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Upregulation of $Ca_v3.2$ T-type calcium channels in adjacent intact L4 dorsal root ganglion neurons in neuropathic pain rats with L5 spinal nerve ligation

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ABSTRACT

Besides the injured peripheral dorsal root ganglion (DRG) neurons, the adjacent intact DRG neurons also have important roles in neuropathic pain. Ion channels including $Ca_v3.2$ T-type calcium channel in the DRG neurons are important in the development of neuropathic pain. In the present study, we aimed to examine the expression of $Ca_v3.2$ T-type calcium channels in the intact DRG neurons in neuropathic pain. A neuropathic pain model of rat with lumbar 5 (L5) spinal nerve ligation (SNL) was established, in which the L4 DRG was separated from the axotomized L5 DRG, and the molecular, morphological and electrophysiological changes of $Ca_v3.2$ T-type calcium channels in L4 DRG neurons were investigated. Western blotting showed that total and membrane protein levels of $Ca_v3.2$ in L4 DRG neurons increased, and voltage-dependent patch clamp recordings revealed an increased T-type current density with a curve shift to the left in steady-state activation in the acutely isolated L4 DRG neurons in neuropathic pain rats. Immunofluorescent staining further showed that the membrane expression of $Ca_v3.2$ increased in CGRP-, IB4-positive small neurons and NF200-positive large ones. In conclusion, the membrane expression and the function of $Ca_v3.2$ T-type calcium channels are increased in the intact L4 DRG neurons in neuropathic pain rats with peripheral nerve injury like SNL.

1. Introduction

Injury to the peripheral nerves induces neuropathic pain symptoms (Campbell and Meyer, 2006). In the neuropathic pain model like lumbar 5 (L5) and L6 spinal nerve ligation (SNL) (Kim and Chung, 1992), partial nerve injury involves both intact and injured peripheral axons. While early studies on neuropathic pain mainly focus on local injury sites and damaged sensory neurons, emerging evidence demonstrate the changes of the L4 dorsal root ganglion (DRG) neurons with intact axons. Some molecules related to nociception are upregulated in the intact L4 DRG neurons after L5 SNL (Campbell and Meyer, 2006), whereas abnormal activities in uninjured nerves (Meyer and Ringkamp, 2008; Wu et al., 2002) and L4 DRG neurons (Ma et al., 2003) trigger and maintain the neuropathic pain by increasing the excitability of primary sensory neurons. So, possible changes of ion channel properties in intact DRG neurons adjacent to the axotomized DRGs are of great interest.

Voltage-gated Ca^{2+} channels fall into two major categories based on their membrane potential properties: high-voltage activated (HVA) or sustained currents and low-voltage activated (LVA) or transient currents (T-type) (Bourinet et al., 2016). T-type Ca^{2+} channels, including $Ca_v3.1$, $Ca_v3.2$ and $Ca_v3.3$ isoforms, are important regulators in nociceptive transmission (Catterall, 2010), and the $Ca_v3.2$ channel is predominantly expressed in primary sensory neurons (Talley et al., 1999; Chen et al., 2003; Bourinet et al., 2005). In DRG neurons from peripheral neuropathic pain animals, T-type calcium channels contribute to pain sensitization and are possible therapeutic targets of neuropathic pain (Jagodic et al., 2008; Takahashi et al., 2010; Wen et al., 2010).

Our previous study found that the accumulated $Ca_v3.2$ channels in the uninjured sural nerve played an important role in peripheral sensitization (Chen et al., 2018). In the present study, we aim to

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investigate the properties of $Ca_v3.2$ channels in the adjacent intact DRG in rats with neuropathic pain. We observed increased membrane expression and function of $Ca_v3.2$ T-type calcium channels in intact L4 DRG neurons of neuropathic pain rats with L5 SNL.

2. Materials and methods

2.1. Animals

Adult male Sprague Dawley rats weighing 180–220 g were provided by the Department of Experimental Animal Sciences, Peking University Health Science Center (Beijing, China). Animals were housed under a standard 12 h reversed light/dark cycle at 22.0 ± 1.0 °C with food and water available *ad libitum*. All animal experimental protocols followed the guidelines of the International Association for the Study of Pain (Zimmermann, 1983) and were approved by the Institutional Animal Care and Use Committee of Peking University Health Science Center.

2.2. Spinal nerve ligation (SNL) model of neuropathic pain in rats

The surgical procedure for SNL was performed as previously described (Kim and Chung, 1992). The rat was anesthetized with 1% pentobarbital sodium (50 mg/kg, *i.p.*). A 2-cm-long skin incision was made on the back at the left side of the L4–S2 spine level. After removing the transverse process of the left paraspinal muscles and L5 vertebra, the L5 spinal nerve was isolated and ligated with 6-0 silk thread. Special care was taken during L5 ligation to avoid possible damage to the L4 spinal nerve. In Sham-operated rats, animals received the same operation except that the L5 nerve was not ligated.

2.3. Behavioral tests

For mechanical allodynia, the 50% paw withdrawal threshold (PWT) was measured by the up-down method (Chaplan et al., 1994) and as in our previous reports (Liu et al., 2012; Zhang et al., 2016). Eight *von* Frey filaments were chosen (0.41–15.10 g, North Coast, Gilroy, CA). Each trial started with a *von* Frey force of 2.00 g delivered perpendicularly to the plantar surface of the left hind paw. An abrupt withdrawal of the foot during stimulation or immediately after the removal of the filament was recorded as a positive response. This procedure was repeated until 6 stimuli after the first change in response had been observed.

