

Burning Cold: Involvement of TRPA1 in Noxious Cold Sensation

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Soon after its discovery ten years ago, the ion channel TRPA1 was proposed as a sensor of noxious cold. Evidence for its activation by painfully cold temperatures (below $\sim 15^{\circ}\text{C}$) has been mixed, however. Some groups found that cold elicits a nonselective conductance in cells expressing TRPA1; others found no activation, or argued that activation is an indirect effect of elevated Ca^{2+} . Sensory cells from the trigeminal and dorsal root ganglia that are activated by cold were sometimes correlated with those cells expressing TRPA1; other times not. Mice lacking TRPA1 showed behavioral deficits for some assays of painful cold sensation, but not others. New evidence tends to support direct activation of TRPA1 by cold, and the slow and relatively weak activation of TRPA1 by cold helps reconcile some conflicting studies.

Ah me! alas, pain, pain ever, forever!
 The crawling glaciers pierce me with the spears
 Of their moon-freezing crystals; the bright chains
 Eat with their burning cold into my bones.
Prometheus Unbound
 Percy Bysshe Shelley

Introduction

Our understanding of the somatosensory system is due in part to the observations of the English neurologist, Sir Henry Head (1861–1940). His experiments—sometimes on himself—allowed him to distinguish several distinct sensations in the general sense of touch. These include our ability to discriminate fine differences in mechanosensation and temperature, as well as the sensation of painful cutaneous stimuli such as extremely hot and cold temperatures. Head also described inflammatory and neuropathic pain based on an experiment in which he severed the radial nerve in his own arm to document the loss and sequential recovery of sensory modalities after nerve damage.

Head realized that even in the acute senses, the sensory transduction of a physical stimulus is highly complex, and the eventual perception is further subjected to the integrative action of both the peripheral and central nervous systems. The sense of temperature is an abstraction from complex afferent processes originating at the

source of the stimuli; in some cases such as noxious cold temperatures, the stimulus evokes several distinct sensations. For instance, the perception of pain may be combined with the perception of cold in the general sensation of noxious cold. To understand such a complex process, many have focused on identifying the molecular thermosensors residing at the nerve terminals using chemical mimetics of hot or cold temperatures. This approach is exemplified by the use of capsaicin, the stimulant found in hot chili peppers, to identify its receptor TRPV1. Studies on TRPV1 have provided a productive entryway to understanding the molecular basis of thermosensation, leading by both analogy and homology to the putative cold sensors TRPM8 and TRPA1.

Complexities of Cold Sensation

Found in species ranging from yeast to human, transient receptor potential (TRP) channels typically contain six transmembrane domains that form a central pore, as well as differing amino and carboxyl domains that impart differential sensitivity to various sensory stimuli (Wang and Woolf, 2005). After the finding that TRPV1 is involved in noxious heat sensation, investigators found other TRP channels that are activated by temperatures ranging from noxious hot to cool (Patapoutian et al., 2003; Tominaga and Caterina, 2004). TRPM8, for instance, is activated at temperatures below $\sim 23^{\circ}\text{C}$ and chemicals that produce a cool sensation, such as menthol and icilin (McKemy et al., 2002; Peier et al., 2002). The sensation of painful cold is distinct from the sensation of cool because it encompasses an additional percept of pain. Painful cold sensations are elicited by temperatures of $\sim 15^{\circ}\text{C}$ and below. TRPA1 (originally known as ANKTM1; Jaquemar et al., 1999) was reported to be activated by temperatures below 16°C and was proposed as a sensor for painful or noxious cold (Story et al., 2003). TRPA1 shares some pharmacology with TRPM8. Both are activated by icilin and menthol, although menthol blocks TRPA1 at higher concentrations (Karashima et al., 2007). Other studies found instead that cold did not activate TRPA1 (Jordt et al., 2004), or that the effect was an indirect activation of TRPA1 through cold-induced

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Abbreviations used in this paper: DRG, dorsal root ganglion; TRP, transient receptor potential.

increases in intracellular Ca^{2+} (Zurborg et al., 2007). Indirect activation via calcium may represent a mechanism of sensing noxious cold (Bandell et al., 2007). Is TRPA1 directly or indirectly activated by painful cold?

