TRPM8 as a target for analgesia

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Abstract:

TRPM8 is a cation channel expressed in a small subpopulation of sensory neurons, which detect innocuous cooling and mostly lack characteristics of nociceptors. Whether TRPM8 normally contributes to noxious cold sensing is debatable as few TRPM8-positive neurons co-express nociceptive markers. TRPM8 is a promising analgesic target. TRPM8 agonists cause analgesia in chronic pain states, by TRPM8-expressing afferents gating-out a wide range of hypersensitive pain responses in spinal cord. TRPM8 antagonists may attenuate exaggerated responses to mild cooling during chronic pain but will not achieve generalised analgesia as they can only block cool detection, not the clinically problematic hypersensitivity to other sensory modalities. Biochemical and physiological properties of TRPM8, the chemistry of TRPM8 ligands, intracellular modulation of TRPM8 and the neurobiological consequences of TRPM8 activation are discussed, with a view to future improvements in therapeutic targeting.

Key words:

TRPM8, pain, analgesia, cool sensing, 5-HT_{1B} receptor, intracellular signalling

Running Title:

TRPM8 and analgesia

Only just over ten years ago, the TRPM8 channel was cloned by several independent groups as the mediator of cool and menthol responsiveness in sensory ganglia and as unknown transcript from prostate that was upregulated in cancer [1-3]. Since that time, evidence has steadily and consistently accumulated to confirm a key physiological role of TRPM8 in the detection of innocuous cool. In addition there is increasing interest in the channel as a potential target for new analgesics, especially in the context of chronic pain states that respond poorly to currently available therapeutics.

TRPM8 structure:

The TRPM8 channel is a non-selective cation channel assembled as a homotetramer of the 1104 amino acid protein, which individually has a molecular weight of around 128 kDa. The monomer has extended intracellular N- and C-termini and six transmembrane domains with a loop between domains 5 and 6 that is thought to contribute to the channel pore. Various reports implicate the C- or N-terminal segments in subunit assembly [4-6]. The C-terminal segment contains a short TRP box motif that is highly conserved throughout the family; including residues 1005 and 1009, involved in binding the allosteric potentiator PIP_2 (phosphatidylinositol 4,5-bisphosphate), and is also necessary for agonist responsiveness and setting the cool temperature response threshold [2, 3, 7-9]. The initial part of the N-terminal segment has also been implicated in moderating responsiveness to cool and menthol [10].

Chemical agonists that activate TRPM8 include menthol and the synthetic compound icilin, both of which evoke a sensation of coolness. However differences in channel residues involved in their recognition indicate that the two agonists bind to distinct sites, consistent with evidence that activation by icilin or cooling but not by menthol shows clear pH dependence and that icilin but not menthol action appears to require an increase in intracellular Ca^{2+} levels [11, 12]. Mutation of residues 745, 842 and 856 in transmembrane

domain 2 and the loop between domains 4 and 5 disrupts menthol activation whereas residues 799, 802 and 805 in transmembrane domain 3 are required for icilin effects [12-15]. TRPM8 also shows voltage sensitivity, with residues 842 and 856 probably contributing to voltage sensing [14, 16, 17]. Interactions between the different modes of activation are observed in that agonists not only increase open probability and conductance of the channel but also shift both its thermal threshold and voltage sensitivity towards more normal physiological levels [14, 16-19]. The overall responses of a cell expressing TRPM8 to its activating stimuli will be further influenced by the effects of other ion channels on membrane potential and by signals from other receptors/intracellular signalling cascades.

Localisation of TRPM8 in the nervous system:

TRPM8 has a highly selective tissue distribution. Apart from primary somatosensory neurons and prostate tissue, from where it was cloned, it is expressed at only low levels in other tissues. These include visceral afferents, bladder, vascular smooth muscle, stomach, liver, colon, lung and airways, sperm and some primary tumours [20-28]. In the nervous system TRPM8 is strongly expressed in trigeminal (TG) and dorsal root ganglia (DRG) and has subsequently been observed in autonomic ganglia [29-31]. However, there appears to be extremely limited TRPM8 expression within the central nervous system (CNS) [32] other than that associated with afferent terminals. This idea was fully corroborated by observations on mutant mice expressing Green Fluorescent Protein (GFP) under the control of the TRPM8 promoter [33, 34]. Furthermore, only 5-10% of DRG and TG neurons display the responsiveness to mild cooling (threshold around 25 ^{o}C), menthol and icilin that is characteristic of TRPM8, emphasizing the highly selective outcome to be expected from any intervention that might usefully target the channel. Early observations indicated that these neurons were small in diameter and distinct from those expressing classical nociceptive markers such as calcitonin

gene-related peptide, TRPV1 or binding sites for the lectin IB4 [3]. Most TRPM8-positive sensory neurons appear to be C-fibres staining for peripherin, with a minor group of Aδ fibres, whose small cell bodies stain for neurofilament antigens [2, 33-36]. Extensive in situ hybridisation experiments in DRG indicated a pattern of cellular expression almost entirely distinct from that of the nociceptive channels TRPV1 and TRPA1 [37]. Other studies combining immunohistochemical and alternative localisation techniques indicate a modest degree of overlap [33, 34, 38], and our own dual immunofluorescence data from TRPM8 and TRPV1 (Table 1) concur in finding TRPV1 expression in a very small proportion of TRPM8-positive cells. A recent study utilising genetically targeted ablation of TRPV1 and TRPM8-expressing cells reinforced the idea of minimal overlap [39]. Technical issues such as differences in sensitivity and selectivity of the reagents and methods used may contribute to the range of findings reported. A further factor to consider is that the extent of overlap may differ between somatic and autonomic sensory neurons where phenotypic subdivisions may differ from those of somatic afferents [20, 24, 40]. Studies exploring functional responses of TRPM8 have also tried to explore the extent to which any overlap is significant.

In vitro studies on the physiological role of TRPM8 in sensing cool / cold temperatures:

When cloned TRPM8 is expressed in oocytes or fibroblasts, the cells are reported to show a temperature activation range from around 22-25 ^{o}C (threshold) to 8-10 ^{o}C (maximal) [2, 3]. Correspondingly, native TG neurons that show Ca^{2+} elevation responses selectively to menthol have thermal activation thresholds around 25 ^{o}C [41]. DRG or TG neurons from TRPM8$^{-/-}$ mice show significantly reduced responses to cooling stimuli, which ranged in different studies from 22 ^{o}C through to 9 ^{o}C, and in C-fibre firing induced by cooling from 32 ^{o}C to 2 ^{o}C in a skin-nerve preparation [42-45]. With such read-outs of responses to imposed progressive cooling, it is not clear whether components of responses in the noxious

temperature range are lacking in TRPM8$^{-/-}$ mice as well as those from innocuous temperatures. These data remain equivocal in deciding whether TRPM8 is involved in physiological cold pain under normal conditions.

