Identification of a cold receptor reveals a general role for TRP channels in thermosensation

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The cellular and molecular mechanisms that enable us to sense cold are not well understood. Insights into this process have come from the use of pharmacological agents, such as menthol, that elicit a cooling sensation. Here we have characterized and cloned a menthol receptor from trigeminal sensory neurons that is also activated by thermal stimuli in the cool to cold range. This cold- and menthol-sensitive receptor, CMR1, is a member of the TRP family of excitatory ion channels, and we propose that it functions as a transducer of cold stimuli in the somatosensory system. These findings, together with our previous identification of the heat-sensitive channels VR1 and VRL-1, demonstrate that TRP channels detect temperatures over a wide range and are the principal sensors of thermal stimuli in the mammalian peripheral nervous system.

The somatosensory system detects changes in ambient temperature over a remarkably wide range. This process is initiated when a hot or cold stimulus excites sensory nerve fibres that project from dorsal root or trigeminal ganglia, which innervate regions of the trunk and head, respectively. These primary afferent neurons convert thermal stimuli into electrochemical signals (that is, action potentials) and relay sensory information to integrative centres in the spinal cord and brain[1,2]. Noxious (painful) heat is detected by sensory neurons that respond with a 'moderate' thermal threshold of about 43 °C or with a 'high' threshold of about 52 °C (refs 3–5). Insights into the mechanisms of heat sensation have come from molecular cloning of the vanilloid receptor (VR1), an excitatory ion channel on sensory neurons that is activated by temperatures exceeding 43 °C, and by capsaicin, the main pungent ingredient in 'hot' chili peppers[6]. Electrophysiological, anatomical and genetic studies show that VR1 contributes to heat sensitivity of moderate-threshold neurons and is essential for the development of thermal hypersensitivity after tissue injury[7,8]. A related ion channel, VRL-1, does not respond to capsaicin, but is activated by temperatures exceeding 50 °C, suggesting that it contributes to heat sensitivity of high-threshold sensory neurons[9]. Both VR1 and VRL-1 belong to the transient receptor potential (TRP) family of ion channels, and we have hypothesized that molecules of this type contribute to thermosensation over a wide temperature range[9].

In contrast to our understanding of noxious heat sensation, little is known about how we detect cold. In mammals, cool sensation is generally believed to be mediated by a small sub-population of unmyelinated C and thinly myelinated Aδ primary afferent fibres that discharge in the innocuous temperature range (15–30 °C)[10,11]. Responses to noxious cold (<15 °C) are also observed in these fibre types, with prevalence ranging from 10% to 100% depending on stimulus intensity and species examined[7,12–15]. This wide variability in the literature may reflect the fact that thermal thresholds for cold-sensitive fibres are not as well defined as they are for heat, and thus fibre types that transduce sensations of innocuous cool versus noxious cold are not as firmly established. Calcium-imaging and patch-clamp studies of dissociated dorsal root ganglion (DRG) neurons have shown that cold (~20 °C) promotes calcium influx, possibly through the direct opening of calcium-permeable ion channels[16,17]. However, several other mechanisms have been proposed to explain cold-evoked membrane depolarization, including inhibition of background K+ channels[18], activation of Na+-selective degenerin channels[19], inhibition of Na+/K+ ATPases[20], and differential effects of cold on voltage-gated Na+ and K+ conductances[21]. Thus it is not clear whether cold excites sensory neurons by activating a discrete 'cold receptor', or by modulating a constellation of excitatory and inhibitory channels on these cells.

To clarify these issues, we looked for a cold transducer using an expression cloning approach. Our strategy was based on the paradigm presented by the vanilloid receptor, namely, to investigate how plant products such as menthol elicit a cool sensation. Fifty years ago, Hensel and Zotterman[10] showed that menthol potentiates responses of trigeminal fibres to cold by shifting their thermal activation thresholds to warmer temperatures. Moreover, they proposed that cooling compounds mediate their psychophysical effects by interacting with a protein that is specifically involved in cold transduction. Subsequent studies suggested that menthol depolarizes sensory neurons by inhibiting voltage-dependent Ca2+ channels[22] (thereby decreasing activation of Ca2+-dependent K+ channels)[23], or by directly activating calcium-permeable channels[16,24]. However, there is currently no direct pharmacological or biochemical evidence to support the existence of a bona fide menthol-binding site on sensory neurons, nor is it clear whether menthol and cold act through the same molecular entity.

Here we show that the molecular site of menthol action is an excitatory ion channel expressed by small-diameter neurons in trigeminal and dorsal root ganglia. The cloned channel is also activated by cold (8–28 °C), proving that menthol elicits a sensation of cool by serving as a chemical agonist of a thermally responsive receptor. This cold- and menthol-sensitive receptor (CMR1) is a member of the long TRP (or TRPM) channel subfamily, making it a close molecular cousin of the heat-activated channels VR1 and VRL-1. Thus we conclude that TRP channels are the primary molecular transducers of thermal stimuli within the mammalian somatosensory system.

