

Acute carbon dioxide avoidance in *Caenorhabditis elegans*

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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 TGF β 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.

behavioral genetics | cGMP signaling | chemosensation

Carbon dioxide is a critical sensory cue for organisms as diverse as humans (1), rodents (2, 3), insects (4, 5), and bacteria (6). The ability to rapidly detect and respond to changing CO₂ concentrations is important for survival: high CO₂ concentrations cause hypoxia, anesthetization, and death. CO₂ perception may be particularly important for soil-dwelling organisms such as *Caenorhabditis elegans*, because bacteria-rich soil microenvironments are often subject to large fluctuations in CO₂ and O₂ levels (7). However, despite the fundamental importance of CO₂ for all living organisms, remarkably little is known about how CO₂ evokes behavioral responses in animals.

Many mammals, including humans, are capable of detecting CO₂. Humans detect CO₂ as a trigeminal stimulus, and different humans show different sensitivities to CO₂ (1). Mice detect and avoid near-atmospheric concentrations of CO₂ via a subset of olfactory sensory neurons that project to the necklace glomeruli in the olfactory bulb (2). Many insects also detect CO₂. For example, *Drosophila* is repelled by CO₂ via a single class of olfactory receptor neurons on the antennae (8), whereas mosquitoes are attracted to CO₂ via olfactory receptor neurons on the maxillary palps (9).

The phylum Nematoda contains both free-living and parasitic species, some of which have been shown to respond to CO₂. Many animal-parasitic nematodes, including the human-parasitic nematodes *Ancylostoma duodenale* and *Necator americanus*, are attracted to CO₂; for these nematodes, CO₂ exhaled by their hosts serves as a host-localization and host-invasion cue (10, 11). Similarly, many plant-parasitic nematodes use CO₂ emission by plant roots for host localization (12, 13). Some species of free-living marine nematodes are also attracted to CO₂, which is used as a sensory cue to locate the decaying animal and plant carcasses that serve as their food sources (14). However, nothing is known about the mechanisms underlying

CO₂ perception in these nematodes, and whether free-living terrestrial nematodes respond to CO₂ was unclear (10, 15, 16).

The well studied model *C. elegans* is a free-living terrestrial nematode that navigates through its environment using a combination of chemosensory, thermosensory, and mechanosensory cues. The nervous system of the *C. elegans* hermaphrodite consists of 302 neurons, >10% of which are chemosensory (17, 18). The perception of volatile and water-soluble chemicals is mediated primarily by 11 pairs of chemosensory neurons that extend ciliated dendrites into the paired amphid sensilla of the head (17). *C. elegans* also senses O₂ via two ciliated and two unciliated sensory neurons that extend dendrites into the pseudocoelomic body fluid (17, 19).

We demonstrate that *C. elegans* display an acute avoidance response to CO₂. We then identify signaling pathways that affect CO₂ avoidance by conducting a screen of existing mutants with neurosensory defects. We find that CO₂ avoidance is mediated by cGMP signaling and requires the receptor guanylyl cyclase DAF-11 and the cGMP-gated channel TAX-2/TAX-4. CO₂ response is modulated by multiple neuronal regulatory molecules. Nutrient deprivation decreases CO₂ avoidance, and this response is mediated by the insulin and TGF β pathways. Finally, acute CO₂ avoidance is mediated primarily by the paired BAG neurons of the head, and cGMP signaling is required in the BAG neurons to mediate CO₂ avoidance.

Results

An Acute Carbon Dioxide Response in *C. elegans*. 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).

C. elegans responds to CO₂ in a dose-dependent manner (Fig.

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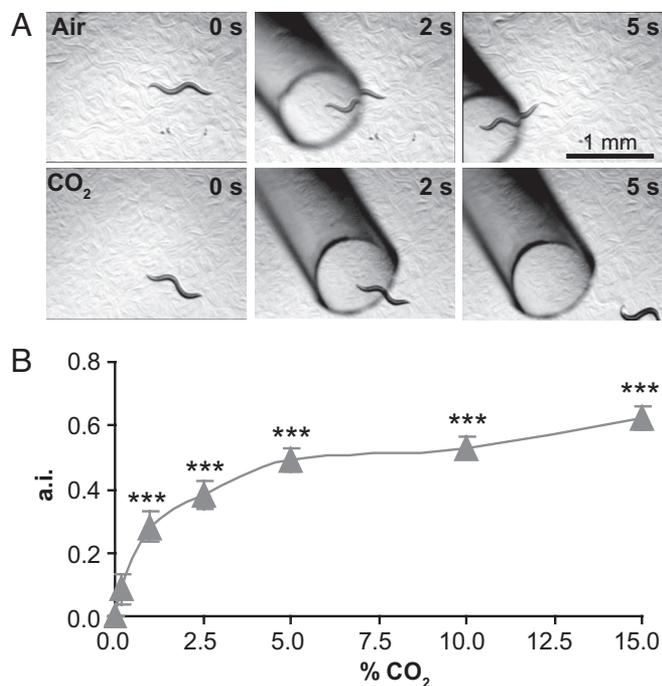