A number of studies on cultured TG or DRG cells describe subpopulations responding to both mild cooling (generally through the range 25-17 ^{o}C) and to menthol that are considered to reflect TRPM8-expressing cells [46-49]. Some caution is needed though as menthol has only modest selectivity for TRPM8 over TRPA1 [50], which also displays sensitivity to reduced temperatures and is expressed in a subset of TRPV1-positive nociceptors [41, 51]. A careful comparison of the temperature sensitivity of individual menthol-responsive / allyl isothiocyanate (TRPA1 agonist)-unresponsive TG neurons, likely to express TRPM8, and those activated by both menthol and allyl isothiocyanate, likely to express TRPA1, points to higher (innocuous range) temperature thresholds in the TRPM8 group but with substantial overlap [41].

Although menthol-responsiveness in itself does not decisively implicate TRPM8, some studies have explored responsiveness of sensory neuron populations to menthol and capsaicin [48]. Neurons with dual responsiveness could be taken as evidence for functionally significant TRPM8 channels in nociceptors, but the debate remains equivocal because of menthol's limited selectivity for TRPM8 over TRPA1. In addition, the electrophysiological responses of menthol-activated (putative TRPM8-positive) DRG neurons to mild cooling to 24 ^{o}C, involving tetrodotoxin-sensitive Na^{+} channels, appeared to be attenuated at the more intense cold level of 10 ^{o}C, whereas the intense cold-induced firing in neurons with tetrodotoxin-insensitive Na^{+} channels (putative nociceptors) remained robust [52].

The development of highly selective agonist and antagonist tools for TRPM8 should help to further elucidate the situation. An additional variable in experiments with cultured sensory

neurons is the possibility of a change in phenotype relating to duration in vitro [34], a parameter that can differ considerably between studies, and may also undergo some transition during chronic pain states [53].

In vivo studies on the physiological role of TRPM8 in sensing cool / cold temperatures:

C-fibre recordings in rodents identify two distinct populations of cold-sensitive neurons [54]. The first of these comprises low temperature threshold, mechano-insensitive, heat-insensitive afferents, sensitive to small reductions in skin temperature of as little as 2 ^{o}C, that are activated by menthol (10% topical) and by evaporative cooling due to acetone; ie non-nociceptive thermoreceptors that may express TRPM8. The second comprises high threshold cold-, mechano- and heat-sensitive nociceptive afferents, firing at 12 ^{o}C or below that are indifferent to menthol. These findings are consistent with a significant role for TRPM8 in innocuous cool detection but not cold pain in normal animals. Similar profiles are reported from microneurography experiments in normal human volunteers [55]. Very high concentrations of menthol (up to 40 %) applied topically to the skin are perceived as noxious [56-58]; a situation mirrored by behavioural experiments in rodents [36], but interpretative caution is needed as TRPM8 selectivity is uncertain at such concentrations. In the special circumstance of the cornea, highly sensitive cool thermoreceptors predominate, which respond vigorously to small reductions in temperature (as little as 0.5 ^{o}C reduction) and contain abundant TRPM8 [59].

Thermal place preference tests with TRPM8$^{-/-}$ mice indicate lack of sensation across the innocuous cooling temperature range [42-44, 60]. While colder temperatures (10 ^{o}C and below) showed return of temperature preference and cold plate paw withdrawal responses (10 ^{o}C and below) were normal in TRPM8$^{-/-}$ mice [42, 44, 45], some attenuation of thermal preference was seen, to a greater or lesser extent, at temperatures as low as 5 ^{o}C. Correspondingly, the

flicking responses due to evaporative cooling from acetone, which can cool the skin to temperatures around 14-18 ^{o}C [43, 61], were substantially reduced in TRPM8$^{-/-}$ mice [42, 44] or by systemic administration of the high affinity TRPM8 antagonist PBMC [61]. As this temperature range corresponds to the loosely-defined border between innocuous and noxious cooling in man [62] and the precise thermal consequences of differing laboratory protocols are uncertain, it is not clear that the test explicitly reflects cold pain as opposed to a response to innocuous cooling.

Intraplantar injection of the selective TRPM8 agonist icilin at high local concentrations (8 mM solution) can evoke flinching behaviour and spinal cord c-Fos expression, which are reduced in TRPM8$^{-/-}$ mice [43-45, 63]. Intraplantar injections of icilin or menthol at high concentrations however cause activation of a wide variety of sensory neurons of different types through apparently TRPM8-independent processes [64]. Furthermore, many agents when injected directly into the skin, presumably adjacent to sensory nerve terminals, can elicit nociceptive responses that they would not normally cause through other routes such as topical administration [65]. While intraplantar icilin-evoked nocifensive behaviour appears to involve TRPM8, it is not clear that this relates to physiological cold sensing.

As adaptive compensatory responses are possible in constitutive knockout animals, a targeted ablation strategy has also been investigated. In TRPM8 neuron-ablated mice (which express a diphtheria toxin (DTx) receptor transgene under the control of the TRPM8 promoter and were treated with DTx) results corroborated those in TRPM8$^{-/-}$ mice [39, 60]. TRPM8 ablation abrogated behavioural responses to acetone-induced evaporative cooling and thermal preference through the innocuous cooling range of 30-10 ^{o}C [39, 60]. However, avoidance of 0-10 ^{o}C cold surfaces and paw withdrawal/flinching to severe noxious cold were also attenuated in TRPM8 neuron-ablated mice and this was to a greater extent than in TRPM8$^{-/-}$

mice [39, 60]. Some of these data were obtained with a new sensitive forepaw-flinching assay but the precise extent of temperature reduction reached in the forepaws may be affected by guarding behaviour. Furthermore because the strategy ablates neuronal populations rather than individual candidate molecular mediators, additional proteins in subsets of TRPM8-expressing cells could be key to noxious cold sensing.

