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Evolution of acid nociception: ion channels and receptors for detecting acid

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Summary

Nociceptors, i.e. sensory neurones tuned to detect noxious stimuli, are found in numerous phyla of the Animalia kingdom and are often polymodal, responding to a variety of stimuli, e.g. heat, cold, pressure and chemicals, such as acid. Due to the ability of protons to have a profound effect on ionic homeostasis and damage macromolecular structures, it is no wonder that the ability to detect acid is conserved across many species. To detect changes in pH, nociceptors are equipped with an assortment of different acid sensors, some of which can detect mild changes in pH, such as the acid-sensing ion channels, proton-sensing G protein-coupled receptors and several two-pore potassium channels, whereas others, such as the transient receptor potential vanilloid 1 ion channel, require larger shifts in pH. This review will discuss the evolution of acid sensation and the different mechanisms by which nociceptors can detect acid.

Nociception Evolution And The Drive For Nociceptor Acid-Sensitivity

In the 160th anniversary of their publication, Charles Darwin's words still ring true, "any variation…if it be in any degree profitable to an individual of any species…will tend to the preservation of that individual, and will generally be inherited by its offspring" (1). It could be argued that one of the most profitable facets of any organism is the ability to detect and react to potentially damaging stimuli in its environment, hence nociception (derived from the Latin *nocere* meaning to hurt/harm), the neural process of encoding noxious stimuli, is common to many species in the Animalia kingdom (2–6). However, not all Animalia have a complex nervous system, for example, Porifera (sponges) contract in response to changes in extrinsic conditions (e.g. turbulent water) and glass sponges transmit electrical signals through their syncytial tissues (7). Although a number of genes associated with neuronal function have been identified in *Amphimedon queenslandica* (8), as is the case with Placozoa (9), the presence of neuronal genes and electrical conductivity does not constitute a nervous system and experimental work to determine if neuronal gene expression is linked to sensory function in Porifera and/or Placozoa remains to be determined (10). By contrast, Cnidaria (e.g. jellyfish and sea anemones), possess diffuse nerve nets (11) and mechanical stimulation of *Calliactus parasitica* produces nervous impulses, strong stimulation (i.e. potentially nociceptive) evoking a closure reflex (12). Similarly, Ctenophores (comb jellies) also possess sensory receptors and nerve cells (13), but

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there has, to our knowledge, been no investigation of the potential nociceptive function of their nervous system. It is therefore in Bilateria (e.g. Animalia other than Porifera, Placazoa, Ctenophores and Cnidaria) where an integrated nervous system has fully evolved (14) and nociception has been most frequently studied. In humans, the importance of a nociceptive system is illustrated by individuals with congenital insensitivity to pain, who often accumulate injuries and whose heightened risk-taking behaviour is thought to contribute to higher early-life mortality (15). There are also genetic variations that result in excessive nociception and studying these variations at a functional level has contributed to understanding of how the nociceptive system works, as well as highlighting points for therapeutic intervention (16). It should be noted that nociception and pain are not the same, even though the terms are often used interchangeably. As above, nociception is the neural process of encoding noxious stimuli, which involves specialised sensory neurones called nociceptors. By contrast, pain is usually defined as an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage. Using the term pain (rather than nociception) for non-mammalian species has produced rigorous discussion in the field due to the debate over which organisms have the capacity for emotional processing, however, this is beyond the scope of this article and has been reviewed elsewhere (17–19).

In many species, nociceptors are polymodal, i.e. they respond to multiple stimuli (e.g. heat, pressure and chemicals such as acid), owing to the expression of different receptors. Polymodality has been determined using a range of electrophysiological and imaging approaches, but recent single-cell RNA-sequencing studies show that sensory neurones usually express a multitude of different receptors that confer polymodality and enable transcriptomic segregation of sensory neurones into subtypes, whose function can be interrogated *in vitro* and *in vivo* (20–24).

Here, we will focus on proton-induced nociceptor activation, others having previously reviewed sensory neurone mechanosensitivity (25–27) and thermosensitivity (28,29). Protons influence ion homeostasis and modulate enzyme activity, and thus organisms have evolved the ability to regulate extracellular and intracellular pH through membrane transporters and a range of proton buffering systems (30–32). Expression of a range of proton-sensitive receptors, summarised in Fig. 1, permits detection of protons by nociceptors, proton-induced activation/inhibition of these receptors can in turn modulate nociceptor excitability. The ability of protons to activate nociceptors and/or evoke nocifensive behaviour has been demonstrated in a wide range of species, including: the nematode worm *Caenorhabditis elegans* (33), the medicinal leech *Hirudo medicinalis* (34), the Northern grass frog *Rana pipiens* (35–37), the rainbow trout *Oncorhynchus mykiss* (38,39), the chicken *Gallus gallus* (40,41), the mouse *Mus musculus* (42,43), the rat *Rattus norvegicus* (44) and the human *Homo sapiens* (45,46); however, acid nociception is not universal, the naked mole-rat (*Heterocephalus glaber)*, the Cape mole-rat (*Georychus capensis*) and the East African root rat (*Tachyoryctes splendens*) displaying no acid-induced nocifensive behaviour (42,47). The presence of acid nociception in such a wide variety of species, both aquatic and terrestrial, demonstrates the likely evolutionary pressure to maintain selection for being able to detect and respond to changes in the pH of an organism's environment, whereas presumably any cost to those organisms that do not display acid nociception is outweighed by some other benefit. A phylogenetic summary depicting the evolution of nociceptors, acid nociception and different acid-sensors is illustrated in Fig. 2.

In humans, perhaps the first demonstration that acid evokes pain was from von Gaza and colleagues who reported that pain and a change in the proton concentration were common to inflammation, and that tissue alkalinisation could reverse pain associated with abscesses (48). Indeed, work in humans (49–52) and rodents (53) supports the fact that tissue acidosis occurs during inflammation, but equally inflammation can occur in the absence of acidosis (54,55). In terms of the mechanisms by which acid causes pain, Krishtal and colleagues were the first to demonstrate that protons could excite sensory neurones by evoking transient inward currents (56). Subsequent analysis of various mammalian

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nociceptors demonstrated that protons produce three main types of excitation: transient inward currents (current inactivation in the presence of protons), sustained inward currents (continuous inward current in the presence of protons) and mixed (both transient and sustained phases) (46,57–61). Underpinning these different responses are a variety of different mechanisms and this review will discuss the different mechanisms and what is understood about their roles in different species.

