

The nociceptive TRPM3 channel as potential therapeutic target for the treatment of chronic pain

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ABSTRACT

Chronic pain is a major health problem affecting millions of people worldwide. Despite decades of research, chronic pain remains poorly understood and notoriously hard to control. Indeed, about half of the chronic pain sufferers report inadequate pain control with current analgetics. Therefore, identification of novel potential drug targets as a starting point in the development of novel painkillers represents an important aim in pain research. Several members of the Transient Receptor Potential (TRP) ion channel superfamily are highly expressed in sensory neurons, where they play a role in the detection of painful stimuli. Here, we will provide a detailed overview of a recently identified nociceptive TRP channel, TRPM3, as a promising target for the treatment of chronic pain.

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GENERAL INTRODUCTION:

Pain – Ion channels

Chronic pain management represents a serious healthcare problem worldwide, as it affects approximately 14.6% of the adult population within the United States (1). Unfortunately, its management in the community remains generally unsatisfactory, as currently available analgesics often show insufficient pain relief. Classical medicines used to relieve pain, including nonsteroidal anti-inflammatory drugs (NSAIDs) and opioids, are among the most used drugs. However, long-term treatment with these analgesics for chronic pain is hampered by inter-individual variability in drug efficacy, unwanted side-effects. Accordingly, for a better control of chronic pain a deeper knowledge of the underlying molecular mechanisms is mandatory.

Nociceptive pain serves as a protective mechanism and an associative condition, as well as an alarm system for a wide range of pathological conditions. The first and foremost process is the peripheral detection and transduction of noxious stimuli, which are conveyed to higher-order structures in the central nervous system (CNS). Typically, nociceptive neurons are either thinly myelinated A δ or unmyelinated small diameter - low conduction velocity C fibers. The cell bodies of these neurons are located in the dorsal root ganglia (DRG) or trigeminal ganglia (TG), and they have one axon with two branches, with one branch extending to the peripheral tissues such as skin and internal organs, and the other branch relaying the detected information to

second-order neurons located in the dorsal horn (2, 3). The neurons are functionally characterized by the type of sensory ion channels expressed on the plasma membrane. These ‘sensory’ ion channels are important for the detection of various noxious stimuli including thermal (e.g. heat or cold), mechanical (e.g. crushing or shearing) and chemical signals (e.g. acid or chemicals released during inflammation). These ion channels, expressed at the plasma membrane of sensory nerve endings, convert the noxious stimuli into electrical signals, which then travel from the periphery to the CNS in the form of action potentials (2, 3).

In recent years, various types of ion channels, including members of the Transient Receptor Potential (TRP) channels, have been identified as highly sensitive molecular pain detectors or ‘nociceptor’ channels. These discoveries have fueled detailed studies examining the molecular and cellular mechanisms of nociception and have led to the development of novel targets for the treatment of pain (4). Here, we provide a brief overview of the role of a recently characterized member of the TRP superfamily in sensory neurons, TRPM3, as nociceptive channel, and of its therapeutic potential as a target for the treatment of chronic pain. In addition, we discuss properties of TRPM3 in comparison to previously defined therapeutic targets like TRPV1.

Nociceptive TRP channels

The superfamily of TRP channels contains 28 mammalian members (27 in humans) and is subdivided based on sequence homology

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into six subfamilies, categorized as Canonical (TRPC), Vanilloid (TRPV), Ankyrin (TRPA), Melastatin (TRPM), Polycystin (TRPP), and MucoLipin (TRPML) (5). Typically, TRP channels contain six transmembrane spanning regions (S5-S6), a pore-forming loop between the fifth (S5) and sixth (S6) regions and intracellular N- and C- termini (Fig. 1A) (6). Assembly of four of these subunits into homo- or heteromultimers results in functional ion channels (Fig. 1B) (7). TRP channels are mostly non-selective cation channels with, except for TRPM4 and TRPM5, significant calcium permeability. They are found in a wide variety of cell types, both on the plasma membrane and in intracellular organelle membranes (8). Several TRP channels act as primary transducers of sensory stimuli, making the TRP superfamily one of the most extensively studied receptor families in sensory biology (9).

