

# Cross-Modulation of Homeostatic Responses to Temperature, Oxygen and Carbon Dioxide in *C. elegans*

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## Abstract

Different interoceptive systems must be integrated to ensure that multiple homeostatic insults evoke appropriate behavioral and physiological responses. Little is known about how this is achieved. Using *C. elegans*, we dissect cross-modulation between systems that monitor temperature, O<sub>2</sub> and CO<sub>2</sub>. CO<sub>2</sub> is less aversive to animals acclimated to 15°C than those grown at 22°C. This difference requires the AFD neurons, which respond to both temperature and CO<sub>2</sub> changes. CO<sub>2</sub> evokes distinct AFD Ca<sup>2+</sup> responses in animals acclimated at 15°C or 22°C. Mutants defective in synaptic transmission can reprogram AFD CO<sub>2</sub> responses according to temperature experience, suggesting reprogramming occurs cell autonomously. AFD is exquisitely sensitive to CO<sub>2</sub>. Surprisingly, gradients of 0.01% CO<sub>2</sub>/second evoke very different Ca<sup>2+</sup> responses from gradients of 0.04% CO<sub>2</sub>/second. Ambient O<sub>2</sub> provides further contextual modulation of CO<sub>2</sub> avoidance. At 21% O<sub>2</sub> tonic signalling from the O<sub>2</sub>-sensing neuron URX inhibits CO<sub>2</sub> avoidance. This inhibition can be graded according to O<sub>2</sub> levels. In a natural wild isolate, a switch from 21% to 19% O<sub>2</sub> is sufficient to convert CO<sub>2</sub> from a neutral to an aversive cue. This sharp tuning is conferred partly by the neuroglobin GLB-5. The modulatory effects of O<sub>2</sub> on CO<sub>2</sub> avoidance involve the RIA interneurons, which are post-synaptic to URX and exhibit CO<sub>2</sub>-evoked Ca<sup>2+</sup> responses. Ambient O<sub>2</sub> and acclimation temperature act combinatorially to modulate CO<sub>2</sub> responsiveness. Our work highlights the integrated architecture of homeostatic responses in *C. elegans*.

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## Introduction

To maintain a constant internal milieu animals use internal sensory receptors to monitor cues such as CO<sub>2</sub>/pH [1], O<sub>2</sub> [2], temperature [3], and osmolality [4]. These interoceptors counter changes in internal milieu by coordinating homeostatic responses that alter physiology and behavior [5]. Cross-talk between different interoceptive systems is likely to be important to ensure an integrated homeostatic response by the animal to multiple homeostatic insults. However, relatively little is known, at the molecular and circuitry levels, about how such cross-talk is encoded.

In vertebrates electrophysiological studies have identified cell populations and circuits that respond to homeostatic imbalance in O<sub>2</sub>, CO<sub>2</sub>/pH and temperature. The neurons comprising these circuits are only beginning to be resolved, and the molecular mechanisms controlling their responses are poorly understood. Nevertheless, studies in several animals suggest that cross-modulation of homeostatic responses is important for survival. In panting mammals, a rise in core body temperature elicits increased ventilation rate to help cooling, even though this causes temporary alkalosis of the blood due to excessive blowing off of CO<sub>2</sub>. This over-ride appears to be achieved by changing the set-point at which CO<sub>2</sub> sensors inhibit ventilation when [CO<sub>2</sub>] decreases, but

the mechanisms involved are unclear [6]. In the mouse, recent work has shown that suppressing the activity of serotonergic neurons impairs both respiratory and body temperature control, although whether the same or different sub-populations of neurons mediate these effects is unclear [7,8]. In mammals, the drive to increase ventilation rate is stimulated more strongly when animals simultaneously experience a drop in O<sub>2</sub> and a rise in CO<sub>2</sub> [9].

In invertebrates, such as the free-living nematode *C. elegans*, behavioral mechanisms that counter homeostatic imbalance are particularly important, since the animal's buffering capacity is limited. *C. elegans* responds to variation in temperature, O<sub>2</sub> and CO<sub>2</sub> by mounting sophisticated behavioral responses. Exposure to temperatures above or below the range in which *C. elegans* can grow elicits strong avoidance responses [10]. When navigating thermal clines in which it can thrive, ~15°C to 25°C, *C. elegans* migrates to the temperature at which it grew recently, as long as this was not associated with starvation [11,12]. These responses require the animal to memorize its recent temperature experience and to change this memory when temperature or nutrient conditions change. A neural circuit that subserves these behaviors has been identified, and involves the thermosensory neurons AFD and AWC [13–16]. Temperature experience alters the thermosensing properties of AFD neurons: in animals acclimated to higher temperatures, the threshold at which a temperature rise

