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Intact A δ -fibers up-regulate TRPA1 and contribute to cold hypersensitivity in neuropathic rats

Guangchen Ji, Shengtai Zhou, and Susan M. Carlton¹

Department of Neuroscience and Cell Biology, University of Texas Medical Branch, Marine Biomedical Institute, Galveston, TX 77555-1069

Abstract

Mechanisms underlying cold hypersensitivity in neuropathic states are unclear. Recent data indicate both TRPM8 and TRPA1 play a role. In relation to TRPA1, there are reported increases in mRNA. However, it is unknown whether TRPA1 mRNA is translated into functional receptors, whether these receptors are found on peripheral nociceptors and what population of primary afferents expresses the receptors. The present study provides several lines of evidence that TRPA1 receptors are expressed on intact primary sensory neurons and contribute to cold hypersensitivity following spinal nerve ligation (SNL). Immunohistochemical studies show that expression of TRPA1 is significantly increased in the ipsilateral compared to the contralateral L4 DRG. Using mustard oil (MO, selective TRPA1 agonist), Ca²⁺ imaging demonstrates an increase in the percentage of MO-sensitive L4 DRG cells in SNL compared to sham and naïve rats. The magnitude of the Ca²⁺ response evoked by MO is also significantly larger in SNL compared to sham and naïve rats. Behavioral studies demonstrate that SNL results in increased nocifensive behaviors to mechanical and cold stimulation that is not seen in sham or naïve rats. Behavioral responses in sham rats are no different from naïve rats. *In vitro* single fiber recordings demonstrate A δ -fibers (intact L4 axons) in the nerve-injured hind paw have conduction velocities no different from naïve rats. In contrast, compared to naïve, mechanical thresholds of the A δ -fibers in SNL rats are significantly decreased, the proportion of cold-sensitive and MO-sensitive A δ -fibers is significantly increased and the response magnitude of A δ -fibers to MO is significantly increased. MO-induced activity in A δ -fibers is significantly reduced by ruthenium red (TRPA1 receptor antagonist). These results demonstrate that TRPA1 is expressed on peripheral nociceptors, and they are up-regulated on intact A δ -fibers following nerve injury, contributing to cold hypersensitivity. Supported by NS27910 and NS40700 to SMC.

Keywords

peripheral neuropathy; sensitization; mustard oil; hyperalgesia

Recently, advances have been made in identifying the transient receptor potential (TRP) ion channels contributing to temperature-sensing. Among the TRP channels identified, TRPM8 and TRPA1 are activated by cold and expressed on dorsal root ganglion (DRG) cells (Reid and Flonta, 2001a; McKemy et al., 2002; Peier et al., 2002; Reid et al., 2002; Story et al., 2003).

¹To Whom Correspondence Should Be Sent: Dr. Susan M. Carlton, Department of Neuroscience and Cell Biology, University of Texas Medical Branch, Marine Biomedical Institute, Galveston, TX 77555-1069, Phone: (409)772-2124, Fax : (409)772-2789, Email : smcarlto@utmb.edu.

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TRPM8 has a threshold of 25 °C and is sensitive to menthol (McKemy et al., 2002; Peier et al., 2002). In contrast, TRPA1 has a threshold near 17 °C, is insensitive to menthol but is activated by mustard oil (MO), (Jordt et al., 2004; Bandell et al., 2004) and is expressed in a subset of sensory neurons expressing nociceptive markers (Story et al., 2003; Bandell et al., 2004).

Cold allodynia is a striking feature not only in patients with various nerve injuries (Wahren and Torebjörk, 1992; Ochoa and Yarnitsky, 1994; Bowsher and Haggett, 2005), but also in animals with a peripheral nerve injury (Choi et al., 1994; Vissers and Meert, 2005). The mechanisms underlying enhanced sensitivity to cold remain unknown. It has been hypothesized that TRPA1 contributes to cold hyperalgesia after inflammation and nerve injury (Colburn et al., 2007). Anatomical studies showed that the percentage of TRPA1 mRNA-positive neurons increased in DRG neurons after spinal nerve injury, and knock-down of TRPA1 using antisense alleviated this cold hyperalgesia (Obata et al., 2005; Katsura et al., 2006). It is unknown whether TRPA1 mRNA is translated into functional receptors, whether these receptors are found on peripheral nociceptors and what population of primary afferents expresses the receptors. Therefore in the present study, we investigated the expression of TRPA1 receptors on DRG neurons and on peripheral axons in the hind paw by immunostaining with TRPA1 specific antibodies in SNL rats. Changes in TRPA1 function on L4 DRGs and A δ -fibers innervating the nerve injured hind paw were assessed using Ca²⁺ imaging and an *in vitro* skin nerve preparation, respectively.

EXPERIMENTAL PROCEDURES

All experiments were approved by the University Animal Care and Use Committee and followed the guidelines of the International Association for the Study of Pain for the ethical care and treatment of laboratory animals (Zimmermann, 1983).

Surgery to induce the peripheral neuropathy

Rats received a unilateral tight ligation of left L5 and L6 spinal nerves using the surgical procedure described by Kim and Chung (1992). Briefly, rats were anesthetized with a gaseous mixture of halothane (4% induction, 1% maintenance) and N₂O and O₂ (1:2 flow ratio). Under anesthesia and using sterile technique, a skin incision was made on the left just lateral to the lumbar vertebral processes. The intraspinal muscles were retracted and the L5 spinal nerve visualized. Using 6-0 sterile silk, 1 tie was placed around the nerve and drawn very tightly, causing an immediate axotomy. The L6 spinal nerve was visualized just caudal to the lateral process of the L6 vertebra and was ligated as described above. The muscle and skin was closed in layers using 3-0 silk suture and wound clips. The rats were returned to their cages and monitored until they were up and moving around. These were designated spinal nerve ligation (SNL) rats. Sham surgery was performed in the same manner as ligated animals, including exposure of the L5 and L6 nerves, but the nerves were not ligated. These were designated “sham” rats. A third group that had no surgery or manipulation was designated naïve rats.

