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Plasticity in Intact A δ - and C-Fibers Contributes to Cold Hypersensitivity in Neuropathic Rats

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Abstract

Cold hypersensitivity is a common sensory abnormality accompanying peripheral neuropathies and is difficult to treat. Progress has been made in understanding peripheral mechanisms underlying neuropathic pain but little is known concerning peripheral mechanisms of cold hypersensitivity. The aim of this study was to analyze the contribution of uninjured primary afferents to the cold hypersensitivity that develops in neuropathic rats. Rats with a lumbar 5 (L5) and L6 spinal nerve ligation (SNL, Chung model) but not sham, developed mechanical allodynia, evidenced by decreased paw withdrawal thresholds and increased magnitude of response to von Frey stimulation. Cold hypersensitivity also developed in SNL but not sham rats, evidenced by enhanced nociceptive behaviors induced by placement on a cold plate (6 °C) or application of icilin (a transient receptor potential M8 (TRPM8)/transient receptor potential A1 (TRPA1) receptor agonist) to nerve-injured hind paws. Single fiber recordings demonstrated that the mean conduction velocities of intact L4 cutaneous A δ - and C-fibers were not different between naive and SNL rats; however, mechanical thresholds of the A δ - but not the C-fibers were significantly decreased in SNL compared with naive. There was a higher prevalence of C-mechanoheat-cold (CMHC) fibers in SNL compared with naive, but the overall percentage of cold-sensitive C-fibers was not significantly increased compared with naive. This was in contrast to the numerous changes in A δ -fibers: the percentage of L4 cold sensitive A δ -, but not C-fibers, was significantly increased, the percentage of L4 icilin-sensitive A δ -, but not C-fibers, was significantly increased, the icilin-induced activity of L4 A δ -, but not C-fibers, was significantly increased. Icilin-induced activity was blocked by the TRPA1 antagonist Ruthenium Red. The results indicate plasticity in both A δ - and C-uninjured fibers, but A δ fibers appear to provide a major contribution to cold hypersensitivity in neuropathic rats.

Keywords

peripheral neuropathy; sensitization; TRPA1; TRPM8; neuropathic pain; cold allodynia

Cold hypersensitivity is a striking feature in patients (Wahren and Torebjörk, 1992; Ochoa and Yarnitsky, 1994; Bowsher and Haggett, 2005), and animals (Choi et al., 1994; Vissers and Meert, 2005) with peripheral nerve injuries. The mechanisms underlying enhanced sensitivity to cold remain unclear. Evidence suggests that receptors expressed on peripheral sensory fibers contribute to the sensation of cold pain. For example, it is well documented that peripheral A δ - and C-fibers are activated by non-painful cooling and a subpopulation of nociceptors is excited by noxious cold (Dubner et al., 1975; Georgopoulos, 1976; Leem et al., 1993a; Simone and Kajander, 1997). In agreement with these physiological findings, two cold-activated ion channels, transient receptor potential M8 and A1 (TRPM8 and TRPA1, respectively) have been shown to be expressed by dorsal root ganglion (DRG) cells (Reid and Flonta, 2001a,b;

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McKemy et al., 2002; Peier et al., 2002; Story et al., 2003) and trigeminal ganglion cells (Madrid et al., 2006).

The indications are that primary afferents contribute to the cold hypersensitivity that develops following peripheral nerve injury (Djouhri et al., 2004; Obata et al., 2005); however, the specific roles played by A δ - and C-fibers in this sensory abnormality are unknown. Therefore in the present study, we behaviorally assessed the presence of cold hypersensitivity in rats with a spinal nerve ligation (SNL) (Kim and Chung, 1992). We then examined the contribution of cutaneous A δ - and C-nociceptors to the cold hypersensitivity by performing single fiber recordings in an *in vitro* skin-nerve preparation in naive and nerve-injured rats. The proportion of nociceptors activated by cold stimulation and the proportion and magnitude of response of nociceptors to icilin (TRPA1/TRPM8 agonist) were determined in the recordings and with live cell calcium imaging. Some data have been previously published in abstract form (Ji and Carlton, 2005).

Experimental Procedures

All experiments were approved by the University Animal Care and Use Committee and followed the IASP guidelines for the ethical care and use of laboratory animals (Zimmermann, 1983). Experiments were designed to minimize the number of animals used and their discomfort.

Behavioral studies

Habituation—Male Sprague–Dawley rats (270–320 g) were housed in groups of three in plastic cages with soft bedding under a reversed 12-h light/dark cycle. Following arrival at the animal care facility, they were acclimated for at least 3 days before behavioral testing was initiated. For testing mechanical or icilin sensitivity, rats were habituated two times by placement on a wire mesh platform in Plexiglas cages (8×8×18 cm) for 1 h each session. On the day of testing, rats were placed in the Plexiglas cages and allowed to acclimate for at least 30 min before either spontaneous or evoked behavioral responses were observed. For testing cold sensitivity, rats were habituated two times by placement on a conductive metal platform in Plexiglas cages (8×8×18 cm) for 10 min. The platform was maintained either at room temperature (24 °C) or 6 °C (ice water was circulated in copper tubing attached to the bottom of the platform). For all behavior testing, the experimenter was blinded to the condition (naïve, sham or SNL) of the animal. Animals were tested pre-surgery and 7–14 days post-surgery.

