Mini-Symposium

New Insight in Cold Pain: Role of Ion Channels, Modulation, and Clinical Perspectives

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Cold temperature detection involves the process of sensory transduction in cutaneous primary sensory nerve terminals, which converts thermal stimuli into depolarizations of the membrane. This transformation into electrical signals is followed by the subsequent propagation of action potentials in cold-sensitive afferent nerve fibers. A large array of ion channels shapes this process; however, the precise contribution of specific ion channel subtypes to cold perception and cold pain remains elusive. This review aims at giving an update on our current understanding of the role played by TRPs, leak K^+ and voltage-gated Na^+ and K^+ channels in the transduction of cold by nociceptors and in cold-induced pain.

Key words: **cold pain; ion channels; nociception**

The perception of cold temperatures starts with the conversion of thermal stimuli into electrical signals by molecular transducers in the plasma membrane of primary sensory nerve terminals, a process known as sensory transduction. Some of the cold-sensitive nerve fibers detect moderate innocuous cold, whereas others detect noxious cold temperatures ~<19°C (Campero et al., 1996, 2001). Ultimately, the intensity and duration of the stimuli are coded into trains of action potentials by voltage-gated ion channels. A large array of ion channels, including thermo-sensitive Transient Receptor Potential (TRP), sodium and potassium channels shape this process. Our current understanding of the mechanisms of thermal transduction is fairly incomplete, especially the transduction of cold stimuli that appears to involve different cold transducers, some of which are yet to be identified, and other ion channels playing an indirect role in this process by setting the electrophysiological environment required by the transduction machinery. Pathological cold pain, a common symptom in a range of neuropathic pain syndromes, may also arise from dysfunctions of this transduction machinery, and presents as cold allodynia, a pain response to cold temperatures that do not normally provoke pain, and/or cold hyperalgesia, an increased sensitivity to painful cold temperature (Yin et al., 2015). The aim of this review is to provide an overview of our current knowledge about the contribution of specific ions channels to physiological and pathological cold transduction and cold-triggered pain, emphasizing on the recent progress made in the understanding of the identity and respective roles and regulation of these channels. New findings about the two thermo-TRP channels TRPM8 and TRPA1 recognized as involved in cold perception will be presented. The contribution of the voltage-gated sodium channel Nav1.9 to cold pain in animals and humans will also be described together with the wide diversity of potassium channels shown to be important for cold sensation. Finally, we will present how different cold pain conditions induced by toxins or chemotherapeutic agents altering cold sensitivity give us insight regarding cold transduction and cold pain sensation.

Ion channels involved in the transduction of cold temperature by nociceptors

TRP channels

TRP cation channel subfamily M member 8 (TRPM8), the receptor for menthol, was the first cold-transducer channel to be described (McKemy et al., 2002; Peier et al., 2002). This nonselective cation channel is directly activated by innocuous cooling (<28°C, $Q_{10} > 20$), and *in vivo* behavioral studies showed an involvement of TRPM8 channels in thermal discrimination <25°C (Bautista et al., 2007; Colburn et al., 2007; Dhaka et al., 2007). More recent studies have also shown a role for TRPM8 channels and TRPM8-expressing neurons in cold-triggered nociception (Knowlton et al., 2011; Pogorzala et al., 2013). Although TRPM8 involvement in non-noxious thermal discrim-

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ination <25°C is undisputable, its involvement in cold-triggered nociception remains debated (Yin et al., 2015). Whereas TRPM8 activation by cold as well as by exogenous substances such as menthol have been the matter of extensive studies (Almaraz et al., 2014), endogenous activators or inhibitors of TRPM8 have more rarely been identified. Interestingly, it was shown previously that androgens increase TRPM8 expression in non-neural cells (L. Zhang and Barritt, 2004; Y. Zhang et al., 2004; Thebault et al., 2005). In addition to this genomic regulation by androgens, testosterone acts directly on the TRPM8 channel at subphysiological concentrations (Asuthkar et al., 2015), and recent unpublished work also shows that, in the presence of the androgen receptor, physiological concentration of testosterone specifically inhibits TRPM8 activity in transfected cells and primary sensory neurons through direct interaction of the channel with the androgen receptor at the plasma membrane. Most interestingly, *in vivo* experiments show that androgens reduce male sensitivity to non-noxious cold temperatures through a TRPM8-dependent mechanism (D.G. et al., unpublished data). This may be consistent with the notion that elevated plasma levels of testosterone, which usually accompany mating behaviors, physical activity, stress, or aggression, by desensitizing TRPM8 would help to diminish the impact of environmental cold as a factor that may impede taking necessary actions.

