Lidocaine reduces pain behaviors by inhibiting the expression of Nav1.7 and Nav1.8 and diminishing sympathetic sprouting in SNI rats

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Abstract

Chronic neuropathic pain is a significant clinical challenge, and the mechanisms of neuropathic pain remain elusive. Previous studies have shown that spontaneous potential, which is triggered by Nav1.7 and Nav1.8 in the dorsal root ganglion (DRG), is crucial for the development of inflammatory and neuropathic pain. Functional coupling between the sympathetic nervous system and somatosensory nerves after a nerve injury has also been noted as an important factor in neuropathic pain. However, the relationship of sympathetic sprouting with Nav1.7 and Nav1.8 remains unclear. Therefore, we dynamically examined the mechanical withdrawal threshold (MWT), changes in Nav1.7 and Nav1.8, and sympathetic sprouting after lidocaine treatment in the spared nerve injury (SNI) model of rats. After lidocaine treatment, the MWT obviously increased, showing that hypersensitivity was significantly relieved and the abnormal expression of Nav1.7 and Nav1.8 caused by SNI was also significantly reduced. In addition, lidocaine distinctly inhibited sympathetic nerve sprouting and basket formation around the Nav1.7 and Nav1.8 neurons in the DRG. These results indicate that lidocaine may alleviate neuropathic pain by inhibiting the expression of Nav1.7 and Nav1.8, and diminishing sympathetic sprouting in DRG.

Keywords

Neuropathic pain, Nav1.7, Nav1.8, sympathetic sprouting, dorsal root ganglion

Introduction

Neuropathic pain (NP) is caused by a lesion or disease of the somatosensory system, including the peripheral fibers and central neurons.[1,2] It affects 7–10% of the general population,[2] and is characterized by allodynia, hyperalgesia, and spontaneous pain.[1,2] Spontaneous activity of myelinated peripheral neurons has been found in past studies, which is a major feature of pain models.[3,4]

Sympathetic sprouting of the dorsal root ganglion (DRG) is the structural basis of peripheral sensitization of NP.[5] Many (but not all) pain models have shown that reducing sympathetic germination can reduce some painful behaviors.[6,7] In a spinal nerve ligation (SNL) model, Chunga[8] found that sympathetic sprouting in the DRG comprised regenerated branches (lateral branches) from the proximal part of the injured spinal nerve. Similar reports have been reported in chronic constriction injury of the sciatic nerve (CCI), inflammatory injury, and sciatic nerve transection injury

models, confirming that sympathetic sprouting occurs preferentially in spontaneously active DRGs, mainly the posterior ganglion fibers.[9-11] There may be a complementary relationship between spontaneous activity and sympathetic sprouting in NP. Spontaneous activity promotes sympathetic sprouting, and inhibition of neuronal activity blocks sprouting. Instead, there is evidence that sympathetic sprouting may contribute to neuronal overexcitability and

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spontaneous activity, creating a vicious cycle of positive feedback. In early stages of the SNL model, stimulation of dorsal branches that have sympathetic nerve sprouting has an excitatory effect on medium and large diameter cells.[6] Xie[11] found that cutting off sympathetic sprouting in the dorsal ramus eliminated spontaneous activity caused by spinal nerve ligation. In another study, sprouting was also shown to be reduced when spontaneous activity was decreased by local knockdown of Nav1.6.[12] Therefore, spontaneous activity may initiate sympathetic sprouting, while sympathetic sprouting may maintain spontaneous activity.

In summary, in past studies of NP, sympathetic sprouting and ectopic electrical activity were found to be closely related, but whether they show correlation with Nav1.7 and Nav1.8 is unclear. Nav1.7 and Nav1.8 are crucial in the process of initiating and disseminating nociceptive action potential to produce pain,[13,14] which may play an important role in the pathogenesis of human NP.[15,16] In our previous study, Nav1.7, Nav1.8, and sympathetic sprouting were co-located on medium and large neurons after SNI surgery. To further explore the relationship between Nav1.7, Nav1.8, and sympathetic sprouting, we treated SNI rats with lidocaine, and observed the dynamic changes in Nav1.7, Nav1.8, and sympathetic sprouting to provide a scientific basis for the clinical treatment of NP.

Materials and methods

Animals

The experimental protocol was approved and implemented by the Laboratory Animal Ethics Committee of Shanghai Jiao Tong University (Ethics No. 20210106–01). Male Sprague-Dawley (SD) rats weighing 180–200 g were provided and bred by the Animal Experimental Center of Shanghai Jiao Tong University. All rats were free to eat and drink under standard laboratory conditions.

