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CICLO XXXII

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Ruolo dei canali *Transient Receptor Potential* (TRP), espressi in cellule neuronali e non-neuronali, nelle patologie dolorose di origine infiammatoria e neuropatica

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Index

I - Introduction

1.1. Pain: definition and classification

The International Association for the Study of Pain (IASP) defines pain as "*an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage*" [1]. Pain is the most common symptom reported to health care providers, representing a driving force of health care utilization and lost productivity, and it also exacts a substantial toll on the afflicted, their loved ones, and society in general [2].

Pain is a sensation in a part or parts of the body, representing a multidimensional sensory experience physically associated with hurting and soreness, and with avoidance motor reflexes and alterations in autonomic output. But what is most important is that pain is always unpleasant, intrinsically distressing, and therefore it also has cognitive and emotional components. It has been claimed that human pain experience is composed of three different dimensions [3]: a) a sensory-discriminative dimension which identifies location, timing, and physical characteristics of the noxious stimulus and activates withdrawal reflexes to prevent or limit tissue damage; b) an affective-motivational dimension, the one most closely linked with emotion, which underlies the unpleasantness associated with exposure to the noxious stimulus and activates defensive behaviours such as escape and recovery; c) a cognitive-evaluative dimension which affects the evaluation of the meanings and consequences of an injury or pain. Moreover, the subcortical circuit that governs defensive responses and involves nonconscious processing of stimuli can interact with the cerebral cortex and yield the conscious experience of fear and anxiety when dysregulated [4]. Sustained activation of these cortical sites may thus contribute to secondary emotional reactions associated with pain, which in turn can contribute to further suffering and disability [5].

Pain may vary in intensity (from mild, to moderate or severe), quality (it can be sharp, burning, or dull), duration (transient, intermittent or persistent), and spatial description (superficial or deep, localized or diffuse). It can be essentially divided in two broad categories: adaptive pain contributes to survival by protecting the organism from injury or promoting healing when injury has occurred; maladaptive pain represents a

pathologic processing of the nervous system and it is what we might interpret as disease. However, there is not a standard taxonomy of pain. Distinctions are frequently made though between acute and chronic pain or cancer and non-cancer pain.

Acute pain is a sensory experience caused by a noxious stimulus and mediated by the nociceptive system: this represents a key early warning tool that acts as an alarm system to disclose to the body the presence of a potentially damaging stimulus. The nociceptive pain system helps the body to associate certain categories of stimuli with danger that must be avoided, by linking them to intense unpleasant sensations. For this system to work, it is required that the sensation of pain is strong enough to demand immediate attention. Thus, it is important for this system not to be chronically disabled but just put under control during specific situations, such as surgery or medical procedures. Nociceptive pain is indeed a vital physiologic sensation, and loss of its function can inevitably lead to tissue damage: reported examples are self-induced mutilation of the tongue or lips, destruction of joints, loss of the fingertips, and pressure ulcers. It has been shown that patients with congenital insensitivity to pain due to a mutation of the Nerve Growth Factor (NGF) tyrosine kinase A receptor (which results in a loss of high-threshold sensory neurons) have a reduced life expectancy [6].

The main difference between chronic and acute pain is duration: chronic pain is generally defined as persistent pain lasting more than 3 months [7]. It appears that persistent pain is associated with altered neuronal activity and sensitization of peripheral primary sensory neurons in dorsal root ganglia (DRG) and trigeminal ganglia (TG) [8-10], as well as the sensitization of central nociceptive neurons in spinal cord, trigeminal nucleus, brain stem and cortex [11-13], particularly when initiated by peripheral injury or stimulation. Such sensitization is derived from long-term changes in morphology, neurochemistry, and gene expression, and is characterized by an enhanced pain response to thermal and mechanical noxious stimuli (hyperalgesia), a decrease in pain threshold to normally non-painful stimuli (allodynia), and an increase in spontaneous activity (spontaneous pain). Spontaneous pain and changes in sensitivity are cardinal features of clinical pain, distinguishing it from normal acute nociceptive pain. Chronic pain is often (but not always) a maladaptive form of pain, and represents a rising health problem, predicted to affect up to 30% of adults worldwide. Strong efforts have been made in pain research during the past decades, but translation of preclinical results into clinical practice has been minor, and very few novel therapeutic opportunities have been offered to patients.

Current treatments, which include opioids and non-steroidal anti-inflammatory drugs (NSAIDs), are still inadequate due to considerable side effects and incomplete efficacy. The resulting condition is that patients are frequently undertreated, and new potent analgesic drugs with a better safety profile may highly be needed.

Pain is one of the cardinal features of inflammation, and is well established that mediators of inflammation are released locally after tissue damage, ranging from bradykinin to prostaglandins, H^+, ATP, NGF, pro-inflammatory cytokines and chemokines. These inflammatory mediators (referred collectively to as "inflammatory soup") can, in turn, stimulate and cause sensitization of pain-sensing nociceptors [8,14]. Hyperactivity of primary sensory neurons will also increase the release of excitatory neurotransmitters (*e.g.* glutamate) and neuromodulators such as substance P (SP), calcitonin gene-related peptide (CGRP) and brain-derived neurotrophic factor (BDNF), causing in this way the hyperactivity of post-synaptic nociceptive neurons as well [15]. However, pain symptoms can be promoted by different etiologic agents, including physical trauma, neurotoxins, infections, cancer and chemotherapeutic treatment, immune and metabolic diseases, and migraine.

1.1.1. Inflammatory and neuropathic pain

There are two main types of chronic pain: inflammatory nociceptive pain and neuropathic pain. Inflammatory pain is associated with tissue damage and the resulting inflammatory process, so it is an adaptive form of pain, which promotes healing. This happens because if tissue damage occurs (*e.g.* through trauma, surgery, or other pathologies) the body shifts from protecting against noxious and potentially damaging stimuli to promoting healing of the injured tissue: in this state there is an increase in sensitivity to stimuli that usually do not cause pain. As a result, we tend to prevent contact with or movement of the injured part to minimize further damage until complete repair is achieved. Endogenous mediators released from the damaged or infected tissues increase the extravasation of the vessels and attract the immune cells, including mast cells, macrophages, neutrophils, and platelets, to the injured site [16]. These mediators can also directly activate the nociceptors, evoking pain or modulating the sensitivity of the primary nociceptors, thus causing a hyperreactive reaction to stimuli [17].

Unlike inflammatory pain, neuropathic pain is not associated with an overt condition of tissue inflammation, but is rather dependent from a damage or dysfunction

of the nervous system. It is most frequently due to peripheral nerve injury or lesions, such as in patients with diabetic neuropathy, polyneuropathy associated with AIDS, post-herpetic neuralgia, and lumbar radiculopathy, but it can also result from lesions to the central nervous system, such as in patients with spinal cord injury or multiple sclerosis and stroke [18]. In another pain condition, called functional pain, no neurologic injury or peripheral deviance can be detected: the pain in this case is due to an abnormal responsiveness or function of the nervous system, so that the symptoms are amplified by heightened gain or sensitivity of the sensory apparatus. The origin of this hyperresponsiveness is currently unknown, but there are several common conditions which have features that may allow us to place them in this category of pain: for example, fibromyalgia, irritable bowel syndrome, some forms of non-cardiac chest pain, and tension-type headache [19-21].

Inflammatory, neuropathic, and functional pain may have different causes, but they all share the main characteristics of chronic pain, which are spontaneous pain, allodynia and hyperalgesia. Pain in these syndromes may indeed arise spontaneously in the apparent absence of any peripheral stimulus, or it may be evoked by low-intensity, normally innocuous stimuli such as a light touch or vibration, or it may be an exaggerated and prolonged response to a noxious stimulus.

1.1.2. Migraine and cancer pain

Migraine pain is a different kind of pain, and is consequently categorized as a separate branch of pain. It is an episodic neurologic condition that has been related to abnormal cortical activity: this status alters sensory input from dural and cerebrovascular sensory fibers and is consequently associated with an abnormal sensory processing in the brainstem. Migraine encompasses features of both inflammatory and functional pain, as well as objective neurologic dysfunction [22,23]. Moreover, migraine attacks are triggered by a variety of provoking irritants agents [24].

According to the last update of the Global Burden of Disease by the World Health Organization, migraine alone seems to be responsible for almost 3% of disability attributable to a specific disease worldwide [25]. Migraine is a pain disorder that affects almost 15% of the adult population worldwide, and its burden in terms of suffering, disability, healthcare, and social and economic costs is unimaginable.

Pain reported by migraine patients is characterized by throbbing and frequently unilateral severe headache attacks, usually associated with nausea, vomiting, and/or sensitivity to light (photophobia), sound (phonophobia) or odors (osmophobia). These attacks typically last for 4-72 h if untreated. Furthermore, in about 30% of patients, migraine attacks can be preceded or accompanied by transient focal neurologic symptoms, commonly known as 'aura'. This phenomenon can be characterized by visual symptoms, but could also consist in paresthesias or language disturbances.

Cancer pain is another type of pain, which is unique and can be caused by a series of different situations. The character and source of pain caused by cancer, indeed, may vary greatly, depending on the type of tumor, its location and its proximity to other tissues. In some cases, tumor cells can produce chemical signals that contribute directly to pain, as in osteosarcomas. In other tumors, though, the pain may be due to mechanical compression or invasion of a nerve, to distention of an organ, to ischemia, or to an inflammatory reaction to tissue necrosis (inflammatory pain). Last but not least, cancer pain may also represent a neurotoxic side-effect of chemotherapy in some patients [26].

1.2. Anatomy of pain: primary afferent nociceptors

The pain pathways form a complex and dynamic sensory, cognitive and behavioural system that evolved to detect, integrate and coordinate protective response to incoming potentially threatening noxious stimuli [27]. This defense system encompasses both the primitive spinal reflexes (the only protection for simple organisms) all the way up to the complex emotional responses which are consciously and subconsciously experienced by humans as pain [28]. The sensory experience of pain begins in the periphery: the peripheral terminals of primary afferent fibers detect many stimuli and translate this information into the dorsal horn of the spinal cord, where the central ends of these fibers terminate.

A nociceptor is a primary sensory neuron that is activated by potentially harmful stimuli, capable of causing tissue damage, and its existence was first proposed by Sherrington nearly a century ago [29]. Indeed, electrophysiological studies have shown the existence of primary sensory neurons that can be excited by noxious heat, intense pressure or irritant chemicals, but not by innocuous stimuli such as warming or light touch [14]. The location, intensity, and temporal pattern of noxious stimuli are transduced

into a recognizable signal through unmyelinated nociceptors at the terminal end of sensory neurons. Normally, the membrane permeability and potential fluctuate through physical deformation or molecular binding. But if depolarization reaches a critical threshold, an action potential is propagated along the sensory nerve toward the spinal cord [28].

Nociceptive receptors located at the peripheral ends of primary afferent fibers have a unique morphology, called pseudo-unipolar: both central and peripheral terminals emanate from a common axon stalk, with cell bodies located in DRG, TG, or nodose ganglia. The peripheral axons of these neurons innervate tissues, such as skin, whose terminals react to sensory stimuli; the central axons enter the spinal cord, where they form synapses with second order neurons to transfer information to the central nervous system (CNS) (Figure I-1). Many neurons innervating the viscera are located in the nodose ganglia, and their peripheral fibers are associated with the vagus nerve, while their central axons project to the area postrema.

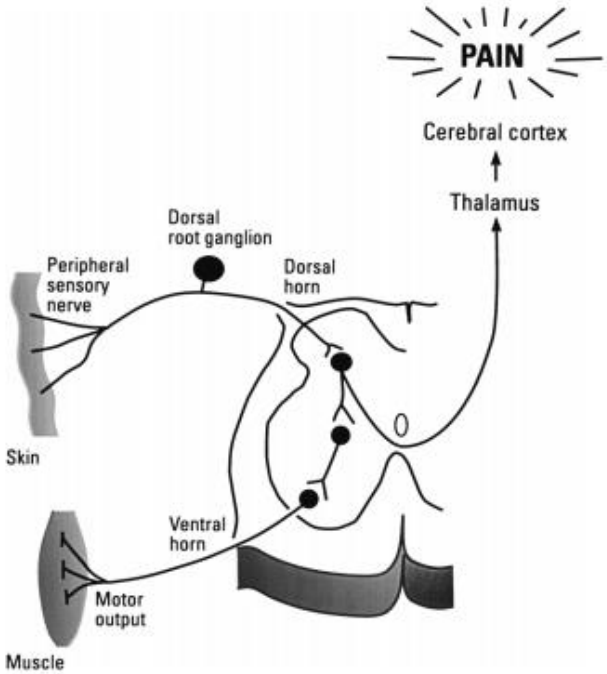

Figure I-1. A simple schematic representation of a spinal nociceptive reflex pathway. Noxious stimuli applied to the region innervated by the fibre causes activation and the release of neuropeptides from the peripheral terminals (resulting in the generation of an inflammatory response) and also from the central terminals within the dorsal horn of the spinal cord. Here the sensory neurons make connections with (i) interneurons and motorneurons, whose pathway carries information to mediate the reflex response, and (ii) neurons whose output ascends via synaptic relays to the thalamus and then to the cerebral cortex, where in humans pain perception is experienced. [30]

Sensory fibers originated from the ganglia represent a heterogeneous population, and can be divided into three main groups based on anatomical and functional criteria. Cell bodies with the largest diameter give birth to myelinated, rapidly conducting Aβ primary sensory fibers. Most, but not all, of these fibers detect innocuous stimuli applied to skin, muscle and joints, and thus do not contribute to pain. Small- and medium-diameter neurons instead, give rise to most of the nociceptors' fibers, including thinly myelinated, more rapidly conducting Aδ fibers, and unmyelinated, slowly conducting C fibers (Figure I-2a). It has been assumed that Aδ and C fibers mediate "first" and "second" pain, respectively, where "first pain" refers to the rapid, acute, sharp pain, and "second pain" to the delayed, more diffuse, dull pain evoked by noxious stimuli (Figure I-2b).

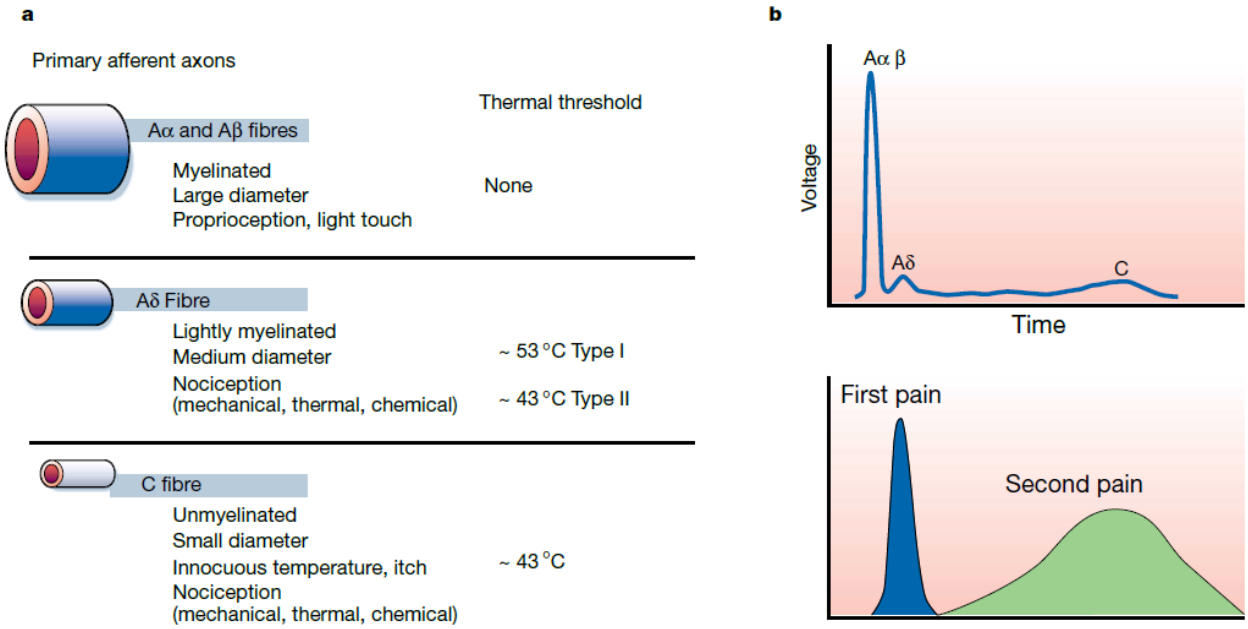

Figure I-2. Different nociceptors detect different types of pain. a) Peripheral nerves include small-diameter (Aδ) and medium- to large-diameter (Aα,β) myelinated afferent fibers, as well as small-diameter unmyelinated afferent fibers (C). **b)** The fact that conduction velocity is directly related to fibre diameter is highlighted in the compound action potential recording from a peripheral nerve. Most nociceptors are either Aδ or C fibres, and their different conduction velocities (6–25 and ~1.0 $m \cdot s^{-1}$, respectively) account for the first (fast) and second (slow) pain responses to injury. [14]

There are two main classes of Aδ nociceptors, that can be distinguished by their differential responsiveness to intense heat, even if both of them respond to intense mechanical stimuli. Moreover, Aδ and C nociceptive fibers can either respond to only one type of physical stimulus ("unimodal receptors"), or can more commonly integrate different stimuli and generate a response to potentially harmful thermal, mechanical

and/or chemical stimuli ("polymodal nociceptors") [14], monitoring the overall tissue condition in this way.

All primary sensory nociceptors make synaptic connections with interneurons in the grey matter of the spinal cord (dorsal horn). Subsets of dorsal horn neurons, in turn, project axons transmitting pain messages to higher brain centers, including the reticular formation, thalamus, and ultimately the cerebral cortex. The dorsal horn of the spinal cord is indeed organized into anatomically and electrophysiologically distinct *laminae*. The spinal cord neurons in lamina I and II are generally responsive to noxious stimulation (*via* Aδ and C fibers), those in lamina III and IV are primarily responsive to innocuous stimuli (*via* Aβ fibers), and neurons in lamina V receive convergent non-noxious and noxious inputs directly *via* Aβ and Aδ (monosynaptic) and indirectly *via* C fibers (polysynaptic) [8] (Figure I-3).

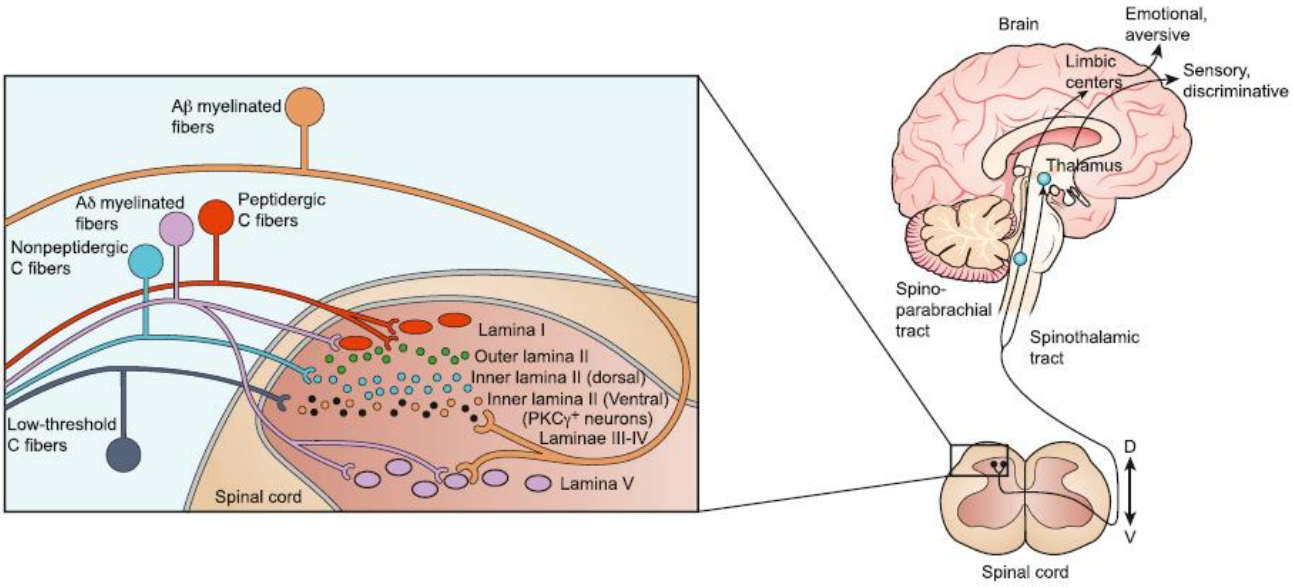

Figure I-3. Spinal Cord Neuroanatomy: Inputs and Projections. The different populations of primary afferent fibers target different regions of the dorsal horn of the spinal cord, with the input from C nociceptors concentrated in the superficial dorsal horn (laminae I and II). The small myelinated Aδ nociceptors target both laminae I and V. The low-threshold C mechanoreceptors, in contrast, target neurons in the ventral part of inner lamina II, which contains many PKCg-expressing populations of interneurons. The terminals of large-diameter (Aβ) afferents are concentrated in laminae III–V. The right side of the figure illustrates the major ascending pathways that derive from the spinal cord dorsal horn, with pathways that derive from projection neurons in laminae I and V predominating. The spinothalamic tract carries sensory-discriminative information to the contralateral thalamus. By contrast, information transmitted via the spinoparabrachial pathway engages limbic system circuits, including amygdala and insular cortex, and contributes to the affective/emotional component of the pain experience. [31]

In animal models, c-Fos protein expression represents a useful marker for monitoring neural activities in central pathways of the sensory system, especially in the pain pathway [32]. It has been shown that spinal neurons expressing c-Fos after noxious stimulation are located in laminae I, II and V of the dorsal horn.

Most C fibers nociceptors are defined as capsaicin-sensitive neurons for they have the ability to respond to noxious chemical stimuli such as capsaicin, which is the pungent ingredient in hot chili peppers. Moreover, histochemical studies on adult DRG neurons revealed two broad classes of unmyelinated C fibers. The first population, so-called peptidergic population, contains the neurotransmitter peptides SP and CGRP, and expresses TrkA, the high-affinity tyrosine kinase receptor for NGF. Neurons in this population most heavily project to laminae I and II. The second population instead, can be labelled with the α-D-Galactosyl-binding lectin, IB4, and expresses P2X3 receptors, a specific subtype of ATP-gated ion channels. This second population project most heavily to the inner lamina II. Both IB4-binding and CGRP-expressing neurons respond to capsaicin in the adult [33].

The most common neurotransmitter synthesized by DRG cells is glutamate [34]. However, the ability of sensing and transmitting noxious stimuli and information is intrinsically associated with the release of neuropeptides from the peripheral terminals. Usually, the ionic gating results in an excitatory effect with the subsequent depolarization of the nerve fibers and the initiation of an action potential propagation that ultimately leads to the transfer of the nociceptive information up to the central nervous system. But calcium (Ca^{2+}) influx into the peptidergic nerve endings causes the local release of proinflammatory neuropeptides, such as CGRP, tachykinins, SP and neurokinin A (NKA). Activation of CGRP and tachykinin receptors (NK1, NK2, NK3) on effector cells, in turn, particularly at the vascular levels, promotes a series of inflammatory responses which are collectively referred to as “neurogenic inflammation” [35]. Sir Thomas Lewis was a pioneer in this field, and he precisely defined the dual “nocifensor” role of these neurons: the ability of a group of the widely branching sensory fibers to respond to the injury and to generate, at the same time, action potentials, which are carried antidromically to other branches of the fibers, where they promote the release of chemical substances that cause the flare and increase the sensitivity of other sensory neurons, responsible for the pain sensation [36]. This phenomenon, firstly described at the somatic level (skin), has now been showed occurring in a variety of visceral organs. In addition, it seems that sensory

neuropeptides release may occur not only from collateral fibers hit by antidromic action potentials, through a tetrodotoxin-sensitive axon reflex, but also by the stimulated terminal itself via a tetrodotoxin-independent mechanism, as in the case of capsaicin stimulation [37].

1.2.1. Neurogenic inflammation

As above mentioned, the expression "neurogenic inflammation" is used to refer to a series of responses that occur peripherally, mainly at the vascular level, after the activation of capsaicin-sensitive nociceptors. In the vascular tissues, the release of CGRP directly induces vasodilation, while the release of SP and NKA induces activation of the SP/NKA NK1 receptor and, consequently, plasma protein extravasation and leukocyte adhesion to the vascular endothelium of postcapillary venules [35] (Figure I-4). In non-vascular tissues CGRP mediates cardiac positive chronotropic effects and relaxation of the bladder dome, while SP/NKA can induce contraction of the smooth muscle of the iris sphincter (mediated by NK2 receptor), of the ureter, bladder neck and urethra (NK2/NK1 receptor), and also exocrine gland secretion (NK1 receptor).

Species-related variations in neurogenic inflammatory responses have been shown, for example, in the airways. SP/NKA release from capsaicin-sensitive nerve terminals causes direct bronchoconstriction in guinea pigs, and indirect nitric oxide/prostanoid-mediated bronchodilatation in rats and mice. In human isolated bronchi, activation of NK2 and, in part, NK1 receptors induces a robust bronchoconstriction just as in guinea pigs [38]. In the human bronchus, tachykinins also have the ability to stimulate seromucous secretion from bronchial glands (NK1) [39], and to excite postganglionic cholinergic terminals (NK3) [40]. However, what is sure is that neurogenic inflammation markedly contributes to inflammatory responses both at the somatic and visceral levels, in different mammal species. In the human skin capsaicin or histamine are able to cause a flare response which is clearly mediated by the stimulation of capsaicin-sensitive terminals and consequent release of neuropeptides, since it is blocked by local anesthetics or by repeated applications of topical capsaicin (inducing capsaicin-mediated desensitization of the terminals) [41]. There is evidence that CGRP is released by capsaicin from human tissues *in vitro* [42] and during migraine attacks [43]. Moreover, a major role of CGRP release from trigeminal perivascular nerve derived from the observation by Olesen and colleagues [44] that BIBN4096BS, a peptide with high affinity

for the CGRP receptor and that is unable to cross the blood brain barrier [45], reduces pain and other symptoms associated with migraine attacks.

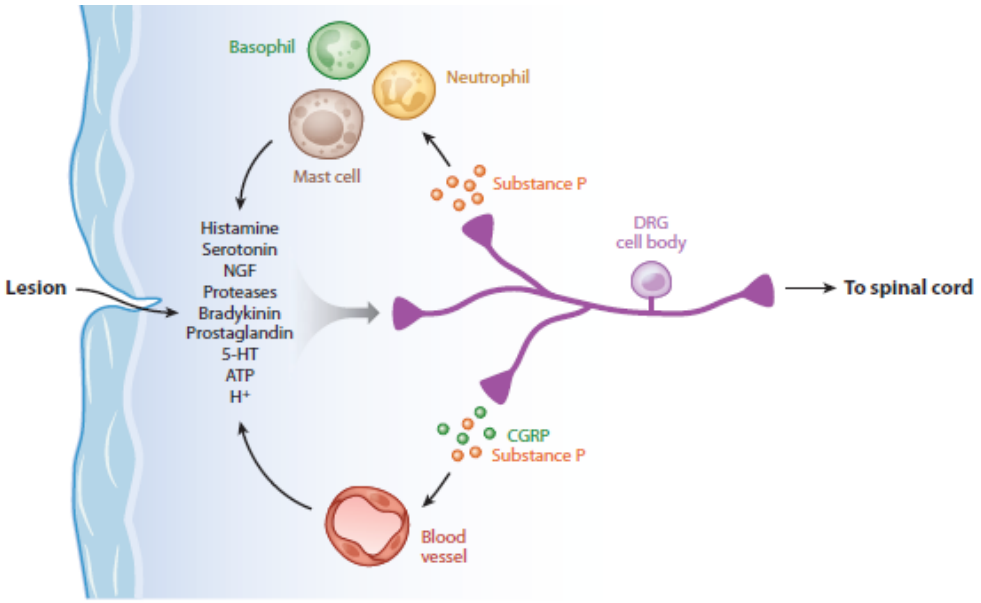

Figure I-4. Sensitization of nociceptors by tissue injury and inflammation. Primary afferent nociceptors (in DRG) are activated by noxious thermal, mechanical, or chemical stimuli and transmit noxious signals to secondary neurons in the spinal cord dorsal horn, and thence to the brain, eliciting a percept of acute discomfort or pain. In addition, nociceptors can signal antidromically and release transmitters also from stimulated peripheral terminals. The release of substance P and CGRP elicits vasodilation, vascular leakage, and other responses from nearby peripheral cell types. These actions, in turn, produce or release a panoply of local signaling molecules (neurogenic inflammation). These include neurotrophins, prostanoids and other bioactive lipids, extracellular protons and nucleotides, and monamines, each of which interacts with receptors on the nociceptor terminal to enhance its sensitivity to physical or chemical stimuli. This phenomenon represents a key peripheral mechanism whereby tissue injury promotes pain hypersensitivity. [46]

1.3. Transient Receptor Potential (TRP) channels

The most important ion channel family that detects and transmits noxious stimuli is the Transient Receptor Potential (TRP) channels family. This family of receptors consists of proteins that are conserved nonselective calcium-permeable channels [46]. TRP channels serve as molecular sensors of multiple stimuli, ranging from chemical agents to temperature, osmolarity and changes in pH [47].

The first TRP channel-encoding gene was discovered in 1977 in the fruit-fly *Drosophyla melanogaster*, where mutants for that gene exhibited impaired vision due to a lack of a specific Ca^{2+} influx-dependent pathway into photoreceptors [48]: in this animal, phototransduction involves the activation of membrane cation channels mediated by

light-sensitive G protein-coupled receptor rhodopsin and phospholipase Cβ. This leads to a depolarizing current (called light-induced current, LIC). A *Drosophyla* mutant was identified, displaying a transient LIC in response to light, in contrast to the sustained LIC which usually occurs in wild type flies: the mutant strain was named *trp*, for "transient receptor potential". Mutations in this gene led to a disruption of a Ca^{2+} entry channel in the photoreceptors, indicating that the TRP channel, the protein encoded by the *trp* gene, might contribute to the Ca^{2+} influx [49].

From that moment on, more than 50 members of the TRP family have been characterized in many tissues and cell types, in both vertebrates and invertebrates, making them one of the largest known groups of ion channels [50]. What all TRP proteins have in common, is that they are characterized by promiscuous activation mechanisms [51], and they all play crucial roles in sensory physiology, including hearing, vision, olfaction, taste, touch, and thermo- and osmo-sensation. In yeasts, TRP channels are used to perceive and respond to hypertonicity [52]; in nematodes (*Caenorhabditis elegans*) to detect and avoid noxious chemicals [53]; in male mice to discriminate between males and females with a specific pheromone-sensing TRP channel [54]. Humans use TRP channels mostly to sense taste (sweet, bitter, umami) and temperature (warmth, heat or cold) [55]. Genetic studies have highlighted the importance of TRP channels in numerous biological processes, and TRP channelopathies are associated with a wide range of human disorders (*e.g.*, polycystic kidney disease, skeletal dysplasia, and familial episodic pain syndrome) [51]. Many TRP channels are also activated by various natural plant products, also acting on pain, so that these herbal compounds have often provided valid pharmacological tools for the study of these signaling receptors [46] (Figure I-5).

TRP channels have been categorized essentially according to their primary amino acid sequences, rather than selectivity, or ligand affinity. Twenty-eight different TRP channels have been described in mammals, and classified in six subfamilies [56] (Figure I-6). The TRPC (Canonical) subfamily consists of seven members (*i.e.* TRPC1-TRPC7), while the TRPM (Melastatin) subfamily consists of eight different channels (TRPM1-TRPM8). The TRPV (Vanilloid) subfamily includes six members (TRPV1-TRPV6). The TRPP (Polycystin) and TRPML (Mucolipin) subfamilies both contain three mammalian members, but they are still not sufficiently characterized, though gaining interest towards them is increasing due to their involvement in several human diseases. The TRPA

(Ankyrin) subfamily is the most recently identified, and comprises only one known mammalian member.

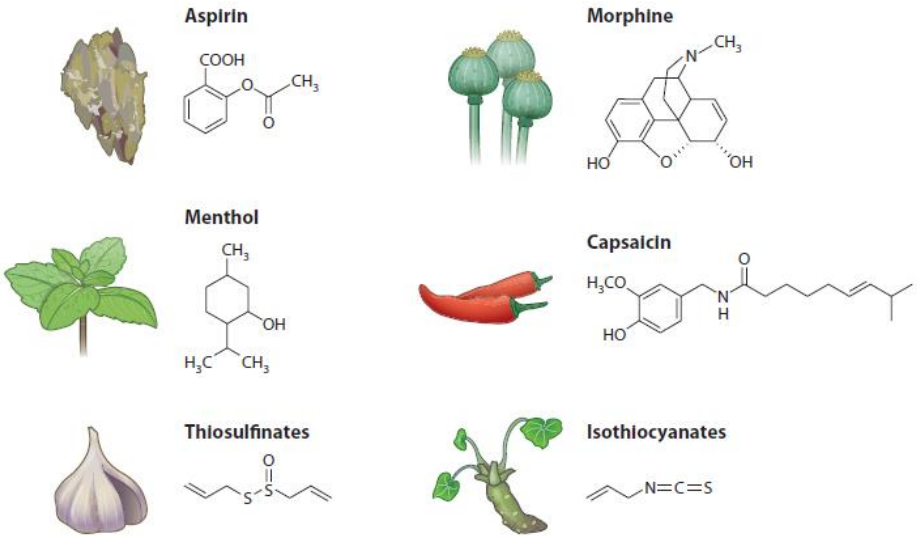

Figure I-5. Natural products as TRP or pain-regulating agents. Herbal products with effects on pain or TRP channels include salicylic acid (related to aspirin) from willow bark; morphine from opium poppies; menthol from mint leaves; capsaicin from chili peppers; thiosulfinates from garlic, onions, and other allium plants; and isothiocyanates from wasabi and other mustard plants. [46]

One of the characteristics all TRP channels have in common is the structure. These receptors are composed of six transmembrane domains (called S1-S6), and the cation-permeable pore region is formed by a short hydrophobic stretch between S5 and S6 (Figure I-7a). Both the N- and C- terminal regions are located intracellularly. Though TRPs share some topographic similarities with voltage-gated channels, these two different classes of channels are only distant relatives. While voltage-gated channels' opening results from the movement of a charged S4 segment upon a change in transmembrane voltage (like in K_v/Na_v/Ca_v channels), S4 in TRP channels lacks the complete set of positively charged aminoacidic residues which are necessary for the voltage sensor [57]. In fact, TRP channel gating is in general not directly regulated by voltage sensing but is affected by energy differences associated with changes in temperature, binding of various molecules, and voltage.

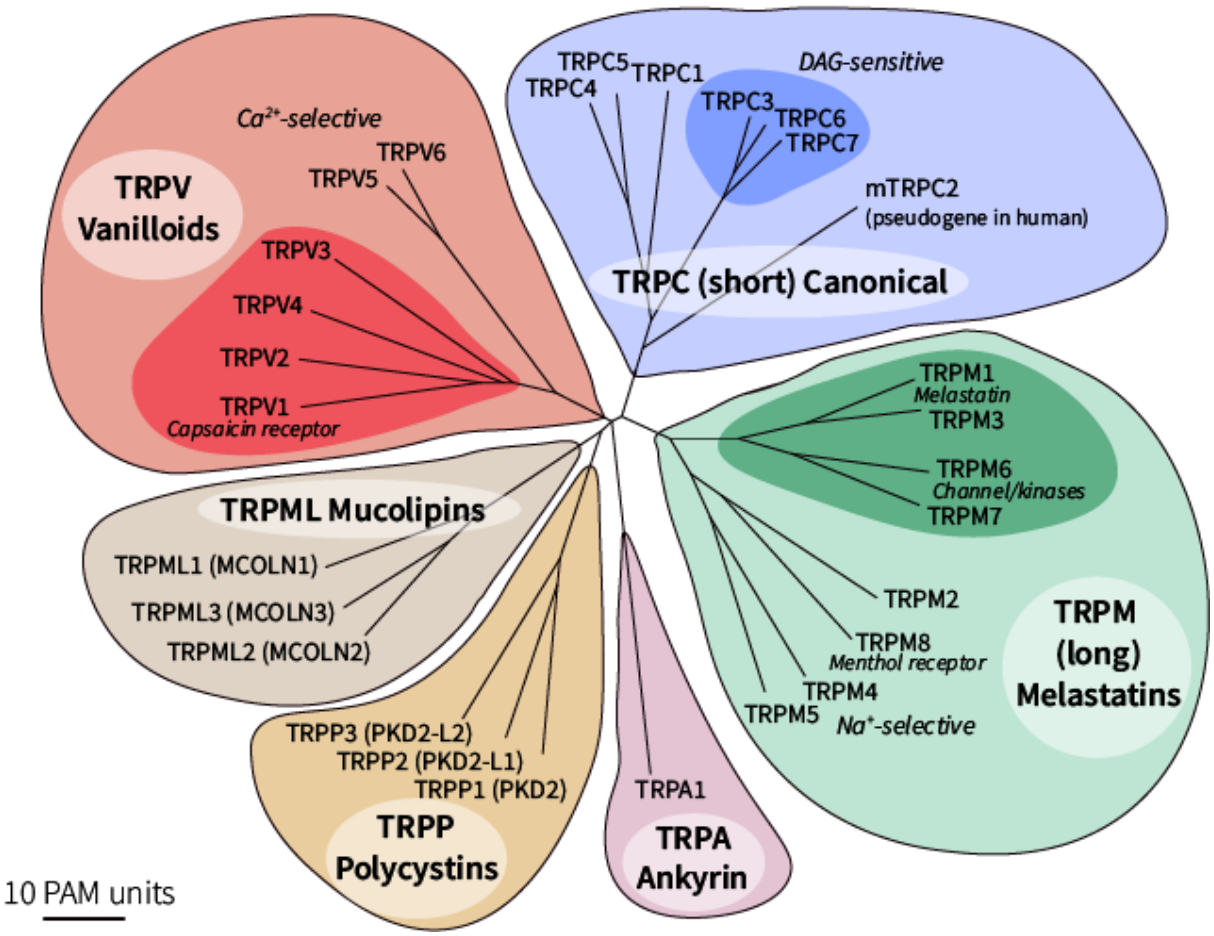

Figure I-6. Mammalian TRP family tree. The evolutionary distance is shown by the total branch lengths in point accepted mutations (PAM) units, which is the mean number of substitutions per 100 residues. Re-adapted from [56].

All functionally characterized TRP channels mediate the transmembrane flux of cations through their electrochemical gradients, thereby raising intracellular concentrations of Ca^{2+} and Na^{+}, and depolarizing the cell. Most Ca^{2+}-permeable TRPs have only a poor selectivity for Ca^{2+}, with a permeability ratio relative to Na^{+} (P_{Ca}/P_{Na}) ranging between 0.3 and 10. The only exceptions are TRPV5 and TRPV6, which are highly Ca^{2+}-selective channels, with a P_{Ca}/P_{Na} >100. Functional TRP channels consists of either homo- or hetero-multimers of four TRP subunits (Figure I-7a). Usually the C-terminus is a highly conserved region, whereas the N-terminus of most of them slightly differs from one another, and can contain a variable number of ankyrin repeats. These are 33-residue motifs, whose variable residues are responsible for mediating specific protein-protein interactions [58]. It is possible that ankyrin domains have a role in the assembling of macromolecular complexes between the plasma membrane and the cytoskeleton, and in transmitting mechanical forces to the gate of the channels.

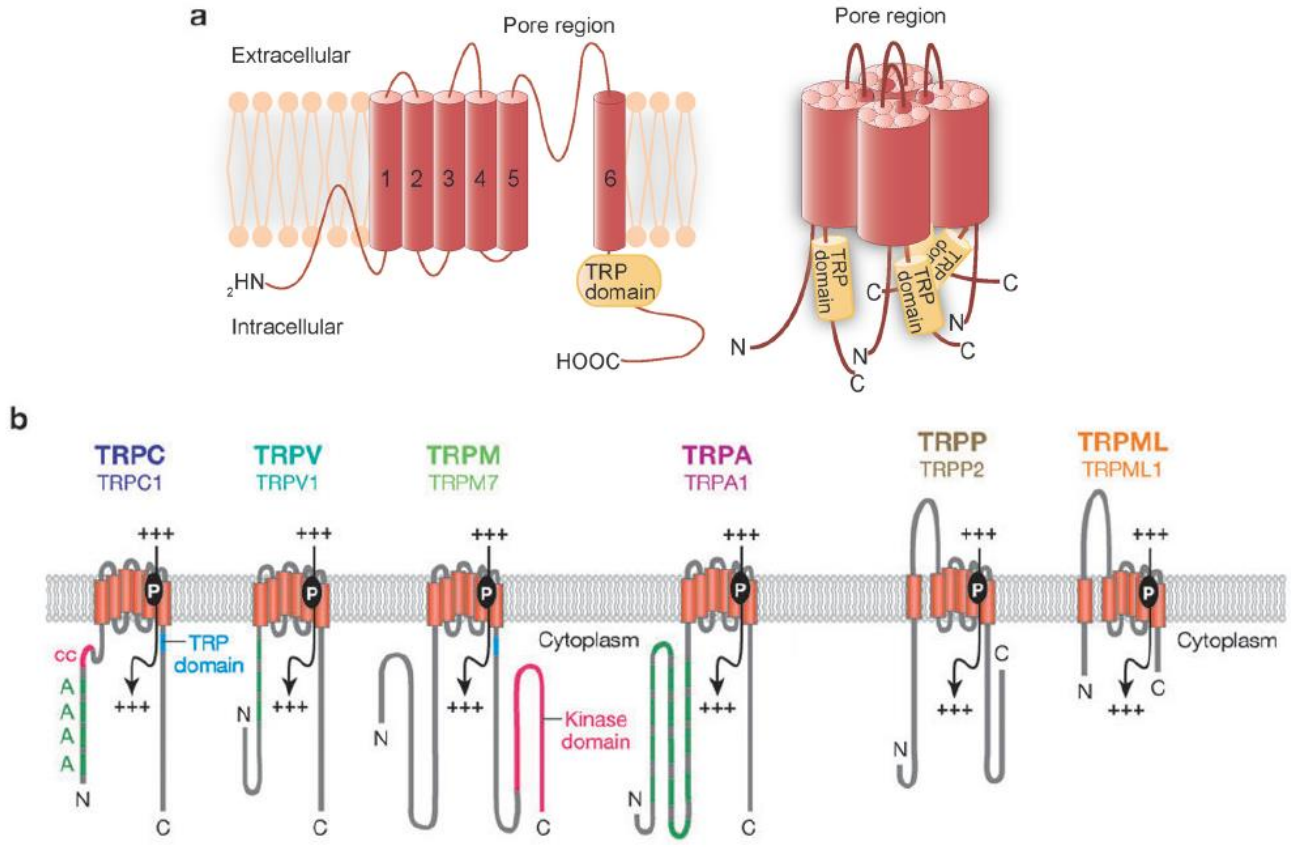

Figure I-7. Transmembrane topology and chemical structures of mammalian TRP channels. a) The TRP protein has six putative transmembrane domains, a pore region between the fifth and sixth transmembrane domains, and a TRP domain in the C-terminal region. The TRP protein assembles into homo-tetramers or hetero-tetramers to form channels in quaternary structure**. b)** The TRP superfamily. Single members from each of the six mammalian subfamilies. The following domains are indicated: A, ankyrin repeats; cc, coiled-coil domain; protein kinase domain; TRP domain. Also shown are transmembrane segments (vertical rectangles) and pore loop (P), allowing the passage of cations (+++). [59,60]

Activation of TRP channels changes the membrane potential, translocates important signaling ions cross the cell membrane, alters enzymatic activity, initiates endocytosis/exocytosis. In doing so, TRP channels are known to play crucial roles in many fundamental processes in life, such as fertilization, sensory transduction, cell survival, and development [61]. TRP channels are widely expressed in mammalian tissues, in both excitable and non-excitable cells, and they are generally described as calcium-permeable channels with polymodal activation properties: indeed, their localization in the plasma membrane of neurons or other cells indicates that they act as sensors of chemical, mechanical (osmotic) and thermal stimuli, and a large body of evidence about this has been collected now, using a plethora of stimuli. This sensitivity to polymodal activation suggests that the specific cellular context (*i.e.*, phosphorylation status, lipid environment, interacting proteins, concentration of relevant ligands) is crucial to the physiologically

relevant stimulus that can activate any given TRP. Ligands that are able to activate TRP channels might be classified as: a) exogenous small organic molecules, including synthetic and natural compounds; b) endogenous lipids or products of lipid metabolism; c) purine nucleotides and their metabolites; d) inorganic ions, with Ca^{2+} itself and Mg^{2+} being the most likely to have physiological relevance [62].

1.3.1. TRPC subfamily

TRP channels classified in TRPC subfamily are the most closely related to *Drosophyla* TRP, and for this reason they are defined as "Canonical" or "Classical" TRP. In this subfamily of TRP channels, there are seven mammalian members (TRPC1-7), among which the permeability ratio P_{Ca}/P_{Na} varies significantly. The activation usually comes after stimulation of G Protein-coupled receptors (GPCR) or tyrosine kinase receptors, that can activate different isoforms of phospholipase C (PLC) [63]. Nevertheless, a relatively recent study reported that TRPC1 can be directly activated by membrane stretch, independently of PLC activity [64].

1.3.2. TRPM subfamily

Some of the eight members of the TRPM ("Melastatin") subfamily can be additionally classified into three subgroups on the basis of sequence homologies: TRPM1 and 3, TRPM 4 and 5, TRPM6 and 7. TRPM2 and TRPM8 represent instead two structurally distinct channels. TRPM channels exhibit highly variable permeability to Ca^{2+} and Mg^{2+}, with TRPM4 and TRPM5 being permeable only to Na^{+}, and TRPM6, TRPM7 and specific splice variants of TRPM3 being highly permeable to Ca^{2+} and Mg^{2+}. In contrast to TRPCs, TRPVs, and TRPA, TRPMs do not contain ankyrin repeats within their N-terminal region. Three of these channels are associated with the transduction of noxious stimuli: TRPM2 is expressed in some cells of the immune system [65], is regulated by some reactive oxygen species (ROS), and is involved in inflammatory pain; TRPM3 is expressed in neural (DRG and TG neurons) and non-neural tissues (kidney and pancreas), and has been linked to neurogenic pain response [47]; TRPM8 is mainly expressed in DRG and TG neurons, and is known to be activated by low temperatures (<25 °C) and by menthol, so it is associated with cold hypersensitivity and neuropathic pain [66].

1.3.3. TRPML subfamily

The TRPML ("Mucolipin") subfamily consists of three different members (TRPML1-3), that are relatively small proteins consisting of ~600 aminoacidic residues. Moreover, coassembly among different TRPML subunits has been reported [67]. TRPML1 appears to be widely expressed, residing in late endosomes/lysosomes. Here, this channel may act as an H^+-sensor, probably to prevent over-acidification [68]. 666666666666666666666

1.3.4. TRPP subfamily

TRPP ("Polycystin") subfamily is quite heterogeneous and can be divided, on structurally criteria, in PKD1-like (TRPP1-like) and PKD2-like (TRPP2-like) proteins. PKD1-like members of this subfamily comprise TRPP1 (previously named PKD1), PKDREJ, PKD1-L1, PKD1-L2, PKD1-L3: all of these receptors are structurally quite different from the classical TRP channels, in that TRPP1 consists of 11 transmembrane domains, and a very long and complex extracellular domain (~3000 aminoacidic residues). PKD2-like members, instead, structurally resemble other TRP channels. There is considerable evidence that TRPP1 and TRPP2 can physically interact through the coiled-coil domains of their C-terminal regions, to form a coupled signaling complex at plasma membrane [69].

1.3.5. TRPV subfamily

The TRPV "Vanilloid" subfamily includes six mammalian members, and TRPV1 is the best characterized so far. Ion channels in this subfamily contain three to five cytosolic ankyrin repeats in their N-termini. TRPVs can be divided into two subgroups.
TRPV1-TRPV4 are all heat-activated channels, as well as chemosensors for a broad range of endogenous and synthetic ligands. They are also non-selective for cations (modestly permeable to Ca^{2+}). In addition, TRPV4 has been recently described as being activated also upon cell swelling [70]. Notably, all of these different chemical and physical stimuli have mostly an additive, or even supra-additive, effect on the gating of TRPV channels, which endows these receptors with the ability to act as signal integrators. This property is of great importance to several pathologies.

The characteristics of the other two TRPV subfamily members, TRPV5 and TRPV6, are slightly different from the aforementioned ones. Their temperature sensitivity is relatively lower than the others', they are actually the only highly Ca^{2+}-selective channels in the TRP family, and they are both tightly regulated by intracellular Ca^{2+} [71]. TRPV5 and TRPV6 play a crucial role as gatekeepers in epithelial Ca^{2+} transport, as well as selective Ca^{2+} influx pathways in non-excitable cells [72].

TRPV1 channel has been commonly referred to as the "capsaicin receptor". It was first described in a subpopulation of small to medium diameter DRG and TG neurons and in nodose ganglia, as a polymodal receptor that can be activated by vanilloid compounds, such as capsaicin, or resiniferatoxin, moderate heat (≥43 °C), and low pH (<5.9) [73,74]. Since then, TRPV1 has been found in many other neuronal but also non-neuronal cells, even though its highest expression levels are in sensory neurons [75]. It can also be activated by camphor [76], allicin [77], nitric oxide [78], and spider toxins [79], potentiated by ethanol [80], and modulated by extracellular cations [81]. Moreover, several studies have shown that inflammatory mediators such as bradykinin, prostaglandin E_2, extracellular ATP, glutamate and NGF can indirectly sensitive TRPV1 to the extent that body temperature can be sufficient to activate nociceptors [73,82]. Various mechanisms could explain how inflammatory mediators can sensitive this receptor: they may increase TRPV1 expression levels in the membrane [83,84], induce TRPV1 phosphorylation by protein kinases [85], or release phosphatidil 4,5-bisphopshate inhibition of TRPV1 [86]. In addition, these inflammatory mediators usually act on receptors that are coupled to G proteins or tyrosine kinase-dependent pathways, thus activating PLC and/or PLA2 which, in turn, can induce the release of arachidonic acid (AA) metabolites: several amide derivatives (like anandamide) and lipoxygenase-induced metabolism products (as 12-(S)-HPETE) of arachidonic acid are actually agonists of TRPV1 [87]. Proteases released during inflammation or nerve injury, such as trypsin and mast cell tryptase, can also sensitize TRPV1 *via* protease activated receptor 2 (PAR2) and PKA/PKCε second messenger pathways [88]. All of these findings demonstrate that TRPV1 not only participates in the acute pain evoked by chemicals and moderate heat, but also strongly contributes to peripheral sensitization. Due to its importance in pain sensation, several compounds have been developed to modulate the activity of TRPV1, in order to eliminate or reduce pain [47].

Another important member of the TRPV subfamily is TRPV4. This polymodal receptor has a wide expression pattern and a corresponding variety of physiological roles

[89]. TRPV4 is indeed widely expressed in non-nervous tissues and cells, including urinary bladder, kidney, vascular endothelium, keratinocytes, cochlear hair cells, and Merkel cells [90-92], but also in TG and DRG sensory neurons, where its activation causes SP and CGRP release, thus evoking neurogenic inflammation [93,94]. TRPV4 was firstly identified as an osmo-transducer activated by a decrease in osmolarity, suggesting its role in cell swelling regulation [89,95]. Later studies showed that TRPV4 is also activated by shear stress [96], innocuous warmth (27-35 °C) [97], low pH and citrate [98], endocannabinoids and AA metabolites [99], and NO [78]. The mechanosensitive nature of this channel, and its implication in shear stress sensing, suggest a role in flow-sensitive cells such as vascular endothelial and renal tubular epithelial cells. However, the mechanism of mechanical stress-mediated activation of TRPV4 is still under debate, and two transduction pathways have been proposed: the PLC/diacylglycerol (DAG) pathway, and the PLA2/AA pathway [100,101]. Evidences suggest that activation of TRPV4 by hypotonicity involves its phosphorylation by Src family of tyrosine kinase [102], and that this channel is not directly activated by mechanotransduction [95]. Hypotonicity becomes painful to the animals when nociceptive fibers are sensitized by PGE_2, whose levels usually increase during inflammation or after a mechanical, thermal or chemical injury. TRPV4 channel also plays a crucial role in mechanical hyperalgesia elicited by inflammatory mediators, since PGE_2 and serotonin can act synergistically to engage this receptor in mechanical and osmotic stimuli-evoked hyperalgesia, through cAMP/PKA and PKCε [103]. In addition, proteases generated during inflammation activate PAR2, which in turn may sensitize TRPV4 through the activation of multiple second messenger pathways, such as PKA, PKC, PKD, PLCβ [93,104].

1.3.6. TRPA subfamily

The TRPA "Ankyrin" subfamily is currently the only TRP subfamily to enlist only one mammalian member, the TRPA1 channel. TRPA1 is mainly expressed in DRG ad TG neurons [105], and is activated by numerous endogenous and exogenous natural compounds. This channel comprises at least 14 ankyrin repeats in its N-terminal region, an unusual structural feature that may be relevant for its role as a mechanosensor. TRPA1 shares structural similarities with TRPN1, the only channel in the TRPN subfamily. TRPN1 is characterized by 29 ankyrin repeats in its N-terminus, and probably acts as a mechanotransduction channel involved in hearing, but is only expressed in non-mammal

organisms (*Caenorhabditis elegans*, *Drosophyla melanogaster*, and zebrafish), though being closely homologous to TRPA1.

1.4. The TRPA1 channel

TRPA1 channel was originally cloned in 1999 from human lung fibroblasts [106], but it was also later found as selectively expressed in a subpopulation of unmyelinated nociceptors, co-expressed with the capsaicin receptor TRPV1, suggesting its role in nociception [51,105,107]. TRPA1 is a non-selective cation channel permeable to both monovalent and divalent cations, including Ca^{2+}, Na^{+}, K^{+}. However, when constitutively open, under physiological conditions, TRPA1 has a unitary conductance ranging from ~70 pS to ~110 pS in the inward and outward directions, making it highly permeable to Ca^{2+}, compared to most of the other TRP channels [108,109]. In presence of TRPA1 activators, the pore of this channel (with a size of 11.0 Å) can undergo dilation, increasing Ca^{2+} permeability and allowing even larger charged molecules to pass through the membrane [110,111].

1.4.1. Structure

TRPA1 structure presents six transmembrane domains, like all other TRP channels, with the pore loop between S5 and S6, and intracellular N- and C-terminal regions. There is no apparent voltage sensor in S4, in contrast to voltage-gated K^{+} channels, but TRPA1 displays some voltage dependency, though being less pronounced than in TRPM8 and TRPV1 [112,113]. The most distinct feature of TRPA1 channels is their long N-terminus, with 14 to 18 ankyrin repeat domains which seems to be important for protein-protein interactions and for the insertion into the plasma membrane [114] (Figure I-8). Moreover, the N-terminal region of TRPA1 contains a large number of cysteine residues which are crucial for this receptor: cysteine residues can in fact form a network of protein disulfide bridges within or between monomers [115]; in addition, cysteine and lysine residues in the N-terminal region represent key targets for electrophilic TRPA1 activators, even though cysteine outside this region may also contribute to channel gating [115] (for example, the potent TRPA1 agonist Zn^{2+} may bind to cysteine and histidine residues in the C-terminus [116,117]). Cvetkov and collaborators provided in 2011 direct

structural insights on TRPA1 channel through a 16 Å resolution structure model of purified, amphipol-stabilized, TRPA1 proteins analyzed by single-particle electron microscopy [118]. This model suggested that the critical N-terminal cysteine residues involved in electrophilic activation are located at the interface between neighboring subunits, and form a "ligand-binding pocket" which allows disulfide bonding between the different cysteine residues. Covalent modifications by thiol-reactive compounds within such pockets may alter interactions between the subunits, thus promoting conformational changes and modifications of the gating mechanism [115,118]. A more recent study used single-particle electron cryomicroscopy to determine the 4 A° resolution structure of full-length human TRPA1. Several unexpected features were revealed in this manner, including an extensive coiled-coil assembly domain and a highly integrated nexus that converges on an unpredicted transient receptor potential (TRP)-like allosteric domain [119] (Figure I-8).

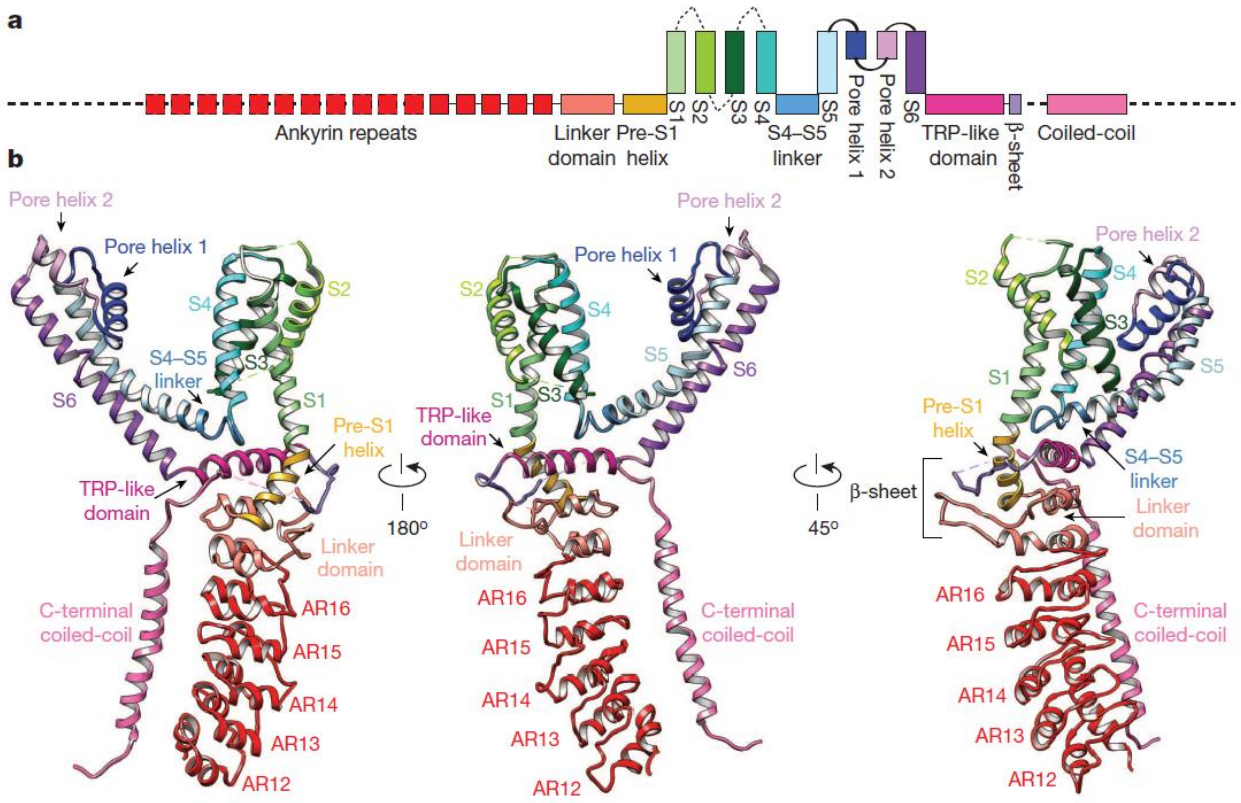

Figure I-8. Structural details of a single TRPA1 subunit. a) Linear diagram depicting major structural domains color-coded to match ribbon diagrams below. Dashed lines and boxes denote regions for which density is insufficient to resolve detailed structure (sequence before AR12, loop containing Cys 665, S1–S2, S2–S3 and S3–S4 linkers, connection between third b-strand and coiled-coil, C terminus subsequent to coiled-coil), or where specific residues cannot be definitively assigned (portion of the linker before and after the coiled-coil). **b)** Ribbon diagrams depicting three views of the TRPA1 subunit. [119]

Some studies have suggested that the N- and C-terminal regions of TRPA1 may contain binding sites for Ca^{2+}, that can both sensitize or desensitize the receptor [107,120,121]. TRPA1 activity is indeed strongly modulated by Ca^{2+}. Micromolar intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) are actually able to activate TRPA1, and also elevations in extracellular Ca^{2+} can transiently increase the channel activity. It has been proposed that this kind of activation may depend on Ca^{2+} binding to a putative N-terminal EF-hand motif, located between ankyrin repeat 11 and 12 [122,123]. However, the importance of this EF-hand motif is questionable, since point mutations in this region have only modest effects on $[Ca^{2+}]_i$ -dependent activation mechanism, while deletion of this region impairs trafficking of the truncated channel to the plasma membrane [109]. Another proposed putative binding domain for Ca^{2+} is a cluster of four conserved acidic residues in the distal C-terminus of TRPA1 (Glu1077, Asp1080, Asp1081, Asp1082) [120]: these may have strong effects on the Ca^{2+}- and voltage-dependent potentiation/inactivation of agonist-induced responses. In fact, deletion of 20 aminoacidic residues from the C-terminus (including the four mentioned) selectively slowed down the Ca^{2+}dependent inactivation without affecting other functional parameters of the channel.

1.4.2. Localization

Soon after the identification of TRPA1 receptor in human pulmonary fibroblasts [106], it was also found in hairy cells of the auditory system [124]. More importantly, abundant expression of this receptor was localized in a subpopulation of peptidergic sensory neurons in DRG and TG (with C and Aδ fibers) [105,107]. These neurons contain and release, upon activation, the neuropeptides SP, NKA, and CGRP, thus signaling nociceptive responses. TRPA1-expressing subpopulation of neurons is mostly also TRPV1-positive, even if a small portion of neurons only expressing TRPA1 exists, including some myelinated Aβ fibers activated by innocuous mechanical force [125]. A study in 2008 identified different mechano-sensitive or insensitive sensory neuronal categories by using radial stretch in live-cell calcium imaging [126]. The group of stretch-sensitive cells could be further divided into two clusters: the first is made by small-diameter cells, sensitive to both hydroxy-α-sanshool (a two pore K^+ channel antagonist) and capsaicin (the TRPV1 agonist), and likely corresponds to high threshold nociceptors; the second is composed of large-diameter cells only responsive to hydroxy-α-sanshool, and probably represents low threshold proprioceptors. Stretch-insensitive neurons could fall into two

subgroups as well, only made by small-diameter cells: the first is made by peptidergic neurons sensitive to both capsaicin and mustard oil, which is a TRPA1 agonist; the second is composed by a small cohort of menthol-sensitive cells. Thus, TRPA1 and TRPV1 co-expressing neurons are apparently insensitive to mechano-stimulation. This could be of crucial importance for their putative role in neurogenic inflammation, given that they contain and release inflammatory neuropeptides.