Anatomical localization of TRPA1 in DRGs and cutaneous nerves

Light microscopic localization in DRGs—Rats were deeply anaesthetized with sodium pentobarbital (80 mg/kg, i.p.) and perfused transcardially with 4% paraformaldehyde and 0.1% picric acid in 0.1 M phosphate buffer (PB, pH 7.4) at 4 °C. The left and right L4 DRGs were removed from SNL rats (n = 3, 11 days post-surgery) and placed in 30% sucrose-PB for cryoprotection overnight at 4 °C. The ganglia were fast frozen in liquid N₂ in a cryo-embedding compound and sectioned on a cryostat (Microm International, GmbH). Sections were incubated in rabbit anti-TRPA1 (1:2000, a generous gift from Dr. David Corey, Harvard Medical School, Boston, MA) overnight at room temperature. After rinsing in 0.1 M phosphate buffered saline

(PBS, pH 7.4), sections were placed in Cyanin 3- (Cy3-) conjugated anti-rabbit IgG (1:400, Jackson ImmunoResearch, West Grove, PA) for 1 h. After final rinsing, slides were cover slipped with Vectashield mounting media (Vector Laboratories, Burlingame, CA). On some sections, the primary antiserum was omitted and the tissue was otherwise treated as described above. Immunoreaction product was absent in this control tissue. Preabsorption controls were previously reported and resulted in no staining (Corey et al., 2004). Subjective measures were used to define a TRPA1-labeled cell. The level of reaction product in a cell was compared to the surrounding clearly, unlabeled cells, to the control tissue that was processed without primary antibody (and thus showed only background levels) and to the absorption control tissue.

Stereological Analysis—Following immunostaining staining, a color digitized image of each section (24 bits per pixel), was captured using an Olympus BX51 microscope with an attached Spot RT digital camera (Diagnostic Instruments, Sterling Heights, MI) and a Cy3 filter cube. Camera exposures were adjusted automatically by Spot software (version 3.1) and brightness and gamma corrections were made manually to achieve the best signal-to-noise ratios. The physical dissector (pairs of sections) was used to achieve unbiased estimates of TRPA1-labeled DRG neurons (Coggeshall, 1992). The total number of neurons in a DRG was also estimated using the physical dissector on cresyl violet stained sections.

The size of most DRG sections and the magnification needed to identify labeled cells necessitated analysis of a randomly chosen subregion or fraction (f) in each section in a pair. Once identified, images in the subregion in each pair were outlined from the computer screen onto clear mylar sheets including all labeled cell profiles and an appropriate number of fiduciary landmarks. The mylar sheets from a pair were matched with one another using the fiduciary landmarks, and all labeled profiles that appeared in both sections were disallowed. Labeled profiles remaining were “tops” or “counts” (identified as Q- in the stereological literature). Using “fractionator sampling” (West and Gundersen, 1990; Gundersen et al., 1998), total numbers of cresyl violet stained neurons and numbers of TRPA1-labeled neurons were estimated by multiplying “Q-” by the reciprocal of the fraction (1/f) of each sampled section pair. These numbers (N) for all pairs in a DRG were summed (ΣN). The total number of neurons in the entire DRG was then estimated by $(\Sigma N \times k)/2$ (divided by two because each section in a pair was used both as reference and look-up sections) (Coggeshall, 1992). Percentages of labeled neurons were calculated by dividing the numbers of labeled neurons by the total DRG cell number $\times 100$. The experimenter was blinded to which ganglia (ipsilateral or contralateral) were being analyzed.

Determining Cell Diameters—The diameters of TRPA1-labeled and cresyl violet stained DRG neurons were calculated by summing the length and width of those neurons with a visible nucleus and dividing by two. Whenever possible, at least 100 neurons were measured in each DRG.

Electron Microscopic localization in digital nerves—Naive rats (n = 3) were deeply anaesthetized with sodium pentobarbital (80 mg/kg, i.p.) and perfused transcardially with 4% paraformaldehyde but no glutaraldehyde was added. The digital nerves were dissected and cut into 2.0–2.5mm segments. Prior to immunostaining, all tissue was rinsed in graded alcohols to increase antibody penetration. The tissue was blocked in normal goat serum (NGS) and then incubated in antiserum directed against TRPA1 (1:1000) for 72h at 4°C. Following incubation, the tissues were rinsed in PBS and immunostained using an ABC kit (Vector Labs) and diaminobenzidine. The tissue was then osmicated, dehydrated and embedded in plastic. Ultrathin sections of the digital nerves were cut at right angles to the long axis of the axons, mounted on formvar coated slot grids and viewed on a JEOL 100CX electron microscope and random fields were photographed. Control tissue (antibody omitted) had no immunostaining.

Ca²⁺ imaging in dissociated DRG cells

Cell Culture—Naive and sham Sprague Dawley rats (80–120g) or SNL rats (7–10 days after surgery) were deeply anesthetized with sodium pentobarbital (80 mg/kg, i.p.). Both L4 DRGs in naive rats and the ipsilateral L4 DRGs in sham and SNL rats were removed, placed in ice-cold oxygenated dissecting solution containing (in mM): 130 NaCl, 5 KCl, 2 KH₂PO₄, 6 MgCl₂, 1.5 CaCl₂, 10 Glucose, 10 HEPES (pH adjusted to 7.2 with NaOH base and osmolality adjusted with sucrose to 300 mOsm) (Xu and Huang, 2002), and minced into smaller pieces. The DRG fragments were then digested in 1mg/ml collagenase D (Roche Diagnostics, Indianapolis, IN) and 0.5mg/ml trypsin (Sigma-Aldrich, St Louis, MO) in a 37 °C water bath for 1 h and then washed 3 times with dissecting solution. Fire-polished glass pipettes were used to mechanically dissociate the DRG cells. The dissociated cells (0.1 ml) were plated on poly-lysine coated glass coverslips inside 35 mm culture dishes. Dissecting solution (2 ml) was added to the wells 15 min after plating the cells and the wells were maintained for 1 h at room temperature prior to experiments. A total of 10 L4 DRG from SNL, 6 from naive and 6 from sham rats were used for this part of the study.