Surgery to induce the peripheral neuropathy—Rats received a unilateral SNL of the 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. With blunt dissection the intraspinal muscles were retracted and the L5 spinal nerve was visualized. Using 6-0 sterile silk (George Tiemann and Co., Hauppauge, NY, USA), one tie was placed around the nerve and knotted 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 incision was closed in layers using 3-0 silk suture. The rats were returned to their cages and monitored until they were up and moving around. As a result of this surgery, axons in the L4 spinal nerve were left intact innervating the plantar hind paw. Some rats (shams) underwent identical surgeries but the spinal nerves were not ligated. Control (naïve) animals did not receive any surgery.

Testing mechanical sensitivity—Two different measures were used to assess changes in mechanical sensitivity using von Frey filaments applied to the plantar surface of the hind paw in sham and SNL rats: changes in paw withdrawal threshold (PWT) and changes in % maximum response frequency. PWT was determined using the Dixon up–down method to establish the threshold at which 50% of the rats withdraw their hind paw (Chaplan et al., 1994). To assess changes in % maximum response, von Frey filaments of three different bending forces (12.9 mN, 22.8 mN and 43.2 mN) were applied 10 times each, with 2–3 s between applications, with a 1–2 min inter-trial interval. For each stimulus, it was noted if the hind paw was moved, and if so, a quality rating was assigned: 1=a mild response where the rat moved the hind paw but did not lose contact with the platform; 2=average response where the hind paw was lifted and then replaced; 3=robust response where the paw was lifted and licked or groomed (Carlton et al., 1995). A % maximum response for each von Frey was then calculated by dividing each animal's score by the total maximum possible (30) and multiplying by 100.

Testing cold-induced responses—Cold stimulation-induced nociception was assessed by quantitating flinching behavior of the left hind paw in naïve, sham and SNL rats during a 5-min trial on a thermo-neutral (room temperature 24 °C) or cold plate (6 °C) (Choi et al., 1994; Jasmin et al., 1998). Flinching was defined as a spontaneous rapid jerk of the whole paw whether the paw was on the platform or held in the air. 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 9 days after SNL or sham surgery.

Testing icilin-induced responses—The TRPA1/TRPM8 agonist icilin, (1 mM made in 1% DMSO), was applied to the plantar skin using a cotton-tip applicator in naïve, sham and SNL rats. As a control, vehicle (1% DMSO) was applied in a similar manner to SNL rats. Icilin-induced nociceptive behaviors were assessed by counting the number of paw flinches in 1 min intervals over a 10 min period.

Electrophysiological studies

In vitro skin-nerve recordings—Single unit recordings from A δ - and C-fibers in glabrous skin were obtained using an *in vitro* skin–nerve preparation (Reeh, 1986; Du et al., 2001, 2006). Male Sprague-Dawley rats (200–300 g) 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.

Heat stimulation—A feedback-controlled lamp (made in house) placed beneath the organ bath supplied radiant heat to the receptive field of each unit. A temperature probe was placed on the corium side in the receptive field and 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 (Fig. 1A, 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 thermal threshold.

Mechanical stimulation—A dual mode lever system (Aurora Scientific Inc., Ontario, Canada) was used to determine mechanical threshold of each unit. The system had a motor-

driven stylus (1.5 mm diameter) that delivered a force in the form of a continuous ascending ramp (from 0 to 250 mN in 20 s). The stylus was placed on the corium side in the most sensitive region of the receptive field of the unit. The gram force eliciting the first spike upon activation of the ramp was considered threshold for that unit (Fig. 1B).

Cold stimulation—A small plastic hollow cylinder (7 mm O.D.×10 mm high), designed to hold chemicals within a circumscribed region was mounted on a micromanipulator and placed over the receptive field of each unit on the corium side. A temperature probe was attached to the inside wall of the cylinder. To apply cold stimulation, the SIF in the cylinder was gently pipetted out and a pellet of frozen SIF was very gently placed into the cylinder, avoiding any mechanical stimulation on the unit. These SIF pellets were made using small capsules (5.6 mm O.D.×10 mm high, BEEM capsules, Electron Microscopy Science, Hatfield, PA, USA) so that their shape and size were consistent from trial to trial. The pellets, stored in the freezer until used, produced rapid cooling of the units from 34.12 ± 0.08 °C to 0.91 ± 0.14 °C 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 was greater than the mean+2 S.D. of background. The mean background rate for a fiber population (A δ or C) was determined by recording unit activity for 2 min prior to application of the cold stimulation and calculating the mean (Du et al., 2003).

Chemical stimulation—A subpopulation of units that displayed a sensitivity to cold (0 °C) was also tested for sensitivity to icilin. Icilin was dissolved in 1% DMSO to make a 10 mM stock solution and then diluted with SIF (buffered to pH 7.40 ± 0.05) to make 0.01, 0.1, 1.0 10.0, and 100.0 μ M icilin. A plastic, hollow cylinder was placed over the receptive fields of the units and the SIF replaced with vehicle (1% DMSO) or the ascending icilin concentrations, each applied for 2 min and unit activity recorded. There was a 30 s SIF wash interval before each subsequent concentration was applied. Cumulative responses of the fibers were analyzed.

To determine if Ruthenium Red (RR) attenuated icilin-induced activity, the SIF in the cylinder was replaced with 50 μ M RR or vehicle. After 5 min, these solutions were replaced with 50 μ M RR+10 μ M icilin, or vehicle+10 μ M icilin for 2 min and activity was recorded. The RR antagonizes TRPA1 (Bandell et al., 2004), but not TRPM8 activation (Weil et al., 2005).

