O₂-Sensing Neurons Control CO₂ Response in *C. elegans*

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Sensory behaviors are often flexible, allowing animals to generate context-appropriate responses to changing environmental conditions. To investigate the neural basis of behavioral flexibility, we examined the regulation of carbon dioxide (CO₂) response in the nematode *Caenorhabditis elegans*. CO₂ is a critical sensory cue for many animals, mediating responses to food, conspecifics, predators, and hosts (Scott, 2011; Buehlmann et al., 2012; Chaisson and Hallem, 2012). In *C. elegans*, CO₂ response is regulated by the polymorphic neuropeptide receptor NPR-1: animals with the N2 allele of *npr-1* avoid CO₂, whereas animals with the Hawaiian (HW) allele or an *npr-1* loss-of-function (*lf*) mutation appear virtually insensitive to CO₂ (Hallem and Sternberg, 2008; McGrath et al., 2009). Here we show that ablating the oxygen (O₂)-sensing URX neurons in *npr-1(lf)* mutants restores CO₂ avoidance, suggesting that NPR-1 enables CO₂ avoidance by inhibiting URX neurons. URX was previously shown to be activated by increases in ambient O₂ (Persson et al., 2009; Zimmer et al., 2009; Busch et al., 2012). We find that, in *npr-1(lf)* mutants, O₂-induced activation of URX inhibits CO₂ avoidance. Moreover, both HW and *npr-1(lf)* animals avoid CO₂ under low O₂ conditions, when URX is inactive. Our results demonstrate that CO₂ response is determined by the activity of O₂-sensing neurons and suggest that O₂-dependent regulation of CO₂ avoidance is likely to be an ecologically relevant mechanism by which nematodes navigate gas gradients.

Introduction

Animals from nematodes to humans respond to environmental gases, such as CO₂ and O₂. CO₂ is aversive for many free-living animals but attractive for many parasitic animals, which rely on CO₂ for host location (Luo et al., 2009; Chaisson and Hallem, 2012). O₂ increases or decreases can evoke avoidance responses in flies and nematodes (Chang et al., 2006; Morton, 2011) and alter foraging and feeding behaviors (Wingrove and O'Farrell, 1999; Cheung et al., 2005; Rogers et al., 2006; Vigne and Frelin, 2010). These responses are critical for survival: exposure to hypercapnia, hyperoxia, or hypoxia can result in reduced neural activity, cell cycle arrest, tumor formation, or death (Wingrove and O'Farrell, 1999; Harris, 2002; West, 2004; Langford, 2005).

The nematode *Caenorhabditis elegans* detects and responds to changes in environmental CO₂ and O₂ (Scott, 2011). *C. elegans* adults migrate away from a CO₂ source and toward ~10% O₂

(Gray et al., 2004; Bretscher et al., 2008; Hallem and Sternberg, 2008). However, CO₂ response can vary with developmental stage and environmental context. For example, CO₂ is repulsive for adults but attractive for dauer larvae (Hallem et al., 2011a), and the behavioral response to simultaneous changes in CO₂ and O₂ levels is indicative of an interaction between the responses to the two gases (Bretscher et al., 2008; McGrath et al., 2009).

The response of *C. elegans* to CO₂ and many other stimuli is regulated by NPR-1, a polymorphic neuropeptide receptor homologous to mammalian neuropeptide Y receptors (de Bono and Bargmann, 1998; Gray et al., 2004; Rogers et al., 2006; Bretscher et al., 2008; Hallem and Sternberg, 2008; Macosko et al., 2009; McGrath et al., 2009; Reddy et al., 2009). The N2 strain of *C. elegans* contains an *npr-1* allele that confers solitary feeding behavior, whereas the CB4856 Hawaiian (HW) strain contains an *npr-1* allele that confers social feeding behavior (de Bono and Bargmann, 1998). N2 animals respond strongly to CO₂ but weakly to O₂ on food, whereas HW animals appear relatively indifferent to CO₂ but respond strongly to O₂ on food (Gray et al., 2004; Bretscher et al., 2008; Hallem and Sternberg, 2008). NPR-1 is thought to act by repressing neural activity (Chang et al., 2006; Macosko et al., 2009).

To investigate the mechanisms of CO₂ response plasticity, we examined the regulation of CO₂ response by NPR-1. We show that HW and *npr-1(lf)* animals do not avoid CO₂ despite showing normal CO₂-evoked activity in BAG neurons. However, ablation of URX neurons in *npr-1(lf)* animals restores CO₂ avoidance, suggesting that NPR-1 enables CO₂ avoidance by decreasing URX activity. URX is activated by increases in ambient O₂ (Persson et al., 2009; Zimmer et al., 2009; Busch et al., 2012), and we show that its O₂-sensing ability is required to inhibit CO₂ avoidance. We also show that HW and *npr-1(lf)* animals avoid CO₂ under low O₂ conditions, when URX is inactive. Our results

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suggest that CO₂ avoidance is regulated by ambient O₂ via a pair of O₂-sensing neurons, allowing flexible responses to fluctuating levels of environmental gases.

