

## Sensitisation of Nociceptors – What are Ion Channels Doing?

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**Abstract:** Nociceptors are peripheral sensory neurones which respond to painful (noxious) stimuli. The terminals of nociceptors, which have a high threshold to stimulation in their native state, undergo a process known as *sensitisation*, or lowering of threshold, following injury or inflammation. Amongst sensory receptors, sensitisation is a property unique to nociceptors. A shift in the stimulus-response function of nociceptors renders them more sensitive, resulting in both a reduction in the activation threshold, such that previously non-noxious stimuli are perceived as noxious (*allodynia*) and an increased response to suprathreshold stimuli (*hyperalgesia*). Sensitisation protects us from harm and is essential for survival, but it can be disabling in conditions of chronic inflammation. This review focuses on three stages in sensitisation: 1) *Inflammatory mediators*, which are released from damaged resident cells and from others that invade in response to inflammation, and include bradykinin, prostaglandins, serotonin, low pH, ATP, neurotrophins, nitric oxide and cytokines; 2) *Intracellular signalling molecules* which are important in transmitting the actions of inflammatory mediators and include protein kinase A and C, Src kinase, mitogen-activated protein kinases and the membrane lipid PIP<sub>2</sub>; and 3) *Ion channel targets* of intracellular signalling which ultimately cause sensitisation and include the temperature-sensitive transient receptor potential channels, acid-sensitive ion channels, purinoceptor-gated channels, and the voltage-sensitive sodium, potassium, calcium and HCN channels.

**Keywords:** Pain, hyperalgesia, inflammation, cell signalling.

### INTRODUCTION

Sensory neurones transduce mechanical, thermal and chemical stimuli into nerve impulses which then travel to higher centres to initiate painful sensations, a process referred to as nociception [1]. The activation of specific receptors or ion channels underlies the transduction of noxious stimuli into action potentials at peripheral nociceptive nerve terminals. Action potentials are then relayed via the dorsal horn of the spinal cord to the primary somatosensory area of the cerebral cortex, where the sensation of pain is experienced. Although there is a positive correlation between stimulus intensity and reported pain intensity, a large degree of plasticity is found at all levels of the nociceptive system which means that the experience of pain is highly variable [2]. Following inflammation or damage, noxious stimuli can evoke an exaggerated and prolonged pain (*hyperalgesia*) and pain thresholds can be lowered so that stimuli that are normally innocuous now become noxious (*allodynia*). Previously ‘silent’ nociceptors can become responsive to thermal or mechanical stimuli when sensitised by inflammatory substances or tissue injury [3, 4]. Most of these mechanisms have a rapid onset and are readily reversible. Longer term changes are found during chronic inflammation and in neuropathic pain, a disabling condition in which partial damage to peripheral nerves causes long-lasting allodynia and hyperalgesia. These long-term changes in gene expression will be discussed in the context of the relevant receptors.

During the process of inflammation, a mixture of *inflammatory mediators* such as peptides (bradykinin), lipids (prostaglandins), neurotransmitters (ATP), protons and neurotrophins (NGF) is released. Some of these mediators directly excite nociceptors, inducing painful sensations, while others sensitise nociceptors. The release of neuropeptides from peripheral neurones supports plasma extravasation of further mediators and chemoattraction of inflammatory cells, a process termed “neurogenic inflammation”. The sensitisation of the primary afferent neurones by local inflammatory mediators can occur by a direct action on the sensory neurones, or it can involve an indirect action on non-neuronal cells, especially but not exclusively on immune cells, from which further inflammatory mediators can then be released.

In this review, peripheral sensitisation is considered to be an increase in the responsiveness of primary afferent nociceptors compared to baseline. Repetitive stimulation, a part of the definition of sensitisation in a behavioural setting, is not required to elicit sensitisation at a molecular level. Sensitisation can arise for a variety of reasons, which can be broadly separated into effects on ion currents directly activated by the noxious stimulus (generator currents) and effects on ion channels involved downstream in the initiation of action potentials. Sensitisation is distinct from the actual process of initiating action potentials, though some of the ion channels responsible for determining the action potential threshold (e.g. hyperpolarisation-activated cyclic nucleotide gated channels, HCN) and for causing excitation (e.g. sodium channels) are also targets for sensitisation (see below).

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Visceral nociceptors are not as well studied as somatic, especially cutaneous, afferents. Many results concerning mediators and receptors have, however, been replicated in visceral pain models including all the mediators and receptors mentioned below [5]. A few differences have been reported, e.g. the IB4-positive population is smaller in visceral compared to somatic afferents, almost all visceral afferents are peptidergic and TRPV1-positive neurones are more prevalent than in somatic nociceptors [6, 7].

The mediators of inflammatory pain are better established than the processes involved in neuropathic pain, for this reason this review will focus on inflammatory pain. In the case of neuropathic pain some similar mechanisms of sensitisation and gene regulation have recently been reviewed [8]. The first part of the review focuses on extracellular mediators released by inflammation and leading to sensitisation. In the second part of this review we discuss how these mediators activate a rather small number of intracellular signalling pathways and mediators in order to induce peripheral sensitisation. The main mechanisms characterised to date are (i) phosphorylation of ion channels already in the surface membrane, and (ii) altered cell surface expression of ion channels, which can be modulated both in the short term by trafficking of ion channels from an intracellular store and in the longer term by altered gene expression. The third part of the review discusses the impact the activation of intracellular signalling pathways has on the most important ion channels engaged in sensitisation.

