Na$_v$1.7 protein and mRNA expression in the dorsal root ganglia of rats with chronic neuropathic pain⋆

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Abstract

Neuropathic pain was produced by chronic constriction injury of the sciatic nerve in rats. Behavioral tests showed that the thresholds for thermal and mechanical hyperalgesia were significantly reduced in neuropathic pain rats 3–28 days following model induction. The results of immunohistochemistry, western blot assays and reverse transcription-PCR showed that Na$_v$1.7 protein and mRNA expression was significantly increased in the injured dorsal root ganglia. These findings indicated that Na$_v$1.7 might play an important role in the model of chronic neuropathic pain.

Key Words

Na$_v$1.7; neuropathic pain; dorsal root ganglia; sodium channel; sensitization; hyperalgesia; regeneration; neural regeneration

Research highlights

Na$_v$1.7 protein and mRNA expression were significantly increased in the dorsal root ganglia of neuropathic pain rats, indicating that Na$_v$1.7 might play an important role in chronic neuropathic pain.

Abbreviations

CCI, chronic constriction injury

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INTRODUCTION

Many recent genetic studies have identified the voltage-gated sodium-channel type IX α-subunit (SCN9A, referred to herein as Na$_v$1.7) as a key player in three conditions in which recurrent pain or the inability to sense pain is a prominent symptom[1-2]. The *SCN9A* gene encodes α sub-unit of the Na$_v$1.7 sodium channel, which is mainly expressed in the dorsal root ganglion and sympathetic ganglion of the peripheral nerve system. Different types of channelopathies (diseases caused by disturbed function of ion channel subunits or the proteins that regulate them), all involve the same Na$_v$1.7 sodium channel: (1) primary erythermalgia[3-4]; (2) paroxysmal extreme pain disorder[1]; (3) and channelopathy-associated insensitivity to pain are typified by different pain phenotypes[5]. In an experimental model of inflammatory pain in which an irritant was injected into the hind paws of rats, Na$_v$1.7 protein expression was up-regulated within dorsal root ganglion neurons that project their axons to the inflamed area[6-9]. However, its role in the model of neuropathic pain remains unclear. The present study observed the changes in behavior and Na$_v$1.7 expression in the chronic constriction injury (CCI)-induced disease model of chronic neuropathic pain.
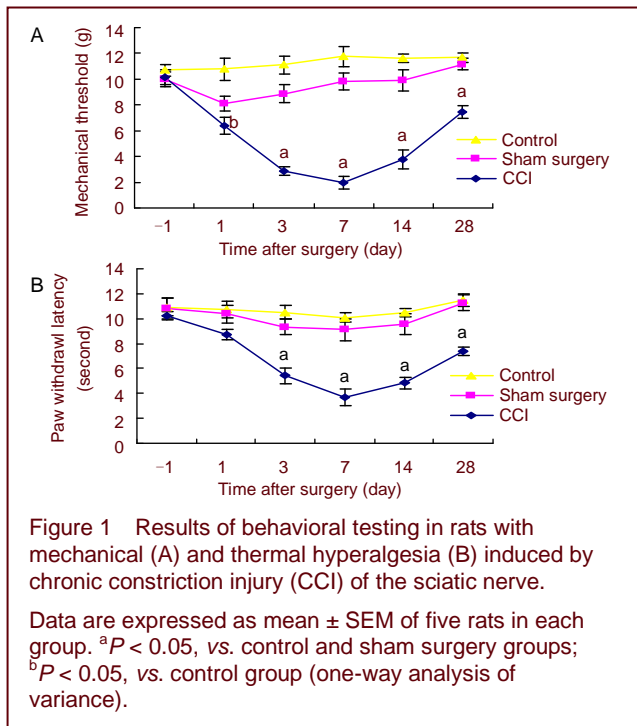
RESULTS

Quantitative analysis of experimental animals

A total of 62 rats were randomly assigned to CCI group ($n = 30$), sham surgery group ($n = 22$) and control group ($n = 10$). Models of neuropathic pain were established by sciatic nerve CCI in the CCI group. The right sciatic nerve was exposed in the sham surgery group. At 1, 7 and 28 days following model induction, seven rats from the CCI group and seven rats from the sham surgery group were used for reverse transcription (RT)-PCR and immunohistochemistry ($n = 3$) and western blot analysis ($n = 4$). There were

three rats and four rats in control group used for RT-PCR/ immunohistochemistry and western blot analysis, respectively. Five rats from each group were used for behavioral testing before model induction, and at 1, 3, 7, 14, 28 days after surgery.