Setting of thermal sensitivity in TRPM8-expressing sensory neurons:

Pre-exposure to chemical agonists such as menthol markedly increases cold-induced Ca^{2+} entry in TRPM8-expressing oocytes or fibroblasts and menthol-sensitive TG cells, as well as cold-induced firing in a skin-nerve preparation [2, 3, 66], a phenomenon also reported with TRPA1 [67]. The effective temperature threshold for cellular responses to cooling can also be influenced by co-expressed K^+ channels acting to hyperpolarise the membrane and oppose TRPM8-mediated depolarisation. Both Kv1 and Kv7 family channels are co-expressed with TRPM8, notably in nociceptors where high K^+ channel : modest TRPM8 expression ratios may drive the cold threshold into the noxious temperature range [66, 68]. In contrast, low threshold non-nociceptive cool-sensing afferents appear to have high ratios of TRPM8 : K^+ channel expression [59, 66, 68]. In addition, K_2P TREK family channels, some of which are directly closed by temperature reductions, are present in subsets of TRPM8-positive cells [69] and may impact on their thermal sensitivity. Effects of cooling on A-type K^+ channels and tetrodotoxin-sensitive or resistant Na^+ channels may also modulate firing in cool thermoreceptors and cold nociceptors [52, 70], while the notable resistance of $Na_v1.8$ to cooling-induced desensitisation is crucial for transmission in cold-sensitive afferents [71]. TRPM8 function (and indeed that of any other threshold-setting channels) is of course also subject to a variety of modulatory influences from intracellular signalling events (see below).

The impact of pain states on TRPM8 function:

Chronic pain states of either inflammatory or neuropathic origin crucially involve central hypersensitivity that is brought about by neurochemical changes ensuing from maintained nociceptor firing [72]. This will manifest as exaggerated responses to noxious stimuli (hyperalgesia) and perception of normally innocuous stimuli as noxious (allodynia). This central re-setting of excitability will most likely apply to thermal, mechanical and cool sensory inputs so in the case of TRPM8-mediated inputs a degree of cool allodynia would be entirely expected. Whether there are any specific adaptive responses in TRPM8-expressing neurons themselves has been investigated by a number of groups, with varying results. There is little evidence that inflammation alters TRPM8 expression but there is clearly amplified responsiveness to acetone-induced cooling in the intraplantar Complete Freund's Adjuvant (CFA) model of inflammatory pain [43]. This could potentially reflect increased expression of TRPA1 [73, 74] or simply the expected central hypersensitivity to inputs from the TRPM8-mediated innocuous cool afferents [60, 61]. In some neuropathic pain models, TRPM8 expression is reported to increase [36, 75-77], although little change, or reduced expression, has been reported in others [73, 78, 79]. Cool allodynia is apparent after nerve injury and both pharmacological and genetic interventions indicate that this is likely to involve TRPM8 [43, 60, 61, 76-78]. This may however reflect simply the TRPM8-mediated reportage of innocuous cooling that, like any other somatosensory input, becomes amplified due to injury-induced central sensitisation. Indeed a recent electrophysiological study reported that acetone-evoked evaporative cooling responses, but not other sensory responses of spinal cord neurons were inhibited by a selective TRPM8 antagonist in nerve injured but not naive rats [80]. Interestingly however, in patients with established cool allodynia due to nerve injury, the topical administration of menthol does not aggravate hypersensitivity [81].

TRPM8-mediated analgesia:

Although cooling and mint extracts containing menthol have been widely used for many years due to their soothing, antinociceptive effects, the molecular basis was long unknown [82-85]. The cloning of TRPM8 and its identification in a subset of DRG/TG sensory neurons provided a likely framework. In 2006, TRPM8 was specifically demonstrated for the first time to mediate analgesia due to cooling or the chemical agonists menthol and icilin, applied topically or intrathecally in animal models of both chronic neuropathic pain (CCI, chronic constriction injury) and inflammatory pain [36]. The pharmacological identification of TRPM8 mediation was corroborated by antisense knockdown experiments indicating that active functional TRPM8 was required as opposed to any potential for an effect due to agonist-induced channel desensitisation. Both thermal (heat) hyperalgesia and mechanical allodynia were reversed, but cool allodynia was not addressed because of the likelihood of a complex, mixed influence. Interestingly, there was no effect on unsensitised responses in naive animals or in unaffected limbs until much higher concentrations, which produced pro-nociceptive effects. Experiments investigating the effects of relatively high concentrations of topically applied menthol in naive animals report attenuation of noxious thermal responses, mixed effects on cool/cold responses and sensitisation of innocuous mechanical responses [86], although mediation by TRPM8 was not ascertained and off-target effects may contribute. The original observations of TRPM8 analgesia were subsequently confirmed in the CCI model of neuropathic pain, where intrathecal menthol was similarly found to strongly reduce thermal hyperalgesia and mechanical allodynia but increase withdrawal responses from a 4 ^{o}C cold plate [77]. Mediator specificity was established by antisense knockdown in this study too. Interestingly the analgesic effects of TRPM8 agonists were not observed in an alternative neuropathic pain model (SNL, spinal nerve ligation) [87] where TRPA1 has been implicated [88]. Evidence for TRPM8-mediated analgesia was also provided in the formalin-induced flinching model where cool-induced analgesia was attenuated in TRPM8$^{-/-}$ mice compared to controls

[44]. Recent work provides robust support for the idea of TRPM8-mediated analgesia, showing that systemic or topical administration of menthol diminishes pain behaviour due to noxious heat, TRPV1 or TRPA1 activators or intraperitoneal acidification as well as attenuating inflammation-induced mechanical hypersensitivity [89]. The critical role of TRPM8 was clearly demonstrated through abrogation of effects in the presence of a highly selective TRPM8 antagonist or in TRPM8$^{-/-}$ mice. Powerful analgesic effects of systemic menthol against formalin-induced flinching and inflammatory hypersensitivity have also been recently described at rather higher dosage levels, although in this case TRPM8-independent mechanisms may also contribute significantly [90]. Key supportive data have also been provided through experiments on targeted ablation of TRPM8-expressing neurons where the cooling-evoked attenuation of mechanical allodynia seen in the CCI neuropathic pain model in control animals was abrogated by toxin-evoked TRPM8 ablation [60]. Corresponding results were seen in TRPM8$^{-/-}$ mice. Further evidence shows that pain state-induced synaptic hypersensitivity not only at the spinal cord but also forebrain levels can be reversed by topical administration of TRPM8 agonist; with the involvement of TRPM8 established through blockade by a highly selective antagonist [91]. Taken together, these observations firmly establish that TRPM8 activation is able to gate-out hypersensitive nociceptive inputs and activation of the CNS in chronic pain statess, most likely through the spinal influence of the TRPM8-expressing subset of sensory afferents.