**Author Summary**

Many animals are either attracted or repelled by carbon dioxide. We show that the way *C. elegans* responds to CO<sub>2</sub> depends on the temperature it has acclimated to and the oxygen tensions it is experiencing. The effects of acclimation temperature are mediated by a temperature-sensing neuron called AFD that also responds to CO<sub>2</sub>. The responses evoked in AFD by a change in CO<sub>2</sub> concentration are reprogrammed by acclimation temperature. This reprogramming does not appear to require synaptic input from other neurons. O<sub>2</sub> modulates CO<sub>2</sub> avoidance by setting the activity of the tonically signalling O<sub>2</sub> sensor URX. A switch from 21% to 19% O<sub>2</sub> is sufficient to convert CO<sub>2</sub> from a neutral stimulus to an aversive one in a *C. elegans* wild strain. Modulation of CO<sub>2</sub> responses by O<sub>2</sub> cues requires the interneuron RIA which itself responds to changes in CO<sub>2</sub> and is directly post-synaptic to URX. CO<sub>2</sub> gradients are likely to be common in rotting fruit where *Caenorhabditis* live. Such gradients could be associated with food, pathogens, conspecifics or predators of *C. elegans*. The value of CO<sub>2</sub> as a sensory cue thus depends crucially on context. This may explain the remarkable complexity of CO<sub>2</sub> sensing in *C. elegans*.

evokes a Ca<sup>2+</sup> response in AFD occurs at correspondingly higher temperatures [17,18]. This plasticity allows animals to respond homeostatically to external temperature fluctuations, by seeking and remaining at temperatures they are acclimated to.

*C. elegans* also displays responses to variation in [O<sub>2</sub>], and avoids both high and low O<sub>2</sub> [19]. Wild-caught *C. elegans* strongly avoids 21% O<sub>2</sub>, both on and off food, and burrow to escape from the surface [20]. This avoidance response is sculpted by O<sub>2</sub>-sensing neurons in the body cavity called AQR, PQR and URX [20,21,22]. When [O<sub>2</sub>] levels rise towards 21% the AQR, PQR and URX neurons become activated, by a mechanism involving the atypical soluble guanylate cyclases GCY-35/GCY-36. The tuning of the O<sub>2</sub> response is sharpened by a neuroglobin expressed in AQR, PQR and URX neurons, called GLB-5, that suppresses neuronal activity when ambient [O<sub>2</sub>] falls just below 21% [20,23]. The AQR, PQR and URX neurons are all tonic receptors: they show sustained signalling as long as [O<sub>2</sub>] is high [24]. This tonic activity stimulates sustained rapid movement until animals encounter a preferred lower [O<sub>2</sub>] environment.

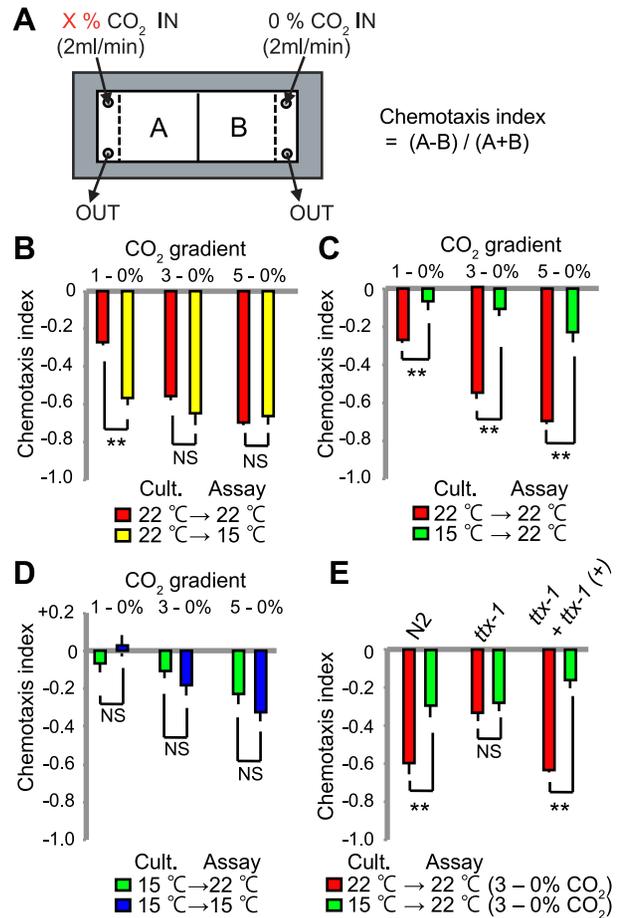
*C. elegans* also avoids elevated CO<sub>2</sub> [25,26]. As in vertebrates, high [CO<sub>2</sub>] is harmful to *C. elegans*, reducing brood size and disrupting muscle structure [27]. An array of sensory neurons mediates CO<sub>2</sub> avoidance behavior [28]. This network includes the temperature sensor AFD, the major gustatory neuron ASE, and the BAG neurons, which are also activated by decreasing O<sub>2</sub> levels [22].

Here we investigate how the temperature and O<sub>2</sub> sensing systems of *C. elegans* modulate the distributed circuit that mediates responses to CO<sub>2</sub>.

