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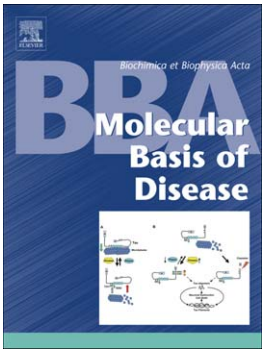

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TRP channels: **T**argets for the **R**elief of **P**ain

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Abstract

Patients with inflammatory or neuropathic pain experience hypersensitivity to mechanical, thermal and/or chemical stimuli. Given the diverse etiologies and molecular mechanisms of these pain syndromes, an approach to developing successful therapies may be to target ion channels that contribute to the detection of thermal, mechanical and chemical stimuli and promote the sensitization and activation of nociceptors. Transient Receptor Potential (TRP) channels have emerged as a family of evolutionarily conserved ligand-gated ion channels that contribute to the detection of physical stimuli. Six TRPs (TRPV1, TRPV2, TRPV3, TRPV4, TRPM8 and TRPA1) have been shown to be expressed in primary afferent nociceptors, pain sensing neurons, where they act as transducers for thermal, chemical and mechanical stimuli. This short review focuses on their contribution to pain hypersensitivity associated with peripheral inflammatory and neuropathic pain states.

Introduction

Pain is normally a transitory unpleasant sensation subsequent to a noxious or potentially injurious stimulus generated in somatic or visceral tissues. Unlike acute pain, inflammatory and neuropathic pain are often persistent, chronic states. Inflammatory pain is caused by irritation, injury or infection of somatic or visceral tissues. Its role is to prevent further injury while neuropathic pain is caused by a primary lesion or dysfunction in the peripheral nervous system. The management of chronic pain is a major unmet medical need in our aging society. The associated rise in the occurrence of many diseases (e.g., arthritis, diabetes, viral infections and side effects of the treatment of cancer and AIDS) and the relative inadequacy of currently available pain therapies (e.g., NSAIDS, opioids, anti-epileptics and tricyclic antidepressants) to produce sustained relief in patients with chronic pain, have generated a growing interest in pursuing novel pharmacological approaches.

Patients suffering from chronic pain often experience hypersensitivity to mechanical, thermal and/or chemical stimulation in the form of hyperalgesia (aggravated pain response to normally painful stimuli) and/or allodynia (pain response to normally innocuous stimuli). Given the diverse etiologies (e.g., physical trauma, neurotoxins, chemotherapy, infections, heredity, immune and metabolic diseases) and the variety of molecular mechanisms underlying pain hypersensitivity (e.g., different second messenger pathways and mitochondrial functions), the approach of targeting ion channels in primary afferent nociceptive neurons that can contribute to the detection of physical stimuli, may be an effective approach for developing more successful therapies for clinical pain syndromes. In the present review, we will focus on the role of mammalian Transient

Receptor Potential (TRP) channels and their function in dorsal root ganglion (DRG) nociceptive sensory neurons.

Hyperalgesia for cold, heat or mechanical stimuli, well documented symptoms of inflammatory and neuropathic pain, is mediated by sensitization of transduction processes in small-diameter unmyelinated C-fibers and medium-diameter myelinated Ad-fibers. These nociceptive neurons either respond to one type of physical stimulus (unimodal nociceptors), or more commonly integrate and generate a response to potentially damaging thermal, mechanical and/or chemical stimuli (polymodal nociceptors). Inflammation and peripheral nerve dysfunction have been associated with increased excitability of nociceptors as a result of changes in their ionic conductance properties leading to the speculation that nociceptive endings detect physical stimuli by means of ion channels responsive to thermal, chemical and/or mechanical stimuli. The search for such molecules was supported by the key finding that both heat and capsaicin, the pungent ingredient in hot pepper, induced influx of cations in nociceptors [1-4]. Because capsaicin induces a burning pain sensation it was hypothesized that capsaicin and heat may evoke painful responses through a common transducer. In 1997, Caterina and colleagues cloned the vanilloid receptor 1, subsequently renamed TRPV1, a capsaicin and heat-sensitive cation channel. TRPV1 is a mammalian relative of the *Drosophila* transient receptor potential (TRP) channel, which along with its homologue TRPL is responsible for phototransduction [5, 6]. TRPV1 is a polymodal receptor, its invertebrate relatives are essential to sensory transduction (phototransduction, thermosensation, mechanosensation, osmosensation [7]) and in mammals its activation by heat and protons results in an influx of cations which can depolarize the cell and

generate action potentials. Such hallmarks initiated an intense interest in the potential role of TRP channels in pain.

TRP family overview

The TRP channel family is one of the largest families of ion channels with representative members across the phylogenetic tree, from yeast to humans. Based on amino acid sequence homology, the mammalian members of this family have been classified into 6 subfamilies; TRPC (**C**anonical), TRPV (**V**anilloid), TRPM (**M**elastatin), TRPP (**P**olycystin), TRPML (**M**ucolipin) and TRPA (**A**nkyrin) [8-11]. Mammalian TRP channels are permeable to cations and their general membrane topology is similar to the superfamily of voltage gated channels. They have 6 transmembrane domains flanked by intracellular N- and C-terminal regions of variable length with a pore loop between transmembrane domain 5 and 6 [7, 8]. Four subunits need to assemble as homo- and/or heterotetramers to form a functional channel [12, 13]. Although several TRPs may be weakly voltage-dependent [14] they lack the hallmark of voltage-gated channels, the *voltage sensor* [15-17]. Beyond their general membrane topology and permeability to cations, TRP channels are strikingly diverse. Unlike other families of ion channels, the sequence homology of mammalian TRP channels is low and they have a wide variety of modes of activation (temperature, chemical compounds, osmolarity, mechanical stimulation, lipids, light, oxidative stress, acid, pheromones), regulation (transcription, alternative splicing, glycosylation, phosphorylation), ion selectivity, broad tissue distribution (virtually all cells tested express at least one member of the family) and physiological functions.

