Knockout of glial channel ACD-1 exacerbates sensory deficits in a *C. elegans* mutant by regulating calcium levels of sensory neurons

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Wang Y, D’Urso G, Bianchi L. Knockout of glial channel ACD-1 exacerbates sensory deficits in a *C. elegans* mutant by regulating calcium levels of sensory neurons. *J Neurophysiol* 107: 148–158, 2012. First published October 12, 2011; doi:10.1152/jn.00299.2011.—Degenerin/epithelial Na$^+$ channels (DEG/ENaCs) are voltage-independent Na$^+$ or Na$^+$/Ca$^{2+}$ channels expressed in many tissues and are needed for a wide range of physiological functions, including sensory perception and transepithelial Na$^+$ transport. In the nervous system, DEG/ENaCs are expressed in both neurons and glia. However, the role of glial vs. neuronal DEG/ENaCs remains unclear. We recently reported the characterization of a novel DEG/ENaC in *Caenorhabditis elegans* that we named ACD-1. ACD-1 is expressed in glial amphid sheath cells. The glial ACD-1, together with the neuronal DEG/ENaC DEG-1, is necessary for acid avoidance and attraction to lysine. We report presently that knockout of *acd-1* in glia exacerbates sensory deficits caused by another mutant; the hypomorphic allele of the cGMP-gated channel subunit *tax-2*. Furthermore, sensory deficits caused by mutations in *G$_i$* protein *odr-3* and guanylate cyclase *daf-11*, which regulate the activity of TAX-2/TAX-4 channels, are worsened by knockout of *acd-1*. We also show that sensory neurons of *acd-1* *tax-2;p694* double mutants fail to undergo changes in intracellular Ca$^{2+}$ when animals are exposed to low concentrations of attractant. Finally, we show that exogenous expression of TRPV1 in sensory neurons and exposure to capsaicin rescue sensory deficits of *acd-1* *tax-2;p694* mutants, suggesting that sensory deficits of these mutants are bypassed by increasing neuronal excitability. Our data suggest a role of glial DEG/ENaC channel ACD-1 in supporting neuronal activity.

Degenerin/epithelial Na$^+$ channels (DEG/ENaCs) are voltage-independent Na$^+$ or Na$^+$/Ca$^{2+}$ channels composed of three identical or homologous subunits (Jasti et al. 2007) that participate in a large variety of physiological functions, including sensory perception (touch, pain, taste; Chandrashekar et al. 2010; Chalfie 2009; Chen et al. 2002; Price et al. 2000; Price et al. 2001; Sluka et al. 2003), learning and memory (Wemmie et al. 2002), and transepithelial Na$^+$ transport (Hummel et al. 1996). DEG/ENaCs have been found in glia: 1) messenger RNA and protein for DEG/ENaC subunits were found in retinal Muller cells (Brockway et al. 2002), and the contribution of DEG/ENaCs in retinal function has been suggested based on the finding that amiloride, a DEG/ENaC blocker, alters electroretinograms (Brockway et al. 2005). 2) DEG/ENaCs are expressed in Schwann cells associated with Ruffini’s endings of the rat incisor (Hitomi et al. 2009), and ASIC2 is expressed in the inner core lamellae (thought to be of glial origin) of the Pacinian corpuscles (Calavia et al. 2010). 3) Several members of the DEG/ENaC family of channels are expressed in astrocytes and gliomas and have been suggested to play a role in glioma malignancy (Berdiev et al. 2003; Vila-Carriles et al. 2006, 2007).

We recently reported the cloning and characterization of a novel DEG/ENaC channel gene from *Caenorhabditis elegans* that we named *acd-1* (Wang et al. 2008). ACD-1 is expressed in amphid sheath cells (and at low levels also in the spermatheca, not shown), a pair of glial cells that ensheath the sensory dendrites of 12 pairs of *C. elegans* sensory neurons. We found that knockout of *acd-1* in glia worsens the reduced sensitivity to acidic environments and lysine of *deg-1* null mutants. We undertook this study to test the prediction that knockout of *acd-1* in glia worsens sensory deficits caused by mutations in other sensory neurons genes and to shed light on the mechanism of ACD-1-mediated glia regulation of the function of sensory neurons. We show in this study that knockout of *acd-1* channel in glia exacerbates sensory deficits of a hypomorphic mutant of *tax-2*, the $\beta$-subunit of a cGMP-gated channel, needed in *C. elegans* for chemotaxis to odors and tastants (Bargmann et al. 1993; Coburn and Bargmann 1996). We also show that knockout of *acd-1* exacerbates sensory deficits caused by mutations in genes that encode signaling molecules, the $G_i$ protein *ODR-3* and the guanylate cyclase protein DAF-11, which have been proposed to regulate the activity of the TAX-2/TAX-4 channel (Bargmann 2006). Finally, we show that exogenous depolarization and increase in intracellular Ca$^{2+}$ of the sensory neurons rescue the sensory deficits of *acd-1* *tax-2;p694* double-mutant animals. Together, these data show that when *acd-1* is knocked out, sensory deficits caused by mutations in sensory neurons genes are
exacerbated, suggesting a role of glial AC-1 in supporting neuronal function. Increasing sensory neurons excitability through another mechanism bypasses the AC-1 requirement.

