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

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Identification of a Cold Receptor Reveals a General Role for TRP Channels in Thermosensation

by

Werner Maria Neuhausser

DISSERTATION

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Werner Maria Neuhausser completed the following work related to this dissertation:

Sensory neuron cell culture and Ca^{2+} microfluorimetry

Mammalian cell electrophysiology (patch clamp) of sensory neurons

Sensory neuron library screening (with David D McKemy)

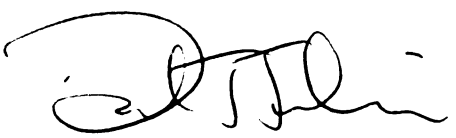
Mammalian cell electrophysiology (patch clamp) of cloned CMR1 channel

Single channel recordings (cloned CMR1 channel)

CMR1 antibody purification

CMR1 immunohistochemistry in the CNS

The contribution of WMN to this work does, in my assessment, constitute a body of work sufficient to fulfill the requirements for a doctoral degree from UCSF.



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Abstract

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, like menthol, that elicit a cooling sensation. 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/TRPM8, 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. We find that CMR1/TRPM8 is expressed in a small subset (~15%) of sensory ganglia neurons and almost exclusively in small diameter cells (~20 μm) that project to lamina I and II of the spinal cord dorsal horn and trigeminal nucleus caudalis. Co-immunolabelling with other sensory neuron specific markers demonstrates that CMR1/TPRM8 is expressed in a capsaicin receptor (VR1/TRPV1), IB4, CGRP, NF200, and substance P negative cell population. Moreover, CMR1/TRPM8 protein is conspicuously absent from cells expressing the small diameter, unmyelinated neuronal marker peripherin. Under conditions of inflammation and tissue damage, expression of CMR1/TRPM8 increased in dorsal horn neurons, suggestive of a role for CMR1/TRPM8 in cold allodynia. Taken together, our data demonstrate that CMR1/TRPM8 defines a distinct subset of sensory neurons that are immunologically distinct from those expressing other thermosensitive channels and nociceptive markers. This result is

consistent with the specificity model for the perception of noxious vs. innocuous stimuli. These findings, together with the previous identification of the heat-sensitive channels, VR1 and VRL-1, demonstrate that TRP channels detect temperature over a wide range and are the principle sensors of thermal stimuli in the mammalian peripheral nervous system.

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I.

Introduction

Making Sense of Thermosensation

One can only imagine what life would be like without our ability to perceive and interact with our environment. Without our ability to taste, smell, hear, see, touch and feel warm or cold our world would be a very dark and boring but even more so a dangerous place. We need to 'sense' and monitor our environment in order to be able to adapt our behavior to constantly changing environmental conditions. We need to feel pain to elicit regulatory, protective behavior to prevent tissue damage and ensure survival.

The molecular characterization of some of these processes has advanced remarkably in recent years with the identification of genes encoding receptor families for smell and taste ligands (Adler, Hoon et al. 2000), (Axel 1995). However, much remains unknown about the transduction mechanisms of other sensory modalities such as touch and the perception of temperature. It remains one of the most exciting endeavors in these areas of sensory neurobiology to identify the receptors and molecules that provide the interface between our mind and the outside world for these modalities.

The ability to sense ambient temperature is an important tool for many organisms from *Paramecium* (Kuriu, Nakaoka et al. 1996) to higher vertebrates. Exposure to noxious (painful) heat or cold elicits a protective withdrawal-reflex program to prevent tissue damage. The perception of physiological, innocuous temperature allows complex adaptive behavior from thermotaxis behavior of the

roundworm *C. elegans* (Ryu and Samuel 2002) to wearing a warm sweater on one of the cold and windy summer days in San Francisco.

Heat Sensation

In vertebrates, the sensing of ambient temperature is mediated by cutaneous thermoreceptors of the somatosensory system. Early studies have shown the existence of subsets of afferent sensory fibers that are activated in a defined temperature range by either a warm, hot or cold stimulus (Hensel and Zotterman 1951) (Kenshalo and Duclaux 1977) (Hensel and Kenshalo 1969). Together these receptors can detect changes in ambient temperature over a remarkably wide range. This process is initiated when a hot or cold stimulus excites sensory nerve fibers 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 (i.e. action potentials) and relay sensory information to integrative centers in the spinal cord and brain (Fields 1987) (Julius and Basbaum 2001). Noxious (painful) heat is detected by sensory neurons that respond with a 'moderate' thermal threshold of ~43°C or with a 'high' threshold of ~52°C (Raja, Meyer et al. 1999) (Nagy and Rang 1999) (Cesare and McNaughton 1996). 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, or by capsaicin, the main pungent ingredient in 'hot' chili peppers. (Caterina, Schumacher et al. 1997). 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 following tissue injury (Caterina, Leffler et al. 2000; Davis, Gray et al. 2000). 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 (Caterina, Rosen et al. 1999). Both VR1 and VRL-1 belong to the transient receptor potential (TRP) ion channel family, and our laboratory has hypothesized that molecules of this type contribute to thermosensation over a wide temperature range (Caterina, Rosen et al. 1999).

The TRP Family of Ion Channels

The TRP channel family consists of 21 related ion channels (in mammals) whose members share significant sequence homology and predicted structural similarities, such as six transmembrane domains and cytoplasmic N- and C termini. Their overall topology relates TRP channels to voltage–gated K^+ channels and cyclic-nucleotide channels. TRP channels are divided into three subfamilies (TRPC, TRPV, TRPM) (Montell, Birnbaumer et al. 2002) (Montell, Birnbaumer et al. 2002) (Clapham, Runnels et al. 2001). These three subfamilies are distinguished according to overall similarity as well as several unique characteristics. TRPV and TRPC members contain N-terminal ankryn repeats possibly encoding linker domains to the cytoskeleton. TRPC and TRPM members encode a "TRP box" (of unknown function) in the C-terminus. In addition to these classical TRP channels, other ion channel classes are now

thought to be (although distantly) related to TRPC, TRPV, TRPM family members. The new subtypes include TRPP for PKD2-like channels (PKD2 is mutated in polycystic kidney disease), TRPML for mucolipidin-like channels (mucolipidin mutations are responsible for some lysosomal storage disorders) (bassi 2000) and TRPN for NOMPC-like channels (NOMPC is required for mechanosensation in flies) (Kim, Chung et al. 2003; Sidi, Friedrich et al. 2003).

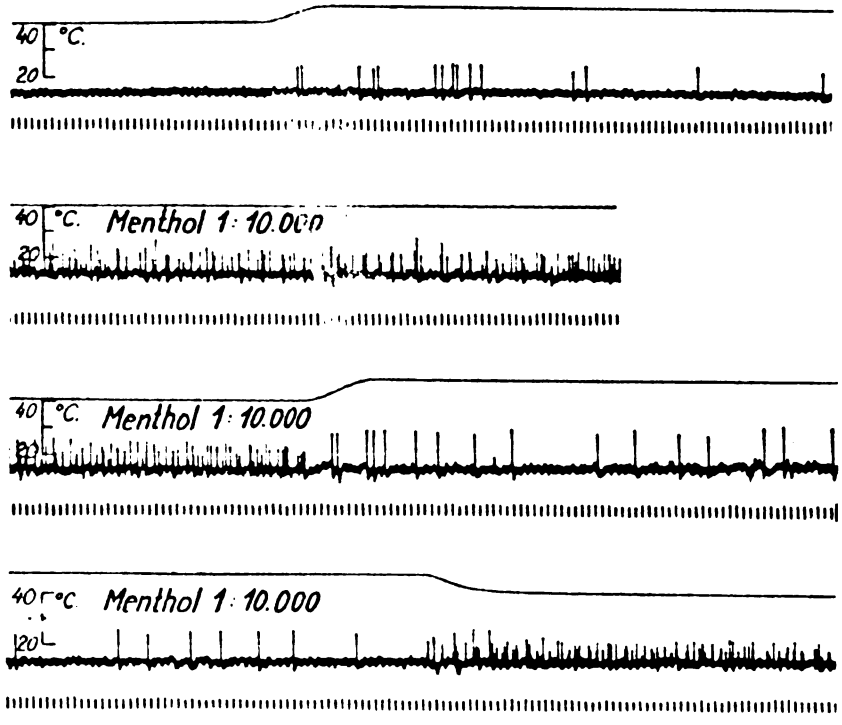
The amino acids that link the fifth and sixth transmembrane segments form a non-selective cation pore permeable to monovalent cations and Ca^{2+}. Thus, activation of these channels shifts the membrane potential to 0mV and raises intracellular Ca^{2+}. Ca^{2+} is one of the most common signal transduction elements in biological systems and membrane depolarization and Ca^{2+} influx may be involved in signaling events downstream of channel activation. TRP channels are widely expressed in the nervous system and in non-excitable cells and exhibit remarkable diversity in their biological roles. Members of this channel family have been implicated in processes ranging from pain perception, vasodilation and taste to male aggression (Montell, Birnbaumer et al. 2002) (Perez, Huang et al. 2002) (Jungnickel, Marrero et al. 2001). Many family members show interesting modes of activation and regulatory mechanisms. In some cases a broad range of stimuli activates a single TRP channel. VR1 (TRPV1) for example is activated by vanilloid compounds (including capsaicin, the active ingredient in chili peppers as well as by anandamide (the endogenous agonist for the cannabinoid receptor CB1), extracellular protons and heat exceeding 42°C (Caterina, Schumacher et al. 1997). The inflammatory mediators

bradykinin and NGF activate this channel through activation of PLC which relieves the channel from PIP_2 mediated inhibition (Chuang, Prescott et al. 2001) establishing a causal link between VR1 and inflammatory pain. Remarkably, other family members (TRPM2, TRPM6 and TRPM7) consist of channel domains linked to an enzymatic domain that may play a role in channel activation (Runnels, Yue et al. 2001) (Perraud, Fleig et al. 2001). However, the physiological role of most family members remains unknown.

Cold Sensation

In contrast to our understanding of noxious heat sensation, little is known about how we detect cold. Previous studies had shown that the sensation of cold is not elicited by an absence of thermal energy (heat) resulting in quiescence of warm or heat activated fibers, but rather by the induction of activity in a subset of fibers specific for cold (Hensel and Zotterman 1951) (Hensel, Andres et al. 1974) (Hensel and Wurster 1969) (Fig I1). In mammals, cool sensation is generally believed to be mediated by a small subpopulation of unmyelinated C and thinly myelinated Aδ primary afferent fibers that discharge in the innocuous temperature range (15-30°C) (Hensel and Zotterman 1951) (Bessou and Perl 1969). Responses to noxious cold (<15°C) are also observed in these fiber types with percentages ranging from 10% to 100%, depending on stimulus intensity and species examined (Kress, Koltzenburg et al. 1992; Simone and Kajander 1996; Simone and Kajander 1997; Caterina, Leffler et al. 2000; Cain, Khasabov et al. 2001). This wide variability in the literature may reflect the fact that thermal

Fig. I1. Cold and menthol sensitive fibers in the cat lingual nerve (from Hensel at al., 1951)



thresholds for cold-sensitive fibers are not as well defined as they are for heat and thus fiber 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 (Suto and Gotoh 1999; Reid and Flonta 2001). However, several other mechanisms have been proposed to explain cold-evoked membrane depolarization, including inhibition of background K^+ channels (Reid and Flonta 2001), activation of Na^+-selective degenerin channels (Askwith, Benson et al. 2001), inhibition of (Na^+/K^+) ATPases (Pierau, Torrey et al. 1974), or differential effects of cold on voltage-gated Na^+ and K^+ conductances (Braun, Bade et al. 1980). Thus it is not clear whether cold excites sensory neurons by activating a discrete "cold receptor" molecule, or by modulating a constellation of excitatory and inhibitory channels on these cells.

Thermal Coding in the Peripheral Nervous System

It has been a central and contentious issue for some time in sensory biology how a specific stimulus is encoded in the activation pattern of receptor cells and how that pattern is decoded by the brain to ensure specificity of stimulus recognition (Buck 1996). More specifically, how do we sense and recognize the different qualities of a stimulus – for example the qualities of 'hot' and 'noxious' for a 50°C stimulus. One model (the 'specificity model') assumes a simple 'labeled line' design where each receptor cell responds to just one quality

of a stimulus (e.g. 'bitter' vs. 'sweet', 'hot' vs. 'cold' or 'noxious' vs. 'innocuous'). Different receptor cells among that population, however, may respond to several different stimuli of that defined quality (e.g. different bitter tasting molecules). The other model (the 'pattern theory') predicts a combinatorial code of overlapping afferent pathways where a receptor cell can be activated by multiple stimulus qualities e.g. different odors in the olfactory epithelium or heat and mechanical stimulation in sensory neurons. This model assumes a topographic map or combinatorial decoding of a neuronal activation pattern in the CNS to determine the qualities of a stimulus. One of the central medical issues with regard to coding is the question whether pain is generated by intense activation of neurons that also encode innocuous stimulus qualities or by activation of neurons that are specifically tuned to detect noxious stimuli (Perl 1998).

Olfactory information seems to be encoded according to the latter model. An individual olfactory neuron expresses only one of thousands of odorant receptor genes, such that neurons are functionally distinct. A specific stimulus quality (a specific 'odor'), however, can activate different types of neurons within the olfactory epithelium. Thus, a topographic map defining either the positions of specific neurons in the epithelium or the positions of their projections has to be employed for odorant recognition (Ngai, Chess et al. 1993; Ngai, Dowling et al. 1993). In some areas of sensory biology a 'labeled line' model may accurately describe stimulus encoding. It has been shown recently that taste receptor cells are not broadly tuned across the different qualities of bitter, sweet and amino acid taste. Individual taste cells are dedicated to transduce only one of these

modalities (Zhang, Hoon et al. 2003). Electrophysiological characterization of DRG neurons has led to the specificity model of pain transduction (Perl 1998). However, such a model might be an oversimplification since the contribution of fibers labeled 'innocuous' to painful sensations is presently unclear e.g. in the case of visceral pain. In addition, a subpopulation of nociceptive neurons (polymodal nociceptors) seem to be generalists in the sense that they can be activated by multiple noxious stimuli e.g. noxious mechanical and thermal stimulation (Fields 1987). This raises the interesting question how we are able to distinguish mechanical pain from heat pain. Decoding of those two overlapping pathways may occur in the CNS and / or input from innocuous fibers may be used by the brain for discrimination.

How a specific thermal stimulus is encoded is presently unclear since some of the thermal transducer molecules that could be used as molecular markers to investigate afferent thermal pathways have not been identified yet. The specificity of thermal sensation could be ensured by either specialized afferent pathways for each thermal modality or a combinatorial code of overlapping afferent pathways. In the latter case a thermal stimulus of defined temperature will activate multiple sensory neuron subtypes whose pattern of activity must then be decoded in the spinal cord or the brain to interpret the actual temperature quality. The identification of taste and olfactory receptor families has opened the door to understanding the basic mechanisms of taste and smell transduction and coding. Similarly, identification of additional thermal detectors for stimulus qualities such as cold will clarify the mechanism of thermal

transduction and will shed light on the cellular and molecular logic of thermal coding in the peripheral nervous system.

The Pharmacology of Cold Sensation and its Use for Expression Cloning

To clarify these issues, we looked for a cold transducer using an expression cloning approach. Our strategy was based on the paradigm set forth by the vanilloid receptor, namely, to ask how plant products such as menthol elicit a cool sensation. It had been known for almost 80 years that the action of menthol is not caused by physical cooling of the skin or mucous membranes via evaporation, but by some chemical action directly on cold receptors. (Goldscheider et al., 1926). Fifty years ago, Hensel and Zotterman (Hensel and Zotterman, 1951) showed that menthol potentiates responses of trigeminal fibers to cold by shifting their thermal activation thresholds to warmer temperatures (Fig. I1). Moreover, they proposed that cooling compounds mediate their psychophysical effects by interacting with a protein that is specifically involved in cold transduction. Previous studies have suggested that menthol depolarizes sensory neurons by inhibiting voltage-dependent Ca^{2+} channels (Swandulla, Carbone et al. 1987) (thereby decreasing activation of Ca^{2+}-dependent K^+ channels) (Schafer, Braun et al. 1986), or by directly activating calcium-permeable channels (Reid and Flonta 2001) (Okazawa, Terauchi et al. 2000). 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. Remarkably, the cloned channel is also activated by cold (8 to 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.