Materials and Methods

Strains. *C. elegans* strains are listed in the order in which they appear in the figures. The following strains were used: N2 (Bristol); DA609 *npr-1(ad609)*; CB4856 (Hawaiian); CX11697 *kyls536[flp-17::p17 SL2 GFP, elt-2::mCherry]*; *kyls538[glb-5::p12 SL2 GFP, elt-2::mCherry]*; EAH2 *gcy-9(tm2816)*; PS6416 *pha-1(e2123)*; *syEx1206[gcy-33::G-CaMP3.0, pha-1(+)]*; EAH117 *npr-1(ad609)*; *syEx1206[gcy-33::G-CaMP3.0, pha-1(+)]*; EAH119 *bruEx89[gcy-33::G-CaMP-3.0, ets-8::GFP]*; MT17148 *flp-21(ok889)*; *flp-18(n4766)*; PR767 *ttx-1(p767)*; GN112 *pgls2[gcy-8::caspase, unc-122::GFP]*; PR679 *che-1(p679)*; MT18636 *nIs326[gcy-33::YC3.60]*; *lin-15AB(n765)*; AX2047 *gcy-8::YC3.60, unc-122::dsRed*; XL115 *flp-6::YC3.60*; CX9592 *npr-1(ad609)*; *kyEx2016[npr-1::npr-1 SL2 GFP, ofm-1::dsRed]*; CX9395 *npr-1(ad609)*; *kyEx1965[gcy-32::npr-1 SL2 GFP, ofm-1::dsRed]*; CX9633 *npr-1(ad609)*; *kyEx2096[flp-8::npr-1 SL2 GFP, ofm-1::dsRed]*; CX9396 *npr-1(ad609)*; *kyEx1966[flp-21::npr-1 SL2 GFP, ofm-1::dsRed]*; CX9644 *npr-1(ad609)*; *kyEx2107[ncs-1::npr-1 SL2 GFP, ofm-1::dsRed]*; CX7102 *lin-15(n765) qals2241[gcy-36::egl-1, gcy-35::GFP, lin-15(+)]*; CX7158 *npr-1(ad609) qals2241[gcy-36::egl-1, gcy-35::GFP, lin-15(+)]*; ZG629 *ials22[gcy-36::GFP, unc-119(+)]*; EAH80 *ials22[gcy-36::GFP, unc-119(+)]*; *npr-1(ad609)*; EAH106 *bruEx86[gcy-36::G-CaMP3.0, coel::RFP]*; EAH114 *npr-1(ad609)*; *bruEx86[gcy-36::G-CaMP3.0, coel::RFP]*; ZG24 *ahr-1(ia3)*; ZG624 *ahr-1(ia3)*; *npr-1(ad609)*; CX6448 *gcy-35(ok769)*; CX7157 *gcy-35(ok769)*; *npr-1(ad609)*; RB1902 *flp-19(ok2460)*; PT501 *flp-8(pk360)*; PT502 *flp-10(pk367)*; EAH123 *npr-1(ad609) flp-19(ok2460)*; EAH141 *npr-1(ad609) flp-8(pk360)*; EAH140 *flp-10(pk367)*; *npr-1(ad609)*; PS5892 *gcy-33(ok232)*; *gcy-31(ok296)*; EAH127 *gcy-33(ok232)*; *gcy-31(ok296) lon-2(e678) npr-1(ad609)*. In addition, CX7376 *kyls511[gcy-36::G-CaMP, coel::GFP]* and EAH115 *kyls511[gcy-36::G-CaMP, coel::GFP]*; *npr-1(ad609)* were used to confirm the results shown in Figure 5A with independent transgenes, and RB1903 *flp-19(ok2461)* and EAH139 *npr-1(ad609) flp-19(ok2461)* were used to confirm the results shown in Figure 5C with an independent deletion allele of *flp-19*. All transgenes were injected into N2, except *bruEx89*, which was injected into CB4856 to generate EAH119. EAH2 was derived from FX2816 by outcrossing to N2 for five generations. Nematodes were cultured on NGM plates containing *Escherichia coli* OP50 according to standard methods (Brenner, 1974). *C. elegans* dauer larvae were collected from the lids of plates from which the OP50 food source had been depleted ("starved plates") and stored in dH₂O at 15°C before use. All nematodes tested were hermaphrodites.

Generation of reporter transgenes and transgenic animals. To generate EAH119, the *gcy-33::G-CaMP3.0* construct from PS6416 was injected into CB4856 at 50 ng/μl along with *ets-8::GFP* at 50 ng/μl as a coinjection marker. To generate EAH106, a *gcy-36::G-CaMP3.0* transcriptional fusion construct was generated by amplifying a 1.0 kb region upstream of the start codon of the *gcy-36* gene from N2 genomic DNA using primers that included the following sequences: 5'-gatgttgtagatgggtttga-3' and 5'-aaattcaacaaggctaccaca-3'. The promoter fragment was then cloned into a modified Fire vector containing the *G-CaMP3.0* coding region (Tian et al., 2009). The *gcy-36::G-CaMP3.0* construct was injected into N2 animals at a concentration of 25 ng/μl along with 50 ng/μl of *coel::RFP* as a coinjection marker.

Acute CO₂ avoidance assays. Acute CO₂ avoidance assays were performed as previously described (Hallem and Sternberg, 2008; Guillermin et al., 2011; Hallem et al., 2011b). Briefly, ~10–15 young adults were tested on 5 cm assay plates consisting of NGM agar seeded with a thin lawn of *E. coli* OP50 bacteria. Gas stimuli consisted of certified industrial mixes (Airgas or Air Liquide). CO₂ stimuli consisted of 10% CO₂, 10% O₂ (unless otherwise indicated), and the rest N₂. Control stimuli consisted of 10% O₂ (unless otherwise indicated) and the rest N₂. Two 50 ml gas-tight syringes were filled with gas: one with CO₂ and one without CO₂. The mouths of the syringes were connected to flexible PVC tubing 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. Gases were delivered blindly, and worms were scored blindly. An avoidance index was calculated by subtracting the fraction of animals that reversed to the air control from the fraction that reversed to the CO₂. Single-worm acute CO₂ avoidance assays were performed on L4 or young adult laser-ablated animals (see Fig. 3C) as described above, except that each animal was tested 12 times with >2 min between trials. For each animal, an avoidance index was calculated by subtracting the fraction of trials in which it reversed to the air control from the fraction of trials in which it reversed to the CO₂ stimulus. The avoidance index for each genotype or treatment was calculated as the mean avoidance index for each animal of the same genotype or treatment.