Cytosolic Ca²⁺ Imaging—The DRG cells were incubated with 3 μM Fura-2 acetomethoxy ester (Molecular Probes, Eugene, OR) in normal bath solution containing 0.2% pluronic acid F-127 at room temperature for 40 min, washed with dissecting solution and left in this solution at room temperature in the dark for another 40 min so the cells could stabilize. Ratiometric Ca²⁺ imaging was performed using an inverted fluorescent Nikon microscope equipped with a 340 nm and 380 nm excitation filter changer (Sutter, Novato, CA), a 510 nm long pass emission filter and a 12-bit digital monochrome cooled CCD camera (Roper Scientific Coolsnap HQ) controlled by Metafluor software v6.5 (Molecular Devices, Sunnyvale, CA). Fluorescent images and ratios were acquired every 2 seconds, and processed using Metamorph software (Molecular Devices). The effect of MO on the cytosolic Ca²⁺ was tested after obtaining a 2 min baseline recording, followed by application of 100μM MO solution for 2 min, followed by wash off with normal bath solution. Cells were considered to respond to MO if their F340/F380 ratio increased by more than 20% during the 2 min MO application. All experiments were performed at room temperature.

Behavioral studies

Habituation—Male Sprague-Dawley rats were housed in groups of three in plastic cages with soft bedding under a reversed light/dark cycle of 12h/12h. Following arrival at the animal care facility, they were acclimated for at least 3 days before behavioral testing was initiated. Each rat was habituated two times in each testing paradigm before being placed in an experimental group.

Testing mechanical sensitivity—Rats were placed in a plexiglass cage (8 × 8 × 18 cm) on a raised, wire mesh platform and von Frey filaments were applied to the plantar surface of the hind paws. Mechanical threshold was determined using the Dixon up-down method (Dixon, 1991; Chaplan et al., 1994) with a series of filaments with bending forces ranging from 2.64 to 489.4 mN. In SNL rats, the ipsilateral and contralateral hind paws were tested before and 7–14 days after SNL surgery.

Testing cold sensitivity—Cold-induced nociception was assessed by quantitating flinching behavior in naive, sham and SNL rats during a 5-minute trial on a thermo-neutral (room temperature 24°C) or cold (6°C) plate (Choi et al., 1994; Jasmin et al., 1998; Ji et al., 2007). On the day of testing, the rat was placed on the plate for a total of 10 min. The first 5 min interval was habituation, however, during the second 5 min interval flinching behavior was recorded. Thermo-neutral and cold plate testing were performed before surgery and nine to fourteen days after SNL or sham surgery.

Testing acetone sensitivity—Application of acetone has been widely used to detect the presence of cold allodynia in humans (Frost et al., 1988; Davis et al., 1991) and animals (Choi et al., 1994; Kim et al., 1997). Each rat was placed in a plexiglass cage on a raised, wire mesh platform. An acetone bubble (diluted to 90% with water) was formed on the end of a 1ml syringe and the bubble gently touched to the heel of the hindpaw. The acetone quickly spread over the proximal half of the plantar surface of the paw. Acetone-induced behavior was assessed in naïve, sham and SNL rats by counting the number of flinches and the number of seconds an animal spent lifting and/or licking (L/L) the paw over a 5 min period. The acetone was applied 5 times to the ipsilateral paw with a 5 min interval between trials and the average response calculated. As a control for the mechanical stimulation that accompanies touching the skin with a bubble of fluid, room temperature water was applied in the same manner as the acetone and behavior assessed.

Testing MO sensitivity—Each rat was placed in a plexiglass cage on a raised, wire mesh platform. The MO (TRPA1 agonist, made in 1% DMSO) was applied to the ipsilateral hind paw plantar skin of naïve, sham and SNL rats using a cotton-tip applicator soaked in 10 mM MO. In a separate group, vehicle (1% DMSO) was applied in a similar manner to SNL rats as a control. The MO-induced nociceptive behaviors were reported as the number of flinches and number of seconds of L/L time per 1 min interval over 10 min.

Electrophysiological studies

In vitro single fiber recordings—Single unit recordings from A δ -fibers in glabrous skin were obtained using an *in vitro* skin-nerve preparation that was described by Reeh (1986) (Reeh, 1986) and is well-established in our lab (Carlton et al., 2001; Du et al., 2001; Du et al., 2003; Du et al., 2006; Ji et al., 2007). Male Sprague Dawley rats (200–300g) were killed with an overdose of CO₂, and the hind paw glabrous skin was dissected from each animal with the medial and lateral plantar nerves intact. The preparation was placed corium side up in an organ bath and superfused (15 ml/min, 34 °C) with an oxygen-saturated, modified synthetic interstitial fluid solution (SIF) (in mM: 123 NaCl, 3.5 KCl, 0.7 MgSO₄, 2.0 CaCl₂, 9.5 Na gluconate, 1.7 NaH₂PO₄, 5.5 glucose, 7.5 sucrose, and 10 HEPES, pH 7.40). The plantar nerves were placed in a separate chamber with a top layer of mineral oil and a bottom layer of SIF. The nerves were desheathed and teased apart on a mirror stage under a dissecting microscope. Small nerve bundles were isolated and repeatedly split with sharpened forceps until single-unit activity was obtained.

Units were identified by manual probing of the glabrous skin with a blunt glass rod and if they had a clearly defined receptive field, they were studied in detail. Neural activity from single units was amplified using a DAM80 Differential Amplifier (World Precision Instruments) attached to a monopolar gold wire electrode, with the reference electrode positioned nearby, and recorded using a PC computer-controlled CED1401 interface and Spike II software (CED, UK). The conduction velocity of each unit was determined by monopolar electrical stimulation (0.1 ms duration, train frequency 1 Hz) at the most mechanosensitive site in the receptive field of each unit using a teflon-coated steel electrode (5 M Ω impedance, 250 μ m shaft diameter). Conduction velocity was determined from the latency of the action potential and the distance from the stimulation electrode to the recording site (Fig 1A, measured in millimeters). Fibers with a conduction velocity of over 1.6m/s and less than 10m/s were classified as A δ -fibers (Koltzenburg et al., 1992; Kress et al., 1992; Ji et al., 2007). A window discriminator was used to isolate units when collecting the raw data and a template matching program (Spike II) was used during the off-line analysis to discriminate a unit of interest from all others.