Acid-Sensing Ion Channels

The acid-sensing ion channels (ASICs) are part of the epithelial sodium channel (ENaC)/degenerin (DEG) ion channel superfamily, which in mammals consists of nine genes, four encode ENaC subunits, four encode ASICs and one encodes the bile acid-sensitive ion channel (BASIC, sometimes termed ASIC5, ~30% homologous to other ASICs, but proton-insensitive(62)). The four ASIC genes encode six ASIC subunits, splice variants of the ASIC1 and ASIC2 genes resulting in: ASIC1a (63), ASIC1b (64,65), ASIC2a (66), ASIC2b (67), ASIC3 (68) and ASIC4 (69,70). Although there was initial debate surrounding the subunit stoichiometry of functional ASICs, both X-ray crystallography (71) and atomic force microscopy (72) have demonstrated that ASICs are trimeric ion channels. However, not all ASIC subunit configurations produce proton-sensitive ion channels, ASIC2b and ASIC4 homomers are proton-insensitive, but can form proton-sensitive heteromers and/or regulate ASIC subunit surface expression (67,73); the naked mole-rat ASIC3 is also proton-insensitive, a potential adaptation to a subterranean lifestyle (74). When proton-sensitive ASICs are activated, an inward cation flux (largely Na^+, although ASIC1a shows Ca^{2+} permeability) leads to neuronal depolarisation and in nociceptors, if of sufficient magnitude to produce action potential firing, would lead to nociception. With regard to their expression profile, in mammals, all ASIC subunits are expressed in sensory neurone cell bodies in the dorsal root ganglia (DRG), albeit that ASIC4 is expressed at comparatively much lower levels (22,23,75); interestingly the ASIC3 transcript is down regulated in sensory neurones of the proton-insensitive rodents, naked mole-rats, Cape mole-rats and the East African root rat (47).

The chicken ASIC1a crystal structure identified a region termed the acidic pocket containing three carboxylate pairs (D238–D350, E 239–D346 and E220–D408; chicken ASIC1a numbering), which were suggested to be the primary sites for proton sensing (71). Mutational analysis shows that these residues, whilst regulating pH sensitivity, do not fully abolish ASIC1a proton sensitivity (76,77). Moreover, the proton-sensitive ASIC2a lacks D350, which might explain why it is the least proton sensitive of the functional mammalian ASIC homomers (78), but ASIC2b, which is proton-insensitive, also only lacks D350 (79). Together, these results suggest that sites outside of the acidic pocket are important for ASIC proton sensitivity and several studies have identified further amino acids that are required for normal proton sensing by ASIC1a (76,77,80,81). Furthermore, comparative analysis of rat ASIC2a/ASIC2b (82) and zebrafish (*Danio rerio*) zASIC4.1 (proton-sensitive) and zASIC4.2 (proton-insensitive) (83) have demonstrated the critical importance of the extracellular domain proximal to the first transmembrane domain for conferring ASIC proton sensitivity and in particular the importance of the histidine residue H73 (mouse ASIC1a numbering) (Fig. 1) (79,84). In first attempting to determine when proton sensitivity arose in ASICs, it was demonstrated that the spiny dogfish (*Squalus acanthias*, a cartilaginous fish) produces proton-sensitive ASICs (85), but that neither the lamprey *Lampetra fluviatilis* (86), nor the tunicate *Ciona intestinalis* (87) do. However, more recent analysis of ENaC/DEG sequences from several phyla has demonstrated that ASICs from a variety of deuterostome lineages are not only expressed in the nervous system, but are also proton-sensitive (including the tunicate *Oikopleura dioica*) (88). It was also demonstrated that the conserved H73 residue (mouse ASIC1a numbering), proximal to the first transmembrane domain, was critical in determining proton sensitivity in both the lancelet *Branchiostoma belcheri* and mice (i.e. distantly related species), suggesting that the appearance of this histidine coincided with the emergence of ASIC proton sensitivity with further lineage specific changes occurring over time (88). Further evidence for the importance of the extracellular domain proximal to the first transmembrane domain comes from studying ASIC4, whereby West Indian Ocean coelacanth (*Latimeria chalumnae*), African clawed frog

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(*Xenopus laevis*) and chicken (*G. gallus*) ASIC4s all respond to protons, but rat ASIC4 does not: 24 amino acids in the β1 strand running from the first transmembrane domain into the extracellular domain (including H73) were shown to confer proton sensitivity and insertion of a single amino acid in mammalian ASIC4 resulted in proton-insensitivity (88). Overall, the extensive recent analysis by Lynagh et al. (88) clearly demonstrates that ASIC proton sensitivity is conserved across many animal phyla, including invertebrates, although little is known about the contribution to nociception of ASICs in these species.

When considering the ENaC/DEG family more broadly, members, such as HaFaNaC from the mollusc *Cornu aspersum* (previously *Helix aspersa*) (89) and HyNaC from the cnidarian *Hydra magnipapillata* (90) are activated by peptides, rather than protons, suggesting a potential role for evolutionary ASIC precursors as peptide sensors; indeed, mammalian ASICs are modulated, but not activated, by a variety of peptides (91–95). Interestingly, the non-proton agonist of ASIC3 2-guanidine-4-methylquinazoline (GMQ) activates HaFaNaC and related mollusc ENaC/DEG channels by a mechanism distinct to their activation by the endogenous agonist FMRFamide (96), which highlights that dual activation/modulation of ASICs and related channels is a conserved feature.

In addition to peptides, ASIC function can be modulated by numerous endogenous mediators and other compounds (see (97,98) for a review), some of which, like arachidonic acid (44,57,99), nitric oxide (100) and protein kinase C (PKC) (101), are, like protons, upregulated in inflammation and thus likely work synergistically to activate ASICs and produce pain. In terms of how protons modulate mammalian nociceptor function, they both activate and sensitise rodent nociceptors (58,102), ASIC3 being particularly important. For example, protons activate mouse C-fibre nociceptors in an ASIC3-dependent manner, mice lacking ASIC3 showing less nociceptor firing at pH 5.0 than wildtype mice (although no difference was observed at pH 4.0 and ASIC3$^{-/-}$ mice showed no difference in acid-evoked licking behaviour) (43). Similarly in rats, blockade of ASIC3 with APETx2 inhibits acid-evoked nociceptor firing and reduces acid-evoked pain behaviour (44,103), but a caveat of interpreting this is that APETx2 also inhibits the voltage-gated sodium channel subunit 1.8, $Na_V1.8$ (104). Considering the role of tissue acidosis in some forms of inflammation, there has been considerable investigation of how ASICs contribute to hyperalgesia in numerous animal models and overall evidence supports the targeting of ASICs to relieve pain (105,106). Although evidence supports targeting of ASIC1a/1b subunits (107,108), there has been more extensive investigation of ASIC3, most likely due to its activation producing a pronounced sustained phase following the initial transient phase, i.e. ASIC3 can likely transduce sustained tissue acidosis into nociceptor activation and pain behaviour (68). For example, arachidonic acid potentiates the sustained phase of ASIC3 (57), arachidonic acid potentiates acid-evoked pain in rats that is reversed by ASIC3 inhibition (44), chronic hyperalgesia induced by repeated intramuscular acid injections is abolished in mice lacking ASIC3 (109) and a peptide from *Conus textile* venom potentiates ASIC3 activity concomitant with enhancing acid-evoked hyperalgesia (94).