**Results**

**Previous temperature experience sets CO<sub>2</sub> avoidance in *C. elegans***

To examine if temperature can modify *C. elegans*' responses to CO<sub>2</sub> we grew N2(Bristol) animals at 22°C and compared their behavior in CO<sub>2</sub> gradients at 15°C and 22°C (Figure 1A, B) [25,28]. CO<sub>2</sub> avoidance at the two temperatures was similar when animals navigated 3%–0% and 5%–0% CO<sub>2</sub> gradients. However,



**Figure 1. CO<sub>2</sub> avoidance is modulated by acclimation temperature.** A. Assay for *C. elegans* CO<sub>2</sub> responses. Animals navigate a defined CO<sub>2</sub> gradient in a microfluidic device. The chemotaxis index is calculated by counting animals in two halves of the device, using the formula shown. B–D. Chemotaxis indices for animals cultivated at either 15°C or 22°C and assayed in different CO<sub>2</sub> gradients at either 15°C or 22°C. \*\*, *p*<0.01; n.s., not significant, Student's *t*-test. E. A mutation in *ttx-1*, which is specifically required to confer AFD neural identity, disrupts modulation of CO<sub>2</sub> avoidance by acclimation temperature. Assays were performed in 3%–0% CO<sub>2</sub> gradients. \*\*, *p*<0.01; n.s., not significant, Student's *t*-test. doi:10.1371/journal.pgen.1004011.g001

animals in a 1–0% CO<sub>2</sub> gradient avoided the high CO<sub>2</sub> half of the microfluidic device more strongly when assayed at 15°C compared to 22°C (Figure 1A).

*C. elegans* can retune its temperature preference according to the temperature to which it is acclimated [13,29]. This behavior is encoded in AFD [17,18], a neuron that also responds to CO<sub>2</sub> [28]. We therefore examined how previous temperature experience altered subsequent CO<sub>2</sub> responses. We grew animals at 15°C or 22°C, and assayed their CO<sub>2</sub> responses at each temperature. Strikingly, previous temperature experience altered CO<sub>2</sub> avoidance. Animals grown at 15°C avoided CO<sub>2</sub> less strongly than animals grown at 22°C, regardless of whether the assay temperature was 15°C or 22°C (Figure 1B–D). Animals grown at 15°C showed weaker CO<sub>2</sub> avoidance even when exposed to relatively high CO<sub>2</sub> levels, 5% (Figure 1B–D). Thus, the temperature to which *C. elegans* has acclimated helps determine the aversiveness of CO<sub>2</sub>.

### Acclimation temperature does not reprogram CO<sub>2</sub> responses in AFD-defective mutants

We investigated if the AFD neurons helped to reprogram CO<sub>2</sub> avoidance behavior according to acclimation temperature. The *ttx-1* (thermotaxis defective) gene encodes a member of the OTD/OTX subclass of homeodomain transcription factors [30]. Mutations in *ttx-1* selectively disrupt AFD specification, and confer a thermotaxis-defective phenotype. Loss of *ttx-1* also reduces CO<sub>2</sub> avoidance in animals navigating CO<sub>2</sub> spatial gradients [28]. If AFD neurons were important for temperature regulation of CO<sub>2</sub> avoidance responses, then *ttx-1* mutants would display similar CO<sub>2</sub> avoidance regardless of cultivation temperature. As shown previously, *ttx-1* mutants grown at 22°C only avoided CO<sub>2</sub> weakly [28], resembling wild-type animals grown at 15°C (Figure 1E). This defect was rescued by a wild-type *ttx-1* transgene (Figure 1E). By contrast, loss of *ttx-1* did not alter the CO<sub>2</sub>-avoidance behavior of animals cultivated at 15°C. These data suggest AFD is required for acclimation temperature to modify CO<sub>2</sub> aversive responses.

### Acclimation temperature re-programs the CO<sub>2</sub> responsiveness of AFD

Acclimation temperature sets the response threshold of AFD neurons to warming [17]. This prompted us to investigate whether acclimation temperature also alters the CO<sub>2</sub> responsiveness of AFD. To measure CO<sub>2</sub>-evoked Ca<sup>2+</sup> responses in AFD we expressed the genetically encoded Ca<sup>2+</sup> sensor cameleon YC3.60 [31] from the *gcy-8* promoter [32]. For our recordings we used animals acclimated to 15°C or 22°C, but maintained animals at 22°C while we imaged them. In animals acclimated to 22°C high CO<sub>2</sub> evoked in AFD the complex Ca<sup>2+</sup> response described previously (Figure 2A) [28]. This typically consisted of an initial slight drop in Ca<sup>2+</sup> when CO<sub>2</sub> levels rose, followed by a rise in Ca<sup>2+</sup> to above pre-stimulus levels, and finally, when the CO<sub>2</sub> stimulus was removed, a Ca<sup>2+</sup> spike that rapidly decayed back to baseline. By contrast, animals acclimated to 15°C exhibited a simple response: a rise in Ca<sup>2+</sup> when CO<sub>2</sub> levels rose, and a fall when CO<sub>2</sub> was removed (Figure 2B). These data suggest that the previous temperature experience of *C. elegans* reconfigures the CO<sub>2</sub> response properties of AFD neurons.

