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Molecular basis of peripheral innocuous cold sensitivity

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Abstract

Of somatosensory modalities cold is one of the more ambiguous percepts, evoking the pleasant sensation of cooling, the stinging bite of cold pain, and welcome relief from chronic pain. Moreover, unlike the precipitous thermal thresholds for heat activation of thermosensitive afferent neurons, thresholds for cold fibers are across a range of cool to cold temperatures that spans over 30°C. Until recently, how cold produces this myriad of biologic effects was unknown. However, recent advances in our understanding of cold mechanisms at the behavioral, physiologic, and cellular level have begun to provide insights into this sensory modality. The identification of a number of ion channels that either serve as the principal detectors of a cold stimulus in the peripheral nervous system, or are part of a differential expression pattern of channels that maintain cell excitability in the cold, endows select neurons with properties that are amenable to electric signaling in the cold. This chapter highlights the current understanding of the molecules involved in cold transduction in the mammalian peripheral nervous system, as well as presenting a hypothetic model to account for the broad range of cold thermal thresholds and distinct functions of cold fibers in perception, pain, and analgesia.

INTRODUCTION

The somatosensory system is able to detect subtle changes in ambient temperature due to the coordinated efforts of functionally and molecularly distinct thermo-sensory neurons. These cells are capable of discerning temperatures that are perceived as painful (extreme cold or burning heat) and innocuously pleasant (mild cooling or comfortable warmth). In humans, the perception of innocuous cold temperatures occurs when the skin is cooled as little as 1°C, with very little deviation in thermal acuity thresholds between subjects (Erpelding et al., 2012). The threshold for painful cold is reported to be near 12°C; however, this modality of pain is felt over a broad temperature range spanning 0–28°C (Morin and Bushnell, 1998). Unlike the precipitous and distinct percept that is painful heat, there are differences in how cold pain is felt, with subjects describing painful cold as encompassing a number of distinct qualities, such as sharp, stinging, aching, or pricking pain (Morin and Bushnell, 1998). Thus, psychophysically cold can be an ambiguous percept, something that has confounded our understanding of the detection and transduction mechanisms of cold sensation. This chapter focuses on the cellular mechanisms that endow cold-sensitive neurons with the capacity to

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detect cold temperatures, primarily those that are pleasant, but also how this system may underlie the rare conditions in which innocuous cool is painful.

BEHAVIORAL RESPONSES TO COLD TEMPERATURES

One of the key means of determining the properties of cold-sensitive peripheral neurons is to correlate their activity and function to an animal's response to cold temperatures. However, for cold this has proven challenging. Classic assays for testing animals' sensitivity to heat traditionally include a heated plate from which the animals' thermal sensitivity is measured by the latency or speed in which they lift or withdraw their hindpaw. The shorter the latency, the more sensitive the animal. For cold, however, the cold plate test tends to be highly variable between studies, even those conducted in the same laboratory (Daniels and McKemy, 2007). For example, in studies of different mouse lines in which the genes of putative molecules involved in cold were disrupted (knockout or gene-nulls), latencies for hindpaw lifts at near freezing temperatures (0 to −1°C) ranged from 5 to 200 seconds in control mice (McKemy, 2010). This high degree of variability has made conclusive analysis of cold difficult. At these temperatures, mice generally assume a posture that largely limits contact with the cold surface by rearing up on their hindlimbs and not exposing their forepaws, rump, and tail to the cold (Blasius et al., 2011). These significant differences in animal behavior have motivated the field to identify and develop novel experimental approaches to assess cold sensitivity.

For example, a variation of the cold plate test used restrained mice allowing for measurements of both paws independently as only one is placed on a cold plate at a time (Gentry et al., 2010). Moreover, this eliminated any confounds caused by whole-body exposure to cold and subsequent reduction in mobility, as seen in the cold plate assay. Using this approach, latencies to lifts at 0°C were less than 10 seconds, a duration consistent with responses to noxious heat (Gentry et al., 2010). However, the acts of habituating the animals to restrainment can be problematic, and this methodology is not commonly used. Alternatively, a dynamic thermal plate approach has been employed, in which an animal is placed on a surface warmed to near 32°C and then the temperature is slowly reduced until the animal performs an escape behavior, which was near 5°C in control mice (Descoeur et al., 2011). The confounding concern for this assay is that it does not account for adaptation that will occur with the gradual change in temperature.

Until recently, the most used cold sensitivity assay employed evaporative cooling, a methodology that uses a droplet of acetone placed on the animal's hindpaw (Choi et al., 1994). As the liquid evaporates the surface skin temperature drops to near 17°C, inducing flinching, licking, and guarding behaviors depending on the animal's sensitivity. While considered a measurement of innocuous cold, the behaviors are reminiscent of nocifensive (pain-like) responses, suggesting that temperatures in this range may indeed be bothersome to the animal. However, acetone has an odor, and a liquid is applied to the animal's skin in this assay, variables that hamper interpretation of exactly what component of the behavior is due to cold and what the other stimulus modalities (olfaction and mechanosensation) are contributing.