Groups

54 SD rats were randomly divided into three groups: Sham group, SNI group, and Li group (lidocaine treated). SNI and Li groups all were given the SNI surgery, rats in the Li group were given an intraperitoneal injection of lidocaine 10 mg/kg every day for 21 consecutive days after the operation, while SNI group and sham groups were administered an intra-peritoneal injection of 0.9% normal saline.

Protocol

Mechanical pain threshold was measured 1 day before the operation and on days 3, 7, 14, and 21 after the operation. DRGs were extracted from three rats in each group on the 7th and 14th day after the operation for Western blotting, quantitative reverse transcription polymerase chain reaction (q-RT-PCR), and immunofluorescence assay (Table 1). Three rats in each group were used for WB, q-RT-PCR, and im-munofluorescence experiments, and each rat underwent the same experiment at least thrice to ensure the rigor and re-peatability of the experiment.

The Spare nerve injury model

According to the Decosterd[17] SNI model, after administering deep anesthesia to the rats, the subcutaneous tissue and femur were bluntly separated from the left thigh skin, about 1 cm along the sciatic nerve. Three branches of the sciatic nerve (tibial nerve, common peroneal nerve, and sural nerve) were exposed in the proximal popliteal fossa. At this level, the tibia and common peroneal nerve were ligated tightly with 4-0 silk, the distal segment was cut off, and 2–4 mm of the distal stump was removed. Care was taken not to damage the fibular nerve. Ensure disinfection of the suture. And in the sham group, only the sciatic nerve was exposed, the rest of the operations are the same as the surgical group.

Table 1. Experiment procedure.
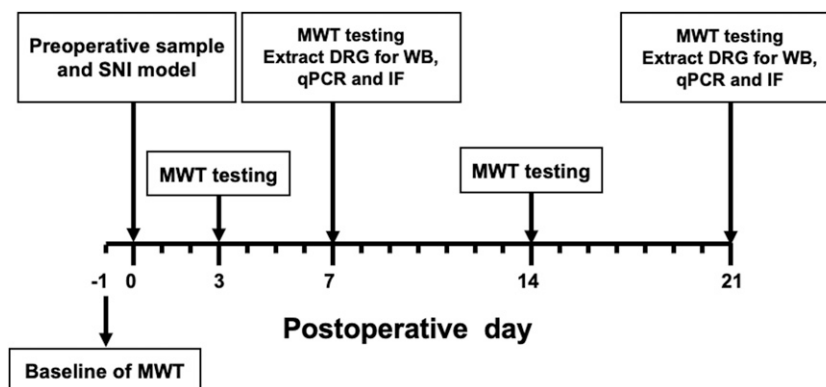
Behavior Testing

Von-frey fiber was used to assess mechanical pain in rats according to the up-and-down method recorded by Chaplan.[18] They were tested before the operation and 3, 7, 14, and 21 days after the operation (18:00–22:00 p.m.). Before each experiment, the rats were allowed to calm down for 30 min. Von Frey filaments, starting at 2 g, vertically act on the center of the affected paw of rats and remain for 6–8 s. If the rats perform a lower limb rapid jump or withdraw the paw completely, it is considered as a positive reaction, which is denoted as X, and otherwise as O. Animals with no response to the highest intensity were set at 15 g. Starting from the change in the fiber strength (X becomes O, or O becomes X) for six consecutive times, and according to Dixon,[19] the value of k was found based on the foot-raising reaction of rats, and the gram number of the last fiber was recorded as X_f, δ = 0.024, 50% MWT (g) = ($10^{[X_f + k\delta]}$)/10000.

Extraction of dorsal root ganglion

L4–L6 DRGs were collected from three rats in each group after the behavioral tests on the 7th and 21st day after the operation. After deep anesthesia, the left thigh muscle was bluntly isolated to expose the sciatic nerve, L4–L6 spinal nerve, and DRGs, which were rice grain in size, pale yellow, oval, and had a smooth surface. The DRGs were cut off and stored in a $-80°$ refrigerator.