Based on their ability to detect and transduce specific nociceptive modalities and their expression in sensory neurons, some members of the TRP superfamily have been grouped into the category of 'nociceptive TRP channels'. Activation of nociceptive TRP channels by specific pain-producing stimuli generates cell depolarization, which can result in activation of voltage-gated ion channels and generation of action potentials (Fig. 1C). Therefore, these channels serve as the primary molecular sensors for the detection and transduction of pain under physiological and pathophysiological conditions (10). In addition, modifications in channel function, trafficking or gene

expression of nociceptive TRP channels are critically implicated in the development of hypersensitivity and pain under a variety of pathological conditions. These findings indicate that pharmacological targeting of TRP channels may be a novel means to treat specific pain pathologies.

During the past decennia, several nociceptive TRP channels have been identified, including TRPV1, TRPM8 and TRPA1 (10). The first molecularly identified nociceptor TRP channel was TRPV1, which was discovered based on its sensitivity to capsaicin, the pungent compound of hot chili peppers (11). Like the majority of TRP channels, TRPV1 is polymodal in terms of its activation. Indeed, in addition to capsaicin, the channel can be directly activated by endovanilloids such as N-arachidonoyl-dopamine and endocannabinoids such as anandamide (12-14), as well as by heat, extracellular acidity, ($\sim \leq \text{pH } 6.0$) (11, 15, 16), and intracellular alkalinisation ($\sim \geq \text{pH } 7.8$) (17). Importantly, *Trpv1* knockout mice not only lack sensitivity to capsaicin, but also fail to develop thermal hyperalgesia after inflammation and injury (18-20).

TRPM8 was identified as the first TRP channel involved in cold detection by the somatosensory system (21, 22). *Trpm8*-deficient mice exhibit a deficit in avoiding cool temperatures (23, 24). Moreover, whereas mild cooling can evoke analgesia in wild-type mice, cooling induced analgesia was absent in *Trpm8*-deficient mice (24).

TRPA1 was originally described as a cold sensitive ion channel (25-28), although this conclusion has been debated by others (29).

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TRPA1 can be activated by a plethora of chemically diverse noxious or irritant compounds (27, 30, 31). Several *in vivo* studies have shown that *Trpa1*-deficient mice have marked deficiencies in the nocifensive responses to such chemical agents as well as to noxious cold (25, 32, 33).

The role of TRPV1, TRPM8 and TRPA1 in pain has been extensively reviewed elsewhere (10). Below we provide a current view of (patho) physiological roles of the more recently identified nociceptor channel TRPM3.

TRPM3

The human *Trpm3* gene is the largest gene on chromosome 9 (9q21.11-q21.12 (34), and TRPM3 was originally described as a volume-regulated, non-selective, Ca^{2+} -permeable, voltage-dependent cation channel in the human kidney (35, 36).

Expression

TRPM3 is widely expressed in different organs and cell types. High expression was confirmed on the mRNA and protein levels in brain, pituitary gland, kidney, pancreas, eye and the nervous system (37). In particular, expression was detected in different brain regions, as well as in peripheral sensory neurons (35, 38-40). Interestingly, RT-qPCR data indicate that TRPM3 expression in neurons of the DRG and TG is as high as that of the other nociceptive TRP channels TRPV1, TRPM8 and TRPA1 (40). At the functional level, TRPM3 expression was demonstrated in pancreatic cells (38, 41-43) and

somatosensory neurons, where TRPM3 is involved in the sensation of painful stimuli (40, 43, 44).

