

# Enhanced Expression of TREK-1 Is Related with Chronic Constriction Injury of Neuropathic Pain Mouse Model in Dorsal Root Ganglion

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## Abstract

Neuropathic pain is a complex state showing increased pain response with dysfunctional inhibitory neurotransmission. The TREK family, one of the two pore domain K<sup>+</sup> (K2P) channel subgroups were focused among various mechanisms of neuropathic pain. These channels influence neuronal excitability and are thought to be related in mechano/thermosensation. However, only a little is known about the expression and role of TREK-1 and TREK-2, in neuropathic pain. It is performed to know whether TREK-1 and/or 2 are positively related in dorsal root ganglion (DRG) of a mouse neuropathic pain model, the chronic constriction injury (CCI) model. Following this purpose, Reverse Transcription Polymerase Chain Reaction (RT-PCR) and western blot analyses were performed using mouse DRG of CCI model and compared to the sham surgery group. Immunofluorescence staining of isolectin-B4 (IB4) and TREK were performed. Electrophysiological recordings of single channel currents were analyzed to obtain the information about the channel. Interactions with known TREK activators were tested to confirm the expression. While both TREK-1 and TREK-2 mRNA were significantly overexpressed in DRG of CCI mice, only TREK-1 showed significant increase (~9 fold) in western blot analysis. The TREK-1-like channel recorded in DRG neurons of the CCI mouse showed similar current-voltage relationship and conductance to TREK-1. It was easily activated by low pH solution (pH 6.3), negative pressure, and riluzole. Immunofluorescence images showed the expression of TREK-1 was stronger compared to TREK-2 on IB4 positive neurons. These results suggest that modulation of the TREK-1 channel may have beneficial analgesic effects in neuropathic pain patients.

**Key Words:** Neuropathic pain, Chronic constriction injury model, Dorsal root ganglion, Isolectin-B4, TREK-1 expression

## INTRODUCTION

The TREK family, one of the two pore domain K<sup>+</sup> (K2P) channel subgroups, is a promising starting point for the pain researchers who study the modulation of pain. While the mechanisms underlying neuropathic pain remains unclear, it is believed that nerve hyperexcitability is the major cause of the pain. Based on this, profound new therapeutic approaches are focused on the nerve excitability control.

Two-pore domain K<sup>+</sup> (K2P) channels are background K<sup>+</sup> channels that have ability to set the resting membrane potential and influence the neuronal excitability. Among the six subfamilies of K2P channels, TREK subfamily is thought to be related in mechano or thermosensation as they are reversely activated by a variety of environmental factors such as heat and mechanical stretch (Kim and Chung, 1992; Duprat *et al.*, 2000). These channels are also being implicated in nociception in inflammatory conditions. TREK-1 mRNA expression in mouse DRG neurons were decreased after induced chemi-

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**Table 1.** Primer sequences used for RT-PCR

Name (Channel)	Primer sequences (5' to 3')	GenBank Accession Nos.	Expected size (bp)
GAPDH	Forward: CTAAAGGGCATCCTGGGC Reverse: TTAATCCTTGGAGGCCATG	NM_017008	201
KCNK2 (TREK-1)	Forward: TGCCAAAGTGGAGGACACAT Reverse: CTCTCCACCTCTTCTTCG	AF325671	361
KCNK10 (TREK-2)	Forward: CAGCCCAAGAGTGCCACTAA Reverse: GGATCCCAAGATGGCGTAT	NM_023096	493

cal colitis (La and Gebhart, 2011). TREK-1 knockout mice displayed increased response to thermal and mechanical stimuli suggesting the development of thermal hyperalgesia and allodynia (Alloui *et al.*, 2006). Selective TREK-2 expression was observed in IB4 binding c-fibers in rat, and TREK-2 knockdown showed decreased spontaneous pain behaviors (Acosta *et al.*, 2014).

Although multiple studies demonstrated that TREKs play an important role in neuronal excitability and pain conduction in neuronal tissues under physiologic conditions (Ocaña *et al.*, 2004; Alloui *et al.*, 2006; Huang and Yu, 2008; Franks and Honoré, 2004), or in trigeminal neuropathy (Takeda *et al.*, 2011), thus far, the expression of TREK in the DRG of a neuropathic pain model has not yet been assessed. We assessed the expression of TREK-1 and TREK-2 in the DRG neurons of CCI mice, obtained immunofluorescence images using markers for IB4 and TREK, and performed electrophysiological studies.

