), whereas P62 is an autophagy substrate, and is thus degraded along with other captured substrates of the autophagosome (Klionsky et al., 2016). The increased LC3-II in the SNL group may therefore simply reflect an increase in autophagosome synthesis, or may indicate a decreased rate of autophagosome degradation. Accordingly, the SNL + HF-SCS group had decreased LC3-II, which may indicate either inhibited autophagy with decreased autophagosome synthesis or autophagosome consumption caused by effective degradation of the autophagosome.

To address this point of contention, we used standard detection techniques by applying CQ combined with autophagic flux monitoring (Klionsky et al., 2016). CQ is an anti-malarial and anti-inflammatory agent that has been widely used to treat malaria and rheumatoid arthritis. CQ is currently used to neutralize lysosomal pH and prevent the degradation of autophagosomes in autophagy-related studies (Klionsky et al., 2016; Turksen, 2019). Considering that neither LC3-II nor P62 were significantly different between SNL rats with and without CQ application, it may be that nerve damage in SNL rats induced a severely defective degradation of autophagosomes. This might be sufficient to trigger the accumulation of LC3-II or P62 in the spinal cord of SNL group rats, regardless of whether nerve injury affects autophagosome synthesis. Conversely, because further accumulations of both LC3-II and P62 were detected in the SNL + HF-SCS + CQ group, an active autophagosome degradation process was confirmed to exist in the SNL rats
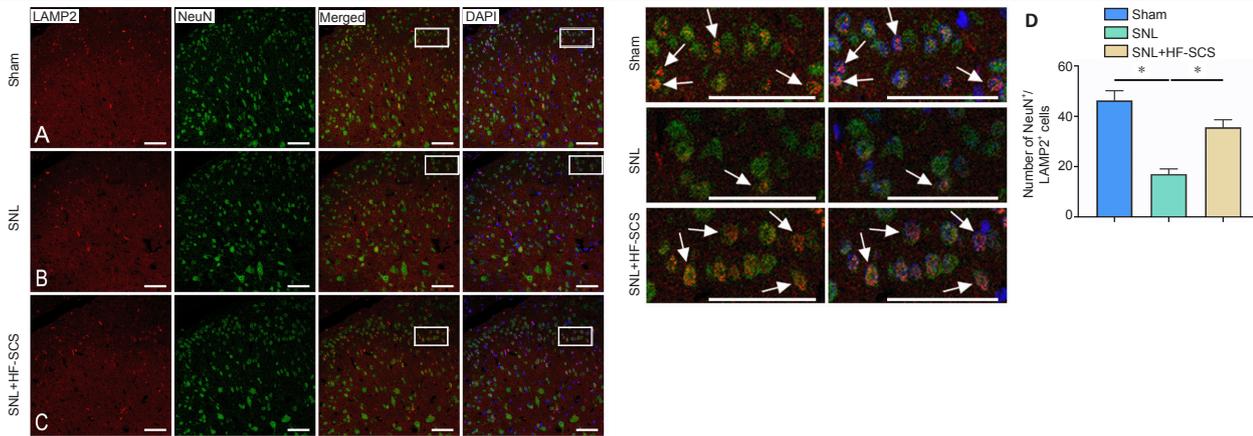
Figure 5 │ HF-SCS attenuates SNL-induced lysosomal dysfunction in neuropathic pain rats, mainly in the neurons of the superficial layers of the ipsilateral dorsal horn.

