

A glial DEG/ENaC channel functions with neuronal channel DEG-1 to mediate specific sensory functions in *C. elegans*

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Mammalian neuronal DEG/ENaC channels known as ASICs (acid-sensing ion channels) mediate sensory perception and memory formation. ASICs are closed at rest and are gated by protons. Members of the DEG/ENaC family expressed in epithelial tissues are called ENaCs and mediate Na⁺ transport across epithelia. ENaCs exhibit constitutive activity and strict Na⁺ selectivity. We report here the analysis of the first DEG/ENaC in *Caenorhabditis elegans* with functional features of ENaCs that is involved in sensory perception. ACD-1 (acid-sensitive channel, degenerin-like) is constitutively open and impermeable to Ca²⁺, yet it is required with neuronal DEG/ENaC channel DEG-1 for acid avoidance and chemotaxis to the amino acid lysine. Surprisingly, we document that ACD-1 is required in glia rather than neurons to orchestrate sensory perception. We also report that ACD-1 is inhibited by extracellular and intracellular acidification and, based on the analysis of an acid-hypersensitive ACD-1 mutant, we propose a mechanism of action of ACD-1 in sensory responses based on its sensitivity to protons. Our findings suggest that channels with ACD-1 features may be expressed in mammalian glia and have important functions in controlling neuronal function.

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Introduction

DEG/ENaC channel subunits (named after the *Caenorhabditis elegans* degenerins, the first member cloned causes degeneration, and the mammalian epithelial Na⁺ channels) are two transmembrane domain proteins that trimerize (Jasti *et al*, 2007) to form Na⁺ (Canessa *et al*, 1993, 1994) and Na⁺/Ca²⁺-selective (Bianchi *et al*, 2004; Xiong *et al*, 2004) ion channels. Neuronally expressed DEG/ENaCs are primarily ASICs (acid-sensing ion channels) and are gated by extracellular protons (Krishtal, 2003). Members of the family that are expressed in epithelial tissues (called ENaCs) are inhibited by intracellular (Garty *et al*, 1987) but not extracellular acidification (Awayda *et al*, 2000) and mediate vectorial Na⁺ transport required for fluid clearance in the lung (Hummler *et al*, 1996; Barker *et al*, 1998) and Na⁺ reabsorption in intestine and kidney essential for Na⁺ homeostasis (Garty and Palmer, 1997).

Several members of the DEG/ENaC family across species have been implicated in sensory processes, including mechanosensation (Chalfie and Sulston, 1981; Price *et al*, 2000, 2001), pain sensation (Chen *et al*, 2002; Sluka *et al*, 2003), thermo-sensation (Askwith *et al*, 2001), pheromone perception (Lin *et al*, 2005) and proprioception (Shreffler *et al*, 1995; Tavernarakis *et al*, 1997). ASICs have been implicated in pain sensation. For example, ASIC3 has been proposed to contribute to angina pectoris and to moderate- to high-intensity pain sensation (Chen *et al*, 2002; Naves and McCleskey, 2005). The role of DEG/ENaC channels in mediating sour taste is controversial. ENaC and ASIC proteins are found in taste buds (Kretz *et al*, 1999; Ugawa *et al*, 2003), but the ASIC2 knockout has no effects on sour taste sensitivity (Richter *et al*, 2004). Prior to this study, no DEG/ENaC channels have been linked to the detection of amino acids. Finally, some members of the DEG/ENaC family including *C. elegans* MEC-4 and mouse ASIC1a can induce neurodegeneration under conditions in which they become hyperactivated (Driscoll and Chalfie, 1991; Xiong *et al*, 2004). MEC-4 is hyperactivated by dominant gain-of-function mutations in the pore (MEC-4(A713), the *d* position) (Driscoll and Chalfie, 1991), ASIC1a is hyperactivated by prolonged acidosis of ischaemic brain (Xiong *et al*, 2004). Despite the difference in the mechanism of hyperactivation, we and others have proposed that the neurotoxic capacity of both these channels is linked to their permeability to Ca²⁺ ions (Bianchi *et al*, 2004; Xiong *et al*, 2004).

DEG/ENaC channels have also been found in glia. One group documented mRNA and protein for ENaC and ASIC family members in retinal Muller cells (Golestaneh *et al*, 2000; Brockway *et al*, 2005) and found that electroretinograms in the presence of the DEG/ENaC blocker amiloride are altered, suggesting that these channels play a role in retinal function (Brockway *et al*, 2005). Another group documented DEG/ENaC expression in astrocytes and gliomas, and proposed a role for these channels in glioma malignancy (Berdiev *et al*, 2003).

We present here the cloning and functional characterization of a novel *C. elegans* DEG/ENaC channel with greatest sequence similarity to mammalian hINaC (human intestinal Na⁺ channel) and BLINaC (brain, liver and intestinal Na⁺ channel) that we named ACD-1 (acid sensitive channel, degenerin-like). We document that ACD-1 is expressed in *C. elegans* amphid glia where it is needed for behavioural function rather than glia, or associated neuron, viability. We show that when *acd-1* is knocked out in conjunction with DEG/ENaC *deg-1*-null mutation, *deg-1* sensory defects in acidic solution avoidance and reduced attraction to lysine acetate are markedly exacerbated. We characterize properties of the ACD-1 channel to show that it is sensitive to both extracellular and intracellular acidification. Lastly, we engineer a mutation in ACD-1 that potentiates the channel sensitivity to extracellular protons and show that this mutant exacerbates acid avoidance behaviour *in vivo*. We propose a mechanism for the glial channel ACD-1 in controlling acid avoidance and lysine chemotaxis behaviours based on its acid sensitivity.

Results

C24G7.2 gene encodes a novel DEG/ENaC channel subunit

The *C. elegans* genome contains 28 predicted genes that encode proteins with similarity to DEG/ENaC subunits (reviewed in Bianchi and Driscoll, 2004). Of these, only three have been characterized using electrophysiological tools in heterologous expression systems and *in vivo* (Garcia-Anoveros *et al*, 1998; Goodman *et al*, 2002; Jospin *et al*, 2004; O'Hagan *et al*, 2005). The three functionally characterized *C. elegans* DEG/ENaCs are expressed in mechanosensory neurons (MEC-4 and MEC-10) (Goodman *et al*, 2002; O'Hagan *et al*, 2005) and muscles (UNC-105) (Garcia-Anoveros *et al*, 1998; Jospin *et al*, 2004), share the highest similarity with mammalian ASIC and are involved in mediating touch sensation and muscle contraction. *C. elegans* degenerins homologous to other types of mammalian DEG/ENaCs have not been functionally studied to date. Of the mammalian DEG/ENaCs, hINaC (human) and BLINaC (mouse and rat) are the least understood. Messenger RNA for hINaC and BLINaC has been found in intestine, liver and brain, but roles of these DEG/ENaCs in the physiology of these tissues remain unexplored (Sakai *et al*, 1999; Schaefer *et al*, 2000). We thus sought to identify *C. elegans* DEG/ENaC subunits with similarity with hINaC and BLINaC. We focused on gene C24G7.2 that shares the highest similarity with human hINaC (29% identity and 45% similarity) and mouse and rat BLINaC (28% identity and 44% similarity) (Figure 1A). We isolated a knockout mutant of C24G7.2 by screening a library of EMS-mutagenized animals using gene-specific primers. We identified a 0.9 kb deletion that removed part of the seventh, the eighth and part of the ninth exons, resulting in a frameshift that prematurely terminates the protein at amino acid 245 and is thus likely a null mutant (Figure 1A). For reasons documented by experiments below, we renamed C24G7.2 as ACD-1. It may be noteworthy that although ACD-1 shares greatest sequence similarity with mammalian hINaC and BLINaC, at the position corresponding to the channel-activating 'd' substitutions in *C. elegans* MEC-4 and DEG-1, ACD-1 encodes a Ser instead of an Ala

(Figure 1A) (a Ser substitution at this site is not expected to be channel hyperactivating; Driscoll and Chalfie, 1991). Other features of the ACD-1 protein include a cysteine-rich region preceding the second transmembrane domain (conserved in DEG/ENaC subunits across species; Benos and Stanton, 1999), and two potential glycosylation sites (Figure 1B). The phylogenetic tree in Figure 1C shows evolutionary relationships between ACD-1 and other DEG/ENaCs.

