

# Receptor-type guanylate cyclase is required for carbon dioxide sensation by *Caenorhabditis elegans*

Elissa A. Hallem<sup>a,1</sup>, W. Clay Spencer<sup>b</sup>, Rebecca D. McWhirter<sup>b</sup>, Georg Zeller<sup>c,d,2</sup>, Stefan R. Henz<sup>d</sup>, Gunnar Rättsch<sup>c</sup>, David M. Miller III<sup>b</sup>, H. Robert Horvitz<sup>e</sup>, Paul W. Sternberg<sup>a,3</sup>, and Niels Ringstad<sup>e,f,3</sup>

<sup>a</sup>Howard Hughes Medical Institute, Division of Biology, California Institute of Technology, Pasadena, CA 91125; <sup>b</sup>Department of Cell and Developmental Biology, Vanderbilt University School of Medicine, Nashville, TN 37232; <sup>c</sup>Friedrich Miescher Laboratory of the Max Planck Society, 72076 Tübingen, Germany; <sup>d</sup>Department of Molecular Biology, Max Planck Institute for Developmental Biology, 72076 Tübingen, Germany; <sup>e</sup>Howard Hughes Medical Institute, Department of Biology and McGovern Institute for Brain Research, Massachusetts Institute of Technology, Cambridge, MA 02139; and <sup>f</sup>Department of Cell Biology and the Helen L. and Martin S. Kimmel Center for Biology and Medicine at the Skirball Institute of Biomolecular Medicine, New York University Langone Medical Center, New York NY 10016

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**CO<sub>2</sub> is both a critical regulator of animal physiology and an important sensory cue for many animals for host detection, food location, and mate finding. The free-living soil nematode *Caenorhabditis elegans* shows CO<sub>2</sub> avoidance behavior, which requires a pair of ciliated sensory neurons, the BAG neurons. Using in vivo calcium imaging, we show that CO<sub>2</sub> specifically activates the BAG neurons and that the CO<sub>2</sub>-sensing function of BAG neurons requires TAX-2/TAX-4 cyclic nucleotide-gated ion channels and the receptor-type guanylate cyclase GCY-9. Our results delineate a molecular pathway for CO<sub>2</sub> sensing and suggest that activation of a receptor-type guanylate cyclase is an evolutionarily conserved mechanism by which animals detect environmental CO<sub>2</sub>.**

guanylyl cyclase | olfaction | transcriptional profiling | regulator of G protein signaling | chemosensation

The ability to detect and respond to changing concentrations of environmental CO<sub>2</sub> is widespread among animals and plays a critical role in locating food, finding hosts and mates, and avoiding danger (1–4). CO<sub>2</sub> exposure can also have profound physiological effects, including altered respiration, motility, fecundity, and emotional state (5–7). CO<sub>2</sub> is sensed as an aversive cue by many free-living animals, including humans (3, 6, 8, 9). By contrast, many parasites and disease vectors are attracted to CO<sub>2</sub>, which serves as a sensory cue for host location (1, 10).

Nematodes constitute a large and highly diverse phylum that includes both free-living and parasitic species. Many parasitic nematodes, including some of the most devastating human- and plant-parasitic nematodes, are attracted to CO<sub>2</sub>. By contrast, adults of the free-living species *Caenorhabditis elegans* are repelled by CO<sub>2</sub> (11–14). CO<sub>2</sub> avoidance by *C. elegans* requires a pair of head neurons called the BAG neurons (13), which also mediate responses to decreases in ambient oxygen levels (15). Whether the BAG neurons directly sense CO<sub>2</sub> is not known, and the signaling pathways that mediate CO<sub>2</sub> detection are poorly understood.

We show here that environmental CO<sub>2</sub> specifically activates the BAG neurons and not other neurons that drive avoidance behavior, suggesting that the BAG neurons are primary sensory neurons that detect CO<sub>2</sub>. Prolonged CO<sub>2</sub> exposure causes desensitization of avoidance behavior and the BAG neurons themselves, indicating that behavioral adaptation to CO<sub>2</sub> occurs at the level of the BAG neurons. In addition, we show that the CO<sub>2</sub>-evoked activity of the BAG neurons requires a cGMP signaling pathway consisting of the receptor guanylate cyclase GCY-9 and the cGMP-gated cation channel TAX-2/TAX-4. Insects detect CO<sub>2</sub> using a pair of gustatory receptors (16, 17), whereas some mammals detect CO<sub>2</sub> using the receptor-type guanylate cyclase, guanylate cyclase D (GC-D), and soluble adenylylase (18–20). Our results show that the mechanism of CO<sub>2</sub> detection in *C. elegans* more closely resembles that of mammals than insects and suggest an evolutionarily ancient role

for receptor-type guanylate cyclases in mediating environmental CO<sub>2</sub> detection by animals.

