



# Distinct neurogenetic mechanisms establish the same chemosensory valence state at different life stages in *Caenorhabditis elegans*

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An animal's preference for many chemosensory cues remains constant despite dramatic changes in the animal's internal state. The mechanisms that maintain chemosensory preference across different physiological contexts remain poorly understood. We previously showed that distinct patterns of neural activity and motor output are evoked by carbon dioxide (CO<sub>2</sub>) in starved adults vs dauers of *Caenorhabditis elegans*, despite the two life stages displaying the same preference (attraction) for CO<sub>2</sub>. However, how the distinct CO<sub>2</sub>-evoked neural dynamics and motor patterns contribute to CO<sub>2</sub> attraction at the two life stages remained unclear. Here, using a CO<sub>2</sub> chemotaxis assay, we show that different interneurons are employed to drive CO<sub>2</sub> attraction at the two life stages. We also investigate the molecular mechanisms that mediate CO<sub>2</sub> attraction in dauers vs adults. We show that insulin signaling promotes CO<sub>2</sub> attraction in dauers but not starved adults and that different combinations of neurotransmitters and neuropeptides are used for CO<sub>2</sub> attraction at the two life stages. Our findings provide new insight into the distinct molecular and cellular mechanisms used by *C. elegans* at two different life stages to generate attractive behavioral responses to CO<sub>2</sub>.

**Keywords:** carbon dioxide response; *Caenorhabditis elegans*; chemosensory behavior; sensory valence; gas sensing; dauer larva

## Introduction

Chemosensation is a critical sensory modality that contributes to locating food, finding mates or hosts, and avoiding pathogens and predators (Hildebrand and Shepherd 1997). In some cases, the valence of a chemosensory stimulus, i.e. whether the stimulus is attractive or aversive, depends on an animal's physiological state (Ribeiro and Dickson 2010; Wasserman et al. 2012; Wasserman et al. 2013; Devineni and Scaplen 2021). However, in other cases, chemosensory valence remains constant despite dramatic changes in internal physiology (Schmidt and Beauchamp 1988; Hansson et al. 2010; Oleszkiewicz et al. 2022; Banerjee et al. 2023). The molecular and cellular mechanisms that enable animals to maintain the same valence state across widely varying physiological conditions remain poorly understood.

The response of the free-living nematode *Caenorhabditis elegans* to carbon dioxide (CO<sub>2</sub>) offers a powerful model system for exploring the mechanisms that determine chemosensory valence (Banerjee and Hallem 2020). While well-fed adults are repelled by CO<sub>2</sub>, starved adults are attracted to CO<sub>2</sub> (Bretscher et al. 2008; Hallem and Sternberg 2008; Bretscher et al. 2011; Hallem, Spencer, et al. 2011; Rengarajan et al. 2019). In addition, dauer larvae—long-lived, nonfeeding, stress-resistant larvae that form in

response to adverse environmental conditions (Hu 2007)—are attracted to CO<sub>2</sub> (Hallem, Dillman, et al. 2011a; Banerjee et al. 2023). Thus, a comparison of the molecular and cellular mechanisms that drive CO<sub>2</sub> attraction in dauers vs starved adults can provide insight into how different life stages establish similar chemosensory preferences.

We previously showed that although both starved adults and dauers are attracted to CO<sub>2</sub>, the neural activity dynamics and the motor outputs evoked by CO<sub>2</sub> differ across the two life stages (Rengarajan et al. 2019; Banerjee et al. 2023). For example, the AIY, RIG, and AIB interneurons show different CO<sub>2</sub>-evoked calcium responses in starved adults vs dauers. AIY displays stochastic excitatory CO<sub>2</sub>-evoked activity in starved adults (Rengarajan et al. 2019) but inhibitory CO<sub>2</sub>-evoked activity in dauers (Banerjee et al. 2023), whereas RIG and AIB do not respond to CO<sub>2</sub> in starved adults but display excitatory CO<sub>2</sub>-evoked activity in dauers (Rengarajan et al. 2019; Banerjee et al. 2023). However, how these distinct CO<sub>2</sub>-evoked patterns of neural activity contribute to CO<sub>2</sub> attraction was unclear.

In this study, we correlate the CO<sub>2</sub>-evoked activities of these interneurons with their roles in promoting CO<sub>2</sub> attraction in starved adults vs dauers. We show that individual interneurons make distinct contributions to CO<sub>2</sub> attraction at the two life stages.

Whereas AIY promotes CO<sub>2</sub> attraction in starved adults but not dauers, RIG and AIB promote CO<sub>2</sub> attraction in dauers but not starved adults. We also show that insulin signaling, which promotes CO<sub>2</sub>-evoked AIB activity in dauers (Banerjee et al. 2023), functions in neurons to selectively drive CO<sub>2</sub> attraction in dauers. In addition, we identify distinct combinatorial codes of neurotransmitters and neuropeptides that promote CO<sub>2</sub> attraction at the two life stages. Our results illuminate the different neurogenetic mechanisms that operate in dauers vs adults to establish the same chemosensory valence state.

## Materials and methods

### C. elegans strains

Worms were cultured on 2% nematode growth media (NGM) plates seeded with *Escherichia coli* OP50 bacteria at ambient temperature (~22°C) and CO<sub>2</sub> (~0.038%) as previously described (Stiernagle 2006; Scott 2011; Banerjee et al. 2023). The temperature-sensitive strains CB1370, DR1565, JT191, EAH404, EAH407, and EAH408 were maintained at 15°C but were moved to ambient temperature (~22°C) at least 24 h prior to experiments to minimize any effects of temperature shifts on behavior, as previously described (Banerjee et al. 2023). The strains JT709 and BQ1 were treated similarly for direct comparison with strain DR1565 in Fig. 2f. A complete strain list is provided in Supplementary Table 1.

