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A carbon dioxide avoidance behavior is integrated with responses to ambient oxygen and food in Caenorhabditis elegans

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Homeostasis of internal carbon dioxide (CO2) and oxygen (O2) levels is fundamental to all animals. Here we examine the CO2 response of the nematode Caenorhabditis elegans. This species inhabits rotting material, which typically has a high CO2 concentration range. We show that well-fed C. elegans avoid CO2 levels above 0.5%. Animals can respond to both absolute CO2 concentrations and changes in CO2 levels within seconds. Responses to CO2 do not reflect avoidance of acid pH but appear to define a new sensory response. Sensation of CO2 is promoted by the cGMP-gated ion channel subunits TAX-2 and TAX-4, but other pathways are also important. Robust CO2 avoidance in well-fed animals requires inhibition of the DAF-16 forkhead transcription factor by the insulin-like receptor DAF-2. Starvation, which activates DAF-16, strongly suppresses CO2 avoidance. Exposure to hypoxia (<1% O2) also suppresses CO2 avoidance via activation of the hypoxia-inducible transcription factor HIF-1. The npr-1 215V allele of the naturally polymorphic neuroepithelial receptor npr-1, besides inhibiting avoidance of high ambient O2 in feeding C. elegans, also promotes avoidance of high CO2. C. elegans integrates competing O2 and CO2 sensory inputs so that one response dominates. Food and allelic variation at NPR-1 regulate which response prevails. Our results suggest that multiple sensory inputs are coordinated by C. elegans to generate different coherent foraging strategies.

Results

C. elegans Avoids Elevated CO2. To investigate how C. elegans responds to CO2, we first exposed N2 (Bristol) wild-type animals to spatial CO2 gradients. Gas gradients were set up over worms on agar surfaces using microfluidic chambers connected to defined gas mixtures (Fig. 1 A and B and ref. 23; see Methods). Within these chambers laminar flow operates such that a linear gas gradient is generated by simple diffusion between the two ends of the chamber. Unless otherwise indicated, O2 was kept at 21% in these mixtures: CO2 was increased at the expense of N2. When only air was pumped into the chamber, N2 animals distributed equally to both sides of the chamber space (Fig. 1A). However, on introduction of a 5% to 0% CO2 gradient, animals rapidly (<10 min) vacated areas of the chamber where CO2 levels were high (Fig. 1B). To examine the concentration dependence of C. elegans CO2 avoidance, we also assayed animals in gradients of 0.25% to 0%, 0.5% to 0%, 1% to 0%, and 3% to 0% CO2. Avoidance of CO2 was concentration-dependent, and animals avoided high CO2 both in the presence and in the absence of a lawn of Escherichia coli food [Fig. 1 C and D and supporting information (SI) Fig. S1]. However, bacteria slightly but significantly reduced the strength of the avoidance response (Fig. 1 C and D and Fig. S1). The significance threshold for C. elegans CO2 response was 1% CO2 on food and 0.5% CO2 off food at the 0.01% significance level (Fig. 1 C and D and Fig. S1). Thus, CO2 is a potent repellent for N2 animals.

To provide a simple measure for the CO2 response we calculated a chemotaxis index by subtracting the number of animals in the low CO2 half of the chamber from the number in the high CO2 half and dividing by the total number of animals in the assay. Chemotaxis indices of +1, 0, and −1 indicate perfect attraction, indifference, and perfect avoidance of CO2, respectively. The chemotaxis indices for CO2 gradients of 1% to 0%, 3% to 0%, and 5% to 0% were...
The speed an animal moves at influences how rapidly it can escape an aversive cue. This led us to examine whether elevated CO\textsubscript{2} avoidance in \textit{C. elegans}, raising CO\textsubscript{2} from 0\% to 5\% led to a doubling of the average speed of feeding N2 animals, from 46 to 92 \textmu m/s (Fig. 2E). Unlike the increase in reversals and turns, which lasted for only 1–2 min (Fig. 2A–D), the increased rate of movement was sustained as long as CO\textsubscript{2} levels remained high (\textgreek{t} > 30 min; Fig. 2F). This endurance suggests that absolute levels of CO\textsubscript{2}, rather than change in its concentration, can signal to control speed of movement.