Thermal stimulation—A feedback controlled lamp placed beneath the organ bath supplied radiant heat to the receptive field of each unit. The beam was focused through the translucent

bottom of the bath onto the epidermal surface of the skin. A standard 10 s heat ramp was applied to each unit, starting from an adapting temperature of 34°C and rising to 47 °C (47 °C on the corium side was equivalent to 51°C on the epidermal side). The temperature at which the second spike was elicited by the heat stimulus was defined as the thermal threshold.

Mechanical stimulation—A Dual Mode Lever System (Aurora Scientific Inc. Ontario, Canada) was used to determine mechanical threshold. The system had a motor-driven stylus that delivered a force in the form of a continuous ascending ramp (from 0–250 mN in 20 s). The stylus (1.5 mm diameter) was placed in the receptive field of the unit on the corium side of the glabrous skin. The first spike occurring once the ramping force was activated was considered threshold for that unit (Fig. 1B).

Cold stimulation—A small plastic, hollow cylinder (7mm O.D. × 10 mm high) mounted on a micromanipulator was placed over the receptive field of each unit. The SIF in the cylinder was carefully drawn out and a pellet of frozen SIF gently placed in the cylinder, avoiding mechanical stimulation. These SIF pellets were made using small capsules (5.6mm O.D. × 10mm high, BEEM capsules, Electron Microscopy Science, Hatfield, PA, USA) and stored in the freezer so that their shape and size were consistent from trial to trial. The pellets produced rapid cooling of the units from 34.1 ± 0.1 °C to 0.9 ± 0.1 °C ($n = 10$) in approximately 1–2 s which was maintained at ~0 °C for 30 s (Fig. 1C). A unit was considered to be cold-sensitive if the discharge rate evoked during cold stimulation exceeded the mean background discharge rate + 2 SDs. (The mean background rate for the fiber population was determined by recording unit activity for 2 minutes prior to application of the cold stimulation and calculating the mean.).

Effects of MO on single fiber activity—To investigate MO-induced activity in cold-sensitive A δ -fibers, a plastic, hollow cylinder was placed over the receptive field of each unit and the SIF in the cylinder replaced with MO made in SIF (pH 7.4). The MO was dissolved in SIF to different concentrations: 0.01, 0.1, 1.0 and 3.0 mM. Ascending concentrations were applied to the receptive fields for 2 min each and unit activity recorded. There was a 30 s interval before each subsequent dose was applied.

Effects of Ruthenium Red (RR) on MO-induced excitation—To determine whether RR attenuated MO-induced activities, cold-sensitive A δ -fibers were pretreated with 50 μ M RR or vehicle for 5 min. These solutions were replaced with 50 μ M RR + 3 mM MO or vehicle + 3 mM MO for 2 min and activity recorded. Ruthenium Red is an antagonist for TRPA1 (Bandell et al., 2004), but not TRPM8 (Weil et al., 2005).

Statistics

Data were expressed as means $\pm$ standard errors (SE) unless otherwise noted and evaluated using SigmaStat (Jandel Corporation). Parametric or nonparametric tests were used depending on the passing of a normality test. For the anatomical studies, differences in percentages of TRPA1-labeled DRGs were evaluated with a t-test. Differences in the populations of dissociated DRG cells were determined using a Chi-Square test. Differences in the change in ratios for the Ca²⁺ imaging were determined using a one-way ANOVA. In behavioral studies, differences between treatment groups were evaluated using a paired t-test or one-way ANOVA. In the electrophysiological studies, differences between percentages of populations in SNL or naïve were determined using a Chi-Square test, differences in discharge rates were evaluated with a Friedman's ANOVA, Wilcoxon or Mann-Whitney U test where appropriate. $P < 0.05$ was considered significant.

RESULTS

Anatomical Studies

Anatomical localization of TRPA1 in DRGs and cutaneous nerves—Light microscopic analysis of L4 DRG sections from three SNL rats immunostained with TRPA1 antibody demonstrated a subpopulation of small and medium diameter fluorescently labeled cells (Fig. 2). Stereological estimates indicated there was a significant increase in the percentage of TRPA1-labeled cells in the ipsilateral (29.5%) compared to the contralateral (17.2%) DRG (Table 1, $p < 0.05$, t-test). Mean diameter of the labeled cells was $21.8 \pm 0.2 \mu\text{m}$, suggesting these were small to medium sized cells. Electron microscopic analysis of cutaneous axons in digital nerves demonstrated the presence of TRPA1 reaction product in small diameter myelinated axons (presumed A δ -fibers) and unmyelinated axons (presumed C-fibers) (Fig. 3), demonstrating that TRPA1 is transported out of the DRG and expressed in the peripheral terminals.

Ca²⁺ imaging in dissociated DRG cells

Increased percentage of L4 DRG cell responding to MO in SNL rats—A total of 305 L4 DRG cells from 6 naïve, 6 sham and 10 SNL rats were examined for their responses to 100 μM MO. Cells demonstrating a $\geq 20\%$ change in the F340/F380 ratio were considered MO-sensitive. Only 14% (15/107) in naïve and 11% (9/81) of DRG cells in the sham group responded to MO. In contrast, 42% (50/117) of DRG cells in the SNL group showed a positive response to MO (Fig. 4A, $p < 0.001$, Chi-square test). The mean size of the cells responding to MO was similar among the naïve ($715 \pm 25 \mu\text{m}^2$), sham ($801 \pm 34 \mu\text{m}^2$) and SNL ($685 \pm 17 \mu\text{m}^2$) groups.

Increased magnitude of response of L4 DRG cells to MO in SNL rats—Analysis of the magnitude of the Ca²⁺ responses in naïve, sham and SNL groups demonstrated that MO induced a significantly greater response in SNL compared to all other groups. The F340/F380 ratio in SNL was 1.52 ± 0.05 , compared to naïve which was 1.26 ± 0.05 and sham which was 1.21 ± 0.1 (Fig. 4B, $p < 0.05$, one-way ANOVA).

Behavioral Studies

Mechanical hypersensitivity—In SNL rats, the mechanical threshold of the ipsilateral and contralateral hind paws were tested before and 7–14 days after surgery. The mechanical threshold of the ipsilateral but not the contralateral paw was significantly decreased compared to pre-surgery levels (Fig. 5A, $p < 0.05$, paired t-test). There was no change in threshold in the sham group, comparing pre- and post-surgery thresholds (Fig. 5B).