More extraneuronal localization and functions of TRPA1 have also been identified. For example, TRPA1 activation inhibited the repair of epithelial wounds in the stomach, probably by the suppression of cell migration, suggesting the involvement of TRPA1 in gastric epithelial restitution [127]. TRPA1 channel is also highly expressed in the bladder epithelium, so it might be involved in the bladder sensory transduction and in the induction of overactive bladder by bladder outlet obstruction [128]. The localization of TRPA1 in nerves that also express TRPV1 and CGRP, and in urothelial and interstitial cells, as well as the findings that TRPA1 agonists can modify tone of human urethral preparations, propose a role for TRPA1 in afferent and efferent sensory signaling functions of the prostate [129]. TRPA1 is also able to regulate intestinal motility, since is highly expressed in rat enterochromaffin cells, and its agonists can stimulate these cells in releasing serotonin (5-HT) [130]. TRPA1 is found in melanocytes [131], mast cells [132]; Yu, 2009 #136}, odontoblasts [133]. TRPA1 has been proposed to contribute to different airway inflammatory diseases, including chronic cough, asthma and chronic obstructive pulmonary disease (COPD), since it was detected in non-neuronal airway cells such as human small cell lung cancer (SCLC) cells, fibroblasts, epithelial cells, and smooth muscle cells. In these cells, TRPA1 induces the release of proinflammatory cytokines like interleukin-8 [134], prevents apoptosis promoting cell survival *via* an extracellular signal-regulated kinase (ERK1/2)-dependent pathway, and favors detrimental processes in epithelial airway cells taken from cystic fibrosis patients. Moreover, TRPA1 inhibition is able to attenuate neurogenic and non-neurogenic inflammatory responses to cigarette smoke or allergens [135]. Finally, there is evidence that TRPA1 channel is expressed in endothelial cells of rat cerebral vessels, where it regulates vascular tone by nitric oxide (NO)- and cyclooxygenase-independent pathways: the relaxing mechanism activated by TRPA1 agonists appears in fact to be mediated by endothelial cells' Ca^{2+}-activated K^+ channels and inwardly rectifying K^+ channels in arterial myocytes [136].

1.4.3. Function

Many different categories of stimuli have been reported to activate the TRPA1 channels either directly or indirectly. These include cold, mechanical displacement, and chemical irritants like exogenous pungent compounds and bradykinin and other endogenous proalgesic agents.

Thermosensation. Several receptors belonging to the TRP channel family exhibit highly temperature-selective gating. Among the TRPs expressed in sensory neurons, TRPV1 is activated by noxious heat, while TRPM8 by cold temperatures and cooling compounds, such as menthol [137]. TRPA1 was also originally reported to mediate detection of noxious cold, since it is expressed in nociceptive neurons, and since heterologously expressed TRPA1 in chinese hamster ovary (CHO) cells is activated by cold temperatures with a lower activation threshold than TRPM8 [105]. However, for long time it has not been quite clear whether or not TRPA1 could act as a noxious cold sensor *via* either direct or indirect mechanism. Many studies had shown TRPA1 activation by noxious range cold temperatures (~17 °C and below) in heterologous systems like human embryonic kidney (HEK) cells or CHO cells [105,138,139]; other studies had shown instead that exogenously expressed TRPA1 is not activated by noxious cold [107,124,140]. A study from Zuborg et al. reports cold-induced activation of TRPA1 in overexpression systems, due to an indirect effect mediated by intracellular Ca^{2+} *via* an EF-hand domain in the N-terminal region, after direct activation of the receptor and Ca^{2+} release from intracellular stores [123]. All of these contradictory findings appear to have been resolved by a more recent work, which showed that TRPA1 null mice were still able to sense cold, but that their behavioural response to noxious cold was significantly reduced in the absence of TRPA1 [141]. Furthermore, mice in which sensory neurons expressing $Na_v1.8$ were eliminated by diphtheria toxin A, exhibit a strong reduction in TRPA1 expression in DRG neurons, and a lack of TRPA1-mediated nociceptive responses to formalin and cold [142]. Thus, noxious cold sensing *in vivo* requires somatosensory neurons co-expressing both $Na_v1.8$ and TRPA1.

Mechanotransduction. Another property for which TRPA1 channel has been proposed is mechanotransduction. Mice with a deletion of the pore domain of TRPA1 exhibit decreased behavioural responses to intense (noxious) mechanical force [143], though in a similar mutant mouse behavioural deficits to mechanical stimuli were not observed [144]. In another study, a modulatory role for TRPA1 in regulating mechanical

firing was described, both in primary sensory neurons and in keratinocytes of the epidermis [145]. In addition, a small molecule inhibitor of TRPA1 reverses mechanical hyperalgesia induced by inflammation in mice [146]. No *in vitro* studies have provided clear evidence that this channel can be directly gated by mechanical force, although a 2008 study shows that heterologously expressed TRPA1 is activated by hypertonic saline solution, suggesting its sensitivity to osmotic stimuli [147]. However, it should be noted that the nature of an osmotic stimulus, and the way it activates channels in a cell membrane, may differ substantially from those of a punctuate mechanical force applied to a localized region of the neuronal membrane.

Chemical irritants. TRPA1 is actually best characterized as a chemosensor, activated by many natural or synthetic chemical agents that are able to cause neurogenic inflammation and pain. It is currently well known that this channel can detect thiol-reactive electrophilic and oxidative compounds, in addition to non-electrophilic compounds, as well as being indirectly regulated by GPCR signaling.

Electrophilic ligands of TRPA1 can be of environmental, dietary, or endogenous origin, and allyl isothiocyanate (AITC) contained in mustard oil is one of the most efficient. Other electrophiles include methyl-, isopropyl-, benzyl-, and phenyletyl-isothiocianate (found in wasabi, mustard, and horseradish), cynnamaldehide (contained in cinnamon), allicin (from garlic), acrolein, iodoacetamide, and methanethiosulfonate ethylammonium (MTSEA, used for cysteine scanning) [51,107] (Figure I-9a). These electrophilic agonists modify nucleophilic cysteine and lysine residues in the N-terminal region [148] (Figure I-9b): in human TRPA1 they modify cysteines Cys619, Cys639, and Cys663 (and, to a lesser extent, lysine K708) [149]; in the mouse TRPA1 homologue, instead, the most reactive cysteines are Cys415, Cys422, and Cys622 [150].

A series of endogenous α,β-unsaturated aldehydes, which are produced by lipid peroxidation in response to oxidative stress at the sites of inflammation and tissue injury, can also activate TRPA1 [144,151,152]. These include 4-oxononenal [153], that has been reported to cause nociceptive behaviour mediated by TRPA1, and 4-hydroxy-2-nonenal (4-HNE), which is an α,β-unsaturated hydroxyalkenal produced in inflamed tissues by peroxidation of omega 6-polyunsaturated fatty acids (such as linoleic and arachidonic acid) [154,155]. 4-HNE, acting *via* covalent modification of the cysteine/lysine residues in the TRPA1 N-terminus [152], evokes the release of SP and CGRP from nerve endings, resulting in extravasation of plasma proteins into the surrounding tissue.

More recently, mediators of oxidative and nitrative stress have been identified as TRPA1 activators, including hydrogen peroxide (H_2O_2), superoxide (O^{2-}), hypochlorite (ClO^-), and peroxynitrite ($ONOO^-$) [138,156,157]. Nitrooleic acid, a byproduct of nitrative stress, is also a TRPA1 agonist [158]. It has been reported that ROS cause cysteine oxidation or disulfide formation; reactive nitrative species (RNS), like nitric oxide (NO), mediate S-nitrosylation; reactive carbonyl species (RCS), like electrophilic prostaglandins (PG) and α,β-unsaturated aldehydes, alkylatively modify cysteine residues. Indeed, cyclopentenone PGs have been shown to produce pain and neurogenic inflammation through TRPA1 activation [159,160]. Moreover, the cyclopentenone isoprostane 8-iso-PGA2, which is synthesized from E-isoprostane in a cyclooxygenase-independent manner, stimulates sensory nerve terminals by targeting TRPA1 [159]. Hydrogen sulfide (H_2S), a RNS, is a malodorous gas that functions as an endogenous transmitter in humans, is involved in a wide variety of processes, including nociceptive processes, and is able to evoke CGRP release from sensory neurons *via* TRPA1 [161]. All of these findings suggest the importance of TRPA1 channel as an unspecific sensor of exogenous stimuli and metabolites of oxidative and nitrative stress, which acts to alert of inflammation and tissue injury.

N-acetyl-p-benzoquinoneimine (NAPQI) is the metabolite of N-acetyl-p-aminophenol (paracetamol, acetaminophen), and represents another example of TRPA1 electrophilic agonist: it can stimulate TRPA1 and cause airway neurogenic inflammation, an effect abolished by the use of TRPA1 antagonists [162].

It has been reported that high concentrations of carbon dioxide (CO_2) can induce a stinging sensation that is dependent on the activation of TG nociceptors expressing TRPA1 that innervate the respiratory, nasal, and oral epithelia. CO_2 can produce this effect by diffusing into cells and producing intracellular acidification, thereby gating TRPA1 channel [163]. This means that acid pH can activate TRPA1, but also alkaline pH causes pain through this mechanism, with Cys422 and Cys622 being responsible for high pH perception: indeed, pain behaviours evoked by intraplantar injection of ammonium chloride are reduced in TRPA1 null mice [164].

Although it is now generally accepted that TRPA1 is activated through covalent modification of specific cysteine residues, the precise mechanism and the chemistry of this phenomenon with unsaturated carbonyl-containing compounds is still unclear. What is known, is that channel activation occurs via alkylative conjugate addiction [165].

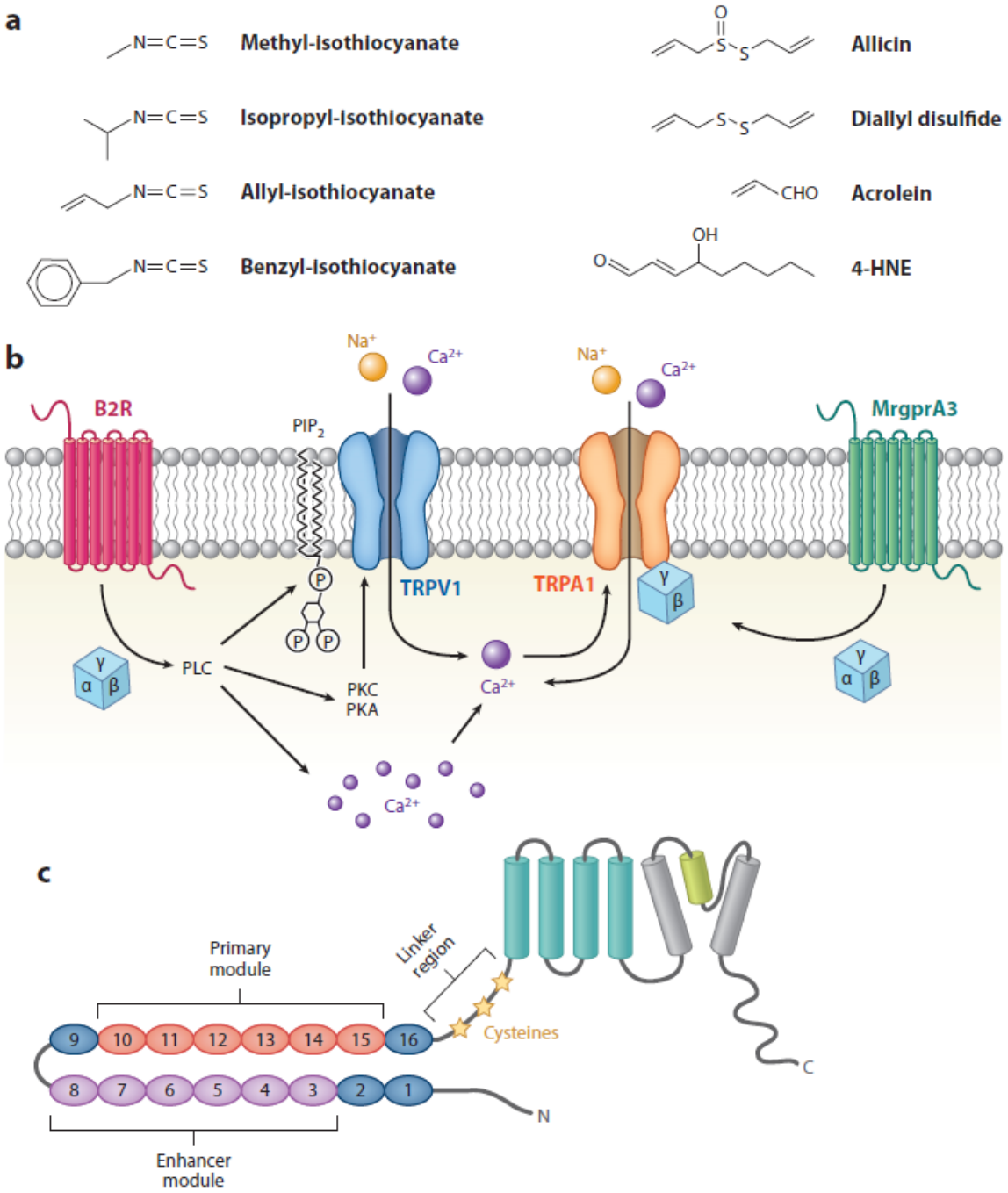

Figure I-9. The wasabi receptor, TRPA1, is a detector of chemical irritants. a) Activators of TRPA1 include exogenous irritants and endogenous products of tissue injury and inflammation. The agents shown here include isothiocyanates and α,β-unsaturated aldehydes, both of which exhibit strong electrophilic reactivity. **b)** In addition to direct activation by electrophilic irritants, TRPA1 functions as a receptor-operated channel that can be activated or sensitized by G protein–coupled signaling pathways. Two such mechanisms have been proposed: (i) a GPCR, such as the BK2 bradykinin receptor, activates PLC to mobilize release of intracellular Ca^{2+}. Increased cytoplasmic Ca^{2+} then activates TRPA1; (ii) activation of a GPCR, such as the MrgprA3 puritogen receptor, promotes the release of free Gβγ, which serves as the downstream cytoplasmic activator of TRPA1. This channel can also be activated or sensitized by other events that enhance cytoplasmic Ca^{2+} levels, such as activation of TRPV1 or other Ca^{2+}-permeable channels. **c)** Electrophilic agonists covalently modify three key cysteine residues within the linker region connecting the ankyrin repeat domain to the transmembrane core of the channel (ankyrin repeats shown as colored ovals). [46]

Different TRPA1 electrophilic agonists have in common the ability to form covalent adducts with thiol moieties, and this is what gives them the characteristic of TRPA1 activators. A variety of known agonists, including acrolein and other α,β-unsaturated aldehydes, possess an electrophilic carbon or sulphur atom that is subject to nucleophilic attack (Micheal addiction) by cysteine, lysine or histidine residues of TRPA1 [166]. Mutagenesis studies have, indeed, confirmed that such reactivity promotes channel gating through covalent modification of residues within the cytoplasmic N-terminal domain [149,151,152].

However, TRPA1 can also be activated by non-electrophilic compounds, that are unlikely to induce covalent modifications of the channel protein. For example, some anesthetic agents can induce activation and sensitization of this receptor: propofol (2,6-diisopropylphenol), a commonly used intravenous anesthetic, elicits intense pain upon injection *via* TRPA1 [167]; lidocaine, which usually inhibits cellular excitability by blocking voltage-gated Na^+ channels, is able to activate the TRPA1 in a concentration-dependent manner. Lidocaine can also act as an inhibitor of the channel, and this effect is more evident in rodent rather than human TRPA1 (probably due to species-specific differences in the pore region between S5 and S6) [168]. Several fenamate non-steroidal anti-inflammatory drugs (NSAIDs) act also as non-electrophilic TRPA1 activators, including flufenamic, niflumic and mefenamic acid, as well as flurbiprofen, ketoprofen, diclofenac and indomethacin [169]. In addition, TPA1 is a non-covalent sensor of polyunsaturated fatty acids (PUFAs), which contain at least 18 carbon atoms and 3 unsaturated bonds. These molecules can non-covalently bind to domains located in the N-terminus, and activate TRPA1 to excite primary sensory neurons and enteroendocrine cells [170].

Many non-electrophilic modulators of TRPA1 work in a bimodal fashion, activating it at low concentrations and inhibiting it at higher concentrations. This is the case of menthol, a known TRPM8 agonist from *Mentha piperita*, which is also a human TRPA1 activator at low (micromolar) concentrations, whereas higher concentrations cause a reversible channel inactivation [171]. Mouse TRPA1 is instead just blocked by menthol. The super-cooling synthetic compound icilin, similarly to menthol, activates not only TRPM8 but also TRPA1 [172,173]. Caffeine, from *Coffea arabica*, activates mouse TRPA1 but suppresses its human homologue [174], and also nicotine from *Nicotinia tabacum* and anabasin from *Nicotinia glauca*, are TRPA1 bimodal modulators (topical application of

nicotine causes irritation of the mucosa and skin *via* TRPA1 activation, but higher concentrations inhibit the channel) [175]. TRPA1 is also activated by Δ9THC, the psychoactive compound contained in marijuana, and by cannabidiol (CBD) and cannabichromene (CBC), two non-psychoactive cannabinoids [176].

High concentrations of zinc, which is an essential biological element required for the structure of over 300 proteins, can have cytotoxic effects and cause inflammation and pain. Surprisingly, TRPA1 is highly sensitive to intracellular zinc (nanomolar concentrations) [117]. Zinc is able to activate TRPA1 through a unique mechanism that requires zinc influx through the channel and subsequent activation *via* specific intracellular cysteine and histidine residues.

Many TRP channels, including TRPA1, are activated or modulated by neurotransmitter or growth factor receptors that stimulate PLC (Figure I-9b). *In vitro* studies raised the possibility that TRPA1, activated in this manner, functions as a "receptor-operated" channel that depolarizes nociceptors in response to many proalgesic or proinflammatory agents that activate PLC [139]. The proinflammatory peptide bradykinin, produced endogenously in response to tissue injury, inflammation, or ischemia, binds to the PLC-coupled bradykinin receptors (BK2) on sensory neurons, and is able to elicit acute pain through immediate excitation of nociceptors, followed by a longer lasting sensitization to thermal and mechanical stimuli [177]. Indeed, mice with a mutation in TRPA1 did not develop hyperalgesia after exposure to bradykinin [144]. Finally, TRPA1 has been shown to be sensitized by nerve growth factor (NGF) and PAR2, both known to play a role in inflammatory pain [178,179].

1.4.4. Pharmacology

Some molecules have been studied as TRPA1 channel antagonists, starting with ruthenium red, gentamicin, gadolinium and amiloride. Ruthenium red and gentamicin are very similar, and both are pore blockers that plug into the channel pore. Amiloride and gadolinium, instead, block the receptor by interacting with an extracellular site of the channel [124]. However, none of these antagonists is TRPA1-selective because they can also block other types of TRP channels, as well as other ion channels. A more selective TRPA1 antagonist was later identified, and called HC-030031: this compound was able to attenuate formalin-induced neuropathic pain, and inflammation- and neuropathy-induced mechanical hypersensitivity [180,181] by selectively inhibiting the TRPA1 channel.

HC-030031 has been a key compound in determining the role of TRPA1 channel in the first and second phase of the nociceptive response induced by formaldehyde, and in general in providing evidences implicating the role of TRPA1 in pain. Further studies showed that HC-030031 reduced somatic and visceral nociception [182], as well as bradykinin-induced mechanical hyperalgesia [146]. This antagonist is currently being used to identify novel roles of TRPA1 in health and diseases, or novel TRPA1 activators, along with TRPA1 deficient mice.

Additional antagonists have been more recently identified. A-967079 is a potent selective inhibitor that was able to attenuate pathological pain without altering noxious cold sensation or body temperature regulation [183]. Chembridge5861528 is an analogue of HC-030031 and has been shown to reduce mechanical hyperalgesia *in vivo* in diabetic mice [184]. AP18 is a small molecule competitive antagonist which showed *in vivo* analgesic activity in well-established animal models of inflammation, such as the complete Freund adjuvant (CFA) and the bradykinin-induced mechanical hyperalgesia [146].

As for clinical applications of TRPA1 antagonists, apart from one positive press release on TRPA1 antagonism in human pain studies, apparently only one paper was published, in which A-967079 failed to inhibit human acidosis-induced pain [185]. However, Eli Lilly recently acquired all assets related to Hydra Biosciences' pre-clinical program of TRPA1 antagonists currently studied for the potential treatment of pain syndromes (indicating strong interest in the field).

1.5. TRPA1 role in pain

The role of TRPA1 in pain and inflammation has been described by a plethora of studies. Reech et al. already showed in 1986 that the topical application of mustard oil was able to induce pain and inflammation [186], and this effect was later proved to be mediated by AITC, the irritant compound contained in mustard oil, and TRPA1 [107]. Intraplantar injection of cinnamaldehyde into the paws of mice causes a clear pain response, which persists in the TRPV1 null mice, indicating that this behaviour is independent of TRPV1 channel activation and is, instead, associated with TRPA1 [139]. TRPA1 is also a specific mediator of the nociceptive response to the volatile compound acrolein, present in tear gas and tobacco smoke and responsible for the neurogenic inflammation that affects airways [144]. The proinflammatory peptide bradykinin itself, as

already explained, is able to indirectly activate TRPA1 channels through BK2 receptor and PLC-dependent hydrolysis of phosphatidil 4,5-bisphopshate, which produces the TRPA1 activators inositol-triphosphate (IP3) and DAG [139]. All of the nociceptive effects of the aforementioned stimuli have been confirmed through the use of TRPA1 knockout mice [143,144], and the fact that this channel can be activated by all of these exogenous and endogenous compounds, including the synthetic inflammatory compound formalin [181], implicate that TRPA1 is unequivocally associated with inflammation processes and/or tissue damage. It has been demonstrated that this channel is involved in mechanical hyperalgesia and that is an important transducer of noxious cold [143].

In addition, TRPA1 has a role in an inherited disorder known as familial episodic pain syndrome (FEPS). This syndrome is characterized by the presence of severe pain in the upper body accompanied by breathing difficulty, tachycardia, sweating, generalized pallor, and stiffness of the abdominal wall, and becomes present if an individual is in prolonged fatigued and exposed to cold and physical stress. This syndrome has been shown to be caused by a point mutation in the TRPA1 channel, which generates a gain of function of the receptor [187].

Third-generation aromatase inhibitors (AIs), which include the triazoles anastrozole and letrozole, and the steroidal agent exemestane, are currently recommended for adjuvant endocrine treatment as primary, sequential, or extended therapy (with tamoxifen) for postmenopausal women diagnosed with estrogen receptor-positive breast cancer [188]. Estrogens are the main hormones involved in the development of breast cancer. Tamoxifen inhibits the growth of breast tumors by competitive antagonism of estrogens at their receptor site, but also having partial agonistic effects. AIs, instead, are able to markedly suppress plasma estrogen levels in post-menopausal women by inhibiting or inactivating aromatase, the enzyme responsible for the synthesis of estrogens from androgenic substrates. However, the use of AIs has been associated with a series of relevant side effects, including the AI-associated musculoskeletal symptoms (AIMSS), characterized by pain of the hands, knees, hips, lower back, and shoulders [189]. Since the chemical structure of exemestane includes a system of highly electrophilic conjugated Michael acceptor groups, which might react with the thiol groups of reactive cysteine residues, exemestane, letrozole and anastrozole may produce neurogenic inflammation, nociception, and hyperalgesia by targeting TRPA1 [190]. Moreover, aromatase inhibition, while reducing downstream estrogens, moderately

increases upstream plasma concentrations of androgens, including androstenedione (ASD), which possesses some of the reactive chemical features of exemestane: androstenedione has been shown to also target TRPA1, thus possibly contributing to AIMSS [191].

1.5.1. TRPA1 and Neuropathic pain

Neuropathic pain is dependent from a damage or dysfunction of the nervous system, frequently due to peripheral nerve injury. Several models of neuropathic pain, such as nerve injury, diabetic neuropathy, and neuropathy induced by some chemotherapeutic agents, are characterized by hypersensitivity to chemical, thermal, and mechanical stimuli. The involvement of TRPA1 in different patterns of neuropathic pain has been proposed by various reports, with supporting data obtained by using pharmacological and genetic tools.

Chemotherapy-induced peripheral neuropathy (CIPN) represents a potentially dose-limiting side effect of commonly used chemoterapeutic agents. These include platinum-based compounds (cisplatin and oxaliplatin), taxanes (paclitaxel), vinca alkaloids (vincristine), and the proteasome inhibitor bortezomib. Symptoms are predominantly sensory, ranging from a mild tingling sensation to spontaneous burning pain and hypersensitivity to stimuli, sometimes accompanied by motor symptoms like weakness and myalgias (muscle cramps and aching) [192]. This syndrome is frequently associated with axonal degeneration, which could be irreversible if there is injury to the DRG and neuronal apoptosis [193]. Moreover, chemotherapeutic agents have the ability to produce oxidative stress, which contributes to their anticancer action but also seems to be responsible for major adverse reactions. In line with this assumption, it has been reported that oxaliplatin-induced mechanical hyperalgesia and heat- and cold-evoked allodynia in rats are attenuated by antioxidants [194]. Due to its primary localization in sensory neurons, and its nociceptive role as a sensor of oxidative stress, TRPA1 appears to be a valid candidate as a contributor to CIPN. Indeed, TRPA1-deficient mice exhibit reduced mechanical allodynia after administration of cisplatin and oxaliplatin [195]. Other TRP channels may have a role in CIPN, as TRPV1 channel deletion worsened cisplatin-induced mechanical allodynia [196], and mechanical hyperalgesia induced in a neuropathy mouse model by paclitaxel derives in part from the activation of the TRPV4 channel [103]. However, another study showed that both cold allodynia and mechanical hyperalgesia

evoked by paclitaxel are entirely mediated by TRPA1 [197]. Platinum-derived drugs (oxaliplatin/cisplatin), paclitaxel, and bortezomib, do not directly gate TRPA1, and there is evidence that cisplatin and oxaliplatin target the channel by producing ROS, since established hypersensitivity is transiently reverted by the antioxidant α-lipoic acid and by the TRPA1 antagonist HC-030031 [198,199]. Hypersensitivity is completely absent in TRPA1 deleted mice, and this implies that TRPA1 is necessary and sufficient for establishing a prolonged (10 days) hypersensitivity condition.

Another important role for TRPA1 in neuropathic pain has been recently proposed by our research group in a mouse model induced by partial sciatic nerve ligation [200]. Before our work, TRPA1 had been identified in oligodendrocytes, with possible detrimental roles in ischemia and neurodegeneration [201]. We extended this observation to Schwann cells, the peripheral analogs of oligodendrocytes, which proved themselves to be able to orchestrate neuroinflammation and ensuing neuropathic pain, *via* TRPA1. Schwann cells are peripheral glial cells which wrap around axons of motor and sensory neurons to form the myelin sheath, supporting neurons in the peripheral nervous system. We found out that TRPA1 is actually expressed by these cells, and our results showed the cellular and molecular events contributing to TRPA1-mediated mechanical allodynia and neuroinflammation in neuropathic pain: partial sciatic nerve ligation induces the release of the CCL2 chemokine, promoting the extravasation of hematogenous monocytes. These generate a rapid NOX2-dependent oxidative burst, which targets the TRPA1 channel localized in Schwann cells. TRPA1 activation in Schwann cells, in turn, evokes a Ca^{2+}-dependent, NOX1-mediated prolonged bidirectional H_2O_2 generation. While the outward H_2O_2 release produces a space-scaled gradient that determines the final macrophage recruitment to the injured nerve trunk, the inward H_2O_2 release targets TRPA1 expressed in the nociceptor wrapped inside the Schwann cells, producing mechanical allodynia [200].

1.5.2. TRPA1 and Migraine

While the proposal that meningeal plasma extravasation, mediated by SP and the NK1 receptor, contributes to migraine was not confirmed by several clinical trials [202], the component of neurogenic inflammation produced by CGRP that is released from perivascular trigeminal nerve endings seems to represent the underlying mechanism of migraine headaches. In fact, many chemical unrelated CGRP receptor antagonists, like telcagepant, have been shown to ameliorate the pain and the associated symptoms of

migraine attacks [44,203]. These findings imply that stimuli that are able to excite peptidergic nociceptors to release CGRP might be expected to trigger migraine attacks. Being expressed in nociceptors, TRPA1 has emerged as a specific target for many migraine triggers, and some antimigraine drugs have proved to have inhibitory effects on this channel.

It is common knowledge that a series of exogenous stimuli, such as environmental agents, food, medicines, can either provoke or favor headache in migraineurs [24,204]. For example, a portion of migraineurs appears to be particularly sensitive to inhalation of cigarette smoke, which increases the frequency of migraine attacks and cluster headache (a severe type of primary headache) attacks [204,205]. Among the thousands of components of cigarette smoke, crotonaldehyde [206], acetaldehyde [207], formaldehyde [181], H_2O_2 [138], nicotine [175], and acrolein [144], have all been identified as TRPA1 activators. Cigarette smoke exposure, indeed, causes in rodents a neurogenic inflammatory response in the airways [208], which is entirely mediated by TRPA1 gating [206]. Application of acrolein to the rat nasal mucosa produces a TRPA1-dependent and CGRP-mediated increase in meningeal blood flow [209]. Acrolein, as a recognized TRPA1 agonist, could also be responsible for the irritant responses evoked by vehicle exhaust and tear gas, which include cough, chest pain and dyspnea, and headache [210]. Additional known migraine triggers which have been identified as TRPA1 activators are ammonium chloride [211] and formalin (from formaldehyde) [181]. Cluster headache-like attacks can be triggered by exposure to the scent of the California bay laurel, *Umbellularia californica*, which is also known as the "headache tree" for this property [212]. Umbellunone is the major constituent of *Umbellularia californica*, and is able to react with the biogenic thiol cysteamine, producing a Michael adduct [213]. Indeed, a study published by our research group showed that umbellunone can gate TRPA1 and release CGRP, producing neurogenic meningeal vasodilation after intranasal application *in vivo* [213].

Nitroglycerine and its analogues are able to exert a cardioprotective effect through the release of nitric oxide (NO), an active vasodilator gaseous molecule, but can also cause an adverse reaction characterized by headache attacks [214,215]. NO-induced intra- and extra-cranial vasodilation is considered to be one possible mechanism of this adverse effect [216]. This hypothesis is strengthened by the observation that sumatriptan (an agonist of the serotonin 5-HT1B receptor) reverses migraine [217,218], probably through a vasoconstrictor effect, but the use of a CGRP receptor antagonist doesn't [219]. Nitroglycerine/NO can

actually release CGRP, *in vitro* [220] and *in vivo* [221], and NO has been identified as a TRPA1 agonist [222]. However, nitroglycerine typically evokes an early and transient headache in both migraineurs and healthy controls, and (only in migraineurs) this headache can be followed by a severe migraine attack after 4-5 hours delay [223]. Vasodilation and CGRP cannot easily account for this postponed migraine onset [220,223], and currently it is unknown if a TRPA1-dependent mechanism could contribute to an eventual neuronal sensitization or other pathways responsible for NO donors-triggered migraine attacks.

Many analgesic and antimigraine drugs have also been shown to act by targeting TRPA1. For example, dipyrone and pyrazolone derivatives are a class of potent analgesic drugs whose mechanism of action remained unclear for decades, despite their therapeutic success against colic pain, post-surgical pain, neuropathic pain, and migraine. However, a recent study proved with i*n vitro* and *in vivo* data that these compounds selectively antagonize TRPA1 activation by reactive channel agonists, thereby producing antinociceptive and antihyperalgesic effects [224].

The herbal extract of butterbur [*Petasites hybridus* (L.) Gaertn] has long been used as an antimigraine preparation, and the major constituents, petasin and isopetasin, are considered responsible for this therapeutic effect [225,226]. There is clinical evidence of the beneficial action of this extract in migraine prevention [227-229], and butterbur is currently recommended at high levels of strength for migraine prophylaxis by the American Headache Society [230]. However, given the instability issues of petasin, which spontaneously turns into isopetasin, the specific analgesic role of each of these sesquiterpenoids has not been clearly identified. Though several hypotheses have been advanced [231-233], none of these actions seems to be relevant in migraine pathophysiology. Petasin and isopetasin both contain electrophilic double bonds that could potentially interact with bionucleophiles, so they could exert their action by TRPA1 targeting.

The extract of saffron crocus, *Crocus sativus* L., has also been used for centuries for its therapeutic effects, which include analgesia in painful conditions and headaches [234,235]. Saffron contains three main bioactive constituents, crocin, safranal, and picrocrocin, which have been shown to have antioxidant [236], anti-inflammatory [237], and antinociceptive [235] properties. However, the underlying mechanisms responsible for the analgesic action of saffron have not been yet elucidated. It could be possible that these compounds as well exert their analgesic action *via* TRPA1 gating.

1.6. Aim of the study

All of the studies cited previously, demonstrate that the TRPA1 channel has gained quite increasing scientific interest for its role as a sensor of pain and cell-damaging agents, since its first cloning. The TRPA1 is a nonspecific calcium-permeable channel expressed in primary sensory neurons of DRG, TG, and vagal ganglia, where it co-localizes with the TRPV1 channel [109,238] and stimulates the release of SP and CGRP [239].

After the identification of the main role of TRPA1 in nociceptive pain models, the discovery that TRPA1 acts as a target of an unprecedented series of chemically different molecules, including the ones generated from oxidative stress, points to the proposition of a novel pathway for producing neurogenic inflammation and for sensing pain, combining TRPA1 and oxidative stress. Moreover, recent reports have emphasized the key function of this channel in models of neuropathic pain and migraine. Growing evidence is in fact robustly building up the hypothesis that TRPA1 plays a major role in the hypersensitivity to chemical, thermal, and mechanical stimuli, which characterizes neuropathic pain. Additionally, while the role of TRPA1 in mechano- and cold-transduction remains to be better investigated, it has been extensively proved that TRPA1 is important for the detection of chemical irritants: this channel is activated by a wide range of pungent and irritant exogenous compounds, many of which also derived from plant and alimentary products [139].

The main purpose of this three years study was to investigate the role of TRPA1, expressed in neuronal and non-neuronal cells, as a sensor of chemical irritants and in different pathological conditions that generate pain.

First of all, we have investigated the possibility that petasin and isopetasin contained in butterbur [*Petasites hybridus* (L.) Gaertn] [225] could selectively target TRPA1. For hundreds of years butterburs (Petasites), herbaceous perennial plants belonging to the large genus of *Asteraceae*, that also encompasses *Tanacetum parthenium* L., have been used by folk medicine of northern Eurasia and America for therapeutic purposes, including treatment of fever, respiratory diseases, spasms, and pain [240]. Among the large number of compounds contained in common butterbur, the major constituents, petasin and isopetasin [226], are considered responsible for the antimigraine effects of the herbal extract [241]. Clinical evidence [227-229] on beneficial action in migraine prevention has been obtained with a preparation that contains standardized amounts (minimum 15%,

corresponding to 7.5 mg) of petasin/isopetasin [226,241]. However, due to the instability of petasin, that spontaneously turns into isopetasin, the specific role of each sesquiterpenoid for bioactivity has not been clearly identified yet. Several hypotheses have been advanced, including inhibition of leukotriene synthesis in leukocytes [231], inhibition of voltage-sensitive calcium channels in arterial smooth muscle cells [232], and antimuscarinic activity [233], but none of these actions seems to be relevant in migraine pathophysiology. Despite this mechanistic uncertainty, butterbur extract is currently recommended with high level of strength for migraine prophylaxis [230]. Petasin and its cross-conjugated isomer, isopetasin, are eremophilane sesquiterpene esters of petasol and angelic acid. Both compounds contain electrophilic double bonds and can potentially interact with bionucleophiles. Given the role of electrophilic compounds for the paradoxical induction/prevention of headache via modulation of the activity of the TRPA1 channel [213,242], we wondered if the butterbur constituents petasin and isopetasin could target this channel in a non-covalent way and affect functional responses relevant to migraine.

Later, we focused on the hypothesis that the saffron constituent safranal could also target TRPA1. *Crocus sativus* L., commonly known as saffron crocus, belongs to the family of *Iridaceae* [243] and is commonly used for flavoring and coloring food preparations. Saffron extracts contain three main bioactive constituents: the carotenoid crocin, responsible for its typical color, the monoterpene aldehyde picrocrocin, and the volatile compound safranal, which accounts for its special flavor [244]. Saffron has been reported to possess beneficial effects against depression, sexual dysfunction, premenstrual syndrome and weight loss [243,245]. Although clinical trials reported headache as one of the most frequent adverse effects of saffron [246], in Indian traditional medicine saffron has also been used to treat headache [234]. Preclinical studies focused on the pharmacological activity of saffron and its purified constituents, suggesting antioxidant [236], anti-inflammatory [237], and antinociceptive [235] properties of the golden spice. In particular, the antinociceptive effect of safranal has been demonstrated in formalin, acetic acid and carrageenan induced pain [235], and in chronic constriction injury and nerve crush injury in rodents [247,248]. The analgesic action of safranal has been attributed to its ability to suppress glial activation and proinflammatory cytokines production in the central nervous system [249]. However, the underlying pharmacological mechanisms responsible for the action of saffron in pain transmission have not been yet elucidated. Given the role of TRP channels in pain signaling [224,239], and given the analgesic action of saffron components [235], we evaluated

whether the three main constituents of saffron affect the function of TRP channels expressed in nociceptors, and in particular that of TRPA1.

We also investigated the role of TRPA1 relatively to a common pain condition, migraine. Occupational exposure or treatment with organic nitrates have long been known to provoke migraine [250,251], and nitroglycerine (or glyceryl trinitrate, GTN) has been used as a clinical reliable provocation test for migraine attacks [252-254]. In most subjects, including healthy controls, GTN administration evokes a mild and short-lived headache; however, migraineurs, after a remarkable time lag (hours) from GTN exposure, also develop severe headaches that fulfill the criteria of a typical migraine attack [214,254]. Furthermore, GTN administration in rodents and humans produces a delayed and prolonged hyperalgesia that temporally correlates with the GTN-induced migraine-like attacks [255-257]. Several studies have proposed mechanisms to explain these GTN-evoked headaches, ranging from degranulation of meningeal mast cells [255], to delayed meningeal inflammation sustained by induction of NO synthase and prolonged NO generation, together with release of the primary migraine neuropeptide CGRP [258,259], which has a recognized role in migraine [260,261]. Considering the involvement of TRPA1 in pain and migraine, since its inhibition attenuates different types of neuropathic [200,262] and inflammatory pain [263], and since several headache triggers target this channel [134], we investigated the role of TRPA1 in a GTN-induced migraine model.

Finally, we investigated whether ibuprofen-acyl glucuronide (IAG), a metabolite of ibuprofen, antagonizes TRPA1. Ibuprofen is the first approved member of propionic acid derivatives, and is a classical non-steroidal anti-inflammatory drug (NSAID) widely used for its analgesic and anti-inflammatory properties [264,265]. It is indicated to relieve inflammation and several types of pain, including headache, muscular pain, toothache, backache, and dysmenorrhea [264]. This drug probably ranks after aspirin and paracetamol in non-prescription over-the-counter use for the relief of symptoms of acute pain, inflammation and fever [264], and is most likely to be the least toxic. Ibuprofen therapeutic effects are attributed to inhibition of prostanoid synthesis by a non-selective, reversible inhibition of both COX1 and COX2 [266,267]. Usually, ibuprofen is almost completely metabolized, through an oxidative reaction, to the inactive metabolites carboxy-ibuprofen and 2-hydroxyibuprofen, which are both eliminated in the urine [268,269]. However, 10–15% of ibuprofen is glucuronidated to ibuprofen-acyl glucuronide [269]. In one study plasma levels of ibuprofen and IAG have been assessed in patients receiving long-term

administration of oral doses of 600/800 mg ibuprofen: the ibuprofen and IAG ratio was ~30 to 1 [270]. While glucuronide metabolites, including those generated from ibuprofen, are generally considered inactive and rapidly excreted compounds [271], acyl glucuronides can undergo hydrolysis, acyl migration and molecular rearrangement. These processes can transform them in reactive metabolites, which may covalently bind various macromolecules [270,272]. Given that TRPA1 has been reported to contribute to inflammation by different pathways, we wondered if IAG is able to antagonize the TRPA1 channel, contributing *via* this mechanism, to the analgesic and anti-inflammatory actions of ibuprofen. This could also be of clinical importance for patients, if it would turn out that IAG may have a different efficacy and safety profile from ibuprofen.

II - The antimigraine butterbur ingredient, isopetasin, desensitizes peptidergic nociceptors via the TRPA1 channel

2.1. Methods

Animals

All animal care and experimental procedures were carried out according to the European Union (EU) guidelines and Italian legislation (DLgs 26/2014, EU Directive application 2010/63/EU) for animal care procedures and were approved under University of Florence research permits #204/2012-B and #194/2015-PR. In addition, the number of animals and intensity of noxious stimuli used were the minimum necessary to demonstrate consistent effects of the treatments used. Animal studies are reported in compliance with the ARRIVE guidelines [273,274].

C57BL/6 mice (male, 20–25 g, 5 weeks; Envigo, Milan, Italy; N = 208), littermate wild-type (*Trpa1*$^{+/+}$, N = 35) and TRPA1-deficient (*Trpa1*$^{-/-}$; N = 50) mice (25–30 g, 5–8 weeks), generated by heterozygotes on a C57BL/6 background (B6.129P–*Trpa1*$^{tm1Kykw/J}$; Jackson Laboratories, Bar Harbor, ME, USA), wild-type (*Trpv4*$^{+/+}$; N = 15) and TRPV4-deficient (*Trpv4*$^{-/-}$; N = 15) mice (25–30 g, 5–8 weeks), generated by heterozygotes on a C57BL/6 background [275] and TRPV1-deficient mice (*Trpv1*$^{-/-}$; B6.129X1-*Trpv1*$^{tm1Jul/J}$; N = 15) backcrossed with C57BL/6 mice (*Trpv1*$^{+/+}$; N = 15) for at least 10 generations (Jackson Laboratories, Bar Harbor, ME, USA; 25–30 g, 5–8 weeks) or Sprague–Dawley rats (male, 75–100 g, Envigo, Milan, Italy; N = 73) were used. Animals were housed in a controlled-temperature environment, 10 per cage (mice) or five per cage (rats), with wood shaving bedding and nesting material, maintained at 22 ± 1 °C. Animals were housed with a 12 h light/dark cycle (lights on at 07:00 h) and fed with rodent chow (Global Diet 2018, Harlan, Lombardy, Italy) and tap water ad libitum. Animals were allowed to acclimatize to their housing environment for at least 7 days prior to experimentation and to the experimental room for 1 h before experiments. Behavioural experiments were done in a quiet,

temperature-controlled (20 to 22°C) room between 09:00 and 17:00 h and were performed by an operator blinded to genotype and drug treatment. Animals were killed with inhaled CO_2 plus 10–50% O_2 under a valid institutional animal protocol.

Cell culture and isolation of primary sensory neurons

Naïve untransfected HEK293 cells (American Type Culture Collection, Manassas, VA, USA; ATCC® CRL-1573™) were cultured according to the manufacturer's instructions. HEK293 cells were transiently transfected with the cDNAs (1 μg) coding for wild-type (wt-hTRPA1) or mutant 3C/KQ human TRPA1 (C619S, C639S, C663S, K708Q; 3C/K-Q hTRPA1-HEK293) (both donated by D. Julius, University of California, San Francisco, CA, USA) [149] using the jetPRIME transfection reagent (Polyplus transfection® SA, Strasburg, France) according to the manufacturer's protocol. HEK293 cells stably transfected with cDNA for human TRPA1 (hTRPA1-HEK293, donated by A.H. Morice, University of Hull, Hull, UK), or with cDNA for human TRPV1 (hTRPV1-HEK293, donated by M. J. Gunthorpe, GlaxoSmithKline, Harlow, UK), or with cDNA for human TRPV4 (hTRPV4-HEK293, donated by N.W. Bunnett, Monash Institute of Pharmaceutical Sciences, Parkville, Australia) were cultured as previously described [224]. Human fetal lung fibroblasts (IMR90; American Type Culture
Collection, Manassas, VA, USA; ATCC® CCL-186™), which express the native TRPA1 channel, were cultured in DMEM supplemented with 10% FBS, 2 mM glutamine, 100 U penicillin and 100 μg/mL streptomycin. Cells were plated on glass coated (poly-L-lysine, 8.3 μM) coverslips and cultured for 2–3 days before being used for recordings. For all cell lines, the cells were used as received without further authentication.

Primary trigeminal ganglion (TG) neurons were isolated from adult Sprague–Dawley rats and C57BL/6 or *Trpa1*$^{+/+}$ and *Trpa1*$^{-/-}$ mice and cultured as previously described [276]. Briefly, TG were bilaterally excised under a dissection microscope and transferred to HBSS containing 1 mg/mL of trypsin plus 2 mg/mL of collagenase type 1A or papain, for rat or mouse ganglia, respectively, for enzymatic digestion (30 min, 37°C). Ganglia were then transferred to warmed DMEM containing: 10% FBS, 10% horse serum, 2 mM L-glutamine, 100 U/mL penicillin and 100 mg/mL streptomycin and dissociated into single cells by several passages through a series of syringe needles (23–25 G). Medium and ganglia cells were filtered to remove debris and centrifuged. The pellet was suspended in DMEM with added 100 ng/mL mouse-NGF and 2.5 mM cytosine-D-

arabinofuranoside free base. Neurons were then plated on glass coverslips coated with poly-L-lysine (8.3 μM) and laminin (5 μM).

Cellular recordings

Mobilization of intracellular calcium ($[Ca^{2+}]_i$) was measured in untransfected or transfected HEK293 cells, in IMR90 and in primary sensory neurons, as previously reported [276]. Plated cells were loaded with 5 μM Fura-2 AM-ester (Alexis Biochemicals; Lausen, Switzerland) added to the buffer solution (37°C) containing the following (in mM): 2 $CaCl_2$; 5.4 KCl; 0.4 $MgSO_4$; 135 NaCl; 10 D-glucose; 10 HEPES and 0.1% bovine serum albumin at pH 7.4. After loading (40 min, 37°C), cells were washed and transferred to a chamber on the stage of an Olympus IX81 microscope for recording. Cells were excited alternately at 340 and 380 nm to indicate relative intracellular calcium changes by the $Ratio_{340/380}$ ($R_{340/380}$) and recorded with a dynamic image analysis system (XCellence Imaging software; Olympus srl, Milan, Italy). Results are expressed as the percentage of increase of $R_{340/380}$ over the baseline normalized to the maximum effect induced by ionomycin (5 μM) (% Change in $R_{340/380}$).

Whole-cell patch-clamp recordings were performed in untransfected and transfected HEK293 cells and in primary sensory neurons plated on coated coverslips as previously reported [276]. Briefly, coverslips were transferred to a recording chamber (1 mL volume), mounted on the platform of an inverted microscope (Olympus CKX41, Milan, Italy) and superfused at a flow rate of $2mL \cdot min^{-1}$ with a standard extracellular solution containing (in mM): 10 HEPES, 10 D-glucose, 147 NaCl, 4 KCl, 1 $MgCl_2$ and 2 $CaCl_2$ (pH adjusted to 7.4 with NaOH). Borosilicate glass electrodes (Harvard Apparatus, Holliston, MA, USA) were pulled with a Sutter Instruments puller (model P-87) to a final tip resistance of 4–7 MΩ. Pipette solution used for HEK293 cells contained (in mM): 134 K-gluconate, 10 KCl, 11 EGTA, 10 HEPES (pH adjusted to 7.4 with KOH). When recordings were performed on rat neurons, 5 mM $CaCl_2$ was present in the extracellular solution and pipette solution contained (in mM): 120 CsCl, 3 Mg_2ATP, 10 BAPTA, 10 HEPES-Na (pH adjusted to 7.4 with CsOH). Data were acquired with an Axopatch 200B amplifier (Axon Instruments, CA, USA), stored and analysed with a pClamp 9.2 software (Axon Instruments, CA, USA). All the experiments were carried out at room temperature (20–22°C). Cell membrane capacitance was calculated in each cell throughout the experiment by integrating the capacitive currents elicited by a ±10 mV voltage pulse. Currents were detected as inward currents activated on cell superfusion with the various stimuli in the

voltage-clamp mode at a holding potential of -60 mV. Peak currents activated in each experimental condition were normalized to cell membrane capacitance and expressed as mean of the current density (pA/pF) in averaged results. Signals were sampled at 10 kHz and low-pass filtered at 10 kHz.

Cells and neurons were challenged with isopetasin (0.1 μM–3 mM), isopetasol (0.1 μM–3 mM) and angelic acid (Santa Cruz Biotechnology Inc.; TX, USA) (0.1 μM–3 mM) to assess their ability to promote a cellular response. To induce TRPA1 channel-dependent responses, cells and neurons were challenged with the selective agonist, AITC (1–10 μM). GSK1016790A (0.05–0.1 μM) was used to induce a selective response from TRPV4 channel. Buffer solution containing DMSO 0.5% was used as vehicle. Capsaicin (0.1–1 μM) was used to induce a selective response of TRPV1 channel and to identify capsaicin-sensitive neurons. The activating peptide for human proteinase-activated receptor 2 (hPAR2-AP; 100 μM) or KCl (50 mM) were used to elicit a TRP-independent cellular response. Some experiments were performed in the presence of selective antagonists for TRPA1, HC-030031 (50 μM), for TRPV1, capsazepine (10 μM), for TRPV4 channels, HC-067047 (30 μM) or their respective vehicles (0.5, 0.1 and 0.3% DMSO respectively).

Organ bath assays

Contractile response studies were performed on rat or *Trpa1*$^{+/+}$ and *Trpa1*$^{-/-}$ mice urinary bladder strips. Briefly, urinary bladder was excised from rat or mouse and longitudinal strips were suspended at a resting tension of 1 g for rat and 0.5 g for mouse tissues in 10mL organ bath bathed in aerated (95% O_2 and 5% CO_2) Krebs–Henseleit solution containing (in mM): 119 NaCl, 25 $NaHCO_3$, 1.2 KH_2PO_4, 1.5 $MgSO_4$, 2.5 $CaCl_2$, 4.7 KCl and 11 D-glucose, maintained at 37°C. After 45 min of equilibration, tissues were contracted twice with carbachol (l μM), with a 45 min washing out period between the first and second administration. Tissues were challenged with isopetasin (10–300 μM), AITC (100 μM), GSK1016790A (10 μM) and capsaicin (0.3 μM) or their vehicles. In some experiments, tissues were pretreated with the TRPA1 channel antagonist, HC-030031 (50 μM), the TRPV1 channel antagonist, capsazepine (10 μM), the TRPV4 channel antagonist, HC-067047 (30 μM) or a combination of NK1 and NK2 receptor antagonists, L-733060 and SR48968 respectively (both 1 μM). Some tissue preparations were desensitized by exposure to capsaicin (10 μM for 20 min, twice). Similarly, some preparations were exposed to isopetasin (100–300 μM for 20 min, twice) before the challenge with various stimuli. Motor activity of tissue preparation was recorded isometrically on a force

transducer (Harvard Apparatus, Ltd, Kent, UK). Responses were expressed as percentage (%) of the maximum contraction induced by carbachol (1 μM).

CGRP-like immunoreactivity (CGRP-LI) assay

For CGRP-LI outflow experiments, 0.4 mm slices of mouse spinal cord (combined cervical, thoracic and lumbosacral segments) were superfused with an aerated (95% O_2 and 5% CO_2) Krebs–Henseleit solution containing 0.1% bovine serum albumin plus the angiotensin-converting enzyme inhibitor, captopril (1 μM), and the neutral endopeptidase inhibitor, phosphoramidon (1 μM), to minimize peptide degradation. Tissues were stimulated with isopetasin (10–100 μM) or its vehicle (1% DMSO) dissolved in modified Krebs–Henseleit solution and superfused for 10 min. Some tissues were pre-exposed to capsaicin (10 μM, 30 min) or superfused with a calcium-free buffer containing EDTA (1 mM). Other tissues were pre-exposed to a high concentration of isopetasin (300 μM, 30 min) and then, after a prolonged washing (40 min), stimulated with AITC (100 μM), capsaicin (0.3 μM), GSK1016790A (10 μM) or KCl (40 mM). Fractions (4 mL) of superfusate were collected at 10 min intervals before, during and after administration of the stimulus and then freeze-dried, reconstituted with assay buffer and analysed for CGRP-LI by using a commercial enzyme-linked immunosorbent assay kit (Bertin Pharma, Montigny le Bretonneux, France). None of the used substances showed any cross reactivity with the CGRP antiserum. CGRP-LI was calculated by subtracting the mean prestimulus value from those obtained during or after stimulation. Detection limits of the assays were 5 pg/mL. Results were expressed as femtomoles of peptide per gram of tissue. Stimuli did not cross-react with CGRP antiserum.

Behavioural experiments (face rubbing)

For behavioural experiments, after habituation, C57BL/6, *Trpa1*$^{+/+}$ and *Trpa1*$^{-/-}$, *Trpv1*$^{+/+}$ and *Trpv1*$^{-/-}$, *Trpv4*$^{+/+}$ and *Trpv4*$^{-/-}$ mice were randomized to treatment groups, consistent with experimental design. Each experiment was repeated two to three times (using two or three animals in each experimental session). For the *in vivo* experiments, the first outcome assessed was acute spontaneous nociception evoked by the s.c. injection (10 μL), into the right mouse whisker pad (perinasal area), of AITC (10–100 nmol), capsaicin (0.5–3 nmol), GSK1016790A (1–5 nmol), isopetasin (5–50 nmol) or their vehicle (2.5% DMSO). Nociception was assessed by measuring the time (seconds) that the animal spent rubbing the injected area of the face with its paws [277]. These observations were made without knowledge of the treatments given (blinded). The nociceptive effects

of s.c. injection (10 μL) of isopetasin (50 nmol) and AITC (50 nmol) or capsaicin (3 nmol) or GSK1016790A (2.5 nmol) were also tested in *Trpa1*$^{+/+}$ and *Trpa1*$^{-/-}$, *Trpv1*$^{+/+}$ or *Trpv1*$^{-/-}$, *Trpv4*$^{+/+}$ or *Trpv4*$^{-/-}$ mice respectively. To assess the pharmacological target of isopetasin, C57BL/6 mice received isopetasin (50 nmol in 10 μL, s.c.) 60 min after i.p. treatment with the selective TRPA1 channel antagonist, HC-030031 (100 mg/kg) or 30 min after the selective TRPV1 channel antagonist, capsazepine (4 mg/kg) or 60 min after the selective TRPV4 channel antagonist, HC-067047 (100 mg/kg) or their vehicle (4% DMSO plus 4% tween 80 in isotonic saline).

Then, the preventive effect of isopetasin on nociceptive responses evoked by known TRPA1, TRPV1 and TRPV4 channel agonists was measured. Isopetasin (5 mg·kg^{-1}) was administered i.g. quod diem (q.d.) for 5 days in C57BL/6 or *Trpa1*$^{-/-}$ mice and each day, 60 min later, AITC (50 nmol) or capsaicin (3 nmol) or GSK1016790A (2.5 nmol) or their vehicle were injected (10 μL, s.c.) and behavioural tests performed, as described above. The dose of isopetasin for preventive purposes was calculated according to the National Institute of Health conversion formula in order to administer to animals a dose similar to that has been proved efficacious for migraine prevention in humans [278].

Dural blood flow

Rats were anesthetized (urethane, 1.4 g in 10 mL/kg, i.p.), and the head fixed in a stereotaxic frame. A cranial window (4 × 6 mm) was opened into the parietal bone to expose the dura mater. Changes in rat middle meningeal artery blood flow were recorded with a Laser Doppler Flowmeter (Perimed Instruments, Milan, Italy). The probe (needle type, tip diameter 0.8 mm) was fixed near a branch of the middle meningeal artery (1mm from the dural outer layer). The window was filled with synthetic interstitial fluid. In a first set of experiments, dural blood flow was monitored for 30min after administration of isopetasin (5 mg in 10 mL/kg, i.p.) or its vehicle. In a second set of experiments, dural blood flow was measured after the administration of acrolein (50 nmol, intranasal), ethanol (140 μL/kg, i.v.), sodium nitroprusside (1 mM, 100 μL, topical to the exposed dura mater) or their vehicles (isotonic saline) in rats treated for 5 days with isopetasin (5 mg/kg, i.g.) or its vehicle 60 min after the treatment. Baseline flow was calculated by the mean flow value measured during a 5 min period prior to stimulus. The increase in blood flow was calculated as change (%) over the baseline.

Data and statistical analysis

The data and statistical analysis in this study comply with the recommendations on experimental design and analysis in pharmacology [279]. Data are reported as mean ± SEM. Statistical analysis was performed by the unpaired two-tailed Student's t-test for comparisons between two groups and the ANOVA, followed by the Bonferroni post hoc test, for comparisons between multiple groups (GraphPadPrism version 5.00, San Diego, CA, USA). Agonist potency was expressed as the EC_{50}. $P < 0.05$ was considered statistically significant.

Materials

^1H (500 and 400 MHz) NMR spectra were measured on Varian INOVA NMR spectrometers (Palo Alto, CA, USA). Chemical shifts were referenced to the residual solvent signal ($CDCl_3$: δH = 7.26). Silica gel 60 (70–230 mesh) used for gravity column chromatography was purchased from Macherey-Nagel (Düren, Germany). Reactions were monitored by TLC on Merck 60 F254 (0.25 mm) plates (Kenilworth, NJ, USA), visualized by staining with 5% H_2SO_4 in methanol and heating. Organic phases were dried with Na_2SO_4 before evaporation. Chemical reagents and solvents were from Sigma-Aldrich (Milan, Italy) and were used without any further purification unless stated otherwise. All natural compounds were finally purified with Jasco-HPLC, Hichrom column 21.2 × 250 mm (Tokyo, Japan), normal phase with PU-2080 binary pump and UV-2075 plus detector to guarantee a purification degree for biological assays.

For petasin isolation, a sample (500 g) of common butterbur [*P. hybridus* (L.) Gaertn.] roots collected in the Swiss Alps was powdered using a kitchen blender. The root powder was extracted twice with 5 L of acetone (ratio plant:solvent 1:10), affording, after filtration and evaporation, 14.36 g (2.9%) of a dark brown oily extract. The latter was purified by gravity column chromatography on silica gel using petroleum ether-EtOAc (from 9:1 to 7:3) as eluant to afford 9 g (1.8%) of petasin as yellow oil, identified by comparison with spectroscopic data. For isomerization of petasin to isopetasin, 500 mg of NaH 95% (36.53 mmol) was added to a solution of petasin (390 mg, 1.23 mmol) in toluene (10 mL). After stirring for 20 h at room temperature, the reaction was quenched by dilution with water; 2N H_2SO_4 was added, and the reaction was extracted with ethyl acetate. The organic phase was dried, filtered and evaporated. The residue, a brown oil, was purified by gravity column chromatography on silica gel using petroleum ether-EtOAc (9:1) as eluant and further purified with HPLC to yield 256 mg (65.6%) of isopetasin (1b) as yellow oil. The hydrolysis of petasin in isopetasol has been obtained by

adding petasin (350 mg, 1.10 mmol) to a 5% KOH methanol (10 mL) solution. After stirring for 20 h at room temperature, the reaction was diluted with 2N H_2SO_4 and extracted with EtOAc. The organic phase was dried, filtered and evaporated. The residue, a brown oil, was purified by gravity CC on silica gel using petroleum-ether (5:5) as eluant and further purified with HPLC to yield 187.7 mg (72.8%) of isopetasol as white crystals.

For *in vitro* experiments, isopetasin and isopetasol were dissolved in 100% DMSO at 10 mM concentration and then diluted in aqueous solution. For systemic *in vivo* treatment, isopetasin (5 mg in 10 mL/kg) was dissolved in 0.5% carboxymethylcellulose solution (CMC) for intragastric (i.g.) administration or in 4% DMSO plus 4% tween 80 in isotonic saline for i.p. administration. HC-030031 was synthesized as previously described [206]. If not otherwise indicated, all other reagents were from Sigma-Aldrich (Milan, Italy) and dissolved in appropriate vehicle solutions.

2.2. Results

2.2.1. Isopetasin targets the human TRPA1 channel

In hTRPA1-HEK293 cells, isopetasin and isopetasol, but not angelic acid, produced concentration-dependent increases in $[Ca^{2+}]_i$ (EC_{50}s 10 and 100 μM respectively), responses that were attenuated by the selective TRPA1 channel antagonist, HC-030031 (Figure II-1A and Supplementary Figure II-S1C). hTRPV1-HEK293 and hTRPV4-HEK293 cells that were activated by capsaicin or GSK1016790A (TRPV1 and TRPV4 channel agonists respectively) did not respond to isopetasin (Figure II-1C, D). In whole-cell patch-clamp recordings, isopetasin elicited inward currents in hTRPA1-HEK293 cells that were abolished by HC-030031 (Figure II-1B). Isopetasin did not evoke any current in hTRPV1-HEK293 and hTRPV4-HEK293 cells (Figure II-1C, D). Isopetasin and isopetasol did not induce any cellular response in naïve HEK293 cells (Supplementary Figure II-S1B, D). 3C/K-Q hTRPA1-HEK293 cells, which express a TRPA1 which lacks three cysteine and one lysine residues, responded to the non-electrophilic
agonist, menthol, as wild-type hTRPA1-HEK293, but failed to respond to isopetasin (50 μM) (Figure II-1E). In IMR90, which constitutively express the TRPA1 channel [106], isopetasin concentration-dependently increased $[Ca^{2+}]_i$, (EC_{50} 8 μM), a response that was abolished by HC-030031 (Figure II-1F and Supplementary Figure II-S1E). Calcium

responses elicited by hPAR2-AP and ion currents evoked by KCl were not affected by HC-030031 indicating selectivity (Figure II-1A, B, F and Supplementary Figure II-S1B, C, D).

2.2.2. Isopetasin selectively activates the rodent and human TRPA1 channel

Exposure of cultures of rat TG neurons to isopetasin evoked a concentration-dependent (EC_{50} 10 μM) $[Ca^{2+}]_i$ increase in neurons that responded to capsaicin (Figure II-2A and Supplementary Figure II-S1F). Responses to isopetasin were abolished by HC-030031, but not by selective TRPV1 (capsazepine) or TRPV4 channel (HC-067047) antagonists (Figure II-2A).

Notably, while isopetasin, AITC and capsaicin activated TG neurons from *Trpa1*$^{+/+}$ mice, neurons from *Trpa1*$^{-/-}$ mice responded only to capsaicin (Figure II-2C). In capsaicin-sensitive rat TG neurons, isopetasin evoked inward currents that were selectively blocked by HC-030031 but unaffected by capsazepine and HC-067047 (Figure II-2B). Thus, calcium and electrophysiology data indicated that TRPA1 channel are necessary and sufficient for rodent nociceptors and human cells to respond to isopetasin.

