Novel TRPM8 Antagonist Attenuates Cold Hypersensitivity after Peripheral Nerve Injury in Rats

Ryan Patel, Leonor Gonçalves, Robert Newman, Feng Li Jiang, Anne Goldby, Jennifer Reeve, Alan Hendrick, Martin Teall, Duncan Hannah, Sarah Almond, Nicola Brice, and Anthony H. Dickenson


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ABSTRACT
Abnormal cold sensitivity is a common feature of a range of neuropathies. In the murine somatosensory system, multiple aspects of cold sensitivity are dependent on TRPM8, both short term and in response to peripheral nerve injury. The specialized nature of cold-sensitive afferents and the restricted expression of TRPM8 render it an attractive target for the treatment of cold hypersensitivity. This current study examines the effect of a novel TRPM8 antagonist (M8-An) in naïve and spinal nerve-ligated rats through behavioral and in vivo electrophysiological approaches. In vitro, M8-An inhibited icilin-evoked Ca2+ currents in HEk293 cells stably expressing human TRPM8 with an IC50 of 10.9 nM. In vivo, systemic M8-An transiently decreased core body temperature. Deep dorsal horn recordings were made in vivo from neurons innervating the hind paw. M8-An inhibited neuronal responses to innocuous and noxious cooling of the receptive field in spinal nerve-ligated rats but not in naïve rats. No effect on neuronal responses to mechanical and heat stimulation was observed. In addition, M8-An also attenuated behavioral responses to cold but not mechanical stimulation after nerve ligation without affecting the uninjured contralateral response. The data presented here support a contribution of TRPM8 to the pathophysiology of cold hypersensitivity in this model and highlight the potential of the pharmacological block of TRPM8 in alleviating the associated symptoms.

Introduction
Cold hypersensitivity and hyperalgesia are often symptoms of several neuropathic conditions, including complex regional pain syndrome and trigeminal neuralgia, peripheral nerve injury (Maier et al., 2010), and also chemotherapy-induced neuropathy where cancer treatment is often limited because of adverse effects and the resulting neuropathy is largely undertreated by currently available drugs (Kaley and DeAngelis, 2009). Cold hypoaesthesia can exist alongside cold hyperalgesia; patients often describe pain elicited by low temperatures as having a burning quality (Ochoa and Yarnitsky, 1994).

Much diversity exists in the sensations evoked by cold; however, relatively little is known about transduction of cold temperatures at the molecular level or how this relates to the properties of cold perception. In rodents, cold sensitivity is predominantly conferred by TRPM8, a member of the transient receptor potential melastatin family, which is gated by cold temperatures below 25°C and cold mimetic compounds such as menthol (McKemy et al., 2002; Peier et al., 2002). TRPM8 is expressed in a subset of Aδ- and C-fibers; mice devoid of TRPM8 display deficits in aversion to innocuous cold temperatures (Bautista et al., 2007; Colburn et al., 2007; Dhaka et al., 2007) and to a certain degree noxious cold temperatures (Knowlton et al., 2010). This points to the likely existence of at least one other noxious cold sensor, proposed by some to be TRPA1 (Kwan et al., 2006; Karashima et al., 2009). However this remains controversial and is disputed by others (Bautista et al., 2006; Knowlton et al., 2010). Sensitivity to noxious cold does, however, appear dependent on Nav1.8 afferents, a subpopulation of which expresses TRPM8 (Zimmermann et al., 2007; Minett et al., 2012). Ablation of TRPM8+ afferents results in insensitivity to cool temperatures, cold-induced analgesia, and noxious cold without affecting other modalities (Knowlton et al., 2013).