CO₂ chemotaxis assays. CO₂ chemotaxis assays were performed on young adults essentially as previously described (Bretscher et al., 2008). Briefly, animals were washed off plates and into a 65 mm Syracuse watch glass using M9 buffer. Animals were washed 3× with M9 and transferred from the watch glass to a 1 cm × 1 cm square of Whatman paper. Animals were then transferred from the filter paper to the center of a 9 cm NGM or chemotaxis plate (Bargmann et al., 1993). Gas stimuli were delivered to the plate through holes in the plate lids as previously described (Hallem et al., 2011a; Dillman et al., 2012), except at a flow rate of 2 ml/min. Assay plates were placed on a vibration-reducing platform for 20 min. The number of worms in a 2-cm-diameter circle centered under each gas inlet was then counted, except for Figures 1B and 2A, B, where the number of worms in an area comprising ~3/10 of the plate under each gas inlet was counted. The chemotaxis index was calculated as follows: (no. of worms at CO₂ – no. of worms at control)/(no. of worms at CO₂ + control). Two identical assays were always performed simultaneously with the CO₂ gradient in opposite directions on the two plates to control for directional bias resulting from room vibration; assays were discarded if the difference in the chemotaxis index for the two plates was ≥0.9 or if <7 worms moved into the scoring regions on one or both of the plates.

For CO₂ chemotaxis assays under different O₂ conditions, assays were performed as described above inside airtight canisters (OGGI; 13.3 cm × 10.1 cm) with four holes drilled into the lids to insert tubing for gas flow. One hole was used to establish the ambient O₂ level, two were used to establish the CO₂ gradient, and one was used as an exhaust. A gas mixture consisting of either 7% O₂ and the balance N₂, or 21% O₂ and the balance N₂, was pumped into the chamber at a rate of 2.5 L/min for 1 min and then 0.5 L/min for the duration of the assay. The CO₂ stimulus (10% CO₂, either 7% O₂ or 21% O₂, balance N₂) and control stimulus (7% O₂ or 21% O₂, balance N₂) were pumped into the chamber at a rate of 2 ml/min using a syringe pump, as described above. The assay duration was 25 min.

Dauer CO₂ chemotaxis assays were performed as previously described (Hallem et al., 2011a; Dillman et al., 2012). Briefly, assays were performed on chemotaxis plates (Bargmann et al., 1993). For each assay, ~50–150 dauers were placed in the center of the assay plate. Gas stimuli and gas delivery to the assay plate were as described above, and a chemotaxis index was calculated as described above.

Calcium imaging. Imaging was performed using the genetically encoded calcium indicators G-CaMP (Zimmer et al., 2009), G-CaMP3.0 (Tian et al., 2009), or yellowameleon YC3.60 (Nagai et al., 2004). Young adult or L4 animals were immobilized onto a cover glass containing a 2% agarose pad made with 10 mM HEPES using Surgi-Lock 2oc instant tissue adhesive (Meridian). A custom-made gas delivery chamber was placed over the cover glass. Gases were delivered at a rate of 0.8–1 L/min. Gas delivery was controlled by a ValveBank4 controller (AutoMate Scientific). Imaging was performed on an AxioObserver A1 inverted microscope (Carl Zeiss) using a 40× EC Plan-NEOFLUAR lens, a Hamamatsu C9100 EM-CCD camera, and AxioVision software (Carl Zeiss). For YC3.60 imaging, the emission image was passed through a DV2 beam splitter (Photometrics) as previously described (Hallem et al., 2011b). Image analysis was performed using AxioVision software (Carl Zeiss) and Microsoft Excel. The mean pixel value of a background region of interest was subtracted from the mean pixel value of a region of interest containing the neuron soma. Fluorescence values were normalized to the average values obtained in the 4 s before CO₂ delivery. For YC3.60 im-

Figure 1. *npr-1* is required for CO₂ avoidance behavior but not CO₂ detection. **A**, *npr-1* is required for CO₂ avoidance by adults. Left, CO₂ chemotaxis assay. Right, Acute CO₂ avoidance assay. *npr-1(lf)* and HW animals do not respond to CO₂ in either assay. ****p* < 0.001, one-way ANOVA with Bonferroni post-test. *n* = 6–9 trials (chemotaxis assay) or 10–27 trials (acute assay) for each genotype. Error bars represent SEM. **B**, Animals that lack BAG neurons and *gcy-9(tm2816)* mutant animals do not respond to CO₂. BAG-ablated animals express a transgene that specifically kills the BAG neurons (Zimmer et al., 2009; Hallem et al., 2011b). Left, CO₂ chemotaxis assay. Right, Acute CO₂ avoidance assay. The CO₂ stimulus was delivered in an airstream containing 10% O₂, which approximates the preferred O₂ concentration for *C. elegans* (Gray et al., 2004); the control airstream also contained 10% O₂. *n* = 18 or 19 trials (chemotaxis assay) or 4–27 trials (acute assay). ****p* < 0.001, Kruskal–Wallis test with Dunn's post-test (chemotaxis assay) or one-way ANOVA with Bonferroni post-test (acute assay). Error bars represent SEM. **C**, *npr-1* is not required for CO₂ detection by BAG neurons. BAG neuron cell bodies of N2 (left), *npr-1(lf)* (middle), and HW (right) animals respond to CO₂. Calcium increases were measured using the calcium indicator G-CaMP3.0. Black lines indicate average calcium responses; white shading represents SEM. Blue lines below the traces indicate the timing of the CO₂ pulse. The peak response amplitudes of all three genotypes were not significantly different (one-way ANOVA with Bonferroni post-test). The decay kinetics of all three genotypes were significantly different (*p* < 0.001 using a polynomial curve fit). However, these differences are not likely to be a result of differences at the *npr-1* locus because recordings from BAG neurons of N2 animals using G-CaMP3.0 showed different decay kinetics from BAG neurons of N2 animals using YC3.60 (Fig. 2C,D). **D**, The NPR-1 ligands FLP-21 and FLP-18 are not required for CO₂ response. ****p* < 0.001, one-way ANOVA with Bonferroni post-test. *n* = 6–9 animals for each genotype. Error bars represent SEM.