So a case can be made for the use of either antagonists or agonists at TRPM8 in the treatment of pain. Antagonists may be useful to treat the cool allodynia associated with chronic pain states. Effects are likely to be limited to this modality however, as they would influence only the sensory detection of cool that involves TRPM8 and not the central sensitisation that leads to parallel problems of mechanical allodynia and thermal hyperalgesia. Antagonists could potentially be considered for treating acute cold pain in naive subjects but any evidence to

validate this is much less strong than that illustrating the role of TRPM8 in innocuous cool thermosensation. It may well be that other factors play a key part in noxious cold sensing so any effect of TRPM8 blockade may be less robust; this will become clear in future work. Agonists show great promise in that they are now well documented to produce efficacious analgesia in hypersensitive pain states where there appear to inhibit central sensitisation and therefore reverse chronic pain of a number of different modalities. Both neuropathic and inflammatory pain hypersensitivity can be effectively targeted. One caveat with this approach would be that cool allodynia may be exacerbated, although it would be predicted that the analgesic effects of TRPM8 agonists suppressing central hypersensitivity act in opposition to any enhancement of peripheral cool sensing and thereby ameliorate any cool allodynia. Clinical evidence in chronic pain patients supports this idea, as cool allodynia does not seem to be a problematic issue [81, 92, 93]. Care is also needed in evaluation of the therapeutic window because of the possibility of noxious sensations if supra-therapeutic concentrations of agonists are reached. Either strategy (as with any analgesic intervention) could potentially encounter on-target side effects in other tissues or off-target effects due to insufficient pharmacological specificity; a medicinal chemistry issue around the particular pharmacophore utilised. As TRPM8 is expressed in relatively few tissues, the on-target side-effect issue may be relatively unproblematic, especially if agents are applied topically to dermatomes around the site of chronic pain to access selectively the relevant TRPM8 afferents and limit the systemic drug load. For both antagonists and agonists a further possible issue that would need to be evaluated might be disruption of central thermoregulation, as identified in the case of TRPV1 antagonists.

TRPM8 in thermoregulation:

When antagonists of the noxious-heat sensing channel TRPV1 were tested in vivo as potential analgesics, significant effects on regulation of core body temperature became

apparent. TRPV1 antagonists caused hyperthermia, thermogenesis and vasoconstriction in wild type but not TRPV1$^{-/-}$ mice [94, 95]. Although in contrast to TRPV1, TRPM8 is absent from central thermoregulatory centres in the hypothalamus, potential effects of TRPM8 agonists or antagonists on core temperature have been investigated. Intraperitoneal injection of icilin at high concentrations produces a characteristic acute shivering behaviour known as "Wet Dog Shakes", presumably by stimulating visceral afferents, and this response is attenuated in TRPM8$^{-/-}$ mice [45, 96, 97]. Systemic or topical administration of menthol or icilin (at relatively high doses) leads to a transient increase in core temperature, presumably an attempt at compensatory thermoregulation [98-103]. Accordingly systemic TRPM8 antagonists produce a transient reduction in core temperature [61, 80, 103, 104]. In both cases, some studies confirmed lack of effects in TRPM8$^{-/-}$ mice. Whether any significant thermoregulatory changes are observed at the doses therapeutically relevant for the treatment of chronic pain remains to be established.

Pharmacology of TRPM8 antagonists and agonists:

TRPM8 antagonists:

For a number of years no highly selective antagonists of TRPM8 were available to help validate the inferred role of the channel in thermosensation and modulation of pain processing. Early studies identified that some TRPV1 antagonists also had affinity for TRPM8, providing the first useful but fairly non-selective tools. Capsazepine, BCTC and SB-452533 were shown to be effective TRPM8 antagonists but with clearly lower potency than at TRPV1, posing difficulties for data interpretation in complex in vivo situations [105, 106]. The antifungal agent clotrimazole was identified as a relatively potent TRPM8 antagonist [107], but it additionally blocks K^+ channels and activates both TRPV1 and TRPA1. Tryptamine derivatives such as 5-benzoyloxytryptamine were also unexpectedly shown to antagonise

TRPM8 but may well impact on 5-HT receptor function and any potential effects on other pain-relevant channels are unknown [108]. The first clearly selective TRPM8 agonist widely disclosed was AMTB, developed by Bayer [109], although its affinity was still only moderate. Other pharma; Glenmark, Amgen, Janssen, Johnson and Johnson have now developed a number of highly potent and selective TRPM8 antagonists with diverse chemical structures. These include benzothiophene sulphonamides and phosphonates, fused oxazoles and thiazoles, benzimidazoles, fused piperidines, aryl glycines, menthylamines and ylidenephthalides (although the last two may have some TRPA1/TRPV1 activity) [110-118]. Additional potent and effective TRPM8 antagonists from further structural series have been produced by Pfizer and Takeda [61, 80] and even endogenous and plant cannabinoids inhibit at submicromolar concentrations [119]; yielding a truly diverse array of pharmacophores as TRPM8 blockers (Figure 1).

TRPM8 agonists:

The prototypical TRPM8 agonist menthol (more precisely (-) menthol or IR, 2S, 5R-2-isopropyl-5-methylcyclohexanol) is an effective but not particularly selective TRPM8 agonist. In our studies measuring Ca^{2+} fluorescence responses of HEK-293 cells stably expressing human TRPM8 it shows a mean EC_{50} of 11.2 μM with efficacy 66% that of the benchmark agonist icilin, but a mean EC_{50} of 29.6 μM and 95% efficacy compared to allyl isothiocyanate (mustard oil) at TRPA1. The stereoisomers show an unremarkable structure-activity relationship [13, 36], but an extensive range of analogues has been produced, notably WS-12 and D-3263, which show greatly increased potency at TRPM8 [120-125]. The tetrahydropyrimidine-2-one, icilin, was reported to produce marked sensations of cold [126] and is established as a potent TRPM8 agonist with an EC_{50} of 0.3 μM and considerable selectivity against TRPA1 (about 300 fold in our assays), and even more against TRPV1 [2, 105]. However

icilin has substantial affinity as an antagonist at TRPV3 [127]. Thienopyrimidine compounds [124] display moderate potency and selectivity, with a hydroxyethyl-substituted example showing an EC_{50} of 10 μM at TRPM8 and at least 20-fold selectivity over TRPA1 and TRPV1 in our hands. Perhaps the most enigmatic of TRPM8 agonists is an aliphatic phosphonate, WS-148, (1-(di-*sec*-butyl-phosphinoyl)-heptane), which is reported to show an EC_{50} of 4.1 μM at TRPM8, although its off-target profile has not been described [121]. A recent report describes a series of isoxazole compounds as potent partial agonists in causing Ca^{2+} elevation in a DRG/neuroblastoma cell line that responds to menthol and expresses TRPM8 mRNA [128]. Further characterisation of these compounds will be interesting, especially as modelling of interactions with a quaternary structural model of the TRPM8 channel [129] indicates a potential for both menthol-like and icilin-like interactions in the series [128] (Figure 2). The development of new highly selective TRPM8 agonists as tools will be an important step forward as the majority of physiological studies carried out to date with TRPM8 agonists have utilised either menthol or icilin and unless additional strategies are incorporated to validate TRPM8 mediation some caution is needed in interpretation.