A relatively new technique uses a measurement of paw withdrawal latencies in freely moving animals to radiant cooling as a gauge of cold sensitivity. This approach, termed the cold plantar assay, is similar to the radiant heat Hargreaves assay (Hargreaves et al., 1988), and employs a compressed pellet of dry ice pressed underneath the surface of a glass plate on which the animal stands (Brenner et al., 2012). Thus, the surface on which the animal's paw is placed is quickly cool and cold sensitivity is determined by the latency taken for the animal to lift its hindpaw. This approach is attractive as it is performed on freely moving animals without the need for restraint or touching of the paw as with the acetone assay. However, the cold plantar assay does not provide a means to measure cold-sensitivity thresholds as animals show similar latencies to lift regardless of the temperature of the surface (Brenner et al., 2014). Animals instead respond to an absolute change in temperature of ~1–2°C. Nonetheless, the analogy of this assay to that of the well-established Hargreaves assay makes this an appropriate approach to measure cold sensitivity.

The most robust tests for cold acuity to date have been the two-temperature choice assay and the thermal gradient assay (Bautista et al., 2007; Colburn et al., 2007; Dhaka et al., 2007). For the former, in this nonreflexive assay, animals are placed in a chamber comprised of two thermally controlled plates and then allowed to freely move across both surfaces. When both are maintained at the same temperature, animals will explore the entire chamber and spend an equal amount of time on each surface. To test thermal sensitivity, one plate is held constant (typically 32–30°C) with the other varied to either warmer or colder temperatures. The animal's preference is determined by the surface on which it spends the majority of the recording period, whereas the animal's avoidance of a temperature is inferred by how often it transitions between temperatures (McCoy et al., 2011). These approaches have been used in various combinations to test the necessity of putative cold-relevant molecules in animal behavior, providing the most compelling data to date.

Lastly, thermal gradient assays are used to establish the absolute preferred temperature of experimental animals, with either rectangular or circular chambers constructed in which the surface temperature ranges from extreme cold to cool to warmth to extreme heat in a linear fashion (Dhaka et al., 2007; Touska et al., 2016). The assay is advantageous as it allows a linear longitudinal ambient temperature gradient common for six separate channels to be established such that behaviors such as cold seeking can be determined along with the preferred ambient temperature of experimental animals (Almeida et al., 2006a, b; Wanner et al., 2017). However, this assay does not measure cold temperature exclusively, but provides evidence as to the animal's preferred temperature over time.

Nonetheless, as with all the assays described, these preference assays have their flaws, namely that they do not establish acute cold sensitivity, nor can they be used to determine altered pain sensitivity in a single location as with the other assays. Thus, there is still great need in the field to establish better animal behavior assays, and investigators are encouraged to employ multiple strategies in order to obtain the clearest representation of an animal's temperature acuity in different experimental paradigms. This is keenly important in the testing of the necessity of candidate molecular and cellular detection and transduction mechanisms for cold sensation.

HUMAN

Properties of cold-sensitive peripheral afferent sensory neurons

Afferent sensory neurons of the dorsal root (DRG) or trigeminal ganglia, with nerve endings in the skin and oral cavity, are the initial detectors of temperature change, with both small-diameter C-fibers and medium-diameter Aδ-fibers responsive to this stimulus modality (McKemy, 2007). Seminal studies conducted in the mid to late 20th century identified cold-responsive single nerve fibers in humans and animals that were loosely categorized as either thermoreceptors involved in the detection of innocuous cool, or cold nociceptors mediating cold pain, all defined by the activation temperature (Hensel, 1973; LaMotte and Thalhammer, 1982; Simone and Kajander, 1996, 1997; Carr et al., 2009). Cold nociceptors are quiescent at normal skin temperature and only become active when the skin is cooled to temperatures in the noxious cold range (LaMotte and Thalhammer, 1982; Simone and Kajander, 1997). Conversely, cold chemoreceptors display tonic activity at skin temperature, with cold leading to an increase in their firing rates and, conversely warmth reducing nerve responses (Brock et al., 2001; Campero et al., 2001; Madrid et al., 2006). However, the exact proportion of thermoreceptors or nociceptors that respond to cold temperatures is not clear, with some reports suggesting that 10–15% of cells within a ganglion are responsive to cool to cold temperatures, or up to 100% if the stimulus intensity drops below freezing (Simone and Kajander, 1996, 1997; Campero et al., 2001).