Neurophysiological recordings—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, Sarasota, FL, USA) attached to a monopolar gold wire electrode, with the reference electrode positioned nearby, and recorded using a PC computer-controlled CED1401 interface and Signal software (Cambridge Electronics, UK). The conduction velocity (CV) 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 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 (measured in millimeters). Fibers with a CV of less than 1.6 m/s were classified as C-fibers. Fibers with a CV of over 1.6 m/s and less than 10 m/s were classified as A δ -fibers. These criteria are in agreement with those previously published using a similar skin-nerve preparation (Koltzenburg et al., 1992; Kress et al., 1992).

Ca²⁺ imaging in dissociated DRG cells

Cell culture—Naive (Sprague–Dawley, 180–280 g) or SNL (7–10 days after surgery) rats were deeply anesthetized with sodium pentobarbital (80 mg/kg, i.p.). Both L4 DRGs in naive rats and the ipsilateral L4 DRG in 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 TRIS base and osmolality adjusted with sucrose to 305 mOsm) (Xu and Huang, 2002), and minced into smaller pieces. The DRG fragments were then digested in 0.1% collagenase D (Roche Diagnostics, Indianapolis, IN, USA) and 0.06% trypsin (Sigma-Aldrich, St. Louis, MO, USA) in a 37 °C waterbath for 1 h and then washed three 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-L-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 eight L4 DRGs from SNL rats and 10 DRGs from naive rats were used for this part of the study. Experiments were replicated with DRGs from SNL and naive rats at least four times.

Cytosolic Ca²⁺ imaging—The DRG cells were incubated with 3 μM Fura-2 acetomethoxy ester (Molecular Probes, Eugene, OR, USA) 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, USA), 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, USA). Fluorescent images and ratios were acquired every 2 s, and processed using Meta-morph software (Molecular Devices). The effect of icilin on the cytosolic Ca²⁺ was tested after obtaining a 2 min baseline recording, followed by application of 1, 10 or 100 μM icilin solution for 2 min each, followed by washoff with normal bath solution. There was at least a 5 min interval between applications of ascending icilin doses. Cells were considered responsive to icilin if their F₃₄₀/F₃₈₀ ratio increased by more than 20% during the 2 min icilin application. Cell surface areas were measured by acquiring light transmission images that were analyzed off-line using Meta-morph software (Molecular Devices, Sunnyvale, CA, USA).

Statistics

Data were expressed as means±standard errors (S.E.) and evaluated using SigmaStat (Jandel Corporation, San Rafael, CA, USA). Parametric and non-parametric analyses were used where appropriate (depending on the passing of a normality test). For the behavioral tests, differences between treatment groups were evaluated using a Wilcoxon signed rank test for von Frey data, a Kruskal Wallis test for cold plate data, a one-way ANOVA for icilin data (normality test was passed). In the electrophysiological studies, differences in discharge rates were evaluated with either a Wilcoxon signed rank or Mann Whitney *U* test and differences in populations were evaluated with a chi-square test. Differences in ratios for the Ca²⁺ imaging were determined using a Mann Whitney *U* test. *P*<0.05 was considered significant.

Results

Behavioral studies

Mechanical allodynia—The PWT and mechanical sensitivity of the ipsilateral paw were tested pre-surgery and 7–14 days post-surgery. Pre-surgery, SNL rats had a mean PWT of 145±52 mN and sham rats had 180±0.0 mN (all went to cutoff) and these values were not significantly different (Fig. 2A). Furthermore, during pre-surgery testing, SNL rats were seldom responsive to the three von Frey filaments used in sensitivity testing (mean response frequencies were 1.5±1.1, 1.7±2.2 and 5.4±5.8 mN respectively, Fig. 2B). Post-surgery, the mean PWT of SNL rats was significantly decreased (14±8 mN, *P*<0.05, Wilcoxon signed rank test, Fig. 2A) but sham PWTs did not change significantly from pre-surgery baselines (135±64

mN). The % maximum response to the three von Frey filaments was significantly increased in SNL rats (35.6 ± 17.0 , 50.9 ± 16.2 and 67.1 ± 13.2 mN, respectively), compared with pre-surgery ($P < 0.05$, Wilcoxon signed rank test, Fig. 2B). These data demonstrate the presence of mechanical allodynia in SNL compared with sham rats.

Cold hypersensitivity

Cold plate-induced behaviors—Prior to surgery, naive, sham and SNL groups showed similar amounts of flinching behavior on the cold plate (6°C), with only ~ 0 – 2 flinches in 5 min (data not shown). Following surgery, placement of these groups on the cold plate induced significant amounts of flinching in the nerve-injured hind paw in the SNL rats compared with naive and sham rats (Fig. 3A, $P < 0.05$, Kruskal-Wallis test). Placing naive, sham or SNL rats on a room temperature (24°C) plate produced little or no flinching behavior (Fig. 3B, $P = 0.34$, Kruskal Wallis test). These data demonstrate that SNL produces an increase in cold sensitivity not seen in naïve or sham rats.

Icilin-induced behaviors—Topical application of icilin (1 mM) to the plantar hind paw of naive or sham rats did not produce any flinching behavior during a 10 min trial, however, applying vehicle to the SNL paw did produce some flinching. When icilin was applied to the SNL paw, the flinching that resulted was significantly different from all other groups (Fig. 4, $P < 0.05$, one-way ANOVA). These data demonstrate that the increased cold sensitivity is related to expression of TRPA1/TRPM8 receptors in the periphery.