Biophysical characteristics

The biophysical characteristics of TRPM3 channels have been extensively investigated in heterologous overexpression systems. Typically, heterologous overexpression of TRPM3 proteins results in a small but significant constitutive conductance (35, 36, 45), which can be further increased by various ligands (see below). Similar properties have been described for endogenously expressed TRPM3 channels in pancreatic cells and primary cultures of mouse DRG and TG (38, 40). Reported single channel conductances are ~65 pS in the presence of Ca^{2+} and ~83 pS when solely carried by Na^{+} (35, 36).

Pharmacology

TRPM3 presents a polymodally-activated channel, being sensitive to a variety of chemical ligands as well as to physical stimuli (46). Reported endogenous activators include the sphingolipid D-erythro-sphingosine ($\text{EC}_{50} \sim 12\mu\text{M}$) (47) as well as the neurosteroid pregnenolone sulfate (PS) ($\text{EC}_{50} \sim 23\mu\text{M}$) (38, 48). However, the EC_{50} values for these substances to activate TRPM3 are in the micromolar range, and it remains unclear whether such levels are ever obtained under physiologically relevant conditions for the substances to be considered as endogenous TRPM3 activators. In addition, the dihydropyridines nifedipine and de-nitro-nifedipine were identified as exogenous activators of

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TRPM3 (38). To date, the most potent TRPM3 activator in the literature is the synthetic small molecule CIM0216, with a potency that greatly exceeds that of currently used agonists ($EC_{50} \sim 0.77\mu M$) (43). TRPM3 channels are also dependent on temperature and voltage (40). While TRPV1, the prototypical heat-activated TRP channel, shows a sharp increase in activity at temperatures above $42^{\circ}C$ (15), TRPM3 channel activity increases at higher temperatures (46). Importantly, increasing the temperature shifted the dose-response curve of the endogenous agonist PS to lower values, effectively increasing the potency of PS. Thus, at the physiological body temperature, responses to PS doses as low as 100nM reliably activated TRPM3 channels (40). Such nanomolar PS concentrations may be within the physiological range of plasma PS concentrations in humans (49).

Several TRPM3 inhibitors have been reported in literature, including endogenous and exogenous compounds such as steroids, PPAR- γ agonists, fenamates and flavanones (42, 46, 50-54). Highly potent TRPM3 blockers include secondary plant metabolites such as the flavonones naringenin ($IC_{50} \sim 500nM$), liquiritigenin ($IC_{50} \sim 500nM$), and isosakuranetin ($IC_{50} \sim 50nM$) (53, 54). Recently, the nonsteroidal anti-inflammatory drug diclofenac ($IC_{50} \sim 6.2\mu M$), the tetracyclic antidepressant maprotiline ($IC_{50} \sim 1.3\mu M$), and the anticonvulsant primidone ($IC_{50} \sim 600nM$) were identified as highly potent TRPM3 blockers (55, 56). These clinically used drugs abrogate the PS-induced increase in intracellular Ca^{2+} concentrations in freshly

isolated DRG neurons of mouse and rat (53, 55). Several of these TRPM3 inhibitors have been used as tool compounds to study the physiological role of TRPM3 in *in vivo* studies, as will be discussed in more detail in the next section of this review.

Two distinct ionic pores in TRPM3 channels

Like in all TRP channels, activating stimuli lead to the opening of a central cation-conducting pore in TRPM3, which is formed by the pore-forming reentrant loop between the fifth (S5) and the sixth (S6) transmembrane domain. This central pore is highly permeable for Ca^{2+} and Mg^{2+} and can be blocked by the aspecific TRP blocker lanthanum (20, 44). Interestingly, recent evidence was given for the existence of an alternative ion pore in TRPM3 distinct from the central pore that can be activated by the combined application of PS and the antifungal drug clotrimazole (Clt), or by application of CIM0216 (43, 44). Typically, this alternative ion permeation pathway allows for a steep inward current at hyperpolarizing potentials, and exhibits low permeability to Ca^{2+} and Mg^{2+} , and low sensitivity to lanthanum. Activation of this pathway may have physiological consequences in sensory neurons, where opening of the alternative pore leads to an increased influx of Na^{+} in the cell, resulting in stronger depolarization and elevated action potential firing rates. Opening of the alternative ion permeation pathway in TRPM3 represents a possible explanation for some unwanted effects of topically applied drugs. For instance, the antifungal drug clotrimazole, when used to locally treat