For thermal hyperalgesia, a radiant heat was used for assessing paw withdrawal latency (PWL). The power of the thermal stimulator was adjusted to basal PWLs to 12–15 s, with a cut-off time of 30 s to prevent possible tissue injury. Three latency measurements were taken with at least 5-min intervals for each trial, and the average value was used to represent the thermal pain threshold of the rat. All behavioral tests were performed in a blinded fashion.

2.4. Western blot

2.4.1. Total protein preparation

L4 DRG tissues were homogenized in lysis buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 0.1% SDS and protease inhibitor cocktail (Roche). After being kept on ice for 1 h, the lysate was centrifuged at 12,000 rpm for 15 min at 4 °C, the supernatant mixing with loading buffer containing 2% SDS, 100 mM dithiothreitol (DTT), 10% glycerol and 0.25% bromophenol blue was denatured at 95 °C for 5 min. The concentration of protein was determined using a bicinchoninic acid (BCA) kit (Pierce Biotechnology Inc.).

2.4.2. Membrane protein preparation

Membrane proteins were prepared following the protocol (Tyrrell et al., 2001). L4 DRGs were homogenized in lysis buffer containing 0.3 M sucrose, 10 mM Tris pH 8.1, 2 mM EDTA, 1 mM phenylmethanesulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT) and protease inhibitor cocktail (Roche). Homogenates were kept on ice for 1 h before centrifugation at 1000*g* for 7 min at 4 °C to remove nuclei and intact cells. One more cycle, the two extracted supernatants were combined and centrifuged at 120,000*g* for 1 h at 4 °C. The pellet, containing the total membrane fraction, was suspended in 0.2 M KCl and 10 mM 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES, pH 7.4). To solubilize the membrane fraction, an equal volume of 5% Triton X-100 and 10 mM HEPES (pH 7.4) was added to the sample, and the suspension was kept on ice for 1 h. Unsolubilized material was pelleted by centrifugation at 10,000*g* for 10 min at 4 °C, and the supernatant was collected for Western blot assay.

2.4.3. Western blot

Equal amounts of proteins from each sample were separated on 8% SDS-PAGE gels and transferred to polyvinylidene fluoride membranes (Merck Millipore). The blots were blocked in 5% nonfat milk at room temperature for 1 h, incubated respectively with rabbit anti-rat $Ca_v3.2$ (1: 500, catalogue #H-300, Santa Cruz Biotechnology), rabbit anti-rat β-tubulin (1: 2000, catalogue #2128S, Cell Signaling Technology), or mouse anti-rat transferrin receptor (TfR, catalogue #13-6800, 1: 1000, Life Technologies) at 4 °C overnight. After washing in Tris-buffered saline and Tween-20, the blots were incubated with a horseradish peroxidase-conjugated goat anti-rabbit (1: 3000, catalogue #111-035-003, Jackson ImmunoResearch Laboratories) or goat anti-mouse (1: 3000, catalogue #115-035-003, Jackson ImmunoResearch Laboratories) antibody at room temperature for 1 h. Protein bands were detected using ECL reagents (Perkinelmer) and visualized by a chemiluminescence imaging system. The band density was quantified with Quantity One software 4.6.2 (Bio-Rad Laboratories).

2.5. Immunofluorescent staining

As described in our previous study (Xiao et al., 2015), rats were transcardially perfused through the aorta with normal saline 200 ml followed by 4% paraformaldehyde (PFA) in 0.1 M PB 300 ml. L4 DRG was dissected and post-fixed in 4% PFA for 4–6 h, cryoprotected in 20% and 30% sucrose solutions at 4 °C in turn. Tissues were cut to 8-μm in thickness with a Leica CM 1800 cryostat and thaw-mounted onto gelatin/chrome alum-coated glass slides. After being baked in an incubator at 37 °C, sections were permeabilized in 0.01 M PBS for 30 min and blocked in 10% normal goat serum with 0.3% Triton X-100 for 1 h at room temperature. The sections were incubated with primary rabbit anti-rat $Ca_v3.2$ (1: 200, catalogue #ACC-025, Alomone) and one of the following marker antibodies: mouse anti-rat calcitonin gene-related peptide (CGRP, 1: 1000, catalogue #C7113, Sigma), FITC-conjugated isolectin B4 (IB4, 1: 1000, catalogue #L2895, Sigma), mouse anti-neurofilament 200 (NF200, 1: 1000, catalogue #N0142, Sigma) for 36 h at 4 °C. After washing with PBS, sections were incubated with secondary antibodies at room temperature for 2 h: Alexa Fluor 488-conjugated goat anti-rabbit IgG (1: 500, catalogue #A-11008, Invitrogen), Alexa Fluor 594-conjugated goat anti-rabbit IgG (1: 500, catalogue #A-11012, Invitrogen) and Alexa Fluor 568-conjugated goat anti-mouse IgG (1: 500, catalogue #A-11004, Invitrogen).

Immunofluorescent photographs were taken by a confocal laser scanning microscope (FV1000, Olympus, Germany). For quantification, 6–8 non-consecutive sections from each DRG were imaged and counted. At least 200 labelled cells per rat were analyzed. For the labeling methods of $Ca_v3.2$ membrane expression of, a neuron was

counted as positive if more than half of its circumference was encircled by $Ca_v3.2$-immunoreactitity (-*ir*) (Liu et al., 2012). The total number of $Ca_v3.2$-, NF200-, CGRP-, or IB4-*ir* neurons in the region of interest was counted by Image J 1.5 (Wayne Rasband National Institutes of Health, USA), and the percentage of the colocalization was determined separately.