Electrophysiological studies

General properties of fiber populations analyzed—Based on our behavioral data, there were no significant differences between naive and sham-operated rats when tested for mechanical or cold sensitivity. Therefore in the physiological studies, responses of fibers in naive rats were compared with that in SNL rats. Recordings were made from 176 C-fibers and 177 A δ -fibers in 57 naive and 38 SNL rats. All fibers were mechano-sensitive since we used mechanical probing as our search stimulus. Fibers were placed into categories depending upon their conduction velocity and their response to mechanical, heat and cold stimulation (Leem et al., 1993a). Fibers that did not respond to either heat (up to 51°C) or cold (down to 0°C) stimuli were classified as C- or A δ -mechanical units (CM or ADM). Fibers that responded to mechanical and heat stimuli were classified as C- or A δ -mechanoheat units (CMH or ADMH) and those that responded to mechanical and cold stimuli were classified as C- or A δ -mechanocold units (CMC or ADMC). Fibers that responded to mechanical, heat and cold were classified as C- or A δ -mechanoheat-cold units (CMHC or ADMHC).

C-fiber properties—Of the 176 C-fibers analyzed in this study, 105 were recorded from naive rats and 71 from SNL rats (Table 1). The distribution of C-fiber conduction velocities in SNL rats was similar to that in naive rats (Fig. 5). The spontaneous activity of C-fibers was elevated in SNL (0.09 ± 0.02 impulses/s, $n = 29$) compared with naive rats (0.05 ± 0.01 impulses/s, $n = 31$) but this change was not significant ($P = 0.24$). Surprisingly, the threshold for C-fiber activation to a mechanical stimulus in SNL rats (69.5 ± 9.4 mN) was not different from naive (60.3 ± 8.4 mN, Fig. 6).

C-fiber responses to 0°C —Pellets of frozen SIF were used to cool receptive fields down to $\sim 0^\circ\text{C}$. Two subtypes of mechanocold-sensitive C-fibers (CMC and CMHC) were identified in this study (Table 1). The percentage of CMHC units was significantly increased in SNL (54%) compared with naive (27%) ($P < 0.001$, chi-square test). In contrast, the percentage of CMH units was significantly decreased compared with naive. Surprisingly, fewer CMC units were activated in SNL (13%) compared with naive rats (26%), but this change did not reach significance ($P = 0.056$, chi-square test). Regardless of the increase in the CMHC population,

the total percentage of mechanosensitive C fibers responding to cold stimulation was not different in SNL (66%) compared with naive (52%) (Table 2) due to the decrease in the CMC population.

C-fiber responses to icilin—A subpopulation of units responding to cold stimulation (0 °C) was also tested for responsiveness to icilin (TRPA1/TRPM8 receptor agonist). An ascending series of icilin concentrations (0.01, 0.1, 1.0, 10, 100 μ M) was applied to the receptive fields of C-fibers and cumulative responses recorded. Exposure to vehicle (1% DMSO) did not change the discharge rate compared with background for either group (naive: vehicle=0.04 $\pm$ 0.02; background=0.04 $\pm$ 0.02; SNL: vehicle=0.13 $\pm$ 0.07; background=0.13 $\pm$ 0.06). Compared with vehicle, icilin produced a dose-dependent increase in activity at 1, 10 and 100 μ M concentrations (Fig. 7A, $P<0.05$, Friedman's ANOVA); however, there was no difference in the icilin-induced C-fiber discharge rates at any dose comparing naive and SNL rats (Fig. 7A) and the percentage of C-fibers activated by icilin did not change in naive compared with SNL rats (Table 2).

A δ -fiber properties—Of the 177 A δ -fiber analyzed in this study, 90 were recorded from naive rats and 87 from SNL rats (Table 1). The distribution of A δ -fiber conduction velocities in SNL rats was similar to that in naive rats (Fig. 5). The spontaneous activity of A δ -fibers was elevated in SNL rats (0.08 $\pm$ 0.02 impulses/s, $n=37$) compared with naive rats (0.03 $\pm$ 0.01 impulses/s, $n=25$) but this change was not significant ($P=0.24$). The threshold for A δ -fiber activation to a mechanical stimulus in SNL rats (31.2 $\pm$ 5.2 mN) was significantly lower compared with naive rats (52.1 $\pm$ 4.9 mN, Fig. 6, $P<0.01$, Wilcoxon signed rank test).

A δ -fiber responses to 0 °C—Two subtypes of mechanocold-sensitive A δ -fibers (ADMC and ADMHC) were identified in this study, and the majority were ADMC (Table 1). The percentage of ADMC units was significantly increased in SNL (55%) compared with naive (30%) ($P<0.01$, chi-square test), but the percentage of ADMHC units did not change (SNL=7%, naive=6%). The percentage of ADM units was significantly decreased in SNL compared with naive rats, ($P<0.01$, chi-square test). As a result, the total percentage of mechanosensitive A δ -fibers responding to cold stimulation was significantly larger in SNL (62%) compared with naive (36%) rats (Table 2, $P<0.05$, chi-square test).