## MATERIALS AND METHODS

### Animals, husbandry and ethical approval

The official permission number for those animal experiments of the Institutional Animal Care and Use Committee of Seoul National University is SNU-110719-3. All experiments were performed on 7-8 week-age ICR mice (Orient Bio Inc., Seoul, Korea). The mice were housed 5 per cage and allowed free access to food and water. The cages were covered with soft bedding and maintained in an environmental room equipped with a programmed 12 h light cycle (7 am/7 pm) at constant temperature (20°C) and humidity (60%). Animals were given anesthesia before surgery using 3% isoflurane and sacrificed according to AVMA Guidelines for the Euthanasia of Animals: 2013 Edition after the completion of experiments. All of the experimental procedures were performed according to the ethical guidelines for the use of animals in research of the International Association for the Study of Pain (IASP) and the Institutional Animal Care and Use Committee of Seoul National University.

### Chronic constriction injury (CCI) model

The CCI model of ICR mice were generated as described previously (Lee *et al.*, 2013). A total of 52 mice were randomly divided into two experimental groups: 1- sham-operated group and 2-CCI group. Mice of two those groups were anaesthetized with 3% isoflurane. The left hindlimb was incised at the level of the middle thigh and a section was cut through the biceps femoris. The muscle was retracted and the common sciatic nerve was exposed. Proximal to the sciatic nerve trifur-

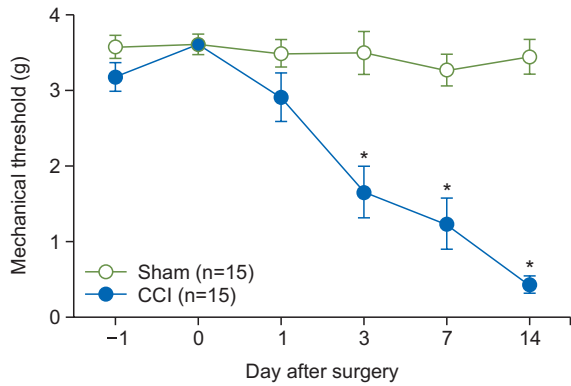
cation, the nerve was freed of adhering muscle and four loose ligatures of 6-0 chromic gut (W812, Ethicon Inc., Somerville, NJ, USA) were tied 1 mm apart. The knots were slowly tightened until a brief twitch in the hindlimb was observed. After nerve ligation, the muscle and skin were closed with 6-0 silk (W802, Ethicon Inc.).

### Behavioral tests

All behavioral tests were performed as described previously (Lee *et al.*, 2010). All behavioral tests were performed and recorded on days-1 (the day before surgery), 0 (before surgery) and 1, 3, 7, 14 after surgery. All tests were conducted by an investigator blinded as to whether the animal was in the CCI or Sham group. To assess the mechanical allodynia, the plantar surface of the left hind paw was stimulated using von Frey monofilaments. The animals were housed in transparent Plexiglass boxes (5×10×5 cm) placed on an elevated floor of metal mesh that allowed von Frey filaments to be applied to the left hind paw from below. At least 30 min after habituation, the von Frey monofilaments were applied perpendicular to the whole plantar surface of the paw. Each filament was tested five times at intervals of 5 s before changing filaments. The threshold was taken as the lowest force that evoked a brisk withdrawal response to one of five repetitive stimuli.

### Isolation of dorsal root ganglia and a culture of DRG neurons

DRGs for culture were dissected from the lumbar spinal cord (L3-L6) of mice. Cultured DRG neurons were prepared as described previously (Oh *et al.*, 1996). Ganglia were collected in cold (4°C) Dulbecco's modified Eagles' medium (DMEM) containing 10% FBS (Invitrogen, Grand Island, NY, USA), 1 mM sodium pyruvate, 10 U/ml penicillin (Invitrogen), and 10 µg/ml streptomycin (Invitrogen). These were washed three times with DMEM and incubated for 30 min in DMEM containing 1 mg/ml collagenase (Type II; Worthington, Freehold, NJ, USA). Ganglia were then washed three times with Mg<sup>2+</sup>- and Ca<sup>2+</sup>-free Hank's Balanced Salt Solution (HBSS) and were incubated with gentle shaking in warm (37°C) HBSS containing 2.5 mg/ml trypsin (Invitrogen). The solution was centrifuged at 1,000 rpm (194 g) for 10 min. Next, the pellet was washed three times with DMEM containing 10% FBS to inhibit enzyme activity. The pellet was then suspended in culture medium and gently triturated with a heat-polished Pasteur pipette. The suspension was plated on glass coverslips coated with poly-L-lysine and placed in a culture dish. Neurons were incubated at 37°C in a 95% air-5% CO<sub>2</sub> gas mixture. The cells were used 1-5 days after plating.