2.6. Patch clamp recording for calcium currents in acutely dissociated DRG neurons

L4 DRG neurons were acutely dissociated as described in our previous studies (Xiao et al., 2015). Briefly, rats were deeply anesthetized and L4 DRG was freshly dissected. After being washed in cold, oxygenated Dulbecco Modified Eagle Medium (DMEM, Gibco), the neurons were was digested with collagenase type IA (3 mg/ml, Sigma) for 55 min and 0.25% trypsin (Gibco) for 13 min at 37 °C. The digestion was terminated with DMEM containing 10% fetal bovine serum. Next, ganglia were gently dissociated by a fire-polished Pasteur pipette and the suspension was placed on poly-D-lysine (0.5 mg/ml, Sigma)–coated glass coverslips. Cells were cultivated in an incubator with 95% air and 5% CO_2 at 37 °C for 3–4 h before patch clamp recording.

Whole-cell patch T-type calcium current recording was performed as described previously (Jagodic et al., 2007; Zhang et al., 2014). Recording was made using an EPC-10 amplifier with PatchMaster software (HEKA, Freiburg, Germany). Series resistance (Rs) and capacitance (Cm) values were taken from readings of the amplifier after electronic subtraction of the capacitive transients. The external solution contained the following components (in mM): 10 $BaCl_2$, 152 tetraethylammonium (TEA)-Cl, and 10 HEPES, pH adjusted to 7.4 with TEA-OH. The internal solution contained the following components (in mM): 135 tetramethylammonium hydroxide (TMA-OH), 10 ethylene glycol tetraacetic acid (EGTA), 40 HEPES, and 2 $MgCl_2$, pH adjusted to 7.2 with hydrofluoric acid. All chemicals were obtained from Sigma unless otherwise noted. To allow studies of well-isolated T-currents in acutely isolated DRG cells, a fluoride (F)-based internal solution was used to facilitate HVA Ca^{2+} current rundown (Jagodic et al., 2007).

To isolate T-type channel currents, the neurons were held at −70 mV and imposed voltage commands of depolarizing pulses from −110 to −40 mV in 10-mV increments. For activation curve, T-type channel currents were evoked by command voltage steps from the holding potential of −110 mV for 40 ms to test potentials from −60 to −20 mV for 300 ms in 10 mV increments. For inactivation curve, currents were evoked by a test potential to −40 mV for 300 ms with 10 mV increments after a 1 s pre-pulse at potentials ranging from −110 to −30 mV. The amplitude of T-current was measured from the peak to the end of the depolarizing pulse. The voltage dependencies of activation and steady-state inactivation were described with single Boltzmann distributions of the following formulas:

$$\text{Activation}: G(V) = G_{max}/(1 + \exp[-(V - V_{50})/k]);$$

$$\text{Inactivation}: I(V) = I_{max}/(1 + \exp[(V - V_{50})/k]).$$

In these formulas, G_{max} is the maximal conductance, I_{max} is the maximal amplitude of current, V_{50} is the voltage at which half the current is activated or inactivated, and k represents the voltage dependence (slop) of the distribution. The data were analyzed using Origin 8.5 (Microcal Software, Northampton, MA).

2.7. Statistical analysis

Statistical analyses were performed with GraphPad Prism 5.0 (GraphPad Software, *Inc.*, USA). All data were expressed as mean ± SEM. Two-tailed unpaired Student's *t*-test was used for comparison of the mean values between two groups. Two-way ANOVA followed by Bonferroni *post hoc* test was used for multiple comparison. χ^2 test was used to identify differences in the incidence of effects. Differences with $p < 0.05$ were considered statistically significant.

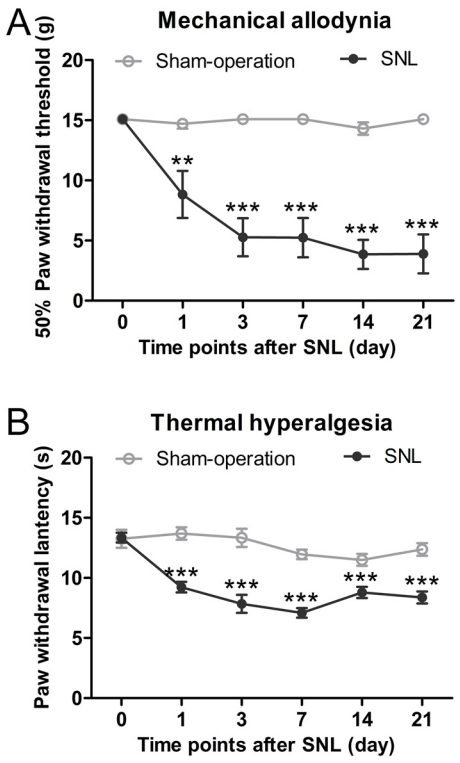

Fig. 1. Establishment of neuropathic pain model of rats with L5 SNL. Mechanical allodynia (A) and thermal hyperalgesia (B) developed on the first day after SNL surgery and lasted for at least 21 days. n = 7–8. $^{**}p < 0.01$, $^{***}p < 0.001$, two-way ANOVA followed by Bonferroni's *post hoc* test.