The key cellular evidence for the transduction processes that underlie cold-dependent changes in cell function came from observations that an increase in intracellular Ca^{2+} concentration occurred in cultured rat DRG neurons when temperatures were lowered from ~30 to 20°C (Suto and Gotoh, 1999). These responses were due to Ca^{2+} influx as they required the presence of external Ca^{2+} and were independent of external sodium concentration, suggesting that Na^{+}-dependent membrane depolarization was not required. In vitro, 10–15% of trigeminal ganglion or DRG neurons respond to cold temperatures at thresholds for activation ranging from ~35 to near 15°C (Suto and Gotoh, 1999; Reid and Flonta, 2001b; McKemy et al., 2002; Babes et al., 2004; Xing et al., 2006; Madrid et al., 2009).

Several studies found the presence of two distinct groups of cold neurons; the first was distinguished by response properties indicative of thermoreceptors with an activation temperature near 30°C. The second had properties suggestive of cold nociceptors with a high-threshold activation temperature of below 20°C (Thut et al., 2003; Xing et al., 2006; Madrid et al., 2009). These different activation thresholds suggest the former cells to be an in vitro model for innocuously cool signaling afferents, while the latter may be analogous to those mediating noxious cold. Indeed, high-threshold cells were largely capsaicin-sensitive, further implicating these cells as nociceptors (Xing et al., 2006). Thus, cold neurons respond over a wide range of cold thermal thresholds and cultured sensory afferents are a useful experimental model and provide insights into the mechanisms of cold signaling.

Natural products produced by plants have been fundamental in our understanding of nervous system function. This is also true for cold in that the vast majority of cold-sensitive neurons respond to the cold-mimetic menthol, a cyclic terpene alcohol found in leaves of the genus

Mentha and commonly included in many commercially available products (Patel et al., 2007). The sensation of pleasantly cool evoked by menthol is well known, but at higher concentrations it can cause burning, irritation, and pain (Green, 1992; Cliff and Green, 1994; Green and Schoen, 2007). Seminal studies by Hensel and Zotterman (1951) examining cold sensitivity in cat lingual nerve recordings found that menthol raised the cold activation temperature of cold fibers, suggesting these effects were due to modulation of an as yet unidentified cold detection mechanism. Indeed, they proposed that menthol would "exert its action upon an enzyme, which is concerned in the thermally conditioned regulation of the discharge of the cold receptors" (Hensel and Zotterman, 1951).

Further validation for this hypothesis came from study of cultured sensory neurons where ~15% of excitable cells were menthol-sensitive (assayed largely with Ca^{2+}-microfluorimetry), and in most reports there was a strong correlation with menthol and cold sensitivity at the cellular level (Reid and Flonta, 2001b, 2002; McKemy et al., 2002; Viana et al., 2002). Moreover, rigorous experiments in which Ca^{2+}-imaging-based screens for cold and menthol-sensitive neurons were coupled with electrophysiology showed that both cold and menthol activated a nonselective cation conductance in sensory afferents (Reid and Flonta, 2001b; McKemy et al., 2002). Menthol-evoked currents are observed in cold-sensitive neurons with both low- and high-threshold temperature responses, but are more robust in the former (Xing et al., 2006; Madrid et al., 2009). Thus, cold-sensitive neurons, both in vivo and in vitro, are largely sensitive to menthol and this sensitivity is used as a chemical cold mimetic to identify cold-sensing nerves. For further insights into afferent neural pathways of thermoeffectors, see Chapters 16 and 19 in this volume.

The role of Na^+ and K^+ conductances in cold signaling

What then allow neurons to respond to a change in ambient temperature? Initial data showing that inhibition of Na^+/K^+ ATPase increased the static discharge frequency of cold fibers suggested that processes controlling neuronal polarity might underlie a cold transduction mechanism (Pierau et al., 1974). This made intuitive sense, as cold will inhibit enzymatic function, consistent with several reports finding that inhibition of Na^+/K^+ ATPase affected cold-fiber responses, leading to the conclusion that its activity mediated neuronal cold sensitivity (Pierau et al., 1974, 1975; Spray, 1974; Schafer and Braun, 1990). However, further study showed that the magnitude of the depolarization was largely sub-threshold, dampening its influence on cold responses and suggesting the Na^+/K^+ ATPase was not part of the cold detection mechanism (Reid and Flonta, 2001a).