ACD-1 subunits form Na⁺-selective channels

We next sought to functionally characterize ACD-1 by electrophysiological analysis in *Xenopus* oocytes. We found that ACD-1 produced large voltage-independent currents (Figure 2A) that reversed at ~ +10 mV and that were sensitive to amiloride (Figure 2B). When we looked at the permeability to NaCl, LiCl and KCl, we found that the ACD-1 is permeable to Na⁺ and to a lower degree to Li⁺ and K⁺ (PNa ≫ PLi > PK) (Figure 2C). As ACD-1 is only marginally permeable to K⁺, we argue that the relatively low reversal potential of the current reflects the phenomenon of Na⁺ overload previously described in oocytes expressing constitutively active DEG/ENaC channels (Garcia-Anoveros *et al*, 1998; Goodman *et al*, 2002; Bianchi *et al*, 2004). In support of this, we found that smaller currents reversed at a potential closer to ENaC (not shown). We conclude that ACD-1 subunits form Na⁺-selective homomeric channels in *Xenopus* oocytes.

ACD-1 is not further activated by hyperactivating mutations and is impermeable to Ca²⁺

C. elegans DEG/ENaC channel MEC-4 is hyperactivated by Ala to Val or Thr substitutions at positions 713 in the channel pore (the (d) mutation) (Driscoll and Chalfie, 1991; Goodman *et al*, 2002). MEC-4(d) channels have higher open probability than MEC-4(+) channel, resulting in larger whole-cell currents (Brown *et al*, 2007). Other members of the family including *C. elegans* UNC-105 and mammalian MDEG, hINaC and BLINaC are hyperactivated by analogous substitutions (Garcia-Anoveros *et al*, 1998; Sakai *et al*, 1999; Schaefer *et al*, 2000). We wondered whether ACD-1 channels, which display large whole-cell currents in the wild-type form, could be further activated by mutating Ser513 (corresponding to Ala713 in MEC-4) to Val. We found that currents produced by injection of ACD-1(S513V) into *Xenopus* oocytes were similar in amplitude to currents generated by wild-type ACD-1 (Figure 2D and F).

We recently reported that MEC-4(d) is Ca²⁺ permeable and may contribute to necrosis initiation by elevating intracellular Ca²⁺ concentration (Bianchi *et al*, 2004). We thus asked whether ACD-1(d) channels were Ca²⁺ permeable. We perfused oocytes expressing ACD-1(d) with a solution in which we substituted NaCl with CaCl₂ and found that the oocyte endogenous Ca²⁺-activated Cl⁻ current, which we have used as a sign of Ca²⁺ permeability through MEC-4(d) (Bianchi *et al*, 2004), was not activated (Figure 2E). Similar results were obtained in oocytes expressing wild-type ACD-1 (not shown). These results indicate that ACD-1 and ACD-1(d) channels are not permeable to Ca²⁺. However, we found that S513V substitution changed amiloride block by reducing the concentration of amiloride needed to inhibit 50% of the current (K_i) from 99 to 17 μM (measured at -160 mV; Figure 2G).

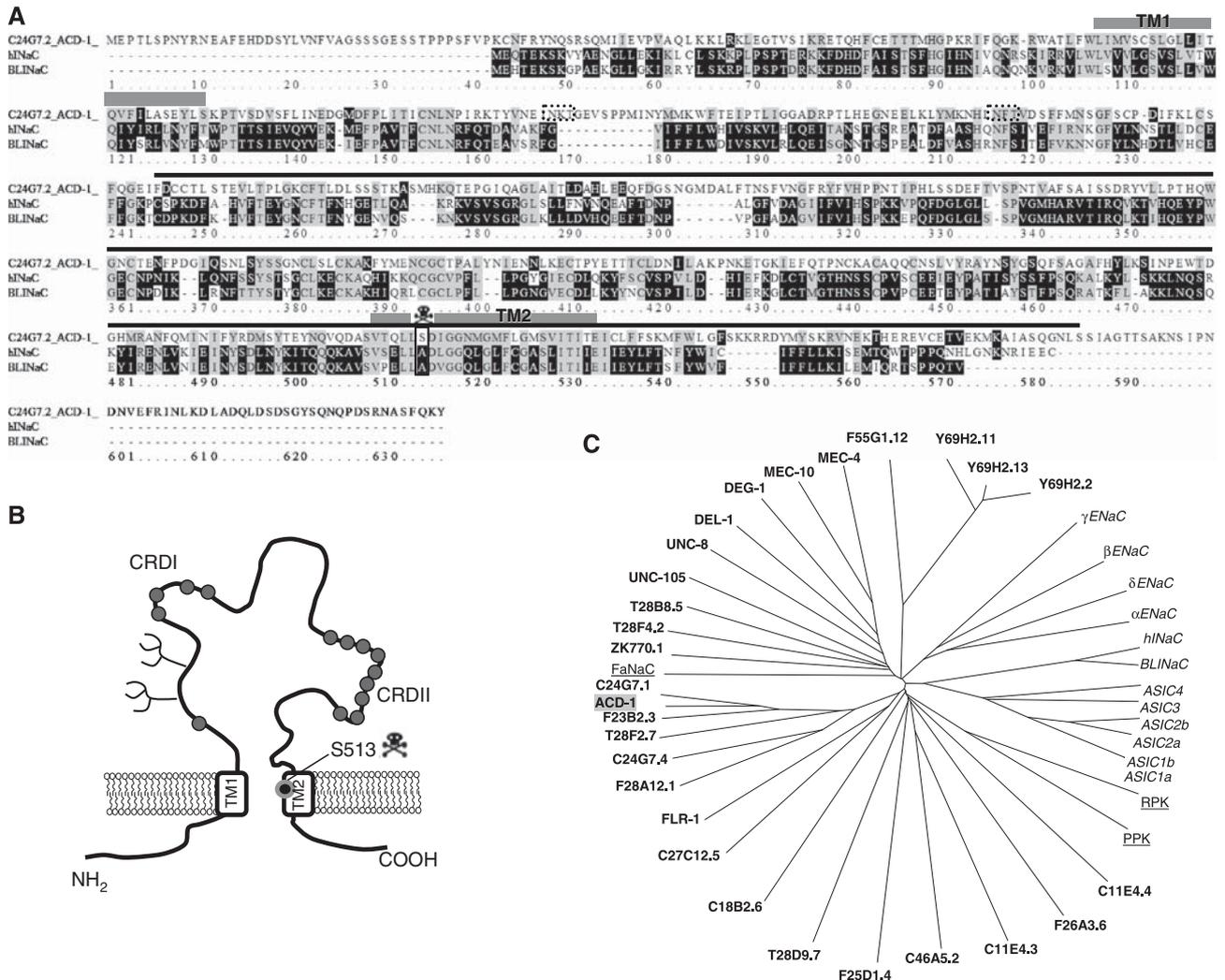


Figure 1 The *C. elegans* DEG/ENaC channel ACD-1 (acid-sensitive channel degenerin-like) shares similarity with mammalian BLINaC and hINaC. (A) We designated C24G7.2 as ACD-1 because of its sensitivity to acidic solutions. C24G7.2 protein sequence and alignments with hINaC (29% identity and 45% similarity) and mouse and rat BLINaC (28% identity and 44% similarity). The transmembrane domains TM1 and TM2 and the region of the protein deleted in *acd-1(bz90)* knockout are indicated by the grey boxes and black line, respectively. The transmembrane domains were identified by threading ACD-1 sequence onto the sequence of chicken ASIC1a (Jasti *et al*, 2007), using PyMOL (www.PyMOL.org). The box and the skull designate position S513 in ACD-1 corresponding to the amino acid that when mutated to Val or Thr in MEC-4 induces hyperactivation of the channel (A713V or T, the (d) mutation). The dotted boxes indicate potential glycosylation sites. Alignment by ClustalW (available at bioweb.pasteur.fr). (B) A schematic diagram representing ACD-1 predicted topology. The potential glycosylation sites are indicated by the branches. The circles depict cysteine residues; a region just preceding TM2 is rich in cysteines. This feature is conserved in DEG/ENaC subunits across species (Benos and Stanton, 1999). (C) Dendrogram of *C. elegans* (bold), *Drosophila* (RPK and PPK, underlined), *Helix apersa* (FaNaC, underlined) and mammalian (italic) DEG/ENaC channel subunits. ACD-1 subunit has a grey background.