A δ -fiber responses to icilin—Activation TRPA1/TRPM8 receptors on a subpopulation of cold sensitive fibers was accomplished by application of an ascending series of icilin concentrations (0.01, 0.1, 1.0, 10, 100 μ M) to the receptive fields. Exposure to vehicle (1% DMSO) did not change the discharge rate compared with background for either group (naive: vehicle=0.04 $\pm$ 0.02; background=0.03 $\pm$ 0.02; SNL: vehicle=0.08 $\pm$ 0.03; background=0.08 $\pm$ 0.02). Icilin concentrations of 10 and 100 μ M produced a significant increase in discharge rate compared with vehicle (Fig. 7B, $P<0.05$, Friedman's ANOVA). Icilin at 10 μ M and 100 μ M induced a significant increase in mean discharge rate in SNL compared with naive (Fig. 7B, $P<0.05$, Mann Whitney U test, Fig. 8) and a greater percentage of A δ -fibers were icilin-sensitive (10 μ M) in SNL compared with naive (Table 2, $P<0.05$, chi-square test). Pre-treatment of the receptive field of A δ -fibers (ADMC) with RR (50 μ M, 5 min, a TRPA1 antagonist) significantly reduced icilin-induced activity such that icilin alone produced a discharge rate of 1.01 $\pm$ 0.34 impulses/s ($n=5$) but icilin + RR produced a lowered discharge rate of 0.20 $\pm$ 0.05 impulses/s ($P<0.05$, Mann Whitney U test).

Calcium imaging studies

Ca²⁺ imaging in dissociated DRG cells—Icilin-induced cytosolic calcium spiking activity in DRG cells was reflected as the changes in the ratio of Fura-2 fluorescence when excited at 340 nm and 380 nm. In randomly chosen fields in wells containing L4 DRG cells

from naive or SNL rats, almost no cells responded to 1 μM icilin while almost every cell responded to the highest dose of 100 μM suggesting a nonspecific response, therefore 10 μM was chosen for analysis. Upon application of icilin, calcium spiking activity was maximum in the cells within the first 1–2 min and then tapered off to a sustained response that was elevated above baseline for at least 5 min (Fig. 9). In SNL rats 65% (17/26) of cells responded to 10 μM icilin compared with 15% (15/102) from naive rats ($P < 0.001$, chi-square test). The magnitude of the response, calculated as a mean change in ratio during the 2 min icilin application, was also significantly increased in SNL (2.09 ± 0.03) compared with naïve (1.27 ± 0.05) (Fig. 9 inset, $P < 0.001$, Mann Whitney test). The mean surface area for icilin-sensitive DRG cells was $721 \pm 56 \mu\text{m}^2$ in SNL and $855 \pm 89 \mu\text{m}^2$ in naive rats and these were not different.

Discussion

This study indicates SNL rats develop mechanical allodynia, evidenced by decreases in PWT and increases in % maximum response to mechanical stimulation. Cold hypersensitivity also develops evidenced by nociceptive behaviors induced by exposure of the nerve-injured paw to 6 °C (cold plate) or icilin (a TRPM8/TRPA1 receptor agonist). Single fiber recordings from intact L4 axons demonstrate that the prevalence of CMHC and ADMC fibers increases in SNL rats compared with naive but CMH and ADM fibers decrease. Furthermore, there is a significant increase in the magnitude of response as well as the percentage of A δ -, but not C-fibers, responding to 0 °C and icilin in the intact L4. Icilin-induced activity is blocked by the TRPA1 antagonist RR. The Ca²⁺ imaging in the DRG cells confirms the single fiber data, demonstrating that the percentage and magnitude of response to icilin are significantly increased in the L4 DRG cells in SNL compared with naive. Thus, the data indicate that uninjured A δ and C primary afferent fibers, expressing TRPA1, contribute significantly to cold hypersensitivity in SNL rats.

Behavioral evidence of allodynia

Mechanical allodynia and cold hypersensitivity are striking features of neuropathic pain in humans (Wahren and Torebjörk, 1992; Ochoa and Yarnitsky, 1994; Dworkin, 2002; Herr, 2004; Samuelsson et al., 2005) and animal models (Kim and Chung, 1992; Carlton et al., 1994; Choi et al., 1994; Baron, 2000). We confirm the presence of mechanical allodynia in SNL rats via two different von Frey testing paradigms. While there is no doubt that the injured fibers in the SNL model (from L5 and L6) contribute to the mechanical allodynia (Sheen and Chung, 1993; Yoon et al., 1996; Jang et al., 2007), there is also behavioral evidence that activity in uninjured L4 afferents aids in the development of neuropathic pain as well (Lee et al., 2003; Jang et al., 2007).

Cold hypersensitivity is also demonstrated by two different testing paradigms in the current study: cold plate and icilin. While the cold plate is a non-selective stimulus for cold receptors, icilin specifically activates TRPA1 and TRPM8 cold receptors presumed to be located on nociceptor terminals in the skin. Each paradigm resulted in enhanced nociceptive behaviors in SNL compared with naïve and sham rats. We could rule out contributions of mechanical stimulation to the enhanced cold responses in SNL rats because their behavior was no different from naive or sham rats when placed on the metal plate kept at room temperature. Furthermore, cotton-tipped applicators were used to apply icilin to the hind paws of the naive, sham and SNL groups but only the SNL group showed prolonged nociceptive behavior.