Western Blotting

Tissue protein was extracted, and strong RIPA cleavage buffer (Biyuntian Biotechnology, China) was added to the samples at the ratio of 1g:10 mL, and PMSF (Biyuntian Biotechnology, China) was added at 1:100. An automatic rapid grinding machine (JX-FSTPRP-24) was used for 60 Hz 30s and repeated five times. Subsequently, the sample was centrifuged at 13500 r/min for 20 min at 4°C, the supernatant collected for total protein measurement, and the BCA method used for protein quantification to

4 μg/μl with a sample buffer (Laemmli buffer 2X, China University) at 70°C heating for 10 min. Then 8% SDS-PAGE electrophoresis (sample size 40 μg) was performed, first at 80 V and then at 120 V, followed by ice bath transfer of the (200 mA, 120 min) PVDF membrane (Corning, USA). Thereafter, the blot was blocked in 5% skim milk at room temperature for 2 h. Primary anti-Nav1.7 (rabbit anti-mouse, 1:200; ASC-008, Alomone) and Nav1.8 (rabbit anti-rat 1:200; ASC-016, Alomone) antibodies were incubated at 4°C centigrade overnight. The second antibody (goat anti-rabbit horseradish peroxidase 1:10000) was incubated at room temperature for 2 h after washing with 1 × TBST for three times, 15 min each time. ECL solution (Millipore, USA) was used for exposure. Image J software analysis was used to obtain the strip gray value.

Quantitative Real-time PCR

Total RNA was extracted with Trizol reagent (Invitrogen, USA). Then mRNA reverse transcription was performed using Hifair ® III first Strand c DNA Synthesis Super Mix for qPCR kit (Yeasen Biotechnology, China). The conditions of the reverse transcription cycle were: 25°C for 5 min, 55°C for 15 min, 85°C for 5 min. The mRNA expressions of Nav1.7 and Nav1.8 were analyzed using specific primer sets (Table 2).

The expression levels of different genes were standardized using β-actin expression as a control. Using AceQ Universal SYBR qPCR Master Mix (Vazyme Biotechnology, China) reagent and Applied Biosystems 7000 real-time PCR detection instrument, the CT value was calculated according to the instructions with relative quantitative mean analysis using $2^{-\Delta\Delta CT}$.

Immunofluorescence

After deep anesthesia, the thoracic cavity was opened in the supine position, the heart was exposed, the right atrial appendage was cut, and the needle was quickly inserted from the left ventricle. The heart was first perfused with saline and then fixed with 4% paraformaldehyde. After fixation, L5 DRG tissue was

Table 2. List of primer sets.

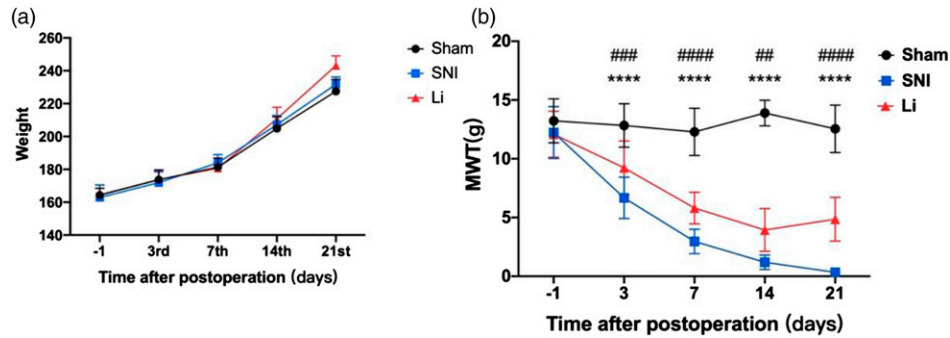| Gene	Primer sequence 5'-3'	Product size(bp)
Nav1.7-F	CCAGTTGCGACTGTAGGGTT	20
Nav1.7-R	AGTCGCGAATCGTGGATACC	20
Nav1.8-F	TCCAGAGAAAGTCGAGTATGCC	22
Nav1.8-R	GTCATGAGGCGGAACAGTGA	20
TH-F	TGTCACGTCCCCAAGGTTCA	20
TH-R	GCAGGTTGAGAACAGCATTCC	21
β-actin-F	GGAGATTACTGCCCTGGCTCCTA	23
β-actin-R	GACTCATCGTACTCCTGCTTGCTG	24