Amiloride inhibits DEG/ENaC channels by interacting with sites within the pore, in the membrane electric field (Palmer, 1985; Garcia-Anoveros *et al*, 1998; Goodman *et al*, 2002). Thus, amiloride block in DEG/ENaCs is usually influenced by membrane voltage. We wondered whether this was the case for ACD-1 as well. We found that the K_i for amiloride is unaffected by the membrane potential in wild-type ACD-1, suggesting that the blocker interacts with an extracellular binding site (Figure 2H). However, the K_i becomes voltage dependent in ACD-1(S513V), suggesting that S513V mutation introduces or uncovers an amiloride-binding site within the channel pore (Figure 2I). These results indicate that S513 is in the pore, similar to analogous residue A432 in chicken ASIC1a (Jasti *et al*, 2007). Taken together, these data suggest ACD-1 pore structure is similar to other DEG/ENaCs.

DEG/ENaC channel ACD-1 is expressed in *C. elegans* glia

To gain insight into ACD-1 physiological function, we sought to determine ACD-1 expression pattern by analysing transgenic animals harbouring a translational ACD-1::GFP construct. We found that transgenic animals strongly expressed ACD-1::GFP in the amphid sheath cells, a pair of glial-like cells located in the head of the worm that can be visualized by the expression of sheath cell-specific marker *VAP-1::RFP* (Figure 3A–H; Perens and Shaham, 2005). Amphid sheath cell processes run down to the tip of the nematode nose and wrap around the sensory neuron dendrites forming a channel through which sensory neurons, cilia (the sensory endings of sensory neurons) pass before reaching the external environment (Shaham, 2006).

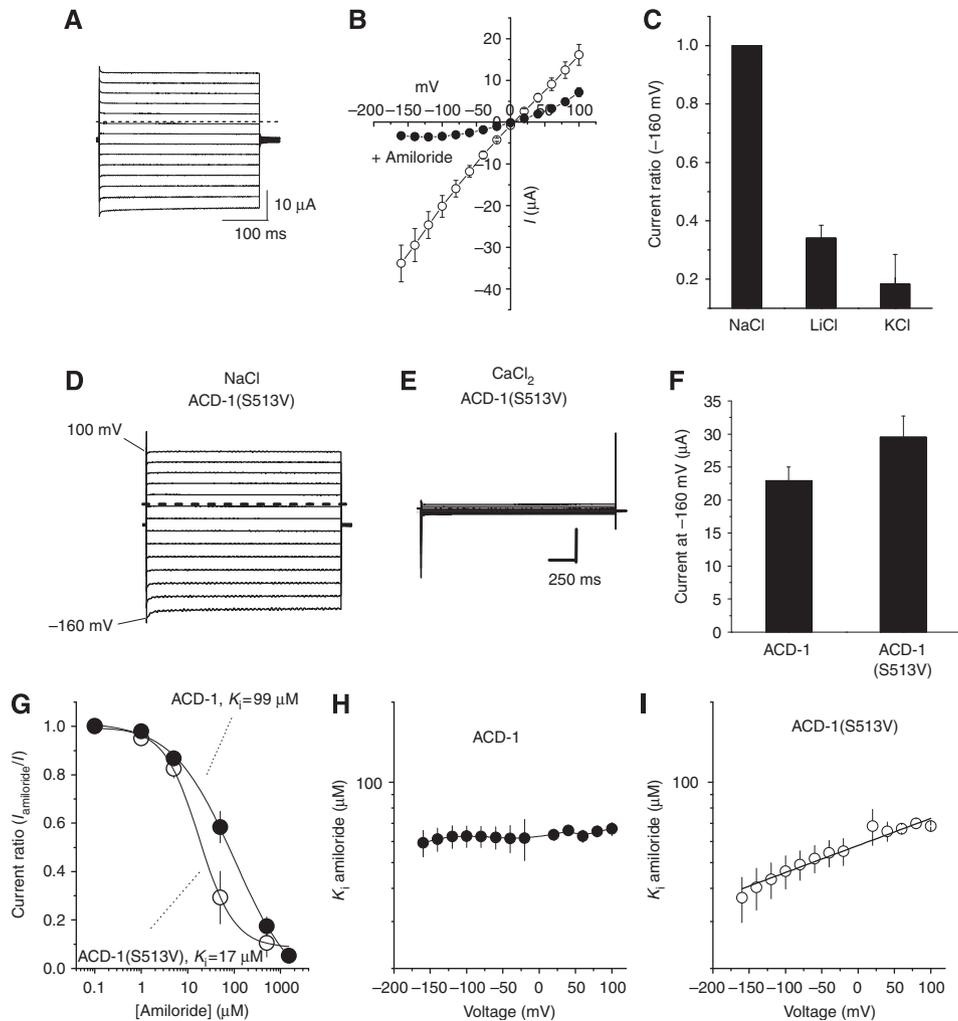


Figure 2 Properties of wild-type and mutated ACD-1 channel. (A) Typical Na⁺ currents in an oocyte injected with *acd-1* cRNA, perfused with a physiological NaCl solution. Voltage steps were from -160 to +100 mV from a holding potential of -30 mV. (B) Current-voltage relationships for oocytes injected with *acd-1* cRNA when the oocytes are perfused with a physiological NaCl solution (open circles, *n* = 15) and with a physiological saline plus 500 μM of DEG/ENaC channel blocker amiloride (filled circles, *n* = 14). (C) Selectivity of ACD-1 channels to monovalent cations, established by determining the ratio of the inward current at -160 mV when perfusing the oocytes with NaCl, LiCl and KCl (*n* = 8). (D) Typical Na⁺ currents in an oocyte injected with *acd-1(S513V)* cDNA, perfused with a physiological NaCl solution. Voltage steps were from -160 to +100 mV from a holding potential of -30 mV. The (S513V) mutation in ACD-1 is analogous to A713V in MEC-4(d) (Driscoll and Chalfie, 1991). (E) We perfused the same oocyte shown in (D) with a solution in which we substituted NaCl with CaCl₂. Note that perfusing the oocytes with the CaCl₂ solution does not activate the oocyte endogenous Ca²⁺-activated Cl⁻ current that we have used in the past as a measure of MEC-4(d) permeability to Ca²⁺ (Bianchi *et al*, 2004). Similar results were obtained for wild-type ACD-1 channels. (F) S513V mutation does not hyperactivate ACD-1. Average current amplitude at -160 mV from oocytes injected with wild-type and S513V mutant ACD-1, *n* = 5. The averages are not significantly different by *t*-test (*P* = 0.12). (G) Amiloride dose-response curves for ACD-1 and ACD-1(S513V) (*K*_i = 99 and 17 μM, respectively, *n* = 5 for both). Data are expressed as mean ± s.e. (H) Lack of voltage dependence of amiloride blockade in ACD-1 channels (*n* = 5). (I) The (S513V) mutation in ACD-1 introduces voltage dependence of amiloride blockade in ACD-1 channels, suggesting that amino acid 513 is located within the membrane electric field (*n* = 5). Data points were fitted with a Woodhull model (*r* = 0.14). Data are expressed as mean ± s.e.