2.2.3. Isopetasin excites and desensitizes rodent peptidergic nociceptors

In cultured rat TG neurons, currents evoked by AITC or capsaicin were unaffected by pre-exposure to AITC but were markedly desensitized after pre-exposure to isopetasin at high (50 μM), but not at low concentration (5 μM) (Figure II-3A). Isopetasin-evoked CGRP release from mouse dorsal spinal cord slices was abolished in tissues desensitized to capsaicin or in the absence of extracellular Ca^{2+} (Figure II-3D), indicating that isopetasin evokes a neurosecretory process from neurons expressing TRPV1 channel. The role of TRPA1 channel in neuronal excitation and the ensuing neurosecretion by isopetasin was demonstrated by its failure to evoke any release in spinal cord slices from *Trpa1*$^{-/-}$ mice (Figure II-3E). Notably, exposure to an elevated concentration of isopetasin attenuated the release of CGRP produced by AITC, capsaicin and the non-specific depolarizing agent KCl (Figure II-3F), indicating heterologous neuronal desensitization.

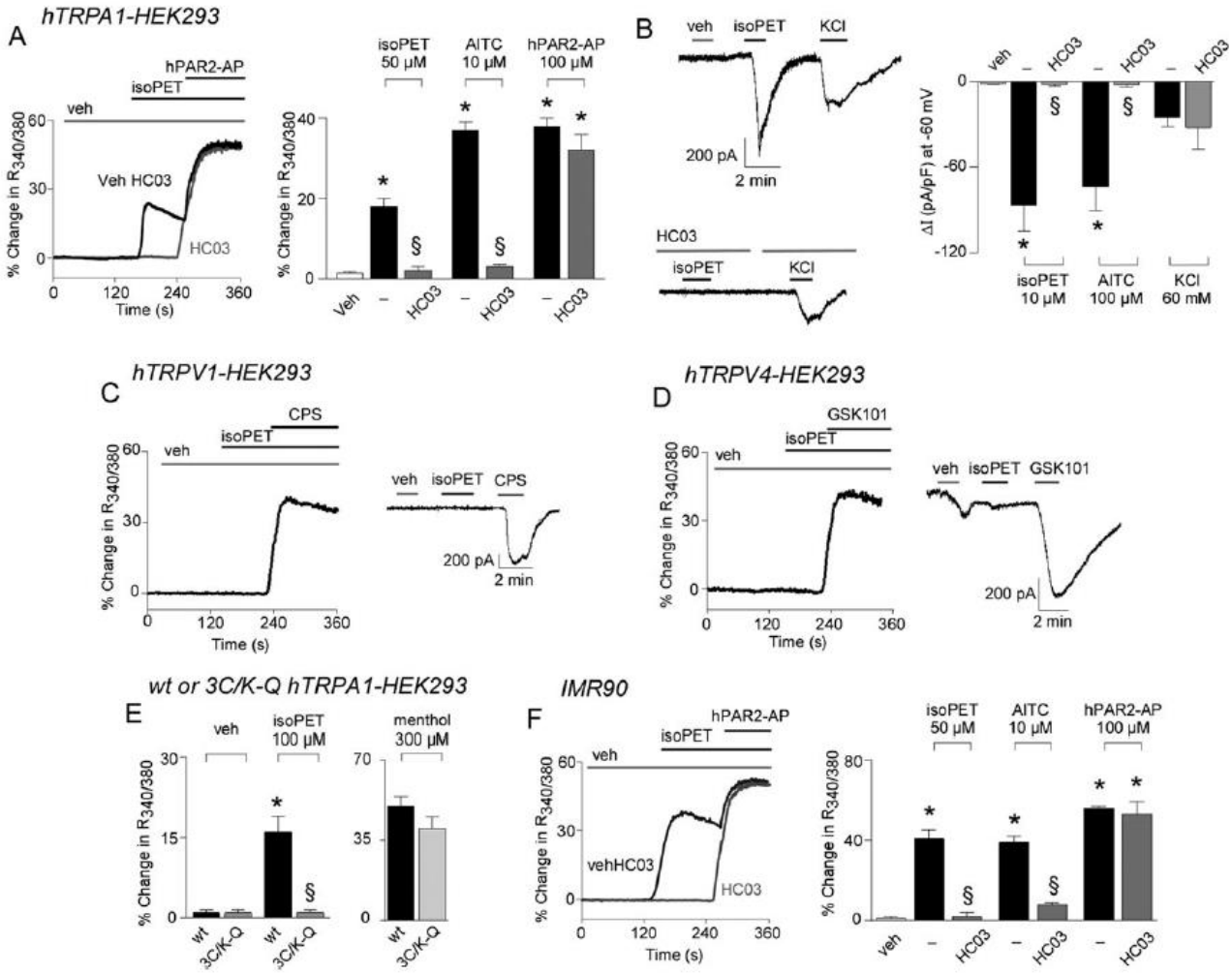

Figure II-1. Isopetasin (isoPET) selectively activates the human TRPA1 channels. A) Representative traces and pooled data of the $[Ca^{2+}]_i$ response evoked by isoPET (50 µM) in HEK293 cells transfected with the cDNA of human TRPA1 (hTRPA1-HEK293). $[Ca^{2+}]_i$ response evoked by isoPET (50 µM) and the selective TRPA1 channel agonist, AITC (10 µM) are abolished by the selective TRPA1 channel antagonist, HC-030031 (HC03; 30 µM), which does not affect the response evoked by the activating peptide for hPAR2 (hPAR2-AP (100 µM). **B)** Representative traces and pooled data of the whole cell patch-clamp inward currents evoked by isoPET (10 µM), AITC (100 µM) and KCl (60 mM) in hTRPA1-HEK293. HC03 abates the response to both isoPET and AITC without affecting the KCl response. **C, D)** Representative traces of $[Ca^{2+}]_i$ responses and whole-cell patch-clamp inward currents in HEK293 cells transfected with the cDNA of human TRPV1 (hTRPV1-HEK293) (C) or with the cDNA of human TRPV4 (hTRPV4-HEK293) (D) show that isoPET (50 µM in $[Ca^{2+}]_i$ imaging or 10 µM in electrophysiological experiments) does not activate either TRPV1 or TRPV4 channels, which are activated by their respective agonist, capsaicin (CPS; 1 µM) and GSK1016790A (GSK101; 100 nM) respectively. **E)** Pooled data of $[Ca^{2+}]_i$ responses evoked by isoPET (100 µM) and menthol (300 µM) in wild-type (wt) and mutant (3C/K-Q) hTRPA1-HEK293 transfected cells. **F)** Representative traces and pooled-data of $[Ca^{2+}]_i$ response induced by isoPET in IMR90. HC03 inhibits the $[Ca^{2+}]_i$ response activated by isoPET (50 µM) but not that evoked by hPAR2-AP (100 µM). Veh is the vehicle of isoPET. Each column represents the mean ± SEM of n > 25 cells from three to six independent experiments for $[Ca^{2+}]_i$ recording or n > 5 cells from four to eight independent experiments for electrophysiological recording. Dash indicates combined vehicles of treatments. **P*<0.05, significantly different from veh, §*P*<0.05, significantly different from isoPET; ANOVA followed by Bonferroni test.

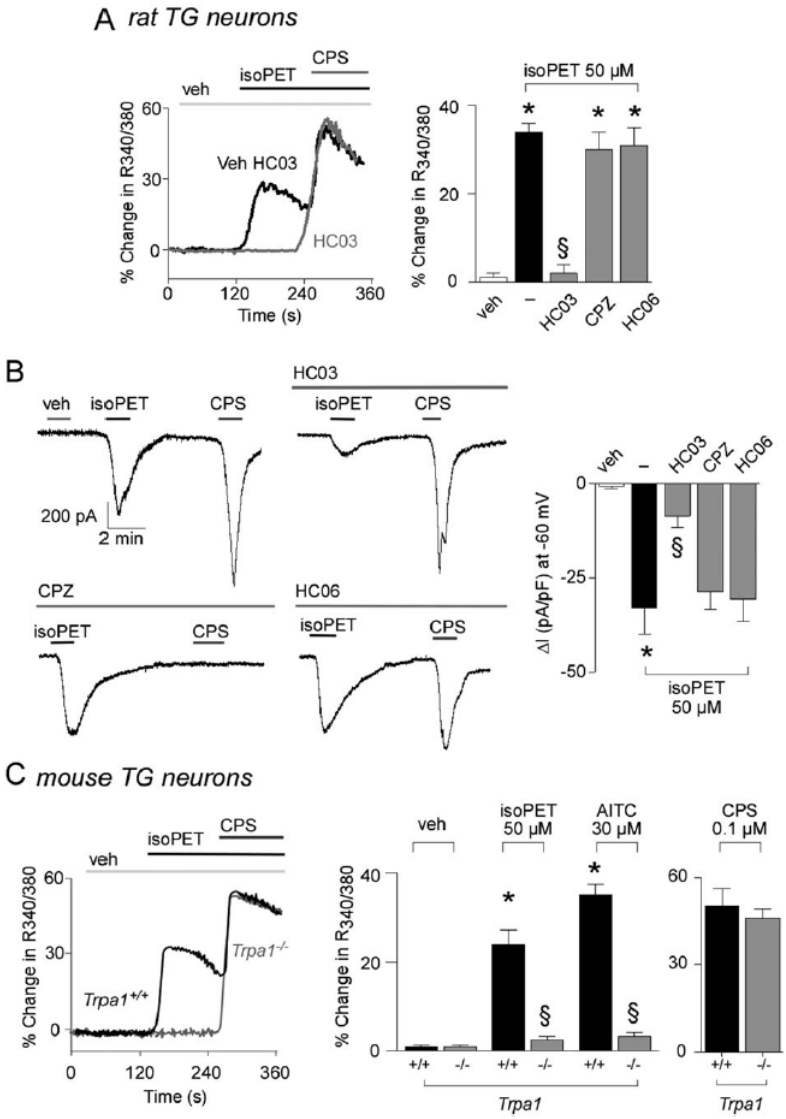

Figure II-2. Isopetasin (isoPET) selectively activates TRPA1 channel in cultures of rodent TG neurons. A, B) Representative traces and pooled data of $[Ca^{2+}]_i$ responses (A) and whole-cell patch-clamp inward currents (B) evoked by isoPET (50 µM) in rat TG neurons responding to capsaicin [CPS; 0.1 µM (A) and 1 µM (B)]. Both $[Ca^{2+}]_i$ responses and ion currents elicited by isoPET are abolished in the presence of the selective TRPA1 channel antagonist, HC-030031 [HC03; 30 µM (A) and 50 µM (B)], and unaffected in the presence of the selective antagonist for TRPV1 channels, capsazepine (CPZ; 10 µM), or TRPV4 channels, HC-067047 (HC06; 30 µM). **C)** Representative traces and pooled data of the $[Ca^{2+}]_i$ response evoked by isoPET (50 µM) or AITC (30 µM) in cultured TG neurons from *Trpa1*$^{+/+}$ mice; both responses are absent in neurons from *Trpa1*$^{-/-}$ mice. Responses to capsaicin (CPS; 0.1 µM) are unchanged. Veh is the vehicle of isoPET. Each column represents the mean ± SEM of n > 20 neurons from three to six independent experiments for $[Ca^{2+}]_i$ recordings or of at least n > 5 cells from four to eight independent experiments for electrophysiological recordings. Dash indicates combined vehicles of treatments. **P*<0.05, significantly different from veh, §*P*<0.05, significantly different from isoPET; ANOVA followed by Bonferroni test.

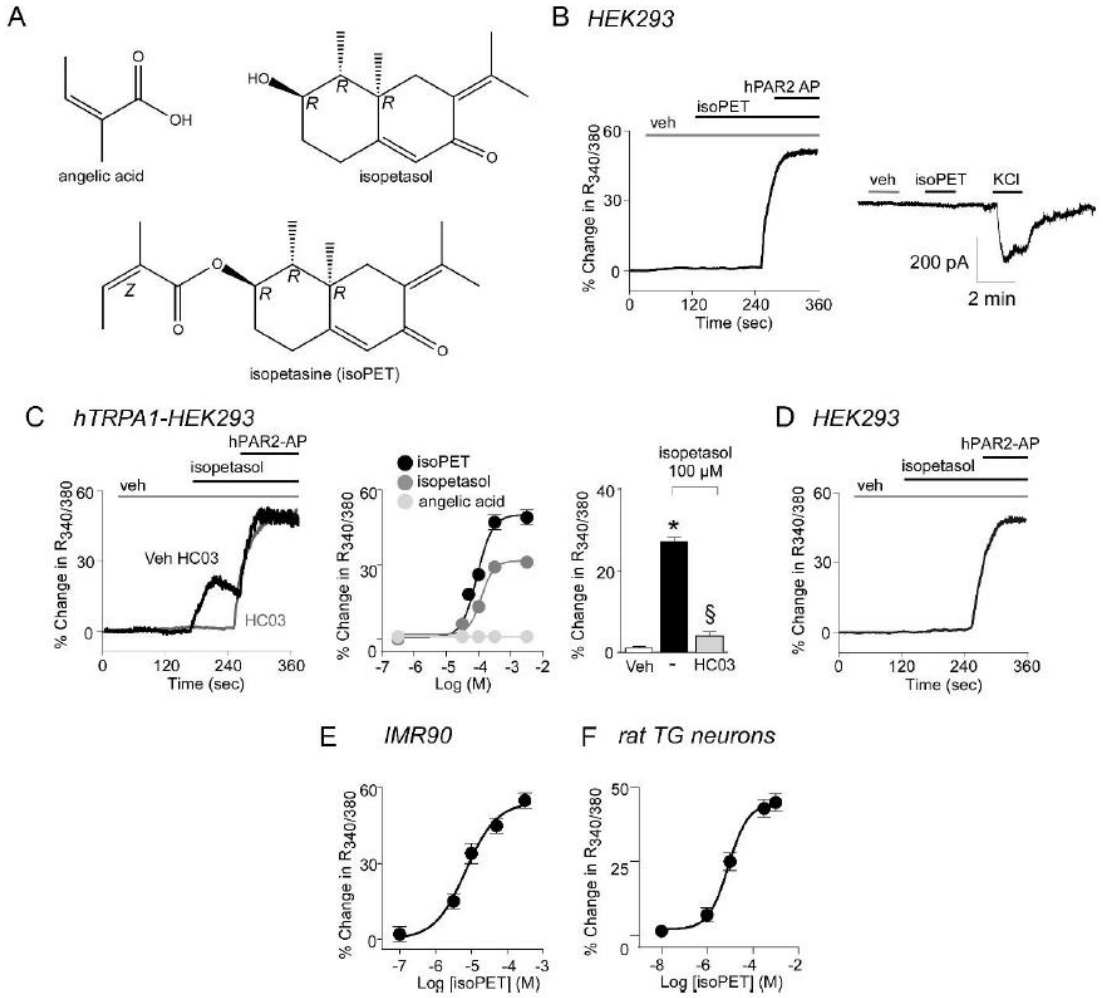

Figure II-S1. A) Chemical structure of isopetasol, angelic acid and isopetasin (isoPET). **B)** Challenge with isoPET (50 µM) does not elicit calcium response nor ion currents in untransfected HEK293 cells which instead responded to the activating peptide for human proteinase activated receptor 2 (hPAR2-AP, 100 µM) or KCl (100 µM). **C)** Representative traces, concentration response curve and pooled data of the calcium response evoked by isopetasol in HEK293 cells transfected with the cDNA codifying for human TRPA1 (hTRPA1-HEK293). isoPET induces a similar concentration response curve in hTRPA1-HEK293 cells with a higher maximum effect at the highest concentration used. Angelic acid does not produce calcium response at any of the concentrations tested (0.5 µM – 3 mM). The calcium response evoked by isopetasol (100 µM) is abolished in the presence of the selective TRPA1 antagonist HC-030031 (HC03; 30 µM) and is absent in **D)** untransfected HEK293 cells. Concentration response curves of the calcium response evoked by isoPET in human foetal lung fibroblasts, IMR90 **(E)**, and in rat cultured trigeminal ganglion (TG) neurons **(F)**. Veh is the vehicle of isopetasol. Each column or point represents the mean ± SEM of n > 20 cells/neurons from 3-6 independent experiments. Dash indicates vehicle of HC03. **P*<0.05 vs. veh, §*P*<0.05 vs. isopetasol; ANOVA followed by Bonferroni test.

Terminals of peptidergic primary sensory neurons are widely expressed in most tissues and organs. The rodent urinary bladder contains a dense network of SP-containing nerve fibres, whose stimulation results in SP release from intramural sensory nerve endings that via NK1/NK2 receptor stimulation contracts the bladder smooth muscle [280]. Isopetasin caused a concentration-dependent contraction of isolated strips of the rat urinary bladder, a response attenuated by desensitization to capsaicin, NK1 and NK2 receptor (L-733060 and SR48968 respectively) antagonism and HC-030031, but not by capsazepine or HC-067047 (Figure II-3B). In addition, isopetasin induced contractile responses in isolated strips of urinary bladders obtained from *Trpa1*$^{+/+}$ mice, but not in those obtained from *Trpa1*$^{-/-}$ mice (Supplementary Figure II-S2A). Thus, isopetasin-evoked contractions were the result of SP release from peptidergic sensory nerve terminals, through activation of TRPA1 channels. Exposure of urinary bladder strips to a high concentration of isopetasin markedly attenuated contractions evoked by AITC and capsaicin, but not by carbachol, suggesting neuronal desensitization that, however, preserved the contractility of the bladder smooth muscle (Figure II-3C). Furthermore, exposure to a high isopetasin concentration attenuated the contractile response to capsaicin in isolated strips of urinary bladders obtained from *Trpa1*$^{+/+}$ mice, but not in those obtained from *Trpa1*$^{-/-}$ mice (Supplementary Figure II-S2B), suggesting that isopetasin induces neuronal desensitization by selective targeting of TRPA1 channels.

2.2.4. Isopetasin inhibits nociception and neurogenic dural vasodilatation via TRPA1 channel

Subcutaneous injection of irritant agents into the upper whisker pad of mice produces a typical nocifensor behavior described as facial rubbing [277]. Injection (s.c.) of AITC into the upper whisker pad evoked a transient (<15 min) and dose-dependent facial rubbing that was abolished by HC-030031 (Figure II-4A). Similarly, facial rubbing produced by capsaicin was attenuated by capsazepine (Figure II-4B), while facial rubbing evoked by GSK1016790A was inhibited by the TRPV4 channel antagonist, HC-067047 (Figure II-4C). Isopetasin produced facial rubbing that reproduced the features of the response to AITC, being abolished by HC-030031 and unaffected by capsazepine or HC-067047 (Figure II-4D). Moreover, similar to AITC, isopetasin failed to provoke any facial rubbing in *Trpa1*$^{-/-}$ mice (Figure II-4E). Isopetasin-evoked facial rubbing in *Trpv1*$^{-/-}$ and

Trpv4$^{-/-}$ mice was similar to that observed in their respective wild-type littermates (Figure II-4F).

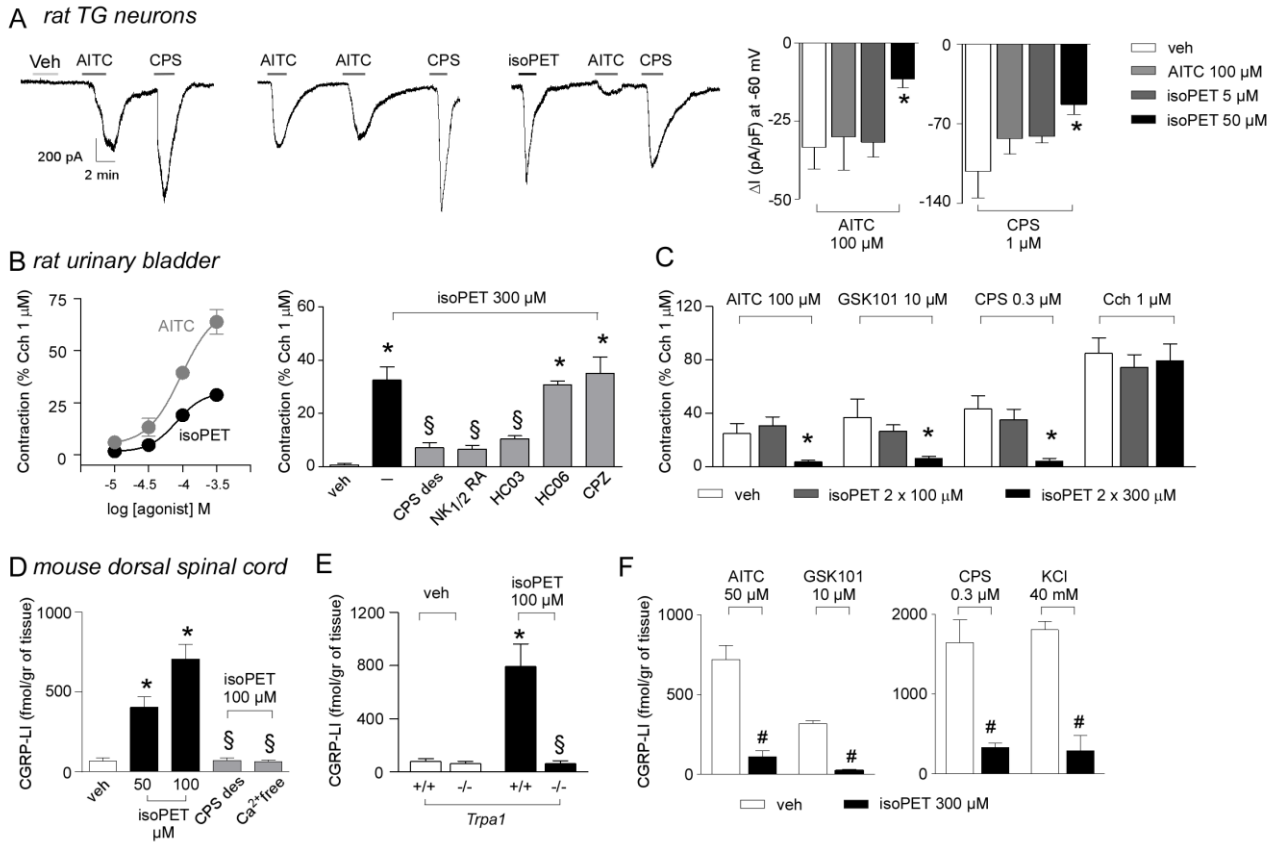

Figure II-3. Isopetasin (isoPET) elicits characteristic desensitization of TRPA1 channel. A) Representative traces and pooled data of whole-cell patch-clamp inward currents through rat TG neurons showing that the initial challenge with isoPET (50 µM), instead of AITC (100 µM), or its vehicle (veh), produces neuronal desensitization with a reduced response to the subsequent exposure to AITC (100 µM) and capsaicin (CPS; 1 µM). Each column represents the mean ± SEM of n > 6 independent experiments. **B)** Concentration–response curve of the contractile response evoked by AITC and isoPET in rat urinary bladder strips. At the highest concentration used, isoPET (300 µM) evokes a contractile response that is selectively inhibited by the selective TRPA1 channel antagonist HC-030031 (HC03; 50 µM) and unaffected by the selective TRPV1 channel antagonist capsazepine (CPZ; 10 µM) or the selective TRPV4 channel antagonist, HC-067047 (HC06; 30 µM). The isoPET contraction is also abolished by a combination of NK1 and NK2 receptor antagonists (NK1/2 RA; L-733060 and SR48968, both 1 µM) and after exposure (20 min, twice) to a high concentration of capsaicin (CPS; 10 µM) that induces neuronal desensitization. **C)** Exposure (20 min, twice) to the highest concentration of isoPET (300 µM) markedly reduces the contractile response evoked by a selective TRPA1, TRPV4 or TRPV1 channel agonist, AITC (100 µM), GSK1016790A (GSK101; 10 µM) or CPS (0.3 µM), but does not affect the response to carbachol (Cch; 1 µM). **D)** isoPET increases the CGRP-LI outflow from mouse dorsal spinal cord slices in a concentration-dependent manner. Effect of isoPET (100 µM) is abolished by calcium removal (Ca^{2+}-free), capsaicin desensitization (CPS-des). **E)** isoPET (100 µM) increases CGRP-LI outflow from spinal cord slices from *Trpa1*$^{+/+}$ mice, but not from *Trpa1*$^{-/-}$ mice. **F)** Exposure to a high concentration of isoPET (300 µM, 30 min) blocks CGRP-LI release evoked by AITC (50 µM), GSK101 (10 µM), CPS (0.3 µM) or KCl (40 mM). Each column represents the mean ± SEM of n = 4 independent experiments. Dash indicates combined vehicles of the treatments. **P*<0.05, significantly different from veh; §*P*<0.05, significantly different from isoPET; ANOVA followed by Bonferroni test. #*P*<0.05, significantly different from isoPET, Student's t-test.

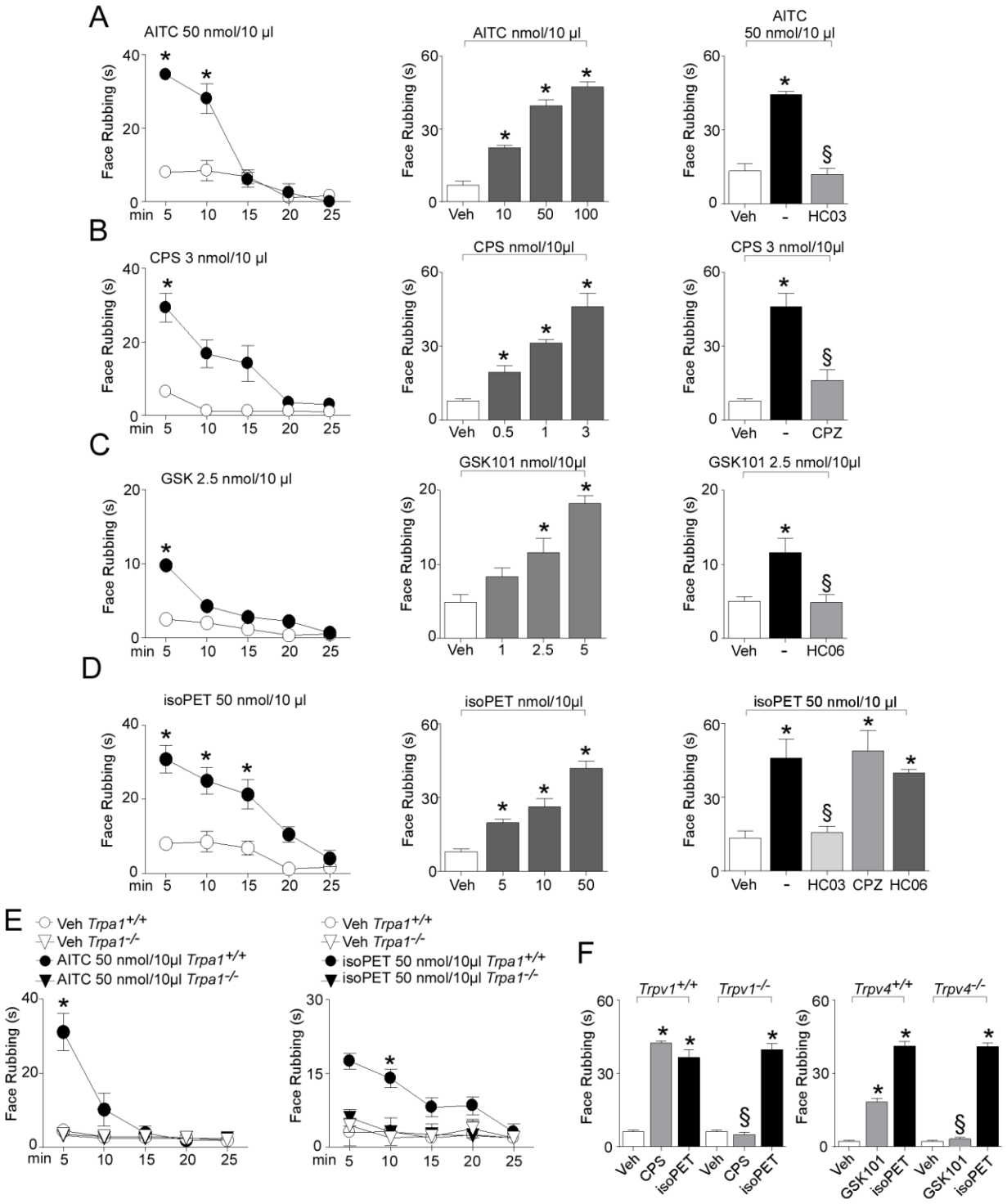

Figure II-4. Local administration of isopetasin (isoPET) evokes irritant pain. A) Time course of facial-rubbing in C57BL/6 mice after s.c. injection (10 µL) of the selective TRPA1 agonist, AITC (50 nmol) or its vehicle (veh) into the whisker pad. Time that mice spent rubbing is plotted for each 5min block over 25 min. The irritant pain evoked by AITC, quantified as the total time spent rubbing in the first 10 min, is dose-dependent and abolished in mice pretreated with the selective TRPA1 antagonist HC-030031 (HC03; 100 mg/kg i.p., 1 h before). Similar to AITC, s.c. injection of **(B)** the selective TRPV1 agonist, capsaicin (CPS) or **(C)** the selective TRPV4 channel agonist, GSK1016790A (GSK101), shows dose-dependent irritant pain behaviours which are blocked by pretreatment with their respective antagonist, capsazepine (CPZ; 4 mg/kg, i.p. 30min before) or HC-067047 (HC06; 10 mg/kg, i.p., 30 min before). **D)** Local injection (10 µL, s.c.) of isoPET into the whisker pad of C57BL/6 mice elicits dose-dependent irritant behaviours. The effect evoked

by isoPET (50 nmol) was blocked in mice pretreated with HC03 but unaffected in HC06 or CPZ pretreated mice. **E)** Time course of the facial-rubbing activity evoked by both AITC (50 nmol in 10 µL, s.c.) and isoPET (50 nmol in 10 µL, s.c.), or their respective veh in *Trpa1*$^{+/+}$ mice. No effect is observed in injected *Trpa1*$^{-/-}$ mice. **F)** isoPET injection (50 nmol in 10 µL, s.c.) into the whisker pad of *Trpv1*$^{+/+}$ and *Trpv1*$^{-/-}$ mice elicits a similar irritant effect, while CPS (3 nmol in 10 µL, s.c.) produces an irritant response only in *Trpv1*$^{+/+}$ mice. Similarly, *Trpv4*$^{+/+}$ and *Trpv4*$^{-/-}$ show a similar irritant response to isoPET, while GSK101 (2.5 nmol in 10 µL, s.c.) produces an irritant response only in *Trpv4*$^{+/+}$ mice. Values are mean ± SEM of at least five mice from three independent experiments. **P*<0.05, significantly different from veh or veh *Trpa1*$^{+/+}$ or veh *Trpv*1$^{+/+}$ or veh *Trpv4*$^{+/+}$, §*P*<0.05 significantly different from AITC or CPS or GSK101 or isoPET or CPS *Trpv1*$^{+/+}$ or GSK101 *Trpv4*$^{+/+}$, ANOVA followed by Bonferroni test or Student's t-test.

A single dose of isopetasin (given i.g.) did not affect the ability of local AITC, capsaicin or GSK1016790A to evoke facial rubbing (Figure II-5A). However, after giving a single dose of isopetasin for three consecutive days, rubbing responses to AITC and GSK1016790A were reduced, whereas that to capsaicin was unaffected (Figure II-5A). Following isopetasin administration for five consecutive days, responses to AITC, GSK1016790A and capsaicin were further attenuated (Figure II-5A). This indicates that longer-term repeated isopetasin i.g. administration leads to a desensitization of nocifensive behaviour specifically evoked by activators of TRPV1, TRPV4 and TRPA1 channels. Moreover, chronic administration of isopetasin for five consecutive days in *Trpa1*$^{-/-}$ mice did not affect facial rubbing evoked by TRPV1 or TRPV4 channel agonists (Supplementary Figure II-S2C). These *in vivo* results support the hypothesis, originated by *in vitro* findings, that desensitization to isopetasin requires selective targeting of the TRPA1 channel. With more direct relevance for headaches and migraine, we next intended to test whether this would also affect CGRP-mediated dilation of meningeal arteries, which we subsequently assessed in rats. Firstly, i.p. administration of isopetasin did not produce any detectable change in meningeal perfusion (Supplementary Figure II-S2D). Secondly, after isopetasin administrations for five consecutive days, increases in blood flow produced by the TRPA1 channel agonist, acrolein [144,209], and the TRPV1 channel agonist, ethanol [80,281], were reduced (Figure II-5B). In contrast, the response to the direct vasodilator sodium nitroprusside remained unchanged (Figure II-5B).

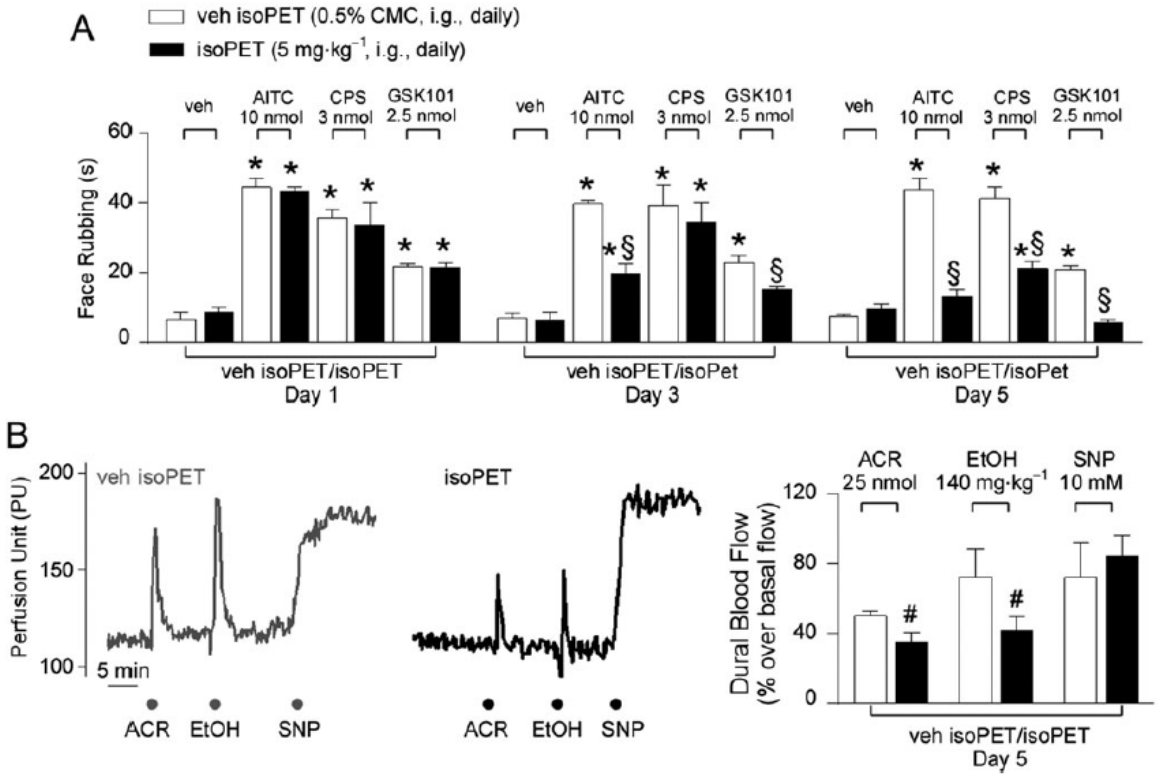

Figure II-5. Chronic administration of isopetasin (isoPET) desensitizes sensory nerve endings. A) Daily i.g. administration of isoPET (5 mg/kg) in naïve C57BL/6 mice gradually reduces the nociceptive response evoked by s.c. injection (10 µL) of AITC (10 nmol), capsaicin (CPS; 3 nmol) or GSK1016790A (GSK101; 2.5 nmol) in mouse whisker pad and measured as the facial-rubbing activity observed in the first 15 min after the injection. Veh is the vehicle of the various stimuli. Values are mean ± SEM of at least five mice per group from three independent experiments. **P*<0.05, significantly different from veh, §P < 0.05, significantly different from veh-isoPET, ANOVA followed by Bonferroni test. **B)** Representative traces and pooled data of the increases in dural blood flow evoked by intranasal acrolein (ACR, 50 nmol in 50 µL), intravenous ethanol (EtOH, 140 µL/kg) or dural application (100 µL) of sodium nitroprusside (SNP; 10 mM) in rat treated for 5 days with systemic isoPET (5 mg/kg, i.g. per day) or its vehicle (0.5% CMC 10 mL/kg, i.g. per day). Chronic treatment with isoPET significantly reduced responses to ACR and EtOH, but not to SNP. Values are mean ± SEM of at least five rats per group from three independent experiments. #*P*<0.05, significantly different from veh-isoPET; Student's t-test.

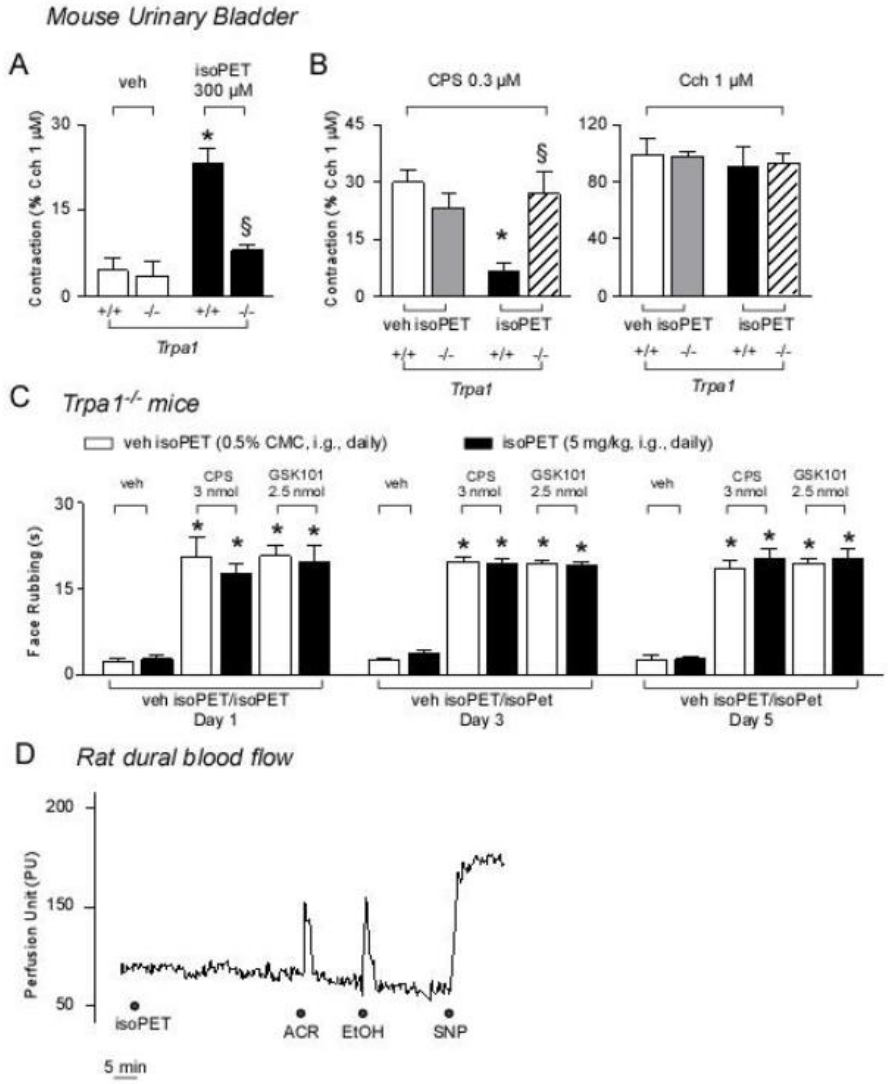

Figure II-S2. A) Isopetasin (isoPET; 300 µM) evokes a contractile response in strips of mouse urinary bladder isolated form *Trpa1*$^{+/+}$ mice, an effect that is absent in urinary bladder from *Trpa1*$^{-/-}$ mice. **B)** Exposure (20 min, twice) to a high concentration of isoPET (300 µM) markedly reduces the contractile response evoked by the selective TRPV1 agonist, capsaicin (CPS; 0.3 µM), in longitudinal strips of mouse urinary bladder isolated from *Trpa1*$^{+/+}$ mice, but not in tissues from *Trpa1*$^{-/-}$ mice. isoPET (300 µM) does not affect the contractile response to carbachol (CCh; 1 µM) in either *Trpa1*$^{+/+}$ or *Trpa1*$^{-/-}$ mouse urinary bladders. Each column represents the mean ± SEM of n = 5 from 3 independent experiments. Veh is the vehicle of isoPET. **P*<0.05 *vs*. *Trpa1*$^{+/+}$ veh or *Trpa1*$^{+/+}$ veh isoPET; ANOVA followed by Bonferroni test. **C)** In *Trpa1*$^{-/-}$ mice daily intragastric (i.g.) administration of isoPET (5 mg/kg) does not affect nociceptive response elicited by subcutaneous (s.c.) injection (10 µl) of capsaicin (CPS; 3 nmol) or GSK1016790A (GSK101; 2.5 nmol) into mouse whisker pad and measured as the facial-rubbing activity observed in the first 15 min after injection. Veh is the vehicle of the various stimuli. Values are mean ± SEM of at least 4 mice per group from 3 independent experiments. **P*<0.05 *vs*. veh, ANOVA followed by Bonferroni test. **D)** Representative trace of the effect in rat dural blood flow evoked by intraperitoneal isoPET (5 mg/kg), intranasal acrolein (ACR, 50 nmol in 50 µl), intravenous ethanol (EtOH, 140 µL/kg) or dural application (100 µl) of sodium nitroprusside (SNP; 10 mM).

2.3. Discussion

Pharmacological and genetic data presented here show that isopetasin activates the universal irritant receptor ion channel, TRPA1. Mouse, rat and human (native and recombinant) TRPA1 channels are gated with similar efficacy and potency by isopetasin, suggesting that responses evoked by the drug *in vivo* in rodents could be reproduced in humans. Furthermore, failure to activate TRPV1 and TRPV4 channels indicates that nociceptor activation by isopetasin is caused by selective targeting of TRPA1 channels. As isopetasin failed to evoke calcium responses in the mutant transfected cells (3C/K-Q hTRPA1-HEK293), the molecular mechanism responsible for channel activation by this compound must be similar to that proposed for electrophilic and reactive agonists that gate TRPA1 channel via the involvement of specific cysteine/lysine residues [149,152]. Moreover, isopetasol, but not angelic acid, induced a calcium response in cells expressing TRPA1 channel. Thus, the hemiterpene moiety of angelic acid is not critical for activity, while the eremophilane sesquiterpenoid framework of isopetasol is essential for channel gating.

Peripheral sensory neurons expressing TRPA1 channel exert a dual function in as much as they both signal nociceptive stimuli to the brain and, by releasing the neuropeptides SP and CGRP, mediate neurogenic inflammation responses in the vasculature and other organs [239,282]. In the rat urinary bladder, AITC, by targeting TRPA1 channel on intramural nerve terminals, elicits neurogenic SP-mediated smooth muscle contractions [283]. Isopetasin produced an effect similar to that of AITC in terms of mechanism of action. However, the efficacy of isopetasin was much lower than that of AITC, indicating that the herbal compound may act as a partial agonist of the channel. Isopetasin, similarly to parthenolide [276], caused desensitization of peptidergic primary sensory neurons either limited to TRPA1 channel, or involving other TRP channel stimulants and non-TRP depolarizing agents. Transition from homologous to heterologous desensitization seems to depend on the concentration and time of exposure to isopetasin. Pre-exposure to isopetasin of cultured TG neurons or their central terminals in the dorsal brainstem reduced AITC-evoked currents or CGRP release respectively. Desensitization of sensory nerve terminals by isopetasin was not limited to trigeminal innervation, as it was observed in nerve terminals of the rat urinary bladder. In this preparation, neurogenic SP-mediated smooth muscle contractions evoked by AITC and capsaicin [283] were markedly reduced by pre-exposure to isopetasin. Inhibition of

neurogenic inflammation by isopetasin in extra-trigeminal innervation may help explain the reported beneficial effects of butterbur in painful and inflammatory conditions, such as arthritis [284] and allergic rhinitis [285,286].

Data obtained *in vitro* were reproduced *in vivo*. The facial rubbing evoked by s.c. AITC was characterized as an effect mediated by TRPA1 channel, while capsaicin or GSK1016790A were effective via TRPV1 or TRPV4 channels respectively. To mimic the clinical use of butterbur in migraine treatment, isopetasin was given via i.g. administration. Results showed a time-dependent increase in the desensitizing effect of i.g. isopetasin that initially involved only TRPA1 channel but, later, involved TRPV4 and TRPV1 channels. Thus, after a single isopetasin dose, the rubbing responses to AITC, capsaicin and GSK1016790A were unaffected, significant inhibition of AITC- or GSK1016790A-mediated responses was observed following a 3-day cycle of isopetasin administration. After a 5-day cycle, the capsaicin mediated nociceptive response was also attenuated. This finding suggests that a more prolonged treatment schedule, providing a non-selective (heterologous) desensitization of peptidergic nerve terminals with TRPV1/TRPA1/TRPV4 channels, might be beneficial in migraine prophylaxis.

The molecular bases of the process that results in channel and neuronal desensitization have been investigated more extensively following activation of TRPV1 channel. Exposure to capsaicin or resiniferatoxin results in a time- and concentration-dependent massive Ca^{2+} influx into the neurons that results in a neurotoxic cation overload, thus attenuating nociceptor functioning. Although neuronal desensitization produced by TRPA1 channel has been explored in less detail, the AITC-evoked heterologous desensitization of CGRP release from the rat hind paw skin is Ca^{2+}-dependent, similar to that produced by capsaicin [287]. Furthermore, in meningeal afferents, selective stimulation of TRPA1 channel increased activation threshold and promoted CGRP release but did not cause propagated action potentials, suggesting that co-expression of TRPV1 channel in peripheral nociceptors is required for TRPA1-mediated pro-nociceptive function [288]. Our present experiments do not contradict this proposal because, as indicated by desensitization studies, isopetasin-evoked responses (bladder contraction, rubbing behaviour, CGRP release and increase in dural blood flow) were all mediated by TRPA1 channel apparently expressed in TRPV1-positive primary afferents.

Clinical studies with low MW compounds that selectively block the CGRP receptor for the acute and preventive treatment of the migraine attack [44,203,289] or, more recently,

with anti-CGRP monoclonal antibodies as preventive anti-migraine medicines [290], have strengthened the hypothesis of a pivotal role of this neuropeptide in the mechanism of migraine headaches. Although the precise CGRP-dependent pathway that results in the pain and associated symptoms of migraine attack has not been completely identified, peptidergic neurons of the trigeminal ganglion appear to play a major role in migraine pain [289]. CGRP released from sensory nerve terminals in rodents and other mammals, including humans, dilates arterial vessels [291], including meningeal arteries [209,281,292]. Thus, CGRP-mediated meningeal vasodilatation following stimulation of TG neurons is considered a good approximation of the neurochemical events that occur during the migraine attack. Previous studies have shown that meningeal vasodilation evoked by administration of either acrolein (intranasal) or ethanol (i.p.) is mediated by CGRP released from perivascular trigeminal nerve endings triggered by activation of TRPA1 or TRPV1 channels respectively [209,281]. The present observation that 5 days of isopetasin, given i.g., attenuated the increase in rat dural blood flow evoked by both acrolein and ethanol suggests that isopetasin, possibly by targeting TRPA1 channels, desensitized trigeminal nociceptors so they were no longer able to release the migraine mediator, CGRP. This novel property of isopetasin may add to the mechanisms previously described [231-233,293] to explain the action of chronically administered butterbur to prevent migraine [227-229].

Migraine therapy consists of different classes of drugs. While acute treatment of attacks is confined to analgesics, non-steroidal anti-inflammatory drugs and triptans, the prevention of attacks is primarily based on β-blockers, antiepileptics, anti-depressants and 5-HT antagonists. It is possible that in the near future, low MW CGRP receptor antagonists (gepants) will be used for acute treatment and anti-CGRP or anti-CGRP receptor monoclonal antibodies will be used for prophylaxis. Our present findings support the view that some drugs and herbal medicines currently prescribed for the acute or prophylactic treatment of migraine can be grouped into a novel class of therapeutics, namely, TRPA1 channel inhibitors. These comprise compounds that target the channel with different modalities. The universally used acetaminophen (paracetamol) via its reactive metabolite, N-acetylbenzoquinoneimine, activates [162] and then desensitizes [294] TRPA1 channel. The well-established, but still widely and successfully prescribed pyrazolone derivatives [295], dipyrone (metamizole) [296] and propyphenazone, are selective TRPA1 channel antagonists [224]. The active component of

feverfew, parthenolide, moderately stimulates TRPA1 channel both *in vitro* and *in vivo*, causing channel desensitization and neuronal dysfunction [276]. Butterbur is indicated as per the American Headache Society guidelines with a level A recommendation for migraine prophylaxis [230]. The present findings would add the butterbur constituent, isopetasin [225], to the list of TRPA1-tropic agents, which, like parthenolide, stimulate the channel, thereby causing desensitization of peptidergic trigeminal nerve terminals, attenuating their ability to release CGRP and to signal pain. Successful treatment and prevention of migraine by targeting of TRPA1 cation channels and the ensuing peptidergic sensory neuron dysfunction by parthenolide and isopetasin provides a solid basis for future basic and translational investigations of migraine treatments based on actions at TRPA1 channels.

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III - TRPA1 mediates the antinociceptive properties of the constituent of *Crocus sativus* L., safranal

3.1. Methods

Animals

In vivo experiments and tissue collection were carried out according to the European Union (EU) directive guidelines (2010/63/EU) and Italian legislation (DLgs 26/2014) for animal care procedures. Studies were conducted under the University of Florence research permit #194/2015-PR. Animals were housed in a temperature- and humidity-controlled vivarium (12-hour dark/light cycle, free access to food and water) and were allowed to acclimatize for at least 7 days prior to experimentation. Animal studies were reported in compliance with the ARRIVE guidelines [273,274]. A group size of six animals for behavioural experiments was determined by sample size estimation using G*Power (v3.1) [297] to detect size effect in a post-hoc test with type 1 and 2 error rates of 5 and 20% respectively. Allocation concealment was performed using a randomization procedure (http://www.randomizer.org/).

The following mouse strains were used: C57BL/6 mice (male, 20-25 g, 5 weeks; Envigo, Milan, Italy); littermate TRPA1 wild-type (*Trpa1*$^{+/+}$) and TRPA1-deficient (*Trpa1*$^{-/-}$) mice (25-30 g, 5-8 weeks) generated by heterozygotes on a C57BL/6 background (B6.129P*Trpa1*$^{tm1Kykw/J}$; Jackson Laboratories, Bar Harbor, ME, USA); wildtype (*Trpv4*$^{+/+}$) and TRPV4-deficient (*Trpv4*$^{-/-}$) mice (25-30 g, 5-8 weeks), generated by heterozygotes on a C57BL/6 background [275] and TRPV1-deficient mice (*Trpv1*$^{-/-}$; B6.129X1-*Trpv1*$^{tm1Jul/J}$) backcrossed with C57BL/6 mice (*Trpv1*$^{+/+}$) for at least 10 generations (Jackson Laboratories; 25-30 g, 5-8 weeks). Sprague-Dawley rats (male, 75-100 g, Envigo) were also used. Animals were killed with inhaled CO_2 plus 10%-50% O_2.

Cell culture

Naïve untransfected HEK293 cells (American Type Culture Collection, Manassas, VA, USA; ATCC® CRL-1573™) were cultured according to the manufacturer's instructions.

HEK293 cells were transiently transfected with the cDNAs (1 µg) codifying for wild-type (wt-hTRPA1) or mutant 3C/K-Q human TRPA1 (C619S, C639S, C663S, K708Q; 3C/K-Q hTRPA1-HEK293) [149] using the jetPRIME transfection reagent (Polyplus-transfection® SA, Strasburg, France) following the manufacturer's protocol. HEK293 cells stably transfected with cDNA for human TRPA1 (hTRPA1-HEK293), or with cDNA for human TRPV1 (hTRPV1-HEK293), or with cDNA for human TRPV4 (hTRPV4-HEK293) were cultured as previously described [224]. Human foetal lung fibroblasts (IMR90; American Type Culture Collection; ATCC® CCL-186™), which express the native TRPA1 channel, were cultured as previously described [298]. Cells were plated on glass-coated (poly-L-lysine, 8.3 µM) coverslips and cultured for 1-2 days before being used for recordings.

Isolation of primary sensory neurons

Primary dorsal root ganglion (DRG) neurons were isolated from adult Sprague-Dawley rats and *Trpa1*$^{+/+}$ and *Trpa1*$^{-/-}$ mice, and cultured as previously described [276]. Briefly, DRG were bilaterally excised and transferred to Hank's Balanced Salt Solution containing trypsin (1 mg/mL) and collagenase type 1A or papain (both, 2 mg/mL), for rat or mouse ganglia respectively (35 minutes, 37°C). Ganglia were then transferred to warm Dulbecco's Modified Eagle Medium supplemented with 10% foetal bovine serum, 10% horse serum, 2 mmol/L L-glutamine, 100 U/mL penicillin and 100 mg/mL streptomycin and dissociated into single cells by several passages through a series of syringe needles (23-25G). Ganglia cells were centrifuged and suspended in medium with the addition of normal growth factor (100 ng/mL) and cytosine-D-arabinofuranoside free base (2.5 mM). Neurons were then plated on glass coverslips coated with poly-L-lysine (8.3 µM) and laminin (5 µM).

Cellular recordings

Mobilization of intracellular calcium ($[Ca^{2+}]_i$) was measured in untrasfected or transfected HEK293 cells, in IMR90 and in DRG neurons. Cells on coated coverslips were loaded with 5 µmol/L Fura-2 AM-ester (Alexis Biochemicals, Lausen, Switzerland) added to the extracellular solution (37°C) containing the following (in mM): 2 $CaCl_2$, 5.4 KCl, 0.4 $MgSO_4$, 135 NaCl, 10 D-glucose, 10 HEPES and 0.1% bovine serum albumin at pH 7.4. After 40 minutes loading, coverslips with cells were washed and then transferred to a chamber on the stage of an Olympus IX81 microscope for recording. Cells were excited alternatively at 340 and 380 nm and recorded with a dynamic image analysis system (XCellence Imaging software; Olympus srl, Milan, Italy). Results were expressed as the percentage of

increase in $R_{340/380}$ over the baseline (% Change in $R_{340/380}$), and each effect was normalized to the maximum effect induced by ionomycin (5 μM) [276]. For whole-cell patch-clamp recordings, coverslips with cells were transferred to a recording chamber (1 mL volume), mounted on the platform of an inverted microscope (Olympus CKX41) and superfused at a flow rate of 2 mL/min with a standard extracellular solution at pH 7.4 (adjusted with NaOH) containing (in mM): 10 HEPES, 10 D-glucose, 147 NaCl, 4 KCl, 1 $MgCl_2$, and 2 $CaCl_2$. Borosilicate glass electrodes (Harvard Apparatus, Holliston, MA, USA) were pulled with a Sutter Instruments puller (model P-87) to a final tip resistance of 4-7 MΩ. Pipette solution used for HEK293 cells contained (in mM): 134 K-gluconate, 10 KCl, 11 EGTA, 10 HEPES (pH adjusted to 7.4 with KOH). When recordings were performed on rat DRG neurons, the extracellular solution contained 5 mM CaCl2, and pipette solution contained (in mM): 120 CsCl, 3 Mg_2ATP, 10 BAPTA, 10 HEPES-Na (pH adjusted to 7.4 with CsOH). Data were acquired with an Axopatch 200B amplifier (Axon Instruments, CA, USA), stored and analysed with a pClamp 9.2 software (Axon Instruments). All the experiments were carried out at room temperature (20-22°C). Currents were detected as inward currents activated on cell superfusion with the various stimuli in the voltage-clamp mode (holding potential of −60 mV). Cell membrane capacitance was calculated in each cell throughout the experiment by integrating the capacitive currents elicited by a ±10 mV voltage pulse. Peak currents were normalized to cell membrane capacitance and expressed as mean of the current density (pA/pF) in averaged results. Signals were sampled at 1 kHz and low-pass filtered at 10 kHz.

Cells and neurons were challenged with safranal (0.1-300 μM), picrocrocin (1-300 μM) and crocin (30-200 μM). Allyl-isothiocyanate (AITC, 1-10 μM) and GSK1016790A (0.05-0.1 μM) were used to induce TRPA1 and a TRPV4 selective response respectively. Capsaicin (0.1-1 μM) was used to induce a TRPV1 selective response and to identify capsaicin-sensitive neurons. Buffer solution containing dimethyl sulfoxide (DMSO, 1%) was used as vehicle. The activating peptide for human proteinase-activated receptor 2 (hPAR2-AP; 100 μM) or KCl (40-80 mM) were used to elicit a TRP-independent cellular response. Some experiments were performed in the presence of TRPA1, TRPV1 and TRPV4 selective antagonists, HC-030031 (50 μM), capsazepine (10 μM) and HC-067047 (30 μM) respectively, or their vehicles (0.5% or 0.1% or 0.3% DMSO respectively).

Organ bath assay

Rat urinary bladder was excised from rat, and longitudinal strips were suspended at a resting tension of 1 g in 10-mL organ bath bathed in aerated (95% O_2 and 5% CO_2) Krebs-Henseleit solution maintained at 37°C containing (in mM): 119 NaCl, 25 $NaHCO_3$, 1.2 KH_2PO_4, 1.5 $MgSO_4$, 2.5 $CaCl_2$, 4.7 KCl and 11 D-glucose. After 40 minutes of equilibration, tissues were challenged twice with carbachol (CCh, 1 μM), with a 45-minute washing out period between the two administrations. Motor activity was recorded on a force transducer isometrically (Harvard Apparatus, Ltd, Kent, UK). Tissues were challenged with safranal (10-300 μM), AITC (100 μM), GSK1016790A (10 μM) and capsaicin (0.3 μM) or their vehicles. In some experiments, tissues were pre-exposed to HC-030031 (50 μM), capsazepine (10 μM), HC-067047 (30 μM) or a combination of NK1 and NK2 receptor antagonists, L-733,060 and SR48968 respectively (both 1 μM). Some preparations were desensitized by treatment with a high concentration of capsaicin (10 μM for 20 minutes, twice) or were exposed to safranal (300 μM for 20 minutes, twice) before the challenge with other stimuli. Responses were expressed as percentage (%) of the maximum contraction, induced by CCh (1 μM).

CGRP-like immunoreactivity assay

For CGRP-like immunoreactivity (CGRP-LI) outflow, 0.4-mm slices of rat or mouse spinal cord were superfused with an aerated (95% O_2 and 5% CO_2) Krebs-Henseleit solution modified with 0.1% bovine serum albumin plus the angiotensin-converting enzyme inhibitor, captopril (1 μM) and the neutral endopeptidase inhibitor, phosphoramidon (1 μM) to minimize peptide degradation. Fractions (4 mL) of superfusate were collected at 10-minute intervals before, during and after administration of the stimuli and then freeze-dried, reconstituted with assay buffer, and analysed for CGRP-LI using a commercial enzyme-linked immunosorbent assay kit (Bertin Pharma, Montigny le Bretonneux, France). Detection limits of the assays were 5 pg/mL. Stimuli did not cross-react with CGRP antiserum. Tissues were stimulated with safranal (10-300 μM) or its vehicle (1% DMSO). Some tissues were pre-exposed to capsaicin (10 μM, 30 minutes) or superfused with a calcium-free buffer containing EDTA (1 mM). Some preparations were pre-exposed to TRPA1 antagonist, HC-030031 (50 μM), capsazepine (10 μM) or HC-067047 (30 μM). Other tissues were pre-exposed to safranal (100 μM, 30 minutes) and then, after a prolonged washing (40 minutes), stimulated with AITC (50 μM) capsaicin (0.1 μM) or GSK1016790A (10 μM). Results were expressed as femtomoles of peptide *per* gram of tissue.

Acute nociceptive response

The acute nociception was assessed in C57BL/6, *Trpa1*$^{+/+}$ and *Trpa1*$^{-/-}$, *Trpv1*$^{+/+}$ and *Trpv1*$^{-/-}$ *or Trpv4*$^{+/+}$ and *Trpv4*$^{-/-}$ mice, after intraplantar (i.pl.) injection (20 μL/paw) of safranal (0.2-20 nmol), AITC (10 nmol), capsaicin (0.2 nmol), GSK1016790A (2 nmol) or their vehicle (7% and 0.5% DMSO). Immediately after injection, mice were placed individually in plexiglas chambers and the amount of time (seconds) spent licking and shaking the injected paw was recorded for a 5-minute time period, as previously described [159]. Nociception induced by safranal (20 nmol) was also evaluated 60 minutes after intraperitoneal (i.p.) treatment with HC-030031 (100 mg/kg) or 30 minutes after capsazepine (4 mg/kg) or HC-067047 (10 mg/kg) or their vehicle (all, 4% DMSO plus 4% tween 80 in isotonic saline, 0.9% NaCl). In another experimental setting, safranal (0.5-1 mg/kg, i.g.) was administered each day for 5 consecutive days [235]. Each day, 60 minutes after safranal i.g. administration, AITC (10 nmol), capsaicin (0.2 nmol) or GSK1016790A (2 nmol) or their vehicles (0.5% DMSO) were administered (20 μL, i.pl.) and acute nociceptive response was recorded.

Drugs and reagents

HC-030031 [2-(1,3-dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-7H-purin-7-yl)-*N*-(4-isopropylphenyl) acetamide] was synthesized as previously described [206]. If not otherwise indicated, all other reagents were obtained from Sigma-Aldrich (Milan, Italy).

Statistical analysis

Statistical analysis was performed using the unpaired two-tailed Student's *t* test for comparisons between two groups and the one- or two-way ANOVA, followed by the post-hoc Bonferroni's test for comparisons of multiple groups (GraphPad Prism version 5.00, San Diego, CA, USA). A $P < 0.05$ was considered statistically significant. Data are reported as mean ± SEM.

3.2. Results

3.2.1. Safranal and picrocrocin, but not crocin, selectively activate the human TRPA1 channel

Safranal and picrocrocin evoked a concentration-dependent calcium response in hTRPA1-HEK293 (EC_{50}s 17 ± 0.3 μM and 56 ± 0.3 μM respectively), but not in naïve HEK293 cells (Figure III-1B). The selective TRPA1 antagonist HC-030031 attenuated the response evoked by safranal and picrocrocin and AITC (Figure III-1B). Crocin, the third main constituent of saffron was not investigated in this test because of its intense yellow color which interferes with the recording system. Thus, the ability of crocin to target TRPA1 was analysed by whole-cell patch-clamp electrophysiology. Crocin did not evoke any measurable inward current in hTRPA1 HEK293, compared to its vehicle (Figure III-1C).

