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Article

Analgesia Mediated by the TRPM8 Cold Receptor in Chronic Neuropathic Pain

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Summary

Background: Chronic established pain, especially that following nerve injury, is difficult to treat and represents a largely unmet therapeutic need. New insights are urgently required, and we reasoned that endogenous processes such as cooling-induced analgesia may point the way to novel strategies for intervention. Molecular receptors for cooling have been identified in sensory nerves, and we demonstrate here how activation of one of these, TRPM8, produces profound, mechanistically novel analgesia in chronic pain states.

Results: We show that activation of TRPM8 in a subpopulation of sensory afferents (by either cutaneous or intrathecal application of specific pharmacological agents or by modest cooling) elicits analgesia in neuropathic and other chronic pain models in rats, thereby inhibiting the characteristic sensitization of dorsal-horn neurons and behavioral-reflex facilitation. TRPM8 expression was increased in a subset of sensory neurons after nerve injury. The essential role of TRPM8 in suppression of sensitized pain responses was corroborated by specific knockdown of its expression after intrathecal application of an antisense oligonucleotide. We further show that the analgesic effect of TRPM8 activation is centrally mediated and relies on Group II/III metabotropic glutamate receptors (mGluRs), but not opioid receptors. We propose a scheme in which Group II/III mGluRs would respond to glutamate released from TRPM8-containing afferents to exert an inhibitory gate control over nociceptive inputs.

Conclusions: TRPM8 and its central downstream mediators, as elements of endogenous-cooling-induced analgesia, represent a novel analgesic axis that can be exploited in chronic sensitized pain states.

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Introduction

Chronic neuropathic pain arising from peripheral nerve damage is a severe clinical problem with limited treatment options [1]. Changes in both damaged and undamaged primary afferent neurons as well as central (spinal cord) sensitization lead to hyperalgesia (accentuated responses to painful stimuli), allodynia (pain in response to normally innocuous stimuli), and spontaneous pain. We hypothesized that elucidating the poorly understood mechanisms underlying cold-induced analgesia might lead the way to novel neuropathic analgesics.

Since Hippocrates and Galen [2, 3], sporadic reports have described the use of cooling to produce analgesia [4]. Clinical trials show beneficial effects of cooling on chronic back pain, dental pain, postoperative pain, and muscle injuries [5]. Preparations containing menthol, which produces a cool sensation, are used topically to relieve neuralgia in traditional Chinese and European medicine [6, 7]. Mint oil has been reported to alleviate thermally elicited pain and postherpetic neuralgia [8, 9], and oral menthol can cause short-term analgesia [10]. Furthermore, in mice, oral or intracerebroventricular application of menthol decreased nociceptive responses to the hot-plate test and acetic-acid writhing test [11]. Despite this history, no definitive mechanism has been established for cool-induced analgesia.

The recent isolation of the transient receptor potential (TRP) cation channels present in primary sensory neurons has revolutionized our understanding of cutaneous temperature detection. The best-characterized example is the capsaicin- and heat-sensitive TRPV1 receptor [12], and although much less is known about cool-sensitive TRPs, they are the target of intensive research [13]. TRPM8 is activated at innocuous cool temperatures (with 50% activation around 18°C–19°C [14]) and by menthol and icilin [15, 16], which act as selective (but not totally specific) activators of the channel [14, 17]. The TRPM8 channel is expressed by a subpopulation of sensory neurons in dorsal root ganglia (DRG) and trigeminal ganglia [15, 16], where responses to cooling correlate well with mRNA expression and menthol sensitivity [18–20].

The TRPA1 channel is also expressed in DRG and trigeminal ganglia [14, 21] and is reportedly activated by cooling temperatures beginning 5°C–6°C lower than that for TRPM8 [14, 22] and by noxious chemicals such as cinnamaldehyde and mustard oil [14, 22, 23]. However, the role of TRPA1 in physiological cold sensation is currently unclear [13], with some reports of TRPA1 not being activated by cold [21, 23] and of normal cold sensitivity in TRPA1 knockout mice [24]. In contrast, another study of TRPA1 knockout mice reported attenuated responses to noxious cold [25], and antisense knockdown studies show a decrease in development of nerve-injury- or inflammation-induced hyperalgesia to intense cold stimuli [26, 27]. On balance, TRPA1 seems a less likely candidate than TRPM8 for the mediator of cooling-induced analgesia.

A central spinal mechanism of analgesia is suggested, because cutaneous cooling can prevent pain produced by afferent stimulation [4]. Glutamate-receptor-dependent plasticity in spinal cord neurons commonly underlies chronic pain states with both ionotropic and metabotropic receptors participating at pre- and postsynaptic sites. Although most glutamate receptors are excitatory, the Group II/III metabotropic receptor (mGluR) subtypes exert inhibitory influences, suggesting the hypothesis that they could potentially underpin cooling-induced analgesia. Indeed, Group II and III mGluRs are present in the spinal cord largely on afferent terminals, but with some glial and postsynaptic expression [28, 29], and their activation inhibits both nerve-injury- and inflammation-induced sensitization of neuronal and behavioral responses [30–32].

Here we demonstrate marked analgesic effects of peripherally or centrally applied TRPM8 activators (such as icilin or menthol), or mild cooling of the skin, in a model of neuropathic pain. TRPM8 levels in DRG and superficial dorsal horn were increased after nerve injury. Analgesia was restricted to injury-sensitized responses and abolished after antisense knockdown of TRPM8. Peripheral application of icilin or menthol also activated slowly conducting afferents (in a TRPM8-antisense-sensitive manner) and suppressed the increased responsiveness of single dorsal-horn neurons ipsilateral to nerve injury. In contrast, activation of TRPA1 produced hyperalgesia (in both naive animals and nerve-injured animals). TRPM8-mediated analgesia was selectively reversed by intrathecal administration of Group II/III mGluR antagonists and mimicked by agonists, and iontophoresis of a Group III mGluR antagonist, as an example, reversed the inhibitory effect of icilin on sensitized single neurons. Sensitization-specific analgesia from TRPM8 activation was also observed in inflammatory afferent demyelination and TRPA1 activator-induced pain models.

Results

Reversal of Nerve-Injury-Induced Reflex Sensitization by Peripheral Activation of TRPM8 Channels

In order to model potential clinical usage, we administered icilin topically to the paws by placing rats with chronic constriction injury (CCI) to sciatic nerve in a bath with 1-cm-deep drug solution, kept at 30°C to avoid any effects on local skin temperature. After 5 min, icilin (80 μM), but not vehicle (0.2% dimethylformamide in water), caused striking reversal of CCI-induced behavioral-reflex sensitization to thermal and mechanical stimuli (Figure 1A). Concentration-dependent effects were observed from 2.5 μM up to a maximum of 500 μM, with no effect on contralateral or naive responses (Figure 1B). At much higher concentrations of icilin, we observed the beginning of a trend toward increasing reflex sensitivity, ipsilateral and contralateral to CCI and in naive animals (Table 1), that was statistically significant in mechanical and (after a delay) in thermal tests, only at the highest concentration tested, 5 mM. This effect was distinct from the prominent analgesia at low concentrations of icilin because it was not specific to a sensitized state and may be due to weak interaction with other targets or nonspecific actions. Specific reversal of sensitized responses was also caused by another selective TRPM8 activator, (−)-menthol (4 mM). The stereoisomers isomenthol and (+)-menthol, which are several-fold-less-potent agonists of TRPM8 [33, 34], also produced reversal of sensitized responses at concentrations of 8 mM and 16 mM (Figure 1C).

Icilin is expected to activate TRPM8-containing afferents, so we recorded firing activity in saphenous-nerve afferents after topical application of icilin (Figure 1D). The nerve was dissected to produce small-number preparations of fine afferents, with conduction velocities of up to 2.6 m/s (representing C- and Aδ-fiber afferents [35]). Icilin (200 μM) applied to the receptive field on the hind limb caused a significant increase in firing frequency in 21.6% (40 out of 185) of recorded fine afferents with a mean 7-fold increase in firing frequency from baseline of 4.5 ± 2.5 Hz to 31.6 ± 3.4 Hz and a mean time to peak effect of 3.3 ± 0.5 min. Icilin did not produce rapid desensitization, agreeing with some reports [36] but not others [37]. Recovery was consistently observed. Similar results were obtained from both hairy and glabrous skin. Large myelinated mechanoreceptors (conduction velocities 6.8–15 m/s, n = 43) were unaffected.

Consistent with a TRPM8-mediated mechanism, paw immersion at 16°C–20°C for 5 min also produced statistically significant mechanical analgesia (Figure 1E). Recordings from a subcutaneous thermistor probe showed that deep skin temperatures were 0.5°C above bath temperatures after 5 min in similar conditions. This temperature is in the range expected to activate TRPM8 and stimulate innocuous cool-sensitive fibers. Immersion temperatures below 14°C (in the range where other cold sensors, in addition to TRPM8, are also likely to be activated) elicited active nociceptive withdrawal reflexes limited to the period of hind paw immersion, in agreement with the known temperature activation range for nociceptive cold fibers [38].

Localization of TRPM8 in Afferents and Superficial Dorsal Horn: Increased Expression after Nerve Injury

The presence and localization of TRPM8 in DRG and the spinal cord were investigated by immunoblotting and immunohistochemistry. After rapid homogenization of DRG in Laemmli lysis buffer and SDS-PAGE, immunoblots probed with a rabbit polyclonal antibody raised to TRPM8 residues 278–292 and 1090–1104 (human) [39] showed a single, strong band at approximately 128 kDa (the predicted molecular weight of TRPM8), with faint bands observed at approximately 170, 60, and 50 kDa (Figure 2A). Both antigen-preabsorbtion and antisense-knockdown controls were consistent with specificity of this antibody in recognition of TRPM8 at approximately 128 kDa under the conditions used. Preincubation of the antibody with membranes from COS7 cells transfected with TRPM8 expression plasmid abolished the band at 128 kDa, whereas sham preabsorbtion with membranes from cells with empty vector did not (Figure 2A). Correspondingly, intrathecal delivery of a TRPM8 antisense oligonucleotide to naive (non-CCI) rats over 5 days also resulted in almost complete knockdown of the 128 kDa band (Figure 2A), whereas a

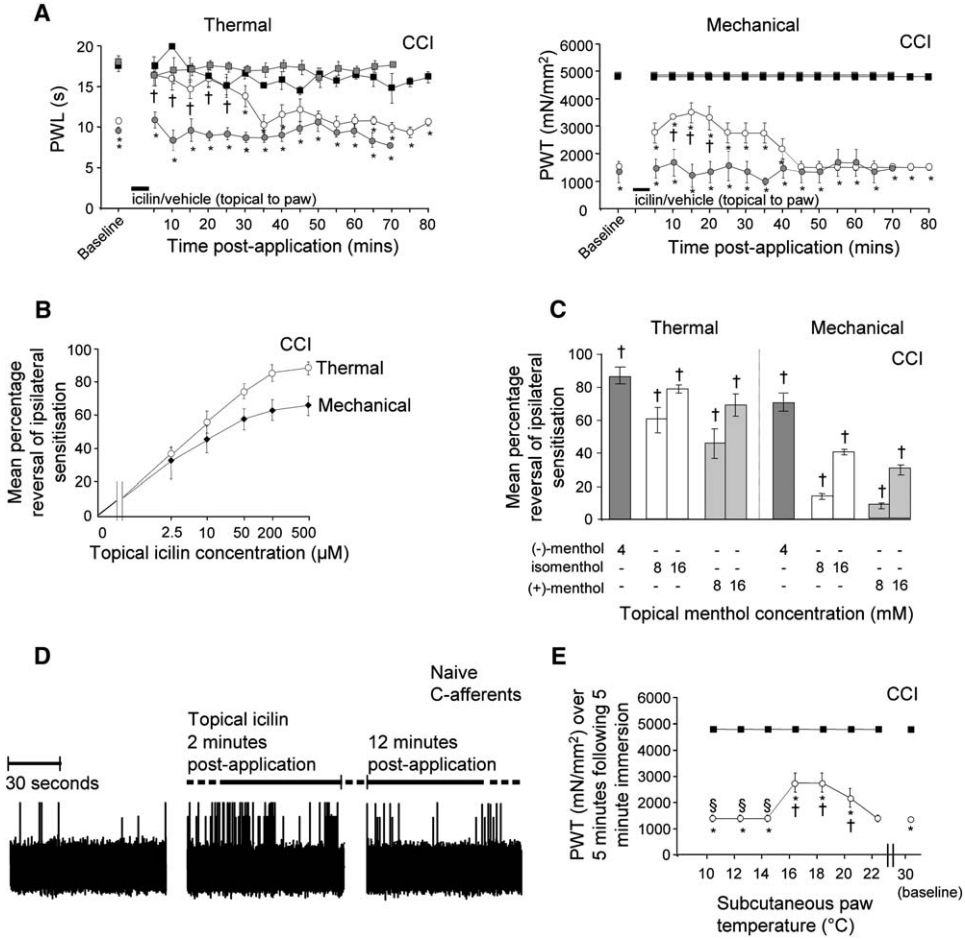

Figure 1. Peripheral TRPM8 Activation and Moderate Cooling Are Analgesic after CCI

(A, B, C, and E) Behavioral data from CCI animals, shown as mean ± SEM; each graph represents n of six animals.

