Responses of intact and injured sural nerve fibers to cooling and menthol

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Teliban A, Bartsch F, Struck M, Baron R, Jänig W. Responses of intact and injured sural nerve fibers to cooling and menthol. J Neurophysiol 111: 2071–2083, 2014. First published February 26, 2014; doi:10.1152/jn.00287.2013.—Intact and injured cutaneous C-fibers in the rat sural nerve are cold sensitive, heat sensitive, and/or mechanosensitive. Cold-sensitive fibers are either low-threshold type 1 cold sensitive or high-threshold type 2 cold sensitive. The hypothesis was tested, in intact and injured afferent nerve fibers, that low-threshold cold-sensitive afferent nerve fibers are activated by the transient receptor potential melastatin 8 (TRPM8) agonist menthol, whereas high-threshold cold-sensitive C-fibers and cold-insensitive afferent nerve fibers are menthol insensitive. In anesthetized rats, activity was recorded from afferent nerve fibers in strands isolated from the sural nerve, which was either intact or crushed 6–12 days before the experiment distal to the recording site. In all, 77 functionally identified afferent C-fibers (30 intact fibers, 47 injured fibers) and 34 functionally characterized A-fibers (11 intact fibers, 23 injured fibers) were tested for their responses to menthol applied to their receptive fields either in the skin (10 or 20%) or in the nerve (4 or 8 mM). Menthol activated all intact (n = 12) and 90% of injured (n = 20/22) type 1 cold-sensitive C-fibers; it activated no intact type 2 cold-sensitive C-fibers (n = 7) and 1/11 injured type 2 cold-sensitive C-fibers. Neither intact nor injured heat- and/or mechanosensitive cold-insensitive C-fibers (n = 25) and almost no A-fibers (n = 2/34) were activated by menthol. These results strongly argue that cutaneous type 1 cold-sensitive afferent fibers are nonnociceptive cold fibers that use the TRPM8 transduction channel.

cutaneous afferents; cold sensitivity; hairy skin; rat; nerve injury; menthol

THE SKIN OF THE RAT HINDLIMB is innervated by several functionally distinct types of afferent unmyelinated (C) nerve fibers. In rat sural nerve, they consist of three large groups (Jänig et al. 2009): 1) mechano- and/or heat-sensitive nerve fibers that are not cold sensitive, most of them probably being nociceptive (~60%); 2) nonnociceptive low-threshold cold-sensitive C-fibers (~20%; called type 1 cold-sensitive fibers); type 1 cold-sensitive fibers are mechanosensitive, and a few of them are also activated by heat stimuli (see also Kress et al. 1992; Leem et al. 1993); and 3) nociceptive high-threshold cold-sensitive C-fibers, most of them also being heat and/or mechanosensitive (~20%; called type 2 cold-sensitive C-fibers). Regenerating and regenerated cutaneous afferent C-fibers of the injured rat sural nerve up to 15 mo after injury consist of these three functional groups, occurring at similar frequencies. Thus the functional characteristics as described by their responsiveness to thermal or mechanical stimuli are preserved in injured cutaneous nerve fibers that can be activated only from the nerve, and not from the skin (Gorodetskaya et al. 2009; Grossmann et al. 2009a, 2009b; Jänig et al. 2009). However, there are quantitative differences between intact and injured afferent nerve fibers in the sense that the responses to the physiological stimuli are smaller and that the frequencies of type 2 cold-sensitive and cold-insensitive C-fibers with (ectopically generated) ongoing activity are significantly higher in injured C-fibers than in noninjured C-fibers (Gorodetskaya et al. 2009; Jänig et al. 2009; Jänig and Kirillova 2013).

Low-threshold cold sensitivity in cutaneous afferent neurons is suggested to be mediated by the TRP8 receptor potential melastatin 8 (TRPM8) agonist, whereas high-threshold cold-sensitive C-fibers and cold-insensitive afferent nerve fibers are menthol insensitive. In anesthetized rats, activity was recorded from afferent nerve fibers in strands isolated from the sural nerve, which was either intact or crushed 6–12 days before the experiment distal to the recording site. In all, 77 functionally identified afferent C-fibers (30 intact fibers, 47 injured fibers) and 34 functionally characterized A-fibers (11 intact fibers, 23 injured fibers) were tested for their responses to menthol applied to their receptive fields either in the skin (10 or 20%) or in the nerve (4 or 8 mM). Menthol activated all intact (n = 12) and 90% of injured (n = 20/22) type 1 cold-sensitive C-fibers; it activated no intact type 2 cold-sensitive C-fibers (n = 7) and 1/11 injured type 2 cold-sensitive C-fibers. Neither intact nor injured heat- and/or mechanosensitive cold-insensitive C-fibers (n = 25) and almost no A-fibers (n = 2/34) were activated by menthol. These results strongly argue that cutaneous type 1 cold-sensitive afferent fibers are nonnociceptive cold fibers that use the TRPM8 transduction channel.