MATERIALS AND METHODS

Molecular biology. The construction of P_acd::AC-1 has been described previously (Wang et al. 2008). We constructed P_cry-6:: TRPV1 using a previously described construct that drives expression of rat TRPV1 in ASE neurons (Suzuki et al. 2008). To target expression of TRPV1 in AWC neurons, we replaced the promoter of gcy-6, functional in ASES (Yu et al. 1997), with the promoter of the odr-3 gene (1.5 kb upstream of the start codon) upstream of rat TRPV1 in the pd115.118 Fire vector. To construct P_odr-3:: TAX-2, we swapped the TRPV1 sequence with TAX-2 cDNA sequence.

The construction of AC-1::GFP plasmid was cloned by RT-PCR. To drive expression of GCaMP in amphid sheath glia, we inserted the GCaMP1.0 sequence in pPD95.75 Fire vector downstream of the promoter of the odr-3 gene, which functions in glial amphid sheath cells (Perens and Shaham 2005).

C. elegans strains and growth. Nematode strains were maintained at 20°C on standard nematode growth medium (NGM) seeded with Escherichia coli strain OP50 (Brenner 1974). Wild-type animals were N2 Bristol. Other strains used were ZB90 acd-1(bz90) I; PR691 tax-2(p691) I; PR694 tax-2(p694) I; CX2205 odr-3(n2150) V; DR47 def-11(m47) V; BLC20 acd-1(bz90) I tax-2(p694) I; BLC135 acd-1(bz90) I; odr-3(n2150) V; BLC136 acd-1(bz90) I def-11(m47) V; BLC139 excEx77 [P_acd::V-1; P_str-2::GFP]; BLC140 acd-1(bz90) I tax-2(p694) I; excEx76 [P_odr-3::V-1; P_str-2::GFP]; BLC145 acd-1(bz90) I tax-2(p694) I; kyEx87 [str-2::GCaMP1.0, unc-122::GFP]; excEx69 [P_odr-3::V-1; pRF4]; BLC147 acd-1(bz90) I tax-2(p694) I; excEx19 [P_Vap-1::ACD-1::RFP; P_str-2::GFP; P_vap-1::ACD-1::GFP]; BLC134 acd-1(bz90) I tax-2(p694) I; kyEx87 [str-2::GCaMP1.0, unc-122::GFP]; excEx66 [P_Vap-1::ACD-1; pRF4]; DA1297 lin-15B(n765) X; adEx1297 [lin-15[+] gcy-6::GFP]; BLC187 tax-2(p694) I; adEx1288 [lin-15[+] gcy-7::GFP]; BLC137 acd-1(bz90) I tax-2(p694) I; adEx1288 [lin-15[+] gcy-6::GFP]; BLC167 acd-1(bz90) I tax-2(p694) I; kyEx87 [str-2::GCaMP1.0, unc-122::GFP]; BLC176 acd-1(bz90) I tax-2(p694) I; kyEx87 [str-2::GCaMP1.0, unc-122::GFP]; BLC187 klyIs150 IV [tax-2::delta::GFP]; BLC115 acd-1(bz90) I; klyIs150 IV [tax-2::delta::GFP]; OS1984 lin-15B(n765) X; nsEx1111 [lin-15[+] P_Vap-1::GCaMP1.0]; and BLC40 acd-1(bz90) I; nsEx1111 [lin-15[+] P_Vap-1::GCaMP1.0]. Germline transformation was carried out as described previously (Mello et al. 1991), and double mutants were generated by standard genetic crosses. Mutations were followed through the crosses by PCR. acd-1(bz90) is referred to as “acd-1” in figures and text for simplicity.

Behavioral assays. For chemotaxis and avoidance assays, we followed previously described procedures (Bargmann et al. 1993; Bargmann and Horvitz 1991; Troemel et al. 1995). For NaCl avoidance assays, a chunk of agar 1 cm in diameter was removed from 10-cm plates and soaked in the attractant for 3 h. Chunks were put back in the plate overnight to allow equilibration and formation of a gradient. For isoamyl alcohol and trimethylthiazole assays, 1 μl of odor at the indicated dilution was placed on one side of the plate. Thirty worms were placed between the test spot and a control spot on the opposite side of the plate. Ten microliters of 20 mM Na3N were placed on both spots to anesthetize animals once they reached the spot. After 1 h, animals on each side of the plate were counted and an attraction index was determined as follows: (number of animals at attractant — number of animals at control)/(total number of animals). When capsaicin was used, 200 μl of 50 μM capsaicin diluted in water were spread on the plate 10 min before the animals were transferred. For octanol avoidance assays, we dipped an eyelash hair glued on a toothpick in octanol and placed it in front of a forward-moving animal on a plate without food. We measured the time it took for the animal to respond to the odor by reversing direction (Troemel et al. 1995). Odors were diluted in ethanol. Statistical significance was determined by ANOVA.

Ca2+ imaging. Animals were glued on glass coverslips using surgical glue (Glutue or Nexcare 3M) and immediately immersed in M13 buffer. Coverslips were then transferred to a perfusion chamber mounted on a Nikon Eclipse E600FN microscope equipped with green fluorescent protein (GFP) filters. We imaged the cell body of AWC neurons and of amphid sheath cells (Chalasani et al. 2007). Animals were perfused using a gravity perfusion system at a rate of ~75 μl/s with S-Basal buffer (100 mM NaCl, 50 mM K-phosphate, pH 6) or S-Basal plus isoamyl alcohol and capsaicin at the indicated dilutions and concentrations. Based on the size of the perfusion tubes and chamber, the level of solution in the chamber and the level of solution in the syringes, it took 20–40 s to completely exchange the solution in the bath. Images were acquired using TILLvisION version 3.3 software and the Imago charge-coupled device camera (T.I.L.L. Photonics). Images were acquired at 1 Hz, and typical exposure time was 30–100 ms. The image stacks were then analyzed using ImageJ. First, images were registered using ImageJ TurboReg to correct for movement. The region of interest corresponding to each cell was then selected according to the shape and position of the cells. The fluorescence intensity of the region of interest was measured and output to a data log file. Origin 6.1 was used to plot the data created by ImageJ.