(A) Paw withdrawal latency (PWL; s) to noxious heat and paw withdrawal threshold (PWT; mN/mm^2) to mechanical stimuli before and after 5 min paw immersion in a shallow 30°C water bath containing 80 μM icilin or vehicle. ○: ipsilateral paw plus icilin; ●: ipsilateral + vehicle; ■: contralateral plus icilin, ■: contralateral plus vehicle. * indicates significant ipsilateral-contralateral differences; † indicates significant difference from predrug baseline ($p < 0.05$).

(B) Concentration-response curve for mean ± SEM percentage reversal of ipsilateral sensitization for thermal (○) or mechanical (◆) tests calculated over 10–15 min after paw immersion in 2.5–500 μM icilin.

(C) Reversal of ipsilateral sensitization by paw immersion in (−)-menthol at 4 mM, and by higher concentrations of the less-potent stereoisomers (+)-menthol and isomenthol (8 and 16 mM). Values were calculated over 10–25 min after paw immersion from experiments as shown in (A). † indicates significant differences from predrug baseline ($p < 0.05$).

(D) Typical electrophysiological recording from the subpopulation of icilin-responsive C fiber afferent fibers before, 2 min after topical application of icilin to the receptive field (peak effect), and 12 min later.

(E) Mechanical analgesia measured over 5 min after 5 min immersion of paws at the indicated temperature range. § denotes spontaneous withdrawal responses during the immersion period, * denotes significant difference from contralateral paw, and † denotes significant difference from preimmersion baseline ($p < 0.05$).

missense control oligonucleotide was ineffective (see below). Intrathecally delivered fluorescent-labeled oligonucleotides have been shown to effectively penetrate the DRG, as soon as 4 hr after initial delivery [40]. The faint bands, at 50 and 60 kDa at least, remained present in each case and so are likely to represent nonspecific interactions of the antibody under these conditions. As further controls, we showed that TRPV1 immunoreactivity was unaffected by treatment with the TRPM8 antisense reagent and that the housekeeping enzyme GAPDH (36 kDa) was evenly present in each lane (Figure 2A). After nerve injury, there was a marked increase in expression of the 128 kDa TRPM8-immunoreactive band specifically in ipsilateral, but not contralateral, DRG (Figure 2A), whereas immunoreactivity for GAPDH was unaltered. Densitometric ratios for TRPM8 expression as percentage of GAPDH were 80.7 ± 4.1 ipsilateral to CCI, which was significantly greater than that seen contralateral to CCI (49.3 ± 3.2) and in naive DRG (50.7 ± 2.7) (mean ± standard error of the mean [SEM], n = 5–6). L4–5 spinal cord extracts showed that TRPM8 immunoreactivity was present centrally, and after preparation of a crude particulate fraction (centrifugation at 11,000 × g for 45 min), these also showed consistent increases in expression ipsilateral to injury. Densitometric values ipsilateral to CCI were 198% ± 6.7% of those from naive tissue ($p < 0.05$; mean ± SEM, n = 5), whereas contralateral values were 125% ± 7.1% ($p > 0.05$).

Table 1. Analgesic Effects of Topically Administered Icilin against Neuropathic Sensitization Revert at Very High Concentrations to a General Nociceptive Effect

Reflex Response Sensitivity at Different Times after Icilin Application to Paw						
Thermal PWL (s)						
Drug Concentration (μM)	Naive		CCI Ipsi		CCI Con	
Predrug Baseline	15.5 ± 0.2		9.7 ± 0.2		16.4 ± 0.2	
	15 min	50 min	15 min	50 min	15 min	50 min
0 (vehicle)	15.1 ± 1.2	14.9 ± 0.8	9.8 ± 1.1	9.7 ± 0.9	15.2 ± 0.6	15.1 ± 0.7
1000	15.1 ± 1.2	14.8 ± 0.9	16.4 ± 0.8[a]	8.6 ± 0.2	16.1 ± 0.5	15.2 ± 0.4
2500	14.9 ± 0.3	13.2 ± 0.8	16.4 ± 0.9[a]	8.4 ± 0.6	16.8 ± 0.9	15.3 ± 0.7
5000	15.3 ± 0.5	13.1 ± 0.7[b]	16.7 ± 1.0[a]	7.2 ± 0.6[b]	16.1 ± 0.9	13.4 ± 0.7[b]
Mechanical PWT (mN/mm^2)						
Drug Concentration (μM)	Naive		CCI Ipsi		CCI Con	
Predrug Baseline	4830.6 ± 0.0		805.7 ± 26.3		4793.6 ± 37.5	
	15 min	50 min	15 min	50 min	15 min	50 min
0 (vehicle)	4830.6 ± 0.0	4830.6 ± 0.0	805.7 ± 26.3	805.7 ± 26.3	4830.6 ± 0.0	4830.6 ± 0.0
1000	4830.6 ± 0.0	4830.6 ± 0.0	702.8 ± 185.3	702.8 ± 185.3	4530.1 ± 300.5	4530.1 ± 300.5
2500	4830.6 ± 0.0	4830.6 ± 0.0	651.9 ± 193.0	651.9 ± 193.0	4454.9 ± 245.9	4454.9 ± 245.9
5000	3828.6 ± 316.8[b]	4830.6 ± 0.0	412.2 ± 24.3[b]	736.0 ± 26.3	2040.2 ± 115.2[b]	4830.6 ± 0.0

Significant effects of drug on reflex responses are indicated:
[a] Significant increase from baseline, indicating analgesic effect of icilin.
[b] Significant decrease from baseline values, indicating hyperalgesic effects.

Immunohistochemistry was carried out with a rabbit polyclonal antibody raised to TRPM8 residues 656–680 (rat) [41], the specificity of which was addressed by antigen-preabsorbtion and antisense-knockdown controls. The labeling observed in a discrete subpopulation of DRG cells was abolished after preincubation with the peptide antigen (no positive cells seen, counted over twelve 500 μm^2 sections, compared with a mean of 5.3 ± 0.4 TRPM8-positive cells per 500 μm^2 DRG section with sham treatment of antibody, and 5.1 ± 0.5 TRPM8-positive cells per 500 μm^2 DRG section with untreated antibody, counted over 12 sections each). In immunoblots, the antibody also labeled in naive DRG tissue a single band, at approximately 128 kDa, that was abolished either by preabsorbtion with the peptide antigen or by prior 5 day intrathecal infusion of TRPM8 antisense (Figure 2B). Immunohistochemical staining in the spinal cord showed that TRPM8 was largely expressed in the superficial dorsal horn, like the C fiber marker peripherin (Figure 2C), and that after dorsal rhizotomy (L2–6), the vast majority of TRPM8 (and peripherin) immunoreactivity was lost ipsilaterally (reductions of approximately 80%–90%), suggesting that spinal TRPM8 originates largely from afferents. In confirmation of the immunoblot findings, levels of TRPM8-like immunoreactivity were increased in the dorsal horn ipsilateral to injury (by approximately 70%–80%), but retained a similar distribution to that in naive animals (Figure 2D).

To establish whether the increases in afferent TRPM8 expression occurred in specific subpopulations of DRG cells, we investigated TRPM8 colocalization with markers of myelinated afferents (neurofilament-200; NF-200 [42]) and unmyelinated afferents (peripherin [43]). In naive rats, TRPM8 immunoreactivity was largely confined to a subpopulation of unmyelinated DRG cells (8.3% ± 0.2% of peripherin-positive cells; 34 of 408 cells) and only minimally expressed in myelinated, NF-200-positive cells (1.3% ± 0.5%; 6 of 445 cells). However, after CCI, TRPM8 expression was significantly increased ipsilaterally in both NF-200- and peripherin-positive cells, to 7.9% ± 1.2% (31 of 390 cells) and 15.5% ± 0.8% (64 of 412 cells), respectively. Corresponding contralateral values were unaltered from naive values, at 2.0% ± 0.4% (14 of 346 cells) and 9.2% ± 0.4% (42 of 452 cells) (Figures 2E and 2F). Data were taken from 3 CCI and 3 naive animals and counted across 15–21 sections. The additional TRPM8-expressing NF-200-positive cells were small (average diameter, 19.7 ± 0.8 μm), presumed Aδ myelinated neurons [35]. There were no significant differences in the diameters of NF-200- or peripherin-positive cells or in the numbers of NF-200- or peripherin-positive DRG neurons per section.

Molecular Identification of TRPM8 as the Mediator of Icilin-Induced Analgesia

To define the specific involvement of TRPM8 in icilin analgesia, we further utilized the antisense-oligonucleotide knockdown strategy. TRPM8 antisense or mismatched control oligonucleotides were delivered intrathecally over 13 days to parallel the sensitization developing after CCI. The development of CCI-induced behavioral-reflex sensitization was unaffected, including thermal hyperalgesia and mechanical allodynia (Figures 3A and 3B) and cold allodynia (control CCI animals showed elevation of the paw ipsilateral to nerve injury out of 4°C water for 8.1 ± 0.5 s at peak, 9–11 days after surgery, whereas corresponding values in antisense-treated CCI animals were 7.6 ± 0.6 s). In contrast, the reversal of neuropathic reflex sensitization produced normally by 80 μM icilin applied to the paws (Figure 1A) was abolished by treatment with antisense (Figure 3A), but not missense (Figure 3B), reagents. The mean ± SEM reversals of ipsilateral sensitization over 10–25 min after icilin treatment in antisense- and missense-treated animals were 7.7% ± 7.4% and 82.8% ± 6.9%, respectively, for paw withdrawal latency (PWL), and

9.4% ± 8.2% and 58.7% ± 8.2% for paw withdrawal threshold (PWT), with missense-treated, but not antisense-treated, animals retaining significant effects of icilin ($p < 0.05$). When antisense osmotic pumps were depleted, but animals were still neuropathic (18 days after surgery for insertion of 14 day minipumps and CCI), responses to icilin were restored to 83.7% ± 10.1% reversal of sensitization for PWL and 54%.0 ± 7.2% for PWT. Effectiveness of knockdown was assessed by SDS-PAGE/immunoblotting. Expression of the 128 kDa TRPM8-immunoreactive band in both ipsilateral and contralateral DRG was greatly reduced by the antisense reagent, and the increase in TRPM8 expression normally seen ipsilateral to nerve injury was prevented (Figure 3C). The missense reagent had no effect (Figure 3C), showing TRPM8 expression similar to that in untreated CCI animals (Figure 2A). In missense-treated animals, TRPM8:GAPDH ratios were 77.9% ± 2.0% ipsilateral to CCI and 52.9% ± 2.1% contralateral, similar to corresponding control values (Figure 2A and above), whereas in antisense-treated animals, values were much lower (19.8% ± 2.2% and 14.9% ± 2.1%, respectively, mean ± SEM, n = 5).