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fungal infections, is known to induce local irritation, redness and burning sensation in a significant fraction of patients, and it may be speculated that this irritation results from nociceptor neuron activation following the opening of the non-canonical pore in TRPM3. Possibly, mediators released during inflammation or other endogenously released compounds may also be able to open the alternative TRPM3 pore, but such compounds remain to be identified.

In vivo role of TRPM3

Endogenously expressed TRPM3 channels were first studied in the insulinoma cell line Ins1 and in mouse pancreatic cells in primary culture, where it was shown that activation of TRPM3 by PS increased glucose-induced insulin release (38) and Zn^{2+} uptake (41), leading to the hypothesis that TRPM3 may play a role in the insulin signaling and glucose homeostasis (38). However, no obvious effects are observed in resting blood glucose concentrations in *Trpm3*^{-/-} mice, suggesting that TRPM3 is not essential for normal glucose-induced insulin release in mice (40). Later on, TRPM3 was shown to be functionally expressed in around 60% of neurons of the somatosensory system, and a role as nociceptor channel was proposed (40). Indeed, intraplantar injection of the TRPM3 agonists PS (40) and CIM0216 (43) induces nocifensive behavioral responses such as licking or lifting of the injected paw in wild type mice, whereas these nocifensive responses are lacking in *Trpm3*^{-/-} mice. Oppositely, systemic application of the TRPM3 inhibitors isosakuranetin, hesperetin

and primidone significantly attenuated nocifensive responses to chemical pain induced by PS (53, 55). All together, these arguments point towards a possible role of TRPM3 as chemosensitive nociceptive channel.

Heterologously expressed TRPM3 is activated by heat, with a current-temperature relationship curve that is shifted towards higher temperatures compared to that of TRPV1 (Fig. 2B). The heat sensitivity of TRPM3 was further validated in a subset of sensory neurons, which showed a strong correlation between heat responsiveness and sensitivity to PS. Moreover, the number of heat sensitive neurons is significantly reduced in *Trpm3*^{-/-} mice (40). Further *in vivo* analysis indicated that *Trpm3*^{-/-} mice have a specific deficit in sensing elevated temperatures in a variety of behavioral assays, including tail immersion, hot plate and the thermal preference tests (40). Similarly, pharmacological inhibition of TRPM3 by isosakuranetin (53) and primidone (55) reduces the sensitivity of mice to acute noxious heat in hot plate (52 °C) and tail immersion assays.

Interestingly, *Trpm3*^{-/-} mice also displayed a deficit in inflammation-induced hyperalgesia toward noxious heat provoked by injection of complete Freud's adjuvant (40). In addition, pretreatment with primidone prevents signs of CFA-induced sensitization, suggesting a clinically relevant role for TRPM3 in treating inflammatory hyperalgesia (55). Importantly, activation of TRPM3 can also lead to release of pro-inflammatory calcitonin gene-related peptide (CGRP) from peripheral nociceptor nerve

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terminals, which can be blocked by the inhibitor isosakuranetin and is absent in *Trpm3*^{-/-} tissue (43). The ability of TRPM3 activators to induce acute nociceptive pain and the reduced pain behavior in *Trpm3*^{-/-} mice, suggested that inhibitors of TRPM3 may be useful analgesics.

TRPM3 as ‘hot’ therapeutic target

Several in-depth studies have been conducted to characterize the role of nociceptive TRP channels in multiple pain pathologies, establishing them as potential targets for new-generation analgesics (an overview is given by (57)). In particular, numerous small molecule inhibitors of TRPV1 have been developed and tested as drugs for treatment for various pain conditions; yet, translation of this research to actual clinical applications has not yet been achieved. Below, we will briefly discuss the lessons learnt from TRPV1 as a pharmaceutical target, and make a case for TRPM3 as a potentially safer alternative.