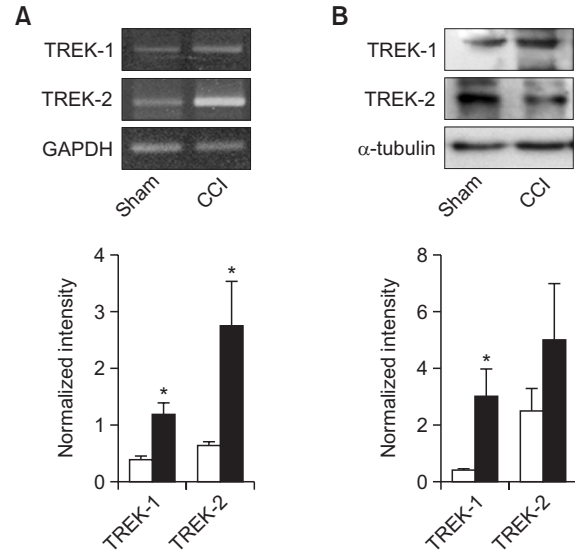
**Fig. 1.** Behavior test of CCI and sham mice. The CCI mice showed a significantly reduced threshold level to mechanical stimuli after surgery compared to the sham group, indicating the development of allodynia. \* $p < 0.05$  vs. the sham group.

**Reverse transcription polymerase chain reaction (RT-PCR) analysis**

RT-PCR was performed as described previously (Gardener *et al.*, 2004). Total RNA was isolated respectively from DRGs of sham group and CCI group using TRIzol Reagent (Invitrogen). First-strand cDNAs were synthesized from total RNA isolated from DRGs using oligo (dT) (RT-&GO Mastermix, Qiogene, Cambridge, UK) and were then used as a template for PCR amplification. Specific primers for TREK-1 and TREK-2 channels were used in PCR reactions with Taq polymerase (G-Taq™, Cosmo Genetech, Seoul, Korea). Table 1 lists the DNA sequences of the primers used to detect the expression of the TREK-1 and TREK-2 channels. PCR was conducted in a final reaction volume of 30  $\mu$ l containing 1  $\mu$ l (~50 ng) of diluted first-strand cDNA. The PCR conditions included an initial denaturation at 94°C for 5 min, followed by 30 cycles at 94°C for 30 s, 57°C for 45 s, and 72°C for 45 s, and a final extension step at 72°C for 10 min. The PCR products were directly sequenced with the ABI PRISM® 3100-Avant Genetic Analyzer (Applied Biosystems, CA, USA).

**Western blot analysis**

Western blot was performed as described previously (Acosta *et al.*, 2014). The DRGs of CCI mice, respectively, were homogenized in lysis buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 10% glycerol, 100 mM NaF, 0.2 mM Na-orthovanadate, 0.5% NP-40, 1.5 mM MgCl<sub>2</sub>, 1 mM EGTA, 1 mM dithiothreitol, 1 g/ml leupeptin, 10 mM benzamide, 1 g/ml pepstatin A, 1 mM phenylmethylsulfonyl fluoride, and 10.5 g/ml aprotinin. The samples were incubated for 20 minutes on ice with intermittent vortexing. The extracts were clarified by centrifugation at 14,000 rpm (19,300 g) for 15 min at 4°C. The resulting supernatant was separated using a 10% SDS-polyacrylamide gel and transferred to a nitro-cellulose membrane for 30 min using a semi-dry transfer (Bio-Rad, CA, USA). The membranes were blocked with 5% fat-free dry milk and then incubated with a TREK-1 (1:500 dilution, Chemicon, CA, USA) or TREK-2 (1:1000 dilution, Alomone Labs, Jerusalem, Israel) polyclonal antibody and  $\alpha$ -tubulin polyclonal antibody (1:1000 dilution, Sigma, MO, USA). This was followed by incubation with a secondary peroxidase-conjugated anti-rabbit antibody at 1:2000 (Sigma, MO, USA). Immuno-positive bands were



**Fig. 2.** Upregulation of TREK expression in the DRG isolated from CCI mice. (A) The expression of TREK-1 and TREK-2 mRNA. PCR was performed on cDNA templates obtained from the DRG of sham and CCI mice. (B) Western blot analysis in DRG. The TREK-1 and TREK-2 protein levels increased in the DRG obtained from the CCI mice. Equal amounts (25  $\mu$ g) of total protein were loaded in each lane. The bar graphs summarize the levels of TREK mRNA and protein expression, which changed after injury. Each bar represents the mean  $\pm$  SD of four repeated experiments. The asterisks indicate significant differences from the corresponding control value (sham-operated groups, \* $p < 0.05$ ).