Further investigation was limited to safranal, the most potent of the three compounds. The non-electrophilic agonist, menthol, evoked a robust calcium response in 3C/K-Q hTRPA1 HEK293 cells, which express a mutant form of the TRPA1 receptor, lacking three key cysteine (C619, C639, C663) and one lysine (K708) residues, which are required for channel activation by electrophilic agonists [149,151] (Figure III-1D). However, safranal failed to induce any calcium response in 3C/K-Q hTRPA1 HEK293 cells (Figure III-1D). Finally, in IMR90 cells, which constitutively express the native human TRPA1 receptor [106], safranal and picrocrocin evoked concentration-dependent calcium responses (EC_{50}s 9 ± 0.2 μM and 44 ± 0.4 μM respectively) that were attenuated by HC-030031, alike the response evoked by AITC (Figure III-1E).

Similar results were obtained in whole-cell patch-clamp recording experiments. Safranal elicited concentration-dependent inward currents in hTRPA1-HEK293 cells, an effect that was attenuated by HC-030031 and was absent in untransfected HEK293 cells (Supplementary Figure III-S1A). Either safranal or picrocrocin failed to evoke calcium responses or inward currents (safranal) in HEK293 cells transfected with the human TRPV1 (hTRPV1 HEK293) or TRPV4 (hTRPV4 HEK293) that, however, were efficiently stimulated by their selective agonists, capsaicin and GSK1016790A respectively (Supplementary Figure III-S1B,C,D,E). In all experiments, calcium responses elicited by hPAR2-AP and ion currents evoked by KCl were not affected by HC-030031, indicating selectivity (Figure III-1B,E and Supplementary Figure III-S1A).

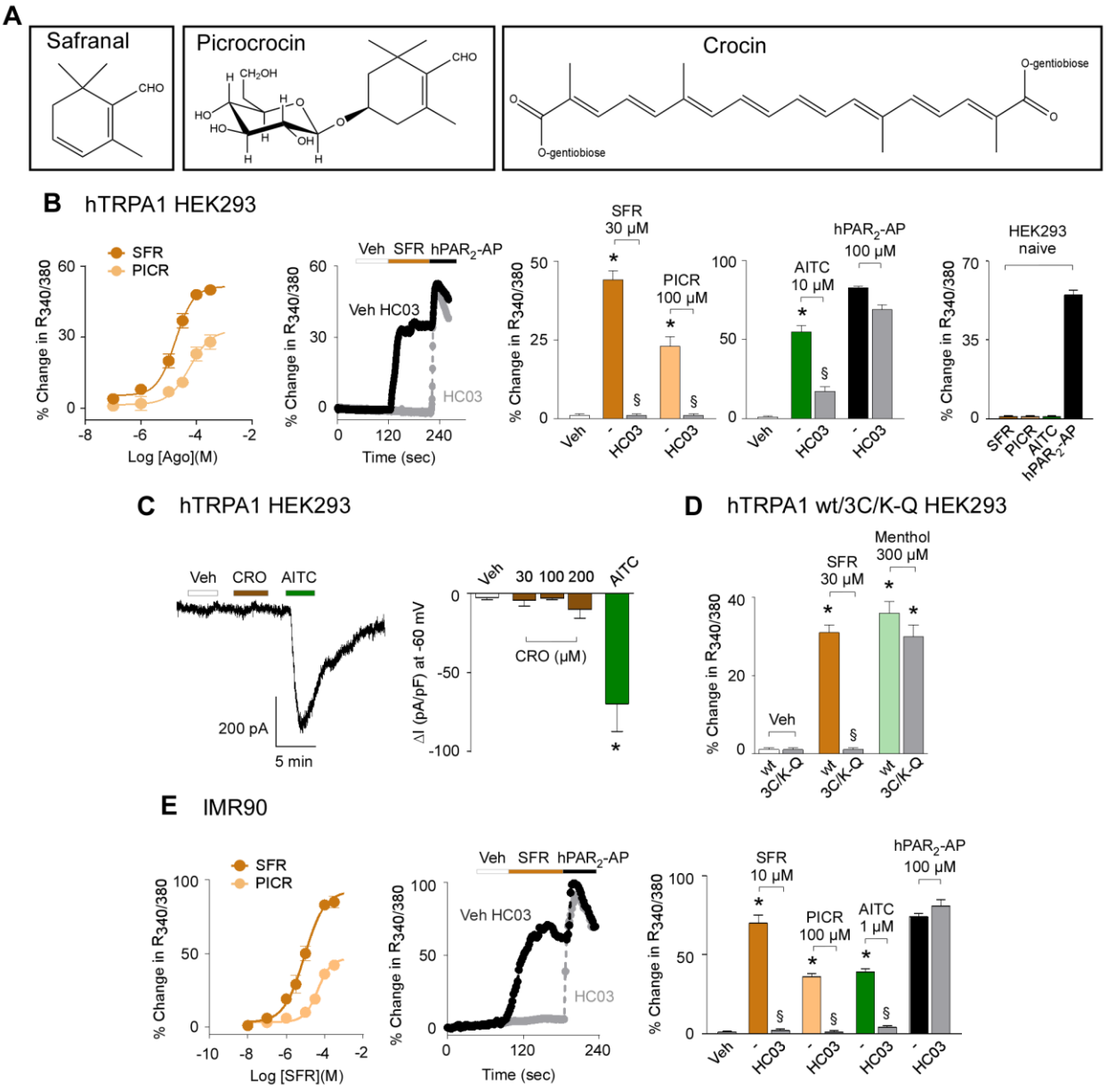

Figure III-1. Safranal (SFR) and picrocrocin (PICR) selectively target the human TRPA1 channel. A) Chemical structures of SFR, PICR and crocin (CRO). **B)** Concentration response curves of the calcium mobilization evoked by SFR and PICR in hTRPA1 HEK293 cells. Representative traces and pooled data of calcium response evoked by SFR, PICR and AITC in hTRPA1 HEK293 pre-exposed to HC-030031 (HC03, 30 µM) or its vehicle (-) and in naïve HEK293 cells. **C)** Representative traces and pooled data of whole-cell patch-clamp inward currents evoked by CRO and AITC (100 µM) in hTRPA1 HEK293. **D)** Pooled data of calcium responses evoked by SFR and menthol in wild-type (wt) and mutant (3C/K-Q) hTRPA1 HEK293 transfected cells. **E)** Concentration response curves of the calcium mobilization evoked by SFR and PICR in IMR90 cells. Representative traces and pooled data of calcium responses evoked by SFR, PICR and AITC pre-exposed HC03 (30 µM) or its vehicle (-) in IMR90 cells. HC03 does not affect the response evoked by the hPAR2-AP (100 µM). Veh is the vehicle of SFR, dash (-) indicates the vehicle of HC03. Data are mean ± SEM of n > 20 cells from 4 to 6 independent experiments (B, D, E) and n > 3 cells from 3 to 5 independent experiments (C). **P*<0.05 vs veh, §*P*<0.05 vs SFR, PICR or AITC; one-way ANOVA with Bonferroni post-hoc correction.

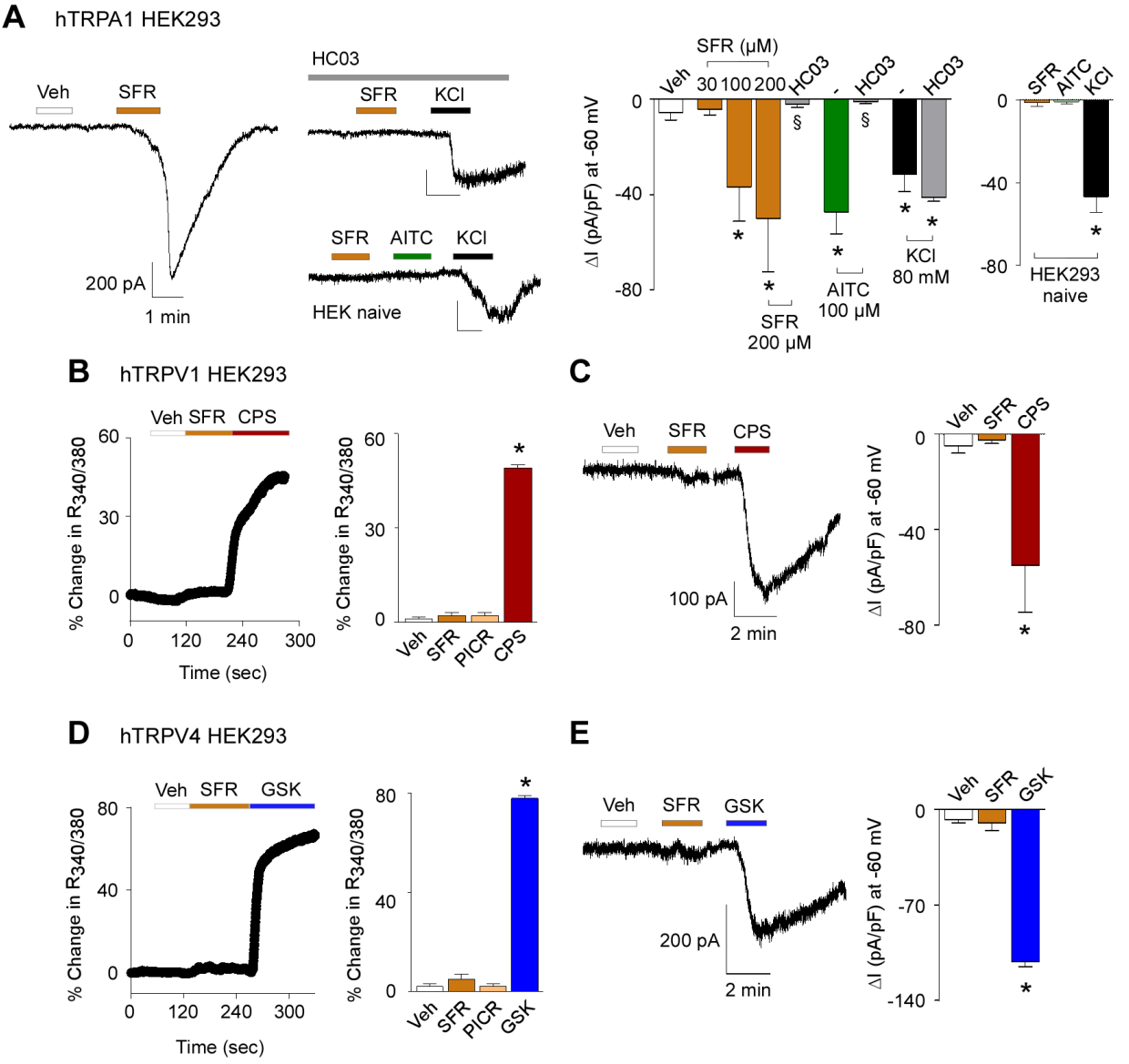

Figure III-S1. A) Representative traces and pooled data of whole-cell patch-clamp inward currents evoked by SFR, AITC and KCl in hTRPA1 HEK293 cells pre-exposed to HC-030031 (HC03, 30 μM) or its vehicle (-) and in naïve HEK293 cells. **B, C)** Representative traces and pooled data of calcium responses and whole-cell patch-clamp inward currents evoked by SFR (100 μM), PICR (200 μM) and capsaicin (CPS; 1 μM) in hTRPV1 HEK293. **D, E)** Representative traces and pooled data of calcium responses and whole-cell patch-clamp inward currents evoked by SFR (100 μM), PICR (200 μM) and GSK1016790A (GSK; 0.5-1 μM) in hTRPV4 HEK293. Veh is vehicle of SFR. Data are mean ± SEM of n > 4 cells from 4-6 independent experiments (A, C, E) and n > 30 cells from 4-6 independent experiments (B, D). **P*<0.05 *vs.* veh; §*P*<0.05 *vs.* SFR and AITC; one-way ANOVA followed by Bonferroni test.

3.2.2. Safranal selectively excites TRPA1 in rodent sensory neurons

Exposure to safranal of cultured rat DRG neurons evoked a concentration-dependent calcium response in a subset of cells, identified as nociceptors by their ability to respond to KCl, capsaicin and AITC [159] (Figure III-2A). In rat DRG neurons maximum calcium responses to safranal or AITC were 35.8% ± 8.5% and 56.0% ± 6.0% of ionomycin (n = 25, $P < 0.01$) respectively, and EC_{50}s were 38 ± 0.03 μM and 5 ± 0.3 μM respectively. Calcium responses were attenuated by HC-030031, and unaffected by the respective TRPV1 and TRPV4 selective antagonists, capsazepine and HC-067047 (Figure III-2A). Safranal or AITC elicited inward currents in rat DRG neurons, that were blocked by HC-030031 (Figure III-2B), which, however, did not affect currents evoked by capsaicin, indicating selectivity (Figure III-2A,B). Safranal and AITC produced calcium responses in DRG neurons isolated from *Trpa1*$^{+/+}$ mice, but not in neurons isolated from *Trpa1*$^{-/-}$ mice (Figure III-2C), while the response to capsaicin was unchanged in both mice strains.

3.2.3. Safranal causes neuropeptides release and acute pain via TRPA1 activation in nociceptors

There is evidence that TRPA1 is localized in peptidergic primary sensory neurons [105,126]. Central and peripheral endings of primary sensory neurons are widely expressed in most tissues and organs, including rat urinary bladder and rat or mouse spinal cord, where upon stimulation they release proinflammatory neuropeptides, such as CGRP and SP. SP released upon stimulation of TRPV1 or TRPA1 results in a contractile response of isolated strips of rat urinary bladder that is mediated by activation of the NK1 and NK2 receptors for SP in bladder smooth muscle cells [283].

Safranal caused a concentration-dependent contractile response of isolated strips of rat urinary bladder with a slightly lower potency than AITC (EC_{50}s were 76 ± 0.07 μM and 56 ± 0.02 μM respectively) (Figure III-3A). Efficacy of safranal was also lower than that of AITC (maximum response, 15.5% ± 3.0% of carbachol, n = 6, and 35.1% ± 6.2% of carbachol respectively, n = 6, $P < 0.05$) (Figure III-3A). The response of safranal was attenuated by pre-exposure to a combination of NK1 and NK2 receptor antagonists (L-733,060 and SR48968 respectively), by pre-exposure to a high concentration of capsaicin able to desensitize the nociceptors and by pre-exposure to the TRPA1 selective antagonist, HC-030031, while it was unaffected by pre-exposure to capsazepine (TRPV1

antagonist) and HC-067047 (TRPV4 antagonist) (Figure III-3A). This finding supports the hypothesis that safranal behaves as a partial agonist at the TRPA1 channel.

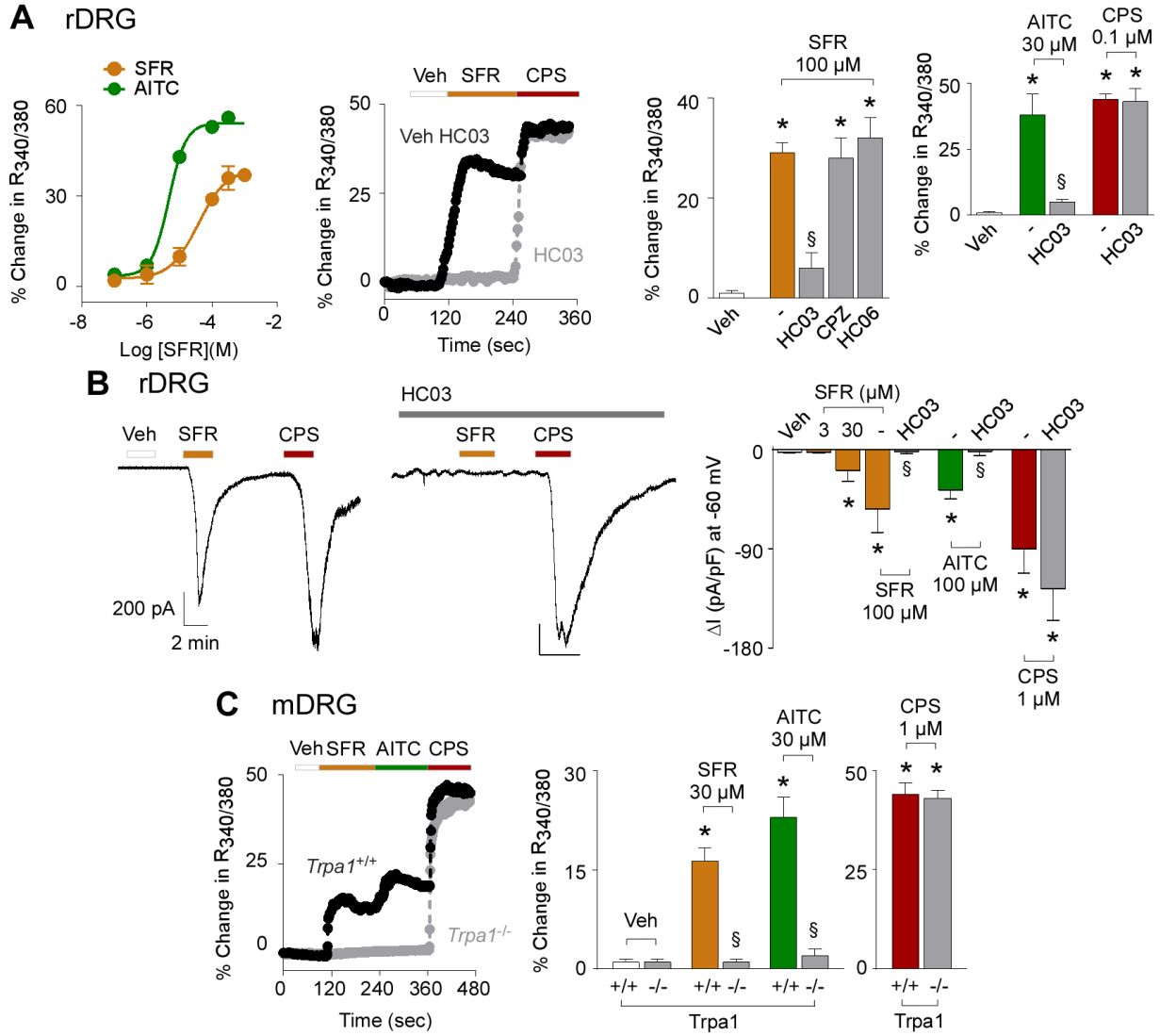

Figure III-2. Safranal (SFR) selectively activates TRPA1 in rodent primary sensory neurons. **A)** Concentration response curves of the calcium mobilization evoked by SFR and AITC in rat DRG (rDRG) neurons. Representative traces and pooled data of calcium response evoked by SFR, AITC and capsaicin (CPS) in rDRG neurons pre-exposed to HC-030031 (HC03, 50 µM), capsazepine (CPZ; 10 µM), HC-067047 (HC06; 30 µM) or their vehicles (-). **B)** Representative traces and pooled data of whole-cell patch-clamp inward currents evoked by SFR, AITC and CPS in rDRG neurons. HC03 does not affect the responses evoked by CPS. **C)** Representative traces and pooled data of the calcium responses evoked by SFR or AITC in mouse DRG (mDRG) neurons from *Trpa1*$^{+/+}$ and *Trpa1*$^{-/-}$ mice. Veh is the vehicle of SFR. Dash (-) indicates vehicles of the different treatments. Data are mean ± SEM of n > 20 cells from 4 to 6 independent experiments (A, C) and n > 3 cells from 3 to 5 independent experiments (B). **P*<0.05 vs veh; §*P*<0.05 vs SFR or AITC; one-way ANOVA with Bonferroni post-hoc correction.

Safranal elicited a concentration-dependent increase in CGRP-LI outflow from rat spinal cord slices which was absent in a calcium-free medium or in tissues pre-exposed to a desensitizing concentration of capsaicin and in the presence of HC-030031 (Figure III-3B). Safranal-evoked CGRP release was unaffected by capsazepine or HC-067047 (Figure III-3B). Exposure to safranal increased CGRP outflow from dorsal spinal cord slices obtained from *Trpa1*$^{+/+}$ mice, but not from tissues taken from *Trpa1*$^{-/-}$ mice (Figure III-3C). Thus, safranal elicits CGRP release from a subset of TRPV1-positive neurons via a neurosecretory process, mediated by TRPA1.

These *ex vivo* findings were replicated in an *in vivo* setting. Injection of safranal (0.2-20 nmol) into the mouse paw (20 µL, i.pl.) evoked a dose-dependent acute nociceptive response (Figure III-3D). The response evoked by the highest dose (20 nmol) of safranal was attenuated by pretreatment with systemic administration HC-030031 (100 mg/kg, i.p.) and was absent in *Trpa1*$^{-/-}$ mice (Figure III-3E,F). Selectivity of safranal action for TRPA1 was strengthened by the observation that the nociceptive response evoked by i.pl. safranal (20 nmol) in *Trpv1*$^{+/+}$ and *Trpv4*$^{+/+}$ was maintained in *Trpv1*$^{-/-}$ and *Trpv4*$^{-/-}$ mice (Figure III-3G,H). Similarly, the nociceptive response to safranal was unaffected by pretreatment with TRPV1 and TRPV4 antagonists (capsazepine and HC-067047 respectively) (Figure III-3E).

3.2.4. *In vitro* and *in vivo* exposure to safranal attenuated TRPA1-mediated responses

In hTRPA1-HEK293 transfected cells, inward currents evoked by AITC underwent a concentration-dependent attenuation after preexposure to increasing concentrations of safranal (Figure III-4A), whereas KCl-evoked currents were not affected (Figure III-4A). In cultured rat DRG neurons, pre-exposure to safranal reduced inward currents evoked by AITC, but not currents evoked by capsaicin, suggesting that safranal promotes selective TRPA1 desensitization (Figure III-4B). Specific desensitization of TRPA1 was also observed in organ bath experiments. Pre-exposure to safranal reduced contractile responses of rat urinary bladder strips evoked by safranal and AITC, but not those evoked by capsaicin, GSK1016790A or carbachol (Figure III-4C). Finally, pre-exposure to safranal was able to markedly reduce CGRP release from rat spinal cord evoked by AITC, without affecting the release evoked by capsaicin or GSK1016790A (Figure III-4D).

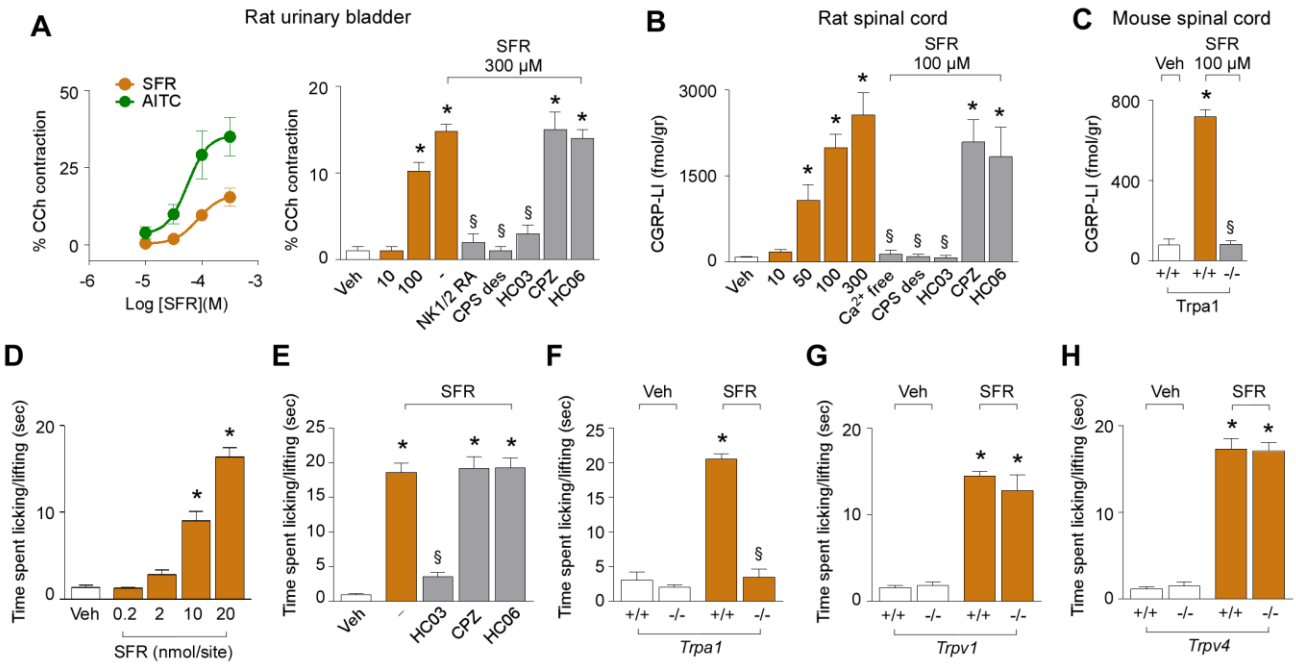

Figure III-3. Safranal (SFR) activating TRPA1 in nociceptors causes neuropeptides release and pain. A) Concentration response curves of the contractile response induced by SFR and AITC in rat urinary bladder. Pooled data of the contractile response evoked by SFR in rat urinary bladder pre-exposed to a combination of NK1/NK2 receptor antagonists (NK1/2 RA; L-733,060 and SR48968, both 1 µM), to a high concentration of capsaicin (CPS des; 10 µM), HC-030031 (HC03, 50 µM), capsazepine (CPZ; 10 µM), HC-067047 (HC06; µM) or their vehicles (-). **B)** Pooled data of CGRP release evoked by SFR from rat dorsal spinal cord after calcium removal (Ca^{2+} free), or after capsaicin desensitization (CPS des; 10 µM), or exposed to HC03 (50 µM), CPZ (10 µM) or HC06 (30 µmol/L). **C)** CGRP release evoked by SFR from dorsal spinal cord from *Trpa1*$^{+/+}$ and *Trpa1*$^{-/-}$ mice. **D)** Dose-dependent acute nociceptive response evoked by intraplantar (i.pl.) injection (20 µL) of SFR in C57BL/6 mice. **E)** Pooled data of the acute nociceptive response evoked by SFR (20 nmol, i.pl.) after intraperitoneal (i.p.) HC03 (100 mg/kg), CPZ (4 mg/kg), HC06 (10 mg/kg) or their vehicles (-, 4% DMSO, 4% tween 80 in 0.9% NaCl). **F-H)** Acute nociceptive response evoked by SFR (20 nmol, i.pl.) in *Trpa1*$^{+/+}$ and *Trpa1*$^{-/-}$, *Trpv1*$^{+/+}$ and *Trpv1*$^{-/-}$, and *Trpv4*$^{+/+}$ and *Trpv4*$^{-/-}$ mice. Veh is the vehicle of SFR. Dash (-) indicates vehicles of treatments. Data are mean ± SEM of 4-6 independent experiments (A-C) and n = 6 mice per group (D-H). **P*<0.05 vs Veh; §*P*<0.05 vs SFR; one-way ANOVA with Bonferroni post-hoc correction.

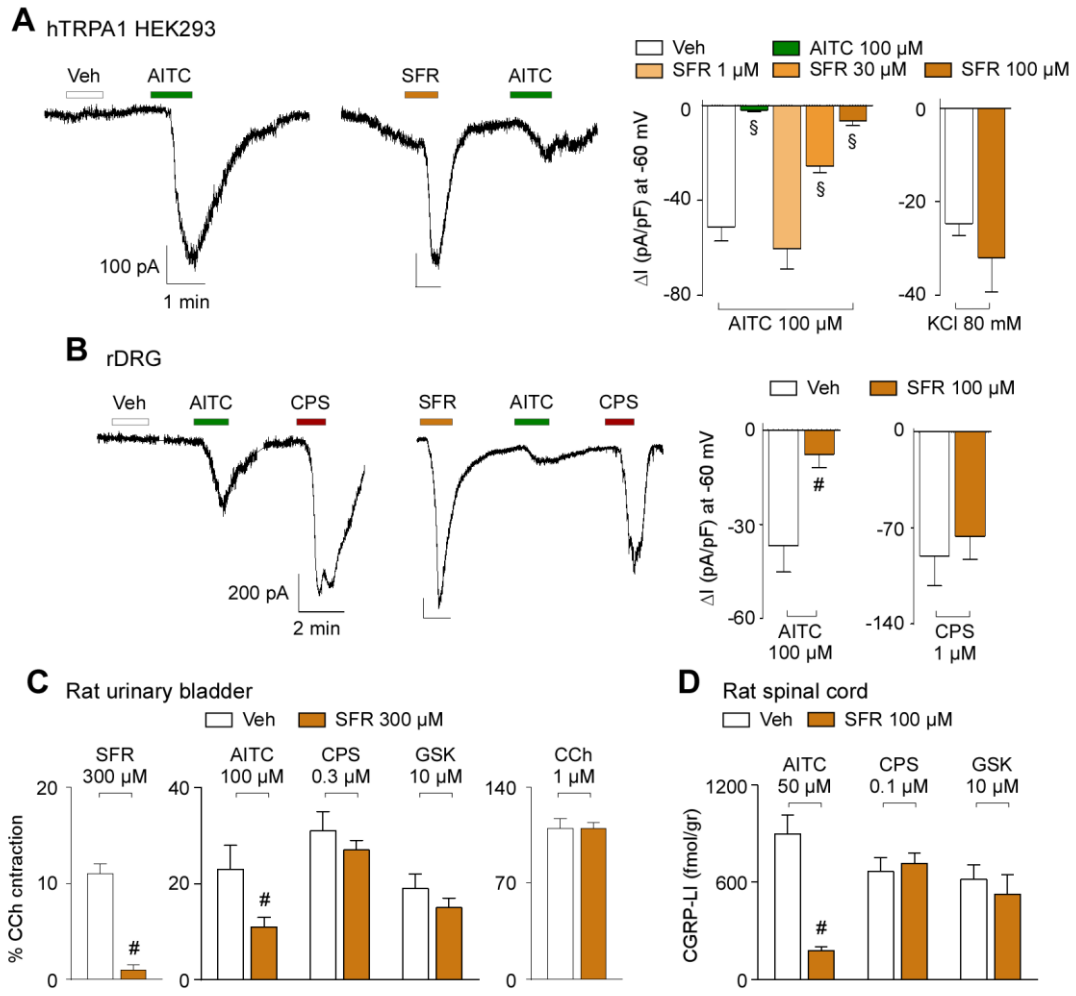

Figure III-4. Safranal (SFR) causes desensitization. A) Representative traces and pooled data of whole-cell patch-clamp inward currents of the concentration dependant desensitization induced by SFR in response to AITC and KCl in hTRPA1 HEK293. **B)** Representative traces and pooled data of whole-cell patch-clamp inward currents of the desensitization induced by SFR in response to AITC and capsaicin (CPS) in rat DRG (rDRG) neurons. **C)** Pooled data of the desensitization induced by SFR in the contractile response evoked by SFR, AITC, CPS, GSK and carbachol (CCh). **D)** Pooled data of the desensitization induced by SFR in CGRP-LI release from rat spinal cord evoked by AITC, CPS and GSK. Veh is the vehicle of SFR. Data are mean ± SEM of n > 3 cells from 3-5 independent experiments (A, B) and 4-6 independent experiments (C, D). §P<0.05 vs AITC, SFR; one-way ANOVA followed by Bonferroni test. #P<0.05 vs AITC and SFR, Student's t test.

To test whether pre-exposure to safranal could desensitize TRPA1-mediated responses *in vivo*, safranal was administered by the intragastric route (i.g.) at two different doses (0.5-1 mg/kg). One single i.g. administration of either doses (day 1) did not affect the ability of local (i.pl.) AITC, capsaicin or GSK1016790A to evoke acute nociceptive responses (Figure III-5A, B, C). After administration of the two doses of safranal for 3 consecutive days the nociceptive responses evoked by i.pl. AITC were slightly reduced, but not those evoked by capsaicin and GSK1016790A (Figure III-5A, B, C). After administration of the two doses of safranal for 5 consecutive days the nociceptive responses evoked by i.pl. AITC were markedly attenuated, the effect of the highest dose of safranal being more pronounced (Figure III-5A). Nociceptive responses evoked by both capsaicin and GSK1016790A were unaffected (Figure III-5B,C).

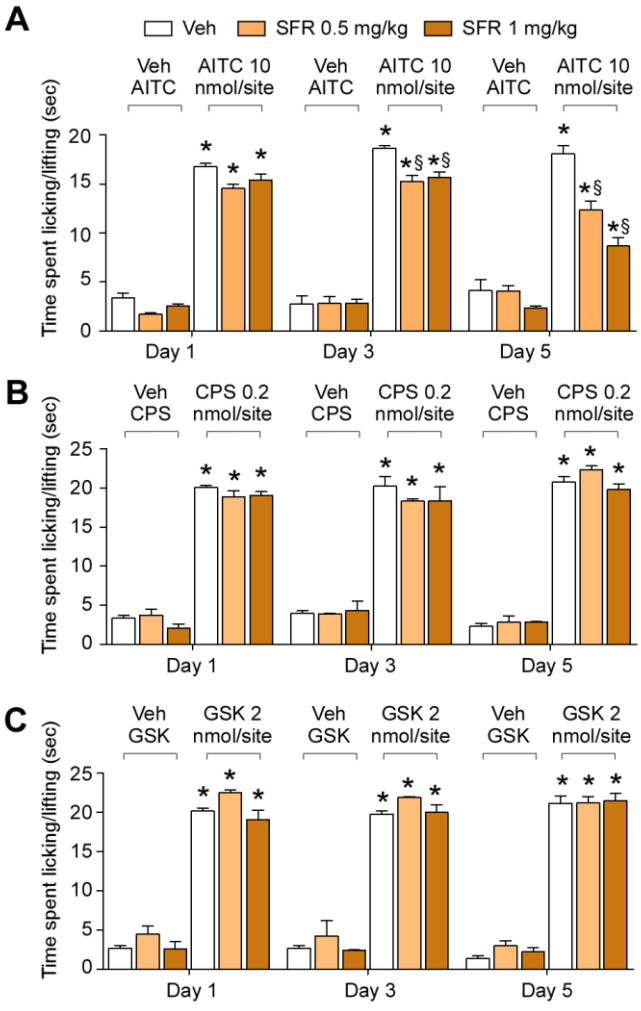

Figure III-5. Repeated treatment with systemic safranal (SFR) causes TRPA1 desensitization. A-C) Pooled data of the acute nociceptive response induced by intraplantar (20 µL) AITC (A), capsaicin (CPS) (B) or GSK1016790A (GSK) (C) after daily intragastric administration of SFR (0.5-1 mg/kg) in C57BL/6 mice. Veh is vehicle of SFR. Data are mean ± SEM of n = 6 mice per group. **P*<0.05 vs veh; §*P*<0.05 vs AITC; two-way ANOVA with Bonferroni post-hoc correction.

3.3. Discussion

Present results show that two of the three main constituents of saffron, safranal and its precursor, picrocrocin, target the mouse, rat and human (native and recombinant) TRPA1, whereas the third constituent, crocin, was completely inactive. The lower potency of picrocrocin compared to safranal may depend on various factors. The presence of two β substituents on the double bond and chemical instability as compared to safranal [299] could explain the lower reactivity of picrocrocin. The failure to stimulate TRPV1 or TRPV4 channels indicates that nociceptor activation by safranal is selectively mediated by TRPA1. Interestingly, safranal failed to evoke any calcium response in HEK293 cells transfected with mutant TRPA1 (3C/K-Q) channel, indicating that, similarly to other electrophilic and reactive agonists [149,151], channel activation by the saffron constituent is mediated by specific cysteine/lysine residues. Both potency and efficacy of the calcium response evoked by safranal in cultured rat DRG neurons were lower than that of AITC. In particular, the lower efficacy supports the hypothesis that safranal may act as a partial TRPA1 agonist.

Peptidergic sensory neurons expressing TRPA1 exert a dual afferent and efferent role, because upon stimulation they can both signal pain to the brain and release from their peripheral terminals the neuropeptides SP and CGRP, which mediate neurogenic inflammatory responses [239,282]. One of these responses is the SP-mediated contraction of rat urinary bladder smooth muscle [300], which can be also produced by TRPA1 activation in intramural sensory nerve terminals [283]. Here, we found that safranal produced a contraction mechanistically similar to that induced by AITC, because it was abrogated after exposure to a high, desensitizing, concentration of capsaicin, in the presence of a TRPA1 selective antagonist and in the presence of a combination of NK1 and NK2 receptor antagonists. Notably, the efficacy of safranal to target TRPA1 in the urinary bladder was lower than that of AITC, further supporting a possible partial agonism of the compound.

Results obtained *in vitro* were recapitulated in an *in vivo* setting, where the proalgesic action of safranal was mediated exclusively by TRPA1. Intraplantar injection of safranal elicited a concentration-dependent acute nociceptive response that was TRPA1-dependent, being selectively attenuated by TRPA1 antagonism and gene deletion. However, there is also indication that saffron has beneficial effect in certain pain conditions [245,301]. Furthermore, safranal has been reported to attenuate pain-like responses in animal models of inflammatory and neuropathic pain [235,247,248]. In particular,

safranal attenuated mechanical allodynia and thermal hyperalgesia in a chronic constriction injury model [248], suppressed the second phase of the orofacial pain induced by formalin [302] and the late phase of the carrageenan-induced paw oedema [235]. The apparent contradiction between these findings and the ability of safranal to excite the proalgesic TRPA1 may be explained considering that pre-exposure to safranal of cultured DRG neurons selectively attenuated AITC-evoked responses, without affecting TRPA1-independent responses. *In vitro* results were recapitulated by *in vivo* experiments, where repeated administration (5 days) of i.g. safranal reduced the acute nociceptive responses evoked by local AITC, leaving unaffected the nociceptive responses evoked by TRPV1 or TRPV4 stimulation. Thus, safranal attenuates the afferent function of nociceptors apparently by promoting a process of homologous desensitization of the TRPA1 channel, which might contribute to the antinociceptive properties of saffron. More recently, it has been demonstrated that TRPA1 is expressed by Schwann cells, where it can amplify and sustain macrophage- dependent neuropathic pain [200]. Thus, it is possible that safranal exerts its partial agonist and desensitizing activities at the Schwann cell TRPA1, contributing in this manner to reduce neuropathic pain.

Clinical investigations with saffron has reported headache as a possible adverse reaction [246]. In contrast with this observation, saffron has been used by Indian traditional medicine to treat headaches [234]. TRPA1 is preferentially expressed by peptidergic sensory neurons, and upon its activation evokes the simultaneous release of the proinflammatory and proalgesic neuropeptides, SP and CGRP [152,179]. CGRP is now considered a major contributor of migraine pain as small molecule CGRP receptor antagonists and monoclonal antibodies against CGRP or its receptor have marked beneficial effects in migraine [239,289]. The observation that in rat urinary bladder strips and in rat dorsal spinal cord slices pre-exposure to safranal reduced AITC-evoked SP-mediated contractile responses and CGRP release respectively, without affecting TRPA1-independent responses further suggests that safranal attenuates the efferent and pro-migraine function of peptidergic nociceptors.

We previously showed that upon *in vitro* or *in vivo* exposure to other herbal preparations, such as isopetasin, contained in butterbur [*Petasites hybridus* (L.) Gaertn.] [298], and parthenolide, a major constituent of *Tanacetum parthenium* [276], TRPA1-expressing trigeminal neurons undergo concentration- or dose-dependent desensitization. However, while isopetasin and parthenolide evoked non-selective

desensitization of peptidergic nociceptors as they also attenuated responses mediated by TRPV1 and TRPV4 activation [276,298], safranal seems to selectively reduce responses elicited by TRPA1 agonism. The mechanism of the selective activity of safranal is unknown. Different pharmacokinetic properties or distinct activation of intracellular signalling mechanisms may be the causes of the diverse ability of the three herbal derivatives to desensitize the channel, and these deserve further investigation. Nevertheless, safranal, one major constituent of saffron extract, targets TRPA1 with a lower potency than full agonists and attenuates responses mediated by TRPA1 activation by other stimuli. The dual action of safranal on TRPA1 might contribute to the reported either detrimental or beneficial actions of the compound in animal models of pain and of saffron in humans.

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IV - TRPA1/NOX in the soma of trigeminal ganglion neurons mediates migraine-related pain of glyceryl trinitrate in mice

4.1. Methods

Animals

In vivo experiments were in accordance with the European Union Directive 2010/63/EU guidelines, the Italian legislation (DLgs 26/2014), and the University of Florence research permit #194/2015-PR. The following mouse strains were used: C57BL/6 (male, 20–25 g, 5–6 weeks; Envigo); littermate wild-type (*Trpa1*$^{+/+}$) and TRPA1-deficient (*Trpa1*$^{-/-}$) mice (25–30 g, 5–8 weeks) [143]; wild-type (*Trpv4*$^{+/+}$) and TRPV4-deficient (*Trpv4*$^{-/-}$) mice (25–30 g, 5–8 weeks) [275]; and TRPV1-deficient mice (*Trpv1*$^{-/-}$; B6.129X1-*Trpv1*$^{tm1Jul/J}$) backcrossed with C57BL/6 mice (*Trpv1*$^{+/+}$) for at least 10 generations (Jackson Laboratories, 25–30 g, 5–8 weeks). To selectively delete the *Trpa1* gene in primary sensory neurons, 129STrpa1$^{tm2Kykw/J}$ mice (*floxed TRPA1*, *Trpa1*$^{fl/fl}$, Stock No: 008649; Jackson Laboratories), which possess loxP sites on either side of the S5/S6 transmembrane domains of the *Trpa1* gene, were crossed with hemizygous *Advillin-Cre* male mice [303,304]. The progeny was genotyped by standard PCR for Trpa1 (PCR Protocol 008650, Jackson Laboratories; www.jax.org) and *Advillin-Cre* [303]. Mice negative for *Advillin-Cre* (*Adv-Cre*$^{-}$; *Trpa1*$^{fl/fl}$) were used as control. Successful *Advillin-Cre* driven deletion of TRPA1 mRNA was confirmed by RT-qPCR [305]. Mice were housed in a temperature- and humidity-controlled vivarium (12-h dark/light cycle, free access to food and water, 10 animals per cage). Mice were acclimatized in a quiet, temperature-controlled room (20–22°C) for 1 h before behavioural studies between 9 am and 5 pm. A randomization procedure (http://www.randomizer.org/) was used to allocate animals to treatments. Investigators were blinded to genotype and drug treatments. For logistical reasons up to 18 mice could be studied on any one day. In all cases, control and experimental groups were studied on the same day, and experiments were replicated on

different days to generate results from the required number of mice. Animals were anaesthetized with intraperitoneal (i.p.) ketamine (90 mg/kg) and xylazine (3 mg/kg) and euthanized with inhaled CO_2 plus 10–50% O_2.

Based on our experience in similar experimental settings and on data published by others, we anticipated a standardized difference of 1.8 standard deviations (SDs) between wild-type and *Trpa1*$^{-/-}$ animals 1 h after exposure to GTN and control treatments. Using G*Power (v3.1) [297], we determined that six animals per group were the minimum necessary to detect the size effect in a post hoc t-test with type 1 and 2 error rates of 5 and 20%, respectively. Initial data acquisition then showed that the effect size in some comparisons remained meaningful but smaller than anticipated, ~1.6 SDs. Sample sizes of subsequent experiments were increased in order to maintain power at 80%.

Reagents

Glyceryl trinitrate (GTN 50mg/50ml, Bioindustria L.I.M. S.p.A.) or its vehicle (5% glucose and 1.5% propylene glycol in sterile water) were used. HC-030031 [2-(1,3-dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-7H-purin-7-yl)-N-(4-isopropylphenyl) acetamide] was synthesized as previously described [159]. If not otherwise indicated, reagents were obtained from Sigma-Aldrich. The vehicle, except for GTN or where expressly indicated, was 4% dimethyl sulfoxide (DMSO) plus 4% Tween 80 in isotonic saline, NaCl 0.9%.

Behavioural tests

Mechanical allodynia. Mechanical allodynia was evaluated by applying von Frey filaments to the periorbital region over the rostral portion of the eye (periorbital mechanical allodynia, PMA) [306] or to the posterior hind paw of mice, before (basal threshold) and 24 h after GTN (1, 5 and 10 mg/kg) or vehicle. Some C57BL/6, *Trpa1*$^{+/+}$ and *Trpa1*$^{-/-}$ mice were injected [10 ml/site, subcutaneously (s.c.)] with allyl isothiocyanate (AITC, 10 nmol in 2.5% DMSO in 0.9% NaCl), GTN (10 mg/site), S-nitroso-N-acetylpenicillamine (SNAP, 40 mg/site in 2.5% DMSO in 0.9% NaCl), CGRP (0.5–5 mg/site in 0.9% NaCl) or their vehicles, and PMA was assessed before and 6 h after treatment. Additional C57BL/6 mice received resiniferatoxin (50 mg/kg, s.c.) or its vehicle (10% ethanol and 10% Tween 80 in 0.9% NaCl) [307]. Seven days after resiniferatoxin, when responses (the eye wiping test, see below) to capsaicin were abolished, GTN (10 mg/kg, i.p.)-evoked PMA and H_2O_2 levels were evaluated. TRPA1 antisense (50-TATCGCTCCACATTGCTAC-30) or mismatch control (50-

ATTCGCCTCACATTGTCAC-30) oligonucleotide (TRPA1 antisense or mismatch oligonucleotide)
were administered to C57BL/6 mice by intrathecal injection (5 nmol/5 ml) [308] for four consecutive days. On Day 5, the efficiency of TRPA1 silencing was tested by eye wiping responses to AITC and the Trpa1 mRNA content in trigeminal ganglion neurons, and PMA evoked by GTN (10 mg/kg, i.p.) and H_2O_2 levels were evaluated. *Adv-Cre*$^{+}$; *Trpa1*$^{fl/fl}$ and *Adv-Cre*$^{-}$; *Trpa1*$^{fl/fl}$ received GTN (10 mg/kg, i.p.) or its vehicle and PMA and H_2O_2 levels in trigeminal ganglion neurons (collected 2 h after GTN/vehicle) were evaluated.

The mechanical threshold was calculated by the up-and down paradigm [309]. All the drugs, at the maximum used doses, did not evoke any direct nociceptive/allodynic responses or locomotor impairment. Doses and routes of administration of drugs and their targets are reported in Table IV-1. Times of administration are reported in boxes placed above graphs.

Eye wiping test. The number of eye wiping movements, following the instillation of ye drops of capsaicin (1 nmol/5 ml), AITC (10 nmol/5 ml) or vehicle (2% and 4% DMSO, respectively) to the conjunctiva, was recorded for a 10-min time period [213].

Cell culture and isolation of primary sensory neurons

HEK293 cells (American Type Culture Collection; ATCC® CRL-1573™), cultured according to the manufacturer's instructions, were transiently transfected with the cDNAs (1 mg) codifying for wild-type (hTRPA1-HEK293) or mutant 3C/K-Q human TRPA1 (C619S, C639S, C663S, K708Q; 3C/K-Q hTRPA1-HEK293) [149] using the jetPRIME® transfection reagent (Polyplus-transfection® SA) according to the manufacturer's protocol. Primary trigeminal ganglion neurons were isolated from C57BL/6 or *Trpa1*$^{+/+}$ and *Trpa1*$^{-/-}$ mice, and cultured as previously described [213]. To obtain trigeminal neuronal and satellite glial cell (SGC) mixed cultures or SGC-enriched cultures, the protocol reported previously was used [310].

Calcium imaging assay

Intracellular calcium mobilization, $[Ca^{2+}]_i$, was measured in transfected HEK293 cells and in trigeminal ganglion neurons, as reported previously [213]. Trigeminal ganglion neurons were challenged with GTN (10–300 mM), SNAP (30 mM), AITC (10 mM) or their respective vehicle. Capsaicin (0.1 mM) was used to identify capsaicin-sensitive neurons. Some experiments were performed in the presence of HC-030031 (50 mM), capsazepine (10 mM), HC-067047 (30 mM), carboxy-PTIO (cPTIO, 100 mM) and disulfiram (1 mM)..

Drug	Action	Dose
HC-030031	TRPA1 antagonist	100 mg/kg i.p. [180]; 100 µg/10 µl s.c. [311]; 10 µg/5 µl i.th. [311]
A967079	TRPA1 antagonist	100 mg/kg i.p. [262];10 µg/5 µl i.th. [312]
Capsazepine	TRPV1 antagonist	4 mg/kg i.p. [199]
HC-067047	TRPV4 antagonist	10 mg/kg i.p. [197]
Disulfiram	Aldehyde dehydrogenase inhibitor	100 mg/kg i.p. [313]; 10 µg/10 µl s.c. §; 5 µg/5 µl i.th. §
cPTIO	Nitric oxide scavenger	0.6 mg/kg i.p. [314]; 60 µg/10 µl s.c. [315]; 30 µg/5 µl i.th. [316]
α-lipoic acid	Antioxidant	100 mg/kg i.p. [194]; 5 µg/10 µl s.c. [194]; 10 µg/5 µl i.th. §
PBN	Free-radical spin trap	100 mg/kg i.p. [200]
NAC	HNE sequestering agent	250 mg/kg i.p.; 20 µg/10 µl s.c.; 50 µg/5 µl i.th. [317]
L-Carnosine	HNE sequestering agent	250 mg/kg i.p. [318]
Apocynin	Non-selective NOX inhibitor	100 mg/kg i.p. [319]
Gp91ds-tat	NOX2 inhibitor	10 mg/kg i.p. [200]
ML171	NOX1 inhibitor	60 mg/kg i.p. [200]
GKT137831	NOX1/4 inhibitor	60 mg/kg i.p. [200]
$CGRP_{8-37}$	CGRP receptor antagonist	2 µmol/kg i.p. [162]; 10 nmol/10 µl s.c. §; 5 nmol/5 µl i.th. §
BIBN4096BS	CGRP receptor antagonist	1 mg/kg i.p. [45]; 4 nmol/10 µl s.c. §; 1 µg/5 µl i.th. [320]

Table IV-1. Doses and routes of administration of drugs. § Preliminary experiments showed that this dose did not evoke *per se* any behavioural response.

Buffer solution containing 0.5% DMSO was used as vehicle for SNAP and all inhibitors/scavenger. Wild-type or mutant hTRPA1-HEK293 cells were challenged with GTN (100 mM), menthol (100 mM) or their vehicle

H_2O_2 assay

H_2O_2 was determined by using the Amplex Red® assay (Invitrogen), in C57BL/6 mice, in trigeminal ganglion neurons before or after (0.5–6 h) GTN (10 mg/kg, i.p.), and 2 h after GTN/vehicle in mice pretreated (0.5 h before GTN) with cPTIO (0.6 mg/kg, i.p.) and disulfiram (100 mg/kg, i.p.) or treated (1 h after GTN) with HC-030031 (100 mg/kg, i.p.) and alpha lipoic acid (100 mg/kg i.p.), or desensitized with resiniferatoxin or silenced with TRPA1 antisense or mismatch oligonucleotide; in dorsal horn of brain stem before and after (1 and 2 h, respectively) GTN. In *Trpa1*$^{+/+}$ and *Trpa1*$^{-/-}$, and in *Adv-Cre*$^{+}$; *Trpa1*$^{fl/fl}$ and *Adv-Cre*$^{-}$; *Trpa1*$^{fl/fl}$ mice H_2O_2 was 2 h after GTN. Increase in H2O2 release was quantified in trigeminal neuron-SGCs mixed and SGC-enriched primary culture after challenge with GTN (10, 50 and 100 mM), AITC (30 mM), SNAP (100 mM) or their vehicle (0.3% DMSO for AITC and SNAP) in the presence of HC-030031 (50 mM) or its vehicle (0.5% DMSO in Krebs-Ringer phosphate buffer), or in a Ca^{2+}-free Krebs-Ringer phosphate (KRP) buffer containing EDTA (1mM) or after a pre-exposure to a high concentration of capsaicin (10 mM, 20 min) [321].

Immunofluorescence

Anaesthetized C57BL/6 and *Trpa1*$^{+/+}$ and *Trpa1*$^{-/-}$ mice treated with GTN (10 mg/kg, i.p.) or its vehicle were transcardially perfused with PBS, followed by 4% paraformaldehyde. Trigeminal ganglion neurons and brainstem were removed, postfixed for 24 h, and paraffin embedded or cryoprotected (4°C, overnight) in 30% sucrose. Cryosections (10 mm) of brainstem and formalin fixed paraffin-embedded sections (5 mm) of trigeminal ganglion neurons were incubated with the following primary antibodies: 4-HNE (ab48506, 1:40, HNEJ-2, Abcam), TRPA1 (ab58844, 1:400, Abcam), NOX1 (SAB2501686, 1:250), NOX2 (ab80897, 1:200, Abcam), NOX4 (ab109225, 1:200, Abcam), glutamine synthetase (G2781, 1:400) (1 h, room temperature) diluted in phosphate-buffered saline (PBS) and 2.5% normal goat serum (NGS). Sections were then incubated with fluorescent secondary antibodies: polyclonal Alexa Fluor® 488, and polyclonal Alexa Fluor® 594 (1:600, Invitrogen) (2 h, room temperature) and coverslipped. The analysis of negative controls (non-immune serum) was simultaneously performed to exclude the presence of non-specific immunofluorescent staining, cross-

immunostaining, or fluorescence bleed-through. Tissues were visualized, and digital images were captured using an Olympus BX51 (Olympus srl). 4-HNE staining in trigeminal ganglion was evaluated as the fluorescence intensity measured by an image processing software (ImageJ 1.32 J, National Institutes of Health). Data are expressed as mean fluorescence intensity (% of basal).

4-HNE staining was determined in trigeminal ganglion neurons collected from C57BL/6 mice before (basal level) or after (0.5, 1, 2, 3, 4, 6 h) GTN (10 mg/kg, i.p.) and 4 h after GTN/vehicle administration in C57BL/6 mice pretreated with cPTIO (0.6 mg/kg, i.p. given 0.5 h before GTN) or N-acetyl-L-cysteine (NAC, 250 mg/kg, i.p., given 0.5 h before GTN) or treated with HC-030031 or alpha lipoic acid (both 100 mg/kg i.p., given 3 h after GTN) and in *Trpa1*$^{+/+}$ and *Trpa1*$^{-/-}$ mice, and in dorsal horn of brain stem collected from C57BL/6 mice before and after (1 and 2 h) GTN.

Mouse trigeminal neuron-SGC mixed and SGC-enriched cultures were cultured for 2–3 days, fixed in ice-cold methanol/acetone (5 min, 20°C), washed with PBS and blocked with NGS (10%) (1 h, room temperature). The cells were then incubated with the primary antibodies (NeuN, 1:600, and glutamine synthetase, 1:300) (1 h, room temperature), with fluorescent secondary antibodies (1:600, polyclonal Alexa Fluor® 488, and polyclonal Alexa Fluor® 594, Invitrogen) (2 h, room temperature), mounted and digital images were captured using an Olympus BX51.

Proximity ligation assay

Co-localization of TRPA1 and NOX2 in mouse trigeminal ganglion was obtained using an *in situ* PLA detection kit (Duolink, Olink Biosciences Inc.) as previously described [322]. In trigeminal ganglion sections (5 mm) fluorescence images were obtained using Olympus BX51 and a 100x oil immersion objective. Negative control was performed by omitting primary antibodies or proximity ligation assay (PLA) probes.

Real-time PCR

RNA was extracted from trigeminal ganglion of C57BL/6 mice after TRPA1 antisense or mismatch oligonucleotide (i.th.) and of *Adv-Cre*$^{+}$; *Trpa1*$^{fl/fl}$ and *Adv-Cre*$^{-}$; *Trpa1*$^{fl/fl}$. The standard TRIzol® extraction method was used. The SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad) was used for amplification, and the cycling conditions were the following: samples were heated to 95°C for 1 min followed by 40 cycles of 95°C for 10 sec, and 65°C for 20 sec. PCR reaction was carried out in triplicate, and 18S was the chosen reference gene.

Blood flow experiments

Cutaneous blood flow was assessed using a laser Doppler flowmeter (Perimed Instruments) in anaesthetized in C57BL/6, *Trpa1*$^{+/+}$ and *Trpa1*$^{-/-}$ mice. Cutaneous blood flow was monitored by a probe (cutaneous type) fixed to the shaved periorbital area, before and after the systemic administration of GTN (10 mg/kg, i.p.) or its vehicle. Before (0.5 h) GTN injection, mice were treated with intraperitoneal HC-030031, disulfiram (both, 100 mg/kg) and BIBN4096BS (1mg/kg) or their vehicles and cutaneous blood flow was monitored for at least 0.5 h. Baseline blood flow was calculated by the mean flow value measured during a 5-min period before the stimulus. The increase in cutaneous blood flow was calculated as the percentage change over the baseline.

Statistical analysis

Statistical analysis was performed by the two-tailed Student's *t*-test for comparisons between two groups. A one-way ANOVA followed by the post hoc Bonferroni's test was used for comparisons of multiple groups. For behavioural experiments with repeated measures, the two-way mixed model ANOVA followed by the post hoc Bonferroni's test was used. $P < 0.05$ was considered statistically significant (GraphPad Prism version 5.00).

4.2. Results

4.2.1. GTN evokes NO-mediated TRPA1-independent vasodilatation and TRPA1-dependent allodynia

Administration of GTN (1–10 mg/kg, i.p.) to C57BL/6 mice induced a dose-dependent and prolonged PMA (Figure IV-1A). GTN (10 mg/kg, i.p.) also produced an early and transient (0–10 min) cutaneous increase in blood flow in the periorbital skin (Figure IV-1B). GTN (1 and 10 mg/kg, Figure IV-1C and D, respectively) evoked PMA in *Trpa1*$^{+/+}$ mice, whereas *Trpa1*$^{-/-}$ mice were fully protected. However, the increase in cutaneous blood flow evoked by GTN (10 mg/kg) in *Trpa1*$^{+/+}$ mice was maintained in *Trpa1*$^{-/-}$ mice (Figure IV-1E). Genetic deletion of TRPV1 or TRPV4 channels did not affect GTN-evoked PMA (Supplementary Figure IV-S1A,B). Systemic GTN (10 mg/kg) also induced sustained mechanical allodynia in the hind paw of C57BL/6 and *Trpa1*$^{+/+}$, but not *Trpa1*$^{-/-}$ mice (Supplementary Figure IV-S1C and D). This response, which indicates a general proalgesic action of GTN, was not further investigated.

The TRPA1 antagonist, HC-030031, given (i.p.) 0.5 h before and 1, 3, 4 and 5 h after GTN, transiently and completely reversed PMA at all time points (Figure IV-1F and Supplementary Figure IV-S1E). HC-030031 (1 h after GTN) reversed PMA induced by the lowest dose of GTN (1 mg/kg) (Supplementary Figure IV-S1F). Another channel antagonist, A967079, given 0.5 h before and 1 h after GTN (10 mg/kg) also reversed allodynia (Supplementary Figure IV-S1G). Antagonists of TRPV1 (capsazepine) and TRPV4 (HC-067047), administered before GTN did not affect GTN-induced PMA (Supplementary Figure IV-S1H, I).

Mitochondrial ALDH-2 is known to generate NO from GTN [323]. To determine the contribution of NO to GTN-evoked PMA, mice were treated with the ALDH-2 inhibitor, disulfiram, or the specific NO scavenger, cPTIO. Both disulfiram and cPTIO, when administered (i.p.) 0.5 h before, but not 1 h after, GTN, attenuated GTN-evoked PMA (Figure IV-1F). The ability of disulfiram and cPTIO to prevent PMA if given before GTN indicates that NO is needed to initiate GTN-evoked PMA. However, failure of disulfiram and cPTIO to reverse established PMA suggests that additional mechanisms are required to sustain PMA. Pretreatment with disulfiram, but not with HC-030031 (both i.p., 0.5 h before GTN), attenuated the increase in periorbital blood flow evoked by GTN (10 mg/kg) (Figure IV-1G).

4.2.2. NO, but not GTN, directly targets TRPA1

To determine whether TRPA1 is directly gated by GTN and/or NO, responses to different NO donors were investigated *in vitro*. GTN caused a concentration-dependent increase in $[Ca^{2+}]_i$ in trigeminal ganglion neurons from C57BL/6 and *Trpa1*$^{+/+}$, but not from *Trpa1*$^{-/-}$ mice (Figure IV-2A,B). The TRPA1 antagonist (HC-030031), but not the TRPV1 or TRPV4 antagonists (capsazepine or HC-067047, respectively), abolished responses (Figure IV-2A). Key intracellular cysteine and lysine residues of TRPA1 interact with oxidants and electrophilic agents [149]. Whereas GTN increased $[Ca^{2+}]_i$ in hTRPA1-HEK293, HEK293 cells expressing a mutant 3C/K-Q hTRPA1 were unresponsive (Figure IV-2C). The NO scavenger (cPTIO) or ALDH-2 inhibitor (disulfiram) did not affect GTN signals in neurons from C57BL/6 mice (Figure IV-2D). In contrast, the HC-030031-dependent Ca^{2+}-response to a different NO donor, SNAP, was inhibited in the presence of cPTIO (Figure IV-2D). Thus, GTN activates TRPA1 by targeting specific cysteine and lysine

residues. However, conversely to SNAP, the *in vitro* action of GTN is not mediated through NO release.

Results obtained *in vivo* by local administration of NO donors recapitulated the *in vitro* findings. Injection (s.c.) in the periorbital area of the TRPA1 agonist, AITC, evoked PMA that was reversed by local (s.c.) pretreatment with HC-030031 (Supplementary Figure IV-S1J). Local (s.c.) NO donors, GTN or SNAP, induced PMA in *Trpa1*$^{+/+}$, but not *Trpa1*$^{-/-}$ mice (Figure IV-2E,F). Both local (s.c.) or systemic (i.p.) TRPA1 antagonism by HC-030031 reversed PMA evoked by local GTN or SNAP (Figure IV-2G,H). Systemic (i.p.) disulfiram or cPTIO did not affect PMA evoked by local (s.c.) GTN (Figure IV-2G). However, cPTIO (i.p.) attenuated PMA evoked by local (s.c.) SNAP (Figure IV-2H). Thus, GTN evokes allodynia by mechanisms that depend from the route of administration. Local GTN directly regulates TRPA1 gating, whereas systemic GTN indirectly regulates TRPA1 by a process that involves ALDH-2-mediated liberation of NO.

4.2.3. Oxidative stress and TRPA1 sustain GTN-evoked mechanical allodynia

The NO scavenger (cPTIO) or ALDH-2 inhibitor (disulfiram) prevented GTN-evoked allodynia when administered before GTN, but were ineffective when given after. Thus, NO is necessary to initiate the hypersensitivity condition, but is not sufficient for its maintenance. Since GTN stimulates oxidative stress [324], we hypothesized that NO, liberated from GTN, initiates the process that subsequently sustains TRPA1-dependent PMA via reactive oxygen species generation. Two different reactive oxygen species scavengers, alpha lipoic acid and *N*-tert-butyl-α-phenylnitrone (PBN), reversed PMA only when given (i.p.) 1 h after, but not 0.5 h before GTN (Figure IV-3A,B).

GTN-evoked PMA persisted for ~8 h, but alpha lipoic acid or PBN were unable to reverse PMA 5 h post-GTN (Figure IV-3C), indicating that mediators other than reactive oxygen species are required to sustain hypersensitivity beyond 5 h. 4-HNE is a major electrophilic aldehyde that is generated by free radical attack of polyunsaturated fatty acids [166], and is a TRPA1 agonist [152]. In contrast to reactive oxygen species, which are short-lived, the biological activity of 4-HNE may last for hours [325]. NAC and L-carnosine efficiently scavenge α,β-unsaturated aldehydes, including 4-HNE. NAC or L-carnosine (i.p.), administered before GTN, did not attenuate the first phase of GTN-evoked PMA (2

h), but strongly inhibited later phases (5 h) (Figure IV-3B,C), indicating that carbonylic derivatives more stable than reactive oxygen species sustain the final phase of PMA.