To investigate if this retuning was driven by the intrinsic temperature-sensing properties of AFD neurons, or required pre-synaptic input, we imaged the Ca<sup>2+</sup> responses of AFD neurons to CO<sub>2</sub> in *snb-1* (*synaptobrevin-1*) mutants, which are defective in synaptic transmission [33]. CO<sub>2</sub>-evoked responses in AFD neurons were not altered in *snb-1* animals compared to wild type, regardless of acclimation temperature (Figure 2C, D). These data suggest that the temperature experience can retune the CO<sub>2</sub> response properties of AFD neurons when synaptic signalling is defective.

We characterized the response properties of the AFD neurons further. Previously, we had only exposed animals to sharp changes in CO<sub>2</sub> that occurred within 1–2 s, and we always returned animals to 0% CO<sub>2</sub> between stimuli [29]. To examine AFD responses to rises in CO<sub>2</sub> from non-zero levels, we subjected animals acclimated to 22°C to a stimulus train involving multiple CO<sub>2</sub> switches, namely 0%–1%–3%–5%–3%–1%–0%. Whenever CO<sub>2</sub> levels increased, we observed an initial drop in Ca<sup>2+</sup> followed by a rise in Ca<sup>2+</sup> (Figure 2E). Whenever CO<sub>2</sub> levels decreased, we observed a spike of Ca<sup>2+</sup> that rapidly returned to baseline. This pattern of CO<sub>2</sub> evoked Ca<sup>2+</sup> response suggests that AFD can encode whether an animal is moving towards higher or lower CO<sub>2</sub>.

Previous work has identified one potential molecular sensor for CO<sub>2</sub>, the transmembrane guanylate cyclase *gcy-9* [34]. We compared CO<sub>2</sub>-evoked responses in AFD neurons in wild type and *gcy-9* mutants. We observed no difference in the response, suggesting that molecules other than GCY-9 confer CO<sub>2</sub>-responsiveness to AFD neurons (Figure S1).