cutaneous afferents; cold sensitivity; hairy skin; rat; nerve injury; menthol

METHODS

Anesthesia and Animal Maintenance

Thirty-three male Wistar rats (body weight 300–560 g) were used for this study. In 10 rats, the sural nerve was intact, and in 23 rats, the sural nerve was crushed 6–12 days before the experiments, about 22–25 mm proximal to the heel as described by Grossman et al. (2009a, 2009b). During the experiments, anesthesia and maintenance of the rats were the same as described previously (Gorodetskaya et al. 2003; Grossmann et al. 2009a, 2009b; Teliban et al. 2011). In brief, the rats were anesthetized with pentobarbital sodium (50 mg/kg ip initially; 10 mg/kg iv per hour during surgery and the experiments). During the experiments, the rats were ventilated through a cannula inserted into the trachea and immobilized by pancuronium injected through a catheter in the right jugular vein (pancuronium bromide, Pancuronium Organon; initial dose 2.5 mg/kg, maintenance 2
Mean arterial blood pressure measured via a catheter in the tail artery was always ≥80 mmHg. Acid-base status was regularly determined and was in the range of pH 7.4, PCO2 35–40 mmHg, and PO2 ≥100 mmHg. At the end of the experiments, the animals were killed under deep anesthesia by an intravenous injection of a saturated potassium chloride solution. All experiments were approved by the local animal care committee of the state administration and were conducted in accordance with German Federal Law.

Recording and Electrical Stimulation

The left sural nerve was exposed from the ankle to its junction with the sciatic nerve. The sciatic nerve containing the sural nerve was isolated from the surrounding tissue for about 10–15 mm at its proximal end. A pool was formed from the skin flaps and filled with warm (30°C) paraffin oil. The most proximal site of the sciatic nerve was placed on a rigidly fixed small black Perspex platform.

Fine strands were teased out from the most proximal site of the sciatic nerve and put on a platinum electrode for recording; the indifferent electrode was connected to the nearby tissue. The signals of theafferent nerve fibers in the nerve strands were differentially amplified, filtered (120 Hz to 1-1.2 kHz for unmyelinated fibers; 120 Hz to 12 kHz for identifying myelinated fibers), and fed through a window discriminator. The sural nerve was positioned on a pair of stimulation electrodes 19–33 mm distal (26 ± 0.7 mm) to the recording electrode but about 14–15 mm proximal to the crush site and was electrically stimulated with square-wave pulses of 0.1 (A-fibers) or 0.5 ms in duration (C-fibers) at strengths of up to 40 V. All fibers were identified electrically on the basis of their conduction velocities as A- or C-fibers (>2 m/s and ≥2 m/s, respectively; Lawson and Waddell 1991; Waddell et al. 1989).

Physiological Stimulation of Skin or Nerve

Mechanical stimuli. Mechanosensitivity of intact and injured sural nerve fibers was tested with a fine-tipped blunt glass rod applied either to the skin surface or to the nerve at or distal to the lesion site. The surface area of contact was in the range of 1 mm². In this study we did not measure the strength of the mechanical stimuli applied to the nerve injury site (or the skin), but we did so in previous studies (Grossmann et al. 2009b; Jänig et al. 2009). Strong (noxious) mechanical stimuli were applied to the skin with the use of a forceps (pinch stimuli) or a fine-tipped glass rod (strong pressure stimuli).

Thermal stimuli. Thermal sensitivity of the intact nerve fibers was tested using ramp stimuli generated by a custom-built Peltier thermode attached to the skin. The maximal area of contact of the Peltier thermode was 0.72 cm² (0.8 × 0.9 cm) and therefore larger than the size of the receptive fields of the afferent fibers. The temperature was changed at a rate of 1°C/s, starting from an adaptation temperature of about 30°C. The maximal plateau stimuli used were 0 and about 55°C for about 30- and 15-s duration, respectively.

Thermal sensitivity of the injured nerve fibers was tested using a water-perfused thermode that was positioned on the nerve at or distal to the injury site according to the receptive field of the afferent fiber. The length of the contact site with the nerve was 3 mm. The temperature was measured at this contact site (see Gorodetskaya et al. 2003). The nerve fibers were stimulated either with a cold stimulus of about 5°C or with a heat stimulus of about 50°C. Graded cold and heat stimuli were used to study the stimulus-response functions of the afferent neurons before and after application of menthol. All thermal stimuli lasted for ~30 s, starting from a baseline temperature of 30°C.

Application of Menthol to Skin or Nerve

Small pieces of cotton wool soaked in menthol [10 or 20% (wt/vol) in 96% ethanol] were applied to the surface of the skin for 5 min. These concentrations of menthol correspond to 0.640 and 1.280 molar solutions. In eight experiments, ethanol was applied to the skin as vehicle. To protect the solution of menthol from evaporation (a radiant lamp was used to heat the skin for suppression of ongoing activity of the cold fibers), the cotton wool was covered by a piece of parafilm.

Small pieces of filter paper soaked in menthol [dissolved in ethanol (0.2 or 0.4% in Tyrode solution)] in concentrations of 4 or 8 mM (corresponding to solutions of 0.0625 and 0.125%, respectively) were applied to the nerve. In eight experiments, 0.2 or 0.4% ethanol was applied as control for menthol.

The pieces of cotton wool and filter paper were in the range of 48–60 mm² and about 1 mm thick. This size guaranteed that the concentration of menthol on the skin surface or nerve surface was the same as in the piece of cotton wool or filter paper. It is of course impossible to estimate the concentration of menthol in the skin or crush injury site where the terminals of the nerve fibers are located. The time interval between two applications of menthol was about 120 min or longer to avoid tachyphylaxis.