In humans, there is some evidence to support acid-evoked pain being ASIC dependent, (45,110), but not all studies support these findings (111). Experimentally, acute application of acid is associated with certain limitations, for example, not being sure of what pH nerve terminals actually encounter and how acute acid application corresponds to the acid stimulation that nociceptors encounter under pathological conditions. Moreover, pharmacological targeting of ASICs for the treatment of pain is complicated by the fact that ASICs are expressed throughout the mammalian nervous system (75) as homo- and heterotrimers and they are implicated in many physiological processes, e.g. mechanosensation (112), proprioception (113) and synaptic plasticity (114).

In summary, ASICs are proton-sensitive in a wide range of phyla with more distantly related ion channels being activated by peptides, whereas ASICs undergo peptide modulation. Alongside roles

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in normal neurophysiology, ASICs play a key role in inflammatory pain in mammals, which warrants further investigation as potential sites for therapeutic intervention.

Transient Receptor Potential Ion Channels

The *trp* (transient receptor potential) gene was first identified from a *Drosophila melanogaster* mutant which exhibited insensitivity to light despite retaining normal eye structure (115), the protein product of this gene was later shown to be a cationic ion channel (116). Several homologous genes have since been identified in *D. melanogaster*, leading to the emergence of the TRPs as an independent family of ion channels comprising several subgroups (117). TRP channels appear to have emerged before the divergence of fungi and animals, with TRPP and TRPV homologs being identified in the protist *Thecamonas trachens* and ancestral genes representing five of the mammalian TRP subfamilies arising by the speciation of choanoflagellates (118). Given TRPs can be traced back to unicellular eukaryotes, and their common expression at the plasma membrane, they likely evolved as sensors of the extracellular environment. Over evolutionary time, the number and diversity of TRP channels has increased: a total of thirteen TRP genes have been discovered for *D. melanogaster*, *C. elegans'* genome contains seventeen, there are twenty-eight mouse TRPs and twenty-seven human TRPs have been identified. TRPs are generally considered to function as homotetramers, each subunit comprises intracellular N- and C- termini and 6 transmembrane domains, a re-entrant loop between the fifth and sixth transmembrane domains forms the channel pore (119); it should be noted that there is however recent evidence of heteromeric TRP channel configurations (120). Variations in motifs and modalities present within the receptors permit them to respond to a diverse range of stimuli including both chemicals and physical properties such as pressure, light and temperature. Additionally, TRPs are heavily influenced by levels of plasma membrane phospholipids and exhibit extensive phosphoregulation, enabling integration of external and internal signals. Given the sensitivity of TRPs to numerous stimuli it is perhaps unsurprising that many are expressed throughout the nervous system, particular by sensory neurones where they confer a high degree of polymodality (121).

The ability of TRPs to respond to protons was first demonstrated for TRPV1: acidic pH potentiating capsaicin-induced inward currents (122) and protons later being shown to directly activate TRPV1 (123). Since this finding other TRPs have been shown to be activated or positively modulated by extracellular pH, including TRPV4 (124), TRPM7 (125), TRPP2, TRPP3 (126), TRPC4, TRPC5 (127) and TRPA1 (128). There is evidence that all of these TRPs are expressed in nociceptors, albeit at differing expression levels (22,23), and whereas some TRPs are inhibited by extracellular acidosis (127) or activated by intracellular acidosis (129), in most instances proton-induced TRP activation on nociceptors results in cation influx, depolarisation and nociception activation. While the ability of most of these TRPs to respond to protons was discovered with rodent variants of the receptors, interestingly, only the human variant of TRPA1 (hTRPA1) displays proton sensitivity. hTRPA1 was found to be active within an extracellular pH range of 7.0 – 5.4, while the closely related Rhesus monkey (*Macaca mulatta*) TRPA1, which shares 98% sequence homology with human *TRPA1*, was shown to be proton-insensitive. Comparisons of the primary sequences of the two channels identified four non-conserved amino acids distributed around the start of the sixth transmembrane domain, which when mutated reduced hTRPA1 proton sensitivity (128). Molecular studies of TRPV1 have also pinpointed residues around transmembrane domain six as conferring proton sensitivity independently of capsaicin- and heat-sensitivity (130). Similarly, the mutation of glutamate residues present in the re-entrant loop of TRPC5 abolishes acid-induced activation (127). Although the specific residues differ, the importance of the re-entrant loop and transmembrane domain six in conferring proton sensitivity of TRPs is evident (Fig. 1). Taken together, the fact that the residues important for proton sensitivity are not conserved and the finding that only the hTRPA1 is proton-sensitive, it is likely that the ability of TRPs to respond to protons evolved separately within each subfamily, highlighting the evolutionary importance of acid-sensation.

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Avoidance of acidic environments by *C. elegans* suggests an ability of the nematode to detect extracellular pH and avoid acidic areas. This has been shown to be mediated by *osm-9*, a proposed homolog of mammalian TRPV channels, as reduced acid-avoidance behaviour was observed in *osm-9* mutants and following treatment of wildtype nematodes with the broad-spectrum TRP inhibitor ruthenium red (33). Evidence explicitly linking proton-induced TRP signalling as contributing to the manifestation of pain is relatively scarce for higher-order organisms, perhaps due to the promiscuous nature of TRP activation, and there is conflicting evidence for an involvement of TRPV1 in acute acid-induced nociception in humans (110,111); however, a large body of evidence implicates TRPs in thermal hyperalgesia and the role of TRPs in pain has been comprehensively reviewed (131). Indeed, studies of knockout mice suggest that both TRPV1 and ASIC3 are relatively redundant in the development of acute pain, but significantly contribute to hypersensitivity (132). Given the prominence of acidosis in many conditions associated with pain, particularly at chronic stages following influx of immune cells, the establishment of a hypoxic environment (133,134), and the high expression of proton-sensitive TRPs by nociceptors (135) it is widely accepted that acidosis likely potentiates TRPs resulting in hyperalgesia, something well supported by *in vitro* evidence with substantiation needed *in vivo* (123,127,128). In addition to TRP potentiation priming nociceptors, leading to more frequent action potential discharge, activation of TRPs in sensory neurones has been shown to coordinate release of the neuropeptides substance P and calcitonin gene-related peptide, which can in turn prime other neurones leading to hyperalgesia as well as contribute to central sensitisation (136–138). To summarise, TRP channels are clearly implicated in nociception, but the promiscuous nature of these receptors makes it difficult to specifically attribute proton-activation as causing TRP-mediated nociception. However, given the correlation between localised acidosis and inflammation it is likely proton-induced TRP-signalling is important in the manifestation of inflammatory pain.