Changes of behavior in neuropathic pain rats

Compared with the sham surgery group and control group, the thresholds for thermal and mechanical hyperalgesia were significantly reduced in the CCI group at 3–28 days following surgery ($P < 0.05$; Figure 1). Compared with the control group, there were no significant changes in the thresholds for thermal and mechanical hyperalgesia in the sham surgery group ($P > 0.05$; Figure 1). These results indicate that the CCI model was established successfully.

Figure 1 Results of behavioral testing in rats with mechanical (A) and thermal hyperalgesia (B) induced by chronic constriction injury (CCI) of the sciatic nerve.

Data are expressed as mean ± SEM of five rats in each group. [a]$P < 0.05$, *vs.* control and sham surgery groups; [b]$P < 0.05$, *vs.* control group (one-way analysis of variance).

$Na_v1.7$ expression in the L_5 dorsal root ganglion on the lesion side in neuropathic pain rats

Immunohistochemistry experiments revealed $Na_v1.7$-positive neurons in injured L_5 dorsal root ganglion specimens (Figure 2). There was a significant increase ($P < 0.05$) in the expression of $Na_v1.7$ in injured L_5 dorsal root ganglion specimens of CCI groups at 1, 7, 28 days after surgery (Figure 2H), compared with the sham surgery and control groups. These results indicate that CCI induced long-lasting $Na_v1.7$ activation in the rat dorsal root ganglion. Compared with the control and sham surgery groups, $Na_v1.7$ levels were significantly increased in the CCI group ($P < 0.05$ or $P < 0.01$; Figure 3). Western blot results indicated that the $Na_v1.7$ expression level changed with behavioral alterations in the rat right foot pain model in CCI groups.
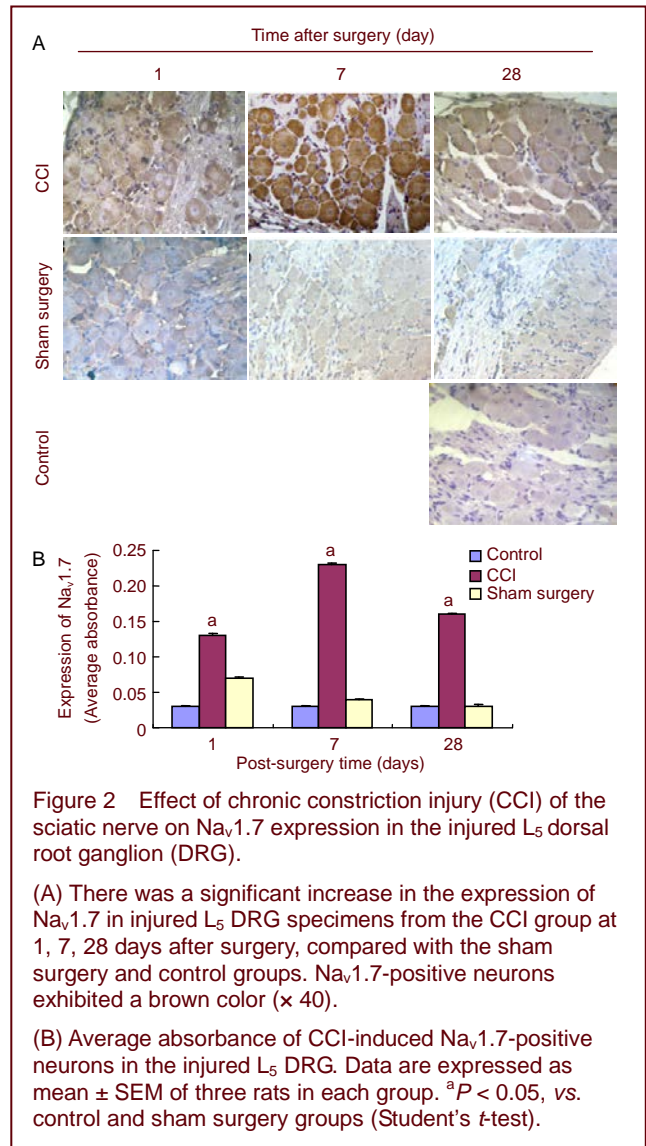