Based on the role of TRPV1 in various painful syndromes, several classes of potent and selective TRPV1 antagonists have been developed as potential new analgesic drugs. Many of these TRPV1 inhibitors showed encouraging analgesic effects in thermal and mechanical sensitivity in animal models of inflammatory, neuropathic, bone-cancer and post-operative pain (58-60) as well as in studies of post-operative pain in humans (61). However, many of these candidate drugs caused significant hyperthermia in both, model animals and in humans, which greatly hampered the progress of the first

generation of TRPV1-targeting drugs in clinical trials (61-64).

The TRPV1 antagonist-induced hyperthermia is an on-target effect, as it is observed in rodents, dogs, primates and humans but not in *Trpv1*-deficient mice, whereas injection of capsaicin causes TRPV1-dependent hypothermia.(62, 65, 66). A novel generation of TRPV1 antagonists aims to inhibit activity induced by heat or capsaicin, while leaving activation by protons intact. These second-generation inhibitors do no longer markedly affect body temperature (67), but whether such compounds are equally effective as analgesics remains to be established. Note that, comparable to TRPV1 antagonism, inhibition of the cold-activated TRPM8 also affects the body temperature, namely causing transient hypothermia (68, 69). Thus, TRPV1 and TRPM8 both play a central role in thermal homeostasis, which has put important constraints on the possible therapeutic use of antagonists of these channels. In contrast, available results suggest that TRPM3 does not play a major role in the regulation of the body temperature, as systemic application of the TRPM3 agonist PS or of TRPM3 antagonists such as isosakuranetin and primidone did not affect body temperature in mice (Fig. 2A) (40, 53, 55). Altogether and thereby blockers of TRPM3 will not alter the body temperature.

A second concern of inhibition of a temperature sensitive ion channel is the possible impairment of the acute thermosensory function, which may result in inadvertent cutaneous or oral burns, for

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instance when taking a shower or drinking hot coffee. Pharmacological blockade of TRPV1 receptors indeed attenuates response to noxious heat stimulation in uninjured animals (58, 70-72) and in humans (73). Similar observations are also reported for the TRPM3 inhibitor primidone, in that injection of primidone induced prolonged response latencies to a noxious thermal stimulus in the hot plate assay (55). However, *in vitro* modelling of the simulated inward TRPV1 and TRPM3 currents in function of temperature indicates that the current-temperature relation for TRPM3 is shifted towards higher temperatures compared to TRPV1 (Fig. 2B). This would suggest that the onset of the noxious stimulation of sensory neurons will largely depend on TRPV1, and that pharmacological inhibition of TRPM3 will not lead to dramatic changes in acute noxious heat sensing as long as the activity of TRPV1 is left intact.

A third concern is the broad expression pattern of TRPM3 outside the sensory system, which may lead to unwanted on-target side-effects (74). However, considering the generally healthy phenotype of *Trpm3*^{-/-} mice, and the absence of observed neurological problems in the studies using systemic treatment with flavanones and primidone (53, 55), it can be assumed that TRPM3 blockade is, at least on a short-term basis, without major adverse effects.

A growing number of studies has confirmed the pain-relieving action of TRPM3 inhibitors in animal pain models *in vivo*. However, to date, the available compounds

do not represent ideal tools for medical use, considering *i)* the suboptimal pharmacokinetic properties of some compounds (e.g. naringenin and isosakuranetin), *ii)* the agonistic effect on ER-beta (e.g. liquiritigenin), *iii)* the possible aspecific modulation of the blockers towards other proteins like the cytochrome P450 family (e.g. naringenin and liquiritigenin) and *iv)* the dual mechanistic effects due to products of hepatic metabolism (e.g. primidone). Furthermore, it was proposed that off-target effects due to the anti-inflammatory and antioxidant properties of flavanones might have influenced the results obtained in inflammatory pain models (53-55). Therefore, novel TRPM3 antagonists suitable for clinical use need to be identified or designed.