Physiological correlates to behavioral changes

Primary afferent fiber contributions to mechanical hypersensitivity—There is no doubt that changes in the CNS contribute to the manifestations of mechanical allodynia. Dorsal horn neurons show increased background activity and enhanced activity following mechanical

stimulation in nerve-injured limbs (Palecek et al., 1992a,b; Laird and Bennett, 1993). However, there is substantial evidence that primary afferents, both injured and uninjured, play a major role in the expression of the sensory abnormalities following nerve injury (Ringkamp and Meyer, 2005). For example, the development of ongoing activity or altered excitability in injured peripheral nerves or their DRG is demonstrated following nerve injury in humans (Campbell et al., 1988; Gracely et al., 1992) and animals (Kajander et al., 1992; Kajander and Bennett, 1992; Ali et al., 1999; Liu et al., 2000a,b; Ma et al., 2003; Sapunar et al., 2005). *In vivo* preparations show that intact fibers also develop a spontaneous activity (Ali et al., 1999; Wu et al., 2001; Shim et al., 2005). In the present *in vitro* study, the results show an elevation in spontaneous activity in intact A δ - and C-fibers after SNL, although these elevations did not reach significance. Differences in preparation (*in vitro* versus *in vivo*) or recording period for spontaneous activity (2 min [present study] versus 5 min [Wu et al., 2001]) may account for this discrepancy. Shim et al. (2007) reported an enhancement of activity-dependent slowing of CV and supranormal CV in uninjured nerves following L5 SNL, providing further evidence that the excitability of uninjured primary afferent fibers can be altered in the presence of injured afferents. We did not analyze activity-dependent slowing of CVs in this study; however, given that the fibers appeared to be changing categories, we compared the distribution of CVs to provide some assurance that we were studying comparable A δ - and C-fiber populations in the SNL and naïve groups.

Shim et al. (2005) report that response thresholds of C- and A δ -fibers to mechanical stimuli is lower in SNL rats compared with shams, providing the first evidence that uninjured nociceptors innervating the hind paw skin become sensitized to mechanical stimuli. The present study confirms a lowered threshold for intact A δ -fibers in SNL rats, but we find no change in mechanical thresholds of C-fibers. This discrepancy may be due to differences in paradigms and methodology since the former study uses an *in vivo* preparation and von Frey filaments while the present study uses an *in vitro* preparation and a motor driven stylus to deliver the mechanical stimulus. Nevertheless, both *in vivo* (Shim et al., 2005) and *in vitro* (current study) data demonstrate that intact primary afferents could contribute to the processes leading to central sensitization and the development of mechanical hypersensitivity that arises following SNL. Finally, due to the fact mechanical thresholds of A δ fibers were significantly lowered in SNL compared with naïve in this study, it is likely that the percentage of mechano-sensitive A δ fibers is inflated in relation to the entire A δ population. Some of these fibers may have been mechano-insensitive fibers pre-surgery.

Primary afferent fiber contributions to cold sensitivity—It is well known that innocuous and noxious cold sensations in normal rats are mediated by primary afferent fibers, including both A δ - and C-fibers (Georgopoulos, 1976, 1977; LaMotte and Thalhammer, 1982; Leem et al., 1993b; Simone and Kajander, 1996; Takahashi et al., 2003). In the present study, 36% of the A δ -fiber and 52% of the C-fiber population are excited by noxious ($\sim 0^\circ\text{C}$) cold and this is consistent with previous reports in rat (Simone and Kajander, 1997) and monkey (Georgopoulos, 1976, 1977). Simone and Kajander (1996) reported that stimulus temperatures $\leq 0^\circ\text{C}$ were required to excite A δ fibers thus, the population reported here is a conservative estimate of A δ -cold sensitive fibers. Leem et al. (1993) using an electrical search stimulus reported that $\sim 31\%$ of C- but no A δ -fibers responded to cold stimulation in the rat plantar nerve, however, the stimulus was 12°C and thus above the threshold for activation of normal A δ fibers.

Phenotypic switch of uninjured cutaneous afferent fibers

The present results show for the first time that dramatic changes in uninjured C- and A δ -fibers underlie the cold sensitivity that arises in the neuropathic condition. Even though the ligation surgery reduces the absolute number of axons in SNL paws compared with naïve and sham

paws, the proportions of the various fiber types would remain the same. Nevertheless, in SNL rats the percentage of CMH and ADM units significantly decreases while the percentage of CMHC and ADMC units significantly increases compared with naive. The most likely explanation is that a phenotypic switch occurs where some CMH and ADM units begin to express ion channels/receptors activated by cooling or cold stimulation (i.e. TRPM8 or TRPA1) and thus are now classified as CMHC and ADMC, respectively. This hypothesis is supported by the calcium imaging studies which demonstrate a significant increase in the population of icilin-sensitive L4 DRG cells after SNL. These data are also consistent with reported increases in cold-responsive uninjured DRG neurons following SNL (Djouhri et al., 2004) and a reported increase in TRPA1 mRNA in uninjured L4 DRG cells in a similar neuropathic model (Obata et al., 2005).

In the present study, mechanical probing was used as a search stimulus, therefore changes in properties of fibers responding only to cold (Leem et al., 1993a) were not assessed and no conclusion can be made concerning any changes in their sensitivity to cold or to other modalities. It is highly unlikely that any high threshold mechanoreceptors were missed in our analysis since our search stimulus was a suprathreshold stimulus (>100 mN), exceeding the threshold of all mechanoreceptors with the exception of silent nociceptors.

It has been reported that intact primary afferent axons in the vicinity of injured fibers can undergo phenotypic switches. De novo synthesis of tumor necrosis factor- α receptors (TNF- α , Schafers et al., 2003b), brain-derived neurotrophic factor (BDNF) (Fukuoka et al., 2001; Obata et al., 2003), TRPV1 (Hudson et al., 2001), or SNS (Nav1.8) sodium channels (Porreca et al., 1999) can occur in uninjured DRG neurons following nerve injury. Wallerian degeneration may be a driving force for the phenotypic shifts (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 intact L4 fibers to byproducts of Wallerian degeneration. This process induces an increase in nerve growth factor (NGF) (Fukuoka et al., 2001) and cytokines including TNF- α (Shamash et al., 2002). When intact L4 axons are exposed to this altered environment, NGF induces up-regulation of TRPA1 in L4 DRG neurons (Obata et al., 2005) and TNF- α sensitizes the fibers to mechanical (and probably cold) stimulation (Schafers et al., 2003a,b).