Cold hypersensitivity

Cold plate-induced behavior—Placement of naïve or sham rats on a cold plate (6 °C) resulted in minor flinching behavior and these groups were not different from each other. However, SNL rats were very sensitive to this temperature, indicated by the significant increase in number of flinches (Fig. 6A, one-way ANOVA). As a control, all groups were tested on a room temperature plate (24 °C) and very little flinching behavior was observed in any group, ruling out mechanical stimulation as a driving force of the SNL behavior on the cold plate (Fig. 6B).

Acetone-induced behavior—Topical application of acetone to the plantar skin of the hind paw produced minor flinching and lifting/licking (L/L) in naïve and sham rats and these groups were not different from each other. However, acetone induced significant amounts of flinching and L/L behaviors in the nerve-injured hind paw in SNL rats (Figs. 7A and B, $p < 0.05$, one-

way ANOVA). As a control, water was topically applied to the SNL paw but this did not result in any behavior.

MO-induced behavior—Topical application of the TRPA1 agonist MO (10 mM) to the plantar hind paw in naïve and sham rats produced little or no flinching or L/L behaviors (Fig. 8A and B). Vehicle (1% DMSO) did produce minor flinching when applied to the SNL paw but this was not different from the naïve and sham MO groups. However, application of MO to the SNL paw induced significant amounts of flinching and L/L behaviors (Fig. 8A and B, $p < 0.05$, one-way ANOVA) when compared to all other groups.

Electrophysiological Studies

General properties of fibers analyzed—In our previous study, the total percentage of cold-sensitive A δ -fibers, but not C-fibers, were increased significantly in the nerve-injured paw in SNL rats (Ji et al., 2007), therefore we concentrated on analysis of A δ -fibers only in the present study. Also, based on the behavior data in this study as well as in our previous study (Ji et al., 2007), there was no significant difference between naïve and sham-operated rats when tested for mechanical and cold sensitivity. Therefore, in the physiological studies, responses of fibers from naïve rats were compared to that in SNL rats. Consequently, 72 A δ -fibers were recorded in 10 naïve rats and 15 SNL rats. The mean CV for A δ -fibers in naïve rats was 3.51 ± 0.31 m/s and in SNL rats was 4.50 ± 0.44 m/s and these were not different (Fig. 9A).

All fibers included in this analysis were mechanosensitive fibers since we used mechanical probing as our search stimulus. Fibers were placed into one of four categories based on their responses to additional stimuli such as heat and cold. Fibers that did not respond to either heat (51 °C) or cold (~0 °C) stimuli were classed as A δ -mechanical units (ADM). Fibers that responded to heat were classified as A δ -mechanoheat units (ADMH) and those that responded to cold were classed as A δ -mechanocold units (ADMC). Fibers that respond to both heat and cold were classed as A δ -mechanoheat-cold units (ADMHC) (Leem et al., 1993a).

Based on our criteria described above, the population of A δ -fibers ($n = 36$) in naïve rats was distributed in the following manner: 64% ADM, 19% ADMC, 3% ADMHC and 14% ADMH. The population of A δ -fibers ($n = 36$) in the SNL rats consisted of 28% ADM, 61% ADMC, 3% ADMHC and 8% ADMH. Compared to naïve, the percentage of ADM fibers in SNL rats was significantly decreased and the percentage of ADMC fibers was significantly increased ($p < 0.01$ and $p < 0.001$ respectively, Chi-square test). There was no difference in the percentage of ADMHC or ADMH fibers between the groups (summarized in Table 2). The data indicated that 24/36 units in SNL rats had spontaneous activity compared to 7/36 units in naïve rats. The level of spontaneous activity of the A δ -fibers was clearly increased in SNL rats (0.035 ± 0.014 impulses/sec, ranging from 0 to 0.26 impulses/sec, $n = 24$) compared to naïve rats (0.006 ± 0.006 impulses/sec, ranging from 0 to 0.042 impulses/sec, $n = 7$). Furthermore, the mean mechanical threshold of A δ -fibers in SNL rats (55.8 ± 6.7 mN, $n = 33$) was significantly lower compared to naïve rats (104.3 ± 8.8 mN, $n = 28$, Fig. 9B, $p < 0.05$, Wilcoxon Test).

Cold-induced activity—The percentage of fibers responding to cold stimulation (ADMC + ADMHC) was significantly larger in SNL (64%, 23/36) compared to naïve (22%, 8/36) rats (Table 2, Fig. 9C, $p < 0.001$, Chi-square test). The main change occurred in the cold-sensitive ADMC population because there was no difference in ADMHC in naïve and SNL rats.

Mustard oil-induced activity—A δ -fibers responding to cold were also exposed to MO. In naïve rats, only 17% (6/36) of the ADMC fibers were MO-sensitive, but in SNL rats, 42% (15/36) were MO-sensitive and this increase was significant (Fig. 9D, $p < 0.05$, Chi-square test). Application of an ascending series of MO concentrations (0.01, 0.1, 1.0, 3 mM) to the

receptive fields of cold-sensitive A δ -fibers produced a dose-dependent effect with MO inducing a significant increase in discharge rate at 1 mM and 3 mM in SNL and at 3 mM in naïve, compared to vehicle (Fig. 10 A and B, $p < 0.05$, Friedman's ANOVA). The discharge rate of A δ -fibers from SNL rats was significantly higher following 3 mM MO compared to naïve ($p < 0.05$, Mann-Whitney U). Pre-treatment of the receptive field of A δ -fibers with Ruthenium Red (50 μ M, 5 minutes) significantly reduced the fiber activity induced by 3mM MO (Fig. 10C, $p < 0.05$, Mann-Whitney U test).