To confirm that antisense knockdown of TRPM8 resulted in associated functional changes in afferents, we made saphenous-nerve recordings from naive animals receiving intrathecal delivery of TRPM8 antisense or missense oligonucleotides, 4–5 days after insertion of the pump. The increase in firing frequency evoked by topical icilin (200 μM) was strongly reduced in animals receiving antisense. Only 3 out of 34 recorded slowly conducting fibers (8.8%) showed a partial activation in response to the drug, increasing firing by approximately 2-fold from a baseline of 5.8 ± 1.4 to 12.7 ± 0.6 Hz, compared with the 7-fold increase observed in over 20% of fine afferents in naive animals. In contrast, missense animals showed a 7-fold increase in firing frequency in 25% of 40 recorded fibers from a baseline of 3.3 ± 0.7 to 23.1 ± 3.2 Hz, similar to results from naive animals. Similarly, in missense-treated animals, topical (−)-menthol (4 mM) produced an approximately 8-fold increase in mean firing frequency (from 4.5 ± 2.9 to 38.9 ± 7.6 Hz), activating 20% of fibers (35 identified afferents recorded). This compared with no obviously activated afferents in antisense-treated animals (mean firing frequency 4.0 ± 1.8 Hz at background, 4.8 ± 1.9 Hz after drug application, 28 identified afferents recorded). However, TRPM8 antisense treatment did not alter the effect of topically applied resiniferatoxin (1 mM), a potent TRPV1 agonist acting as a control. In antisense-treated animals, resiniferatoxin evoked a 6-fold increase in firing frequency in activated afferents (from 4.5 ± 0.7 baseline to 25.9 ± 1.8 Hz at peak response, 16 afferents activated out of 28 recorded), which was similar to responses in missense-treated animals (showing a mean 5-fold change in firing frequency from 4.6 ± 2.8 to 24.8 ± 3.1 Hz).

Central Intrathecal Administration of TRPM8 Activators Also Inhibits Neuropathic Sensitization

Because TRPM8 is present on central terminals of primary sensory neurons as well as their peripheral terminals (Figures 2B and 2C, [44, 45]), we investigated whether intrathecal application of TRPM8 activators near the central terminals would also produce analgesia. Intrathecal injection of icilin (10 nmol) produced robust reversal of CCI-induced behavioral-reflex sensitization in thermal and mechanical tests (Figure 4A, $p < 0.05$ for up to 55 min). Intrathecal injection of (−)-menthol (200 nmol) in CCI rats also caused a significant reversal of the sensitized responses, lasting 35–40 min (Figure 4B). Because of the higher potency and efficacy of icilin at TRPM8 [15], further experiments mainly utilized icilin as the representative TRPM8 activator. Icilin produced dose-dependent analgesic effects restricted to the nerve injury side in both thermal and mechanical tests that were statistically significant by 0.125 nmol and increased to almost complete reversal of sensitization by 10 nmol. Nonlinear curve-fitting indicated that maximal effects of icilin were similar for PWL and PWT (91.6% ± 9.9% and 82.6% ± 6.8% reversal of sensitization, respectively), as were EC_{50} values (dose for 50% of maximal effect; 0.17 ± 0.02 nmol and 0.31 ± 0.02 nmol, respectively).

In complete contrast to the effects of TRPM8 activators, the TRPA1 activator, cinnamaldehyde [22] (75 nmol injected intrathecally), significantly increased reflex responsiveness in thermal and mechanical tests and was effective contralateral as well as ipsilateral to nerve injury (Figure 4C). The sensitizing effects of cinnamaldehyde were prevented by coinjection of Ruthenium Red (0.25 nmol), which can block TRPA1 channels [21, 46], whereas the analgesic effect of intrathecally injected icilin was unaffected (Figure 4D). Sensitizing effects of cinnamaldehyde were also seen in naive animals, with a 36.9% ± 7.9% reduction in PWL and a 41.9% ± 6.7% reduction in PWT. Similar effects were produced by two further TRPA1 activators [46, 47], allicin (25 nmol), where corresponding reductions were 33.2% ± 5.9% and 20.7% ± 8.2%, respectively, and diallyl disulphide (50 nmol), with equivalent values of 25.9% ± 7.0% and 28.5% ± 6.2% (mean ± SEM, n = 3–6). In contrast, the TRPM8 activators icilin (10 nmol) and (−)-menthol (200 nmol) were without effect in naive animals (data not shown). Topical application of cinnamaldehyde (1.5 mM) also produced bilateral sensitization of behavioral reflexes in naive animals (mean decrease of 32.0% ± 8.6% in PWL and 20.2% ± 8.2% in PWT, $p < 0.05$, n = 6). This corresponds to the licking and shaking behavior as well as the decrease in PWL reported after intraplantar injection of cinnamaldehyde [22].

In further experiments, we investigated whether the sensitized pain behaviors caused by TRPA1 activators or other pain models were susceptible to icilin-induced analgesia. Sensitization caused by intrathecal or topical cinnamaldehyde was markedly attenuated by intrathecal icilin (Table 2). The effect of topical cinnamaldehyde was additionally reversed by topical icilin (200 μM, data not shown). Sensitization caused by focal demyelination of the sciatic nerve [43] or intraplantar injection of Complete Freund's Adjuvant (CFA) was also significantly inhibited by intrathecal icilin (Table 2).

Central Mechanism of Icilin Reversal of Neuropathic Sensitization Involves mGlu Group II/III Receptors

Because topical icilin increases activity in fine afferents (Figure 1D) and both intrathecal and topical icilin reverse nerve-injury-induced sensitization, centrally mediated

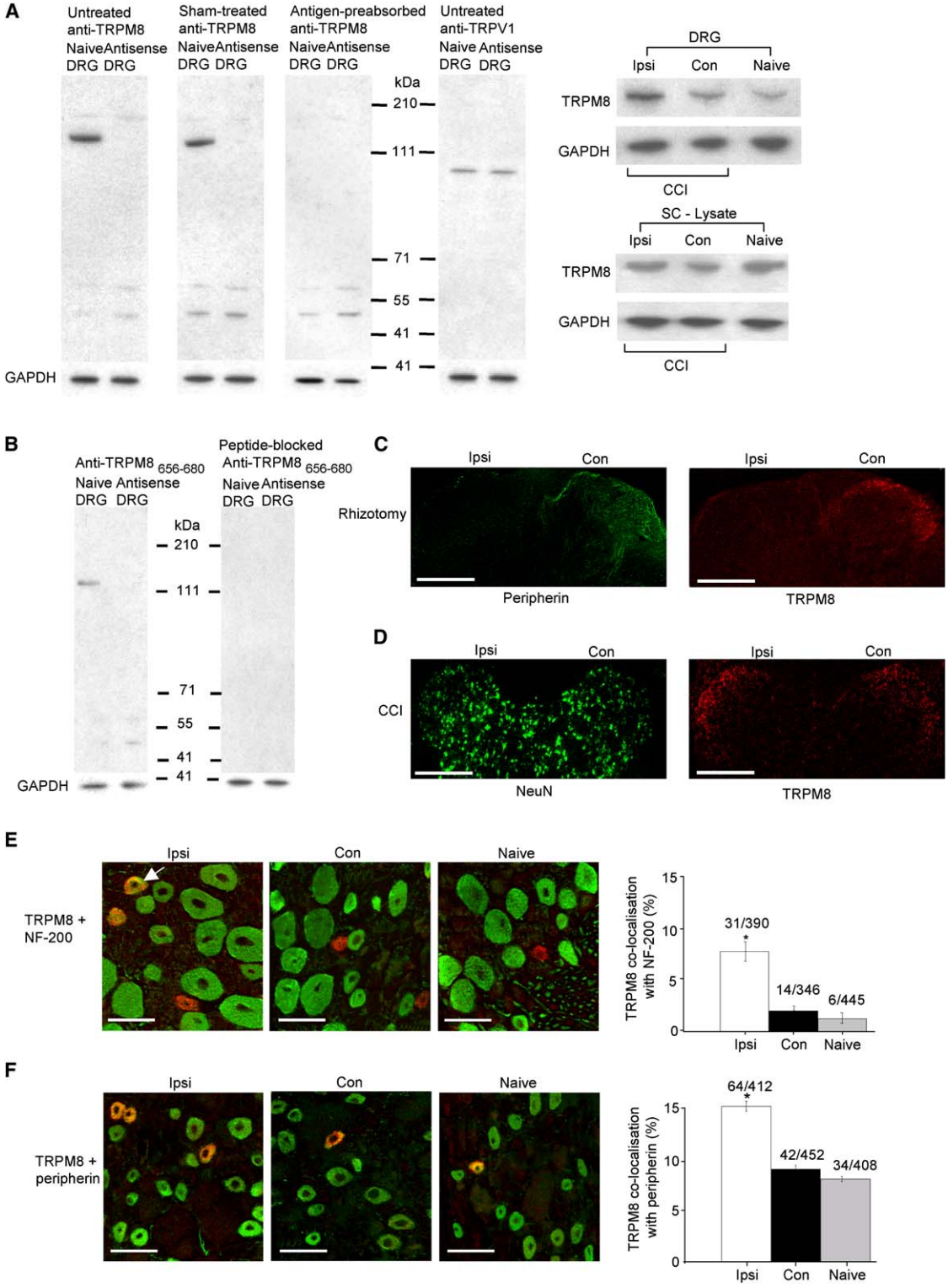

Figure 2. TRPM8 Immunoreactivity Is Present in DRG and Spinal Cord, Arises from Afferents, and Is Increased Ipsilateral to CCI

(A) Immunoblots of DRG show whole gels with TRPM8 protein running at 128 kDa and additional faint bands at approximately 170, 60, and 50 kDa, in normal rat DRG with specific knockdown of the 128 kDa band in DRG from antisense-treated animals. In additional controls, when the TRPM8 antibody was preincubated with membranes from TRPM8-expressing cells, the 128 kDa immunoreactive band was removed, whereas sham treatment had no effect. Blots additionally show GAPDH loading controls. TRPV1 expression (single band at ~90 kDa) was unaltered in DRG from TRPM8 antisense-treated animals. Immunoblots for TRPM8 protein showed a clear increase in expression of the specific 128 kDa band in DRG ipsilateral ("ipsi") to nerve injury compared to contralateral ("con") and naive DRG, with no change in GAPDH. Spinal cord (SC) whole lysates showed no discernable changes in TRPM8 levels; however, increased levels ipsilateral to nerve injury were seen in crude particulate fractions.

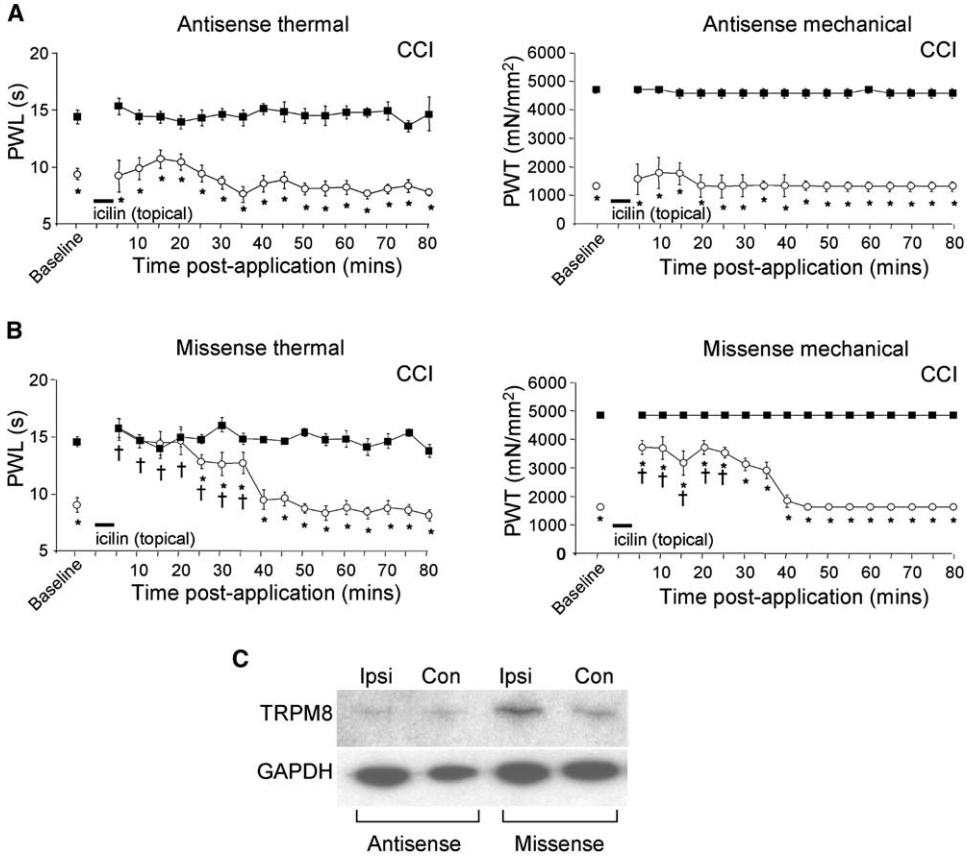

Figure 3. Specific TRPM8 Knockdown by Antisense Oligonucleotide Prevents Icilin-Induced Analgesia after CCI

(A and B) Paw withdrawal latency (PWL, s) to noxious heat and paw withdrawal threshold (PWT; mN/mm^2) to mechanical stimuli are shown ipsilateral (○) or contralateral (■) to CCI animals with antisense (n = 12) or missense (n = 10) treatments. Data show mean ± SEM.