Today, ten years after the publication of the cloning of TRPV1, several other TRPs have been described in dorsal root ganglia; TRPV2, TRPV3, TRPV4, TRPA1 and TRPM8. These channels are emerging as sensory transducers that may participate in the generation of pain sensations evoked by chemical, thermal and mechanical stimuli. TRPV1, TRPV2, TRPV3 and TRPM8 are commonly referred to as thermoreceptors and TRPV4 and TRPA1 as mechanoreceptors. However, the hallmark of TRP channels is their polymodality and TRPV1, TRPV3, TRPM8 and TRPA1 are also recognized as chemoreceptors, respectively responsive to capsaicin and endocannabinoids, camphor [18], menthol [19, 20], mustard and cinnamon oil [21, 22], and TRPV4 and TRPA1 as thermoreceptors [23-25]. Recent studies in mice deficient in TRP channels indicates that TRP channels may play a crucial role in the hypersensitivity to thermal, chemical and mechanical stimuli that is associated with peripheral inflammation and neuropathies. The purpose of this review is to give an overview of the emerging role of TRP channels in the peripheral mechanisms of pain hypersensitivity associated with inflammatory and neuropathic states.

TRPV1

TRPV1, originally named vanilloid receptor 1 (VR1) and commonly referred as the capsaicin receptor, was first described as a polymodal receptor activated by three pain-producing stimuli; vanilloid compounds (capsaicin, resiniferatoxin), moderate heat (=43 ºC) and low pH (<5.9) [26, 27]. Since then, TRPV1 has been reported to be also activated by camphor [28], allicin [29, 30], nitric oxide [31], spider toxins [32], potentiated by ethanol [33] and modulated by extracellular cations [34]. TRPV1 was initially described in a subpopulation of small- to medium-diameter neurons in dorsal

root, trigeminal and nodose ganglia [26, 27]. While TRPV1 has since been described in many other neuronal and non neuronal cells [35-44], its highest expression level is in sensory neurons [43]. The initial expectation was that TRPV1 was *the* heat transducer in sensory neurons because its thermal activation threshold was comparable to: 1) the threshold for the perception of pain in human skin, 2) the threshold recorded *in vivo* for C-fiber nociceptors, and 3) the threshold for endogenous heat-evoked cationic current recorded in small-diameter dissociated DRG (For review, [45]). Mice lacking a functional TRPV1 gene were generated and the initial prospect of TRPV1 as *the* heat pain transducer in sensory neurons was tested. Sensory neurons from mice lacking TRPV1 did not respond to capsaicin, resiniferatoxin, protons or temperature (<50ºC) *in vitro* and behavioral response to capsaicin were absent and responses to acute thermal stimuli were diminished [46, 47]. In contrast, mice lacking functional TRPV1 showed normal physiological and behavioral responses to noxious mechanical stimuli. However, the most striking feature of these mice was the virtual absence of thermal hypersensitivity in the setting of inflammation; while wild-type mice had decreased threshold or latencies of withdrawal from mechanical and thermal stimuli, respectively, following mustard oil, complete Freund's adjuvant, carrageenan or inflammatory mediators (i.e., bradykinin, nerve growth factor, adenosine triphosphate), mice lacking functional TRPV1 only displayed hypersensitivity to mechanical stimuli [46-49]. Consistent with this finding, several studies have now demonstrated, *in vitro* and *in vivo*, that inflammatory mediators (bradykinin, prostaglandin E_2, extracellular ATP, glutamate and nerve growth factor) indirectly sensitize TRPV1 [50-52]; following exposure of sensory neurons to inflammatory mediators, responses to capsaicin or heat are dramatically enhanced to the

extent that body temperature can be sufficient to activate nociceptors [27, 53]. Inflammatory mediators sensitize TRPV1 function by various mechanisms; they may increase TRPV1 expression levels in the membrane [54, 55], induce TRPV1 phosphorylation by protein kinases [48, 56, 57] or release the inhibition of TRPV1 by phosphatidylinositol 4,5-bisphosphate, which render the channel more responsive to agonist stimulation [48, 58]. In addition, these inflammatory mediators act on receptors that are coupled to G proteins or tyrosine kinase pathways thus activating phospholipase C and/or phospholipase A2 which, in turn, induce the release of arachidonic acid metabolites. Several amide derivatives of arachidonic acid (anandamide) and lipoxygenase products of arachidonic acid, such as 12-(S)-HPETE, are agonists of TRPV1 and therefore are candidates for endogenous capsaicin like substances [59, 60]. In addition to inflammatory mediators, proteases released during inflammation or nerve injury, such as trypsins and mast cell tryptase, can also sensitize TRPV1; these proteases cleave the protease-activated receptor 2 to sensitize TRPV1 to induce thermal hyperalgesia through PKA and PKCε second messenger pathways [61, 62]. These findings demonstrate that TRPV1 not only participates in pain evoked by chemical and moderate heat but that TRPV1 contributes to peripheral sensitization, acting as the final substrate for multiple inflammatory mediators that operate via distinct intracellular signaling pathways.

This important realization initiated pre-clinical investigations of a potential role of TRPV1 in models of acute and chronic pain including: 1) monitoring of the development of acute or chronic hyperalgesia either in mice lacking functional TRPV1 gene or in rats receiving intrathecal injection of antisense oligodeoxynucleotides or silencer RNA

(resulting in a specific and reversible knock-down of TRPV1 protein) to further unravel TRPV1 function, and 2) massive chemical efforts to identify novel TRPV1 antagonists with the hope that these molecules would have analgesic properties.