Similarly, sodium and potassium currents are fundamental in the cell's ability to fire action potentials, and both types of conductance have been associated with cold responses (Kang et al., 2005; Zimmermann et al., 2007; Abrahamsen et al., 2008; Madrid et al., 2009; Noel et al., 2009). Nav1.8, a tetrodotoxin-resistant voltage-gated sodium channel, is expressed in approximately 75% of mouse peripheral sensory neurons, including those considered nociceptors (Shields et al., 2012). The channel's involvement in cold signaling was first reported when it was shown to be resistant to inhibition by cold (Zimmermann et al., 2007). Normally, cold temperatures gradually increase voltage-dependent slow inactivation of tetrodotoxin-sensitive Nav channels, thereby limiting excitability. However, inactivation of

Nav1.8 channels is unaffected by cold. Moreover, cold reduces the voltage activation threshold for Nav1.8, properties suggesting that this channel is the primary impulse generator in the cold (Zimmermann et al., 2007). Consistent with these in vitro data, mice lacking the Nav1.8 gene, or in which Nav1.8-expressing neurons are ablated, are reportedly unresponsive to noxious cold, yet show normal responses to measures of innocuous cool sensitivity (Zimmermann et al., 2007; Abrahamsen et al., 2008). Thus, Nav1.8 clearly plays a role in cold transduction, presumably specifically involved in noxious cold signaling.

Nav1.9 has also been shown to play a key role in cold responses (Lolignier et al., 2015). Channel expression and function are upregulated after injury and suggested to enhance cellular responses of cold transducers under pathologic conditions. Both acute cold responses and cold hypersensitivity after injury were dampened in mice and rats with reduced Nav1.9 expression, indicating that it is involved in the transduction of cold signals in vivo. Similarly, neuropathic cold hypersensitivity is attenuated in mice lacking functional Nav1.6 channels and by treatment with Nav1.6 antagonists (Sittl et al., 2012; Deuis et al., 2013). Thus, cold transduction is tuned by select Na^+ conductances.

Similarly, potassium conductances are also critical in regulating excitability and several mechanisms have been proposed for K^+-channel-mediated cold signaling (Belmonte et al., 2009). For example, one of the earliest indicators for a cold transduction mechanism was that cooling induced the closing of a background K^+ current (Reid and Flonta, 2001a; Viana et al., 2002), causing depolarization and firing via an ever-present cationic inward current, termed Ih, which was poorly inhibited by cold. In cold-insensitive neurons, cold-evoked firing is prevented by a slow, transient, 4-AP-sensitive K^+ current (IKD) that acts as an excitability brake, and its absence, or minimal expression, in cold-sensitive neurons allows for depolarization in the cold (Viana et al., 2002). Pharmacologic blockade of IKD induced thermosensitivity in cold-insensitive neurons, suggesting that cold activation was not the product of cold directly acting on a cold thermosensor, but that these cells were electrogenically tuned by the differential expression of select K^+ conductances (Reid and Flonta, 2001a; Viana et al., 2002). Reports also find that cells with a high-threshold cold response express high levels of K^+ brake currents, whereas low-threshold neurons express low levels of IKD (Madrid et al., 2009). However, the molecular identity of IKD has yet to be elucidated, but is suggested to be a member of the voltage-gated Kv channel family.

In addition, the two-pore domain K^+ channels TREK-1 and TRAAK are heat-sensitive when expressed in heterologous expression systems (Maingret et al., 2000; Kang et al., 2005). In regard to their role in cooling, it is proposed that heat-evoked activity of these channels polarizes cells, whereas cooling leads to channel closing, depolarization, and then subsequent action potential firing. Thus, as described for IKD currents, the absence of this K^+-mediated brake current in the cold may contribute to cold neuronal activity (Maingret et al., 2000; Kang et al., 2005; Noel et al., 2009). Double-knockout mice for both channels are reported to be more sensitive to cold on the cold plate at temperatures ranging from 10 to 20°C, as well as show deficits in thermal preference in the cool range (from 21 to 15°C) (Noel et al., 2009). While all of the mechanisms are clearly critical for cold signaling, as well as other sensory modalities, it remains to be validated if these serve as cold detectors rather than transducers.

Detectors of cold in sensory neurons

As described above, the mechanisms that control cell excitability are clearly important for cold signaling, as they are for all neuronal function, but they do not provide a clear molecular rationale for what enables specific subsets of afferents to be selectively cold-sensitive. The first evidence for specific cold detection came from the analysis of cell responsivity to the cold mimetic menthol. Cold- and menthol-evoked activity of a biophysically identifiably cation conductance suggested the presence of a specific protein (as Hensel and Zotterman (1951) proposed) mediating cold sensitivity. This was validated with the cloning of transient receptor potential melastatin 8 (TRPM8, also called Trp-p8 or CMR1) as a cold and menthol-gated ion channel (McKemy et al., 2002; Peier et al., 2002). In sensory ganglia, TRPM8 is expressed in <15% of small-diameter (~20 μm) sensory neurons, consistent with the proportion of neurons shown to be cold- and menthol-sensitive in neuronal cultures (McKemy et al., 2002; Peier et al., 2002; Takashima et al., 2007). TRPM8 is found in a heterogeneous population of neurons, with a subset expressing nociceptive markers such as TRPV1 and calcitonin gene-related peptide, as well as labels for both Aδ- and C-fibers, the receptor tyrosine kinase TrkA and the artemin-receptor GFRα3 (Takashima et al., 2007, 2010; Dhaka et al., 2008).