## INFLAMMATORY MEDIATORS OF SENSITISATION

There are many extracellular mediators which contribute to nociceptor sensitisation. The importance of any specific factor has been proven difficult to dissect, since many of these release others, generating a multi-focussed web of factors. Ryan and Majno noted that inflammation is most likely mediated by vascular leakage, due to the actions of the kinins, vasoactive amines (histamine, serotonin) and the prostaglandins [9]. Many more groups of compounds might be involved, based on measurements from human inflamed tissue and pharmacological studies [10]. The multifactorial nature of inflammation led to the idea of an 'inflammatory soup' or 'inflammatory mediators' [11]. Unfortunately, this term is used for different compositions and concentrations, usually involving bradykinin, prostaglandin  $E_2$ , histamine and serotonin, often at a lowered pH. The composition is clearly artificial and omits some important mediators found subsequently.

### Bradykinin

Bradykinin is amongst the most potent sensitizing agents. Injection of bradykinin into human skin produces a dose-dependent pain and heat hyperalgesia, suggesting that bradykinin is capable of exciting and sensitising nociceptors to heat [12]. Bradykinin is formed by cleavage of low molecular weight tissue kininogen by the protease kallikrein to liberate lys-bradykinin (kallidin) which is then cleaved further to bradykinin by an aminopeptidase [13]. Kallikreins are serine proteases activated from prekallikrein by activated Factor XII of the coagulation cascade [14]. Bradykinin and kallidin are both potent  $B_2$  receptor activators, and are responsible for acute sensitisation and activation [15].

Functional  $B_2$  receptors are widely expressed, both in the nervous system and in non-neuronal tissues [16]. Kallikrein has also been shown to activate the  $B_2$  receptor directly [17]. Further cleavage of the C-terminal arginine of bradykinin liberates des-arg<sup>9</sup> bradykinin, which has little action at the  $B_2$  receptor but is a potent activator of the  $B_1$  receptor. The  $B_1$  receptor is absent or expressed at low levels under normal conditions, but during prolonged inflammation  $B_1$  receptors are induced in primary nociceptors by glial cell derived neurotrophic factor and can sensitise the heat evoked current in dorsal root ganglia (DRG) neurones [18, 19]. Both bradykinin receptors are G-protein coupled and activate  $G_q$ /phospholipase  $C_\beta$  ( $PLC_\beta$ ) and  $G_i$ /phospholipase  $A_2$  [20]. Downstream of phospholipase  $C_\beta$  are the mediators  $IP_3$ /diacylglycerol and protein kinase C (PKC), as discussed below. Downstream of phospholipase  $A_2$ , arachidonic acid from the cell membrane is converted to leukotrienes, hydroperoxyeicosatetraenoic acids (HPETEs), epoxyeicosatrienoic acids (EETs) and, most importantly, prostaglandins which lead to activation of protein kinase A (PKA). Bradykinin is also a good example of an inflammatory mediator which causes the secondary release of transmitters from neurones, including calcitonin gene-related peptide, substance P and acetylcholine [21], and also the release of further inflammatory mediators from immune cells, including NGF, interleukins, tumour necrosis factor, prostaglandins and leukotrienes [22, 23]. Bradykinin decreases the threshold of thermally-activated ion channels such as TRPV1, and it also sensitises nociceptors by modulating other ion channels, for example by reducing the activity of potassium channels, which makes cells more excitable by decreasing the resting potential.

### Prostaglandins

Prostaglandins (PG) are derived from arachidonic acid, which is released from membrane phospholipids by the esterase action of phospholipase  $A_2$ .  $PGE_2$  and  $PGI_2$  have been found to be more potent sensitizers than  $PGF_{2\alpha}$ ,  $PGD_2$  or  $TxA_2$  in humans [24] and in animal models [25]. Both have similar effects but different time courses of action [26]. The actions of  $PGE_2$  and  $PGI_2$  are transduced by the G-protein coupled EP and IP receptors, respectively [27, 28], and in both cases the main action in primary sensory neurones is to couple to  $G_s$ , which in turn activates adenylate cyclase to release cyclic adenosine monophosphate (cAMP) and thus activate PKA, as discussed below [29]. Production of prostaglandins via cyclooxygenases 1 and 2 is the target of the most common used painkiller family. Cyclooxygenase inhibition reduced the activation of sensory neurones [30]. Prostaglandins sensitise sodium and calcium channels and suppress outward potassium currents [31]. An alternative cAMP-dependent pathway mediated via  $PKC\epsilon$  has been described in IB4-positive neurones [32]; the observed sensitisation in inflamed tissue is conveyed by  $Epac1$  (the exchange protein directly activated by cAMP) from cAMP to  $PKC\epsilon$  [33]. Given the convergence of so many pathways on  $G_s$  proteins, it is not surprising that opioids, which have been shown to be potent peripheral analgesics in addition to their central actions, owe their peripheral actions to a counterbalancing activation of  $G_i$ , which inhibits adenylate cyclase [34]. Arachidonic acid is not only a substrate for production of prostaglandins but is also processed by

lipoxygenases and cytochrome p450 epoxygenase. Injection of the leukotrienes lowered nociceptive thresholds [35] and inhibition of 5- and 12-lipoxygenase reduced development of hyperalgesia [36]. There is also some indication of modulation of pain by EETs [37].

