Acute carbon dioxide avoidance in *Caenorhabditis elegans*

Elissa A. Hallem* and Paul W. Sternberg

Howard Hughes Medical Institute and Division of Biology, California Institute of Technology, Pasadena, CA 91125

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Carbon dioxide is produced as a by-product of cellular respiration by all aerobic organisms and thus serves for many animals as an important indicator of food, mates, and predators. However, whether free-living terrestrial nematodes such as *Caenorhabditis elegans* respond to CO2 was unclear. We have demonstrated that adult *C. elegans* display an acute avoidance response upon exposure to CO2 that is characterized by the cessation of forward movement and the rapid initiation of backward movement. This response is mediated by a cGMP signaling pathway that includes the cGMP-gated heteromeric channel TAX-2/TAX-4. CO2 avoidance is modulated by multiple signaling molecules, including the neuropeptide Y receptor NPR-1 and the calcineurin subunits TAX-6 and CNB-1. Nutritional status also modulates CO2 responsiveness via the insulin and TGFβ signaling pathways. CO2 response is mediated by a neural circuit that includes the BAG neurons, a pair of sensory neurons of previously unknown function. TAX-2/TAX-4 function in the BAG neurons to mediate acute CO2 avoidance. Our results demonstrate that *C. elegans* senses and responds to CO2 using multiple signaling pathways and a neural network that includes the BAG neurons and that this response is modulated by the physiological state of the worm.

**Results**

To determine whether *C. elegans* responds to CO2, we developed a CO2 avoidance assay based on the osmotic avoidance assay (20, 21). Specifically, the head of a forward-moving worm is exposed to an air stream containing CO2, and a response is scored if the worm reverses direction within 4 seconds [Fig. 1A and supporting information (SI) Movies S1 and S2]. Reversals are characteristic of avoidance responses in *C. elegans*: exposure to 1-octanol, hyperosmolarity, and nose touch elicit rapid reversals (17, 22, 23).

Wild-type N2 worms respond to CO2; for example, 76% reverse in response to an air stream containing 10% CO2, whereas only 23% reverse when the air stream does not contain CO2 (Table S1). We calculated an avoidance index (a.i.) for CO2 by subtracting the fraction of worms that reversed in response to an air stream that does not contain CO2 from the fraction that reversed in response to an air stream containing CO2. N2 worms show an a.i. of 0.53 in response to 10% CO2 (Fig. 1B).

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*To whom correspondence should be addressed: Division of Biology 156-29, California Institute of Technology, 1200 East California Boulevard, Pasadena, CA 91125. E-mail: ehallem@caltech.edu.*

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Acute CO2 Avoidance Varies Among Strains of C. elegans and Species of Free-Living Nematodes. We next asked whether CO2 aversion is conserved across six strains of C. elegans (24). Strains N2, CB3191, and TR389 show robust CO2 avoidance, whereas strains AB1, CB4853, and CB4856 are essentially unresponsive to CO2 in this assay (Fig. S1A). This suggests that CO2 avoidance is a rapidly evolving behavior. Different strains of C. elegans have been isolated from diverse ecological niches (25, 26), raising the possibility that this behavior may be advantageous in only some niches.

We then examined CO2 avoidance in five phylogenetically and ecologically diverse species of nematodes from the order Rhabditida (27). C. elegans and Pristionchus pacificus show robust CO2 avoidance, whereas Caenorhabditis briggsae, Caenorhabditis species 3, and Panagrellus redivivus show little or no CO2 avoidance (Fig. S1B). Thus, acute CO2 avoidance is found in some but not all free-living nematode species. However, species that do not avoid CO2 in our assay may exhibit CO2 responses under conditions not tested here.

The Neuropeptide Y Receptor NPR-1 Modulates CO2 Avoidance. Different wild isolates of C. elegans show polymorphic feeding behavior: some are solitary feeders that disperse on bacterial lawns, and others are social feeders that aggregate into clumps at the edge of the bacterial lawn (28). The three CO2-sensitive strains of C. elegans are solitary feeders, whereas the three CO2-insensitive strains are social feeders (Fig. S1A) (28). Thus, solitary feeding correlates with CO2 avoidance, suggesting that both behaviors may be subject to common regulatory mechanisms.

Feeding behavior is modulated by the neuropeptide Y receptor gene npr-1: loss-of-function mutations in npr-1 can convert a solitary strain into a social strain (28). We therefore investigated whether npr-1 also modulates acute CO2 avoidance. A hypomorphic mutation in npr-1 in an N2 background results in reduced CO2 avoidance, and a null mutation eliminates acute CO2 avoidance (Fig. 2A). Thus, npr-1 modulates CO2 avoidance.