## Results

**BAG Neurons Are Activated by CO<sub>2</sub>.** BAG neurons are located in the head, extend ciliated dendrites to the tip of the nose (21), and are required for avoidance of CO<sub>2</sub> across concentrations (Figs. S1 and S2). To determine whether the BAG neurons respond to environmental CO<sub>2</sub>, we monitored the activity of BAG neurons using the genetically encoded ratiometric calcium indicator cameleon (22). The *gcy-33* promoter was used to drive expression of cameleon specifically in the BAG neurons (23). We first confirmed that animals expressing *gcy-33::cameleon* show normal CO<sub>2</sub> avoidance behavior (Fig. S3). We then imaged these animals and found that CO<sub>2</sub> exposure evoked rapid and reversible calcium transients in the cell bodies of BAG neurons (Fig. 1A and Fig. S4). By contrast, other sensory neurons known to mediate *C. elegans* avoidance behavior, the amphid neurons ASH, ADL, and AWB, did not respond to a 10% CO<sub>2</sub> stimulus, indicating that the CO<sub>2</sub> response of BAG neurons is cell type-specific (Fig. 1B). This result suggests that the BAG neurons are the primary sensory neurons that detect CO<sub>2</sub>.

**Prolonged CO<sub>2</sub> Exposure Desensitizes BAG Neurons.** Prolonged exposure to many sensory stimuli results in behavioral adaptation, which can occur either in the primary sensory neuron or in circuitry downstream of the sensory neuron (24–28). We tested whether CO<sub>2</sub> avoidance behavior adapts to prolonged CO<sub>2</sub> exposure by exposing animals to 1%, 5%, or 10% CO<sub>2</sub> for either 1 or 5 min and then testing their ability to respond to 5% or 10% CO<sub>2</sub> in an acute avoidance assay. We found that prolonged exposure to either 5% or 10% CO<sub>2</sub> greatly reduced subsequent behavioral responses to the same concentration of CO<sub>2</sub>, and responses to 10% CO<sub>2</sub> were significantly decreased after a 1-min

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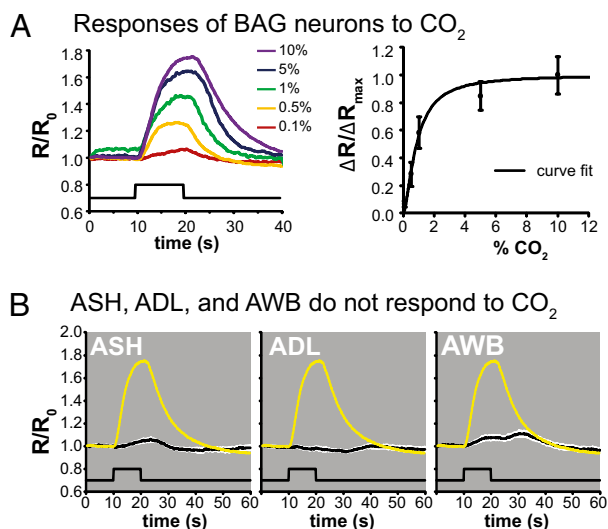
Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. [HQ636455](http://www.ncbi.nlm.nih.gov/nuclseq/HQ636455)). The gene expression data have been deposited in the Gene Expression Omnibus database (accession no. [GSE23769](http://www.ncbi.nlm.nih.gov/geo/GSE23769)).

<sup>1</sup>Present address: Department of Microbiology, Immunology, and Molecular Genetics, University of California, Los Angeles, CA 90095.

<sup>2</sup>Present address: Structural and Computational Biology Unit, European Molecular Biology Laboratory, 69117 Heidelberg, Germany.

<sup>3</sup>To whom correspondence may be addressed. E-mail: [pws@caltech.edu](mailto:pws@caltech.edu) or [niels.ringstad@med.nyu.edu](mailto:niels.ringstad@med.nyu.edu).