### Histamine

The main source of histamine is mast cells, the degranulation of which is stimulated by substance P and vasoactive intestinal peptide [38, 39]. Extracellular levels of histamine are substantially increased in inflammation, but its contribution to sensitisation is unclear, since histamine evokes the sensation of itch rather than pain and directly activates only a minimal fraction of neurones [40].

### Serotonin

Serotonin, or 5-hydroxytryptamine (5-HT), is present at increased levels in inflamed tissues, and is released mainly from mast cells and platelets [41]. Serotonin can directly excite neurones via activation of the 5-HT<sub>3</sub> receptor, which is an ion channel, and can in addition sensitise via the G<sub>q</sub>-coupled 5-HT<sub>2A</sub> receptors found in neurones [42, 43]. Inflammatory pain was shown to be reversed by an antagonist for 5-HT<sub>2A</sub> [44]. In trigeminal pain 5-HT<sub>1</sub> receptor subtypes are involved, and antagonists can be used to treat migraine [45]. At a molecular level, sensitisation by 5-HT has been demonstrated for sodium channels [46]. Indirectly, serotonin sensitises by activating monocytes [47] and by interleukin 6 release from endothelial cells [48].

### Protons

Acidic conditions occur in inflammation and ischemia. Protons can directly activate transducer channels, as discussed below, but the necessary pH levels are rarely found even in infections or severe tissue damage [49, 50]. Only intradermal infusion of pH 5 and below induces pain in humans, although owing to the high tissue buffering capacity the actual tissue pH attained may be less acidic than this and the corresponding pH at the nerve terminals remains unknown [51]. Furthermore, these extreme proton concentrations reduced conduction of many channels [52] and can lead to conduction block [53]. There might however be exceptions such as stomach afferents which are continuously exposed to extreme proton concentrations in the vicinity of lesions [54]. Protons also commonly act as subthreshold sensitising agents, acting cooperatively with bradykinin, ATP and inflammatory mediators [55-58].

### ATP

ATP activates both the ionotropic P2X purinoceptors and the metabotropic P2Y receptors. High levels of ATP are required for purinoceptor activation, and are seldom attained by tissue damage or ischemia, but can be achieved by release from (sympathetic) neurones [59]. We discuss the P2Y receptors in this section and the P2X receptors in the final section on ion channels. DRG neurones express P2Y1 and P2Y2 receptors [60]. P2Y1 activation in DRG neurones inhibits Ca<sub>v</sub>2.2 and P2X3 channels [61]. Activation of PKC $\epsilon$  following P2Y1 receptor activation sensitises TRPV1 [62]. P2Y2 receptor activation also sensitises TRPV1 in expression systems and leads to TRPV1-mediated thermal hyperalgesia [63]. Following tissue injury, activation of

extracellular signal-regulated kinases (ERKs) is mediated by P2Y receptors [64] and gene regulation is triggered via the cAMP response element binding protein CREB [65]. In contrast to these actions mediated by ATP and the P2 family of receptors, activation of the P1 receptors by adenosine decreases cellular excitability and nociception, and adenosine has analgesic actions in behavioural experiments [66].

### Neurotrophins

Neurotrophins, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin 3-5, activate the tyrosine kinase-couples receptors of the Trk family. Neurotrophin effects can be separated into short-term effects mediated by phosphorylation and trafficking of ion channels to the membrane, and long-term changes mainly caused by changes in gene expression [67, 68]. Injection of NGF has a rapid hyperalgesic effect, and also causes a hyperalgesia lasting for many days [69]. The short term action of NGF in promoting hyperalgesia is thought to be predominantly mediated via TrkA receptors [70]. NGF acutely sensitised the activation of sensory neurones by capsaicin, an effect mediated by TRPV1 [71]. NGF binds to TrkA, causing phosphorylation of tyrosine Y760 and thus activating the PI3K-Src signalling pathway to phosphorylate Y200 of TRPV1, which causes enhanced insertion of TRPV1 into the cell membrane [72, 73]. There have also been reports that NGF alters the activation threshold of TRPV1 via p42/44 mitogen-activated protein kinases (MAPK) [74]. NGF is an important link to stimulation of the immune system, including mast cell degranulation, cytokine production and B- and T-cell proliferation [75, 76]. The importance of NGF as an inflammatory mediator is also due to retrograde transport to the cell nucleus, where it induces a long term upregulation of gene expression, including TRPV1, acid-sensing ion channels (ASIC) and sodium channels, neuropeptides such as substance P and calcitonin gene-related peptide (CGRP), and bradykinin receptors [77-80].

### Nitric Oxide

Nitric oxide (NO) is produced by the nitric oxide synthases, of which there are three isoforms, endothelial neuronal and inducible (eNOS, nNOS and iNOS, respectively). NO producing enzymes can be activated by noxious irritants, by NO itself, or in DRG neurones following nerve injury [81-83]. Local inhibition of NO synthesis reduces inflammatory hyperalgesia [84]. Nitric oxide is a short-lived inflammatory mediator and therefore has only very local effects, but it can pass readily through cell membranes and therefore often has effects in cells adjacent to those in which the NO is produced. The most well-known downstream pathway is the direct activation of soluble guanylate cyclase by NO, leading to enhanced levels of cGMP and consequent activation of protein kinase G in target cells. Bradykinin can also stimulate the formation of cGMP in nociceptive neurones [85], and while for central sensitisation cGMP seems to be important [86], in primary afferents the relevance of cGMP seems minor in comparison to cAMP [87, 88]. NO can also act directly on membrane proteins to S-nitrosylate cysteine residues, leading to the breaking of Cys-Cys bonds and a consequent change in

protein structure [89]. An important additional long-term effect of NO is the upregulation of cyclooxygenase 2 [90].