aging, the YFP/CFP ratio was calculated as previously described (Hallem et al., 2011b). Images were baseline corrected using a linear baseline correction. Traces with unstable baselines before the onset of the CO₂ stimulus were discarded.

Laser ablation. Ablations were performed on L2 and L3 animals as previously described (Hallem and Sternberg, 2008). Briefly, animals were mounted on glass slides for DIC microscopy on a pad consisting of 5% Noble agar in dH₂O with 5% sodium azide as anesthetic. Ablations were performed on a Zeiss AxioImager A2 microscope with an attached MicroPoint laser (Carl Zeiss). Neurons were ablated by focusing a laser microbeam on the cell. Mock-ablated animals were mounted similarly but were not subjected to a laser microbeam. Neurons were identified by both cell position and GFP expression. Loss of the ablated cell was confirmed by observing loss of fluorescence in the adult animal.

Fluorescence microscopy. Nematodes were anesthetized with 3 mM levamisole and mounted on a pad consisting of 5% Noble agar in dH₂O. Epifluorescence images were captured using a Zeiss AxioImager A2 microscope with an attached Zeiss AxioCam camera and Zeiss AxioVision software (Carl Zeiss). To quantify epifluorescence in Figure 4D, all images were taken with the same exposure time. Average pixel intensities in the region of interest were quantified using AxioVision software (Carl Zeiss). Relative intensities were normalized by setting the highest mean intensity value to 1.

Statistical analysis. Statistical analysis was performed using GraphPad Instat and Prism. All significance values reported are relative to the N2 control, unless otherwise indicated.

Results

NPR-1 regulates CO₂ avoidance behavior

To investigate the role of *npr-1* in mediating CO₂ response, we examined the CO₂-evoked behavior of N2, HW, and *npr-1(lf)* animals in both a chemotaxis assay and an acute avoidance assay. We found that N2 animals displayed robust CO₂ avoidance in

both assays, whereas HW and *npr-1(lf)* animals were essentially unresponsive to CO₂ in both assays (Fig. 1A). Thus, the N2 allele of *npr-1* is required for the behavioral response to CO₂. CO₂ avoidance behavior also requires the CO₂-detecting BAG neurons and the receptor guanylate cyclase gene *gcy-9*, which encodes a putative receptor for CO₂ or a CO₂ metabolite (Fig. 1B) (Hallem and Sternberg, 2008; Hallem et al., 2011b; Brandt et al., 2012). To test whether *npr-1* is required for CO₂ detection, we imaged from BAG neurons using the genetically encoded calcium indicator G-CaMP3.0 (Tian et al., 2009). We found that the BAG neurons of N2, *npr-1(lf)*, and HW animals all showed CO₂-evoked activity (Fig. 1C), suggesting that *npr-1* regulates the behavioral response to CO₂ downstream of the calcium response of BAG neurons. The *flp-21* and *flp-18* genes, which encode NPR-1 ligands, are not required for CO₂ avoidance, suggesting that other ligands are required for the regulation of CO₂ response by *npr-1* (Fig. 1D).

In addition to the BAG neurons, the salt-sensing ASE neurons and the temperature-sensing AFD neurons have been implicated in CO₂ detection and avoidance (Bretscher et al., 2011). However, we found that *che-1* mutant animals, which lack functional ASE neurons (Uchida et al., 2003), displayed normal CO₂ avoidance in both a chemotaxis assay and an acute assay (Fig. 2A) (Hallem and Sternberg, 2008). Both AFD-ablated animals and *ttx-1* mutant animals, which lack functional AFD neurons (Satterlee et al., 2001), showed defective CO₂ avoidance in a chemotaxis assay but not an acute assay (Fig. 2A) (Hallem and Sternberg, 2008). These results suggest that ASE neurons are not required for CO₂ avoidance under our assay conditions and that AFD neurons are required for some but not all CO₂-evoked be-