Data Analysis

Neural activity, temperature of the Peltier thermode, temperature of the thermode for stimulation of the sural nerve, arterial blood pressure, electrocardiogram, and endotracheal pressure were simultaneously fed into a computer using the Cambridge Electronic Design (CED) data acquisition system. Data analysis was performed off-line using the general purpose capture and analysis Spike II System (CED, Cambridge, UK). Data are means ± SE. For statistical analysis, the χ² test, Student’s t-test, Spearman correlation test, ANOVA, or nonparametric Wilcoxon signed-rank test was used.

RESULTS

Responses of Intact Functionally Identified C-Fibers to Menthol Applied to Their Receptive Field

Type 1 cold-sensitive C-fibers. Twelve intact C-fibers with type 1 cold sensitivity were investigated. At a constant skin temperature of 30°C, these fibers had an ongoing discharge of 6.5 ± 0.7 impulses/s (n = 12), which was increased by cooling of the skin and showed both dynamic and static responses (Figs. 1A and 3B). The dynamic responses were graded with decreasing temperature and had a maximal mean discharge rate of 22.2 ± 4 impulses/s at 10–25°C when tested with ramp-shaped stimuli. The discharge rate during static activation (in the last 10 s of the stimulus plateau) was also graded with temperature and had a maximal response of 16 ± 1.9 impulses/s at about 5°C (not shown). In all type 1 cold-sensitive C-afferents, warming the skin above 30°C inhibited ongoing activity (Figs. 1B and 3C). In one C-fiber, heating-induced inhibition of activity was followed by mild heating-induced activation. In 11 C-fibers, rewar ming to 30°C after the cold stimulus resulted in a transient depression of the activity that lasted up to 1 min (Figs. 1A and 3B), and in 10 C-fibers, the warming-induced inhibition was followed by a rebound excitation when the receptive field was recooled to 30°C (Figs. 1B and 3C). The threshold for activation by cooling (increase of frequency by about 20% above baseline during the ramp-shaped stimuli at 1°C/s) was 28.5 ± 0.4°C, starting from an adaptation temperature of 30°C. The high cold sensitivity of these C-fibers is also demonstrated by the observation that noncontact cold and heat stimuli applied with a metal rod of 5 or 50°C positioned 2–3 mm above their receptive fields activated or inhibited most of the fibers (Fig. 1, D and E). Activation by acetone applied to the skin was observed in 12
C-fibers tested (Fig. 1C). All type 1 cold-sensitive C-fibers were mechanoinsensitive. Type 1 cold-sensitive fibers exhibited no bursting discharges during cooling (see interval histogram in Fig. 2A).

All 12 type 1 cold-sensitive C-fibers tested were activated by menthol (10 or 20% in 96% ethanol) applied to their receptive fields in the skin. Figure 2 illustrates a typical example. To visualize the activating and sensitizing effect of menthol on these fibers, the ongoing activity was suppressed by increasing the temperature of the skin surface to 33–34°C with radiant heat before and during application of menthol. The response to the menthol-ethanol solution consisted of an immediate transient activation and a delayed slow increase of activity (Fig. 2E). Application of the vehicle to skin (96% ethanol) generated only the immediate transient but not the delayed response (Fig. 2C). The immediate response was induced by transient cooling of the skin generated by temporary interruption of radiant heat stimulation during application of the menthol-ethanol- or ethanol-soaked cotton wool swab to the cutaneous receptive field of the afferent fiber. The ongoing activity before test period 1 (TP1) and after TP2 was measured at a skin temperature of 30°C generated by the Peltier thermode. A fiber was activated by menthol when its activity increased after application of menthol and remained increased throughout the application of menthol. The latency of the response was measured after three evoked action potentials. The increase of activity in presence of menthol was significantly larger in the second minute compared with either the activity immediately before application of menthol ($P < 0.001$, paired $t$-test) or the activity during application of vehicle (2–5 min, $P < 0.001$, ANOVA). The latency of the response was 12–194 s ($58.0 ± 16.5$ s; $n = 11$). The activity was still significantly increased 2 min after removal of menthol ($P < 0.01$, paired $t$-test) and after TP2 (about 20–30 min after application of menthol; $P < 0.05$, paired $t$-test) compared with the ongoing activity before TP1. Bursting discharges during application of menthol were not observed in the type 1 cold-sensitive afferent neurons (see interval histogram in Fig. 2E).

Figure 3, B and C, demonstrates the population response of the type 1 cold-sensitive C-fibers to cold and heat stimuli measured before (in TP1 in A) and about 10–20 min after (in TP2 in A) application of menthol to the skin. The threshold of activation by cooling after menthol was $28.0 ± 0.4^\circ C$, starting from an adaptation temperature of $30^\circ C$, and was not significantly different from the threshold before application of menthol. The response profile did not change qualitatively. However, after application of menthol, the peak response appeared later and activation was stronger during the plateau phase of the cold stimulus ($P < 0.01$, paired $t$-test; Fig. 3B). When the receptive field was recooled after a heat stimulus, after appli-
cation of menthol, the fibers started to discharge at higher temperatures and in some cases even during the plateau phase of the heat stimulus (Fig. 3C). This illustrates the menthol-induced sensitization to thermal stimulation in these fibers.