### Cytokines

Immune cells contribute to sensitisation via a complex network [91]. TNF $\alpha$  is released from monocytes and tissue macrophages by several stimuli including microbial products, complement and tissue damage, and induces sensitisation with both rapid and long-lasting components [92]. TNF $\alpha$  activates the TNFR1 and TNFR2 receptors, which are upregulated during inflammation [93]. The importance of TNF $\alpha$  in chronic inflammation such as arthritis is illustrated by the success of monoclonal antibodies against it [94]. TNF $\alpha$  is the trigger for the release of a whole cascade of cytokines, including NGF and the interleukins 1 $\beta$ , 6 and 8, which are amongst the strongest proinflammatory cytokines [95]. Among these interleukin 1 $\beta$  and NGF are especially potent in inducing lasting sensitisation [96]. These cascades also finally lead to prostaglandin formation. A link to the p38 MAPK pathway has been demonstrated [97]. In addition to this indirect action through a downstream cascade, there is also evidence for a direct action of cytokines on nociceptors [98, 99]. Members of the CCL family also trigger hyperalgesia, and an effect in sensitizing TRPV1 has been shown for CCL2 and CCL3 [100, 101]. Many other chemokines have been described [102].

### Prokineticins

Two human proteins named prokineticin 1 and 2 and their corresponding G-protein-coupled receptors have been identified [103]. Systemic injection of a prokineticin receptor agonist induces a biphasic hyperalgesia [104], and the heat and capsaicin sensitivity of prokineticin receptor 1 knockout mice is lower than in wildtype, implying a tonic sensitizing effect on TRPV1 *in vivo* [105]. Prokineticin receptors are expressed in neurones and their activation sensitised TRPV1, suggesting a role for prokineticins in physiological inflammation and hyperalgesia [106].

### Proteases

Proteases are released during injury and blood clotting, and act upon protease-activated receptors (PARs), a family of four G protein-coupled receptors, to regulate hemostasis, inflammation, pain, and repair. PARs are cleaved by proteases at a specific site near the extracellular N-terminus, unmasking a tethered ligand which is then able to bind to and activate the receptor. PAR2 seems responsible for sensitisation, it is localised in most nociceptive neurones, and PAR2 in particular seems to be important in sensitising neurones via PKC-dependent phosphorylation of TRPV1 [107].

## INTRACELLULAR SIGNALLING MECHANISMS

The previous section has dealt with a plethora of mediators released during inflammation. The number of intracellular signalling pathways activated by these mediators is, by contrast, much less. Phosphorylation of target membrane ion channels is a key driver of sensitisation; the key players here seem to be the serine/threonine protein kinases PKA and PKC, and the tyrosine kinases Src and MAPK, though important roles for other kinases no doubt

remain to be discovered. Principal phosphatases currently known to drive dephosphorylation include the calcium-dependent phosphatase calcineurin (PPP3, formerly PP2B) and the SHP tyrosine phosphatases [108]. Dephosphorylation normally follows activation and is often driven by increased intracellular calcium levels [109, 110]. A change in phosphorylation state can be accompanied with trafficking to or from the plasma membrane [73, 111, 112].

### Protein Kinase A

PKA is activated by pathways which activate adenylate cyclase, leading to the production of cAMP. Stable analogs of cAMP cause sensitisation in behavioural models [113] and in *in vitro* models using rat nociceptive afferent neurones [88, 114]. PKA catalytic subunits were shown to translocate to the plasma membrane when activated [115]. Phosphorylation by PKA sensitises TRP channels, and sodium, calcium and potassium channels (Table 1).

### Protein Kinase C

Activation of PKC can sensitise as well as activate nociceptors [116]. PKC has several calcium-dependent and -independent isoforms with different contributions to sensitisation [117]. Of particular importance is PKC $\epsilon$ , which sensitises heat-evoked activation of DRG neurones [118]. Subtype-specific antagonists and PKC $\epsilon$  deficient mice support that this isoform is vital for acute inflammation [119]. One report found that PKC $\alpha$  was also involved in sensitisation of TRPV1 [120]. Systemic inhibition of PKC $\beta$  reduced diabetic hyperalgesia through reduction of cGMP in DRG neurones [121]. PKC activation sensitises capsaicin-stimulated neuropeptide release [122]. It was demonstrated that PKC activation can reduce the temperature threshold of TRPV1, even to below body temperature, thus leading to tonic activation of pain-sensitive afferents [58, 123]. PKC is also involved in sensitisation in neuropathic pain models, including alcoholic, diabetic and chemotherapy-induced neuropathy [124-126].

Members of the A-kinase anchoring protein (AKAP) family associate both PKA and PKC with their targets [127]. It has recently been shown that the AKAP79 isoform binds to both TRPV1 and TRPV4 and in this way maintains PKA, PKC and the phosphatase calcineurin in close proximity with their target serines and threonines. Downregulation or inhibition of binding of AKAP79 completely disrupts sensitisation via both PKA and PKC [128, 129]. These observations suggest that the binding of AKAP79 to TRPV1 may be a “master switch” whose inhibition could be used to antagonise sensitisation via both the PKA and the PKC pathways.