(A–C) Immunofluorescence studies showing double labeling with LAMP2 (lysosomal marker, red, stained by Alexa Fluor 594) and NeuN (neuronal marker, green, stained by Alexa Fluor 488) in the sham (A), SNL (B), and SNL + HF-SCS groups (C). Nuclei are stained with DAPI (blue). In the SNL group, there was a marked downregulation of LAMP2 immunoreactivity in spinal neurons (arrows); this effect was significantly attenuated in the SNL + HF-SCS group, especially in the superficial layers of the ipsilateral spinal cord. The enlarged images of the boxes in A–C were shown in the right. Scale bars: 50 μm. (D) Quantitative result of NeuN$^+$/LAMP2$^+$ cells. Data are presented as the mean ± SEM (n = 5 per group). *P < 0.05 (one-way analysis of variance with Dunnett's multiple comparisons test). DAPI: 4,6-Diamidino-2-phenylindole; HF-SCS: high-frequency spinal cord stimulation; LAMP2: lysosome-associated membrane protein-2; SNL: spinal nerve ligation.
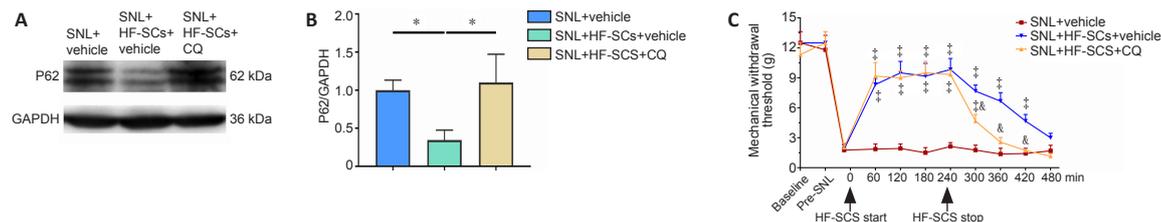
Figure 6 │ HF-SCS induces a long-lasting analgesic effect that can be inhibited by CQ pretreatment via the blocking of autophagic flux in the spinal cord of neuropathic pain rats.

(A, B) Dorsal horn P62 protein levels. Data are presented as the mean ± SEM (n = 5 per group). *P < 0.05 (one-way analysis of variance followed by Dunnett's multiple comparisons test). (C) Changes in mechanical withdrawal thresholds. Data are presented as the mean ± SEM (n = 6 per group). ‡P < 0.05, $vs.$ SNL + vehicle group; &P < 0.05, $vs.$ SNL + HF-SCS + vehicle group (two-way analysis of variance followed by Tukey's $post hoc$ test). CQ: Chloroquine; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; HF-SCS: high-frequency spinal cord stimulation; P62: sequestosome 1; SNL: spinal nerve ligation.

that received HF-SCS treatment. The relatively low levels of P62 in the SNL + HF-SCS group certainly suggest that HF-SCS treatment may increase the rate of autophagosome degradation in nerve injury rats. Consequently, the decreased LC3-II in the SNL + HF-SCS group is likely to be a result of the consumption of LC3-II in the process of more effective autophagy degradation.

In the later processes of autophagy, autophagosomes fuse with and are degraded by lysosomes. Lysosomal dysfunction is implicated in several neurodegenerative diseases (Hossain et al., 2020). We therefore investigated whether lysosomal dysfunction was involved in our neuropathic pain study, and revealed that SNL injury indeed downregulated LAMP2 and matu-CTSD, indicating the inadequate amount and low efficiency of lysosomes under these conditions. In contrast, both LAMP2 and matu-CTSD were markedly increased following HF-SCS treatment. Furthermore, immunofluorescence results demonstrated that the cellular localization of HF-SCS-induced lysosomal functional was mainly in neurons. Moreover, HF-SCS treatment significantly increased the numbers of NeuN$^+$/LAMP2$^+$ cells in the superficial layers of the spinal dorsal horn, where nociceptive inputs are received from primary afferent fibers and conveyed to associative and second-order neurons.

We next explored the possible relationship between the autophagic regulation of HF-SCS and the analgesic effects of such stimulation. Notably, the inhibition of autophagy had no significant influence on the analgesic effects of ongoing HF-SCS, indicating that autophagic regulation may not be the

main mechanism of stimulation-induced analgesia. However, after inhibiting autophagy by suppressing autophagosome degradation with CQ, the analgesic effects of HF-SCS after stimulation cessation were greatly reduced and shortened, suggesting that the regulation of lysosomes and autophagy participates in the analgesic maintenance of HF-SCS treatment.

In clinical applications, the analgesic maintenance effects of SCS have attracted increasing attention (Guan, 2012; Kapural et al., 2016). In addition, paresthesia, or tingling sensations that are induced by traditional SCS through the modulation of neuronal activity to overlap with a patient's painful region and replace the pain (Miller et al., 2016; Geurts et al., 2017), still considerably impacts the daily life of patients (Al-Kaisy et al., 2014, 2015). In severe cases, satisfactory analgesia inevitably demands higher amplitudes of stimulation, which adversely affect the motor function of limbs. Even in high-frequency conditions, a large amplitude also generates an uncomfortable "burning sensation" (Abejón et al., 2016; Linderoth and Foreman, 2017). For these reasons, patients usually turn off the electrical stimulation generator while they sleep or are driving; nonetheless, they are usually forced to restart the stimulator because of pain recurrence shortly after. Some previous studies have also reported that 50 Hz SCS or HF-SCS also has analgesic effects after cessation, but the timing of the electrical stimulation varied in each study, ranging from day 1 to day 16 after nerve injury (Truin et al., 2011; Shechter et al., 2013; Song et al., 2015). Furthermore, the duration of electrical stimulation varied from 30 to 120 minutes (Truin et