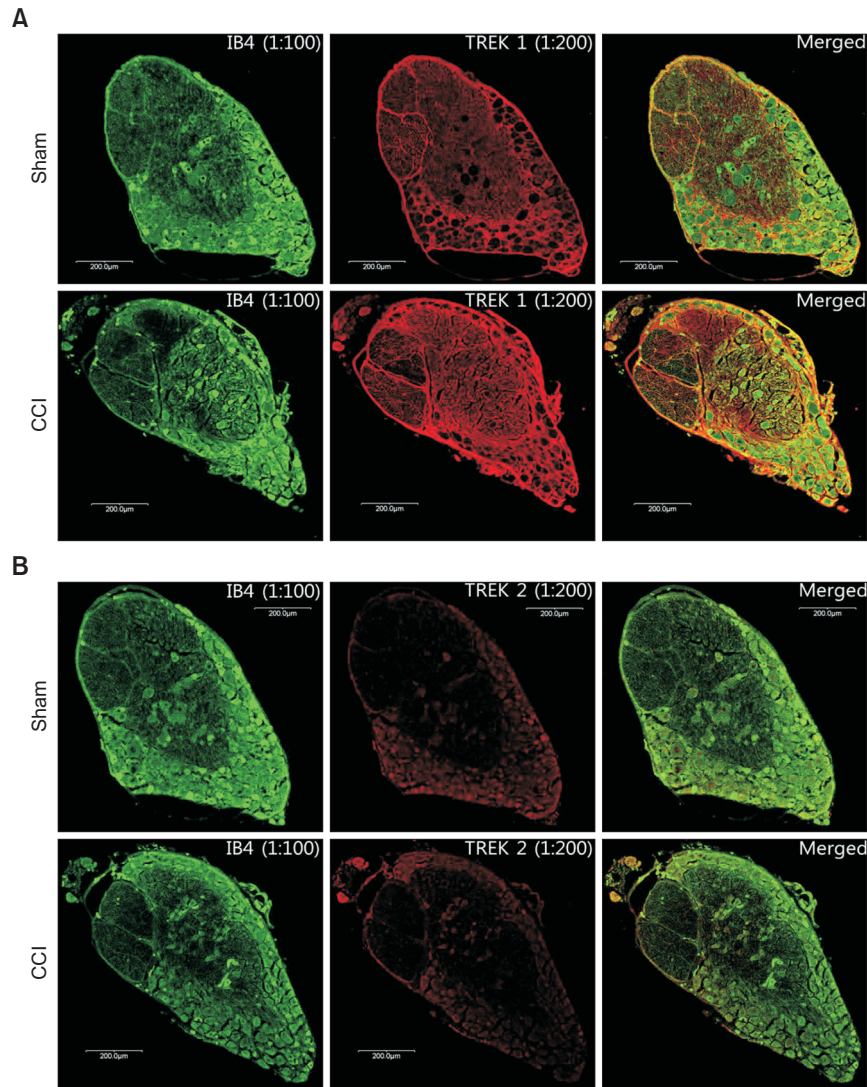
visualized by enhanced chemiluminescence (ECL Plus kit, ELPIS, Daejeon, Korea) following the manufacturer’s instructions.

**Immunofluorescence**

Immunofluorescence was performed as described previously (Acosta *et al.*, 2014). DRG neurons of CCI mice were harvested and postfixed with 4% PFA overnight. Tissues were cryoprotected with 30% sucrose in PBS for one day at 4°C. Cryoblock was made of OCT embedding compound on dry ice with 99% ethyl alcohol. Cryosections were cut to 8  $\mu$ m in thickness. For TREK-1, TREK-2, IB4 immunofluorescence staining, the sections were rinsed in PBS and incubated with biotin conjugated IB4 mixture (1:100, invitrogen) for 1h. After rinsing, H<sub>2</sub>O<sub>2</sub> block and UltraVblock treated to the each section for 20 min. Then IB4 treated sections were incubated with a mixture of TREK-1 or TREK-2 (1:100, santa cruz) overnight at 4°C. After rinsing in PBS, they were incubated 1h with secondary antibodies; Anti-rabbit IgG conjugated with Cy3 (1:1000) to the primary TREK antibody and Streptavidin conjugated with DTAF (1:1000, Jackson Lab, Sacramento, USA) to the IB4. All sections were rinsed in PBS and mounted onto cover glass with per mount solution. Images were taken by a confocal laser scanning microscope (Olympus FV-300, Tokyo, Japan).