### Src Kinase

The tyrosine kinase Src has recently been shown to play a key role in sensitisation of TRPV1. Binding of NGF to the TrkA receptor initiates several signalling cascades, amongst them the ras-MAPK pathway (see next section) and the PI3K-PKB-Src pathway. Src phosphorylates an N-terminal tyrosine in TRPV1, leading to a rapid increase in trafficking to the neuronal cell membrane and therefore to an enhanced response to activators of TRPV1 [73]. This pathway seems likely to explain the rapid increase (minutes) in membrane TRPV1 following exposure to NGF, while increased

**Table 1. Summary of the regulation of the ion channels and intracellular pathways discussed in this review. ↑ denotes an activation or sensitisation, ↓ an inhibition or desensitisation, ↔ evidence that there is no regulation. ∅ indicates that we have found no report of modulation. In the case of conflicting evidence a combination of symbols is given. For calcium and potassium channels the affected channel subtypes are mentioned when known, or marked as NI (not identified) if currents were measured without clear identification of subtypes**

	PKA	PKC	Src	MAPK	PIP <sub>2</sub>	Inflammation
TRPV1	↑[115, 155]	↑ [154, 156, 252]	↑[72-74, 153, 158]	↑ [74, 78]	↑[158, 253-256] ↓[257]	↑[258]
TRPV4	↑ [129]	↑[129, 259]	↑[260, 261]	∅	∅	↑[262, 263]
TRPM8	↓[177, 264]	↓[114, 177]	∅	∅	↑[143, 178, 265]	↔[266]
TRPA1	↑ [170]	∅	∅	↑ [266]	↑[267] ↓[171, 268]	↑[266, 269]
ASIC	∅	↑[197, 270]	∅	↑[77]	∅	↑ [271, 272]
P2X	[33]	↑indirect [33, 202, 273] ↓[274]	∅	∅	↑ P2X1 [275]	↑[276, 277]
Nav1.7	↑[278] ↓ [221]	↑ [221]	∅	∅	∅	↑[208, 209, 279]
Nav1.8	↑[221, 230, 280]	↑[230, 280, 281] ↓[221]	∅	∅	∅	↑ [208, 209, 279]
Nav1.9	∅	↑[230, 282]	∅	∅	∅	↑[279] ↔ [208]
Potassium	↑ K <sub>v</sub> 1.2 [283] K <sub>ir</sub> 1.1 [284] K <sub>ir</sub> 2.1 [285] ↓ NI [233] K <sub>2P</sub> [286]	↑ K <sub>v</sub> 3.4[287] ↓ K <sub>ir</sub> 2.1[285] K <sub>ir</sub> 4.1/5.1[288] K <sub>2P</sub> [286]	↓ K <sub>v</sub> 1.3 [289]	∅	↑ K <sub>ir</sub> 1-3 [290]	↑ K <sub>v</sub> 1.2 [235]
Calcium	∅	↑ NI[241, 291] Cav2.2 and Cav2.3[242] ↓ [243, 244, 292]	∅	∅	↑ [293, 294]	∅
HCN	∅	↑ [295]	↑[296, 297]	↑[295]	↑ [298]	↑[299]

expression following activation of the ras-MAPK pathway is responsible for more long-term sensitisation (days).

### Mitogen-Activated Protein Kinases (MAPK)

Kinases of the MAPK family (ERK, p38, and JNK) can contribute to nociceptor sensitisation, although their role in central pain processing is more prominent [130]. MAPK inhibitors alleviate hyperalgesia and allodynia in inflammatory pain models, though basal sensory threshold are little affected [131]. ERKs are activated in DRG neurones by NGF [132], by chemical and electrical stimulation [133] and by inflammation by Freund's adjuvant

[134]. Similarly, p38 MAPK in DRG neurones is also activated by NGF [135], by capsaicin and by thermal stimulation [136]. P38 and c-Jun are both activated in DRG neurones by inflammation [137, 138]. Both p38 and c-Jun aminoterminal kinases are activated by TNF $\alpha$  [139], and c-Jun regulates expression of neuropeptides vasoactive intestinal peptide (VIP) and neuropeptide Y (NPY) in DRG neurones [140].

### PIP<sub>2</sub>

Phosphatidyl-inositol 4,5-bisphosphate (PIP<sub>2</sub>) is an ubiquitous component in the plasma membrane. PIP<sub>2</sub> levels

in the plasma membrane have been found to regulate the activities of several neuronal transporters and receptors [141], including sodium-proton exchangers, inward rectifier potassium channels, epithelial sodium channels, ryanodine-sensitive calcium channels, several TRP channels, P2X channels and HCN channels (see Table 1). Probably the most significant regulation in the context of nociception is the enhancement of activity of TRP channels caused by elevations of PIP<sub>2</sub> [142], and the consequent loss of activity when PIP<sub>2</sub> levels are depleted, for instance by an elevation of intracellular calcium [143]. See further discussion below.

## MODULATION OF ION CHANNELS

The following section discusses ion channels thought to be of major importance for nociceptor sensitisation. Interestingly, many of them are rather selectively expressed in sensory neurones [144]. The IUPHAR nomenclature <http://www.iuphar-db.org> is used.

### TRP Channels

Members of the transient receptor potential (TRP) family have received a great deal of attention in the context of pain processing [145]. The name originates from the transient receptor potential found in the visual receptors of *Drosophila melanogaster* with the *trp* mutations. The defective gene was later identified as the canonical TRP receptor, an ion channel with a characteristic ankyrin repeat motif in the N-terminal cytoplasmic domain [146]. A large family of TRP channels has been identified [147]. This review focuses on the three thermally sensitive TRP channels TRPV1, TRPA1 and TRPM8, which are expressed in somatosensory neurones [148] and are thought to be the main TRP ion channels involved in sensitisation.