When returned from 5\% CO\textsubscript{2} to 0\%, feeding animals showed a further transient increase in speed before slowing down to the speed they exhibited before the CO\textsubscript{2} rise (Fig. 2E). In contrast to our observations in the presence of food, raising CO\textsubscript{2} levels from 0\% to 5\% in the absence of food caused a decrease in the average speed of movement, from 235 to 183 \textmu m/s (Fig. 2G). Returning animals to atmospheric CO\textsubscript{2} levels reversed this inhibition. In summary, our data suggest that \textit{C. elegans} can respond both to absolute levels of CO\textsubscript{2}, which can regulate speed, and to changes in CO\textsubscript{2} levels, which modulate reversals and turns and, to some extent, speed too.

**CO\textsubscript{2} Avoidance Is Distinct from Avoidance of Acid pH.** CO\textsubscript{2} is potentially a complex sensory stimulus. \textit{C. elegans} lives in aqueous films and responds to chemical stimuli dissolved in these films. CO\textsubscript{2} is highly soluble in water, reacting to form carbonic acid that dissociates to yield H\textsuperscript{+} and HCO\textsubscript{3}\textsuperscript{-} (Fig. 2H). HCO\textsubscript{3}\textsuperscript{-} can dissociate further to yield H\textsuperscript{+} and CO\textsubscript{3}\textsuperscript{2}\textsuperscript{-}, but CO\textsubscript{3}\textsuperscript{2}\textsuperscript{-} concentrations are negligible at physiological pH. Thus at the air–water interface an equilibrium is set up between gaseous CO\textsubscript{2} and its solvation products (Fig. 2H).

Previous studies have indicated that \textit{C. elegans} avoids acid pH (24). This raised the possibility that CO\textsubscript{2} avoidance reflects escape from acid pH. We therefore examined how a 5\% to 0\% CO\textsubscript{2} gradient changed agar pH across the microfluidic chamber (Fig. S2). We observed a pH change of <0.1 pH units across the chamber, from pH 6.22 to pH 6.29. The small size of the pH change was expected because the agar substrate is buffered (see Methods). This small pH change and the previous observation that \textit{C. elegans} avoids acid only below pH 4 (24) suggest that changes in external pH are unlikely to explain CO\textsubscript{2} avoidance.

\textit{C. elegans} could also avoid CO\textsubscript{2} by responding to changes in HCO\textsubscript{3}\textsuperscript{-} levels in the medium. To test this we examined CO\textsubscript{2} responses on agars buffered at different pH values, from 4.9 to 7.1. The concentrations of HCO\textsubscript{3}\textsuperscript{-} generated by any given partial pressure of CO\textsubscript{2} should vary 100-fold across this pH range. We saw no substantial differences in avoidance of 5\% CO\textsubscript{2} at different pH values (Fig. 2F). These data suggest that changes in external H\textsuperscript{+} and HCO\textsubscript{3}\textsuperscript{-} are unlikely to be the sensory stimuli that trigger CO\textsubscript{2} avoidance. However, the permeability of CO\textsubscript{2} across lipid bilayers is high (\textgreek{t} = 0.35 cm s\textsuperscript{-1}) (25), and the \textit{C. elegans} genome encodes several genes with homology to carbonic anhydrases, the enzymes that catalyze hydration of CO\textsubscript{2} (www.wormbase.org). \textit{C. elegans} could therefore sense CO\textsubscript{2} fluctuations by monitoring internal (extracellular or intracellular) H\textsuperscript{+} or HCO\textsubscript{3}\textsuperscript{-} levels. Alternatively, \textit{C. elegans} could respond to molecular CO\textsubscript{2}.