We find that CMR1/TRPM8 is expressed in a small subset of capsaicin receptor (VR1/TRPV1)(-), IB4(-), CGRP(-), NF200(-), and substance P (-) population of sensory neurons, and almost exclusively in small diameter cells. Moreover, CMR1/TRPM8 protein is conspicuously absent from cells expressing the small diameter, unmyelinated neuronal marker peripherin. Under conditions of inflammation and tissue damage, expression of CMR1/TRPM8 increased in dorsal horn neurons, suggestive of a role for CMR1/TRPM8 in cold allodynia. Taken together, our data demonstrate that CMR1/TRPM8 defines a distinct subset of sensory neurons that are immunologically distinct from those expressing any other thermosensitive channels and nociceptive markers. This result is consistent with the specificity model for the perception of noxious vs. innocuous stimuli.

More Candidate Thermal Detectors Among the TRP Channel Family

Since completion of this work several additional temperature sensitive members of the extended TRP ion channel family have been identified. TRPV 4, originally identified as an osmosensitive channel in kidney, brain and inner ear (Strotmann, Harteneck et al. 2000) (Wissenbach, Bodding et al. 2000) (Liedtke, Choe et al. 2000), has recently been shown to be activated by temperatures > 25°C. Since TRPV4 is expressed in neurons of the hypothalamus this channel may participate in the central regulation of body temperature (Guler, Lee et al. 2002). TRPV3, another member of the VR1 related TRPV channel family has been shown to respond to heat with a threshold between 31°C and 39°C (Xu, Ramsey et al. 2002) (Smith, Gunthorpe et al. 2002) (Peier, Reeve et al. 2002). At this point it is not clear whether TRPV3 and TRPV4 contribute to somatic thermosensation in sensory neurons or are even expressed in primary afferent cells. However, both channels are expressed in keratinocytes (Guler, Lee et al. 2002) (Peier, Reeve et al. 2002) and have been proposed to constitute warm sensors in the skin by inducing activity in adjacent nerve terminals via release of signaling factors from keratinocytes.

Closure of background (leak) K^+ channels has been suggested to contribute to the transduction of cold in sensory neurons (Reid and Flonta 2001) (Maingret, Lauritzen et al. 2000). Recent data suggest that cold sensitive TG neurons lack a 4-AP sensitive voltage-gated K^+ conductance (IK_D) that

hyperpolarizes the cell and prevents cold-initiated depolarization and action potentials after closure of background K^+ channels in cold insensitive neurons. TREK-1, a two-pore K^+ channel (Maingret, Lauritzen et al. 2000) has been proposed as a possible candidate for the cold inhibited background conductance. However, the true molecular identity of IK_D and background conductance awaits future experimental studies.

Another TRP-like channel (ANKTM1) has been shown recently to be gated by cold below ~17°C and to be expressed in a small (4%) subpopulation of sensory neurons (Story, Peier et al. 2003). This channel is not activated by menthol compounds that convey a pleasant/cool feeling and has a lower threshold of activation than CMR1. Thus, ANKTM1 may be involved in transmitting a noxious cold stimulus, a suggestion that is consistent with the channel being expressed in nociceptive (VR1, CGRP positive) neurons. ANKTM1 has little sequence similarity to the three mammalian subfamilies (TRPC, TRPV, TRPM) of TRP channels and is most closely related to invertebrate TRP-like channels of the TRPN subfamily (Jaquemar, Schenker et al. 1999).

II.

Identification of a Cold Receptor Reveals a General Role for TRP Channels in Thermosensation

Menthol and Cold Activate an Outwardly Rectifying, Ca^{2+}-permeable Channel on Trigeminal Sensory Neurons

Body surfaces innervated by trigeminal fibers, such as the eye and tongue, are particularly sensitive to cold and cooling compounds (Eccles 1994). 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 increases in intracellular free-calcium in a relatively small sub-population of trigeminal neurons (Fig. 1a), consistent with work from others using DRG cultures (Suto and Gotoh 1999; Okazawa, Terauchi et al. 2000; Reid and Flonta 2001). Menthol and cold excited a largely overlapping subset of neurons, a significant fraction of which (54.5 ± 6.1%) 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 approximately half of the menthol/cold sensitive cells may be categorized as such. Since menthol is also called 'pepper'mint and might increase intracellular Ca^{2+} by activating the capsaicin receptor VR1 we next investigated whether menthol functions as an agonist for the cloned capsaicin receptor VR1 and whether VR1 is required for menthol/cold responses. Menthol could not activate VR1 expressed in HEK cells and we observed robust menthol/cold responses in DRG neurons from VR1 -/- mice (Fig. 1b,c). Thus, we concluded that the capsaicin receptor is neither necessary nor sufficient for menthol evoked activation of sensory neurons.

Whole-cell patch-clamp recordings from trigeminal neurons showed that menthol or cold elicited rapidly developing membrane currents (Fig. 2a,b) that were characterized by pronounced outward rectification (i.e. responses at positive holding potentials were substantially greater than those at negative voltages) (Fig. 2c). These currents reversed close to 0 mV (E_{rev} = -3.6 ± 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 (Reid and Flonta 2001). Indeed, ion substitution experiments showed little discrimination among monovalent cations, but revealed significantly higher permeability for calcium ions (P_{Ca}/P_{Na} = 3.2; P_K/P_{Na} = 1.1; P_{Cs}/P_K = 1.2; n = 6) (Fig. 2d). We found these biophysical properties particularly interesting because they are reminiscent of VR1 and other TRP channels (Clapham, Runnels et al. 2001).

Room temperature menthol evoked responses in a dose-dependent manner (Fig. 2e) with a half-maximal effective concentration (EC_{50}) of 80 ± 2.4 μM, a potency similar to that determined for DRG neurons using calcium imaging (Okazawa, Terauchi et al. 2000). Fitting these data with the Hill equation suggests that receptor activation requires the binding of more than one menthol molecule (η = 2.2). In addition to menthol, the mint plant synthesizes structural analogues that also elicit a cooling sensation, albeit 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. 2a). Cold also elicited membrane currents in a dose-dependent manner, with a

characteristic temperature threshold of 27.1 ± 0.5°C (n = 4) (Fig. 2f). As reported for DRG neurons, menthol potentiated cold responses and shifted the thermal threshold to higher temperatures (29.6 ± 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. 2a). 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 both stimuli activate currents with very similar biophysical properties, supporting the idea of a common molecular site of action.

Expression Cloning of a Receptor for Menthol and Other Cooling Compounds

Because menthol and cold activate native conductances with intrinsic and significant permeability to calcium ions, we reasoned that a calcium imaging-based screening strategy (Caterina, Schumacher et al. 1997) could be used to isolate a functional cDNA encoding a menthol or cold-sensitive receptor. In light of the higher prevalence of these responses in trigeminal cultures (14.8% versus 7.4% for DRG, n = 745 and 1425 cells, respectively), we constructed a cDNA expression library from this tissue. Pools containing ~10,000 clones were transfected into human embryonic kidney-derived HEK293 cells, which were subsequently loaded with the calcium-sensitive fluorescent dye, Fura-2, and microscopically examined for changes in intracellular calcium upon exposure to

room temperature menthol (500 μM). In this way, we identified a single cDNA that conferred menthol sensitivity to transfected cells (Fig.3).

As noted above, menthol is one of several naturally occurring or synthetic cooling compounds whose relative potencies in psychophysical assays span a wide range (Eccles 2000). To assess their effects 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) (Wei and Seid 1983), which showed approximately 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. 4a, b). Eucalyptol, the main constituent of oil of *Eucalyptus*, also elicited membrane currents, but with decreased efficacy and potency (3.4 ± 0.4 mM) compared to 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. 5a). Like native menthol-evoked responses in trigeminal neurons, these currents showed relatively high permeability to calcium, little selectivity among monovalent cations (P_{Ca}/P_{Na} = 3.3; P_K/P_{Na} = 1.2; P_{Cs}/P_K = 1.1; n = 4 - 9) (Fig. 5b) and could be

partially blocked by 200 μM La^{2+} (Fig. 5b, insert). Menthol-evoked currents also showed significant desensitization (53.9 ± 1.7% decrease in peak current between the first and second application, n = 3), but interestingly, little desensitization (9.1 ± 7%, n = 3) was observed in nominally calcium-free bath solution (Fig. 5c). 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 (not shown). When measured at the single channel level, menthol-evoked currents also showed pronounced outward rectification. These responses were characterized by brief, transient openings and had a slope conductance of 83 ± 3 pS at positive potentials (Fig. 5d, 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.

The Menthol Receptor is also Activated 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 ~35°C to ~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 (ranging from 0.2 to 1°C/second) did not influence threshold or saturation temperature (Fig. 6a, 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 ± 0.4°C and saturated at 8.2 ± 0.3°C. (n = 12) (Fig. 6c). Consistent with the behavior of native cold currents, addition of a sub-activating concentration of menthol (20 μM) to the perfusate increased threshold and saturation temperature to 29.7 ± 0.3°C and 15.6 ± 0.4°C, respectively (n = 7) (Fig. 6c). Interestingly, we found that menthol is a more efficacious agonist than cold since saturating cold-evoked currents were smaller than those obtained with a maximal dose of room temperature menthol (67.4 ± 1.9%, n = 7) (Fig. 6d).

We also examined cold-evoked currents in menthol receptor-expressing HEK293 cells. As observed for native cold responses (Fig. 2c), current-voltage relationships for the cloned channel showed steep outward rectification (Fig. 6e) and were markedly potentiated by a sub-activating dose of menthol (10 μM). 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 (Chuang, Prescott et al. 2001). Native cold-evoked responses in sensory fibers or cultured DRG neurons show adaptation to a prolonged thermal stimulus lasting several minutes (Kenshalo and Duclaux 1977; Reid and Flonta 2001). 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. 6f). This observation suggests that the cloned receptor also adapts to thermal challenges, and that this effect is reversed upon heating. Desensitization to cold differed from that observed with menthol because it was independent of extracellular calcium (not shown).

Interestingly, VR1 shows similar behavior in that desensitization to chemical (capsaicin) or thermal (heat) stimuli are calcium-dependent or -independent, respectively. Taken together, our observations show that the cloned receptor, which we now designate cold-menthol receptor subtype 1 (CMR1), has properties identical to endogenous cold/menthol currents observed in sensory neurons (Suto and Gotoh 1999; Okazawa, Terauchi et al. 2000; Reid and Flonta 2001).

CMR1 is a Member of the TRP Channel Family

The CMR1 cDNA contains an open reading frame of 3312 bp that is predicted to encode a protein of 1104 amino acids with a molecular mass of 128 kD (Fig. 7a). 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 N- and C-terminal cytoplasmic tails (Clapham, Runnels et al. 2001). 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 (Perraud, Fleig et al. 2001; Runnels, Yue et al. 2001; Sano, Inamura et al. 2001). In contrast, CMR1 has a significantly shorter C-terminal region (Fig. 7b) 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 (Tsavaler, Shapero et al. 2001). This presumptive TRP channel, called trp-p8 (or TRPM8), may therefore be the human orthologue of rat CMR1. Aside from prostate epithelium, TRPM8 was found to be expressed by a variety of tumors, including prostate, melanoma, colorectal, and breast carcinoma (Tsavaler, Shapero et al. 2001).

III.

Expression of the Cold-menthol Receptor CMR1 in the PNS and CNS Defines a Unique Subpopulation of Sensory Neurons Involved in Cold Sensation

CMR1 is Expressed in DRG and TG Sensory Neurons

The presence of CMR1in sensory neurons was not assessed by Tsavaler, L. et al. 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 ~6 and 4.5 kb were detected in both neuronal tissues (Fig. 8a), similar to that reported for TRPM8 expression in human prostate (Tsavaler, Shapero et al. 2001). At the cellular level, CMR1 transcripts were found in a subset of sensory neurons with small-diameter cell bodies (18.2 ± 1.1 μm and 21.6 ± 0.5 μm in dorsal root and trigeminal ganglia, respectively) (Fig. 8b), similar in size to VR1-expressing cells (19.2 ± 0.3 μm) (Caterina, Rosen et al. 1999). CMR1 transcripts were more prevalent in trigeminal versus 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 suggested that CMR1 is expressed by a sub-population of C-fibers (and possibly Aδ fibers) representing <20% of all primary afferent neurons.

CMR1 Expression Defines a Unique Subpopulation of Sensory Neurons

For a more detailed analysis of CMR1 expression in the nervous system, a peptide matching to the C-terminus of rat CMR1/TRPM8 (a.a. 1089-LNDLKGLLKEIANKIK -a.a. 1104) was generated and inoculated into rabbits (AnaSpec, Inc., California and Strategic Biosolutions, California). Crude bleeds

(data not shown) and affinity-purified antibodies were tested by Western analysis using protein extracts prepared from pcDNA3 (control) and rCMR1 transfected HEK293 cells (Fig. 9). A 125 kDa protein band, corresponding to the predicted polypeptide size of rat CMR1 (128 kDa) was observed in CMR1 expressing cells, but not in those transfected with vector control (Fig. 9). In addition, antibody specificity was determined by the absence of this protein band when the primary antibody was pretreated with the peptide used to generate the antibody but not with a peptide generated from an N-terminal region (data not shown).

We next examined CMR1 expression in paraformaldehyde fixed trigeminal and dorsal root ganglia from adult rats. Immunofluorescence was detected in a small fraction of cells in both types of sensory ganglia and corresponds to small diameter neurons (mean diameter 21.6 ± 0.5 μm and 18.2 ± 1.1 μm for TG and DRG, respectively). Moreover, the percentage of total ganglionic cells expressing CMR1 protein (~15 and ~8 % for TG and DRG, respectively) are similar to those found functionally to be menthol and cold sensitive in ganglionic primary cultures. (McKemy, Neuhausser et al. 2002) (Viana, de la Pena et al. 2002). These data provide supportive evidence for the notion that cold sensation is mediated by small diameter afferent C fibers, as shown by several ex vivo studies using mammalian afferent nerve recordings (Bessou and Perl 1969) (Kress, Koltzenburg et al. 1992). In addition, we observed immunofluorescence in a small number of neurons of the nodose ganglion indicating that CMR1 is expressed in the visceral nervous system (Fig. 11c).

To further characterize the cells expressing CMR1 in sensory ganglia, we next examined co-localization of CMR1 with other neuronal markers. Initially, the expression profiles of CMR1 and the heat-sensitive TRP channels VR1 were examined. Several groups have described a population of sensory cells in primary culture that are responsive to both menthol and capsaicin, suggestive that these cells express both CMR1 and VR1. In addition, fiber recordings using the so-called skin-nerve prep have demonstrated capsaicin- and menthol-induced increases in spike frequency in a single afferent (M. Koltzenburg, communication). In conflict with this pharmacological and functional data, Peier et al. (Peier, Moqrich et al. 2002) combined immunochemistry and in situ analysis to show that CMR1 and VR1 were not co-expressed in the same cell in sensory ganglia. To resolve this contradiction, we used our CMR1 and VR1 specific antibodies to examine the expression patterns in 15 μm sections of trigeminal and dorsal root ganglia. We found, as suggested by Peier et al, that CMR1 and VR1 were not co-expressed in either adult or neonatal (P0-2) sensory neurons in either TG or DRG (Fig. 10a, b). We next examined co-expression of CMR1 and VR1 cultures of neonatal TG and DRG neurons. In acute cultures, up to six hours post-plating, CMR1 and VR1 are segregated into different cell populations. However, upon prolonged culture, ~16 hrs, approximately 50% of neurons isolated from both TG and DRG that are immunoreactive for CMR1 also express VR1 (Fig. 10 c). Since expression of the capsaicin receptor has been shown to

be regulated by NGF (Winter, Forbes et al. 1988) this observation may be the result of the exposure of sensory neurons to NGF in the culture medium.

Our colocalization experiments did not detect expression of CGRP, substance P or IB4 in sensory neurons expressing CMR1 (Fig. 11a-c). We next investigated the presence of NF 200, a marker for large diameter myelinated fibers, and peripherin which labels small diameter, unmyelinated neurons. Interestingly, CMR1/TRPM8 protein is absent from cells expressing both NF200 and peripherin and double staining for peripherin and NF 200 reveals a small subset of unlabeled sensory neurons (Fig.11d-f). This result is interesting since cold sensitive fibers are generally believed to comprise a subgroup of the C-fiber population.