Activation of a Ca²⁺-permeable channel by menthol and cold

Body surfaces innervated by trigeminal fibres, such as the eye and tongue, are particularly sensitive to cold and cooling compounds[25]. We therefore used calcium imaging and electrophysiological methods to examine responses of dissociated rat trigeminal neurons to menthol and cold. Indeed, these stimuli produced robust
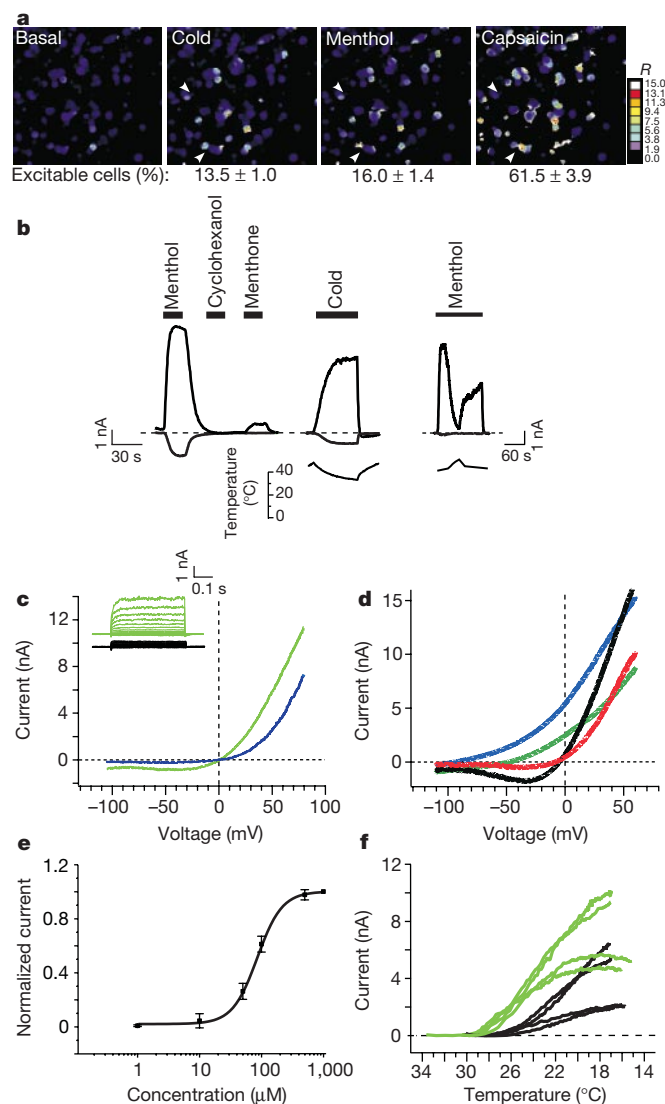
Figure 1 A subset of trigeminal neurons express an outwardly rectifying Ca^{2+}-permeable channel activated by menthol and cold. **a**, Responses of dissociated trigeminal neurons to cold (7 °C), menthol (500 µM) and capsaicin (1 µM) were assessed by calcium imaging. Arrowheads mark menthol-responding cells that were insensitive to capsaicin. Relative calcium concentrations are indicated by the Fura-2 ratio (R). The percentage ($\pm$s.e.m.) of excitable (potassium-sensitive) cells responding to each stimulus is indicated below. **b**, Electrophysiological responses of trigeminal neurons to menthol (50 µM), cyclohexanol (100 µM), menthone (100 µM) or cold (16 °C) were measured in both inward and outward directions ($V_{hold} = -60$ and $+80$ mV, respectively). Increasing temperature of perfusate (from room temperature to 30 °C) completely antagonized currents evoked by 100 µM menthol (right). Perfusate temperatures are indicated below each current trace. **c**, Responses evoked by menthol (50 µM, green) and cold (16 °C, blue) show strong outward rectification. Inset, baseline currents (black) and menthol-evoked responses (green) in nominally Ca^{2+}-free bath solution at room temperature. **d**, Voltage ramps (-120 to $+80$ mV in 200 ms) were used to establish current–voltage relationships in different extracellular solutions. Composition of the bath and electrode solutions and calculated reversal potentials are detailed in Supplementary Information. **e**, Concentration-response curve for menthol-evoked inward currents ($V_{hold} = -60$ mV) in trigeminal neurons. Membrane currents in each neuron were normalized to 1 mM menthol at room temperature. Each point represents mean value ($\pm$s.e.m.) from six independent neurons and were fit with the Hill equation. **f**, Temperature-response curves (from 33 to 16 °C) were determined for trigeminal neurons in the presence (green) or absence (black) of 10 µM menthol. Menthol potentiated the size of cold-evoked currents and shifted thermal thresholds from 27.1 $\pm$ 0.5 °C to 29.6 $\pm$ 0.3 °C ($n = 4$).

increases in intracellular free calcium in a relatively small sub-population of trigeminal neurons (Fig. 1a), consistent with work from others using DRG cultures[16,17,24]. Menthol and cold excited a largely overlapping subset of neurons, a significant fraction of which (54.5% $\pm$ 6.1, mean $\pm$ s.e.m.) were also activated by capsaicin (Fig. 1a). Sensitivity to capsaicin is considered a functional hallmark of nociceptors (primary sensory neurons that detect noxious stimuli) and thus about half of the menthol/cold-sensitive cells may be categorized as such.

Whole-cell patch-clamp recordings from trigeminal neurons showed that menthol or cold elicited rapidly developing membrane currents (Fig. 1b) that were characterized by pronounced outward rectification (that is, responses at positive holding potentials were substantially greater than those at negative voltages) (Fig. 1c). These currents reversed close to 0 mV ($E_{rev} = -3.6 \pm 1.9$ mV and -0.8 ± 0.3, respectively; $n = 5$), suggesting that they result from the opening of non-selective cation channels, consistent with recent observations in DRG neurons[16]. Indeed, ion substitution experiments showed little discrimination among monovalent cations, but revealed significantly higher permeability P for calcium ions ($P_{Ca}/P_{Na} = 3.2$; $P_K/P_{Na} = 1.1$; $P_{Cs}/P_K = 1.2$; $n = 6$) (Fig. 1d). We found these biophysical properties particularly interesting because they are reminiscent of VR1 and other TRP channels[26].

Room-temperature menthol evoked responses in a dose-dependent manner (Fig. 1e) with a half-maximal effective concentration (EC_{50}) of 80 $\pm$ 2.4 µM, a potency similar to that determined for DRG neurons using calcium imaging[24]. Fitting these data with the Hill equation suggests that receptor activation requires the binding of more than one menthol molecule ($\eta = 2.2$). In addition to menthol, the mint plant synthesizes structural analogues that also elicit a cooling sensation, although with reduced potency. One of these, menthone, elicited very small currents in trigeminal neurons, and cyclohexanol, an inactive synthetic menthol analogue, had no effect (Fig. 1b). Cold also elicited membrane currents in a dose-dependent manner, with a characteristic temperature threshold of 27.1 $\pm$ 0.5 °C ($n = 4$) (Fig. 1f). As reported for DRG neurons, menthol potentiated cold responses and shifted the thermal threshold to higher temperatures (29.6 $\pm$ 0.3 °C at 10 µM menthol). Conversely, increasing the temperature of the perfusate (from room temperature to 30 °C) completely antagonized currents evoked by 100 µM menthol (Fig. 1b). Taken together, our findings and those of others demonstrate that menthol and cold activate a calcium-permeable channel on trigeminal and DRG sensory neurons. Moreover, our electrophysiological data show that these stimuli activate currents with very similar biophysical properties, supporting the idea of a common molecular site of action.

Expression cloning of a receptor for cooling compounds

Because menthol and cold activate native conductances with intrinsic and significant permeability to calcium ions, we reasoned that a screening strategy based on calcium imaging[6] could be used to isolate a functional complementary DNA coding for a menthol- or cold-sensitive receptor. Because these responses are more prevalent in trigeminal cultures (14.8% versus 7.4% for DRG, $n = 745$ and 1,425 cells, respectively), we constructed a cDNA expression library from this tissue. Pools containing about 10,000 clones were transfected into HEK293 cells (derived from human embryonic kidney). We subsequently loaded the cells with the calcium-sensitive fluorescent dye Fura-2, and microscopically examined them for changes in intracellular calcium on exposure to room-temperature menthol (500 µM). In this way, we identified a single cDNA that conferred menthol sensitivity to transfected cells.

As noted above, menthol is one of several naturally occurring or synthetic cooling compounds that together span a wide range of relative potencies in psychophysical assays[25]. To assess the effects of the compounds on the newly identified receptor, we expressed the cloned cDNA in *Xenopus* oocytes and measured evoked responses

using whole-cell voltage-clamp methods. Clearly, the most robust responses were elicited by the super-cooling agent icilin (AG-3-5)[27], which showed about 2.5-fold greater efficacy and nearly 200-fold greater potency than menthol (EC_{50} = 0.36 ± 0.03 μM and 66.7 ± 3.3 μM, respectively) (Fig. 2a, b). Eucalyptol, the main constituent of oil of *Eucalyptus*, also elicited membrane currents, but with lower efficacy and potency (3.4 ± 0.4 mM) than menthol or icilin. Menthone, camphor and cyclohexanol had little or no effect, even when applied at concentrations approaching their limits of solubility in aqueous buffers (>500 μM). Finally, the vanilloid-receptor agonist, capsaicin, did not elicit responses in these cells.