### AFD responses to CO<sub>2</sub> are reconfigured by the steepness of the CO<sub>2</sub> gradient

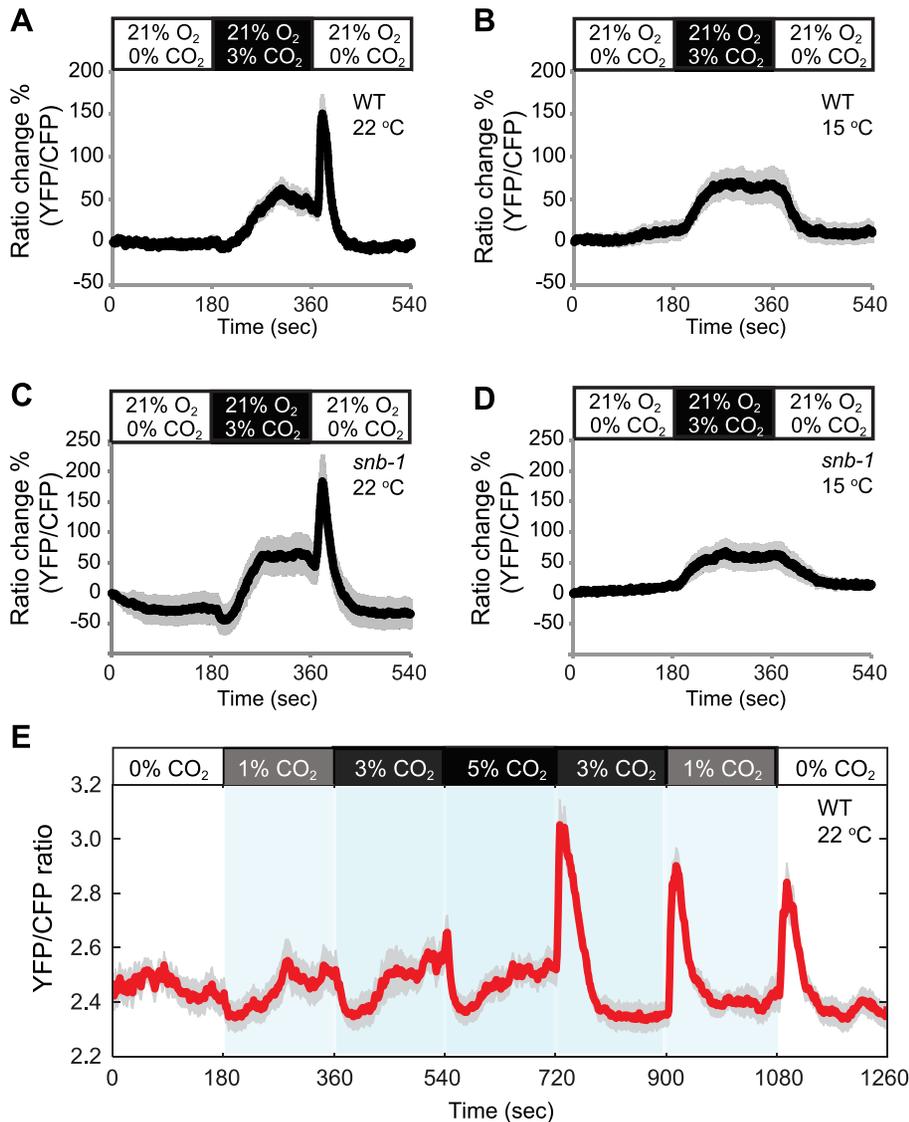
The ubiquity of CO<sub>2</sub> suggests that its value as a cue is likely to depend not only on context (such as temperature) but also on the shape of the CO<sub>2</sub> stimulus. Very rapid change in CO<sub>2</sub> levels may convey a different meaning from a very gradual change. In our behavioral experiments, animals navigated shallow CO<sub>2</sub> gradients and encountered changes in the order of 0.01% CO<sub>2</sub> per second (depending on speed and direction of travel in the gradient). To examine if AFD could respond to such shallow CO<sub>2</sub> gradients, we exposed animals cultivated at 22°C to gradual linear increases and decreases in CO<sub>2</sub> concentration at rates of 0.04% and 0.01% per second (Figure 3A,B). AFD responded to both these CO<sub>2</sub> gradients, but with very different response patterns. Gradients of 0.04% CO<sub>2</sub>/second evoked AFD Ca<sup>2+</sup> responses reminiscent of those elicited by sharp changes in CO<sub>2</sub> (>1% CO<sub>2</sub>/second; see Figure 2): Ca<sup>2+</sup> levels decreased while CO<sub>2</sub> was slowly rising to 5%, then rose sharply as CO<sub>2</sub> levels stabilized at 5%. When we gradually reduced CO<sub>2</sub> levels back to 0%, Ca<sup>2+</sup> levels spiked, returning to baseline when animals were in 0% CO<sub>2</sub> (Figure 3A). By contrast, gradients of 0.01% CO<sub>2</sub>/second evoked a series of Ca<sup>2+</sup> spikes while CO<sub>2</sub> levels were rising (Figure 3B). Ca<sup>2+</sup> levels tended to return to baseline when CO<sub>2</sub> levels stopped rising, but spiking resumed when CO<sub>2</sub> levels started falling. This spiking pattern disappeared when we imaged Ca<sup>2+</sup> responses evoked by the same 0.01% CO<sub>2</sub>/second gradient in animals acclimated to 15°C (Figure 3C). In these animals responses were more similar to those evoked by steeper CO<sub>2</sub> gradients in animals acclimated to 15°C (compare Figure 3C to Figure 2B). These data indicate that AFD neurons respond to both rapid and slow changes in CO<sub>2</sub>, but with different response patterns. The also highlight complexity in how AFD encodes CO<sub>2</sub> stimuli.

### Ambient O<sub>2</sub> levels regulate *C. elegans* CO<sub>2</sub> avoidance behaviour

To investigate further how different homeostatic responses are integrated, we examined if CO<sub>2</sub> avoidance behavior was modulated by different background ambient [O<sub>2</sub>]. In body fluids and many ecological niches low [O<sub>2</sub>] coincides with high [CO<sub>2</sub>], and, conversely, 21% O<sub>2</sub> is associated with low CO<sub>2</sub>. Cross-talk between the two gas sensing circuits could enable *C. elegans* to recognize and respond appropriately to such environments.

To examine this possibility, we placed N2 animals in microfluidic chambers containing gradients of CO<sub>2</sub> at different fixed concentrations of O<sub>2</sub>. As expected, increasing [CO<sub>2</sub>] elicited increasing avoidance behavior: *C. elegans* avoided 5% CO<sub>2</sub> more strongly than 3% or 1% CO<sub>2</sub> (Figure 4A) [25,26]. Moreover, CO<sub>2</sub> avoidance was influenced by the background ambient O<sub>2</sub> concentration. N2 animals navigated down CO<sub>2</sub> gradients more strongly when ambient O<sub>2</sub> concentration was 11%, than when it was 21%. Increased avoidance was particularly striking when animals navigated shallow gradients of 1–0% CO<sub>2</sub> (Figure 4A). Such shallow CO<sub>2</sub> gradients are likely to be ecologically relevant in the rotting habitats where *C. elegans* thrives.