In addition to compounds that mimic cold temperatures, pungent compounds that produce a burning sensation, such as mustard oil (derived from plants of the *Brassica* family) and cinnamon oil (from the cinnamon tree *Cinnamomum verum*), also activate TRPA1 (Macpherson et al., 2005). Thus compounds that produce both cool and pungent/burning sensations activate TRPA1. Moreover, TRPA1 is found in small-diameter nociceptive neurons that express TRPV1 (Story et al., 2003). Why would the very same neurons be activated by compounds that are variously sensed as hot, cool, or pungent?

Studies from fruit flies have contributed to understanding the function of the TRPA family. *Drosophila melanogaster* has five distinct TRPA orthologs, of which three (Pyrexia, Painless, and dTRPA1) play a role in thermosensation. Pyrexia is activated in vitro by noxious heat for flies ($\geq 40^\circ\text{C}$), whereas Painless—expressed in the multidendritic sensory neurons—confers sensitivity to temperatures $\geq 55^\circ\text{C}$ to mechanical stimuli and to mustard oil (Tracey et al., 2003; Al-Anzi et al., 2006). dTRPA1 is expressed in the corpus callosum in central brain neurons as well as in neuroendocrine cells of the corpus cardiacum (Rosenzweig et al., 2005). Knockdown of dTRPA in larval flies reduces thermotaxis. Thus, TRPA channels in flies are also variously involved in sensation of temperature, pungent chemicals, and mechanical pain. Are fly TRPA channels fundamentally the same as mammalian TRPA1, or has function diverged during evolution?

From psychophysical experiments, noxious cold evokes multiple sensory percepts. When the palm is placed on a cooling surface, a human subject can reliably distinguish distinct cold-evoked sensations as the temperature is dropped from 32 to 3°C . The first and longest lasting sensation is cold. As the temperature drops into the noxious range below 15°C , sensations of pain and ache are elicited. Toward the end of the cold ramp and during the maintained stimulus at 3°C , a prickle sensation is evoked akin to the sensation of mechanical prodding with pins and needles. As the temperature is returned to 32°C , a mild heat sensation appears (Davis and Pope, 2002). Can some of these perceptions be attributed to TRPM8, TRPA1, or to both?

Here, we sort through the rapidly growing literature on TRPA1 and attempt to reconcile some of the conflicting evidence on cold pain sensation by TRPA1.

Chemical Activation of TRPA1

TRPA1 is an unusual TRP channel in that it has an extended intracellular N terminal of 17 ankyrin repeats preceding the first transmembrane domain. It is the only mammalian TRP channel with so many ankyrin repeats; TRPN1 has more but is found only in invertebrates and

some fish and amphibians. The C terminus of TRPA1 contains a highly conserved 160 amino acids composing a putative coiled-coil domain. TRPA1 is activated by at least three distinct and overlapping paradigms: pain-causing chemicals, intracellular calcium, and G protein-coupled receptors.

TRPA1 is activated by a variety of irritating chemicals that elicit painful sensations. These include cinnamaldehyde, mustard oil, *N*-methyl maleimide and formaldehyde, and all aldehyde-containing compounds that form covalent adducts with electrophilic amino acids such as cysteines or lysines. Although there is some dispute about which modified cysteines are critical between human and mouse TRPA1, a key cysteine is located in the last ankyrin repeat. Mutations of critical cysteines abolish the activation by these compounds (Hinman et al., 2006; Macpherson et al., 2007a). Physiologically relevant compounds, such as lipid peroxidation products related to oxidative damage, 4-hydroxynonenal, and the cyclopentenone prostaglandin 15-deoxy-12,14-prostaglandin J₂, contain aldehyde groups and can also activate TRPA1 by forming covalent adducts with cysteines in the N-terminal portion of TRPA1 (Macpherson et al., 2007b; McNamara et al., 2007; Cruz-Orengo et al., 2008).