A more detailed biophysical analysis of the cloned receptor was carried out in transfected HEK293 cells, where menthol or icilin produced currents with nearly time-independent kinetics and steep outward rectification (Fig. 3a). Like native menthol-evoked responses in trigeminal neurons, these currents showed relatively high permeability to calcium and little selectivity among mono-valent cations (P_{Ca}/P_{Na} = 3.3; P_K/P_{Na} = 1.2; P_{Cs}/P_K = 1.1; n = 4–9) (Fig. 3b). Menthol-evoked currents also showed significant desen-sitization (53.9% ± 1.7 decrease in peak current between the first and second application, n = 3), but little desensitization (9.1% ± 7,

n = 3) was observed in nominally calcium-free bath solution (Fig. 3c). Icilin showed even stronger desensitization, but unlike menthol this agonist was essentially inactive in the absence of extracellular calcium. Similar observations were obtained in oocytes (data not shown). When measured at the single-channel level, menthol-evoked currents also showed pronounced outward recti-fication. These responses were characterized by brief, transient openings and had a slope conductance of 83 ± 3 pS at positive potentials (Fig. 3d, e). We also observed events with smaller unitary currents, which may represent subconductance states of the channel or openings that were simply too brief to be resolved in our analysis.
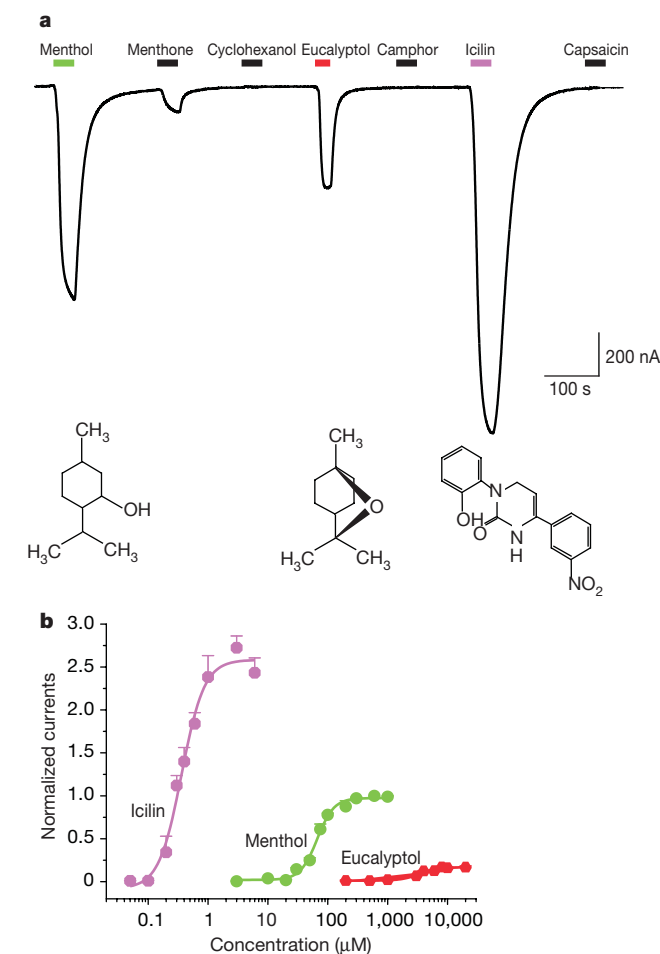

Figure 2 Cooling compounds activate the cloned receptor. **a**, An oocyte expressing the cloned receptor was exposed to consecutive applications of menthol (100 μM), menthone (500 μM), cyclohexanol (500 μM), eucalyptol (20 mM), camphor (1 mM), icilin (300 nM) and capsaicin (1 μM). Resulting membrane currents were measured under voltage clamp at −60 mV. Bars denote the duration of agonist application. Chemical structures for menthol, eucalyptol and icilin are shown below their respective responses. **b**, Concentration-response curves for icilin (pink), menthol (green) and eucalyptol (red). Responses were normalized to those evoked by 500 μM menthol. Each point represents mean values (±s.e.m.) from 4–9 independent oocytes.
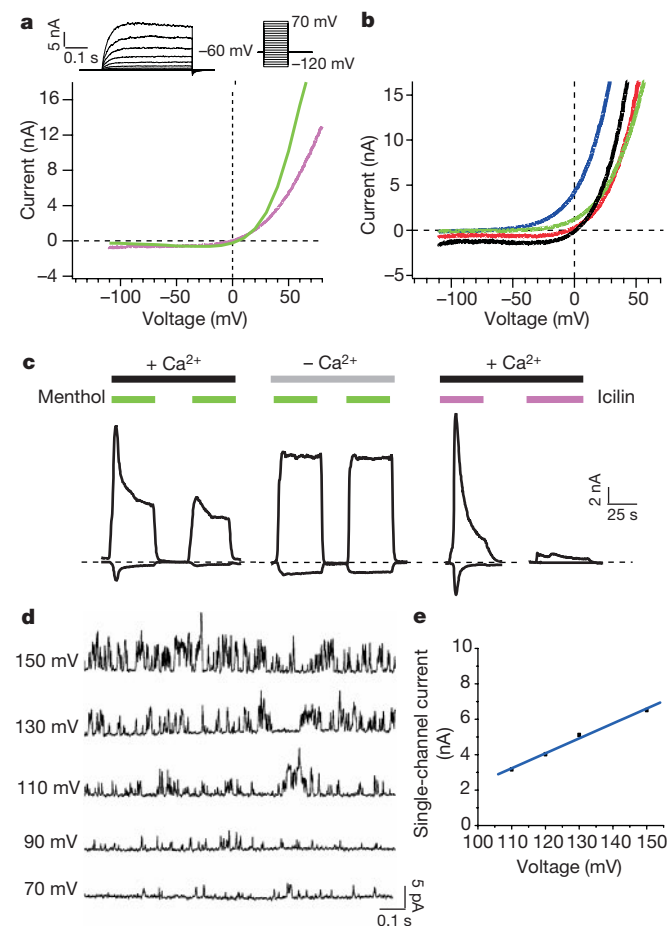

Figure 3 Electrophysiological properties of menthol-induced currents in transfected HEK293 cells. **a**, Time dependence of menthol-induced whole-cell currents were analysed using 400-ms voltage step pulses ranging from −120 to +70 mV in 10-mV steps (top right). Traces show current response induced by menthol (50 μM) at room temperature in nominally Ca^{2+}-free bath solution (top left). The current−voltage relationship (bottom) was obtained from the same pulse protocol using 200 μM menthol (room temperature) by plotting the time-independent current component as a function of membrane voltage. Menthol currents reversed at −4.5 ± 1.1 mV (±s.e.m., n = 4) and show strong outward rectification (green). A similar current−voltage relationship was obtained for 2 μM icilin (pink) from a 200-ms voltage ramp (−120 to +80 mV). **b**, Voltage ramps (200 ms) ranging from −120 to +80 mV were used to record current−voltage curves in different extracellular solutions. Composition of the bath and electrode solutions and calculated reversal potentials are detailed in Supplementary Information. **c**, Current desensitization evoked by menthol (50 μM) and icilin (2 μM) is dependent on calcium. **d**, Single-channel traces were recorded from transfected HEK293 cells in the cell-attached patch configuration at different pipette potentials. **e**, Slope conductance of the amplitudes of single-channel currents was obtained by linear regression, yielding a single-channel conductance of 83 ± 3 pS (n = 3).