Figure 1. Changes of MWT and weight after surgery. (a–b) Changes of MWT between three groups. ****$p<0.0001$, compared with Sham; ####$p<0.0001$, Li group compared with Spared nerve injury group.
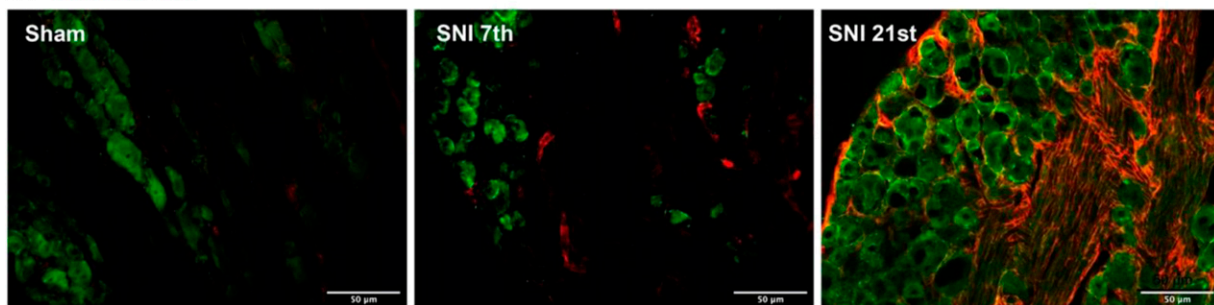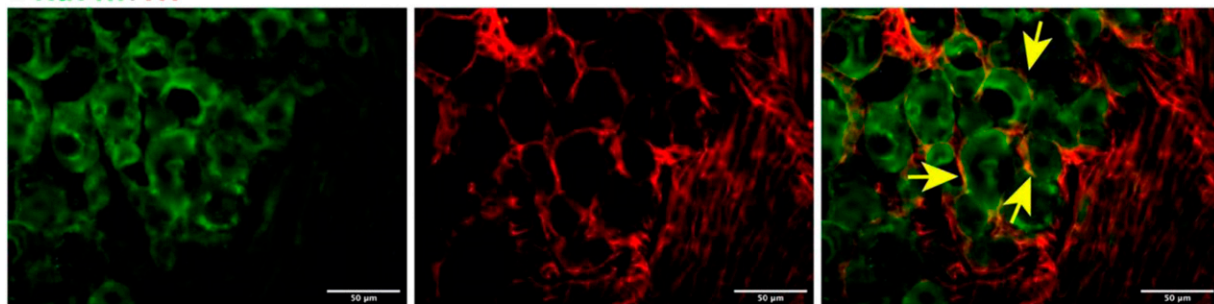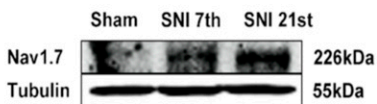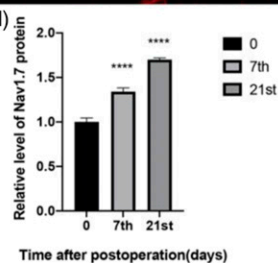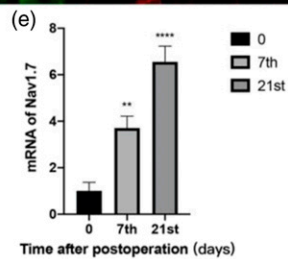

Figure 2. Spared nerve injury promotes the expression of Nav1.7 and induces abnormal basket-like structure. (a) Immunofluorescence showed that the expression of Nav1.7 in DRG increased significantly on the 7th and 21st after SNI. (b) Double immunofluorescence of basket structure in DRG after SNI, which surrounded Nav1.7 positive neurons by TH. Scale = 50 μm. Basket-like structure (yellow arrow), sympathetic sprouting (red) Nav1.7positive (green) (c–d) Western blotting: SNI promoted the expression of Nav1.7 in DRG. (e) Q-PCR results. Compared with Sham group, *$p<0.05$, **$p < 0.01$, ****$p<0.0001$.