Menthol can exert a wide range of TRPM8-independent effects, generally at somewhat higher concentrations than those that adequately activate TRPM8. These include potentiation of GABA and glycine currents, inhibition of α4β2- and α7-nicotinic cholinergic receptors, inhibition of 5-HT_3 receptors, inhibition of Ca^{2+} and Na^{+} channels and local anaesthetic-like suppression of action potential firing and even at very high concentrations, desensitisation of TRPV3 and cell cycle arrest in proliferating cells [90, 130-143]. Systemic menthol at low doses produces analgesia that principally involves TRPM8 [89] while effects at considerably higher doses appear to involve disruption of Ca^{2+} and Na^{+} channels [90].

Apart from its significant affinity for TRPV3, icilin has a number of additional effects. Inhibition of L-type Ca^{2+} channels is reported at similar sub-micromolar concentrations similar to those causing activation of TRPM8 but the basis is rather unclear as the effect increased slowly, without saturating, over 5 orders of magnitude of concentration [87]. Icilin at somewhat higher concentrations is also reported to activate epithelial Na^{+} channels, evoke a distinct inhibitory effect on TRPM8 gating that is separate from Ca^{2+}-dependent channel desensitisation and even lead to cell cycle arrest in proliferating cells [15, 144-146].

Modulation of TRPM8 function by cellular signalling:

Lipids:

Like other TRP channels, TRPM8 is prominently modulated by PIP_2, which slows channel rundown in isolated membrane patches and acts to facilitate channel activation by cooling or chemical agents [147-149]. Mutation of TRP box PIP_2-binding residues greatly reduces TRPM8 responses to stimuli including chemical agonists [91, 148]. Enzymatic activity of phospholipase C (PLC), including that evoked by agonist stimulation of PLC-coupled receptors, can lead to dynamic local depletion of PIP_2 concentrations and suppression of TRPM8 function [147, 148, 150, 151]. A PIP_2 binding protein, PIRT, is widely expressed in DRG cells and enhances menthol responses in HEK-293 cells co-transfected with TRPM8, while $PIRT^{-/-}$ mice show partially attenuated avoidance of cool and cold surfaces [152].

Lysophospholipids and polyunsaturated fatty acids, products of phospholipase A_2 (PLA_2) action on membrane phospholipids, reciprocally enhance or inhibit TRPM8 function respectively [153-155]. Inhibition of PLA_2 enzyme activity attenuated the hypersensitivity to 10 ^{o}C cold induced by the intraplantar injection of icilin at a high concentration, but not due to menthol [63]. Corresponding experiments in mutant mice indicated the involvement of TRPM8 in hypersensitivity due to intraplantar icilin but TRPA1 in that induced by menthol. PLA_2

activation downstream of receptor activation and kinase cascades could therefore lead to complex profiles of TRPM8 modulation. Changes in membrane lipid composition may also influence the localisation of TRPM8 within cholesterol-rich lipid rafts, which appears to normally down-regulate its responsiveness to stimuli [156]. Volatile general anaesthetics have been reported to affect TRPM8 with a transient enhancement followed by a sustained inhibition of function, effects that may reflect alterations in the membrane protein:lipid interface [157].

Kinases:

The intracellular domains of TRPM8 contain a number of potential regulatory phosphorylation sites that match more or less closely to the consensus target sequences of several common kinases [158]. Phorbol ester or receptor-mediated activation of protein kinase C (PKC) inhibits TRPM8 function [159-162] but there is little firm evidence that direct phosphorylation is responsible, rather a downstream calcineurin-dependent dephosphorylation mechanism [159]. Protein kinase A (PKA) activation brought about by forskolin or 8-Br-cAMP is reported to reduce chemical agonist-evoked TRPM8 responses or DRG neuronal responses to cooling [119, 161], although in our experiments with TRPM8-expressing HEK-293 cells we observe significant inhibitory effects of phorbol 12,13-dibutyrate but not forskolin. A similar lack of effect of forskolin or 8-Br-cAMP was reported in cultured DRG cells [162] and another study even indicated that basal function of TRPM8 was dependent on patent phosphorylation by PKA [163].

TRPM8-positive sensory neurons are thought to be dependent on the tyrosine kinase receptor for Nerve Growth Factor, TrkA during development and during cell culture [164, 165] so tyrosine phosphorylation-dependent signalling cascades might modulate TRPM8. Although TrkA appears not to be generally co-expressed with TRPM8 in adult sensory neurons [165], around

half of the TRPM8-positive TG and DRG cells express the GFRα3 receptor for another neuronal growth factor, artemin, and a subset of these also contain TrkA [166]. Intraplantar injection of artemin enhanced behavioural responses to evaporative cooling with acetone, a response that was absent in TRPM8$^{-/-}$ mice, but also reduced withdrawal latencies to noxious heat [166]. Intraplantar NGF had a similar but less marked effect on cooling responses. The intracellular signalling cascades underlying these modulatory effects remain to be elucidated.