Figure 2 Effect of chronic constriction injury (CCI) of the sciatic nerve on $Na_v1.7$ expression in the injured L_5 dorsal root ganglion (DRG).

(A) There was a significant increase in the expression of $Na_v1.7$ in injured L_5 DRG specimens from the CCI group at 1, 7, 28 days after surgery, compared with the sham surgery and control groups. $Na_v1.7$-positive neurons exhibited a brown color (× 40).

(B) Average absorbance of CCI-induced $Na_v1.7$-positive neurons in the injured L_5 DRG. Data are expressed as mean ± SEM of three rats in each group. [a]$P < 0.05$, *vs.* control and sham surgery groups (Student's *t*-test).
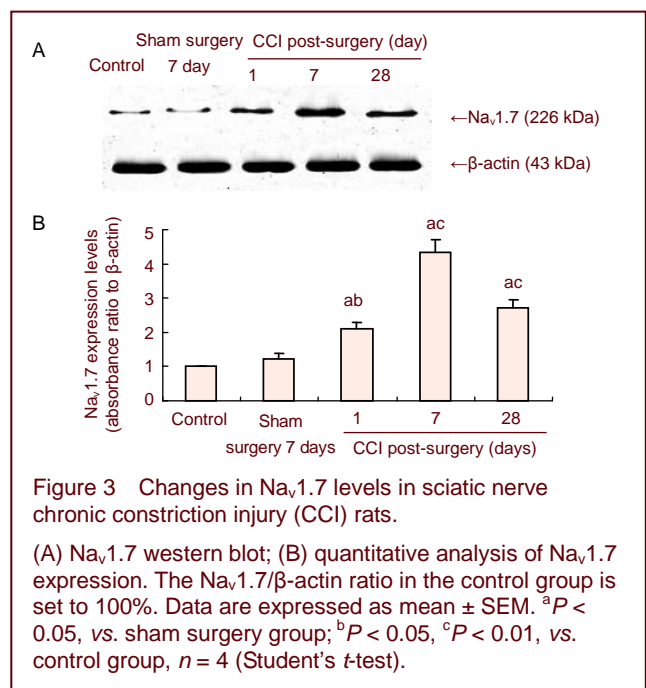

Figure 3 Changes in $Na_v1.7$ levels in sciatic nerve chronic constriction injury (CCI) rats.

(A) $Na_v1.7$ western blot; (B) quantitative analysis of $Na_v1.7$ expression. The $Na_v1.7$/β-actin ratio in the control group is set to 100%. Data are expressed as mean ± SEM. [a]$P < 0.05$, *vs.* sham surgery group; [b]$P < 0.05$, [c]$P < 0.01$, *vs.* control group, $n = 4$ (Student's *t*-test).

$Na_v1.7$ mRNA expression in the L_5 dorsal root ganglion on the lesion side in neuropathic pain rats