**Signaling Through cGMP-Gated Ion Channels Contributes to CO\textsubscript{2} Avoidance.** Two major chemosensory pathways have been defined in \textit{C. elegans}. One is mediated by a cGMP-gated ion channel encoded by the tax-2 and tax-4 genes (26, 27). A second is mediated by transient receptor potential V-like (TRPV-like) ion channels encoded by osm-9 and its associated subunits encoded by ocr genes (28, 29). We tested whether mutations in these genes disrupted CO\textsubscript{2} avoidance. Loss of osm-9 did not cause a carbon dioxide avoidance defective (Cdad) phenotype in the presence or absence of food (Fig. 3A). In contrast, mutations in tax-2 or tax-4 completely disrupted CO\textsubscript{2} avoidance on food but only partially disrupted avoidance off food (Fig. 3A). Thus, cGMP pathways contribute to CO\textsubscript{2} avoidance, but other signal transduction pathways may also be important.

**Starvation Suppresses CO\textsubscript{2} Avoidance.** \textit{C. elegans} thrives in decaying organic matter where microbial activity can significantly raise local CO\textsubscript{2} levels (21, 22). It was therefore surprising that N2 animals avoided CO\textsubscript{2}. Studies of other nematodes, both free-living bacteriophagous species (e.g., \textit{Panagrellus rilius}) and plant (e.g., \textit{Meloidogyne incognita}) and animal (e.g., \textit{Stenonema sp.}) parasites, have reported chemoattraction not chemorepulsion to CO\textsubscript{2}. This led us to examine whether \textit{C. elegans} avoidance of CO\textsubscript{2} is context-dependent. We began by asking whether starvation alters CO\textsubscript{2} avoidance. We removed N2 animals from food for 1, 3, or 5 h and then tested their responses in a 5\% to 0\% CO\textsubscript{2} gradient off food. Food deprivation suppressed CO\textsubscript{2} avoidance: N2 animals...
showed no significant CO2 avoidance after 3 h without food and weak attraction toward CO2 after 5 h without food (Fig. 3B). Thus, whereas well fed or feeding animals strongly avoid CO2, starved animals do not.

**Insulin-Like Signaling Sustains CO2 Avoidance.** Several neuroendocrine pathways signal feeding state in *C. elegans* (32–35). These include the *daf-2* insulin-like receptor pathway: high DAF-2 signaling is associated with the well fed state, whereas low signaling is associated with food deprivation. We speculated that starvation might suppress CO2 avoidance by inhibiting DAF-2 signaling. This hypothesis predicts that mutants in this pathway would behave like starved wild-type animals even when they are well fed. Consistent with this, mutants in the insulin-like signaling pathway, including the *daf-2* insulin-like receptor, the 3-phosphoinositide-dependent kinase *pdk-1*, and the protein kinase B serine/threonine kinase *akt-1* showed reduced CO2 avoidance or even weak attraction (Fig. 3 C and D). Insulin-like signaling thus sustains avoidance of high CO2.

The effects of food deprivation on CO2 responses occurred over several hours (Fig. 3B), a timescale consistent with a transcriptional reconfiguration of CO2-sensing circuits. Reduced DAF-2 signaling activates the DAF-16 Forkhead transcription factor (32, 36). We therefore asked whether DAF-16 was responsible for suppressing CO2 avoidance in *daf-2* mutants. Consistent with such a scenario, *daf-2; daf-16* double mutants strongly avoided high CO2 and behaved indistinguishably from N2 animals (Fig. 3C). Together these data are consistent with a model in which starvation reconfigures CO2 responses, at least in part, by down-regulating insulin-like signaling and activating the DAF-16 forkhead transcription factor.