### Molecular biology and transgenesis

The strains where specific neurons were genetically ablated or silenced were generated in previous studies (Kunitomo et al. 2013; Luo et al. 2014; Calhoun et al. 2015; Jin et al. 2016; Guillermin et al. 2017). The *inx-6<sup>AIB OFF</sup>* strain, where *inx-6* function was eliminated specifically in AIB, was generated in a previous study (Bhattacharya et al. 2019). For tissue-specific rescue of *daf-2*, the following plasmids containing the *daf-2* cDNA were obtained from Addgene and individually injected into the CB1370 *daf-2(e1370)* strain at 30 ng/μL, along with *Pmyo-2::dsRed* (20 ng/μL) as a coinjection marker and pBlueScript (50 ng/μL): pJH4531 (*Prgef-1::daf-2*) for pan-neuronal rescue (Addgene #132366), pJH4723 (*Pges-1::daf-2*) for intestinal rescue (Addgene #178899), and pJH4733 (*Pmyo-3::daf-2*) for muscle rescue (Addgene #178898) (Hung et al. 2014).

### CO<sub>2</sub> chemotaxis assays

CO<sub>2</sub> chemotaxis assays on starved adults and dauers were performed as previously described (Rengarajan et al. 2019; Banerjee et al. 2023). For assays with starved adults, young adults (~1 day old) were washed in a watch glass and then starved on a 9 cm 2% NGM plate without bacteria for 3 h. Animals were placed within an annular ring of Whatman paper soaked in 20 mM copper chloride (CuCl<sub>2</sub>) solution to prevent the worms from migrating off the plate, since copper is aversive to *C. elegans* (Campbell et al. 2017). After 3 h of starvation, the paper ring was removed. Animals were then washed off the plate into M9 buffer and then washed twice in M9 and once in ddH<sub>2</sub>O in a watch glass. For testing, animals were transferred from the watch glass onto the center of a 9 cm 2% NGM plate using a piece of Whatman paper. For assays with dauer larvae, dauers were generated by transferring 8–10 young adults onto 2% NGM plates with a lawn of OP50 bacteria and leaving the plate at room temperature for 10–14 days until the bacterial lawn was consumed. Dauers were isolated from other life stages on the plate using a sodium dodecyl sulfate (SDS) resistance assay (Karp 2016) as previously described

(Banerjee et al. 2023). The dauers were then transferred in water drops onto 2% NGM plates for assays.

CO<sub>2</sub> chemotaxis assays were performed as previously described (Guillermin et al. 2017; Rengarajan et al. 2019; Banerjee et al. 2023). Animals were placed onto the center of a 9 cm 2% NGM plate. The CO<sub>2</sub> stimulus (the test concentration of CO<sub>2</sub>, 21% O<sub>2</sub>, balance N<sub>2</sub>) and air stimulus (21% O<sub>2</sub>, balance N<sub>2</sub>) were pumped through holes in opposite sides of the plate lid at a rate of 2 mL/min (for adults) or 0.5 mL/min (for dauers) to generate a CO<sub>2</sub> gradient (Supplementary Fig. 1a) using a syringe pump (PHD 2000, Harvard Apparatus), with the syringe output connected to the plate lid via ¼-inch flexible PVC tubing. Assay duration was 20 min (for adults) or 1 h (for dauers). After the assay, the number of animals within a 20-mm diameter circle under each gas inlet (for adults) or within 30-mm segments on the side of the plate (for dauers) were counted (Supplementary Fig. 1b and c). For transgenic strains with extrachromosomal arrays, only animals expressing the fluorescent transgene were counted. A chemotaxis index (CI) was then calculated as

$$CI = \frac{\# \text{ animals at CO}_2 - \# \text{ animals at air control}}{\# \text{ animals at CO}_2 + \# \text{ animals at air control}}$$

To account for directional bias due to vibration or other stimuli, assays were conducted in pairs, with the gas gradient in opposite orientations. If the absolute value of the difference in CI between the 2 assays in the pair was ≥0.9, both assays were discarded as behavior was assumed to result from directional bias. Assays were also discarded if fewer than 7 animals navigated into the combined scoring regions. For strain RB2575, which showed decreased motility, single assays within a pair that had more than 7 animals in the combined regions were scored and included in the analysis even if fewer than 7 animals moved in the other assay in the pair, provided there was no directional bias within the pair. In the case of the neuropeptide genes *ins-1*, *flp-2*, and *flp-17*, 2 independent alleles of each gene were tested in CO<sub>2</sub> chemotaxis assays.

### Histamine assays with dauers

Transgenic dauers expressing the histamine-gated chloride channel HisCl1 (Pokala et al. 2014) were isolated using 1% SDS treatment as described above. Dauers were then incubated in 20 mM histamine solution (in dH<sub>2</sub>O) for 1 h prior to assays. For the no-histamine controls, dauers were incubated in dH<sub>2</sub>O without histamine for the same duration. Dauers were then transferred onto 2% NGM plates without bacteria, with or without 20 mM histamine, and CO<sub>2</sub> chemotaxis assays were performed as described above.

### Microscopy

For starved adults, 1-day-old adults were starved for 3 h prior to imaging. Dauers for imaging were isolated by SDS treatment as described above. Animals were anesthetized using 10 mM levamisole and placed onto 2% agarose pads on glass slides. Imaging was performed with a Zeiss Axio Observer inverted wide-field fluorescent microscope equipped with a Colibri 7 for LED fluorescence illumination, a Plan-APOCHROMAT 20x objective lens, a Hamamatsu ORCA-Flash 4.0 camera, and Zen software (Zeiss). For Supplementary Fig. 3, images were captured as z-stacks and maximum intensity projections were constructed using Fiji (Schindelin et al. 2012).

## Statistical analysis

Statistical tests were performed using GraphPad Prism v9.3.1. Normality was determined using the D'Agostino–Pearson omnibus normality test. If data were not normally distributed, non-parametric tests were used. Power analyses were performed using G\*Power v3.1.9.6 (Faul et al. 2007).