To test the dynamic range of O<sub>2</sub> regulation, we asked if increasing [O<sub>2</sub>] to above 21% could further suppress CO<sub>2</sub>



**Figure 2. Acclimation temperature alters CO<sub>2</sub>-evoked Ca<sup>2+</sup> responses in AFD neurons.** In animals cultivated at 22°C a rise and fall in CO<sub>2</sub> evokes a complex Ca<sup>2+</sup> response in AFD neurons (A). Ca<sup>2+</sup> initially falls when CO<sub>2</sub> begins to increase, then rises. When CO<sub>2</sub> levels fall, there is a Ca<sup>2+</sup> spike. By contrast, animals cultivated at 15°C show a simple response to the same stimulus (B). C–D The effect of acclimation temperature on CO<sub>2</sub>-evoked Ca<sup>2+</sup> responses in AFD neurons is unaltered in *snb-1* mutants defective in synaptobrevin. E. Ca<sup>2+</sup> responses evoked in AFD by a 0%–1%–3%–5%–3%–1%–0% CO<sub>2</sub> stimulus train in animals acclimated to 22°C. Shading highlights switch times. Acclimation temperature is shown for each panel under genotype.

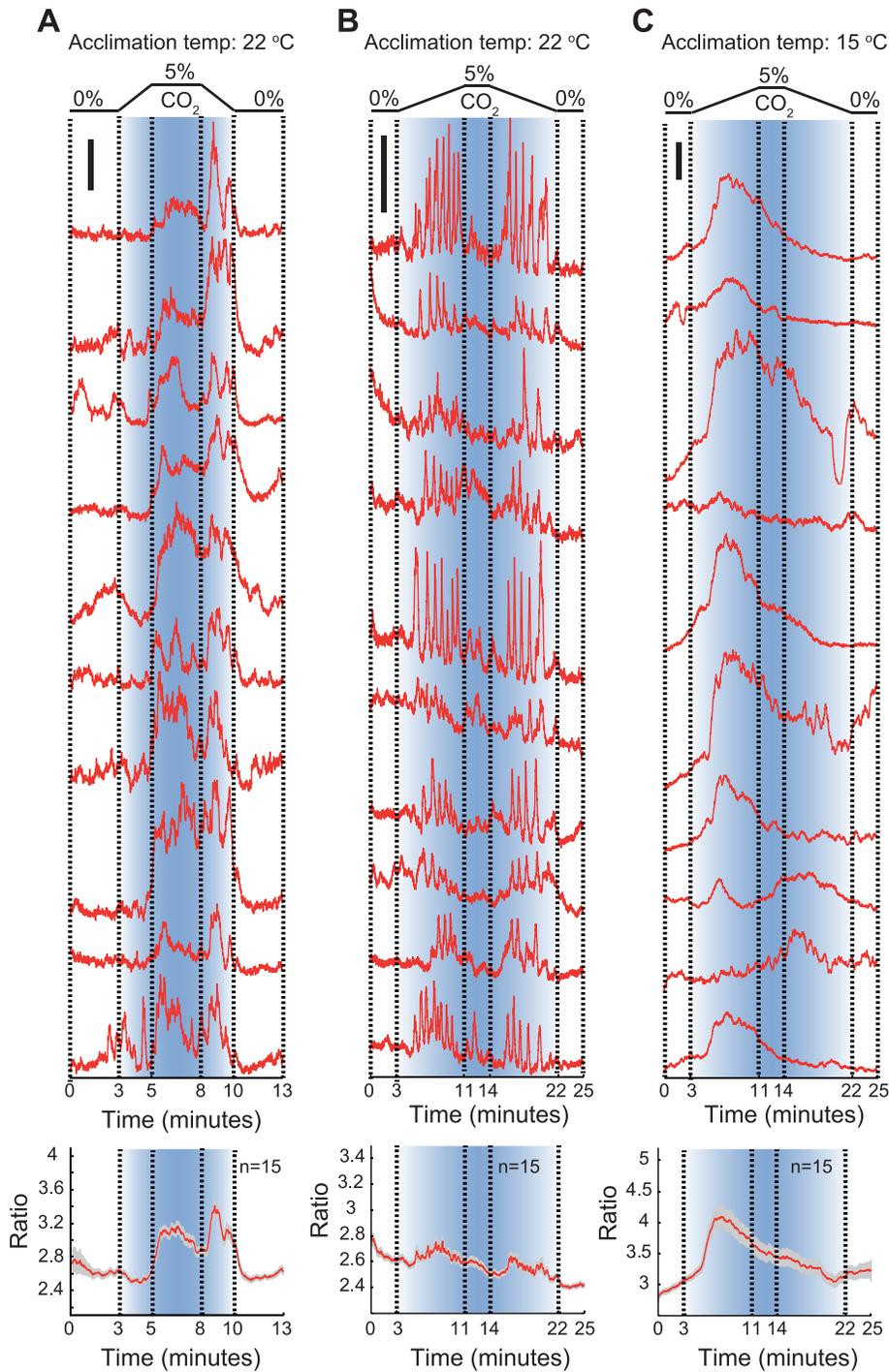
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avoidance. Although this is unphysiological, previous studies have shown that *C. elegans* can grow and reproduce in even 100% O<sub>2</sub> without any apparent adverse effects [35]. Since *C. elegans* only weakly avoided 1% CO<sub>2</sub> in 21% O<sub>2</sub>, we used a steeper 3–0% CO<sub>2</sub> gradient, to improve our dynamic range. Increasing ambient [O<sub>2</sub>] to 50% significantly suppressed avoidance of 3% CO<sub>2</sub> (Figure 4B). These data suggest that ambient O<sub>2</sub> concentration provides a contextual cue to modulate *C. elegans* avoidance of CO<sub>2</sub>.