Genetic ablation of TRPM8 abolishes cold-evoked behaviors after peripheral inflammation or nerve injury (Colburn et al., 2007) and in models of chemotherapy-induced neuropathy (Descoeur et al., 2011). Several lines of evidence support the therapeutic potential of TRPM8 antagonists, which have been demonstrated to be efficacious in neuropathy and inflammatory models (Knowlton et al., 2011; Calvo et al., 2012) in addition to inhibiting bladder reflexes (Lashinger et al., 2008). This has raised the prospects that pharmacological...
modulation of TRPM8 could be beneficial in the treatment of aberrant cold sensitivity. In this study, we used a novel TRPM8 antagonist to examine the role of TRPM8 in cold sensitivity in naive and spinal nerve-ligated (SNL) rats using behavioral and in vivo electrophysiological techniques to examine responses to threshold and suprathreshold cold stimulation respectively.

Materials and Methods

Animals. Male Sprague-Dawley rats (250–300 g) were used for behavioral and electrophysiological experiments, and male Wistar rats (230–300 g) were used for thermal telemetry. Animals were group housed on a 12-hour/12-hour light-dark cycle; food and water were available ad libitum. All procedures described here were approved by the appropriate governmental authorities and were designed with commitment to reduce numbers and undue suffering in accordance with International Association for the Study of Pain ethics guidelines (Zimmermann, 1983).

Calcium Imaging. TRPM8 antagonist activity was determined by measuring changes in intracellular calcium levels using a Ca2+-sensitive fluorescent dye. The changes in fluorescent signal were monitored by FLIPR (Molecular Devices, Sunnyvale, CA). TRPM8-mediated increases in intracellular Ca2+ concentration were readily detected upon activation with icilin. Twenty-four hours prior to the assay, HEK293 cells stably expressing human TRPM8 were seeded in cell culture medium in black, clear-bottom poly-L-lysine-coated 384-well plates (BD Biosciences, Oxford, UK) and grown overnight at 37°C, 5% CO2. On the day of the assay, cell culture media were removed, and cells were loaded with Calcium 5 Dye (Molecular Devices) for 1 hour at 37°C, 5% CO2. M8-An [6-(2-[2-fluoro-6-(trifluoromethyl)phenoxyl]-2-methylpropyl}carbamoyl)pyridine-3-carboxylic acid; synthesized in house] was added to cells for 15 minutes prior to the addition of icilin (0.32 μM) to all wells to achieve a final concentration that produces an approximately 80% maximal response. The IC50 values were determined from a seven-point concentration response curve generated using the average of two wells for each data point.

Thermal Telemetry. G2 E-mitter telemetry probes (Mini Mitter, Portland, OR) were surgically implanted and secured into the peritoneal cavity under general anesthesia [ketamine (75 mg/kg i.p.) and xylazine (10 mg/kg i.p.)]. The animals were housed singly in cages after surgery and were allowed to recover for at least 1 week before drug testing. All animals were tested at the same time of day (10:30 AM). On the day of testing, each animal in its own home cage was placed individually in the telemetry receiver to acclimatize for at least 3 hours before baseline data collection. The baseline temperature was determined as the mean of 60 minutes recording immediately before intraperitoneal dosing of either vehicle [85% normal saline, 10% Cremaphor (Sigma-Aldrich, St. Louis, MO) and 5% dimethylsulfoxide (Sigma-Aldrich)] or 30 or 100 mg/kg M8-An. Core body temperature was measured using Vital View software (Mini Mitter) for 360 minutes postdosing.

Spinal Nerve Ligation Surgery. SNL surgery was performed as described by Kim and Chung (1992). Rats (125–135 g) were maintained under 2% v/v isoflurane anesthesia delivered in a 3:2 ratio of nitrous oxide and oxygen. Under aseptic conditions a paraspinal incision was made and the left tail muscle was incised. Part of the L5 transverse process was removed to expose the L5 and L6 spinal nerves, which were then isolated with a glass nerve hook (Ski-Ry Ltd, London, UK) and ligated with a nonabsorbable 6-0 braided silk thread proximal to the formation of the sciatic nerve. The surrounding muscle was closed with absorbable 3-0 sutures and the skin with Michel clips. All rats groomed normally and gained weight in the following days after surgery.