CMR1 Fibers Project to the Spinal Cord Dorsal Horn and Trigeminal Nucleus

Lamina I of the spinal cord dorsal horn and the trigeminal nucleus caudalis of the medulla are considered to be the target for thermoreceptors receiving direct input from Aδ and C primary afferent fibers. Thus, our immunocytochemical analysis of CMR1 expression in the CNS focused on the distribution of CMR1 in the spinal cord and brainstem, which receive their central projections from DRG and TG, respectively. We observed intense immunoreactivity in the terminals of afferent fibers projecting to the superficial layers of the trigeminal nucleus caudalis in the medulla (Fig. 12a,b) and the spinal cord dorsal horn at all levels (cervical, thoraic, lumbar and sacral) (Fig. 12c-f). In contrast to VR1 (+) fibers

which occasionally extend to lamina V and X (especially in the sacral cord), CMR1 staining was confined to lamina I & II (Fig. 13d). Within the spinal cord dorsal horn CMR1 (+) fibers terminate preferentially in lamina I, lamina IIo and the medial part of lamina IIi, similar to VR1 and substance P expressing fibers (Fig. 13a-e). However, using confocal microscopy we did not observe costaining of individual fibers expressing CMR1 and VR1 or Subst P, confirming the staining pattern of corresponding cell bodies in the gangion. In addition, we did not observe colocalization of CMR1 (+) and IB4 (+) fibers which project mostly to lamina IIi. A similar pattern of immunoreactivity was observed in the trigeminal nucleus caudalis of the medulla. Consistent with the staining of nodose ganglion cells we observed a small number of CMR1 + fibers in the nucleus of the solitary tract, a nucleus of the medulla that receives vagal projections from visceral organs via the nodose (inferior) ganglion (Fig. 13f).

CMR1 Expression is Upregulated in Inflammation

Tissue injury and inflammation will induce both hyperalgesia and allodynia to thermal stimuli. Indeed, it has been demonstrated that inflammation induced by injection of CFA into the hind paw will induce cold allodynia. To test for a potential role of CMR1 in cold allodynia, we compared the expression levels of CMR1 in spinal cord sections of adult rats with CFA-induced tissue inflammation. At 3 days post-injection, immunoreactivity for CMR1 was markedly increased on the ipsalateral side in comparison to the contralateral side (Fig. 14). This increase in CMR1 protein levels was maintained at ten days post injection with

an increase in immunoreactive density of 2-fold. Similar increases in VR1 immunoreactivity have been observed under similar conditions. We tested other injury paradigms such as nerve ligation and as with inflammation, CMR1 levels are upregulated at the level of the spinal cord. Interestingly, VR1 is down regulated in this injury paradigm.

IV.

Discussion

A General Role for TRP Channels in Thermosensation

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. Indeed, as few as three ion channels (CMR1, VR1, and VRL-1) may provide coverage for a remarkably wide range of temperatures (8 to 28°C, >43°C, 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 to 40°C), suggesting that additional molecules or mechanisms are involved in mediating thermosensation in these temperature ranges. This notion may be supported by the recent identification of other TRP channels that could act as thermal transducers in the noxious cold (ANKTM1) and warm range (TRPV3, TRPV4) (Guler, Lee et al. 2002; Smith, Gunthorpe et al. 2002; Xu, Ramsey et al. 2002; Story, Peier et al. 2003).

However, the contribution of TRPV3 and TRPV4 to thermosensation is presently unclear since neither channel seems to be expressed in sensory neurons (W.M.N. unpublished observation, (Guler, Lee et al. 2002)). However, both channels are expressed in keratinocytes (Guler, Lee et al. 2002) (Peier, Reeve et al. 2002) and warm currents that resemble TRPV4 currents in

transfected cells have been recorded from a mouse keratinocyte cell line (Chung, Lee et al. 2003). Thus, they may constitute warm transducers in the skin by inducing activity in adjacent nerve terminals via release of signaling factors from keratinocytes. Since TRPV4 is expressed in the hypothalamus it may also play a role in regulation of body temperature. ANKTM1 is expressed in only a tiny fraction of DRG and TG neurons (Story, Peier et al. 2003) and if it is involved in the transduction of noxious cold, this channel cannot mediate activation of the large number of sensory fibers by cold < 15 °C that has been reported (Simone and Kajander 1996). This suggests the involvement of other channels in the transduction of noxious cold. Furthermore, thermosensitive TRP channels may play additional roles other than thermotransduction for example in inflammation, response to thermal injury, cell proliferation and wound healing. These issues will most likely be investigated by future studies using TRPV3, TRPV4 and ANKTM1 null mice.

A Cellular Model for Cold Transduction

The ion channel encoded by the CMR1 gene can be activated in neurons and in CMR1 transfected cells by cold and several natural (menthol, eucalyptol, camphor) and synthetic (icilin) agents that elicit cold sensations in humans. Together with the isolation of a cDNA by expression cloning from sensory neurons and the expression pattern of the CMR1 protein in sensory neurons, this makes a strong case for a crucial role of this channel in the transduction of cold.

In addition, the projection pattern of CMR1 fibers to the CNS is consistent with expression of CMR1 in sensory neurons that mediate cold transduction in vivo and the proposed role for CMR1 as a thermosensor. Sensory fibers expressing CMR1 terminate almost exclusively in lamina I and II of the spinal cord dorsal horn and the trigeminal nucleus caudalis and these superficial layers have traditionally been considered to be the target for temperature sensitive fibers (Fruhstorfer and Hensel 1973; Han, Zhang et al. 1998).

However, on the cellular level other non-TRP channels may take part in the transduction of a cold stimulus into an excitatory signal. Cold sensitive fibers fire single or multiple bursts of action potentials in a pattern that reflects the speed and intensity of cooling (Kenshalo and Duclaux 1977). What are the molecular players that shape the pattern of these action potentials? Cold-sensitive neurons (and other neurons) respond to cooling with closure of a resting K+ conductance (Reid and Flonta 2001) causing membrane depolarization and increased excitability to a depolarizing current. TREK-1 (a two pore domain K+ channel) has been proposed as a candidate but the true molecular identity of this leak channel remains unknown. In addition, cold sensitive sensory neurons seem to lack a slow transient voltage gated 4-AP sensitive potassium current that is present in cold insensitive neurons and acts as an excitability brake during cooling by opposing depolarization (Viana, de la Pena et al. 2002). Expression and activation of CMR1 in this background of K+ channels may provide the decisive depolarizing force that causes sufficient membrane depolarization to cause action potentials and stimulus transduction.

The pattern of these action potentials may be shaped by the presence of a hyperpolarization-activated cyclic-nucleotide gated K+ channel (HCN) that influences membrane excitability in excitable cells of the brain and heart by promoting membrane repolarization (Ludwig, Zong et al. 1998). Cooling causes inhibition of this cationic inward current and the resulting hyperpolarization may limit the range of firing and cause the characteristic burst(s) of action potentials during a cold stimulus in CMR1 expressing cells.

Coding of Cold Stimuli

Our immunohistochemistry data show that expression of CMR1 is limited to a unique subpopulation of sensory neurons that do not express other neuronal markers like VR1, peripherin, NF200, IB4, CGRP and substance P. Thus, CMR1 represents a novel marker for a previously uncharacterized subpopulation of sensory neurons and a valuable tool to decipher coding and specificity in sensory transduction.

The specificity theory of pain transduction (Perl 1998) assumes the existence of sensory neurons that specialize in the detection of a unique sensory modality. According to this theory, innocuous cold, which can readily be distinguished from noxious cold and heat, should activate a subgroup of sensory neurons that are not activated by any noxious stimulus. Previous functional studies of DRG and TG neurons in culture (McKemy, Neuhausser et al. 2002) (Viana, de la Pena et al. 2002) (Reid, Babes et al. 2002) have demonstrated that

a significant proportion of menthol responsive neurons also respond to capsaicin, implying coexpression of CMR1 and the heat receptor VR1. Our analysis of CMR1 protein expression did not detect overlap of CMR1 and VR1 immunoreactivity in either adult or neonatal TG or DRG sensory neurons or in acute cultures, up to six hours post-plating. However, in prolonged culture a proportion of CMR1 expressing neurons also expresses VR1. We conclude that expression of one or both thermosensitive channels might be upregulated by the culture conditions, possibly via the effects of NGF or other growth factors. It is unclear at this point whether co-expression in culture is indicative of a physiological paradigm found in vivo or represents a culture artefact. Since the recently identified low-threshold cold sensitive ion channel ANKTM1 is expressed in VR1 (+) neurons, we infer that CMR1 (+) sensory neurons are also ANKTM1 negative (Story, Peier et al. 2003). Thus, expression of CMR1 is mutually exclusive with any known nociceptive marker and thermosensitve channel and our results are consistent with the specificity model for the perception of noxious vs. innocuous stimuli. CMR1 activity may, however, modulate activity in nociceptive neurons centrally and we cannot exclude the possibility that continued activity in CMR1 expressing fibers at temperatures below the threshold for ANKTM1 activation may contribute to painful sensations at these temperatures. However, a subset of VR1 (+) fibers that are activated by noxious heat is expressing ANKTM1 and will therefore also be activated at painful cold temperatures. Since we can clearly distinguish between a noxious cold and a

noxious heat stimulus, integration of information in the CNS (patterning) must also play a role in the discrimination of suprathreshold noxious modalities.

For the future, analysis of the differential neural connections of thermosensitve fibers expressing CMR1, VR1, VRL-1 and ANKTM1/VR1 to spinal cord neurons and the different projection pathways of these second order neurons to higher centers in the CNS will provide a more comprehensive picture of how we perceive temperature.

Possible Role for CMR1 in Noxious Cold Transduction and Cold Allodynia

While CMR1 can clearly underlie activity in the relatively small subpopulation of cool/menthol sensitive fibers, it cannot account for the reportedly large proportion of nociceptive afferent fibers that respond to sub-zero temperatures (Simone and Kajander 1996; Simone and Kajander 1997; Cain, Khasabov et al. 2001) since its expression is restricted to ≤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). If one considers 15°C as an approximate demarcation between cool and cold (Hensel and Zotterman 1951; Rainville, Chen et al. 1999), then CMR1 clearly encodes temperatures that encompass all of the innocuous cool (15 to 28°C) and part of the noxious cold (8 to 15°C) range. Furthermore, CMR1 could contribute to depolarization of fibers at temperatures in the ultra-cold range (<8°C) if the

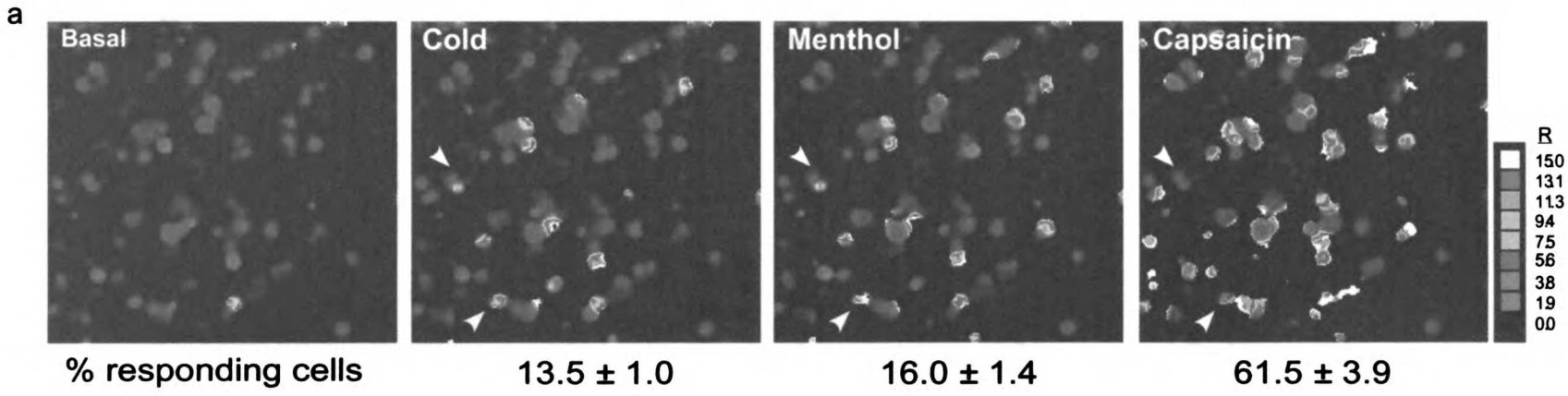
channel is modulated in a manner that extends its sensitivity range *in vivo*. Indeed, several TRP channels are regulated by receptors that couple to phospholipase C (PLC) and the thermal activation threshold for VR1 can be markedly shifted to lower temperatures by inflammatory agents (e.g. bradykinin and nerve growth factor) that activate this pathway (Clapham, Runnels et al. 2001) (Harteneck, Plant et al. 2000; Premkumar and Ahern 2000; Chuang, Prescott et al. 2001). Other inflammatory products, such as protons and lipids, appear to interact with VR1 directly (Zygmunt, Petersson et al. 1999; Hwang, Cho et al. 2000; Jordt, Tominaga et al. 2000). Thus, CMR1 might be modulated in a similar manner, expanding its range of temperature sensitivity under normal or pathophysiological conditions. Interestingly, our results show that expression of CMR1 is upregulated in CFA induced inflammation. CMR1 may therefore transduce innocuous cold under physiological conditions but may take part in painful sensations under inflammatory conditions. CMR1 may play a role in cold allodynia/hyperalgesia by either increasing the number of cold sensitive cells or the extent of cold-induced neural activity due to increased channel activity.

A Possible Role for CMR1 in Other Tissues

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 (Eccles, 1994; Eccles, 2000). 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 (Wei 1981), and while 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 (Takeuchi, Tamaoki et al. 1994), an observation that is intriguing in light of the observation that TRPM8 transcripts are expressed in normal prostate epithelium (Tsavaler, Shapero et al. 2001). Physiological roles for TRP channels in the prostate are currently unknown, but expression or repression of several TRP genes in tumor cells suggests that these proteins influence cell proliferation, possibly through their ability to regulate intracellular calcium levels (Clapham, Runnels et al. 2001). As 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, 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.

Fig. 1. Menthol and cold increase intracellular Ca2+ in a subset of DRG and TG neurons independent from the capsaicin receptor VR1.



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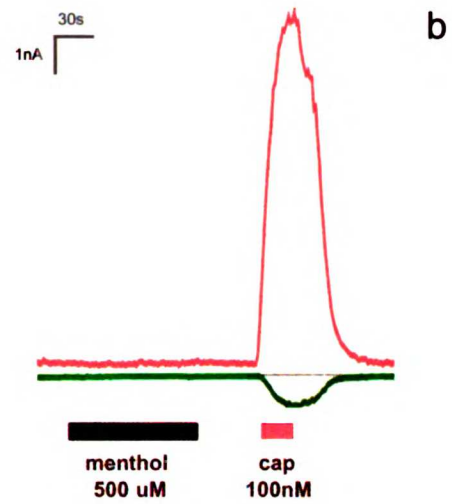

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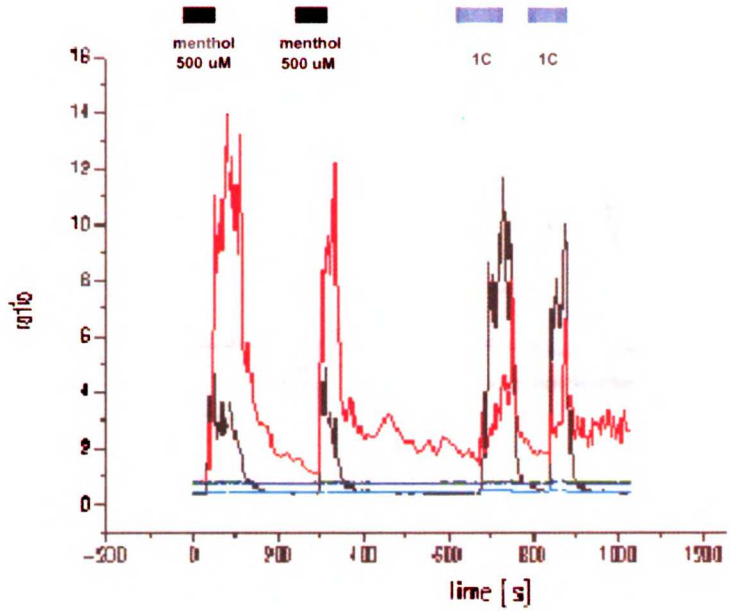
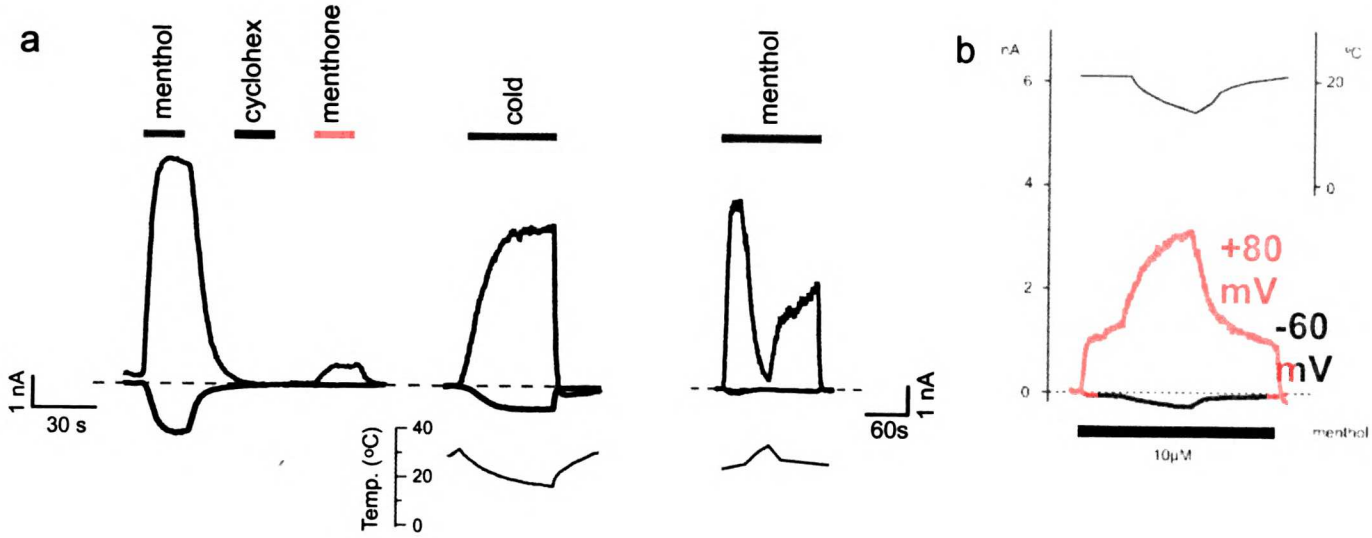


Fig. 2. A subset of trigeminal neurons express an outwardly rectifying Ca2+-permeable channel activated by menthol and cold.