Activation of the menthol receptor by cold

To determine whether the menthol receptor is also a cold sensor, we tested its thermal responsiveness in oocytes by lowering the temperature of the perfusate from about 35 °C to about 5 °C. This elicited a robust and rapidly activating inward current (at negative holding potentials) that was remarkably consistent since the rate of temperature change ($0.2–1 °C s^{-1}$) did not influence threshold or saturation temperature (Fig. 4a, b). Moreover, cold-evoked currents were directly proportional to temperature regardless of the direction of the temperature change (not shown). Cold-activated currents had a thermal threshold of $25.8 \pm 0.4 °C$ and saturated at $8.2 \pm 0.3 °C$ ($n = 12$) (Fig. 4c). Consistent with the behaviour of native cold currents, addition of a sub-activating concentration of menthol ($20 \mu M$) to the perfusate increased threshold and saturation temperature to $29.7 \pm 0.3 °C$ and $15.6 \pm 0.4 °C$, respectively ($n = 7$) (Fig. 4c). We found that menthol is a more efficacious agonist than cold because saturating cold-evoked currents were smaller than those obtained with a maximal dose of room-temperature menthol ($67.4\% \pm 1.9$, $n = 7$) (Fig. 4d).

We also examined cold-evoked currents in HEK293 cells expressing the menthol receptor. As observed for native cold responses (Fig. 1c), current–voltage relationships for the cloned channel showed steep outward rectification (Fig. 4e) and were markedly potentiated by a sub-activating dose of menthol ($10 \mu M$). Menthol increased cold-evoked currents in both the outward and inward direction, but the effect on the inward component was more pronounced, reminiscent of the effect of capsaicin on VR1 (ref. 28). Native cold-evoked responses in sensory fibres or cultured DRG neurons show adaptation to a prolonged thermal stimulus lasting several minutes[16,29]. We found that receptor-transfected cells showed small, outwardly rectifying basal currents at room temperature (~22 °C), but responses to a subsequent 22 °C stimulus were markedly larger after the cell had first been warmed to 31 °C (Fig. 4f). This observation suggests that the cloned receptor also adapts to thermal challenges, and that this effect is reversed on heating. Desensitization to cold differed from that observed with menthol because it was independent of extracellular calcium (data not shown). VR1 shows similar behaviour in that desensitization to chemical (capsaicin) or thermal (heat) stimuli are dependent or independent, respectively, of calcium. Taken together, our observations show that the cloned receptor, which we now designate cold–menthol receptor type 1 (CMR1), has properties identical to endogenous cold/menthol currents observed in sensory neurons[16,17,24].

CMR1, member of the TRP channel family

The CMR1 cDNA contains an open reading frame of 3312 base pairs (bp) that is predicted to code for a protein of 1,104 amino acids with a relative molecular mass of 128,000 (M_r 128 K) (Fig. 5a). Database searches revealed significant homology between this deduced sequence and members of the TRP ion channel family, particularly with the subgroup of long TRP (or TRPM) channels, so named for their characteristically large amino- and carboxy-terminal cytoplasmic tails[26]. Among members of this subfamily, TRPM2 and TRPM7 have been electrophysiologically characterized and shown to behave as bifunctional proteins in which enzymatic activities associated with their long C-terminal domains are believed to regulate channel opening[30–32]. In contrast, CMR1 has a significantly shorter C-terminal region (Fig. 5b) and does not contain any obvious enzymatic domains that might be associated with channel regulation.

Further search of the literature showed that CMR1 is 92% identical to a human sequence originally identified as a prostate-specific transcript[33]. This presumptive TRP channel, called trp-p8 (or TRPM8), is thus likely to be the human orthologue of rat CMR1. Aside from prostate epithelium, TRPM8 was found to be expressed

by a variety of tumours, including prostate, melanoma, colorectal and breast carcinoma[33]. The presence of this channel in sensory neurons was not assessed and we therefore carried out northern blot and *in situ* hybridization studies to examine expression of CMR1 in rat trigeminal and dorsal root ganglia. Indeed, transcripts of about 6 and 4.5 kilobases (kb) were detected in both neuronal tissues (Fig. 6a), similar to that reported for TRPM8 expression in human prostate[33]. At the cellular level, CMR1 transcripts were found in a subset of sensory neurons with small-diameter cell bodies ($18.2 \pm 1.1 \mu m$ and $21.6 \pm 0.5 \mu m$ in dorsal root and trigeminal ganglia, respectively) (Fig. 6b), similar in size to VR1-expressing cells ($19.2 \pm 0.3 \mu m$)[9]. CMR1 transcripts were more prevalent in trigeminal than dorsal root ganglia and were conspicuously absent from the vast majority of larger-diameter cells, consistent with our physiological observations using neuronal cultures. These findings suggest that CMR1 is expressed by a subpopulation of C fibres (and possibly Aδ fibers) representing less than 20% of all primary afferent neurons.
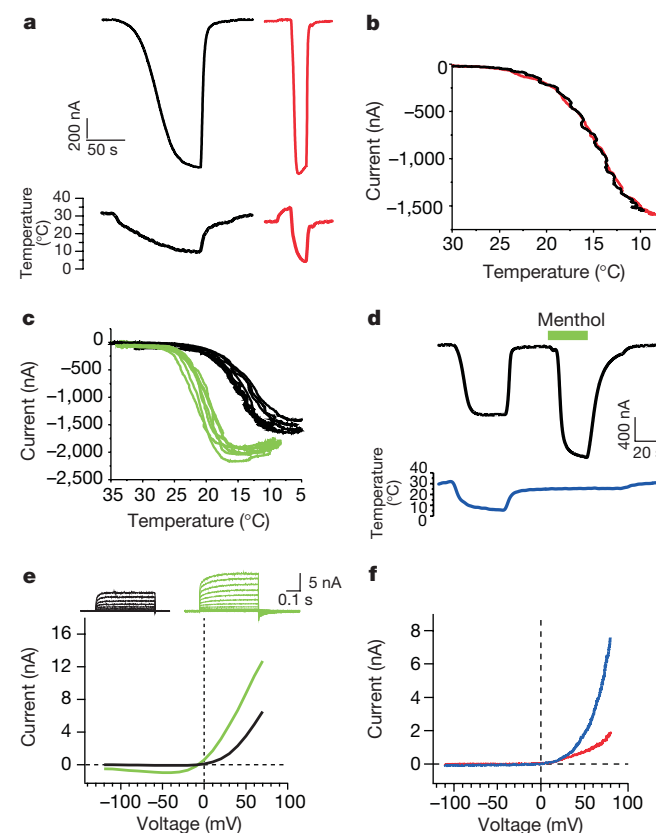