Consistent with the psychophysical sensation of cold induced by menthol, TRPM8 channels are directly gated when temperatures drop below 26°C, with activity increasing in magnitude down to 8°C (McKemy et al., 2002; Peier et al., 2002; Zakharian et al., 2010). Interestingly, this broad range spans what are considered both innocuous cool (~30–15°C) and noxious cold temperatures (<15°C). A number of cold-mimetic compounds activate TRPM8 channels, including Cool-actP, Cooling Agent 10, FrescolatMGA, FrescolatML, geraniol, hydroxycitronellal, linalool, PMD38, WS-3, and WS-23 (McKemy et al., 2002; Behrendt et al., 2004; Weil et al., 2005). Many of these compounds, such as icilin, will induce characteristic shivering or “wet dog” shakes when given intravenously, a process that requires TRPM8 channels and neurons (Wei and Seid, 1983; Dhaka et al., 2007; Knowlton et al., 2013). Similarly, a number of antagonists have been identified, including PBMC, M8-B, BCTC, thio-BCTC, CTPC, and capsazepine, with PBMC and M8-B shown to induce a dramatic hypothermic response in mice, likely due to inhibition of cold-sensing afferents (Behrendt et al., 2004; Knowlton et al., 2011; Almeida et al., 2012).

The mechanisms underlying gating of TRPM8 channels by cold are still unresolved, as is the case for all thermosensitive TRP channels. Purified TRPM8 channels, when reconstituted into a planar lipid bilayer, are activated by cold, the most convincing evidence that this channel is directly gated by temperature (Zakharian et al., 2010). TRPM8 channels are weakly voltage-sensitive and show characteristic outward rectification (McKemy et al., 2002). This voltage sensitivity, along with the topologic similarity between TRP channels and voltage-gated K^+ channels, suggested that activation by temperature and voltage are linked (Brauchi et al., 2004; Voets et al., 2004, 2007). However, there is evidence that temperature-, agonist-, and voltage-dependent gating are independent processes, since distinct activation domains for each have been identified, suggesting that the effect of one gating mechanism acts on another in an allosteric fashion (Brauchi et al., 2004, 2006, 2007; Matta and Ahern, 2007; Daniels et al., 2009).

A key modulator of TRPM8 channel activity is the phosphoinositide PIP2, which has been shown to be obligatory for TRPM8 channel function. TRPM8 channels adapt to prolonged stimulation in a manner that requires the presence of external Ca^{2+} (McKemy et al., 2002), and channel activity in excised membrane patches rapidly runs down after excision, but can be recovered by the presence of exogenous PIP2 on the cytoplasmic face of the channel (Liu and Qin, 2005; Rohacs et al., 2005). Activation of phospholipase C (PLC) also leads to channel adaptation, suggesting that Ca^{2+} entry through the TRPM8 pore activates Ca^{2+}-sensitive PLCδ isoforms to cleave PIP2 and thereby adapt TRPM8 channels (Daniels et al., 2009). Moreover, residues in the proximal region of the C-terminus near the TRP domain of TRPM8 have been proposed to interact with PIP2 (Rohacs et al., 2005).

In addition to modulation by PIP2, the GTP-binding protein Gαq directly binds to TRPM8 channels and inhibits activity (Klasen et al., 2012; Zhang et al., 2012). Gβγ had no effect on channel function, and a Gαq chimera that could not activate downstream signaling cascades was equally capable of inhibiting TRPM8. These data suggested that TRPM8 is inhibited under conditions of inflammation that activate Gαq, thereby exacerbating heat responses. However, in vivo evidence also shows that inflammatory mediators such as the glial cell line-derived neurotrophic factor receptor ligand artemin and nerve growth factor can potentiate cold responses in a TRPM8-dependent manner (Lippoldt et al., 2013). In addition, mice lacking the receptor for artemin, GFRα3, do not exhibit cold allodynia in a variety of nerve injury models, suggesting that activation of this neurotrophin pathway leads to alterations in TRPM8 channel or neuronal function to promote cold pain (Lippoldt et al., 2016). Thus, TRPM8 likely plays a fundamental role in cold pain.