ACD-1 channel functions with neuronal DEG/ENaC channel DEG-1 in acid avoidance behaviour

As *C. elegans* amphid sheath cells encircle the dendritic endings of sensory neurons, we considered the possibility that the glial channel ACD-1 might be involved in sensory perception. Because mammalian ASICs are gated by protons, we hypothesized that ACD-1 may have a function in acid sensation. *C. elegans* exhibit a strong aversive response to low pH environments on agar pH gradient plates (Sambongi *et al*, 2000). To investigate the contribution of ACD-1 channels to acid avoidance, we assayed acute responses in individual animals using ‘drop tests’, in which a drop of acidic

solution (either HCl or acetic acid) is delivered to a single animal (Hilliard *et al*, 2004). We observed that wild-type *C. elegans* showed pronounced escape responses to solutions of pH 3.5 in the drop test assay (Figure 3I). This response was partially dependent on the type of acid used for equal pH, as acetic acid, which induces intracellular acidification (Thomas, 1984; Zampighi *et al*, 1988), elicited a stronger avoidance behaviour than HCl, suggesting that intracellular acidification may have a key function in eliciting the response (Speake and Elliott, 1998). Interestingly, we also observed that the response was partially amiloride-sensitive (Figure 3I) and that the robustness of the escape response was dependent on

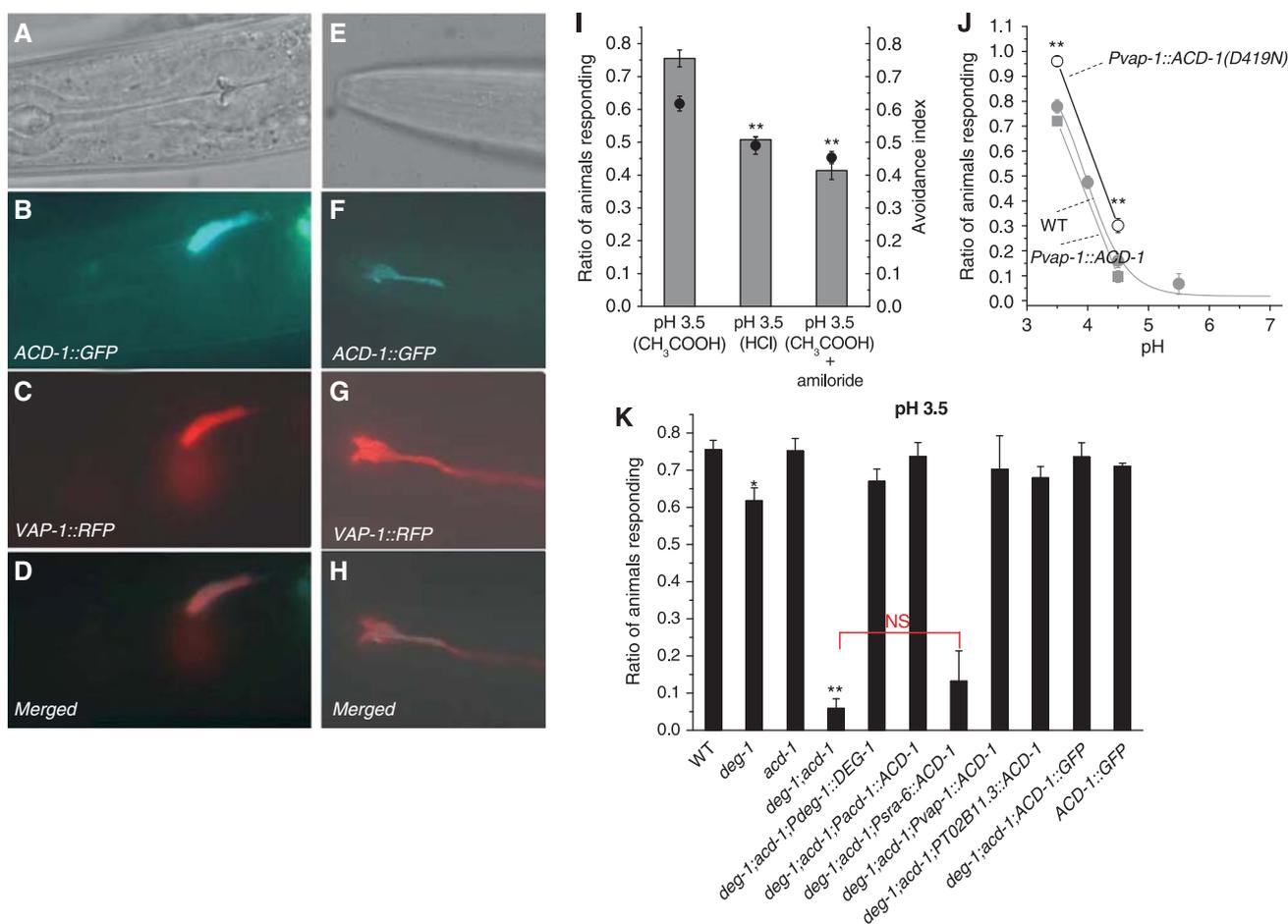


Figure 3 *ACD-1::GFP* is expressed in the glial amphid sheath cells where it is needed for acid avoidance behaviour. (A, B) DIC and fluorescent (GFP channel) micrographs of a transgenic animal expressing *Ex[ACD-1::GFP;VAP-1::RFP]* showing the expression of *ACD-1::GFP* in two large cells in the head of the worm (the other cell is on another focal plane) whose cell bodies are on either side of the pharyngeal bulb. *vap-1* encodes for a venom-allergen-like-protein expressed in amphid sheath cells (Perens and Shaham, 2005). (C) The same animal was photographed using the rhodamine filter. This revealed overlapping of green (*ACD-1::GFP*) and red (*VAP-1::RFP*) signals (see (D), merged), establishing that *ACD-1::GFP* is expressed in amphid sheath cells. (E–H) DIC and fluorescent micrographs taken with GFP and rhodamine filters of the tip of the nose of the same animal shown in (A–D), showing the expression of *ACD-1::GFP* in amphid sheath cell processes (only one is shown here, the other one is on another focal plane). Front is left. (I, J) *C. elegans* exhibit an amiloride-sensitive aversive behaviour towards acidic solutions. (I) Average avoidance index to solutions in which pH was adjusted to 3.5 using acetic acid (CH₃COOH) or HCl, measured using our drop test assay (see Materials and methods). For experiments in which we added amiloride, we preincubated animals in 1 mM amiloride for 30 min and then assayed adding amiloride to the pH 3.5 solution. Number of experiments performed was 8, 5 and 3, respectively. In each experiment, at least 10 animals were tested. (J) Quantification of ratio of animals responding (grey circles) at different pHs for wild-type *C. elegans*, *deg-1;acd-1* mutants expressing wild-type *ACD-1*, and *deg-1;acd-1* mutants expressing *ACD-1(D419N)*. *ACD-1* expression was driven in the glial amphid sheath cells by the *vap-1* promoter (Perens and Shaham, 2005). We fitted the wild-type *C. elegans* pH/avoidance curve by sigmoidal equation, which gave pH_{0.5} values of 3.8. Data are expressed as mean ± s.e. *n* of trials was 6–9 at pH 3.5 and 4.5 and 4–11 at the other pHs; at least 10 animals in each trial were tested. At least two transgenic lines were generated per construct and no statistical difference was found between data obtained from these lines. (K) *ACD-1* functions with *DEG-1* to mediate acid sensation. Ratio of animals responding to M13 solution buffered to pH 3.5 with acetic acid for wild-type, *deg-1*, *acd-1*, *deg-1;acd-1* double mutants and *deg-1;acd-1* double mutants expressing exogenous *ACD-1* cDNA under the control of *acd-1* promoter, *sra-6* promoter (Troemel *et al*, 1999) and glial-specific *vap-1* and T02B11.3 promoters (Perens and Shaham, 2005) and (Supplementary Figure 2). *ACD-1* expression in sheath cells but not ASH neurons rescues the acid insensitivity of *deg-1;acd-1* mutants. *ACD-1* construct used for the determining *ACD-1* expression pattern, which includes *acd-1* promoter and genomic sequence (B, F), also rescued acid avoidance defect of *deg-1;acd-1* mutants but did not enhance acid avoidance when expressed in wild-type *C. elegans*. The figure also shows that a construct containing the *deg-1* promoter that we have used for our expression pattern studies (Figure 4A) and *deg-1* cDNA sequence (*Pdeg-1::DEG-1*) rescues the acid avoidance deficit of *deg-1;acd-1* mutant. Number of experiments was from 4 to 8 with at least 10 animals tested each. Data are expressed as mean ± s.e. **, **P* < 0.01 and 0.05 by comparison with wild-type (WT) animals by *t*-test, respectively. NS stands for nonsignificant difference between the averages indicated by the red bracket by *t*-test.

the proton concentration (pH_{0.5} ~4; Figure 3J). We next assayed *acd-1* knockout animals (*acd-1(bz90)*) but found that they responded to pH 3.5 (Figure 3K).