3. Results

3.1. Establishment of neuropathic pain model of rats with L5 SNL

We first established neuropathic pain model. After L5 SNL, for mechanical allodynia measurement (Fig. 1A), it was found that 50% PWTs decreased significantly in the ipsilateral hindpaw (group effect: $F_{(1,70)} = 63.28$, $p < 0.001$; time effect: $F_{(5,70)} = 12.93$, $p < 0.001$; interaction: $F_{(5,70)} = 11.99$, $p < 0.001$; two-way ANOVA followed by Bonferroni's *post hoc* test). For thermal hyperalgesia measurement (Fig. 1B), the PWLs also decreased in the ipsilateral hindpaw (group effect: $F_{(1,65)} = 114.60$, $p < 0.001$; time effect: $F_{(5,65)} = 11.65$, $p < 0.001$; interaction: $F_{(5,65)} = 6.68$, $p < 0.001$; two-way ANOVA followed by Bonferroni's *post hoc* test). These results suggest a successful neuropathic pain model in rats with L5 SNL.

3.2. Increased total and membrane $Ca_v3.2$ protein expression in intact L4 DRGs after L5 SNL

We examined total $Ca_v3.2$ protein expression with Western blotting in intact L4 DRGs at 3, 7, 14 and 21 days after L5 SNL. As shown in Fig. 2A and C, $Ca_v3.2$ total protein expression increased significantly at day 7 and day 14 after SNL (group effect: $F_{(1,36)} = 9.67$, $p < 0.05$; time effect: $F_{(3,36)} = 2.28$, $p > 0.05$; interaction:

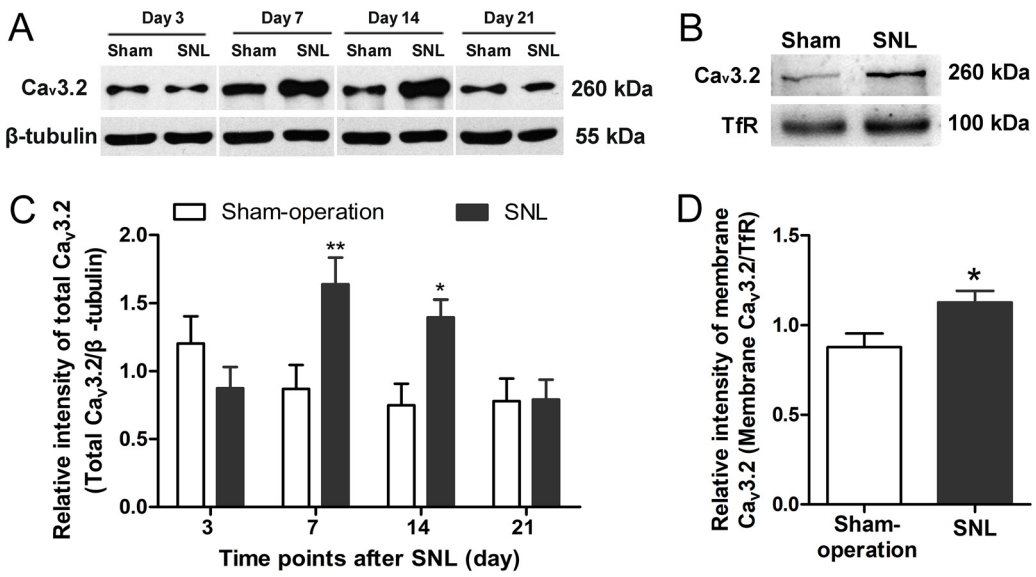

Fig. 2. Increased total and membrane $Ca_v3.2$ protein expression in intact L4 DRGs in rats with SNL-induced neuropathic pain. Representative Western blot bands illustrated total (A) and membrane (B) $Ca_v3.2$ protein levels in L4 DRG, respectively. (C) Total $Ca_v3.2$ protein in L4 DRG increased at day 7 and day 14 after in SNL rats. n = 7. $^*p < 0.05$, $^{**}p < 0.01$, two-way ANOVA with Bonferroni's *post hoc* test. (D) Membrane $Ca_v3.2$ protein in L4 DRG increased 7 days after SNL. n = 6. $^*p < 0.05$, unpaired *t* test.

$F_{(3,36)} = 4.18$, $p < 0.01$; two-way ANOVA followed by Bonferroni's *post hoc* test).

In addition, we selected 7 days after operation as a representative to examine the membrane expression of $Ca_v3.2$ proteins. This process was detected with Western blot on the cell membrane sample which was isolated by high-speed centrifugation from the L4 DRGs. It was shown that the membrane expression of $Ca_v3.2$ increased in intact L4 DRG 7 days after SNL (1.13 ± 0.06 *vs.* 0.88 ± 0.08, SNL group *vs.* Sham-operation group, $t = 2.54$, $p < 0.05$, unpaired *t* test, Fig. 2B and D).

3.3. Increased T-type calcium channel currents in intact L4 DRG neurons

The $Ca_v3.2$ subtype is the predominant T-type channel expressed in DRG neurons and implicated in nociceptive signaling (Sierra et al., 2017). Next, we examined T-type calcium channel currents in intact L4 DRG neurons. The evoked T-type currents were measured in small (<30 μm diameter) DRG neurons including 12 cells from Sham-operated rats and 11 cells isolated at 7 days after SNL. There was no significant difference in the percentage of DRG neurons showing T-type current between SNL rats and Sham-operation rats (SNL: 11/30, 36.67%; Sham-operation: 12/35, 34.29%; $p > 0.05$, χ^2 test). The capacitance of the neurons in SNL group (24.98 ± 2.20 pF) was similar to that in Sham-operation group (21.77 ± 2.20 pF, $t = 1.03$, $p > 0.05$, unpaired *t* test). Representative traces in SNL and Sham-operation groups were shown in Fig. 3A. Consistent with the increase of $Ca_v3.2$ membrane protein, T-type calcium current densities of L4 DRG neurons were higher in SNL group than those in Sham-operation group (7.09 ± 0.93 pA/pF *vs.* 4.00 ± 0.34 pA/pF, SNL group *vs.* Sham-operation group, $t = 3.23$, $p < 0.01$, unpaired *t* test, Fig. 3B).