Two-Pore Potassium Channels

The two-pore (K2P) domain ion channel family comprises membrane proteins, encoded by the *kcnk* genes, that share a common molecular architecture, consisting of four transmembrane domains (TM1-4), two pore-forming domains (P1 and P2) and a extracellular cap between the TM1 and the P1 domains, assembling as either homo- or heterodimers (Fig. 1) (139–141). K2P channels underlie the background K^+ current observed in excitable and non-excitable cells, playing a key role in setting the resting membrane potential and input resistance in neurones, therefore regulating cellular excitability (142,143). Additionally, K2P channel activity is influenced by many physicochemical factors including extra- and intracellular pH, temperature, membrane stretch, as well as being modulated by membrane lipids and volatile anaesthetics, i.e. like ASICs and TRP channels, K2P channels integrate a number of external and internal signals. In mammals, 15 different K2P subunits have been identified and grouped into 6 different subclasses (TWIK, TREK, TASK, TALK, THIK and TRESK) based on their sequence similarity and functional properties (142), however, transcript processing and post-translational modifications further increase their diversity (144,145). Nevertheless, K2P channels are not restricted to mammals, being highly conserved during evolution. The first ion channel presenting two pore-forming domains per subunit was identified in the yeast *Saccharomyces cerevisiae* and named TOK1 (YORK), however, this channel differs from the mammalian K2P channels by having eight TMs (146), rather than the four observed in mammalian K2Ps. Furthermore, K2P channels with a 2P/4TM architecture have been identified in a range of different animal species, including: the marine sponge *A. queenslandica* (147), the marine opisthobranch *Aplysia californica* (148), *D. rerio* (149), *D. melanogaster* (150), *C. elegans* (151), *M. musculus* (152), *R. norvegicus* (153) and *H. sapiens* (139), i.e. K2P channels are an ancient ion channel family.

Most mammalian K2P channels are modulated by extra- and intracellular acidification. In particular, the inhibition of proton-sensitive K2Ps by extracellular acidic pH reduces constitutive K^+ efflux produced by these channels, thus contributing to membrane depolarisation and acid-induced

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nociception. Within the K2P channel family, TASK1 and TASK3 channels from human, mouse, rat and guinea pig (TASK3) (153–156) are markedly sensitive to extracellular acidification. The protonation of H98 (Fig. 1), immediately following the K^+ selectivity filter sequence (GYG) in the P1 loop, primarily confers proton sensitivity to these channels (155–157), although mutation of this residue does not completely abolish proton sensitivity and the participation of other residues in the extracellular domain (H72 and K210: mTASK1 numbering) has also been demonstrated (158). Moreover, TASK1 and TASK3 can form heteromeric channels, presenting intermediate properties in terms of proton sensitivity (159). In *D. melanogaster*, 10 putative K2P channels have been identified by homology screening, but only two of them share a significant sequence identity with mammalian K2P. dTASK6 (52-57% identity with hTASKs (160)), appears to be proton-sensitive, however, mutation of conserved histidines (H98 and H72 in hTASKs) does not produce proton-insensitive channels, and it has been suggested that other residues in the M1-P1 loop are involved in this process (161). Moreover, dTASK7 (49-55% identity with hTASKs (160)) does not form homomeric functional channels due to two non-conserved residues (A92 and M93) in the P1 domain and although mutation of these residues to conserved threonines produces functional channels, they are still proton-insensitive, even though dTASK7 presents the conserved histidines (H98 and H72) in its sequence (161), reaffirming the involvement of other residues/regions in the proton sensitivity of these channels. In *C. elegans*, forty-seven genes encode K2P channels (160,162,163). Among them, SUP-9 and TWK-20 exhibit significant sequence identity with hTASKs (43-57%) (160), however, their proton sensitivity has not yet been tested. Members of the TALK family are mainly activated in the alkaline pH range; however, they are markedly inhibited by protons, being less active (TASK2), largely inhibited (TALK1) or completely inhibited (TALK2) at pH 7.4 (164,165). Similarly, zebrafish TASK2 (zTASK2) displays a comparable proton sensitivity profile as its mouse homologue (166). Human TWIK1 channels are also inhibited by extracellular acidosis due to the protonation of a homologous histidine (H122) in the P1 domain (167), nonetheless, TWIK1 produce only a very small current in heterologous systems and native conditions due to post-translational modifications (sumoylation) (167,168). In addition, it has been shown that during extracellular acidification, TWIK1, TASK1 and TASK3 become permeable to Na^+ (169,170), a trait also observed in TWIK1 in hypokalaemic conditions (171). TRESK, another member of the K2P family regulated by intracellular Ca^{2+} (172), is also sensitive to extracellular acidosis, however, whereas mouse/rat TRESK are inhibited by extracellular low pH due to a homologous histidine (H132 in mTRESK), the presence of tyrosine in the same position makes hTRESK proton-insensitive (173,174); an arginine residue in the zebrafish TRESK homologue might result in proton-insensitivity, but this has not been tested (175). With regard to TREKs, mutation of a different histidine in the M1P1 extracellular loop of murine TREK1 (H126), TREK2 (H151) and TRAAK (H85) channels demonstrated its involvement in their pH sensitivity. However, whereas TREK1 and TRAAK are inhibited by extracellular acidification, TREK2 is activated, this differential pH modulation involved other charged residues in the P2M4 domain (176). Moreover, both TREK1 and TREK2 are activated by intracellular acidification, with residues in the C-terminus responsible for this activation (177,178). Interestingly, another study showed that the proton sensitivity of hTREK1 follows a different mechanism (C-type inactivation), involving two different surfaced-exposed histidines (H87 and H141; not conserved in mTREK1 and TREK2) in the turret loop (179). Altogether, K2P channel proton sensitivity is common to most subfamilies, involving protonatable histidine residues in the extracellular domain (Fig.1), a trait conserved across mammalian species and in *D. melanogaster*, however, the lack of functional data with regard to the proton sensitivity of K2P channels in other species, including model organisms such as *C. elegans*, prevents a more extensive evolutionary analysis of K2P channel proton sensitivity.

When considering physiological roles of K2P channels, their key role in setting neuronal excitability has resulted in attempts to elucidate their participation in nociception (143,180), largely through manipulation of gene expression due to the lack of specific K2P channel agonists and antagonists. For instance, $TASK1^{-/-}$ and $TASK3^{-/-}$ mice have altered thermal perception, whereas $TASK1^{-/-}$ display