Figure 4 The menthol receptor is sensitive to cold. **a**, Inward currents (top) were evoked in the same menthol receptor-expressing oocyte by repetitive decreases in perfusate temperature. Cooling ramps (bottom) were applied at two different rates ($0.2 °C s^{-1}$, black; $1 °C s^{-1}$, red). **b**, Temperature-response profile of the cold-evoked currents shown in **a**. **c**, Response profiles of cold-evoked currents in seven independent oocytes in the absence (black) or presence of a sub-activating concentration of menthol ($20 \mu M$; green). **d**, Inward currents evoked in a menthol receptor-expressing oocyte by a saturating cold stimulus (35 to 5 °C, blue trace) were smaller than those evoked by a maximal dose of menthol at room temperature ($500 \mu M$, green bar). **e**, Current–voltage relationship for a stimulus evoked by cold (14 °C) in HEK293 cells transfected with the menthol receptor in the absence (black) or presence (green) of a sub-activating dose ($10 \mu M$) of menthol. Menthol-induced potentiation and outward rectification of cold-evoked currents are also evident in the accompanying current traces (above) obtained at various voltage steps (-120 to 70 mV). **f**, Current–voltage relationship in transfected HEK293 cells for basal current at 22 °C before (red) and after (blue) warming to 31 °C.

Discussion

Menthol has long been known to evoke a sensation of cold. Our findings now provide a molecular explanation for this psychophysical response by demonstrating that cooling compounds and cold are detected by the same molecular entity on primary afferent neurons of the somatosensory system. Moreover, our results show that thermosensation is mediated by a common molecular mechanism that uses TRP ion channels as primary transducers of thermal stimuli. As few as three ion channels (CMR1, VR1 and VRL-1) may provide coverage for a remarkably wide range of temperatures (8–28, >43 and >50 °C, respectively) (Fig. 7a). Still, these channels do not respond to all commonly experienced temperatures, such as ultra-cold (<8 °C) or warm (~30–40 °C), suggesting that additional molecules or mechanisms are involved in mediating thermosensation in these temperature ranges.

If one considers 15 °C as an approximate demarcation between cool and cold[10,34], then CMR1 clearly enables detection of temperatures that encompass all of the innocuous cool (15–28 °C) and part of the noxious cold (8–15 °C) range. Furthermore, CMR1 could

contribute to depolarization of fibres at temperatures in the ultra-cold range (<8 °C) if the channel is modulated in a manner that extends its sensitivity range *in vivo*. Indeed, several TRP channels are regulated by receptors that couple to phospholipase C (PLC), and the thermal activation threshold for VR1 can be markedly shifted to lower temperatures by inflammatory agents (for example, bradykinin and nerve growth factor) that activate this pathway[26,28,35,36]. Other inflammatory products, such as protons and lipids, seem to interact with VR1 directly[37–39]. Thus, CMR1 might be modulated in a similar manner, expanding its range of temperature sensitivity under normal or pathophysiological conditions. CMR1 may also work in concert with cold-sensitive background potassium channels, which have been suggested to alter the duration or kinetics of cold-evoked action potentials[18]. Although CMR1 can clearly underlie activity in the relatively small sub-population of cool/menthol-sensitive fibres, it cannot account for the reportedly large proportion of nociceptive afferent fibres that respond to sub-zero temperatures[13–15] because its expression is restricted to no more than 15% of trigeminal or DRG neurons. If these latter responses to ultra-cold stimuli are, indeed, physiologically relevant, then they must be mediated by another transducer(s). Finally, it is interesting to note that, unlike CMR1, the heat-sensing receptors VR1 and VRL-1 are activated only in the noxious (pain-producing) range. These biophysical differences in channel sensitivities may help to explain why psychophysical thresholds for cold-evoked pain are not as distinct as they are for heat.

When expressed together, CMR1 and VR1 can endow a cell with distinct thermal thresholds and temperature response ranges for cold and hot, respectively (Fig. 7b). Our calcium-imaging data suggest that a significant proportion (~50%) of CMR1-expressing small-diameter neurons also express VR1 and can therefore be categorized as cold- and heat-responsive nociceptors. These observations provide a plausible molecular explanation for the so-called 'paradoxical' activation of cool (25 °C)-sensitive thermoreceptors by noxious heat[40,41], and for the fact that noxious cold is sometimes perceived as burning pain[42]. However, they also raise two interesting questions: how do we distinguish hot from cold, and why doesn't activation of these neurons by an innocuous cool stimulus always cause discomfort or pain? This conundrum might be explained at the cellular level if activation of CMR1 by a cold stimulus elicits action potentials of lower firing frequencies and/or reduced Ca^{2+} influx than VR1-mediated heat responses. Indeed, noxious heat is reported to be a much stronger excitatory stimulus for cat trigeminal C fibres than noxious cold[11].
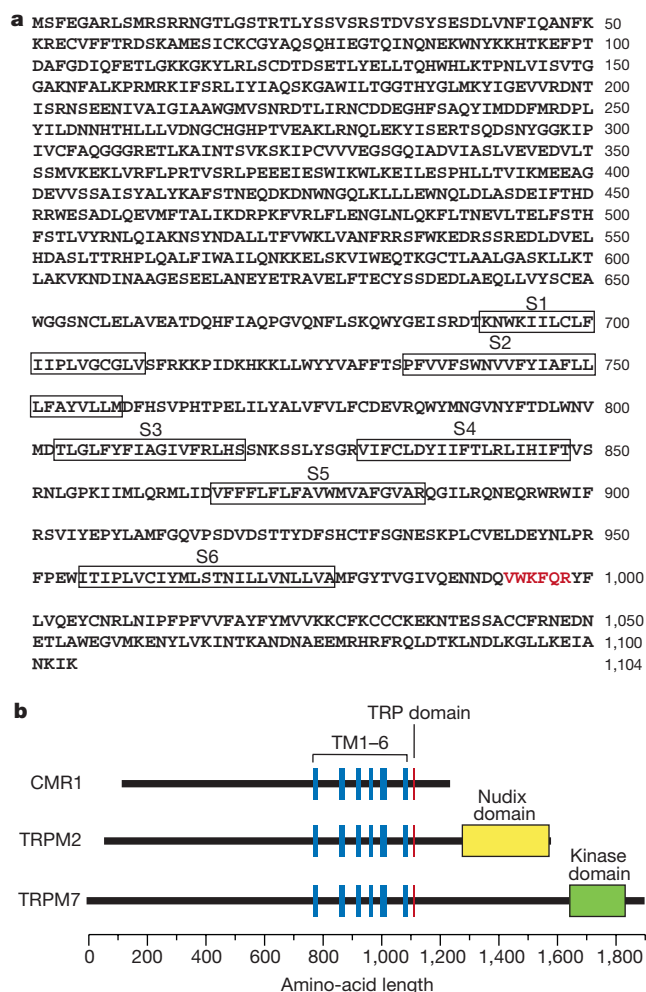
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NKIK 1,104