G protein-coupled receptor signalling:

Intracellular signalling from the mainly G_i/G_o-coupled α2A-adrenoreceptor (most likely the inhibition of adenylyl cyclases) has been reported to inhibit TRPM8 function both in a heterologous cell expression system and in a small subpopulation of menthol-sensitive sensory neurons [163], with reducing phosphorylation status of PKA target sites thought to be responsible. The mainly G_q/G_{11}-coupled M3 muscarinic receptor also inhibits TRPM8 in co-transfected HEK-293 cells [155]. Rather than through anticipated candidate mechanisms such as PLC-dependent PIP_2 depletion, this appears to involve PLA_2-dependent arachidonic acid production through a process unaffected by a selective PLC inhibitor. In addition the mainly G_q/G_{11}-coupled B2 bradykinin receptor can reduce TRPM8 channel currents and Ca^{2+} elevation in co-transfected cells and in a subpopulation of DRG cells, through a process not significantly affected by inhibitors of PLC or PKC [167]. The direct activator of PKC phorbol 12-myristate, 13-acetate had no apparent effect on TRPM8 activity in these experiments, in contrast to earlier reports [159-162]. Evidence was provided in support of a PLC-independent mechanism of G_q binding to the channel to cause its inhibition, centring on effects of a chimeric $G\alpha_q/G\alpha_i$ construct to avoid PLC activation as wild-type, constitutively active and a previously described PLC-disabled mutant $G\alpha_q$ were able to trigger the earlier proposed inhibitory mechanism of PIP_2 depletion. Wild type and constitutively active $G\alpha_q$ were

reported to be associated with TRPM8 in chelation-affinity and immunoprecipitation experiments; controls were provided using cells transfected with a single construct alone. In addition $G\alpha_q$ from cell lysates was captured by GST fusion constructs of both N- and C-terminal domains of TRPM8. An additional protein that may be of relevance to these findings is G protein-coupled receptor kinase 2 (GRK2), which is recruited to the vicinity of activated receptors and is known to sequester $G\alpha_q$ [168]. Interestingly the region of $G\alpha_q$ replaced in the $G\alpha_q/G\alpha_i$ chimera is important for GRK2- as well as PLC-β-interaction [169]. GRK2 could potentially be associated with the B2 receptor in transfected cells or in vivo and affect $G\alpha_q$ localisation and availability. An activated construct of the $G\alpha_q$ congener $G\alpha_{11}$, is reported to have a lesser effect on TRPM8 compared to $G\alpha_q$ [170] and the same pattern is seen in the impairment of inositol phosphate signalling caused by GRK2 binding to the two G proteins [171]. Whether or not GRK2 plays any part in $G\alpha_q$: TRPM8 interactions is yet to be explored. Surprisingly though, the intraplantar injection of bradykinin (at doses effective in causing heat hypersensitivity, [172] had no effect at all on behavioural responses to evaporative cooling in mice [166]. Additional data consistent with $G\alpha_q$ inhibition of TRPM8 was provided in experiments investigating the pruritogenic receptor MrgprA3 in a small minority of DRG neurons that respond to capsaicin, allyl isothiocyanate and menthol [173]. MrgprA3 activation partially reduced subsequent menthol responses in around half of these cells, whereas capsaicin responses were enhanced through a distinct mechanism [173]. The difficulties in distilling a simple unified model of TRPM8 channel regulation are emphasised by data indicating a completely contrasting process; the activation of $G\alpha_q$ and PLC by TRPM8 itself [174]. This report describes menthol-evoked Ca^{2+} mobilisation from intracellular stores through a TRPM8-dependent process involving PLC activation, although menthol at high concentrations is also known to exert TRPM8-independent effects on endoplasmic reticulum

[175]. Correspondingly, fluorescence resonance energy transfer (FRET) measurements indicated close proximity between fluorophore-tagged TRPM8 and $G\alpha_q$ that was considered to underlie G protein and PLC activation. The overall picture remains to be clarified, although potentially multiple processes could operate concurrently.

A recent report describes a novel mechanism for the upregulation of TRPM8 function, which centres on a newly discovered molecular signalling complex between the 5-HT_{1B} receptor and TRPM8 [91] (Figure 3). The channel associates directly with the 5-HT_{1B} receptor but not with other G protein-coupled receptors, in HEK-293 cells and in small DRG neurons. 5-HT_{1B} receptor activation amplifies TRPM8 responses to icilin or menthol through a mechanism dependent on phospholipase D1 (PLD1), which is also incorporated into the signalling complex. Evidence was provided that the process by which PLD can enhance channel function involves a stimulatory effect on phosphatidylinositol 4-phosphate (PIP) 5-kinase, leading to elevated levels of PIP_2, the allosteric enhancer of TRPM8. In chronic pain models the combined treatment with 5-HT_{1B} agonist and TRPM8 agonist was shown to amplify icilin (or menthol) -induced reversal of synaptic hypersensitivity in the CNS and enhance TRPM8 analgesia.

CNS processes activated by TRPM8-positive afferents:

In the superficial dorsal horn of the spinal cord there are various categories of supraspinally projecting neurons including polymodal nociceptors and non-nociceptive cool receptors, indicating at least partially distinct spinal processing and onward transmission of innocuous cool sensation [176-179]. TRPM8-expressing afferents, visualised in mice with TRPM8 promoter-targeted GFP constructs, terminate in outer laminae II and laminae I of spinal dorsal horn [33, 34]. These terminals overlap with the zone of peptidergic nociceptor termination as exemplified by CGRP and substance P immunofluorescence (but show minimal co-

staining at the single fibre level), while essentially avoiding the region of IB4-reactive non-peptidergic nociceptor termination in inner laminae II. Cadherin-8 is expressed in laminae I, IIouter and especially IIinner of the dorsal horn and is also present in a subset of small DRG cells, many of which co-express TRPM8 [180]. Ultrastructural analysis indicated both pre- and post-synaptic cadherin-8 expression within complex glomerular synapses. Recording from dorsal horn neurons in slices of wild-type mice, menthol-evoked increased mEPSP frequency and this effect was abolished in cadherin-$8^{-/-}$ animals, indicating that cadherin-8 may be required for functional connectivity between TRPM8 afferents and target neurons in superficial dorsal horn [180]. A further ultrastructural study showed that TRPM8-positive C- and Aδ-fibre terminals entered into synaptic connections that were generally simple dendritic, less commonly complex glomerular and occasionally axo-axonic in nature [181]. In the trigeminal sensory nuclei there was evidence for a segregated localisation of TRPM8 afferents that were positive for CGRP and those that expressed TRPM8 alone.