Behavioral Testing. Behavioral testing of SNL rats was performed 14 days postsurgery. Rats were placed inside Perspex chambers on a wire mesh floor and allowed to acclimatize. Fifty percent withdrawal thresholds were determined using the up-down method described by Chaplan et al. (1994) with von Frey filaments (Touch-Test, North Coast Medical, Gilroy, CA), proving forces of 1.4, 2, 4, 6, 8, 10, and 15g. Filaments were applied until they buckled for 5–6 seconds. All rats had mechanical hypersensitivity defined as at least a 50% difference in paw withdrawal threshold between ipsilateral and contralateral paws. Cold hypersensitivity was tested by applying a drop of acetone to the plantar surface of the paw using a modified 1-ml syringe. Acetone was applied five times, rats were allowed time to recover between applications. Flinching, licking, biting, and shaking were considered positive responses to cold or mechanical stimulation. Only rats with significant cold hypersensitivity were used for analysis, which was defined as three or more withdrawals out of five on the nerve-injured side (3/14 rats were excluded). Rats were then dosed intraperitoneally with vehicle or 30 mg/kg M8-An. Behavioral testing was repeated 30, 60, and 90 minutes postdosing. The experimenter was blind to the treatment during behavioral testing.

In Vivo Electrophysiology. In vivo electrophysiology was conducted as previously described (Urch and Dickinson, 2003). Spinal nerve-ligated rats were used between days 15 and 18 postsurgery. Animals were anesthetized with 3.5% v/v isoflurane delivered in 3:2 ratio of nitrous oxide and oxygen. Once areflexic, a tracheotomy was performed, and rats were subsequently maintained at 1.5% v/v isoflurane for the remainder of the experiment. Rats were secured in a stereotaxic frame, and a luminoestetomy was performed to expose L2–L4 segments of the spinal cord. Extracellular recordings were made from deep dorsal horn wide dynamic range (WDR) spinal neurons (lamina V/VI) with receptive fields on the glabrous skin of the toes using parylene-coated tungsten electrodes (A-M Systems, Sequim, WA).

Electrical stimulation of WDR neurons was delivered transcutaneously via needles inserted into the receptive field. A train of 16 electrical stimuli (2-ms pulses, 0.5 Hz) was applied at three times the threshold current for C-fiber activation. Responses evoked by Aβ (0–20 ms), Aδ (20–90 ms), and C-fibers (90–350 ms) were separated and quantified on the basis of latency. Neuronal responses occurring after the C-fiber latency band were classed as postdischarge. The input and the wind-up were calculated as input = (action potentials evoked by first pulse) × total number of pulses (16), wind-up = (total action potentials after 16 train stimulus) – input. The receptive field was also stimulated using a range of natural stimuli (brush, von Frey filaments—2, 8, 15, 26, and 60 g—and heat—35, 42, 45, and 48°C) applied over a period of 10 seconds per stimulus and the evoked response quantified. The heat stimulus was applied with a constant water jet onto the center of the receptive field. Acetone (100 μl) and ethyl chloride (Miller Medical Supplies, Newport, UK) were applied as an evaporative innocuous cooling and noxious cooling stimulus, respectively. Neuronal response to room temperature water was subtracted from acetone and ethyl chloride response to control for concomitant mechanical stimulation during application.

Data were captured and analyzed by a Cambridge Electronic Design 1401 interface coupled to a computer with Spike 2 software (Cambridge, UK) with poststimulus time histogram and rate functions. After three consecutive stable baseline responses to natural stimuli (<10% variation, data were averaged to give control values), animals were injected subcutaneously into the contralateral flank with either 30 or 100 mg/kg M8-An. Responses to electrical and natural stimuli were measured 30 minutes postsedation and then every 20 minutes for the following 80 minutes. One neuron was characterized per rat.

Statistics. Statistical analyses were performed using SPSS v21 (IBM, Armonk, NY). For in vivo electrophysiology measures, statistical significance was tested using a one-way or two-way repeated-measures (RM) analysis of variance (ANOVA), followed by a Bonferroni post hoc test for paired comparisons. Sphericity was tested using Mauchly’s test; the Greenhouse-Geisser correction was applied if violated. Behavioral time courses were tested with the
Friedman test, followed by a Wilcoxon test and Bonferroni correction for paired comparisons. Area under curve (AUC) values were calculated using the trapezoid rule and compared with a one-way ANOVA followed by a Bonferroni post hoc test.