Furthermore, the activation curve was hyperpolarized, *i.e.*, the V_{50} left-shifted from -32.65 ± 1.01 mV in the Sham-operation group to -36.86 ± 1.59 mV in the SNL group ($t = 2.24$, $p < 0.05$, unpaired *t* test, Fig. 3C). There was no obvious difference in inactivation curves between SNL (-65.47 ± 2.11 mV) and Sham-operation groups (-62.33 ± 1.41 mV, $t = 1.28$, $p > 0.05$, unpaired *t* test, Fig. 3D). These results indicate that $Ca_v3.2$ channels are functionally unregulated in intact L4 DRG neurons after SNL.

3.4. Increased $Ca_v3.2$ membrane expression in L4 DRG neurons

In order to visually observe the $Ca_v3.2$ membrane expression, we next examined the cellular localization of the $Ca_v3.2$ proteins in L4 DRG neurons 7 days after SNL. Immunofluorescent staining showed that $Ca_v3.2$ protein was abundant in DRG neurons in both Sham-operation and SNL groups. More importantly, there was a much higher expression level of $Ca_v3.2$ on the neuronal membrane in SNL rats in comparison to the Sham-operation rats (Fig. 4A, B). After quantitative analysis, the percentage of neurons with $Ca_v3.2$ membrane expression increased significantly in SNL group ($19.66 \pm 1.67\%$) than that in Sham-operation group ($9.39 \pm 0.96\%$, $t = 5.33$, $p < 0.001$, unpaired *t* test, Fig. 4C).

We further identified the neurochemical cell subtypes of increased $Ca_v3.2$ trafficking to membrane, double immunofluorescent staining of $Ca_v3.2$ with CGRP, IB4 or NF200 was performed. Small unmyelinated C fiber DRG neurons can be classified into two subtypes: the peptidergic neurons marked by CGRP and the non-peptidergic ones marked by IB4; large myelinated A fiber neurons can be marked with NF200 (Liu et al., 2012). Immunofluorescent staining showed that membrane expression of $Ca_v3.2$ increased significantly in all three subtypes of L4 DRG neurons in SNL rats compared with that in the Sham-operation rats. The percentage of membrane expressing $Ca_v3.2$ in CGRP-, IB4- and NF200-positive neurons in the Sham-operation group was $1.88 \pm 0.38\%$, $2.34 \pm 0.28\%$ and $4.15 \pm 0.56\%$, respectively; while in the SNL group, the percentage significantly increased to $5.37 \pm 1.01\%$ ($t = 3.24$, $^*p < 0.05$, unpaired *t* test), $7.36 \pm 1.85\%$ ($t = 2.68$, $^*p < 0.05$, unpaired *t* test) and $7.87 \pm 0.85\%$ ($t = 3.66$, $^{**}p < 0.01$, unpaired *t* test), respectively (Fig. 5D–F).

4. Discussion

In this study, we examined molecular and functional changes of $Ca_v3.2$ T-type calcium channels in the L4 intact neurons adjacent to the injured nerve in neuropathic pain rats with SNL. Our results demonstrate increased membrane expression of $Ca_v3.2$ proteins and T-type currents in L4 DRG neurons. These findings provide evidence for a novel ionic mechanism of peripheral neuropathic pain in the L4 DRG neurons.