In a transgenic mouse line that expresses GFP in a subset of GABAergic laminae II interneurons, tonic central cells [182], menthol or icilin increase mEPSP frequency in these neurons, consistent with a direct input from TRPM8 positive afferents and a role in cool sensing [183]. Interneuron cross-connections [184, 185] could then lead to inhibitory effects on vertical and transient central excitatory interneurons involved in processing Aδ- and C-fibre nociceptive inputs [183]. Such pathways could contribute to the gating-out of hypersensitive responses to nociceptive inputs and reversal of pain behaviour seen when icilin or menthol are administered at low doses in chronic pain models [36, 91]. In those studies any major contribution of opioid receptors at the spinal level was excluded but a key mediating role of Group II and Group III metabotropic glutamate receptors was identified, each known to be expressed at both pre- and post-synaptic locations. There is evidence however to support an

involvement of κ-opioid receptors in the acute analgesic effects of systemic menthol but the site of action is likely to be an unknown supraspinal location as effects were attenuated when κ-opioid antagonist was delivered intracerebroventricularly [85]. A further study reported that acute analgesic effects of the menthol congener WS-12 (at a rather high intraperitoneal dose) in the hot plate test were diminished by a systemic naloxone, a general opioid receptor antagonist [89]. These reports could both be consistent with a supraspinal role of κ-opioid receptors in TRPM8 ligand-induced analgesia but in neither case was it shown explicitly that effects were TRPM8-mediated. Mice in which the developmental transcription factor Bhlhb5 is constitutively deleted lack a further subpopulation of inhibitory GABAergic interneurons in superficial dorsal horn and display pathological itch [186]. When these neurons were genetically tagged they were shown to co-express either galanin/dynorphin or neuronal nitric oxide synthase and be activated by capsaicin, allyl isothiocyanate and menthol each of which is reported to exert antipruritic influences [187]. Thus these cells may receive input from TRPM8 afferents and may influence itch through dynorphin/κ-opioid receptor mechanisms, although preprodynorphin$^{-/-}$ mice do not replicate the phenotype [187]. Importantly though, Bhlhb5$^{-/-}$ mice do not exhibit any pain phenotype [186], so perhaps are dissociated from pathways underpinning TRPM8 analgesia. A further recent study reported that in isolated DRG neurons, the μ-opioid receptor agonist morphine, at a relatively high concentration, causes a small (15 %) attenuation of menthol-induced currents and disruption of menthol-induced Ca^{2+} entry [188]. Data were provided to suggest that μ-opioid receptor immunoreactivity was found in TRPM8 immunoprecipitates from HEK-293 cells transfected with TRPM8 plus μ-opioid receptor but not cells transfected with TRPM8 alone and also that morphine may promote TRPM8 internalisation. Evidence that μ-opioid receptors are expressed in native TRPM8-positive afferents is yet to be provided by immunofluorescence

or equivalent approaches. Morphine-evoked analgesia was tested on cold responses of TRPM8$^{-/-}$ mice and found to be less marked than in the wild type, but the marked re-setting of pain thresholds in the mutant mouse line means that it is hard to interpret the comparison with certainty. The robust antinociceptive effects of μ-opioid receptors that are known to be exerted at spinal dorsal horn and brainstem levels would need to be taken into account for a holistic analysis.

A further report relating to the idea of functional interaction between different groups of afferent inputs describes the effects of toxin ablation of CGRP-expressing nociceptive afferents [189]. Interestingly, their removal amplifies behavioural responses to an evaporative cooling stimulus provided by acetone and reduces withdrawal latency from a cold plate at noxious temperatures. This is consistent with the general concept of crossover gating between different classes of afferent pathways, and indicates that concurrent activation of analgesic inputs associated with innocuous cool sensation and blockade of nociceptive inputs may bring about a synergistic, greater than additive, therapeutic outcome.

Conclusions:

Over the small number of years since its discovery, the TRPM8 ion channel has become established as a promising target for analgesic intervention. Abundant evidence indicates that TRPM8 is key to the sensing of innocuous cool temperatures and is expressed in a subpopulation of small DRG/TG neurons, which course to the superficial dorsal horn in parallel to nociceptive afferents. In chronic pain states, the central sensitisation that underpins hypersensitive pain behaviour is attenuated by this TRPM8-positive input, acting to gate-out nociceptive processing and produce an analgesic effect. TRPM8 agonists can usefully target this mechanism to provide efficacious analgesia in chronic neuropathic or inflammatory pain. TRPM8 agonists are thought to exert mixed effects on one particular sign of chronic pain,

that is cool allodynia, where they may both amplify innocuous cool sensing and attenuate central sensitisation; the overall outcome therefore being to ameliorate any allodynia. TRPM8 antagonists are being investigated for the treatment of cool allodynia; while they are likely to be effective in reducing the cool sensing input itself, there is no basis to expect any effect on central sensitisation or any impact on other modalities of chronic hypersensitive pain. Any participation of TRPM8 in noxious cold sensing in naive subjects is less clearly documented at present, with only a minority of TRPM8-positive DRG/TG cells expressing nociceptive markers, so any potential for intervention there remains to be validated. In the future the development of new highly selective chemical ligands for TRPM8 will be crucial, not only in providing tools to help unravel the complex neurobiology underlying its impact on pain processing, but also in the search for new drugs that are efficacious in the treatment of chronic pain.

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Table 1:

Condition	TRPV1 alone	TRPM8 alone	TRPV1 + TRPM8	Colocalised as % TRPV1	Colocalised as % TRPM8
Naïve	211	61	8	3.7	11.6
CFA	119	42	5	4.0	10.6
CCI	155	55	8	4.9	12.7

Immunofluorescence assessment of TRPV1/TRPM8 colocalisation in small DRG neurons: Sixteen μm unfixed cryostat sections (12-19 from 4-5 rats in each case) were incubated with guinea pig anti-TRPV1 and rabbit anti-TRPM8 primary antibodies, then appropriate secondaries labelled with distinct ALEXA-fluorophores. Cells were counted at x40 magnification from confocal images using a Zeiss LS510 Axiovert microscope.

Figure 1. Examples of recently produced TRPM8 antagonists reported to be active at submicromolar concentrations in vitro. Compounds represent several distinct structural series. While certainly potent TRPM8 antagonists, the pharmacological selectivity profiles are generally not described in detail.