TRPA1 is activated as well by intracellular Ca^{2+} but in a calmodulin-independent manner (Doerner et al., 2007; Zurborg et al., 2007). In searching for a Ca^{2+} binding site, an EF-hand-like sequence (DISDTRLLNEGDL) was recognized at the end of the 12th ankyrin repeat (Hinman et al., 2006). Mutations that alter the negatively charged amino acids thought to coordinate Ca^{2+} were found to abolish the Ca^{2+} -dependent activation of TRPA1 (Zurborg et al., 2007). Although the structure of the ankyrin repeats makes it unlikely that this sequence forms a true EF-hand (Gaudet, 2008), it nevertheless seems to control Ca^{2+} activation of TRPA1. Moreover, TRPA1 is weakly voltage dependent and voltage activation interacts with that of Ca^{2+} ; specifically, increasing intracellular Ca^{2+} to the low micromolar range shifts the voltage-dependent activation to more hyperpolarized potentials by nearly 150 mV (Zurborg et al., 2007).

Finally, activation of some G protein-coupled receptors activates TRPA1. Bradykinin, binding to the bradykinin 2 receptor, results in release of intracellular calcium through a phospholipase C-mediated cascade (Bandell et al., 2004). Perhaps TRPA1 is activated by the rise in intracellular calcium, or perhaps by another part of the second messenger pathway.

Activation of TRPA1 by Noxious Cold In Vitro

Because TRPA1 is a nonselective cation channel and passes Ca^{2+} , calcium imaging is often used to assess activation of the channel. By this measure, or by measuring receptor currents during cooling, TRPA1 expressed in Chinese hamster ovary cells was activated by cold temperatures below $\sim 16^\circ\text{C}$ (Story et al., 2003). TRPM8, in

the same assay, was activated at a cool temperature of 21°C. Moreover, TRPA1 was activated by icilin (100 mM), but not by the TRPM8 agonist menthol (500 mM). Later, it was found that menthol (like Ca²⁺) activates TRPA1 by shifting the voltage dependence to more negative potentials, but also blocks with a K_D of ~50 mM so that low but not high concentrations of menthol allow current through TRPA1 (Karashima et al., 2007). These initial observations lead to the proposal that TRPA1 mediates noxious cold sensation.

In similar calcium-imaging experiments, however, TRPA1 expressed in HEK cells did not show activation by cold stimuli, at least for brief (15–20-s) exposures, whereas TRPM8 produced a Ca²⁺ influx within seconds (Jordt et al., 2004). Perhaps the short stimuli were not sufficient to produce a second messenger required for activation of TRPA1. Indeed, Zurborg et al. (2007) found that cooling HEK cells produced a rise in intracellular Ca²⁺, whether or not they expressed TRPA1, at temperatures below ~17°C. They argued that activation of TRPA1 by noxious cold is an indirect effect of the rise in intracellular Ca²⁺. Moreover, mutation of key residues in the EF-hand-like sequence abolished both Ca²⁺ activation and cold activation (Zurborg et al., 2007), suggesting that cold activation occurs through Ca²⁺ activation, but not ruling out that the putative EF-hand domain is central to both Ca²⁺ and cold activation. More recently, Karashima et al. (2009) found that cooling to 10°C elicited nonselective currents in Chinese hamster ovary cells expressing TRPA1. Currents were smaller but were still present in the absence of extracellular Ca²⁺ and with the depletion of intracellular Ca²⁺ stores by thapsigargin, indicating an intrinsic sensitivity of TRPA1 to cold that is augmented by secondary Ca²⁺ activation.

To eliminate the confounding effects of second messengers—at least freely diffusible second messengers—ion channels can be studied with excised patch recordings. When excised patches from HEK cells expressing TRPA1 were cooled to temperatures below 16°C, the single-channel open probability increased. These channels were identified as TRPA1 by activation with mustard oil, by reversal potential and single-channel conductance, and by block by the TRPA1 antagonist camphor (Sawada et al., 2008). Importantly, cold still activated TRPA1 in Ca²⁺-free solutions, suggesting a direct effect of noxious cold on channel gating. Activation of TRPA1 in single-channel recordings, in the absence of Ca²⁺, was confirmed by Karashima et al. (2009). Noxious cold activation of TRPA1 may still be mediated by a membrane-associated second messenger, but it is probably not Ca²⁺. Moreover, the single-channel recordings illuminated a potential confounding effect of cold in some experiments. Cooling increased the open probability but, as for most other channels, reduced the single-channel conductance. Thus, in some circumstances cooling might increase open probability

but still decrease the total current by a larger effect on conductance (Karashima et al., 2009).