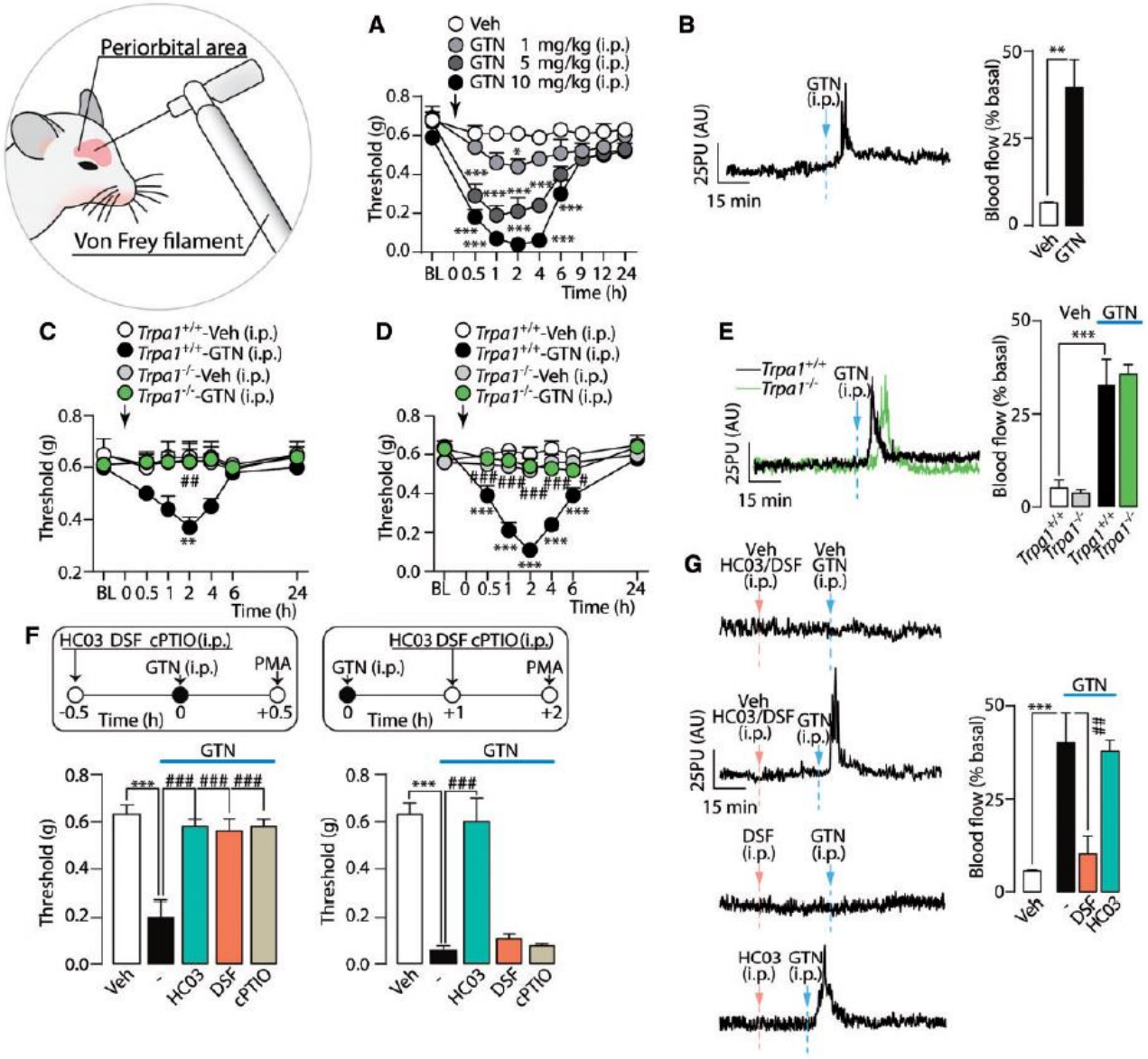

Figure IV-1. GTN induces PMA via a TRPA1-dependent mechanism. A) Dose- and time-dependent PMA evoked by GTN in C57BL/6 mice. **B)** Representative traces and pooled data of the increases in periorbital skin blood flow evoked by GTN (10 mg/kg). PMA evoked by **C)** low (1 mg/kg, i.p.) and **D)** high (10 mg/kg, i.p.) dose of GTN, in *Trpa1*$^{+/+}$ and *Trpa1*$^{-/-}$ mice. **E)** Representative traces and pooled data of the increases in periorbital skin blood flow evoked by GTN (10 mg/kg) in *Trpa1*$^{+/+}$ and *Trpa1*$^{-/-}$ mice. **F)** Pretreatment with systemic (i.p.) HC-030031 (HC03; 100 mg/kg), disulfiram (DSF, 100 mg/kg) or cPTIO (0.6 mg/kg) abates PMA measured 0.5 h after GTN (10 mg/kg). HC03 but not DSF and cPTIO (given after GTN) reduces PMA measured 2 h after GTN. **G)** Representative traces and pooled data of the increases in periorbital skin blood flow evoked by GTN (10 mg/kg). DSF but not HC03, both given before GTN abolishes the increase in blood flow induced by GTN. BL = baseline mechanical threshold; Veh = the vehicle of GTN. Dash (-) indicates combined vehicles of treatments. Arrows indicate times of drug administration. AU = arbitrary units; PU = perfusion units. Error bars indicate mean ± SEM, 6–8 mice per group. **P*<0.05, ***P*<0.01, ****P*<0.001 versus vehicle, *Trpa1*$^{+/+}$-vehicle and #*P*<0.05, ##*P*<0.01, ###*P*<0.001 versus *Trpa1*$^{+/+}$-GTN or GTN; one-way or two-way ANOVA with Bonferroni post hoc correction, and Student's t-test.

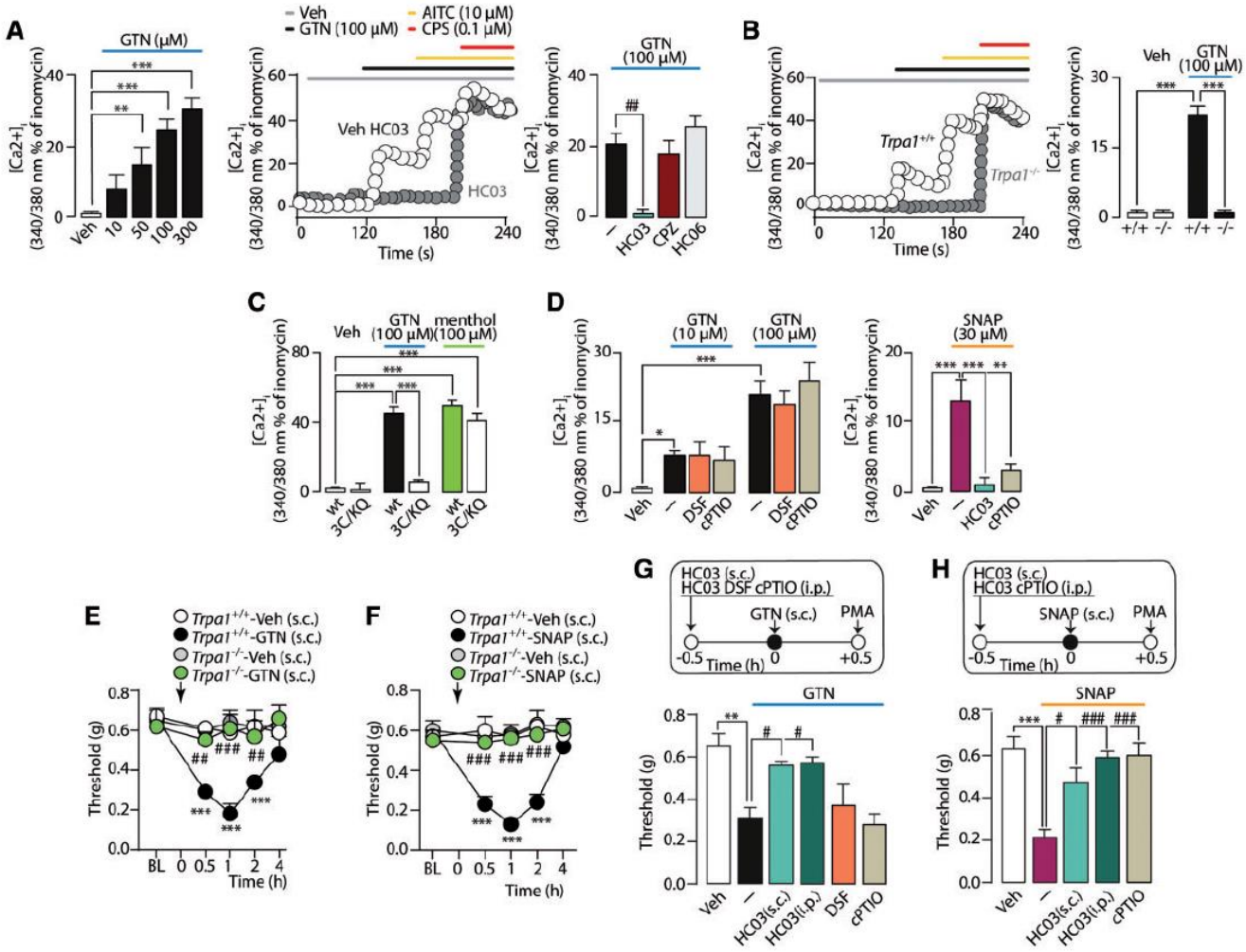

Figure IV-2. GTN-evoked PMA is mediated by NO. A) Ca^{2+}-response to GTN in mouse trigeminal ganglion neurons, which also respond to AITC or capsaicin (CPS), is attenuated by HC-030031 (HC03), but not by HC-067047 (HC06, TRPV4 antagonist) or capsazepine (CPZ, TRPV1 antagonist), and **B)** abated by TRPA1 deletion. **C)** Ca^{2+}-response to GTN or menthol in HEK293-cells expressing wild-type (wt) or mutant (3C/K-Q) human TRPA1. **D)** GTN-evoked Ca^{2+}-response in trigeminal ganglion neurons is unaffected by disulfiram (DSF) or cPTIO while SNAP-evoked Ca^{2+}-response is abated by cPTIO and HC03. Veh = the vehicle of GTN. Dash (-) indicates combined vehicles of treatments. Error bars indicate mean ± SEM of n>15 neurons or 50 cells (A–D). **P*<0.05, ***P*<0.01, ****P*<0.001; one-way ANOVA with Bonferroni post hoc correction. **E, G)** Local (10 ml s.c.) GTN (10 mg) evokes a time-dependent PMA that is abated in *Trpa1*$^{-/-}$ mice and by the pretreatment with local (s.c.) and systemic (i.p.) HC03 (100 mg and 100 mg/kg, respectively), but not with systemic DSF (100 mg/kg, i.p.) and cPTIO (0.6 mg/kg, i.p.). **F, H)** Local (10 ml s.c.) SNAP (40 mg) induces a time-dependent PMA that is abated in *Trpa1*$^{-/-}$ mice and prevented by pretreatment with local (s.c.) and systemic (i.p.) HC03 (100 mg and 100 mg/kg, respectively) and systemic cPTIO (0.6 mg/kg, i.p.). BL = baseline mechanical threshold; Veh = the vehicle of GTN. Dash (-) indicates combined vehicles of treatments. Arrows indicate time of drug administration. Error bars indicate mean ± SEM, 6–8 mice per group. ***P*<0.01, ****P*<0.001 versus *Trpa1*$^{+/+}$-Veh, vehicle and #*P*<0.05, ##*P*<0.01, ###*P*<0.001 versus *Trpa1*$^{+/+}$-GTN, GTN, *Trpa1*$^{+/+}$-SNAP or SNA, one-way or two-way ANOVA with Bonferroni post hoc correction.

4.2.4. GTN does not produce periorbital allodynia by a local mechanism

Local injections of the TRPA1 agonist, AITC, and the NO donors, GTN or SNAP, caused TRPA1-dependent PMA, suggesting the involvement of TRPA1 on cutaneous nerve terminals. However, PMA evoked by systemic GTN was unaffected by local treatment with disulfiram, cPTIO, HC-030031, alpha lipoic acid and NAC (Figure IV-3D,E,F), excluding that systemic GTN activates TRPA1 on cutaneous afferent nerve fibres to induce PMA. Centrally administered TRPA1 antagonists, HC-030031 or A967079 (pre- or post-GTN, i.th.), attenuated PMA-evoked by systemic GTN (Figure IV-3G,H and Supplementary Figure IV-1K). Disulfiram or cPTIO (pre-, but not post-GTN), alpha lipoic acid (post-, but not pre-GTN) and NAC (pre- or post-GTN) (all i.th.) (Figure IV-3G,H,I) also attenuated PMA.

4.2.5. GTN/NO targets TRPA1 in the soma of trigeminal ganglion neurons to generate oxidative stress

Failure of subcutaneous and ability of intrathecal drugs to attenuate GTN-evoked PMA suggested the involvement of central anatomical areas, including the terminals in the dorsal horn of the brainstem and nociceptor cell bodies in the trigeminal ganglion. To determine whether systemic GTN induces oxidative stress in these locations, we measured two markers of oxidative and carbonylic stress, H_2O_2 and 4-HNE, respectively. GTN, which failed to increase H_2O_2 and 4-HNE in brainstem, caused a rapid and transient increase in H2O2 (1–3 h) and a gradual and sustained increase in 4-HNE (4–6 h) in the trigeminal ganglion neurons (Figure IV-4A,B). cPTIO and disulfiram (both pre-GTN) and alpha lipoic acid (post-GTN) blunted the H_2O_2 increase (at 2 h) (Figure IV-4C). cPTIO and NAC (pre-GTN), and alpha lipoic acid (post-GTN) blunted the 4-HNE signal (at 4h) (Figure IV-4D). Unexpectedly, antagonism (HC-030031) or genetic deletion of TRPA1 also attenuated GTN-induced increases in H_2O_2 or 4-HNE (Figure IV-4 C,D,E). These data suggest that oxidative stress generation, which seems to mediate GTN-evoked PMA, is initiated by NO-induced activation of TRPA1 within the trigeminal ganglion.

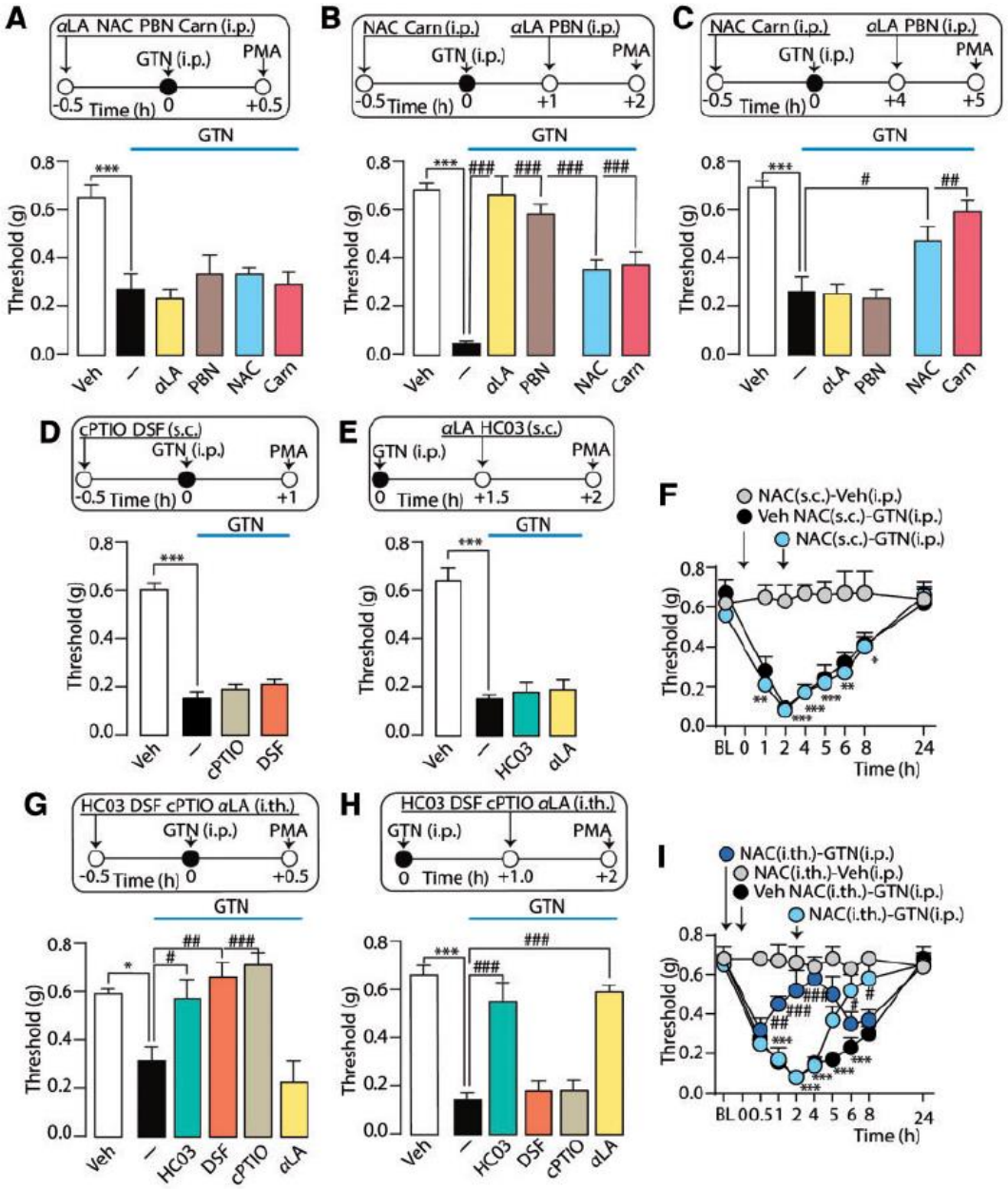

Figure IV-3. Prolonged PMA induced by GTN is mediated by oxidative stress. A) PMA evoked by GTN (10 mg/kg) in C57BL/6 mice is not affected by the alpha lipoic acid (αLA, 100 mg/kg), NAC (250 mg/kg), PBN (100 mg/kg) or L-carnosine (Carn, 200 mg/kg) (all pre-GTN). **B, C)** Alpha lipoic acid and PBN (all post-GTN) reduce PMA measured at 2 h not at 5 h. Pretreatment with NAC and Carn reach the maximum effect in the reduction of PMA, 5 h after GTN. **D)** PMA evoked by systemic (i.p.) GTN (10 mg/kg) is unaffected by local (10 ml, s.c.) cPTIO (60 mg) or disulfiram (DSF; 10 mg) (all pre-GTN), and by **E)** HC-030031 (HC03; 50 mg) and alpha lipoic acid (αLA, 5 mg) (all post-GTN). **F)** Local (10 ml, s.c.)
administration of NAC (20 mg, post-GTN) does not affect GTN-evoked PMA. **G)** PMA evoked by systemic (i.p.) GTN (10 mg/kg) is reversed by intrathecal (5 ml, i.th.) HC03 (10 mg), DSF (5 mg) and cPTIO (30 mg) but not with alpha lipoic acid (10 mg) (all pre-GTN). **H)** Intrathecal (i.th.) HC03 (10 mg) and alpha lipoic acid (10 mg), but not DSF (5 mg) and cPTIO (30 mg), (all post-GTN) reduce PMA measured 2 h after GTN. **I)** Intrathecal (i.th.) NAC (50 mg) (pre- or post-GTN) reduces GTN-evoked PMA. BL = baseline mechanical threshold; Veh = the vehicle of GTN. Dash (-) indicates combined vehicles of treatments. Arrows indicate time of drug administration. Error bars indicate mean ± SEM, 6–9 mice per group. **P*<0.05, ***P*<0.01, ****P*<0.001 versus vehicle, NAC-Veh. #*P*<0.05, ##*P*<0.01, ###*P*<0.001 versus GTN, vehicle NAC-GTN; one-way or two-way ANOVA with Bonferroni post hoc correction.

Sensory neurons and SGCs are present in the trigeminal ganglion (Figure IV-5A). To determine which cell type generates GTN/NO-evoked oxidative stress, C57BL/6 mice were treated with resiniferatoxin, which is known to defunctionalize TRPV1-expressing neurons [307], which co-express TRPA1 [200,326]. Treatment with resiniferatoxin, which suppressed eye wiping evoked by TRPV1 and TRPA1 agonists (capsaicin and AITC, respectively) (Supplementary Figure IV-S1L), attenuated both GTN-evoked PMA and H2O2 generation (Figure IV-5B), supporting a role of TRPV1/TRPA1-positive neurons in these responses. GTN stimulated release of H_2O_2 from mixed cultures of trigeminal ganglion neurons/SGCs (Figure IV-5C), but not from primary cultures of isolated SGCs (Figure IV-5D). Removal of extracellular Ca^{2+} or pre-exposure to a high capsaicin concentration that, similar to resiniferatoxin, desensitizes TRPV1/TRPA1-positive neurons [224], attenuated GTN-evoked increase in H_2O_2 in mixed cultures of trigeminal ganglion neurons/SGCs (Figure IV-5C). GTN, AITC and SNAP increased H_2O_2 release from trigeminal ganglion neurons/SGCs mixed cultures in a HC-030031-dependent manner (Figure IV-5C).

Intrathecal administration of TRPA1 antisense oligonucleotide downregulated Trpa1 mRNA expression in trigeminal ganglion neurons (Figure IV-5E) and reduced AITC-evoked eye wiping (Supplementary Figure IV-S1M). TRPA1 antisense oligonucleotide attenuated GTN-evoked PMA and H_2O_2 increase in trigeminal ganglion neurons (Figure IV-5E). Further evidence for the role of neuronal TRPA1 in GTN-evoked oxidative stress and allodynia was obtained by studying *Advillin-Cre*$^{+}$; *Trpa1*$^{fl/fl}$ mice, which exhibited reduced TRPA1 mRNA in trigeminal ganglion neurons (Figure IV-5F) and TRPA1-mediated eye wiping evoked by AITC (Supplementary Figure IV-S1M). GTN failed to evoke PMA or H_2O_2 generation in trigeminal ganglion neurons in *Advillin-Cre*$^{+}$; *Trpa1*$^{fl/fl}$ mice (Figure IV-5F), thus supporting that GTN/NO initiates a TRPA1-dependent and oxidative stress-mediated mechanism that perpetuates nociceptor activation by an autocrine pathway that is confined to the trigeminal ganglion.

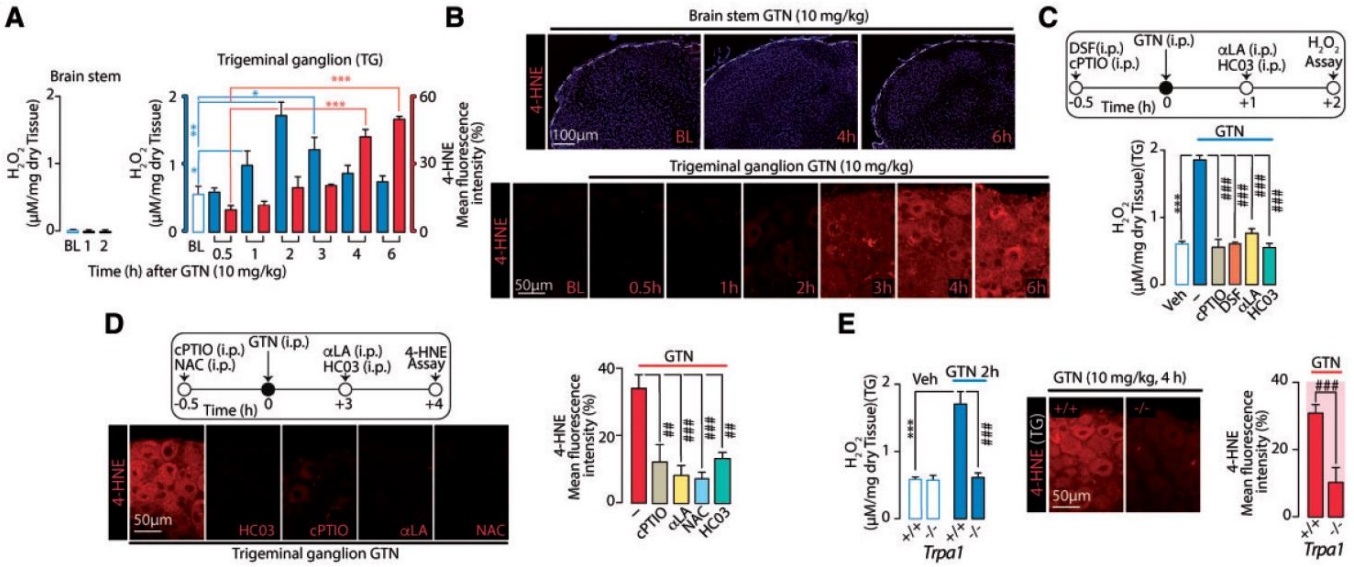

Figure IV-4. GTN generates oxidative stress in trigeminal ganglion via NO and TRPA1. A, B) Systemic (i.p.) GTN increases H_2O_2 levels and 4-HNE staining in trigeminal ganglion, but not in brain stem of C57BL/6 mice. **C)** Two hours after GTN the increase in H_2O_2 in trigeminal ganglion neurons, is abolished by systemic (i.p.) cPTIO (0.6 mg/kg) and disulfiram (DSF, 100 mg/kg) (all pre-GTN) or alpha lipoic acid (αLA) or HC-030031 (HC03, both 100 mg/kg) (all post-GTN). **D)** Representative images and pooled data of 4-HNE staining in trigeminal ganglion neurons, 4 h after GTN (10 mg/kg) administration in C57BL/6 mice treated systemically (i.p.) with cPTIO (0.6 mg/kg) or NAC (250 mg/kg) (all pre-GTN), or with HC03 and alpha lipoic acid (both, 100 mg/kg) (all post-GTN). **E)** Systemic (i.p.) GTN (10 mg/kg) increases H_2O_2 levels and 4-HNE staining in trigeminal ganglion neurons from *Trpa1*$^{+/+}$, but not *Trpa1*$^{-/-}$ mice. BL = baseline level of H_2O_2 or 4-HNE; Veh = the vehicle of GTN. Dash (-) indicates combined vehicles of treatments. Error bars indicate mean ± SEM, 4–6 mice per group. **P*<0.05, ***P*<0.01, ****P*<0.001; ###*P*<0.001; one-way ANOVA with Bonferroni post hoc correction and Student's t-test.

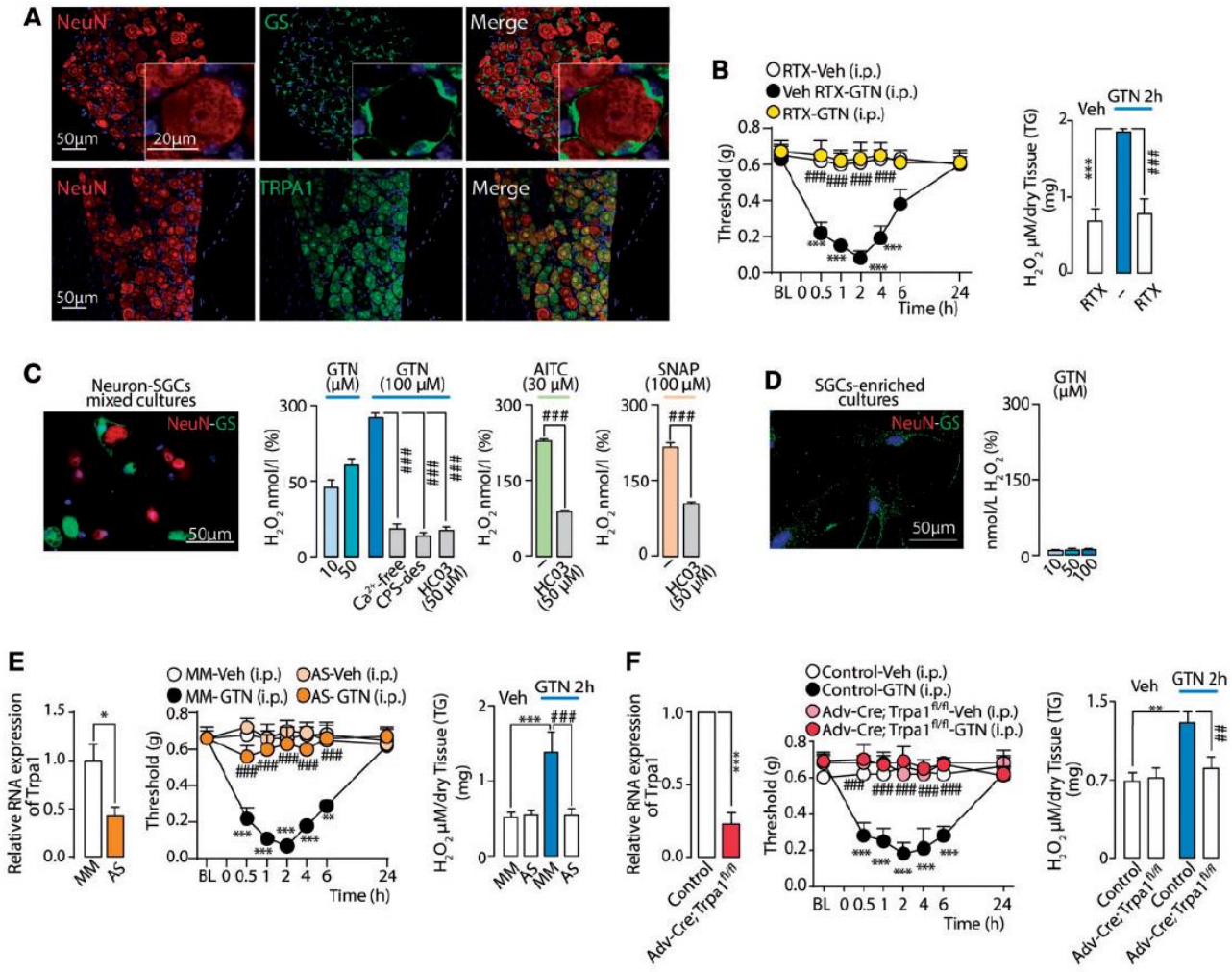

Figure IV-5. GTN targets neuronal TRPA1 to generate periorbital oxidative stress and PMA. **A)** Neurons (identified by neuronal nuclei, NeuN), SGCs (identified by glutamine synthetase, GS) and TRPA1 staining in trigeminal ganglion. **B)** Neuronal defunctionalization with resiniferatoxin prevents systemic (i.p.) GTN-evoked PMA and H2O2 increase in trigeminal ganglion neurons of C57BL/6 mice. **C)** H_2O_2 release elicited by GTN from trigeminal ganglion neurons-SGCs mixed cultures (see staining for NeuN/GS) is inhibited by extracellular Ca^{2+} removal (Ca^{2+}-free), pre-exposure to capsaicin (CPS-des) or HC-030031 (HC03), which also inhibits H_2O_2 release elicited by AITC or SNAP. **D)** In SGCenriched cultures (see staining for GS, but not NeuN) GTN does not release H_2O_2. **E)** Intrathecal TRPA1 antisense oligonucleotide inhibits TRPA1 mRNA expression, systemic (i.p.) GTN-evoked PMA and H_2O_2 increase in trigeminal ganglion neurons. **F)** TRPA1 mRNA expression in trigeminal ganglion neurons from control and *Advillin-Cre*; *Trpa1*$^{fl/fl}$ (*Adv-Cre*; *Trpa1*$^{fl/fl}$). Systemic (i.p.) GTN-evoked PMA and H_2O_2 increase in trigeminal ganglion neurons are reduced in *Adv-Cre*; *Trpa1*$^{fl/fl}$ mice. BL = baseline mechanical threshold; Veh = the vehicle of GTN. Dash (-) indicates vehicles of treatments. Error bars indicate mean ± SEM, 6–8 mice per group or 2–7 replicates from three independent experiments. **P*<0.05, ***P*<0.01, ****P*<0.001 versus resiniferatoxin-vehicle (RTX-Veh), mismatch oligonucleotide-vehicle (MM-Veh) and control-Veh; ##*P*<0.01, ###*P*<0.001 versus vehicle resiniferatoxin-GTN, MM-GTN, control-GTN, GTN, AITC or SNAP, one-way or two-way ANOVA with Bonferroni post hoc correction and Student's t-test.

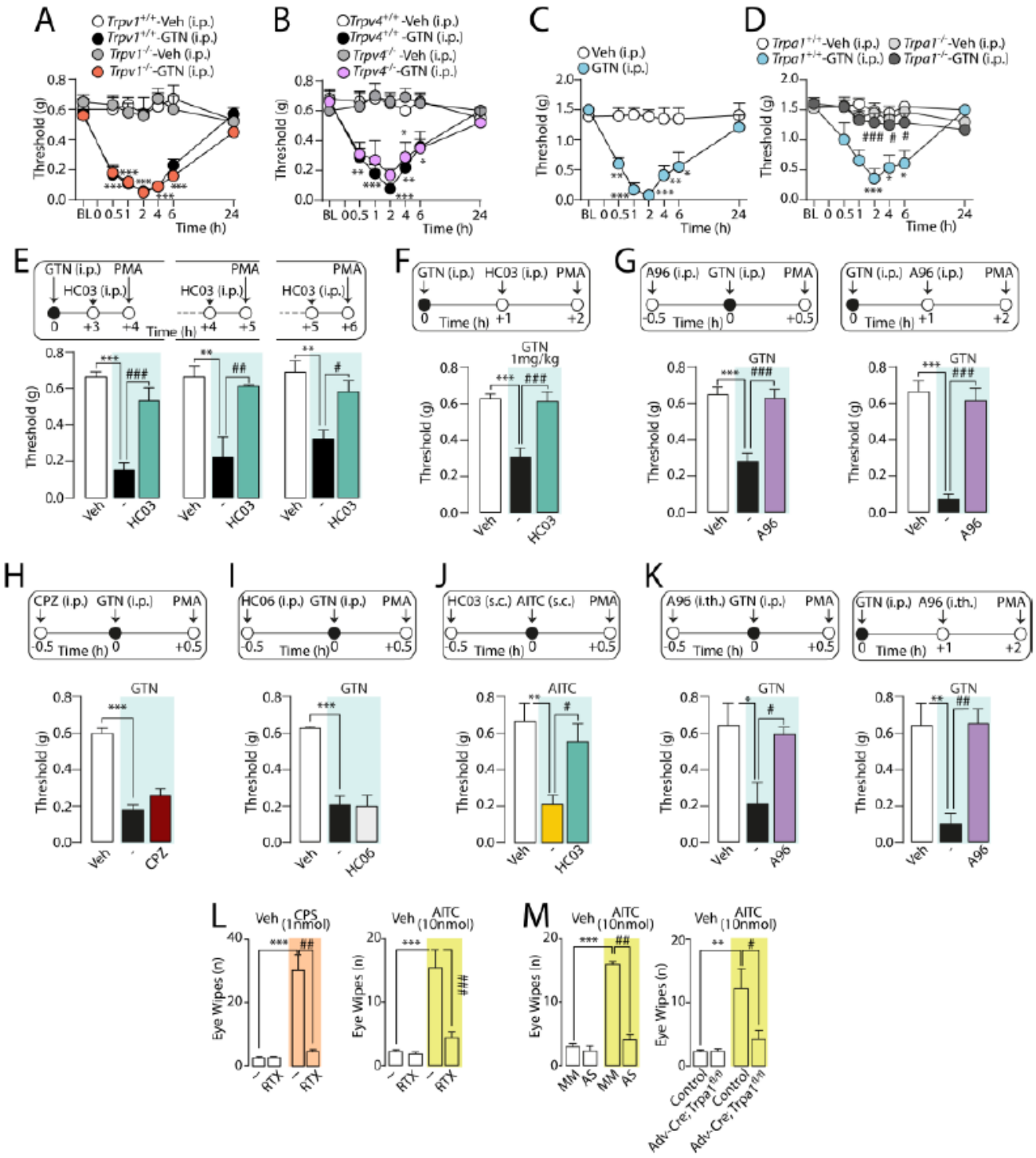

Figure IV-S1. A, B) Periorbital mechanical allodynia (PMA) evoked by GTN (10 mg/kg, i.p.) in *Trpv1*$^{+/+}$ or in *Trpv4*$^{+/+}$ is unaffected in *Trpv1*$^{-/-}$ or in *Trpv4*$^{-/-}$ mice. Time-course of hind paw mechanical allodynia evoked by GTN (10 mg/kg, i.p.) in **C)** C57BL/6 mice or **D)** *Trpa1*$^{+/+}$ and *Trpa1*$^{-/-}$ mice. **E)** TRPA1 antagonism by HC-030031 (HC03; 100 mg/kg, i.p.) transiently reverses GTN-evoked (10 mg/kg, i.p.) PMA in C57BL/6 mice. **F)** PMA evoked by GTN (1 mg/kg, i.p.) is attenuated by (HC03 (100 mg/kg, i.p). **G)** GTN (10 mg/kg, i.p.)-evoked PMA is inhibited by A967079 (A96; 100 mg/kg, i.p), but not by **H)** capsazepine (CPZ, 4 mg/kg, i.p.) or **I)** HC-067047 (HC06, 10 mg/kg, i.p.). **J)** PMA evoked by subcutaneous (s.c.) injection (10 µl/site) into the periorbital area of AITC (10 nmol) is inhibited by s.c. HC03 (100 µg/site). **K)** GTN (10 mg/kg, i.p.)-evoked PMA is inhibited by intrathecal (i.th.) A96 (10 µg). **L)** Eye wiping response evoked by ocular instillation (5 µl/drop eye) of capsaicin (CPS) or AITC in RTX desensitized C57BL/6 mice. **M)** Eye wiping response evoked by ocular instillation (5 µl/drop eye) of AITC in C57BL/6 mice

treated (i.th.) with TRPA1 AS/MM ODN or in *Advillin-Cre+*;*Trpa1*fl/fl (*Adv-Cre+;Trpa1fl/fl*) or *Advillin-Cre-*; *Trpa1*fl/fl (Control) mice. BL, baseline mechanical threshold. Veh is the vehicle of GTN. Dash (-) indicates combined vehicles of treatments. Arrows indicate time of drug administration. Error bars indicate mean ± SEM, 6-7 mice *per* group. **P*< 0.05, ***P*<0.01, ****P*<0.001 *vs*. *Trpv1*+/+-Veh, *Trpv4*+/+- Veh, *Trpa1*+/+-Veh, Veh. #*P*<0.05, ##*P*<0.01, ###*P*<0.001 *vs*. *Trpa1*+/+-GTN, GTN, AITC, CPS; one-way or two-way ANOVA with Bonferroni post-hoc correction.

4.2.6. TRPA1 and NOXs in the soma of trigeminal ganglion nociceptors maintain GTN-evoked allodynia

To explore the mechanism by which oxidative stress sustains GTN-evoked allodynia within trigeminal ganglion neurons we localized immunoreactivity for NOX1, NOX2, and NOX4 in mouse trigeminal ganglion neurons but not in SGCs (Supplementary Figure IV-2A), confirming previous studies [327]. Immunoreactivities for the three NOX isoforms colocalized with that for TRPA1 (Figure IV-6A). The non-selective NOX inhibitor, apocynin (i.p., post- but not pre-GTN) reversed GTN-evoked allodynia (Figure IV-6B,C). Selective NOX2 (gp91ds-tat) or NOX1 (ML171) inhibitors (i.p., post- but not pre-GTN) attenuated, and their combination abolished, GTN-evoked PMA (Figure IV-6B,C). The NOX4 inhibitor GKT137831 reduced, and the combination of GKT137831 and gp91ds-tat abolished, GTN-evoked allodynia (Supplementary Figure IV-S2B,C). However, since GKT137831 also inhibits NOX1, the role of NOX4 remains uncertain. Furthermore, a proximity ligation assay showed that NOX2 and TRPA1 are closely located in trigeminal ganglion neuronal cell bodies (Figure IV-6D), suggesting that their interaction could underlie efficient reactive oxygen species release. Thus, the soma of TRPA1-expressing trigeminal ganglion neurons possesses the biochemical machinery required to initiate and sustain oxidative stress evoked by GTN.

4.2.7. CGRP contributes only in part to GTN-evoked allodynia

As CGRP is a key mediator of migraine headaches [260,261] the contribution of CGRP to GTN-induced vasodilation and PMA was explored. Two different CGRP receptor antagonists, CGRP8-37 and BIBN4096BS (olcegepant) given (i.p.) after, but not before GTN, partially inhibited PMA (Figure IV-7A). CGRP8-37 or BIBN4096BS administered centrally (i.th.) either before or after GTN did not affect GTN-evoked PMA (Figure IV-7B). Local CGRP (0.5–5 mg/10 ml s.c.) in the periorbital area induced a dose-dependent and sustained (4 h) PMA (Figure IV-7C). Local (s.c.) pretreatment with CGRP8-37 or

BIBN4096BS prevented CGRP-induced PMA (Figure IV-7D). Local administration of CGRP8-37 or BIBN4096BS after, but not before GTN, partially attenuated PMA in a manner similar to that produced by their systemic administration (Figure IV-7E). GTN-evoked increase in cutaneous blood flow was unaffected by the pretreatment with BIBN4096BS (Figure IV-7F). Thus, the early vasodilation evoked by GTN is unrelated to CGRP.

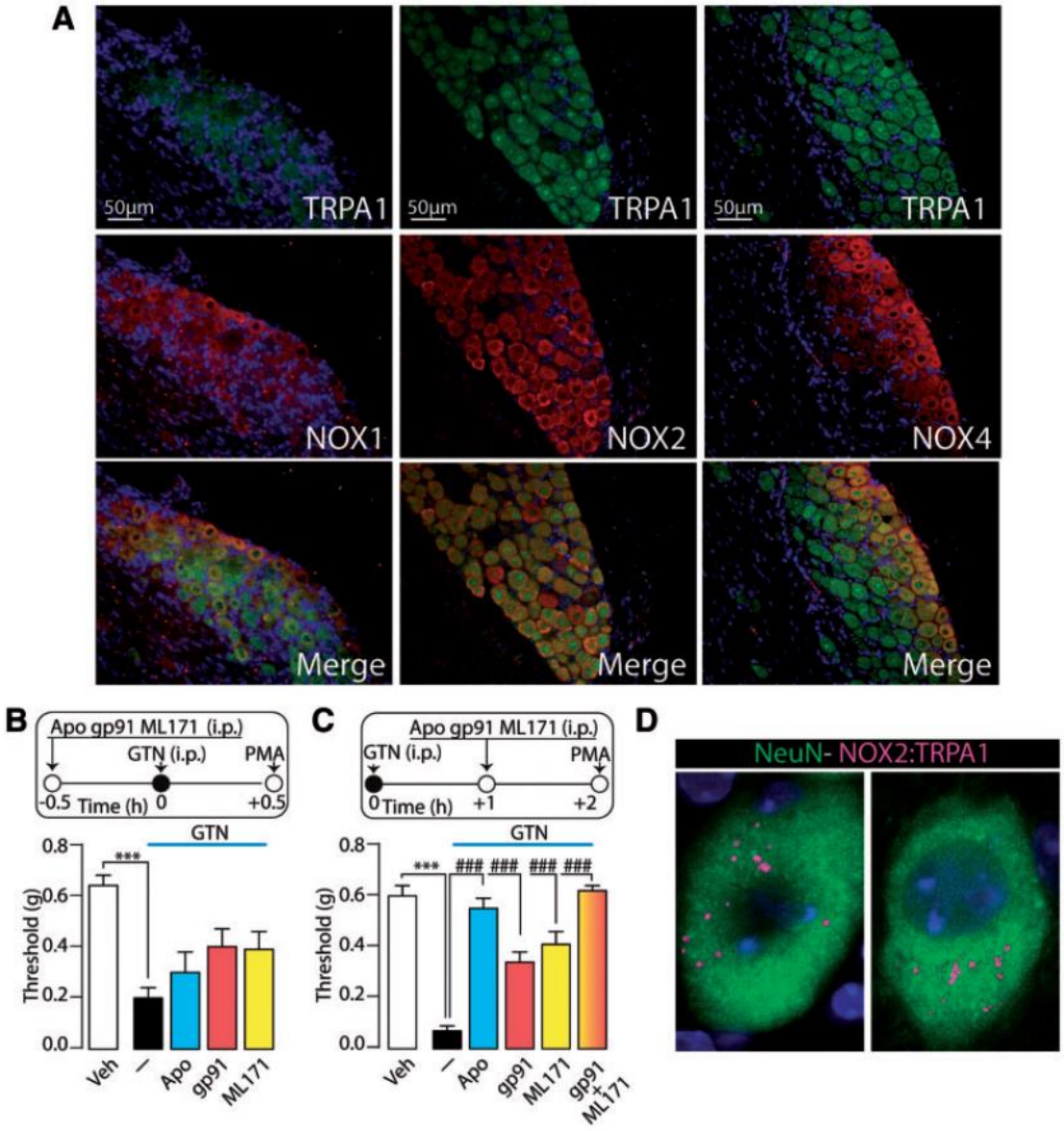

Figure IV-6. GTN evokes PMA via NADPH oxidase dependent mechanism. A) Representative images of TRPA1, NOX1, NOX2 and NOX4 staining in mouse trigeminal ganglion. **B)** The unselective NOX inhibitor, apocynin (APO; 100 mg/kg), the selective NOX2 (gp91ds-tat peptide, gp91; 10 mg/kg) or the selective NOX1 (ML171; 60 mg/kg) (i.p., all pre-GTN) do not affect PMA evoked by systemic (i.p.) GTN (10 mg/kg). **C)** APO (100 mg/kg), gp91 (10 mg/kg) or ML171 (60 mg/kg) (i.p., all post-GTN) partially reduce PMA. The combination of gp91 and ML171 (i.p., post-GTN) reverses PMA measured 2 h after GTN. **D)** In situ proximity ligation assays (PLAs) for TRPA1: NOX2 in mouse trigeminal ganglion labelled with NeuN. Veh = the vehicle of GTN. Dash (-) indicates vehicles of treatments. Error bars indicate mean ± SEM, 7–8 mice per group. ***P<0.001 versus vehicle. ###P<0.001 versus GTN; one-way ANOVA with Bonferroni post hoc correction.

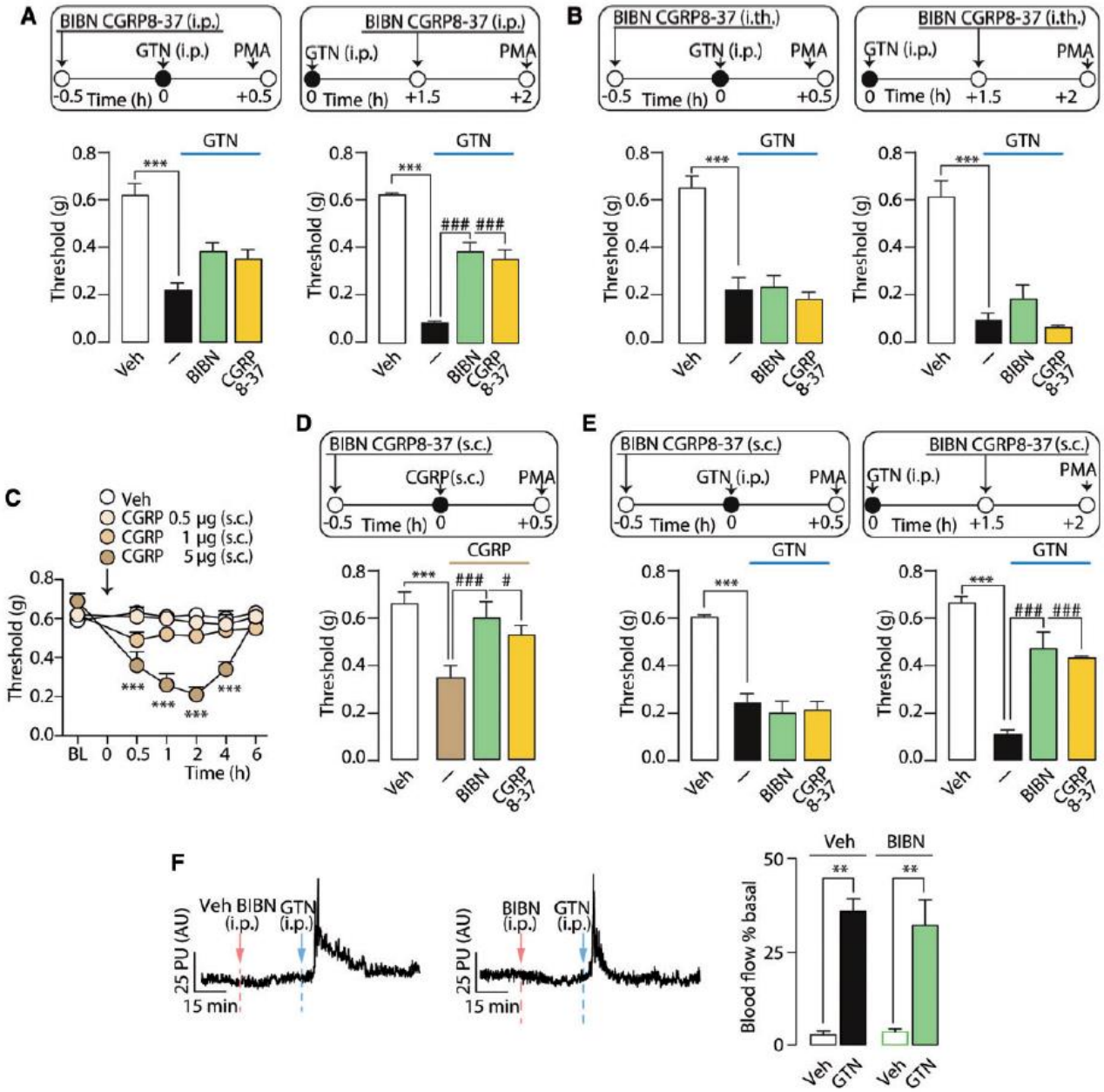

Figure IV-7. CGRP released from periorbital nerve terminals contributes to GTN evoked PMA. A) PMA evoked by systemic (i.p.) GTN (10 mg/kg) is partially reduced by i.p. administration of CGRP8-37 (4 mmol/kg) or BIBN4096BS (BIBN; 1 mg/kg), when given post-, but not pre-GTN. **B)** Intrathecal (i.th.) administration of CGRP8-37 (5 nmol) or BIBN (1 mg) pre- and post-GTN (10 mg/kg) does not affect GTN-evoked PMA. **C)** Local (s.c.) CGRP (0.5-5 mg) evokes a dose- and time-dependent PMA. **D)** Pretreatment with local (s.c.) BIBN (4 nmol) or CGRP8-37 (10 nmol) prevents CGRP (5 mg)-evoked PMA. **E)** Local (s.c.) CGRP8-37 (10 nmol) or BIBN (4 nmol) reduce PMA when given post-, but not pre-GTN (i.p., 10 mg/kg). BL = baseline mechanical threshold; Veh = the vehicle of GTN or CGRP. Dash (-) indicates vehicles of treatments. Data are presented as mean ± SEM of 6–8 mice per group. ***P<0.001 versus Veh. #P<0.05, ###P<0.001 versus GTN or CGRP; one-way and two-way ANOVA with Bonferroni post hoc correction. **F)** Representative traces and pooled data of the increases in periorbital skin blood flow evoked by GTN (i.p., 10 mg/kg). Pretreatment with systemic (i.p.) BIBN (1 mg/kg) does not affect the early increase in blood flow. Veh = the vehicle of GTN. Data are presented as mean ± SEM of 6–7 mice per group. **P<0.01 versus vehicle; one-way ANOVA with Bonferroni post hoc correction.

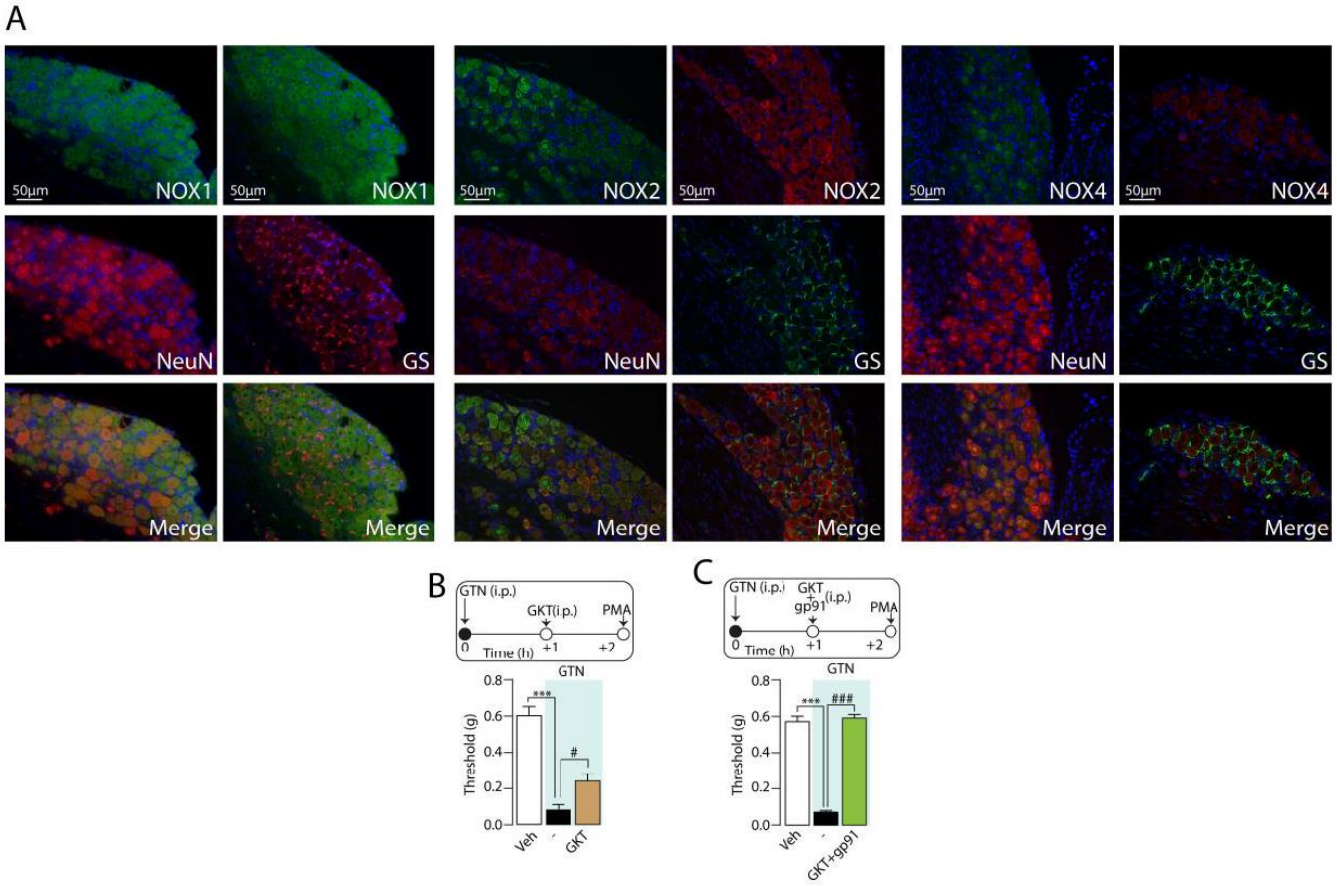

Figure IV-S2. A) Representative images of staining for NOX1, NOX2 and NOX4 and neuronal (neuronal nuclei, NeuN) or satellite glial cells (glutamine synthetase, GS) in mouse trigeminal ganglion (TG) from C57BL/6 mice. (Scale bars: 100 µm). **B, C)** Periorbital mechanical allodynia (PMA) evoked by GTN (10 mg/kg, i.p.) in C57BL/6 mice is reduced by the NOX1/4 inhibitor, GKT137831 (GKT, 60 mg/kg, i.p.), and completely abated by the combination of GKT and the NOX2 selective inhibitor, gp91ds-tat peptide (gp91, 10 mg/kg, i.p.). Veh is the vehicle of GTN. Dash (-) indicates combined vehicles of treatments. Error bars indicate mean ± SEM, 8 mice *per* group. ***P<0.001 *vs*. Veh. #P<0.05, ###P<0.001 *vs*. GTN; one-way ANOVA with Bonferroni post-hoc correction.

4.3. Discussion

We report that the delayed and prolonged GTN-evoked PMA in mice is entirely TRPA1-dependent. Two chemically unrelated TRPA1 antagonists, but not TRPV1 or TRPV4 antagonists, completely reversed PMA. Furthermore, deletion of *Trpa1*, but not *Trpv1* or *Trpv4*, prevented the development of PMA. A recent report that TRPA1 antagonism attenuates GTN potentiation of formalin-evoked periorbital allodynia in rats is in line with our findings [328]. We exposed mice to a dose of GTN (10 mg/kg) that has been

used by others [256,329,330], but which exceeds the dose used in humans (~40 mg/kg, i.v.) [214,218]. However, genetic deletion or pharmacological blockade of TRPA1 also suppressed PMA evoked by a low dose of GTN (1 mg/kg). The observation that, after correction for the mouse to man conversion factor [278], this dose is only 2-fold higher than the human dose, supports the translational relevance of the role of TRPA1 in GTN-evoked allodynia in mice.

NO activation of TRPA1, either directly, or indirectly *via* its by-products, through nitrosylation of cysteinyl residues, is an important post-translational mechanism of channel regulation [222]. NO donors activate TRPA1 in cultured trigeminal ganglion neurons and hTRPA1-HEK293 cells by distinct mechanisms. As Ca^{2+} responses by SNAP, but not those by GTN, were inhibited by the NO scavenger, cPTIO, NO does not seem required for TRPA1 activation by GTN. It is possible that under *in vitro* conditions NO is released with insufficient velocity or in insufficient amounts to elicit TRPA1 gating, while the channel is engaged directly by GTN which binds to the same key cysteine/lysine residues required for channel activation by electrophilic and oxidant molecules [149,331].

In vivo GTN induces PMA *via* diverse anatomical pathways, mechanisms and time courses that depend from the route of administration. As the ALDH-2 inhibitor, disulfiram, and the NO scavenger, cPTIO, blocked PMA elicited by systemic, but not local administration of GTN, local GTN likely causes allodynia by targeting TRPA1 by a direct NO-independent mechanism, whereas systemic GTN by an indirect, NO dependent pathway. As a consequence, the ability of NO released from GTN to target TRPA1 requires that the conversion occurs distant [323] from the site where NO engages the channel. Evidence that NO must have spent some time in the extracellular environment before leading to S-nitrosothiol formation [332] supports this explanation. Furthermore, the observation that cPTIO and disulfiram prevent allodynia when administered before, but not after systemic GTN implies that NO is initially necessary, but is not subsequently sufficient, to sustain the allodynia. Attenuation by two different TRPA1 antagonists, HC-030031 or A967079, of GTN-evoked allodynia was a transient phenomenon, probably because of the limited half-lives of the antagonists in mice. These findings suggest that to maintain allodynia, TRPA1 must be engaged continuously by one or more mediators generated by GTN/NO and whose identity and source are unknown.

GTN generates oxidative stress [324]. The observation that pretreatment with the reactive oxygen species scavengers, alpha lipoic acid or PBN, did not prevent GTN-evoked

PMA, but attenuated allodynia from for 3–4 h after GTN, indicates that reactive oxygen species do not initiate the response, but mediate the ensuing phase. However, additional TRPA1 agonists must contribute to the final phase of PMA, when antioxidants were ineffective. Carbonylic by-products of oxidative stress, including 4-HNE, have half-lives longer than reactive oxygen species [325], and are known to target TRPA1 [152]. PMA attenuation by NAC and L-carnosine, which efficiently quenches aldehydes, indicates that 4-HNE and/or related aldehydes engage TRPA1 to mediate the terminal phase of PMA, from 3–4 to 8 h after GTN.

Failure of local and efficacy of intrathecal antioxidants and TRPA1 antagonists to block PMA indicate that systemic GTN/NO does not target TRPA1 on cutaneous terminals of nociceptor and suggests the involvement of TRPA1 in a central site, such as the soma and central terminals in the brainstem of trigeminal ganglion neurons. Assessment of GTN-induced oxidative stress showed no change in the dorsal brain stem, whereas a marked increase in H_2O_2 and 4-HNE levels were found in trigeminal ganglion neurons. Remarkably, the time course of H_2O_2 formation (1–3 h) paralleled the ability of reactive oxygen species scavengers to attenuate PMA, and the time course of increased 4-HNE staining (3–6 h) paralleled the ability of aldehyde scavengers to inhibit PMA.

Primary sensory neurons and associated SGCs are most abundant cell types in trigeminal ganglion. The observation that resiniferatoxin, which defunctionalizes TRPV1/TRPA1 expressing nociceptors [307], attenuated GTN-evoked H_2O_2 generation *in vivo*, suggests that trigeminal ganglion neurons rather than SGCs generate oxidative stress. As pharmacological blockade of TRPA1, global TRPA1 deletion, selective deletion of TRPA1 in peripheral sensory neurons and TRPA1 knockdown all abrogated GTN-evoked oxidative stress and PMA, TRPA1 expressed by trigeminal ganglion sensory neurons seems to be a critical step for PMA. Although various TRP channels can promote reactive oxygen species release [333], to our knowledge this is the first evidence that TRPA1 expressed by cell bodies of primary sensory neurons increase the tissue burden of oxidative stress. Sensory neurons express several NOX isoforms that may mediate the GTN/NO/TRPA1 signal [327]. Trigeminal ganglion neurons, but not SGCs, co-express TRPA1 and NOX1, NOX2 and NOX4, and the NOX inhibitor apocynin reverses GTN-evoked mechanical allodynia, thus suggesting that NOXs expressed by trigeminal ganglion neurons and located downstream from TRPA1 generate the pro-allodynic oxidative burden. NOX1 and NOX2 isoforms provide a major contribution, since the combination of

selective NOX1 and NOX2 inhibitors afforded complete inhibition of allodynia. Notably, similar to alpha lipoic acid, the capacity of NOX inhibitors to suppress allodynia faded with time and was absent when the drugs were administered 3–4 h after GTN.