Sambongi *et al* (2000) reported that combined laser ablation of ADF, ASE, ASK and ASH chemosensory neurons as well as of ADF/ASE, ASK/ASH, ADF/ASK and ASE/ASH pairs of sensory neurons diminishes *C. elegans* sensitivity

to acidic solutions. We hypothesized that *acd-1* knockout may exacerbate sensory defects caused by reduced sensory neuron function. The DEG/ENaC channel *DEG-1* was previously reported to be expressed in ASH chemosensory neurons (Hall *et al*, 1997) and thus we first looked at the contribution *DEG-1* to acid avoidance behaviour. We found *deg-1*-null mutant animals (*deg-1(u38u421)*) escaped pH 3.5 with

reduced frequency compared with wild type, although the effect of the *deg-1*-null mutation was modest (Figure 3K). Strikingly, however, when we combined the *deg-1*-null mutation with *acd-1* deletion, we found that more than 90% of the *deg-1;acd-1* double mutants failed to respond to acidic solutions (Figure 3K). Thus, we conclude that ACD-1 and DEG-1 channels function in concert to mediate acid avoidance behaviour.

ACD-1 channel functions cell-autonomously in the glial cells to orchestrate acid avoidance behaviour

We found that ACD-1 is expressed in the amphid sheath cells. Oddly, neurons shown to contribute to acid sensation, including ASH (Sambongi *et al*, 2000), did not express ACD-1::GFP. However, there are caveats to GFP expression experiments (reviewed in Rhoads *et al*, 2005) that leave open questions about whether ACD-1 might in fact act in the glial cells. To better test where ACD-1 channels function to control acid sensation, we expressed ACD-1::GFP in ASH neurons and amphid sheath cells of *deg-1;acd-1* mutants using cell-specific promoters. We found that although the expression of ACD-1 driven in ASH neurons by the *sra-6* promoter (Troemel *et al*, 1995) did not rescue the acid

avoidance defective phenotype of the *deg-1;acd-1* double mutant, ACD-1 expression driven in amphid glia by two distinct and independent promoters (the promoters of the *vap-1* and T02B11.3 genes Perens and Shaham, 2005) and Supplementary Figure 2) did (Figure 3K). Taken together, these data indicate that ACD-1 acts in glia to orchestrate acid avoidance behaviour in *C. elegans*.

DEG-1 is expressed in ASK sensory neurons

We expected that precise evaluation of the DEG-1 expression pattern would help clarify the molecular and cellular mechanisms of DEG-1-mediated acid sensing. We thus analysed transgenic animals harbouring a translational DEG-1::GFP construct. We found that DEG-1::GFP was expressed in ASK and ASG chemosensory neurons (Figure 4A). We did not detect expression of DEG-1::GFP in ASH neurons (Hall *et al*, 1997) (although as our GFP construct did not include all *deg-1* sequences, we cannot rule out that it might be). Interestingly though, we found that the exogenous expression of DEG-1 in *deg-1;acd-1* double mutants under the control of the promoter that we used in our expression pattern studies, rescued acid avoidance behaviour (Figure 3K), suggesting that perhaps DEG-1 acts in ASK to contribute to acid

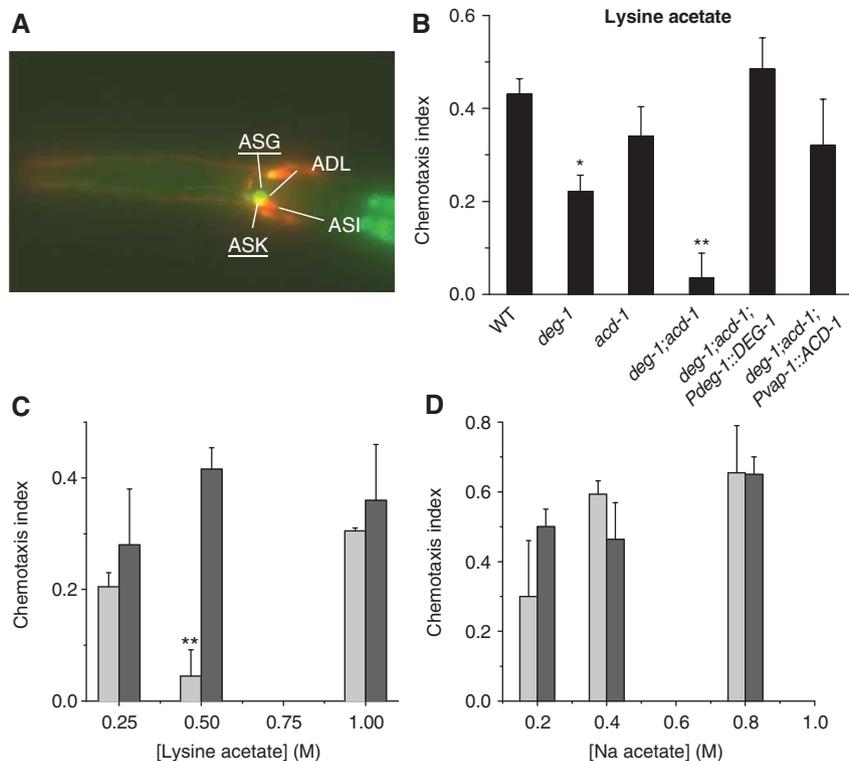


Figure 4 DEG/ENaC channels ACD-1 and DEG-1 are also needed for lysine chemotaxis. (A) DEG-1 is expressed in ASK and ASG chemosensory neurons. Merged photographs of a transgenic *C. elegans* expressing DEG-1::GFP stained with lipophylic dye DiO. Overlapping of green and red fluorescence identifies ASK sensory neurons as one of the neurons that expresses DEG-1::GFP. Another nearby neuron that expresses DEG-1::GFP was tentatively identified as ASG. DiO staining (red) is also evident in ADF and ASI sensory neurons. Dorsal view, front to the left. (B) Chemotaxis index towards lysine acetate was determined for wild-type, *deg-1*, *acd-1* mutants and *deg-1;acd-1* double mutant, *deg-1;acd-1* double mutants expressing exogenous ACD-1 in amphid glia and *deg-1;acd-1* double mutants expressing DEG-1 in sensory neurons. ACD-1 expression was under the control of glial-specific *vap-1* promoter (Perens and Shaham, 2005), whereas DEG-1 expression was under its native promoter (see Materials and methods). The compromised chemotaxis of *deg-1;acd-1* to lysine is rescued by the exogenous expression of ACD-1 in the glial amphid sheath cells and DEG-1 in sensory neurons. Number of trials was 5–14, with at least 30 animals assayed in each trial. (C) The chemotaxis index of wild-type (dark grey) and *deg-1;acd-1* double mutant animals (light grey) was determined at different concentrations of lysine acetate (0.25, 0.5 and 1 M). $n=4-11$. (D) We determined the chemotaxis index to Na acetate for wild-type (dark grey) and *deg-1;acd-1* double mutant animals (light grey) using different concentrations of Na acetate (0.2, 0.4 and 0.8 M). $n=4-11$. Data are expressed as mean \pm s.e. **, * $P < 0.01$ and 0.05 by comparison with wild-type (WT) animals by *t*-test, respectively.

avoidance (Sambongi *et al*, 2000). Importantly, ASK neurons mediate both acid avoidance and chemotaxis to lysine (Bargmann and Horvitz, 1991; Sambongi *et al*, 2000)—thus, our expression studies suggested one neuronal type that we could further probe for function in *deg-1*, *acd-1* and *deg-1;acd-1* mutants.

Because *deg-1* is not thought to be expressed in the amphid sheath cells, our results also suggest that DEG-1 and ACD-1 are not part of the same channel complex; rather they function in different cell types to mediate acid sensation. Further support for this is that the addition of DEG-1 cRNA to oocytes expressing ACD-1 does not change ACD-1 currents in any measurable way (not shown).