Recent studies have reported that TRPV1 plays a pronociceptive role in some models of acute inflammatory pain. Mice lacking a functional TRPV1 gene ($TRPV1^{-/-}$) did not display nocifensive behavior following intraplantar injection of phorbol 12-myristate 13-acetate (activator of protein kinase C) suggesting that PMA-induced nociceptive behavior was exclusively dependent on TRPV1 [63]. Of note, PKC plays a prominent role in hypersensitivity to thermal stimuli after inflammation [64]. In a model of mild heat injury, $TRPV1^{-/-}$ mice had markedly reduced thermal and mechanical hyperalgesia [63], this finding has clinical relevance because cutaneous thermal injury induces heat and mechanical hyperalgesia in human skin [65, 66]. In contrast, formalin-induced nocifensive behavior, which is composed of two phases, the first supposedly due to the chemonociceptive effect of formalin and the second mainly mediated by inflammatory mediators, was similar in both TRPV1 genotypes. While carrageenan-induced heat hyperalgesia is mediated by TRPV1 [47], the clinically important mechanical hyperalgesia that is also induced was similar in $TRPV1^{-/-}$ and wild-type mice [63]. Similarly, Caterina and colleagues (2000) reported that mechanical hyperalgesia in $TRPV1^{-/-}$ mice, one day after the injection of complete Freund's adjuvant (CFA, model of inflammation) into the hind paw, was similar to that in wild-type mice while chemical and thermal hyperalgesia were markedly reduced [47]. However, Szabo and colleagues (2005) reported that 16 days after subcutaneous injection of CFA in rat hind paw and tail (model of chronic arthritis), mechanical hyperalgesia is attenuated in $TRPV1^{-/-}$ mice.

These results suggest a complex role of TRPV1 in inflammatory hyperalgesia induced by CFA; TRPV1 participates in the development of chemical and thermal hyperalgesia in the acute phase, possibly from the action of low pH, heat (*calor*) and inflammatory mediators [67], but can also participate in the mechanical hyperalgesia associated with the chronic phase of adjuvant arthritis possibly from the activation/sensitization of TRPV1 receptors by bradykinin, prostaglandins and lipoxygenases products that are released in arthritic joints [68]. Taken together, these findings suggest that the role of TRPV1 may vary within the different stages of inflammation and therefore between different inflammatory diseases.

TRPV1 function has also been investigated in models of neuropathic pain. TRPV1 plays an important role in chemical and thermal hyperalgesia in a model of diabetic neuropathy [69, 70], its role may be associated with altered cell-specific expression (decrease of TRPV1 protein expression in C-fibers paralleled by an increase in A-fibers) coupled to an increase in its function (oligomerization, reallocation of channels to cell surface plasma membrane and/or increase of TRPV1 phosphorylation coupled to impaired desensitization). However, in contrast to its pronociceptive role in thermal and chemical hyperalgesia in diabetic mice, TRPV1 may have a protective role in the development of mechanical hyperalgesia, which is greater and starts earlier in $TRPV1^{-/-}$ compared to wild-type mice [63]. Similarly, mechanical hyperalgesia associated with cisplatin-induced toxic neuropathy (chemotherapy-induced neuropathy) starts 4 weeks earlier in $TRPV1^{-/-}$ mice but once it has started, there is no difference between the TRPV1 genotypes [63].

The contribution of TRPV1 has also been tested in models of neuropathic pain associated with nerve lesion; after traumatic mononeuropathy caused by ligation of sciatic nerve, the induced cold allodynia can be markedly reduced after treatment with silencer RNA for TRPV1 [71] while both the induced mechanical and heat hyperalgesia is comparable in $TRPV1^{-/-}$ and $TRPV1^{+/+}$ mice [46, 63]. In contrast, TRPV1 antisense oligodeoxynucleotides reduce the mechanical hyperalgesia associated with spinal nerve ligation [72]. Finally, supporting a role of TRPV1 in neuropathic pain, an increase in TRPV1 expression level has been reported in uninjured DRG following peripheral nerve injury [73, 74] and molecular phenotype of non-injured C-fiber afferents is functionally important in the maintenance of neuropathic pain induced by partial nerve injury [75, 76].

These studies suggest that TRPV1 may play a role in the development and maintenance of chronic pain. Its contribution goes beyond its role as a thermoreceptor and while it plays an essential role in the transduction of thermal hyperalgesia it also contributes to mechanical hyperalgesia. Surprisingly, TRPV1 may not only be pronociceptive but may also play a protective role in mechanical hyperalgesia. While not all the studies have been performed under comparable conditions or used similar behavioral tests, one common conclusion emerges; TRPV1 is an important contributor to pain although its role is obviously more complex than first reported. The validation of the contribution of TRPV1 in inflammatory and neuropathic pain has generated a major interest in the development of specific vanilloid antagonists. These molecules have been reported to act as analgesics in different models of chronic pain [77-87]. However, the development of TRPV1 antagonists as analgesic drugs raises the issues of specificity and side effects, 1) TRPV1 tissue expression clearly indicates that the role of TRPV1 is not

restricted to inflammatory and neuropathic pain and antagonists may well affect physiological and pathological functions of TRPV1, and 2) TRPV1 may play both a pronociceptive and protective role in a model of chronic pain (i.e, diabetic neuropathy). Current clinical trials with TRPV1 receptor antagonists and future studies on the contribution of TRPV1 in rodent models of acute and chronic pain will hopefully soon provide a more definitive answer as to the role of TRPV1 in inflammatory and neuropathic pain syndromes

TRPV2

TRPV2, originally named vanilloid receptor-like protein 1 (VR-L1), was discovered as a structural homologue of TRPV1 with 50% amino acid identity [88]. It is insensitive to capsaicin or protons but is activated by high temperature (~52ºC), swelling and 2-aminoethoxydiphenylborate (2-APB) [88-90]. While regulatory mechanisms of TRPV2 gating are still poorly understood, reports suggest that growth factor (insulin-like growth factor-I) and PI3-kinase signaling pathways enhance TRPV2 activity [91, 92]. TRPV2 is widely expressed in neuronal and non neuronal cells [93-96] and intense TRPV2 immunolabeling is detected in medium-diameter DRG neurons that are associated with myelinated Ad-fibers and in a small percentage of C-fibers [88, 97]. The threshold for thermal activation of TRPV2 in a heterologous expression system (~52ºC) is similar to that of a subset of Ad-fibers recorded *in vivo* [98] and *in vitro* [46, 99]. Therefore, TRPV2 has been suggested to act as a high-threshold temperature sensor in Ad nociceptors [100, 101]. TRPV2 can heteromultimerize with TRPV1 *in vitro* and *in vivo* [102, 103], but the co-localization of these two channels in the same cell only