**Results**

**M8-An Inhibits Icilin Evoked Ca\(^{2+}\) Currents In Vitro.** The ability of M8-An (Fig. 1A) to block icilin-evoked currents was examined in HEK293 cells expressing hTRPM8 channels. M8-An inhibited Ca\(^{2+}\) currents in a dose-dependent manner with an IC\(_{50}\) of 10.9 ± 2.6 nM (Fig. 1B). M8-An also activated hTRPA1 but with an EC\(_{50}\) of 2.8 μM and inhibited hTRPV1 with an IC\(_{50}\) of >3 μM (data not shown). Our data confirm M8-An as an inhibitor of chemical activation of TRPM8 channels in vitro. Pharmacokinetic analysis revealed that after an intraperitoneal injection of 10 mg/kg M8-An in rats, the average peak blood concentration was 24.77 μg/ml (61.87 μM) after 33 minutes with a half-life of 1.8 hours (data not shown). We next sought to examine the ability of M8-An to inhibit cold activation of TRPM8 in vivo.

**M8-An Decreases Core Body Temperature.** Several lines of evidence implicate TRPM8 in thermoregulation. Menthol and icilin both increase core body temperature (Ding et al., 2008; Masamoto et al., 2009), whereas several novel TRPM8 antagonists have been reported to have significant hypothermic effects (Knowlton et al., 2011; Almeida et al., 2012; Gavva et al., 2012). The effect of 30 and 100 mg/kg M8-An on core body temperature was investigated using implanted telemeters. Vehicle-treated rats exhibited a short-lasting likely stress-related hyperthermic effect after dosing, whereas M8-An caused a transient decrease in body temperature (two-way ANOVA \(P < 0.001\), followed by Bonferroni post hoc test) (Fig. 2A). M8-An (30 and 100 mg/kg) resulted in average peak decreases of 1.35 and 2.43°C, respectively, and overall decreases in temperature compared with vehicle treatment in the 6 hours after dosing (one-way ANOVA \(P < 0.001\), followed by Bonferroni post hoc test) (Fig. 2B).

**M8-An Does Not Inhibit Neuronal Responses to Innocuous or Noxious Cooling in Naïve Rats.** In vivo electrophysiology was performed to examine the effect of TRPM8 inhibition on spinal neuronal responses to cold stimulation and its effect, if any, on other modalities under normal or neuropathic conditions. Table 1 summarizes neuronal depths from the surface of the cord and pre-drug baseline responses to mechanical, thermal, and electrical stimulation. Neurons were characterized from depths corresponding to lamina VVI (Watson et al., 2009) and had receptive fields on the glabrous skin of the hind toes. Deep dorsal horn neurons displayed graded firing to natural stimuli and were identified as wide dynamic range by confirming responses to dynamic brushing, noxious punctate mechanical,
and heat stimulation. No statistically significant difference was observed in electrical thresholds for activation of A- or C-fibers or evoked responses of lamina VVI neurons between naive and spinal nerve-ligated rats (unpaired Student’s t test, P < 0.05).

After isolating single dorsal horn neurons and after stable baseline recordings, naive rats were dosed subcutaneously with 100 mg/kg M8-An. Acetone and ethyl chloride were applied as evaporative cooling stimuli. From recordings of skin temperature, these solvents have been described previously to represent innocuous and noxious stimuli, respectively, because the latter induces a withdrawal reflex in normal animals (Leith et al., 2010). Compared with baseline, 100 mg/kg M8-An did not significantly reduce the number of action potentials evoked by innocuous or noxious cold stimulation (one-way RM ANOVA, P > 0.05) (Fig. 3A). Furthermore, M8-An did not alter neuronal responses to punctate mechanical stimulation (two-way RM ANOVA, P > 0.05) (Fig. 3C), heat stimulation (two-way RM ANOVA, P > 0.05) (Fig. 4B), or dynamic brushing of the receptive field (one-way RM ANOVA, P > 0.05) (Fig. 4D). Measures of neuronal excitability and total spinal neuronal events evoked by global stimulation of Aβ-, Aδ-, and C-fibers were also unaffected in SNL rats, indicating blocking TRMP8 does not alter electrical function of primary afferent fibers under normal or pathologic conditions (one-way RM ANOVA, P > 0.05) (Fig. 4E).