Figure 2. The role of AFD, ASE, and BAG neurons in CO₂ response. **A**, Animals that lack functional ASE neurons respond normally to CO₂ in both a chemotaxis assay (left graph) and an acute avoidance assay (Hallem and Sternberg, 2008). Animals that lack functional AFD neurons respond normally to CO₂ in an acute avoidance assay (right) but not a chemotaxis assay (left). AFD-ablated animals (AFD⁻) express a transgene that specifically kills the AFD neurons (Glauser et al., 2011). The *ttx-1* and *che-1* genes encode transcription factors that are required for normal development of the AFD and ASE neurons, respectively (Satterlee et al., 2001; Uchida et al., 2003; Hobert, 2010). ***p* < 0.01, Kruskal–Wallis test with Dunn’s post-test. ****p* < 0.001, Kruskal–Wallis test with Dunn’s post-test. *n* = 10–18 trials (chemotaxis assay) or *n* = 9–27 trials (acute assay) for each genotype. Error bars represent SEM. **B**, Animals that lack BAG neurons do not respond to CO₂ when the CO₂ stimulus is delivered in an airstream containing 21% O₂, which approximates the atmospheric O₂ concentration. The control airstream also contained 21% O₂. ****p* < 0.01, Mann–Whitney test. ****p* < 0.001, one-way ANOVA with Bonferroni post-test. *n* = 4–11 trials (chemotaxis assay) or *n* = 10–27 trials (acute assay). Error bars represent SEM. **C, D**, Calcium responses of BAG, AFD, and ASE neurons to 10% CO₂ (**C**) and 5% CO₂ (**D**), measured using the ratiometric calcium indicator yellowameleon YC3.60. Black lines indicate average calcium responses; white shading represents SEM. Blue lines below the traces indicate the timing of the CO₂ pulse. Calcium increases were observed in BAG neuron cell bodies but not AFD and ASE neuron cell bodies. *n* = 5–13 animals for each genotype.

haviors. By contrast, animals lacking BAG neurons showed a complete loss of CO₂ response in both assays, regardless of whether CO₂ was delivered in combination with 10% O₂, which approximates the preferred O₂ concentration of *C. elegans* (Gray et al., 2004), or 21% O₂, which approximates atmospheric O₂ concentration (Figs. 1B and 2B). We then imaged from BAG, ASE, and AFD neurons using the calcium indicator yellowameleon YC3.60 (Nagai et al., 2004). We observed CO₂-evoked activity in BAG neurons but not AFD and ASE neurons in response to a 20 s pulse of either 5% or 10% CO₂ (Fig. 2C,D). Thus, BAG neurons are the primary sensory neurons that contribute to CO₂ response under our assay conditions.

NPR-1 regulates URX neuron activity to control CO₂ avoidance behavior

NPR-1 is not expressed in BAG neurons but is expressed in a number of other sensory neurons as well as some interneurons (Macosko et al., 2009). To identify the site of action for the regulation of CO₂ response by *npr-1*, we introduced the N2 allele of

npr-1 into *npr-1(lf)* mutants in different subsets of neurons and assayed CO₂ response. We found that expressing *npr-1* in neuronal subsets that included the O₂-sensing URX neurons (Cheung et al., 2004; Gray et al., 2004) restored CO₂ response (Fig. 3A). These results suggest that NPR-1 activity in URX neurons is sufficient to enable CO₂ avoidance. However, we cannot exclude the possibility that NPR-1 function in other neurons also contributes to CO₂ avoidance.

To further investigate the role of the URX neurons in regulating CO₂ response, we ablated URX neurons in both the N2 and *npr-1(lf)* backgrounds and assayed CO₂ avoidance behavior. We found that either genetic ablation of a neuronal subset that includes URX or specific laser ablation of URX in the N2 background had no effect on CO₂ avoidance (Fig. 3B,C). However, both genetic and laser ablation of URX in *npr-1(lf)* mutants restored CO₂ avoidance (Fig. 3B,C). Moreover, the response of URX-ablated *npr-1(lf)* animals was not significantly different from the response of URX-ablated N2 animals in our laser ablation experiment (Fig. 3C). Thus, in *npr-1(lf)* mutants, URX neu-

Figure 3. *npr-1* appears to act in URX neurons to regulate CO₂ avoidance. **A**, Expression of *npr-1* cDNA from N2 animals in subsets of neurons that include URX restores CO₂ avoidance to *npr-1(lf)* mutants in an acute CO₂ avoidance assay. * $p < 0.05$, relative to the *npr-1(lf)* mutant (one-way ANOVA with Bonferroni post-test). ** $p < 0.01$, relative to the *npr-1(lf)* mutant (one-way ANOVA with Bonferroni post-test). $n = 16$ –27 trials for each genotype. Full expression patterns for each transgene were previously described (Macosko et al., 2009). **B**, Genetic ablation of a subset of neurons that includes URX in *npr-1(lf)* mutants restores CO₂ avoidance. Left, CO₂ chemotaxis assay. Right, Acute CO₂ avoidance assay. *** $p < 0.001$, relative to the *npr-1(lf)* mutant (one-way ANOVA with Bonferroni post-test). $n = 7$ or 8 trials (chemotaxis assay) or 8–27 trials (acute assay) for each genotype. **C**, Specific laser ablation of URX neurons in *npr-1(lf)* mutants restores CO₂ avoidance in an acute CO₂ avoidance assay. Ablations were performed on animals expressing a *gcy-36::GFP* transgene to verify the identity of the URX neurons. *** $p < 0.001$ (one-way ANOVA with Bonferroni post-test). The responses of mock-ablated N2 animals, URX-ablated N2 animals, and URX-ablated *npr-1* animals were not significantly different ($p > 0.05$). $n = 7$ –10 trials for each treatment. For all graphs, error bars represent SEM.

rons inhibit CO₂ avoidance and removal of URX neurons is sufficient to restore CO₂ avoidance. Our results suggest a model in which CO₂ avoidance behavior is regulated by URX neuron activity. In N2 animals, NPR-1 reduces URX neuron activity, thereby enabling CO₂ avoidance. In *npr-1(lf)* animals, increased activity of URX neurons inhibits the CO₂ circuit, resulting in a loss of CO₂ avoidance.