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**Fig. 1.** BAG neurons are activated by CO<sub>2</sub>. (A *Left*) BAG neuron cell bodies respond to CO<sub>2</sub>. R/R<sub>0</sub> is the YFP to CFP ratio (R) divided by the average YFP to CFP ratio of the first 10 frames (R<sub>0</sub>). (A *Right*) Dose-response curve for CO<sub>2</sub>. ΔR/ΔR<sub>max</sub> is the maximal ratio change caused by presentation of a given CO<sub>2</sub> stimulus normalized to the maximal ratio change measured (evoked by 10% CO<sub>2</sub>). (B) ASH, ADL, and AWB do not respond to 10% CO<sub>2</sub>. The yellow trace is the average response of the BAG neurons to 10% CO<sub>2</sub>. The white area around each trace represents the SEM; the lower black traces indicate the stimulus onset and duration (n = 5–18 animals for each condition or genotype). For all graphs, error bars represent SEMs.

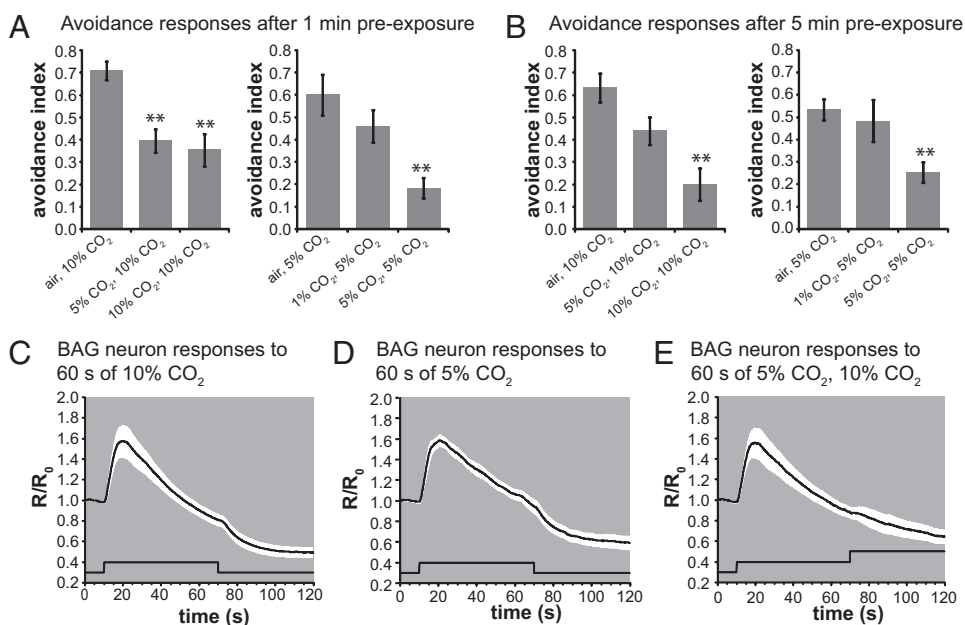
exposure to 5% CO<sub>2</sub> (Fig. 2 A and B). Thus, *C. elegans* displays behavioral adaptation in response to prolonged CO<sub>2</sub> exposure. To test whether behavioral adaptation to 5% or 10% CO<sub>2</sub> occurs at the level of the BAG neurons, we recorded the calcium response of BAG neurons evoked by prolonged exposure to

CO<sub>2</sub>. We found that a 1-min exposure to either 5% or 10% CO<sub>2</sub> caused an initial increase in BAG neuron calcium, which then dropped to below-baseline levels (Fig. 2 C and D). This drop in BAG neuron calcium was the result of desensitization; a 1-min exposure to 5% CO<sub>2</sub> blocked the calcium response to a subsequent 10% CO<sub>2</sub> stimulus (Fig. 2E). These results show that the BAG neurons desensitize during prolonged exposure to CO<sub>2</sub> and suggest that behavioral adaptation to CO<sub>2</sub> occurs at the level of the BAG sensory neuron.