(A) The analgesia normally elicited by topical icilin was not observed after antisense knockdown of TRPM8 receptor indicated by the persistence of significant ipsilateral-contralateral differences in PWL and PWT (* $p < 0.05$).

(B) In contrast, after missense treatment, icilin produced a significant reversal of CCI-induced ipsilateral sensitization in PWL and PWT responses in comparison to baseline values († $p < 0.05$), as in untreated CCI animals (Figure 1A).

(C) Immunoblots of DRG tissue probed for TRPM8 and GAPDH protein levels after TRPM8 antisense or missense treatment. TRPM8 expression (and the increase in expression normally seen ipsilateral to CCI, Figure 2A) was selectively reduced by antisense, but not missense, infusion, whereas GAPDH levels were unchanged.

events are likely to be important in icilin action. Icilin-responsive afferents are expected to release glutamate, so we hypothesized that inhibitory glutamate receptors in the dorsal horn might underlie icilin-induced analgesia. Group II/III mGluRs could subserve such a role because they are antinociceptive in models of inflammatory, neuropathic, and acute pain [30–32] and inhibit transmission between primary afferent and spinal cord neurons in sensitized states [48, 49]. Group II mGluRs are localized on primary afferent terminals in lamina II, particularly in small nociceptive afferents [28, 50], although some are found postsynaptically and on glia [29]. Group III mGluRs are also found presynaptically in the dorsal horn and are 45% coexpressed with either IB4 or Substance P (markers of small nociceptive neurons [51]). To assess whether activation of Group II/III mGluRs might mimic icilin reversal of neuropathic sensitization, we intrathecally injected the selective Group II

(B) shows western blots of DRG from naive or TRPM8 antisense-treated rats probed with the TRPM8 antibody used for immunohistochemistry in (C)–(F) below. Pretreatment of the antibody with the antigenic peptide or TRPM8 antisense treatment removed the single specific band at ≈128 kDa.

(C) L5 spinal cord sections taken 8 days after dorsal rhizotomy were immunostained for peripherin (green) and TRPM8 (red) and showed marked reduction of both proteins ipsilateral to rhizotomy.

(D) Immunostaining for TRPM8 (red) and the neuronal marker NeuN (green) in the spinal cord dorsal horn from CCI animals showed that TRPM8 was increased ipsilateral to CCI with no change in distribution, whereas NeuN levels were unchanged. Scale bar for (C) and (D) represents 500 μm.

(E and F) Immunohistochemical colocalization in DRG sections ipsilateral or contralateral to nerve injury and in naive animals of TRPM8 (red) with (E) NF-200 (green) or (F) peripherin (green). In naive animals, TRPM8 is mainly located in peripherin-positive C fibers, with little or no apparent expression in myelinated (NF-200) cells. Ipsilateral to nerve injury, TRPM8 expression was increased markedly in small NF-200-positive cells, whereas a lesser increase in TRPM8:peripherin coexpression was also observed. Scale bar represents 50 μm. The bar charts in (E) and (F) show the percentage coexpression (mean ± SEM) for TRPM8:NF-200 and TRPM8:peripherin, respectively; actual cell counts are shown above columns. Statistically significant increases in the percentage coexpression values were seen in both cases ipsilateral to CCI, $p < 0.05$ (*).

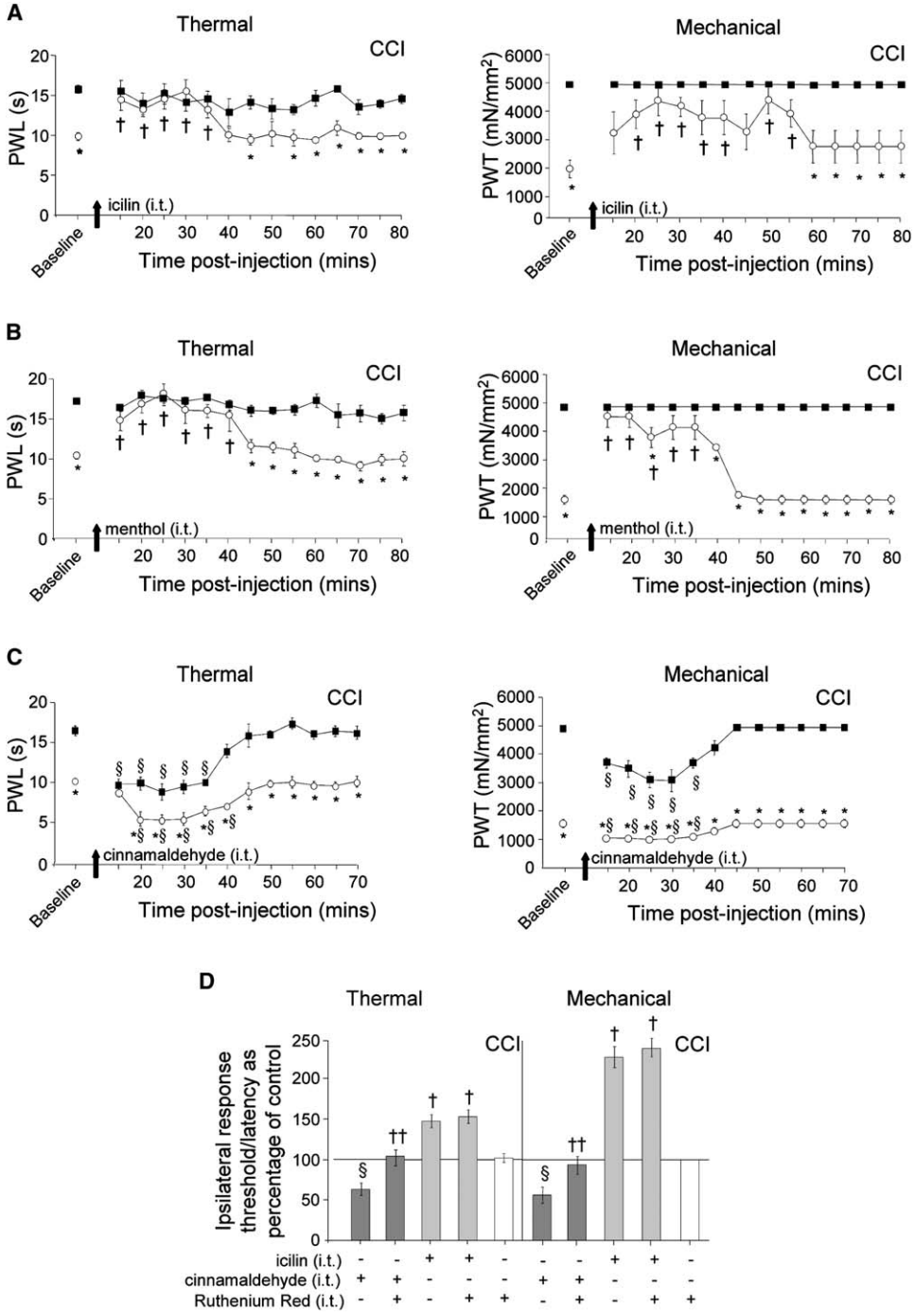

Figure 4. Central TRPM8 Activation Is Analgesic after CCI, whereas TRPA1 Activation Is Hyperalgesic

Paw withdrawal latency (PWL, s) to noxious heat and paw withdrawal threshold (PWT, mN/mm^2) to mechanical stimuli are shown ipsilateral (○) or contralateral (■) to CCI. * denotes significant ipsilateral-contralateral differences (* $p < 0.05$). Data show mean ± SEM, and each test represents n of six animals unless otherwise indicated. Rats were intrathecally injected (at arrow). The TRPM8 activators (A) icilin (10 nmol) and (B) (−)-menthol (200 nmol) both significantly reversed ipsilateral thermal and mechanical sensitization in comparison to preinjection values (†, $p < 0.05$). (C) In contrast, intrathecal application of the TRPA1 activator cinnamaldehyde (75 nmol) produced bilateral hyperalgesia and allodynia in CCI animals. § ($p < 0.05$) shows statistically significant increases in thermal and mechanical reflex responsiveness of both ipsilateral and contralateral paws.

(D) shows that Ruthenium Red (0.25 nmol) inhibits cinnamaldehyde (75 nmol)-induced hypersensitivity, but not icilin (10 nmol)-mediated analgesia, ipsilateral to CCI. Values are mean ± SEM, n = 4. Statistically significant changes in PWL/PWT values as a result of cinnamaldehyde or icilin compared to predrug baseline are shown as § and †, respectively ($p < 0.05$), and for Ruthenium Red-reversal of the effect of cinnamaldehyde (††, $p < 0.05$).

Table 2. Reversal of Sensitization by Intrathecal Icilin Administration in Different Pain Models

Pain Model	PWL (s) Difference from Control (no drug)	PWL (s) Difference from Control (+ icilin)	Mean % Reversal of Thermal Sensitization (after 15–30 min)	PWT (mN/mm^2) Difference from Control (no drug)	PWT (mN/mm^2) Difference from Control (+ icilin)	Mean % Reversal of Mechanical Sensitization (after 15–30 min)
CCI	5.9 ± 0.9[a]	0.9 ± 1.2[b]	84.7 ± 6.0	3347.4 ± 126.3 [a]	775.4 ± 467.7[b]	76.8 ± 5.3
CFA	4.7 ± 0.9[a]	1.6 ± 1.6[b]	66.0 ± 7.5	2975.6 ± 245.5[a]	1707.6 ± 149.9[b]	42.6 ± 2.8
Lysolecithin	7.8 ± 1.0[a]	2.4 ± 1.2[b]	69.2 ± 5.1	3003.8 ± 182.4[a]	1102.5 ± 422.5[b]	63.3 ± 6.4
Cinnamaldehyde (intrathecal)	5.9 ± 0.9[a]	0.5 ± 1.8[b]	91.5 ± 4.1	2022.6 ± 375.4[a]	150.3 ± 125.2[b]	92.6 ± 2.8
Cinnamaldehyde (topical)	5.0 ± 1.5[a]	0.4 ± 1.6[b]	92.0 ± 6.1	1002.0 ± 316.8[a]	0.0 ± 0.0[b]	100.0 ± 0.0

[a] Significant ipsilateral-contralateral differences in surgical pain models or, in the case of cinnamaldehyde-induced responses, differences from prior baseline, are indicated ($p < 0.05$).
[b] Significant icilin (10 nmol, intrathecal)-induced reversal of sensitization is indicated ($p < 0.05$).