(S1, S2, S3, S4, S5, S6 transmembrane domain labels shown above boxed regions)

b

Figure 5 CMR1 is a member of the TRP family of ion channels. **a**, The predicted amino-acid sequence determined from the CMR1 cDNA. Boxes designate predicted transmembrane domains, and amino acids encompassing the conserved TRP family motif are shown in red. **b**, Schematic comparison of CMR1 with other TRPM family members, TRPM2 and TRPM7. Proteins are aligned by putative transmembrane domains (blue boxes) and the TRP motif (red boxes) as landmarks. The numeric label is based on the TRPM7 sequence. CMR1 has a significantly shorter C-terminal tail and does not contain any conserved domains indicative of enzymatic activity associated with TRPM2 (ADP ribose pyrophosphatase, Nudix motif, yellow box) or TRPM7 (protein kinase, green box).
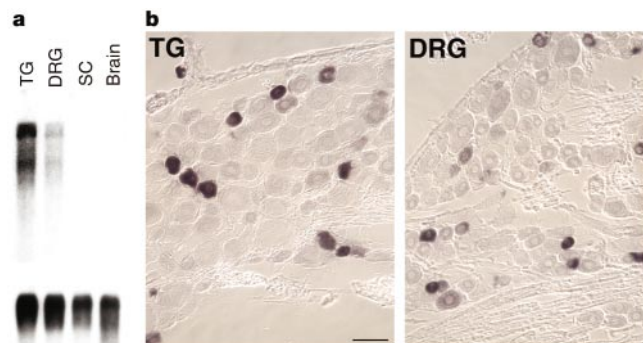
Figure 6 CMR1 is expressed by small-diameter neurons in trigeminal and dorsal root ganglia. **a**, Poly(A)+ RNA from adult rat trigeminal ganglia (TG), dorsal root ganglia (DRG), spinal cord (SC) and brain were analysed by northern blotting, revealing two predominant transcripts of roughly 6 and 4.5 kilobases. The blot was re-probed with a rat cyclophilin cDNA (bottom) to control for sample loading. **b**, Histological sections from adult rat trigeminal or dorsal root ganglia showed selective staining (brown) with a digoxygenin-labelled CMR1 probe over neurons with small-diameter (~19 μm) cell bodies. Scale bar, 50 μm.
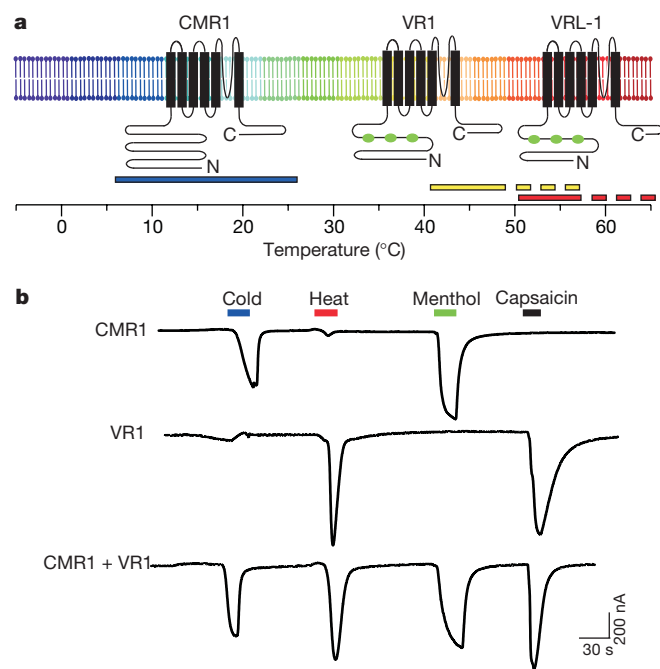
Figure 7 TRP-like channels mediate thermosensation from cold to hot. **a**, Schematic representation of the thermal sensitivity ranges of CMR1, VR1 and VRL-1. The range of each protein's temperature sensitivity is denoted by bars (CMR1, blue; VR1, yellow; VRL-1, red). Other molecules may contribute to temperature sensation in zones not necessarily covered by these channels, in particular warm (30–40 °C) or extremely cold (<8 °C) zones. **b**, Oocytes co-expressing CMR1 and VR1 demonstrate that these channels are sufficient to confer thermal responsiveness to both cold (menthol) and heat (capsaicin) independently. Bars above traces indicate application of thermal or chemical stimuli (cold, 35 to 8 °C; heat, 25 to 50 °C; menthol, 100 μM; capsaicin, 1 μM).

Our ability to discriminate among thermal stimuli must also involve decoding of sensory information at the level of the spinal cord and brain, where inputs from afferent fibres having different thermal sensitivities (for example, heat only, cold only, or both) are integrated. For instance, excitatory input from hot- and cold-sensitive (CMR1$^+$, VR1$^+$) nociceptors may be modulated at the central level by concurrent input from cold-specific (CMR1$^+$, VR1$^-$) fibres. Such action is supported by two observations. First, central unmasking, or disinhibition of, innocuous cool-induced activity in polymodal C-fibre nociceptors has been proposed as an explanation of the thermal grill illusion[42], in which simultaneous contact with warm and cool surfaces evokes a sensation of burning pain. Second, an innocuous cool stimulus can evoke the sensation of burning pain in humans after blocking conduction in A fibres or after loss of these fibres from injury (large-fibre neuropathy)[43,44]. Clearly, answers to these and related questions will require a more detailed understanding of where functionally defined sub-populations of primary afferent neurons make synaptic connections in the spinal cord. The cloning of CMR1 now makes it possible to identify cold-sensitive fibres and to assess the contribution of this ion channel to cold sensation *in vivo*.

When applied to skin or mucous membranes, menthol produces a cooling sensation, inhibits respiratory reflexes, and, at high doses, elicits a pungent or irritant effect that is accompanied by local vasodilation[25,45]. Most, if not all, of these physiological actions can be explained by excitation of sensory nerve endings, but CMR1 receptors elsewhere might contribute to these or other effects of cooling compounds or cold. For example, intravenous administration of icilin induces intense shivering in mammals[46], and although this may be mediated by a sympathetic reflex in response to sensory nerve stimulation, it will be interesting to determine whether CMR1 or homologues are expressed in brain regions, such

as the hypothalamus, that regulate core body temperature. Outside of the nervous system, menthol has been reported to increase intracellular calcium in canine tracheal epithelium[47], an observation that is intriguing in light of the observation that TRPM8 transcripts are expressed in normal prostate epithelium[33]. Physiological roles for TRP channels in the prostate are currently unknown, but expression or repression of several TRP genes in tumour cells suggests that these proteins influence cell proliferation, possibly through their ability to regulate intracellular calcium levels[26]. Because cold is unlikely to be the natural stimulus for TRPM8 in this context, other modulators of the channel may exist, such as an endogenous menthol-like ligand or a bioactive lipid. In any case, to our knowledge CMR1 is now the first member of the long TRP channel family to be associated with a known physiological function or extracellular ligand, making it an important tool for uncovering contributions of these channels to cell growth and sensory perception. □