URX neurons are not required for CO₂ attraction by dauers

In contrast to *C. elegans* adults and developing larvae, *C. elegans* dauer larvae are attracted to CO₂ (Fig. 4A) (Guillermin et al., 2011; Hallem et al., 2011a). The dauer is a developmentally arrested, alternative third larval stage that is thought to be analogous to the infective juvenile stage of parasitic nematodes (Hotez et al., 1993). The mechanism responsible for the change in CO₂ response valence that occurs at the dauer stage is not yet known. BAG neurons and the putative CO₂ receptor GCY-9 are required for CO₂ attraction by dauers (Fig. 4A) (Hallem et al., 2011a), suggesting that the same mechanism of CO₂ detection operates at the dauer and adult stages. However, *npr-1(lf)* and HW dauers are also attracted to CO₂, indicating that *npr-1* is not required for CO₂ attraction (Fig. 4B). The lack of requirement for *npr-1* at the dauer stage is not the result of altered *npr-1* expression in URX neurons because *npr-1* is expressed at comparable levels in N2 dauers and developing third-stage larvae (L3s) (Fig. 4C,D). To test whether URX neuron activity is required for CO₂ attraction by dauers, we tested whether dauers that lack URX neurons are still attracted to CO₂. We found that URX-ablated N2 and *npr-1(lf)* dauers display normal CO₂ attraction (Fig. 4E), indicating that URX neurons are not required to promote CO₂ attraction by dauers. Thus, URX neurons control whether CO₂ is a repulsive or neutral stimulus in adults, but other mechanisms are required to promote CO₂ attraction by dauers.

O₂ sensing by URX neurons is required for regulation of CO₂ avoidance

The URX neurons are O₂-sensing neurons that express O₂ receptors of the soluble guanylate cyclase (sGC) family

Figure 4. Mechanisms of CO₂ attraction by dauers. **A**, BAG neurons and the receptor guanylate cyclase gene *gcy-9* are required for CO₂ attraction by dauers. * $p < 0.05$ (Kruskal–Wallis test with Dunn's post-test). ** $p < 0.01$ (Kruskal–Wallis test with Dunn's post-test). $n = 4$ –12 trials. **B**, *npr-1* is not required for CO₂ attraction by dauers. $n = 4$ –8 trials. **C**, Epifluorescence images of *npr-1* expression in the URX neurons of L3 (left) and dauer (right) larvae in the N2 background. *npr-1* expression was assayed in *npr-1* animals containing an *npr-1::SL2 GFP* transgene (Macosko et al., 2009). Arrowheads indicate the location of the URX neuron cell body. Anterior is to the left. **D**, *npr-1* expression in the URX neurons of L3 and dauer larvae is not significantly different (unpaired *t* test). $n = 17$ –20 animals. **E**, URX neurons are not required for CO₂ attraction by dauers. Both N2 and *npr-1(lf)* dauers containing a genetic ablation of the URX, AQR, and PQR neurons display normal CO₂ attraction. $n = 4$ –8 trials for each genotype. For all graphs, error bars represent SEM.

(Cheung et al., 2004; Gray et al., 2004). Whether the URX neurons are also activated by CO₂ is unclear (Bretscher et al., 2011; Brandt et al., 2012). To test whether URX neurons regulate CO₂ response by directly responding to CO₂, we imaged

Figure 5. URX neurons mediate O₂-dependent regulation of CO₂ avoidance. **A**, URX neurons do not respond to CO₂ in either N2 or *npr-1(lf)* animals under our imaging conditions. Calcium transients in URX neuron cell bodies were measured using G-CaMP3.0. Black lines indicate average calcium responses; white shading represents SEM. Blue lines below the traces indicate the timing of the CO₂ pulse. *n* = 8 or 9 animals for each genotype. To verify the lack of CO₂ response in URX neurons, we also imaged from N2 and *npr-1(lf)* animals containing an independently generated construct that expressed G-CaMP in URX (McGrath et al., 2009); these animals also did not display CO₂-evoked activity in URX (data not shown). **B**, O₂ sensing by URX neurons is required for regulation of CO₂ avoidance. Mutation of *ahr-1* or *gcy-35* rescues the CO₂ response defect of *npr-1(lf)* mutants. **p* < 0.05 (Kruskal–Wallis test with Dunn’s post-test). ****p* < 0.001 (Kruskal–Wallis test with Dunn’s post-test). *n* = 4–9 trials for each genotype. Error bars represent SEM. **C**, Neuropeptide signaling regulates CO₂ avoidance. Mutation of the URX-expressed neuropeptide genes *flp-8* and *flp-19* significantly rescues the CO₂ response defect of *npr-1(lf)* mutants. ***p* < 0.01, relative to the *npr-1(lf)* mutant (one-way ANOVA with Bonferroni post-test). ****p* < 0.001, relative to the *npr-1(lf)* mutant (one-way ANOVA with Bonferroni post-test). *n* = 6–14 trials. Error bars represent SEM.

from the URX neurons of N2 and *npr-1(lf)* animals during CO₂ exposure using the calcium indicator G-CaMP3.0. We found that URX neurons are not activated by CO₂ (Fig. 5A). URX neurons did appear to show a slight decrease in calcium levels in response to CO₂, but whether this decrease is biologically relevant is not yet clear. These results indicate that URX neurons do not regulate CO₂ response as a result of CO₂-induced activation.