**Type 2 Cold-Sensitive and Cold-Insensitive Afferent Fibers**

Seven (high threshold) type 2 cold-sensitive C-fibers (Figs. 4 and 5), 7 C-fibers that were only either heat sensitive or heat and mechanosensitive, and 4 C-fibers that were only mechanosensitive were tested for their responses to menthol (20%) applied to their cutaneous receptive fields for 5 min (Table 1, C-fibers). The type 2 cold-sensitive C-fibers were not activated by menthol. The cold activation threshold of these cold-sensitive C-fibers was 12.0 ± 2.2°C before and 11.6 ± 2.6°C after application of menthol (10%). The total activity during a cold stimulus of 5°C and 55-s duration (ramp plus plateau; see Fig. 4) was 60 ± 21 impulses before and 44 ± 14 impulses after application of menthol (Figs. 4 and 5). Five type 2 cold-sensitive afferent C-fibers tested with 20% menthol applied to their receptive fields were not activated by this dose of menthol, and the latency and magnitude of their responses to cold stimuli did not change. The 11 cold-insensitive afferent C-fibers were not activated, and their responses to mechanical or heat stimuli were not changed, by menthol (Table 1).

**Responses of Injured Functionally Identified Afferent C-Fibers to Menthol Applied to the Injury Site of the Nerve**

Injured type 1 cold-sensitive C-fibers. Twenty-two regenerating type 1 cold-sensitive C-fibers were studied for their responses to menthol applied to the site of their receptive fields in the sural nerve at or distal to the injury site. These injured C-fibers had ongoing activity at 30°C (1.9 ± 0.4 impulses/s, n = 19) and showed typical responses to cold or heat stimuli applied to the nerve as reported recently (Grossmann et al. 2009a, 2009b; Jänig et al. 2009). Their activity was increased by cooling and depressed by heating (Fig. 6, A and B). Five injured type 1 cold-sensitive C-fibers were also activated by heat stimuli after an initial inhibition of their activity. This phenomenon was more common in the population of injured fibers than in the population of intact fibers (23 vs. 8%; see Discussion and Table 2). The threshold for activation by cold stimuli was 22.7 ± 0.6°C, starting from a baseline temperature...
of 30°C \( (n = 21) \). This activation threshold was significantly lower than the activation threshold of the intact type 1 cold-sensitive fibers \( (28.4 \pm 0.6°C; \ P < 0.001, t\text{-test}) \). The maximal mean activity during a cold stimulus of 3–5°C was 8.3 \( \pm 1.3 \) impulses/s. All injured type 1 cold-sensitive C-fibers were mechanoinsensitive.

Twenty of the 22 injured type 1 cold-sensitive C-fibers were activated by menthol \( (19 \text{ fibers to } 4 \text{ mM menthol, 1 fiber to } 8 \text{ mM menthol}) \) applied to their receptive fields in the sural nerve \( (\text{Fig. 6C}) \). The two type 1 cold-sensitive fibers not activated by 4 mM menthol were not tested for their responses to 8 mM menthol. A fiber was activated by menthol when its activity increased after application of menthol and remained increased throughout the application of menthol. 

The ongoing activity during a cold stimulus of 3–5°C was significantly lower than the activation threshold of the intact type 1 cold-sensitive fibers \( (28.4 \pm 0.6°C; \ P < 0.001, t\text{-test}) \). The maximal mean activity during a cold stimulus of 3–5°C was 8.3 \( \pm 1.3 \) impulses/s. All injured type 1 cold-sensitive C-fibers were mechanoinsensitive.

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Figure 7, B–D, demonstrates the population responses of the injured type 1 cold-sensitive C-fibers to cold and heat stimuli before and after application of menthol. The total responses were quantitatively the same before and after application of menthol to the sural nerve. The total responses were measured in 11 C-fibers before, during, and after application of menthol (TP1 and TP2 application). The total responses were measured in 11 C-fibers before, during, and after application of menthol (TP1 and TP2 application). The total responses were measured in 11 C-fibers before, during, and after application of menthol (TP1 and TP2 application). The total responses were measured in 11 C-fibers before, during, and after application of menthol (TP1 and TP2 application).

The response profiles did not change qualitatively. However, activation during nerve cooling was slightly (but not significantly) enhanced and the rebound activation after heating was somewhat delayed (shown in Fig. 7D for type 1 cold-sensitive C-fibers that were only depressed by heat stimuli).

Figure 8 illustrates the relation between the ongoing activity at 30°C \( (\text{abscissa scale}) \) and the maximal responses to menthol of the intact type 1 cold-sensitive cutaneous small afferent fibers \( (10\% \text{ menthol to skin}) \) and the injured type 1 cold-sensitive cutaneous small afferent fibers \( (4 \text{ mM menthol to nerve}) \). The response to menthol was measured over 1 min, usually about 3 min after the start of menthol application. The two parameters
The cold ramp stimuli were delivered at a rate of 1°C/s, starting from a preset temperature of 30°C and decreasing to 5°C. The duration of the static cold stimulus was 15 s. Inset: action potentials of the C-fibers several times superimposed.

are significantly correlated with each other (r = 0.69, P < 0.001, Spearman’s rank correlation). Furthermore, both are significantly lower in injured than in intact C- afferents (maximal response to menthol: 11.7 ± 1.5 vs. 3.1 ± 0.5 impulses/s, P < 0.000; ongoing activity: 6.5 ± 0.8 vs. 1.6 ± 0.5 impulses/s, P < 0.000; Wilcoxon signed-rank test; compare large open circle with large filled circle in Fig. 8).