How would cold directly activate TRPA1? A general two-state model has been proposed to explain the temperature effects on thermosensitive TRP channels (Brauchi et al., 2004; Voets et al., 2004). According to the model, temperature reduces the activation energy associated with voltage-dependent open and closed states. For heat-activated channels, the transition to the open state is facilitated by hot temperatures, whereas for cold-activated channels, the closed state is prolonged by cold temperatures; chemical agonists serve as gating modifiers. For TRPM8, with a Q₁₀ for activation of 24, a decrease in enthalpy and entropy accompany channel activation, suggesting that opening involves large conformational changes of the channel protein (Brauchi et al., 2004). For TRPA1, similarly, cooling shifts the voltage dependence of the channel toward more negative potentials; the data can also be fitted well with a model based on a decrease in entropy and enthalpy associated with activation (Karashima et al., 2009). Whether the modulatory domains of *Drosophila* TRPAs allow for warm temperatures (Viswanath et al., 2003; Lee et al., 2005) instead of cold to alter voltage-dependent gating is an intriguing question that will allow some evolutionary insight into the role TRPA1 plays in thermosensation.

TRPM8 Knockout Mice

To separate the responses to cold mediated by TRPM8 and by TRPA1, we can first look at residual function in trigeminal and dorsal root ganglion (DRG) neurons of TRPM8 knockout mice. TRPA1 is expressed in a population of nociceptors that also express TRPV1 and that is largely separate from TRPM8 neurons. In wild-type animals, two populations of DRG neurons that respond to cooling can be distinguished by their threshold for activation. The neurons activated by innocuous cooling tend to respond to menthol as well, suggesting that these express TRPM8.

TRPM8 knockout animals show a large reduction in the number of cells sensitive to icilin and to menthol, as assayed by Ca²⁺ imaging (Bautista et al., 2007; Colburn et al., 2007; Dhaka et al., 2007). Cells that responded to cooling to 22°C were absent in the knockout. However, a small population of dissociated DRG neurons still responded to noxious cold temperatures below 15°C. Because these neurons appeared insensitive to mustard oil, it was argued that the residual response to noxious cold in the TRPM8 knockout is not mediated by TRPA1 (Bautista et al., 2007).

Dissociation of DRG neurons may not preserve their natural sensitivity, particularly if cold-sensitive channels are mainly in peripheral processes that are detached during dissociation. Thus the *ex vivo* skin/nerve preparation was used to deliver short (20-s) cold temperature ramps from 32 to 2°C and to record firing rates in peripheral fibers.

In this preparation, a striking reduction in both the number and the firing rate of cold-responsive fibers was observed (Bautista et al., 2007). There was not complete elimination of cold-sensing fibers, however. Both these assays suggest that another cold sensor besides TRPM8 mediates response to noxious cold. It may be TRPA1 or it may be quite different from TRP channels. For instance, nearly half of superior cervical ganglion neurons are activated by cooling, but very few respond to either menthol or mustard oil, suggesting neither TRPM8 nor TRPA1 is involved (Munns et al., 2007).

Behavioral assays of TRPM8 knockout mice show a clear deficit in avoidance of cold (Bautista et al., 2007; Colburn et al., 2007; Dhaka et al., 2007). In a binary temperature-choice assay, wild-type mice avoid a cold plate that is cooled to 25°C or less, whereas TRPM8^{-/-} mice avoid the cold plate only when it is cooled to 15°C or less (Bautista et al., 2007). In a continuum temperature assay, wild-type mice largely stay at locations above 30°C, whereas TRPM8^{-/-} mice venture down to 20°C (Dhaka et al., 2007). These studies suggest that TRPM8 is mainly responsible for detecting cool temperatures between 23 and 10°C. However, these assays primarily assessed temperature preference, a behavior distinct from the percepts of noxious cold such as pain, ache, and pricking. These animals also suggest the presence of another system for detecting noxious cold in the absence of TRPM8.