Xenopus oocytes expression and electrophysiology. acd-1 cRNA synthesis and expression in Xenopus oocytes was achieved as previously described (Wang et al. 2008; Wang and Bianchi 2009). Briefly, capped RNA was synthesized using the T7 mMESSAGE mMAChINE kit (Ambion), purified (Qiagen RNAeasy columns), and run on denaturing agarose gels to check for size and cRNA integrity. cRNA quantification was then performed spectroscopically. Stage V and VI oocytes were selected among multistaged oocytes dissected by 2-h collagenase treatment (2 mg/ml in Ca2+-free OR2 solution) from Xenopus laevis ovaries. Oocytes were incubated in OR2 medium, which consists of 82.5 mM NaCl, 2.5 mM KCl, 1 mM CaCl2, 1 mM MgCl2, 1 mM NaH2PO4, 0.5 g/l polyvinyl pyroloide, and 5 mM HEPES (pH 7.2), supplemented with penicillin and streptomycin (0.1 mg/ml) and 2 mM Na-pyruvate. Oocytes were then injected with 60 nl of cRNA 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 (0.2–0.5 M) were filled with 3 M KCl, and oocytes were perfused with a solution containing (in mM) 100 NaCl, 2.5 mM KCl, 1 mM CaCl2, 1 mM MgCl2, 1 mM NaH2PO4, 0.5 g/l polyvinyl pyrolidone, and 5 mM HEPES (pH 7.2), supplemented with penicillin and streptomycin (0.1 mg/ml) and 2 mM Na-pyruvate. Oocytes were then injected with 69 nl of cRNA for a final amount of 5 ng/oocyte. Oocytes were incubated in OR2 at 20°C for 2–4 days before recording.

RESULTS

Knockout of glial acd-1 exacerbates sensory deficits caused by a hypomorphic allele of tax-2. We previously reported the cloning and characterization of ACD-1, a novel DEG/ENaC channel subunit expressed in C. elegans amphid glia (Wang et al. 2008). We found that knockout of acd-1 in glia exacerbates sensory deficits caused by a deg-1 null mutation (Chalfie and...
We thus acquired the tax-2(p694) mutant strain, crossed it with acd-1 knockout to generate acd-1 tax-2(p694) double-mutant animals, and tested the function of sensory neurons. tax-2 encodes for a β-subunit of a cGMP-gated channel expressed in several C. elegans sensory neurons. tax-2, together with the α-subunit gene tax-4, is necessary for chemotaxis to water-soluble attractants and odors, including Na\(^+\) and isoamyl alcohol, and for avoidance of repellents (Bargmann et al. 1993; Coburn and Bargmann 1996). tax-2(p694) is a hypomorphic allele in which part of the promoter region and the first exon of tax-2 are deleted. Indeed, the expression of a tax-2 GFP transgene that mimics the molecular lesion of tax-2(p694) allele, termed tax-2(delta)::GFP, is strongly reduced in AQR, AFD, ASE and BAG neurons (Coburn and Bargmann 1996).

When we assayed attraction to 0.2 M Na\(^+\), we found that tax-2(p694) animals were less attracted than wild-type animals to this concentration of Na\(^+\) [Fig. 1A, means ± SE (SD): N2, 0.55 ± 0.04 (0.16); tax-2(p694), 0.24 ± 0.04 (0.16); P < 0.01]. This was not surprising, since tax-2(p694) strongly reduces expression of TAX-2 in ASE neurons, the principal chemosensory neurons that drive attraction to Na\(^+\) (Bargmann and Horvitz 1991; Suzuki et al. 2008). Knockout of acd-1 exacerbated the reduced sensitivity to Na\(^+\) of tax-2(p694) animals. acd-1 tax-2(p694) double-mutant animals were completely insensitive to 0.2 M Na\(^+\) [less sensitive than the sum of tax-2(p694) and acd-1 reduced sensivities] and distributed randomly on a plate in which a Na\(^+\) gradient with a peak of 0.2 M was established [Fig. 1A, −0.019 ± 0.040 (0.12); P < 0.05 compared with tax-2(p694); P < 0.01 compared with acd-1]. This phenotype was rescued by expression of ACD-1 in amphid sheath cells of acd-1 tax-2(p694) mutants [Fig. 1A, 0.380 ± 0.03 (0.12)]. At 0.4 M Na\(^+\), we found that tax-2(p694), acd-1, and acd-1 tax-2(p694) single- and double-mutant animals were attracted to Na\(^+\) similarly to wild-type animals. The strong allele tax-2(p691) had severely impaired chemotaxis to 0.4 M Na\(^+\) (Coburn and Bargmann 1996) [Fig. 1A, N2, 0.525 ± 0.04 (0.153); tax-2(p694), 0.418 ± 0.028 (0.076); acd-1, 0.468 ± 0.062 (0.177); acd-1 tax-2(p694), 0.488 ± 0.048 (0.127); tax-2(p691), 0.120 ± 0.057 (0.11); P < 0.01 compared with N2].