### Tonically signalling O<sub>2</sub> sensors inhibit CO<sub>2</sub> avoidance at high ambient [O<sub>2</sub>]

Our results suggested that O<sub>2</sub>-sensing neurons or neuroendocrine cells persistently signal O<sub>2</sub> concentration to modify the activity of CO<sub>2</sub> transducing circuits. Previous studies have shown

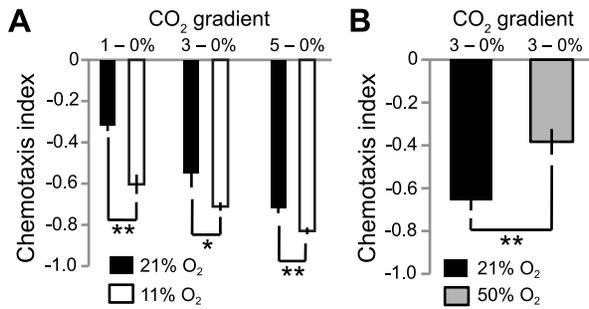
that the AQR, PQR and URX O<sub>2</sub> sensors signal tonically when ambient [O<sub>2</sub>] is close to 21%, and become progressively less active as [O<sub>2</sub>] falls [24]. The O<sub>2</sub>-evoked Ca<sup>2+</sup> responses of these neurons requires the atypical soluble guanylyl cyclases GCY-35 and GCY-36, which appear to be O<sub>2</sub> sensors [20,22,36]. In *gcy-35* or *gcy-36* loss-of-function mutants the Ca<sup>2+</sup> levels in the O<sub>2</sub> sensing neurons reported by cameleon YC3.60, are low, resembling those found in wild type animals kept at low [O<sub>2</sub>] [24]. To test if tonic signalling by AQR, PQR and URX neurons persistently repressed CO<sub>2</sub> avoidance in high [O<sub>2</sub>], we compared the CO<sub>2</sub> avoidance of wild type, *gcy-35*, and *gcy-36* mutants at 21% and 11% O<sub>2</sub>. In 11% O<sub>2</sub> *gcy-35* and *gcy-36* mutants avoided CO<sub>2</sub> like N2 controls. However, whereas increasing background O<sub>2</sub> levels to 21% inhibited the CO<sub>2</sub> avoidance behavior of wild type animals, it had no effect on *gcy-35* or *gcy-36* mutant animals (Figure 5A). These



**Figure 3. Shallow and steep CO<sub>2</sub> gradients evoke qualitatively different Ca<sup>2+</sup> responses in AFD.** A. Ca<sup>2+</sup> responses evoked in AFD by CO<sub>2</sub> switches indicated at top, involving linear 0–5% and 5%–0% CO<sub>2</sub> gradients occurring over 2 minutes. This corresponds to a rate of change of 0.04% CO<sub>2</sub>/second. The upper part of the panel shows traces obtained from 10 randomly selected individual AFD neurons; an average trace is plotted at the bottom. Animals imaged in this panel were acclimated to 22°C. B, C. Ca<sup>2+</sup> responses evoked in AFD by CO<sub>2</sub> switches indicated at top, involving linear switches from 0–5% and 5%–0% CO<sub>2</sub> occurring over 8 minutes. This corresponds to a change of 0.01% CO<sub>2</sub>/second. The upper part of the panels shows traces obtained from 10 randomly selected individual AFD neurons; average traces are plotted at the bottom. Animals imaged in (B) were acclimated to 22°C; those in (C) were acclimated at 15°C. For each panel, individual and average traces are at the same scale. The scale bar in each panel represents 0.4 YFP/CFP ratio unit.  
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data suggest that tonic signalling from one or more of the AQR, PQR and URX O<sub>2</sub> sensors represses CO<sub>2</sub> avoidance at high O<sub>2</sub> concentrations.

To confirm our results, we rescued the *gcy-36* mutant phenotype using cell-specific promoters. Expressing *gcy-36* cDNA from its own upstream sequence, which drives expression in AQR, PQR



**Figure 4. Ambient O<sub>2</sub> levels set CO<sub>2</sub> avoidance.** A. *C. elegans* avoids shallow gradients of CO<sub>2</sub> more strongly when O<sub>2</sub> levels are low. The CO<sub>2</sub> gradients used are indicated above the graph. B. Artificially high O<sub>2</sub> levels can reduce CO<sub>2</sub> avoidance further. \*\*,  $p < 0.01$ ; \*,  $p < 0.05$ , Student's *t*-test.  
doi:10.1371/journal.pgen.1004011.g004