represents a very small percentage of DRG neurons both in control or inflammatory states [102, 104] and nociceptors lacking both TRPV1 and TRPV2 have normal heat responses [105], bringing into question the relevance of TRPV1/TRPV2 heteromers as a nociceptive heat sensor in DRG neurons. The role of TRPV2 in sensory neurons is not clear but a recent study by Shimosato and colleague (2005) reports the upregulation of TRPV2 protein level in medium-sized DRG neurons after intraplantar injection of CFA, leading these authors to suggest a role for TRPV2 in peripheral sensitization during inflammation, possibly in the transduction of pain hypersensitivity to high noxious temperature. Probably because of its very high heat threshold as well as its differential distribution compared to TRPV1, there have been fewer studies related to pain that focus on TRPV2. However its predominant distribution in the neurotrophin-3 dependent subpopulation of DRG neurons [106], its protein level upregulation following inflammation, its potential to heteromultimerize and its properties to be activated by 2-APB may be clues to its contribution to pain associated with inflammation or neuropathy. The generation of mice lacking functional TRPV2 gene would be very useful to further investigate its role in inflammatory and neuropathic pain.

TRPV3

TRPV3, which shares 40-50% homology with TRPV1, is activated by warm temperature (= 34ºC), with increased responses to higher noxious thermal stimuli and enhanced current following repetitive heat stimulation [20, 107, 108]. TRPV3 is also strongly activated and sensitized by camphor, irritants extracted from thyme, oregano, savory and cloves [109] and 2-APB [90]. Strong activation by either 2-APB or thermal

stimuli leads to the appearance of a secondary current; the initial gradually sensitizing current is followed by a current of larger amplitude with altered biophysical properties (loss of outward rectification, altered permeability and altered temperature and voltage-dependence) [110]. TRPV3 activity is strongly potentiated by G protein-coupled receptor stimulation linked to phospholipase C [109], arachidonic acid and other unsaturated fatty acids directly potentiate TRPV3 responses to 2APB in heterologous expression systems [111] and nitric oxide activates TRPV3 by cysteine S-nitrosylation [31].

Adding to the complexity of understanding the role of TRPV3 in pain, its tissue expression varies depending on the species considered; while it is specific to skin in mice [20], in humans it is expressed in trigeminal ganglia, spinal cord, brain, keratinocytes, tongue and DRG neurons [108]. Because of its restricted distribution in mice, studies on TRPV3 have focused on its role in keratinocytes where this protein plays a major role in detecting innocuous as well as noxious heat stimuli [18]. TRPV3 is a candidate transducer contributing to pain hypersensitivity associated with inflammatory states: 1) colocalizing with TRPV1 in human DRG where it could presumably form heteromultimeric channels exhibiting varying sensitivities to noxious stimuli [107, 112], 2) activated and potentiated by phospholipase C as well as the PKC second messenger pathway, which are both important events downstream from receptor activation by inflammatory mediators in sensory neurons, 3) being directly activated by nitric oxide, which when produced within sensory neurons acts as a second messenger mediating nociceptors sensitization [113] and 4) arachidonic acid and other fatty acids, which are produced during inflammation, potentiate TRPV3 function [111]. Of note, TRPV3 can also form heteromeric channels with TRPV2 *in vitro* [13]; whether these heteromeric

channels have any functional relevance *in vivo* remains to be demonstrated. The low or none detection of TRPV3 protein in DRG of rodents has limited the study of its role in nociceptor function, however its distribution in DRG nociceptors in human and its gating properties sustain an interest in a possible role in peripheral pain mechanisms.

TRPV4

Initially cloned as a mammalian osmo-transducer activated by a decrease in osmolarity of as little as 30 mOsm [114, 115], TRPV4 is a polymodal receptor not only activated by hypotonicity and shear stress [116-119] but also by innocuous heat with a threshold >27ºC [23, 24, 119], the phorbol ester 4 a- phorbol 12,13-didecanoate (4aPDD) [120, 121], low pH and citrate [122], endocannabinoids and arachidonic acid metabolites [123, 124], the active compound of *Andrographis paniculata*, bisandrographolide A, a Chinese herbal plant [125] and by nitric oxide [31]. Interestingly, unlike TRPV1, TRPV2 and TRPV3, TRPV4 is not activated by 2-APB [90].

While TRPV4 is widely expressed [114, 115, 126, 127], its distribution in cochlear hair cells, vibrissal Merkel cells, sensory ganglia [23, 114, 117, 122, 128] as well as in free nerve endings and cutaneous A and C-fibers terminals [129] suggested a role in mechano-transduction, beyond osmosensation. This idea was also supported by the finding that while the mutation of the osmosensing TRPV gene, *Osm9*, in *C. elegans* resulted in the absence of response to osmotic and mechanical stimuli in these worms, transgenic expression of mammalian TRPV4 in ASH nociceptive neurons of *Osm-9* mutant worms restored both osmotic and mechanical avoidance [130]. This important discovery suggested an evolutionarily conserved role for both osmo- and

mechanotransduction for TRPV4. The same year, mice lacking functional TRPV4 gene became available and these mice showed impaired sensitivity to acid, an increase in mechanical nociceptive threshold and altered thermal selection behavior [122, 131, 132]. In contrast, mice lacking functional TRPV4 have normal response to noxious heat and low-threshold mechanical stimuli [122, 131]. Finally, it was demonstrated that agonists of TRPV4 promote the release of the neuropeptides substance P and CGRP from the central projections of primary afferents in the spinal cord [128]. These studies suggest a role of TRPV4 in nociception.