## Results

### Distinct sets of interneurons promote CO<sub>2</sub> attraction in dauers vs starved adults

In both starved adults and dauers, CO<sub>2</sub> is detected by the paired BAG sensory neurons in the head (Hallem and Sternberg 2008; Hallem, Spencer, et al. 2011; Smith et al. 2013; Rengarajan et al. 2019; Banerjee et al. 2023). However, many of the interneurons downstream of BAG show different patterns of CO<sub>2</sub>-evoked neural activity in starved adults vs dauers (Fig. 1a) (Banerjee et al. 2023), which may in part reflect differences in synaptic connectivity of these interneurons with the BAG neurons at the two life stages (Fig. 1a) (White et al. 1986; Varshney et al. 2011; Bhattacharya et al. 2019; Yim et al. 2023). To understand the contribution of these different interneurons to CO<sub>2</sub> attraction at the two life stages, we used a CO<sub>2</sub> chemotaxis assay (Supplementary Fig. 1) to examine the behavioral responses of strains where individual interneurons downstream of BAG were either genetically ablated or silenced (Carrillo et al. 2013; Guillermin et al. 2017; Katz et al. 2018; Rengarajan et al. 2019). We focused on the RIG, AIY, AVE, AIB, and RIA interneurons; strains lacking RIB and AVA function were not tested because they did not produce enough dauers for chemotaxis assays, indicating a possible role for these neurons in promoting dauer entry.

We found that RIG- and AIB-ablated dauers showed reduced CO<sub>2</sub> attraction compared with wild type, whereas AVE-silenced dauers were repelled by instead of attracted to CO<sub>2</sub> (Fig. 1b, Supplementary Fig. 2a and b). In contrast, the AIY and RIA neurons were not required for CO<sub>2</sub> attraction in dauers across all tested concentrations (Fig. 1b, Supplementary Fig. 2c and d). Thus, RIG, AIB, and AVE promote CO<sub>2</sub> attraction in dauers, whereas AIY and RIA do not. We note that whereas the RIG, AIY, and AIB promoters we used to genetically ablate these interneurons showed the expected cell-specific expression pattern in starved adults and dauers, the promoter used to genetically silence AVE (*opt-3*) showed faint expression in several neurons in addition to AVE in dauers (Supplementary Fig. 3). Thus, we cannot exclude the possibility that one or more of these *opt-3*-expressing neurons also contribute to CO<sub>2</sub> response in dauers.

To further investigate the roles of CO<sub>2</sub> microcircuit interneurons in regulating CO<sub>2</sub> attraction across life stages, we compared the behavioral responses of interneuron-ablated starved adults and dauers using a CO<sub>2</sub> chemotaxis assay. We found that wild-type and AIB-ablated starved adults were similarly attracted to CO<sub>2</sub>, suggesting that these neurons drive CO<sub>2</sub> attraction in dauers but not starved adults (Fig. 1c). RIG is also required for CO<sub>2</sub> attraction in dauers but not starved adults (Fig. 1d). In contrast, AIY is required for CO<sub>2</sub> attraction in starved adults (Rengarajan et al. 2019) but not dauers (Fig. 1e), whereas RIA is not required to establish CO<sub>2</sub> attraction at either life stage (Fig. 1b) (Rengarajan et al. 2019). To confirm the role of AIB in regulating CO<sub>2</sub> response in dauers, we examined the behavioral responses of dauers in which the AIB neurons were chemogenetically silenced using the histamine-gated chloride channel HisCl1 (Pokala et al. 2014). Dauers expressing HisCl1 specifically in AIB showed a significant

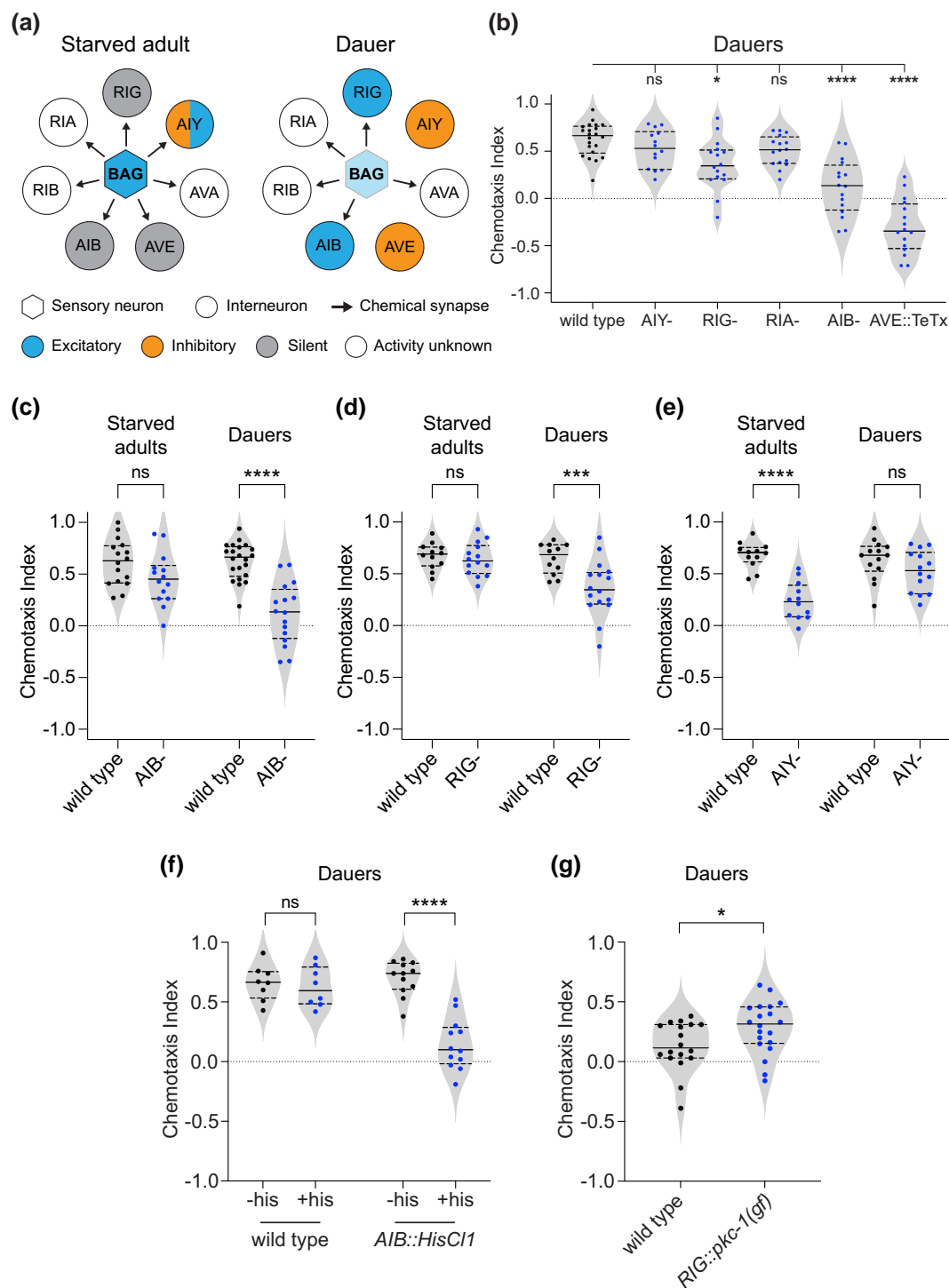
reduction in CO<sub>2</sub> attraction when treated with exogenous histamine compared with untreated control dauers (Fig. 1f), confirming that AIB promotes CO<sub>2</sub> attraction in dauers. To further confirm the role of RIG in promoting CO<sub>2</sub> attraction in dauers, we hyperactivated RIG by expressing a gain-of-function allele of the protein kinase C (*pkc-1*) gene (Sieburth et al. 2005; Sieburth et al. 2007) specifically in RIG (Guillermin et al. 2017). We found that hyperactivation of RIG results in enhanced CO<sub>2</sub> attraction in dauers (Fig. 1g), providing additional evidence that RIG promotes CO<sub>2</sub> attraction in dauers. Thus, both AIB and RIG promote CO<sub>2</sub> attraction in dauers but not starved adults. Together, our results demonstrate that distinct interneurons are required for establishing the same valence state at 2 different life stages.