Discussion

Several lines of evidence are provided demonstrating that TRPA1 plays a critical role in cold hypersensitivity following nerve injury including anatomical, Ca²⁺ imaging, behavioral and electrophysiological studies. For the first time, definitive evidence is provided that TRPA1 receptors, expressed by uninjured A δ -fibers, contribute to the aberrant sensory coding of cold stimulation in nerve-injured rats. The data demonstrate that expression of TRPA1 protein is significantly increased in the un-injured ipsilateral compared to contralateral L4 DRG neurons in SNL rats. Our *in vitro* recordings from intact A δ -fibers demonstrate, for the first time, that both the proportion and the magnitude of response of these fibers to cold stimulation and to MO are significantly increased in SNL rats compared to naïve. This response is significantly reduced by a TRPA1 receptor antagonist ruthenium red. The increased expression of TRPA1 in intact A δ -fibers is likely to be a mechanism underlying the enhanced pain behaviors in the cold plate, acetone and MO tests. The Ca²⁺ imaging results confirm our anatomical and physiological data, demonstrating a significant increase in the percentage and an increase in magnitude of response of un-injured L4 neurons responding to MO in SNL compared to sham and naïve rats. Taken together, the data strongly suggest that increased expression of TRPA1 in A δ -fibers innervating the nerve-injured hind paw contribute to the cold hypersensitivity induced by SNL.

Anatomical localization of TRPA1 in primary afferent neurons

Two cold-activated ion channels, TRPM8 and TRPA1, expressed by DRG cells have been identified and cloned (Reid and Flonta, 2001a,b; McKemy et al., 2002; Peier et al., 2002; Story et al., 2003). There are clearly other cold-activated channels in the DRG that have been identified but are waiting to be cloned (Munns et al., 2006). Regardless, the data indicate that TRPA1 plays a key role in the cold hyperalgesia which arises in this nerve-injury model. This receptor is activated around 17 °C, a temperature reported by humans as painfully cold (Davis and Pope, 2002). It has been co-localized in sensory neurons expressing nociceptive markers such as transient receptor potential vanilloid 1 (TRPV1), calcitonin gene-related peptide (CGRP) and substance P (SP) (Story et al., 2003). Previous studies have hypothesized that TRPA1 contributes to cold hyperalgesia after nerve injury based on the findings that the percentage of TRPA1 mRNA-positive neurons increase in the L4 DRG following SNL (Obata et al., 2005) and knock down of TRPA1 relieves the cold hyperalgesia (Katsura et al., 2006). The current study provides the first evidence that the increased TRPA1 mRNA is translated into protein and also provides the first localization of TRPA1 in peripheral axons in rat cutaneous digital nerves. After SNL, approximately 29.5% of L4 DRG neurons on the nerve-injured side express TRPA1 and this percentage is slightly lower than that reported for TRPA1 mRNA expression (43.1%). This antibody was particularly sensitive to fixation and as a result, minimal exposure to aldehydes was necessary in order for the immunostaining to be successful. Thus, we consider the estimates reported here for TRPA1-labeled DRG cells to be very conservative. Counts of TRPA1-labeled axons in the periphery were not performed but the data showed that both unmyelinated and small myelinated axons were positively stained indicating that both C and A δ -fibers expressed TRPA1. Localization of TRPA1 on C and A δ fibers is consistent with the single fiber recordings reported here and previously (Ji et al., 2007).

Behavioral evidence for TRPA1 involvement in cold hypersensitivity

The behavioral results indicated that SNL rats developed mechanical allodynia and cold hypersensitivity, confirming previous reports using this model (Kim and Chung, 1992; Choi et al., 1994; Ji et al., 2007). Cold hypersensitivity was demonstrated by three different testing paradigms. Using a cold plate test, we confirmed that SNL rats show increased nocifensive behaviors compared to naïve and sham rats. However, it has been reported that nocifensive behaviors were still observed in the cold plate test in SNL rats following complete sensory denervation of the nerve-injured hind paw, making it likely that this method of testing is not specific for demonstrating evoked cold sensitivity in the hind paw (Choi et al., 1994). We also employed an acetone test since it has been used to test cold allodynia in humans (Frost et al., 1988; Davis et al., 1991) where it elicits a sense of coolness upon evaporation. Acetone is now widely used to test changes in cold sensitivity in rats following nerve injury (Choi et al., 1994; Kim et al., 1997; El-Khoury et al., 2002; Vissers and Meert, 2005). Although not selective for any cold receptor, acetone did increase flinching and L/L behaviors when applied to the hind paw in nerve-injured rats but not naïve or sham rats. Applying water in the same manner did not induce nociceptive behaviors, indicating that the “mechanical” stimulation accompanying the application of acetone did not contribute to the nociceptive behaviors. Mustard oil selectively activates TRPA1 cold receptors (Jordt et al., 2004; Bautista et al., 2006). When applied to the nerve-injured hind paw it produced enhanced nocifensive behaviors in SNL but not naïve or sham rats and vehicle had no effect. It is unknown if TRPA1 is expressed by other components in the skin but our anatomical data confirmed their presence on cutaneous axons, including small diameter, unmyelinated and thinly myelinated axons. Hence, we propose that MO was having a direct effect on these nociceptors and that their activation resulted in the nocifensive behaviors.

Physiological correlates underlying the cold hypersensitivity

It has been previously demonstrated that both innocuous and noxious cold sensation were mediated by primary afferent fibers, including A δ - and C-fibers, in normal animals (Georgopoulos, 1976; Georgopoulos, 1977; LaMotte and Thalhammer, 1982; Leem et al., 1993b; Simone and Kajander, 1996; Takahashi et al., 2003; Ji et al., 2007). We have recorded from intact A δ - and C-fibers innervating the nerve-injured hind paw in SNL rats and demonstrated that the intact (un-injured) A δ population plays a critical role in the cold hypersensitivity detected in the paw (Ji et al., 2007). For example, the percentage of L4 cold-sensitive and icilin-sensitive A δ -fibers was significantly increased but there was no comparable change in C-fibers. Icilin-induced activity was significantly increased in L4 A δ -fibers but not C-fibers. We do not discount a role for intact C-fibers in cold allodynia/hyperalgesia since there was a higher prevalence of C-mechanoheat-cold (CMHC) fibers in SNL compared to naïve rats, but the overall percentage of cold-sensitive C-fibers in SNL rats was not different from naïve. For these reasons, we focused on recording from the A δ -fiber population in the present study. As expected, there was a significant increase in the percentage of cold-sensitive A δ -fibers (ADMC) and a concomitant decrease in the mechanoreceptive A δ -fibers (ADM) in the SNL compared to naïve rats. This change in percentage suggests a phenotypic shift in the intact primary afferents as a result of the injury and we have discussed this at length in a previous paper (Ji et al., 2007). There is the slight possibility that the cold hypersensitivity was related to the selective degeneration of A δ -cold fibers innervating the hind paw, thereby unmasking the burning sensation associated with cold stimulation of polymodal C-fibers (LaMotte and Thalhammer, 1982; Wahren et al., 1989; Yarnitsky and Ochoa, 1990). However, the degeneration which occurs following tight ligation of the L5 and L6 spinal nerves is not selective but includes A β -, A δ - and C-fibers. Clearly A δ -cold responsive fibers were left intact since we were recording from such a population.