The contribution of TRPV4 in the detection of warm temperatures and chemically-induced thermal hyperalgesia has also been investigated [133]; inflammatory and thermal hyperalgesia induced by capsaicin or carrageenan injection was markedly reduced in TRPV4$^{-/-}$ mice, the number and activity level of neurons in response to warm stimuli was also decreased in TRPV4$^{-/-}$ mice, which displayed a longer latency to escape from a hot-plate stimulus set at 35-45ºC. Of note, TRPV4, as TRPV3, is highly expressed in skin keratinocytes and thermosensation may not be restricted to sensory neurons; activation of TRPV4 and TRPV3 channels in keratinocytes may signal to sensory neuron terminals deeply embedded in the epidermis to contribute to temperature sensitivity [23, 45, 132, 134, 135]. Further studies are needed to determine the relative contribution of TRPV4 function in sensory neurons and/or keratinocytes to temperature sensation.

We reported using two models (i.e., mice lacking functional TRPV4 and transient down-regulation of the level of TRPV4 protein in the rat), that in the presence of prostaglandin E_2 TRPV4 mediates nocifensive behaviors to small increases or decreases in osmolarity [117, 136]. This finding is relevant for a role of TRPV4 in pathological

pain states because small changes in osmolarity have been described in various diseases (diabetes, alcoholism, aquadynia and asthma) and increase in osmolarity jointly with pH decreases are believed to contribute to inflammatory pain [137, 138]. TRPV4 also plays a crucial role in mechanical hyperalgesia following the exposure to inflammatory mediators [139]; in this study we demonstrated that: 1) concerted action of inflammatory mediators was necessary to reach the threshold level of cAMP necessary to engage TRPV4 in mechanical hyperalgesia, and 2) TRPV4 is engaged in hyperalgesia to mechanical and osmotic stimuli by two key intracellular second-messenger pathways of inflammatory hyperalgesia, protein kinase A (PKA) and protein kinase Cε (PKCε). In addition to inflammatory mediators such as PGE_2 or serotonin, protease-activated receptor 2 agonists were also demonstrated to sensitize TRPV4 [128]. Proteases generated during inflammation and injury cleave protease-activated receptor 2 on primary afferent neurons to activate second messenger pathways (PLCβ, PKA, PKC and maybe PKD) which, in turn, sensitize TRPV4. The authors demonstrate that the protease-activated receptor 2 sensitizes both TRPV4-mediated release of substance P and CGRP in the spinal cord and TRPV4-induced mechanical hyperalgesia [128]. These findings demonstrate the important role of TRPV4 in the development of acute inflammatory hyperalgesia.

The contribution of TRPV4 in chronic pain has been investigated in a rat model of painful small-fiber peripheral neuropathy, Taxol chemotherapy-induced neuropathy [140]. Taxol treatment enhanced nociceptive behavioral responses to mechanical and hypotonic stimulation of rat hind paw. Treatment with TRPV4 antisense oligodeoxynucleotides reversed the Taxol-induced mechanical hyperalgesia and

markedly reduced the hyperalgesia to hypotonic stimuli. The integrin antagonist hexapeptide GRGDTP and the Src specific inhibitor, PP_1, inhibited Taxol-induced hyperalgesia to the same extent as TRPV4 antisense suggesting that Taxol-induced TRPV4-mediated hyperalgesia depends on an integrin/Src tyrosine kinase signaling pathway. Specific integrins play a role in the maintenance of neuropathic and inflammatory hyperalgesia [141, 142]. Taxol-induced mechanical hyperalgesia also depends on both PKCε and PKA second-messenger signaling [143]. Of note, mechanical hyperalgesia induced by direct activation of PKCε and PKA is decreased by TRPV4 antisense and is absent in mice lacking functional TRPV4 [139]. Moreover, Src tyrosine kinase can directly interact with both cAMP/PKA and PKCε pathways [144-147]. Taken together these data suggest that TRPV4 may be engaged in neuropathic pain via a second messenger pathway involving integrin/Src tyrosine kinase/PKA/PKCε signaling pathway. The use of mice lacking functional TRPV4 and/or rats treated with TRPV4 antisense has demonstrated the role of this channel in the development of thermal and mechanical hyperalgesia associated with inflammation and neuropathy. Thus, current knowledge on TRPV4 function suggests that it may play a role complementary to that of TRPV1 in producing peripheral sensitization, also acting as a final substrate for multiple inflammatory mediators that operate via distinct intracellular signaling pathways.

TRPM8

Although first identified in prostate gland as an androgen-responsive channel [148] TRPM8 has since been described as a cold and menthol-activated channel with prominent voltage dependent gating properties [19, 149-151]. Cold and menthol both

induce membrane depolarization and firing of action potentials in a subpopulation of nociceptors [152-155].

When expressed in heterologous cells, the temperature threshold and biophysical properties of the TRPM8 current are similar to those recorded in sensory neurons [19, 151]. In addition, TRPM8 is expressed in ~ 15% of small-diameter DRG neurons, which is consistent with the percentage of cultured sensory neurons responsive to cold and menthol [149, 151, 153]. TRPM8 is also activated by numerous other cooling compounds such as eucalyptol, spearmint, WS-3 and icilin; this activation is dependent on different factors such as intra and extracellular Ca^{2+} concentration and pH [22, 156, 157]. The activity of TRPM8 is down-regulated by the activation of PKC [158] and inhibited by ethanol in a PIP2-dependent manner [159]. TRPM8 is expressed in prostate and in small-diameter trigeminal [160] and dorsal root ganglion neurons, suggesting its specific expression in C- and possibly Ad- fibers [19, 161, 162]. The expression of TRPM8 in nociceptive neurons has been controversial, thought to be a culturing artifact due to the addition of NGF [25] but there is now evidence for an expression in both nociceptive and non-nociceptive neurons [152, 163, 164]. The contribution of TRPM8 channels to innocuous cold transduction has recently been reported in primary sensory neurons [165] but its role in nociception remains to be demonstrated (for reviews on TRPM8, [166-168]). However, the range of temperature over which it responds including both innocuous and noxious temperatures, the regulatory role of intracellular acidity [169] and its property of adaptation to prolonged stimuli [19] suggest that TRPM8 is a candidate as a sensory transducer contributing to pain hypersensibility associated with inflammation or neuropathy. However, two recent studies investigated a potential role of TRPM8 in