Research Article

al., 2011; Song et al., 2014, 2015). In addition, none of the aforementioned studies conducted a detailed exploration of the mechanisms of analgesia after the electrical stimulation was stopped. Our study demonstrated that HF-SCS produced about 3 hours of lasting analgesia when 4 hours of HF-SCS treatment was applied within 24 hours after SNL surgery. This may be regarded as an early intervention. Many studies have reported that the mechanisms of neuropathic pain evolve dynamically in all stages of its occurrence and development (Jay and Barkin, 2014), and the state of autophagy also changes over different nerve injury courses (Lipinski et al., 2015). Therefore, the therapeutic effects of the same treatment may be different when it is applied at different times. However, many studies have indicated that early interventions for neuropathic pain may lead to better outcomes (Harney et al., 2005; Saranita et al., 2009; Williams et al., 2009; Cohen and Mao, 2014; Vallejo et al., 2017). We might thus be able to extend the lasting analgesic time in the clinical application of HF-SCS through the further implementation of relevant research, a better understanding of the mechanisms of lasting analgesia, and more reasonable timing for the application of HF-SCS. We may therefore be able to provide more comfortable analgesia and further improve the quality of life in patients with neuropathic pain.

Lysosomes play an important role in autophagic processes and participate in the secretion of neurotransmitters by exocytosis, which has been considered as a therapeutic target in several neurodegenerative disorders (Coutinho et al., 2015). In the present study, we demonstrated that the spinal cord dorsal horn of rats had lysosomal function damage on the second day after peripheral nerve injury. However, 4 hours of HF-SCS treatment partially restored lysosomal function, activated autophagic flux, and relieved pain sensitivity. Therefore, the regulation of lysosomal function may be a potential target for the early treatment of neuropathic pain. Moreover, the effects of HF-SCS on lysosomal function and autophagic flux may be one of the mechanisms by which HF-SCS can interfere with the central sensitization of neuropathic pain; this concept is worthy of further exploration in follow-up studies.

However, the detailed mechanisms underlying nerve injury-related neuropathic pain and lysosomal dysfunction remain poorly understood. Accordingly, there is a need for further investigations of lysosomal regulation in the mechanisms of analgesia maintenance that is induced by the application of 10 kHz HF-SCS. A better understanding of these concepts may allow for improved neuromodulation strategies, which might lead to better or even curative treatment of intractable neuropathic pain, rather than the temporary relief of pain symptoms.

The present experiment had a number of limitations. First, we were unable to measure the expression levels of proteins in specific cell types in the spinal cord using western blot. Different cells may play different roles in the developing process of neuropathic pain. Second, the current LC3B antibody and detection technology were unable to clearly distinguish LC3-II from LC3-I in the immunofluorescence of tissue sections. Third, although CQ greatly shortened the maintenance of analgesia after the cessation of HF-SCS, autophagy regulation may not be the only mechanism by which HF-SCS produces long-lasting analgesia. Fourth, in Experiment 2, we did not detect the six groups of samples in one western blot at the same time, making it difficult to compare the differences between the sham + CQ, SNL + CQ, and SNL + HF-SCS + CQ groups, which may have led to missing data regarding the different statuses of autophagy initiation in these groups. Fifth, because we lacked some control groups (such as a sham + HF-SCS group in Experiments 1 and 3, and an SNL + CQ group in Experiment 4), the molecular signaling mechanism by which HF-SCS restores lysosomal function

remains far from clear, and requires further exploration. However, inhibition of the mammalian target of rapamycin pathway can lead to the activation of lysosomal gene transcription, and thus increase lysosomal biogenesis (Lipinski et al., 2015). Whether this pathway also participates in HF-SCS treatment needs future investigation.

In conclusion, the present study demonstrated that peripheral nerve injury led to lysosomal dysfunction and impaired autophagic flux in the spinal cords of rats. Four hours of HF-SCS treatment was able to alleviate the SNL-induced accumulation of autophagosomes by restoring lysosomal function, mainly in neurons in the superficial layer of the dorsal horn, and led to the long-lasting analgesic effects of HF-SCS.

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