TRPA1 Knockout Mice

To address the role of TRPA1 in noxious cold sensation, two independent knockout animals were made (Bautista et al., 2006; Kwan et al. 2006). Both knockout alleles contain a deletion in the same region of TRPA1 (the fifth and sixth transmembrane domains, required for ion conduction), so differences in phenotype probably cannot be attributed to differences in genotype. In one study, trigeminal neurons were briefly (~30 s) cooled to noxious cold temperatures (6–16°C) and assessed with Ca²⁺ imaging. In wild-type animals, ~16% of the cells showed robust responses to cooling, and most of those also responded to menthol, suggesting that they are TRPM8-expressing cells. The remainder did not respond to mustard oil, suggesting that they do not express TRPA1. These experiments gave no evidence of a cold-sensitive, TRPA1-expressing population of cells, and so it is perhaps not surprisingly that the percentages did not change in neurons from TRPA1^{-/-} animals (Bautista et al., 2006). Similar results were seen in DRG neurons.

However, parallel studies of the vagus nerve, which carries fibers of the nodose and jugular ganglia, do suggest a role for TRPA1 in noxious cold sensation (Fajardo et al., 2008). Sensory neurons from the nodose ganglion and jugular ganglion innervate the viscera and body wall, respectively. In Ca²⁺ imaging experiments, nearly half of the nodose ganglion neurons were cold sensi-

tive, and most cold-sensitive neurons displayed the pharmacological properties of TRPA1 and not TRPM8; they were activated by the TRPA1 agonist cinnamaldehyde and blocked by camphor and HC03001 (Xu et al., 2005). Importantly, TRPA1^{-/-} mice had only half the number of cold-sensitive neurons compared with wild type; lost was the population of nodose neurons that are sensitive to both cold and to cinnamaldehyde. This suggests that TRPA1 is activated by noxious cold to produce visceral pain sensations (Fajardo et al., 2008).

Similarly, cold-sensitive neurons from trigeminal ganglia were studied with Ca²⁺ imaging (Karashima et al., 2009). About 20% of trigeminal neurons responded to cooling. Some apparently expressed TRPM8 based on sensitivity to menthol and insensitivity to mustard oil; others apparently expressed TRPA1 based on mustard oil sensitivity. In TRPA1^{-/-} animals, only 10% of the neurons responded to cooling, half that of wild-type animals; lacking were the cold-sensing neurons that were also mustard oil sensitive. Moreover, the missing population tended to have thresholds in the painful cold range of 10 to 20°C, consistent with TRPA1 mediating responses to painful cold while TRPM8 responds to cooling. The difference with the Bautista et al. (2006) results might be explained by latency of activation. TRPA1-expressing neurons respond to cold about three times more slowly than TRPM8-expressing neurons, requiring >100 s to reach full response (Karashima et al., 2009), so that the short ~30-s stimuli of the Bautista experiments may have missed responses from this population of cells.

For both knockout animals, differences in behavior were sought to represent deficits in cold pain sensation in the TRPA1^{-/-} animals. When acetone was applied to a paw to cause evaporative cooling, Bautista et al. (2006) found no difference in flinches per minute, but Kwan et al. (2006) found the duration of paw lifting or shaking was almost halved in the knockouts. When mice were placed on a cold plate that was cooled below 0°C, Bautista et al. (2006) found no difference in the latency (~40 s) to the first paw lift or first shiver. Kwan et al. (2006) instead counted the number of paw lifts over an extended period (300 s) and found the total number was almost halved in knockouts.

To follow up on this difference, Bautista et al. (2007) used a temperature preference assay in which mice chose between two surfaces that varied in temperature from 30 to 5°C, with one surface always 5–10°C warmer than the other. At all temperatures the mice chose the warmer plate ~80% of the time (300-s test duration). The choice behavior was unchanged in the TRPA1 knockout mice, suggesting that TRPA1 does not mediate temperature preference.