pain hypersensitivity; Katsura and colleagues (2006) reported that cold hyperalgesia induced by L5 spinal nerve ligation is not affected by TRPM8 antisense, and Proudfoot and colleagues (2006) reported that activation of TRPM8 by icilin in sensory neurons elicited *analgesia* in three different models of pain; a model of chronic neuropathic pain (chronic constriction injury of the sciatic nerve, CCI), a model of inflammatory pain (CFA) and a model of peripheral demyelination (focal application of lysolecithin to the sciatic nerve). They found that the level of expression of TRPM8 is upregulated in DRG and spinal cord following CCI nerve injury and that treatment with TRPM8 antisense oligodeoxynucleotides prevents icilin-induced analgesia. The authors show evidence for a centrally-mediated activation of TRPM8 that relies on metabotropic glutamate receptors and suggest that these glutamate receptors would respond to glutamate released from afferents expressing TRPM8, to inhibit the nociceptive inputs [162]. Mice lacking a functional TRPM8 gene have yet to be reported; while current knowledge on TRPM8 distribution and function suggest a potential protective role in neuropathic pain, further studies are needed to confirm that finding.

TRPA1

TRPA1 was first identified as a protein overexpressed in a liposarcoma cell line (ANKTM1, [170]) but was later recognized as a member of a new TRP subfamily characterized by the presence of a large number of ankyrin repeat motifs located on the cytosolic amino terminal domain (TRPAnkyrin). TRPA1 is expressed in the inner ear and in trigeminal and DRG neurons [25, 171]. Expressed in heterologous systems it is activated by pungent ingredients of mustard oil, garlic, wintergreen oil, clove oil, ginger

and cinnamon oil [21, 22, 29, 172] all of which induce acute painful burning or pricking sensation. Consistent with a role in nociception TRPA1 is highly co-expressed with TRPV1 in small-diameter peptidergic nociceptors while it is rarely co-expressed with TRPM8 [25, 161, 172], and TRPA1 is localized at free nerve endings in mouse nociceptors. Behavioral studies in mice lacking TRPA1 ($TRPA1^{-/-}$) confirmed its role in nociception to irritants such as mustard oil, acrolein and garlic [173, 174]. TRPA1 has also been suggested to be a sensor of noxious cold stimuli, but this property is still controversial; temperature below 18ºC activates recombinant TRPA1 [22, 25], treatment with TRPA1 antisense oligodeoxynucleotides reduced behavioral hypersensitivity to cold after CFA-induced inflammation or sciatic nerve injury [175]; treatment with TRPA1 antisense also alleviated cold hyperalgesia induced by L5 spinal nerve ligation, probably resulting from an increase in TRPA1 protein level in the nearby uninjured L4 DRG [176], and one group demonstrated that $TRPA1^{-/-}$ mice have impaired behavioral responses to a cold plate maintained at 0ºC [173]; however, 2 other groups failed to demonstrate a response of TRPA1 to noxious cold[174, 177]. The disparity between the studies might result from: 1) the different conditions of *in vitro* recording (i.e., DRG from newborn mice compared with DRG from adult mice), 2) difference in the specific behavioral test used (i.e., latency before the first paw lift in response to a cold plate stimulus versus number of paw lifts during the first 5 min after mice were placed on the cold plate), and 3) sexual dimorphism; Kwan and colleagues (2006) observed the highest difference in cold sensitivity in female mice while Jordt and colleagues (2004) only used males. Our laboratory has demonstrated that sexual dimorphism is an essential factor in the modulation of pain pathways in nociceptors [178-180] and gender differences in pain

sensibility is well documented, therefore the sex of the experimental animals should be carefully considered.

TRPA1 has also been suggested to be a sensor for mechanical stimuli for several reasons: 1) its *Drosophila* homologue, *painless*, participates in mechanical nociception and an evolutionarily conserved role has been shown within the TRP family [171, 181], 2) its functional properties suggest that it is one component of the mechanosensory channel in hair cells [171, 173, 182-184], and 3) some of its biophysical properties may match that of a high-threshold mechanoreceptor. However, its role in mechanical nociception still remains controversial. Kwan and colleagues (2006) reported that TRPA1$^{-/-}$ mice showed a deficiency in sensing noxious punctate cutaneous mechanical stimuli; these mice had higher mechanical thresholds and reduced response to a series of suprathreshold stimuli when compared to TRPA1 wild-type mice, suggesting a potential role in the transduction of high-threshold mechanical stimuli. On the other hand, Bautista and colleagues (2006) reported no difference in mechanical thresholds between TRPA1$^{-/-}$ and wild-type mice. Again, this discrepancy could arise from the difference in the two behavioral measurements, one group measuring values of mechanical threshold, the other the percentage of withdrawals in response to increasing mechanical stimuli. However, in contrast to the probable participation of TRPA1 in the hypersensitivity to cold following sciatic nerve injury [175], TRPA1 does not appear to participate in the associated mechanical hypersensitivity [173].