Fig. 1. Acute CO₂ avoidance in *C. elegans*. (A) When the head of a forward-moving N2 worm is exposed to an air mixture, the worm continues forward locomotion (Upper). When the worm is exposed to 10% CO₂, the worm halts forward locomotion and rapidly reverses (Lower). (B) The response of N2 worms to CO₂ is dose-dependent. ***, $P < 0.001$. $n = 22$ –44 trials. For all graphs, error bars represent SEM.

1B). The response to 1% CO₂ was significantly different from the response to 0% CO₂, demonstrating that *C. elegans* can detect CO₂ concentrations as low as 1% above ambient levels (Fig. 1B). The average atmospheric concentration of CO₂ is $\approx 0.04\%$. A CO₂ concentration of 10% was used for subsequent experiments because of the robust response to this concentration.

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).

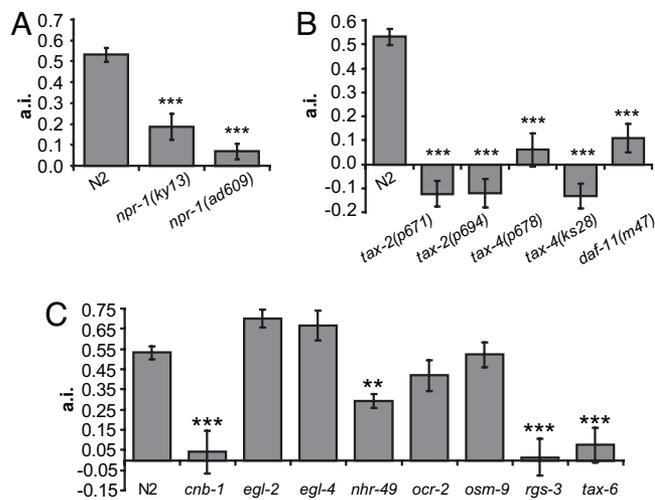


Fig. 2. Signaling pathways that affect acute CO₂ avoidance. (A) NPR-1 modulates CO₂ response. *npr-1(ky13)* hypomorphs show greatly reduced CO₂ avoidance, and *npr-1(ad609)* null mutants show essentially no CO₂ avoidance. ***, $P < 0.001$. $n = 12$ –44 trials. For all graphs, error bars represent SEM. (B) cGMP signaling is required for CO₂ response. Mutants of *tax-2*, *tax-4*, and *daf-11* do not show acute CO₂ avoidance. ***, $P < 0.001$. $n = 11$ –44 trials. (C) The *cnb-1*, *rgs-3*, and *tax-6* mutants do not show acute CO₂ avoidance, whereas the *nhr-49* mutant shows reduced CO₂ avoidance. **, $P < 0.01$; ***, $P < 0.001$. $n = 11$ –44 trials. Data for N2 are from Fig. 1.

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*,

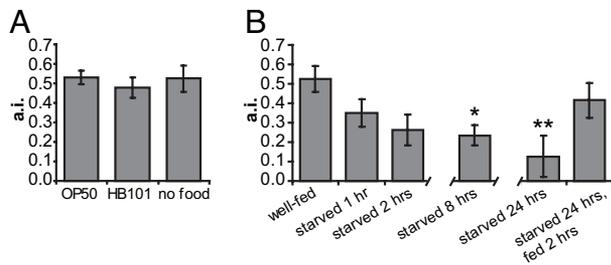


Fig. 3. The response to CO₂ is modulated by starvation. (A) Avoidance of CO₂ is not modulated by the presence of food. No significant differences were observed between worms tested on OP50, the *Escherichia coli* strain typically used as a food source for *C. elegans*; HB101, a different strain of *E. coli*; or off food. $n = 12$ –44 trials. For all graphs, error bars represent SEM. The data for N2 tested on OP50 are from Fig. 1. (B) Starvation results in a reversible decrease in CO₂ avoidance. *, $P < 0.05$; **, $P < 0.01$. $n = 7$ –13 trials. As a control for the 24-h time point off food, N2 worms were left on assay plates for an additional 24 h and then tested on food; no difference was observed between these worms and worms left on assay plates overnight and then tested.