Injured type 2 cold-sensitive C-fibers and injured cold-insensitive afferent fibers. Eleven injured type 2 cold-sensitive C-fibers and 14 injured cold-insensitive C-fibers that were heat sensitive or heat and mechanosensitive were tested for their responses to menthol (8 mM) applied to their receptive fields at the nerve injury site for 5 min. Only one of the type 2 cold-sensitive C-fibers was activated by menthol. The activation threshold of the type 2 cold-sensitive C-fibers was 13.6 ± 1.2°C (n = 11) before and 11.8 ± 2.1°C (n = 8) after application of menthol. The total activity during a cold stimulus of 5°C and 20-s duration was 24 ± 5 impulses before and 21 ± 5 impulses after application of menthol. The 14 cold-insensitive afferent C-fibers were not activated, and their responses to mechanical or heat stimuli were not changed, by menthol (Table 1).

**Responses of Afferent A-Fibers to Menthol**

Eleven intact and 23 injured functionally identified A-fibers were tested for their responses to menthol. We used the same criteria to classify the A-fibers as for the C-fibers. Twenty-six fibers were mechanosensitive, 7 were heat and mechanosensitive, and one was type 2 cold sensitive (Table 1). Two injured A-fibers were activated by menthol; one was a type 2 cold-sensitive afferent A-fiber and the other a heat-sensitive afferent fiber. The remaining A-fibers were not activated or influenced in their responsiveness to mechanical or heat stimuli by menthol (Table 1).

**DISCUSSION**

In this study we have shown for the first time that almost all innocuous cold-sensitive intact or injured afferent nerve fibers of the rat hindlimb skin, which have been functionally characterized on the basis of their responses to physiological mechanical or thermal stimuli, were activated by menthol, whereas almost all other functional types of afferent fibers, including high-threshold cold-sensitive C-fibers, were insensitive to menthol. Only 2/34 nonnociceptive (type 1) cold-sensitive afferent fibers, all of them unmyelinated, were not activated by menthol, whereas only 1 of 43 afferent C-fibers, which were type 2 cold sensitive or cold insensitive, was excited by menthol. Two of 34 A-fibers tested were excited by menthol; one was a type 2 cold-sensitive fiber and one a heat-sensitive fiber per our physiological criteria. The five afferent fibers showing “abnormal” responses to menthol (i.e., either absence of activation in type 1 cold-sensitive afferents or activation by menthol of type 2 cold-sensitive afferents or cold-insensitive afferents) were injured afferents (Table 1).

The concentrations of menthol applied to the receptive fields in the skin (10–20% in ethanol) or to the injury site of the nerve (4 and/or 8 mM) were high. Lower concentrations of menthol (5% applied to skin; 100 μM or 1 mM applied to the nerve injury site) either did not activate type 1 cold-sensitive afferents or produced weak responses. The highest concentrations of menthol we used (20%, 8 mM) were very unlikely to

**Table 1. Numbers of functional types of afferent neurons activated or not activated by menthol**

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<th>Response to Menthol</th>
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<td>Activation</td>
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<td>Intact</td>
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<tr>
<td>C-fibers</td>
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<tr>
<td>Type 1 cold sensitive</td>
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<tr>
<td>Type 2 cold sensitive</td>
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<td>Heat and/or</td>
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Values are numbers of functional types of intact or injured afferent C-fibers or A-fibers either activated or not affected by menthol. All intact afferent fibers were tested with 10–20% menthol (in ethanol). All injured type 1 cold-sensitive C-fibers were tested with 4 mM menthol. All injured type 2 cold-sensitive and cold-insensitive afferent fibers were tested with 8 mM menthol. The two injured type 1 cold-sensitive C-fibers not activated by menthol (*) were only tested with 4 mM menthol.
Responses of intact type 1 cold-sensitive afferent fibers to cold stimuli were enhanced 10–20 min after application of menthol (Fig. 5). We cannot exclude the possibility that this enhancement would probably have been larger if tested directly under application of menthol. This enhancement may show this behavior to menthol: activation at a low concentration of menthol and inhibition of ongoing activity at higher concentrations (Kirillova-Woytke I, Baron R, and Jänig W, unpublished observation). It is debated whether the TRPA1 channel is specifically responsible for the transduction of noxious cold stimuli (Bautista et al. 2006; Belmonte and Viana 2008; Belmonte et al. 2009; Kwan et al. 2006; Reid 2005; Zurborg et al. 2007). In fact, many afferent cold-sensitive neurons do not seem to express either the TRPM8 or the TRPA1 protein, and it is suggested that other channels, in particular potassium channels, may be involved in the responses of afferent neurons to cold stimuli (Babes 2009; Munns et al. 2007).