We then tested the effects of dauer-specific changes in neuronal connectivity on the life stage-specific requirement for interneurons in establishing CO<sub>2</sub> attraction. AIB was previously shown to form gap junctions with BAG in dauers but not adults—the innexin *INX-6* is expressed in AIB specifically in dauers, where it forms a gap junction complex with *CHE-7*, a gap junction subunit expressed in BAG (Fig. 2a) (Bhattacharya et al. 2019). Consistent with previous results (Bhattacharya et al. 2019), *che-7* mutant dauers, as well as dauers where *inx-6* expression is eliminated specifically in AIB (*inx-6<sup>AIB OFF</sup>*), showed significantly reduced CO<sub>2</sub> attraction compared with wild-type dauers (Fig. 2b). In contrast to *inx-6<sup>AIB OFF</sup>* dauers, *inx-6<sup>AIB OFF</sup>* starved adults showed no defects in CO<sub>2</sub> attraction (Fig. 2c). Further, ectopic expression of *inx-6* in AIB did not affect CO<sub>2</sub> responses in starved adults (Fig. 2d), indicating that additional mechanistic differences underlie CO<sub>2</sub> attraction in dauers vs starved adults.

### Insulin signaling promotes CO<sub>2</sub> attraction in dauers but not starved adults

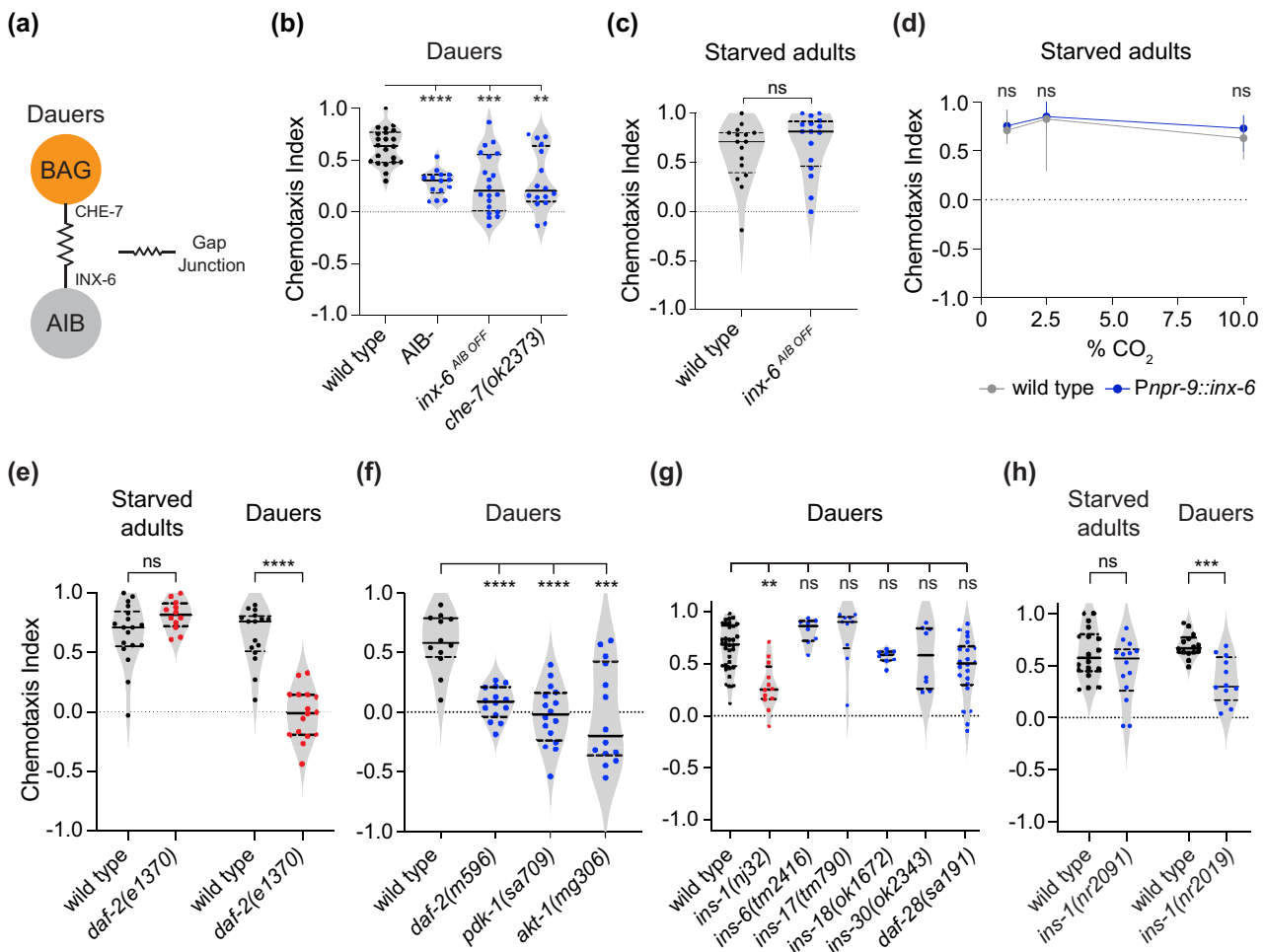
We then investigated the molecular mechanisms that regulate CO<sub>2</sub> attraction in dauers and starved adults. Insulin signaling regulates entry into the dauer state (Hu 2007) as well as a diverse array of chemosensory behaviors in adults (Tomioka et al. 2006; Hallem and Sternberg 2008; Adachi et al. 2010). Moreover, we previously showed that insulin signaling modulates the CO<sub>2</sub>-evoked neuronal activity of AIB in dauers (Banerjee et al. 2023). We therefore examined the role of insulin signaling in regulating CO<sub>2</sub> attraction in starved adults and dauers.