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an increased sensitivity to hot temperatures (181), TASK3$^{-/-}$ mice are hypersensitive to cold temperatures (182), suggesting roles for both channels in thermosensation. Moreover, TASK3 is enriched in TRPM8-positive cold-sensitive neurones, which display a decreased thermal threshold after TASK3 silencing and in neurones from TASK3$^{-/-}$ mice (182), and expression of TASK3 in $Na_V1.8$-negative neurones has been shown to be involved in innocuous and acute noxious cooling (183). During inflammation, the mRNA levels of TASK1 and TASK3 are reduced and this reduction has been correlated to spontaneous pain behaviours (184). Furthermore, after spared sciatic nerve injury, TASK3 and TWIK1 are downregulated in lumbar 4 and 5 DRG, whilst TASK1 expression remains constant, however, TASK3 expressions return to baseline levels in weeks, whereas downregulation of TWIK1 persisted for months (185). In a similar fashion, TRESK, the most highly expressed K2P channel in DRG neurones, which together with TREK2 mediates most of the background K^+ current in small- and medium-sized DRG neurones, i.e. likely nociceptors (173), is downregulated in inflammation partially underlying spontaneous pain behaviour observed in rats (184). In addition, it has been shown rat TRESK (rTRESK) is inhibited by arachidonic acid and hypertonic medium (174,186), as well as protons, all mediators found in the inflammatory soup, and these effects appear to be additive (174), highlighting the role of TRESK on neuronal excitability during inflammation. Corroborating the idea of TRESK being important in sensory neuronal excitability, overexpression of TRESK in trigeminal neurones reduces neuronal excitability (187) and TRESK overexpression in DRG and spinal cord after nerve injury alleviates neuropathic pain in rats (188). Moreover, analysis of migraine genetics supports a role for TRESK. The dominant negative mutation F139WfsX24 (TRESK-MT) downregulates TRESK wildtype channels, inducing hypersensitivity of trigeminal neurones (189) and occurs in patients experiencing familial migraine with aura (190). Intriguingly, a further TRESK mutation (C110R), found in control and migraine patients (191) that produces a complete loss of TRESK function, does not however induce trigeminal neurone hyperexcitability (192). A recent study has described a novel transcriptional mechanism, frameshift mutation-induced alternative translation initiation (fsATI), that resolves the role of TRESK. During TRESK-MT transcription, fsATI leads to the production of a second protein fragment (MT2) that inhibits TREK1/TREK2 activity increasing sensory neuronal excitability in trigeminal neurones, i.e. both non-functional TRESK-MT and inhibition of TREK1/TREK2 are required to induce migraine-like pain states in mice (193). Lastly, members of the TREK channel family are modulated by a wide range of physicochemical factors, including temperature and mechanical stretch, and they have been implicated in polymodal pain perception. TREK1$^{-/-}$ mice are more sensitive to painful heat near the threshold between warmth and noxious heat, exhibit greater mechanical sensitivity and enhanced inflammatory hypersensitivity, suggesting a role for TREK1 in peripheral nociceptor sensitisation in inflammation (194). Moreover, extracellular acidosis and lysophosphatidic acid, two inflammatory mediators, inhibit TREK1 activity (195). TREK2$^{-/-}$ mice show enhanced sensitivity to warmth and cool temperatures and reduced mechanical threshold in normal conditions and additionally display an absence of nocifensive behaviours in response to hypertonic saline injections after PGE_2 sensitization (196).

In summary, the role of K2P in different aspects of nociception and pain pathophysiological states has been clearly confirmed by the use of animal models, however, their specific role in acid nociception has yet to be tested.

Proton-Sensing G Protein-Coupled Receptors

Rather than binding complex extracellular ligands, proton-sensing G protein-coupled receptors (PS-GPCRs) engage heterotrimeric G-proteins in response to mild increments in the extracellular proton concentration. To date, four mammalian PS-GPCRs have been identified: the proton sensitivity of GPR68 was described first, quickly followed by GPR4, GPR65 and GPR132, which were studied due to high sequence similarity (197–199). Homologous genes for PS-GPCRs have been identified across vertebrate subphyla, all of which exhibit strong conservation of histidine residues in extracellular portions of the receptor, amino acids that confer proton sensitivity (Fig. 1) (197–200). PS-GPCRs

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exhibit widespread tissue distribution (201) and are expressed in mammalian sensory neurones (22,23), thus they likely evolved as sensors of the extracellular environment, being engaged in response to local pH perturbations and functioning to maintain homeostasis. This has been reaffirmed by studies of knockout mice, which are in the most part phenotypically normal (202), however issues regarding autoimmunity have been reported, implicating these receptors in immune cell function (203,204). Many PS-GPCRs have also been shown to respond to various lipids, such as GPR65 which responds to psychosine (205), whether the receptors first arose as proton sensors or receptors for lipids is also unknown, but may be addressed by studying evolutionary older variants. PS-GPCRs are expressed throughout the central and peripheral nervous systems. Importantly, a high degree of co-expression with peripherin and TRPV1, both markers of small diameter nociceptors has been observed (201), suggesting a role of these receptors in acid nociception. Further to this, expression of GPR4, GPR65 and GPR132 has been shown to be upregulated at the transcriptional level in various rodent models of inflammation (206). Indeed, a body of evidence exploring the roles of PS-GPCRs in inflammation has started to amass in recent few years.

Taking each PS-GPCR in turn, GPR4 preferentially couples to $G\alpha_s$ proteins following acidic challenge, resulting in the accumulation of cAMP, half maximal activation of this pathway occurs in response to pH 7.55 in HEK293 cells transiently expressing GPR4 (197). Although unlikely to result in acute nociceptor activation, downstream signalling from $G\alpha_s$ plays a key role in nociceptor sensitisation (207,208), i.e. proton-induced activation of GPR4 can sensitise nociceptor function, which may be important to sensitisation process during chronic pathological conditions associated with tissue acidosis. Elevated GPR4 mRNA has been detected in a murine model of inflammatory pain (206), as well as in the colon and intestinal tissues of human ulcerative colitis and Crohn's disease patients. Moreover, $GPR4^{-/-}$ mice responded less severely to the colitis model in terms of weight loss, histological damage and leukocyte infiltration (209,210). Accordingly, acid-induced activation of GPR4 coordinates increased expression of pro-inflammatory genes and enhances immune cell recruitment following acidosis (211). Miltz and colleagues have recently developed a novel antagonist of GPR4, which as well as preventing proton-induced cAMP accumulation in cell lines, was found to be orally active and could reduce swelling and prevent joint damage in an arthritis model as well as reduce mechanical hyperalgesia following complete Freund's adjuvant (CFA)-induced inflammation (212).

GPR65, also referred to as the T cell death-associated gene 8 (TDAG8) receptor, is activated by protons, as well as the glycosphingolipid psychosine and the synthetic compound BTB09089. While protons and BTB09089 cause GPR65-mediated accumulation of cAMP (198,213) and thus could lead to nociceptor sensitisation as described above, psychosine inhibits adenylate cyclase and mobilises intracellular Ca^{2+} (205) suggesting an inherent G-protein bias; mobilisation of intracellular Ca^{2+} both depolarises neurones and activates PKC and thus proton-mediated GPR65 activation could both activate and sensitise nociceptors. Genome wide association studies have reported correlation between single nucleotide polymorphisms in GPR65 and the inflammatory conditions chronic obstructive pulmonary disease-asthma overlap and ankylosing spondylitis (214,215). $GPR65^{-/-}$ mice are more susceptible to developing colitis, which has been linked to an influence of GPR65 on lysosomal function and pathogen clearance (216), however, no link between the gene and inflammatory bowel disease was observed in a Chinese population (217). Neuronal expression of GPR65 has been shown to increase in murine models of bone cancer pain and following carrageenan- or CFA-induced inflammation, and when siRNA targeting GPR65 is administered to animals before the induction of these models less mechanical hyperalgesia is observed (218,219). Direct activation of GPR65 with BTB09089 also produces mechanical allodynia (219). Studies of cultured DRG and HEK293T cells have also shown that GPR65 is able to enhance capsaicin-induced Ca^{2+} fluxes through TRPV1, indicative of a pro-inflammatory role of the receptor (206,220). A study of TRPV1, ASIC3 and GPR65 knockout animals has shown that while all three receptors are important for the manifestation of chronic inflammation following injection of CFA, only loss of GPR65 prevented the acute phase,