**cGMP-Gated Channel TAX-2/TAX-4 Is Required for BAG Neuron Responses to CO<sub>2</sub>.** CO<sub>2</sub> avoidance behavior by *C. elegans* requires multiple signaling pathways, including a cGMP, a G protein, an insulin, and a TGF-β pathway (13, 14). To test whether any of these pathways are required for the CO<sub>2</sub>-evoked calcium response of the BAG neurons, we measured CO<sub>2</sub>-evoked calcium transients of BAG neurons in animals that are mutant for these pathways and behaviorally defective in acute CO<sub>2</sub> avoidance. We found that mutations in *tax-2* and *tax-4*, which encode subunits of a cGMP-gated cation channel (29), eliminated the CO<sub>2</sub> calcium response of BAG neurons, indicating a direct requirement for TAX-2/TAX-4 in the signal transduction cascade that leads to CO<sub>2</sub> detection (Fig. 3).

We also found that G-protein signaling modulated BAG neuron responses to CO<sub>2</sub>; mutation of *rgs-3*, which encodes a regulator of G protein signaling (30), resulted in reduced BAG neuron responses to CO<sub>2</sub> (Fig. 3). To test whether *rgs-3* functions in BAG neurons to regulate CO<sub>2</sub> responses, we expressed *rgs-3* specifically in the BAG neurons of *rgs-3* mutants and found that this expression restored normal CO<sub>2</sub> avoidance behavior (Fig. S5A). Because regulator of G protein signaling (RGS) proteins negatively regulate G-protein signaling (31), our results indicate that a G-protein signaling pathway negatively regulates BAG neurons.

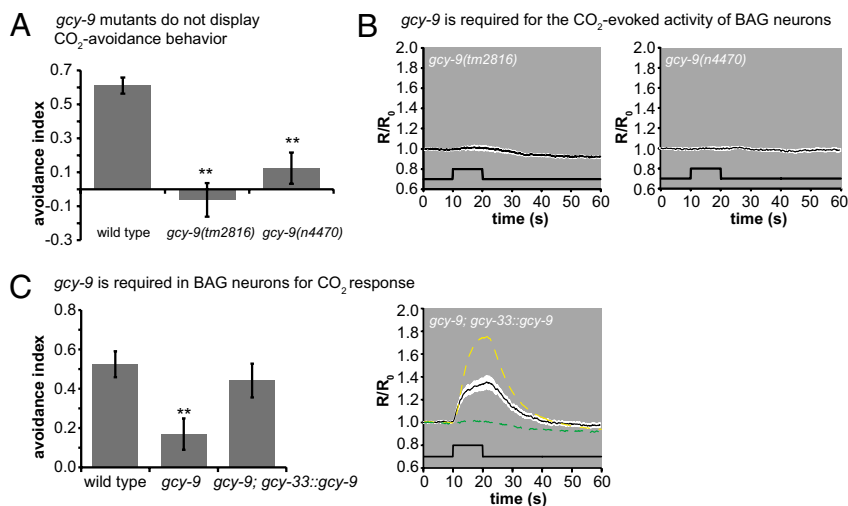
A number of genes that are required for behavioral avoidance of CO<sub>2</sub> (13) were not required for CO<sub>2</sub>-evoked calcium responses of the BAG neurons. These genes include *npr-1*, which



**Fig. 2.** Prolonged CO<sub>2</sub> exposure desensitizes BAG neurons. (A and B) Acute CO<sub>2</sub> avoidance after either a (A) 1-min or (B) 5-min exposure to CO<sub>2</sub>. For each treatment condition, the stimulus used during the preexposure is indicated followed by the stimulus used for the acute avoidance assay (n = 11–15 trials for each treatment). (C and D) Prolonged pulses of either (C) 10% or (D) 5% CO<sub>2</sub> results in BAG neuron desensitization (n = 7 animals for each condition). (E) Prior exposure to 5% CO<sub>2</sub> blocks the response to 10% CO<sub>2</sub> (n = 7 animals). The white area around each trace represents the SEM; the lower black traces indicate the stimulus onset and duration.







**Fig. 5.** *gcy-9* is required for CO<sub>2</sub> sensing by BAG neurons. (A) *gcy-9* mutants do not respond to CO<sub>2</sub> ( $n = 10$ – $32$  trials for each genotype). (B) *gcy-9* mutants do not show CO<sub>2</sub>-evoked activity in the BAG neurons ( $n = 9$  animals). (C) Expression of *gcy-9* specifically in the BAG neurons completely rescues CO<sub>2</sub> avoidance behavior (Left) and partially rescues CO<sub>2</sub>-evoked calcium transients of BAG neurons (Right). Left has  $n = 22$ – $39$  trials for each genotype, and Right has  $n = 8$  animals. The yellow dashed line shows the mean response of WT animals, and the green dashed line shows the mean response of *gcy-9(tm2816)* animals. The white area around each trace represents the SEM; the lower black traces indicated the stimulus onset and duration. For this experiment, CO<sub>2</sub> avoidance behavioral assays were performed on all three genotypes in parallel.