Methods

Neuronal cell culture and Ca^{2+} microfluorimetry

Trigeminal ganglia were dissected from newborn Sprague Dawley rats and dissociated with 0.125% collagenase P (Boehringer) solution in CMF Hank's solution at 37 °C for 20 min (P0 animals) to 30 min (P4 animals), pelleted, and resuspended in 0.05% trypsin at 37 °C for 2 min. Ganglia were triturated gently with a fire-polished Pasteur pipette in culture medium (MEM Eagle's/Earle's BSS with 10% horse serum, vitamins, penicillin–streptomycin, L-glutamine and 100 ng ml^{-1} nerve growth factor 7S, Invitrogen), then enriched by density gradient centrifugation as described[48]. Cells were resuspended in culture medium and plated onto coverslips coated with polyornithine (PLO) (1 mg ml^{-1}; Sigma) and laminin (5 μg ml^{-1}; Invitrogen). Cultures were examined 1–2 d after plating by Ca^{2+} microfluorimetry as described[6]. For cell selection before patch-clamp analysis, CaCl$_2$ and pluronic acid were omitted from the loading buffer.

Mammalian cell electrophysiology

Trigeminal neurons responding to 50 μM menthol with an increase in intracellular Ca^{2+} were selected for patch-clamp recordings. HEK293 cells were cultured in DMEM with 10% fetal bovine serum and co-transfected (Lipofectamine 2000, Invitrogen) with 1 μg CMR1 plasmid and 0.1 μg enhanced green fluorescence reporter plasmid to identify transfected cells. Cells were plated onto PLO-coated coverslips the next day and examined 2 d after transfection. Gigaseals were formed with pipettes (Garner Glass 7052, internal diameter 1.1 mm, outer diameter 1.5 mm) having a resistance of 3–5 MΩ in standard pipette solution. Liquid junction potentials (measured in separate experiments) did not exceed 3 mV and thus no correction for this offset was made. Whole-cell voltage clamp was performed at a holding potential of −60 mV with a 200-ms voltage ramp from −120 mV to +80 mV at 3.6 Hz. Data were acquired using Pulse and Pulsefit (HEKA GmbH) software. Recordings were sampled at 20 kHz and filtered at 2 kHz. Pipette and recording solutions for neuronal and mammalian cell experiments are detailed in Supplementary Information. Recordings were performed at 22 °C unless noted otherwise. Temperature ramps were generated by cooling or heating the perfusate in a jacketed coil (Harvard) connected to a thermostat. Temperature in the proximity of the patched cell was measured with a miniature thermocouple (MT-29/2, Physitemp) and sampled using an ITC-18 A/D board (Instrutech) and Pulse software. Permeability ratios for monovalent cations to Na (P_X/P_{Na}) were calculated according to $P_X/P_{Na} = \exp(\Delta E_{rev(Na-X)}F/RT)$, where $\Delta E_{rev(Na-X)}$ is the reversal potential change, F the Faraday constant, R the universal gas constant and T the absolute temperature. For measurements of Ca^{2+} permeability, P_{Ca}/P_{Na} was calculated according to ref. 49 (equation A6).

Cloning, northern blot and *in situ* hybridization

Trigeminal neurons from newborn rats were dissociated and enriched as described[48]. Polyadenylated RNA (~2 μg) was isolated from these cells (PolyATract Kit, Promega) and used to construct a cDNA library in pcDNA3 (Invitrogen) as described[50]. Library sub-pools consisting of about 10,000 clones were transiently transfected into HEK293 cells by lipofection and split 24 h later into 8-well glass chamber slides coated with Matrigel (Becton Dickinson). Responses to chemical or thermal stimuli were assessed 6–24 h later using Fura-2 Ca^{2+} imaging. Iterative subdivision and re-screening of a positive pool yielded a single menthol-responsive clone. Northern blotting and *in situ* hybridization histochemistry were performed as described[6] using the entire CMR1 cDNA to generate ^{32}P- and digoxygenin-labelled probes, respectively.

Oocyte electrophysiology

Complementary RNA transcripts were synthesized and injected into *Xenopus laevis* oocytes as described[50]. Two-electrode voltage-clamp recordings were performed 2–7 d after injection. Dose-response curves for cooling compounds were performed at room temperature (22–24 °C). Icilin (AG-3-5) was provided by E. Wei. Temperature ramps were generated by heating (~35 °C) or cooling (~4 °C) the perfusate in a Harvard coil and monitoring temperature changes with a thermistor placed near the oocyte.