The TRP cation channel subfamily A member 1 (TRPA1), or ANKTM1, is another thermo-TRP channel expressed in nociceptive DRGs and trigeminal neurons. TRPA1 is activated by pungent compounds and was initially characterized as a cold-sensitive ion channel (Story et al., 2003), but this has remained contentious ever since, with a number of studies presenting supporting (Fajardo et al., 2008; Karashima et al., 2009; Moparthi et al., 2014) or conflicting evidence (Jordt et al., 2004; McKemy, 2005; Bautista et al., 2006; Knowlton et al., 2010). However, pharmacological inhibition or genetic inactivation of TRPA1 clearly revealed the importance of TRPA1 for the behavioral response to noxious cold (≤5°C) *in vivo* (Kwan et al., 2006; Karashima et al., 2009; Gentry et al., 2010). In contrast, the absence of TRPA1 does not influence the behavior of mice in thermal preference tests designed to evaluate comfort temperature preferences (Knowlton et al., 2010), suggesting that TRPA1 most specifically controls the responsiveness to noxious cold, but not to innocuous cool. Several reports have also identified TRPA1 as a physiological sensor of critical importance for cold hypersensitivity associated with inflammatory and neuropathic pain (Zygmunt and Högestätt, 2014). Recent unpublished observations suggest a novel mechanism for TRPA1 regulation of cold nociception and cold pain. It is hypothesized that TRPA1 regulates cold sensitivity *in vivo* indirectly, rather than by simply acting as a sensory transduction molecule (D.A. et al., unpublished data).

The discovery of the cold-triggered activation of the TRP cation channel subfamily C member 5 (TRPC5) has expanded the list of cold-sensitive TRP channels expressed in DRG neurons (Zimmermann et al., 2011). However, it is not clear whether TRPC5 plays a role in somatosensory cold sensation as, although *TRPC5*$^{-/-}$ fibers have an increased responsiveness to cooling, *TRPC5*$^{-/-}$ mice show normal response to noxious cold and cold preference over wild-type mice.

These findings also point to another aspect of cold transduction by DRG neurons because 30%–50% of isolated DRG neurons in culture are sensitive to cold but unresponsive to either agonists of TRPA1 or TRPM8, indicating that a fraction of sensory neurons most probably relies on a different, yet unidentified, mechanism to transduce cold stimuli (Alloui et al., 2006; Munns et al., 2007).

Potassium channels

Potassium channels are hyperpolarizing inhibitory channels and therefore key determinants of neuronal excitability, regulating resting membrane potential, spike threshold and duration, and repetitive firing activity. Primary sensory neurons express a complex complement of potassium channels that modulates their transducing properties and shape their excitability (Gold et al., 1996; Belmonte and Viana, 2008; Tsantoulas and McMahon, 2014). The diversity of K^+ channels expressed in DRG neurons was confirmed at the single-cell transcriptome level (Usoskin et al., 2015). A number of studies have highlighted the role of different potassium channels in the modulation of cold sensitivity (for review, see Belmonte and Viana, 2008). Their expression and function change following neuronal injury, which contributes to nociceptors hyperexcitability and pain symptoms, such as mechanical and cold allodynia (Chien et al., 2007; Descoeur et al., 2011; Pollema-Mays et al., 2013; Pereira et al., 2014; Tsantoulas and McMahon, 2014).