Neither cooling nor menthol applied to the receptive fields of intact or injured type 1 cold-sensitive C-fibers produced, with one exception, bursting discharges as reported for innocuous cold-sensitive fibers innervating hairy or hairless skin of the limbs or face in primates (Darian-Smith et al. 1973; Dubner et al. 1975; Iggo 1969; Kenshalo and Duclaux 1977) or for the trigeminal innocuous cold-sensitive afferent neurons in rodents, guinea pigs, bats, cats, and avians (Carr et al. 2003; Parra et al. 2010; Schäfer et al. 1986, 1988). However, as far as we know, bursting patterns have not been reported in the discharge of cold-sensitive fibers innervating hairy or hairless skin of limbs in rodents (Kress et al. 1992; Leem et al. 1993).

In vitro studies on isolated dorsal root or trigeminal ganglion cells have shown that menthol activates neurons expressing the TRPM8 channel, which is specifically activated by small temperature decreases (Babes et al. 2004; Belmonte et al. 2009; Dhaka et al. 2008; Madrid et al. 2006, 2009; Peier et al. 2002; Story et al. 2003; Viana et al. 2002; Xing et al. 2006). This corresponds to in vivo studies on low-threshold cold-sensitive primary afferent neurons innervating the cat cornea (Gallar et al. 1993), the cat tongue (Hensel and Zotterman 1951), the guinea pig nasal mucosa ( Sekizawa et al. 1996), or the dog laryngeal mucosa (Sant’Ambrogio et al. 1991) and to ex vivo studies of murine mechanosensitive cold receptors in the skin of limbs in rodents (Kress et al. 1992; Leem et al. 1993).
saphenous nerve preparation (Zimmermann et al. 2011) showing that the TRPM8 agonist menthol activates these afferent neurons. Our results on intact and injured type 1 cold-sensitive afferent fibers (Table 1), practically all of them being activated by menthol, fully agree with these in vitro and in vivo studies.

In the rat and mouse, 5–12% of the cells in the dorsal root ganglia (Babes et al. 2004; Dhaka et al. 2008; Peier et al. 2002; Reid et al. 2002; Xing et al. 2006, 2007) or the trigeminal ganglion (Abe et al. 2005; Dhaka et al. 2008; Thut et al. 2003; Viana et al. 2002) are cold sensitive, menthol sensitive, and/or express TRPM8. The frequency of type 1 cold-sensitive afferent C-fibers in the population of intact or injured sural nerve C-fibers is about 20% (see Gorodetskaya et al. 2009; Grossmann et al. 2009a; Jänig et al. 2009). This percentage is fully in agreement with the percentages obtained in in vitro studies if one takes into account that many dorsal root ganglion cells innervate deep somatic tissues and some visceral tissues.

Table 2. Mechanosensitivity and heat sensitivity of cold-sensitive C-fibers

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<th>Type 1 Cold Sensitive</th>
<th>Type 2 Cold Sensitive</th>
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<tr>
<td></td>
<td>(Menthol Sensitive)</td>
<td>(Menthol Insensitive)</td>
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Values are frequencies of mechano- and heat sensitivity of intact and injured type 1 (menthol sensitive) and type 2 (menthol insensitive) cold-sensitive C-fibers; \( n \) = no. of fibers. Heat sensitivity was tested to stimuli of up to 52°C. Data are from the present study and as reported by Gorodetskaya et al. (2009), Jänig et al. (2009), and Teliban et al. (2011). \( *P < 0.000 \), comparison of type 2 vs. type 1 injured or intact cold-sensitive neurons; \( \dagger P < 0.000 \), comparison of injured vs. intact type 1 cold-sensitive neurons; \( \ddagger P < 0.000 \), comparison of injured vs. intact type 2 cold-sensitive neurons; \( \dagger \dagger P = 0.067 \), comparison of injured vs. intact type 2 cold-sensitive neurons (\( \chi^2 \) test, Fisher’s correction).

Fig. 6. Responses of a single injured type 1 cold-sensitive C-fiber to menthol (4 mM). Response to a cold (about 5°C; A and C) or heat stimulus (about 50°C; B and D) applied by a water-perfused thermode to the receptive field in the nerve before (A and B) and after (C and D) application of menthol to the receptive field in the nerve. The temperature stimuli started from a preset temperature of about 30°C. E: response to menthol applied to the nerve. Application of ethanol (0.2%) alone had no effect. Insets: interval histograms of the activity during cooling (A, \( n = 92 \) intervals; C, \( n = 98 \) intervals) show only one peak, indicating the absence of bursting (time resolution, 20 ms).

Table 2. Mechanosensitivity and heat sensitivity of cold-sensitive C-fibers

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Frequencies of Mechanosensitivity and Thermosensitivity in Afferent Neurons

Table 2 shows the percentages of type 1 (low threshold and menthol sensitive) cold-sensitive and type 2 (high threshold and menthol insensitive) cold-sensitive afferent neurons projecting in the sural nerve that are heat sensitive or mechanosensitive from our present study and three recent studies.
conducted in our laboratory (Gorodetskaya et al. 2009; Jänig et al. 2009; Teliban et al. 2011). The afferent fibers were divided into intact (noninjured) afferents and injured afferents (up to 15 mo after sural nerve crush). The injury was a crush lesion of the sural nerve, allowing the nerve fibers to regenerate. The comparison shows that mechanosensitivity and heat sensitivity have a significantly lower representation in the populations of intact or injured type 1 cold-sensitive afferent neurons than in the populations of intact or injured type 2 cold-sensitive afferent neurons (\(P < 0.000\), \(\chi^2\) test). The results show furthermore that heat sensitivity has a higher representation in injured than in intact cold-sensitive afferent neurons, although this did not reach significance in the population of type 2 cold-sensitive afferents (\(P < 0.05\) for type 1 cold-sensitive afferents, \(P = 0.67\) for type 2 cold-sensitive afferents).