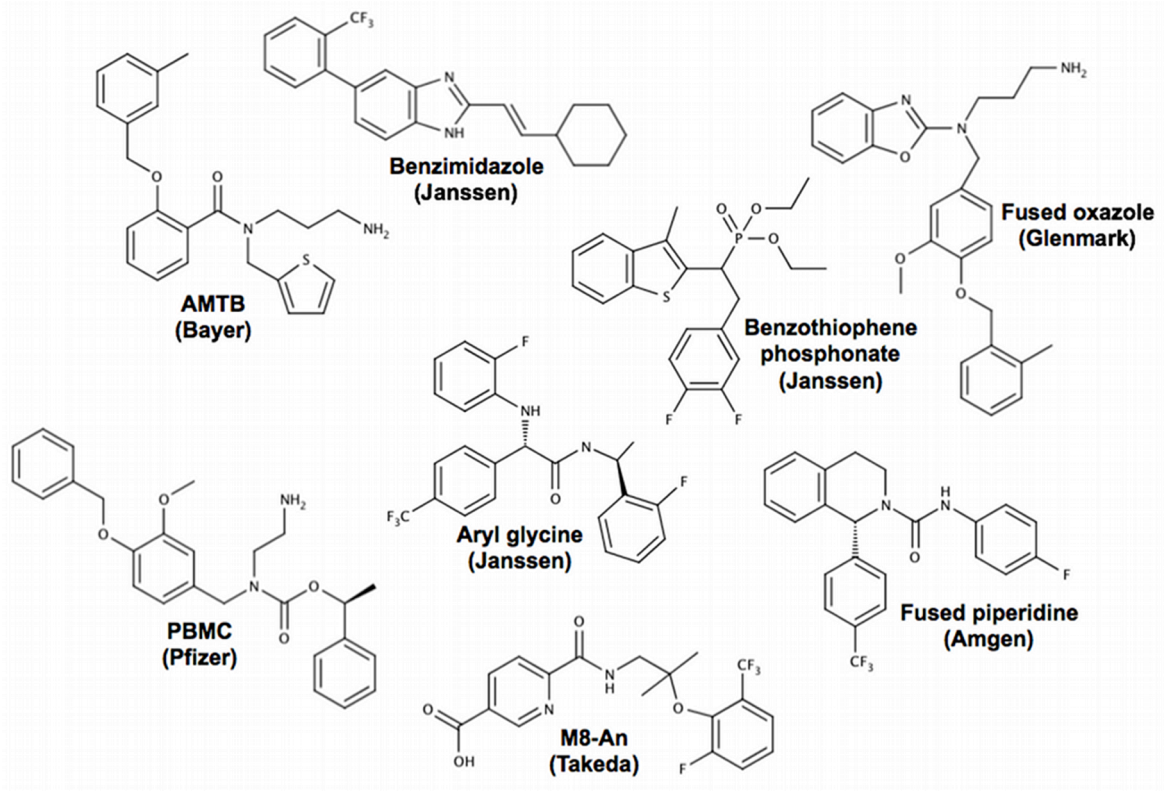

Figure 2. TRPM8 agonists of different structural series. WS-12 and D-3263 are analogues of menthol with considerably increased potency, showing EC_{50} at submicromolar concentrations. D-3263 has undergone a Phase 1, Open Label Trial in relation to potential anti-tumour activity. Icilin shows submicromolar potency and moderate selectivity. Thienopyrimidines, phosphonates and isoxazoles need to be further assessed to determine target selectivity.

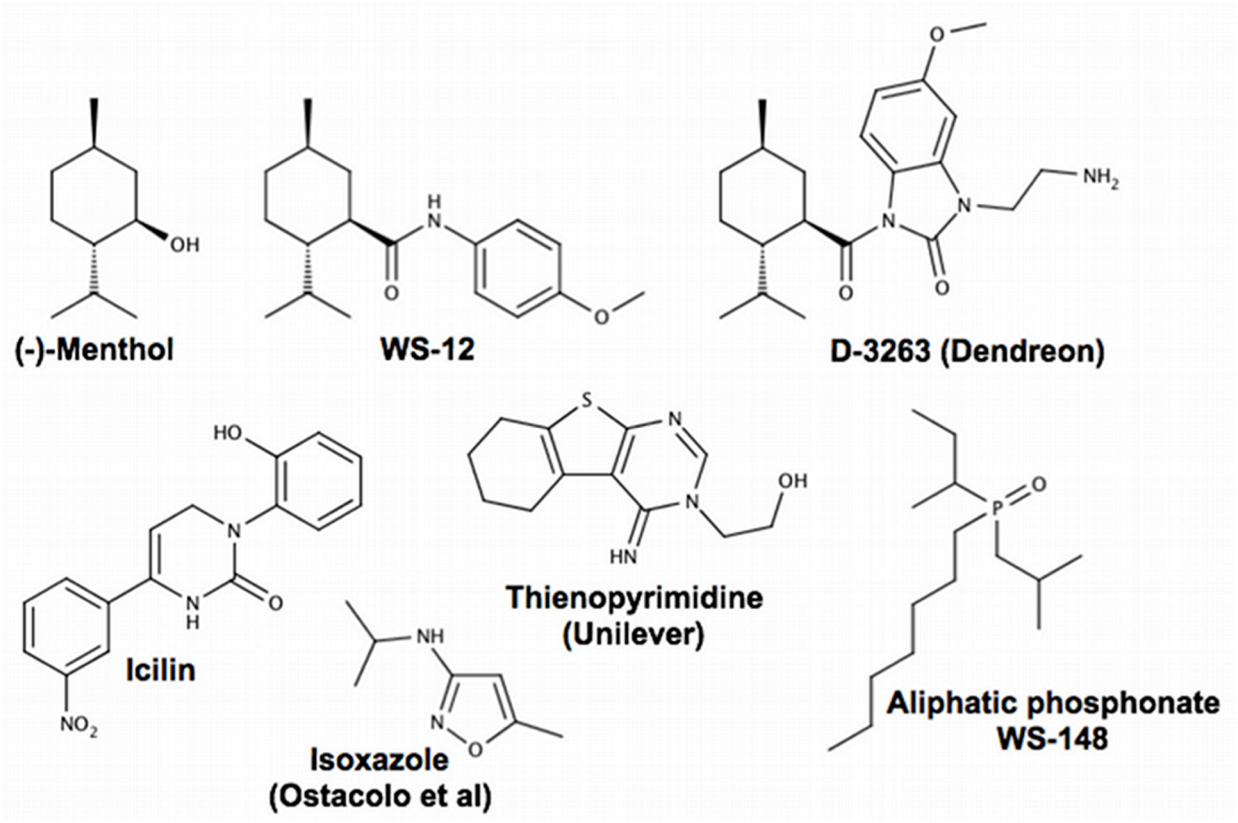

Figure 3. Schematic diagram illustrating the analgesic gating-out of hypersensitive spinal nociceptive transmission in chronic pain by TRPM8-expressing non-nociceptive afferents and the molecular mechanism through which 5-HT_{1B} receptors act to enhance this process.