Contributions of cold receptors (TRPA1/TRPM8) to neuropathic pain

Important progress had been made in understanding the molecular basis of cold transduction following cloning of cold-activated ion channels in the transient receptor potential (TRP) family. At least two cold-activated ion channels, TRPM8 and TRPA1, are expressed by DRG cells and activated by icilin. There is some controversy concerning TRPA1 and whether it participates in responses to painful cold stimuli since one TRPA1 knockout strain shows little change compared with wild type in sensitivity to intense cold (Bautista et al., 2006), while another TRPA1 knockout strain shows a reduced sensitivity to noxious cold (Kwan et al., 2006). Compensatory processes occurring during embryologic development of these knockout mice may account for the differences. It has been shown that a significant proportion of DRG cells responding to cold stimulation (5 °C) do not express either TRPM8 or TRPA1 receptor, indicating that additional receptors are important in cold transduction (Munns et al., 2006; Babes et al., 2006). One study reports that only 5% of DRG cells in mouse co-express TRPM8 and TRPA1 (Munns et al., 2006). In the present study RR, a TRPA1 antagonist (Bandell et al., 2004; Weil et al., 2005), significantly reduced icilin-induced nociceptor activity in the SNL rats, suggesting that TRPA1 activation contributed in part to the cold hypersensitivity. In agreement with this, expression of TRPA1 mRNA, but not TRPM8, was significantly increased in DRG cells after SNL (Obata et al., 2005) and knockdown of TRPA1 but not TRPM8, alleviated cold hyperalgesia after SNL (Katsura et al., 2006). The present study demonstrates

that SNL rats had increased percentages of CMHC and ADMC fibers compared with naive. These fiber types would arise from small and medium diameter DRG cells, respectively. Our findings are consistent with reports of an increased expression of TRPA1 mRNA in small and medium diameter DRG cells (Obata et al., 2005). It is suggested that TRPA1 is minimally expressed in A δ fibers (Kobayashi et al., 2005). In the present study, 38% of A δ fibers in naive rats responded to icilin, indicating the presence of TRPA1 and/or TRPM8. Ongoing studies in the laboratory using more selective TRPA1 ligands (mustard oil) will clarify the cutaneous fiber types expressing these cold receptors in the naive and nerve-injured state. However, our current data and the studies cited above strongly indicate that TRPA1 receptors are important contributors to the cold hypersensitivity that develops following peripheral nerve injury, but we do not rule out contributions from TRPM8 in SNL rats at this time.

Conclusion

The behavioral studies indicate that cold and mechanical allodynia is induced after SNL. The electrophysiological studies indicate that a phenotypic switch occurs in both the intact C- and A δ -fiber populations. While there are some changes in uninjured C-fibers, such as a higher prevalence of CMHC in the SNL compared with naive, the overall percentage of cold-sensitive C-fibers is not significantly increased. Thus, it appears that A δ -fibers in particular, play a pivotal role in the expression of cold hypersensitivity after nerve injury. The percentage of L4 cold-sensitive A δ -, but not C-fibers, is significantly increased; the percentage of L4 icilin-sensitive A δ -, but not C-fibers, is significantly increased, the icilin-induced activity of L4 A δ -, but not C-fibers, is significantly increased. The antagonist RR reduces nociceptor responses to icilin suggesting involvement of TRPA1. Finally, calcium imaging studies confirm an increase in percentage and magnitude of response of L4 DRG cells to icilin in SNL compared with naive rats. Although dorsal horn neurons in neuropathic rats show increased activity to mechanical and cold stimulation of the nerve-injured limb (Palecek et al., 1992a,b; Laird and Bennett, 1993), the present data indicate that changes in primary afferent fibers are the driving force for these central changes.

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Abbreviations

ADM	A δ -mechanical unit
ADMC	A δ -mechanocold unit
ADMH	A δ -mechanoheat unit
ADMHC	A δ -mechanoheat-cold unit
CM	C-mechanoreceptor unit
CMC	C-mechanocold unit
CMH	C-mechanoheat unit
CMHC	C-mechanoheat-cold unit
CV	conduction velocity
DRG	dorsal root ganglion
NGF	nerve growth factor

PWT	paw withdrawal threshold
RR	Ruthenium Red
SIF	synthetic interstitial fluid
SNL	spinal nerve ligation
TNF-α	tumor necrosis factor- α
TRPA1	transient receptor potential A1
TRPM8	transient receptor potential M8

Fig. 1.

Traces showing standard heat, mechanical and cold stimulation. (A) Action potentials (upper trace) evoked from an A δ -fiber by the standard 10 s heat ramp starting from 34 and rising to 51 °C on the epidermal surface (lower trace, 51 °C on the epidermal side=47 °C on the corium side). (B) Action potentials (upper trace) evoked from an A δ -fiber by the standard 20 s mechanical ramp (from 0 to 250 mN) stimulation (lower trace). (C) Action potentials (upper trace) evoked from an A δ -fiber by the 30 s cold (from 34 to ~0 °C) stimulation (lower trace).