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suggesting that different acid sensors play different roles in inflammatory hyperalgesia (132). Despite the associations between GPR65 and inflammatory conditions, studies of GPR65 and immune cells have reported decreases in the production of pro-inflammatory cytokines and upregulation of protective factors, following proton-induced activation (213,221,222). Similarly, the expression of pro-inflammatory cytokines was elevated in GPR65$^{-/-}$ mice compared to wildtypes in a colitis model, the same was observed for a T-cell transfer colitis model when the T-cells were harvested from GPR65$^{-/-}$ mice, however, the differential expression of inflammatory mediators between GPR65$^{-/-}$ and wildtype animals did not ameliorate the disease pathology (223). Taken together there appears to be a paradox surrounding the role of GPR65 in inflammation with the role of GPR65 as pro- or anti-inflammatory being highly dependent on the cellular context. GPR65 thus represents an interesting receptor for studying the neuroimmune axis of inflammation.

GPR68, or the ovarian cancer G protein-coupled receptor 1 (OGR1), so called as it was first cloned from an ovarian cancer cell line (224), stimulates the accumulation of inositol phosphates and mobilisation of intracellular Ca^{2+} in response to extracellular acidosis within the range of pH 7.6 – 6.8 (225). This suggests a $G\alpha_q$ coupling, which would both directly activate nociceptors (through Ca^{2+} mobilisation and depolarisation) and coordinate sensitisation (through PKC activation). More recently, GPR68 has also been shown to be activated by benzodiazepines and physical stress (226,227). GPR68 has been implicated in the production of pro-inflammatory cytokines by a number of cell types in response to extracellular acidification, including pancreatic β-cells, osteoblasts and aortic smooth muscle cells (228–230). Fittingly, GPR68 has been postulated to be the molecular mediator behind asthma-associated inflammation due to its coordinating role in the production of interleukin-6 in response to bronchial acidosis (231). Additionally, GPR68 has been highlighted as a potential target in the treatment of heartburn associated pain given the high expression of this receptor compared to other PS-GPCRs within oesophageal C-fibres (232). Hypoxia has been reported to contribute to increased expression of GPR68 by intestinal macrophages and colonic tissue (233) and further to this, elevated GPR68 mRNA was observed in intestinal mucosa from ulcerative colitis and Crohn's disease patients (234). Studies of GPR68$^{-/-}$ mice have identified several genes whose expression is dependent on activation of GPR68 by extracellular acidosis. Loss of GPR68 was also shown to be protective in a murine model of spontaneous colitis, with less inflammation and myeloperoxidase activity as well as a lower incidence of colonic prolapse being observed (234).

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The last of the PS-GPCRs is GPR132, also referred to as G2A, shows more restricted expression than other PS-GPCRs and is comparatively less studied. Following acidic challenge cells expressing GPR132 can activate Rho GTPases, however minimal activity in cAMP and inositol phosphate accumulation assays has been reported (199,235) and thus proton-induced GPR132 activation has the potential to both activate and sensitise nociceptors. Enhanced Ca^{2+} signals in response to acid challenge have been described for cells co-expressing GPR132 and GPR68, suggesting the PS-GPCRs may form oligomers to increase signalling diversity (236). In a neuropathic pain model, G2A$^{-/-}$ mice exhibit less mechanical hypersensitivity, however oxaliplatin, the drug used to manifest the model, was shown to increase levels of oxidised lipids which could sensitise TRPV1 via G2A and PKC dependent mechanisms (237). In contrast, overexpression of G2A in mice reduced mechanical hypersensitivity following CFA-induced inflammation, while knockdown prolonged hyperalgesia (238). Given that we have recently reported that the CFA model does not result in acidosis, this suggests protons are not agonising G2A to confer the observed pain relief (55). The role of G2A in acid nociception thus remains elusive.

While less is known about the nociceptive roles of PS-GPCRs compared to the other proton-sensitive receptors, the associations between the genes encoding these receptors and conditions associated with pain, as well as observations that loss of PS-GPCRs leads to reduced pain phenotypes in various

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animal models, shows that PS-GPCRs are rightfully of considerable interest in understanding the molecular mechanisms underpinning nociception.

Other Acid Sensors

In this review, we have focused on the main proton sensors with regard to nociceptor function. However, numerous ion channels are modulated by pH that we have not discussed here, such as inhibition of NMDA receptors, voltage-gated Ca^{2+} channels, the voltage-gated proton channel H_V1 and ionotropic purinergic receptors but these have been reviewed elsewhere (97,239).

Integrating Nociceptor Acid Sensitivity

As mentioned earlier, the naked mole-rat does not respond to acid as a noxious stimulus, despite a comparable expression pattern of ASICs to mice (75), functional ASIC-like and TRPV1-like currents being recorded from isolated naked mole-rat sensory neurones (58,74) and similar proton-sensitivities of cloned mice and naked mole-rat ASICs and TRPV1 (58), with the exception of ASIC3 (74); the proton-sensing properties of K2Ps and PS-GPCRs remain unknown in this species. Recent RNA-sequencing analysis has also demonstrated that proton-sensitive ASIC3 and TWIK1 (as well as sepiapterin reductase) are both commonly downregulated in the naked mole-rat and the other proton-insensitive rodents, the Cape mole-rat and East African root rat) (47). Downstream of proton-detection, the transducer channel has to mediate sufficient depolarisation to reach the activation threshold for Na_V subunits to initiate an action potential. However, protons can negatively regulate Na_V subunits, blocking the channel pore and altering the voltage-sensor movement that changes gating, as has been extensively reviewed (240), and thus Na_V modulation can also play a role in nociceptor acid-sensitivity. In naked mole-rat DRG neurones, macroscopic voltage-gated inward currents are significantly more susceptible to proton inhibition than those in mouse DRG neurones (58), attributed to a difference in the amino acids involved in proton inhibition, EKE replacing the relatively conserved KKV, and thus enhancing proton block. Swapping KKV for EKE in human $Na_V1.7$ enhanced the degree of proton block and thus acid acts like an anaesthetic in naked mole-rat acid-sensing nociceptors to prevent action potential firing (58). This EKE motif is also found in the proton-insensitive Cape mole-rat, whereas EKD (which has a similar -+- charge constellation) is present in other, proton-sensitive mole-rats suggesting that additional factors are likely involved in determining proton-induced nociceptor excitability and further negative charges in $Na_V1.7$ have been identified as common only to the naked and Cape mole-rats (47). The selection of $Na_V1.7$ variants likely results from the evolutionary pressure of living in a hypercapnic, but relatively safe, environment and thus the change in $Na_V1.7$ may represent an adaptation to prevent somatic nociceptor activation by hypercapnia-induced acidosis. Indeed, recent computational analysis has identified evidence of convergent evolution in the $Na_V1.7$ amino acid variation associated with naked mole-rat acid-insensitivity in hibernating (but not closely related, non-hibernating) species (241) such that selection for this motif has occurred at least 6-times independently i.e. the change in the $Na_V1.7$ sequence may represent a form of convergent evolution to enable resistance to acid-induced nociceptor activation in species living in hypercapnic environments. However, the proton-insensitive East Africa root rat has $Na_V1.7$ motifs common to proton-sensitive rodents, suggesting that in addition to selective pressure on $Na_V1.7$ that there are further, divergent mechanisms responsible for proton-insensitivity in this species (47). It should also be noted that acid nociception has usually been measured in response to subcutaneous acid administration, but responses observed are perhaps unrepresentative of a whole organism's proton sensitivity. For example, in the naked mole-rat, subcutaneous acid administration fails to induce nociceptive behaviour and skin-innervating nociceptors are proton-insensitive (42) and yet in the same species, sensory nerves innervating the distal colon are both activated and sensitised by acid (whether this correlate with visceral acid nociception is unknown) (242). This finding correlates with the fact that whilst $Na_V1.7$ is vital for somatic pain, it is not required for visceral pain in mice (243). Therefore, it might well be that divergent evolutionary pressures have led to differential proton sensitivity within a single species, in the case of the naked mole-rat, life in a safe, but hypercapnic environment has