There is little doubt that a phenotypic switch in un-injured A δ -fibers underlies the cold hypersensitivity that arises in the neuropathic condition. In SNL rats, the percentage of cold-sensitive A δ -fibers (64%) was significantly larger than in naïve (22%). Furthermore, the percentage of ADMC units significantly increased but the percentage of ADM units significantly decreased in SNL compared to naïve. The most likely explanation for the change in percentages is that some ADM units began to express ion channels/receptors activated by cooling or cold stimulation (i.e. TRPA1) and thus were now classified as ADMC. This hypothesis was supported by our Ca²⁺ imaging studies which demonstrated a significant increase in the proportion of MO-sensitive L4 DRG cells after SNL compared to sham and naïve. The MO-sensitive cell size corresponded to that population which would give rise to small myelinated axons (A δ -fibers) (Harper and Lawson, 1985a, b). It has been suggested that the driving force for the observed phenotypic shift is Wallerian degeneration (Li et al., 2000; Wu et al., 2002; Shamash et al., 2002). Injury of the L5 and L6 spinal nerves results in partially denervated Remak bundles, exposing the intact L4 fibers to toxic byproducts of degeneration. There are reported increases in NGF (Fukuoka et al., 2001) and cytokines including TNF- α (Shamash et al., 2002) following nerve injury. Obata et al., (2005) has demonstrated that exposure of intact L4 axons to NGF results in up-regulation of TRPA1 mRNA in L4 DRG neurons. Furthermore, TNF- α sensitizes fibers to mechanical (and most likely cold) stimulation (Schafers et al., 2003a; Schafers et al., 2003b).

In the present study, 64% of the A δ -fibers in SNL rats were cold-sensitive and this percentage was higher than that obtained for L4 TRPA1 immunostained DRG cells (29.5%). This discrepancy may be explained by the fact that other cold receptors, such as TRPM8 (McKemy et al., 2002; Peier et al., 2002) and those cold receptors not yet cloned (Munns et al., 2006) were involved in cold transduction in primary afferents fibers. The percentages of MO-sensitive A δ -fibers (41.67%) and MO-sensitive L4 DRG cells (45.3%) in SNL rats were in agreement but they were higher than the percentage of L4 TRPA1 immunostained DRG cells (29.5%). This difference could be explained by the signal-to-noise ratio in the immunostained tissue leading to false-negative staining. As previously discussed, we considered the TRPA1 counts to be conservative estimates of the DRG population expressing this receptor.

TRPA1 versus TRPM8 involvement in cold hypersensitivity

In our previous study, we used icilin to study cold sensitivity of intact L4 primary afferents in SNL rats. There were clear changes in the responses of DRGs and peripheral nociceptors to icilin and increases in the percentages of DRG neurons and peripheral nociceptors activated by icilin but icilin will activate both TRPM8 and TRPA1. In the present study MO, a selective ligand for TRPA1 (Jordt et al., 2004; Bautista et al., 2006), was used and this activated intact A δ -fibers, identifying them as playing a critical role in the cold hypersensitivity in this model. Application of 1 mM MO produced a significant increase in discharge rate in A δ -fibers but this concentration was most likely diluted at least 10 – 100 fold by the time it diffused through the connective tissue and reached its target receptors in the skin. Concentrations of 10 – 100 μ M are similar to that used in cell culture studies (Bautista et al., 2006). A 3 mM MO concentration produced the greatest effect on the A δ -fibers but again this would be greatly diluted before it reached its target receptors. At this higher concentration, we cannot rule out the possibility of non-selective activation of other receptors by MO. However, the activity evoked by 3mM MO was significantly reduced by RR, a TRPA1 antagonist, suggesting that TRPA1 contributed to the single fiber responses. In support of a role for TRPA1 in cold transduction, it was recently reported that there was direct gating of TRPA1 channels by cold temperatures (Swada et al., 2007). Also, TRPA1 mRNA but not TRPM8 mRNA was up-regulated in SNL rats and knockdown of TRPA1 but not TRPM8, blocked cold hypersensitivity in SNL rats (Katsura et al., 2006).

We do not rule out a role for TRPM8 in the cold hypersensitivity that develops following nerve injury. On the contrary, TRPM8 has been shown to be a very important thermosensor, sensing both innocuous and noxious cold temperatures in naïve rats and mice (Madrid R et al., 2006; Bautista et al., 2007; Colburn et al., 2007; Dhaka et al., 2007). Furthermore, WT mice with sciatic nerve injury responded in the acetone test with enhanced nocifensive behaviors but TRPM8 knock out mice did not (Colburn et al., 2007). An extensive study by Xing et al., (2007) demonstrated: 1) blockade of TRPM8 in sciatic nerve-injured rats reduced cold allodynia, 2) increased TRPM8 expression in the ipsilateral DRG compared to sham, 3) an increased number and response of L5 DRG cells to menthol (TRPM8 agonist) compared to sham, 4) membrane currents evoked by menthol and cold were significantly increased in nerve-injured compared to sham. Thus, there is clearly a role for TRPM8 in cold transduction and enhanced sensitivity to cold following nerve injury. TRPA1 does not appear to be co-localized with TRPM8 to any great extent (Story et al., 2003; Babes A et al., 2004). Thus, these receptors reside on separate populations of primary afferents and thus probably have distinct roles in encoding cold perception.