Several findings of the current study support the hypothesis that TRPA1/NOXs, reactive oxygen species and aldehydes sustain GTN-evoked PMA by an action that is confined to trigeminal ganglion neurons. First, GTN induced reactive oxygen species/4-HNE in trigeminal ganglion but not the brainstem. In particular, failure to identify any increase in HNE staining in any specific brainstem area supports the hypothesis that oxidative stress is not generated at this level. Second, intrathecal administration of TRPA1 channel antagonists or antisense oligonucleotides attenuated this response. Third, both GTN-evoked PMA and reactive oxygen species/4-HNE generation were attenuated by resiniferatoxin, which selectively desensitizes TRPV1-expressing primary sensory neurons, a population that encompasses the TRPA1-positive subpopulation [200,326]. Finally, similar attenuation was found in mice expressing Cre-recombinase from the locus of the primary sensory neuron-specific gene Advillin [303,304,334], resulting in a selective *Trpa1* mRNA attenuation in nociceptors. Although our findings suggest a key role for TRPA1 in trigeminal ganglion neurons, the present study did not systematically investigate the possibility that TRPA1 activation and oxidative stress in the CNS also contribute to GTN-evoked PMA. The ability of centrally administered NO donors to affect the function of neurons of the spinal trigeminal nucleus [335,336] suggests that GTN can also act centrally. Furthermore, the cell bodies of trigeminal ganglion neurons that mediate allodynia via TRPA1/NOX/oxidative stress may belong to meningeal nociceptors, which are known to contribute to the sensitization process observed after exposure to inflammatory mediators or GTN [337-339].

The beneficial action of CGRP/CGRP receptor blockade by small molecules or monoclonal antibodies indicate that CGRP is a major mediator of migraine headaches [260,261]. The observation that two different CGRP receptor antagonists, when administered by systemic or local routes, but not central administration, reduced GTN-evoked PMA suggests that CGRP acts at a peripheral site. One possible explanation is that excitation of the NO/TRPA1/NOX pathway in the soma of trigeminal ganglion neurons generates antidromic action potentials, which ultimately invade cutaneous nerve terminals to promote local CGRP release. The observation that a CGRP monoclonal antibody, which does not cross the blood–brain barrier and should act peripherally, attenuates PMA-

induced by sodium nitroprusside plus sumatriptan in rats [340], supports the existence of a peripheral mechanism. However, further studies are required to examine the effects of GTN on antidromic transmission in trigeminal ganglion neurons. Our findings that CGRP provides a limited and delayed contribution to the allodynia may explain the failure of BIBN4096BS to reduce GNT-evoked migraine-like pain in patients [219]. Notably, we report that the early periorbital vasodilation elicited by GTN is entirely due to ALDH-2-dependent NO formation, but neither TRPA1 nor CGRP are involved. Thus, vasodilatation and allodynia are temporally and mechanistically distinct. While vasodilatation is probably due to a direct NO action in the vascular smooth muscle, allodynia is a neuronal phenomenon mediated by TRPA1 activation and the ensuing oxidative stress.

Our present results suggest that systemic GTN is converted by ALDH-2 to NO that causes a transient vasodilatation unrelated to TRPA1 and most probably due to a direct action of the gaseous mediator on smooth muscle guanylyl cyclase (Figure IV-8). NO and/or its by-products also target TRPA1/NOXs in the soma of trigeminal ganglion neurons to increase reactive oxygen species/reactive carbonylic species, thereby sustaining mechanical allodynia. In this view, the channel that triggers the oxidative burst is expressed by the same sensory neurons that potentially mediate the allodynia. Thus, it is not possible to discriminate whether TRPA1, in addition to generating the proalgesic oxidative stress, is also the final mediator of the hypersensitivity. Nevertheless, since TRPA1 is a major sensor of oxidative and carbonylic stress [152,156,159], it is possible that reactive oxygen species/4-HNE, generated by TRPA1 activated by GTN/NO, target the same neuronal channel to signal allodynia. TRPA1 antagonists, which are currently in clinical development, or established antimigraine drugs, which have recently been identified as selective channel inhibitors [224,296], may be used to test whether a mechanism similar to the present one mediates the delayed attack evoked by GTN in migraineurs.

A limitation of our study is that GTN-evoked PMA in naïve mice may not fully replicate migraine in patients. However, periorbital allodynia has been successfully used in rodents to identify neural pathways that are relevant for migraine pathophysiology, such as those responsible for headache triggered by overuse of medications [340,341]. In addition, GTN provocation studies report that healthy volunteers experience both an early headache during GTN infusion [253], and also a delayed headache, although usually milder than in migraineurs [254,342,343]. Moreover, cutaneous allodynia, which is usually

localized to the temporal and periocular areas [344] occurs in ~80% of migraine attacks and can be detected also in extracephalic areas [22].

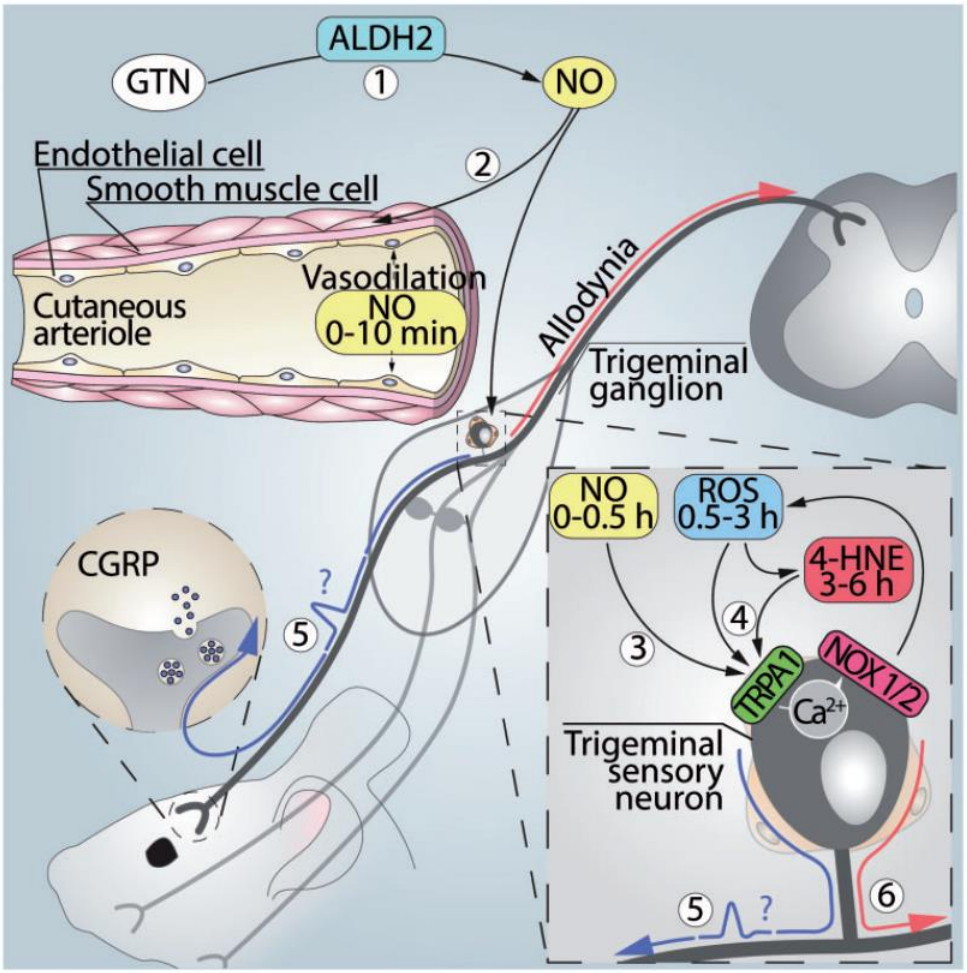

Figure IV-8. Signalling pathway that mediates TRPA1-dependent PMA induced by GTN. 1) Systemic GTN is converted to NO by ALDH-2 at sites distant to the trigeminal ganglion. **2)** NO produces via TRPA1- and CGRP-independent mechanisms a rapid and transient increase in periorbital blood flow. **3)** NO also targets TRPA1 in the soma of trigeminal ganglion neurons to evoke a Ca^{2+}-dependent NOX1/NOX2 activation, thereby increasing reactive oxygen (H_2O_2) and carbonylic (4-HNE) species. **4)** H_2O_2 and 4-HNE by an apparently autocrine pathway in trigeminal ganglion neurons sustain periorbital mechanical allodynia. **5)** Activation of the NO/TRPA1/NOX pathway in the soma of trigeminal ganglion neurons could possibly (?) generate antidromically-propagated action potentials, which ultimately invade cutaneous nerve terminals to promote local CGRP release that partially contributes to PMA. Glyceryl trinitrate administration causes prolonged mechanical allodynia in rodents, which correlates temporally with delayed migraine attacks in patients. Marone et al. show that the allodynia is mediated by TRPA1 activation in cell bodies of trigeminal neurons and ensuing oxidative stress. This neuronal pathway may be of relevance to migraine-like headaches.

The report that GTN evokes a series of typical premonitory symptoms [345], in addition to headaches, strengthens the value of studying allodynia as part of the various symptoms of the migraine attack. Such sensitization, deriving from activation of

meningeal nociceptors by locally-released inflammatory mediators [339], may lead to sensitization of central trigeminal neurons that receive convergent input from the dura and skin [346]. Endogenous mediators and exogenous chemicals, including GTN, elicit delayed sensitization that, via dynamic changes in meningeal arteries [337] and central second order trigeminovascular neurons [346], may be observed in periorbital skin and other cutaneous areas. Our data suggest that the TRPA1/NOX pathway in the soma of trigeminal neurons, in addition to the previously reported peripheral or central sites, contributes to GTN-evoked allodynia.

This work has been published in Brain

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V – The acyl-glucuronide metabolite of ibuprofen has analgesic and anti-inflammatory effects *via* the TRPA1 channel

5.1. Methods

Animals

In vivo experiments and tissue collection were carried out according to European Union (EU) guidelines and Italian legislation (DLgs 26/2014, EU Directive application 2010/63/EU) for animal care procedures, and under the University of Florence research permit #194/2015-PR. C57BL/6 J mice (male, 20–22 g, 6 weeks; Envigo, Milan, Italy); TRPA1-deficient (*Trpa1*$^{-/-}$) mice (25–30 g, 5–8 weeks) [143] or Sprague-Dawley rats (male, 75–100 g, Envigo, Milan, Italy) were used. Animals were housed in a temperature- and humidity-controlled *vivarium* (12-hour dark/light cycle, free access to food and water). Animal studies were reported in compliance with the ARRIVE guidelines [274]. Group size of n=6 animals for behavioral experiments were determined by sample size estimation using G*Power (v3.1) [297] to detect size effect in a post-hoc test with type 1 and 2 error rates of 5 and 20%, respectively. Allocation concealment was performed using a randomization procedure (http://www.randomizer.org/). Experiments were done in a quiet, temperature-controlled (20–22 °C) room between 9 a.m. and 5 p.m. and were performed by an operator blinded to drug treatment. Animals were euthanized with inhaled CO_2 plus 10–50% O_2. For the *in vitro* experiments we used a total of 10 rats and 42 mice.

Reagents and cells

HC-030031 [2-(1,3-dimethyl-2,6-dioxo-1,2,3,6-tetrahydro-7Hpurin-7-yl)-N-(4-isopropylphenyl) acetamide] was synthesized as previously described [197]. If not otherwise indicated, reagents were obtained from Sigma-Aldrich (Milan, Italy). Human embryonic kidney 293 (HEK293, American Type Culture Collection; ATCC® CRL-1573™) cells, HEK293 cells stably transfected with cDNA for human TRPA1 (hTRPA1-HEK293),

or with the cDNA for human TRPV1 (hTRPV1-HEK293), or with the cDNA for human TRPV4 (hTRPV4-HEK293), or with cDNA for both human TRPA1 and human TRPV1 (hTRPA1/V1-HEK293) channels, were cultured as previously described [80,93,347,348]. HEK293 cells were transiently transfected with the cDNAs (1 μg) codifying for wild type (Wt) (hTRPA1-HEK293) or mutant human TRPA1 (C619S, C639S, C663S, K708Q; 3C/K-Q hTRPA1-HEK293) [149] using the jetPRIME transfection reagent (Poliyplus-transfection® SA, Thermo Scientific, Monza, Milan), according to the manufacturer's protocol.

Human embryonic lung fibroblasts (IMR90; ATCC® CCL-186™) were used as a model of human cells constitutively expressing the TRPA1 channel and were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin, according to the manufacturer's instructions. Normal human bronchial epithelial cells (NHBE; Lonza Group Ltd, Basel, Switzerland) were cultured in NHBE growth medium, according to the manufacturer's instructions. All cells were cultured in an atmosphere of 95% air and 5% CO_2 at 37 °C. For all
cell lines, the cells were used when received without further authentication.

Rodent primary sensory neurons were isolated from DRGs taken from Sprague-Dawley rats and cultured as previously described [197]. Briefly, ganglia were bilaterally excised under a dissection microscope and transferred in Hank's balanced salt solution (HBSS) containing 2 mg/ml collagenase type 1A and 1 mg/ml trypsin, for enzymatic digestion (30 min, 37 °C). Ganglia were then transferred to warmed DMEM containing 10% FBS, 10% horse serum, 2mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin and dissociated in single cells by several passages through a series of syringe needles (23–25 G). Medium and ganglia cells were filtered to remove debris and centrifuged. The pellet was resuspended in DMEM with added 100 ng/ml mouse-nerve growth factor and 2.5mM cytosine-b-Darabino-furanoside free base. Neurons were then plated on 25 mm diameter glass coverslips coated with poly-L-lysine (8.3 μM) and laminin (5 μM). DRG neurons were cultured for 3–4 days before being used for calcium imaging experiments.

Calcium imaging assay

Single cell intracellular calcium was measured in untransfected and in hTRPA1-HEK293, hTRPV1-HEK293, hTRPV4-HEK293, hTRPA1/V1-HEK293, 3C/K-Q hTRPA1-HEK293 cells, IMR90 fibroblasts, NHBE cells, or in rat DRG neurons. Plated cells were

loaded with 5 μM Fura-2 AM-ester (Alexis Biochemicals; Lausen, Switzerland) added to the buffer solution (37 °C) containing the following (in mM): 2 $CaCl_2$; 5.4 KCl; 0.4 $MgSO_4$; 135 NaCl; 10 D-glucose; 10 HEPES and 0.1% bovine serum albumin at pH 7.4. After loading (40 min), cells were washed and transferred to a chamber on the stage of an Olympus IX81 microscope for recording. Cells were excited alternatively at 340 and 380 nm and recorded with a dynamic image analysis system (XCellence Imaging software; Olympus srl, Milan, Italy). To evoke a TRPA1-dependent calcium response, cells and neurons were challenged with AITC (1–1000 μM), acrolein (10 μM), hydrogen peroxide (H_2O_2, 500 μM), icilin (30 μM), zinc chloride ($ZnCl_2$, 1 μM) or menthol (100 μM). Buffer solution containing 1% dimethyl sulfoxide (DMSO) was used as vehicle. The selective TRPV1 agonist, capsaicin (0.1 μM), was used in hTRPV1-HEK293, in hTRPA1/V1-HEK293 and, to identify TRPV1-expressing neurons and KCl (50 mM), to identify the entire neuronal population [224]. The selective TRPV4 agonist, GSK1016790 A (0.1 μM), was used in hTRPV4-HEK293 cells. hTRPA1-HEK293, hTRPV1-HEK293, hTRPV4-HEK293, hTRPA1/V1-HEK293, IMR90 fibroblasts and NHBE cells were challenged with the activating peptide (AP) of the human proteinase activated receptor 2 (hPAR2) (hPAR2-AP, SLIGKV-NH2, 100 μM).

Cells or neurons were pre-exposed (10 min) to IAG (1–300 μM), ibuprofen (100 μM), HC-030031 (0.1–30 μM), capsazepine (10 μM), HC-067047 (10 μM) or vehicle (0.3% DMSO) before the addition of the TRPA1, TRPV1 or TRPV4 agonists. Results were expressed as the percentage of the increase in $R_{340/380}$ over baseline, normalized to the maximum effect induced by ionomycin (5 μM) added at the end of each experiment (% Change in $R_{340/380}$); or as the percentage of the inhibitory effect on the calcium response evoked by AITC (% AITC response) for constructing the concentration-response curves in the presence of IAG.

Behavioural experiments

Treatment protocols. C57BL/6 J mice were injected in the plantar surface of the hind paw (intraplantar, i.pl., 20 μl/paw) with a mixture of AITC, acrolein or $ZnCl_2$ (all, 10 nmol) [224] and IAG (0.3–300 nmol) or HC-030031 (0.3–300 nmol) or ibuprofen (300 nmol), or capsaicin (1 nmol) and capsazepine (300 nmol), or hypotonic solution (0.27% NaCl) and HC-067047 (300 nmol), or their vehicle (4% DMSO and 4% tween 80 in 0.9% NaCl), and acute nociceptive responses were recorded over the next 10 min [224,349]. Some C57BL/6 J mice were treated intraperitoneally (i.p.) with IAG (1, 10 and 100 mg/kg), HC-

030031 (1, 10 and 100 mg/kg), ibuprofen (1, 10 and 100 mg/kg) or their vehicle (4% DMSO and 4% tween 80 in 0.9% NaCl) and 30 min after treatment the acute nociceptive response to i.pl. injection of AITC (10 nmol) was recorded over the next 10 min [224]. Other C57BL/6 J mice were treated intraperitoneally (i.p.) with IAG (10 and 100 mg/kg), ibuprofen (10 and 100 mg/kg), HC-030031 (100 mg/kg) [40], capsazepine (4 mg/kg) [224], HC-067047 (10 mg/kg) [350], or their vehicle (4% DMSO and 4% tween 80 in 0.9% NaCl) and 30 min after treatment the acute nociceptive responses to i.pl. injection of acrolein and $ZnCl_2$ (all, 10 nmol), capsaicin (1 nmol) or hypotonic solution (0.27% NaCl) were recorded over the next 10 min [224].

For the carrageenan model, C57BL/6 J mice were injected (i.pl., 20 µl/paw) with carrageenan (300 µg), or its vehicle (0.9% NaCl), and mechanical allodynia was recorded 180 min after injection [308]. Some C57BL/6 J mice were treated (150 min after carrageenan) by i.pl. (20 µl/paw) injection with IAG, ibuprofen (all, 100 nmol), or a mixture of IAG or ibuprofen and HC-030031 (all, 100 nmol), or their vehicle (all 4% DMSO and 4% tween 80 in 0.9% NaCl). Additional C57BL/6 J mice were treated (150 min after carrageenan) with i.p. IAG, ibuprofen (both 10 and 100 mg/kg), HC-030031 (100 mg/kg), indomethacin (30 mg/kg) or their vehicles (4% DMSO and 4% tween 80 in 0.9% NaCl) [224]. Some *Trpa1*$^{-/-}$ mice were treated (150 min after carrageenan) with i.p. IAG (100 mg/kg).

For the formalin test, C57BL/6J mice were injected (i.pl., 20 µl/paw) with formalin (0.5% in 0.9% NaCl) and the acute nociceptive response was monitored over the next 60 min and reported as phase I (0–10 min) and phase II (11–60 min) [181]. Some animals were pretreated by i.pl. (20 µl/paw) injection (10 min before) with IAG and ibuprofen (both 100 nmol) or their vehicle (all 4% DMSO and 4% tween 80 in 0.9% NaCl) or with i.p. IAG, ibuprofen (both 10 and 100 mg/kg, 30 min before), HC-030031 (100 mg/kg, 60 min before) [181] and indomethacin (30 mg/kg, 30 min before) [224].

Acute nociceptive test and Von Frey hair test. Immediately after the i.pl. (20 µl/paw) injection with tested compounds, mice were placed inside a plexiglass chamber and the total time spent in lifting/licking the injected hind paw, as an indicative time of acute nociceptive response, was recorded for 10 min. The i.pl. injection with vehicles of tested compounds produced nociceptive behavior for a maximum of 2 s. Mechanical allodynia was measured in mice by the up-and-down paradigm [351]. Briefly, mice were placed individually in a plexiglass chamber designed for the evaluation of mechanical

thresholds [351] and were habituated to the room temperature for at least 1 h before the test. Then, a series of 7 Von Frey hairs in logarithmic increments of force (0.07, 0.16, 0.4, 0.6, 1, 1.4, 2 g) was used to stimulate the injected hind paw. The response was considered positive when the mouse strongly withdrew the paw. The stimulation started with the 0.6 g filament. The von Frey hairs were applied with sufficient force to cause slight buckling and held for approximately 2–4 s. Absence of response after 5 s led to the use of a filament with increased weight, whereas a positive response led to the use of a weaker (i.e. lighter) filament. Six measurements were collected for each mouse or until four consecutive positive or negative responses occurred. The 50% mechanical withdrawal threshold (expressed in g) response was then calculated from these scores, as previously described [309,351]. Mechanical nociceptive threshold was determined before (basal level) and after different treatments.

Prostaglandin E_2 assay

C57BL/6 J or *Trpa1*$^{-/-}$ mice were injected (i.pl. 20 µl/paw) with carrageenan (300 µg) or its vehicle (0.9% NaCl) and 180 min after treatment the injected paws were collected, weighed, frozen in liquid nitrogen and homogenized in sodium phosphate buffer (PBS 0.1 M, pH 7.4) containing indomethacin (20 µM) to avoid further activation of COX. Homogenates were centrifuged at 9000×g for 20 min at 4 °C [352]. Supernatants were collected and PGE_2 levels were measured by enzyme immunoassay (Abcam, Cambridge, UK), according to the manufacturer's instructions. Some C57BL/6 J were treated (150 min after carrageenan) by i.pl. (20 µl/paw) injection with IAG, ibuprofen (both, 100 nmol) or their vehicles (4% DMSO and 4% tween 80 in 0.9% NaCl). Other animals were treated (150 min after carrageenan) by i.p. injection with IAG, ibuprofen (both, 100 mg/kg), HC-030031 (100 mg/kg, i.p.), indomethacin (30 mg/kg, i.p.), or their vehicles (4% DMSO and 4% tween 80 in 0.9% NaCl).

Molecular modeling

Protein structure refinement. Molecular modeling studies were performed using the structure of the human TRPA1 ion channel determined by electron cryo-microscopy (PDB code 3J9P) [119]. The missing side chains of partially resolved residues as well as the missing loop sequences within the protein core structure were automatically reconstructed by using Modeller software [353]. The refined structure was then energy minimized in explicit water environment, after being embedded in a lipid bilayer. The creation of the phospholipid bilayer constituted by POPC (1-palmitoyl-2-oleoyl-

snglycero- 3-phosphocholine) molecules and the insertion of the protein inside it were performed using Visual Molecular Dynamics (VMD) software [354]. The energy minimization was then carried out with AMBER software, version 16. The system was solvated with a 15 Å water cap on both the "intracellular" and the "extracellular" sides using the TIP3P solvent model, while chloride ions were added as counterions to neutralize the system. The Lipid14 parameters [355] were assigned to POPC molecules. Three sequential minimization stages, each consisting of 8000 steps of steepest descent followed by conjugate gradient, were thus performed. In the first stage, a position restraint of 100 kcal/mol·Å^2 was applied on the whole protein and phospholipid bilayer in order to uniquely minimize the positions of the water molecules. In the second stage, the same position restraint was only applied on the protein residues, thus leaving the phospholipid molecules free, while in the last stage only the protein α carbons were restrained with a harmonic potential of 30 kcal/mol·Å^2.

IAG-TRPA1 covalent binding analysis. Molecular docking studies were performed on the structurally refined and energy minimized structure of hTRPA1 using the covalent docking protocol implemented in Gold software [356]. The calculations were performed selecting C621, C641 and C665 as the covalently modified residues and the acyl portion of IAG belonging to (S)-ibuprofen as the ligand moiety covalently bound to the residues. For each of the three S-acyl-cysteine thioester adducts, 100 different ligand binding orientations were evaluated, and the top-scored disposition was considered for further analyses. The three ligand-protein complexes obtained were then subjected to molecular dynamic (MD) simulations with AMBER 16. Each complex was initially subjected to three stages of energy minimization as performed for protein refinement. Subsequently, the temperature of the system was gradually raised from 0 to 300 K through a brief constant-volume MD simulation where a position restraint of 30 kcal/mol·Å^2 was applied on the protein α carbons. The system was then relaxed through a 500 ps constant-pressure MD simulation in which the harmonic potential applied on the protein α carbons was gradually removed and a Langevin thermostat was used to keep the temperature at 300 K. Finally, 20 ns of constant-pressure MD simulation production were performed by leaving the whole system free and using the Monte Carlo barostat with anisotropic pressure scaling for pressure control. All simulations were performed using particle mesh Ewald electrostatics with a cutoff of 10 Å for non-bonded interactions and periodic boundary conditions. A simulation step of 2.0 fs was employed, as all bonds

involving hydrogen atoms were kept rigid using SHAKE algorithm. The Lipid14 parameters were assigned to POPC molecules, while GAFF parameters were used for the ligand, whose partial charges were calculated with the AM1-BCC method as implemented in the Antechamber suite of AMBER 16. Linear interaction energy (LIE) evaluations were performed between the ligand (i.e. the atoms constituting the S-acyl moiety belonging to ibuprofen of the covalent adduct) and the protein residues located within a 12 Å radius from it. The *ccptraj* analysis program module of AMBER 16, was employed for the calculations, using the trajectories extracted from the last 10 ns of MD simulation, for a total of 100 snapshots (with a time interval of 100 ps).

IL-8 release assay

For IL-8 ELISA assay, NHBE cells were seeded in complete culture medium in 48-well plates, grown to ~80-90% confluence, and incubated overnight in serum-free medium before treatments. All the treatments were then performed in serum free medium. Cells were retreated (30 min) with HC-030031 (50 μM), IAG and ibuprofen (both, 100 μM) or vehicle (1% DMSO) before incubation (18 h at 37 °C in 5% CO2) with freshly prepared AITC (10–30 μM) and TNF-α (0.2 nM). Supernatants were then collected, and the human IL-8 content was assayed using a paired antibody quantitative ELISA kit (Invitrogen, Milan, Italy) (detection limit: 5 pg/ml). The assay was performed according to the manufacturer's instructions.

Data and statistical analysis

All data were expressed as mean ± s.e.m or confidence interval (CI). Statistical analysis was performed by the one-way analysis of variance (ANOVA) followed by the post-hoc Bonferroni's test for comparisons of multiple groups. For behavioural experiments with repeated measures, the two-way ANOVA followed by the post-hoc Bonferroni's test was used. Statistical analysis was performed on raw data using GraphPad software (GraphPad Prism version 6.00, San Diego, CA, USA). $P < 0.05$ was considered statistically significant.

5.2. Results

5.2.1. IAG antagonizes human and rodent TRPA1

The ability of IAG to affect TRPA1-mediated calcium responses was studied by using a single cell assay in human and rodent cells expressing TRPA1. IAG did not evoke per se any calcium response in hTRPA1-HEK293 cells (Figure V-1B,I). However, IAG, like the selective TRPA1 antagonist, HC-030031, inhibited in a concentration-dependent manner calcium response evoked by AITC [IC_{50}, 30 (CI, 22–40) μM and 3 (CI, 1.4–6) μM, respectively] (Figure V-1C). IAG reduced calcium responses evoked by additional reactive TRPA1 agonists, such as acrolein or hydrogen peroxide (H_2O_2) (Figure V-1D), but did not affect the responses by non-reactive agonists, icilin and zinc chloride ($ZnCl_2$) (Figure V-1D), which do not act by binding key cysteine residues of TRPA1 [117,172]. HC-030031 abolished the calcium responses evoked by both reactive and non-reactive agonists (Figure V-1D). IAG did not attenuate the rapid calcium responses evoked by acute exposure to the activating peptide (AP) of the human protease-activated receptor 2 (hPAR2) (hPAR2-AP) (Figure V-1D). This finding supports the selectivity of IAG. The ability of IAG to inhibit TRPA1 by binding key cysteine and lysine residues was further proved by the study of the mutated human TRPA1 (3C/K-Q hTRPA1), which lacks the cysteine and lysine residues, required for channel activation by reactive agonists, and which responds to menthol [149,151,152]. Calcium responses to menthol (100 μM) were unaffected by IAG in 3C/K-Q hTRPA1-HEK293 cells (Figure V-1E).

Selectivity of IAG for TRPA1 was robustly confirmed by a series of observations. In hTRPV1-HEK293, calcium responses to the TRPV1 agonist, capsaicin, were ablated by the TRPV1 selective antagonist, capsazepine, but were unaffected by IAG (Figure V-1F). In hTRPA1/TRPV1-HEK293 co-expressing cells, responses to capsaicin were attenuated by capsazepine, but not by IAG, whereas responses to AITC were ablated by IAG and HC-03003, but not by capsazepine (Figure V-1G). Moreover, in hTRPV4-HEK293 cells, calcium responses to the selective TRPV4 agonist, GSK1016790 A, were ablated by a TRPV4 antagonist, HC-067047, but were unaffected by IAG (Figure V-1H). Ibuprofen did not evoke per se any calcium responses and did not affect the calcium responses evoked by AITC, acrolein or H_2O_2 in hTRPA1-HEK293 cells (Figure V-1D,I). The glucuronidated metabolite of indomethacin, acyl-β-D-glucuronide, neither evoked calcium response nor reduced the calcium response evoked by AITC in hTRPA1-HEK293 cells (Figure V-1I).

IAG also inhibited the AITC-evoked calcium response in IMR90 cells, a cell line where TRPA1 was originally cloned [106], (Figure V-2A), and which constitutively expresses the channel. IAG [IC_{50}s, 60 (CI, 45–88) μM] and HC-030031 [IC_{50}s, 3 (CI, 2–6) μM] reduced AITC-evoked calcium responses (Figure V- 2B,C). IAG failed to attenuate rapid calcium responses evoked by acute exposure to hPAR2-AP (Figure V-2C). IAG [IC_{50}, 50 (CI, 40–70) μM] and HC-030031 [IC_{50}, 1 (CI, 0.3–1.8) μM] reduced AITC-evoked calcium responses in cultured rat dorsal root ganglion (rDRG) neurons, which express the native TRPA1 (Figure V-2D,E,F). IAG did not affect calcium responses to other excitatory stimuli, such as capsaicin and high potassium chloride (KCl) (Figure V-2G). Thus, IAG was able to selectively block the human and rodent TRPA1 channel.

5.2.2. Mode of TRPA1 targeting by IAG

A covalent docking approach was applied to evaluate the binding mode of the possible covalent adducts formed by transacylation of IAG with residues C621, C641 and C665, since the mutation of these residues abolished the inhibitory activity of the ligand on TRPA1. Moreover, these solvent accessible residues are located in an allosteric nexus of the TRPA1 channel, suitable for the detection of electrophile agonists [119], and have been demonstrated to exert a fundamental role in TRPA1 activation by reactive agonists like AITC [149]. The structure of the human TRPA1 ion channel recently determined by electron cryo-microscopy (PDB code 3J9P) was employed for this analysis [119]. After refining the protein structure (see Methods for details), the covalent docking protocol implemented in Gold software was applied to evaluate the binding orientations of the thioester adducts formed by reaction of IAG with C621, C641 and C665, corresponding to the acylation of cysteine thiol groups with the ligand acyl moiety belonging to ibuprofen. For each S-acyl-cysteine adduct, the top-scored binding disposition of the ligand was taken into account and further analyzed through MD simulation studies. After embedding the covalently modified protein in a lipid bilayer and solvating the system with explicit water molecules, 20 ns of MD simulation were performed (see Methods for details). The results were then analyzed in terms of ligand-protein interaction energy, in order to evaluate the reliability of the predicted covalent adducts from an energetic point of view. For this purpose, the linear interaction energy (LIE) approach was employed.

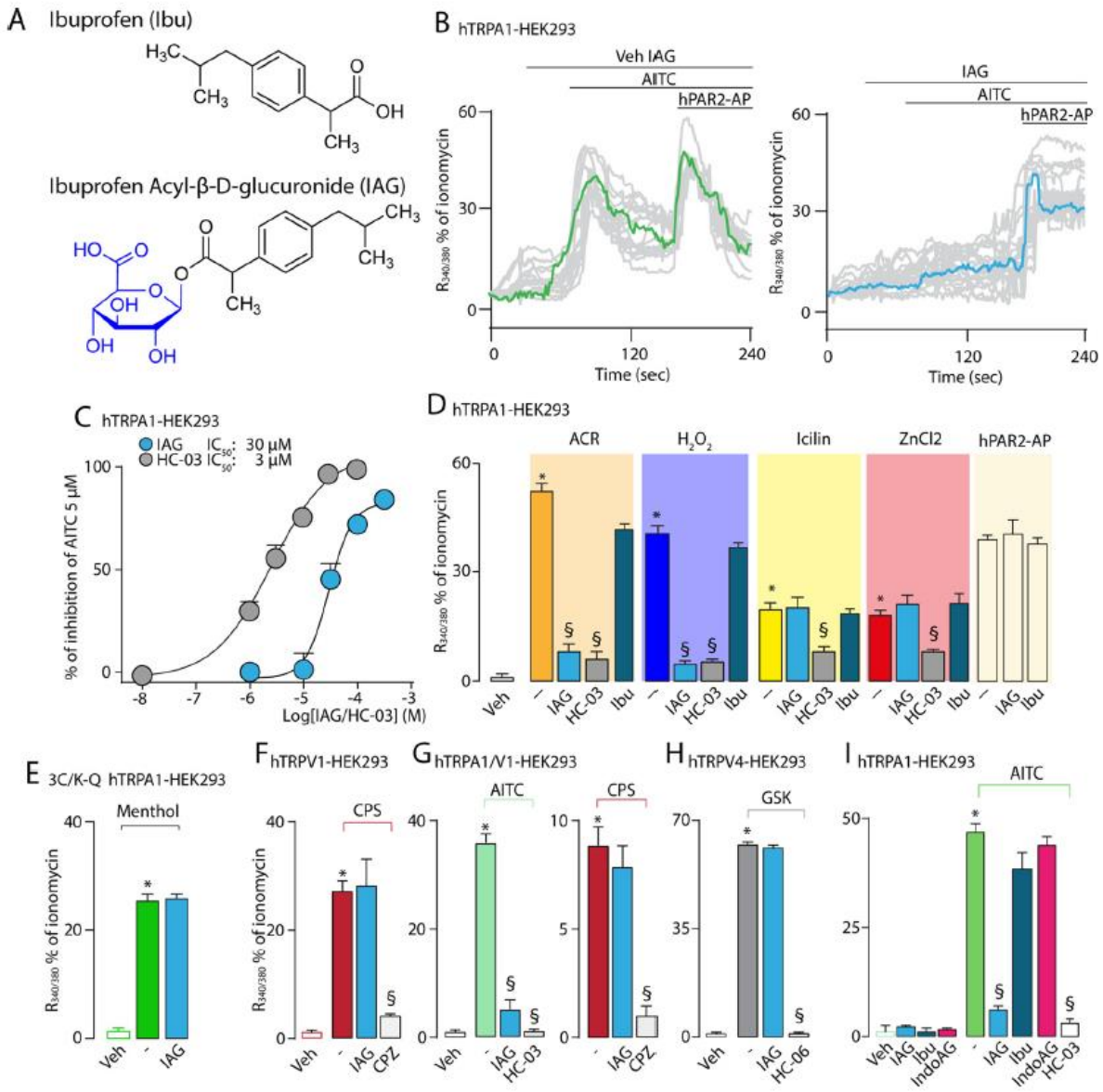

Figure V-1. IAG antagonizes the human recombinant TRPA1. A) Chemical structure of ibuprofen (Ibu) and ibuprofen acyl-β-D-glucoronide (IAG). **B)** Typical traces of the effect of IAG (100 µM) or its vehicle (Veh IAG) on calcium responses evoked by AITC (5 µM) in hTRPA1-HEK293. **C)** Concentration-response curves of the inhibitory effect of IAG and HC-030031 (HC-03), on the calcium response evoked by AITC (5 µM) in hTRPA1-HEK293 cells. **D)** Effect of IAG (100 µM), HC-030031 (HC-03, 30 µM) and Ibu (100 µM) on the calcium responses evoked by acrolein (ACR, 10 µM), hydrogen peroxide (H_2O_2, 500 µM), icilin (30 µM), zinc chloride ($ZnCl_2$, 1 µM) and the activating peptide (AP) of the human proteinase activated receptor 2 (hPAR2) (hPAR2-AP, 100 µM). **E)** Effect of IAG (100 µM) on the 3C/K-Q hTRPA1-HEK293 cells evoked by menthol (100 µM). **F)** Effect of IAG (100 µM) and capsazepine (CPZ, 10 µM) on the calcium responses evoked by capsaicin (CPS, 0.1 µM) in hTRPV1-HEK293 cells. **G)** Effect of IAG (100 µM), CPZ (10 µM) and HC-03 (30 µM) on the calcium response evoked by CPS (0.1 µM) and AITC (10 µM) in hTRPA1/V1-HEK293 cells. **H)** Effect of IAG (100 µM) and HC-067047 (HC-06, 10 µM) on the calcium response evoked by GSK1016790 A (GSK, 0.1 µM) in hTRPV4-HEK293 cells. **I)** Effect of IAG (100 µM), Ibu (100 µM) and indomethacin acyl-β-D-glucuronide (IndoAG, 100 µM) on the calcium response evoked by AITC (5 µM) in hTRPA1-HEK293 cells. Values are mean ± s.e.m of n>50 cells from at least 3 different experiments for each condition. Veh indicates vehicle of AITC, ACR, H_2O_2, icilin, $ZnCl_2$ and hPAR2-AP, dash (-) indicates vehicles of IAG, HC-03, ibu, CPZ, and HC-06. *P<0.05 vs. Veh; §P<0.05 vs. AITC, ACR, H_2O_2, icilin, $ZnCl_2$, CPS or GSK. One-way ANOVA and post-hoc Bonferroni's test.

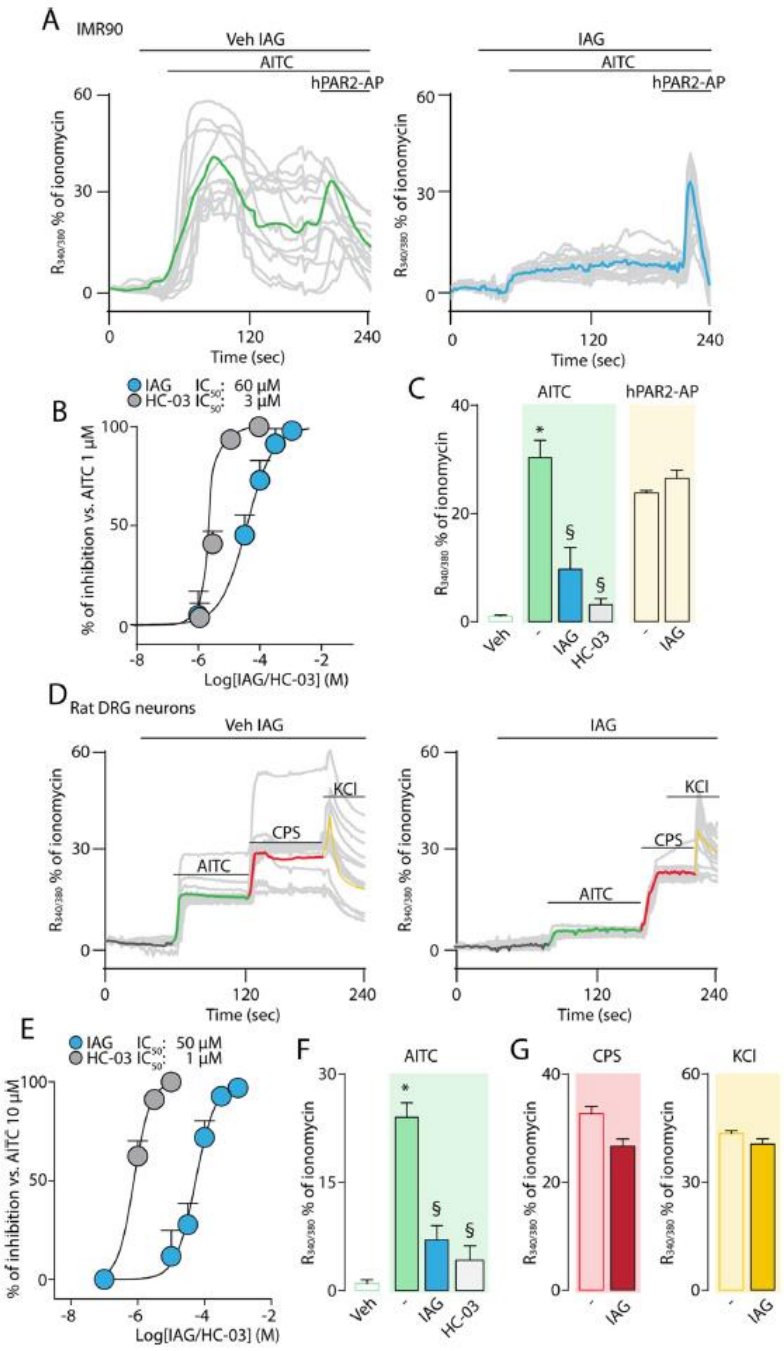

Figure V-2. IAG antagonizes the human and rat native TRPA1. A) Typical traces of the effect of pre-exposure (10 min) to Veh (vehicle) IAG/IAG (100 µM) on the calcium response evoked by AITC (1 µM) and the hPAR2-AP (100 µM) in IMR90 cells. **B)** Concentration-response curves of the inhibitory effect of IAG and HC-030031 (HC-03), on the calcium response evoked by AITC (1 µM) in IMR90 cells. **C)** Pooled data of the effect of IAG and HC-03 on the calcium response evoked by AITC (1 µM) in IMR90 cells. **D)** Typical traces of the inhibitory effect of pre-exposure (10 min) to Veh IAG/IAG (100 µM) on the calcium response evoked by AITC (10 µM), capsaicin (CPS, 0.1 µM) and KCl (50 mM) in rDRG neurons. **E)** Concentration-response curves of the inhibitory effect of IAG and HC-03 on the calcium response evoked by AITC in rDRG neurons. **F)** Pooled data of the effect of IAG and HC-03 on the calcium response evoked by AITC (10 µM) in rDRG neurons. **G)** Pooled data of the effect of IAG (100 µM) on the responses evoked by capsaicin (CPS, 0.1 µM) or high potassium chloride (KCl, 50 mM) in rDRG neurons. Values are mean ± s.e.m of n>25 cells from at least 3 different experiments for each condition. Veh indicates vehicle of AITC, dash (-) indicates vehicles of IAG and HC-03. **P*<0.05 vs. Veh; §*P*<0.05 vs. AITC. One-way ANOVA and post-hoc Bonferroni's test.

LIE evaluations allow the calculation of the non-bonded interactions between the ligand and the surrounding protein residues from the trajectories generated through MD simulations. Electrostatic and van der Waals energetic contributions are calculated for each MD snapshot and the obtained values are then used to derive the average total ligand-protein interaction energy. In this case, LIE evaluations were performed between the atoms constituting the acyl moiety belonging to ibuprofen of the three predicted S-acyl-cysteine covalent adducts and the protein residues located within a radius of 12 Å. The MD trajectories extracted from the last 10 ns of MD simulation were used for the calculations, for a total of 100 snapshots (with a time interval of 100 ps). The average LIE values (aLIE) were obtained for the three different covalent complexes as the sum of the average electrostatic (EELE) and van der Waals (EVDW) energy contributions expressed as kcal/mol (Figure V-3A).

The linear interaction energy evaluations highlighted the S-acyl-C621 thioester as the most energetically favored covalent adduct, presenting a linear interaction energy value (-31.9 kcal/mol) exceeding those estimated for the S-acyl-C641 and S-acyl-C665 covalent complexes by about 12 and 18 kcal/mol, respectively. Interestingly, the homolog of C621 in mouse TRPA1 (C622) was found to be the cysteine residue that most affected TRPA1 activation by reactive agonists, since its mutation completely abolished the responsiveness of TRPA1 to AITC [151]. The average binding disposition of ibuprofen within the S-acyl-C621 thioester adduct obtained from the last 10 ns of molecular dynamics simulation was obtained (Figure V-3B). The acyl chain belonging to ibuprofen perfectly fits a small hydrophobic pocket constituted by I611, F612, P617, V678, I679 and Y680, delimited by K610 and D677 from one side and T684 from the other. In particular, the aromatic moiety of the ligand lies on the P617 side chain, forming lipophilic interactions with this residue, as well as with I622 and I679, while the p-isobutyl group is sandwiched between F612 and V678, showing strong hydrophobic contacts with this latter residue. Moreover, the ligand carbonyl oxygen forms a hydrogen bond with the backbone nitrogen of Y680 that is maintained for about 80% of the entire molecular dynamics simulation, thus contributing to the anchoring of the ligand to the hydrophobic pocket. Interestingly, the S-acyl-C621 thioester was the only covalent complex in which a stable hydrogen bond between the ligand portion and the surrounding protein residues was observed (Figure V-3B).

A

Complex	EELE	EVDW	aLIE
S-acyl-C621	-12.6	-19.3	-31.9
S-acyl-C641	-8.9	-10.7	-19.6
S-acyl-C665	-2.5	-11.3	-13.8

B

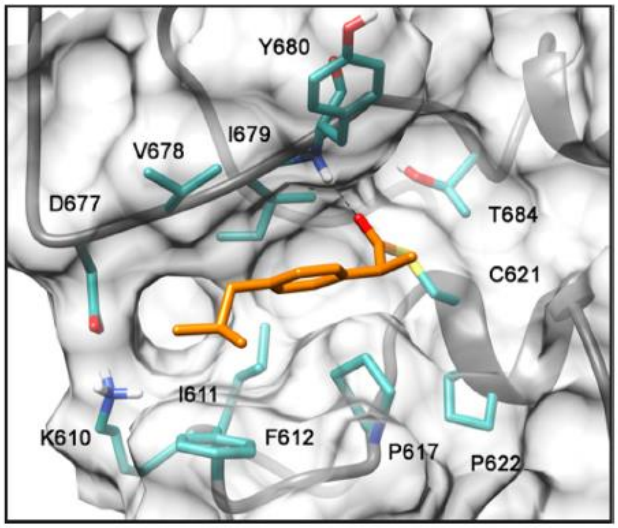

Figure V-3. IAG interact with hTRPA1 in molecular dynamic model. A) Linear Interaction Energy (LIE) results for the three covalent complexes of hTRPA1 obtained by transacylation of C621, C641 and C665 by IAG. Data are expressed as kcal/mol. **B)** Minimized average structure of the S-acyl-C621 hTRPA1 ion channel. The covalent ligand is shown in orange, while the protein residues are colored dark cyan.

5.2.3. IAG selectively inhibits TRPA1-mediated nocifensor responses

Next, we speculated that IAG produces *in vivo* antinociceptive effects via TRPA1 antagonism. The intraplantar (i.pl., 20 µl/paw) administration of IAG or HC-030031 dose-dependently reduced [ID_{50} of 4 (CI, 2–9) nmol, and ID_{50}, 8 (CI, 3–23) nmol, respectively] the acute nociceptive response evoked by the injection of AITC (i.pl.). Maximum inhibition on the nociceptive responses evoked by AITC (i.pl.) was 72%±2% for IAG and, 89%±1.7% for HC-030031 (n=6, $P < 0.05$) (Figure V-4A). Acute nociceptive responses induced by i.pl. capsaicin and hypotonic solution (TRPV1 and TRPV4-mediated responses, respectively) were attenuated by injection of the respective channel antagonists, capsazepine and HC-067047, but were unaffected by IAG (all i.pl.) (Figure V-4B). The nociceptive response evoked by acrolein (i.pl.) was inhibited by IAG and HC-030031 (both i.pl.) (Figure V-4C). In contrast, IAG (i.pl.) failed to affect nociceptive response evoked by the noncovalent agonist, $ZnCl_2$ (i.pl.), which, however, was attenuated by HC-030031 (i.pl.) (Figure V-4C). Ibuprofen i.pl. administration failed to affect the acute nociceptive response evoked by either AITC, acrolein or $ZnCl_2$ (all i.pl.) (Figure V-4C,D).

The systemic (intraperitoneal, i.p.) administration of HC-030031, IAG and ibuprofen dose-dependently [ID_{50}s 7 (CI, 4–14) mg/kg, 10 (CI, 4–20) mg/kg and ID_{50}s 27

(CI, 8–90) mg/kg, respectively] reduced the nociceptive responses to AITC (i.pl.) (Figure V-4E). Maximum inhibition by ibuprofen (42%±3%) was lower than those produced by IAG (76%±4%) and HC-030031 (83 ± 4%) (all 100 mg/kg, n=6 each, $P < 0.05$ ibuprofen vs. both IAG and HC-030031) (Figure V-4E). Systemic (i.p.) IAG did not affect the nociceptive responses evoked by either capsaicin or a hypotonic solution, which, however, were attenuated by the TRPV1 and TRPV4 antagonists, capsazepine and HC067047, respectively (Figure V-4F). Systemic (i.p.) IAG (both, 10 and 100 mg/kg) reduced the nociception evoked by acrolein but not that evoked by $ZnCl_2$ (Figure V-4G,H), whereas only 100 mg/kg, but not 10 mg/kg (both i.p.) ibuprofen reduced the nociceptive responses evoked by acrolein (Figure V-4G). IAG at both 10 and 100 mg/kg was more effective than the respective doses of ibuprofen (Figure V-4E,G). Finally, systemic (i.p.) HC-030031 inhibited the nociceptive responses evoked by both acrolein and $ZnCl_2$ (Figure V-4H).

5.2.4. IAG reduces TRPA1-dependent hyperalgesia and nociception in models of inflammatory pain

We tested the ability of IAG to reduce mechanical allodynia evoked by i.pl. carrageenan injection in the mouse hind paw. Carrageenan induces a prolonged mechanical allodynia that is in part mediated by TRPA1 [308,357]. IAG (i.pl., 2.5 h after carrageenan) almost completely attenuated mechanical allodynia (Figure V-5A), whereas an identical dose of ibuprofen produced a partial inhibition (Figure V-5B). A combination of HC-030031 and ibuprofen (both i.pl.) increased the effect of ibuprofen alone, but did not further affect the inhibitory response to IAG alone (Figure V-5A,B).

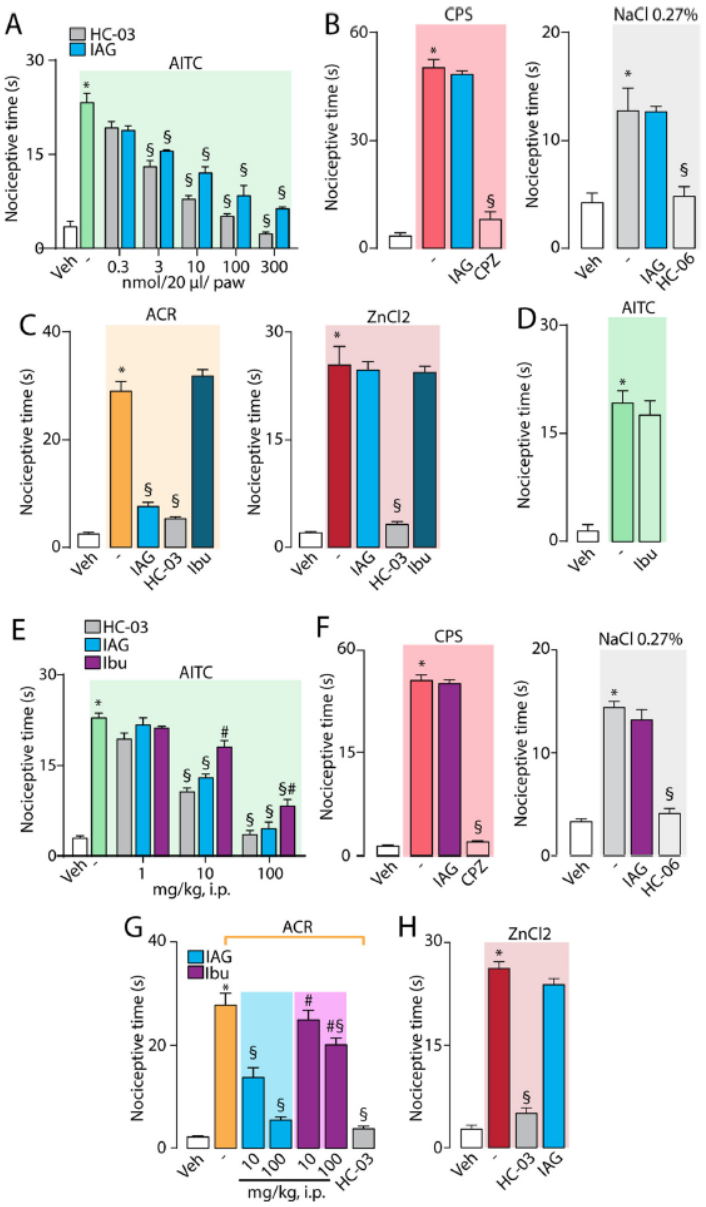

Figure V-4. IAG inhibits nociceptive responses evoked by reactive TRPA1 agonists in mice. **A)** Dose-dependent inhibitory effect of intraplantar (i.pl., 20 µl/paw) administration of IAG (0.3–300 nmol) and HC-030031 (HC-03, 0.3–300 nmol) on the acute nociceptive response evoked by i.pl. allyl isothiocyanate (AITC, 20 nmol) in C57BL/6 J mice. **B)** Effect of IAG (300 nmol), capsazepine (CPZ, 300 nmol) and HC-067047 (HC-06, 300 nmol) on the acute nociceptive response evoked by i.pl. CPS (1 nmol) and NaCl 0.27% in C57BL/6 J mice. **C)** Effect of i.pl. IAG (300 nmol), HC-03 (300 nmol) and ibuprofen (Ibu, 300 nmol) on the nociceptive response evoked by i.pl. acrolein (ACR, 10 nmol) and zinc chloride ($ZnCl_2$, 10 nmol) in C57BL/6 J mice. **D)** Effect of Ibu (300 nmol) on the nociceptive response evoked by i.pl. AITC (20 nmol) in C57BL/6 J mice. **E)** Dose-response inhibitory effect of intraperitoneal (i.p.) administration of IAG, Ibu and HC-03 (all, 1–100 mg/kg) on the acute nociceptive response evoked by i.pl. AITC (20 nmol) in C57BL/6 J mice. **F)** Effect of i.p. IAG (100 mg/kg) CPZ (4 mg/kg) and HC-06 (10 mg/kg) on the acute nociceptive response evoked by i.pl. CPS (1 nmol) and NaCl 0.27% in C57BL/6 J mice. **G)** Effect of i.p. IAG, Ibu (both, 10 and 100 mg/kg) and HC-03 (100 mg/kg) on the acute nociceptive response evoked by i.pl. ACR (10 nmol). **H)** Effect of IAG (100 mg/kg) and HC-03 (100 mg/kg) on the acute nociceptive response evoked by i.pl. $ZnCl_2$ (10 nmol). Values are mean ± s.e.m of n=6 mice for each experimental condition. Veh indicates vehicle of CPS, NaCl 0.27%, ACR, $ZnCl_2$ and AITC, dash (-) indicates vehicles of IAG, HC-03, ibu, CPZ and HC-06. **P*<0.05 vs. Veh; §*P*<0.05 vs. CPS or NaCl 0.27%, ACR and $ZnCl_2$, #*P*<0.05 vs. HC-03 and IAG. Oneway ANOVA and post-hoc Bonferroni's test.

A low systemic (i.p.) dose (10 mg/kg) of IAG, but not ibuprofen, significantly reduced carrageenan- evoked mechanical allodynia (Figure V-5C). A systemic (i.p.) high dose (100 mg/kg) of IAG or ibuprofen attenuated the mechanical allodynia induced by carrageenan, but the effect of IAG resulted higher than that of ibuprofen (Figure V-5D,E). The combination of systemic (both i.p.) HC-030031 and ibuprofen increased the inhibitory action of ibuprofen alone, but did not affect the inhibitory response to IAG alone (Figure V-5D,E). Systemic (i.p.) indomethacin partially inhibited carrageenan-induced mechanical allodynia, and its combination with HC-030031 completely reversed mechanical allodynia (Figure V-5F). Prostaglandin E_2 (PGE_2) assay from paw homogenates of mice receiving carrageenan and treated by local (i.pl., both 100 nmol) or systemic (i.p., both 100 mg/kg) IAG or ibuprofen revealed that both drugs produced a similar and complete reduction in the tissue content of PGE_2 (Figure V-5G,H). Systemic (i.p.) indomethacin, but not HC-030031, reduced PGE_2 content in paw homogenates (Figure V-5H). Finally, ibuprofen-acyl glucuronide attenuated carrageenan-evoked PGE_2 release in TRPA1 deleted (*Trpa1*$^{-/-}$) mice (Figure V-5I). Thus, IAG maintains the ability of the parent compound to inhibit COXs.

Formalin injection (i.pl.) into the hind paw of the mouse classically induces a biphasic nociceptive response, with phase I being entirely dependent on TRPA1 [181], whereas phase II involves different mechanisms, including the release of prostanoids. However, during phase II, ongoing diffusion and spread of formalin along TRPA1-expressing nerves may elicit release of a large variety of different mediators, among which prostanoids [358], which may sensitize TRPA1 [359]. IAG injection (i.pl.) attenuated both phase I and phase II of the response (Figure V-6A). In contrast, ibuprofen failed to affect phase I, but reduced phase II of the formalin test (Figure V-6A). Systemic (i.p.) administration of IAG (10 and 100 mg/kg) reduced phase I of the formalin test (Figure V-6B). However, only 100 mg/kg, but not 10 mg/kg (i.p.), of ibuprofen inhibited phase I of the formalin test (Figure V-6B). HC-030031 [181], but not indomethacin [360], inhibited phase I of the formalin test (Figure V-6B), whereas phase II was attenuated by both drugs (Figure V-6B).

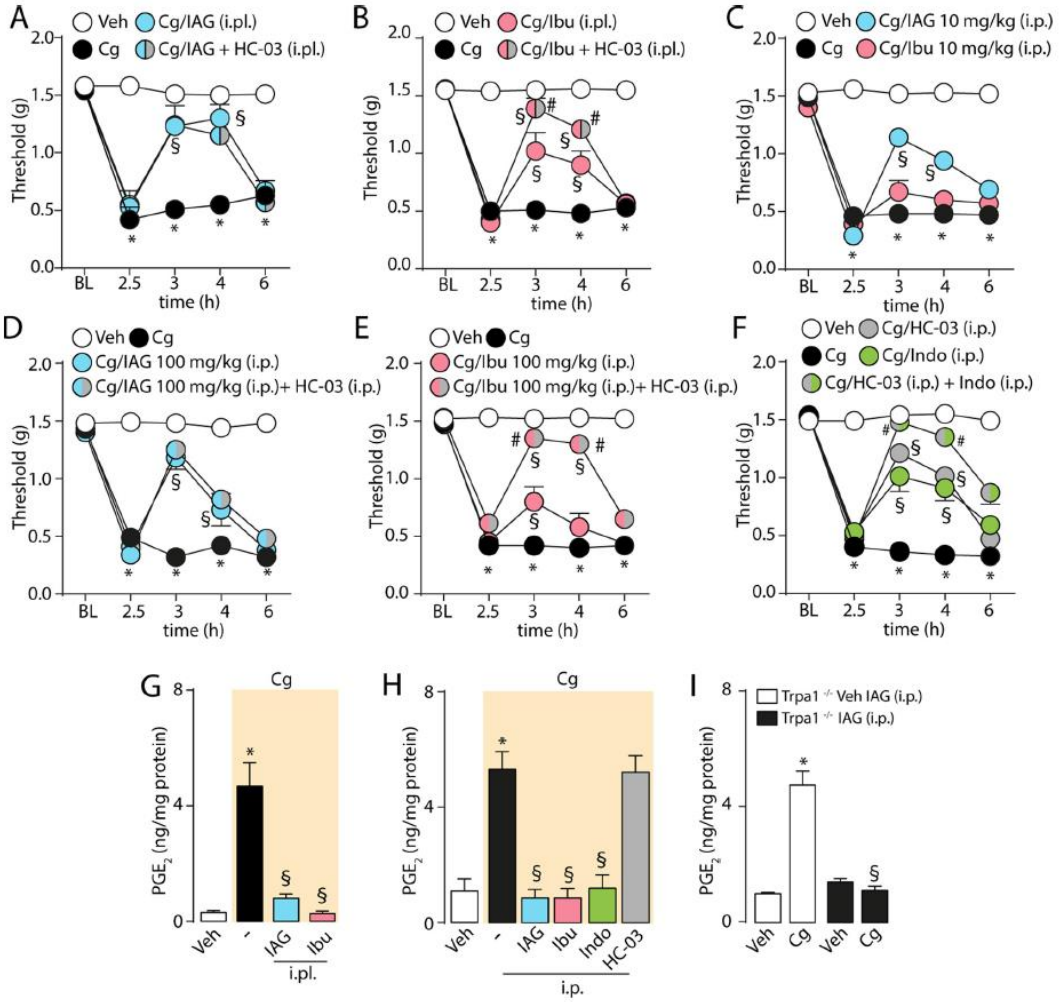

Figure V-5. IAG produces anti-hyperalgesic effect in the carrageenan model of inflammatory pain. A, B) Time course of the inhibitory effect of intraplantar (i.pl., 20 µl/paw) administration of IAG, Ibuprofen (Ibu) (both, 100 nmol) or of a mixture of IAG and HC-030031 (HC-03) or Ibu and HC-03 (all, 100 nmol) on the mechanical allodynia evoked by i.pl. carrageenan (Cg, 300 µg) in C57BL/6 J mice. **C–E)** Time course of the inhibitory effect of intraperitoneal (i.p.) administration of IAG, Ibu (both, 10 and 100 mg/kg) or a combination of IAG (100 mg/kg) or ibu (100 mg/kg) and HC-03 (100 mg/kg) on the mechanical allodynia evoked by i.pl. injection of Cg (300 µg) in C57BL/6 J mice. **F)** Time course of the inhibitory effect of i.p. HC-03 (100 mg/kg) and indomethacin (indo, 30 mg/kg) or a combination of both HC-03 (100 mg/kg) and indo (30 mg/kg) on the mechanical allodynia evoked by i.pl. injection of Cg (300 µg) in C57BL/6 J mice. **G)** PGE_2 levels in paw homogenates measured 180 min after i.pl. Cg (300 µg) in C57BL/6 J mice treated with IAG or Ibu (both, 100 nmol, i.pl.). **H)** PGE_2 levels in paw homogenates measured 180 min after i.pl. Cg (300 µg) in C57BL/6 J mice treated with IAG, Ibu, HC-03 (all, 100 mg/kg, i.p.) or indo (30 mg/kg, i.p.). **I)** PGE_2 levels in paw homogenates measured 180 min after i.pl. Cg (300 µg) in *Trpa1*$^{-/-}$ mice after IAG (100 mg/kg, i.p.). Values are mean ± s.e.m of n=6 mice for each experimental condition. Veh indicates vehicle of Cg, dash (-) indicates vehicles of IAG, Ibu, HC-03 and indo. **P*<0.05 vs. Veh; §*P*<0.05 vs. Cg. #*P*<0.05 vs. Cg/Ibu or Cg/HC-03 or Cg/indo. One- and two-way ANOVA and post-hoc Bonferroni's test.