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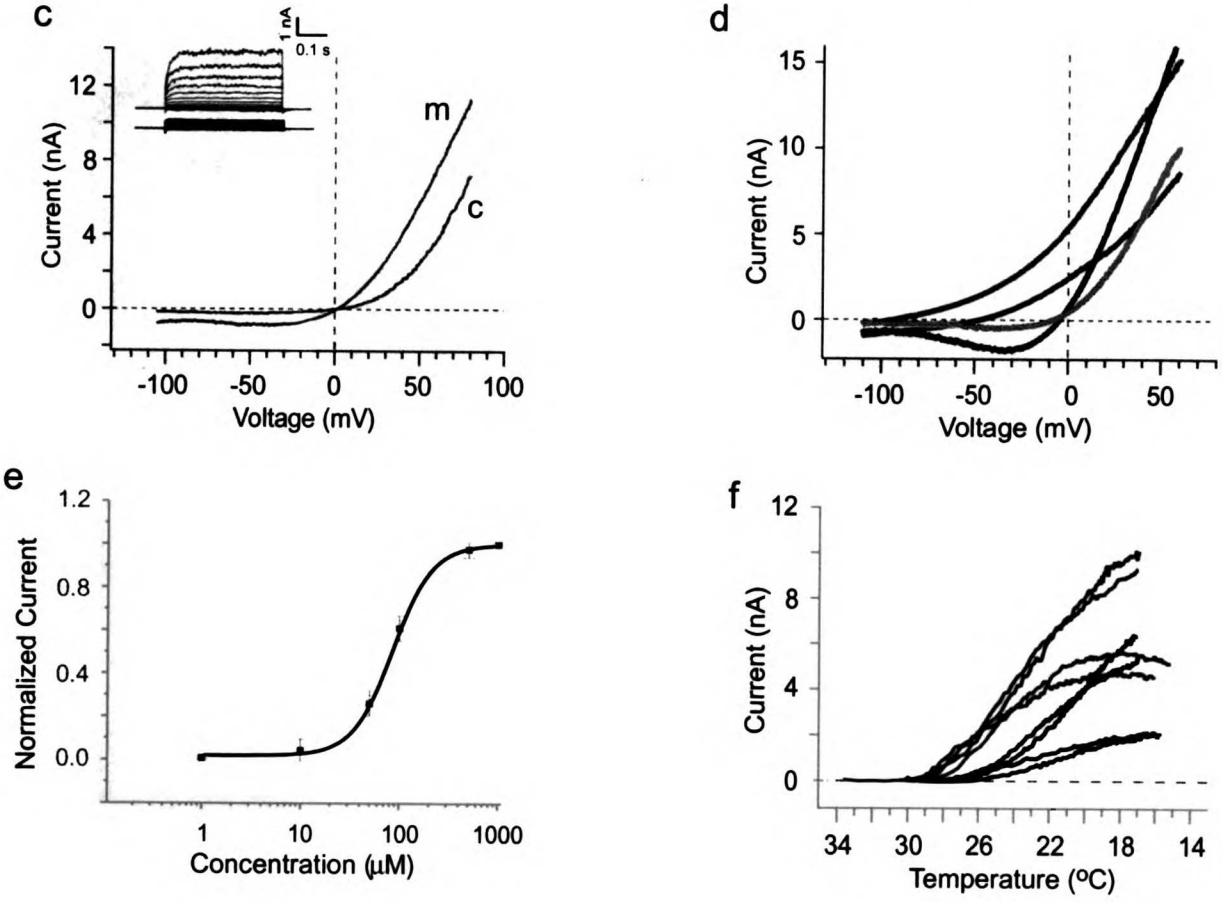


Fig. 3. Expression cloning strategy for a menthol receptor.

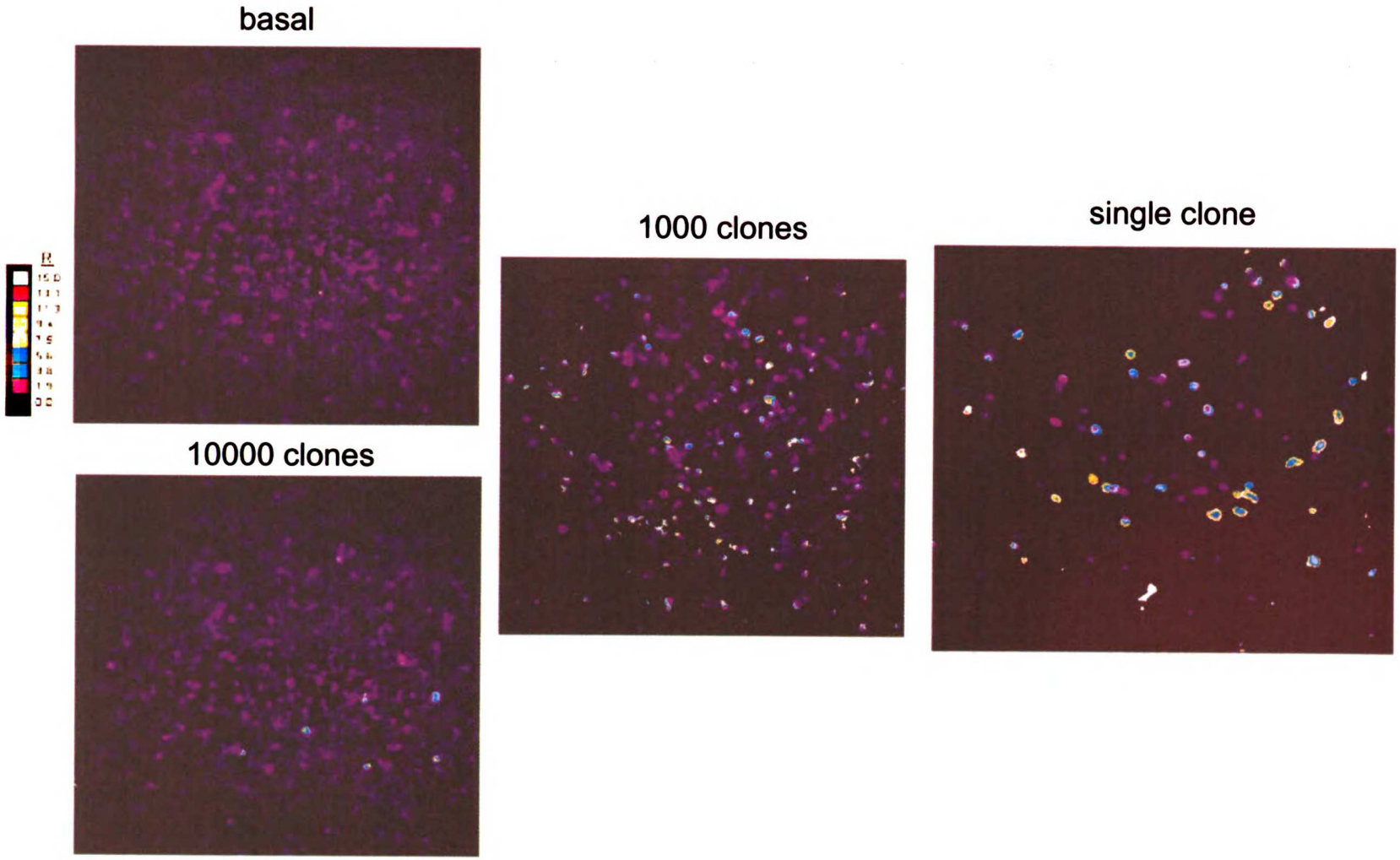
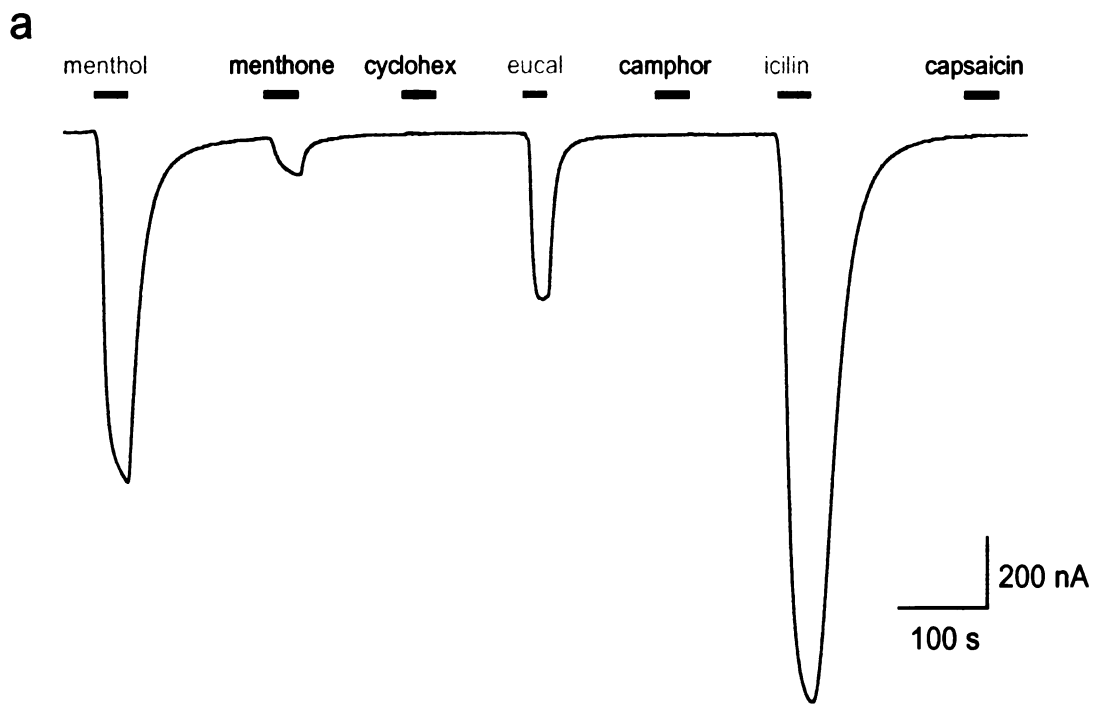


Fig. 4. Cooling compounds activate the cloned receptor.



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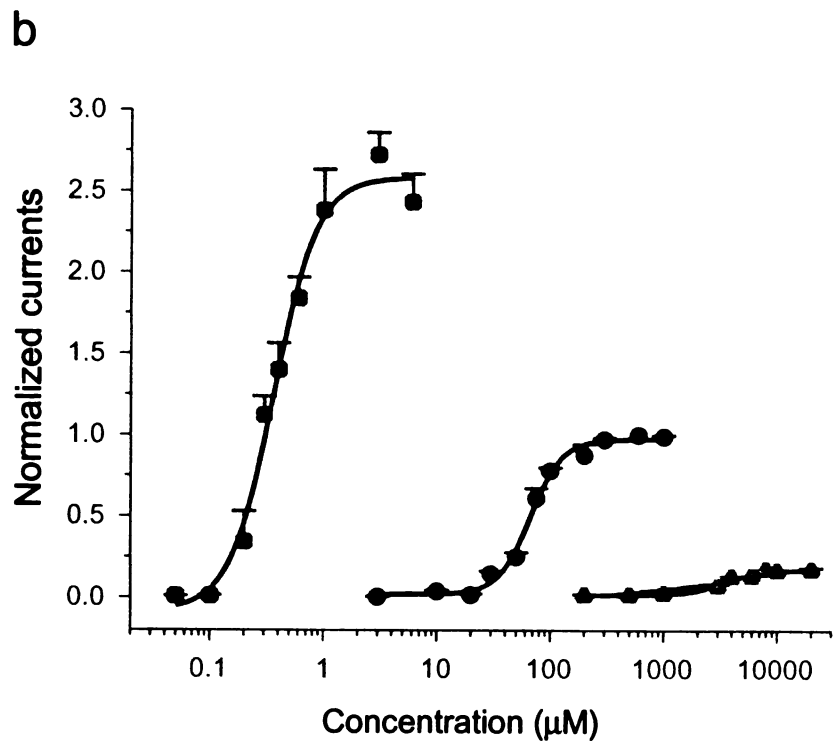
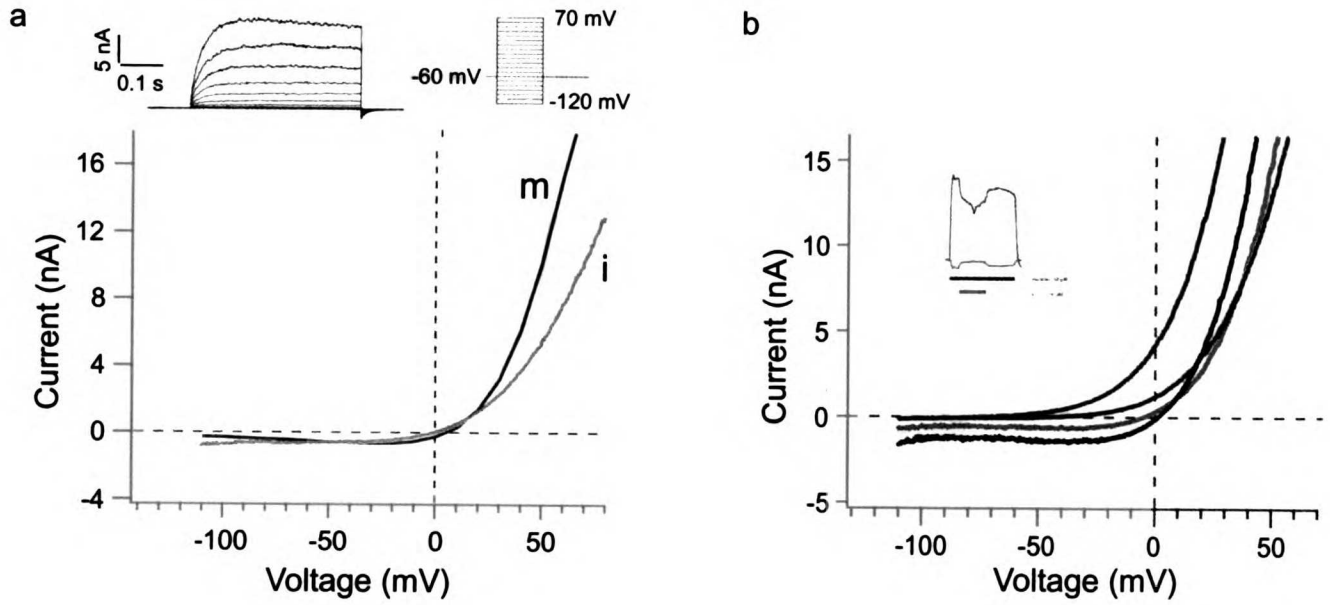


Fig. 5. Electrophysiological properties of menthol-induced currents in transfected HEK293 cells.