Bandell and colleagues (2004) demonstrated that in addition to pungent compounds, TRPA1 is also activated by the inflammatory mediator bradykinin. They demonstrated *in vitro* the coupling of TRPA1 with the G-protein-coupled bradykinin

receptor 2 and the role of phospholipase C on TRPA1 activity. This finding suggests that TRPA1 could function as a receptor that depolarizes nociceptors in response to inflammatory agents that activate PLC [21, 22]. Consistent with this finding, Kwan and colleagues (2006) reported that $TRPA1^{-/-}$ mice have impaired responses to injection of bradykinin as well as a markedly reduced bradykinin-induced mechanical hyperalgesia. Bautista and colleagues (2006) also reported that $TRPA1^{-/-}$ mice have impaired cellular and behavioral response to bradykinin and while the existence and contribution of TRPV1/TRPA1 heteromeric channels cannot be eliminated, they suggested a model of functional interaction between bradykinin, bradykinin receptor 2 and TRPV1; bradykinin binding to G-protein coupled receptors activates PLC/PKC signaling pathway, resulting in the release of Ca^{2+} from intracellular stores, therefore sensitizing TRPV1 leading to Ca^{2+} entry which jointly with Ca^{2+} release from intracellular stores opens TRPA1. A possible "cooperation" between TRPA1 and TRPV1 channels has also been reported in another recent *in vitro* study in which the cannabinoid agonist WIN 55,212-2 dephosphorylates, therefore desensitizing, TRPV1 in trigeminal sensory neurons via the activation of TRPA1; WIN 55,212-2 directly activates TRPA1 which leads to entry of Ca^{2+}, activation of calcineurin and subsequent dephosphorylation of TRPV1 [185, 186].

If many questions remain on the exact modalities of TRPA1 activation its contribution to nociception is fairly established. Moreover, its co-expression and putative functional interaction with TRPV1 suggest that it also contributes to inflammatory pain. Further studies are needed to elucidate its role in chronic pain.

TRP channels: complex sensory integrators participating in pain hypersensitivity

The available data provide a compelling argument for a contribution of TRP channels to pain hypersensitivity associated with inflammation and neuropathy. However, reports of multiple, and sometimes, contradictory functions for a TRP channel, depending on the inflammatory mediator or the model of neuropathic pain used, are problematic (Table 1). Diversity in experimental conditions and specific behavioral tests used can explain some of the differences but these dissimilarities might also reflect the complexity of the functional properties of TRP channels.

One recurrent strategy has been to correlate a chemical or physical stimulus that activates functionally distinct subsets of DRG neurons *in vitro* with the *in vivo* expression pattern of certain TRP channels (i.e., neurons responding to capsaicin or menthol are, respectively, correlated to the expression of TRPV1 or TRPM8 channels). However recent studies suggest that there might be more overlap in the modalities to which TRP channels respond than initially appreciated. While menthol, camphor and cinnamaldehyde were specifically associated with TRPM8, TRPV3 and TRPA1 respectively, a recent study performed *in vitro* reported that menthol activates TRPM8 (30 µM) and TRPV3 (20 mM) while it inhibits TRPA1 (68 µM); camphor activates both TRPV3 (40 mM) and TRPV1 (4.5 mM) while it inhibits TRPA1 (68 µM) and cinnamaldehyde activates TRPA1 (9.5 µM) and inhibits TRPM8 (1.5 mM) [30]. Similarly, the 6 TRP channels expressed in DRG neurons are activated by thermal stimuli, encompassing the whole spectrum of temperature from noxious cold to noxious heat with each member activated at a distinct thermal threshold (TRPA1= 18ºC, TRPM8~ 23-28ºC, TRPV4= 27ºC, TRPV3 ~ 31-39ºC, TRPV1= 43 ºC and TRPV2= 52 ºC). However, these thresholds are known to vary with cellular context, which most likely

will result in some overlap *in vivo* (i.e., TRPV1's thermal threshold lowers to body temperature following sensitization through a phospholipase C-dependent pathway and both TRPV3 and TRPV4 are also active at that temperature). In addition, TRPs are polymodal channels, they bind *multiple* ligands and the binding of one ligand can influence the binding of another (i.e., pH, levels of Ca^{2+} and temperature have been shown to modulate TRPV4 response to hypotonic stimulation). Therefore, *in vivo*, the response of each TRP channel to thermal, chemical and mechanical stimuli may be less unique than what has been described. In addition, TRP channels can be co-expressed in the same DRG neuron, for example both TRPA1 and TRPV3 have been shown to co-localize with TRPV1, and TRPV4 is expressed in small-diameter capsaicin sensitive DRG [25, 107, 112, 117, 161, 172]. Co-localization of these TRP channels may lead to the formation of heteromeric channels with unique or unexpected sensitivities to physical stimuli. Bautista and colleagues (2006) also suggested that, depending on the cellular context, some TRP channels may not function mainly as ligand-gated channels but rather mediate increases in neuronal excitability for various stimuli through activation of intracellular signaling pathways. They reported a functional interaction between G-protein coupled receptors, PLC/PKC signaling pathway, TRPV1 and TRPA1 [174]. If this model is correct then multiple G-protein coupled receptors, coupled to PLC, may induce a similar effect. This is important since TRP channels expressed in DRG neurons are all modulated by the PLC/PKC signaling pathway (Figure 1). In agreement with this idea of "teamwork" between TRP channels is the finding that the cannabinoid WIN 55,212-2 regulates TRPV1 phosphorylation through TRPA1 [185].

This property of TRP channels may partly explain the discrepancy between studies on the contribution of TRP channels to pain hypersensibility. The complex role of TRPV1 in CFA-induced hyperalgesia [47, 68], for example, may reflect a switch in TRPV1 function. TRPV1 participation in the chemical and thermal hyperalgesia associated with the acute phase of CFA-induced inflammation may be as a *ligand-gated* ion channel activated by low pH and heat [67], while its contribution to the mechanical hyperalgesia associated with the chronic phase of adjuvant arthritis may be as a "*teammate"*, increasing neuronal excitability through activation of intracellular signaling pathways by bradykinin, prostaglandins and lipoxygenase products that are released at the site of inflammation, in arthritic joints. Supporting this idea of cooperation, TRP channels have been reported to function in signaling microdomains; TRP channels are clustered in spatially organized membrane microdomains where interactions between signaling molecules and receptors lead to specific cellular response [187-189]. Depending on the partners in the signaling complex, the cellular context, intrinsic regulation (glycosylation, alternative splicing, transcription), the activation and the contribution of a specific TRP channel may vary not only in different subpopulations of nociceptors but also under different inflammatory or neuropathic states.