TRPM8-null ($Trpm8^{-/-}$) mouse lines have severe deficits in cold sensation (Bautista et al., 2007; Colburn et al., 2007; Daniels and McKemy, 2007; Dhaka et al., 2007). One consistent measure of cold deficits in $Trpm8^{-/-}$ mice has been the evaporative cooling assay which has clearly shown a lack of cold sensitivity in these mice (Bautista et al., 2007; Dhaka et al., 2007; Blasius et al., 2011). However, results using the cold plate have proved to be less clear. Two groups reported no difference between wild-type and $Trpm8^{-/-}$ mice using the cold plate tests at 10, 0, −1, −5, or −10°C (Bautista et al., 2007; Dhaka et al., 2007), while a third found a significant difference in withdrawal latency at 0°C, results suggesting the animals were deficient in noxious cold sensation (Colburn et al., 2007). When tests were done with lightly restrained $Trpm8^{-/-}$ mice they showed a significantly longer withdrawal latency than wild-type when their hindpaws were placed on a 10°C plate (Gentry et al., 2010). Moreover, $Trpm8^{-/-}$ mice were completely unresponsive in the dynamic cold plate assay, suggesting they do not perceive cold temperatures at all (Descoeur et al., 2011).

Lastly, while mice rarely show representative nocifensive behaviors with their hindpaws under these conditions, two robust and reproducible behaviors involving the forepaw are readily visible (Knowlton et al., 2013). The first presents as a flinching of both forepaws, which occurs shortly after wild-type animals are placed on a 0°C surface, and a second manifests as a wringing or licking of the forepaw, suggestive of intense irritation. Control animals respond with latencies of less than 10 seconds, whereas $Trpm8^{-/-}$ mice rarely show these behaviors up to the 60-second cut-off time for this noxious stimulus (Knowlton et al.,

2011). Thus, depending on the experimental approach, cold plate behavioral data suggest these animals are at least partially deficient in cold nociception.

Interpretation of cold deficits in Trpm8$^{-/-}$ mice using the two-plate choice assay has also been problematic. These animals show a robust deficit in thermal preference and are unable to discern the difference between the 30°C surface and when the test plate is held at temperatures down to 15°C (Bautista et al., 2007; Colburn et al., 2007; Dhaka et al., 2007; Knowlton et al., 2010), demonstrating that they cannot discriminate between warm and putatively innocuously cool temperatures. However, once temperatures drop below 15°C, Trpm8$^{-/-}$ mice do prefer the warm side, albeit less than what is observed for wild-type mice (Bautista et al., 2007; Knowlton et al., 2010), results implying that other transduction mechanisms predominate below 15°C. It is not clear if this preference for warmth is due to a drive to avoid an unpleasant stimulus, or if it is prompted by a drive to seek out a comfortably warm environment, something that is critical for the maintenance of core body temperatures. Therefore, Trpm8$^{-/-}$ mice may not be able to detect the noxious cold signal when given the choice of two surfaces held at 30°C and <15°C, but are able to discern that the warm surface is preferable due to input from warm fibers (McCoy et al., 2011).

In support of this hypothesis, when the number of times an animal crosses from the 30°C surface to the test surface and back again is counted, wild-type mice show a significant drop in the number of crossing events as one plate is cooled (Knowlton et al., 2010). Indeed, when the test plate is held at 5°C, wild-type mice on average cross over and back from the cold surface once and largely never return, whereas Trpm8$^{-/-}$ mice freely transition between plates, showing no signs of aversion (Knowlton et al., 2010). For further insights into cold pain, see Chapter 6.

While there is some residual cold sensitivity in Trpm8$^{-/-}$ mice, those in which TRPM8 neurons were ablated in the adult animal showed a much more dramatic cold-deficient phenotype (Knowlton et al., 2013; Pogorzala et al., 2013). These animals displayed no preference even at plate temperature below 15°C, suggesting that the molecular properties of TRPM8 neurons are the key determinant of cold sensitivity.

Topical application of cold or cooling compounds produces a temporary analgesic effect mediated by TRPM8-expressing afferents (Proudfoot et al., 2006; Knowlton et al., 2013). In a rodent model of neuropathic pain, paw withdrawal latencies in response to mechanical or heat stimuli were significantly attenuated in animals first treated with cold or cooling compounds such as icilin. Analgesia persists for over 20–30 minutes, after which the animals regained their hypersensitivity similar to before the cool stimuli were applied and at levels of those not pretreated with cold or cooling-compounds. Only modest cooling and low doses of cooling compounds produced analgesia and this is dependent on TRPM8 channels and neurons (Proudfoot et al., 2006; Knowlton et al., 2013).

Moreover, using formalin (a compound that evokes acute pain followed by inflammation) injections into wild-type mouse hindpaws it was found that cooling to 17°C and 24°C produced a marked decrease in pain behaviors (licking and lifting hindpaws) during the acute pain phase (Dhaka et al., 2007). However, mice lacking TRPM8 did not behave

similarly in that they continued to show nocifensive responses even after exposure to the cool surface and were indistinguishable from wild-type animals that were not exposed to cool temperatures. Together these data indicate that TRPM8 is mediating the analgesia provided by cool temperatures and cooling compounds, suggesting that modest activation of TRPM8 afferent nerves can serve as an endogenous mechanism to promote pain relief.