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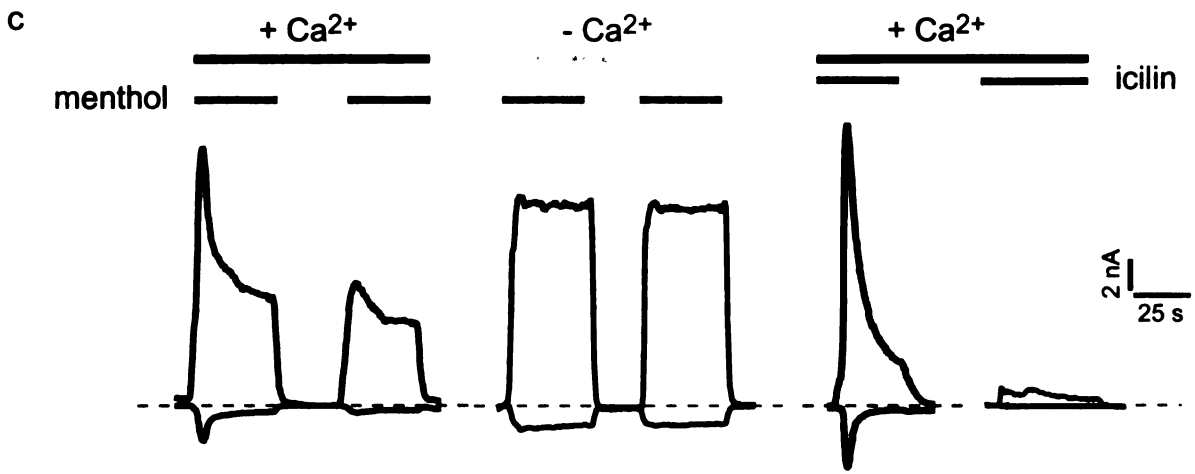

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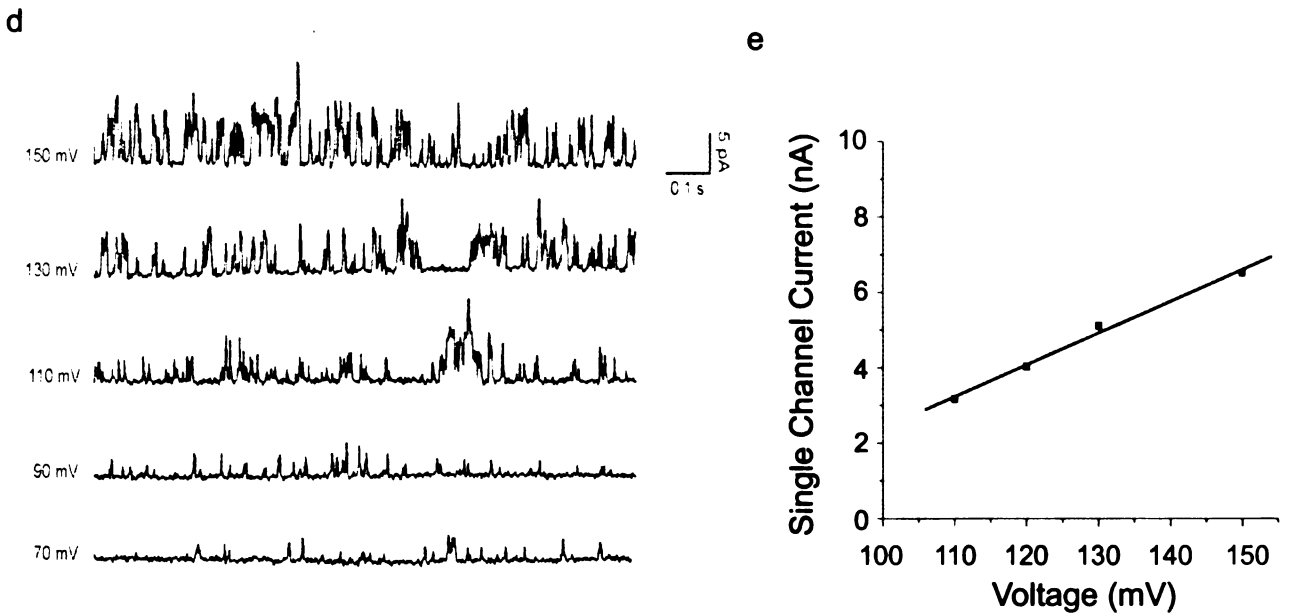
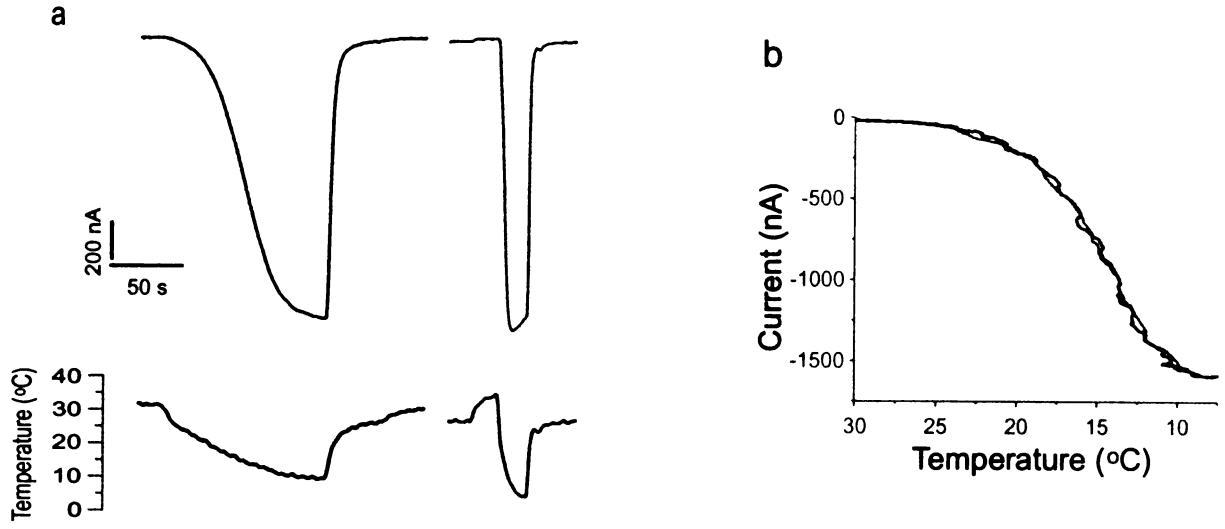


Fig. 6. The menthol receptor is cold sensitive.



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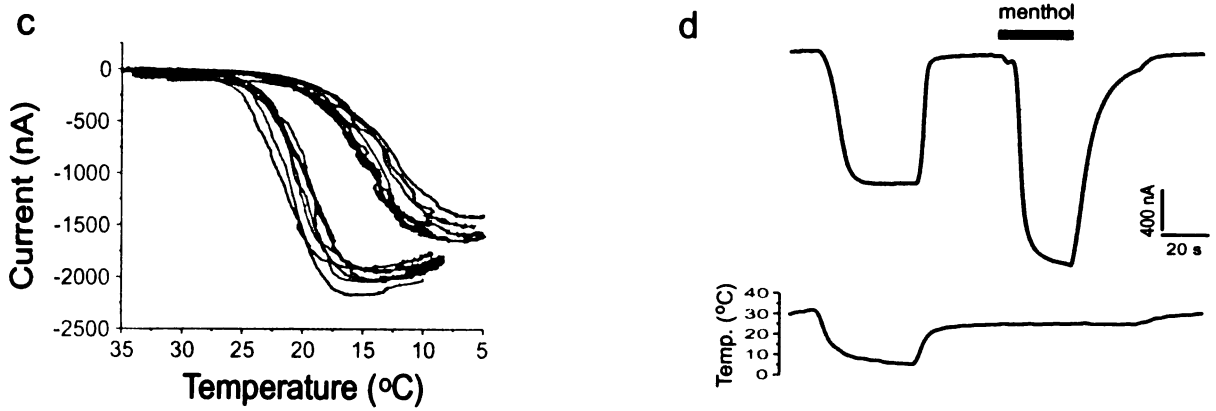

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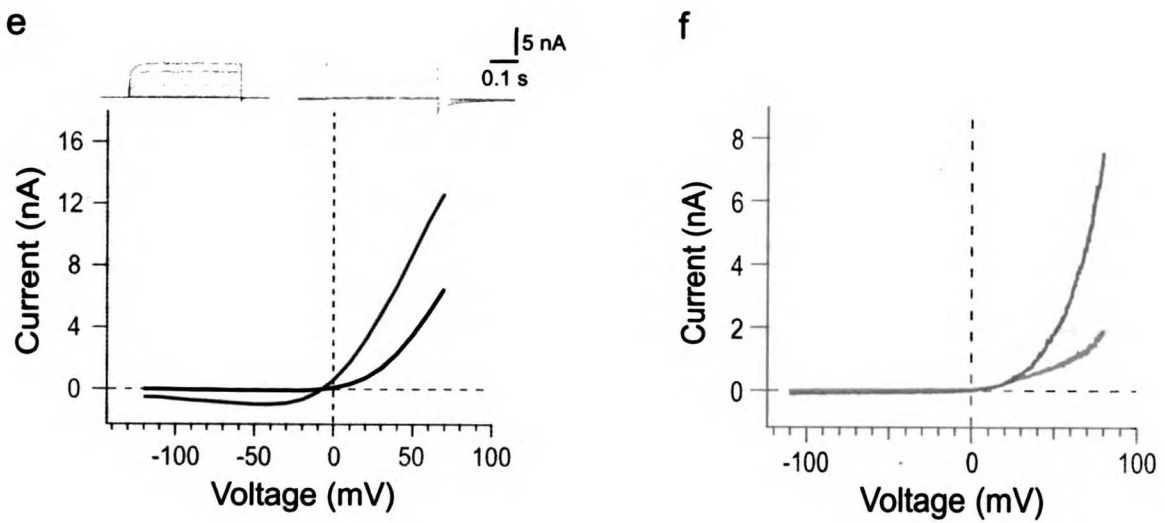


Fig. 7. CMR1 is a member of the TRP family of ion channels.

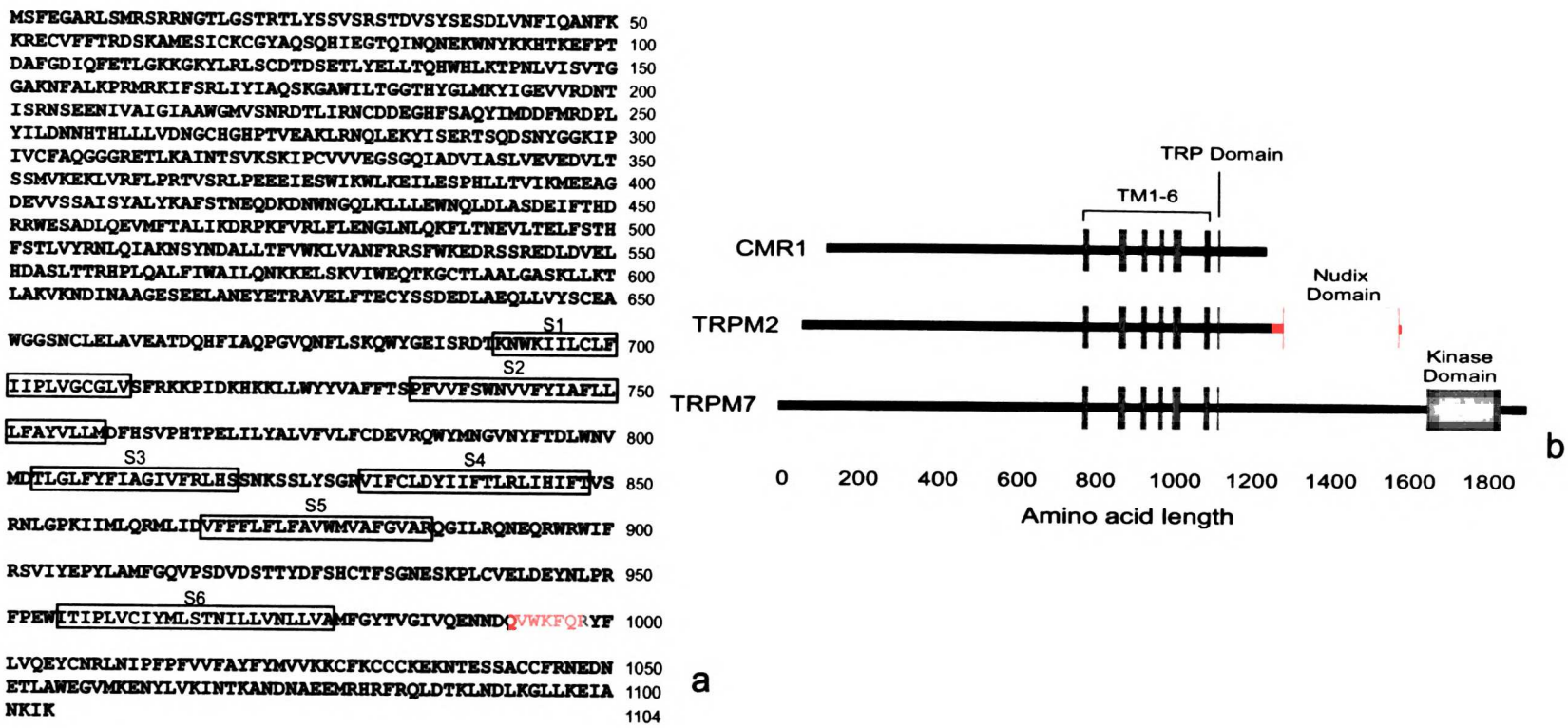


Fig. 8. CMR1 is expressed by small-diameter neurons in trigeminal and dorsal root ganglia.