1. Fields, H. L. *Pain* 13−78 (McGraw-Hill, New York, 1987).
2. Julius, D. & Basbaum, A. I. Molecular mechanisms of nociception. *Nature* **413,** 203−210 (2001).
3. Raja, S. N., Meyer, R. A., Ringkamp, M. & Campbell, J. N. in *Textbook of Pain* (ed. Wall, P. D.Melzack, R.) 11−57 (Churchill Livingstone, Edinburgh, 1999).
4. Nagy, I. & Rang, H. Noxious heat activates all capsaicin-sensitive and also a sub-population of capsaicin-insensitive dorsal root ganglion neurons. *Neuroscience* **88,** 995−997 (1999).
5. Cesare, P. & McNaughton, P. A novel heat-activated current in nociceptive neurons and its sensitization by bradykinin. *Proc. Natl Acad. Sci. USA* **93,** 15435−15439 (1996).
6. Caterina, M. J. *et al.* The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature* **389,** 816−824 (1997).
7. Caterina, M. J. *et al.* Impaired nociception and pain sensation in mice lacking the capsaicin receptor. *Science* **288,** 306−313 (2000).
8. Davis, J. B. *et al.* Vanilloid receptor-1 is essential for inflammatory thermal hyperalgesia. *Nature* **405,** 183−187 (2000).
9. Caterina, M. J., Rosen, T. A., Tominaga, M., Brake, A. J. & Julius, D. A capsaicin-receptor homologue with a high threshold for noxious heat. *Nature* **398,** 436−441 (1999).
10. Hensel, H. & Zotterman, Y. The effect of menthol on the thermoreceptors. *Acta Physiol. Scand.* **24,** 27−34 (1951).
11. Bessou, P. & Perl, E. R. Response of cutaneous sensory units with unmyelinated fibers to noxious stimuli. *J. Neurophysiol.* **32,** 1025−1043 (1969).
12. Kress, M., Koltzenburg, M., Reeh, P. W. & Handwerker, H. O. Responsiveness and functional attributes of electrically localized terminals of cutaneous C-fibers in vivo and in vitro. *J. Neurophysiol.* **68,** 581−595 (1992).
13. Simone, D. A. & Kajander, K. C. Excitation of rat cutaneous nociceptors by noxious cold. *Neurosci. Lett.* **213,** 53−56 (1996).
14. Simone, D. A. & Kajander, K. C. Responses of cutaneous A-fiber nociceptors to noxious cold. *J. Neurophysiol.* **77,** 2049−2060 (1997).
15. Cain, D. M., Khasabov, S. G. & Simone, D. A. Response properties of mechanoreceptors and nociceptors in mouse glabrous skin: an in vivo study. *J. Neurophysiol.* **85,** 1561−1574 (2001).
16. Reid, G. & Flonta, M. L. Cold current in thermoreceptive neurons. *Nature* **413,** 480 (2001).
17. Suto, K. & Gotoh, H. Calcium signalling in cold cells studied in cultured dorsal root ganglion neurons. *Neuroscience* **92,** 1131−1135 (1999).
18. Reid, G. & Flonta, M. Cold transduction by inhibition of a background potassium conductance in rat primary sensory neurones. *Neurosci. Lett.* **297,** 171−174 (2001).
19. Askwith, C. C., Benson, C. J., Welsh, M. J. & Snyder, P. M. DEG/ENaC ion channels involved in sensory transduction are modulated by cold temperature. *Proc. Natl Acad. Sci. USA* **98,** 6459−6463 (2001).
20. Pierau, F. K., Torrey, P. & Carpenter, D. O. Mammalian cold receptor afferents: role of an electrogenic sodium pump in sensory transduction. *Brain Res.* **73,** 156−160 (1974).
21. Braun, H. A., Bade, H. & Hensel, H. Static and dynamic discharge patterns of bursting cold fibers related to hypothetical receptor mechanisms. *Pflugers Arch.* **386,** 1−9 (1980).
22. Swandulla, D., Carbone, E., Schafer, K. & Lux, H. D. Effect of menthol on two types of Ca currents in cultured sensory neurons of vertebrates. *Pflugers Arch.* **409,** 52−59 (1987).
23. Schafer, K., Braun, H. A. & Isenberg, C. Effect of menthol on cold receptor activity. Analysis of receptor processes. *J. Gen. Physiol.* **88,** 757−776 (1986).
24. Okazawa, M., Terauchi, T., Shiraki, T., Matsumura, K. & Kobayashi, S. l-Menthol-induced $[Ca^{2+}]_i$ increase and impulses in cultured sensory neurons. *Neuroreport* **11,** 2151−2155 (2000).
25. Eccles, R. Menthol and related cooling compounds. *J. Pharm. Pharmacol.* **46,** 618−630 (1994).
26. Clapham, D. E., Runnels, L. W. & Strubing, C. The TRP ion channel family. *Nature Rev. Neurosci.* **2,** 387−396 (2001).
27. Wei, E. T. & Seid, D. A. AG-3-5: a chemical producing sensations of cold. *J. Pharm. Pharmacol.* **35,** 110−112 (1983).
28. Chuang, H. H. *et al.* Bradykinin and nerve growth factor release the capsaicin receptor from PtdIns(4,5)P2-mediated inhibition. *Nature* **411,** 957−962 (2001).
29. Kenshalo, D. R. & Duclaux, R. Response characteristics of cutaneous cold receptors in the monkey. *J. Neurophysiol.* **40,** 319−332 (1977).
30. Perraud, A. L. *et al.* ADP-ribose gating of the calcium-permeable LTRPC2 channel revealed by Nudix motif homology. *Nature* **411,** 595−599 (2001).
31. Sano, Y. *et al.* Immunocyte Ca^{2+} influx system mediated by LTRPC2. *Science* **293,** 1327−1330 (2001).
32. Runnels, L. W., Yue, L. & Clapham, D. E. TRP-PLIK, a bifunctional protein with kinase and ion channel activities. *Science* **291,** 1043−1047 (2001).
33. Tsavaler, L., Shapero, M. H., Morkowski, S. & Laus, R. Trp-p8, a novel prostate-specific gene, is up-regulated in prostate cancer and other malignancies and shares high homology with transient receptor potential calcium channel proteins. *Cancer Res.* **61,** 3760−3769 (2001).
34. Rainville, P., Chen, C. C. & Bushnell, M. C. Psychophysical study of noxious and innocuous cold discrimination in monkey. *Exp. Brain Res.* **125,** 28−34 (1999).
35. Harteneck, C., Plant, T. D. & Schultz, G. From worm to man: three subfamilies of TRP channels. *Trends Neurosci.* **23,** 159−166 (2000).
36. Premkumar, L. S. & Ahern, G. P. Induction of vanilloid receptor channel activity by protein kinase C. *Nature* **408,** 985−990 (2000).
37. Zygmunt, P. M. *et al.* Vanilloid receptors on sensory nerves mediate the vasodilator action of anandamide. *Nature* **400,** 452−457 (1999).
38. Hwang, S. W. *et al.* Direct activation of capsaicin receptors by products of lipoxygenases: endogenous capsaicin-like substances. *Proc. Natl Acad. Sci. USA* **97,** 6155−6160 (2000).
39. Jordt, S. E., Tominaga, M. & Julius, D. Acid potentiation of the capsaicin receptor determined by a key extracellular site. *Proc. Natl Acad. Sci. USA* **97,** 8134−8139 (2000).
40. Campero, M., Serra, J., Bostock, H. & Ochoa, J. L. Slowly conducting afferents activated by innocuous low temperature in human skin. *J. Physiol.* **535,** 855−865 (2001).
41. Dodt, E. & Zotterman, Y. The discharge of specific cold fibres at high temperatures (the paradoxical cold). *Acta Physiol. Scand.* **26,** 358−365 (1952).
42. Craig, A. D. & Bushnell, M. C. The thermal grill illusion: unmasking the burn of cold pain. *Science* **265,** 252−255 (1994).
43. Wahren, L. K., Torebjork, E. & Jorum, E. Central suppression of cold-induced C fibre pain by myelinated fibre input. *Pain* **38,** 313−319 (1989).
44. Yarnitsky, D. & Ochoa, J. L. Release of cold-induced burning pain by block of cold-specific afferent input. *Brain* **113,** 893−902 (1990).
45. Eccles, R. Role of cold receptors and menthol in thirst, the drive to breathe and arousal. *Appetite* **34,** 29−35 (2000).
46. Wei, E. T. Pharmacological aspects of shaking behaviour produced by TRH, AG-3-5, and morphine withdrawal. *Fed. Proc.* **40,** 1491−1496 (1981).
47. Takeuchi, S., Tamaoki, J., Kondo, M. & Konno, K. Effect of menthol on cytosolic Ca^{2+} levels in canine airway epithelium in culture. *Biochem. Biophys. Res. Commun.* **201,** 1333−1338 (1994).
48. Eckert, S. P., Taddese, A. & McCleskey, E. W. Isolation and culture of rat sensory neurons having distinct sensory modalities. *J. Neurosci. Methods* **77,** 183−190 (1997).
49. Lewis, C. A. Ion-concentration dependence of the reversal potential and the single channel conductance of ion channels at the frog neuromuscular junction. *J. Physiol.* **286,** 417−445 (1979).
50. Brake, A. J., Wagenbach, M. J. & Julius, D. New structural motif for ligand-gated ion channels defined by an ionotropic ATP receptor. *Nature* **371,** 519−523 (1994).

Supplementary Information accompanies the paper on *Nature*'s website (http://www.nature.com).

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(e-mail: julius@socrates.ucsf.edu). The GenBank accession number for rat CMR1 is AY072788.