In addition to the roles of TRPM8 in somatosensation and nociception, the channel is involved in thermoregulation and homeostatic mechanisms. The channel's involvement in thermoregulation would not be entirely unexpected in light of evidence that other temperature-sensitive ion channels, particularly TRPV1, play a role in regulating body temperature as well (Iida et al., 2005; Montell and Caterina, 2007; Gavva, 2008; Gavva et al., 2008). Moreover, intravenous administration of icilin induces a robust thermogenic-type behavior characterized as "wet dog shakes" (Wei and Seid, 1983), as well as inducing a significant increase in core body temperature, mimicking exposure to cold, effects that are absent in TRPM8$^{-/-}$ animals (Dhaka et al., 2007; Ding et al., 2008; Knowlton et al., 2011; Tajino et al., 2011). The TRPM8 dependence of the icilin-induced changes in core temperature are not due to a general deficiency in the ability of these animals to respond to chemical thermal mimetics as TRPM8-nulls do mount an appropriate hypothermic response to capsaicin injections (Knowlton et al., 2011).

The observation that the cold activation threshold of TRPM8 is approximately 23–26°C suggested to many that the channel mediates innocuous cool, and that other transduction mechanisms are needed to account for noxious cold responses (McKemy, 2005). In screens for TRP channels with robust expression in sensor neurons, TRPA1 was identified and shown to be activated by the TRPM8 agonist icilin when expressed in heterologous expression systems (Story et al., 2003). This original description of TRPA1 also reported that the channel is activated by cold. Calcium imaging and voltage clamp recordings of recombinant TRPA1 channels found that cold temperatures ranging between 8 and 28°C (average threshold of 17°C) evoked an increase in intracellular Ca^{2+} and nonselective cation currents, respectively, that were blocked by ruthenium red, a blocker of several Ca^{2+}-permeable channels (Story et al., 2003). TRPA1 is almost exclusively in nociceptive afferents that also express TRPV1, substance P, and calcitonin gene-reltated peptide, but distinct from those expressing TRPM8 (Story et al., 2003). Thus the expression pattern and activation threshold for TRPA1 made it attractive as the mediator of cold pain and, when coupled with the activation threshold of TRPM8, suggested a similar dual-sensor model for cold transduction as was known for heat (Patapoutian et al., 2003; McKemy, 2005).

However, this hypothesis has proven controversial (Jordt et al., 2004; McKemy, 2005; Nagata et al., 2005; Zurborg et al., 2007; Caspani and Heppenstall, 2009; Knowlton et al., 2010). TRPA1 was found to be the receptor for several pungent irritants such as allyl isothiocyanate (the pungent ingredient in mustard oil and wasabi), allicin (the irritant in raw garlic), and cinnamaldehyde (the spicy component of cinnamon) (Bandell et al., 2004; Jordt et al., 2004; Bautista et al., 2005; Macpherson et al., 2005), all substances which do not evoke the psychophysical sensation of cold. As with menthol and cold responses (Hensel and Zotterman, 1951), the prediction would be that TRPA1 agonists such as cinnamaldehyde or mustard oil should sensitize cold responses, yet studies found no differences in cold

withdrawal threshold when these were topically applied to anesthetized rats (Sawyer et al., 2009; Dunham et al., 2010). Similarly, when mustard oil was topically applied, no increases in cold-evoked spikes were seen in spinal wide dynamic range neurons. However, when cinnamaldehyde was injected into the rat paw, a significant drop in paw lick/jump latency was seen in response to both 0°C and 5°C plate tests in one report, continuing the ambiguity (Tsagareli et al., 2010). In addition, several groups were unable to reproduce TRPA1 activation by cold in both heterologous and native cells innervating the periphery (Jordt et al., 2004; Zurborg et al., 2007). However, cellular context seems critical in that one intriguing report found that TRPA1 channels expressed in dissociated neurons from DRG were not cold-sensitive, whereas cold responses were recorded in isolated vagal afferents, and these were TRPA1-dependent (Fajardo et al., 2008). Lastly, the putative cold activation of TRPM8 does not cross species as other orthologues of the channel are activated by heat (Rosenzweig et al., 2005; Sokabe et al., 2008; Gracheva et al., 2010), furthering the complexity of this channel's role in cold.