The absence of mechanosensitivity in the population of intact type 1 cold-sensitive afferents corresponds to what has been described in the literature for trigeminal or spinal cutaneous nonnociceptive afferents in different species (Cain et al. 2001; Fleischer et al. 1983; Gallar et al. 1993; Kress et al. 1992; Leem et al. 1993; Shea and Perl 1985; for discussion see Hensel 1981; Spray 1986), although mechanosensitivity of nonnociceptive cold-sensitive afferent fibers has not been systematically studied to date. The only study in which the sensitivity of injured cutaneous afferents to menthol and to mechanical stimulation was tested, to our knowledge, was conducted by Roza et al. (2006). These authors investigated afferent fibers ending in a 21-day neuroma of the saphenous nerve of mice in vitro. Nine of 17 cold-sensitive afferent fibers were activated by menthol or showed changes in their threshold to cold stimuli. The authors did not discriminate between high- and low-threshold cold-sensitive afferents. Furthermore, more than half of the menthol-sensitive fibers were mechanosensitive, and almost all menthol-insensitive but cold-sensitive afferents were mechanoinsensitive. Heat sensitivity was not tested in this study. These results obtained on injured afferent fibers by Roza et al. (2006) are at variance with our results. This difference may be related to difference in preparation (in vitro, in vivo) and/or difference in species (mouse, rat).

The frequency of heat sensitivity of our menthol-sensitive intact C-fibers is comparable to the measurements reported by Dhaka et al. (2008), Peier et al. (2002), and Story et al. (2003),...
showing that menthol-sensitive and/or low threshold cold-sensitive dorsal root or trigeminal ganglion cells are either insensitive to capsaicin or do not express TRPV1, except that some 12% of the cells expressing TRPM8 also express TRPV1. These results also agree with Kobayashi et al. (2005), who showed that <2% dorsal root ganglion cells expressing TRPM8 coexpress TRPV1. All other in vitro studies reporting the capsaicin sensitivity of cold- and menthol-sensitive spinal or trigeminal afferent neurons (Babes et al. 2004; Hjerling-Leffler et al. 2007, Okazawa et al. 2004; Reid et al. 2002; Viana et al. 2002; Xing et al. 2006, 2007) or the coexpression of TRPM8 and TRPV1 (McKemy et al. 2002; Okazawa et al. 2004; Takashima et al. 2007) have shown a significantly higher frequency of cold-/menthol-sensitive afferent neurons activated by capsaicin or a significantly higher frequency of neurons coexpressing TRPM8 and TRPV1. Dhaka et al. (2006, 2008) argued that the high percentage of dorsal root and trigeminal ganglion cells activated in vitro by small cooling steps and/or menthol as well as by heat and/or capsaicin may be related to changes of the expression of the TRPV1 channel after dissociation. Indeed, our injured cutaneous type 1 cold-sensitive C-fibers show a significantly higher incidence of heat sensitivity than the intact type 1 cold-sensitive C-fibers innervating skin (Table 2; Jänig et al. 2009; Teliban et al. 2011), which may be related to regulatory changes during regeneration resulting in differential axonal expression of TRP-channels, and probably under the influence of growth factors. In this respect, the high frequency of neurons coexpressing TRPM8 and TRPV1 may relate to particular culture conditions (see Dhaka et al. 2006). However, there may be species differences between mouse and rat (Reid 2005). Finally, not all low-

threshold cold-sensitive and menthol-sensitive dorsal root ganglion cells may innervate skin. Recently, we showed that 40–60% of injured afferent A- and C-fibers innervating skeletal muscle develop cold sensitivity (Kirillova et al. 2011). Most of these cold-sensitive afferent fibers are heat sensitive and mechanosensitive and can also be activated by menthol (Kirillova-Woytte I, Baron R, and Jänig W, unpublished observation).