We first examined the CO<sub>2</sub> response of animals carrying a loss-of-function mutation in the *daf-2* gene, which encodes the sole *C. elegans* insulin receptor (Hu 2007). We found that loss of *daf-2* function had no effect on CO<sub>2</sub> attraction in starved adults (Fig. 2e). In contrast, *daf-2* mutant dauers were unresponsive to CO<sub>2</sub>, suggesting that *daf-2* promotes CO<sub>2</sub> attraction in dauers (Fig. 2e–f). The *daf-2* gene is broadly expressed in multiple tissues in both adults and dauers (Murphy and Hu 2013; Martinez et al. 2020). To identify the tissues where *daf-2* might function to regulate CO<sub>2</sub> attraction in dauers, we performed CO<sub>2</sub> chemotaxis assays on *daf-2* mutant dauers in which *DAF-2* function was specifically restored in either neurons, intestine, or muscle. Restoring *DAF-2* function in neurons, but not intestine or muscle, partially yet significantly rescued the chemotaxis defect of *daf-2* mutant dauers (Supplementary Fig. 4). These results suggest that *DAF-2* primarily functions in neurons to mediate CO<sub>2</sub> attraction in dauers. In addition, we found that dauers carrying loss-of-function mutations in the 3-phosphoinositide-dependent-kinase-1 gene *pdh-1* and the protein kinase B (Akt/PKB) gene *akt-1*, both of which act downstream of *daf-2* (Hu 2007), showed significantly reduced CO<sub>2</sub> attraction (Fig. 2f). Thus, insulin signaling promotes CO<sub>2</sub> attraction in dauers but not starved adults.



**Fig. 1.** Distinct interneurons establish CO<sub>2</sub> attraction in starved adults vs dauers. **a)** Synaptic connectivity of the BAG neurons with downstream interneurons and their CO<sub>2</sub>-evoked neuronal activity in adults vs dauers (White et al. 1986; Rengarajan et al. 2019; Banerjee et al. 2023; Yim et al. 2023). The lighter shade of the BAG neurons in dauers indicates a reduced CO<sub>2</sub>-evoked calcium response, as previously reported (Banerjee et al. 2023). Adapted from Banerjee et al. (2023). **b)** The interneurons RIG, AIB, and AVE promote CO<sub>2</sub> attraction in dauers. Strains where specific pairs of interneurons were genetically ablated (AIY-, RIG-, RIA-, or AIB-) or silenced with tetanus toxin (AVE::TeTx) were used. \*\*\*\*P < 0.0001, \*P < 0.05, ns = not significant, 1-way ANOVA with Dunnett's posttest. n = 14–22 trials per genotype. Responses shown are to 10% CO<sub>2</sub>. **c–e)** Distinct interneurons establish CO<sub>2</sub> attraction in starved adults vs dauers. Behavioral responses of starved adults and dauers with genetically ablated AIB (c), RIG (d), and AIY (e) neurons in CO<sub>2</sub> chemotaxis assays. \*\*\*\*P < 0.0001, \*\*\*P < 0.001, ns = not significant, 2-way ANOVA with Sidak's posttest. n = 12–22 trials per genotype. Responses shown are to 10% CO<sub>2</sub>. **f)** Behavioral responses of wild-type dauers and transgenic dauers with AIB-specific expression of the histamine-gated chloride channel HisCl1 (Pokala et al. 2014). Dauers expressing HisCl1 in AIB (AIB::HisCl1) show significantly reduced CO<sub>2</sub> attraction when treated with exogenous histamine (+his) compared with untreated controls (–his), indicating that transiently silencing AIB reduces CO<sub>2</sub> attraction in dauers. \*\*\*\*P < 0.0001, ns = not significant, 2-way ANOVA with Sidak's posttest. n = 8–12 assays per genotype and condition. Responses are to 10% CO<sub>2</sub>. **g)** Dauers with RIG-specific expression of a *pkc-1* gain-of-function (*gf*) allele show significantly enhanced CO<sub>2</sub> attraction compared with wild-type dauers. \*P < 0.05, Welch's t-test. n = 18–20 assays per genotype. Responses are to 1% CO<sub>2</sub>. For b–g, each data point represents a single chemotaxis assay; solid lines indicate medians and dashed lines indicate interquartile ranges.