RT-PCR showed that, compared with the sham surgery and control groups, $Na_v1.7$ mRNA expression levels were significantly increased in the injured dorsal root ganglion of the CCI group ($P < 0.05$; Figure 4).
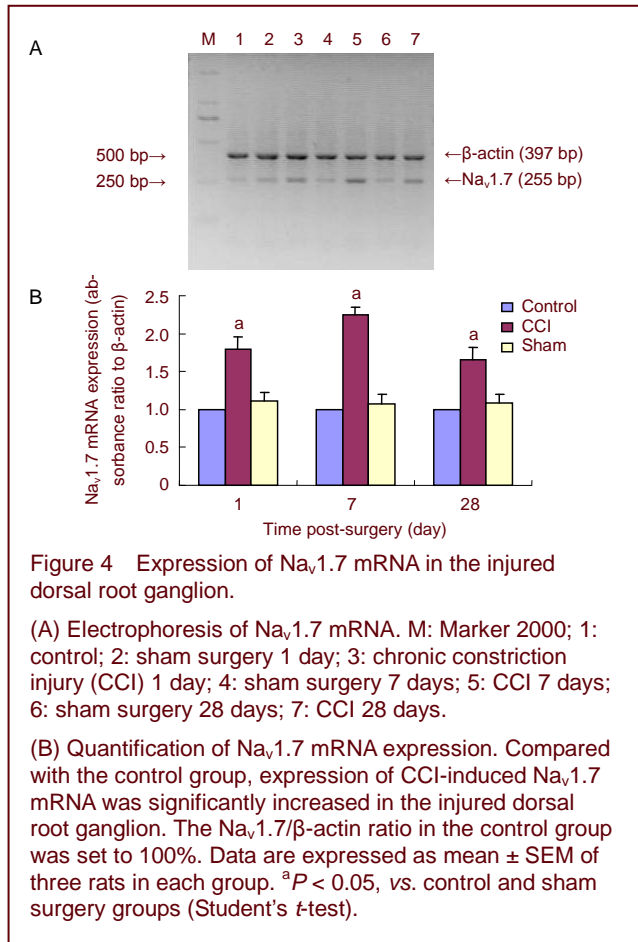

Figure 4 Expression of $Na_v1.7$ mRNA in the injured dorsal root ganglion.

(A) Electrophoresis of $Na_v1.7$ mRNA. M: Marker 2000; 1: control; 2: sham surgery 1 day; 3: chronic constriction injury (CCI) 1 day; 4: sham surgery 7 days; 5: CCI 7 days; 6: sham surgery 28 days; 7: CCI 28 days.

(B) Quantification of $Na_v1.7$ mRNA expression. Compared with the control group, expression of CCI-induced $Na_v1.7$ mRNA was significantly increased in the injured dorsal root ganglion. The $Na_v1.7$/β-actin ratio in the control group was set to 100%. Data are expressed as mean ± SEM of three rats in each group. [a]$P < 0.05$, *vs.* control and sham surgery groups (Student's *t*-test).

DISCUSSION

To obtain insights into the physiological role of $Na_v1.7$, Nassar *et al* [7] generated targeted knockout mice that lack $Na_v1.7$ within nociceptive dorsal root ganglion neurons. Selective deletion of $Na_v1.7$ in the nociceptors of mice produces animals showing a general failure to develop pain or hypersensitivity in response to inflammatory stimuli, while neuropathic pain (chronic pain resulting from injury to the nervous system) remains intact. These results are consistent with an important role of $Na_v1.7$ in setting the inflammatory pain threshold. A previous study confirmed that there was a trend toward an increase in $Na_v1.7$ level in burning mouth syndrome, considered a neuropathic pain disorder, but this increase was not statistically significant[10]. Our immunohistochemistry and western blot results indicated that $Na_v1.7$ expression was significantly increased in the injured dorsal root ganglion of rats during neuropathic pain.

The results of this study show that CCI induced long-lasting $Na_v1.7$ activation in the rat dorsal root ganglion. Increased $Na_v1.7$ expression could contribute to nociceptor activity-induced neural plasticity and the development of dorsal root ganglion neuronal sensitization and persistent pain[6]. In addition, increased $Na_v1.7$ expression was accompanied by increased mechanical and thermal hyperalgesia in CCI rats. These results suggested that activation of peripheral nerve injury nociceptive signals may increase $Na_v1.7$ expression following sciatic nerve CCI. Activation of ion channels, which serve as a linkage bridge between intracellular and extracellular spaces, results in the transduction of noxious stimuli into diverse intracellular responses, including changes in gene expression or transcriptional levels[11-13]. These observations suggest that blockade of $Na_v1.7$ is a promising therapeutic option for the treatment of pain, but emphasize the need to study the $Na_v1.7$-specific blockers that were used with a highly focused design. Our studies will provide information for the treatment of pain in the future. The results of the present study suggest that the $Na_v1.7$ sodium channel may play a significant role in chronic neuropathic pain.