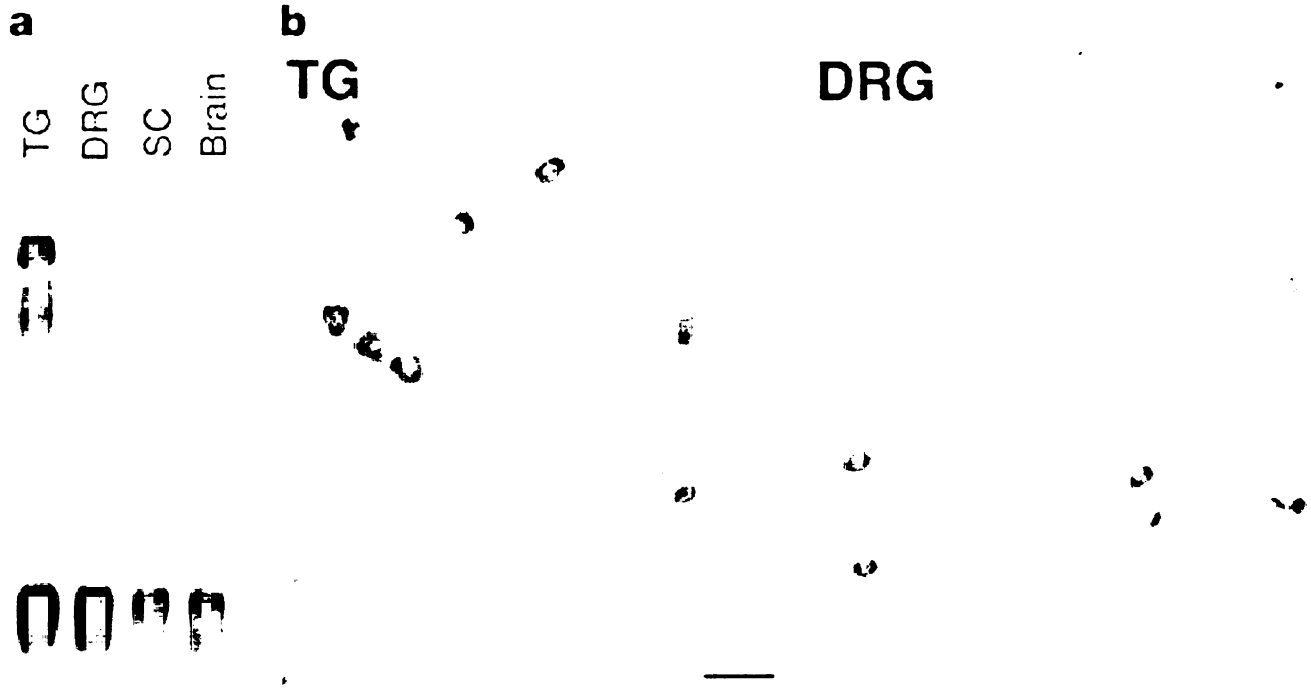


Fig. 9. Immunoblot Analysis of CMR1 transfected HEK cells

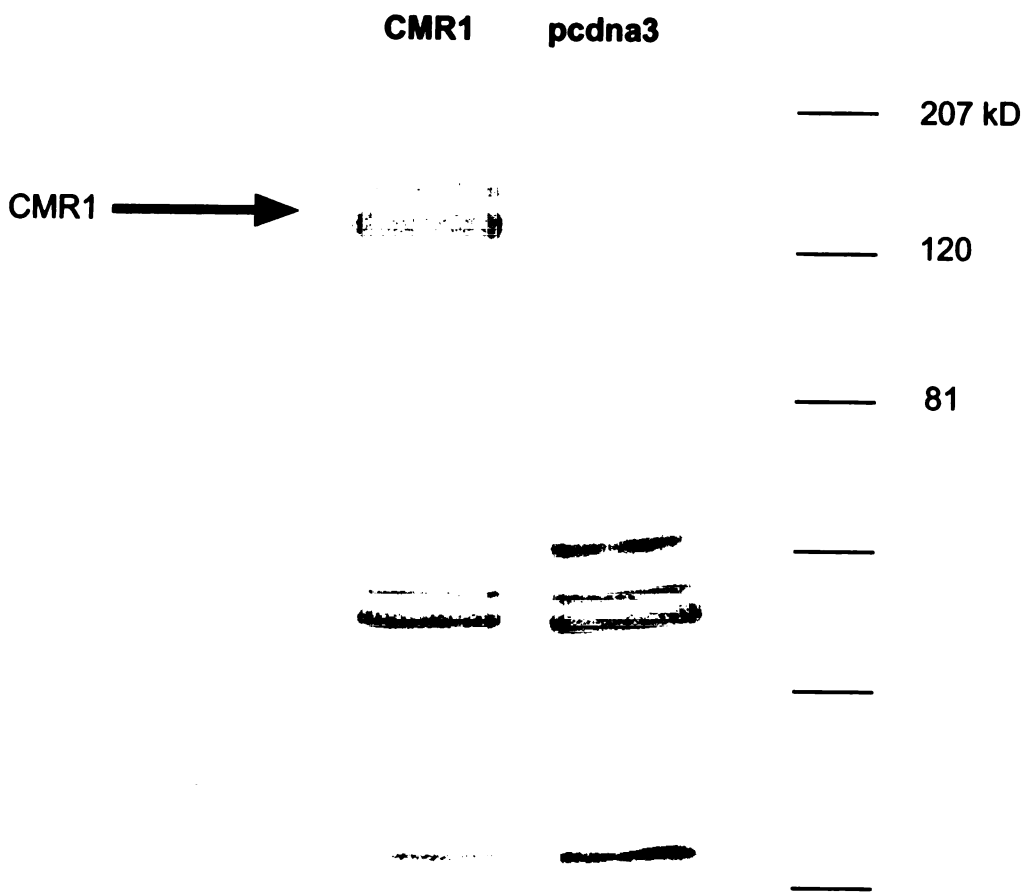
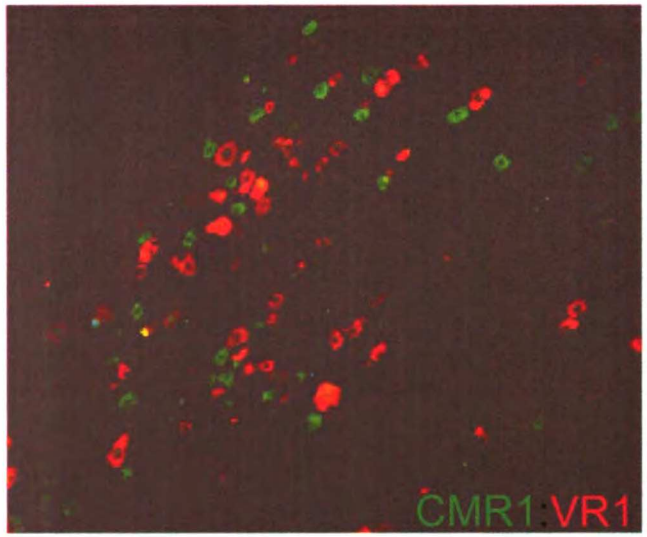


Fig. 10. Expression of CMR1 and VR1 in TG ganglia and primary cultures



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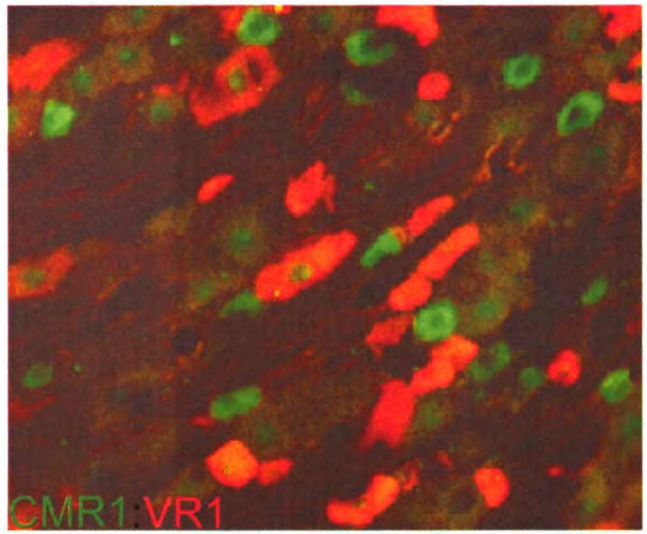

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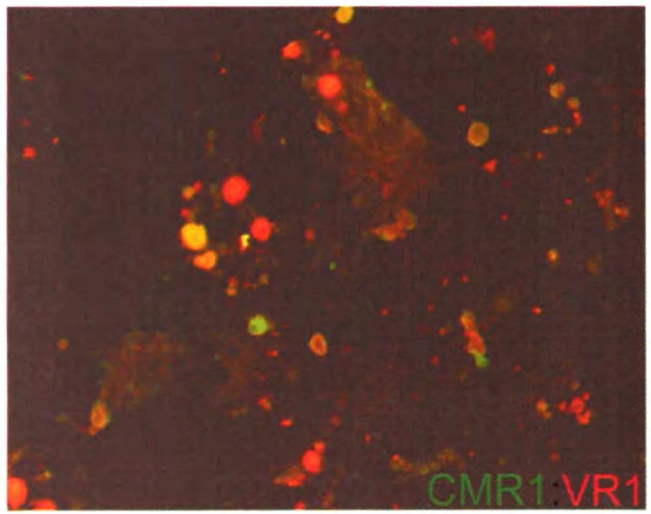

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Fig. 11. CMR1 expression in sensory ganglia defines a distinct subset of sensory neurons

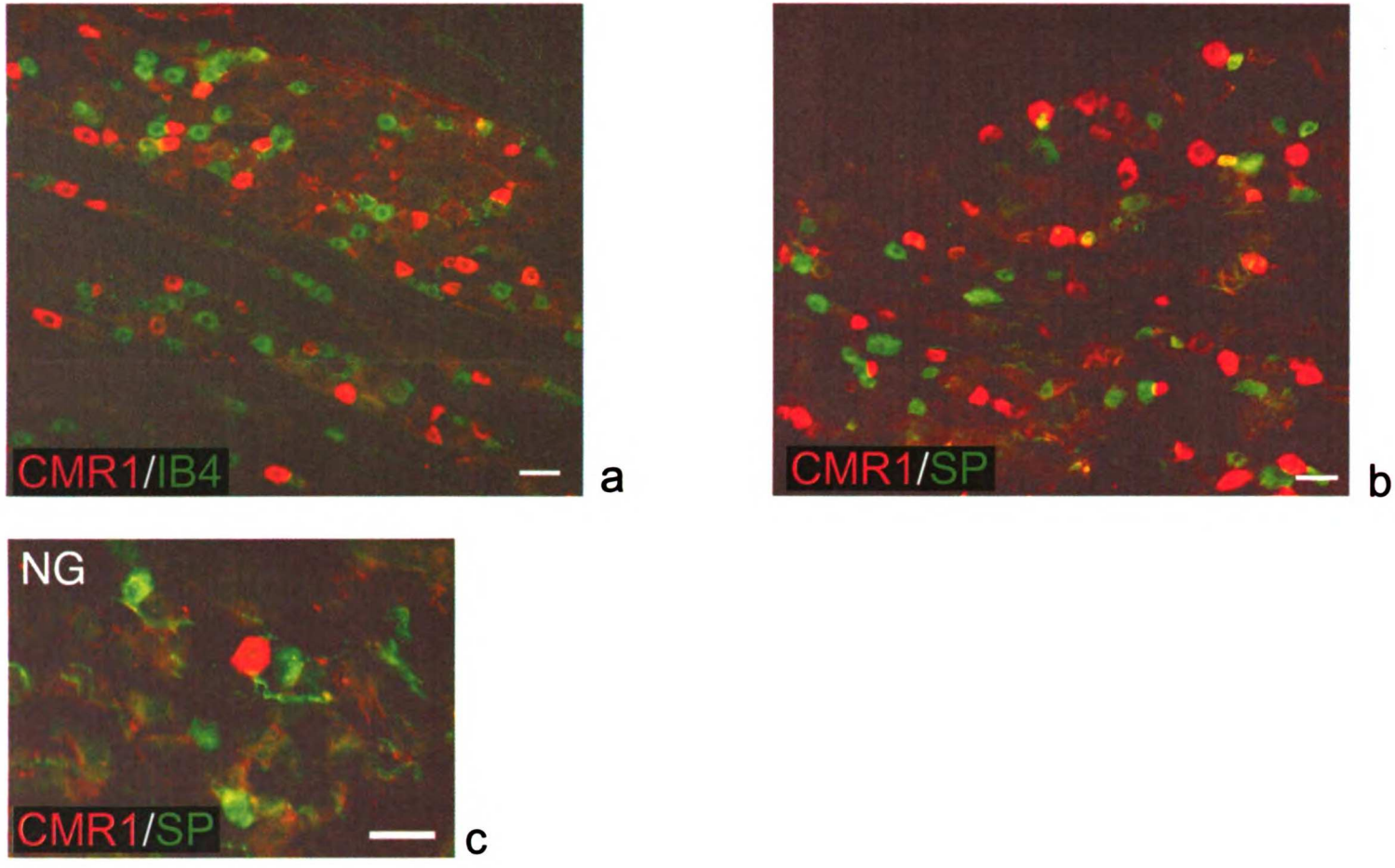
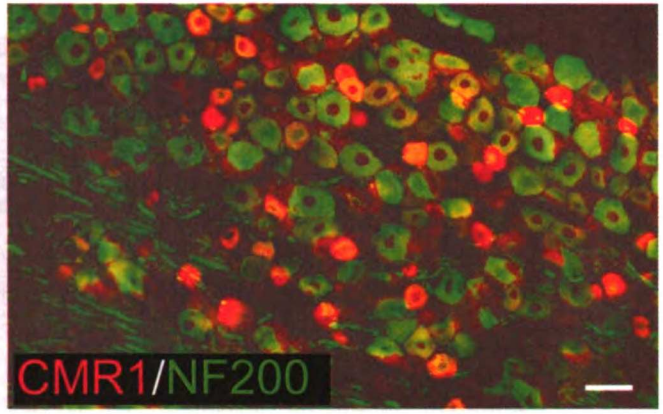


Fig. 11. CMR1 expression in sensory ganglia defines a distinct subset of sensory neurons



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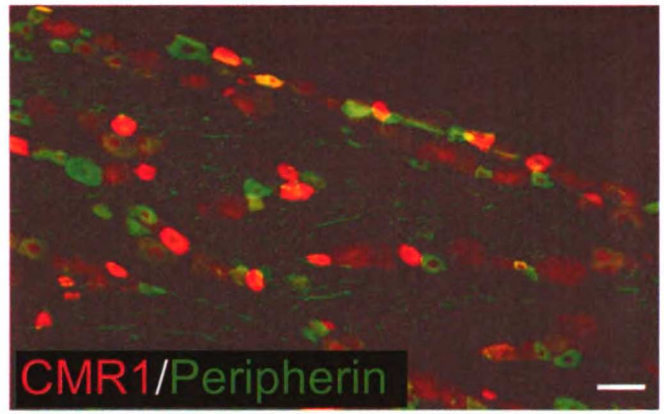

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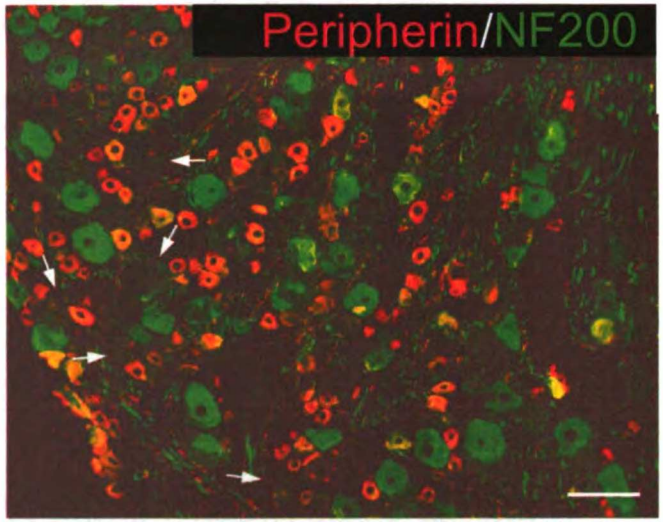

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Fig. 12. Expression of CMR1 in axons along the neuraxis