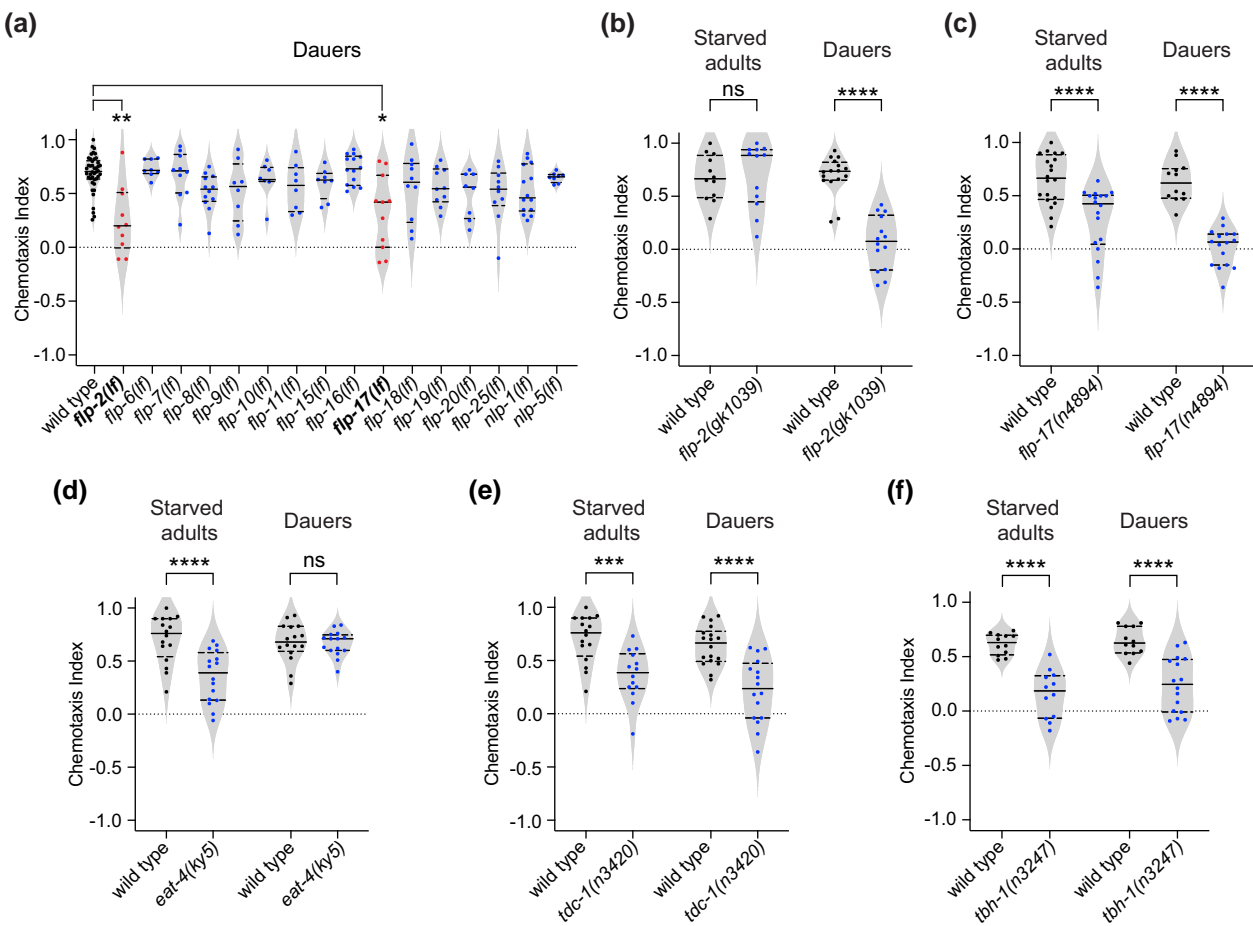


**Fig. 2.** A BAG-AIB gap junction complex and insulin signaling are required for CO<sub>2</sub> attraction in dauers but not starved adults. a) A gap junction complex is formed between the BAG sensory neurons and the AIB interneurons in dauers by selective expression of the innexin subunit *INX-6* in AIB (Bhattacharya et al. 2019). *INX-6* partners with the innexin subunit *CHE-7*, which is expressed in BAG, to form gap junctions (Bhattacharya et al. 2019). b) Behavioral responses of AIB-ablated (*AIB-*) dauers, dauers containing an AIB-specific knockout of *inx-6* (*inx-6<sup>AIB OFF</sup>*) (Bhattacharya et al. 2019), and *che-7* mutant dauers. \*\*\*\**P* < 0.0001, \*\*\**P* < 0.001, \*\**P* < 0.01, 1-way ANOVA with Dunnett's posttest. *n* = 14–22 trials per genotype. c) CO<sub>2</sub> attraction in starved adults remains unaffected in *inx-6* (*inx-6<sup>AIB OFF</sup>*) mutants. ns = not significant, Mann-Whitney test. *n* = 16 trials per genotype. d) Ectopic expression of *INX-6* in AIB does not alter CO<sub>2</sub> responses in starved adults. Behavioral responses of starved adults expressing *inx-6* specifically in AIB under the control of the *npr-9* promoter (*Prnr-9::inx-6*) to CO<sub>2</sub> across concentrations. *n* = 12–16 trials per genotype and condition. Lines show medians and interquartile ranges. ns = not significant, 2-way ANOVA with Sidak's posttest. e) Behavioral responses of wild-type vs *daf-2* mutant starved adults and dauers. *DAF-2* promotes CO<sub>2</sub> attraction in dauers but not starved adults. *n* = 12–18 trials per life stage and genotype. \*\*\*\**P* < 0.0001, ns = not significant, 2-way ANOVA with Sidak's posttest. f) Behavioral responses of *daf-2*, *pdk-1*, and *akt-1* mutant dauers. *n* = 12–16 trials per genotype. \*\*\*\**P* < 0.0001, \*\*\**P* < 0.001, 1-way ANOVA with Dunnett's posttest. g) Behavioral responses of dauers carrying loss-of-function mutations in candidate ILP genes. \*\**P* < 0.01, ns = not significant, Kruskal-Wallis test with Dunn's posttest. *n* = 8–34 trials per genotype. h) Behavioral responses of starved adults and dauers containing a loss-of-function mutation in the insulin-like peptide gene *ins-1*. *INS-1* promotes CO<sub>2</sub> attraction in dauers but not starved adults. \*\*\**P* < 0.001, ns = not significant, 2-way ANOVA with Sidak's posttest. *n* = 12–16 trials per life stage and genotype. For b–c) and e–h), each data point represents a single chemotaxis assay; solid lines indicate medians and dashed lines indicate interquartile ranges. Responses shown are to 10% CO<sub>2</sub>.