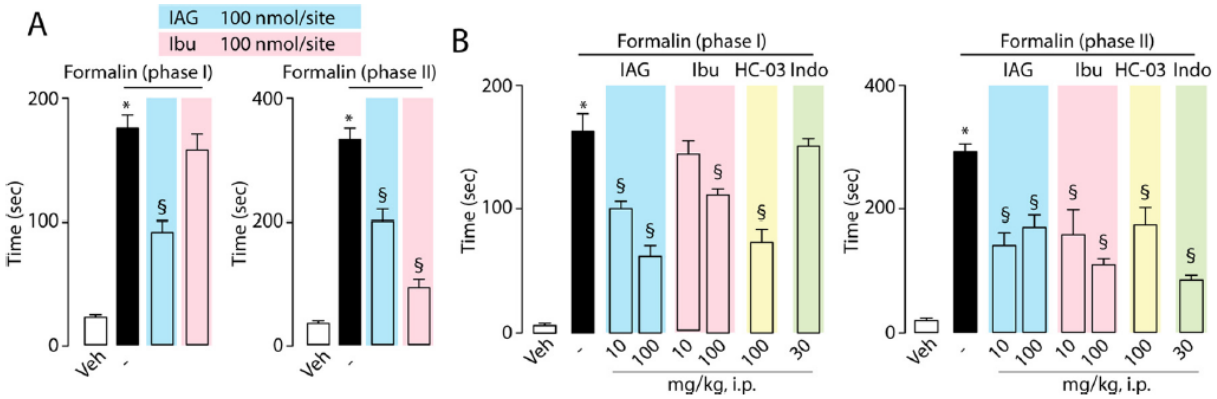

Figure V-6. IAG produces antinociception effect in the formalin model of inflammatory pain. A) Effect of intraplantar (i.pl., 20 µl/paw) administration of IAG and ibuprofen (Ibu) (both, 100 nmol) on phase I and phase II of the formalin test. **B)** Effect of intraperitoneal (i.p.) administration of IAG, Ibu (both, 10 and 100 mg/kg), HC-030031 (HC-03, 100 mg/kg) and indomethacin (Indo, 30 mg/kg) on phase I and phase II of the formalin test. Values are mean ± s.e.m of n=6 mice for each experimental condition. Veh indicates vehicle of formalin, dash (-) indicates vehicles of IAG, Ibu, HC-03 and Indo. **P*<0.05 vs. Veh; §*P*<0.05 vs. formalin. One-way ANOVA and post-hoc Bonferroni's test.

5.2.5. IAG reduces interleukin-8 release evoked by TRPA1 stimulation from bronchial epithelial cells

TRPA1 expressed by various non-neuronal cells of the airways elicits calcium responses and the release of proinflammatory cytokines, including interleukin-8 (IL-8) [134,361,362]. The calcium responses evoked by AITC in NHBE cells, which constitutively express TRPA1 [361], were attenuated in a concentration-dependent manner by IAG [IC_{50}, 20 (CI, 13–40) µM] and HC-030031 [IC_{50},10 (CI, 8–12) µM] (Figure V-7A,B,C). IAG failed to attenuate the rapid calcium responses evoked by acute exposure to hPAR2-AP (Figure V-7C). Exposure to AITC induced a concentration-related release of IL-8 from cultured NHBE cells. This effect was attenuated in the presence of both IAG and HC-030031, but not in the presence of ibuprofen (Figure V-7D). The observation that HC-030031, IAG or ibuprofen did not affect IL-8 release evoked by TNF-α indicated selectivity of IAG and HC-030031 for the AITC-evoked effects (Figure V-7D).

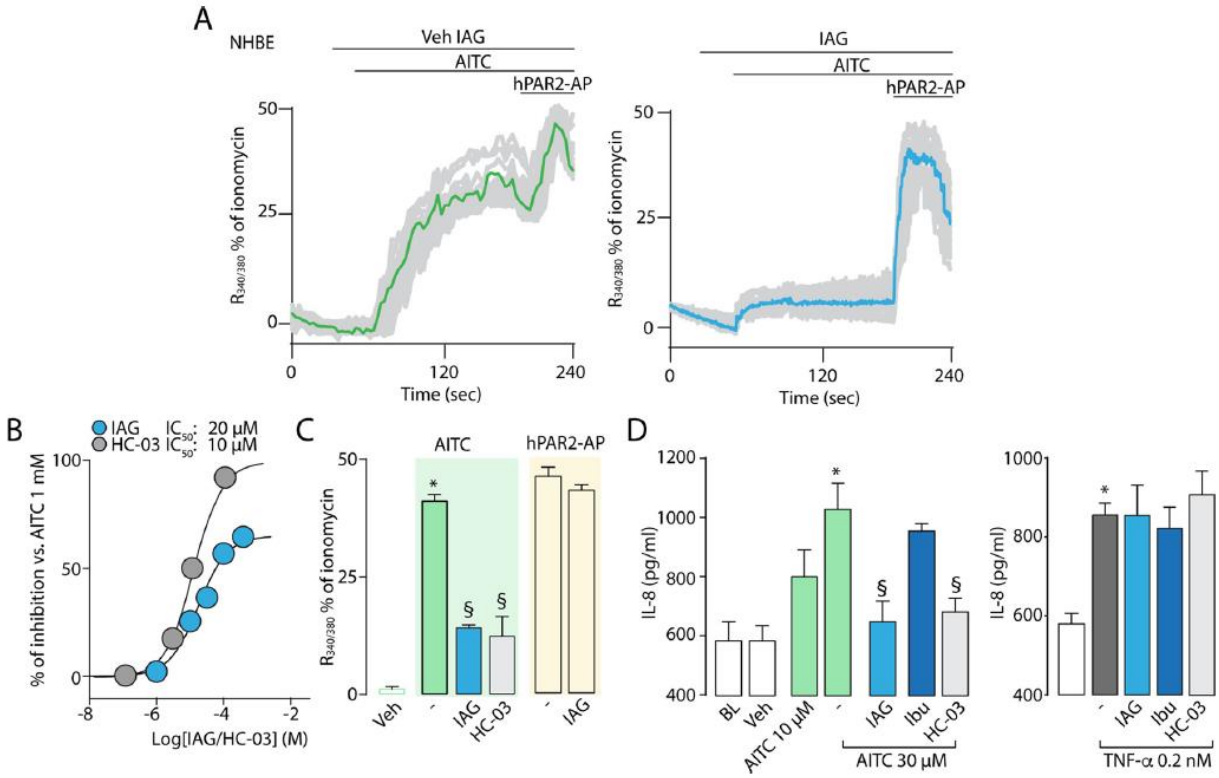

Figure V-7. IAG antagonizes human native TRPA1 in NHBE cells reducing the IL-8 release. **A)** Typical traces of the effect of pre-exposure (10 min) to Veh (vehicle) IAG/IAG (100 µM) on the calcium response evoked by AITC (1 mM) and the hPAR2-AP (100 µM) in NHBE cells. **B,C)** Concentration-response curves and pooled data of the inhibitory effect of IAG (0.1–1000 µM) and HC-030031 (HC-03, 0.1–1000 µM) on the calcium response evoked by AITC (1 mM) in NHBE cells. **D)** IL-8 release from NHBE cells exposed to AITC (10 and 30 µM) or TNF-α (0.2 nM) and pretreated with IAG and ibuprofen (Ibu) (both, 100 µM) and HC-03 (30 µM). Values are mean ± s.e.m. of n>25 cells from at least 3 different experiments for each condition or at least 3 independent experiments. Veh indicates vehicle of AITC and TNF-α, dash (-) indicates vehicles of IAG, Ibu and HC-03. **P*<0.05 vs. Veh; §*P*<0.05 vs. AITC. One-way ANOVA and post-hoc Bonferroni's test.

5.3. Discussion

The COX inhibitor ibuprofen is widely used as a first line treatment for the relief of pain and inflammation [264]. Glucuronide metabolites, including those generated from ibuprofen, are generally considered inactive and rapidly excreted compounds [271]. However, acyl glucuronides, undergoing hydrolysis, acyl migration and molecular rearrangement, exhibit chemical reactivity that allow them to covalently bind various macromolecules [271,363,364]. TRPA1 belongs to such macromolecules that, through Michael addition, undergo nucleophilic attack via specific cysteine/lysine residues [149,151]. Therefore, we hypothesized that, as with various reactive compounds, IAG may react with

TRPA1 [149,151]. Our major finding is that IAG, but not its parent compound, ibuprofen, antagonizes the proalgesic TRPA1 channel. This conclusion derives, primarily, from the *in vitro* pharmacological profile of IAG, which, unlike ibuprofen, selectively inhibits the recombinant and native human TRPA1 and the native rodent channel in nociceptors. Failure of the acyl derivative of indomethacin to affect channel activity underlines the unique ability of IAG to target TRPA1.

Indication that the reactive property of IAG is needed for efficient TRPA1 targeting is based on functional experiments with the mutated form of the human TRPA1 channel, and on docking and molecular dynamic simulations. The mutant hTRPA1-3C/K-Q has the unique property of responding to non-reactive agonists, such as menthol and icilin [149,151,152], but not to reactive agonists, including AITC. In hTRPA1-3C/K-Q expressing cells, IAG did not affect the calcium response evoked by menthol. Thus, the ability of IAG to inhibit TRPA1 depends on the cysteine/lysine residues required for channel activation by electrophilic/reactive agonists. Acyl-glucuronides are known to react by transacylation with nucleophilic residues, leading to the formation of a covalent adduct in which the acyl group, linked to the glucuronide, is transferred to the nucleophilic atom of the residue [271,363,364]. To explore the interaction between IAG and the human TRPA1 channel we performed computational studies, including molecular docking and dynamic simulations, which predicted the formation of covalent adducts between IAG and TRPA1. Computational results with the mutated channel confirm that the inhibitory activity of IAG should be ascribed to its interaction with one of the mutated residues. *In vivo* results that IAG attenuated nociception evoked by reactive TRPA1 agonists, but not those produced by non-reactive agonists, such as icilin and zinc chloride, further supported the *in vitro* data and simulation experiments, underlining that chemical reactivity is required for TRPA1 targeting by IAG.

Additional *in vivo* data strengthen the conclusion obtained from *in vitro* findings. Local injection of IAG in the mouse hind paw prevented acute nociception elicited by local administration of the reactive TRPA1 agonists, AITC and acrolein, but was ineffective against TRPV1 or TRPV4 agonists, indicating selectivity. Notably, local injection of ibuprofen in the mouse hind paw did not affect AITC or acrolein-evoked nociception. It is possible that following i.pl. ibuprofen no IAG is generated locally, and the action of TRPA1 agonists remains unopposed. However, about 10–15% of systemic ibuprofen is converted into IAG [269]. Thus, liver metabolism of a high dose of ibuprofen may produce IAG levels

such as to guarantee a local concentration sufficient for inhibiting TRPA1. This hypothesis is supported by the observation that a high dose of systemic ibuprofen produced a partial attenuation of the nociception evoked by AITC.

Other NSAIDs, which derive from propionic acid, are known to generate acyl glucuronides through hepatic metabolism. These acyl derivatives could potentially possess anti-TRPA1 properties similar to those of IAG. However, acyl glucuronidation does not warrant per se that the metabolites possess the chemical requirements for effective TRPA1 antagonism. For example, we failed to detect any effect of the acyl derivative of indomethacin, acyl-β-D-glucuronide, in antagonizing AITC evoked calcium response *in vitro*, and systemic indomethacin pretreatment did not affect the acute nociception of phase I of the formalin test.

The analgesic action of ibuprofen derives from its ability to inhibit COXs, and the ensuing blockade of prostaglandin generation [266,267]. This feature also justifies the anti-inflammatory activity of ibuprofen. While we provided evidence that IAG targets TRPA1, we wondered whether it maintains the ability of the parent drug to inhibit COXs. We also wondered whether IAG ability to inhibit TRPA1 may exert anti-inflammatory activity in an ibuprofen-independent manner. Carrageenan injection in rodent paw evokes inflammation and prolonged allodynia that are in part mediated by prostaglandins and in part by TRPA1 [224,308,357,365]. When allodynia was analyzed, local (intraplantar) IAG elicited a more robust inhibitory effect than that of an identical dose of ibuprofen, and the combination with HC-030031 potentiated inhibition by ibuprofen, but not that by IAG. Because both IAG and ibuprofen ablated PGE_2 levels it is possible that the effect of locally administered IAG is due to both COX inhibition and TRPA1 antagonism, whereas locally administered ibuprofen solely inhibits COXs.

The study of systemic administered drugs strengthens this hypothesis. A low dose of IAG, but not ibuprofen, attenuated carrageenan-evoked allodynia. A high dose of IAG or ibuprofen completely reversed or partially inhibited allodynia, respectively. Furthermore, the combination of the high dose of ibuprofen and HC-030031 potentiated the effect of ibuprofen alone. Similar results were obtained with indomethacin (its metabolite, acyl-β-D-glucuronide-indomethacin, does not target TRPA1) alone or in combination with HC-030031. Thus, TRPA1 stimulation by endogenous agonists generated by carrageenan-evoked inflammation cannot be completely surmounted by the amount of IAG generated by systemic metabolism of 100 mg/kg ibuprofen. The observation that both systemic

ibuprofen and IAG completely inhibited PGE_2 generation evoked by carrageen, indicates that the metabolite maintains the COX inhibitory activity of the parent drug, and justify the complete attenuation of carrageenan-evoked allodynia by IAG which may simultaneously inhibit COXs and TRPA1. TRPA1 has been reported to contribute to inflammation by different pathways, including the release of proinflammatory cytokines, such as IL-8 [134,362]. The present *in vitro* observation that IAG, but not ibuprofen, attenuates the TRPA1-dependent ability of NHBE cells to release IL-8 underlines the contribution of the COX-independent anti-inflammatory activity of the metabolite.

Our findings add new insights into the antinociceptive/anti-hyperalgesic and anti-inflammatory activity of ibuprofen which, in addition to COX inhibition, attenuates TRPA1 activity *via* IAG generation. This novel mechanism of ibuprofen/IAG indirectly underlines the TRPA1 contribution to acute nociception and delayed allodynia in various models of inflammatory pain. Further studies are needed to establish whether TRPA1 antagonism by IAG contributes to the therapeutic effect of ibuprofen in pain and inflammation in humans, and whether IAG may have an efficacy and safety profile different from its parent drug.

This work has been published in Pharmacological Research

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VI - Conclusions

The TRPA1 represents a key target in pain modulation. This receptor can be activated by a wide series of exogenous chemicals, but in primary sensory neurons it exhibits the distinctive property to detect and to be sensitized by a series of endogenous molecules, which play a major role in inflammation and tissue injury. TRPA1 activators are generally irritant molecules derived from diet and environment [139], or they can be endogenously produced during an inflammatory response, such as the byproducts of oxidative carbonylic and nitrative stress [138,156,158]. TRPA1 activation by oxidative/nitrative/carbonylic stress byproducts is emerging as a major pathway in signaling pain and neurogenic inflammation in the body, involved in different types of pain, ranging from inflammatory, to neuropathic and migraine headache pain. Migraine is a debilitating and widespread neurovascular pain disorder, with an estimated heritability as high as 50% [366]. Migraine affects almost 15% of the adult worldwide population, with an enormous social and economic cost, and with not so effective therapies [367]. This pathology alone is responsible for almost 3% of disability attributable to a specific disease worldwide, and it is ranked first among neurological disorders, seventh among non-communicable diseases and eighth among most burdensome diseases [368]. In the light of this, it is important to find novel therapeutic strategies and targets that would make it possible to ameliorate migraineurs pain condition.

In the first study presented, we examined the effect of the butterbur component isopetasin on TRPA1. Butterbur [*Petasites hybridus* (L.) Gaertn.] is an herbaceous perennial plant belonging to the family of *Asteraceae* (which includes also *Tanacetum parthenium* L. [276]), that has been used by folk medicine of northern Eurasia and America for therapeutic purposes, including treatment of fever, respiratory diseases, spasms, and pain [240]. Butterbur is currently indicated as level A recommendation for migraine prophylaxis by the American Headache Society guidelines [230] and its major constituents, petasin and isopetasin [226], are considered responsible for the antimigraine effects of the herbal extract [241]. Our results show that isopetasin is able to gate TRPA1 channel. Mouse, rat and human (native and recombinant) TRPA1 are gated with similar efficacy and potency by isopetasin, suggesting that responses evoked by the drug *in vivo* in rodents

could be reproduced in humans. Moreover, failure to activate TRPV1 and TRPV4 channels, as well as failure to gate mutant TRPA1 in transiently transfected cells (3C/K-Q hTRPA1-HEK293), indicates that isopetasin is a selective, electrophilic [149], TRPA1 agonist. Petasin stimulated TRPA1 with similar potency, although with less efficacy than isopetasin, so the hemiterpene moiety of angelic acid is not critical for activity. In addition, isopetasin acts as a partial agonist on the channel. A chronic treatment with isopetasin demonstrated that this molecule can desensitize TRPA1, and defunctionalize peptidergic sensory neurons, as mice were not responsive not only to TRPA1, but also TRPV1 and TRPV4, agonists after five days of treatment. Thus, as already seen with parthenolide [276], isopetasin targets TRPA1 and induces defunctionalization of trigeminal nerve terminals, attenuating their ability to release CGRP and signal pain. From these observations, we can conclude that successful treatment and prevention of migraine with isopetasin, may provide a solid basis for future basic and translational-medical investigations of TRPA1-tropic approaches for migraine.

In the second part, we investigated the possibility that some constituents of *Crocus sativus* L. (saffron crocus) could as well target TRPA1. Saffron belongs to the family of *Iridaceae* [243] and has been used for centuries not only for food flavoring and coloring, but also to treat headache in Indian traditional medicine [234]. Furthermore, saffron extract has been reported to possess many beneficial effects, including anti-depressive, antioxidant, anti-inflammatory and antinociceptive properties [235]. The three major constituents of saffron are crocin, picrocrocin and safranal, and analgesic actions have been attributed in particular to safranal in various animal models of pain [247,248]. With our data, we showed that while crocin does not target TRPA1 channel, safranal and picrocrocin gate this receptor, the former with much more efficacy and potency than the latter. Nevertheless, safranal happened to be less effective than AITC, in gating TRPA1. The *in vitro* and *ex vivo* findings that this molecule failed to activate TRPV1 and TRPV4 channels, and that it also failed to generate a $[Ca^{2+}]_i$ increase in HEK293 transfected with the mutant TRPA1 (3C/K-Q hTRPA1-HEK293), led us to theorize that safranal could behave as a selective electrophilic [149] partial agonist on the TRPA1 channel, similarly to isopetasin [298]. Desensitization protocols with rat urinary bladder strips and with rat and mouse spinal cord demonstrated, indeed, that safranal is able to desensitize TRPA1, reducing AITC-induced bladder contraction and CGRP release. In contrast to isopetasin, this desensitization appeared to be homologous, since TRPV1 and TRPV4 agonists were still

able to elicit responses in tissues. Moreover, chronic treatment with safranal in mice confirmed this hypothesis. Thus, safranal is able to homologously desensitize TRPA1 channel, and this mechanism is probably the basis for the understanding of its antinociceptive properties. This was another evidence of the important role that the TRPA1 channel fulfills in pain sensation.

Another part of this three years study aimed at investigating the mechanisms of nitroglycerin (GTN)-induced migraine-like attacks. Occupational exposure or treatment with organic nitrates have long been known to provoke migraine [250,251], and GTN has been used as a clinical provocation test for migraine [252-254]. In most migraineurs subjects, GTN administration evokes (hours later) headaches that fulfill the criteria of a typical migraine attack [214,254]. Moreover, many studies have shown that GTN administration in rodents and humans induces a delayed and prolonged hyperalgesia which seem to temporally correlate with the GTN-induced migraine-like attacks [255-257]. GTN is also able to release the active vasodilator gaseous compound, nitric oxide (NO), which is believed to be the main cause of this migraine-like attacks. The major finding of our study was that the delayed and prolonged GTN-evoked allodynia in mice is entirely TRPA1-dependent, and this evidence is supported by both genetic deletion and pharmacological blockade of the channel. It is known that NO is capable of activating TRPA1, either directly or indirectly *via* its byproducts through nitrosylation of cysteinyl residues [222]. We observed that GTN activates TRPA1 in cultured trigeminal neurons and in human transfected cells (hTRPA1-HEK293). However, under *in vitro* circumstances, the NO-scavenger cPTIO failed to inhibit GTN-induced $[Ca^{2+}]_i$ increase, so the conversion of GTN to NO is not required for GTN to activate TRPA1, probably due to insufficient release velocity or insufficient amount of NO. Moreover, TRPA1 is directly engaged by GTN through the same cysteine/lysine residues required for channel activation by electrophilic agonists [149,331]. On the contrary, *in vivo* systemic treatment with GTN was inhibited by cPTIO, indicating that NO, liberated from GTN by ALDH-2, activates TRPA1 in the soma of TG neurons to induce allodynia. TG, though being outside the blood brain barrier, are contained within the meningeal membranes, and thus are accessible to drugs given both systemically and intrathecally. Our results revealed also the existence of an autocrine pathway within TG nociceptors that is initiated by GTN-released NO. By gating TRPA1 in TG nociceptors, NO leads in fact to activation of NADPH oxidase-1 (NOX1)/NOX2, which generate ROS and the ensuing aldehyde endproducts. Unfortunately, it is not possible to discriminate whether TRPA1,

in addition to generating the proalgesic oxidative stress, is the final mediator of the hypersensitivity, since it is expressed by the same sensory neurons that trigger the oxidative burst and then mediate the allodynia. Nevertheless, TRPA1 is a major sensor of oxidative and carbonylic stress [152,159], so it is possible that TG-expressed TRPA1 is the final target of the ROS/4-HNE generated by GTN/NO. Noteworthy, the CGRP receptor antagonist BIBN4096BS failed to reduce GTN-evoked migraine-like attacks in migraineurs [219]. Our study confirmed this observation, as CGRP provided only a limited contribution to mice allodynia, and GTN caused an early CGRP-independent vasodilation in the mouse periorbital area. Taken together, these results provide new insights on GTN-induced migraine-like attacks mechanisms. TRPA1 antagonists, which are currently in clinical development, or established antimigraine drugs, which have recently been identified as selective channel inhibitors [224] may be used to verify if a mechanism similar to the present one mediates the effects induced by GTN in migraineurs.

Finally, we investigated the possibility that ibuprofen-acyl glucuronide (IAG), a metabolite of ibuprofen, could antagonize TRPA1. Ibuprofen is a classical non-steroidal anti-inflammatory drug (NSAID), widely used for its analgesic and anti-inflammatory properties [264,265], in particular to relieve inflammation and several types of pain, including headache, muscular pain, and others [264]. The therapeutic effects of ibuprofen are attributed to inhibition of prostanoid synthesis by a non-selective, reversible inhibition of both COX1 and COX2 [266,267]. Most of ibuprofen is usually metabolized to inactive metabolites through an oxidative reaction [268,269]; however, 10–15% of ibuprofen is glucuronidated to ibuprofen-acyl glucuronide [269], which may covalently bind various macromolecules [270,272], including TRPA1. Our major finding is that IAG, but not ibuprofen, antagonizes the proalgesic TRPA1 channel. *In vitro*, IAG was able, unlike ibuprofen, to selectively inhibit the recombinant and native human TRPA1 and the native rodent channel in nociceptors. This unique ability was underlined by the fact that the acyl derivative of indomethacin failed to reproduce the same effects. Moreover, IAG did not affect responses evoked by the non-electrophilic agonist menthol in hTRPA1-3C/K-Q mutant cells, indicating that the reactive property of IAG with specific cysteine/lysine residues [149] is required for channel blocking. Computational studies with the mutated channel confirmed this hypothesis. The *in vitro* results were strengthened by *in vivo* experiments, were IAG was able to attenuate nociception evoked by the reactive TRPA1 agonists AITC and acrolein, but not the effects produced by the non-reactive agonists icilin

and zinc chloride. Furthermore, local injection of IAG in the mouse hind paw prevented acute nociception elicited by local administration of reactive TRPA1 agonists, but was ineffective against TRPV1 or TRPV4 agonists, indicating selectivity. A systemic administration of ibuprofen also produced a partial attenuation of AITC-evoked nociception, probably due to the formation of IAG through liver metabolism [269]. Finally, we observed that in a carrageenan-induced model of inflammation, local injection of IAG produced a more potent inhibitory effect than ibuprofen, probably due to a combination of COX-inhibition and TRPA1-antagonism by IAG, since PGE_2 levels were ablated by both compounds in the same way. Our results show that the antinociceptive and anti-inflammatory activity of ibuprofen can be attributed to TRPA1 inhibition, in addition to COX inhibition, *via* IAG generation. Further studies could establish whether IAG may have an efficacy and safety profile different from its parent drug.

The findings of this three years study indicate that TRPA1 expressed in neuronal and non-neuronal cells is a key mediator in inflammatory and neuropathic pain modulation. Our results suggest novel therapeutic approaches to treat pain syndromes, in particular migraine, involving TRPA1 antagonists and TRPA1-desensitizing agents. These analgesic molecules may represent a major advancement for the treatment of pain.

References

1. Pain terms: a list with definitions and notes on usage. Recommended by the IASP Subcommittee on Taxonomy. *Pain.* Jun 1979;6(3):249.

2. Lumley MA, Cohen JL, Borszcz GS, et al. Pain and emotion: a biopsychosocial review of recent research. *J Clin Psychol.* Sep 2011;67(9):942-968.

3. Casey RMKL. Sensory, Motivational and Central Control Determinants of Pain. *The Skin Senses*: Charles C. Thomas; 1968.

4. Johnson MK, Nolen-Hoeksema S, Mitchell KJ, Levin Y. Medial cortex activity, self-reflection and depression. *Soc Cogn Affect Neurosci.* Dec 2009;4(4):313-327.

5. Ericsson M, Poston WS, Linder J, Taylor JE, Haddock CK, Foreyt JP. Depression predicts disability in long-term chronic pain patients. *Disabil Rehabil.* Apr 15 2002;24(6):334-340.

6. Miranda C, Selleri S, Pierotti MA, Greco A. The M581V mutation, associated with a mild form of congenital insensitivity to pain with anhidrosis, causes partial inactivation of the NTRK1 receptor. *J Invest Dermatol.* Oct 2002;119(4):978-979.

7. Treede RD, Rief W, Barke A, et al. A classification of chronic pain for ICD-11. *Pain.* Jun 2015;156(6):1003-1007.

8. Basbaum AI, Bautista DM, Scherrer G, Julius D. Cellular and molecular mechanisms of pain. *Cell.* Oct 16 2009;139(2):267-284.

9. Aley KO, Levine JD. Role of protein kinase A in the maintenance of inflammatory pain. *J Neurosci.* Mar 15 1999;19(6):2181-2186.

10. Gold MS, Levine JD, Correa AM. Modulation of TTX-R INa by PKC and PKA and their role in PGE2-induced sensitization of rat sensory neurons in vitro. *J Neurosci.* Dec 15 1998;18(24):10345-10355.

11. Ossipov MH, Dussor GO, Porreca F. Central modulation of pain. *J Clin Invest.* Nov 2010;120(11):3779-3787.

12. Cao H, Gao YJ, Ren WH, et al. Activation of extracellular signal-regulated kinase in the anterior cingulate cortex contributes to the induction and expression of affective pain. *J Neurosci.* Mar 11 2009;29(10):3307-3321.

13. Woolf CJ. Evidence for a central component of post-injury pain hypersensitivity. *Nature.* Dec 15-21 1983;306(5944):686-688.

14. Julius D, Basbaum AI. Molecular mechanisms of nociception. *Nature.* Sep 13 2001;413(6852):203-210.

15. Woolf CJ, Salter MW. Neuronal plasticity: increasing the gain in pain. *Science.* Jun 9 2000;288(5472):1765-1769.

16. Marchand F, Perretti M, McMahon SB. Role of the immune system in chronic pain. *Nat Rev Neurosci.* Jul 2005;6(7):521-532.

17. Yeu-Shiuan Su W-HS, Chih-Cheng Chen Molecular mechanism of inflammatory pain. *World Journal of Anesthesiology.* 2014 March 27 2014;3(1):71-81.

18. Koltzenburg M, Scadding J. Neuropathic pain. *Curr Opin Neurol.* Oct 2001;14(5):641-647.

19. Bennett RM. The rational management of fibromyalgia patients. *Rheum Dis Clin North Am.* May 2002;28(2):181-199, v.

20. Bolay H, Reuter U, Dunn AK, Huang Z, Boas DA, Moskowitz MA. Intrinsic brain activity triggers trigeminal meningeal afferents in a migraine model. *Nat Med.* Feb 2002;8(2):136-142.

21. Sarkar S, Aziz Q, Woolf CJ, Hobson AR, Thompson DG. Contribution of central sensitisation to the development of non-cardiac chest pain. *Lancet.* Sep 30 2000;356(9236):1154-1159.

22. Burstein R, Cutrer MF, Yarnitsky D. The development of cutaneous allodynia during a migraine attack clinical evidence for the sequential recruitment of spinal and supraspinal nociceptive neurons in migraine. *Brain.* Aug 2000;123 (Pt 8)(Pt 8):1703-1709.

23. Rainville P, Duncan GH, Price DD, Carrier B, Bushnell MC. Pain affect encoded in human anterior cingulate but not somatosensory cortex. *Science.* Aug 15 1997;277(5328):968-971.

24. Kelman L. The triggers or precipitants of the acute migraine attack. *Cephalalgia.* May 2007;27(5):394-402.

25. Murray CJ, Vos T, Lozano R, et al. Disability-adjusted life years (DALYs) for 291 diseases and injuries in 21 regions, 1990-2010: a systematic analysis for the Global Burden of Disease Study 2010. *Lancet.* Dec 15 2012;380(9859):2197-2223.

26. Mantyh PW, Clohisy DR, Koltzenburg M, Hunt SP. Molecular mechanisms of cancer pain. *Nat Rev Cancer.* Mar 2002;2(3):201-209.

27. Melzack R. From the gate to the neuromatrix. *Pain.* Aug 1999;Suppl 6(6):S121-126.

28. Bourne S, Machado AG, Nagel SJ. Basic anatomy and physiology of pain pathways. *Neurosurg Clin N Am.* Oct 2014;25(4):629-638.

29. S. SC. *The Integrative Action of the Nervous System*. New York: Scribner; 1906.

30. Longmore J, Hill RG, Hargreaves RJ. Neurokinin-receptor antagonists: pharmacological tools and therapeutic drugs. *Can J Physiol Pharmacol.* Jun 1997;75(6):612-621.

31. Braz J, Solorzano C, Wang X, Basbaum AI. Transmitting pain and itch messages: a contemporary view of the spinal cord circuits that generate gate control. *Neuron.* May 7 2014;82(3):522-536.

32. Hunt SP, Pini A, Evan G. Induction of c-fos-like protein in spinal cord neurons following sensory stimulation. *Nature.* Aug 13-19 1987;328(6131):632-634.

33. Snider WD, McMahon SB. Tackling pain at the source: new ideas about nociceptors. *Neuron.* Apr 1998;20(4):629-632.

34. Rosenow JM, Henderson JM. Anatomy and physiology of chronic pain. *Neurosurg Clin N Am.* Jul 2003;14(3):445-462, vii.

35. Geppetti P, Holzer P. *Neurogenic inflammation*. Boca Raton: CRC Press; 1996.

36. Lewis T. The nocifensor system of nerves and its reactions. *Br Med J.* 1937.

37. Szolcsanyi J. Capsaicin and nociception. *Acta Physiol Hung.* 1987;69(3-4):323-332.

38. Amadesi S, Moreau J, Tognetto M, et al. NK1 receptor stimulation causes contraction and inositol phosphate increase in medium-size human isolated bronchi. *Am J Respir Crit Care Med.* Apr 2001;163(5):1206-1211.

39. Geppetti P, Bertrand C, Baker J, Yamawaki I, Piedimonte G, Nadel JA. Ruthenium red, but not capsazepine reduces plasma extravasation by cigarette smoke in rat airways. *Br J Pharmacol.* Mar 1993;108(3):646-650.

40. Myers AC, Goldie RG, Hay DW. A novel role for tachykinin neurokinin-3 receptors in regulation of human bronchial Ganglia neurons. *Am J Respir Crit Care Med.* Feb 1 2005;171(3):212-216.

41. Crimi N, Polosa R, Maccarrone C, Palermo B, Palermo F, Mistretta A. Effect of topical application with capsaicin on skin responses to bradykinin and histamine in man. *Clin Exp Allergy.* Oct 1992;22(10):933-939.

42. Geppetti P, Del Bianco E, Cecconi R, Tramontana M, Romani A, Theodorsson E. Capsaicin releases calcitonin gene-related peptide from the human iris and ciliary body in vitro. *Regul Pept.* Sep 3 1992;41(1):83-92.

43. Goadsby PJ, Edvinsson L, Ekman R. Vasoactive peptide release in the extracerebral circulation of humans during migraine headache. *Ann Neurol.* Aug 1990;28(2):183-187.

44. Olesen J, Diener HC, Husstedt IW, et al. Calcitonin gene-related peptide receptor antagonist BIBN 4096 BS for the acute treatment of migraine. *N Engl J Med.* Mar 11 2004;350(11):1104-1110.

45. Doods H, Hallermayer G, Wu D, et al. Pharmacological profile of BIBN4096BS, the first selective small molecule CGRP antagonist. *Br J Pharmacol.* Feb 2000;129(3):420-423.

46. Julius D. TRP channels and pain. *Annu Rev Cell Dev Biol.* 2013;29:355-384.

47. Gonzalez-Ramirez R, Chen Y, Liedtke WB, Morales-Lazaro SL. TRP Channels and Pain. 2017:125-147.

48. Minke B. Drosophila mutant with a transducer defect. *Biophys Struct Mech.* Apr 21 1977;3(1):59-64.

49. Montell C, Rubin GM. Molecular characterization of the Drosophila trp locus: a putative integral membrane protein required for phototransduction. *Neuron.* Apr 1989;2(4):1313-1323.

50. Vriens J, Watanabe H, Janssens A, Droogmans G, Voets T, Nilius B. Cell swelling, heat, and chemical agonists use distinct pathways for the activation of the cation channel TRPV4. *Proc Natl Acad Sci U S A.* Jan 6 2004;101(1):396-401.

51. Nilius B. Transient receptor potential (TRP) cation channels: rewarding unique proteins. *Bull Mem Acad R Med Belg.* 2007;162(3-4):244-253.

52. Denis V, Cyert MS. Internal Ca(2+) release in yeast is triggered by hypertonic shock and mediated by a TRP channel homologue. *J Cell Biol.* Jan 7 2002;156(1):29-34.

53. de Bono M, Tobin DM, Davis MW, Avery L, Bargmann CI. Social feeding in Caenorhabditis elegans is induced by neurons that detect aversive stimuli. *Nature.* Oct 31 2002;419(6910):899-903.

54. Kiselyov K, van Rossum DB, Patterson RL. TRPC channels in pheromone sensing. *Vitam Horm.* 2010;83:197-213.

55. Zhang Y, Hoon MA, Chandrashekar J, et al. Coding of sweet, bitter, and umami tastes: different receptor cells sharing similar signaling pathways. *Cell.* Feb 7 2003;112(3):293-301.

56. Clapham DE. TRP channels as cellular sensors. *Nature.* Dec 4 2003;426(6966):517-524.

57. Clapham DE, Runnels LW, Strubing C. The TRP ion channel family. *Nat Rev Neurosci.* Jun 2001;2(6):387-396.

58. Sedgwick SG, Smerdon SJ. The ankyrin repeat: a diversity of interactions on a common structural framework. *Trends Biochem Sci.* Aug 1999;24(8):311-316.

59. Takahashi N, Kozai D, Mori Y. TRP channels: sensors and transducers of gasotransmitter signals. *Front Physiol.* 2012;3(324):324.

60. Venkatachalam K, Montell C. TRP channels. *Annu Rev Biochem.* 2007;76:387-417.

61. Zheng J. Molecular mechanism of TRP channels. *Compr Physiol.* Jan 2013;3(1):221-242.

62. Ramsey IS, Delling M, Clapham DE. An introduction to TRP channels. *Annu Rev Physiol.* 2006;68:619-647.

63. Montell C. Drosophila visual transduction. *Trends Neurosci.* Jun 2012;35(6):356-363.

64. Maroto R, Raso A, Wood TG, Kurosky A, Martinac B, Hamill OP. TRPC1 forms the stretch-activated cation channel in vertebrate cells. *Nat Cell Biol.* Feb 2005;7(2):179-185.

65. Perraud AL, Fleig A, Dunn CA, et al. ADP-ribose gating of the calcium-permeable LTRPC2 channel revealed by Nudix motif homology. *Nature.* May 31 2001;411(6837):595-599.

66. Benemei S, De Cesaris F, Fusi C, Rossi E, Lupi C, Geppetti P. TRPA1 and other TRP channels in migraine. *J Headache Pain.* Aug 13 2013;14(71):71.

67. Curcio-Morelli C, Zhang P, Venugopal B, et al. Functional multimerization of mucolipin channel proteins. *J Cell Physiol.* Feb 2010;222(2):328-335.

68. Soyombo AA, Tjon-Kon-Sang S, Rbaibi Y, et al. TRP-ML1 regulates lysosomal pH and acidic lysosomal lipid hydrolytic activity. *J Biol Chem.* Mar 17 2006;281(11):7294-7301.

69. Hanaoka K, Qian F, Boletta A, et al. Co-assembly of polycystin-1 and -2 produces unique cation-permeable currents. *Nature.* Dec 21-28 2000;408(6815):990-994.

70. Vriens J, Owsianik G, Fisslthaler B, et al. Modulation of the Ca2 permeable cation channel TRPV4 by cytochrome P450 epoxygenases in vascular endothelium. *Circ Res.* Oct 28 2005;97(9):908-915.

71. Vennekens R, Hoenderop JG, Prenen J, et al. Permeation and gating properties of the novel epithelial Ca(2+) channel. *J Biol Chem.* Feb 11 2000;275(6):3963-3969.

72. den Dekker E, Hoenderop JG, Nilius B, Bindels RJ. The epithelial calcium channels, TRPV5 & TRPV6: from identification towards regulation. *Cell Calcium.* May-Jun 2003;33(5-6):497-507.

73. Tominaga M, Caterina MJ, Malmberg AB, et al. The cloned capsaicin receptor integrates multiple pain-producing stimuli. *Neuron.* Sep 1998;21(3):531-543.

74. Caterina MJ, Schumacher MA, Tominaga M, Rosen TA, Levine JD, Julius D. The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature.* Oct 23 1997;389(6653):816-824.

75. Doly S, Fischer J, Salio C, Conrath M. The vanilloid receptor-1 is expressed in rat spinal dorsal horn astrocytes. *Neurosci Lett.* Mar 4 2004;357(2):123-126.

76. Xu H, Blair NT, Clapham DE. Camphor activates and strongly desensitizes the transient receptor potential vanilloid subtype 1 channel in a vanilloid-independent mechanism. *J Neurosci.* Sep 28 2005;25(39):8924-8937.

77. Macpherson LJ, Geierstanger BH, Viswanath V, et al. The pungency of garlic: activation of TRPA1 and TRPV1 in response to allicin. *Curr Biol.* May 24 2005;15(10):929-934.

78. Yoshida T, Inoue R, Morii T, et al. Nitric oxide activates TRP channels by cysteine S-nitrosylation. *Nat Chem Biol.* Nov 2006;2(11):596-607.

79. Siemens J, Zhou S, Piskorowski R, et al. Spider toxins activate the capsaicin receptor to produce inflammatory pain. *Nature.* Nov 9 2006;444(7116):208-212.

80. Trevisani M, Smart D, Gunthorpe MJ, et al. Ethanol elicits and potentiates nociceptor responses via the vanilloid receptor-1. *Nat Neurosci.* Jun 2002;5(6):546-551.

81. Ahern GP, Brooks IM, Miyares RL, Wang XB. Extracellular cations sensitize and gate capsaicin receptor TRPV1 modulating pain signaling. *J Neurosci.* May 25 2005;25(21):5109-5116.

82. Tominaga M, Tominaga T. Structure and function of TRPV1. *Pflugers Arch.* Oct 2005;451(1):143-150.

83. Zhang X, Huang J, McNaughton PA. NGF rapidly increases membrane expression of TRPV1 heat-gated ion channels. *Embo J.* Dec 21 2005;24(24):4211-4223.

84. Ji RR, Samad TA, Jin SX, Schmoll R, Woolf CJ. p38 MAPK activation by NGF in primary sensory neurons after inflammation increases TRPV1 levels and maintains heat hyperalgesia. *Neuron.* Sep 26 2002;36(1):57-68.

85. Mohapatra DP, Nau C. Desensitization of capsaicin-activated currents in the vanilloid receptor TRPV1 is decreased by the cyclic AMP-dependent protein kinase pathway. *J Biol Chem.* Dec 12 2003;278(50):50080-50090.

86. Chuang HH, Prescott ED, Kong H, et al. Bradykinin and nerve growth factor release the capsaicin receptor from PtdIns(4,5)P2-mediated inhibition. *Nature.* Jun 21 2001;411(6840):957-962.

87. Hwang SW, Cho H, Kwak J, et al. Direct activation of capsaicin receptors by products of lipoxygenases: endogenous capsaicin-like substances. *Proc Natl Acad Sci U S A.* May 23 2000;97(11):6155-6160.

88. Amadesi S, Cottrell GS, Divino L, et al. Protease-activated receptor 2 sensitizes TRPV1 by protein kinase Cepsilon- and A-dependent mechanisms in rats and mice. *J Physiol.* Sep 1 2006;575(Pt 2):555-571.

89. Liedtke W, Choe Y, Marti-Renom MA, et al. Vanilloid receptor-related osmotically activated channel (VR-OAC), a candidate vertebrate osmoreceptor. *Cell.* Oct 27 2000;103(3):525-535.

90. Suzuki M, Mizuno A, Kodaira K, Imai M. Impaired pressure sensation in mice lacking TRPV4. *J Biol Chem.* Jun 20 2003;278(25):22664-22668.

91. Delany NS, Hurle M, Facer P, et al. Identification and characterization of a novel human vanilloid receptor-like protein, VRL-2. *Physiol Genomics.* Jan 19 2001;4(3):165-174.

92. Wissenbach U, Bodding M, Freichel M, Flockerzi V. Trp12, a novel Trp related protein from kidney. *FEBS Lett.* Nov 24 2000;485(2-3):127-134.

93. Grant A, Amadesi S, Bunnett NW. Protease-Activated Receptors: Mechanisms by Which Proteases Sensitize TRPV Channels to Induce Neurogenic Inflammation and Pain. 2007.

94. Guler AD, Lee H, Iida T, Shimizu I, Tominaga M, Caterina M. Heat-evoked activation of the ion channel, TRPV4. *J Neurosci.* Aug 1 2002;22(15):6408-6414.

95. Strotmann R, Harteneck C, Nunnenmacher K, Schultz G, Plant TD. OTRPC4, a nonselective cation channel that confers sensitivity to extracellular osmolarity. *Nat Cell Biol.* Oct 2000;2(10):695-702.

96. Gao X, Wu L, O'Neil RG. Temperature-modulated diversity of TRPV4 channel gating: activation by physical stresses and phorbol ester derivatives through protein kinase C-dependent and -independent pathways. *J Biol Chem.* Jul 18 2003;278(29):27129-27137.

97. Watanabe H, Vriens J, Suh SH, Benham CD, Droogmans G, Nilius B. Heat-evoked activation of TRPV4 channels in a HEK293 cell expression system and in native mouse aorta endothelial cells. *J Biol Chem.* Dec 6 2002;277(49):47044-47051.

98. Buday T, Kovacikova L, Ruzinak R, Plevkova J. TRPV4 antagonist GSK2193874 does not modulate cough response to osmotic stimuli. *Respir Physiol Neurobiol.* Feb 2017;236:1-4.

99. Watanabe H, Vriens J, Prenen J, Droogmans G, Voets T, Nilius B. Anandamide and arachidonic acid use epoxyeicosatrienoic acids to activate TRPV4 channels. *Nature.* Jul 24 2003;424(6947):434-438.

100. Pedersen S, Lambert IH, Thoroed SM, Hoffmann EK. Hypotonic cell swelling induces translocation of the alpha isoform of cytosolic phospholipase A2 but not the gamma isoform in Ehrlich ascites tumor cells. *Eur J Biochem.* Sep 2000;267(17):5531-5539.

101. O'Neil RG, Leng L. Osmo-mechanically sensitive phosphatidylinositol signaling regulates a Ca2+ influx channel in renal epithelial cells. *Am J Physiol.* Jul 1997;273(1 Pt 2):F120-128.

102. Xu H, Zhao H, Tian W, Yoshida K, Roullet JB, Cohen DM. Regulation of a transient receptor potential (TRP) channel by tyrosine phosphorylation. SRC family kinase-dependent tyrosine phosphorylation of TRPV4 on TYR-253 mediates its response to hypotonic stress. *J Biol Chem.* Mar 28 2003;278(13):11520-11527.

103. Alessandri-Haber N, Dina OA, Joseph EK, Reichling D, Levine JD. A transient receptor potential vanilloid 4-dependent mechanism of hyperalgesia is engaged by concerted action of inflammatory mediators. *J Neurosci.* Apr 5 2006;26(14):3864-3874.

104. Chen Y, Yang C, Wang ZJ. Proteinase-activated receptor 2 sensitizes transient receptor potential vanilloid 1, transient receptor potential vanilloid 4, and transient receptor potential ankyrin 1 in paclitaxel-induced neuropathic pain. *Neuroscience.* Oct 13 2011;193:440-451.

105. Story GM, Peier AM, Reeve AJ, et al. ANKTM1, a TRP-like channel expressed in nociceptive neurons, is activated by cold temperatures. *Cell.* Mar 21 2003;112(6):819-829.

106. Jaquemar D, Schenker T, Trueb B. An ankyrin-like protein with transmembrane domains is specifically lost after oncogenic transformation of human fibroblasts. *J Biol Chem.* Mar 12 1999;274(11):7325-7333.

107. Jordt SE, Bautista DM, Chuang HH, et al. Mustard oils and cannabinoids excite sensory nerve fibres through the TRP channel ANKTM1. *Nature.* Jan 15 2004;427(6971):260-265.

108. Nilius B, Appendino G, Owsianik G. The transient receptor potential channel TRPA1: from gene to pathophysiology. *Pflugers Arch.* Nov 2012;464(5):425-458.

109. Nilius B, Owsianik G. The transient receptor potential family of ion channels. *Genome Biol.* 2011;12(3):218.

110. Banke TG, Chaplan SR, Wickenden AD. Dynamic changes in the TRPA1 selectivity filter lead to progressive but reversible pore dilation. *Am J Physiol Cell Physiol.* Jun 2010;298(6):C1457-1468.

111. Chen J, Kim D, Bianchi BR, et al. Pore dilation occurs in TRPA1 but not in TRPM8 channels. *Mol Pain.* Jan 21 2009;5(3):3.

112. Karashima Y, Prenen J, Meseguer V, Owsianik G, Voets T, Nilius B. Modulation of the transient receptor potential channel TRPA1 by phosphatidylinositol 4,5-biphosphate manipulators. *Pflugers Arch.* Oct 2008;457(1):77-89.

113. Voets T, Droogmans G, Wissenbach U, Janssens A, Flockerzi V, Nilius B. The principle of temperature-dependent gating in cold- and heat-sensitive TRP channels. *Nature.* Aug 12 2004;430(7001):748-754.

114. Gaudet R. A primer on ankyrin repeat function in TRP channels and beyond. *Mol Biosyst.* May 2008;4(5):372-379.

115. Wang L, Cvetkov TL, Chance MR, Moiseenkova-Bell VY. Identification of in vivo disulfide conformation of TRPA1 ion channel. *J Biol Chem.* Feb 24 2012;287(9):6169-6176.

116. Andersson DA, Gentry C, Moss S, Bevan S. Clioquinol and pyrithione activate TRPA1 by increasing intracellular Zn2+. *Proc Natl Acad Sci U S A.* May 19 2009;106(20):8374-8379.

117. Hu H, Bandell M, Petrus MJ, Zhu MX, Patapoutian A. Zinc activates damage-sensing TRPA1 ion channels. *Nat Chem Biol.* Mar 2009;5(3):183-190.

118. Cvetkov TL, Huynh KW, Cohen MR, Moiseenkova-Bell VY. Molecular architecture and subunit organization of TRPA1 ion channel revealed by electron microscopy. *J Biol Chem.* Nov 4 2011;286(44):38168-38176.

119. Paulsen CE, Armache JP, Gao Y, Cheng Y, Julius D. Structure of the TRPA1 ion channel suggests regulatory mechanisms. *Nature.* Apr 23 2015;520(7548):511-517.

120. Sura L, Zima V, Marsakova L, Hynkova A, Barvik I, Vlachova V. C-terminal acidic cluster is involved in Ca2+-induced regulation of human transient receptor potential ankyrin 1 channel. *J Biol Chem.* May 25 2012;287(22):18067-18077.

121. Wang YY, Chang RB, Waters HN, McKemy DD, Liman ER. The nociceptor ion channel TRPA1 is potentiated and inactivated by permeating calcium ions. *J Biol Chem.* Nov 21 2008;283(47):32691-32703.

122. Doerner JF, Gisselmann G, Hatt H, Wetzel CH. Transient receptor potential channel A1 is directly gated by calcium ions. *J Biol Chem.* May 4 2007;282(18):13180-13189.

123. Zurborg S, Yurgionas B, Jira JA, Caspani O, Heppenstall PA. Direct activation of the ion channel TRPA1 by Ca2+. *Nat Neurosci.* Mar 2007;10(3):277-279.

124. Nagata K, Duggan A, Kumar G, Garcia-Anoveros J. Nociceptor and hair cell transducer properties of TRPA1, a channel for pain and hearing. *J Neurosci.* Apr 20 2005;25(16):4052-4061.

125. Hjerling-Leffler J, Alqatari M, Ernfors P, Koltzenburg M. Emergence of functional sensory subtypes as defined by transient receptor potential channel expression. *J Neurosci.* Mar 7 2007;27(10):2435-2443.

126. Bhattacharya MR, Bautista DM, Wu K, Haeberle H, Lumpkin EA, Julius D. Radial stretch reveals distinct populations of mechanosensitive mammalian somatosensory neurons. *Proc Natl Acad Sci U S A.* Dec 16 2008;105(50):20015-20020.

127. Hayashi S, Nakamura E, Endo T, Kubo Y, Takeuchi K. Impairment by activation of TRPA1 of gastric epithelial restitution in a wound model using RGM1 cell monolayer. *Inflammopharmacology.* Oct 2007;15(5):218-222.

128. Du S, Araki I, Kobayashi H, Zakoji H, Sawada N, Takeda M. Differential expression profile of cold (TRPA1) and cool (TRPM8) receptors in human urogenital organs. *Urology.* Aug 2008;72(2):450-455.

129. Gratzke C, Weinhold P, Reich O, et al. Transient receptor potential A1 and cannabinoid receptor activity in human normal and hyperplastic prostate: relation to nerves and interstitial cells. *Eur Urol.* May 2010;57(5):902-910.

130. Nozawa K, Kawabata-Shoda E, Doihara H, et al. TRPA1 regulates gastrointestinal motility through serotonin release from enterochromaffin cells. *Proc Natl Acad Sci U S A.* Mar 3 2009;106(9):3408-3413.

131. Bellono NW, Kammel LG, Zimmerman AL, Oancea E. UV light phototransduction activates transient receptor potential A1 ion channels in human melanocytes. *Proc Natl Acad Sci U S A.* Feb 5 2013;110(6):2383-2388.

132. Hox V, Vanoirbeek JA, Alpizar YA, et al. Crucial role of transient receptor potential ankyrin 1 and mast cells in induction of nonallergic airway hyperreactivity in mice. *Am J Respir Crit Care Med.* Mar 1 2013;187(5):486-493.

133. Son AR, Yang YM, Hong JH, Lee SI, Shibukawa Y, Shin DM. Odontoblast TRP channels and thermo/mechanical transmission. *J Dent Res.* Nov 2009;88(11):1014-1019.

134. Nassini R, Pedretti P, Moretto N, et al. Transient receptor potential ankyrin 1 channel localized to non-neuronal airway cells promotes non-neurogenic inflammation. *PLoS One.* 2012;7(8):e42454.

135. Caceres AI, Brackmann M, Elia MD, et al. A sensory neuronal ion channel essential for airway inflammation and hyperreactivity in asthma. *Proc Natl Acad Sci U S A.* Jun 2 2009;106(22):9099-9104.

136. Earley S, Gonzales AL, Crnich R. Endothelium-dependent cerebral artery dilation mediated by TRPA1 and Ca2+-Activated K+ channels. *Circ Res.* Apr 24 2009;104(8):987-994.

137. Caterina MJ. Transient receptor potential ion channels as participants in thermosensation and thermoregulation. *Am J Physiol Regul Integr Comp Physiol.* Jan 2007;292(1):R64-76.

138. Sawada Y, Hosokawa H, Matsumura K, Kobayashi S. Activation of transient receptor potential ankyrin 1 by hydrogen peroxide. *Eur J Neurosci.* Mar 2008;27(5):1131-1142.

139. Bandell M, Story GM, Hwang SW, et al. Noxious cold ion channel TRPA1 is activated by pungent compounds and bradykinin. *Neuron.* Mar 25 2004;41(6):849-857.

140. Bautista DM, Siemens J, Glazer JM, et al. The menthol receptor TRPM8 is the principal detector of environmental cold. *Nature.* Jul 12 2007;448(7150):204-208.

141. Karashima Y, Talavera K, Everaerts W, et al. TRPA1 acts as a cold sensor in vitro and in vivo. *Proc Natl Acad Sci U S A.* Jan 27 2009;106(4):1273-1278.

142. Abrahamsen B, Zhao J, Asante CO, et al. The cell and molecular basis of mechanical, cold, and inflammatory pain. *Science.* Aug 1 2008;321(5889):702-705.

143. Kwan KY, Allchorne AJ, Vollrath MA, et al. TRPA1 contributes to cold, mechanical, and chemical nociception but is not essential for hair-cell transduction. *Neuron.* Apr 20 2006;50(2):277-289.

144. Bautista DM, Jordt SE, Nikai T, et al. TRPA1 mediates the inflammatory actions of environmental irritants and proalgesic agents. *Cell.* Mar 24 2006;124(6):1269-1282.

145. Kwan KY, Glazer JM, Corey DP, Rice FL, Stucky CL. TRPA1 modulates mechanotransduction in cutaneous sensory neurons. *J Neurosci.* Apr 15 2009;29(15):4808-4819.

146. Petrus M, Peier AM, Bandell M, et al. A role of TRPA1 in mechanical hyperalgesia is revealed by pharmacological inhibition. *Mol Pain.* Dec 17 2007;3(40):40.

147. Zhang XF, Chen J, Faltynek CR, Moreland RB, Neelands TR. Transient receptor potential A1 mediates an osmotically activated ion channel. *Eur J Neurosci.* Feb 2008;27(3):605-611.

148. Bang S, Hwang SW. Polymodal ligand sensitivity of TRPA1 and its modes of interactions. *J Gen Physiol.* Mar 2009;133(3):257-262.

149. Hinman A, Chuang HH, Bautista DM, Julius D. TRP channel activation by reversible covalent modification. *Proc Natl Acad Sci U S A.* Dec 19 2006;103(51):19564-19568.

150. Macpherson LJ, Dubin AE, Evans MJ, et al. Noxious compounds activate TRPA1 ion channels through covalent modification of cysteines. *Nature.* Feb 1 2007;445(7127):541-545.

151. Macpherson LJ, Xiao B, Kwan KY, et al. An ion channel essential for sensing chemical damage. *J Neurosci.* Oct 17 2007;27(42):11412-11415.

152. Trevisani M, Siemens J, Materazzi S, et al. 4-Hydroxynonenal, an endogenous aldehyde, causes pain and neurogenic inflammation through activation of the irritant receptor TRPA1. *Proc Natl Acad Sci U S A.* Aug 14 2007;104(33):13519-13524.

153. Taylor-Clark TE, McAlexander MA, Nassenstein C, et al. Relative contributions of TRPA1 and TRPV1 channels in the activation of vagal bronchopulmonary C-fibres by the endogenous autacoid 4-oxononenal. *J Physiol.* Jul 15 2008;586(14):3447-3459.

154. Esterbauer H, Schaur RJ, Zollner H. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. *Free Radic Biol Med.* 1991;11(1):81-128.

155. Benedetti A, Comporti M, Esterbauer H. Identification of 4-hydroxynonenal as a cytotoxic product originating from the peroxidation of liver microsomal lipids. *Biochim Biophys Acta.* Nov 7 1980;620(2):281-296.

156. Andersson DA, Gentry C, Moss S, Bevan S. Transient receptor potential A1 is a sensory receptor for multiple products of oxidative stress. *J Neurosci.* Mar 5 2008;28(10):2485-2494.

157. Bessac BF, Sivula M, von Hehn CA, Escalera J, Cohn L, Jordt SE. TRPA1 is a major oxidant sensor in murine airway sensory neurons. *J Clin Invest.* May 2008;118(5):1899-1910.

158. Taylor-Clark TE, Ghatta S, Bettner W, Undem BJ. Nitrooleic acid, an endogenous product of nitrative stress, activates nociceptive sensory nerves via the direct activation of TRPA1. *Mol Pharmacol.* Apr 2009;75(4):820-829.

159. Materazzi S, Nassini R, Andre E, et al. Cox-dependent fatty acid metabolites cause pain through activation of the irritant receptor TRPA1. *Proc Natl Acad Sci U S A.* Aug 19 2008;105(33):12045-12050.

160. Taylor-Clark TE, Undem BJ, Macglashan DW, Jr., Ghatta S, Carr MJ, McAlexander MA. Prostaglandin-induced activation of nociceptive neurons via direct interaction with transient receptor potential A1 (TRPA1). *Mol Pharmacol.* Feb 2008;73(2):274-281.

161. Miyamoto R, Otsuguro K, Ito S. Time- and concentration-dependent activation of TRPA1 by hydrogen sulfide in rat DRG neurons. *Neurosci Lett.* Jul 20 2011;499(2):137-142.

162. Nassini R, Materazzi S, Andre E, et al. Acetaminophen, via its reactive metabolite N-acetyl-p-benzo-quinoneimine and transient receptor potential ankyrin-1 stimulation, causes neurogenic inflammation in the airways and other tissues in rodents. *Faseb J.* Dec 2010;24(12):4904-4916.

163. Wang YY, Chang RB, Liman ER. TRPA1 is a component of the nociceptive response to CO2. *J Neurosci.* Sep 29 2010;30(39):12958-12963.

164. Fujita F, Uchida K, Moriyama T, et al. Intracellular alkalization causes pain sensation through activation of TRPA1 in mice. *J Clin Invest.* Dec 2008;118(12):4049-4057.

165. Sadofsky LR, Boa AN, Maher SA, Birrell MA, Belvisi MG, Morice AH. TRPA1 is activated by direct addition of cysteine residues to the N-hydroxysuccinyl esters of acrylic and cinnamic acids. *Pharmacol Res.* Jan 2011;63(1):30-36.

166. Dalle-Donne I, Aldini G, Carini M, Colombo R, Rossi R, Milzani A. Protein carbonylation, cellular dysfunction, and disease progression. *J Cell Mol Med.* Apr-Jun 2006;10(2):389-406.

167. Fischer MJ, Leffler A, Niedermirtl F, et al. The general anesthetic propofol excites nociceptors by activating TRPV1 and TRPA1 rather than GABAA receptors. *J Biol Chem.* Nov 5 2010;285(45):34781-34792.

168. Leffler A, Lattrell A, Kronewald S, Niedermirtl F, Nau C. Activation of TRPA1 by membrane permeable local anesthetics. *Mol Pain.* Aug 23 2011;7(62):62.

169. Hu H, Tian J, Zhu Y, et al. Activation of TRPA1 channels by fenamate nonsteroidal anti-inflammatory drugs. *Pflugers Arch.* Mar 2010;459(4):579-592.

170. Motter AL, Ahern GP. TRPA1 is a polyunsaturated fatty acid sensor in mammals. *PLoS One.* 2012;7(6):e38439.

171. Karashima Y, Damann N, Prenen J, et al. Bimodal action of menthol on the transient receptor potential channel TRPA1. *J Neurosci.* Sep 12 2007;27(37):9874-9884.

172. McKemy DD, Neuhausser WM, Julius D. Identification of a cold receptor reveals a general role for TRP channels in thermosensation. *Nature.* Mar 7 2002;416(6876):52-58.

173. Peier AM, Moqrich A, Hergarden AC, et al. A TRP channel that senses cold stimuli and menthol. *Cell.* Mar 8 2002;108(5):705-715.

174. Nagatomo K, Kubo Y. Caffeine activates mouse TRPA1 channels but suppresses human TRPA1 channels. *Proc Natl Acad Sci U S A.* Nov 11 2008;105(45):17373-17378.

175. Talavera K, Gees M, Karashima Y, et al. Nicotine activates the chemosensory cation channel TRPA1. *Nat Neurosci.* Oct 2009;12(10):1293-1299.

176. Morales P, Hurst DP, Reggio PH. Molecular Targets of the Phytocannabinoids: A Complex Picture. *Prog Chem Org Nat Prod.* 2017;103:103-131.

177. Dray A, Perkins M. Bradykinin and inflammatory pain. *Trends Neurosci.* Mar 1993;16(3):99-104.

178. Diogenes A, Akopian AN, Hargreaves KM. NGF up-regulates TRPA1: implications for orofacial pain. *J Dent Res.* Jun 2007;86(6):550-555.

179. Dai Y, Wang S, Tominaga M, et al. Sensitization of TRPA1 by PAR2 contributes to the sensation of inflammatory pain. *J Clin Invest.* Jul 2007;117(7):1979-1987.

180. Eid SR, Crown ED, Moore EL, et al. HC-030031, a TRPA1 selective antagonist, attenuates inflammatory- and neuropathy-induced mechanical hypersensitivity. *Mol Pain.* Oct 27 2008;4(48):48.

181. McNamara CR, Mandel-Brehm J, Bautista DM, et al. TRPA1 mediates formalin-induced pain. *Proc Natl Acad Sci U S A.* Aug 14 2007;104(33):13525-13530.

182. Kerstein PC, del Camino D, Moran MM, Stucky CL. Pharmacological blockade of TRPA1 inhibits mechanical firing in nociceptors. *Mol Pain.* Apr 21 2009;5(19):19.

183. Chen J, Joshi SK, DiDomenico S, et al. Selective blockade of TRPA1 channel attenuates pathological pain without altering noxious cold sensation or body temperature regulation. *Pain.* May 2011;152(5):1165-1172.

184. Wei H, Hamalainen MM, Saarnilehto M, Koivisto A, Pertovaara A. Attenuation of mechanical hypersensitivity by an antagonist of the TRPA1 ion channel in diabetic animals. *Anesthesiology.* Jul 2009;111(1):147-154.

185. Schwarz MG, Namer B, Reeh PW, Fischer MJM. TRPA1 and TRPV1 Antagonists Do Not Inhibit Human Acidosis-Induced Pain. *J Pain.* May 2017;18(5):526-534.