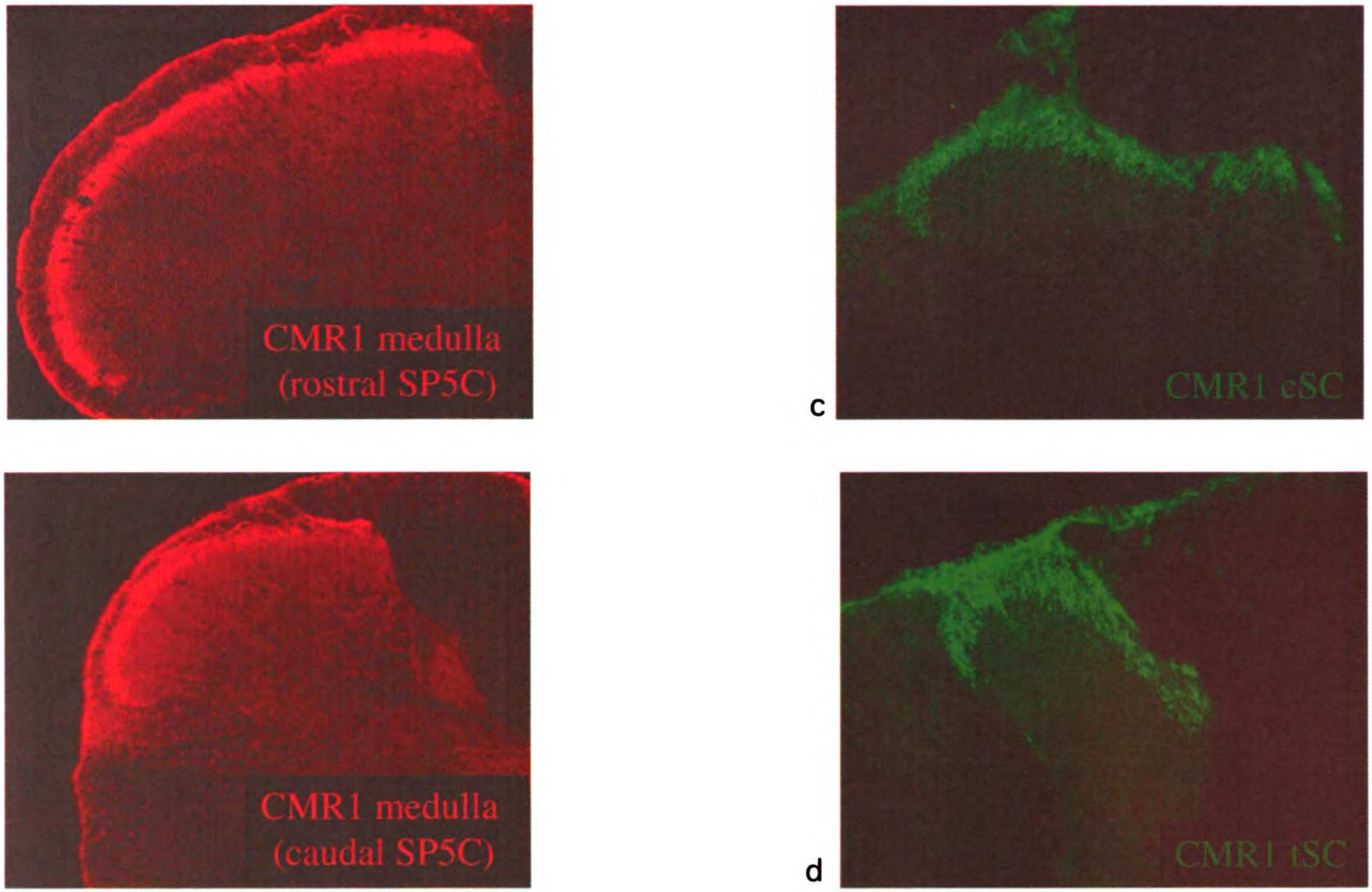


Fig. 12. Expression of CMR1 in axons along the neuraxis

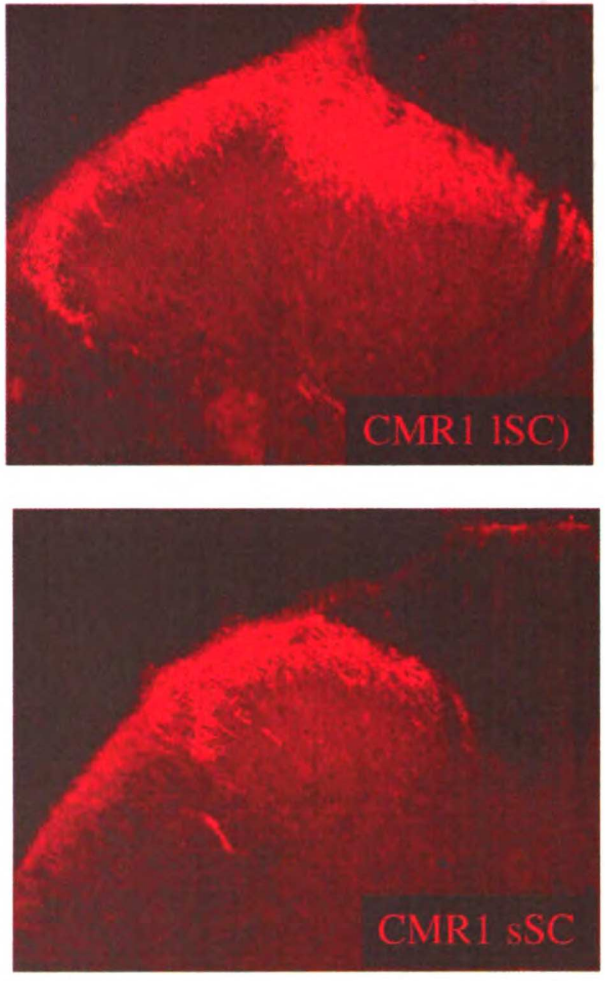
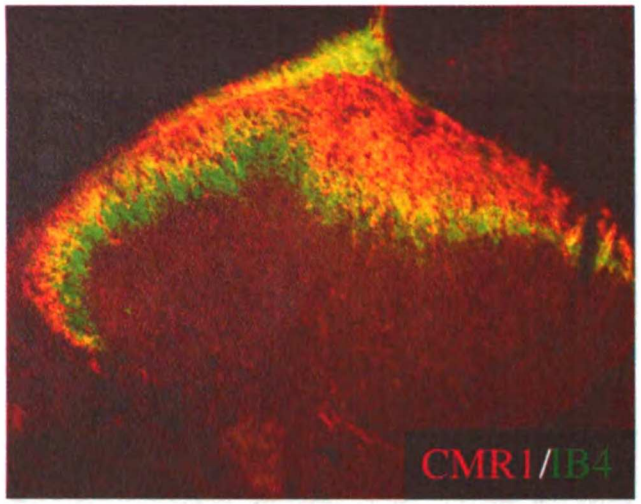


Fig.13. Projection pattern for CMR1, IB4, substance P and VR1 in the spinal cord dorsal horn.



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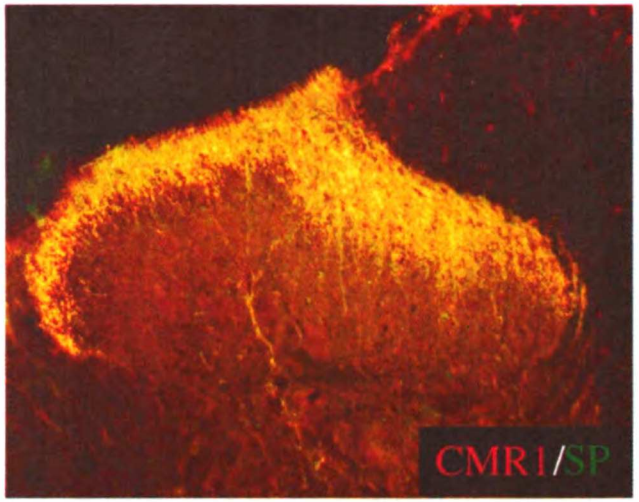

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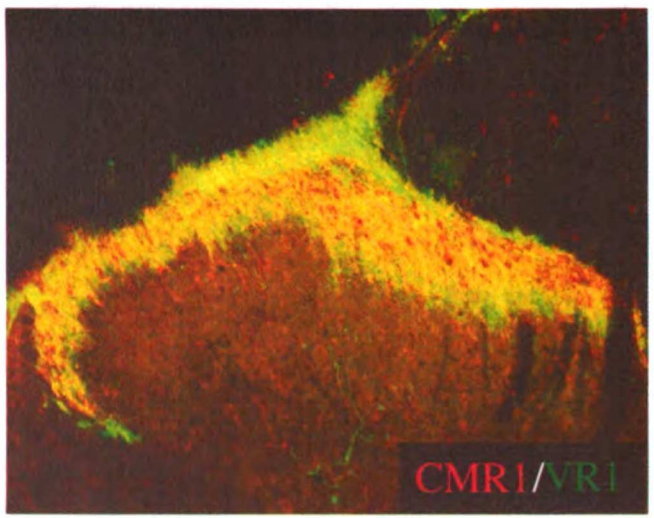

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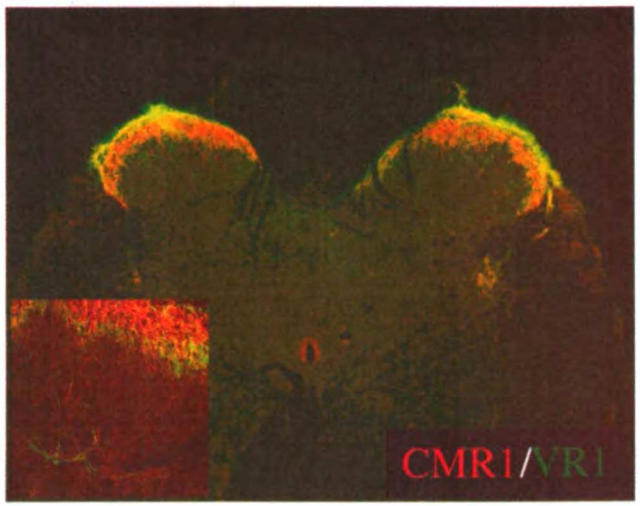

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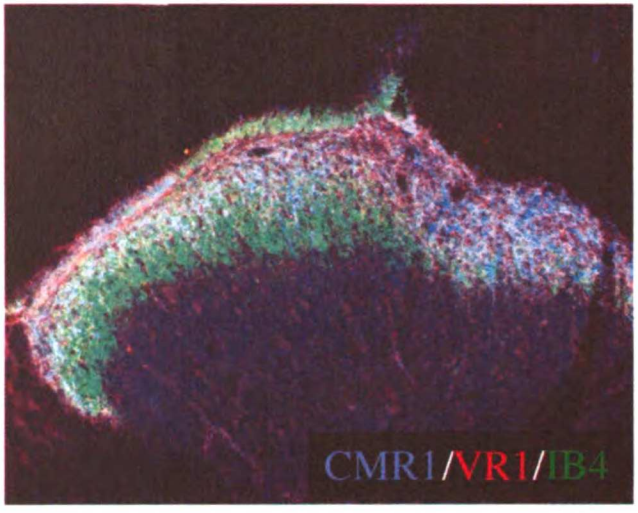
Fig.13. Projection pattern for CMR1, IB4, substance P and VR1 in the spinal cord dorsal horn.



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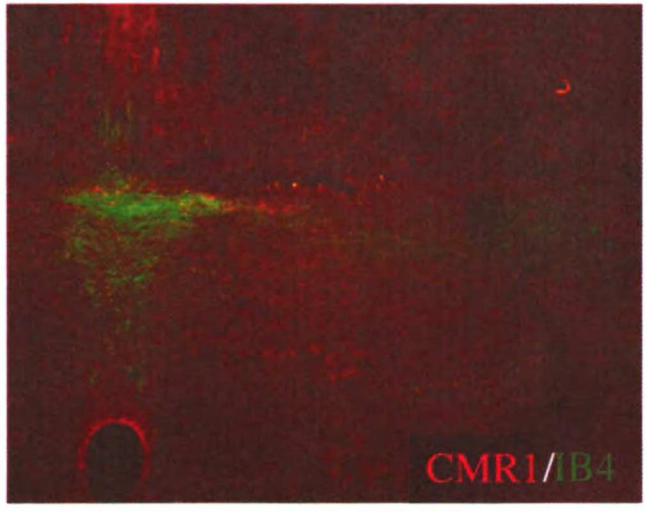

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Fig.14. Upregulation of CMR1 in the dorsal horn after CFA induced inflammation.

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Fig 15. TRP-like channels mediate thermosensation from cold to hot.

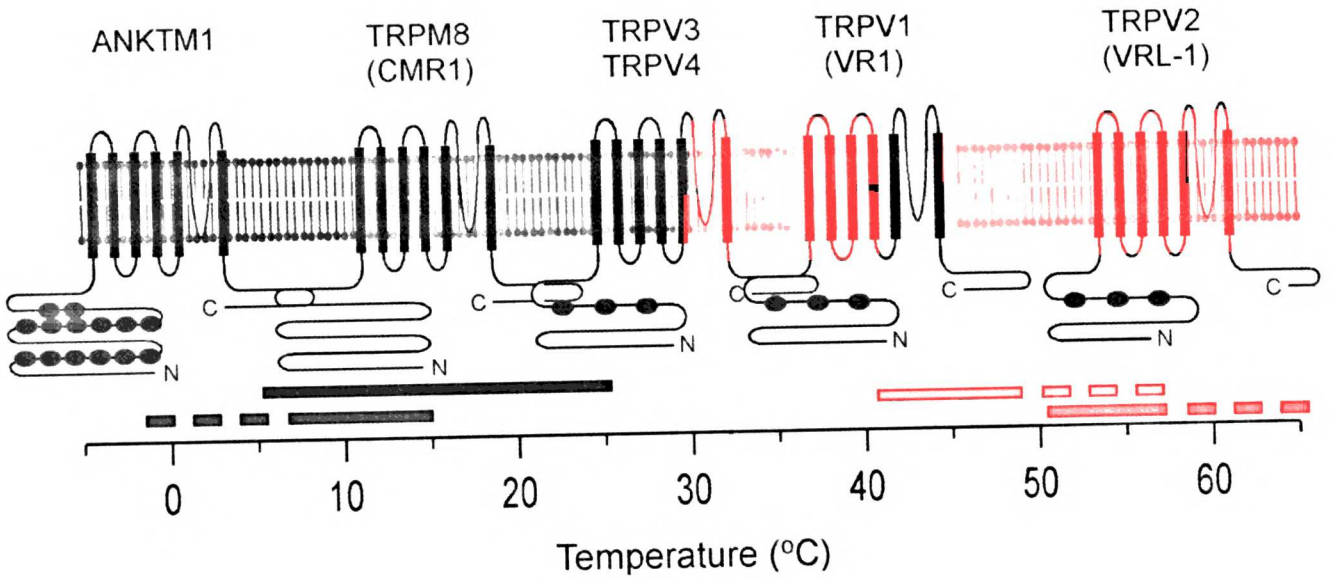

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Fig 15. TRP-like channels mediate thermosensation from cold to hot.

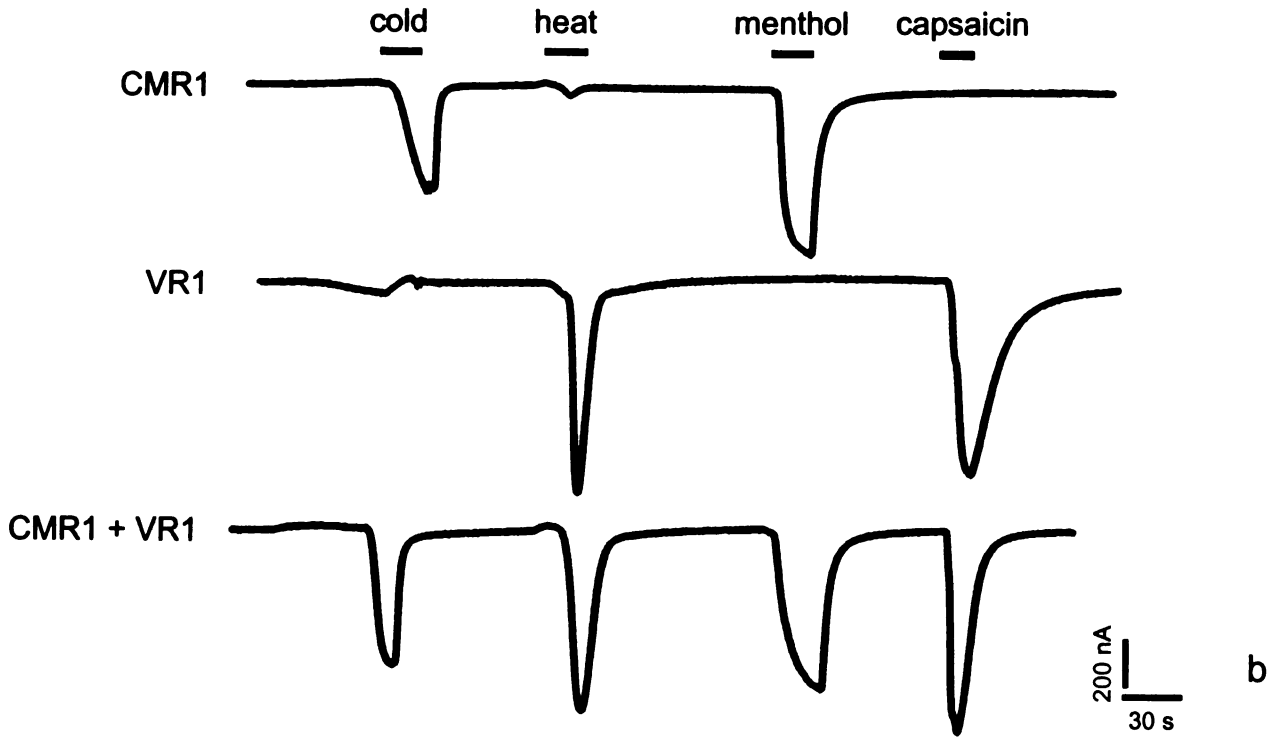

Figure 1. Menthol and cold increase intracellular Ca^{2+} in a subset of DRG and TG neurons independent from the capsaicin receptor VR1. **a,** Responses of dissociated trigeminal neurons to cold (7°C), menthol (500 μM), and capsaicin (1 μM) were assessed by calcium imaging. Arrowheads mark menthol responding cells that were insensitive to capsaicin. Relative calcium concentrations are indicated by Fura-2 ratio (color bar). The percentage (± s.e.m.) of excitable (potassium-sensitive) cells responding to each stimulus is indicated below. **b,** Menthol (500μM) does not activate the cloned capsaicin receptor in HEK cells. **c,** Menthol (500μM) and cold (1°C) evoke Ca^{2+}-responses in DRG neurons from VR1 -/- mice

Figure 2. A subset of trigeminal neurons express an outwardly rectifying Ca^{2+}-permeable channel activated by menthol and cold. **a,** 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 RT to 30°C) completely antagonized currents evoked by 100 μM menthol (right). Perfusate temperatures are indicated below each current trace. **b,** Cold potentiates inward and outward current evoked by 100μM menthol **c,** Menthol (50 μM, green)- and cold (16°, blue)-evoked responses show strong outward rectification. Inset shows baseline currents (black) and menthol-evoked responses (green) in nominally Ca^{2+} free bath solution at RT. **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} = -60mV) in trigeminal neurons. Membrane currents in each neuron were normalized to 1 mM menthol at room temperature. Each point represents mean value (± s.e.m.) from six independent neurons and were fit with the Hill equation. **f,** Temperature-response curves (from 33°C 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 ± 0.5°C to 29.6 ± 0.3°C (n = 4).