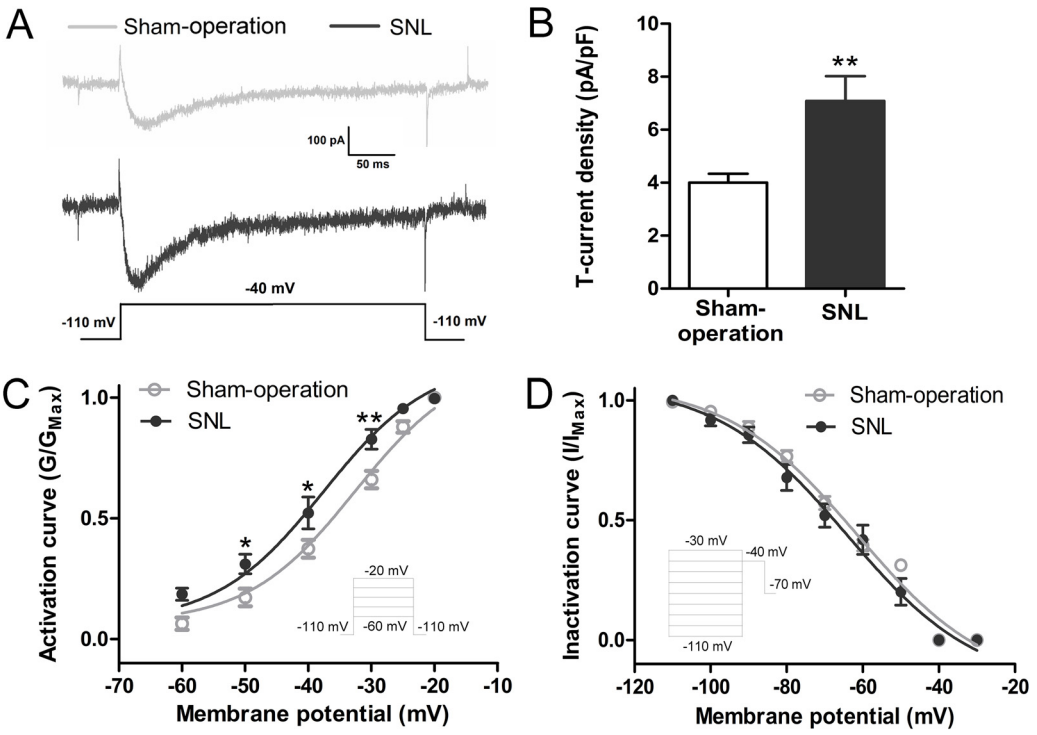

Fig. 3. Upregulation of the $Ca_v3.2$ T-type calcium currents in intact L4 DRG neurons in SNL rats. (A) Representative traces of T-type calcium currents in the L4 DRGs in Sham-operation group and 7 days after SNL. The T-type channel currents were elicited by a 300-ms depolarizing step pulse from the holding potential of −110 mV to a test potential of −40 mV. (B) T-type calcium current density (in pA/pF) increased in SNL rats compared with that in Sham-operation rats. Sham-operation group: n = 12; SNL group: n = 11. $^{**}p < 0.01$, unpaired *t* test. (C) The activation curve showed a shift to left of steady-state activation, suggesting the channel availability increased after SNL. n = 8. $^{*}p < 0.05$, $^{**}p < 0.01$, unpaired *t*-test. (D) The inactivation curve showed no obvious difference between SNL group and Sham-operation group. Sham-operation group: n = 11; SNL group: n = 9.

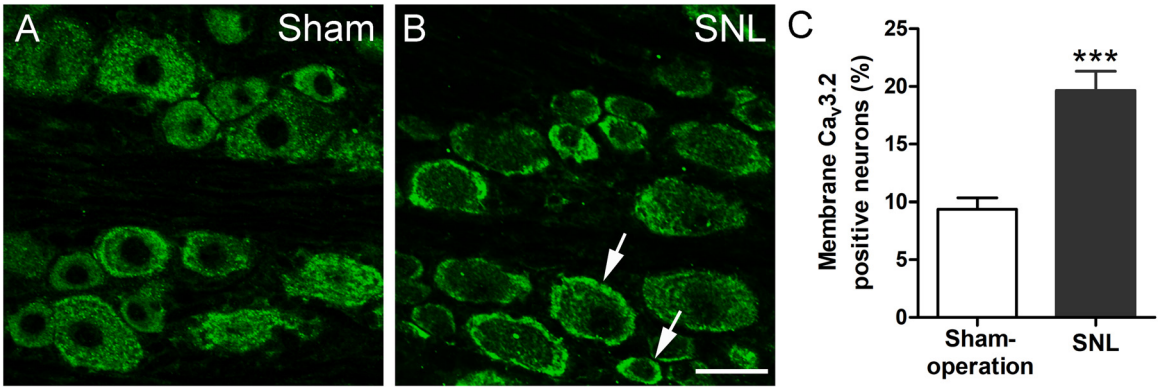

Fig. 4. Increased membrane expression of $Ca_v3.2$ in L4 DRG neurons in neuropathic pain rats 7 days after L5 SNL. (A–B) Representative immunofluorescent images. (C) Quantification of the membrane $Ca_v3.2$ positive neurons in total number of neurons, the expression of $Ca_v3.2$ in cell surface increased in SNL group compared to Sham-operation group. Arrows indicate representative neurons with membrane expression of $Ca_v3.2$. Scale bar = 50 μm. n = 5. $^{***}p < 0.001$, unpaired *t* test.

In L5 spinal nerve injury model of rats, L4 DRG neurons and its fibers play roles in neuropathic pain. Owing to L4 fibers comingle with the degenerating L5 axons in the distal nerves, Wallerian degeneration of injured nerves may sensitize the adjacent uninjured afferent fibers and induce phenotypic and functional changes in the L4 DRG neurons (Meyer and Ringkamp, 2008; Wu et al., 2002). The incidence of abnormal spontaneous activity is higher from L4 DRGs (Ma et al., 2003) and uninjured C-fiber nociceptors (Wu et al., 2002) after L5 nerve injury. Furthermore, injured and uninjured nerves have overlapping projecting regions in the dorsal horn, which show augmented responses to activation of the intact L4 nerves (Shehab et al., 2015; Shehab et al., 2008). Prior elimination of neighboring L4C afferents by local capsaicin treatment prevents mechanical hyperalgesia in rats with SNL (Jang et al., 2010).

The underlying mechanisms of peripheral sensitization in uninjured neurons are not fully understood, ion channels that regulate excitability of neurons may participate this process. For example, an increase expression of HCN2 channels (Smith et al., 2015) and TRPV1 (Fukuoka et al., 2002) in undamaged L4 DRG neurons contribute to the generation of spontaneous activity. The $Na_v1.8$ redistributed in uninjured axons also plays an important role in pain hypersensitivity (Gold et al., 2003).