Concluding remarks:

Current knowledge of TRP channels expressed in primary afferent nociceptors suggest that these channels all have functional properties compatible with a role in nociception. However, compelling data for a contribution to pain and more specifically to pathological pain associated with inflammatory and neuropathic states has only been

reported for TRPV1, TRPV4 and TRPA1. Many questions remain such as; how is the activity of these channels tuned to integrate and respond accurately to multiple stimuli, how do these channels interact not only with each other but also with other receptors contributing to pain transduction in nociceptors, what is the role of TRPV2 in nociceptors, why is TRPV3 expressed in human DRG neurons, and is TRPM8 responsible for the analgesic effect of cooling component? Future studies are needed to elucidate the role of these multifunctional ligand-gated channels in primary afferent nociceptors. Furthermore, while primary heat, chemical and mechanical hyperalgesia is the result of sensitization of previously responsive C- and Ad-fibers, it is established that newly recruited previously "silent" C nociceptors also play a role [190-192]. The molecular mechanisms underlying the participation of these "silent" nociceptors are still unknown; do TRP channels contribute to their sudden "chattiness" in pathological conditions? Will better knowledge of TRP channels help us crack the code of chronic pain? TRP channels certainly are promising *T*argets for the *R*elief of *P*ain.

Figure legend

Figure 1- Inflammatory mediator regulation of TRP channels by phosphorylation in sensory neurons. Inflammatory mediators (bradykinin, ATP, NGF, PGE_2, serotonin) bind to either G-protein-coupled receptors (GPCR) or tyrosine-kinase-coupled receptors (TRK) to activate phospholipase C (PLC), protein kinases A (PKA) and C (PKC), Ca^{2+}-calmodulin-dependent kinase II (CAMKII) and PI3 kinase (PI3K) which, in turn, activate/sensitize (+) or desensitize (-) TRP channels to physical stimuli, and increase Ca^{2+} release from the endoplasmic reticulum (ER). Increase in the concentration of intracellular Ca^{2+} activates PKC, CAMKII and TRPA1. GPCRs can also activate phospholipase A2 (PLA_2) inducing the release of arachidonic acid metabolites such as HPETE or 5,6-EET which, in turn, act as TRP channel agonists. Nociceptor sensitization to thermal, chemical and mechanical stimuli by a specific inflammatory mediator varies depending on which TRP channel is expressed in a DRG neuron. This scheme illustrates how TRP channels may not only act as ligand-gated ion channels but may also increase neuron excitability through the activation of intracellular signaling pathways. Putative heteromeric channels (?) may also increase the complexity. Red lines represent processes engaging TRPV1; green lines, processes engaging TRPV4; blue lines, processes engaging TRPA1 and purple lines, processes regulating TRPM8.

Table 1: Function of TRP channels in DRG neurons

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Table 1: Function of TRP channels in DRG neurons

TRP family	Name	heat threshold	Agonists	regulatory signaling pathways	nociception phenotype in null mice	Nociception in rat treated with antisense or siRNA
TRPV	***TRPV1***	=43º C	capsaicin various vanilloids compounds, camphor, allicin, nitric oxide, protons, spider toxins arachidonic acid metabolites, lipoxygenase products,	PLA2, lipoxygenases, PKA, PLC, PKC, PI3K, p38, CAMKII, ethanol NGF, ATP, PGE2 BK, glutamate,	- impaired thermal avoidance - absence of nocifensive response to capsaicin - impaired inflammatory-induced thermal hyperalgesia - impaired chemical and thermal hyperalgesia in TRPV1 null diabetic mice	- reduced cold allodynia induced by ligation of sciatic nerve - reduced mechanical hyperalgesia

		endocannabinoids, 2-APB	prokineciti n-1 PIP2 (-) calmodulin (-) calcineurin (-)	- mechanical hyperalgesia associated with cisplatin chemotherapy or diabetic neuropathy is enhanced in TRPV1 null mice	induced by spinal nerve ligation
TRPV2	=52ºC	cell swelling and 2-APB	PI 3K insulin-like growth factor	N/A	N/A
TRPV3	~31 to 39ºC	camphor, carvacol, eugenol, thymol, 2-APB, nitric oxide, arachidonic acid, unsaturated fatty acids	PLC PKC	- impaired thermal avoidance	N/A

	TRPV4	=27º C	osmolarity, shear stress, phorbol esters, 4 alpha-PDD, low pH, citrate, bisandrographolide A, nitric oxide arachidonic metabolites, endocannabinoids	PLA2, cytochrome 450, cAMP, PKA, PKC, Src tyrosine kinase integrins PGE2, inflammatory soup	- impaired response to high threshold mechanical stimuli - impaired sensitivity to acid - altered thermal selection behavior - impaired osmotic and mechanical hyperalgesia induced by inflammatory soup - impaired inflammatory-induced thermal hyperalgesia	- reversal of osmotic and mechanical hyperalgesia in a model of Taxol chemotherapy-induced neuropathy - reversal of osmotic and mechanical hyperalgesia induced by inflammatory soup
TRPM						
	TRPM8	~ 23 to 28ºC	menthol, icilin, eucalyptol, spearmint,	PI P2 ethanol	N/	- prevention of illicin-induced analgesia in 3 models of pain: chronic constriction

			WS-3		A	injury of the sciatic nerve, CFA-induced inflammation demyelination of the sciatic nerve
				PKC (-)		- TRPM8 antisense does not affect the cold hyperalgesia induced by spinal nerve ligation
TRPA						
	TRPA1	=18º C	eugenol, gingerol, methyl salicylate, allyl isothiocyanate, cinnamaldehyde, mustard oil,	PL C B K	- impaired responses to irritants and bradykinin - impaired response to cold plate set at 0ºC - impaired responses to high-threshold mechanical stimuli	- reduced cold hyperalgesia induced by CFA or sciaticnerve ligation - reduced cold hyperalgesia induced by spinal nerve ligation

			cannabinoid agonist WIN 55212-2			

(-) inhibitory regulatory signaling pathways
N/A Not available
Ankyrin repeat
TRP-box
Transmembrane domain
Pore region