Figure 3. Expression cloning strategy for a menthol receptor. A calcium imaging-based screening strategy is used to isolate a functional cDNA encoding a menthol or cold-sensitive receptor from a cDNA expression library constructed from TG. Pools containing ~10,000 clones are transfected into HEK293 cells, which are subsequently loaded with the calcium-sensitive fluorescent dye, Fura-2, and microscopically examined for changes in intracellular calcium upon exposure to room temperature menthol (500 μM). A positive pool is amplified until a single cDNA is obtained that confers menthol sensitivity to transfected HEK cells.

Figure 4. 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 boxes), menthol (green circles), and eucalyptol (red triangles). Responses were normalized to those evoked by 500 μM menthol. Each point represents mean values (± s.e.m.) from 4 to 9 independent oocytes.

Figure 5. Electrophysiological properties of menthol-induced currents in transfected HEK293 cells. **a,** Time dependence of menthol induced whole-cell currents were analyzed using 400 ms voltage step pulses ranging from -120 to +70mV in 10mV steps (top). Traces show current response induced by menthol (50 μM) at RT in nominally Ca^{2+} free bath solution. Current-voltage relationship (bottom) was obtained from the same pulse protocol using 200 μM menthol (RT) by plotting the time-independent current component as a function of membrane voltage. Menthol currents reversed at -4.49 ± 1.07 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 +80mV). **b,** 200 ms voltage ramps 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. Currents could be partially blocked by 200 μM La^{2+} (insert) **c,** Menthol- and icilin-evoked current desensitization is calcium-dependent. **d,** Single channel traces were recorded from transfected HEK293 cells in the cell-attached patch configuration at different pipette potentials. **e,** Slope conductance of single channel current amplitudes was obtained by linear regression fit, yielding a single channel conductance of 83 ± 3 pS (n = 3).

Figure 6. The menthol receptor is cold sensitive. **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, black; 1°C/s, red). **b,** Temperature-response profile of the cold-evoked currents shown in panel (a) (color coding corresponds to that of current traces). **c,** Response profiles of cold-evoked currents in seven independent oocytes in the absence (black lines) or presence of a sub-activating concentration of menthol (20 μM, green lines). **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 room temperature menthol (500 μM, green bar). **e,** Current-voltage relationship for a cold (14°C)-evoked stimulus in menthol receptor-transfected cells in the absence (black) or presence (green) of sub-activating dose (10 μM) 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° before (red) and after (blue) warming to 31°C.

Figure 7. CMR1 is a member of the TRP family of ion channels. **a,** The predicted amino acid sequence determined from the CMR1 cDNA. Open boxes designate predicted transmembrane domains and amino acids encompassing the conserved TRP family motif is indicated in red. **b,** Schematic comparison of CMR1 with other TRPM family members, TRPM2 and TRPM7. Proteins are aligned using putative transmembrane domains (blue boxes) and TRP motif (red boxes) as landmarks. 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 8. 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 analyzed by Northern blotting, revealing two predominant transcripts of ~6 and 5 kb. 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-labeled CMR1 probe over neurons with small-diameter (~19 micron) cell bodies (scale bar = 50 micron).

Fig. 9. Immunoblot Analysis of CMR1 transfected HEK cells. Western blot analysis reveals a specific 125 kD band in lysates from CMR1 transfected HEK 293 cells but not from pcDNA3-transfected control cells.

Fig. 10. Expression of CMR1 and VR1 in TG ganglia and primary cultures

a Double immunofluorescence for CMR1 (green) and VR1 (red) in TG ganglia. CMR1 immunoreactivity is present in ~15 % of small diameter neurons and does not colocalize with VR1. **b** high magification of a. **c** Double immunofluorescence for CMR1 (green) and VR1 (red) in primary cultures of sensory neurons from TG (~16 hrs). CMR1 immunoreactivity colocalizes with VR1 in about 50% of CMR1 (+) cells.

Fig. 11. CMR1 expression in sensory ganglia defines a distinct subset of sensory neurons. Double immunofluorescence was used to localize expression of CMR1 (red) and IB4 (green, **a**), substance P in TG (green, **b**), substance P in nodose ganglia (green, **c**), NF200 (green, **d**), peripherin (green, **e**). **f** Double staining for peripherin (red) and NF 200 (green) reveals a small subset of unlabeled sensory neurons (arrows).

Fig. 12. Expression of CMR1 in axons along the neuraxis

Immunoreactivity in the terminals of afferent fibers projecting to the trigeminal nucleus caudalis in the medulla (a, b) and laminae I & II of the spinal cord dorsal horn (cervical (c), thoraic (d), lumbar (e) and sacral (f)).

Fig.13. Projection pattern for CMR1, IB4, substance P and VR1 in the spinal cord dorsal horn. Double labeling for CMR1 (red) and IB4 (green, **a**), substance P (green, **b**) and VR1 (green, **c**) and triple labeling for CMR1 (blue), VR1 (red), IB4 (green) (**e**) reveals projection pattern overlap with VR1 and SP but not IB4. **d** Double labeling for CMR1 (red) and VR1 (green) in the sacral spinal cord reveals arborization of axons into laminae V and X for VR1 but not CMR1. **d (insert)** Confocal image of double labeled fibers (CMR1 red, VR1, green) shows no overlap of individual fibers f Double labeling for CMR1 (red) and IB4 (green) in the solitary tract reveals a small number of CMR1 (+) fibers. **f** Double labeling for CMR1 (red) and IB4 (green) in the nucleus of the solitary tract.

Fig.14. Upregulation of CMR1 in the dorsal horn after CFA induced inflammation. CMR1 immunoreactivity (DAB) in the L5 segment ipsi and contra to the CFA injection into the paw. At 3 days post-injection immunoreactivity for CMR1 in lamina I & II was markedly increased on the ipsilateral side in comparison to the contralateral side.

Figure 15. TRP-like channels mediate thermosensation from cold to hot. **a,** Schematic representation of the thermal sensitivity ranges of CMR1, VR1, and VRL-1. The ranges of each proteins' temperature sensitivity are 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 particularly those of a warm (30 to 40°C) or extremely cold (<8°C) nature. **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).

V.

Experimental Procedures

Neuronal cell culture and Ca^{2+} microfluorimetry.

Trigeminal ganglia were dissected from newborn S/D rats and dissociated with 0.125% collagenase P (Boehringer) solution in CMF Hank's, at 37 °C, for 20 (P0 animals) to 30 min (P4 animals), pelleted, and resuspended in 0.05% STV 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 NGF 7S (Invitrogen)), then enriched by density gradient centrifugation as described (Eckert, Taddese et al. 1997). Cells were resuspended in culture medium and plated onto PLO- (1 mg/ml, Sigma) and laminin-coated (5 µg/ml, Invitrogen) coverslips. Cultures were examined 1-2 days after plating using Ca^{2+} microfluorimetry as described (Caterina, Schumacher et al. 1997). For cell selection prior to 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 two days after transfection. Gigaseals were formed with pipettes (Garner Glass type 7052, ID

1.1, OD 1.5) 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 -60mV with a 200 ms voltage ramp from -120 mV to +80 mV at 3.6 Hz. Data were acquired using Pulse-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 Inst.) connected to a thermostat. Temperature in the proximity of the patched cell was measured using a miniature thermocouple (type 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 $\Delta E_{rev(Na-X)}$ equals the reversal potential change, F the Faraday's 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 Lewis (Lewis 1979) (equation A6).

Solution composition for mammalian cell electrophysionlogy

Pipette solution for neuronal experiments contained (in mM) 140 CsCl, 1 EGTA, 0.6 $MgCl_2$, 10 HEPES, pH 7.4 (adjusted with CsOH). Standard Ca^{2+}-freebath solution for whole-cell recordings contained (in mM) 140 NaCl, 4 KCl, 2

$MgCl_2$, 100 nM TTX (Sigma), 10 TRIS, pH 7.4 (adjusted with HCl) with or without 100 nM dendrotoxin I (Sigma). For ion replacement experiments pipette solution contained (in mM) 140 KCl, 5 CsCl, 1 EGTA, 0.6 $MgCl_2$, 1 4-aminopyridine, 10 HEPES, pH 7.4 (adjusted with KOH). Bath solution contained (in mM) 140 NaCl, 4 KCl, 2 $MgCl_2$, 100 nM TTX, 100 nM dendrotoxin I, 100 nM apamine (Sigma), 100 nM carybdotoxin (Sigma), 1 μM ω-conotoxin MVIIC, 1 μM GVIA (Latoxan), 10 TRIS, pH 7.4 (adjusted with HCl). For monovalent cation permeability experiments perfusate contained (in mM) 140 NaCl (or 140 KCl or 140 NMDG), 100 nM TTX, 1 μM nitrendipine (Sigma), 10 HEPES, 10 glucose, pH 7.4 (adjusted with NaOH, KOH or HCl, respectively). For divalent cation permeability experiments perfusate contained (in mM) 125 NMDG, 10 $CaCl_2$, 100 nM TTX, 1 μM nitrendipine, 10 HEPES, 10 glucose, pH 7.4 (adjusted with HCl). Liquid junction potentials (measured directly in separate experiments) did not exceed 3 mV and thus no correction for this offset was made. Bath solution for on-cell single channel experiments contained (in mM): 150 NaCl, 1 $MgCl_2$, 10 TRIS. Pipette solution for on-cell single channel experiments contained (in mM): 150 NaCl, 1 $MgCl_2$, 0.1 EGTA, 10 TRIS and 5 or 25 μM menthol.

Solution compositions and reversal potentials for neuronal ion replacement experiments

Recording electrodes were filled with standard pipette solution. Replacement of extracellular NaCl (140 mM, red) with equimolar KCl (black) or NMDG (blue) shifted the reversal potential from $E_{rev(Na)}$ = -5.1 ± 3.1 mV (n = 6) to $E_{rev(K)}$ = -4.5 ±

2.0 mV (n = 3) and $E_{rev(NMDG)}$ = -85.0 ± 11.5 mV (n = 7). Replacement of extracellular NaCl with 125 mM NMDG and 10 mM $CaCl_2$ (green) shifted the reversal to $E_{rev(CaNMDG)}$ = -43.3 ± 4.6 mV (n=6, P_{Ca}/P_{Na} = 3.2). Change of pipette solution from standard (140 KCl, 5 CsCl) to 140 CsCl in nominally Ca^{2+} free bath solution shifted the reversal from 0.5 ± 0.6 mV (n = 5) to -3.6 ± 1.9 mV (n = 6, not shown).

Solution compositions and reversal potentials for neuronal ion replacement experiments in HEK cells

Recording electrodes were filled with standard pipette solution. Replacement of extracellular NaCl (140 mM, red) with equimolar KCl (black) or NMDG (blue) shifted the reversal potential from $E_{rev(Na)}$ = -3.8 ± 2.4 mV (n = 13) to $E_{rev(K)}$ = 0.8 ± 1.0 mV (n = 8) and $E_{rev(NMDG)}$ = -77.2 ± 5.9 mV (n = 11), respectively (P_K/P_{Na} = 1.2, P_{NMDG}/P_{Na} = 0.1). Replacement of extracellular NaCl with 125 mM NMDG and 10 mM $CaCl_2$ (green) shifted the reversal to $E_{rev\ (CaNMDG)}$ = -35.2 ± 8.00 mV (n = 9; P_{Ca}/P_{Na} = 3.3). Change of pipette solution from standard (140 KCl, 5 CsCl) to 140 CsCl in nom. Ca^{2+} free bath solution shifted the reversal from -2.3 ± 1.0 mV (n = 3) to -4.5 ± 1.1 mV (n = 4, P_{Cs}/P_K = 1.1, not shown).

Expression cloning, Northern blot analysis and in situ hybridization.

Trigeminal ganglia from newborn rats were dissociated and enriched for neurons by Percoll gradient centrifugation as described (Eckert, Taddese et al. 1997). Polyadenylated RNA (~2 μg) was isolated from these cells (PolyATract Kit, Promega) and used to construct a cDNA library in pcDNA3 (Invitrogen) as described (Brake, Wagenbach et al. 1994). Library subpools consisting of ~10,000 clones were transiently transfected into HEK293 cells by lipofection and split 24 hours later into 8-well glass chamber slides coated with Matrigel (Becton-Dickinson). Responses to chemical or thermal stimuli were assessed 6-24 hours 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 (Caterina, Schumacher et al. 1997) using the entire CMR1 cDNA to generate ^{32}P- or digoxygenin-labeled probes, respectively.

The Genbank accession number for rat CMR1 is AY072788.

Oocyte electrophysiology.

cRNA transcripts were synthesized and injected into Xenopus laevis oocytes as described (Brake, Wagenbach et al. 1994). Two-electrode voltage-clamp recordings were performed 2-7 days post-injection. Dose-response curves for cooling compounds were performed at room temperature (22-24°C).

Icilin (AG-3-5) was generously provided by Dr. E. Wei, UC Berkeley. Temperature ramps were generated by heating (~35°C) or cooling (~4°C) the perfusate in a Harvard coil and monitoring temperature changes with a thermister placed near the oocyte.

Antibody generation and purification

A peptide corresponding to the predicted carboxyl terminus of rat and human CMR1/TRPM8 (LNDLKGLLKEIANKIK) was coupled to keyhole limpet hemocyanin, via an amino terminal cysteine, and used to immunize rabbits (AnaSpec; Strategic Biosolutions). Crude serum was affinity purified using affinity chromatography and antibody was further purified of non-specific antibodies by treatment with powdered liver extract.

Western analysis

Human embryonic kidney (HEK) cells were transiently transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA.) (McKemy, Neuhausser et al. 2002) and total cell lysates were prepared 48-72 hours post-transfection in 150 mM NaCl, 50 mM Tris-HCl (pH 7.4), and 1% CHAPS. Solubilized protein was separated on 12% acrylamide/bis gels and blotted on to PVDF membranes. Blots were pre-treated with 5% powered milk in phosphate buffered saline plus 0.05% Tween-20 (PBST) and then incubated in the same buffer with CMR1/TRPM8 crude and affinity purified antibody. After extensive washing in PBST, blots were probed with goat anti-rabbit secondary antibodies coupled to horseradish

peroxidase and immunoreactive proteins were detected with chemiluminescence. For experiments analyzing neuronal tissues, trigeminal ganglia were dissected from euthanized P2-P7 rats and placed in to ice-cold PBS. Tissue was minced and then homogenized in the lysis buffer described above. 100 μg of solubilized protein was loaded in each lane and blotted as described above.

Immunochemistry

Adult (200-250g) and neonatal Sprague/Dawley rats were anesthetized and subsequently perfused first with PBS then ice-cold 4% paraformaldehyde in PBS. Tissue was dissected and post-fixed for one hour at 4° C in paraformaldehyde solution and then cryo-protected with 30% sucrose in PBS overnight at 4° C. Samples were placed in OCT media and rapidly frozen and stored at -80° C until used. Fifteen μm tissue sections were cut on a microtome and affixed to glass slides then allowed to dry for 2 hours. Primary cultures of TG neurons were prepared as previously described [McKemy, 2002 #6] and fixed with 4% paraformaldeheyde in PBS at 4° C for 30 minutes. Both sensory ganglionic tissue and cultured cell samples were rinsed with PBS and then permeabilized with 0.1% Triton X-100 in PBS for 10 minutes then blocked with 5% of the appropriate animal sera, depending on the species source of the primary antibody used, in PBS plus 0.05% Triton X-100 (PBSTX) for 30 minutes at 4° C. Primary antibody was diluted into PBSTX plus 0.5% serum and 0.5% bovine serum albumen (BSA) and incubated overnight at 4°C. Samples were washed 6 X 5 minutes in PBSTX and then incubated with fluorescently labeled

secondary antibodies (Molecular Probes, OR) in PBSTX plus 0.5% serum and 0.5% BSA for 1 hour at room temperature. Slides were washed as above and then affixed under a coverslip with Fluormount. Floating spinal cord sections were cut on a microtome and placed in to small volume wells treated and washed as described above before being mounted on slides. Fluorescent images were visualized on a inverted Nikon microscope and captured using a CCD camera and Adobe Photoshop software. For the studies regarding tissue inflammation, Sprague-Dawley rats (200-250g) were anesthetized before injection of 100 µl of Complete Freund's Adjuvant in the rear left paw.

VI.

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