Among the different families of K^+ channels, several members of the KCNK channel family, also known as 2-pore domain (K_{2P}) potassium channels, were found to play a key modulatory role in cold sensation. These channels mediate voltage-independent background leak K^+ currents and participate in setting the resting membrane potential of many neurons (Chemin et al., 2003). There are 15 members of this family, with different biophysical properties and distinct expression profiles in sensory neurons (Mathie and Veale, 2015). Some of these channels (e.g., TREK-1, TREK-2, and TRAAK) are highly temperature-sensitive (Maingret et al., 2000; Kang et al., 2005). Analysis of TREK and TRAAK deficient mice suggests their participation in nociception and thermal sensitivity (Alloui et al., 2006; Noël et al., 2009; Pereira et al., 2014). More specifically, *TREK2*$^{-/-}$ mice show enhanced responses to moderate cold temperatures but no signs of abnormal cold pain. In contrast, *TREK1*$^{-/-}$ and *TRAAK*$^{-/-}$ mice have augmented responses to noxious cold. The expression of TREK1, TREK2, and TRAAK channels in DRGs was also found to be diminished in a mice model of neuropathic cold hypersensitivity induced by the antineoplastic agent oxaliplatin (Descoeur et al., 2011; Pereira et al., 2014). Thus, in some forms of neuropathic pain conditions, reduced background potassium activity may contribute to heightened cold sensitivity.

Transcriptome analysis of fluorescent-activated cell sorting of TRPM8-expressing mouse cold thermoreceptors revealed the differential expression of TASK-3, a pH-sensitive K_{2P} leak channel, a finding confirmed by immunocytochemistry and RT-PCR (Morenilla-Palao et al., 2014). Application of a selective TASK-3 blocker shifted the temperature threshold of cold-sensitive TRPM8-expressing DRG neurons to warmer temperatures. Moreover, analysis of cold thermoreceptors in *TASK*-3 KO mice revealed a higher sensitivity to cooling and augmented responses to electrical and thermal stimulations. This argues that combinatorial expression of cold-sensitive hyperpolarizing and depolarizing channels in primary sensory nerve terminals can lead to functional diversity.

The voltage-gated potassium channels Kv1 and Kv7.2/7.3 have been implicated in the physiology of cold sensing and in the pathophysiology of cold pain. In TRPM8-expressing cold-sensitive neurons, differential expression of a fast activating, slowly inactivating, dendrotoxin- and 4-aminopyridine-sensitive, voltage-gated K^+ current (Kv1.1 and Kv1.2 subunits), known as IKD, plays a major role in modulating threshold temperatures to cold stimuli (Madrid

et al., 2009). Activation of IKD dampens the depolarizing effect of the cold-activated TRPM8-dependent current, shifting the temperature thresholds of individual neurons to colder values and reducing their overall response to temperature drops. In a fraction of sensory neurons, application of micromolar doses of 4-aminopyridine can transform their functional phenotype leading to abnormal cold sensitivity (Belmonte and Viana, 2008), an effect also found in intact and damaged peripheral sensory axons (Roza et al., 2006). Moreover, after local blockade of IKD in the hindpaw of mice, normally innocuous cool stimuli elicited nociceptive behaviors, suggesting that IKD acts as a brake to cold sensitivity *in vivo* (Madrid et al., 2009). Thus, IKD sharpens the tuning of sensory neurons to relevant stimuli.

KCNQ channels Kv7.2/3, the molecular components of the M-current, also modulate the response of nociceptors to cold in synergy with TRPM8 channels. Indeed, pharmacological blockade of the M-current increases the excitability of a large fraction of C fibers in response to cold, in which TRPM8 channels activation is required (Vetter et al., 2013). Sensitization of nociceptors to cold by the cooling agents camphor or menthol has also been shown to involve concomitant Kv7.2/3 blockade and TRPM8 activation.

Voltage-gated sodium channels

Nav1.7, Nav1.8, and Nav1.9 are the most abundant voltage-dependent Na^+ channel isoforms in peripheral afferent fibers. Genetic variants of these channels are associated with a spectrum of distinct inherited pain disorders, ranging from congenital pain insensitivity to severe neuropathic pain syndromes. Two of these voltage-gated Na^+ channels, the tetrodotoxin-resistant Nav1.8 and Nav1.9 channels, are expressed in nociceptors and involved in the response of cold-sensitive fibers to noxious cold. The inactivation properties of Nav1.8 and Nav 1.9 channels are less affected by cooling than the tetrodotoxin-sensitive channels, which makes them able to contribute to action potentials initiation in cold-sensitive fibers at low temperatures.