or III mGluR agonists 2R, 4R-APDC or ACPT-III and AP-4, respectively. 2R, 4R-APDC (15 nmol) caused 72.1% ± 6.4% reversal of thermal and 56.0% ± 10.9% reversal of mechanical reflex sensitization ipsilateral to CCI (15–30 min postinjection) with no effect on contralateral responses. ACPT-III and AP-4 (150 nmol each) also reversed thermal sensitization (by 83.6% ± 6.3% and 60.8% ± 6.7%, respectively), as well as mechanical sensitization (65.7% ± 11.4% and 60.7% ± 8.0%), again with no effects contralaterally ($p < 0.05$ in each case). Furthermore, selective Group II and Group III mGluR antagonists LY 341495 (5 nmol, Figure 5A) and UBP 1112 (10 nmol, Figure 5B) each prevented the effect of icilin (10 nmol, Figure 4A). Similarly, the analgesia produced by intrathecal (−)-menthol (200 nmol, Figure 4B) was reversed by intrathecal LY 341495 and UBP 1112. The mean percentage reversal of sensitization over 20–30 min postinjection was 86.1% ± 8.1% for PWL and 80.6% ± 4.2% for PWT with (−)-menthol alone, 22.0% ± 6.9% for PWL and 7.1% ± 7.1% for PWT with menthol and LY 341495, and 9.2% ± 6.9% for PWL and 0.0% ± 0.0% for PWT with (−)-menthol and UBP 1112 (n = 6). Neither LY 341495 nor UBP 1112 had any effects alone (data not shown), suggesting that Group II/III mGluRs show little tonic activation after CCI, but are specifically utilized downstream of icilin. In contrast, intrathecal coadministration of the μ-opioid receptor antagonist naloxone (25 nmol) with icilin had no effect (Figure 5C), indicating that icilin analgesia is opioid independent. To avoid any possibility of nonspecific drug interactions, we also administered icilin (200 μM) topically, but the mGluR antagonists intrathecally. Figure 5D shows that the icilin reversal of thermal and mechanical sensitization in this case was again prevented by LY 341495 or UBP 1112. The analgesic effect of skin cooling to 16°C (Figure 1E) was also prevented by intrathecally applied LY 341495 (5 nmol) or UBP 1112 (10 nmol). The mean percentage reversal of ipsilateral CCI-induced reductions in PWT caused by cooling was 0.0% ± 0.0% in the presence of either drug (n = 5).

To confirm the analgesic effect of icilin at the level of single spinal cord neurons, we made in vivo extracellular recordings of large lamina I and III/IV neurons (which integrate nociceptive and nonnociceptive inputs). Topical administration of icilin (200 μM) to the peripheral receptive field area ipsilateral to CCI caused inhibition of the elevated neuronal responses to motorized rotating brush (Figure 5E). In the eight neurons out of 12 that were affected by icilin (two in lamina I and six in laminae III/IV) brush-induced responses were reduced to 37.4% ± 5.5%, $p < 0.001$). Vehicle had no effect. Contralateral neurons were unaffected (111.9% ± 8.9% of control; n = 6). As an example of one of the Group II/III mGluR antagonists investigated on reflexes, UBP 1112 was ionophoresed in the vicinity of recorded dorsal-horn neurons at currents of 20–60 nA. UBP 1112 reversed the effect of icilin; the brush-induced firing rate reverted to 80.2% ± 9.3% of control values (Figure 5E), but UBP 1112 had no effect alone, and nor did saline current controls.

Because some Group II/III mGluRs may be postsynaptic, we asked whether icilin could reverse the additional sensitization of behavioral-reflex responsiveness caused by intrathecally applied NMDA in CCI animals. Icilin (10 nmol) clearly attenuated the additional ipsilateral sensitization induced by 3.75 nmol of NMDA plus 0.75 nmol of its coagonist site activator, ACPC, injected intrathecally. Ipsilateral PWL values in thermal tests were 10.1 ± 0.6 s at baseline and decreased to 7.9 ± 0.3 s (15–30 min after injection of NMDA/ACPC), but increased to 14.9 ± 0.6 s in the additional presence of icilin. Contralateral values were unaltered by icilin or NMDA/ACPC. Ipsilateral PWT values in mechanical tests were 1504.2 ± 105.3 mN/mm^2 at baseline, 891.6 ± 27.3 mN/mm^2 after injection of NMDA/ACPC, and 3482.7 ± 174.3 mN/mm^2 with coinjection of icilin (mean ± SEM, n = 6). Contralateral values again were unaltered. A component of the central events elicited by icilin may therefore be postsynaptic, although it is important to note that functional NMDA receptors may also be present on afferent terminals [52].

Discussion

Little is known of the mechanism underlying cooling-induced analgesia, but a number of cool-sensitive ion channels, including TRPM8, have recently been identified in somatosensory afferents [13]. We now show that TRPM8 activation reverses nerve-injury-induced hypersensitivity. TRPM8 can be activated by menthol [15, 16], which is analgesic in hot-plate and acetic-acid writhing tests [11], although menthol can produce pain at very high doses [53, 54]. Here, either topical or intrathecal application of (−)-menthol produced behavioral analgesia in the CCI model of neuropathic pain, most

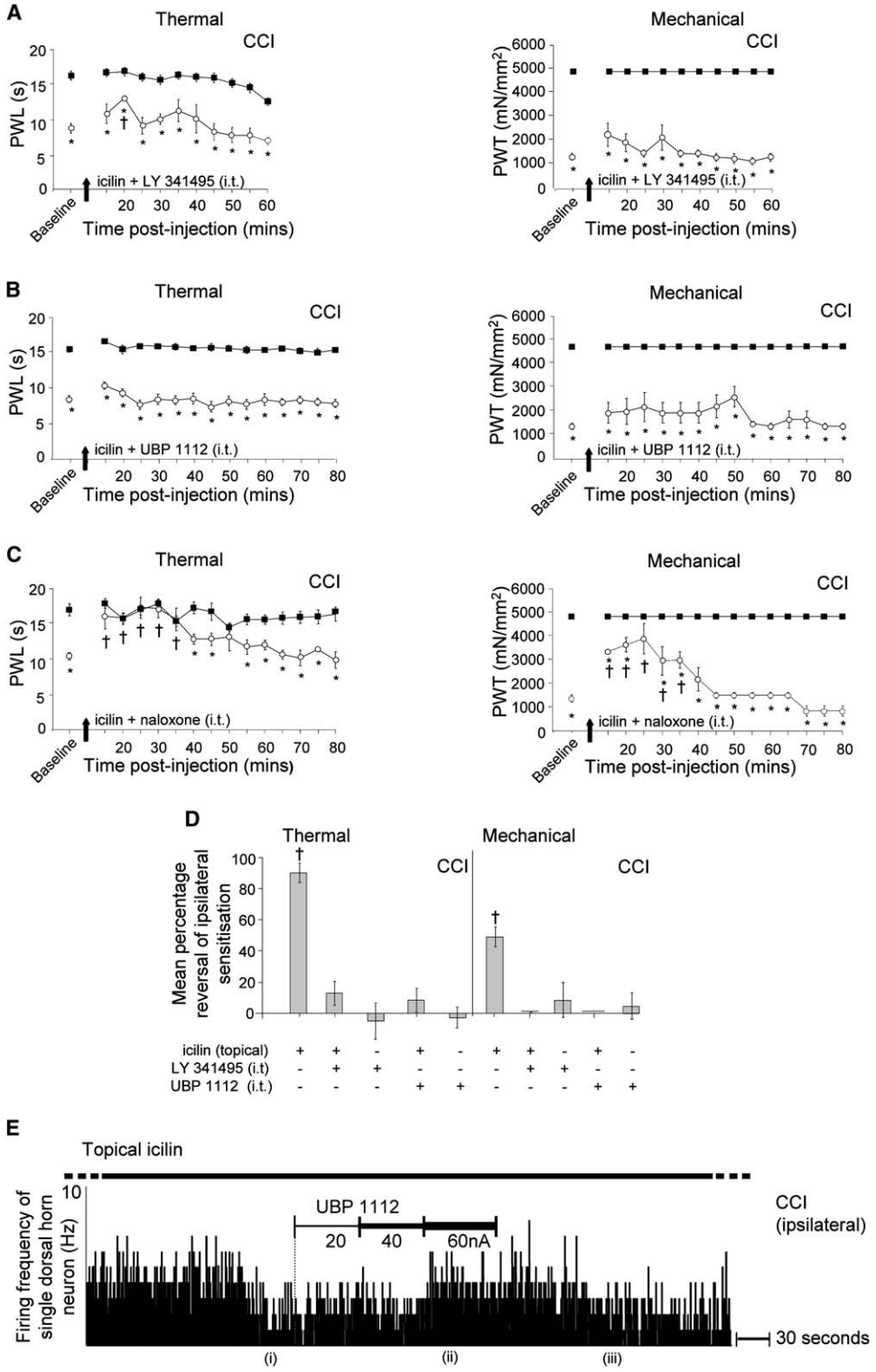

Figure 5. Icilin-Induced Analgesia after CCI Is Prevented by Group II and III mGluR Antagonists

(A–D) Paw withdrawal latency (PWL, s) to noxious heat and paw withdrawal threshold (PWT, mN/mm^2) to mechanical stimuli ipsilateral (○) or contralateral (■) to CCI. Data represent mean ± SEM, with an n of six animals in each case. Rats were intrathecally injected (at arrow). Icilin (10 nmol) was coadministered with (A) the Group II mGluR antagonist LY 341495 (5 nmol), (B) the Group III mGluR antagonist UBP 1112 (10 nmol), or (C) the opioid receptor antagonist, naloxone (25 nmol).

(A and B) When either LY 341495 or UBP 1112 was coadministered with icilin, it abolished the analgesic effect of icilin as indicated by the persistence of sensitized (*, $p < 0.05$) PWT/PWL responses ipsilateral to nerve injury.

(C) Naloxone coadministration with icilin did not prevent the icilin-induced analgesia seen in PWT and PWL responses ipsilateral to nerve injury (†, $p < 0.05$). * indicates significant ipsilateral-contralateral differences, and † indicates significant ipsilateral analgesic effect of drug ($p < 0.05$).

likely by activation of TRPM8. Similar effects were seen with another TRPM8 activator, icilin [15]. As with menthol, very high doses of icilin were found to cause a generalized increase in sensitization, affecting CCI animals bilaterally and naive animals in a similar fashion (Table 1). Importantly, analgesic effects of icilin were seen at 200-fold-lower concentrations than those causing nonspecific sensory changes. Specific involvement of TRPM8 in the reversal of neuropathic sensitization was confirmed by the abrogation of icilin analgesia after intrathecal infusion of a TRPM8 antisense oligonucleotide to knock down TRPM8 expression. Furthermore, the analgesic profile was mimicked by cutaneous cooling to 20°C–16°C, a range activating the TRPM8 channel [14]. Icilin and menthol were applied cutaneously in solution at 30°C, so any possible drug effects on skin temperature were avoided. TRPM8 antisense had no effect alone on CCI-induced sensitized responses to noxious heat, mechanical stimuli, or intense cold (Figure 3), similar to observations made in an alternative neuropathic pain model [27]. A role for the TRPA1 channel in analgesia seems unlikely because selective TRPA1 activators, cinnamaldehyde, allicin, and diallyl disulphide, caused contrasting sensitization/hyperalgesia not only after CCI, but also in naive animals. The analgesic effects of icilin were only seen in the sensitized pain state, but were not restricted to nerve injury, because sensitization due to peripheral inflammation, afferent demyelination, and TRPA1 activation was also reduced. The significant behavioral and electrophysiological effects of topically applied icilin demonstrate that icilin can cross the skin sufficiently to excite peripheral afferents, and point to the likely clinical utility of this or related drugs.