**Electrophysiological studies**

Electrophysiological studies were performed as described previously (Kim *et al.*, 2011). Electrophysiological recording was performed using a patch clamp amplifier (Axopatch 200, Axon Instruments, Union City, CA, USA) at 24°C. Single-chan-



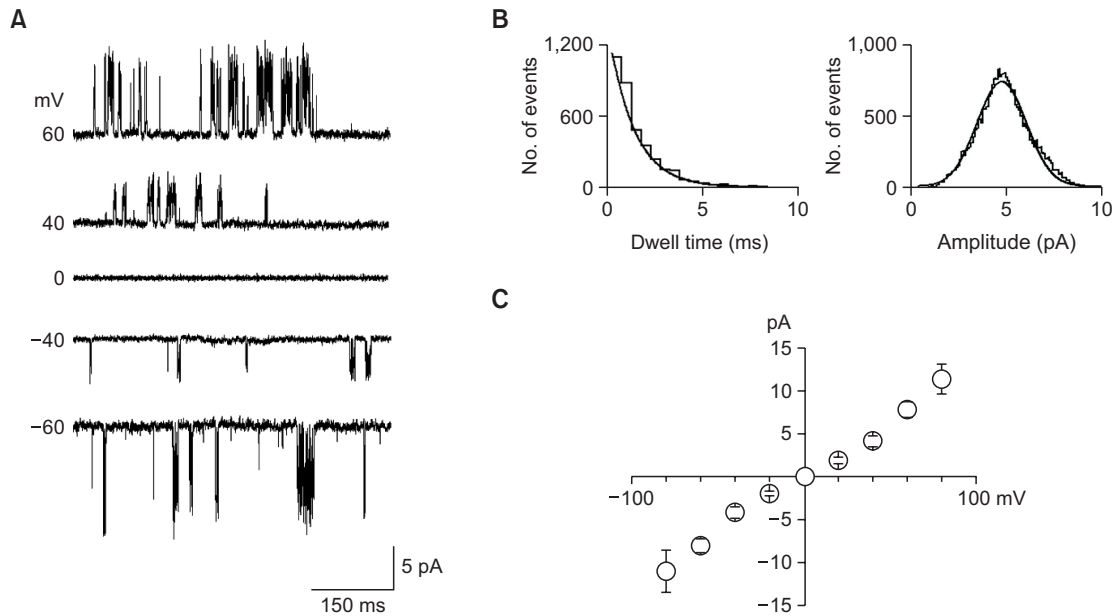
**Fig. 3.** Immunofluorescence staining of DRG neurons. DRG neurons from the control and the CCI groups are stained using a marker for IB4 (green) and TREK (red). (A) Double immunofluorescence indicates a partial colocalization between the IB4+ neuron and TREK-1. (B) The expression of TREK-2 was weak on both control and CCI groups, and little colocalization was observed between IB4+ and TREK-2. The expression of TREK-1 was stronger compared to TREK-2 on IB4+ CCI DRG neurons.

nel currents were digitized with a digital data recorder (VR10, Instrutech, Great Neck, NY, USA) and stored on videotape. The recorded signal was filtered at 2 kHz using an 8-pole Bessel filter (-3 dB; Frequency Devices, Haverhill, MA, USA) and transferred to a computer using the Digidata 1320 interface (Axon Instruments, Union City, CA, USA) at a sampling rate of 20 kHz. The threshold detection of the channel openings was set at 50%. Single channel currents were analyzed with the pCLAMP program (version 9, Axon). The filter dead time was 100  $\mu$ s (0.3/cutoff frequency) for single-channel analysis; therefore, events lasting less than 50  $\mu$ s were not detected. Data were then analyzed to obtain a duration histogram, amplitude histogram, and description of the channel activity ( $NP_o$ , where N is the number of channels in the patch and  $P_o$  is the probability of a channel being open).  $NP_o$  was determined from ~1-2 min of current recording. The single-channel current tracings shown in the figures were filtered at 2 kHz. In experi-

ments using cell-attached patches and excised patches, the pipette and bath solutions contained the following (mM): 150 KCl, 1 MgCl<sub>2</sub>, 5 EGTA, and 10 HEPES (pH 7.3). The pH was adjusted to 7.3. Stock solutions of riluzole (0.1 M) were made in dimethylsulfoxide; the final solutions were made regularly before use. Channel sensitivities were tested using known TREK-1 activators; low pH (pH<sub>i</sub> 6.3), negative pressure (-40 mmHg), and riluzole. All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise stated.

#### Data analysis

The bands obtained from PCR and western blot were quantified by Sigma Gel image analysis software (version 1.0, Jandel Scientific, CA, USA) and Quantity One software (version 4.6.3) attached to GS-800 Calibrated densitometer (Bio-Rad, CA, USA). The relative mRNA and protein levels were calcu-



**Fig. 4.** Single-channel kinetics of TREK-1-like  $K^+$  channels in DRG neurons isolated from CCI mice. (A) Cell-attached patches were formed, and single-channel openings were recorded at the different membrane potentials, as noted on the left. The pipette and bath solutions contained 150 mM KCl. The patches showed a noisy pattern in the open state with large current fluctuations. (B) Histograms of the open time duration (left) and amplitude (right) of the TREK-1-like  $K^+$  channel were obtained from openings at -40 mV. They were fitted by single exponential and Gaussian functions, respectively. (C) Single-channel amplitudes were determined from the amplitude histogram for each membrane potential to construct the current-voltage relationship. Each point represents the mean  $\pm$  SD of five repeated experiments. A relatively linear current-voltage relationship was obtained, which is also typical of TREK-1.

lated by reference to the amount of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and  $\alpha$ -tubulin, respectively.