M8-An Attenuates Cold but Not Mechanical Hypersensitivity in SNL Rats. We next examined the ability of M8-An to alleviate cold hypersensitivity in SNL rats. Behavioral testing was performed 14 days after unilateral ligation of the L5 and L6 spinal nerves. Rats were confirmed to exhibit cold hypersensitivity as withdrawals to acetone-induced evaporative cooling were significantly increased on the injured ipsilateral paw compared with the uninjured contralateral paw (Wilcoxon test, P < 0.05) (Fig. 5, A and B). Rats also exhibited mechanical hypersensitivity, because paw withdrawal thresholds were significantly reduced on the ipsilateral hind paw compared with the contralateral (Wilcoxon test, P < 0.05) (Fig. 5, E and F). Rats were dosed with either 30 mg/kg M8-An or vehicle and tested for 90 minutes postdosing. M8-An reduced the behavioral response to cooling of the injured paw compared with pre-drug values (Friedman test P < 0.01, followed by Wilcoxon’s test and Bonferroni correction) (Fig. 5A), whereas the vehicle alone has no significant effect (Friedman test, P > 0.05) (Fig. 5A). Contralateral responses to cooling were minimal and were not affected by M8-An (Friedman test, P > 0.05) (Fig. 5B). AUC analysis between 30 and 90 minutes postdosing confirms behavioral signs of cold hypersensitivity are reduced by M8-An compared with vehicle treatment (one-way ANOVA P < 0.01, followed by Bonferroni post hoc test) (Fig. 5C). M8-An did not alter mechanically evoked responses on either the ipsilateral (Fig. 5E) or contralateral (Fig. 5F) paw compared with predrug values (Friedman test, P > 0.05). AUC analysis between 30 and 90 minutes postdosing also confirms no

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<tr>
<th>Depth (μm)</th>
<th>Naive (n = 14)</th>
<th>SNL (n = 12)</th>
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<tr>
<td>Depth</td>
<td>747 ± 19.72 (630–870)</td>
<td>611 ± 31.29 (515–750)</td>
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<td>A threshold (mA)</td>
<td>0.03 ± 0.01</td>
<td>0.05 ± 0.01</td>
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<tr>
<td>Aβ evoked (APs)</td>
<td>119 ± 8.12</td>
<td>124 ± 10.49</td>
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<tr>
<td>Aδ evoked (APs)</td>
<td>216 ± 15.77</td>
<td>225 ± 19.15</td>
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<tr>
<td>C evoked (APs)</td>
<td>489 ± 44.66</td>
<td>561 ± 52.85</td>
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<tr>
<td>Postdischarge (APs)</td>
<td>344 ± 34.78</td>
<td>376 ± 38.44</td>
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<tr>
<td>Brush (APs)</td>
<td>443 ± 44.86</td>
<td>494 ± 50.21</td>
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<tr>
<td>2g (APs)</td>
<td>33 ± 7.39</td>
<td>43 ± 8.42</td>
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<tr>
<td>8g (APs)</td>
<td>347 ± 28.64</td>
<td>394 ± 36.69</td>
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<td>15g (APs)</td>
<td>647 ± 38.19</td>
<td>702 ± 52.86</td>
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<td>26g (APs)</td>
<td>950 ± 54.00</td>
<td>1011 ± 54.42</td>
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<tr>
<td>60g (APs)</td>
<td>1346 ± 59.70</td>
<td>1336 ± 67.77</td>
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<tr>
<td>35°C (APs)</td>
<td>305 ± 51.78</td>
<td>322 ± 46.27</td>
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<tr>
<td>42°C (APs)</td>
<td>593 ± 61.28</td>
<td>486 ± 54.70</td>
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<td>45°C (APs)</td>
<td>823 ± 74.55</td>
<td>740 ± 78.18</td>
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<td>48°C (APs)</td>
<td>1238 ± 168.70</td>
<td>1202 ± 97.39</td>
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<td>Acetone (APs)</td>
<td>76 ± 18.54</td>
<td>62 ± 24.13</td>
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<tr>
<td>Ethyl chloride (APs)</td>
<td>534 ± 86.72</td>
<td>536 ± 62.25</td>
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overall effect of either drug or vehicle treatment on mechanical hypersensitivity (one-way ANOVA, $P < 0.001$, followed by Bonferroni post hoc test) (Fig. 5G). The behavioral observations were corroborated by electrophysiological recordings of neuronal responses to punctate mechanical stimulation and acetone-induced cooling. AUC analysis was performed between 30 and 110 minutes postdosing to compare the effects of M8-An on total neuronal events in uninjured and nerve-ligated conditions. M8-An (30 mg/kg) did not affect mechanical coding of WDR neurons to below threshold and threshold stimuli in SNL or naive rats (two-way ANOVA, $P > 0.05$) (Fig. 5H) or to suprathreshold stimuli (data not shown), whereas cooling-evoked responses were significantly reduced in SNL rats (unpaired Student’s $t$ test, $P < 0.05$) (Fig. 5D).