ACD-1 and DEG-1 are needed for lysine chemotaxis

We assayed chemotaxis to lysine acetate on gradient plates (Bargmann and Horvitz, 1991). We found that, similar to acid avoidance, *deg-1*-null mutants are less attracted than wild-type animals to lysine and that *acd-1* knockout greatly exacerbates *deg-1*-null phenotype (Figure 4B). We wondered whether the severe defect of *deg-1;acd-1* could be explained by a reduced sensitivity to lysine acetate across a range of concentrations. We found that *deg-1;acd-1* double mutant animals respond similarly to wild type to 0.25 and 1 M lysine acetate but they are severely impaired in detecting 0.5 M lysine acetate (Figure 4C). These results suggest that channels or receptors other than DEG-1 and ACD-1 may contribute to the mediation of lysine chemotaxis at concentrations lower and higher than 0.5 M. As lysine chemotaxis and acid avoidance are assayed using solutions in which acetic acid is present, we wondered whether reduced sensitivity to acetic acid could only explain the behavioural deficits that we observed in *deg-1;acd-1* mutant animals. To address this question, we determined chemotaxis to a range of Na acetate concentrations. We found that *deg-1;acd-1* chemotax to Na acetate, similar to wild-type animals at all the concentrations tested (Figure 4D).

Importantly, in *deg-1;acd-1* mutant animals, glia and sensory neurons are viable and structurally normal, and *deg-1;acd-1* mutants respond normally to all other attractants and noxious stimuli tested (Supplementary Figure 1). Thus, these results identify the first gene in *C. elegans* that specifically affects chemotaxis to lysine and acid avoidance and highlight glial channel ACD-1 as a modulator of these sensory functions.

ACD-1 channels are inhibited by protons

Given our demonstration that *C. elegans* DEG/ENaC channels DEG-1 and ACD-1 are needed for responding to acid, we investigated the possibility that protons directly affect DEG-1 and ACD-1 channels, similar to the mammalian ASICs (Krishtal, 2003). When we perfused oocytes expressing ACD-1 channels with a solution that was adjusted to pH 5.5 with acetic acid or HCl, we observed a strong suppression of ACD-1 currents (Figure 5A and B). The effect was completely reversible (Figure 5C). To establish whether ACD-1 channels are sensitive to intracellular acidification (Garty *et al*, 1987; Awayda *et al*, 2000), we assayed current amplitude after incubation of the oocytes expressing ACD-1 in a physiological saline at neutral pH containing 20 mM Na acetate known to cause intracellular acidification (the solution was washed away prior to electrophysiological recordings; Thomas,

1984; Zampighi *et al*, 1988). We found that ACD-1 currents in these oocytes were suppressed as compared with control oocytes (Figure 5D). This result suggests that intracellular acidification can inhibit the channel as well. In principle, lysine acetate should produce a similar effect because acetic acid acidifies the intracellular environment by entering the cell as uncharged HAcetate (Speake and Elliott, 1998) and this chemical species is present in both Na acetate and lysine acetate solutions. Indeed, when we assayed oocytes that were incubated in 20 mM lysine acetate, we found that ACD-1 currents were suppressed (Figure 5D). We did not observe any effect of lysine acetate at neutral pH (2–200 mM) acutely perfused onto the oocytes expressing ACD-1. By testing different extracellular proton concentrations, we observed that 6.4 is the pH that gives half-maximal inhibition and that the channel appears to have a single extracellular binding site for H⁺ (Figure 5E; fit with Hill's equation: $K_i = 6.4$, $n = 0.9$).

What are the properties of DEG-1 channel? When we expressed *deg-1* cDNA in *Xenopus* oocytes, we did not detect any current. Introducing the hyperactivating (d) mutation, or addition of postulated accessory subunits did not change the outcome of our experiments (Rajaram *et al*, 1998; Chelur *et al*, 2002; Goodman *et al*, 2002), thus we conclude that DEG-1 does not function in *Xenopus* oocytes probably because this system lacks nematode proteins/factors needed for DEG-1 function.

Analysis of an ACD-1 mutant uncovers a link between the channel pH sensitivity and *C. elegans* behaviour

If glial ACD-1 pH sensitivity has a function in acid avoidance behaviour in *C. elegans*, then the whole animal behaviour should be affected by changes in ACD-1 pH sensitivity. To test this hypothesis, we engineered mutation D419N in the postulated extracellular proton-binding region of ACD-1 (Jasti *et al*, 2007) (Supplementary Figure 3). D419 corresponds to D346 in chicken ASIC1a that when mutated to N renders the channel less sensitive to protons (Jasti *et al*, 2007). We expressed ACD-1(D419N) in *Xenopus* oocytes and assayed pH sensitivity. We found that ACD-1(D419N) generates currents with amplitude and properties similar to wild-type ACD-1 (not shown), but surprisingly, these currents are more rather than less sensitive to acidic pH, being inhibited with a K_i of pH 7.3 instead of 6.4 (Figure 5E, open circles). These data suggest that ACD-1 proton-binding site may be different from ASIC1a. We postulate that the increased acid sensitivity of ACD-1(D419N) may be due to rearrangements of the charges involved in proton binding resulting in higher net negative charge exposed in the extracellular domain of the mutant channel. Regardless of the structure/function implications of this finding, this mutant afforded us the possibility to establish whether a link exists between ACD-1 acid sensitivity and animal behaviour. We thus tested the effect of a more acid-sensitive ACD-1 on animal behaviour by expressing ACD-1(D419N) in the glial amphid sheath cells of *deg-1;acd-1* mutant. We found that a higher percentage of *deg-1;acd-1* animals that express ACD-1(D419N) respond to acidic solutions than wild-type animals (N2) or *deg-1;acd-1* mutant animals that express wild-type ACD-1 (Figure 3J, open circles and filled squares). These results support that there is a link between ACD-1 acid sensitivity and *C. elegans* acid avoidance