Differences in feeding behavior among strains appear to be due to differences in O2 response, and npr-1 mutants display altered O2 preference in the presence of food (29). The fact that O2 and CO2 responses are both modulated by NPR-1 raises the possibility that the same receptor proteins confer responses to both gases. O2 receptors in C. elegans comprise a family of soluble guanylyl cyclases (sGCs) (29, 30). However, sGC mutants respond normally to CO2 (Fig. S2A). Moreover, mutation of the transcription factor AHR-1, which regulates expression of the sGC genes, does not affect CO2 avoidance (Fig. S2A). Thus, CO2 and O2 response are conferred by different receptors despite being subject to the same neuromodulatory control by npr-1.

Acute CO2 Avoidance Is Mediated by cGMP Signaling. To identify the signaling pathways that mediate CO2 avoidance, we screened candidate mutants that had been previously isolated and that display a wide variety of defects in neuronal development and function. TAX-2 and TAX-4 are subunits of a cGMP-gated channel required for normal chemosensory and thermosensory responses (17). We found that mutations in tax-2 and tax-4 eliminate acute CO2 avoidance (Fig. 2B). Also, mutation of the receptor guanylyl cyclase DAF-11 eliminates CO2 response (Fig. 2B). Thus, a cGMP signaling pathway(s) that includes DAF-11, TAX-2, and TAX-4 is required for acute CO2 avoidance.

Multiple Signaling Molecules Modulate Acute CO2 Avoidance. Our screen of candidate genes also identified a number of additional
signaling molecules that modulate CO2 avoidance: tax-6, cnb-1, rgs-3, and nhr-49. The calcineurin subunits TAX-6 and CNB-1 are required for CO2 avoidance: tax-6 and cnb-1 mutants are unresponsive to CO2 in our assay (Fig. 2C). These results suggest that CO2 response requires calcium signaling.

rgs-3 mutants fail to avoid CO2 (Fig. 2C). RGS-3 is a regulator of G protein signaling that is expressed in nine types of sensory neurons: ASH, ADL, AWB, AWC, ASI, ASJ, ASK, PHA, and PHB (31). The fact that rgs-3 mutants do not show acute CO2 avoidance suggests that CO2 response is modulated by signaling through G proteins acting in one or more of the neurons that express rgs-3. Animals with mutations in individual G protein subunits, as well as gpa-1 gpa-2 gpa-3 triple mutants, responded normally to CO2 (Fig. S2B), suggesting that multiple G proteins act redundantly to regulate CO2 response.

Mutation of the nuclear hormone receptor gene nhr-49 results in reduced CO2 avoidance (Fig. 2C). NHR-49 regulates transcriptionally the response to starvation, including expression of fat and energy metabolism genes (32). Thus, acute CO2 avoidance and the starvation response share a common regulatory mechanism involving nhr-49.

Mutants defective in the synthesis and reception of nonessential excitatory neurotransmitters, as well as mutants defective in neuropeptide synthesis and secretion, respond normally to CO2 (Fig. S2 C and D). CO2 avoidance may be mediated by both neuromodulators and neurotransmitters or by multiple neuromodulators or neurotransmitters acting in parallel.

Starvation Modulates CO2 Sensitivity in C. elegans. The regulation of CO2 response by NHR-49 raised the possibility that starvation affects CO2 avoidance. We therefore first asked whether CO2 avoidance is affected by the presence of bacterial food. Many sensory behaviors in C. elegans are affected by food, including O2 aerotaxis and 1-octanol avoidance (29, 33). However, worms show equally robust CO2 avoidance in the presence and absence of food (Fig. 3A). We then asked whether CO2 avoidance is modulated by starvation. Well fed worms tested off food showed an a.i. of 0.53; however, after 24 h of food deprivation the a.i. was reduced to 0.13 (Fig. 3B). When animals deprived of food for 24 h were placed back on food for 2 h, the a.i. was restored to the level of well fed animals (Fig. 3B). Thus, CO2 response is reversibly modulated by nutritional status.

The Insulin and TGFβ Pathways Modulate CO2 Avoidance. The insulin and TGFβ pathways are key regulators of starvation in C. elegans (34). Given that starvation reduces CO2 avoidance, we asked whether insulin and TGFβ signaling might also affect CO2 response. Mutation of the insulin receptor DAF-2 eliminates CO2 avoidance, and mutation of the forkhead transcription factor DAF-16 suppresses the daf-2 phenotype (Fig. 4A). Thus, DAF-16 acts downstream of DAF-2 in the regulation of CO2 response. Also, different alleles of daf-2 confer different CO2 sensitivities (Fig. S3A). The strength of the different daf-2 alleles with respect to CO2 avoidance correlates with the strength of the alleles with respect to hypoxia resistance (Fig. S3B) (35) but not lifespan and dauer formation (36, 37), suggesting that CO2 avoidance and hypoxia resistance may be subject to similar mechanisms of regulation by daf-2.