**Menthol Sensitivity and Nociception**

Separation of our menthol-sensitive afferents into nonnociceptive and potentially nociceptive cold-sensitive afferent C-fibers, based on their responses to heat stimuli or their response thresholds to cold stimuli (see Belmonte et al. 2009; Madrid et al. 2009; Takashima et al. 2007), does not appear to be justified. The intact menthol-sensitive afferents are low-threshold (type 1) cold sensitive with no mechanosensitivity and very little heat sensitivity. In fact, only 7 of 61 intact type 1 cold-sensitive afferent fibers of the present study and two recent studies (Jänig et al. 2009; Teliban et al. 2011) were heat sensitive (i.e., responsive to heat stimuli of 50–52°C; Table 2). Activation of these cold-sensitive afferent neurons by heat could be responsible for triggering the paradoxical cold sensation in humans (Dodt and Zottermann 1952; Thunberg 1901; von Frey 1895). Our results obtained on injured type 1 cold-sensitive C-fibers are seemingly at variance with several recent studies. These studies show that almost 50% of acutely plated menthol-sensitive dorsal root ganglion cells are capsaicin sensitive (Xing et al. 2006), that the percentage of menthol-sensitive dorsal root ganglion cells increases from 7 to 15% in the chronic constriction injury model of the sciatic nerve (Xing et al. 2007), that 90% of cold-sensitive corneal afferents are excited by heating (Hirata and Meng 2010), that cold hyper-sensitivity to mild cold stimulation in mice with chronic constriction injury of the sciatic nerve is blocked by a specific TRPM8 ion channel blocker (Knowlton et al. 2011), and that cold allodynic behavior generated by the TRPM8 agonist icilin is increased in mice treated by the chemotherapy drug oxaliplatin (Gauchan et al. 2009). At present we cannot explain these discrepant results if we do not assume species differences, differences in classification of neurons, or differences in data collection. We have shown for these afferents that heat sensitivity of noninjured type 1 cold-sensitive afferent fibers is rare, that the incidence of this heat sensitivity increases moderately after nerve injury (Table 2), that the percentage of type 1 cold-sensitive afferents does not increase after nerve injury, and that the responsiveness to innocuous cold stimuli decreases in injured afferents (ongoing activity, maximal responses to cooling; Fig. 6). However, we emphasize that 1) we recorded from the primary afferent neurons under conditions similar to the in vivo conditions in nonanesthetized animals or humans, and 2) cold-allodynic behavior may well be dependent on changes in the processing of activity of the innocuous cold-sensitive afferents by the second-order neurons in the spinal or trigeminal dorsal horn. Zanotto et al. (2007) have shown in rats that menthol applied to the tongue activates cold-sensitive second-order wide dynamic range neurons in the trigeminal subnucleus caudalis, enhances their responses to noxious and innocuous cooling of the tongue, and inhibits their responses to noxious heating (but only at a concentration of 40%). The same...
group has also studied the modulation of heat- and cold-evoked responses of lumbar wide dynamic range neurons to menthol applied to the plantar hindpaw in rats. Menthol increases the heat threshold at all concentrations, enhances the cold response at low concentration of 1–10%, and depresses the cold responses at a high concentration (Klein et al. 2012).

**Afferent Menthol Sensitivity in Humans**

How do our results compare with those obtained on human beings? Cutaneous (nonpainful) cold sensations in primates and humans are primarily generated by activation of cutaneous Aδ-fibers (Campero et al. 2009; Darian-Smith et al. 1973; Dubner et al. 1975; Hensel and Boman 1960; Iggo 1969; Kenshalo and Duclaux 1977; Long 1977; see Hensel 1981, 1982; Spray 1986 for review). The functional characteristics of our type 1 cold-sensitive afferents compare closely to those of cutaneous cold-sensitive Aδ-fibers in primates. Campero et al. (2001, 2009) described a population of C-fibers innervating hairy skin of the human lower limb that are low-threshold cold sensitive, menthol sensitive, and mechanoinensitive. The physiological response profile of these cutaneous C-fibers in humans also comes close to the response profile of our type 1 cold-sensitive C-fibers. However, most of these cold-sensitive fibers in humans can be activated by heat stimuli. Campero et al. (2009) argued that these cold-sensitive C-fibers play little role in cold sensation but are instead involved in cold discom- fort (together with the polymodal cold-sensitive C-fibers) and burning hot and heat pain (together with the polymodal cold-sensitive C-fibers). The type 2 cold-sensitive C-fibers in our study compare to the cutaneous C-polymodal nociceptors in humans, which are activated by noxious low temperatures of 10 to 0°C (Campero et al. 1996, 2009). Both show rather low discharge rates to these cold stimuli and are menthol insensitive.

Recent studies on healthy human subjects show that menthol applied to hairy skin elicits ongoing pain and punctate hyperalgesia and enhances cold pain (Hatem et al. 2006; Namen et al. 2005; Wasner et al. 2004). Ongoing pain and cold pain are further enhanced after A-fiber block (Wasner et al. 2004). Furthermore, menthol applied to the tongue significantly enhances lingual cold pain and weakly reduces lingual heat pain (Albin et al. 2008). The authors of these interesting studies hypothesized that menthol sensitizes and activates cold-sensitive peripheral afferent nociceptive neurons with C-fibers. Our study on intact and injured cutaneous afferent neurons in rats does not support this contention, i.e., that nociceptive high-threshold cold-sensitive afferents can be activated or sensitized by menthol. However, two points must be kept in mind. These authors used rather high concentrations of 30–40% menthol in ethanol. In most experiments on intact afferents we used concentrations of 10% menthol. Finally, we cannot exclude differences between species.

In conclusion, intact and injured cold-sensitive afferents innervating the rat hindlimb skin consist of two types: 1) low-threshold afferents that are menthol sensitive; these afferents are mechanoinensitive, and only a few of them are heat sensitive; and 2) high-threshold afferents that are menthol insensitive and mostly also heat sensitive and/or mechanosensitive.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**AUTHOR CONTRIBUTIONS**

A.T., R.B., and W.J. conception and design of research; A.T., F.B., M.S., and W.J. performed experiments; A.T., F.B., and M.S. analyzed data; A.T. and F.B. prepared figures; F.B., M.S., and W.J. interpreted results of experiments; R.B. and W.J. edited and revised manuscript; R.B. and W.J. approved final version of manuscript; W.J. drafted manuscript.

**REFERENCES**


COLD AND MENTHOL SENSITIVITY OF CUTANEOUS NERVE FIBERS