**Statistics**

Student's *t*-test was used for comparison, and  $p < 0.05$  is statistically significant. Data are represented as mean  $\pm$  SD.

**RESULTS**

**Neuropathic pain development of CCI mice**

The mean baseline mechanical threshold of both the CCI and sham groups on the day of surgery was 3.6 g. This value is reasonable because the 4.0 g filament of von Frey filaments has been used as a cut-off for normal ICR mice usually (Hayyan *et al.*, 2013). The threshold level for the CCI surgery group was significantly reduced compared to sham group (Fig. 1), which represents the development of mechanical allodynia.

**Increased mRNA expression of TREK-1 and TREK-2**

Stronger signals were observed for TREK-1 and TREK-2 in the DRG of CCI model group than in the sham mice (Fig. 2A). Semi-quantitative RT-PCR data showed that both the TREK-1 and TREK-2 mRNA expression levels in the DRG of CCI mice were significantly higher than in the sham mice ( $p < 0.05$ ,  $n = 4$ ).

**Increased protein expression of TREK-1**

The TREK-1 protein (45 kD) expression level was significantly increased by  $\sim 9$ -fold in the DRG of CCI model ( $p < 0.05$ ,  $n = 4$ , Fig. 2B). TREK-2 (60 kD) also showed a trend towards increased protein expression level, but this was not significantly

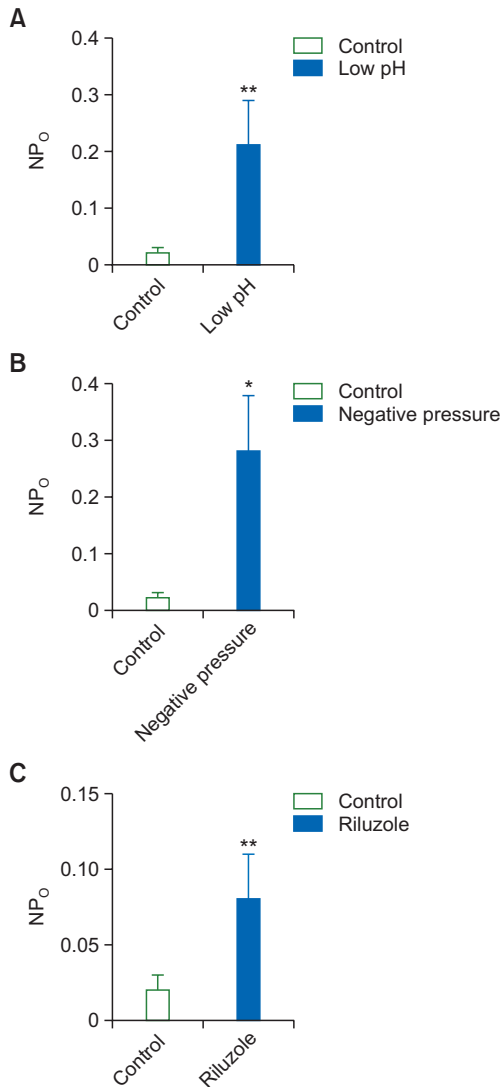
different ( $p > 0.05$ ,  $n = 4$ , Fig. 2B).

**Partial Colocalization between IB4+ and TREK-1**

TREK-1 staining intensity was increased in the DRG of CCI mice compared to the DRG of sham mice. The merged image of DRG of CCI mice revealed partial colocalization between IB4 and TREK-1. TREK-1 expression was also observed on IB4- neurons (Fig. 3A). The expression of TREK-2 was weak on both control and the CCI groups, only slightly increased in the DRG of CCI mice. There was little colocalization between IB4 and TREK-2, much weaker than that of TREK-1 and IB4 (Fig. 3B).