extracted and dehydrated overnight in 25% sucrose solution. OCT embedded frozen section machine (Leica RM 2016, Germany) was used for obtaining 10 μm sections. The sections were incubated with Nav1.7 (1:200, ASC-08, Alomone), Nav1.8 (1:200, ASC-016, Alomone), and TH (Tyrosine hydroxylase, 1:1000, Sc25269, Santa) antibodies overnight at 4°C, and then with fluorescent secondary antibody (Alex-488 labeled ass anti-rabbit, Alex-569 labeled ass anti-rat secondary antibody) at room temperature in the dark for 2 h, sealed with glycerol, and photographed under a fluorescence microscope.
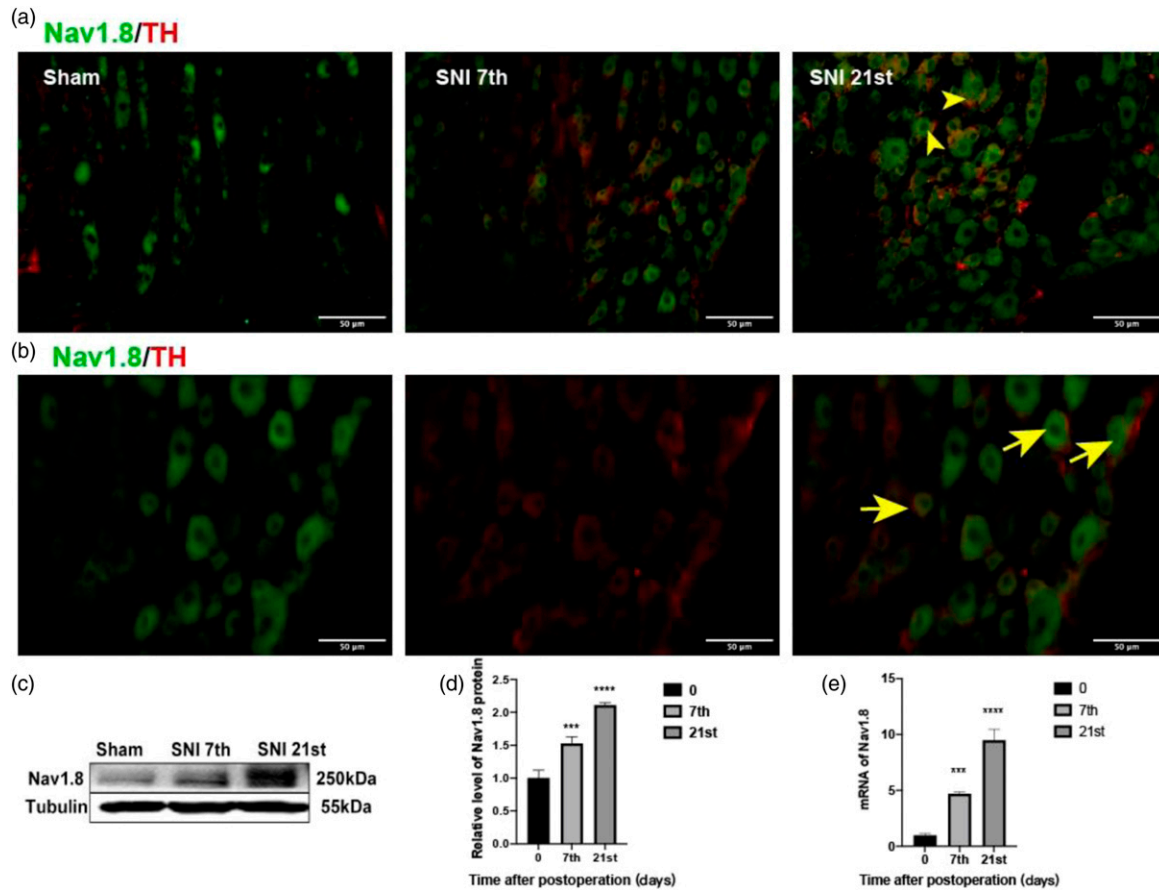
Figure 3. Spared nerve injury stimulates the expression of Nav1.8 and induces an abnormal basket-like structure in DRG. (a) Immunofluorescence showed that SNI induced Nav1.8 protein increased significantly. (b) Double immunofluorescence of the basket-like structure of Nav1.8 and TH in DRG after SNI. Scale = 50 μm. Basket-like structure (yellow arrow), sympathetic sprouting (red), Nav1.8 positive (green). (c–e) Western blotting and q-PCR: SNI promoted the expression of Nav1.8 in DRG. Compared with Sham, *$p<0.05$, **$p < 0.01$, ****$p<0.0001$.

Data analysis

Results are expressed as mean ± SEM, and were processed using GraphPad Prism 5.0. Statistical analysis was performed using one-way ANOVA followed by repeated measurements, along with Dunnett's multiple comparisons or Fisher's least significant difference (LSD) test. All immunofluorescence images were obtained on a fluorescence microscope and analyzed by Image J software.

Results

Spared nerve injury induces hyperalgesia and lidocaine partially reverses mechanical hyperalgesia

There was no significant difference in the body weight between the two groups ($p>0.05$, Figure 1(a)). Compared to the sham group, the MWT of rats in the SNI group was significantly decreased ($p<0.0001$, Figure 1(b)). MWT began to decrease on the third day after the operation ($p<0.0001$,

Figure 1(b)), and decreased to the lowest value on the 21st day after the operation ($p<0.0001$, Figure 1(b)). MWT decreased in a time-dependent manner in the SNI group ($p<0.0001$, Figure 1(b)). Early administration of lidocaine remarkably alleviated the pain allergy on the third day after SNI, and most significantly on the 21st day after the operation, inhibiting the development of NP, although this inhibition was not complete (Figure 1(b)).