Conclusion

In conclusion, the present study shows for the first time an upregulation of TRPA1 protein in the ipsilateral L4 DRG neurons in SNL rats. We also demonstrate that this protein is transported out of the DRG to the peripheral axons where it is localized in unmyelinated and finely myelinated cutaneous axons. Calcium imaging studies confirm an increase in the percentage of MO-sensitive (TRPA1-expressing) DRG cells and an increase in the magnitude of their response to MO. At the single fiber level, physiological correlates for the behavioral allodynia and cold hyperalgesia are identified in a population of A δ -fibers that show a lowered threshold to mechanical activation as well as an increase in percentage and response to cold (0°C) and MO. The data identify a subpopulation of A δ -fibers as a new target in the periphery for therapeutic intervention in pain control in the nerve-injured state.

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A Electrical stimulation

B Mechanical stimulation

C Cold stimulation

Fig. 1. Traces showing the conduction velocity test, standard mechanical ramp and cold stimulation used in the *in vitro* skin nerve preparation

A) The conduction velocity (CV) of each isolated fiber was determined by measuring the distance between stimulating and recording electrodes divided by the latency of the evoked action potential. B) Action potentials (upper trace) of an A δ -fiber evoked by the standard 20 s mechanical ramp (from 0 to 250 mN, lower trace). C) Action potentials (upper trace) of an A δ -fiber evoked by a 30 s cold stimulation (from 34 to $\sim 0^{\circ}\text{C}$, lower trace).

Fig. 2. TRPA1-labeled, L4 DRG cells in SNL rats
Immunostaining with a TRPA1 antibody 11 days post-surgery resulted in an increased percentage of labeled cells on the ipsilateral (A) compared to the contralateral (B) side. Arrows identify small and medium diameter labeled cells. Bar = 25 μ m

Fig. 3. TRPA1-labeled axons in digital nerves from naïve rats

Electron micrographs of axons in hind paw digital nerves from naïve rats, demonstrating reaction product (arrowheads) for TRPA1 in both small myelinated (A, C) and unmyelinated (D, E) axons. Unlabeled axons are also present in B, D and E (*). Bar = 25 μ m

Fig. 4. MO-induced cytosolic Ca^{2+} responses in L4 DRG cells

A) The percentage of MO-sensitive cells was significantly larger in the L4 DRG of SNL compared to naïve and sham rats (** $p < 0.001$, Chi-square test). B) The responses of L4 DRG cells during the 2 min application of MO were significantly increased in SNL rats compared to naïve and sham (* $p < 0.05$, one-way ANOVA).

Fig. 5. Mechanical allodynia in SNL rats

A) The von Frey threshold was significantly decreased on the ipsilateral, but not the contralateral side following SNL surgery (* $p < 0.05$, paired t-test). B) In contrast, there was no change in threshold in sham rats.

Fig. 6. Cold plate-induced behaviors in SNL rats

A) Placing SNL rats on a cold plate (6 °C) resulted in a significant amount of flinching compared to naïve and sham rats ($p < 0.05$, *indicates significant difference from all other groups, one-way ANOVA). B) Placement on a 24 °C plate (room temperature) resulted in little flinching behavior in any group.

Fig. 7. Acetone-induced behaviors in SNL rats

After SNL, the nerve-injured hind paw became much more sensitive to acetone, which evoked significant amounts of flinching (A) and L/L (B) behavior compared to naïve and sham rats ($p < 0.05$, * indicates significant difference from all other groups, one-way ANOVA).

Fig. 8. Mustard oil-induced behavior in SNL rats

After SNL, application of MO (10 mM) to the nerve-injured paw induced significant amounts of flinching (A) and L/L behavior (B) compared to vehicle and compared to MO applied to hind paws of naïve or sham rats. ($p < 0.05$, *indicates significant difference from all other groups, one-way ANOVA).

Fig. 9. Characters of A δ -fibers in naive and SNL rats

A) There was no significant difference in mean conduction velocities comparing naive to SNL rats. B) The mechanical threshold of A δ -fibers was significantly lower in SNL compared to naive rats (* $p < 0.05$, Wilcoxon test). C) The percentage of cold-sensitive A δ -fibers was significantly larger in SNL compared to naive rats (* $p < 0.05$, Chi-square test). D) The percentage of cold/MO-sensitive A δ -fibers was significantly larger in SNL compared to naive rats. (* $p < 0.05$, Chi-square test).

Fig. 10. Responses of A δ -fibers to MO

A) Traces showing MO-induced activity in A δ -fibers. Nociceptors respond following exposure of their receptive fields to MO (0.01, 0.1, 1.0, 3 mM) for 2 min at each concentration. Raw, unfiltered data: upper trace – naive rat, lower trace – SNL rat. B) Application of an ascending series of MO concentrations to the receptive field of A δ -fibers produced a dose-dependent increase in activity ($p < 0.05$, +significantly different from vehicle, Friedman's ANOVA). At 3 mM, MO-induced activity in SNL A δ -fibers was significantly increased compared to naive (* $p < 0.05$, Mann-Whitney U test). C) Pre-treatment with the TRPA1 antagonist ruthenium red (50 μ M, 5 minutes) significantly reduced the 3 mM MO-induced activity in A δ -fibers compared to pre-treatment with vehicle in SNL rats (* $p < 0.05$, Mann-Whitney U test).

Table 1
Stereological estimates of TRPA1-labeled L4 DRG cells

Animal	TRPA1-labeled cells (% of total)	
	ipsilateral	contralateral
1	28.2	14.3
2	25.5	14.1
3	34.9	23.1
Mean $\pm$ SD	29.5 $\pm$ 4.8 *	17.2 $\pm$ 5.1

*
(p<0.05, t-test, mean $\pm$ SD)

Table 2Populations of A δ -fibers recorded in plantar nerves

Unit type	Naïve N = 36 fibers	SNL N = 36 fibers	P value
ADM	23 (64%)	10 (28%)	p<0.01
ADMC	7 (19%)	22 (61%)	p<0.001
ADMHC	1 (3%)	1 (3%)	NS
ADMH	5 (14%)	3 (8%)	NS
Total cold fibers	8 (22%)	23 (64%)	p<0.001

Values are numbers with percents in parentheses. ADM, A δ -mechanical nociceptive units; ADCM, A δ -mechanocold nociceptive units; ADMHC, A δ -mechanoheat-cold nociceptive units; ADMH, A δ -mechanoheat nociceptive units, NS = not significant.