**Up-regulation of TREK-1 in electrophysiological studies**

To determine whether TREK-1 is functionally expressed, single-channel recordings were performed in mouse DRG neurons ranging from 10 to 38  $\mu$ m in diameter. TREK-1- and TREK-2-like currents were recorded in mouse DRG neurons. At +60 and -60 mV, the single-channel conductances were as follows: TREK-1 (132 pS and 134 pS) and TREK-2 (50 pS and 135 pS). From cell-attached and excised patches, TREK-1-like channels (6.7%, 4 of 60 patches) and TREK-2-like channels (3.3%, 2 of 60 patches) were observed in DRG neurons isolated from the sham mice. In the CCI mice, TREK-1-like channels (28.1%, 18 of 64 patches) were highly expressed compared to the TREK-2-like channels (18.8%, 12 of 64 patches). Fig. 4A shows the single-channel opening of TREK-1-like  $K^+$  channels at different membrane potentials in cell-attached patches. In the DRG neurons of CCI mice, TREK-1-like  $K^+$  channel activity was increased after the formation of inside-out patches. To observe the channel kinetics, patches



**Fig. 5.** TREK-1 activators (low pH (pHi 6.3), negative pressure (-40 mmHg), and riluzole (0.1 M) were applied to TREK-1-like channel. For comparison, the control group was tested at pH 7.3, without any pressure or activator. The TREK-1-like channel was easily activated by TREK-1 activators. \* $p < 0.05$ , \*\* $p < 0.01$  vs. the control value ( $n = 5$ , Low pH:  $p = 0.005$ , Negative pressure:  $p = 0.02$ , Riluzole treatment:  $p = 0.003$ , Fig. 3).

at a single level were selected and analyzed. As shown in Fig. 4B, amplitude histograms and open-time histograms of the channel openings were determined from patches at a single level of current and -40 mV in DRG neurons. Single-channel amplitudes were determined from the amplitude histogram for each membrane (Fig. 4C). A further test for TREK-2 was not performed because TREK-2 expression based on the protein level gave an insignificant result. The single-channel conductance and the mean open times of the TREK-1-like channel at -40 mV were  $103 \pm 18$  pS and  $0.9 \pm 0.1$  ms, respectively, similar to previous studies (Oh *et al.*, 1996; Kim *et al.*, 2011). The noisy pattern in the open state showing large current fluctuations and a relatively linear current-voltage relationship are also typical properties of TREK-1 (Maingret *et al.*, 1999; Patel and Honoré, 2002). The TREK-1-like channel was easily and

significantly activated by low pH, negative pressure, and riluzole ( $p < 0.05$ ,  $n = 5$ , Fig. 5). These results provide strong evidence that the up-regulated  $103 \pm 18$  pS channel in the CCI mice is TREK-1.

## DISCUSSION

A large part of the mechanism remains unclear, but the DRG is a major anatomical site for the development and maintenance of neuropathic pain. The DRG develops hyperexcitability after multiple ion channels in injured afferent neurons were altered to produce a bigger generator potential of transduction channel to sensitize to a given natural stimulus or decrease threshold of sodium channels responsible for spike initiation, resulting in their transductions to DRG of those signals. This is one of the possible mechanisms of neuropathic pain (Saad *et al.*, 2002; Campbell and Meyer, 2006).

In neuropathic pain, damaged primary afferent fibers appear to have altered excitability and conduction patterns during the initiation and maintenance phases. These altered patterns reflect changes in multiple ion channels, in density or in operating characteristics. Differential alterations of specific ion channels may be an important determinant of primary afferent discharge and conduction (Gold *et al.*, 2003). Unlike other potassium channels, the K2P channels have two pore forming (2P) domains and four transmembrane (4TM) domains (Lesage *et al.*, 2000). K2P channels have unique background K<sup>+</sup> channel properties influencing the resting membrane potential in neuronal tissues. These channels control resting membrane potential upon activation, thereby reducing excitability (Honoré, 2007).

K2P channels consist of six subfamilies, TWIK, THIK, TASK, TALK, TREK, and TRESK. Under physiologic conditions in the DRG, TREKs and TRESK together contribute 95% of the resting K<sup>+</sup> current at 37°C (Ocaña *et al.*, 2004). Compared to TREK<sup>+/+</sup> mice, TREK-1 deleted mice were more sensitive to low threshold mechanical and thermal stimuli, showing allodynia-like responses (Alloui *et al.*, 2006). In inflammatory conditions, a  $3.0 \pm 0.7$ -fold increase in the TREK-1 mRNA level was observed (Alloui *et al.*, 2006). However, in neuropathic pain conditions, expression levels of TREK subtypes in the DRG have not been studied. Following this background, we examined the molecular and functional expression of TREK-1 and TREK-2 in the DRG using the CCI model. The CCI model has been extensively used for many neuropathic studies because it closely mimics the clinical nerve injury conditions and pain nature such as complex regional pain syndrome type 2 (Bennett and Xie, 1988; Decosterd and Woolf, 2000; Mukhida *et al.*, 2007).