T-type calcium channels in cell distribution and functional roles have been well described in chick sensory neurons (Fox et al., 1987). *In situ* hybridization analysis of the $Ca_v3.x$ gene family indicated that $Ca_v3.2$ exhibited high level in nociceptive sensory neurons (Talley et al., 1999), and $Ca_v3.2$ channels modulated nociception at various peripheral nerve injury models (Jagodic et al., 2008;

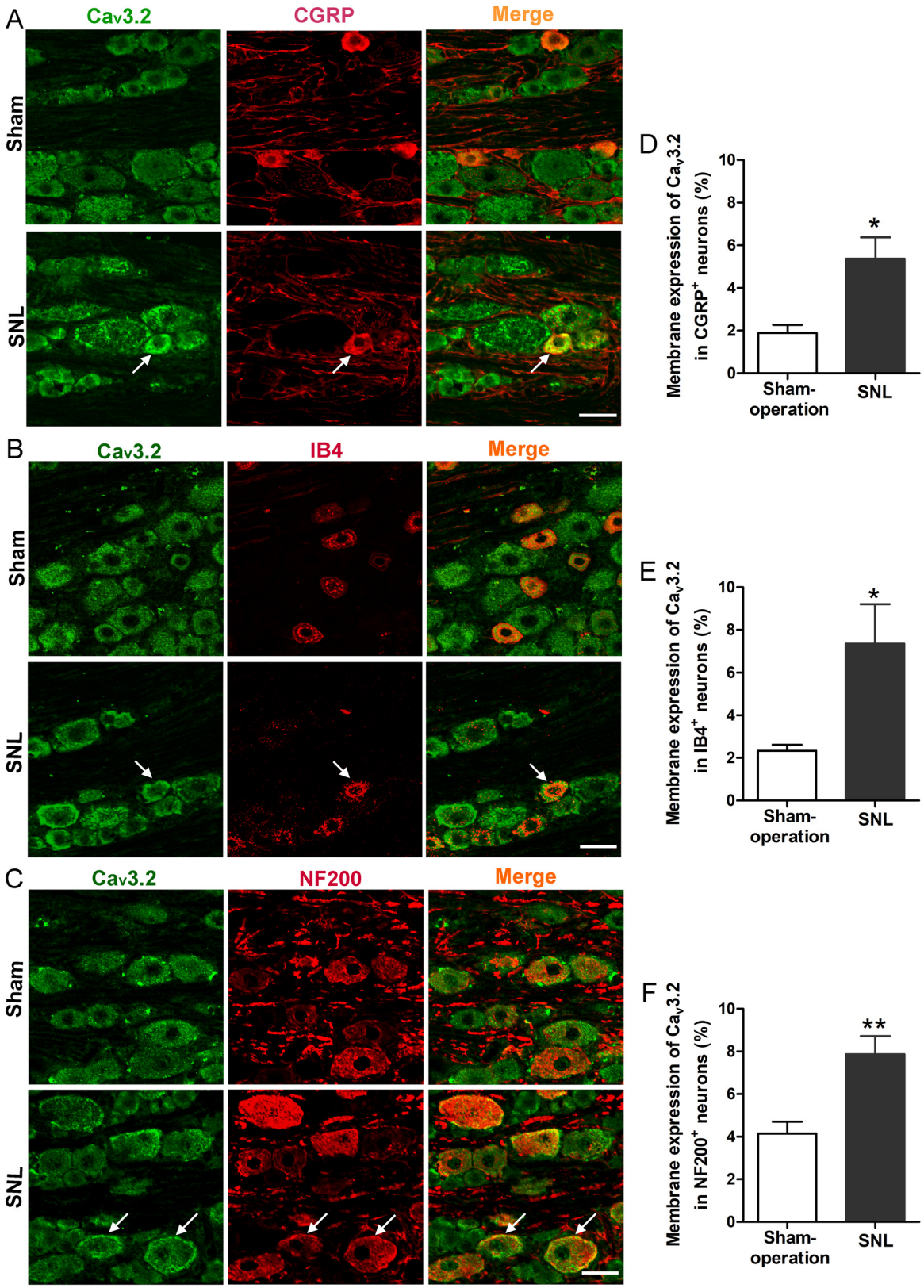

Fig. 5. Increased co-localization of membrane expressing $Ca_v3.2$ in three types of L4 DRG neurons 7 days after L5 SNL. (A–C) Examples of membrane $Ca_v3.2$ co-localized in CGRP-, IB4- and NF200-*ir* neurons, respectively, in the L4 DRGs of Sham-operation and SNL rats. (D–F) Statistical results. In SNL rats, the co-locolization of membrane $Ca_v3.2$ increased in all three subtypes of L4 DRG neurons. Arrows indicate representative neurons with double staining. Scale bar = 50 μm. n = 5. $^*p < 0.05$, $^{**}p < 0.01$, unpaired *t* test.

Takahashi et al., 2010; Wen et al., 2010). In rats subjected to L5 spinal nerve cutting, the protein levels of $Ca_v3.2$ in the ipsilateral L4, L5 and L6 DRG neurons were upregulated (Takahashi et al., 2010). Following SNL, functional T-type Ca^{2+} channels increased in the injured L5 DRG, and blockade or genetic silencing of $Ca_v3.2$ channels reduced neuronal hyperexcitability and nociceptive behaviors

in rats with nerve injury (Snutch and Zamponi, 2017; Yue et al., 2013). Recently, we reported that accumulation of $Ca_v3.2$ T-type Ca^{2+} channels, which are active near resting membrane potential and treated as a "threshold" ion channels for producing and boosting neuronal excitability (Bourinet et al., 2016), in the uninjured sural nerve play important roles in peripheral sensitization and contribute to neuropathic pain (Chen et al., 2018). Here, we focused on $Ca_v3.2$ channels in the intact L4 neurons after L5 SNL.