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resulted in loss of somatic proton-induced nociception, but the homeostatic role of acidification in the gastrointestinal tract (e.g. pathogen elimination) has led to maintained sensory neurone proton sensitivity.

Overall, the co-expression of different proton-sensitive receptors by nociceptors allows integration of localised acidosis to a number of intracellular signalling events within nociceptors. For example, in response to a decrease in extracellular pH both ASICs and TRPs coordinate cation influx, contributing to depolarisation. The permeance of TRPs to Ca^{2+} also allows them to coordinate release of neuropeptides which may act on other receptors to increase nociceptor excitability, as well as promoting inflammation. Similarly, several K2Ps expressed in nociceptors are inhibited by extracellular protons and thus constitutive K^+ efflux is reduced, further contributing to membrane depolarisation and increasing the likelihood of action potential firing. In addition to this, activation of PS-GPCRs has been shown to sensitise certain TRP channels and the role of GPCRs in transcriptional regulation may also serve to increase expression of proton-sensitive ion channels or expression of other proteins that may positively modulate nociceptor activity. The combined effect of this nociceptor priming is greater action potential discharge and heightened sensitivity to harmful stimuli, which likely manifests in pain. An overview of the integration of nociceptor acid-sensation is depicted in Figure 3.

Conclusion

Detection of acid as a noxious stimulus is present in a wide variety of phyla in the Animalia kingdom, thus demonstrating that evolutionary pressure has maintained selection for this facet of nociceptor function. A wide variety of proton-sensitive receptors are expressed by sensory neurones to enable detection of the environmental pH. The range across which these different receptors are activated, as well as their modulation by a variety of other inflammatory mediators, enables sensory neurones to integrate information regarding tissue pH in both physiological and pathophysiological conditions and the hope is that by studying the evolution of nociceptor proton sensitivity the key molecular players can be identified resulting in therapeutic interventions for conditions associated with tissue acidosis and pain. It is most likely this requirement of detecting a pH range that has led to the evolution of a variety of different proton-sensitive receptors, but at the same time, it is clear that many proton-sensitive receptors are modulated by other stimuli. Therefore, it could be that proton sensitivity is a remnant of a precursory role of that particular receptor, but alternatively it could be that proton sensitivity is the key role and that sensitivity to other stimuli is a feature that has remained, but with limited selection pressure, e.g. ASIC precursors are activated by peptides, whereas mammalian ASICs are proton sensors whose function is modulated by peptides.

As much as our understanding of how protons regulate nociceptors has accelerated dramatically in recent years, certain questions remain to be answered:

- Many proton-sensors are activated/modulated by other stimuli, what is the physiological role of such sensors *in vivo* – detection of protons or different stimuli and what evolutionary pressures drove this dual sensitivity?
- Are the ‘proton-sensitive’ residues identified in certain proton sensor families common to all proton-sensitive receptors of that family throughout evolution?
- What evolutionary pressure(s) maintained selection for the variety of acid sensors nociceptors express and how does an individual nociceptor integrate their signals?
- What is the molecular basis of nociceptor proton sensitivity in those species where no pharmacology or genetics has yet been applied, e.g. frogs and leeches, and does this help to explain the evolution of proton-sensor function?

Figure Legends

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Figure 1. Membrane topologies of proton-sensitive receptor subunits. Schematic diagram of the basic structure of proton-sensitive receptors, with residues or regions important for proton sensitivity annotated (yellow – highly conserved among family members; white – less conserved or important in some, but not all, family members). Functional ASICs, K2Ps and TRPs are multimeric, but for simplicity only one subunit of each receptor is shown.

Figure 2. Phylogeny of general nociception and acid nociception. Annotated phylogenetic tree indicating the presence of general nociceptors, observation of acid nociception and functional expression of proton sensitive receptors. Annotation is limited by the rarity of molecular studies focussing on lower-order species. Expression of proton sensitive receptors is only acknowledged for those species where proton sensitivity of at least one member of the group in question has been empirically proven. For simplicity only species addressed in this review are shown.

Figure 3. Proton-sensation at the peripheral terminal of a typical nociceptor. Following localised acidosis, the increased extracellular concentration of protons is sensed by several receptors which act in concert to increase neuronal excitability and release mediators which may sensitise other neurones. Increased extracellular proton concentration induces the activation of proton-sensitive depolarising channels (ASICs and TRPs) causing cation influx and membrane depolarisation. Simultaneously, proton-induced inhibition of K2P channels reduces constitutive K^+ efflux further facilitating membrane depolarisation. Activation of PS-GPCRs can drive changes in gene expression and coordinate phosphorylation and sensitisation of TRP channels. Altogether, nociceptor membrane depolarisation activates Na_V subunits resulting in generation of action potentials that transmit nociceptive signals to the spinal cord (*Amino acid variations in Na_V1.7 of some species renders the channel hypersensitive to proton-block resulting in an absence of proton-induced nociception).

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Author contributions
All authors were involved equally in the writing of this article.