Spared nerve injury increased Nav1.7 and Nav1.8 expression, and sympathetic sprouting in dorsal root ganglion

It was found that compared to the sham group, the sympathetic nerves surrounding the positive neurons of Nav1.7 and Nav1.8 increased after the operation, especially 21 days after the operation (Figure 2(a) to (b), Figures 3(a) and (b)). After SNI, protein expression levels of Nav1.7 and Nav1.8 were measured, which are indicators of action potential. Western
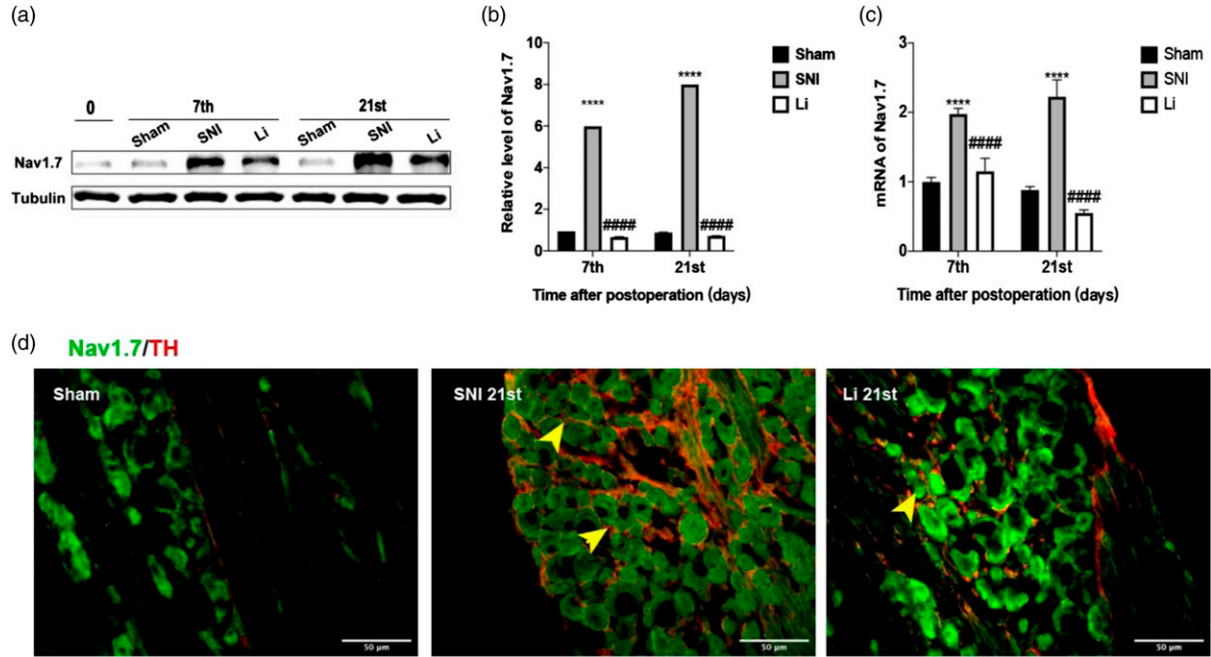
Figure 4. Lidocaine inhibited Nav1.7 expression and reducing basket-like structure. (a–b) Lidocaine did not completely reverse Spared nerve injury-induced increase in Nav1.7 protein expression. (c) Lidocaine reduced the increase in Nav1.7 mRNA. (d) Lidocaine inhibits basket structure. Scale = 50 μm, basket-like structure (yellow arrow), sympathetic sprouting (red), Nav1.7 positive (green). Compared with Sham group, ***p<0.001; Compared with SNI group ###p<0.0001.
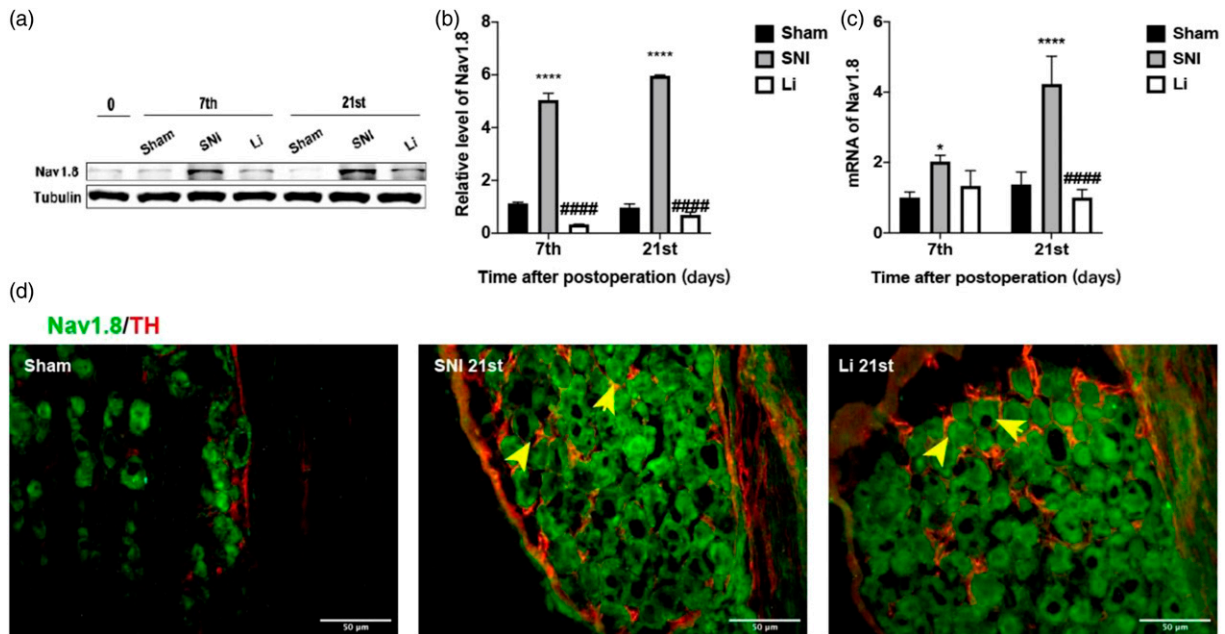