Two independently generated Trpa1$^{-/-}$ lines were analyzed for deficits in cold transduction with mixed results. In one, no deficits in cold-induced behaviors were observed, including responses to evaporative cooling, cold plate withdrawal behaviors, shivering, and thermal preference (Bautista et al., 2006, 2007). However, the other Trpa1$^{-/-}$ line was reported to have deficits in evaporative cooling and cold withdrawal behaviors, although curiously these differences were only significant in female and not male mice (Kwan et al., 2006). Moreover, this line of Trpa1$^{-/-}$ mice was reported to have reduced escape behaviors (jumping when placed on a 0°C cold plate) as well as longer latencies to tail flicks when the tail was immersed in −10°C liquid (Karashima et al., 2009). However, in mice in which both TRPM8 and TRPA1 genes were disrupted, there was no difference in preference or avoidance behaviors beyond those already present in Trpm8$^{-/-}$ mice (Knowlton et al., 2010).

However, TRPA1 is important for cold hypersensitivity after injury (Obata et al., 2005; Dai et al., 2007; da Costa et al., 2010). Inflammation-induced increase in cold sensitivity was blocked using a TRPA1 antagonist, as well as reduced by a decrease in TRPA1 transcript expression (da Costa et al., 2010; del Camino et al., 2010). Moreover, cold hypersensitivity was induced by injection of an endogenous TRPA1 agonist in wild-type but not Trpa1$^{-/-}$ mice (del Camino et al., 2010). An informative result was the observation that TRPA1 channels, both native and heterologously expressed, were not sensitive to cold temperatures, but cold did amplify agonist-evoked TRPA1 currents (del Camino et al., 2010). Thus, under the context of inflammation or nerve injury, when TRPA1 channels are likely stimulated by endogenous mediators, cold may further potentiate these responses and lead to cold-evoked pain. Indeed, TRPA1 likely serves in this context to all modalities and is now considered a "gatekeeper" for chronic pain (see Bautista et al. (2013) for review). As cold hypersensitivity is also reduced in Trpm8$^{-/-}$ mice (Colburn et al., 2007; Knowlton et al., 2011), what remains to be determined is how TRPA1 activation leads to cold hypersensitivity and if this process works through TRPM8 or other transduction channels.

Lastly, homomeric recombinant TRPC5 channels are cold-sensitive in the innocuous range when tested in heterologous systems (Zimmermann et al., 2011). In cellular recordings, TRPC5 was constitutively active at 32°C (skin temperature) with currents increasing with

cooling, and decreasing with warmth. In addition, activation of PLC pathways augmented cold activation of TRPC5. The channel is expressed in DRG neurons, but in a population distinct from TRPM8. However, cold-activated TRPC5 current was not detected in cultured neurons, but in cells from Trpc5$^{-/-}$ mice there was a reduction in the number of cold-sensitive cells measured by changes in intracellular Ca^{2+} levels. These data are further confounded as expression of TRPM8 was reduced in Trpc5$^{-/-}$ mice and no deficits in cold behaviors were observed in these animals. This study was the lone examination of TRPC5 and cold, suggesting an as yet undetermined modulatory role for this channel in cold sensation. For further insights see Chapter 2.

A model for the cellular basis of cold sensation

Our current understanding of the peripheral mechanisms of cold sensation is complicated by the diverse array of molecules that appear to be integral in this stimulus modality. At the core is TRPM8 which, unlike the other molecular candidates for cold transduction, appears to be exclusively involved in cold signaling and not mediating other modalities of somatosensation. What still needs to be resolved is how TRPM8 channels can generate responses in high-threshold cold-sensitive nerves if it is activated initially at temperatures in the innocuous range? Mechanistically thermoceptors such as TRPM8 provide the receptor potential to initiate firing, much in the way ionotropic glutamate receptors mediate post-synaptic potentials. Thus, other electrogenic properties of the nerve, such as channel density, current magnitude, and neuronal excitability are fundamental in impulse generation. Cold cells are readily excitable as expression of K^+ brake conductances is low, thereby limiting the contribution of hyperpolarizing conductances (Viana et al., 2002; Madrid et al., 2009; Noel et al., 2009). In high-threshold cells, expression of TRPM8 is reduced, whereas high levels of IKD currents, attributed to K_v1 channels, are expressed, thereby necessitating a more robust thermal stimulus to activate sufficient TRPM8 currents in order to overcome the excitability brake established by the K^+ conductances. Moreover, Nav1.8, Nav 1.9, and Nav1.6 are likely key in axonal conduction under these cold conditions (Zimmermann et al., 2007; Abrahamsen et al., 2008). Lastly, both TRPM8 and TRPA1 are required for cold pain after injury, and signal independently due to their reported expression in distinct cell types, via an as yet undefined nociceptor cell type expressing both channels, or through an indirect mechanism whereby activation of TRPA1 afferents sensitizes TRPM8-expressing high-threshold nerves such that they fire at a lower (warmer) temperature threshold.

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