tax-2(delta)::GFP is not expressed in AQR, AFD, ASE, and BAG neurons, which normally express TAX-2 (Coburn and Bargmann 1996). However, TAX-2 expression may be reduced in other sensory neurons in tax-2(p694) mutants. Indeed, the tax-2(delta)::GFP and tax-2::GFP expression levels cannot be strictly compared for small changes because different transgenes could be present in different copy number, thus influ-

![Fig. 1. Knockout of glial channel acd-1 worsens reduced attraction to Na\(^+\) of tax-2(p694) mutants. A: chemotaxis to 0.2 and 0.4 M Na\(^+\) was determined for wild-type Caenorhabditis elegans (N2) and for acd-1, tax-2(p694), acd-1 tax-2(p694), acd-1 tax-2(p694); Propal-1::ACD-1, and tax-2(p691) mutants. Number of assays was 15, 11, 7, 7, and 12 for 0.2 M Na\(^+\) and 14, 7, 8, 7, and 4 for 0.4 M Na\(^+\), respectively, with 30 animals used in each assay. tax-2(p694) mutant animals were less attracted to 0.2 M Na\(^+\) than other animals, and knockout of acd-1 worsened this chemotaxis defect. The chemotaxis index for acd-1 knockout animals is not statistically different from that of wild type. Data are means ± SE. *P < 0.05; **P < 0.01 (ANOVA). Chemotaxis to Na\(^+\) is mediated primarily by ASE neurons and to a lesser extent by ASG, ASI, and ADF neurons (Bargmann and Horvitz 1991). B: repulsion by octanol was normal in acd-1 tax-2(p694) double mutants but defective in tax-2(p691) mutants. The time that animals took to respond to octanol at 3 dilutions was recorded. Number of animals tested was 10, 10, and 16 for 10% octanol; 38, 20, and 25 for 30% octanol, and 25, 20, and 26 for 100% octanol. Data are means ± SE. *P < 0.05; **P < 0.01 (ANOVA). Octanol is sensed by AWB, ASH, and ADL neurons (Troemel et al. 1997). C: attraction to trimethylthiazole mediated by AWA neurons was normal in tax-2(p691) and acd-1 tax-2(p694) mutant animals. Attraction to trimethylthiazole at 3 dilutions was assayed. Wild type and mutants did not respond to the most diluted concentration. Number of assays was 4, 3, and 3 for 1E-5 dilution; 6, 3, and 3 for 1E-4 dilution; and 3, 4, and 3 for 1E-3 dilution with 30 animals used in each assay. Data are means ± SE.](http://jn.physiology.org/lookup/doi/10.1152/jn.00299.2011)
enceling GFP expression level. We thus considered the possibility that TAX-2 expression level in tax-2(p694) could be reduced in other neurons, resulting in a effect of glial -expression level on the sensitivity to other sensory cues. For example, TAX-2 is expressed in AWB neurons (Coburn and Bargmann 1996) that mediate avoidance of octanol (together with ASH and ADL neurons that do not express TAX-2) (Troyemel et al. 1997). We found that cad-1 tax-2(p694) double-mutant animals responded normally to three different dilutions of this odor. However, tax-2(p691) showed reduced repulsion by octanol [Fig. 1B, at 10%: N2, 3.57 ± 0.040 (1.20); cad-1 tax-2(p694), 3.20 ± 0.35 (1.13); tax-2(p691), 7.93 ± 1.3 (5.23); P < 0.05; at 30%: N2, 2.26 ± 0.21 (1.32); cad-1 tax-2(p694), 2.60 ± 0.18 (0.82); tax-2(p691), 3.32 ± 0.19 (0.94); P < 0.01; at 100%: N2, 1.60 ± 0.14 (0.70), cad-1 tax-2(p694), 1.70 ± 0.20 (0.92); tax-2(p691), 2.65 ± 0.19 (1.00); P < 0.01]. These results show that TAX-2 functions in AWB neurons to mediate response to octanol. They also suggest that glial cad-1 has no effect on the activity of these sensory neurons. As a control, we also tested attraction to the odor trimethylthiazole, which is sensed by AWA neurons [Bargmann 1993]. AWA neurons do not express TAX-2 (Coburn and Bargmann 1996). Attraction to three dilutions (1E-5, 1E-4, and 1E-3) of trimethylthiazole was similar to wild type in both cad-1 tax-2(p694) and tax-2(p691) mutants [Fig. 1C, at 1E-5: N2, 0.025 ± 0.040 (0.81); cad-1 tax-2(p694), −0.003 ± 0.093 (0.16); tax-2(p691), 0.023 ± 0.049 (0.086); at 1E-4: N2, 0.40 ± 0.03 (0.06); cad-1 tax-2(p694), 0.42 ± 0.11 (0.20); tax-2(p691), 0.390 ± 0.008 (0.01); at 1E-3: N2, 0.71 ± 0.05 (0.08); cad-1 tax-2(p694), 0.79 ± 0.05 (0.09); tax-2(p691), 0.69 ± 0.06 (0.11)]. In conclusion, we found that knockout of cad-1 in glia worsened the reduced sensitivity of tax-2(p694) animals to 0.2 M Na+ mediated primarily by ASE, where TAX-2 expression level is strongly reduced in tax-2(p694) mutants. However, it did not affect sensory perception mediated by neurons that do not express TAX-2 (AWA). Our data on octanol avoidance also suggest that knockout of cad-1 does not indiscriminately affect the function of all sensory neurons in which TAX-2 is expressed and that are ensheathed by amphid glia, as also shown by Baca et al. (2008).