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. S2C 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 TGF β Pathways Modulate CO₂ Avoidance. The insulin and TGF β pathways are key regulators of starvation in *C. elegans* (34). Given that starvation reduces CO₂ avoidance, we asked whether insulin and TGF β signaling might also affect CO₂

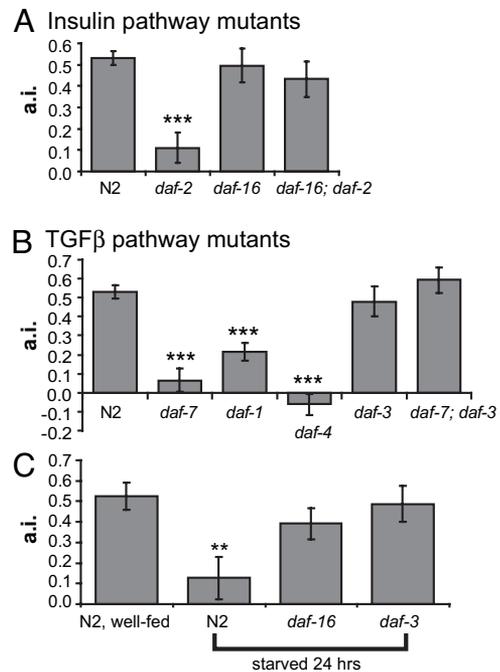


Fig. 4. Insulin and TGF β signaling modulate CO₂ response. (A) *daf-2(e1370)* mutants do not respond to CO₂. This defect is rescued by mutation of *daf-16*. ***, $P < 0.001$. $n = 12$ –44 trials. For all graphs, error bars represent SEM. (B) The TGF β pathway mutants *daf-7*, *daf-1*, and *daf-4* show little or no CO₂ avoidance. The *daf-7* phenotype is rescued by mutation of *daf-3*. ***, $P < 0.001$. $n = 12$ –44 trials. Data for N2 are from Fig. 1. (C) Epistatic interactions between insulin and TGF β signaling and starvation. A 24-h starvation decreases acute CO₂ avoidance. The *daf-16(mgDf47)* and *daf-3(e1376)* mutations restore CO₂ response to starved animals, indicating that starvation acts via the insulin and TGF β pathways. **, $P < 0.01$. $n = 7$ –15 trials. Error bars represent SEM. Data for N2 are from Fig. 3.

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 TGF β pathway also mediates acute CO₂ avoidance: mutations in the TGF β ligand DAF-7, as well as the TGF β 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 TGF β 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

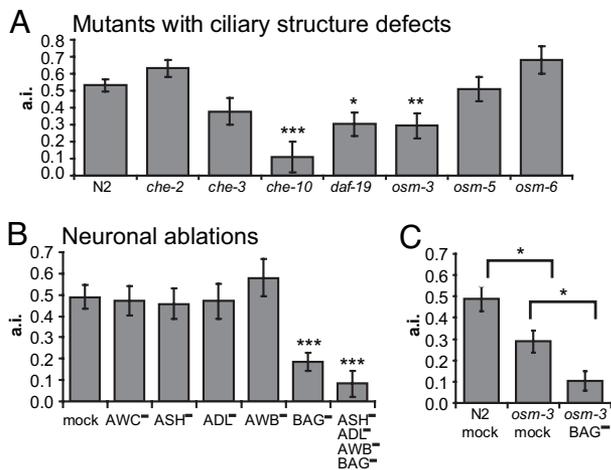


Fig. 5. CO₂ response is mediated primarily by the BAG neurons. (A) Among mutants with defects in ciliary structure, *osm-3* and *daf-19* mutants show reduced CO₂ avoidance, whereas *che-10* mutants show essentially no CO₂ avoidance. *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001. *n* = 11–44 trials. For all graphs, error bars represent SEM. Data for N2 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, AWB, and BAG neurons were ablated. ***, *P* < 0.001. *n* = 13–36 animals for each condition. (C) *osm-3* mock-ablated animals show reduced CO₂ avoidance compared with N2 mock-ablated animals, and *osm-3* BAG-ablated animals show reduced CO₂ avoidance compared with *osm-3* 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. *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 development of specific subsets of sensory neurons do not affect acute CO₂ avoidance (Fig. S4).