**Discussion**

The data presented here demonstrate that a novel TRPM8 antagonist inhibits icilin-evoked currents in vitro, decreases body temperature, reduces spinal neuronal firing to cold stimulation under nerve injured conditions, and attenuates cold-evoked behaviors after a neuropathic insult. To date the effects of TRPM8 antagonists have been examined through behavioral measures and recordings from peripheral neurons. We extended these studies by recording from an integrated system and examining the influence of TRPM8 block on sensory neuronal processing within the spinal cord. We observed that rats display few withdrawal behaviors to innocuous cold stimulation in the absence of injury, which is mirrored by the low neuronal responses to cooling. Behavioral assays of cold sensitivity in normal animals, unlike heat tests, are often ambiguous, unreliable, and display large variability complicating studies of cold sensitivity (McKemy, 2013). Several studies have demonstrated that TRPM8 is required for the aversion to innocuous cold in temperature preference assays (Bautista et al., 2007; Colburn et al., 2007; Dhaka et al., 2007; Knowlton et al., 2013). From a pharmacological perspective, it may prove difficult to discern between cold aversion and warm preference, because efficacious doses of TRPM8 antagonists are often associated with an undesired thermoregulatory effect. Therefore, we performed in vivo electrophysiology under homeothermic conditions to examine the contribution of TRPM8 to spinal neuronal responses to cold stimulation without the confounding impact of changes in body temperature. Although TRPM8-positive afferents terminate superficially in the dorsal horn (Takashima et al., 2007), deep dorsal horn WDR neurons receive these inputs from all fiber types either directly or indirectly through interneurons. We examined nociceptive processing in second-order neurons in the deep dorsal horn, which respond to a wide range of stimuli in an intensity-dependent manner, a feature that correlates with nociceptive processing in humans (Sikandar et al., 2013). In naive rats, neither 30 nor 100 mg/kg M8-An significantly reduced lamina V/VI WDR neuronal responses to innocuous or noxious cold stimulation. Despite the canonical view that TRPM8 is the primary transducer of cool temperatures, surprisingly innocuous cooling-evoked responses were conserved at the highest dose examined. Additional cold sensitive ion channels may be expressed in TRPM8-positive peripheral terminals, which