- cad-1 tax-2(p694) double mutants have reduced sensitivity to the odor isoamyl alcohol. We next tested the sensitivity of tax-2(p694) and cad-1 single- and double-mutants to the odor isoamyl alcohol. AWC sensory neurons sense isoamyl alcohol (Bargmann et al. 1993). tax-2(delta):: GFP is expressed in AWC neurons, suggesting that the p694 allele does not eliminate the expression of TAX-2 in these neurons (Coburn and Bargmann 1996). However, based on the argument made above about GFP reporters, TAX-2 expression in AWC neurons of tax-2(p694) animals could still be reduced, rendering AWC neurons of this mutant susceptible to knockout of cad-1 in glia. We assayed chemotaxis to isoamyl alcohol at three different dilutions: 1E-3, 1E-2, and 1E-1. We found that tax-2(p694) and cad-1 single mutants responded to all the three dilutions of isoamyl alcohol similarly to wild type [Fig. 2A, at 1E-3: N2, 0.446 ± 0.042 (0.120); tax-2(p694), 0.520 ± 0.029 (0.060); cad-1, 0.497 ± 0.010 (0.020); at 1E-2: N2, 0.640 ± 0.033 (0.12); tax-2(p694), 0.680 ± 0.062 (0.170); cad-1, 0.662 ± 0.044 (0.140); at 1E-1: N2, 0.657 ± 0.052 (0.100); tax-2(p694), 0.581 ± 0.090 (0.220); cad-1, 0.703 ± 0.091 (0.150)]. However, cad-1 tax-2(p694) double mutants were less attracted to isoamyl alcohol at the 1E-3 and 1E-2 dilutions compared with wild type and single mutant animals [at 1E-3: 0.157 ± 0.029 (0.270), P < 0.01; at 1E-2: 0.340 ± 0.029 (0.120), P < 0.01]. The severe allele tax-2(p691) had strongly compromised sensitivity to isoamyl alcohol at all the dilutions tested [Fig. 2A, at 1E-3: −0.007 ± 0.046 (0.09), P < 0.01; at 1E-2: 0.120 ± 0.048 (0.120), P < 0.01; at 1E-1: 0.070 ± 0.057 (0.100), P < 0.01]. We conclude that knockout of glial DEG/ENaC channel cad-1 combined with neuronal tax-2(p694) mutation renders animals less sensitive to isoamyl alcohol. Notably, the reduced sensitivity to isoamyl alcohol of cad-1 tax-2(p694) mutants was rescued by expression of ACD-1 under the control of glial promoter vcp-1 (Wang et al. 2008) and by expression of TAX-2 under the control of the promoter of the odr-3 gene expressed in AWC in addition to AWA, AWB, ASH, and ADF neurons (Roayaie et al. 1998) [Fig. 2A, cad-1 tax-2(p694); Pcp-1::ACD-1, 0.647 ± 0.044 (0.110); cad-1 tax-2(p694); Podr-3::TAX-2, 0.860 ± 0.047 (0.110)]. These data show that the reduced attraction to diluted isoamyl alcohol observed in cad-1 tax-2(p694) double-mutant animals is due to impaired and lack of function of TAX-2 and ACD-1 in AWC neurons and amphid glia, respectively, in these mutants. To streamline our approach and interpretations, for the remaining of the study we mostly focused on chemotaxis to isoamyl alcohol, which is mediated by only one pair, and not multiple pairs, of sensory neurons, the AWC neurons.

Knockout of cad-1 worsens sensory deficits caused by mutations in genes that regulate TAX-2 function. Genetic studies suggest that the cyclic nucleotide-gated channel encoded by the tax-4 and tax-2 genes is a sensory transduction channel downstream of G protein signaling. The proposed pathway for sensory transduction is that signaling by G protein odr-3 regulates the function of either a cGMP phosphodiesterase or a membrane guanylate cyclase encoded by the gene daf-11, resulting in closing or opening of the GMP-gated channel (Roayaie et al. 1998; Vowels and Thomas 1994). Given that ODR-3 and DAF-11 regulate the activity of TAX-2/TAX-4, we hypothesized that knockout of cad-1 in glia worsens sensory deficits caused by mutations in odr-3 and daf-11. We crossed the cad-1 knockout strain with odr-3(n2150) and daf-11(m47) mutants and assayed attraction to isoamyl alcohol (dilution 1E-2). Both these mutants have strongly reduced but not complete loss of sensitivity to isoamyl alcohol (Roayaie et al. 1998; Vowels and Thomas 1994). We found that the cad-1;odr-3(n2150) and cad-1;daf-11(m47) double-mutant animals displayed an exacerbated phenotype [Fig. 2B, odr-3(n2150), 0.279 ± 0.075 (0.270); cad-1;odr-3(n2150), 0.099 ± 0.037 (0.130); P < 0.05; daf-11(m47), 0.297 ± 0.043 (0.140); cad-1;daf-11(m47), 0.047 ± 0.100 (0.330); P < 0.05]. Thus knockout of cad-1 in glia worsens sensory deficits caused not only by mutations in tax-2 but also by mutations in G protein gene odr-3 and guanylate cyclase gene daf-11, whose gene products regulate the activity of TAX-2/TAX-4 channels.

TAX-2 expression level and AWC neuron structure are normal in cad-1 tax-2(p694) mutants. We previously showed that knockout of glial cad-1 does not cause structural defects in the glial amphid sheath cells and ASH neuron dendrites, including the cilia (Wang et al. 2008). However, we wanted to confirm that AWC sensory neurons are intact in tax-2(p694) and cad-1 single and double mutants. We also analyzed tax-2(delta)::GFP in AWC neurons to establish whether knockout...
of glial acd-1 causes changes in its expression level. In this case the comparison of GFP expression levels is appropriate because the same transgene is analyzed in wild-type and acd-1 knockout genetic backgrounds, so changes in GFP fluorescence are attributable only to the difference in genetic makeup of the strains and not to the number of copies of the transgene.