General conclusion

Identification and cloning of nociceptive TRP channels has tremendously advanced our understanding of the biology of nociception and multi-modal pain sensation. The development of pharmacological interventions targeting nociceptive TRP channels has not only been closing in on new-generation analgesic drug developments, but also providing vital information on the *in vivo* mechanisms of sensory signal processing. Recently, evidence was shown that inhibition of TRPM3 might be a viable strategy to combat pain. We expect that the availability of potent and selective TRPM3 antagonists may be exploited to assess the channel's physiological and pathophysiological

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functions *in vivo*, and can be used to determine whether TRPM3 inhibition represents a safe and effective means to combat persistent pain in humans.

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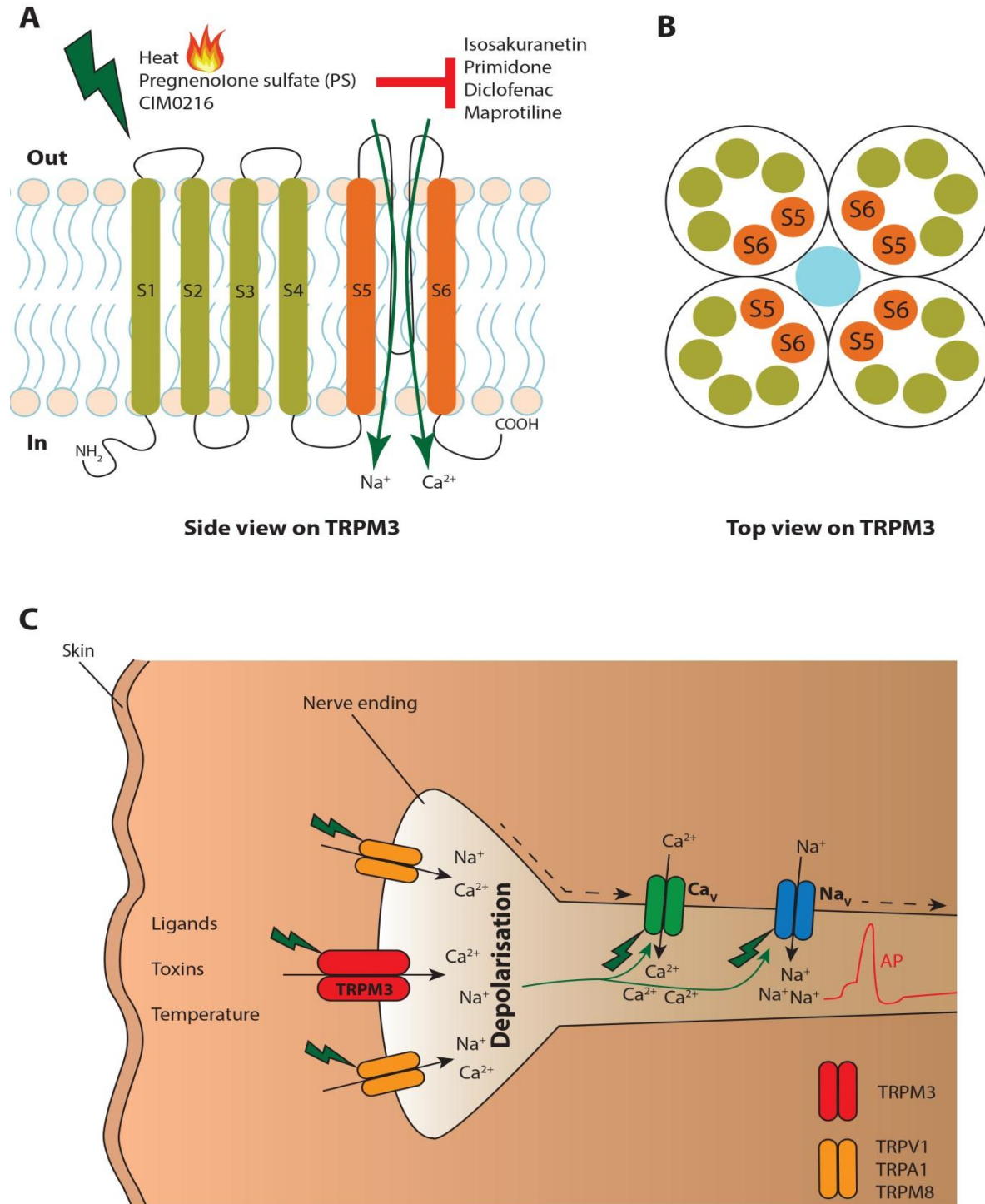
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Figure 1: The TRPM3 ion channel is expressed in sensory neurons.