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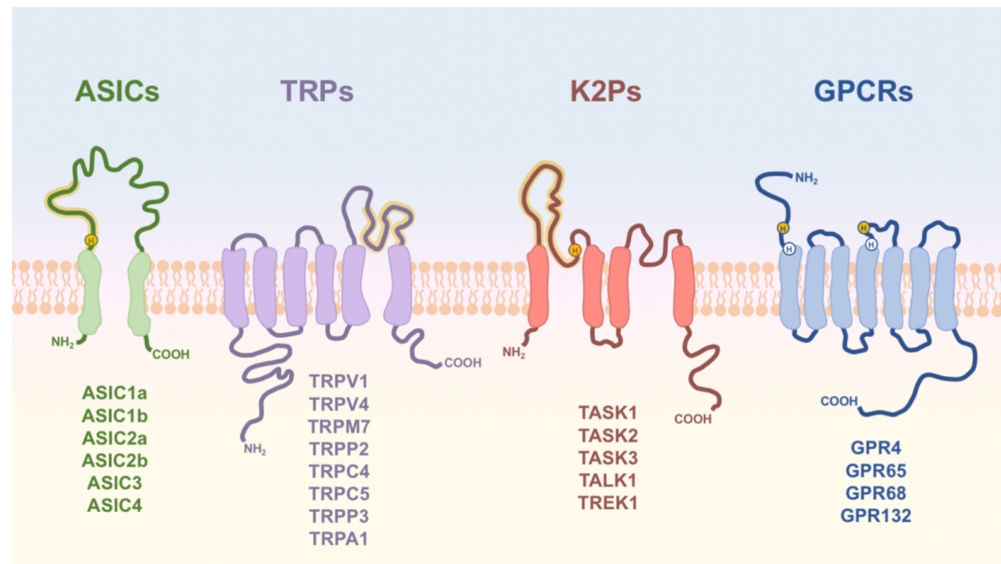

Figure 1. Membrane topologies of proton-sensitive receptor subunits. Schematic diagram of the basic structure of proton-sensitive receptors, with residues or regions important for proton sensitivity annotated (yellow – highly conserved among family members; white – less conserved or important in some, but not all, family members). Functional ASICs, K2Ps and TRPs are multimeric, but for simplicity only one subunit of each receptor is shown.

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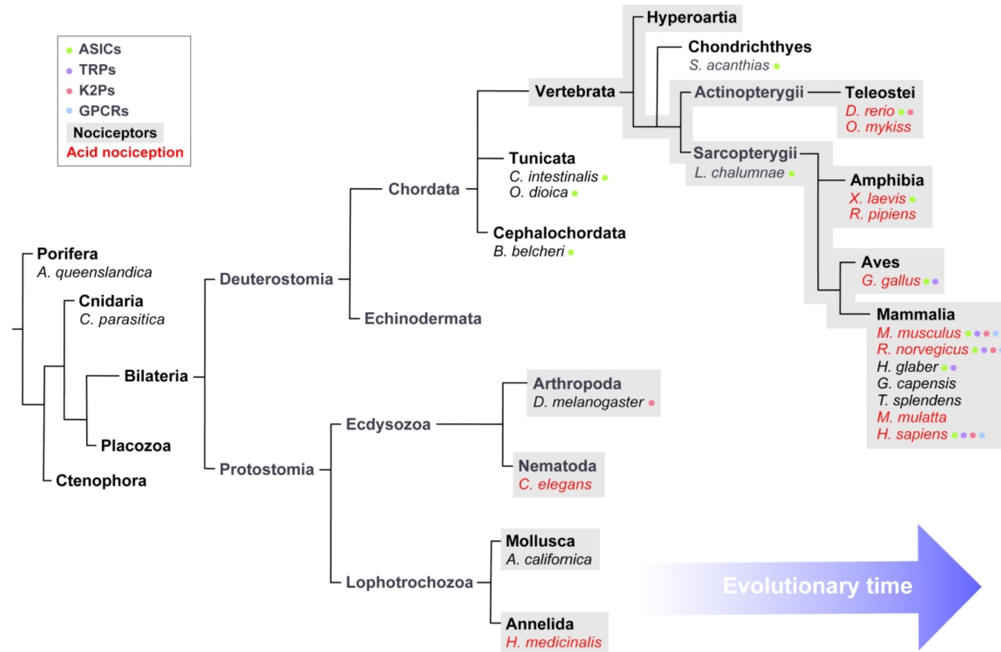

Figure 2. Phylogeny of general nociception and acid nociception. Annotated phylogenetic tree indicating the presence of general nociceptors, observation of acid nociception and functional expression of proton sensitive receptors. Annotation is limited by the rarity of molecular studies focussing on lower-order species. Expression of proton sensitive receptors is only acknowledged for those species where proton sensitivity of at least one member of the group in question has been empirically proven. For simplicity only species addressed in this review are shown.

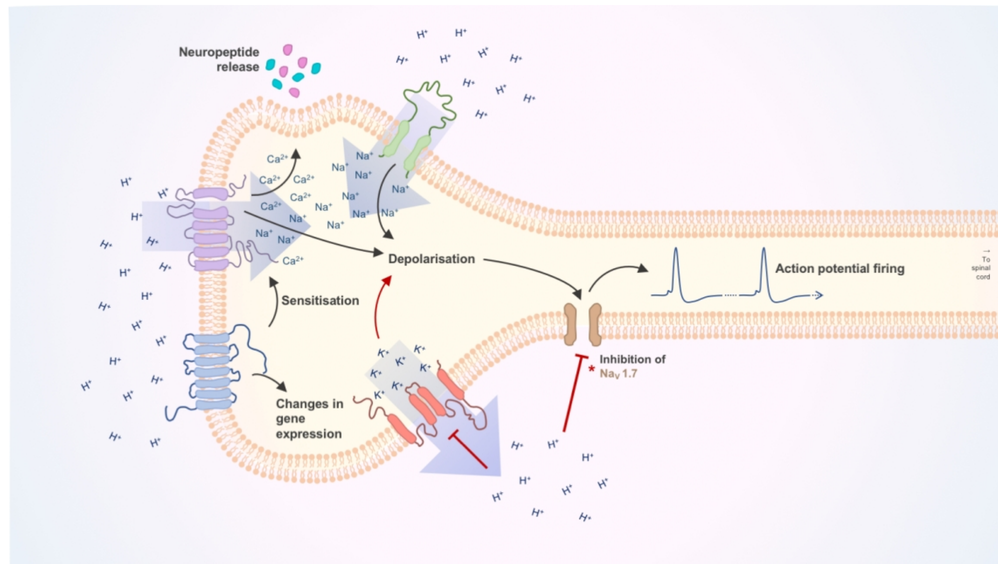

Figure 3. Proton-sensation at the peripheral terminal of a typical nociceptor. Following localised acidosis, the increased extracellular concentration of protons is sensed by several receptors which act in concert to increase neuronal excitability and release mediators which may sensitise other neurones. Increased extracellular proton concentration induces the activation of proton-sensitive depolarising channels (ASICs and TRPs) causing cation influx and membrane depolarisation. Simultaneously, proton-induced inhibition of K2P channels reduces constitutive K+ efflux further facilitating membrane depolarisation. Activation of PS-GPCRs can drive changes in gene expression and coordinate phosphorylation and sensitisation of TRP channels. Altogether, nociceptor membrane depolarisation activates NaV subunits resulting in generation of action potentials that transmit nociceptive signals to the spinal cord (*Amino acid variations in NaV1.7 of some species renders the channel hypersensitive to proton-block resulting in an absence of proton-induced nociception).

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