Nav1.8 is important for nociceptors' ability to remain excitable at low temperature, whereas the cold-induced inactivation of other channels in other afferent fibers appears to contribute to the loss of other sensory modalities (numbness) observed with cooling of peripheral tissues (Zimmermann et al., 2007). Genetic ablation of Nav1.8 in mice results, among other impairments of pain sensitivity, in a decreased sensitivity to noxious cold (Akopian et al., 1999; Zimmermann et al., 2007).

The ultra-slow inactivating tetrodotoxin-resistant Nav1.9 channel, whose expression pattern largely overlaps with Nav1.8 in DRG neurons, has also recently been found to be resistant to cooling and to be involved in the painful response to cold (Lolignier et al., 2015). *Nav1.9*$^{-/-}$ mice have an increased tolerance to noxious cold (from $\leq$12°C) and nociceptors from *Nav1.9*$^{-/-}$ mice show a decreased activation in response to cooling. In cold-sensitive nociceptors, specifically, a strong increase in Nav1.9 current was observed. This increased inward Na^+ current is necessary for the firing of cold-sensitive neurons in response to cooling, in which the large and persistent Nav1.9 current amplifies depolarizations generated by cold transducer channels. The identity of the cold transducer channel(s) expressed by Nav1.9-positive/cold-responding sensory fibers is however still unclear as the vast majority of these fibers appear not to express TRPM8 or TRPA1. Nav1.9 is also involved in cold hypersensitivity in mice (at 15°C-20°C) following treatment with oxaliplatin. This places Nav1.9, together with Nav1.8, at the center of physiological and pathological painful responses to cold. These two channels, together with leak and voltage-gated potassium channels, would provide an ideal electrophysiological environment for the coding and transmission of cold nociceptive information.

Inherited pain disorders associated with Nav1.9 genetic variants

Chronic pain syndromes associated with the Nav isoforms Nav1.7, 1.8, and 1.9, include primary erythromelalgia, paroxysmal extreme pain disorder (PEPD), and small-fiber neuropathies (Dib-Hajj et al., 2010). In particular, Nav1.7-dependent primary erythromelalgia, a condition characterized by severe pain attacks preferentially in the lower legs and arms as well as in the adjacent joints, is recognized for its marked temperature dependence because warmth is often a strong trigger for painful episodes and exacerbates symptoms while patients consistently experience pain relief on cooling of affected body areas.

A heterozygous gain-of-function mutation in *SCN11A*, caused by the missense mutation p.V1184A in Nav1.9 channels, has recently been identified in a family with a history of early-onset chronic peripheral pain (Leipold et al., 2015). Affected family members suffer from pain attacks in their lower and upper extremities lasting ~20–30 min, which are reminiscent of erythromelalgia-associated pain episodes caused by hyperactive Nav1.7 channels. However, patients exhibit a reversed temperature sensitivity of pain sensation because pain is aggravated by cold and partially relieved by warmth. Electrophysiological evaluation of heterologously expressed Nav1.9-V1184A channels revealed that the mutation increases the basal activity of Nav1.9 such that mutant channels require less depolarization to open, effectively increasing the fraction of active mutant channels. In agreement with these gain-of-function features, mutant channels increase the resting membrane potential of murine DRG neurons and subsequently render the neurons hyperexcitable. A reduced cold-dependent attenuation of the excitability of neurons transfected with p.V1184A compared with neurons transfected with wild-type Nav1.9 was observed, which is in line with the temperature dependence of the patients' pain sensation and suggests that the contribution of hyperactive Nav1.9-V1184A channels to nociceptors excitability is more prominent at lower temperatures. Thus, this study corroborates the link between Nav1.9 and cold-pain sensation initially demonstrated by Lolignier et al. (2015). However, the intrinsic temperature dependence of Nav1.9 was not affected by mutation p.V1184A, suggesting that the role of Nav1.9 in cold pain requires additional cellular factors. As discussed in the previous paragraph, yet unidentified cold transducers are possible candidate proteins. According to Lolignier et al. (2015), Nav1.9 acts as an amplifier of cold transducer subthreshold signals. Hyperactive Nav1.9-V1184A mutant channels may as a consequence cause cold-aggravated peripheral pain by signal overamplification in cold-sensitive nociceptors.