The TGFβ pathway also mediates acute CO2 avoidance: mutations in the TGFβ ligand DAF-7, as well as the TGFβ receptors DAF-1 and DAF-4, show severely reduced CO2 avoidance (Fig. 4B). The daf-7 phenotype is rescued by mutation of the SMAD gene daf-3, demonstrating that DAF-3 acts downstream of DAF-7 in the regulation of CO2 avoidance (Fig. 4B). DAF-7 is thought to be expressed specifically in the ASI chemosensory neurons (38). However, ablation of the ASI neurons did not affect CO2 avoidance (data not shown). This may be because daf-7 expression in ASI is required for CO2 response only transiently during early development or because daf-7 is expressed at low levels in other cells required for CO2 response.

To investigate the epistatic relationship between starvation and insulin and TGFβ signaling, we examined whether starved daf-16 and daf-3 mutants respond to CO2. In contrast to wild type, daf-16 and daf-3 mutants that had been starved for 24 h responded normally to CO2 (Fig. 4C). Thus daf-16 and daf-3 rescue the CO2 response defect of starved worms. Starvation
observed between N2 BAG-ablated and osm-3 mutants. Data for N2 mock-ablated are from Fig. 1. (B) BAG-ablated animals show greatly reduced CO2 avoidance, whereas mock-, AWC-, ASH-, ADL-, and AWB-ablated animals respond normally to CO2. No significant difference was observed between BAG-ablated animals and animals in which ASH, ADL, AWC, and AWB were ablated. **P < 0.01; ***P < 0.001. n = 11–44 trials. For all graphs, error bars represent SEM. Data for N2 are from Fig. 1. (C) osm-3 mock-ablated animals show reduced CO2 avoidance compared with N2 mock-ablated animals. Data for N2 mock-ablated are from B. No significant difference was observed between N2 BAG-ablated and osm-3 BAG-ablated animals. *, P < 0.05; **, P < 0.01; ***, P < 0.001. n = 22–33 animals for each condition.

Therefore modulates CO2 response via the insulin and TGFβ pathways.

**CO2 Response Is Mediated by a Neural Circuit That Includes the BAG Neurons.** To gain insight into the neural circuitry underlying CO2 perception, we examined the CO2 sensitivity of mutants with sensory neuron defects. We first tested mutations that affect the development of ciliated sensory neurons. osm-3 and daf-19 mutants showed reduced CO2 response, whereas a che-10 mutant was essentially unresponsive to CO2 (Fig. 5A). osm-3 encodes a kinesin subunit required for normal formation of amphid cilia, and daf-19 encodes an RFX transcription factor required for the formation of all sensory cilia (39). These mutants implicate ciliated sensory neurons in CO2 avoidance. A che-10 mutation causes degeneration of amphid and phasmid neurons, however, the IL1, OLO, and BAG neurons are also affected (40). These results suggest that one or more of the amphid or phasmid neurons, as well as one or more of IL1, OLO, and BAG, play a role in CO2 perception. Mutations that affect developement of specific subsets of sensory neurons do not affect acute CO2 avoidance (Fig. S4).

We also tested the tax-2 allele tax-2(p9694), a cis-regulatory mutation that disrupts tax-2 expression in the AOR, AFD, ASE, and BAG neurons (19). tax-2(p9694) mutants do not respond to CO2 in our assay (Fig. 2B). Because mutations and transgenes that compromise the function of AOR, AFD, and ASE respond normally to CO2 (Fig. 5 and Fig. S4), tax-2 is likely required in the BAG neurons (Fig. S5) for acute CO2 avoidance.

To further test the role of the BAG neurons in CO2 response, we ablated them using a laser microbeam and measured the ability of ablated worms to respond to CO2. As a control, we ablated the AWC olfactory neurons, which mediate attraction (17). Ablation of the BAG neurons resulted in greatly reduced CO2 avoidance (Fig. 5B). By contrast, mock-ablated and AWC-ablated animals responded normally to CO2 (Fig. 5B). Therefore, the BAG neurons are important components of the neural circuit that mediates CO2 response.