The precise identity of the TRPM8-containing cool-responsive afferents is not clear. Subpopulations of Aδ fibers and C fibers are responsive to different ranges of cool temperatures: ~15°C–30°C and <15°C [55, 38]. Innocuous cooling (15°C–30°C) activates a subpopulation of Aδ fibers and C fibers in primates, but almost solely unmyelinated fibers in rodents [55]. In contrast, intense noxious cold is signaled by unmyelinated polymodal nociceptors, which also respond to heat and mechanical stimuli [38]. The TRPM8 activator menthol activates cool-sensitive fibers and sensitizes stimulus-induced responses in the range 20°C–30°C [56, 57]. Studies of TRPM8 in vitro identify this channel as a likely transducer of moderate cool temperatures [20]. In DRG and trigeminal cultures, responses to menthol, cooling (15°C–30°C), and TRPM8 mRNA expression all correlate closely [18–20]. TRPM8 is expressed in 5%–20% of DRG cell bodies that are small and presumed Aδ fibers or C fibers [15, 16], but not in large myelinated fibers. We observed that TRPM8 immunoreactivity is normally associated with a subpopulation of peripherin-positive C fibers, but only minimally with NF-200-positive afferents, whereas high-sensitivity cRNA hybridization suggests the presence of some TRPM8 mRNA in up to 19% of NF-200-positive afferents [58]. The capsaicin- and heat-sensitive TRPV1 channel, which contributes to thermal nociception and inflammatory sensitization [12], is found both in peptidergic afferents (~85%) and in nonpeptidergic (isolectin-B4, IB4-positive) cells in the rat [59]. TRPM8 is not categorically associated with either peptidergic or IB4-positive afferents [15] but is often present in those containing the NGF receptor, Trk A [58]. Different groups have reported coexpression of mRNAs for TRPM8 and TRPV1 or menthol/capsaicin responsiveness of DRG at 29%–50% [15, 20, 60] or close to zero [14, 16, 58]. Overall, it seems likely that TRPM8 is normally expressed in a distinct population of cool-responsive afferents and possibly also to an extent in some nociceptors. Our findings further identified increased expression in peripherin-positive C fibers, but induction in small NF-200-positive presumed Aδ fibers [35], suggesting that plasticity in TRPM8 expression may participate in icilin analgesia in neuropathic pain. No changes in DRG expression of TRPM8 were reported in an alternative neuropathic pain model (ligation of L5 spinal nerve or indeed after CFA) [26, 27], suggesting that specific aspects of the particular model, such as the coexistence of injured and uninjured afferents in DRG after CCI, may be important in TRPM8 upregulation.

TRPA1, which has also been proposed as a cool receptor [14], appears to play an entirely different role, eliciting reflex pain behaviors in naive animals, as well as increasing thermal and mechanical responsiveness in the neuropathic state. This may correspond to clinical observations after nerve injury in which moderately cool stimuli are perceived as painful [1]. TRPA1 is present mainly in small cells in sensory ganglia [14, 21] and may increase ipsilateral to nerve injury and inflammation [26, 27]. Nerve-injury- and inflammation-induced hyperalgesia to noxious cold (5°C) is reported to be decreased by antisense knockdown of TRPA1 [26, 27]. Correspondingly, mutant mice homozygous for targeted disruption of the TRPA1 gene show reduced reflex withdrawal responses to selective TRPA1 activators and reduced sensitization of noxious heat and innocuous mechanical responses caused by these agents [24]. However, the role of TRPA1 in noxious cold responses is disputed, with results from different lines of TRPA1$^{-/-}$ animals showing either attenuated or unaltered coldplate withdrawal responses [24, 25]. Thus the precise role of TRPA1 in cold sensation remains unclear, but here as in other studies TRPA1 clearly acts in a pronociceptive manner [21, 22, 46, 47]. Although icilin may interact with low

(D) The analgesic effects of topically applied icilin (200 μM at 30°C) were also reversed by intrathecal injection of Group II/III mGluR antagonists. The figure shows mean % reversal of the ipsilateral/contralateral difference in either PWL or PWT measured over 15–30 min after 5 min topical application of icilin, with or without concurrent intrathecal injection of LY 341495 (5 nmol) or UBP 1112 (10 nmol), or after mGluR antagonists alone.

(E) Typical extracellular recording of a single dorsal-horn neuron ipsilateral to CCI, responding to continuous motorized brushing of the cutaneous receptive field on the hind paw and the effects of icilin (200 μM at 30°C) topically applied to an adjacent area of the receptive field. Similar results were observed in 8 out of 12 neurons with examples in both laminae III/IV and lamina I. Neuronal firing is displayed as action potentials per second (Hz) plotted against time. (E_i) Brush-evoked firing in neurons ipsilateral to nerve injury was consistently inhibited by topically applied icilin; (E_{ii}) this effect was reversed by ionophoresis of UBP 1112 at 20–60 nA; (E_{iii}) recovery was observed after removal of the UBP 1112 ejection current. Neurons contralateral to nerve injury were unaffected by icilin, and topical vehicle had no effect. In addition, ionophoresis of UBP 1112 alone or saline current controls showed no discernable effect.

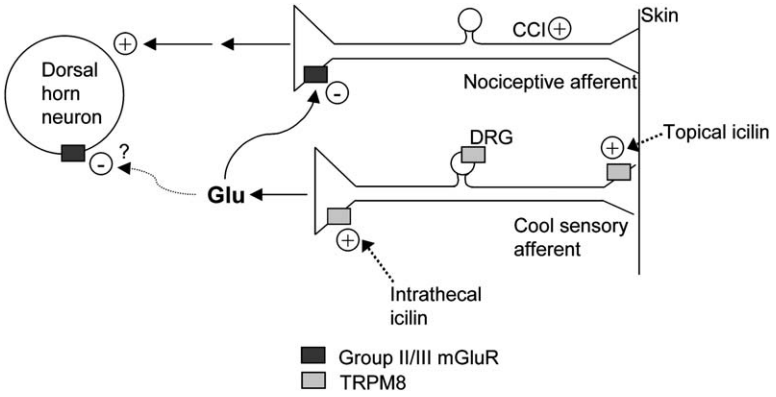

Figure 6. Schematic Representation of a Possible Mechanistic Basis for TRPM8-Mediated Analgesia after CCI

In this simplified hypothetical model, activation of TRPM8 in a subpopulation of afferents by icilin, menthol, or moderate cooling leads to central synaptic release of glutamate (Glu), which then acts through inhibitory Group II/III mGluR receptors located either presynaptically on injury-activated nociceptive afferents or perhaps also postsynaptically on dorsal-horn neurons, thereby attenuating neuropathic sensitization.

potency at the TRPA1 channel [14], the analgesic profile of TRPM8 activators here is entirely different from the pronociceptive profile of TRPA1 activators. The extent of TRPM8/TRPA1 coexpression in afferents is reported to be minimal [14, 58].

The analgesia induced by icilin and menthol and by skin cooling to 16°C was shown to be centrally mediated and dependent on Group II/III mGluRs. The lack of effect of naloxone suggests independence from classical opioid analgesia. Furthermore, at the doses used, mGluR Group II/III antagonists selectively reversed icilin and menthol analgesia in sensitized responses, without any effects alone. Group II/III mGluRs are known to inhibit nociceptive responses [30–32, 48, 49], and we showed that Group II/III mGluR agonists selectively inhibit sensitized responses in neuropathic pain. Both Group II and III mGluR subtypes are expressed in primary afferents, especially IB4-positive cells [28, 50, 51]. Activation of Group II/III mGluRs can inhibit afferent-evoked potentials in the dorsal horn [48], because Group II mGluRs are both pre- and postsynaptic at primary afferent synapses [29], whereas Group III mGluRs are largely presynaptic [51]. Menthol is reported to increase mEPSC frequency at some synapses between DRG and dorsal-horn neurons in culture and in slices [44, 45], presumably corresponding to the activation of TRPM8-containing afferents (Figure 1D) and increased release of glutamate. Figure 6 shows a schematic outline of a model in which glutamate released from TRPM8-expressing afferents could mediate icilin-induced analgesia by acting on Group II/III mGluRs (located presynaptically on nociceptive afferents and possibly also postsynaptically) to result in attenuation of pain-related sensitization (Figure 5E) and behavioral analgesia (Figure 5D).

Conclusions

In summary, these novel findings show that both peripheral and central activation of TRPM8 can produce an analgesic effect that specifically reverses the sensitization of behavioral reflexes elicited by peripheral nerve injury. This effect is produced by very low concentrations of topically applied TRPM8 activators, pointing to the likelihood of its ready utility in a clinical context. Other sensitized pain states, in addition to that induced by nerve injury, are similarly sensitive to reversal by TRPM8 activation, emphasizing the likely value of TRPM8 activators and downstream central mediators of TRPM8 action, such as Group II/III mGluRs, as targets for the development of novel analgesics.

Experimental Procedures

Animals

All experiments were conducted in accordance with the UK Animals (Scientific Procedures) Act, 1986, and guidelines of the University of Edinburgh. Adult male Wistar rats (Harlan, United Kingdom) weighing 120–250 g were used for all experiments.

Neuropathic and Inflammatory Pain Models

Pain models were generated under halothane anesthesia (Zeneca, Cheshire, United Kingdom). For the chronic constriction injury (CCI) model of neuropathic pain, four ligatures were tied loosely to constrict the sciatic nerve at midthigh level (as described previously [61]). An inflammatory pain model was generated by injecting 100 μl of Complete Freund's Adjuvant (CFA, Sigma-Aldrich) into the ventral surface of the right hind paw [61]. A model of peripheral demyelination-induced pain was produced by focal application of lysolecithin to the sciatic nerve [43]. Peak behavioral sensitization was observed postsurgically between days 10 and 16 for CCI, 1 and 3 for CFA, and 7 and 14 for lysolecithin, when pharmacological and electrophysiological experiments and tissue removal were conducted.

Behavioral Testing

Thermal sensitivity was assessed by measuring paw withdrawal latency (PWL, s) in response to a noxious thermal stimulus (Hargreaves' thermal stimulator, Linton Instrumentation, Diss, United Kingdom) directed to the hind paw midplantar glabrous surface. Mechanical sensitivity was recorded as the paw withdrawal threshold (PWT, mN/mm^2) to calibrated von Frey filaments (Stoelting, Illinois), as previously described [61]. Sensitivity to noxious cold was assessed by placing animals in a water bath with an aluminium floor containing 1-cm-deep 4°C water and counting the time the paw was held suspended over a 20 s period.

Intrathecal Application of Drugs

The following drugs were applied intrathecally in a 50 μl volume of saline-based vehicle at 37°C: icilin (2.5–200 μM in saline with 0.2% dimethylformamide, DMF); LY 341495 (100 μM in saline); UBP 1112 (200 μM in saline); 2R, 4R-APDC [(2R, 4R)-4-aminopyrrolidine-2,4-dicarboxylate, 300 μM in saline]; ACPT-III [(1R,3R,4S)-1-aminocyclopentane-1,3,4-tricarboxylic acid, 3 mM in saline]; AP-4 [(L-(1)-2-amino-4-phophonobutyric acid, 3 mM in saline); naloxone (0.5 mM in saline); NMDA (75 μM in saline) and ACPC (1-aminocyclopropanecarboxylic acid, 15 μM in saline) (Tocris Cookson, Bristol, United Kingdom); (−)-menthol [1R, 2S, 5R-(−)-menthol, 4 mM in saline]; cinnamaldehyde (1.5 mM in saline); Ruthenium Red (5 μM in saline) (Sigma-Aldrich, United Kingdom); allicin (0.5 mM in saline with 0.5% DMF); and diallyl disulphide (DADS, 1 mM in saline with 0.5% DMF) (LKT Laboratories, St. Paul, Minnesota). Drugs were injected into the L5–6 intrathecal space under brief halothane anesthesia, with a 25-gauge needle microsyringe (BD Biosciences, Oxford), as described previously [61] in animals that were at peak levels of

behavioral sensitization. Behavioral-reflex testing commenced 15 min after injection to allow recovery from anesthesia [43, 62, 63] and continued every 5 min thereafter until readings returned to baseline levels (n = 6 in each case). We, and others [43, 62, 63], find complete recovery from anesthetic by 15 min. All appropriate controls were carried out to eliminate the possibility of effects due to vehicle or to injection procedure.