**Hypoxia Suppresses CO2 Avoidance via Activation of HIF-1.** Because CO2 is the by-product of aerobic respiration, we speculated that O2-sensing pathways might regulate CO2 responses. One pathway regulated by O2 is the hypoxia-inducible pathway. In both *C. elegans* and mammals, severe hypoxia (<1% O2) induces hypoxia-inducible factor (HIF) transcription factors. In high O2 HIFs are targeted for degradation by prolyl hydroxylases. These enzymes use molecular O2 as a cosubstrate and are active in high, but not low, O2. *C. elegans* encodes a single HIF, called HIF-1 (37), which is targeted for degradation by the prolyl hydroxylase EGL-9 (38). Loss of *egl-9* leads to high levels of HIF-1 irrespective of ambient O2. *egl-9* mutants were attracted to CO2 (Fig. 3E). To investigate whether this reversal of CO2 chemotaxis was due to high HIF-1 activity, we examined the behavior of *egl-9; hif-1* double mutants. Loss of *hif-1* restored strong CO2 avoidance to *egl-9* mutant animals (Fig. 3E). Finally, we asked whether wild-type animals suppress CO2 avoidance after experiencing hypoxia. After 1 h in 1% O2, N2 animals, but not *hif-1* mutant animals, suppressed CO2 avoidance (Fig. 3F). Taken together, these data suggest that hypoxia signals through HIF-1 to reconfigure CO2-sensing circuits, leading to indifference or even attraction to high CO2.

**The NPR-1 Neuropeptide Receptor Promotes CO2 Avoidance.** We chose to extend our studies on the interplay between O2 and CO2 sensing. Previous work has shown that natural variation in the
feeding N\textsubscript{2} animals to hypoxia (1\% O\textsubscript{2}) for 1 h inhibits CO\textsubscript{2} avoidance in a results in strong defects in CO\textsubscript{2} avoidance. Reduced DAF-2 signaling inhibits CO\textsubscript{2} avoidance, compared with N\textsubscript{2}; nd, not determined. (B) The CO\textsubscript{2} avoidance defect in npr\textsubscript{-1} mutants is not a consequence of their aggregation behavior, npr\textsubscript{-1} animals grown in isolation (GII) retain a strong defect in avoidance of 5\% CO\textsubscript{2} compared with similarly reared N\textsubscript{2} animals. The weighted chemotaxis index was calculated by recording the position of each animal in a CO\textsubscript{2} gradient at 1-s intervals for 5 min and weighting this according to location in the CO\textsubscript{2} gradient (see Methods). "N\textsubscript{2} air" represents a negative control with no CO\textsubscript{2} gradient. (\textcopyright\textregistered\textsuperscript{23, 39}). We asked whether npr\textsubscript{-1} regulated not only O\textsubscript{2} but also CO\textsubscript{2} responses. Consistent with this hypothesis, npr\textsubscript{-1} loss-of-function mutants showed striking defects in CO\textsubscript{2} avoidance both on and off food (Fig. 4A). This CO\textsubscript{2} avoidance defect could be rescued by an npr\textsubscript{-1} 215V transgene (Fig. 4A).

To test whether the natural npr\textsubscript{-1} 215F allele also modified CO\textsubscript{2} avoidance, we compared the behavior of N\textsubscript{2} animals to a near isogenic strain, AX613, which bears the npr\textsubscript{-1} 215F allele from the German wild strain RC301 backcrossed 20 times into N\textsubscript{2}. Animals bearing npr\textsubscript{-1} 215F showed a significant reduction in CO\textsubscript{2} avoidance compared with N\textsubscript{2} on food but not off food (Fig. 4A). Thus, animals having high npr\textsubscript{-1} activity strongly avoid CO\textsubscript{2} whereas animals with low npr\textsubscript{-1} activity exhibit weaker avoidance.

Under normal cultivation conditions npr\textsubscript{-1} mutant animals aggregate strongly. One explanation for their reduced CO\textsubscript{2} avoidance is a difference in experience compared with N\textsubscript{2}. To explore this possibility we grew N\textsubscript{2} and npr\textsubscript{-1} animals in isolation. npr\textsubscript{-1}(ad609) animals grown in isolation retained a strong defect in CO\textsubscript{2} avoidance (Fig. 4B). Together, these data suggest that signaling from the NPR\textsubscript{-1} neuropeptide receptor promotes CO\textsubscript{2} avoidance, particularly when food is present.