The fact that CO2 response is severely reduced but not completely eliminated in BAG-ablated animals suggests that other sensory neurons play a role in acute CO2 avoidance. In an attempt to identify these neurons, we ablated the ASH, ADL, and AWB neurons, which mediate olfactory repulsion (17). However, ablation of these neuron pairs individually did not affect acute CO2 avoidance, and ablation of the ASH, ADL, AWB, and BAG neurons in the same animal resulted in a response that was not significantly different from the response of BAG-ablated animals (Fig. 5B). Thus, ASH, ADL, and AWB play at most a minor role in acute CO2 avoidance.

We then ablated the BAG neurons in osm-3 mutants. We found that the response of mock-ablated osm-3 mutants is reduced compared with mock-ablated wild-type animals, and the response of BAG-ablated osm-3 mutants is further reduced (Fig. 5C). These results suggest that, in addition to BAG, one or more of the ciliated sensory neurons affected by the osm-3 mutation play a role in CO2 avoidance.

We note that acute CO2 avoidance could be either a chemosensory or a nociceptive response. However, chemical nociception is mediated primarily by the ASH neurons (17), and ASH-ablated animals respond normally to CO2 (Fig. 5B). Thus, acute CO2 avoidance is likely to be a chemosensory response.

Finally, we generated dose–response curves for four mutants that showed defective CO2 avoidance when tested with 10% CO2: osm-3 and nhr-49, which showed reduced avoidance of 10% CO2; and tax-4 and npr-1, which failed to avoid 10% CO2. We found that the CO2 response of all four mutants is defective across a broad range of concentrations (Fig. S6). These results suggest that acute CO2 avoidance is mediated by the same signaling mechanisms across concentrations.

cGMP Signaling Is Required in the BAG Neurons for Acute CO2 Avoidance. tax-2 and tax-4 are coexpressed in 12 neurons: AWC, AFD, ASE, ASG, ASJ, ASI, AWB, ASK, BAG, AQR, PQR, and URX (41). To identify the neuron(s) in which tax-2/TAX-4 is required for acute CO2 avoidance, we performed a series of cell-specific rescue experiments with tax-4. We first tested whether CO2 avoidance requires tax-4 expression in AQR, PQR, and URX because tax-4 is required in these neurons for normal O2 response (19, 29, 42). We found that tax-4 mutants in which tax-4 is specifically rescued in AQR, PQR, and URX (42) do not respond to CO2 (Fig. 6A). Moreover, animals containing a genetic ablation of AQR, PQR, and URX (42) respond normally to CO2 (Fig. 6A). Thus, AQR, PQR, and URX do not mediate acute CO2 avoidance.

We then examined tax-4 mutants containing an odr-4::tax-4 transgene, in which tax-4 is expressed in AWA, AWB, AWC, ADF, ASG, ASH, ASJ, ASI, ASK, ADL, PHA, and PHB (19). These worms show essentially no CO2 avoidance (Fig. 6B). However, expression of tax-4 in these neurons as well as AQR, PQR, URX, AFD, and BAG using the odr-4, gcy-8, gcy-32, and gcy-33 promoters (19) is sufficient to rescue the CO2 response defect of tax-4 mutants (Fig. 6B). Given that ablation of the BAG neurons results in greatly reduced CO2 avoidance, we then asked whether tax-4 expression in the BAG neurons is sufficient for CO2 avoidance by expressing tax-4 under the control of only the gcy-33 promoter. We found that tax-4 expression in BAG rescues the CO2 response defect of tax-4 mutants (Fig. 6B). Thus, a cGMP signaling pathway involving TAX-2/TAX-4 operates within the BAG neurons to mediate acute CO2 avoidance.

**Discussion**

We have found that *C. elegans* exhibits acute avoidance of CO2. This response requires a cGMP signaling pathway acting within
Under conditions of starvation it may be beneficial to down-regulate CO2 avoidance, and this effect is mediated by insulin and TGFβ signaling. The fact that CO2 response is reduced by starvation contrasts with most olfactory responses in C. elegans, which are enhanced by starvation (17), presumably so as to maximize the worm’s chance of finding food. The starvation-induced decrease in CO2 avoidance may offer a similar ecological advantage: In nature, C. elegans presumably encounters CO2 emitted by both viral food and predators. Under conditions of starvation it may be beneficial to down-regulate CO2 avoidance so as to maximize the probability of encountering food, even if this incurs an increased risk of predation.