We next sought to identify the *DAF-2* ligands that modulate CO<sub>2</sub> response. The *C. elegans* genome encodes 40 insulin-like peptides (ILPs) (Li and Kim 2008). To identify ILPs that may be involved in regulating CO<sub>2</sub> response in dauers, we examined a subset of ILP genes known to be transcriptionally upregulated upon dauer entry (Lee et al. 2017). We found that dauers carrying two independent loss-of-function mutations in the *ins-1* gene showed significantly reduced CO<sub>2</sub> attraction compared with wild-type dauers (Fig. 2g and h). In contrast, CO<sub>2</sub> attraction in starved adults was unaffected in *ins-1* mutants (Fig. 2h). Thus, the modulatory effects of insulin signaling on CO<sub>2</sub> attraction in dauers are mediated at least in part by *INS-1* peptides. Together, our results demonstrate that insulin signaling regulates CO<sub>2</sub> response valence in a life stage-dependent manner.

## Both shared and life stage-specific neuropeptides and neurotransmitters regulate CO<sub>2</sub> attraction

Neuropeptide signaling plays a critical role in modulating neuronal function and behavior across species (Li and Kim 2008) and was previously shown to modulate the CO<sub>2</sub>-evoked behavioral responses of *C. elegans* adults (Guillermin et al. 2017; Rengarajan et al. 2019). To examine whether CO<sub>2</sub> responses in dauers are regulated by neuropeptides, we performed a candidate screen of dauers carrying loss-of-function mutations in 16 neuropeptide genes (Fig. 3a). We focused on neuropeptide genes that are either expressed in BAG (Hallem, Spencer, et al. 2011), transcriptionally upregulated upon dauer entry (Lee et al. 2017), known to regulate CO<sub>2</sub> response in adults (Guillermin et al. 2017;













**Fig. 3.** Both shared and life stage-specific neurotransmitters and neuropeptides shape CO<sub>2</sub> attraction in starved adults vs dauers. a) A reverse genetic screen for neuropeptides that regulate CO<sub>2</sub> attraction in dauers. Behavioral responses of dauers with loss-of-function mutations in neuropeptide genes. \*\**P* < 0.01, \**P* < 0.05, Kruskal–Wallis test with Dunn’s posttest. *n* = 8–42 trials per genotype. The specific loss-of-function (*lf*) alleles used for *flp-2* and *flp-17* were *ok3351* and *ok3587*, respectively. Loss-of-function (*lf*) alleles used for other neuropeptide genes are indicated in the Materials and methods and [Supplementary Table 1](#). b) and c) Behavioral responses of starved adults and dauers containing loss-of-function mutations in the neuropeptide genes *flp-2* (b) and *flp-17* (c). \*\*\*\**P* < 0.0001, ns = not significant, 2-way ANOVA with Sidak’s posttest. *n* = 12–20 trials per genotype and life stage. d–f) Behavioral responses of starved adults and dauers containing loss-of-function mutations in the vesicular glutamate transporter gene *eat-4* (d), the tyrosine decarboxylase gene *tdc-1* (e), and the tyramine β-hydroxylase gene *tbh-1* (f). \*\*\*\**P* < 0.0001, \*\*\**P* < 0.001, ns = not significant, 2-way ANOVA with Sidak’s posttest. *n* = 12–18 trials per genotype and life stage. For a–f, each data point represents a single chemotaxis assay; solid lines indicate medians and dashed lines indicate interquartile ranges. Responses shown are to 10% CO<sub>2</sub>.

Rengarajan et al. 2019), or known to encode neuropeptides that are dependent on the neuropeptide-processing gene *sbt-1*, since *sbt-1* promotes dauer entry and regulates CO<sub>2</sub> attraction in dauers (Lee et al. 2017).

















We found that the FMRFamide-like neuropeptide gene *flp-16* and the neuropeptide-like protein gene *nlp-1*, which were previously shown to regulate CO<sub>2</sub> attraction in starved adults (Rengarajan et al. 2019), are not required to promote CO<sub>2</sub> attraction in dauers (Fig. 3a). Thus, NLP-1 and FLP-16 neuropeptides play adult-specific roles in regulating CO<sub>2</sub> response. However, dauers lacking the FMRFamide-like neuropeptide gene *flp-2* showed significantly reduced CO<sub>2</sub> attraction (Fig. 3a and b), whereas starved adults lacking *flp-2* responded normally to CO<sub>2</sub> (Fig. 3b). Thus, FLP-2 neuropeptides are required to establish CO<sub>2</sub> attraction in dauers but not starved adults. In addition, we found that both dauers and starved adults carrying loss-of-function mutations in the BAG-expressed neuropeptide gene *flp-17* showed significantly reduced CO<sub>2</sub> attraction (Fig. 3a and c). Thus, our results demonstrate that FLP-17 promotes CO<sub>2</sub> attraction in both dauers and starved adults.