*TRPV1* is the founder member of the vanilloid group [149], and is activated by a wide variety of physical (heat >42°C, membrane stretch) and chemical stimuli [150]. Chemical activators are structurally diverse and include vanilloids such as capsaicin and resiniferatoxin, low pH, endogenous lipids and ethanol. The sensory role of TRPV1 in intact animals is still not completely clear, because although it is clearly heat-activated and activation causes a burning sensation, deletion of TRPV1 has rather little effect on thermal thresholds *in vivo* [151, 152]. A pivotal role for TRPV1 in inflammatory heat hyperalgesia is, however, supported by a large body of evidence using TRPV1-deficient mice [151, 152]. Phosphorylation of TRPV1 by PKA, PKC and src leads to sensitisation to a wide range of activating stimuli, including heat, capsaicin, low pH and the endogenous cannabinoid anandamide [118, 153, 154]. The respective phosphorylation sites for PKA and PKC have been identified [155, 156]. A previous proposal that sensitisation of TRPV1 could be explained by removal of PIP<sub>2</sub> following activation of phospholipase C by inflammatory mediators such as bradykinin or NGF [157] is now not thought to be correct because application of PIP<sub>2</sub> to inside out patches was found to enhance rather than reduce the activity of TRPV1 channels [158]. In addition to sensitisation of channels already located in the cell membrane, new TRPV1 channels can also be rapidly trafficked to the cell membrane via exocytosis from an intracellular store, following activation of the PI3K-Src cascade by mediators such as NGF and insulin [73, 112,

159]. Long-term upregulation of expression of TRPV1, driven by growth factors such as NGF, and downstream by the ras-MAPK signalling pathway, are also likely to play a role in long-term thermal hyperalgesia [78, 160]. Evidence has been accumulating for the involvement of TRPV1 in a wide range of diseases, particularly in gastrointestinal, respiratory and bladder diseases [161]. Sensitisation of TRPV1 to a threshold below body temperature could explain ongoing nociceptive input and therefore pain in inflammatory conditions such as arthritis. TRPV1 has sufficient calcium conductance on its own to trigger calcium-dependent mechanism like neuropeptide release without the contribution of voltage-gate calcium channels [162].

*TRPA1* detects chemical irritants such as acrolein, a constituent of smoke, and agonists such as allyl isothiocyanate (mustard oil), allicin or formalin, all of which induce a burning or pricking sensation [163, 164]. Unsaturated electrophilic structures are a common motif and a corresponding covalent and irreversible activation mechanism, targeting intracellular N-terminal cysteine residues, has been described [165, 166]. The expression of TRPA1 is regulated by NGF [163]. Animals deficient in TRPA1 were less sensitive in several pain models [167]. A unique feature of TRPA1 is its direct gating by intracellular calcium, rendering TRPA1 a potential amplifier of other stimuli which reach the necessary calcium threshold [168, 169]. Sensitisation of TRPA1 by bradykinin appears to involve both PLC and PKA pathways in sensory neurones [170]. The protease-activated receptor 2 sensitises TRPA1 via reduction of membrane PIP<sub>2</sub> levels [171]. The sensitivity of TRPA1 to cold has been debated [164, 168], but recent evidence supports a slow activation at low temperatures, which may have been missed by some temperature protocols [163, 172]. Both TRPV1 and TRPA1 are sensors of chemical irritants, and some algogens activate both. Such agonists still activate nociceptive neurones when either channel is individually deleted, but in double knockout animals the activation completely vanishes [173, 174].

*TRPM8* was cloned by two groups [175, 176]. It is activated physically by cooling and by a variety of chemicals e.g. menthol. Recent behavioural studies show that activation of TRPM8 antagonises capsaicin-induced nociception [114]. TRPM8 is co-expressed with bradykinin and prostaglandin receptors, and application of the respective mediators reduced the TRPM8 response to cooling and lowered the threshold temperature via pathways thought to involve PKC and PKA [177]. PKC-dependent dephosphorylation of TRPM8 via protein phosphatase 1 (PP1) has also been proposed to inhibit TRPM8 [114]. PIP<sub>2</sub> cleavage desensitises and PIP<sub>2</sub> addition recovers TRPM8 activation, with the C-terminal TRP box appearing to be the critical PIP<sub>2</sub> interaction site [178]. Mice deficient in TRPM8 have an impaired thermoception at least at near-ambient cool temperatures [179, 180], suggesting a role for TRPM8 in mediating innocuous cold perception. TRPM8 might also play a role in the nocifensive response to noxious cold [179]. In inflammation the temperature threshold of TRPM8 may be elevated and thus could explain cold pain at normally innocuous temperature [181]. The clinical role of TRPM8 is largely unknown. TRPM8 regulation has been reported in urogenital syndromes [182], suggesting a role for TRPM8 in these pathological states.