Fig. 2.

Mechanical allodynia in SNL rats. (A) Compared with pre-surgery, the post-surgery mean mechanical threshold of the nerve-injured paw was significantly decreased but there was no change in threshold in sham rats. (B) The mean % maximum response frequency to von Frey filament stimulation (12.9 mN, 22.8 mN and 43.2 mN) was significantly increased in SNL rats comparing pre- to post-surgery (* $P < 0.05$ compared with pre-surgery, Wilcoxon signed rank test).

Fig. 3.

Cold plate-induced pain behaviors in SNL rats. (A) Compared with pre-surgery, post-surgery flinching was significantly increased for the nerve-injured paw of SNL rats when placed on a 6 °C cold plate. In contrast, sham rats showed no change in flinching behavior post-surgery and were no different than naive rats. (B) Placement on a 24 °C plate resulted in little flinching behavior in any group. (* $P < 0.05$ compared to sham and naive, Kruskal Wallis test).

Fig. 4.

Icilin-induced pain behaviors in SNL rats. Application of 1 mM icilin to the plantar surface of the SNL hind paw induced significant amounts of flinching behavior compared with naïve and sham rats and compared with vehicle in SNL rats (* $P < 0.05$, indicates significant difference from all other groups, one-way ANOVA). (Rats were tested at 7 days post-surgery.)

Fig. 5.

Conduction velocities of A δ - and C-fibers in naive and SNL rats. There appeared to be no difference in the distribution of conduction velocities for A δ - and C-fibers comparing naive to SNL rats. Furthermore, the C-fiber conduction velocities in SNL rats (median=0.75, 25th percentile=0.64, 75th percentile=0.99) were similar to naive (median=0.70; 25th percentile=0.58, 75th percentile=0.91) and the A δ -fiber conduction velocities in SNL rats (median=2.77 m/s, 25th percentile=2.12 m/s, 75th percentile=3.78 m/s) were similar to naive (median=2.71 m/s, 25th percentile=2.12 m/s, 75th percentile=3.71 m/s).

Fig. 6. Mechanical threshold of Aδ- and C-fibers in naive and SNL rats. The mechanical threshold of Aδ-fibers was significantly lower in SNL compared with naive rats, but there was no difference in C-fiber mechanical sensitivity (** $P < 0.01$, Wilcoxon signed rank test).

Fig. 7.

Icilin-induced dose response for A δ - and C-fibers. (A) Application of an ascending series of icilin concentrations (0.01, 0.1, 1.0, 10, 100 μM) to the receptive field of C-fibers (A) and A δ -fibers (B) produced a dose-dependent increase in activity in both fiber populations (* $P < 0.05$, indicates significant difference from vehicle for that group, Friedman's ANOVA). Icilin-induced activity in C-fibers was similar in naive and SNL rats. In contrast, icilin-induced activity in A δ -fibers was significantly increased at 10 and 100 μM in SNL compared with naive rats (* $P < 0.05$, indicates significant difference from naive at same concentration, Mann Whitney U test).

Fig. 8. Icilin-induced activity in A δ -fibers. Examples of nociceptor activity evoked in SNL and naive hind paws following exposure of the receptive field to icilin (10 μ M) for 2 min.

Fig. 9.

Icilin-induced cytosolic Ca^{2+} responses in L4 DRG cells. Responses to icilin are reflected as changes in FURA-2 F340/F380 nm ratio. During the 2 min application of icilin, the cytosolic Ca^{2+} change was significantly larger in SNL compared with naive (inset, *** $P < 0.001$, Mann-Whitney U test).

Table 1

Populations of units recorded in the plantar nerves

Unit type	Naive	SNL	<i>P</i> value
C-fibers	Total=105	Total=71	
CMH	25% (26)	11% (8)	<i>P</i> <0.05
CMHC	27% (28)	54% (38)	<i>P</i> <0.001
CMC	26% (27)	13% (9)	NS
CM	22% (24)	22% (16)	NS
Aδ-fibers	Total=90	Total=87	
ADM	58% (53)	36% (31)	<i>P</i> <0.01
ADMC	30% (27)	55% (48)	<i>P</i> <0.01
ADMHC	6% (5)	7% (6)	NS
ADMH	6% (5)	2% (2)	NS

Due to our search stimulus, these populations represent units that are mechanosensitive. Values are percents with numbers of fibers in parentheses. CMH, C-mechanoheat units; CMHC, C-mechanoheat cold units; CMC, C-mechanocold nociceptive units; CM, C-mechanoreceptor units; ADM, Aδ-mechanoreceptor units; ADCM, Aδ-mechanocold units; ADMHC, Aδ-mechanoheat cold units; ADMH, Aδ-mechanoheat nociceptive units. Naive was compared to SNL using a chi-square test; NS=not significant.

Table 2

Cold and icilin responsive units

Unit type	Naive	SNL	<i>P</i> value
Total Cold-sensitive fibers			
C (CMHC, CMC)	52% (55/105)	66% (47/71)	NS
A δ (ADMC, ADMHC)	36% (32/90)	62% (54/87)	<i>P</i> <0.05
Icilin-sensitive fibers (subpopulation of cold-sensitive fibers)			
C (CMHC, CMC)	83% (5/6)	70% (7/10)	NS
A δ (ADMC, ADMHC)	38% (6/16)	77% (17/22)	<i>P</i> <0.05

Due to our search stimulus, these populations represent units that are mechanosensitive. Values are percents with numbers of fibers in parentheses.