MATERIALS AND METHODS

Design
A randomized, controlled, animal experiment.

Time and setting
This study was performed at the Laboratory of Anatomy, Department of Basic Medical Sciences, Zhengzhou University, China from December 2010 to August 2011.

Materials
A total of 62 adult male Sprague-Dawley rats of clean grade, aged 2 months and weighing 180–220 g, were provided by the Experimental Animal Center of Henan Province (license No. SYXK2005-0012). The rats were housed with a 12-hour light-dark cycle and free access to food and water. They were kept for 1 week under these conditions before surgery. All procedures were performed in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China[14].

Methods
Establishment of sciatic nerve CCI
The CCI group received anesthesia using 10% chloral hydrate (3 mL/kg, intraperitoneally). Povidone iodine solution was utilized to sterilize the skin before the right sciatic nerve was exposed. The sciatic nerve was ligated

with nonabsorbable surgical sutures (6.0 nylon), in total, four knots with an interval of 1–2 mm. Subsequently, the skin was sutured with 6.0 nylon sutures[15]. The right sciatic nerve was exposed only in the sham surgery group. The control group did not receive any surgery.

Mechanical withdrawal and paw withdrawal latency to evaluate mechanical hyperalgesia and thermal hyperalgesia

Mechanical hyperalgesia was assessed using von Frey filaments as previously described[16]. Rats were placed in a transparent plastic box containing a wire mesh floor. The rats were acclimated to the surroundings for 30 minutes prior to testing. Each filament was perpendicularly applied to the mid-plantar surface of the injured hindpaw. Withdrawal thresholds were determined using sequentially increasing and decreasing stimulus strength ("up-and-down" method[17]).

Thermal hyperalgesia was assessed using a radiant heat apparatus, according to previously described protocols[18]. Rats were placed in a plastic box. The rats were acclimated to the surroundings for 30 minutes prior to testing. A high-intensity light beam was focused onto the plantar surface of the hindpaw through the plastic plate. The nociceptive endpoints in the radiant heat test were represented by a characteristic hindpaw lift or lick, and the paw withdrawal latency (in seconds) was measured by the apparatus. To avoid tissue damage, a cut-off time was established at 30 seconds. There were three trials per rat with 5-minute intervals between trials.

$Na_v1.7$ expression levels in the dorsal root ganglion, as detected by immunohistochemistry

For immunohistochemistry, the rats were deeply anesthetized with 10% chloral hydrate (3 mL/kg, intraperitoneally), followed by sternotomy, transcardial aortic needle cannulation, and perfusion with 100 mL heparinized saline. Then, the rats were fixed with 400 mL of 4% paraformaldehyde, and the right L_5 dorsal root ganglion was removed and postfixed in 4% paraformaldehyde in phosphate buffer for 3 hours. The dorsal root ganglion was embedded in paraffin, and cut into 4-μm thick sections using a slicing machine. Tissue sections were dewaxed and washed, and then maintained in 3% H_2O_2 for 20 minutes at 37°C, followed by blocking in goat serum for 20 minutes at 37°C. The sections were incubated at 37°C for 1 hour in rabbit anti-Nav1.7 polyclonal antibody (1:1 000; Sigma, St Louis, MO, USA), at 4°C for 24 hours, washed three times with PBS to remove excess antibodies, incubated in goat anti-rabbit IgG conjugated to biotin (1:100; Biosynthesis Biotechnology, Beijing, China) at 37°C for 30 minutes, washed three times with PBS for 5 minutes each, incubated in horseradish peroxidase-labeled

streptavidin (1:100; Biosynthesis Biotechnology) at 37°C for 30 minutes, washed three times with PBS for 5 minutes each, and then developed using diaminobenzidine (Biosynthesis Biotechnology). The specimens were counterstained with hematoxylin. Images were collected using a DMI3000 B Leica microscope (Leica, Wetzlar, Germany).