We also tested the *tax-2* allele *tax-2(p694)*, a cis-regulatory mutation that disrupts *tax-2* expression in the AQR, AFD, ASE, and BAG neurons (19). *tax-2(p694)* mutants do not respond to CO₂ in our assay (Fig. 2B). Because mutations and transgenes that compromise the function of AQR, AFD, and ASE respond normally to CO₂ (Fig. 6 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, ASI, ASJ, 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

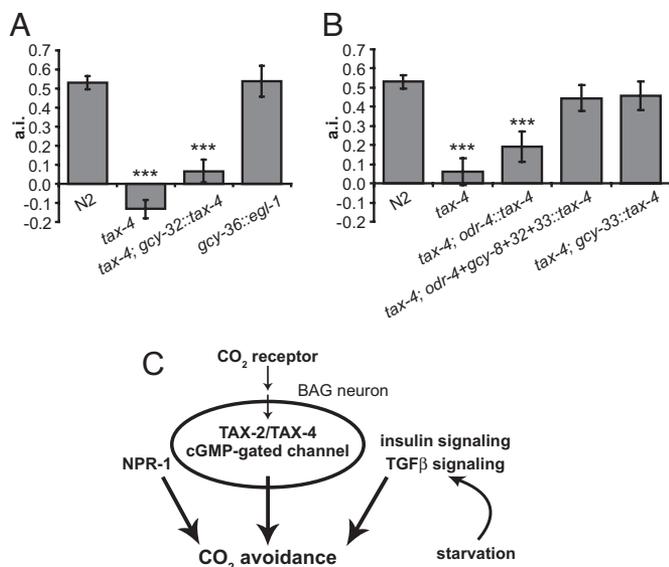


Fig. 6. cGMP signaling is required in the BAG neurons for acute CO₂ avoidance. (A) The URX, AQR, and PQR neurons are not required for acute CO₂ avoidance. Animals containing a *gcy-32::tax-4* transgene in the *tax-4(ks28)* mutant background, in which *tax-4* is expressed specifically in URX, AQR, and PQR (42), do not respond to CO₂. Animals containing a *gcy-36::egl-1* transgene, which kills URX, AQR, and PQR (42), respond normally to CO₂. ***, $P < 0.001$. $n = 11$ –44 trials. For all graphs, error bars represent SEM. (B) *tax-4* is required in the BAG neurons for acute CO₂ avoidance. Animals containing an *odr-4::tax-4* transgene in the *tax-4(p678)* mutant background (19), in which *tax-4* is expressed in 12 sensory neurons, do not respond to CO₂. Animals containing *odr-4+gcy-8+gcy-32+gcy-33::tax-4* transgenes in the *tax-4(p678)* mutant background (19), in which *tax-4* is expressed in 17 neurons including BAG, respond normally to CO₂. Animals containing a *gcy-33::tax-4* transgene in the *tax-4(p678)* mutant background, in which *tax-4* is expressed specifically in the BAG neurons, also respond normally to CO₂. ***, $P < 0.001$. $n = 12$ –44 trials. Data for N2 are from Fig. 1, and data for *tax-4* mutants are from Fig. 2. (C) A model for acute CO₂ avoidance in *C. elegans*. CO₂ avoidance is mediated by a cGMP signaling pathway involving TAX-2/TAX-4 acting within the BAG neurons. This response is modulated by NPR-1. CO₂ response is decreased by starvation, which acts via the insulin and TGF β pathways.

the BAG neurons and is modulated by additional neuronal regulatory molecules as well as by insulin and TGF β signaling (Fig. 6C).

Acute CO₂ avoidance is exhibited by some but not all wild isolates of *C. elegans* and some but not all species of free-living terrestrial nematodes. Thus, acute CO₂ avoidance is a rapidly evolving behavior that has either arisen or been lost multiple times during the course of nematode evolution, raising the possibility that it is advantageous only under some ecological conditions.

Starved worms show reduced CO₂ avoidance, and this effect is mediated by insulin and TGF β 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 bacterial 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.

Acute 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* (29). 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

calculated as the mean a.i. for each worm of the same genotype or treatment. Values obtained for each genotype or treatment are listed in Table S2. References for mutant strains are listed in Table S3.

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