### Other TRPs and Heteromers

In addition to TRPV1, TRPA1 and TRPM8, other TRPs appear to work in concert to induce sensitisation in pathological states. TRPV2 and TRPV3 both sensitise on repeated activation in the absence of intracellular calcium [183, 184], and can be sensitised, as is TRPV1, by 2-aminoethoxydiphenyl borate [183, 185, 186]. TRPV4 can be sensitised by activation of PKC [129, 187], by low pH [188] and by nitric oxide [189]. Intracellular calcium levels trigger the calcineurin-dependent desensitisation of many TRP channels [109, 190]. Recently, TRPC channels were found to contribute to intracellular calcium homeostasis [191].

TRP channels are tetramers. Due to their similarity, the possibility of heteromer formation has been investigated [192]. In many neurones TRP channels are coexpressed, and heteromers could give rise to channels with novel properties, as demonstrated for TRPC channels [193]. Several TRP channels with currently unclear function but strong changes in post-injury expression could alter neuronal sensitivity by such a mechanism [194].

### ASIC Channels

Members of the ASIC family are activated by protons [195]. Local acidosis is observed during ischemia and inflammation and it is therefore tempting to attribute the pain caused by inflammation to activation of ASICs. Sensitivity to protons is not exclusive to members of this family. In skin, a lower pH sensitises all unmyelinated nociceptors to mechanical stimuli, whereas low-threshold mechanosensors are unaffected [51]. Proton-induced currents in DRG neurones are sensitised by nitric oxide, and the sensitisation was shown to depend on S-nitrosylation of ASICs at an external location rather than activation of soluble guanylate cyclase [196]. ASIC channels have been shown to be sensitised by PKC [197].

### P2X Purinoceptors

Both P2X ion channels and P2Y G-protein coupled receptors (see discussion above) can modulate neuronal excitability [198]. In this section we discuss only the former. Application of ATP to human blisters caused pain [199]. P2X receptor isoforms 1–3 are expressed in sensory neurones and in the heart in humans [200]. Peripheral inflammation enhances both the potency and effectiveness of locally administered P2X receptor agonists [201]. Although all P2X receptors have a consensus sequence for PKC phosphorylation, several studies argue against a direct phosphorylation by PKC [202]. C-terminal Src inhibitory kinase inhibits P2X3 receptor by Y393 phosphorylation [203]. An indirect sensitisation via interleukin 1 $\beta$  is also caused by the P2X7 receptor, which is expressed in immune cells [204], which may be the basis of the reduced pain-related behaviour and allodynia seen in P2X7 knockout animals [205].

### Sodium Channels

Nine sodium channels are found in humans, but only those important in DRG neurones are discussed in this review. The properties of sodium channels differ in their activation threshold and their time constants for inactivation and for recovery from inactivation [206]. In native DRG

neurones Na<sub>v</sub> subtypes 1.1 and 1.6 were found preferentially in A-fibre neurones, while 1.7, 1.8 and 1.9 were preferentially located in C-fibre populations [207]. Inflammation increases the expression of subtypes 1.3, 1.7 and 1.8 [208, 209]. Action potentials of nociceptors have a striking long duration and low maximum frequency [210].

Some sodium currents have been recognised for many years to be insensitive to tetrodotoxin (TTX), which provides a useful tool to distinguish the involvement of different isoforms [211, 212]. The TTX-resistant sodium currents are carried by Na<sub>v</sub>1.8 and Na<sub>v</sub>1.9, which are found only in a subpopulation of sensory neurones, and Na<sub>v</sub>1.5 which is only expressed in cardiac cells [213]. TTX-resistant sodium currents in nociceptive neurones are enhanced by inflammatory mediators [214]. The alpha subunits of sodium channels have several phosphorylation motifs for PKA and PKC [215]. In sensory neurones the peak sodium currents are increased and the current voltage-relationship was reported to shift to lower membrane voltages (i.e. in such a way as to enhance the ease of excitation of the neurone) following phosphorylation by PKA [46, 216].

Na<sub>v</sub>1.1 channels are inhibited by PKA activation [217]. Na<sub>v</sub>1.2 expression in sensory neurones is so low that the relevance of these channels is unclear, and in addition the sodium currents are inhibited by PKA and PKC, which does not favour a role in hyperalgesia [207, 218]. Na<sub>v</sub>1.7 produces a fast activating and inactivating current [219]. Due to its slow inactivation from the closed state, Na<sub>v</sub>1.7 is more likely to be activated by slowly rising generator potentials than other sodium channels and is therefore well suited to mediate the slow firing activity in nociceptors [220]. Na<sub>v</sub>1.7 currents are attenuated by activation of PKA and PKC. PKA does not change the steady-state voltage dependence, while PKC shifts it in depolarizing direction [221]. Na<sub>v</sub>1.7 DRG-specific knockouts showed reduced pain-related behaviour compared to wildtype mice [222]. Na<sub>v</sub>1.8 activates only upon strong depolarisation, as its threshold is some 20 mV positive to the thresholds of other sodium currents [223]. Na<sub>v</sub>1.8 has been shown to be the major contributor to the upstroke of action potentials in nociceptors and to account for a large part of the current in trains of action potentials [224]. Na<sub>v</sub>1.8 currents are potentiated by activation of PKA [221, 225]. In contrast, PKC decreases Na<sub>v</sub>1.8 currents, and shifts the voltage dependence in a depolarizing direction [221]. Based on the phenotype of Na<sub>v</sub>1.8 knockout mice, the channel seems to be of minor importance for pain responses [226]. Na<sub>v</sub>1.9 is partially activated at the resting membrane potential, generating a small and persistent current; PGE<sub>2</sub> reduces the time-constant of activation and shifts the current-voltage relationship of activation and inactivation in a negative direction [227, 228]. Na<sub>v</sub>1.9 knockout animals had normal mechanical and thermal thresholds, but had reduced inflammatory hyperalgesia and sensitisation by inflammatory mediators [229]. The sensitisation of Na<sub>v</sub>1.9 is mediated by PKC [230].