Figure 5. Lidocaine inhibited expression of Nav1.8 and declined basket-like structure around Nav1.8 positive neurons. (a–c) lidocaine inhibited increase of Nav1.8 protein and mRNA. (d) Lidocaine reduced Spared nerve injury-induced abnormal structure. Size = 50 μm, basket-like structure (yellow arrow), sympathetic sprouting (red), Nav1.8 positive (green). Compared with sham group, *p<0.05, ****p<0.0001; Compared with SNI group ####p<0.0001.

blot and q-PCR experiments confirmed that Nav1.7 and Nav1.8 expression increased 7 days after SNI, with a significant increase at 21 days compared to the sham group ($p < 0.05$, Figure 2(c) to (e), Figures 3(c) to (e)). To quantify the germination of sympathetic sprouting after a nerve lesion, basket structures were measured by immunostaining, which were co-localized and stained by Nav1.7, Nav1.8, and TH.

Lidocaine inhibits expression of Nav1.7 and reduces sympathetic nerve germination around Nav1.7 positive neurons

Results of Western blot and q-PCR showed that the expression of Nav1.7 was significantly increased in the SNI group on days 7 and 21, while in the lidocaine group, it was significantly lowered in a time-dependent manner ($p<0.0001$, Figures 4(a) to (c)) and did not fully recover to the baseline value. Compared to the sham group, immunofluorescence results showed that the expression level of Nav1.7 in DRGs was significantly increased 21 days after SNI and was remarkably diminished after lidocaine treatment (Figure 4(d)). Immunofluorescence colocalization showed that the basket-like structure was significantly raised on the 21tst day after SNI, with TH surrounded the Nav1.7 positive neurons, while lidocaine reduced this abnormal basket-like structure (Figure 4(d)).

Lidocaine inhibits the expression of Nav1.8 and decreases the basket-like structure around Nav1.8 positive neurons

Results of Nav1.8 were like that of Nav1.7. Western blot and q-PCR showed that lidocaine significantly inhibited SNI-induced abnormal expression of Nav1.8 in a time-dependent manner ($p<0.0001$, Figures 5(a) to (c)). Immunolocalization also revealed that lidocaine significantly reduced the abnormal basket-like structure induced by SNI, in which the Nav1.8 positive neurons were surrounded by TH (Figure 5(d)).

Discussion

The spare nerve injury NP model is more sensitive to mechanical pain,[20,21] and is regarded as the most reliable model that reflects the characteristics of clinical pathology and helps in understanding the mechanism of NP.[17,22,23] Therefore, we established the SNI model and observed the changes in the behavior of rats after surgery. It was found that the SNI rats developed mechanical pain sensitization, rats in the SNI group exhibited spontaneous foot lifting, laming, and autophagy, while those in the sham group were normal. Furthermore, lidocaine significantly relieves mechanical pain sensitivity. Compared to the sham group, MWT of rats in the SNI group was significantly decreased in a time-dependent manner ($p < 0.0001$, Figure 2(a)). The results were consistent with those of Decosterd and Woof,[17] indicating that the

modeling was successful. After treatment with lidocaine, MWT increased significantly ($p < 0.0001$, Figure 5(a)), although it did not return to the baseline. Lidocaine did not completely reverse NP caused by SNI, but it significantly relieved the pain.