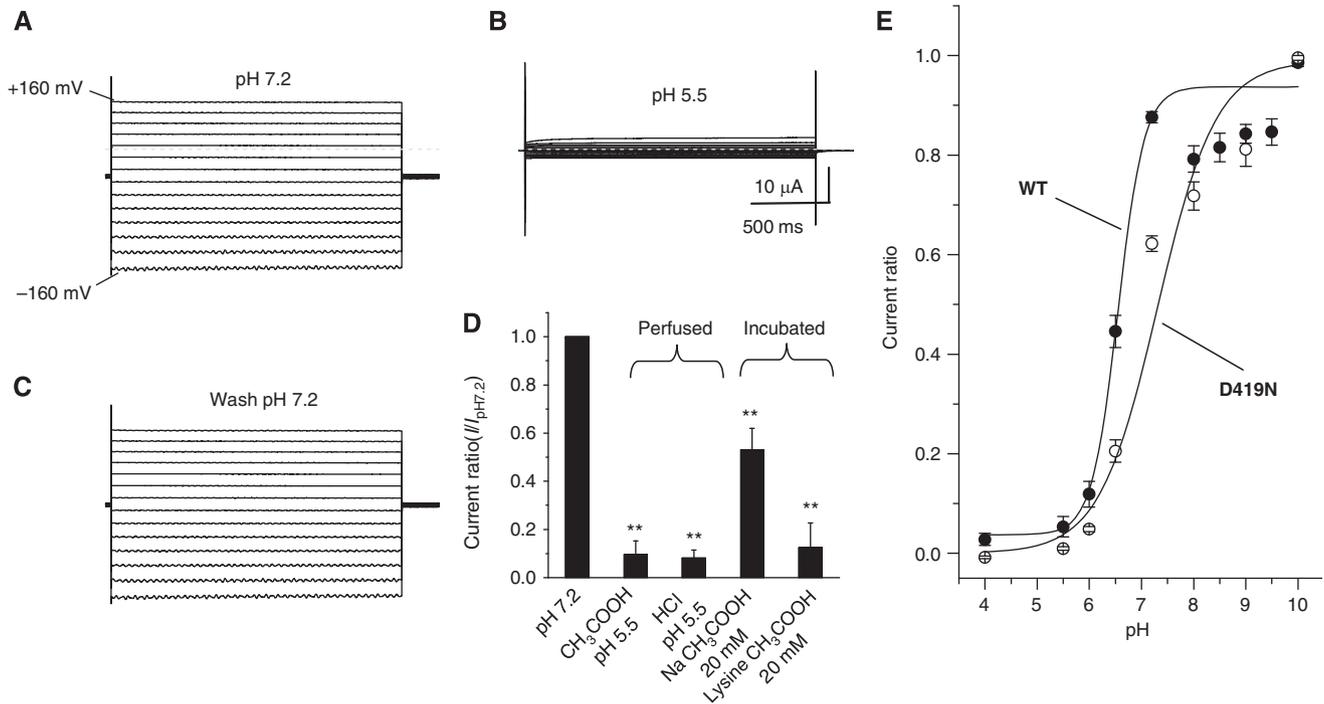


Figure 5 DEDG/ENaC channel ACD-1 exhibits a strong sensitivity to acidic solutions. (A) Example of currents elicited by voltage steps from -160 to $+100$ mV from a holding potential of -30 mV in a *Xenopus* oocyte injected with *acd-1* cRNA and exposed to a physiological NaCl saline solution at pH 7.2. (B) The same oocyte was exposed to a solution at pH 5.5, which resulted in the inhibition of ACD-1 currents. (C) When we returned to physiological pH 7.2, ACD-1 currents returned to control levels. (D) ACD-1 currents are equally suppressed by extracellular solutions buffered with acetic acid and HCl and are reduced by incubation with 20 mM extracellular Na acetate and lysine acetate, suggesting that both extracellular and intracellular acidification cause channel inhibition (Thomas, 1984; Zampighi *et al*, 1988). For incubation with 20 mM Na acetate and 20 mM lysine acetate, we incubated oocytes in the NaCl solution in which we added Na acetate or lysine acetate for 1 h at room temperature. Control oocytes were incubated under the same conditions in NaCl physiological saline. Oocytes were then transferred to control NaCl solution for 15 min prior to recordings. We could not recover currents to control levels after 3 h of wash in Na acetate- and lysine acetate-treated oocytes. This is not unusual and it has been observed by others using this technique for intracellular acidification (Thomas, 1984; Zampighi *et al*, 1988). n was 8 for each bar. (E) Sensitivity of wild-type ACD-1 and ACD-1(D419N) channels to extracellular pH. Currents were recorded at -160 mV, normalized to maximal currents and best fitted with the Hill's equation ($K_i = \text{pH } 6.4$, $n = 0.9$ for wild-type ACD-1; K_i for ACD-1(D419N) was 7.3). n is 4–27. Data are expressed as mean \pm s.e. **Indicates $P < 0.01$ by comparison with untreated oocytes by t -test.

behaviour, suggesting that ACD-1 acid sensitivity plays a role in controlling acid avoidance.

Discussion

We have reported here the characterization of novel *C. elegans* DEG/ENaC channel ACD-1, which shares similarity with mammalian hINaC and BLINaC. We have shown that ACD-1 is a Na^+ -selective, constitutively open ion channel that cannot be further activated by hyperactivating (d) mutations and that is not permeable to Ca^{2+} ions. Our *in vivo* analysis of ACD-1 reveals that ACD-1 is expressed in *C. elegans* glia and that *acd-1* knockout exacerbates sensory defects caused by *deg-1* null, another DEG/ENaC ion channel that we show is expressed in ASK chemosensory neurons (among other neurons). We propose a mechanism of regulation of sensory activity by ACD-1 channels dependent on its sensitivity to intracellular and extracellular acidification.

A role for DEG/ENaC channels in chemosensation

Members of the DEG/ENaC channel family have been suggested as potential transduction channels for acid chemosensation in other systems. In particular, the mammalian ASIC channels, which are proton-activated cation channels, have been suggested as possible sensors in sour taste (Ugawa *et al*,

2003). However, studies of knockout mice lacking one such candidate taste receptor, ASIC2, failed to observe sensory defects *in vivo* (Richter *et al*, 2004). Although additional ASIC channel knockouts remain to be tested in this way, evidence is still lacking to definitively implicate this channel family in sour taste transduction. It is interesting that in *C. elegans*, a proton-inactivated DEG/ENaC channel with very different physiological properties from mammalian ASICs has a function in what can be viewed as the nematode counterpart of the sour taste sense. The physiological properties of ACD-1 are perhaps surprising given its role in regulating sensory perception. DEG/ENaC channels previously implicated in sensory transduction, such as the mechanosensory channel MEC-4, appear to share certain common characteristics: they are closed in the resting state, are gated by a sensory stimulus and in some cases are permeable to Ca^{2+} as well as Na^+ (Bianchi *et al*, 2004; Xiong *et al*, 2004; O'Hagan *et al*, 2005). Channels similar to the canonical ENaC channel were thought to function only in epithelial cells and serve a primarily homeostatic function (Canessa *et al*, 1994). Our findings here suggest that the functional distinctions between these DEG/ENaC subtypes may not be so clear cut, as a member of the latter class appears to have an important (perhaps non-canonical) function in sensory perception. Interestingly, Kretz *et al* (1999) reported the presence of

mRNA for the α -, β - and γ -subunits of ENaCs as well as amiloride-binding sites in rat tongue, raising the possibility that a similar mechanism for sour (and perhaps salt) sensation, mediated by ENaCs, exists in mammals.

Glial cells and chemosensation in *C. elegans*

In the adult *C. elegans* hermaphrodite, there are 56 glial cells. The study of glia in *C. elegans* is still in its infancy, but elegant work to date has shown that glia are important for neuronal development and assembly (Shaham, 2006; Colon-Ramos *et al*, 2007; Yoshimura *et al*, 2008). For example, laser ablation of the amphid sheath cells leads to morphological abnormalities at the dendritic tips of several sensory neurons (T Bacaj and S Shaham, personal communication) (Shaham, 2006). We found that knockout of the glial sheath cell-expressed DEG/ENaC channel *acd-1* does not have an effect on viability or morphology of associated neurons; rather, deficiency specifically affects acid and lysine sensitivity.

How might glia control sensory function in nematodes through the activity of an ion channel? One possibility is that the glial ACD-1 may help DEG-1-expressing neurons ASK and ASH, and other acid-sensing sensory neurons (i.e. ASE and ADL; Sambongi *et al*, 2000), to respond to sensory cues and that ACD-1 regulation of the function of these neurons may depend on the pH of the environment. In support of this hypothesis are our data that show acid insensitivity of the *deg-1;acd-1* mutants (in which the activity of most acid-sensing neurons is possibly lost) being more severe than acid insensitivity of *deg-1* null, and our data that link behaviour to the pH sensitivity of ACD-1. Another question is how to reconcile the fact that *acd-1* knockout (loss of function) combined with *deg-1* null causes behavioural loss of sensitivity to acidic solutions, when ACD-1 channels are actually inhibited by acidic pH. We speculate that transient changes in ACD-1 activity such as those caused by exposure to acidic environments mediate sensory response, whereas permanent loss of the channel causes acid insensitivity through a mechanism similar to sensory adaptation.

How might ACD-1 help sensory neurons respond to sensory cues? One possibility is that ACD-1 inhibition causes amphid sheath cell membrane hyperpolarization that in turn affects sensory neuron function through reduced release of neuromodulators or other factors by the sheath cells. This model implies that amphid sheath cells in *C. elegans* would actually contain and use neuromodulators. Although this has not been shown so far, amphid sheath cells do contain vesicles suggestive of secretion (Perkins *et al*, 1986). Another possibility is that proton block of ACD-1 increases the extracellular K^+ concentration near sensory neuron dendrites through reduced activity of the Na^+/K^+ pump that relies on intracellular Na^+ concentration for function and that this localized change in K^+ environment changes sensory perception. Although our experiments do not distinguish between these possibilities, they clearly suggest a role of a glial-expressed channel in acid and lysine sensation. Future experiments in which the activity of sensory neurons and glia is probed by calcium imaging or electrophysiology will clarify the physiological consequences of *acd-1* channel knockout on glia and neuronal function. Could mammalian hINaC and BLINaC be expressed in glia and function similarly? Mouse and rat BLINaC messenger RNA is found in the forebrain, cerebellum and brainstem (Sakai *et al*, 1999)—this

channel may also be expressed in glia where it could have a function in controlling neuronal function.

Intracellular acidification and sour taste

Richter *et al* (2003) have shown that pH dose–response curves for Ca^{2+} transients elicited by sour stimuli in the mouse tongue differ depending on whether the acid stimulus used is HCl or citric acid, with the weaker citric acid being more potent. They argued that this is because weaker acids can more easily permeate the plasma membrane as undissociated species, thus causing more efficient acidification of the cytosol (Speake and Elliott, 1998). Thus, they conclude that taste receptor cells are responsive to both intracellular and extracellular acidification and suggest that intracellular acidification may have a key function in sour taste signal, as hypothesized earlier by Lyall *et al* (2001).