The TRPM8 channel activators icilin and (−)-menthol were tested in CCI and naive animals. The effects of icilin were additionally assessed in animals with CFA-induced inflammation or with lysolecithin-induced demyelination. Icilin was also coadministered with the μ-opioid receptor antagonist naloxone, the Group II metabotropic glutamate receptor (mGluR) antagonist LY 341495, or the Group III mGluR antagonist UBP 1112. The effects of these antagonists alone, as well as effects of the Group II mGluR agonist 2R, 4R-APDC and the Group III mGluR agonists ACPT-III and AP-4, were assessed in CCI animals. The TRPA1 channel activator cinnamaldehyde, alone and with icilin, was tested in CCI animals and in naive animals. Further TRPA1 channel activators, allicin and diallyl disulphide, were assessed in naive animals. Both icilin and cinnamaldehyde effects in CCI animals were also investigated in the additional presence of Ruthenium Red.

Topical Application of Drugs

Icilin was applied at concentrations of 2.5–500 μM (in water with 0.2% DMF), by placing CCI or naive rats unrestrained for 5 min in a 1-cm-deep water bath (sufficient to cover paws), which was thermostatically controlled to a temperature of 30°C, or by very lightly anesthetizing rats and immersing hind paws in small tubes containing 5 ml icilin (500 μM–5 mM, with a vehicle of 45% dimethylformamide in 0.2% aqueous Tween 80) for 5 min, followed by sensory testing for 60–80 min. The effects of (−)-menthol and its stereoisomers isomenthol (1S, 2R, 5R-menthol,) and (+)-menthol [1S, 2R, 5S (+)-menthol] (4–16 mM in 80% ethanol) were also assessed. Relevant vehicles were always assessed in similar experiments. As a contrast, the effect of cinnamaldehyde (1.5 mM in water) and the effect of cinnamaldehyde with additional icilin (80 μM in water with 0.2% DMF) were assessed in naive animals. The effects of icilin (80 μM) were further assessed in CCI animals that had undergone antisense and missense treatment for knockdown of TRPM8 or immediately after intrathecal injection of either LY 341495 or UBP 1112. Actual skin temperatures were measured by a subcutaneous thermistor probe and were found to equilibrate to around 0.5°C above bath temperature. The effects of brief paw immersion at different temperatures (10°C–22°C for 5 min) on CCI rats were assessed by mechanical testing. The effects of intrathecal LY 341495 or UBP 1112 on the reversal of ipsilateral sensitization in CCI after a 16°C cool challenge for 5 min were measured. Reflex testing commenced 5 min after challenge, unless animals had been anesthetized, in which case 15 min was allowed for recovery. Six replicate animals were tested in all pharmacological experiments.

Dorsal Rhizotomy

To establish whether TRPM8 expression in the spinal cord was predominantly pre- or postsynaptic, we performed a unilateral L2–6 dorsal rhizotomy under anesthesia, following laminectomy to expose the dorsal roots. Eight days later, tissue was removed and processed for immunohistochemistry.

Western Blots

Experiments were performed by standard procedures as previously described [61]; for more details see the Supplemental Data available online.

Immunohistochemistry

Experiments were performed as previously described [43]; for more details, see Supplemental Data.

Antisense Knockdown of TRPM8

Antisense and missense oligonucleotides were 22 mers with phosphorothioate bonds at the last two positions at the 5′ and 3′ ends (MWG Biotech, Ebersberg, Germany). Antisense extended from base −10 to base +12 relative to the start of the open reading frame for the rat TRPM8 gene: 5′ C*T*CGAAGGACATCTTGCCGT*G*G 3′, where * represents phosphorothioate linkages. Missense was designed with four inversions of C/G or A/T as appropriate at residues 3, 11, 14, and 22, preserving overall G/C content. BLAST searches of both oligonucleotides indicated no significant complementarity to any known gene sequence. Fourteen day or seven day osmotic minipumps (for CCI experiments or naive electrophysiology experiments, respectively—Alzet Minipump, models 2002, 2001; Charles River, United Kingdom) containing oligonucleotides (1 μg/μl in sterile saline) were connected to canulae inserted under the dura of the spinal cord to level L5–6 and produced a predicted infusion rate of 0.5 μl/hr. CCI surgery was performed at the same time as minipump implantation. Sensory tests were carried out to assess the time course of behavioral sensitization in animals that had also undergone a CCI injury. Icilin was applied topically rather than intrathecally so as to prevent any interference with the infusion canula. Peripheral nerve recordings and tissue harvesting were carried out after an interval of 4–5 days to allow time for protein knockdown.

Electrophysiology

Peripheral

Recordings of saphenous (sensory) nerve were made in naive animals (n = 7) to assess the effects of topical icilin on primary afferents. In addition, recordings were carried out on animals that had undergone TRPM8 antisense or missense treatment beginning 4–5 days previously. Rats were anesthetized (with 0.6 ml 25% urethane/100 g, intraperitoneal), and the saphenous nerve was exposed and dissected from its associated vein and artery. Further dissection under liquid paraffin enabled identification of afferent preparations comprising a small number of fibers. The conduction velocity of single identified afferent fibers was determined by using bipolar electrodes and the peripheral stimulus technique [64]. After isolation of preparations, icilin (200 μM in water with 0.2% DMF), (−)-menthol (4 mM in 25% ethanol), resiniferatoxin (1 mM in ethanol), or vehicle alone was applied to the hind limb receptive fields, and neuronal responses were recorded with the Chart program (version 3.6).

Central

Recordings of spinal dorsal-horn neurons were made in CCI animals, as described previously [61]. After halothane induction, the jugular vein and trachea were canulated and intravenous anesthetic was delivered: α-chloralose (0.6 mg/kg, Fisher) and urethane (1.2 mg/kg, Sigma), with small supplementary doses of α-chloralose as required throughout the experiment. Core body temperature was maintained at 37°C–38°C by means of a thermostatically controlled heated blanket. The animal was placed in a stereotaxic frame, and the thoracolumbar spinal column was supported by three pairs of swan-necked clamps. A laminectomy was performed at L2–L5, and agar (2% in saline at 37°C) was delivered over exposed cord to increase mechanical stability. Above the recording region, the agar and spinal cord dura were removed, and liquid paraffin was poured into the pool. Extracellular recordings were made from single multireceptive neurons in laminae I–IV through the center barrel of a seven-barrelled glass microelectrode filled with 4 M NaCl (tip-diameter 4–5 μm, DC resistance 5–8 MΩ). The receptive fields of hair-follicle innervated neurons on the distal hind limb were identified by an innocuous brush stimulus [61]. Icilin (200 μM in water with 0.2% DMF) was applied peripherally to the receptive field of individual recorded neurons, and the effect on neuronal response to a rotating brush was recorded and analyzed with Spike2 program (Version 3.2, CED). The Group III mGluR antagonist UBP 1112 (20 mM in water), pH 8.5, and control 1 M NaCl, pH 8.5, were ionophoresed from the side barrels of the electrode by using currents of between 20 nA and 80 nA (Neurophore BH2 ionophoresis system, Medical Systems, Great Neck, New York) to measure effects on neuronal response to icilin.

Statistics

All data were analyzed for statistical significance by using Sigmastat software (version 2.03) with p values < 0.05 being considered significant. Differences in thermal sensitivity between the paw ipsilateral to nerve injury and the contralateral paw were assessed with Student's t test. Any effect of drug treatment was analyzed by one-way repeated-measures ANOVA followed by Dunnett's post-hoc multiple-comparisons test. The equivalent nonparametric tests for mechanical sensitivity were Wilcoxon rank test for

ipsilateral:contralateral differences and Friedman repeated-measures ANOVA followed by Dunn's test for changes from predrug control values. Western blot-densitometric values were compared by using the Wilcoxon test, immunohistochemistry cell counts were analyzed by one-way ANOVA, and electrophysiological spike frequencies were analyzed by one-way ANOVA on ranks.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures and are available with this article online at: http://www.current-biology.com/cgi/content/full/16/16/1591/DC1/.

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References

1. Woolf, C.J., and Mannion, R.J. (1999). Neuropathic pain: Aetiology, symptoms, mechanisms, and management. Lancet *353*, 1959–1964.
2. Sprengell, C. (1735). Hippocrates: The Aphorisms of Hippocrates, and The Sentences of Celsus; with Explanations and References to the Most Considerable Writers … to which Are Added, Aphorisms upon Several Distempers, Not Well Distinguished by the Ancients, Second Edition (London: Sine nomine).
3. Siegel, R.E. (1970). Galen on Sense Perception: His Doctrines, Observations and Experiments on Vision, Hearing, Smell, Taste, Touch and Pain, and Their Historical Sources (Basel: Karger).
4. Bini, G., Cruccu, G., Hagbarth, K.E., Schady, W., and Torebjork, E. (1984). Analgesic effect of vibration and cooling on pain induced by intraneural electrical stimulation. Pain *18*, 239–248.
5. Sauls, J. (1999). Efficacy of cold for pain: Fact or fallacy? Online J. Knowl. Synth. Nurs. *6*, 8.
6. Wright, A. (1870). Oil of peppermint as a local anaesthetic. Lancet *2464*, 726.
7. American Botanical Council (1998). The Complete German Commission E Monographs: Therapeutic Guide to Herbal Medicines, M. Blumenthal, W.R. Busse, A. Goldberg, J. Gruenwald, T. Hall, C.W. Riggins, and R.S. Rister, eds. (Boston: Integrative Medicine Communication).
8. Gobel, H., Schmidt, G., and Soyka, D. (1994). Effect of peppermint and eucalyptus oil preparations on neurophysiological and experimental algesimetric headache parameters. Cephalalgia *14*, 228–234.
9. Davies, S.J., Harding, L.M., and Baranowski, A.P. (2002). A novel treatment of postherpetic neuralgia using peppermint oil. Clin. J. Pain *18*, 200–202.
10. Green, B.G., and McAuliffe, B.L. (2000). Menthol desensitization of capsaicin irritation. Evidence of a short-term anti-nociceptive effect. Physiol. Behav. *68*, 631–639.
11. Galeotti, N., Di Cesare Mannelli, L., Mazzanti, G., Bartolini, A., and Ghelardini, C. (2002). Menthol: A natural analgesic compound. Neurosci. Lett. *322*, 145–148.
12. Caterina, M.J., Leffler, A., Malmberg, A.B., Martin, W.J., Trafton, J., Petersen-Zeitz, K.R., Koltzenburg, M., Basbaum, A.I., and Julius, D. (2000). Impaired nociception and pain sensation in mice lacking the capsaicin receptor. Science *288*, 306–313.
13. McKemy, D.D. (2005). How cold is it? TRPM8 and TRPA1 in the molecular logic of cold sensation. Mol. Pain *1*, 16.
14. Story, G.M., Peier, A.M., Reeve, A.J., Eid, S.R., Mosbacher, J., Hricik, T.R., Earley, T.J., Hergarden, A.C., Andersson, D.A., Hwang, S.W., et al. (2003). ANKTM1, a TRP-like channel expressed in nociceptive neurons, is activated by cold temperatures. Cell *112*, 819–829.
15. McKemy, D.D., Neuhausser, W.M., and Julius, D. (2002). Identification of a cold receptor reveals a general role for TRP channels in thermosensation. Nature *416*, 52–58.
16. Peier, A.M., Moqrich, A., Hergarden, A.C., Reeve, A.J., Andersson, D.A., Story, G.M., Earley, T.J., Dragoni, I., McIntyre, P., Bevan, S., et al. (2002). A TRP channel that senses cold stimuli and menthol. Cell *108*, 705–715.
17. Eccles, R. (1994). Menthol and related cooling compounds. J. Pharm. Pharmacol. *46*, 618–630.
18. Nealen, M.L., Gold, M.S., Thut, P.D., and Caterina, M.J. (2003). TRPM8 mRNA is expressed in a subset of cold-responsive trigeminal neurons from rat. J. Neurophysiol. *90*, 515–520.
19. Thut, P.D., Wrigley, D., and Gold, M.S. (2003). Cold transduction in rat trigeminal ganglia neurons in vitro. Neuroscience *119*, 1071–1083.
20. Babes, A., Zorzon, D., and Reid, G. (2004). Two populations of cold-sensitive neurons in rat dorsal root ganglia and their modulation by nerve growth factor. Eur. J. Neurosci. *20*, 2276–2282.
21. Nagata, K., Duggan, A., Kumar, G., and Garcia-Anoveros, J. (2005). Nociceptor and hair cell transducer properties of TRPA1, a channel for pain and hearing. J. Neurosci. *25*, 4052–4061.
22. Bandell, M., Story, G.M., Hwang, S.W., Viswanath, V., Eid, S.R., Petrus, M.J., Earley, T.J., and Patapoutian, A. (2004). Noxious cold ion channel TRPA1 is activated by pungent compounds and bradykinin. Neuron *41*, 849–857.
23. Jordt, S.E., Bautista, D.M., Chuang, H.H., McKemy, D.D., Zygmunt, P.M., Hogestatt, E.D., Meng, I.D., and Julius, D. (2004). Mustard oils and cannabinoids excite sensory nerve fibres through the TRP channel ANKTM1. Nature *427*, 260–265.
24. Bautista, D.M., Jordt, S.E., Nikai, T., Tsuruda, P.R., Read, A.J., Poblete, J., Yamoah, E.N., Basbaum, A.I., and Julius, D. (2006). TRPA1 mediates the inflammatory actions of environmental irritants and proalgesic agents. Cell *124*, 1269–1282.
25. Kwan, K.Y., Allchorne, A.J., Vollrath, M.A., Christensen, A.P., Zhang, D.S., Woolf, C.J., and Corey, D.P. (2006). TRPA1 contributes to cold, mechanical, and chemical nociception but is not essential for hair-cell transduction. Neuron *50*, 277–289.
26. Obata, K., Katsura, H., Mizushima, T., Yamanaka, H., Kobayashi, K., Dai, Y., Fukuoka, T., Tokunaga, A., Tominaga, M., and Noguchi, K. (2005). TRPA1 induced in sensory neurons contributes to cold hyperalgesia after inflammation and nerve injury. J. Clin. Invest. *115*, 2393–2401.
27. Katsura, H., Obata, K., Mizushima, T., Yamanaka, H., Kobayashi, K., Dai, Y., Fukuoka, T., Tokunaga, A., Sakagami, M., and Noguchi, K. (2006). Antisense knock down of TRPA1, but not TRPM8, alleviates cold hyperalgesia after spinal nerve ligation in rats. Exp. Neurol. *200*, 112–123.
28. Carlton, S.M., Hargett, G.L., and Coggeshall, R.E. (2001). Localization of metabotropic glutamate receptors 2/3 on primary afferent axons in the rat. Neuroscience *105*, 957–969.
29. Tamaru, Y., Nomura, S., Mizuno, N., and Shigemoto, R. (2001). Distribution of metabotropic glutamate receptor mGluR3 in the mouse CNS: Differential location relative to pre- and postsynaptic sites. Neuroscience *106*, 481–503.
30. Fisher, K., Lefebvre, C., and Coderre, T.J. (2002). Antinociceptive effects following intrathecal pretreatment with selective metabotropic glutamate receptor compounds in a rat model of neuropathic pain. Pharmacol. Biochem. Behav. *73*, 411–418.
31. Simmons, R.M., Webster, A.A., Kalra, A.B., and Iyengar, S. (2002). Group II mGluR receptor agonists are effective in persistent and neuropathic pain models in rats. Pharmacol. Biochem. Behav. *73*, 419–427.
32. Chen, S.R., and Pan, H.L. (2005). Distinct roles of group III metabotropic glutamate receptors in control of nociception and dorsal horn neurons in normal and nerve-injured Rats. J. Pharmacol. Exp. Ther. *312*, 120–126.
33. Behrendt, H.J., Germann, T., Gillen, C., Hatt, H., and Jostock, R. (2004). Characterization of the mouse cold-menthol receptor