Our results show increased expression of $Ca_v3.2$T type calcium channels in the L4 neurons after L5 SNL. Western blot showed that $Ca_v3.2$ increased in L4 DRG mainly in the mid-term of SNL. Protein levels of $Ca_v3.2$ increased at day 7 and day 14, and returned to the basal levels at day 21 after SNL. Several other researchers reported similar changes. For example, the spontaneous activity develops 8–10 days in uninjured C-fibers after L5 ventral rhizotomy (Wu et al., 2002), L4 Aβ-nociceptive neurons become sensitized in rats 7 days after L5 spinal nerve axotomy (Djouhri, 2016). Our previous study also showed that on day 14 after SNI, the Aβ-, Aδ- and C-fibers in uninjured nerves were sensitized, which were accompanied by accumulation of $Ca_v3.2$ (Chen et al., 2018). These evidence suggest $Ca_v3.2$ increased in the mid-term after SNL in L4 fibers or DRG neurons and thus might contribute to the maintenance of neuropathic pain.

Increased membrane expression of $Ca_v3.2$ parallels functional upregulation of $Ca_v3.2$ in L4 DRG neurons, as confirmed by our electrophysiological results: the densities of T-type currents are increased, and shifting to left of the activation curve of T-type channels indicates that the channels became easier to open to enhance neuronal excitability (Fig. 3). In our study, we found the hyperpolarizing shifting of activation curve of $Ca_v3.2$ ion channels (Fig. 3). The possible molecular mechanisms underlying this regulation of T-type channel activity are interesting. It has been reported that T-type Ca^{2+} channels can be regulated by several protein kinases such as CaMKII and rho-associated kinase. The intracellular domain II–III linker of the T type Ca^{2+}-channel is considered to be a key site for modulation by several types of serine threonine/kinases, the serine residue 1198 in this linker can be phosphorylated by CaMKII. Thus the function of the channels is subsequently potentiated (Welsby et al., 2003). The serine and threonine sites in the II–III linker region of the $Ca_v3.1$ T-type calcium channel are also regulated by rho-associated kinase (Iftinca et al., 2007). Whether these kinases have regulatory effects on the T-type Ca^{2+} channels in DRG neurons in neuropathic pain rats is interesting and needs further investigation.

In the processing of nociceptive information, small-diameter DRG neurons and its C fibers are crucial in physiological and pathological pain conditions (Sun et al., 2005a,b). Large-diameter DRG neurons and its fibers usually process touch information in physiological conditions and are also important in tactile allodynia following peripheral nerve injury (Djouhri, 2016; Xu et al., 2015). We also showed the percentage of neurons with membrane expressing $Ca_v3.2$ increased in small C fiber (CGRP and IB4) neurons and large A fiber (NF200) neurons (Fig. 5).

In the present study, we provide preliminary evidence for the possible role of $Ca_v3.2$ T-type channels in L4 DRG neurons after L5 SNL. By the surgery procedure, the L4 spinal nerve did not experience ligation, or in other words, the L4 DRG was "intact". However, L4 DRG neurons are not completely spared after L5 SNL, since a small portion of the adjacent L4 DRGs is immunostained with ATF3, a neuronal injury marker (Fukuoka et al., 2012). We also observed approximately 10% ATF3-immunostaining nuclei in L4 DRG after L5 SNL (data not shown). Thus, it is not possible to completely exclude the role of the injured neurons in intact L4 DRG neurons and their nerve fibers in neuropathic pain.

In our electrophysiological patch clamp recording on the isolated L4 DRG, the neurons we recorded were all ATF3-negative staining (data not shown). This is probably for two reasons: first, the low percentage of the injured neurons; two, those injured neurons could not bear the mechanical dissociation, collagenase or trypsin digestion and thus were broken during the neuron isolation procedure.

The $Ca_v3.2$ is the predominant subtype of T-type channels in DRG neurons (Talley et al., 1999; Chen et al., 2003; Bourinet et al., 2005). Our present study also found that upregulation of expression of the $Ca_v3.2$ T-type calcium channel in L4 DRG neurons in neuropathic pain rats with L5 SNL. In addition to the $Ca_v3.2$, $Ca_v3.1$ and $Ca_v3.3$ subtypes may also contribute to the increased T-type currents. Our results cannot exclude whether $Ca_v3.1$ and $Ca_v3.3$ are involved, and it needs further investigation.

Further pharmacological blockade experiments using $Ca_v3.2$ blockers or gene silencing siRNA agents directly to L4 DRG or its spinal nerve should be performed. But in the present study we failed to do so because of the technical limitations: L4 spinal nerve ligation in rats produced conspicuous flexor weakness (Rigaud et al., 2008), intra-DRG injection had potential injury to DRG neurons histologically (Chang et al., 2016), and even induced hyperalgesia behaviorally in rats (Fischer et al., 2011; Puljak et al., 2009). Once the movement ability is affected, it is impossible to access the movement-based pain behavior in SNL model.

In conclusion, our study demonstrates that membrane expression and function of $Ca_v3.2$ T-type calcium channels increases in the intact L4 DRG neurons in neuropathic pain rats with L5 SNL.

Conflict of interests

Authors declare no conflicts of interests.

Authors' contribution

QYL, FYL and YW designed the experiment; QYL, WC, SC and FFL performed the experiments; QYL, FYL and MY analyzed the data; QYL, FYL and YW wrote the manuscript.

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