![Image](https://example.com/image.png)
may include TRPC5 (Zimmermann et al., 2011). The TRPM8 antagonist BCTC [4-(3-chloro-2-pyridinyl)-N-[4-(1,1-dimethyl-ethyl)phenyl]-1-piperazinecarboxamide] inhibits menthol-evoked currents but has minimal impact on cold-evoked activity in peripheral nerve endings in the cornea (Madrid et al., 2006). A proportion of cold-sensitive neurons are TRPM8 negative where the transduction mechanisms are less clear (Munns et al., 2007). Mechanical coding and heat-evoked responses were unaffected by TRPM8 inhibition as were total spinal neuronal events evoked by global Aδ- or C-fiber activation after suprathreshold electrical stimulation of the receptive field, consistent with the restricted expression of TRPM8 in subsets of these fibers and the absence of effect on overall afferent excitability.

Although cross-reactivity with other TRP channels was not comprehensively addressed, heat-evoked responses of spinal neurons were unaffected at the highest dose tested in vivo, suggesting no significant modulation of other thermo TRPs. Mechanical coding was also unaffected, suggesting little or no activity of M8-An at putative mechanoreceptors TRPC3 and TRPC6 (Quick et al., 2012). We have also confirmed that M8-An does not inhibit TRPA1. These data support previous observations that TRPM8 is not required for behavioral manifestations to heat or mechanical stimulation (Knowlton et al., 2013).

Ideally, a novel therapeutic would alleviate abnormal pain in affected areas without perturbing normal somatosensory function. By use of behavioral assays in neuropathic rats, we examined the systemic effect of a dose of M8-An that had minimal effects on body temperature and neuronal responses to cold stimulation in naive rats. M8-An (30 mg/kg) reversed cold but not mechanical hypersensitivity in SNL rats without affecting behaviors in the uninjured contralateral paw. Furthermore, the same dose attenuated neuronal responses to noxious and innocuous cold stimulation in SNL but not naive rats. As observed in naive rats, M8-An had no impact on heat or mechanical coding of neurons in SNL rats. Measures of neuronal excitability, “input” (the nonpotentiated response), “wind-up” (the potentiated response), and postdischarge also did not differ postdosing of M8-An, consistent with a peripheral inhibition rather than reduced spinal neuronal excitability. The effect of M8-An appears dependent on pathophysiological state, is confined to the damaged nerve territory, and supports previous conclusions that TRPM8 contributes to cold hypersensitivity and hyperalgesia (Colburn et al., 2007).

In general, little is known about the mechanisms of cold hypersensitivity and the relative contributions of peripheral and central components. In healthy volunteers, A-fiber nerve block can unmask a C-fiber-mediated burning pain after cooling, leading to the hypothesis that a central disinhibition of primary afferent activity could underlie cold hyperalgesia (Yarnitsky and Ochoa, 1990). High-concentration menthol applied to areas of cold allodynia appears to reduce rather than exacerbate cold-evoked pain, presumed to be a restoration of this A-fiber inhibition (Wasner et al., 2008). Disrupting the integrity of spinal circuits modifies gating of cold stimuli; ablation of calcitonin gene-related peptide α-positive primary
afferents results in a loss of inhibition of cold-sensitive spinal neurons (McCoy et al., 2013). Spinal neuronal responses to cold are also subject to dynamic supraspinal modulation via the periaqueductal gray and rostral ventromedial medulla, which may sustain abnormal behaviors under pathologic conditions (Rahman et al., 2006; Taylor et al., 2007; Leith et al., 2010). The role of NMDA receptors in mediating central sensitization is well established (Woolf and Thompson, 1991). Correspondingly, ketamine reduces spinal neuronal excitability after peripheral injury (Suzuki et al., 2001) and also reduces pain intensity scores to cold stimulation in neuropathic subjects without affecting cold pain thresholds, implicating a role for central sensitization in cold hypersensitivity, whereas NMDA independent mechanisms are likely involved in perceiving cold temperatures (Jørum et al., 2003). Future studies could further examine central upstream mechanisms driven by TRPM8.