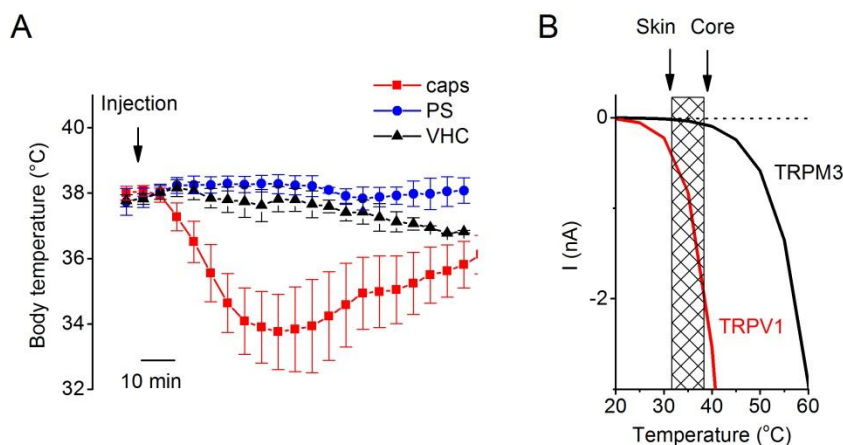


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(A) Cartoon of a side view of the TRPM3 ion channel expressed in the plasma membrane (PM). TRPM3 can be activated by noxious heat temperature, pregnenolone sulphate (PS) and the small molecule CIM0216. The channel can be blocked by isosakuranetin, primidone, diclofenac and maprotiline. (B) Top view on TRPM3 expressed in PM. Four TRPM3 subunits generate a functional ion channel in which the pore loop is turned to each other to form a central pore. (C) Cartoon illustrating the molecular mechanism of peripheral sensory transduction. Sensory ion channels like TRPV1, TRPM8, TRPA1 and TRPM3 are expressed in the PM of free nerve endings and can be activated by ligands, toxins or noxious temperatures ($<10\text{ }^{\circ}\text{C}$ or $>42\text{ }^{\circ}\text{C}$). Opening of these sensory TRP channels will lead to the influx of Na^{+} and Ca^{2+} ions, which will induce a membrane depolarization resulting in the opening of voltage-gated sodium (Na_v) and calcium (Ca_v) channels. Opening of these voltage-gated ion channels will cause the generation of action potentials, which are transduced via second-order sensory neurons of the spinothalamic tract to the CNS where it results in the sensation of pain.

Figure 2: Comparison between TRPV1 and TRPM3



(A) Core temperature is monitored in time after subcutaneous injection of vehicle (black), pregnenolone sulphate (PS, 28 mg/kg, blue) and capsaicin (0.4 mg/kg, red). Adapted from (40) (B) Comparison of simulated inward TRPV1 and TRPM3 currents at -80 mV in function of temperature, using pre-calculated parameters as shown elsewhere (40) and assuming 1000 channels/cell.