To test whether URX neurons instead regulate CO₂ response by responding to O₂, we examined the CO₂-evoked behavior of *aryl hydrocarbon receptor-1* (*ahr-1*) mutants. AHR-1 is a transcription factor that regulates aggregation behavior and that is required for normal expression of sGC O₂ receptors in URX neurons (Qin et al., 2006). We found that *ahr-1* mutants respond normally to CO₂ and that the *ahr-1* mutation rescues the CO₂ response defect of *npr-1(lf)* mutants (Fig. 5B). Thus, regulation of CO₂ avoidance by URX neurons of *npr-1(lf)* animals depends on their ability to sense O₂. Furthermore, mutation of the sGC gene *gcy-35*, which encodes an O₂ receptor that is expressed in URX and required for its O₂ response (Zimmer et al., 2009), also rescues the CO₂ response defect of *npr-1* mutants (Fig. 5B). Thus,

GCY-35-mediated activation of URX neurons by ambient O₂ is required for regulation of CO₂ avoidance behavior. Together, these results demonstrate that CO₂ response is regulated by ambient O₂.

To investigate the mechanism by which URX neurons regulate CO₂ response in *npr-1* mutants, we examined the role of neuropeptide signaling in the regulation of CO₂ avoidance behavior. The URX neurons are known to express FMRFamide-related neuropeptide genes, including *flp-8*, *flp-10*, and *flp-19* (Li and Kim, 2008). To test whether these neuropeptide genes are required for the regulation of CO₂ response, we examined the CO₂-evoked behavior of neuropeptide mutants in the *npr-1(lf)* mutant background. We found that mutation of either *flp-8* or *flp-19*, but not *flp-10*, significantly rescued the CO₂ response defect of *npr-1* mutants (Fig. 5C). These results are consistent with the hypothesis that URX neurons modulate CO₂ response via a neuropeptide signaling pathway involving *flp-8* and *flp-19*. However, we cannot exclude the possibility that release of *flp-8* and *flp-19* from other neurons also contributes to the O₂-dependent regulation of CO₂ response.

npr-1(lf) and HW animals avoid CO₂ under low O₂ conditions

The URX neurons are activated when the ambient O₂ concentration increases from 10% to 21% (Zimmer et al., 2009; Busch et al., 2012). This response consists of both phasic and tonic components: a large initial increase in calcium transients is followed by a smaller sustained increase that continues until O₂ levels return to 10% (Busch et al., 2012). The fact that URX neurons remain active at high O₂ levels but are inactive at low O₂ levels led us to hypothesize that *npr-1(lf)* and HW animals might avoid CO₂ under low O₂ conditions, when URX neurons are inactive. We therefore examined the responses of *npr-1(lf)* and HW animals to CO₂ under low O₂ conditions by reducing the ambient O₂ concentration to 7% for the duration of the CO₂ chemotaxis assay. We found that, at 7% ambient O₂, both *npr-1(lf)* and HW animals displayed CO₂ avoidance behavior that was comparable with that of N2 animals (Fig. 6A). Thus, *npr-1(lf)* and HW animals are indeed capable of responding robustly to CO₂. However, CO₂ response in these animals is regulated by ambient O₂ such that CO₂ is repulsive at low O₂ concentrations and neutral at high O₂ concentrations.

The BAG neurons, which are activated by CO₂, are also activated by decreases in ambient O₂ from 21% to <10% (Zimmer et al., 2009). This raised the possibility that BAG neurons could cell-autonomously integrate responses to O₂ and CO₂, thus contributing to the O₂-dependent regulation of CO₂ response. To test this possibility, we examined the ability of animals that lack the soluble guanylate cyclase genes *gcy-31* and *gcy-33*, which are expressed in BAG neurons and are required for the O₂-evoked activity of BAG neurons (Zimmer et al., 2009), to respond to CO₂ at low ambient O₂. We found that *gcy-33*; *gcy-31* mutants responded normally to CO₂ at low ambient O₂ in both N2 and *npr-1(lf)* animals (Fig. 6B), indicating that the O₂-sensing ability of BAG is not required for the O₂-dependent regulation of CO₂ response. Consistent with these results, the BAG neurons were recently shown to play only a minor role in the chronic response to ambient O₂ (Busch et al., 2012). Thus, regulation of CO₂ response by ambient O₂ is not a result of cell-intrinsic signaling within BAG but instead requires a pair of designated O₂-sensing neurons.

Figure 6. *npr-1(lf)* and HW animals avoid CO₂ under low O₂ conditions. **A**, *npr-1(lf)* and HW animals do not respond to CO₂ at 21% ambient O₂ but avoid CO₂ at 7% ambient O₂. ****p* < 0.01, relative to N2 control (Kruskal–Wallis test with Dunn’s post-test). *****p* < 0.001, relative to N2 control (Kruskal–Wallis test with Dunn’s post-test). *n* = 7–26 trials for each genotype and condition. Error bars represent SEM. **B**, CO₂ response at low ambient O₂ does not require O₂ sensing by BAG neurons. Animals that lack the soluble guanylate cyclase genes *gcy-31* and *gcy-33*, which are required for the O₂ response of BAG neurons (Zimmer et al., 2009), respond normally to CO₂ at 7% ambient O₂ in both the N2 and *npr-1(lf)* mutant backgrounds. The response of N2 animals is not significantly different from the response of *gcy-33; gcy-31* animals, and the response of *npr-1* animals is not significantly different from the response of *gcy-33; gcy-31 npr-1* animals (Kruskal–Wallis test with Dunn’s post-test). *n* = 8–26 trials for each genotype. Error bars represent SEM. **C**, A model for O₂-dependent regulation of CO₂ avoidance. Our results suggest that, in N2 animals, NPR-1 maintains URX in a low activity state, thus enabling CO₂ avoidance even at high ambient O₂. In *npr-1(lf)* mutant animals, reduced activity of URX at low ambient O₂ allows CO₂ avoidance, and increased activity of URX at high ambient O₂ inhibits CO₂ avoidance. Our results also suggest that CO₂ avoidance by URX neurons may be mediated by the URX-expressed neuropeptide genes *flp-8* and *flp-19*.