186. Reeh PW, Kocher L, Jung S. Does neurogenic inflammation alter the sensitivity of unmyelinated nociceptors in the rat? *Brain Res.* Oct 1 1986;384(1):42-50.

187. Kremeyer B, Lopera F, Cox JJ, et al. A gain-of-function mutation in TRPA1 causes familial episodic pain syndrome. *Neuron.* Jun 10 2010;66(5):671-680.

188. Gibson L, Lawrence D, Dawson C, Bliss J. Aromatase inhibitors for treatment of advanced breast cancer in postmenopausal women. *Cochrane Database Syst Rev.* Oct 7 2009;7(4):CD003370.

189. Henry NL, Giles JT, Stearns V. Aromatase inhibitor-associated musculoskeletal symptoms: etiology and strategies for management. *Oncology (Williston Park).* Nov 15 2008;22(12):1401-1408.

190. Fusi C, Materazzi S, Benemei S, et al. Steroidal and non-steroidal third-generation aromatase inhibitors induce pain-like symptoms via TRPA1. *Nat Commun.* Dec 8 2014;5(5736):5736.

191. De Logu F, Tonello R, Materazzi S, et al. TRPA1 Mediates Aromatase Inhibitor-Evoked Pain by the Aromatase Substrate Androstenedione. *Cancer Res.* Dec 1 2016;76(23):7024-7035.

192. Cavaletti G, Marmiroli P. Chemotherapy-induced peripheral neurotoxicity. *Curr Opin Neurol.* Oct 2015;28(5):500-507.

193. Windebank AJ, Grisold W. Chemotherapy-induced neuropathy. *J Peripher Nerv Syst.* Mar 2008;13(1):27-46.

194. Joseph EK, Chen X, Bogen O, Levine JD. Oxaliplatin acts on IB4-positive nociceptors to induce an oxidative stress-dependent acute painful peripheral neuropathy. *J Pain.* May 2008;9(5):463-472.

195. Nakagawa T, Kaneko S. Roles of Transient Receptor Potential Ankyrin 1 in Oxaliplatin-Induced Peripheral Neuropathy. *Biol Pharm Bull.* 2017;40(7):947-953.

196. Bolcskei K, Helyes Z, Szabo A, et al. Investigation of the role of TRPV1 receptors in acute and chronic nociceptive processes using gene-deficient mice. *Pain.* Oct 2005;117(3):368-376.

197. Materazzi S, Fusi C, Benemei S, et al. TRPA1 and TRPV4 mediate paclitaxel-induced peripheral neuropathy in mice via a glutathione-sensitive mechanism. *Pflugers Arch.* Apr 2012;463(4):561-569.

198. Trevisan G, Materazzi S, Fusi C, et al. Novel therapeutic strategy to prevent chemotherapy-induced persistent sensory neuropathy by TRPA1 blockade. *Cancer Res.* May 15 2013;73(10):3120-3131.

199. Nassini R, Gees M, Harrison S, et al. Oxaliplatin elicits mechanical and cold allodynia in rodents via TRPA1 receptor stimulation. *Pain.* Jul 2011;152(7):1621-1631.

200. De Logu F, Nassini R, Materazzi S, et al. Schwann cell TRPA1 mediates neuroinflammation that sustains macrophage-dependent neuropathic pain in mice. *Nat Commun.* Dec 1 2017;8(1):1887.

201. Hamilton NB, Kolodziejczyk K, Kougioumtzidou E, Attwell D. Proton-gated Ca(2+)-permeable TRP channels damage myelin in conditions mimicking ischaemia. *Nature.* Jan 28 2016;529(7587):523-527.

202. Goldstein DJ, Wang O, Saper JR, Stoltz R, Silberstein SD, Mathew NT. Ineffectiveness of neurokinin-1 antagonist in acute migraine: a crossover study. *Cephalalgia.* Nov 1997;17(7):785-790.

203. Ho TW, Ferrari MD, Dodick DW, et al. Efficacy and tolerability of MK-0974 (telcagepant), a new oral antagonist of calcitonin gene-related peptide receptor, compared with zolmitriptan for acute migraine: a randomised, placebo-controlled, parallel-treatment trial. *Lancet.* Dec 20 2008;372(9656):2115-2123.

204. Lima AM, Sapienza GB, Giraud Vde O, Fragoso YD. Odors as triggering and worsening factors for migraine in men. *Arq Neuropsiquiatr.* 2011;69(2B):324-327.

205. Rozen TD. Cluster headache as the result of secondhand cigarette smoke exposure during childhood. *Headache.* Jan 2010;50(1):130-132.

206. Andre E, Campi B, Materazzi S, et al. Cigarette smoke-induced neurogenic inflammation is mediated by alpha,beta-unsaturated aldehydes and the TRPA1 receptor in rodents. *J Clin Invest.* Jul 2008;118(7):2574-2582.

207. Bang S, Kim KY, Yoo S, Kim YG, Hwang SW. Transient receptor potential A1 mediates acetaldehyde-evoked pain sensation. *Eur J Neurosci.* Nov 2007;26(9):2516-2523.

208. Lundberg JM, Saria A. Capsaicin-induced desensitization of airway mucosa to cigarette smoke, mechanical and chemical irritants. *Nature.* Mar 17-23 1983;302(5905):251-253.

209. Kunkler PE, Ballard CJ, Oxford GS, Hurley JH. TRPA1 receptors mediate environmental irritant-induced meningeal vasodilatation. *Pain.* Jan 2011;152(1):38-44.

210. Anderson PJ, Lau GS, Taylor WR, Critchley JA. Acute effects of the potent lacrimator o-chlorobenzylidene malononitrile (CS) tear gas. *Hum Exp Toxicol.* Jun 1996;15(6):461-465.

211. Bessac BF, Jordt SE. Sensory detection and responses to toxic gases: mechanisms, health effects, and countermeasures. *Proc Am Thorac Soc.* Jul 2010;7(4):269-277.

212. Benemei S, Appendino G, Geppetti P. Pleasant natural scent with unpleasant effects: cluster headache-like attacks triggered by Umbellularia californica. *Cephalalgia.* Jun 2010;30(6):744-746.

213. Nassini R, Materazzi S, Vriens J, et al. The 'headache tree' via umbellulone and TRPA1 activates the trigeminovascular system. *Brain.* Feb 2012;135(Pt 2):376-390.

214. Olesen J. The role of nitric oxide (NO) in migraine, tension-type headache and cluster headache. *Pharmacol Ther.* Nov 2008;120(2):157-171.

215. Thomsen LL, Olesen J. Nitric oxide in primary headaches. *Curr Opin Neurol.* Jun 2001;14(3):315-321.

216. Shevel E. The extracranial vascular theory of migraine--a great story confirmed by the facts. *Headache.* Mar 2011;51(3):409-417.

217. Amin FM, Asghar MS, Ravneberg JW, et al. The effect of sumatriptan on cephalic arteries: A 3T MR-angiography study in healthy volunteers. *Cephalalgia.* Sep 2013;33(12):1009-1016.

218. Iversen HK, Olesen J. Headache induced by a nitric oxide donor (nitroglycerin) responds to sumatriptan. A human model for development of migraine drugs. *Cephalalgia.* Oct 1996;16(6):412-418.

219. Tvedskov JF, Tfelt-Hansen P, Petersen KA, Jensen LT, Olesen J. CGRP receptor antagonist olcegepant (BIBN4096BS) does not prevent glyceryl trinitrate-induced migraine. *Cephalalgia.* Nov 2010;30(11):1346-1353.

220. Wei EP, Moskowitz MA, Boccalini P, Kontos HA. Calcitonin gene-related peptide mediates nitroglycerin and sodium nitroprusside-induced vasodilation in feline cerebral arterioles. *Circ Res.* Jun 1992;70(6):1313-1319.

221. Fanciullacci M, Alessandri M, Figini M, Geppetti P, Michelacci S. Increase in plasma calcitonin gene-related peptide from the extracerebral circulation during nitroglycerin-induced cluster headache attack. *Pain.* Feb 1995;60(2):119-123.

222. Miyamoto T, Dubin AE, Petrus MJ, Patapoutian A. TRPV1 and TRPA1 mediate peripheral nitric oxide-induced nociception in mice. *PLoS One.* Oct 29 2009;4(10):e7596.

223. Iversen HK. Experimental headache in humans. *Cephalalgia.* 1995;15(4):281-287.

224. Nassini R, Fusi C, Materazzi S, et al. The TRPA1 channel mediates the analgesic action of dipyrone and pyrazolone derivatives. *Br J Pharmacol.* Jul 2015;172(13):3397-3411.

225. Aydin AA, Zerbes V, Parlar H, Letzel T. The medical plant butterbur (Petasites): analytical and physiological (re)view. *J Pharm Biomed Anal.* Mar 5 2013;75:220-229.

226. Avula B, Wang YH, Wang M, Smillie TJ, Khan IA. Simultaneous determination of sesquiterpenes and pyrrolizidine alkaloids from the rhizomes of Petasites hybridus (L.) G.M. et Sch. and dietary supplements using UPLC-UV and HPLC-TOF-MS methods. *J Pharm Biomed Anal.* Nov 2012;70:53-63.

227. Pothmann R, Danesch U. Migraine prevention in children and adolescents: results of an open study with a special butterbur root extract. *Headache.* Mar 2005;45(3):196-203.

228. Lipton RB, Gobel H, Einhaupl KM, Wilks K, Mauskop A. Petasites hybridus root (butterbur) is an effective preventive treatment for migraine. *Neurology.* Dec 28 2004;63(12):2240-2244.

229. Grossman W, Schmidramsl H. An extract of Petasites hybridus is effective in the prophylaxis of migraine. *Altern Med Rev.* Jun 2001;6(3):303-310.

230. Holland S, Silberstein SD, Freitag F, Dodick DW, Argoff C, Ashman E. Evidence-based guideline update: NSAIDs and other complementary treatments for episodic migraine prevention in adults: report of the Quality Standards Subcommittee of the American Academy of Neurology and the American Headache Society. *Neurology.* Apr 24 2012;78(17):1346-1353.

231. Thomet OA, Wiesmann UN, Blaser K, Simon HU. Differential inhibition of inflammatory effector functions by petasin, isopetasin and neopetasin in human eosinophils. *Clin Exp Allergy.* Aug 2001;31(8):1310-1320.

232. Wang GJ, Shum AY, Lin YL, et al. Calcium channel blockade in vascular smooth muscle cells: major hypotensive mechanism of S-petasin, a hypotensive sesquiterpene from Petasites formosanus. *J Pharmacol Exp Ther.* Apr 2001;297(1):240-246.

233. Ko WC, Lei CB, Lin YL, Chen CF. Mechanisms of relaxant action of S-petasin and S-isopetasin, sesquiterpenes of Petasites formosanus, in isolated guinea pig trachea. *Planta Med.* Apr 2001;67(3):224-229.

234. E. A-SA. The pharmacology of Crocus sativus- A review. *IOSRPHR.* June 2016 2016;6(6):08-38.

235. Tamaddonfard E, Farshid AA, Eghdami K, Samadi F, Erfanparast A. Comparison of the effects of crocin, safranal and diclofenac on local inflammation and inflammatory pain responses induced by carrageenan in rats. *Pharmacol Rep.* 2013;65(5):1272-1280.

236. Assimopoulou AN, Sinakos Z, Papageorgiou VP. Radical scavenging activity of Crocus sativus L. extract and its bioactive constituents. *Phytother Res.* Nov 2005;19(11):997-1000.

237. Boskabady MH, Tabatabaee A, Byrami G. The effect of the extract of Crocus sativus and its constituent safranal, on lung pathology and lung inflammation of ovalbumin sensitized guinea-pigs. *Phytomedicine.* Jul 15 2012;19(10):904-911.

238. Staruschenko A, Jeske NA, Akopian AN. Contribution of TRPV1-TRPA1 interaction to the single channel properties of the TRPA1 channel. *J Biol Chem.* May 14 2010;285(20):15167-15177.

239. Nassini R, Materazzi S, Benemei S, Geppetti P. The TRPA1 channel in inflammatory and neuropathic pain and migraine. *Rev Physiol Biochem Pharmacol.* 2014;167:1-43.

240. Sutherland A, Sweet BV. Butterbur: an alternative therapy for migraine prevention. *Am J Health Syst Pharm.* May 1 2010;67(9):705-711.

241. Danesch U, Rittinghausen R. Safety of a patented special butterbur root extract for migraine prevention. *Headache.* Jan 2003;43(1):76-78.

242. Nilius B, Szallasi A. Transient receptor potential channels as drug targets: from the science of basic research to the art of medicine. *Pharmacol Rev.* Jul 2014;66(3):676-814.

243. Khazdair MR, Boskabady MH, Hosseini M, Rezaee R, A MT. The effects of Crocus sativus (saffron) and its constituents on nervous system: A review. *Avicenna J Phytomed.* Sep-Oct 2015;5(5):376-391.

244. Abdullaev FI. Biological effects of saffron. *Biofactors.* May 1993;4(2):83-86.

245. Hausenblas HA, Heekin K, Mutchie HL, Anton S. A systematic review of randomized controlled trials examining the effectiveness of saffron (Crocus sativus L.) on psychological and behavioral outcomes. *J Integr Med.* Jul 2015;13(4):231-240.

246. Moshiri M, Vahabzadeh M, Hosseinzadeh H. Clinical Applications of Saffron (Crocus sativus) and its Constituents: A Review. *Drug Res (Stuttg).* Jun 2015;65(6):287-295.

247. Tamaddonfard E, Farshid AA, Maroufi S, et al. Effects of safranal, a constituent of saffron, and vitamin E on nerve functions and histopathology following crush injury of sciatic nerve in rats. *Phytomedicine.* Apr 15 2014;21(5):717-723.

248. Amin B, Hosseinzadeh H. Evaluation of aqueous and ethanolic extracts of saffron, Crocus sativus L., and its constituents, safranal and crocin in allodynia and hyperalgesia induced by chronic constriction injury model of neuropathic pain in rats. *Fitoterapia.* Jul 2012;83(5):888-895.

249. Zhu KJ, Yang JS. Anti-allodynia effect of safranal on neuropathic pain induced by spinal nerve transection in rat. *Int J Clin Exp Med.* 2014;7(12):4990-4996.

250. Thadani U, Rodgers T. Side effects of using nitrates to treat angina. *Expert Opin Drug Saf.* Sep 2006;5(5):667-674.

251. Trainor DC, Jones RC. Headaches in explosive magazine workers. *Arch Environ Health.* Feb 1966;12(2):231-234.

252. Thomsen LL, Kruuse C, Iversen HK, Olesen J. A nitric oxide donor (nitroglycerin) triggers genuine migraine attacks. *Eur J Neurol.* Sep 1994;1(1):73-80.

253. Iversen HK, Olesen J, Tfelt-Hansen P. Intravenous nitroglycerin as an experimental model of vascular headache. Basic characteristics. *Pain.* Jul 1989;38(1):17-24.

254. Sicuteri F, Del Bene E, Poggioni M, Bonazzi A. Unmasking latent dysnociception in healthy subjects. *Headache.* Apr 1987;27(4):180-185.

255. Ferrari LF, Levine JD, Green PG. Mechanisms mediating nitroglycerin-induced delayed-onset hyperalgesia in the rat. *Neuroscience.* Mar 11 2016;317:121-129.

256. Tassorelli C, Greco R, Wang D, Sandrini M, Sandrini G, Nappi G. Nitroglycerin induces hyperalgesia in rats--a time-course study. *Eur J Pharmacol.* Mar 19 2003;464(2-3):159-162.

257. Thomsen LL, Brennum J, Iversen HK, Olesen J. Effect of a nitric oxide donor (glyceryl trinitrate) on nociceptive thresholds in man. *Cephalalgia.* May 1996;16(3):169-174.

258. Ramachandran R, Bhatt DK, Ploug KB, et al. Nitric oxide synthase, calcitonin gene-related peptide and NK-1 receptor mechanisms are involved in GTN-induced neuronal activation. *Cephalalgia.* Feb 2014;34(2):136-147.

259. Strecker T, Dux M, Messlinger K. Nitric oxide releases calcitonin-gene-related peptide from rat dura mater encephali promoting increases in meningeal blood flow. *J Vasc Res.* Nov-Dec 2002;39(6):489-496.

260. Edvinsson L. The Journey to Establish CGRP as a Migraine Target: A Retrospective View. *Headache.* Oct 2015;55(9):1249-1255.

261. Ho TW, Edvinsson L, Goadsby PJ. CGRP and its receptors provide new insights into migraine pathophysiology. *Nat Rev Neurol.* Oct 2010;6(10):573-582.

262. Trevisan G, Benemei S, Materazzi S, et al. TRPA1 mediates trigeminal neuropathic pain in mice downstream of monocytes/macrophages and oxidative stress. *Brain.* May 2016;139(Pt 5):1361-1377.

263. Trevisan G, Hoffmeister C, Rossato MF, et al. TRPA1 receptor stimulation by hydrogen peroxide is critical to trigger hyperalgesia and inflammation in a model of acute gout. *Free Radic Biol Med.* Jul 2014;72:200-209.

264. Rainsford KD. Ibuprofen: pharmacology, efficacy and safety. *Inflammopharmacology.* Dec 2009;17(6):275-342.

265. Davies NM. Clinical pharmacokinetics of ibuprofen. The first 30 years. *Clin Pharmacokinet.* Feb 1998;34(2):101-154.

266. Gierse JK, Koboldt CM, Walker MC, Seibert K, Isakson PC. Kinetic basis for selective inhibition of cyclo-oxygenases. *Biochem J.* May 1 1999;339 (Pt 3)(Pt 3):607-614.

267. Boneberg EM, Zou MH, Ullrich V. Inhibition of cyclooxygenase-1 and -2 by R(-)- and S(+)-ibuprofen. *J Clin Pharmacol.* Dec 1996;36(12 Suppl):16S-19S.

268. Kepp DR, Sidelmann UG, Hansen SH. Isolation and characterization of major phase I and II metabolites of ibuprofen. *Pharm Res.* May 1997;14(5):676-680.

269. Rudy AC, Knight PM, Brater DC, Hall SD. Stereoselective metabolism of ibuprofen in humans: administration of R-, S- and racemic ibuprofen. *J Pharmacol Exp Ther.* Dec 1991;259(3):1133-1139.

270. Castillo M, Lam YW, Dooley MA, Stahl E, Smith PC. Disposition and covalent binding of ibuprofen and its acyl glucuronide in the elderly. *Clin Pharmacol Ther.* Jun 1995;57(6):636-644.

271. Kroemer HK, Klotz U. Glucuronidation of drugs. A re-evaluation of the pharmacological significance of the conjugates and modulating factors. *Clin Pharmacokinet.* Oct 1992;23(4):292-310.

272. Sallustio BC, Sabordo L, Evans AM, Nation RL. Hepatic disposition of electrophilic acyl glucuronide conjugates. *Curr Drug Metab.* Sep 2000;1(2):163-180.

273. McGrath JC, Lilley E. Implementing guidelines on reporting research using animals (ARRIVE etc.): new requirements for publication in BJP. *Br J Pharmacol.* Jul 2015;172(13):3189-3193.

274. Kilkenny C, Browne W, Cuthill IC, Emerson M, Altman DG. Animal research: reporting in vivo experiments: the ARRIVE guidelines. *Br J Pharmacol.* Aug 2010;160(7):1577-1579.

275. Liedtke W, Friedman JM. Abnormal osmotic regulation in trpv4-/- mice. *Proc Natl Acad Sci U S A.* Nov 11 2003;100(23):13698-13703.

276. Materazzi S, Benemei S, Fusi C, et al. Parthenolide inhibits nociception and neurogenic vasodilatation in the trigeminovascular system by targeting the TRPA1 channel. *Pain.* Dec 2013;154(12):2750-2758.

277. Luccarini P, Childeric A, Gaydier AM, Voisin D, Dallel R. The orofacial formalin test in the mouse: a behavioral model for studying physiology and modulation of trigeminal nociception. *J Pain.* Dec 2006;7(12):908-914.

278. Reagan-Shaw S, Nihal M, Ahmad N. Dose translation from animal to human studies revisited. *Faseb J.* Mar 2008;22(3):659-661.

279. Curtis MJ, Bond RA, Spina D, et al. Experimental design and analysis and their reporting: new guidance for publication in BJP. *Br J Pharmacol.* Jul 2015;172(14):3461-3471.

280. Steinhoff MS, von Mentzer B, Geppetti P, Pothoulakis C, Bunnett NW. Tachykinins and their receptors: contributions to physiological control and the mechanisms of disease. *Physiol Rev.* Jan 2014;94(1):265-301.

281. Nicoletti P, Trevisani M, Manconi M, et al. Ethanol causes neurogenic vasodilation by TRPV1 activation and CGRP release in the trigeminovascular system of the guinea pig. *Cephalalgia.* Jan 2008;28(1):9-17.

282. Benemei S, Fusi C, Trevisan G, Geppetti P. The TRPA1 channel in migraine mechanism and treatment. *Br J Pharmacol.* May 2014;171(10):2552-2567.

283. Andrade EL, Ferreira J, Andre E, Calixto JB. Contractile mechanisms coupled to TRPA1 receptor activation in rat urinary bladder. *Biochem Pharmacol.* Jun 28 2006;72(1):104-114.

284. Arnold E, Benz T, Zapp C, Wink M. Inhibition of Cytosolic Phospholipase A2alpha (cPLA2alpha) by Medicinal Plants in Relation to Their Phenolic Content. *Molecules.* Aug 17 2015;20(8):15033-15048.

285. Lee DK, Carstairs IJ, Haggart K, Jackson CM, Currie GP, Lipworth BJ. Butterbur, a herbal remedy, attenuates adenosine monophosphate induced nasal responsiveness in seasonal allergic rhinitis. *Clin Exp Allergy.* Jul 2003;33(7):882-886.

286. Schapowal A. Randomised controlled trial of butterbur and cetirizine for treating seasonal allergic rhinitis. *Bmj.* Jan 19 2002;324(7330):144-146.

287. Ruparel NB, Patwardhan AM, Akopian AN, Hargreaves KM. Homologous and heterologous desensitization of capsaicin and mustard oil responses utilize different cellular pathways in nociceptors. *Pain.* Apr 2008;135(3):271-279.

288. Denner AC, Vogler B, Messlinger K, De Col R. Role of transient receptor potential ankyrin 1 receptors in rodent models of meningeal nociception - Experiments in vitro. *Eur J Pain.* May 2017;21(5):843-854.

289. Edvinsson L. CGRP receptor antagonists and antibodies against CGRP and its receptor in migraine treatment. *Br J Clin Pharmacol.* Aug 2015;80(2):193-199.

290. Bigal ME, Edvinsson L, Rapoport AM, et al. Safety, tolerability, and efficacy of TEV-48125 for preventive treatment of chronic migraine: a multicentre, randomised, double-blind, placebo-controlled, phase 2b study. *Lancet Neurol.* Nov 2015;14(11):1091-1100.

291. Sinclair SR, Kane SA, Van der Schueren BJ, et al. Inhibition of capsaicin-induced increase in dermal blood flow by the oral CGRP receptor antagonist, telcagepant (MK-0974). *Br J Clin Pharmacol.* Jan 2010;69(1):15-22.

292. Dux M, Will C, Vogler B, Filipovic MR, Messlinger K. Meningeal blood flow is controlled by H2 S-NO crosstalk activating a HNO-TRPA1-CGRP signalling pathway. *Br J Pharmacol.* Feb 2016;173(3):431-445.

293. Wang GJ, Wu XC, Lin YL, et al. Ca2+ channel blocking effect of iso-S-petasin in rat aortic smooth muscle cells. *Eur J Pharmacol.* Jun 12 2002;445(3):239-245.

294. Andersson DA, Gentry C, Alenmyr L, et al. TRPA1 mediates spinal antinociception induced by acetaminophen and the cannabinoid Delta(9)-tetrahydrocannabiorcol. *Nat Commun.* Nov 22 2011;2(551):551.

295. Ramacciotti AS, Soares BG, Atallah AN. Dipyrone for acute primary headaches. *Cochrane Database Syst Rev.* Apr 18 2007;18(2):CD004842.

296. Bigal ME, Bordini CA, Tepper SJ, Speciali JG. Intravenous dipyrone in the acute treatment of migraine without aura and migraine with aura: a randomized, double blind, placebo controlled study. *Headache.* Oct 2002;42(9):862-871.

297. Faul F, Erdfelder E, Buchner A, Lang AG. Statistical power analyses using G*Power 3.1: tests for correlation and regression analyses. *Behav Res Methods.* Nov 2009;41(4):1149-1160.

298. Benemei S, De Logu F, Li Puma S, et al. The anti-migraine component of butterbur extracts, isopetasin, desensitizes peptidergic nociceptors by acting on TRPA1 cation channel. *Br J Pharmacol.* Sep 2017;174(17):2897-2911.

299. Sanchez AM, Carmona M, Jaren-Galan M, Mosquera MI, Alonso GL. Picrocrocin kinetics in aqueous saffron spice extracts (Crocus sativus L.) upon thermal treatment. *J Agric Food Chem.* Jan 12 2011;59(1):249-255.

300. Maggi CA, Patacchini R, Santicioli P, Giuliani S. Tachykinin antagonists and capsaicin-induced contraction of the rat isolated urinary bladder: evidence for tachykinin-mediated cotransmission. *Br J Pharmacol.* Jun 1991;103(2):1535-1541.

301. S. RJLRMCGRMM. An update review of saffron and its active constituents. *Phytother Res.* 1996;10:189-193.

302. Erfanparast A, Tamaddonfard E, Taati M, Dabbaghi M. Effects of crocin and safranal, saffron constituents, on the formalin-induced orofacial pain in rats. *Avicenna J Phytomed.* Sep-Oct 2015;5(5):392-402.

303. Guan Z, Kuhn JA, Wang X, et al. Injured sensory neuron-derived CSF1 induces microglial proliferation and DAP12-dependent pain. *Nat Neurosci.* Jan 2016;19(1):94-101.

304. Zurborg S, Piszczek A, Martinez C, et al. Generation and characterization of an Advillin-Cre driver mouse line. *Mol Pain.* Sep 11 2011;7(66):66.

305. Zappia KJ, O'Hara CL, Moehring F, Kwan KY, Stucky CL. Sensory Neuron-Specific Deletion of TRPA1 Results in Mechanical Cutaneous Sensory Deficits. *eNeuro.* Jan-Feb 2017;4(1):0069-0016.

306. Elliott MB, Oshinsky ML, Amenta PS, Awe OO, Jallo JI. Nociceptive neuropeptide increases and periorbital allodynia in a model of traumatic brain injury. *Headache.* Jun 2012;52(6):966-984.

307. Pecze L, Pelsoczi P, Kecskes M, et al. Resiniferatoxin mediated ablation of TRPV1+ neurons removes TRPA1 as well. *Can J Neurol Sci.* Mar 2009;36(2):234-241.

308. Bonet IJ, Fischer L, Parada CA, Tambeli CH. The role of transient receptor potential A 1 (TRPA1) in the development and maintenance of carrageenan-induced hyperalgesia. *Neuropharmacology.* Feb 2013;65:206-212.

309. Dixon WJ. Efficient analysis of experimental observations. *Annu Rev Pharmacol Toxicol.* 1980;20:441-462.

310. Chung MK, Asgar J, Lee J, Shim MS, Dumler C, Ro JY. The role of TRPM2 in hydrogen peroxide-induced expression of inflammatory cytokine and chemokine in rat trigeminal ganglia. *Neuroscience.* Jun 25 2015;297:160-169.

311. da Costa DS, Meotti FC, Andrade EL, Leal PC, Motta EM, Calixto JB. The involvement of the transient receptor potential A1 (TRPA1) in the maintenance of mechanical and cold hyperalgesia in persistent inflammation. *Pain.* Mar 2010;148(3):431-437.

312. Wei H, Koivisto A, Saarnilehto M, et al. Spinal transient receptor potential ankyrin 1 channel contributes to central pain hypersensitivity in various pathophysiological conditions in the rat. *Pain.* Mar 2011;152(3):582-591.

313. Kim AK, Souza-Formigoni ML. Disulfiram impairs the development of behavioural sensitization to the stimulant effect of ethanol. *Behav Brain Res.* Mar 5 2010;207(2):441-446.

314. Lee EJ, Hung YC, Chen HY, Wu TS, Chen TY. Delayed treatment with carboxy-PTIO permits a 4-h therapeutic window of opportunity and prevents against ischemia-induced energy depletion following permanent focal cerebral ischemia in mice. *Neurochem Res.* Jun 2009;34(6):1157-1166.

315. Nozaki-Taguchi N, Yamamoto T. Involvement of nitric oxide in peripheral antinociception mediated by kappa- and delta-opioid receptors. *Anesth Analg.* Aug 1998;87(2):388-393.

316. Chen SR, Pan HL. Spinal nitric oxide contributes to the analgesic effect of intrathecal [d-pen2,d-pen5]-enkephalin in normal and diabetic rats. *Anesthesiology.* Jan 2003;98(1):217-222.

317. Rossato MF, Velloso NA, de Oliveira Ferreira AP, de Mello CF, Ferreira J. Spinal levels of nonprotein thiols are related to nociception in mice. *J Pain.* Jun 2010;11(6):545-554.

318. Alsheblak MM, Elsherbiny NM, El-Karef A, El-Shishtawy MM. Protective effects of L-carnosine on CCl4 -induced hepatic injury in rats. *Eur Cytokine Netw.* Mar 1 2016;27(1):6-15.

319. Li YQ, Li XB, Guo SJ, et al. Apocynin attenuates oxidative stress and cardiac fibrosis in angiotensin II-induced cardiac diastolic dysfunction in mice. *Acta Pharmacol Sin.* Mar 2013;34(3):352-359.

320. Mogil JS, Miermeister F, Seifert F, et al. Variable sensitivity to noxious heat is mediated by differential expression of the CGRP gene. *Proc Natl Acad Sci U S A.* Sep 6 2005;102(36):12938-12943.

321. Holzer P. Capsaicin: cellular targets, mechanisms of action, and selectivity for thin sensory neurons. *Pharmacol Rev.* Jun 1991;43(2):143-201.

322. Sullivan MN, Gonzales AL, Pires PW, et al. Localized TRPA1 channel Ca2+ signals stimulated by reactive oxygen species promote cerebral artery dilation. *Sci Signal.* Jan 6 2015;8(358):ra2.

323. Beretta M, Gruber K, Kollau A, et al. Bioactivation of nitroglycerin by purified mitochondrial and cytosolic aldehyde dehydrogenases. *J Biol Chem.* Jun 27 2008;283(26):17873-17880.

324. Wenzl MV, Beretta M, Gorren AC, et al. Role of the general base Glu-268 in nitroglycerin bioactivation and superoxide formation by aldehyde dehydrogenase-2. *J Biol Chem.* Jul 24 2009;284(30):19878-19886.

325. Brame CJ, Salomon RG, Morrow JD, Roberts LJ, 2nd. Identification of extremely reactive gamma-ketoaldehydes (isolevuglandins) as products of the isoprostane pathway and characterization of their lysyl protein adducts. *J Biol Chem.* May 7 1999;274(19):13139-13146.

326. Karai L, Brown DC, Mannes AJ, et al. Deletion of vanilloid receptor 1-expressing primary afferent neurons for pain control. *J Clin Invest.* May 2004;113(9):1344-1352.

327. Bedard K, Krause KH. The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. *Physiol Rev.* Jan 2007;87(1):245-313.

328. Demartini C, Tassorelli C, Zanaboni AM, et al. The role of the transient receptor potential ankyrin type-1 (TRPA1) channel in migraine pain: evaluation in an animal model. *J Headache Pain.* Sep 7 2017;18(1):94.

329. Farkas S, Bolcskei K, Markovics A, et al. Utility of different outcome measures for the nitroglycerin model of migraine in mice. *J Pharmacol Toxicol Methods.* Jan-Feb 2016;77:33-44.

330. Bates EA, Nikai T, Brennan KC, et al. Sumatriptan alleviates nitroglycerin-induced mechanical and thermal allodynia in mice. *Cephalalgia.* Feb 2010;30(2):170-178.

331. Kozai D, Kabasawa Y, Ebert M, et al. Transnitrosylation directs TRPA1 selectivity in N-nitrosamine activators. *Mol Pharmacol.* Jan 2014;85(1):175-185.

332. Zhang Y, Hogg N. Formation and stability of S-nitrosothiols in RAW 264.7 cells. *Am J Physiol Lung Cell Mol Physiol.* Sep 2004;287(3):L467-474.

333. Shimizu S, Takahashi N, Mori Y. TRPs as chemosensors (ROS, RNS, RCS, gasotransmitters). *Handb Exp Pharmacol.* 2014;223:767-794.

334. Hasegawa H, Abbott S, Han BX, Qi Y, Wang F. Analyzing somatosensory axon projections with the sensory neuron-specific Advillin gene. *J Neurosci.* Dec 26 2007;27(52):14404-14414.

335. Koulchitsky S, Fischer MJ, Messlinger K. Calcitonin gene-related peptide receptor inhibition reduces neuronal activity induced by prolonged increase in nitric oxide in the rat spinal trigeminal nucleus. *Cephalalgia.* Apr 2009;29(4):408-417.

336. Koulchitsky S, Fischer MJ, De Col R, Schlechtweg PM, Messlinger K. Biphasic response to nitric oxide of spinal trigeminal neurons with meningeal input in rat--possible implications for the pathophysiology of headaches. *J Neurophysiol.* Sep 2004;92(3):1320-1328.

337. Zhang X, Kainz V, Zhao J, Strassman AM, Levy D. Vascular extracellular signal-regulated kinase mediates migraine-related sensitization of meningeal nociceptors. *Ann Neurol.* Jun 2013;73(6):741-750.

338. Levy D, Jakubowski M, Burstein R. Disruption of communication between peripheral and central trigeminovascular neurons mediates the antimigraine action of 5HT 1B/1D receptor agonists. *Proc Natl Acad Sci U S A.* Mar 23 2004;101(12):4274-4279.

339. Strassman AM, Raymond SA, Burstein R. Sensitization of meningeal sensory neurons and the origin of headaches. *Nature.* Dec 12 1996;384(6609):560-564.

340. Kopruszinski CM, Xie JY, Eyde NM, et al. Prevention of stress- or nitric oxide donor-induced medication overuse headache by a calcitonin gene-related peptide antibody in rodents. *Cephalalgia.* May 2017;37(6):560-570.

341. De Felice M, Ossipov MH, Wang R, et al. Triptan-induced latent sensitization: a possible basis for medication overuse headache. *Ann Neurol.* Mar 2010;67(3):325-337.

342. Perrotta A, Serrao M, Tassorelli C, et al. Oral nitric-oxide donor glyceryl-trinitrate induces sensitization in spinal cord pain processing in migraineurs: a double-blind, placebo-controlled, cross-over study. *Eur J Pain.* May 2011;15(5):482-490.

343. Olesen J, Iversen HK, Thomsen LL. Nitric oxide supersensitivity: a possible molecular mechanism of migraine pain. *Neuroreport.* Aug 1993;4(8):1027-1030.

344. Drummond PD. Scalp tenderness and sensitivity to pain in migraine and tension headache. *Headache.* Jan 1987;27(1):45-50.

345. Afridi SK, Kaube H, Goadsby PJ. Glyceryl trinitrate triggers premonitory symptoms in migraineurs. *Pain.* Aug 2004;110(3):675-680.

346. Burstein R, Yamamura H, Malick A, Strassman AM. Chemical stimulation of the intracranial dura induces enhanced responses to facial stimulation in brain stem trigeminal neurons. *J Neurophysiol.* Feb 1998;79(2):964-982.

347. Sadofsky LR, Sreekrishna KT, Lin Y, et al. Unique Responses are Observed in Transient Receptor Potential Ankyrin 1 and Vanilloid 1 (TRPA1 and TRPV1) Co-Expressing Cells. *Cells.* Jun 11 2014;3(2):616-626.

348. Bautista DM, Movahed P, Hinman A, et al. Pungent products from garlic activate the sensory ion channel TRPA1. *Proc Natl Acad Sci U S A.* Aug 23 2005;102(34):12248-12252.

349. Tonello R, Fusi C, Materazzi S, et al. The peptide Phalpha1beta, from spider venom, acts as a TRPA1 channel antagonist with antinociceptive effects in mice. *Br J Pharmacol.* Jan 2017;174(1):57-69.

350. Everaerts W, Zhen X, Ghosh D, et al. Inhibition of the cation channel TRPV4 improves bladder function in mice and rats with cyclophosphamide-induced cystitis. *Proc Natl Acad Sci U S A.* Nov 2 2010;107(44):19084-19089.

351. Chaplan SR, Bach FW, Pogrel JW, Chung JM, Yaksh TL. Quantitative assessment of tactile allodynia in the rat paw. *J Neurosci Methods.* Jul 1994;53(1):55-63.

352. Ma Y, Li Y, Li X, Wu Y. Anti-inflammatory effects of 4-methylcyclopentadecanone on edema models in mice. *Int J Mol Sci.* Dec 9 2013;14(12):23980-23992.

353. Eswar N, Webb B, Marti-Renom MA, et al. Comparative protein structure modeling using Modeller. *Curr Protoc Bioinformatics.* Oct 2006;Chapter 5(5):Unit-5 6.

354. Humphrey W, Dalke A, Schulten K. VMD: visual molecular dynamics. *J Mol Graph.* Feb 1996;14(1):33-38, 27-38.

355. Dickson CJ, Madej BD, Skjevik AA, et al. Lipid14: The Amber Lipid Force Field. *J Chem Theory Comput.* Feb 11 2014;10(2):865-879.

356. Verdonk ML, Cole JC, Hartshorn MJ, Murray CW, Taylor RD. Improved protein-ligand docking using GOLD. *Proteins.* Sep 1 2003;52(4):609-623.

357. Moilanen LJ, Laavola M, Kukkonen M, et al. TRPA1 contributes to the acute inflammatory response and mediates carrageenan-induced paw edema in the mouse. *Sci Rep.* 2012;2(380):380.

358. Fischer MJ, Soller KJ, Sauer SK, Kalucka J, Veglia G, Reeh PW. Formalin evokes calcium transients from the endoplasmatic reticulum. *PLoS One.* 2015;10(4):e0123762.

359. Dall'Acqua MC, Bonet IJ, Zampronio AR, Tambeli CH, Parada CA, Fischer L. The contribution of transient receptor potential ankyrin 1 (TRPA1) to the in vivo nociceptive effects of prostaglandin E(2). *Life Sci.* Jun 6 2014;105(1-2):7-13.

360. Malmberg AB, Yaksh TL. Antinociceptive actions of spinal nonsteroidal anti-inflammatory agents on the formalin test in the rat. *J Pharmacol Exp Ther.* Oct 1992;263(1):136-146.

361. Lin AH, Liu MH, Ko HK, Perng DW, Lee TS, Kou YR. Lung Epithelial TRPA1 Transduces the Extracellular ROS into Transcriptional Regulation of Lung Inflammation Induced by Cigarette Smoke: The Role of Influxed Ca(2)(+). *Mediators Inflamm.* 2015;2015(10):148367.

362. Mukhopadhyay I, Gomes P, Aranake S, et al. Expression of functional TRPA1 receptor on human lung fibroblast and epithelial cells. *J Recept Signal Transduct Res.* Oct 2011;31(5):350-358.

363. Spahn-Langguth H, Benet LZ. Acyl glucuronides revisited: is the glucuronidation process a toxification as well as a detoxification mechanism? *Drug Metab Rev.* 1992;24(1):5-47.

364. Faed EM. Properties of acyl glucuronides: implications for studies of the pharmacokinetics and metabolism of acidic drugs. *Drug Metab Rev.* 1984;15(5-6):1213-1249.

365. El-Shitany NA, El-Bastawissy EA, El-desoky K. Ellagic acid protects against carrageenan-induced acute inflammation through inhibition of nuclear factor kappa B, inducible cyclooxygenase and proinflammatory cytokines and enhancement of interleukin-10 via an antioxidant mechanism. *Int Immunopharmacol.* Apr 2014;19(2):290-299.

366. Pietrobon D, Moskowitz MA. Pathophysiology of migraine. *Annu Rev Physiol.* 2013;75:365-391.

367. MacGregor EA. Migraine. *Ann Intern Med.* Apr 4 2017;166(7):ITC49-ITC64.

368. Murray CJ, Lopez AD. Measuring the global burden of disease. *N Engl J Med.* Aug 1 2013;369(5):448-457.

List of publications

De Logu F, Tonello R, Materazzi S, Nassini R, Fusi C, Coppi E, **Li Puma S**, Marone IM, Sadofsky LR, Morice AH, Susini T, Terreni A, Moneti G, Di Tommaso M, Geppetti P, Benemei S. *TRPA1 Mediates Aromatase Inhibitor-Evoked Pain by the Aromatase Substrate Androstenedione.* Cancer Res. 2016 Dec 1; 76(23): 7024-7035.

Benemei S, De Logu F, **Li Puma S**, Marone IM, Coppi E, Ugolini F, Liedtke W, Pollastro F, Appendino G, Geppetti P, Materazzi S, Nassini R. *The anti-migraine component of butterbur extracts, isopetasin, desensitizes peptidergic nociceptors by acting on TRPA1 cation channel.* Br J Pharmacol. 2017 Sep; 174(17): 2897-2911.

De Logu F, Nassini R, Materazzi S, de Carvalho Gonçalves M, Nosi D, Rossi Degl'Innocenti D, Marone IM, Ferreira J, **Li Puma S**, Benemei S, Trevisan G, Souza Monteiro de Araújo D, Patacchini R, Bunnett NW, Geppetti P. *Schwann cell TRPA1 mediates neuroinflammation that sustains macrophage-dependent neuropathic pain in mice.* Nat Comm. 2017 Dec; 8(1):1887.

Marone IM, De Logu F, Nassini R, De Carvalho Goncalves M, Benemei S, Ferreira J, Jain P, **Li Puma S**, Bunnett NW, Geppetti P, Materazzi S. *TRPA1/NOX in the soma of trigeminal ganglion neurons mediates migraine-related pain of glyceryl trinitrate in mice.* Brain. 2018 Aug 1;141(8):2312-2328.

Li Puma S, Landini L, Macedo SJ Jr, Seravalli V, Marone IM, Coppi E, Patacchini R, Geppetti P, Materazzi S, Nassini R, De Logu F. *TRPA1 mediates the antinociceptive properties of the constituent of Crocus sativus L., safranal.* J Cell Mol Med. 2019 Mar;23(3):1976-1986.

De Logu F, Landini L, Janal MN, **Li Puma S**, De Cesaris F, Geppetti P, Nassini R. *Migraine-provoking substances evoke periorbital allodynia in mice.* J Headache Pain. 2019 Feb 14;20(1):18.

De Logu F, **Li Puma S**, Landini L, Tuccinardi T, Poli G, Preti D, De Siena G, Patacchini R, Tsagareli M, Geppetti P, Nassini R. *The acyl-glucuronide metabolite of ibuprofen has analgesic and anti-inflammatory effects via the TRPA1 channel.* Pharmacol Res. 2019 Apr;142:127-139.

Brusco I, **Li Puma S**, Chiepe KB, da Silva Brum E, de David Antoniazzi CT, de Almeida AS, Camponogara C, Silva CR, De Logu F, de Andrade VM, Ferreira J, Geppetti P, Nassini R, Oliveira SM, Trevisan G. *Dacarbazine alone or associated with melanoma-bearing cancer pain model induces painful hypersensitivity by TRPA1 activation in mice.* Int J Cancer. 2019 Aug 28.

De Logu F, **Li Puma S**, Landini L, Portelli F, Innocenti A, Souza Monteiro de Araújo D, Janal MN, Patacchini R, Bunnett NW, Geppetti P, Nassini R. *Schwann cells expressing nociceptive channel TRPA1 orchestrate ethanol-evoked neuropathic pain in mice.* J Clin Invest. 2019 Sep 5.

Cancer Research

TRPA1 Mediates Aromatase Inhibitor–Evoked Pain by the Aromatase Substrate Androstenedione

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Abstract

Aromatase inhibitors (AI) induce painful musculoskeletal symptoms (AIMSS), which are dependent upon the pain transducing receptor TRPA1. However, as the AI concentrations required to engage TRPA1 in mice are higher than those found in the plasma of patients, we hypothesized that additional factors may cooperate to induce AIMSS. Here we report that the aromatase substrate androstenedione, unique among several steroid hormones, targeted TRPA1 in peptidergic primary sensory neurons in rodent and human cells expressing the native or recombinant channel. Androstenedione dramatically lowered the concentration of letrozole required to engage TRPA1. Notably, addition of a minimal dose of androstenedione to physiologically ineffective doses of letrozole and oxidative stress byproducts produces AIMSS-like behaviors and neurogenic inflammatory responses in mice. Elevated androstenedione levels cooperated with low letrozole concentrations and inflammatory mediators were sufficient to provoke AIMSS-like behaviors. The generation of such painful conditions by small quantities of simultaneously administered TRPA1 agonists justifies previous failure to identify a precise link between AIs and AIMSS, underscoring the potential of channel antagonists to treat AIMSS. *Cancer Res; 76(23); 7024–35. ©2016 AACR.*

Introduction

Aromatase inhibitors (AI) are a mainstay in the treatment of estrogen-sensitive breast cancer in postmenopausal women (1). AIs block the activity of aromatase cytochrome P450, which transforms the androgens, androstenedione, and testosterone into the estrogens (estrone and 17β-estradiol, respectively; ref. 2), which are responsible for cancer cell replication and growth (3). Unfortunately, one-third of patients treated with AIs develop muscular and joint pain (AI-associated musculoskeletal symptoms, a condition that affects the quality of life of patients and limits adherence to AI therapy (4–6). The underlying mechanism of AIMSS is unknown and, accordingly, the treatment of AIMSS remains an unmet medical need.

Recently, we reported that the transient receptor potential ankyrin 1 (TRPA1), a cation channel highly expressed by a subpopulation of primary sensory neurons of the dorsal root ganglia (DRG; ref. 7), mediates AIMSS-like behaviors evoked by AIs in mice (8). TRPA1-expressing nociceptors contain the neuropeptides substance P (SP) and calcitonin gene–related peptide (CGRP), which mediate neurogenic inflammation (9). Exogenous compounds, including allyl isothiocyanate (AITC), and endogenously generated reactive oxygen species (ROS) and their derivatives, have been identified as TRPA1 agonists (9–12). Similar to other reactive agonists (7), highly electrophilic conjugated Michael acceptor groups of exemestane (13) and nitrile moieties of letrozole and anastrozole (14) react with the thiol groups of specific cysteine and lysine residues to trigger TRPA1 and activate nociceptors (8).

The ability to gate TRPA1 *in vitro* was confirmed *in vivo* by the observation that the pain-like behaviors evoked by AIs in mice are abrogated by genetic deletion or pharmacologic blockade of the channel (8). However, AI concentrations required for TRPA1 gating *in vitro* (8) are 1–2 order of magnitude higher than those found in patient plasma (15). In addition, an important proportion (30%–40%), but not all, of treated patients develop the painful condition (16, 17). These observations suggest that exposure to AIs is necessary, but not sufficient, to produce AIMSS, and that additional factors should cooperate with AIs to promote pain symptoms.

Aromatase inhibition, while reducing downstream production of estrogens, moderately increases upstream plasma concentrations of androgens, including androstenedione (ASD; ref. 18). Exemestane, a false aromatase substrate, blocks enzymatic activity by accommodating in the binding pocket that snugly encloses ASD (2). We reasoned that ASD, which retains some of the reactive chemical features of exemestane, such as the α,β-carbonyl moiety of the A ring and the ketone group at the 17 position, might target

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RESEARCH PAPER

The anti-migraine component of butterbur extracts, isopetasin, desensitizes peptidergic nociceptors by acting on TRPA1 cation channel

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BACKGROUND AND PURPOSE

The mechanism of the anti-migraine action of extracts of butterbur [*Petasites hybridus* (L.) Gaertn.] is unknown. Here, we investigated the ability of isopetasin, a major constituent of these extracts, to specifically target TRPA1 channel and to affect functional responses relevant to migraine.

EXPERIMENTAL APPROACH

Single-cell calcium imaging and patch-clamp recordings in human and rodent TRPA1-expressing cells, neurogenic motor responses in rodent isolated urinary bladder, release of CGRP from mouse spinal cord *in vitro* and facial rubbing in mice and meningeal blood flow in rats were examined.

KEY RESULTS

Isopetasin induced (i) calcium responses and currents in rat/mouse trigeminal ganglion (TG) neurons and in cells expressing the human TRPA1, (ii) substance P-mediated contractions of rat isolated urinary bladders and (iii) CGRP release from mouse dorsal spinal cord, responses that were selectively abolished by genetic deletion or pharmacological antagonism of TRPA1 channels. Pre-exposure to isopetasin produced marked desensitization of allyl isothiocyanate (AITC, TRPA1 channel agonist)- or capsaicin (TRPV1 channel agonist)-evoked currents in rat TG neurons, contractions of rat or mouse bladder and CGRP release from mouse central terminals of primary sensory neurons. Repeated intragastric administration of isopetasin attenuated mouse facial rubbing, evoked by local AITC or capsaicin, and dilation of rat meningeal arteries by acrolein or ethanol (TRPA1 and TRPV1 channel agonists respectively).

CONCLUSION AND IMPLICATIONS

Activation of TRPA1 channels by isopetasin results in excitation of neuropeptide-containing nociceptors, followed by marked heterologous neuronal desensitization. Such atten uation in pain and neurogenic inflammation may account for the anti-migraine action of butterbur.

Abbreviations

AITC, allyl isothiocyanate; IMR90, human fetal lung fibroblasts; PAR2, proteinase activated receptor 2; SP, substance P; TG, trigeminal ganglion

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nature COMMUNICATIONS

ARTICLE

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Schwann cell TRPA1 mediates neuroinflammation that sustains macrophage-dependent neuropathic pain in mice

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It is known that transient receptor potential ankyrin 1 (TRPA1) channels, expressed by nociceptors, contribute to neuropathic pain. Here we show that TRPA1 is also expressed in Schwann cells. We found that in mice with partial sciatic nerve ligation, TRPA1 silencing in nociceptors attenuated mechanical allodynia, without affecting macrophage infiltration and oxidative stress, whereas TRPA1 silencing in Schwann cells reduced both allodynia and neuroinflammation. Activation of Schwann cell TRPA1 evoked NADPH oxidase 1 (NOX1)-dependent H_2O_2 release, and silencing or blocking Schwann cell NOX1 attenuated nerve injury-induced macrophage infiltration, oxidative stress and allodynia. Furthermore, the NOX2-dependent oxidative burst, produced by macrophages recruited to the perineural space activated the TRPA1–NOX1 pathway in Schwann cells, but not TRPA1 in nociceptors. Schwann cell TRPA1 generates a spatially constrained gradient of oxidative stress, which maintains macrophage infiltration to the injured nerve, and sends paracrine signals to activate TRPA1 of ensheathed nociceptors to sustain mechanical allodynia.

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BRAIN
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TRPA1/NOX in the soma of trigeminal ganglion neurons mediates migraine-related pain of glyceryl trinitrate in mice

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Glyceryl trinitrate is administered as a provocative test for migraine pain. Glyceryl trinitrate causes prolonged mechanical allodynia in rodents, which temporally correlates with delayed glyceryl trinitrate-evoked migraine attacks in patients. However, the underlying mechanism of the allodynia evoked by glyceryl trinitrate is unknown. The proalgesic transient receptor potential ankyrin 1 (TRPA1) channel, expressed by trigeminal nociceptors, is sensitive to oxidative stress and is targeted by nitric oxide or its by-products. Herein, we explored the role of TRPA1 in glyceryl trinitrate-evoked allodynia. Systemic administration of glyceryl trinitrate elicited in the mouse periorbital area an early and transient vasodilatation and a delayed and prolonged mechanical allodynia. The systemic, intrathecal or local administration of selective enzyme inhibitors revealed that nitric oxide, liberated from the parent drug by aldehyde dehydrogenase 2 (ALDH2), initiates but does not maintain allodynia. The central and the final phases of allodynia were respectively associated with generation of reactive oxygen and carbonyl species within the trigeminal ganglion. Allodynia was absent in TRPA1-deficient mice and was reversed by TRPA1 antagonists. Knockdown of neuronal TRPA1 by intrathecally administered antisense oligonucleotide and selective deletion of TRPA1 from sensory neurons in *Advillin-Cre*; *Trpa1*$^{fl/fl}$ mice revealed that nitric oxide-dependent oxidative and carbonylic stress generation is due to TRPA1 stimulation, and resultant NADPH oxidase 1 (NOX1) and NOX2 activation in the soma of trigeminal ganglion neurons. Early periorbital vasodilatation evoked by glyceryl trinitrate was attenuated by ALDH2 inhibition but was unaffected by TRPA1 blockade. Antagonists of the calcitonin gene-related peptide receptor did not affect the vasodilatation but partially inhibited allodynia. Thus, although both periorbital allodynia and vasodilatation evoked by glyceryl trinitrate are initiated by nitric oxide, they are temporally and mechanistically distinct. While vasodilatation is due to a direct nitric oxide action in the vascular smooth muscle, allodynia is a neuronal phenomenon mediated by TRPA1 activation and ensuing oxidative stress. The autocrine pathway, sustained by TRPA1 and NOX1/2 within neuronal cell bodies of trigeminal ganglia, may sensitize meningeal nociceptors and second order trigeminal neurons to elicit periorbital allodynia, and could be of relevance for migraine-like headaches evoked by glyceryl trinitrate in humans.

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WILEY

ORIGINAL ARTICLE

TRPA1 mediates the antinociceptive properties of the constituent of *Crocus sativus* L., safranal

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Abstract

Safranal, contained in *Crocus sativus* L., exerts anti-inflammatory and analgesic effects. However, the underlying mechanisms for such effects are poorly understood. We explored whether safranal targets the transient receptor potential ankyrin 1 (TRPA1) channel, which in nociceptors mediates pain signals. Safranal by binding to specific cysteine/lysine residues, stimulates TRPA1, but not the TRP vanilloid 1 and 4 channels (TRPV1 and TRPV4), evoking calcium responses and currents in human cells and rat and mouse dorsal root ganglion (DRG) neurons. Genetic deletion or pharmacological blockade of TRPA1 attenuated safranal-evoked release of calcitonin gene-related peptide (CGRP) from rat and mouse dorsal spinal cord, and acute nociception in mice. Safranal contracted rat urinary bladder isolated strips in a TRPA1-dependent manner, behaving as a partial agonist. After exposure to safranal the ability of allyl isothiocyanate (TRPA1 agonist), but not that of capsaicin (TRPV1 agonist) or GSK1016790A (TRPV4 agonist), to evoke currents in DRG neurons, contraction of urinary bladder strips and CGRP release from spinal cord slices in rats, and acute nociception in mice underwent desensitization. As previously shown for other herbal extracts, including petasites or parthenolide, safranal might exert analgesic properties by partial agonism and selective desensitization of the TRPA1 channel.

KEYWORDS
calcitonin gene-related peptide, neurogenic inflammation, pain, safranal, transient receptor potential ankyrin 1

1 | INTRODUCTION

Crocus sativus L., known as saffron crocus, belongs to the family of *Iridaceas*[1] and is commonly used for flavouring and colouring food preparations. Saffron extracts contain three main bioactive constituents: the carotenoid crocin, responsible for its typical colour, the monoterpene aldehyde picrocrocin, and the volatile compound safranal, which accounts for its special flavour.[2] Saffron has been reported to possess beneficial effects against depression, sexual dysfunction, premenstrual syndrome and weight loss.[1,3] Although clinical trials reported headache as one possible adverse effect of saffron,[4] in Indian traditional medicine saffron has been

Li Puma and Landini are Equally contributing authors.

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RESEARCH ARTICLE Open Access

Migraine-provoking substances evoke periorbital allodynia in mice

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Abstract

Background: Administration of endogenous mediators or exogenous chemicals in migraine patients provoke early headaches and delayed migraine-like attacks. Although migraine provoking substances are normally vasodilators, dilation of arterial vessels does not seem to be the sole contributing factor, and the underlying mechanisms of the delayed migraine pain are mostly unknown. Sustained mechanical allodynia is a common response associated with the local administration of various proalgesic substances in experimental animals and humans. Here, we investigated the ability of a series of endogenous mediators which provoke or do not provoke migraine in patients, to cause or not cause mechanical allodynia upon their injection in the mouse periorbital area.

Methods: Mechanical allodynia was assessed with the von Frey filament assay. Stimuli were given by subcutaneous injection in the periorbital area of C57BL/6J mice; antagonists were administered by local and systemic injections.

Results: Calcitonin gene related peptide (CGRP), but not adrenomedullin and amylin, pituitary adenylyl cyclase activating peptide (PACAP), but not vasoactive intestinal polypeptide (VIP), histamine, prostaglandin E_2 (PGE_2) and prostacyclin (PGI_2), but not $PGF_{2\alpha}$, evoked a dose-dependent periorbital mechanical allodynia. The painful responses were attenuated by systemic or local (periorbital) administration of antagonists for CGRP (CLR/RAMP1), PACAP (PAC-1), histamine H_1, PGE_2 (EP_4), and PGI_2 (IP) receptors, respectively.

Conclusions: The correspondence between substances that provoke (CGRP; PACAP, histamine, PGE_2, PGI_2), or do not provoke (VIP and $PGF_{2\alpha}$), migraine-like attacks in patients and periorbital allodynia in mice suggests that the study of allodynia in mice may provide information on the proalgesic mechanisms of migraine-provoking agents in humans. Results underline the ability of migraine-provoking substances to initiate mechanical allodynia by acting on peripheral terminals of trigeminal afferents.

Keywords: Migraine, calcitonin gene related peptide, pituitary adenylyl cyclase activating peptide, prostaglandin, histamine, vasoactive intestinal polypeptide, allodynia

Background

Migraine is a pain disorder that affects about 15% of the adult population worldwide. Thus, the burden of migraine is enormous in terms of suffering, disability, healthcare, and social and economic costs [1]. For these reasons, migraine is ranked among the most disabling medical conditions [2]. Although considerable progress has been made in the development of new treatment options [3, 4], our current understanding of the mechanisms underlying migraine pain is incomplete. Migraine attacks are elicited by a variety of provoking agents [5], and this peculiar feature provides an opportunity to explore disease mechanisms by endogenous mediators or exogenous chemicals that provoke migraine-like attacks in patients [6].

A prototypical example of a migraine-provoking agent is glyceryl trinitrate (GTN). Occupational exposure to, or treatment with, organic nitrates has long been known to provoke headaches [7–10]. Typically, GTN causes an early, mild and short-lived headache minutes after administration, followed by a remarkably delayed migraine-like

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The acyl-glucuronide metabolite of ibuprofen has analgesic and anti-inflammatory effects *via* the TRPA1 channel

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ABSTRACT

Ibuprofen is a widely used non-steroidal anti-inflammatory drug (NSAID) that exerts analgesic and anti-inflammatory actions. The transient receptor potential ankyrin 1 (TRPA1) channel, expressed primarily in nociceptors, mediates the action of proalgesic and inflammatory agents. Ibuprofen metabolism yields the reactive compound, ibuprofen-acyl glucuronide, which, like other TRPA1 ligands, covalently interacts with macromolecules. To explore whether ibuprofen-acyl glucuronide contributes to the ibuprofen analgesic and anti-inflammatory actions by targeting TRPA1, we used *in vitro* tools (TRPA1-expressing human and rodent cells) and *in vivo* mouse models of inflammatory pain. Ibuprofen-acyl glucuronide, but not ibuprofen, inhibited calcium responses evoked by reactive TRPA1 agonists, including allyl isothiocyanate (AITC), in cells expressing the recombinant and native human channel and in cultured rat primary sensory neurons. Responses by the non-reactive agonist, menthol, in a mutant human TRPA1 lacking key cysteine-lysine residues, were not affected. In addition, molecular modeling studies evaluating the covalent interaction of ibuprofen-acyl glucuronide with TRPA1 suggested the key cysteine residue C621 as a probable alkylation site for the ligand. Local administration of ibuprofen-acyl glucuronide, but not ibuprofen, in the mouse hind paw attenuated nociception by AITC and other TRPA1 agonists and the early nociceptive response (phase I) to formalin. Systemic ibuprofen-acyl glucuronide and ibuprofen, but not indomethacin, reduced phase I of the formalin response. Carrageenan-evoked allodynia in mice was reduced by local ibuprofen-acyl glucuronide, but not by ibuprofen, whereas both drugs attenuated PGE_2 levels. Ibuprofen-acyl glucuronide, but not ibuprofen, inhibited the release of IL-8 evoked by AITC from cultured bronchial epithelial cells. The reactive ibuprofen metabolite selectively antagonizes TRPA1, suggesting that this novel action of ibuprofen-acyl glucuronide might contribute to the analgesic and anti-inflammatory activities of the parent drug.

1. Introduction

Ibuprofen, the first approved member of propionic acid derivatives, is a classical non-steroidal anti-inflammatory drug (NSAID) widely used for its analgesic and anti-inflammatory properties [1,2]. Ibuprofen is indicated to relieve inflammation and several types of pain, including headache, muscular pain, toothache, backache, and dysmenorrhea [2]. Therapeutic effects of ibuprofen are attributed to inhibition of prostanoid synthesis by a non-selective, reversible inhibition of both cyclooxygenase 1 (COX1) and 2 (COX2) [3,4].

Abbreviations: AITC, allyl isothiocyanate; IAG, ibuprofen-acyl glucuronide; ANOVA, analysis of variance; COX, cyclooxygenase; DRG, dorsal root ganglia; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; H_2O_2, hydrogen peroxide; HBSS, Hank's balanced salt solution; HEK, human embryonic kidney cells; NSAID, non-steroidal anti-inflammatory drug; PG, prostaglandin; PAR-2, proteinase activated receptor 2; PAR2-AP, activating peptide of the PAR-2 receptor; ROS, reactive oxygen species; RNS, reactive nitrogen species; RCS, reactive carbonyl species; TRPA1, transient receptor potential ankyrin 1; TRPV1, transient receptor potential vanilloid 1; TRPV4, transient receptor potential vanilloid 4; $ZnCl_2$, zinc chloride

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Title Page

Dacarbazine alone or associated with melanoma-bearing cancer pain model induces painful hypersensitivity by TRPA1 activation in mice

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Novelty and Impact: Chemotherapy-induced pain syndrome is the major antineoplastic therapy limitation, with no treatment available. Several preclinical studies demonstrate the contribution of TRPA1 in the pathogenesis of this pain syndrome. TRPA1 antagonists have recently advanced into clinical trials for neuropathic pain treatment, and the present study may enhance the investigation related to the TRPA1 blockade as a pharmacological target for treating chemotherapy-induced pain syndrome in cancer patients submitted to antineoplastic treatment.

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Schwann cells expressing nociceptive channel TRPA1 orchestrate ethanol-evoked neuropathic pain in mice

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Conflict of interest. RP is fully employed at Chiesi Farmaceutici SpA, Parma, Italy. NWB is a founding scientist of Endosome Therapeutics Inc. The other authors declare no competing financial interests.