to act through a G-protein signaling pathway (16, 17, 38). By contrast, mammalian olfactory receptor neurons that respond to CO<sub>2</sub> express the receptor-type guanylate cyclase GC-D (8), which is activated by the CO<sub>2</sub> metabolite bicarbonate (20, 39). Because CO<sub>2</sub> sensing by the BAG neurons of *C. elegans* requires the receptor-type guanylate cyclase GCY-9, we propose that GCY-9 might function as a receptor for CO<sub>2</sub> or a metabolite of CO<sub>2</sub>. GCY-9 is one of a large number of *C. elegans* guanylate cyclases (Fig. S10), many of which are expressed in small subsets of sensory neurons and are required for specific chemosensory behaviors (23, 29). Although some GCY proteins seem to function as chemoreceptors, others have been proposed to function downstream of G protein-coupled receptors (23, 40–42). It is, therefore, also possible that GCY-9 acts downstream of a yet to be identified receptor for CO<sub>2</sub>.

GCY-9 orthologs are present in multiple nematode species, including the plant-parasitic nematodes *Heterodera glycines* and *Meloidogyne incognita* (43) and the human-parasitic nematode *Brugia malayi* (44). The requirement for GCY-9 in mediating a behavioral response to CO<sub>2</sub> and the importance of CO<sub>2</sub> as a host-seeking cue for many parasitic nematodes raise the possibility that compounds that block GCY-9 activity might be useful in the development of strategies for nematode control.

## Materials and Methods

Standard techniques are listed in *SI Materials and Methods*.

**Behavioral Assay for Acute CO<sub>2</sub> Avoidance.** CO<sub>2</sub> assays were performed as previously described (13). Briefly, for each assay, about 10–20 L4 hermaphrodites were placed on 5-cm assay plates overnight and tested as young adults. Plates consisted of nematode growth medium (NGM) agar seeded with a thin lawn of *Escherichia coli* OP50 bacteria. Gases were medical grade certified mixes (Air Liquide) consisting of the indicated CO<sub>2</sub> concentration, 10% O<sub>2</sub>, and the remaining percentage of N<sub>2</sub>. A concentration of 10% CO<sub>2</sub> was used unless otherwise indicated. Two 50-mL syringes were filled with gas, one with CO<sub>2</sub> and one without CO<sub>2</sub>. The mouths of the syringes were connected to tubes attached to Pasteur pipettes, and gases were pumped through the Pasteur pipettes using a syringe pump at a rate of 1.5 mL/min. Worms were exposed to gases by placing the tip of the Pasteur pipette near the head of a forward-moving worm, and a response was scored if the worm reversed within 4 s. An avoidance index was then calculated by subtracting the fraction of animals that reversed in response to the air control from the fraction of animals that reversed in response to the CO<sub>2</sub> (Fig. S2A).

**Behavioral Assay for Adaptation to CO<sub>2</sub>.** For each assay, about five L4 hermaphrodites were placed on 9-cm assay plates overnight and tested as young adults. Plates consisted of NGM agar seeded with a thin lawn of OP50 in the center of the plate. Gas was pumped into the plate through a hole on one side

of the lid at a rate of 1 L/min; a hole on the other side allowed gas to escape. Worms were preexposed to gas for either 1 or 5 min and then tested immediately in an acute avoidance assay.

**Imaging BAG Neurons in Restrained Animals.** Young adults were immobilized with cyanoacrylate veterinary glue (Surgi-Lock; Meridian Animal Health) on a cover glass coated with a 2% agarose pad made with 10 mM Hepes (pH 7.4). The cover glass was affixed to a custom-made air chamber. The specimen was illuminated with 435-nm excitation light and imaged using a 40× Nikon long-working distance objective (0.75 numerical aperture). The emission image was passed through a DV2 image splitter (Photometrics), and the CFP and YFP emission images were projected onto two halves of a cooled CCD camera (Andor). Images were acquired at 10 Hz, with exposure times between 10 and 50 ms. Gas perfusion was controlled by three-way valves (Numatics) driven by a custom-made valve controller unit. Excitation light, image acquisition, and hardware control were performed by the Live Acquisition software package (Till Photonics). Custom certified gas mixes used for imaging were obtained from Airgas.