We found that the ACD-1 channel expressed in *Xenopus* oocytes was inhibited by both extracellular and intracellular acidification. When we tested the effect of strong and weak acid on behaviour, we found that acetic acid elicits a stronger response, suggesting that if stronger inhibition of the channel can be achieved by the action of both extracellular and intracellular protons, then the effect on behaviour is more robust. It is interesting though that the dose–response curves for pH effect on ACD-1 currents and animal behaviour do not overlap; this result may indicate that a nematode protein or factor tunes ACD-1 sensitivity to more acidic pH *in vivo* as shown for other DEG/ENaCs (reviewed in Bianchi and Driscoll, 2002). Although we have not identified the receptor for lysine, nor could we test whether DEG-1 is sensitive to this amino acid, we suggest that chemotaxis to lysine may be affected by ACD-1 inhibition by intracellular acidification. Thus, our results support the hypothesis of Richter *et al* (2003) that intracellular acidification may have a function in sour (and possibly umami) taste transduction.

Insights into the role of glia in sensory perception and pain

Our work is in line with this newly discovered role of glia in neuronal function. Indeed, recent studies have shown that glia are critical for the development of the nervous system and have a key function in neuronal disorders, including multiple sclerosis, amyotrophic lateral sclerosis, spinocerebellar ataxia, Parkinson's disease, Huntington's disease, ischaemia, neuropathic pain and epilepsy (reviewed in Iadecola and Nedergaard, 2007; Lobsiger and Cleveland, 2007; Rossi *et al*, 2007; Scholz and Woolf, 2007). Indirectly, glia regulate neuronal function by controlling the concentration of ions, neurotransmitters and modulators in the extracellular space (Kuffler, 1967; Cohen *et al*, 1968). Glial cells also modulate neuronal excitability directly, by releasing transmitters or co-agonists onto neurons (Volterra and Meldolesi, 2005; Vesce *et al*, 2007).

Our study proposes considerations on the mechanism of pain sensation associated with inflammation and tissue damage as well. Both these conditions produce a significant local increase of the proton concentration that causes the excitation of nociceptors that in turn evoke pain (Meyer *et al*, 2005). Our results suggest that the glial ion channels may participate in the amplification of the pain signal and thus may be considered as targets for the treatment of inflammation and neuropathic pain.

Materials and methods

Molecular biology

The *ACD-1::GFP* vector was created by introducing an *MscI* fragment including the *acd-1* promoter (2.55 kb prior to the start codon) and genomic sequence (minus stop codon) in frame with GFP into vector pPD95.77 (constructed by Scott Clark, NYU). To drive the expression of *ACD-1::GFP* in ASH neurons, we used the *sra-6* promoter (Troemel *et al*, 1995) and *acd-1* cDNA sequence, respectively. To construct *Pacd-1::ACD-1*, we substituted *acd-1* genomic sequence in *ACD-1::GFP* vector with *acd-1* cDNA. To drive the expression of ACD-1 in amphid sheath cells, we swapped the *sra-6* promoter with *vap-1* promoter (2 kb upstream of the start codon) and the promoter of the *T02B11.3* gene (2 kb upstream of the start codon) to generate *Pvap-1::ACD-1* and *PT02B11.3::ACD-1*. *DEG-1::GFP* was constructed by the amplification of the promoter (2.5 kb) and most genomic sequence (4.8 kb) of *deg-1* and subcloning into pPD95.75 vector in frame with GFP. This DNA construct is expected to be translated into a DEG-1 protein missing the second transmembrane domain and C terminus. The DEG-1 rescue construct (*Pdeg-1::DEG-1*) was generated by swapping the *deg-1* genomic sequence in *DEG-1::GFP* with *deg-1* cDNA sequence after the *BglIII* restriction site.

deg-1 cDNA was amplified from total *C. elegans* RNA using the RT-PCR Titan kit from Clontech. Restriction sites *BamHI* and *HindIII* were added to sense and antisense primers to allow subcloning into pGEM-HE. The 5' end of *acd-1* cDNA (from the start to 464 bp, corresponding to *SwaI* restriction site) was amplified similarly using the Clontech Titan kit, whereas the 3' end (from 464 bp to the stop codon) was obtained from EST clone yk1553g01, kindly provided by Yuji Kohara. D419N mutation was introduced into ACD-1 using QuikChange from Stratagene. Prior to subcloning into pGEM-HE, pPD95.75 or pPD95.77, RT-PCR products were cloned into TOPO pCR2.1 vector for amplification and sequencing.

C. elegans strains and growth

Nematode strains were maintained at 20°C on standard nematode growth medium seeded with *Escherichia coli* strain OP50 (Brenner, 1974). For GFP expression and ACD-1::GFP rescue studies, plasmids were injected into *lin-15(n765)* and *deg-1(u38u421);acd-1(bz90)* strains, respectively. Mutant strain *deg-1(u38u421);acd-1(bz90)* was obtained by standard crossing. Mutations were followed and confirmed by PCR. *deg-1(u38u421)* allele likely encodes for a non-functional, as it suppresses neurodegeneration caused by neurotoxic hypermorphic *deg-1(u38)* mutant (Chalfie and Wolinsky, 1990; Hart *et al*, 1999). *acd-1(bz90)* deletion mutant strain was outcrossed three times.

Dye-filling experiments

Animals were picked to a plate containing 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine, 4-chlorobenzinesulphonate (DiO; Molecular Probes) diluted in M9 buffer (in mM: 85 NaCl, 42.3 Na₂HPO₄ and 22.2 KH₂PO₄) (0.01 mg/ml) and allowed to stain for 3 h at room temperature. Worms were then transferred to an agar plate and allowed to crawl on the bacterial lawn for 15 min to destain. Photographs were taken with a Leica microscope equipped with digital camera using rhodamine and GFP filters.

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Behavioural assays

A drop of M13 solution (30 mM Tris, 100 mM NaCl, 10 mM KCl) at a specific pH was delivered near the tail of the forward-moving animal on an unseeded plate. When the solution was acidic, the animal reversed; otherwise it continued to move forward. Each animal was tested with five drops. We waited for at least 1 min between each drop. The response to each drop delivered was recorded as either a positive or negative response and the animal avoidance index was calculated (AI = positive/total drops). We also expressed the data as ratio of animals responding (a 'responder' responded to at least three out of five trails). For lysine acetate and Na acetate assays, we followed procedures described in Bargmann and Horvitz (1991). Chemotaxis index (CI) was determined as follows: (number of animals at attractant – number of animals at control)/(total number of animals).

Oocyte expression and electrophysiology

Capped cRNAs were synthesized using T7 mMACHINE kit (Ambion), purified (Qiagen RNeasy columns) and run on denaturing agarose gels to check for size and integrity. cRNA quantification was then performed spectroscopically. Stage V–VI oocytes were manually defolliculated after selecting them among multistaged oocytes dissected by 2 h collagenase treatment (2 mg/ml in Ca²⁺-free OR2 solution) from *Xenopus laevis* ovaries (NASCO). Oocytes were incubated in OR2 media (82.5 mM NaCl, 2.5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 1 mM Na₂HPO₄, 0.5 g/l polyvinyl pyrrolidone and 5 mM HEPES, pH 7.2), supplemented with penicillin and streptomycin (0.1 mg/ml) and 2 mM Na pyruvate. Oocytes were injected with 52 nl of cRNA mix for a final amount of 5 ng/oocyte. Oocytes were incubated in OR2 at 20°C for 2–4 days before recording.

Currents were measured using a two-electrode voltage clamp amplifier (GeneClamp 500B; Axon Instruments) at room temperature. Electrodes (0.3–1 M) were filled with 3 M KCl and oocytes were perfused with a NaCl solution containing (in mM): 100 NaCl, 2 KCl, 1 CaCl₂, 2 MgCl₂, 10 HEPES, pH 7.2 or with a CaCl₂ solution containing 73 CaCl₂, 2 KCl, 10 HEPES, pH 7.2. When we tested solutions at pH lower than 6, we used 10 mM 2-(*N*-morpholino)ethanesulphonic acid instead of 10 mM HEPES to buffer the solutions. We used the pCLAMP suite of programs (Axon Instruments) for data acquisition and analysis. Currents were filtered at 200 Hz and sampled at 1 kHz.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

Acknowledgements

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