We used markers for IB4 and TREK for immunofluorescence because there are previous studies that most C-nociceptors (72%) are strongly IB4<sup>+</sup> (Fang *et al.*, 2006). It is shown that nearly one third of C-nociceptive neurons (28%), A $\alpha/\beta$  and A $\delta$  nociceptive neurons are IB4<sup>-</sup>. Our result showed partial colocalization between IB4<sup>+</sup> and TREK-1 in the DRG of CCI mice. This result suggests that neuropathic C-nociceptor type neurons are upregulated in TREK-1.

TREK-1 and TREK-2 are reversibly activated by heat; mechanical stimulus; polyunsaturated fatty acids, such as arachidonic acids; acidosis; neuroprotective agents, such as riluzole; and some volatile anesthetics (Kim and Chung, 1992;

Saadé *et al.*, 2002). The release of unsaturated free fatty acids, local heat or fever, acidosis, and mechanical stretch due to edema are common findings of the inflammatory process and nerve damage. The TREK subfamily is a group of temperature sensitive K<sup>+</sup> channels, and within the 24-37°C range, there is progressive increase in activity (NPo) for TREK-1 and a 14-fold increase in activity for TREK-2 (Kang *et al.*, 2005). In our study, the electrophysiological properties were tested at room temperature (24°C).

In this study, we confirmed that TREK-1 is upregulated and activated in the DRG of a neuropathic pain model. In normal DRGs, TREK-2 outnumbers TREK-1 in abundance and contributes more to setting the resting IK in rats (La and Gebhart, 2011; Marsh *et al.*, 2012). Although the role of TREK-1 in normal condition is smaller than TREK-2, it is suggested that the upregulation of TREK-1 in the neuropathic pain model might be related to the TRPV1, as TREK-1 and TRPV1 are found extensively co-localized (up to ~90%) in some nociceptive DRGs (Alloui *et al.*, 2006) following our results. In previous studies, it was observed that the hyperexcitability of DRG neurons in inflammatory, chronic constricted and axotomized neurons always accompanies the reduction of overall K currents (Takeda *et al.*, 2011).

However, TREK-1 and TREK-2 showed different expression pattern in the CCI model. TREK-2 down-regulation was more dramatic than TREK-1 up-regulation in the CCI model, especially for colocalization between IB4 and TREK-2 (Fig. 2, 3). Thus, the functional role of TREK-2 was sincerely considered in the context of neuropathic pain. Recently, it has been demonstrated that TREK-2 channels were selectively expressed in IB4-binding C-fiber nociceptors and limits spontaneous pain through siRNA-induced TREK-2 knockdown (Acosta *et al.*, 2014). But, recently, it is suggested that TREK-1 is more involved in neuropathic pain and even targeted for neuropathic pain (Heurteaux *et al.*, 2004; Alloui *et al.*, 2006; Noel *et al.*, 2009; Devilliers *et al.*, 2013; Rodrigues *et al.*, 2014). TREK-1<sup>-/-</sup> mice are more sensitive to ischemia and epilepsy, show lower sensitivity to the effects of inhaled anesthetics, and display an increased sensitivity to thermal and mechanical pain (Heurteaux *et al.*, 2004; Alloui *et al.*, 2006; Noel *et al.*, 2009). A recent study has suggested that the pain-reducing actions of morphine may be linked to TREK-1 channel activity through TREK-1 knockout and several behavioural assessment of analgesic effect of morphine including CCI animal model. TREK-1 knockout animals showed significantly less morphine-induced analgesia than WT animals, but there was no difference in the three main adverse effects produced by morphine (constipation, respiratory depression and dependence) between WT and KO animals. This suggests that direct activation of TREK-1 by morphine acting downstream from  $\mu$  opioid receptor stimulates strong analgesic effects without underlying any of the adverse side effects of morphine (Devilliers *et al.*, 2013). Following this study, compounds to modulate TREK-1 channel were synthesized and their antinociceptive effects were demonstrated through electrophysiology and an *in vivo* study, an acetic acid induced-writhing assay (Rodrigues *et al.*, 2014). Those studies strongly suggest that TREK-1 might play a more role in neuropathic pain and modulation of TREK-1 might be a good therapeutic target of neuropathic pain, rather than TREK-2.

In conclusion, our study showed that the expression of TREK-1 is increased in the mouse DRG showing neuropathic

pain behaviors. Both IB4<sup>+</sup> and IB4<sup>-</sup> neurons showed colocalization with TREK-1, but the staining intensity was stronger in IB4<sup>+</sup> DRG neurons, indicating that the expression of TREK-1 was higher in nociceptive C fibers. These findings suggest that TREK-1 may play an important role in the mechanisms of neuropathic pain and modulation of the TREK-1 channel may have beneficial analgesic effects in neuropathic pain patients. However, validation of this finding needs to perform studies with TREK-1 knockout or comparison studies with TREK-2 knockout in CCI model.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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