**Image Analysis.** The mean pixel value of a background region of interest was subtracted from the mean pixel value of a region of interest that circumscribed the specimen. A correction factor, which we measured in images of samples that express only CFP, was applied to the YFP channel to compensate for bleed through of CFP emissions into the YFP channel ( $YFP_{adjusted} = YFP - 0.86 \times CFP$ ). YFP to CFP ratios were normalized to the average value of the first 10 frames (1 s), and a boxcar filter of 5 frames (0.5 s) was applied to the time series.

**Generating a Gene Expression Profile of Embryonic BAG Neurons.** A synchronized population of *nls242[gcy-33::GFP]* hermaphrodites was treated with hypochlorite to release embryos. Embryos were dissociated with chitinase as previously described (45, 46). GFP-labeled BAG neurons were isolated from the freshly dissociated suspension of embryonic cells by FACS; sorted cells were confirmed to be >80% BAG neurons by direct inspection in a fluorescent microscope. A reference population comprised of all viable embryonic cells was also collected by FACS. Dead cells were marked with propidium iodide and excluded from these preparations. Sorting was performed with a Becton-Dickinson FACS-Aria (75- $\mu$ m nozzle; ~15,000 events/s). Approximately 30,000 cells were obtained per sort. Cells were sorted directly into TRIzol LS for extraction with phenol chloroform. RNA was precipitated with isopropanol and purified using a ZYMO DNA-free RNA kit. RNA integrity and concentration were evaluated using an Agilent Bioanalyzer. RNA (1–2 ng) was amplified with the WT-Ovation Pico RNA Amplification System (NuGEN). Double-stranded (ds) cDNA was generated with a WT-Ovation Exon module (NuGEN), and it was fragmented and labeled with an Encore Biotin module (NuGEN) for application to the *C. elegans* Affymetrix 1.0R whole-genome tiling array; ds cDNA targets were used for hybridization, because all probes on the Affymetrix 1.0R array match a single DNA strand, whereas transcripts are derived from both strands. Tiling array results were obtained from three independent replicates (inter-se Pearson correlations  $\geq 0.89$ ). Methods for tiling array analysis are briefly summarized here. A more detailed de-

scription will be reported elsewhere. Unique PM (Perfect Match) probes from exonic regions of gene models were selected to generate a probe set for each gene listed in WormBase (WS199). Intensity values were quantile-normalized, and probe-specific effects were reduced by Robust Multichip Analysis (RMA) (47). An empirical null model of background expression was derived from intergenic probes, and genes with intensity values exceeding this threshold at  $\leq 5\%$  FDR were scored as significantly expressed genes (EGs). Transcripts that were significantly elevated in BAG neurons were identified by comparison with a reference dataset obtained from all viable embryonic cells. Differentially expressed genes were estimated using a linear model and Bayes-moderated  $t$  statistic (48, 49); 850 transcripts with FDR  $\leq 10\%$  and 1.5-fold elevated vs. the reference dataset were scored as significantly enriched in BAG neurons (Dataset S1). Gene ontology analysis was performed with the gene ontology (GO) enrichment analysis widget on the modENCODE intermine website (<http://intermine.modencode.org>). The 850 enriched BAG transcripts were uploaded to the modMINE website on July 5, 2010; 464 transcripts were annotated with GO terms and compared with all genes for enrichment using the hypergeometric test with FDR  $\leq 1\%$ . All transcripts annotated with enriched GO terms are listed in Dataset S2.

**Statistical Analysis.** Statistical tests were performed using GraphPad Instat. Statistical comparisons were made using a one-way ANOVA with Dunnett's posttest, except that Fig. 1A used a paired  $t$  test and Fig. 5A used an unpaired  $t$  test. For Fig. 1A, the dose-response curve shows the least squares fit

of a Hill equation to the data points, with a  $K_d$  of 0.9%  $\text{CO}_2$  and a Hill coefficient of 1.6 (for all graphs,  $***P < 0.001$ ,  $**P < 0.01$ , and  $*P < 0.05$ ). The GCY dendrogram was generated using ClustalW. The *gcy-9* intron-exon structure was generated using Exon-Intron Graphic Maker by Nikhil Bhatla (<http://www.wormweb.org/exonintron>).

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