TRPM8 and vanilloid receptor type-1 VR1 using a fluorometric imaging plate reader (FLIPR) assay. Br. J. Pharmacol. *141*, 737–745.

34. Bandell, M., Dubin, A.E., Petrus, M.J., Orth, A., Mathur, J., Hwang, S.W., and Patapoutian, A. (2006). High-throughput random mutagenesis screen reveals TRPM8 residues specifically required for activation by menthol. Nat. Neurosci. *9*, 493–500.
35. Harper, A.A., and Lawson, S.N. (1985). Conduction velocity is related to morphological cell type in rat dorsal root ganglion neurones. J. Physiol. *359*, 31–46.
36. Weil, A., Moore, S.E., Waite, N.J., Randall, A., and Gunthorpe, M.J. (2005). Conservation of functional and pharmacological properties in the distantly related temperature sensors TRVP1 and TRPM8. Mol. Pharmacol. *68*, 518–527.
37. Chuang, H.H., Neuhausser, W.M., and Julius, D. (2004). The super-cooling agent icilin reveals a mechanism of coincidence detection by a temperature-sensitive TRP channel. Neuron *43*, 859–869.
38. Georgopoulos, A.P. (1976). Functional properties of primary afferent units probably related to pain mechanisms in primate glabrous skin. J. Neurophysiol. *39*, 71–83.
39. Thebault, S., Lemonnier, L., Bidaux, G., Flourakis, M., Bavencoffe, A., Gordienko, D., Roudbaraki, M., Delcourt, P., Panchin, Y., Shuba, Y., et al. (2005). Novel role of cold/menthol-sensitive transient receptor potential melastatine family member 8 (TRPM8) in the activation of store-operated channels in LNCaP human prostate cancer epithelial cells. J. Biol. Chem. *280*, 39423–39435.
40. Lai, J., Gold, M.S., Kim, C.S., Bian, D., Ossipov, M.H., Hunter, J.C., and Porreca, F. (2002). Inhibition of neuropathic pain by decreased expression of the tetrodotoxin-resistant sodium channel, NaV1.8. Pain *95*, 143–152.
41. Brauchi, S., Orio, P., and Latorre, R. (2004). Clues to understanding cold sensation: Thermodynamics and electrophysiological analysis of the cold receptor TRPM8. Proc. Natl. Acad. Sci. USA *101*, 15494–15499.
42. Lawson, S.N., and Waddell, P.J. (1991). Soma neurofilament immunoreactivity is related to cell size and fibre conduction velocity in rat primary sensory neurons. J. Physiol. *435*, 41–63.
43. Wallace, V.C., Cottrell, D.F., Brophy, P.J., and Fleetwood-Walker, S.M. (2003). Focal lysolecithin-induced demyelination of peripheral afferents results in neuropathic pain behavior that is attenuated by cannabinoids. J. Neurosci. *23*, 3221–3233.
44. Baccei, M.L., Bardoni, R., and Fitzgerald, M. (2003). Development of nociceptive synaptic inputs to the neonatal rat dorsal horn: Glutamate release by capsaicin and menthol. J. Physiol. *549*, 231–242.
45. Tsuzuki, K., Xing, H., Ling, J., and Gu, J.G. (2004). Menthol-induced Ca2+ release from presynaptic Ca2+ stores potentiates sensory synaptic transmission. J. Neurosci. *24*, 762–771.
46. Bautista, D.M., Movahed, P., Hinman, A., Axelsson, H.E., Sterner, O., Hogestatt, E.D., Julius, D., Jordt, S.E., and Zygmunt, P.M. (2005). Pungent products from garlic activate the sensory ion channel TRPA1. Proc. Natl. Acad. Sci. USA *102*, 12248–12252.
47. Macpherson, L.J., Geierstanger, B.H., Viswanath, V., Bandell, M., Eid, S.R., Hwang, S., and Patapoutian, A. (2005). The pungency of garlic: Activation of TRPA1 and TRPV1 in response to allicin. Curr. Biol. *15*, 929–934.
48. Gerber, G., Zhong, J., Youn, D., and Randic, M. (2000). Group II and group III metabotropic glutamate receptor agonists depress synaptic transmission in the rat spinal cord dorsal horn. Neuroscience *100*, 393–406.
49. Neugebauer, V., Chen, P.S., and Willis, W.D. (2000). Groups II and III metabotropic glutamate receptors differentially modulate brief and prolonged nociception in primate STT cells. J. Neurophysiol. *84*, 2998–3009.
50. Jia, H., Rustioni, A., and Valtschanoff, J.G. (1999). Metabotropic glutamate receptors in superficial laminae of the rat dorsal horn. J. Comp. Neurol. *410*, 627–642.
51. Li, H., Ohishi, H., Kinoshita, A., Shigemoto, R., Nomura, S., and Mizuno, N. (1997). Localization of a metabotropic glutamate receptor, mGluR7, in axon terminals of presumed nociceptive, primary afferent fibers in the superficial layers of the spinal dorsal horn: An electron microscope study in the rat. Neurosci. Lett. *223*, 153–156.
52. Liu, H., Wang, H., Sheng, M., Jan, L.Y., Jan, Y.N., and Basbaum, A.I. (1994). Evidence for presynaptic N-methyl-D-aspartate autoreceptors in the spinal cord dorsal horn. Proc. Natl. Acad. Sci. USA *91*, 8383–8387.
53. Wasner, G., Schattschneider, J., Binder, A., and Baron, R. (2004). Topical menthol–a human model for cold pain by activation and sensitization of C nociceptors. Brain *127*, 1159–1171.
54. Namer, B., Seifert, F., Handwerker, H.O., and Maihofner, C. (2005). TRPA1 and TRPM8 activation in humans: Effects of cinnamaldehyde and menthol. Neuroreport *16*, 955–959.
55. Iggo, A. (1969). Cutaneous thermoreceptors in primates and sub-primates. J. Physiol. *200*, 403–430.
56. Hensel, H., and Zotterman, Y. (1951). The response of the cold receptors to constant cooling. Acta Physiol. Scand. *22*, 96–105.
57. Schafer, K., Braun, H.A., and Isenberg, C. (1986). Effect of menthol on cold receptor activity. Analysis of receptor processes. J. Gen. Physiol. *88*, 757–776.
58. Kobayashi, K., Fukuoka, T., Obata, K., Yamanaka, H., Dai, Y., Tokunaga, A., and Noguchi, K. (2005). Distinct expression of TRPM8, TRPA1, and TRPV1 mRNAs in rat primary afferent neurons with Aδ/C-fibers and colocalization with Trk receptors. J. Comp. Neurol. *493*, 596–606.
59. Tominaga, M., Caterina, M.J., Malmberg, A.B., Rosen, T.A., Gilbert, H., Skinner, K., Raumann, B.E., Basbaum, A.I., and Julius, D. (1998). The cloned capsaicin receptor integrates multiple pain-producing stimuli. Neuron *21*, 531–543.
60. Okazawa, M., Inoue, W., Hori, A., Hosokawa, H., Matsumura, K., and Kobayashi, S. (2004). Noxious heat receptors present in cold-sensory cells in rats. Neurosci. Lett. *359*, 33–36.
61. Garry, E.M., Delaney, A., Blackburn-Munro, G., Dickinson, T., Moss, A., Nakalembe, I., Robertson, D.C., Rosie, R., Robberecht, P., Mitchell, R., et al. (2005). Activation of p38 and p42/44 MAP kinase in neuropathic pain: Involvement of VPAC(2) and NK(2) receptors and mediation by spinal glia. Mol. Cell. Neurosci. *30*, 523–537.
62. Soliman, A.C., Yu, J.S., and Coderre, T.J. (2005). mGlu and NMDA receptor contributions to capsaicin-induced thermal and mechanical hypersensitivity. Neuropharmacology *48*, 325–332.
63. Garry, E.M., Moss, A., Delaney, A., O'Neill, F., Blakemore, J., Bowen, J., Husi, H., Mitchell, R., Grant, S.G., and Fleetwood-Walker, S.M. (2003). Neuropathic sensitization of behavioral reflexes and spinal NMDA receptor/CaM kinase II interactions are disrupted in PSD-95 mutant mice. Curr. Biol. *13*, 321–328.
64. Iggo, A. (1958). The electrophysiological identification of single nerve fibres, with particular reference to the slowest-conducting vagal afferent fibres in the cat. J. Physiol. *142*, 110–126.