It has been noted previously that WDR neuronal responses to mechanical and heat stimulation do not differ between SNL, sham-operated, and naive rats (Brignell et al., 2008). We have also observed that evoked neuronal responses were identical between nerve-injured and uninjured states. At first, this appears incongruous with increased behavioral sensitivity to below threshold mechanical and cold stimulation, implicating a role for central sensitization in cold hypersensitivity, whereas NMDA independent mechanisms are likely involved in perceiving cold temperatures (Jørum et al., 2003). Future studies could further examine central upstream mechanisms driven by TRPM8.

Alternatives include a peripheral sensitization of cold fibers; however, the evidence for this is sparse (Serra et al., 2009). Whether TRPM8 is sensitized after nerve injury is currently unclear. TRPM8 expression at the mRNA and protein level in dorsal root ganglia appears to be neither up- or downregulated after spinal nerve ligation (Katsura et al., 2006). Several cellular processes modify temperature sensitivity and gating of TRPM8 in vitro. Post-translational N-glycosylation (Pertusa et al., 2012) and phosphatidylinositol 4,5-bisphosphate modulation (Rohacs et al., 2005) shift the voltage dependence of TRPM8, increasing the probability of opening at physiologic temperatures. Neurotrophic factors such as artemin and nerve growth factor also sensitize cold-evoked behaviors in a TRPM8-dependent manner (Lippoldt et al., 2013). Many gene changes occur after injury that affects neuronal excitability (Wang et al., 2002). Thresholds of

Fig. 5. M8-An selectively reduces behavioral and neuronal responses to cooling after spinal nerve ligation. M8-An (30 mg/kg) reversed the behavioral response to acetone on the nerve-injured ipsilateral side compared with predrug values \((n = 6)\), whereas vehicle alone had no significant effect \((n = 5)\) (A). Contralateral responses were not affected by either treatment (B). AUC analysis confirms a significant attenuation of cold hypersensitivity by M8-An compared with vehicle treatment (C). A corresponding decrease in overall neuronal responses to acetone was also observed in SNL rats \((n = 12)\) compared with naive rats \((n = 7)\) (D). M8-An did not alter mechanically evoked responses on the injured ipsilateral paw (E) or the uninjured contralateral side \((n = 7)\) (F). AUC analysis confirms that rats exhibited significant mechanical hypersensitivity, which was not affected by M8-An treatment (G). Neuronal responses to mechanical stimulation in SNL \((n = 12)\) and naive rats \((n = 7)\) were also unaffected by 30 mg/kg M8-An (H). Data represent mean \pm S.E.M.

* \(P < 0.05\), *** \(P < 0.001\). PWT, paw withdrawal threshold, APs, action potentials.
cold-sensitive neurons have been proposed to be refined by
differential expression of background potassium channels in
subsets of TRPM8+ fibers (Madrid et al., 2009; Descour et al.,
2011). A loss of K+ currents could confer cold sensitivity to
previously unresponsive primary afferents (Viana et al.,
2002). The data presented here do not necessarily discount a
peripheral component in addition to central neuroplastic
changes.

In conclusion, our data suggest that cold-evoked neuronal
responses are conserved in naive rats after TRPM8 inhibition,
supporting the existence of other cold transducers, and that
peripheral and central sensitivity may underlie the con-
tribution of TRPM8 to cold hypersensitivity and hyperalgesia
after nerve injury. The identification of selective antagons-
ist will in the future provide useful tools to investigate further
the role of TRPM8 in the transduction of cold

This study supports the validity of pharmaco-

logical modulation of TRPM8 in treating disorders of cold

sensitivity.

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Authorship Contributions

Participated in research design: Patel, Newman, Jiang, Goldby,
Reeve, Hendrick Teall, Almond, Brice, Dickinson.


Contributed new reagents or analytic tools: Goldby, Reeves, Hendrick,
Teall, Hannah, Almond, Dickenson.


Wrote or contributed to the writing of the manuscript: Patel,
Newman, Brice, Dickinson.

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Address correspondence to: Ryan Patel, University College London, Gower Street, Department of Neuroscience, Physiology and Pharmacology, London, WC1E 6BT, UK. E-mail: ryan.patel.10@ucl.ac.uk.