It was found that protein and transcription levels of Nav1.7 and Nav1.8 increased significantly on days 7 and 21 after the operation ($p < 0.05$, Figures 3 and 4). Nav1.7 and Nav1.8 may be the main factors behind the ectopic electrical activity to explain this phenomenon, and the abnormal expression of Nav1.7 and Nav1.8 promotes the generation of pain allergy.[13-16] In our study, lidocaine was used to treat SNI, and it significantly alleviated mechanical pain sensitization in rats, which was consistent with the clinical use of local anesthetics for the treatment of NP. As a non-selective blocker of the sodium channel, lidocaine may relieve pain by inhibiting the protein expression of Nav1.7 and Nav1.8 clinically.

In addition, we also found that the abnormal basket-like structures formed by Nav1.7 and Nav1.8 that co-stained with TH were significantly increased after SNI (Figure 3 and 4). In the normal state, the sympathetic nerve is not associated with the sensory nerve. During the development of NP, abnormal sympathetic-sensory coupling emerges in the DRG, in the abnormal basket-like structure, which is mainly distributed in medium and large diameter neurons.[5,6,24] In our previous experiment, the basket-like structure was indeed found, mainly around the positive large and medium neurons expressing Nav1.7 and Nav1.8. In the present study, these abnormal structures were significantly reduced by lidocaine (Figure 5).

Currently, it has been reported that in NP, ectopic electrical activity in the DRG may be complementary to sympathetic sprouting.[10,24,25] Sympathetic-sensory coupling around large and medium-diameter DRGs wrapped by sympathetic sprouting leads to higher spontaneous electrical activity. It is well known that large and medium-diameter neurons mainly transmit the tactile sensation from mechanical skin receptors. In our previous study, the large and medium-diameter DRG neurons were found to abnormally express Nav1.7 and Nav1.8, and sympathetic sprouting surrounded these neurons, indicating that Nav1.7 and Nav1.8 are abnormally expressed in large and medium-diameter neurons in the DRG after nerve injury, resulting in abnormal cell membrane potential and formation of a basket-like structure with sympathetic nerves, which may be one of the reasons for abnormal pain. We found that lidocaine treatment not only reduced the expression of Nav1.7 and Nav1.8, but also significantly diminished sympathetic sprouting and the basket-like structure. Therefore, we speculated that after nerve injury, large and medium-diameter DRG neurons abnormally expressed Nav1.7 and Nav1.8, resulting in abnormal high-frequency currents, attracting sympathetic sprouting to wrap the neurons, and forming abnormal synaptic connections at this position. The catecholamine

neurotransmitter released by the sympathetic nerve may further promote the abnormal expression of sodium channels in neurons by activating α-adrenergic receptors, resulting in high-frequency and/or explosive discharges, thereby increasing the excitability of neurons, producing a positive feedback effect, amplifying pain signals, and transmitting them to the center, which may be the cause of clinical NP hypersensitivity.

This study only examined Nav1.7 and Nav1.8 as targets for pain. However, a recent mouse model of pain suggests that some types of NP may not require Nav1.7, such as oxaliplatin-induced pain.[26] The different data may be associated with different types of pain models compared to our study. In addition to Nav1.7 and Nav1.8, a recent study demonstrated that knock out of Nav1.6 reduces pain behaviors, sensory neuron excitability, and sympathetic sprouting, indicating that the sodium channel subtype involved in ectopic electrical activity in primary sensory neurons may also include Nav1.6. Therefore, sodium ions and sympathetic sprouting are regarded as treatment targets for further study of NP.

Author Contributions

Jun Tang conceived the study and supervised project, Xiaoxiao Li and Han Chen designed the experiment, analyzed the data, and wrote the manuscript. Xiaoxiao Li and Yanyan Li were responsible for establishment of the spare nerve injury in rats. Xiaoxiao Li and Han Chen conducted the measurement of pain behaviors. Xiaoxiao Li, Yujing Zhu and Tan Zhang carried out Q-PCR, Western blotting, and immunohistochemistry analysis. All authors approved the version to be published.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Compliance with ethical standard

This article does not contain any studies with human participants. The experiment procedures were approved by the Laboratory Animal Ethics Committee of Shanghai Jiao Tong University.

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