Pathological cold pain conditions

Although many pathological pain conditions, including diabetic neuropathy, peripheral nerve injury, chemotherapy-induced neuropathy, poststroke central pain, or ciguatera poisoning, can result in the development of cold pain, the mechanisms by which cold pain arises are still poorly understood and appear to vary significantly in relation to the disease considered (for review, see Yin et al., 2015).

The mechanisms of action of toxins altering cold sensitivity give us insight regarding cold transduction beyond those provided by animals or humans harboring mutant channels. Pathological cold pain is a frequent symptom of ciguatera poisoning, a form of marine food poisoning arising from the consumption of

tropical and subtropical fish contaminated with ciguatoxins from microscopic algae of the *Gambierdiscus* family accumulated through the marine food chain. Ingestion of contaminated fish causes symptoms of dysaesthesias, paresthesias, and cold allodynia in almost all ciguatera patients (Vetter et al., 2014). At the molecular level, ciguatoxins are the most potent known sodium channel activators and additionally enhance neuronal excitability through inhibition of potassium channels. It is now clear that the pathophysiological effects of ciguatoxins can be attributed directly to their action on peripheral sensory neurons, as local intradermal injection in humans recapitulates spontaneous pain and cold allodynia (Zimmermann et al., 2013). Similar symptoms can be observed after intraplantar injection in mice, an effect that is mediated predominantly through peripheral sensory neurons expressing TRPA1 (Vetter et al., 2012). Ciguatoxin-induced cold allodynia can additionally be blocked by concomitant treatment with selective Nav1.8 and Nav1.6 inhibitors, consistent with profound effects on excitability of nociceptive C- and A-fibers, respectively.

In contrast to ciguatera poisoning, cold allodynia elicited after local injection of the chemotherapeutic agent oxaliplatin is unchanged in *Nav1.8* KO mice and develops independently from cold-sensitive TRP channels (Deuis et al., 2013). Oxaliplatin-induced cold allodynia is blocked completely by the selective Nav1.6 inhibitor GIIIA, suggesting a specific role for Nav1.6-expressing sensory neurons in oxaliplatin-induced cold allodynia. However, Nav1.6-expressing neurons seem not to be involved in physiological cold sensation because intraplantar administration of the Nav1.6-selective activator Cn2 elicits spontaneous pain and mechanical allodynia but does not enhance cold sensitivity. Conversely, intraplantar administration of the K^+ channel blocker 4-aminopyridine mimicks oxaliplatin-induced cold allodynia, which is inhibited by Nav1.6 blockers and potentiated by Nav1.6 activators (Deuis et al., 2013). Therefore, it appears that inhibition of neuronal K^+ channels is a common mechanism underpinning the development of enhanced cold sensitivity in both ciguatoxin- and oxaliplatin-induced cold allodynia. Although the precise mechanisms leading to specific changes in cold sensitivity remain to be determined, it is likely that this effect arises at least in part from biophysical changes induced by cooling, which include a decrease in the activation threshold of the sodium currents, an increase in membrane resistance, and closure of temperature-sensitive background potassium channels. In normally cold-insensitive neurons, these effects are opposed by the continued activity of temperature-insensitive K^+ channels, which act as an excitability break. Pharmacological inhibition, or reduced expression, of these channels can in turn drive normally cold-insensitive neurons to respond to cooling, although it remains to be determined whether temperature-sensitive TRP channels are an absolute requirement for this phenomenon. Although these channels likely include Kv1.1 in some types of neurons, the molecular identity of the K^+ channels contributing to cold sensitivity of Nav1.6-expressing neurons remains to be determined.

In conclusion, these recent findings highlight the complexity of cold transduction and coding of cold stimuli by sensory nerve fibers. An array of ion channels is involved in physiological cold sensation and pain and in pathological cold-pain states, either directly or through the tuning of the electrophysiological properties of the cell membrane. The molecular mechanisms of cold hypersensitivity, such as allodynia, affect multiple ion channels and pathways in different ways depending on the pathology in place, even when they look very similar or indistinguishable at the phenotypic level.

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