Does temperature preference reflect pain sensation? Karashima et al. (2009) assayed two additional behaviors that may be more directly related to pain: jumping and tail flick. When placed on a cold plate chilled to

0°C, wild-type mice almost always jump, with a latency of ~20 s. For TRPA1^{-/-} mice, only 12% of animals jumped at all, and even then it was with a latency three times longer. Similarly, when their tails are immersed in a -10°C solution, wild-type mice flick them out in 10–15 s, but TRPA1^{-/-} mice take 30–40 s, and a third of them do not respond at all (Karashima et al., 2009). Moreover, whereas Kwan et al. (2006) detected statistically significant differences in cold response only for female knockout mice, these more robust responses were significant for both sexes. Importantly, both these tests explore the noxious temperature range where TRPA1 is expected to be most active, and—if we can guess what the mice are feeling—may be more representative of pain.

Reconciliation

There has been significant disagreement about the role of TRPA1 in cold sensation. At least three issues complicate comparison among studies. First, there is the overlapping temperature range of TRPM8 activation and TRPA1 activation, and overlapping pharmacology (with menthol activating both TRPM8 and TRPA1 but inhibiting TRPA1 at higher concentrations). Second, there are differences in the duration of stimulus application, with some studies finding no effects on TRPA1 for short (20–30-s) cooling and others finding that TRPA1 is activated by cold but rather more slowly. Finally, there is the interpretation of behavioral experiments, confounded both by the presence of an entire brain between the stimulus and the assay, and by the uncertainty of what behaviors represent pain and what represent preference.

On balance, we are persuaded by evidence for a role of TRPA1 in sensation of pain associated with noxious cold. The demonstration that TRPA1 can be activated in cell-free patches and in the absence of Ca²⁺ (Sawada et al., 2008; Karashima et al., 2009) is particularly convincing. Although the experiments do not show that TRPA1 is directly activated by cold—there might still be a membrane-associated second messenger system—it shows a very intimate association with the sensation of noxious cold temperatures. Moreover, the ability to describe cold activation of TRPA1 with a biophysical theory similar to that for other TRP channels suggests a direct effect of cold (Karashima et al., 2009). Although some experiments using Ca²⁺ imaging have failed to see cold activation in TRPA1-expressing cultured cells, or failed to see cold sensitivity in DRG neurons known to express TRPA1, it is possible that Ca²⁺ imaging is not sensitive enough to detect weak responses. Indeed, cold was found to be a weaker activator of TRPA1 than mustard oil, so that responses of low-expressing cells might have been missed (Karashima et al., 2009).

Experiments from knockout mice seem to agree that a population of neurons exists in both trigeminal and nodose ganglia that are activated by noxious cold and are not responsive to TRPA1 agonists. Both ganglia,

however, also have neurons that are activated by noxious cold and by TRPA1 agonists, cinnamaldehyde or mustard oil, and that are absent in the knockout (Fajardo et al., 2008; Karashima et al., 2009). Thus, there may be three general populations of cold-sensitive sensory ganglion neurons: those expressing TRPM8, those expressing TRPA1 and also TRPV1, and those that use neither TRPM8 nor TRPA1 for detecting cold. Their relative proportions may vary between different ganglia or even different species. As many have noted, the TRPM8 neurons may convey the quality of cool or cold, while the later two classes may be generally involved in coding for pain produced by a variety of stimuli. Together, these signals may be integrated in the central nervous system to produce the perception of painful cold.

Behavioral experiments have been particularly confusing, with some showing a clear behavioral deficit and others not (Bautista et al., 2006; Kwan et al., 2006; Karashima et al., 2009). In general, the assays that did show a deficit in TRPA1^{-/-} mice in what we think of as pain-associated behavior (flinching, paw withdrawal, jumping, and tail flick) tend to involve tests of longer duration that might allow for the slower TRPA1 activation. A test of long duration that found no deficit in TRPA1 knockouts—the temperature preference assay (Bautista et al., 2007)—was not necessarily testing pain. Thus, a preference for warmer temperatures observed even in the TRPA1 knockout mice might be mediated by TRPM8.

Despite the tortuous path toward resolution, a wealth of recent papers has provided fairly convincing evidence for direct activation of TRPA1 by cold, and has elucidated a central role in pain for this most sensitive of sensory channels.

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