### Potassium Channels

Potassium channels contribute to the background conductance in all neuronal cells. They are the largest family of ion-selective channels and can be categorized into four classes: voltage-gated, inwardly rectifying, tandem pore

domain and calcium-activated potassium channels [231]. A reduction in potassium channel conductance increases neuronal excitability and the probability of repetitive discharge [232]. The conductance of potassium channels can be reduced by pathways stimulating cAMP formation and PKA-mediated phosphorylation in rat sensory neurones [233]. Potassium channels properties have been shown to be modulated by PKA, PKC, Src, PIP<sub>2</sub> (see Table 1) and the protein tyrosine kinase PYK2 [234]. NGF and PKA can also regulate the expression and intracellular distribution of potassium channels [235, 236]. Inward rectifier potassium channels can be modified by the second messenger-sensitive minK potassium channel protein [237].

### Calcium Channels

Modulation of calcium influx into the cytoplasm by voltage gated calcium channels or calcium permeable channels affects neuronal excitability and promotes exocytosis of neuropeptides such as substance P and CGRP from nociceptive neurones. Norepinephrine, gamma-aminobutyric acid (GABA) and 5-HT have been demonstrated to inhibit calcium influx and to shift the voltage-dependence of calcium channels [238, 239]. The ten known calcium channel subtypes are not identically regulated. Ca<sub>v</sub>2.2 is most important for neurotransmission in unmyelinated neurones and is a target for antinociceptive drugs [240]. PKC activation rapidly increases calcium currents in sensory neurones [241], an effect which may have a complex origin as subtypes 2.2 and 2.3 are enhanced by PKC activation while 1.1-1.4 are inhibited [242-244]. Calcium channels can be rapidly inhibited by G<sub>i</sub>-protein coupled receptors activated by opioids [245], which is mediated via the G-protein β/γ subunits [246]. An second mechanism which may play an important role in nociception is the activation of Ca<sub>v</sub>3.1 (T-type) calcium channels by reducing agents and endogenous L-cysteine [247].

### HCN Channels

Hyperpolarisation-activated cyclic nucleotide gated channels generate I<sub>h</sub> pacemaker currents in the heart [248]. The hyperpolarisation-evoked current can be enhanced by cAMP and, to a lesser extent, by cGMP by direct binding to a C-terminal site, which causes a shift in channel activation to more positive membrane potentials [249]. Voltage-clamp studies in DRG neurones showed that large neurones expressed a fast, cAMP-insensitive I<sub>h</sub> in large neurones, and that this current was abolished by genetic deletion of HCN1, while in small neurones a slower, cAMP-sensitive current, consistent with expression of HCN2, was seen [250]. In small neurones the enhanced generation of action potentials caused by exposure to PGE<sub>2</sub> was found to be entirely attributable to the cAMP-sensitivity of I<sub>h</sub>, and other factors such as modulation of Na currents (see above) played at most a marginal role [250]. For HCN2, the voltage-dependent activation is shifted by intracellular pH which might increase the role in tissue acidosis [251]. In addition HCN channels can be sensitised by Src and PIP<sub>2</sub> (see table 1) though the importance of this for nociceptors function is currently unknown.

### CONCLUSION

Sensitisation is a property unique to nociceptors – all other sensory receptors adapt to an intense or prolonged

stimulus. The function of sensitisation is to enhance the urgency of a painful stimulus so that action is taken to preserve the integrity of the organism. As such it has been subjected to many millennia of evolution and has become a complex and multifaceted process. In this review we have outlined our current understanding of this vital process at the level of the peripheral sensory neurones. Sensitisation at the level of central pathways also has an important role to play but is beyond the scope of this review. We have identified three main stages of sensitisation: the production of inflammatory mediators which initiate sensitisation; the activation of intracellular signalling pathways; and the ion channel targets which ultimately modulate the electrical response of the nociceptor. Our knowledge in these areas will guide the development of future analgesics to control the pain associated with tissue damage and inflammation.

### ABBREVIATIONS

5-HT	= 5-hydroxytryptamine, serotonin
AKAP	= A-kinase anchoring protein
ASIC	= Acid-sensing ion channels
cAMP	= Cyclic adenosine monophosphate
cGMP	= Cyclic guanosine monophosphate
CGRP	= Calcitonin gene-related peptide
DRG	= Dorsal root ganglion
EET	= Epoxyeicosatrienoic acid
ERK	= Extracellular signal-regulated kinase
HCN	= Hyperpolarisation-activated cyclic nucleotide-gated
HPET	= Hydroperoxyeicosatetraenoic acid
IB4	= Isolectin B4
MAPK	= Mitogen-activated protein kinase
NGF	= Nerve growth factor
NO	= Nitric oxide
PG	= Prostaglandin
PAR	= Protease-activated receptor
PI3K	= Phosphoinositide 3-kinase
PIP <sub>2</sub>	= Phosphatidyl-inositol 4,5-bisphosphate
PKA	= Protein kinase A
PKC	= Protein kinase C
PLC	= Phospholipase C
TRP	= Transient receptor potential
TNFα	= Tumor necrosis factor alpha
TTX	= Tetrodotoxin

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