We also investigated the role of neurotransmitters in regulating CO<sub>2</sub> attraction in starved adults vs dauers. We first examined the role of glutamate signaling, since the CO<sub>2</sub>-detecting BAG neurons express the vesicular glutamate transporter EAT-4 (Serrano-Saiz et al. 2013) and well-fed adults carrying a loss-of-function mutation in the *eat-4* gene show dramatically reduced CO<sub>2</sub> repulsion (Guillermin et al. 2017). We found that starved adults lacking *eat-4* showed significantly reduced CO<sub>2</sub> attraction (Fig. 3d). In contrast, CO<sub>2</sub> attraction remained unaffected in *eat-4* mutant dauers (Fig. 3d). Thus, glutamate signaling is required for CO<sub>2</sub> attraction in starved adults but not dauers. Finally, we examined the role of biogenic amines in regulating CO<sub>2</sub> attraction. Both the tyrosine decarboxylase gene *tdc-1*, which is required for the biosynthesis of tyramine and octopamine (Chase and Koelle 2007), and the tyramine β-hydroxylase gene *tbh-1*, which is required for the conversion of tyramine to octopamine (Chase and Koelle 2007), promote CO<sub>2</sub> attraction in starved adults (Fig. 3e and f) (Rengarajan et al. 2019). We found that both *tdc-1* and *tbh-1* also promote CO<sub>2</sub> attraction in dauers, since *tdc-1* and *tbh-1* mutant dauers show decreased CO<sub>2</sub> attraction (Fig. 3e

(a)

Life stage / Condition	Sensory neuron	Interneuron				Valence state
Starved adults						CO <sub>2</sub> attraction
Dauers						CO <sub>2</sub> attraction

(b)

Life stage / Condition	Molecular signals								Behavior
Starved adults									CO <sub>2</sub> attraction
Dauers									CO <sub>2</sub> attraction

**Fig. 4.** Distinct mechanisms shape CO<sub>2</sub> response valence across life stages. a) Table showing the requirement for the BAG sensory neurons and downstream interneurons in establishing CO<sub>2</sub> response valence in starved adults vs dauers. Neurons involved in establishing CO<sub>2</sub> attraction are shaded; neurons not required for CO<sub>2</sub> attraction are unshaded. b) Table showing the requirement for different molecular signals in establishing CO<sub>2</sub> response valence across life stages. Neurotransmitters and neuropeptides required to establish CO<sub>2</sub> attraction are indicated by shaded boxes; the lack of requirement for a particular molecular signal in establishing CO<sub>2</sub> attraction is indicated by an unshaded box.

and f). Thus, while glutamate signaling regulates CO<sub>2</sub> attraction specifically in starved adults, biogenic amine signaling regulates CO<sub>2</sub> attraction in both starved adults and dauers. Together, our results identify both shared and life stage-specific neuropeptides and neurotransmitters that regulate CO<sub>2</sub> attraction in starved adults vs dauers, suggesting that distinct combinations of neuropeptides and neurotransmitters regulate CO<sub>2</sub> attraction at the two life stages.

## Discussion

We have shown that the same valence state, CO<sub>2</sub> attraction, is established in dauers and starved adults by distinct cellular and molecular mechanisms. The same CO<sub>2</sub>-detecting sensory neurons are required for CO<sub>2</sub> attraction in both dauers and starved adults, but distinct interneurons mediate CO<sub>2</sub> attraction at the two life stages (Fig. 4a) (Hallem, Dillman, et al. 2011a; Rengarajan et al. 2019; Banerjee et al. 2023). The requirement for different interneurons at the dauer vs adult stage may arise from anatomical as well as functional differences in neuronal connectivity at the two life stages. For example, the AIY neurons promote CO<sub>2</sub> attraction in starved adults but not dauers (Fig. 4a), consistent with the dauer-specific loss of chemical synapses between BAG and AIY (Yim et al. 2023). However, AIY still displays CO<sub>2</sub>-evoked activity in dauers (Banerjee et al. 2023), suggesting that this activity is independent of BAG-AIY chemical synapses. In addition, the AIB and RIG neurons promote CO<sub>2</sub> attraction in dauers but not starved adults despite forming chemical synapses with the BAG neurons at both life stages (Fig. 1a) (White et al. 1986; Yim et al. 2023). The distinct requirement for AIB in mediating dauer CO<sub>2</sub> attraction arises due to dauer-specific BAG-AIB gap junctions (Bhattacharya et al. 2019). The role of RIG in selectively promoting CO<sub>2</sub> attraction in dauers may arise from changes in functional connectivity between BAG and RIG, although differences in anatomical connectivity between RIG and other neurons may also contribute. For example, RIG receives dauer-specific synaptic

input from the IL2 neurons, which are required for nictation, a dauer-specific behavior that promotes dispersal by facilitating phoretic interactions between dauers and larger invertebrates (Lee et al. 2012) (Yim et al. 2023). Our results raise the possibility that RIG activity drives CO<sub>2</sub> attraction specifically in dauers to enable dauers to coordinate CO<sub>2</sub> response with nictation.

Our findings also demonstrate that distinct combinations of neurotransmitters and neuropeptides are required for CO<sub>2</sub> attraction at the two life stages (Fig. 4b). Further studies of the effects of these molecular signals on CO<sub>2</sub> microcircuit function will reveal the regulatory principles that enable the worm to establish the same valence state under distinct physiological conditions. For instance, biogenic amine signaling promotes CO<sub>2</sub> attraction in starved adults by regulating the CO<sub>2</sub>-evoked activity of AIY (Rengarajan et al. 2019). In dauers, biogenic amine signaling is also required for CO<sub>2</sub> attraction despite the lack of requirement for AIY (Fig. 4b), suggesting that biogenic amine signaling promotes CO<sub>2</sub> attraction via distinct neural mechanisms at the two life stages. Taken together, our results demonstrate that different mechanisms may operate at different life stages to promote the same valence state. In future studies, it will be interesting to explore whether life stage-specific chemosensory mechanisms function to couple chemosensory responses to other life stage-specific, ethologically relevant behavioral programs.

## Data availability

Strains and plasmids are available upon request. All raw data and statistical analysis from this study are included in [Supplementary File S1](#).

[Supplemental material](#) available at G3 online.

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## Conflicts of interest

The authors declare no conflict of interest.

## Author contributions

N.B., P.-Y.S., P.W.S., and E.A.H. conceived the study. N.B. and E.J.R.P. performed experiments. N.B. and E.J.R.P. analyzed the data. N.B. and E.A.H. wrote the manuscript. All authors read and approved the final manuscript.

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