Discussion

Our results demonstrate that URX neurons control CO₂ response by coordinating the response to CO₂ with the response to ambient O₂. In *npr-1(lf)* animals, O₂-dependent activation of URX neurons determines CO₂ response such that CO₂ is repulsive at low ambient O₂ but neutral at high ambient O₂ (Fig. 6C). Moreover, our results are consistent with the hypothesis that URX neurons regulate the activity of the CO₂ circuit via a neuropeptide signaling pathway that involves the FMRFamide-related neuropeptide genes *flp-19* and *flp-8*. By contrast, in N2 animals, the URX neurons do not inhibit CO₂ avoidance at high ambient O₂ as a result of the presence of NPR-1 (Fig. 6C). NPR-1 does not constitutively silence the URX neurons of N2 animals because the URX neurons of N2 animals are activated by increases in ambient O₂ and ablation of URX in N2 animals alters O₂ response (Zimmer et al., 2009). However, our results suggest that NPR-1 may reduce URX neuron activity in N2 animals such that URX neurons no longer inhibit the CO₂ avoidance circuit. Alternatively, it is possible that NPR-1 activity is dynamically regulated by its neuropeptide ligands such that it is active under some conditions but not others, or that the URX neurons of N2 animals are sufficiently activated but are incapable of regulating CO₂ avoidance as a result of differences in neural connectivity or signaling between N2 and *npr-1(lf)* animals.

A recent survey of wild *C. elegans* strains revealed that the HW allele of *npr-1* is the natural variant, with the N2 allele having arisen during laboratory culturing (McGrath et al., 2009). HW animals were previously thought to be virtually insensitive to

CO₂ (Hallem and Sternberg, 2008; McGrath et al., 2009), raising the question of whether CO₂ avoidance is exclusively a laboratory-derived behavior. Our results demonstrate that HW animals do indeed display robust CO₂ avoidance, but this behavior is restricted to low O₂ conditions. Wild *C. elegans* adults have been found in fallen rotting fruit and in the soil under rotting fruit, where O₂ levels are lower and CO₂ levels are higher than in the atmosphere (Felix and Duveau, 2012). Inside rotting fruit, *C. elegans* occupies microhabitats replete with bacteria, fungi, worms, insects, and other small invertebrates (Felix and Duveau, 2012). In this context, fluctuating levels of CO₂ and O₂ likely serve as important indicators of food availability, population density, and predator proximity (Bendesky et al., 2011; Milward et al., 2011; Scott, 2011). Suppression of CO₂ avoidance at high ambient O₂ may allow worms to migrate toward rotting fruit, which emits CO₂. Once inside the low O₂ environment of rotting fruit, CO₂ avoidance may allow worms to avoid cohabitating predators or overcrowding. Thus, O₂-dependent regulation of CO₂ avoidance is likely to be an ecologically relevant mechanism by which nematodes navigate gas gradients.

In addition to CO₂ response, a number of other chemosensory behaviors in *C. elegans* are subject to context-

dependent changes in sensory valence (Sengupta, 2012). For example, olfactory and gustatory behavior exhibits experience-dependent plasticity, in which chemicals that are attractive to naive animals become neutral or repulsive after prolonged or repeated exposure in the absence of food (Sengupta, 2012). Olfactory plasticity occurs as a result of altered signaling in the AWC olfactory neurons (Tsunozaki et al., 2008), and salt plasticity occurs as a result of altered signaling in the ASE gustatory neurons and the downstream AIA and AIB interneurons (Tomioka et al., 2006; Adachi et al., 2010; Oda et al., 2011). Similarly, O₂ preference is modulated by prior O₂ exposure and the presence of bacterial food as a result of altered signaling in a distributed network of chemosensory neurons (Cheung et al., 2005; Chang et al., 2006). Our results suggest that CO₂ response is modulated by ambient O₂ via the activity of a pair of O₂-detecting neurons that interact with the CO₂ circuit downstream of CO₂ detection by BAG neurons (Fig. 6C). The neurons that act downstream of BAG and URX to control CO₂ response have not yet been identified. A number of interneurons receive synaptic input from both BAG and URX (White et al., 1986), and it will be interesting to determine whether any of them play a role in CO₂ avoidance.

CO₂-evoked behaviors in insects are also subject to context-dependent modulation. For example, the fruit fly *Drosophila melanogaster* is repelled by CO₂ when walking (Suh et al., 2004) but attracted to CO₂ in flight, a valence change that is modulated by octopamine signaling (Wasserman et al., 2013). In addition, both CO₂ repulsion by walking *D. melanogaster* and CO₂ attraction by mosquitoes can be suppressed by food odorants, which

directly alter the activity of the CO₂ receptor (Turner and Ray, 2009; Turner et al., 2011). Insects as well as many other animals, both free-living and parasitic, occupy microhabitats where environmental levels of O₂ and CO₂ vary greatly as a function of food or host availability, population density, and microorganism composition. Thus, it will be interesting to determine whether the control of CO₂ response by O₂-sensing neurons is a conserved feature of gas-sensing circuits.

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