The sensitivity of CO2 response to nutritional status is not universal among animals. For example, starved larvae of the bloodsucking insect Triatoma infestans respond as robustly to CO2 as well fed larvae, even after 60 days of starvation (43). By contrast, many mosquitoes use CO2 as their primary host-seeking cue, and host-seeking behavior is greatly reduced after a blood meal (44). Thus, CO2 response may be subject to different regulatory mechanisms in organisms with different life cycles and behavioral repertoires. For example, CO2 avoidance is mediated primarily by the BAG neurons, and cGMP signaling mediated by TAX-2/TAX-4 is required in BAG for acute CO2 avoidance. The BAG neurons are ciliated neurons of previously unknown function located in the head but not associated with the amphid sensillum (18). These neurons may sense CO2 directly via one or more CO2 receptors, or they may be indirect modulators of CO2 response. It will be interesting to determine whether the BAG neurons also modulate O2 response and also to identify additional signaling components that operate within the BAG neurons. Of the signaling molecules identified in this study as mediators of CO2 avoidance, only TAX-2/TAX-4 are known to be expressed in the BAG neurons. In particular, expression of the guanylyl cyclase DAF-11 has not been observed in the BAG neurons (45), raising the possibility that the effect of daf-11 on CO2 response is indirect and that a different guanylyl cyclase acts upstream of TAX-2/TAX-4 in the BAG neurons to mediate CO2 avoidance.

A CO2 receptor in C. elegans has not yet been identified. In Drosophila, CO2 avoidance is mediated by two members of the gustatory receptor (Gr) family of serpentine receptors, Gr21a and Gr63a (46–48), which are expressed in a single class of olfactory neurons on the fly antenna (5, 8, 46, 47). C. elegans does not contain orthologs of Gr21a and Gr63a, and thus Drosophila and C. elegans use different receptors for CO2 detection.

The CO2 avoidance we observed in some species of free-living terrestrial nematodes contrasts with the attraction to CO2 exhibited by many parasitic and free-living marine nematodes (10–14, 49). Our study provides a foundation for investigations into how the CO2 response network may have evolved in nematodes with very different life cycles and ecological niches.

Materials and Methods

Standard techniques are listed in the SI Methods.

Population Assay for Acute CO2 Avoidance. For each assay, 10–30 C. elegans L4 hermaphrodites were placed onto assay plates overnight and tested as young adults. Assay plates consisted of NGM agar plates containing a thin lawn of OP50 bacteria grown for 1–2 days at room temperature. Gases were medical-grade certified mixtures (Air Liquide) of 0%, 0.2%, 1%, 2.5%, 5%, 10%, or 15% CO2; 10% O2; and the balance N2. An O2 concentration of 10% was chosen to closely approximate the preferred O2 concentration of C. elegans. Ten percent CO2 was used for all experiments unless otherwise indicated. For the avoidance assay, two 50-ml syringes were filled with gas, one with and one without CO2. The mouth of the syringes were connected to tubes attached to Pasteur pipettes, and gases were pumped through the Pasteur pipettes by using a syringe pump (PHD 2000; Harvard Apparatus) at a rate of 1.5 ml/min. Individual worms were exposed to gases by placing the tip of the Pasteur pipette near the head of a forward-moving worm. A response was scored if the worm initiated backward movement within 4 seconds. The gas mixture to which each plate was exposed was alternated such that half of the plates were exposed to air and half were exposed to CO2. Gases were delivered blindly, and worms were tested blindly. Each plate was considered one trial. Plates were assayed one to two times with at least 1 h between trials, except that worms tested off food were tested only once. An a.i. for each genotype was calculated by subtracting the fraction of worms that reversed in response to air from the fraction that reversed in response to CO2. For assays involving other species, both males and females of dioecious species were tested. Values obtained for each genotype or treatment are listed in Table S1.

Single-Worm Assay for CO2 Avoidance. For each assay, individual L4 or young adult hermaphrodites were placed onto assay plates overnight. Worms were tested as described above, except that each worm was tested 15 times with >2 min between trials. No adaptation was observed during the course of these experiments. For each worm, an a.i. was calculated by subtracting the fraction of trials the worm reversed in response to air from the fraction of trials the worm reversed in response to CO2. The a.i. for each genotype or treatment was
Values obtained for each genotype or treatment are listed in Table S2. Calculated as the mean a.i. for each worm of the same genotype or treatment. Biology, Cambridge, U.K.), C. Bargmann (HHMI and The Rockefeller University, New York, NY), J. Thomas (University of Washington, Seattle, WA), and the Caenorhabditis Genetics Center (University of Minnesota, Minneapolis, MN) for strains; D. Anderson (California Institute of Technology, Pasadena, CA) and members of our laboratory for insightful discussions; and J. Srivasan (California Institute of Technology, Pasadena, CA) for discussion of other species. This work was supported by the Howard Hughes Medical Institute, to which P.W.S. is an investigator, and a Helen Hay Whitney postdoctoral fellowship (to E.A.H.).

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