**Sensory Integration of CO\textsubscript{2} and O\textsubscript{2} Signals in C. elegans.** In our previous experiments we exposed animals to gradients of CO\textsubscript{2} in a background of 21\% O\textsubscript{2}. However, in nature C. elegans is likely to encounter simultaneous gradients of O\textsubscript{2} and CO\textsubscript{2}. To explore how C. elegans navigates these more complex situations, we placed animals in combined gradients of O\textsubscript{2} and CO\textsubscript{2} with 11\% O\textsubscript{2} and 5\% CO\textsubscript{2}; at one end of the chamber and 21\% O\textsubscript{2} and 0\% CO\textsubscript{2} at the other. As controls we tested animals in identical gradients of only O\textsubscript{2} or O\textsubscript{2}. Integration of CO\textsubscript{2} and O\textsubscript{2} stimuli was particularly interesting in the context of different alleles of npr\textsubscript{-1} because natural variation at this receptor modifies both CO\textsubscript{2} and O\textsubscript{2} responses. We therefore tested strains carrying npr\textsubscript{-1} 215V (which occurs in N\textsubscript{2} and all dispersing wild isolates), npr\textsubscript{-1} 215F (which occurs in all aggregating wild isolates), and the loss-of-function mutant npr\textsubscript{-1}(ad609).

The response of N\textsubscript{2} animals in the crossed gradient was dominated by CO\textsubscript{2} avoidance: both on and off food animals accumulated at 21\% O\textsubscript{2}/0\% CO\textsubscript{2} (Fig. 5). Thus, the avoidance of high O\textsubscript{2} by N\textsubscript{2} animals when food is absent was suppressed by avoidance of high CO\textsubscript{2}. By contrast, the response of npr\textsubscript{-1}(ad609) and npr\textsubscript{-1} 215F animals in the crossed gradient depended on context (Fig. 5 and Fig. S3). On food, the behavior of these animals was dominated by the O\textsubscript{2} response: animals ignored high CO\textsubscript{2} to accumulate at low O\textsubscript{2}. Conversely, off food it was the response to CO\textsubscript{2} that dominated.
animals behaved as if they were in a gradient that consisted only of CO₂ (compare Fig. 5 D–F).

Thus, *C. elegans* integrates antagonistic inputs from CO₂- and O₂-sensing pathways to generate a coherent behavioral response in which one input dominates. The activity of the NPR-1 receptor reconfigures which of the two sensory responses dominates within the context of food availability.

**Discussion**

Well fed *C. elegans* avoid elevated CO₂ even though they seek environments where O₂ levels are between 11% and 7% (23, 40). The threshold we observed for CO₂ response is ~0.5%. This is ~10-fold higher than atmospheric CO₂ levels, but decaying organic matter can have much higher CO₂ concentrations, of 10% or more. *C. elegans* can respond both to absolute levels of CO₂ by modifying speed, and to change in CO₂ concentration, by altering direction of movement. Interestingly, *C. elegans* responses to O₂ are also coupled to changes in both concentration and absolute levels (40).

Behavioral and genetic dissection of the *C. elegans* CO₂ response reveals surprising complexity. Several observations are most easily explained if *C. elegans* has several pathways that respond to changes in CO₂. First, single mutations in known sensory transduction pathways are not sufficient to abolish CO₂ avoidance under all feeding conditions. Second, CO₂ responses are switched from repulsion to attraction by mutations in some genes. Third, the effects of CO₂ on speed of movement are complex. Although we have not identified CO₂-responsive sensory neurons in this study, one set of candidate neurons is those expressing the TAX-2/TAX-4 cGMP-gated ion channel.

Avoidance of CO₂ is modulated by contextual cues such as feeding state, exposure to hypoxia, and bacteria (Fig. 3G). Starvation completely suppresses CO₂ avoidance. This may represent a tradeoff in which food-deprived animals ignore an aversive cue to explore a wider range of environments. Previous work has shown that starvation inhibits signaling from the insulin-like receptor daf-2 and promotes entry of the DAF-16 forkhead transcription factor into the nucleus (32). Our data are consistent with high DAF-2 signaling in well fed animals sustaining avoidance of high CO₂ and low DAF-2 signaling in starved animals reducing CO₂ avoidance by activating DAF-16. DAF-2 has been implicated in modulating behavior previously, notably in studies of salt chemotaxis and thermo taxis (33, 35, 41). The daf-2 pathway may therefore act globally to reset behavioral state according to feeding conditions. Suppression of CO₂ avoidance in hypoxia may enable animals to migrate through CO₂-rich environments to reach more aerobic environments. Suggestions for how HIF-1 might alter CO₂ responses come from microarray studies. In both mammals and *C. elegans*, HIF regulates expression of carbonic anhydrases (42).