We found that the typical fanlike structure of AWC neuron cilia (Perkins et al. 1986) was preserved in tax-2(p694) and acd-1 as well as in acd-1 tax-2(p694) mutants (Fig. 3A). We also found that the level of expression of tax-2(delta)::GFP was not affected by knockout of acd-1 in glia (Fig. 3B–D). We conclude that knockout of acd-1 in glia does not cause defects in the structure of AWC sensory neurons or changes in the level of expression of the cGMP-gated channel subunit TAX-2 in AWC neurons.

Ectopic axons in sensory neurons of acd-1 tax-2(p694) mutants. It was previously reported that mutations in tax-2 cause abnormal axonal branching of sensory neurons. Ectopic axons were found in different sensory neurons of tax-2(p691) (Coburn et al. 1998). We found that 24.6% of the acd-1 tax-2(p694) animals had ectopic ASE left (ASEL) axons, and this percentage was higher (50%) in tax-2(p691) animals [Fig. 3, E and F; acd-1 tax-2(p694); podr-3::TAX-2 and acd-1 tax-2(p694); P_vap-1::ACD-1 animals was also determined. Number of assays was 9, 4, 4, 13, and 4 for 1E-3 dilution; 14, 8, 17, 6, 7, and 7 for 1E-2 dilution; and 4, 6, 3, 3, and 3 for 1E-1 dilution with 30 animals used in each assay. AWC neurons mediated attraction to isoamyl alcohol. **P < 0.01 compared with wild type (ANOVA). Data are means ± SE. B: attraction to isoamyl alcohol at 1E-2 dilution was assayed as described in A. Number of assays for wild type (N2), odr-3(n2150), acd-1;odr-3(n2150), daf-11(m47), and acd-1;daf-11(m47) animals was 12, 13, 13, 10, and 11, respectively, with 30 animals used in each assay. Data are means ± SE. *P < 0.05 compared with odr-3 and daf-11 single mutants (ANOVA).
the behavioral phenotypes, corroborating that ectopic axons reflect reduced functionality of the sensory neurons.

In vivo Ca\textsuperscript{2+}/H\textsubscript{11001} imaging reveals that AWC neurons of acd-1 tax-2(p694) mutants do not respond to diluted isoamyl alcohol. Given the reduced attraction of acd-1 tax-2(p694) double-mutant animals expressing gcy-6::GFP in the ASE left (ASEL) neuron, ASEL mediates attraction to Na\textsuperscript{+} (Suzuki et al. 2008). The arrow points to an ectopic axon. Scale bar, 50 \mu m. E: the percentage of animals showing ectopic axons in AWC\textsubscript{m} neurons was also quantified in wild type (N2), tax-2(p694), acd-1, acd-1 tax-2(p694), and tax-2(p691) animals. Total number of animals analyzed was 18, 34, 27, 16, and 40, respectively. AWC\textsubscript{m} neurons express GCaMP1.0 under the control of \textit{str-2} (Chalasani et al. 2007). Data are means ± SE. *P < 0.05; **P < 0.01 (ANOVA). Note that acd-1 is not statistically different from wild type in ASEL ectopic axons quantification.
tax-2(p694) double mutants failed to respond to the 1E-6 dilution (only 1/22 responded with a $\Delta F/F$ of 0.033), whereas 9/20, 7/14, and 7/17 responded in wild-type, tax-2(p694), and acd-1 animals, respectively [Fig. 4B, inset, in $\Delta F/F$: N2, 0.0098 ± 0.0029 (0.0132); tax-2(p694), 0.0108 ± 0.0032 (0.0122); acd-1, 0.0152 ± 0.0056 (0.0234); acd-1 tax-2(p694), 0.0015 ± 0.0015 (0.0077); $P < 0.05$ compared with N2 or single mutants]. We conclude that acd-1 tax-2(p694) reduced attraction to diluted isomyl alcohol is due to reduced responsiveness of AWC neurons to low concentrations of this attractant. Importantly, Ca$^{2+}$ transients in response to 1E-6 isomyl alcohol were restored in acd-1 tax-2(p694) transgenic animals expressing ACD-1 in amphid glia [Fig. 4B, inset, in $\Delta F/F$: 0.0091 ± 0.0019 (0.0061)]. Note that the Ca$^{2+}$ transients elicited in wild-type animals are smaller than the ones observed by Chalasani et al. (2007). This difference may be related to the different perfusion system used in our experiments. Our data also suggest that the 1E-2 dilution of isomyl alcohol in chemotaxis assays probably results in a concentration of 1E-6 of this odor at a 5-cm distance from the assay spot. Behavioral and AWC Ca$^{2+}$ defects in acd-1 tax-2(p694) mutants were detected in chemotaxis and Ca$^{2+}$ imaging assays, respectively, at these two concentrations. As expected, AWC neurons of tax-2(p691) mutants did not respond to the highest concentration of isomyl alcohol [Fig. 4B, in $\Delta F/F$: 0.0076 ± 0.0045 (0.0155)], supporting the possibility that the lack of attraction of tax-2(p691) mutants to isomyl alcohol is due to the insensitivity of AWC neurons in this mutant to this odor.