$Na_v1.7$ protein expression levels in the dorsal root ganglion, as determined by western blot analysis

The rats underwent laminectomy on ice. The right L_5 dorsal root ganglion was extracted, and the L_5 dorsal root ganglion was stored in liquid nitrogen for western blot assays. The L_5 ganglia were isolated and stored at −80°C. Sequential precipitation procedures were used on tissue samples that were lysed using a homogenizer in ice-cold (4°C) lysis buffer containing 0.1% phenylmethyl sulfonylfluoride (100 mM), 0.3% aprotinin, and 0.1% sodium orthovanadate (1 M). The homogenates were centrifuged at 15 000 r/min for 15 minutes at 4°C. The supernatants were then stored at −80°C for further use. Protein concentrations were determined using the Bradford method[19]. Samples were solubilized in 2 × sodium dodecyl sulfate sample buffer and then boiled at 100°C for 5 minutes to denature the proteins and disrupt protein complexes. Lysates equivalent to 30 μg of protein were separated by SDS-PAGE on 8% gels and transferred to nitrocellulose membranes. The membranes were blocked for 30 minutes with 10% fat-free milk and then incubated overnight at 4°C with primary antibodies (rabbit anti-Nav1.7 polyclonal antibody, 1:1 000, Sigma; rabbit anti-beta-actin polyclonal antibody, 1:1 000, Santa Cruz Biotechnology, Santa Cruz, CA, USA). The membranes were extensively washed three times in Tris-buffered saline Tween-20 for 10 minutes each, followed by incubation for 1 hour in goat anti-rabbit secondary antibody conjugated to horseradish peroxidase (1:100, Biosynthesis Biotechnology) at room temperature. Immune complexes were detected using a Super Enhanced Chemiluminescence Plus Assay Kit (Biosynthesis Biotechnology). Image analysis was performed using Image Pro Plus 6.0 software (Media Cybernetics Inc., Bethesda, MD, USA). The relative absorbance of each band corresponding to $Na_v1.7$ was normalized to the β-actin value to determine relative protein expression levels.

Nav1.7 mRNA expression levels in the dorsal root ganglion, as detected by RT-PCR

The rats underwent laminectomy on ice. The right L_5 dorsal root ganglion was extracted, and L_5 dorsal root ganglion was stored in liquid nitrogen for western blot assays. A two-step method was used for mRNA extraction, including RT and PCR[20]. The internal reference was

β-actin mRNA. RT-PCR products for Na$_v$1.7 mRNA and β-actin mRNA were obtained and subjected to electrophoresis and imaging with a gel imaging system (Alpha, Akron, OH, USA). Relative Na$_v$1.7 mRNA expression in each group was observed. The absorbance values per mm^2 of electrophoretic bands were analyzed using Imaging Plus Pro 6.0 software, and the Na$_v$1.7/β-actin absorbance ratio was calculated, representing relative Na$_v$1.7 mRNA expression[21].

Statistical analysis

Experimental data were analyzed using SPSS 12.0 software (SPSS, Chicago, IL, USA). All data were expressed as mean ± SEM. Statistical analysis was performed using one-way analysis of variance or the Student's *t*-test. A *P* value < 0.05 was considered statistically significant.

Author contributions: Chao Liu established the animal model of CCI of the sciatic nerve, performed immunohistochemistry, RT-PCR, western blot assays and wrote the manuscript. Jing Cao carried out the behavioral tests and modified the manuscript. Xiuhua Ren provided technical support for the study. Weidong Zang supervised the experiments and corrected the manuscript. All authors read and approved the final manuscript.

Conflicts of interest: None declared.

Ethical approval: All animal experimental procedures were approved by Zhengzhou University Committee on Animal Research.

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