Bacterial signals also modulate CO₂ sensing: the CO₂ responses of well fed animals, both wild type and mutant, differ depending on whether food is present or not. Perhaps different combinations of sensory neurons mediate responses to CO₂ on and off food. Such a scenario has been described for the response of *C. elegans* to the aversive odorant octanol (43).

Sensory responses to CO₂ and O₂ are integrated by the worm in ways that depend on context and genotype at the naturally varying *npr-1* locus. Previous data have shown that *npr-1* 215F suppresses avoidance of high O₂ in feeding animals. Here we show that *npr-1* 215F also promotes CO₂ avoidance. By coordinately stimulating avoidance of high CO₂ and inhibiting avoidance of high O₂, *npr-1* 215F is likely to promote migration to surface environments. In contrast, the *npr-1* 215SV allele permits strong avoidance of high O₂ and weak avoidance of CO₂, promoting migration to subsurface environments. We speculate that these niche preferences may favor speciation.

Why does *C. elegans* avoid CO₂? One reason may be that high external CO₂ can acidify the body fluid of *C. elegans*. However, there are other possibilities. Comparison of local O₂ and CO₂ levels may allow the animal to monitor aeration and escape from an environment before it becomes anaerobic.

In summary, *C. elegans* CO₂ avoidance defines a novel behavior. CO₂ avoidance is highly integrated with other sensory cues of natural importance to the worm, such as food and ambient O₂. One exciting challenge for the future will be to identify the neuronal substrates of CO₂ avoidance in *C. elegans* and to examine how contextual changes alter cellular behavior, leading to the alterations in organismal behavior patterns that we have observed in this study.

**Methods**

**Strains.** Strains were maintained at 22°C by using standard methods unless otherwise indicated (44). Strains used in this study are listed in *SI Materials and Methods*.

**Behavioral Assays.** Spatial CO₂ gradients were generated by using custom-made 33 × 15 × 0.4-mm microfluidic devices fabricated from polydimethylsiloxane
with OP50 (44). Defined CO2::O2::N2 gas mixtures were obtained from The BOC. Definitions of the presence of food were performed on NGM plates on lawns seeded 2 days earlier by pairwise comparison between different strains and conditions using Pearson's chi-squared test at the P < 0.0001 level. In all figures, error bars denote SEM.

Environmental Manipulations. In Fig. 2I, the pH of the nematode substrate was varied by using different buffers as follows: pH 4.9 (40 mM sodium acetate, pH 4.75), pH 5.7 (40 mM malate, pH 5.33), and pH 7.1 (40 mM phosphate, pH 7.2). In starvation experiments (Fig. 3B), two culture plates of N2 animals were washed three times in M9 before transfer to conditioning plates (6 or 9 cm of unseeded NGM). Animals were left for 0, 1, 3, or 5 h and then washed once before being assayed off food for CO2 avoidance.

In the hypoxia conditioning experiments (Fig. 3P), C. elegans cultures were placed in a glove box (Coy Laboratory Products) at 1% O2 for 1 h before being assayed off food for CO2 avoidance.

In Fig. 4B three animals per plate were grown from the L2/L3 larval stage to adulthood. Pools of 25 animals were then assayed in CO2 gradients in the presence of food. The position of each worm in the M9S chamber was recorded over a 5-min period, beginning 10 min after the onset of the assay, with a CCD camera mounted on a dissecting microscope. Resulting films were analyzed, and the positions of the worms in the chamber were determined with DIAS (Soll Technologies). See SI Materials and Methods for further details.

pH Measurements. We measured CO2-induced pH changes using NGM containing 500 µM pH-sensitive chromophore 8-hydroxypyrene-1,3,6-trisulfonic acid (HPTS; Sigma). For the HPTS fluorescence (P) measurement method, see SI Materials and Methods.

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