Glial amphid sheath cells and ACD-1 channels are sensitive to the odor isomyl alcohol, but at higher concentrations. One simple model of how ACD-1 channel expressed in glia may contribute to AWC neurons sensitivity to isomyl alcohol would be that ACD-1 channels are themselves sensitive to isomyl alcohol. To test this possibility, we assayed ACD-1 sensitivity to isomyl alcohol in the Xenopus oocytes expression system. We found that ACD-1 currents were inhibited by perfusion with isomyl alcohol (Fig. 5, A and B). However, the $K$, was 38 mM, corresponding to a dilution of ~1.7E-3 (Fig. 5C), a concentration that is ~1,700 times higher than the concentration at which AWC neurons of acd-1 tax-2(p694) mutants fail to respond to isomyl alcohol (Fig. 4). Do glial amphid sheath cells respond to isomyl alcohol, and if they do, does ACD-1 channel mediate this response? We looked at Ca$^{2+}$ responses in glial amphid sheath cells on perfusion of animals with isomyl alcohol at 1E-4 and 1E-3 dilutions. We found that isomyl alcohol at 1E-3 dilution caused a reduction in Ca$^{2+}$ concentration. However, knockout of acd-1 did not significantly alter these Ca$^{2+}$ changes, suggesting that acd-1 does not contribute to this response [Fig. 5, D–F, in $\Delta F/F$: N2, −0.111 ± 0.03 (0.10); acd-1, −0.098 ± 0.030 (0.08)]. We conclude that glial amphid sheath cells respond to the odor isomyl alcohol by reduction of intracellular Ca$^{2+}$ concentra-
tion. However, this response is not mediated by ACD-1 channels, and it is elicited by concentrations of the odor that are above the concentrations at which AWC neurons of acd-1 tax-2(p694) mutants fail to respond. The sensitivity of glial amphid sheath cells to odors needs further investigation but does not seem to contribute to the phenotype that we observe in acd-1 tax-2(p694) double-mutant animals.

Activation of rat TRPV1 expressed in AWC neurons by capsaicin ameliorates the chemotaxis defects of acd-1 tax-2(p694) mutants. Our results show that knockout of acd-1 in glia worsens sensory deficits caused by mutations in channel subunit genes. One feature of many ion channels is that they are sensitive to the voltage across the plasma membrane. DEG/ENaCs such as DEG-1 and cGMP-gated channels such as TAX-2/TAX-4 are not classical voltage-gated channels, but they often display rectification properties that render their activity sensitive to the voltage across the membrane (Goodman et al. 2002; Komatsu et al. 1999). In addition, voltage-gated channels could be present in AWC neurons, where they could be necessary for amplification of the signal (Suzuki et al. 2003) or to generate action potentials (Mellem et al. 2008). We thus hypothesized that ACD-1 in glia might be involved directly or indirectly in regulating the membrane potential of sensory neurons, therefore resulting in tuning of sensory channels activity. To test this hypothesis, we designed an experiment in which the membrane potential of AWC sensory neurons could be exogenously manipulated. We expressed the rat TRPV1 channel in AWC sensory neurons of wild-type and acd-1 tax-2(p694) animals (Tobin et al. 2002). TRPV1 is a capsaicin-gated channel that conducts cations into the cell, and thus it induces membrane depolarization in the presence of capsaicin (Caterina et al. 1997). We then assayed attraction to isoamyl alcohol at the dilution of 1E-2 in these transgenic animals in the absence and presence of capsaicin. We found that whereas P<sub>adv.s</sub> TRPV1 animals were less attracted to isoamyl alcohol in the presence of capsaicin, acd-1 tax-2(p694); P<sub>adv.s</sub> TRPV1 animals were more attracted to this odor in the presence of capsaicin [Fig. 6A, P<sub>adv.s</sub> TRPV1, 0.555 ± 0.039 (0.10); P<sub>adv.s</sub> TRPV1 in the presence of capsaicin, 0.310 ± 0.054 (0.15); acd-1 tax-2(p694); P<sub>adv.s</sub> TRPV1, 0.370 ± 0.022 (0.05); acd-1 tax-2(p694); P<sub>adv.s</sub> TRPV1 in the presence of capsaicin, 0.580 ± 0.052 (0.140); P < 0.01]. Activation of TRPV1 in AWC is expected to cause membrane depolarization and an increase in intracellular Ca<sup>2+</sup>. To confirm this, we imaged AWC neurons of wild-type (N2) and acd-1 mutant animals perfused with isoamyl alcohol at 1E-4 and 1E-3 dilutions as indicated. Data are means ± SE; n = 8, 15, and 8, respectively. NS, not significantly different.
combined with the hypomorphic mutation tax-2(p694) directly or indirectly causes hyperpolarization of the membrane potential or reduction of intracellular Ca\(^{2+}\) in AWC neurons, which in turn renders these neurons less responsive to isoamyl alcohol. Our data also suggest that there might be an optimal level of membrane potential (or Ca\(^{2+}\)) in AWC neurons that ensures an efficient response to odors, as also suggested by Ferkey et al. (2007).

DISCUSSION

Glia in C. elegans sensory perception. Recent work highlights the profound importance of glia in sensory perception in C. elegans (Bacaj et al. 2008; Wang et al. 2008). Bacaj and colleagues ablated amphid glia and found 1) defects in sensory cilia structure in AWA and AWC with reduced chemotaxis to AWA- and AWC-sensed odors and 2) reduced chemotaxis to sodium without defects in cilia structure of sodium-sensing neurons ASE. This work establishes that glia play a role not only in neuronal development but also in neuronal function. We have shown previously and in the present study that knockout of acd-1 in glia does not compromise the structure of glia or neurons (Wang et al. 2008) but influences the function of sensory neurons. In particular, the fanlike structure of the cilia in AWC neurons is preserved in acd-1 tax-2(p694) mutant animals (Perkins et al. 1986), yet these animals are less sensitive to isoamyl alcohol.

It should be emphasized that our previous work and present data suggest that knockout of acd-1 does not compromise the activity of all sensory neurons. For example, AWB neurons do not seem affected (Fig. 1, C and D). Typically, neuronal function has to be already altered by a mutation in a neuronal gene in order for the knockout of acd-1 to have an impact on the phenotype (Fig. 1, A and B, and Wang et al. 2008). The exception that we found is attraction to isoamyl alcohol mediated by AWC neurons (Fig. 2A). In this case the attraction to isoamyl alcohol is not compromised in tax-2(p694) animals, at least not under our experimental conditions. However, when tax-2(p694) mutation is combined with acd-1 knockout, then a phenotype becomes apparent.

Insight into the role of DEG/ENaC channels in glia/neuron functional interaction. Our experiments, in which we depolamorized the membrane potential and increased intracellular Ca\(^{2+}\) concentrations of AWC neurons by exogenously expressing the rat capsaicin receptor TRPV1 and exposing the animals to capsaicin, suggest that ACD-1 may contribute in a direct or indirect way to keep the membrane potential of sensory neurons relatively depolarized or their basal Ca\(^{2+}\) level relatively high. Our data showing the deleterious effect of depolarizing and increasing intracellular Ca\(^{2+}\) levels in wild-type sensory neurons suggest that the level of Ca\(^{2+}\) or resting potential must remain within a certain range to ensure optimal sensory neurons function, as shown by Ferkey et al. (2007).

How may ACD-1, which is expressed in glia, influence the membrane potential or basal Ca\(^{2+}\) level of sensory neurons? There are several possible mechanisms, including effects of ACD-1 on the expression level, localization, or function of proteins involved in sensory signaling or in maintaining neuronal excitability or basal Ca\(^{2+}\) levels in sensory neurons, effects on the extracellular ionic composition, and effects on the release of glial factors that influence neuronal excitability (Gourine et al. 2010). More experiments are needed to distinguish between these possibilities. However, analogy with a mammalian sensory structure, the Pacinian corpuscle, and similarity of ACD-1 with mammalian ENaCs suggest a possible mechanism that will be further investigated. In some tissues, such as the collecting duct of the kidney, Na\(^{+}\) transport through ENaCs establishes a favorable driving force for K\(^{+}\)
excretion through K\(^+\) channels. It is possible that glial ENaCs such as ACD-1 facilitate K\(^+\) excretion in the microenvironment between the glia and the neurons, therefore setting the resting potential (or resting Ca\(^{2+}\)). Such a model would fit with our previously reported finding that ACD-1 is enriched at the end of the amphid sheath cell process where ensheathing of the dendrites occurs (Wang et al. 2008).

Ilyinsky et al. (1976) indeed used integrative ultramicro-flame photometry to show that K\(^+\) concentration in the fluid between the nerve fiber and the internal lamellae of the Pacinian corpuscle of the cat is higher than in blood and surrounding tissues. Using electrophysiological techniques, they also showed that a concentration of K\(^+\) between 6 and 12 mM lowers the mechanical threshold of these mechanosensory structures. Interestingly, K\(^+\) concentrations higher than 12 mM lower the sensitivity of Pacinian corpuscles. Thus there is a range of K\(^+\) concentration that is optimal for Pacinian corpuscles function. Interestingly, the DEG/ENaC channel ASIC2 is expressed on the internal lamellae (Calavia et al. 2010).

At this point we do not know what proteins in the sensory neurons are affected by glial ACD-1. Voltage-gated channels and channels with rectification properties are influenced by membrane voltage, and many ion channels and signaling proteins are influenced by intracellular Ca\(^{2+}\). Regardless of whether sensory neurons fire regenerative action potentials (Mellem et al. 2008), they are likely to depend on at least one Ca\(^{2+}\)- or voltage-sensitive component for sensory transduction.

There are a total of 56 glial cells in the adult C. elegans hermaphrodite and 28 genes in C. elegans that encode for DEG/ENaC channel subunits (Goodman and Schwarz 2003); the expression pattern of 20 of these is still unknown. It will be interesting to establish whether DEG/ENaC subunits that are highly homologous to ACD-1 are expressed in glia and play a role in sensory perception. In mammals, DEG/ENaCs have been found in brain astrocytes (Berdiev et al. 2003; Vila-Carriles et al. 2006, 2007), in Schwann cells associated with Ruffini endings (Hitomi et al. 2009), in Muller cells of the retina (Brockway et al. 2002), and in the internal lamellae of the Pacinian corpuscles (Calavia et al. 2010). However, little is known about the role of DEG/ENaC channels in these cells. It is possible that mammalian DEG/ENaCs expressed in glia play roles similar to C. elegans ACD-1. Future studies using glial-targeted DEG/ENaC knockouts are needed to test this possibility.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: Y.W., G.D., and L.B. performed experiments; Y.W., G.D., and L.B. analyzed data; Y.W., G.D., and L.B. edited and revised manuscript; Y.W., G.D., and L.B. approved final version of manuscript; L.B. conception and design of research; L.B. interpreted results of experiments; L.B. prepared figures; L.B. drafted manuscript.
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