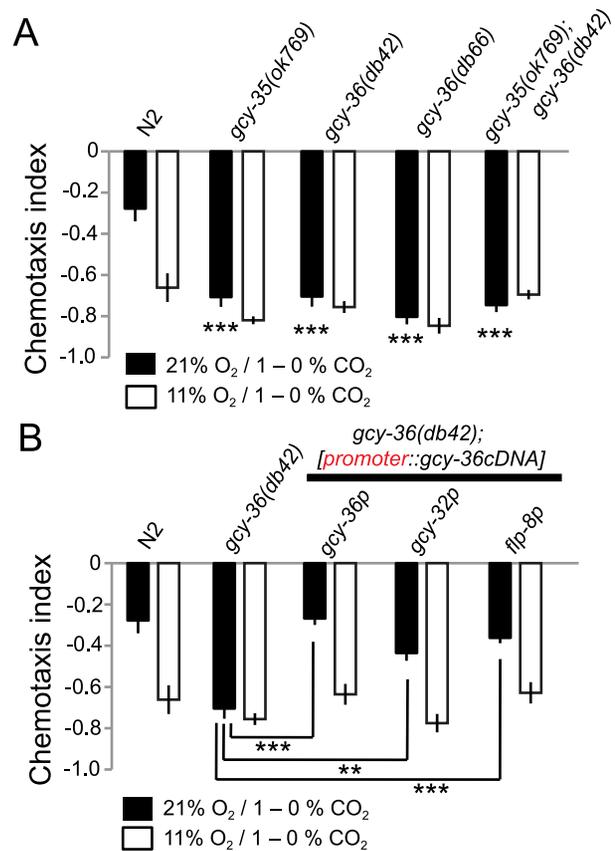
and URX, restored to *gcy-36* mutants reduced CO<sub>2</sub> avoidance at 21% O<sub>2</sub> (Figure 5B). *gcy-36* mutants expressing *gcy-36* cDNA from the *gcy-32* promoter, which also drives expression in AQR, PQR and URX, gave similar rescue (Figure 5B). Expressing *gcy-36* cDNA from the *flp-8* promoter, which drives expression in URX (and AUA and PVM) neurons but not in AQR and PQR also rescued the O<sub>2</sub>-regulated CO<sub>2</sub> avoidance phenotype of *gcy-36* mutants. These results suggest that tonic signalling by the URX O<sub>2</sub>-sensing neuron can persistently suppress CO<sub>2</sub> avoidance while O<sub>2</sub> levels are high.

To extend our results we also examined the consequence of deleting *gcy-32* and *gcy-34*, atypical soluble guanylate cyclases expressed in AQR, PQR and URX neurons whose activities are also likely to be modulated by O<sub>2</sub>, but whose deletion only subtly alters O<sub>2</sub>-evoked behaviors. We observed no effects of these deletions on O<sub>2</sub> regulation of CO<sub>2</sub> avoidance (Figure S2). We did however observe a slight decrease in CO<sub>2</sub> avoidance at 11% O<sub>2</sub> in mutants defective in *gcy-33*, an atypical soluble guanylate cyclase required for the BAG sensory neurons to respond to decreases in O<sub>2</sub> levels (Figure S2) [22]. BAG is also a major CO<sub>2</sub> sensor [28] [34].

### The *npr-1* and *glb-5* genes modulate CO<sub>2</sub> avoidance by O<sub>2</sub>

O<sub>2</sub> responses in the standard laboratory N2 strain differ from those of aggregating wild *C. elegans*, due to genetic differences that have evolved during domestication [19,20,23,36,37]. N2 animals harbor a gain-of-function allele of the *npr-1* neuropeptide receptor that inhibits signalling output from O<sub>2</sub>-sensing circuits in feeding animals. N2 animals also carry a loss-of-function mutation in the neuroglobin *glb-5* that increases the excitability of the AQR, PQR and URX O<sub>2</sub> sensors.

We investigated if variation at *npr-1* and *glb-5* altered O<sub>2</sub> modulation of CO<sub>2</sub> avoidance. In N2 animals, stepwise increases in O<sub>2</sub> from 11% to 21% caused stepwise decreases in CO<sub>2</sub> avoidance (Figure 6A). Animals defective in both the *npr-1* receptor and the *glb-5* neuroglobin (i.e. *npr-1* mutants) were attracted to CO<sub>2</sub> at 21% O<sub>2</sub>, but became progressively more repelled by CO<sub>2</sub> as O<sub>2</sub> concentrations fell. A functional *glb-5*(*Hawaii*) allele made CO<sub>2</sub> more aversive to *npr-1* defective animals: decreasing [O<sub>2</sub>] still stimulated CO<sub>2</sub> avoidance, but at each concentration tested *glb-5*; *npr-1* animals avoided CO<sub>2</sub> more strongly than *npr-1* animals (Figure 6A). Adding the functional *glb-5*(*Hawaii*) allele to N2 animals bearing the *npr-1* gain-of-function allele did not significantly change their CO<sub>2</sub> avoidance behaviour

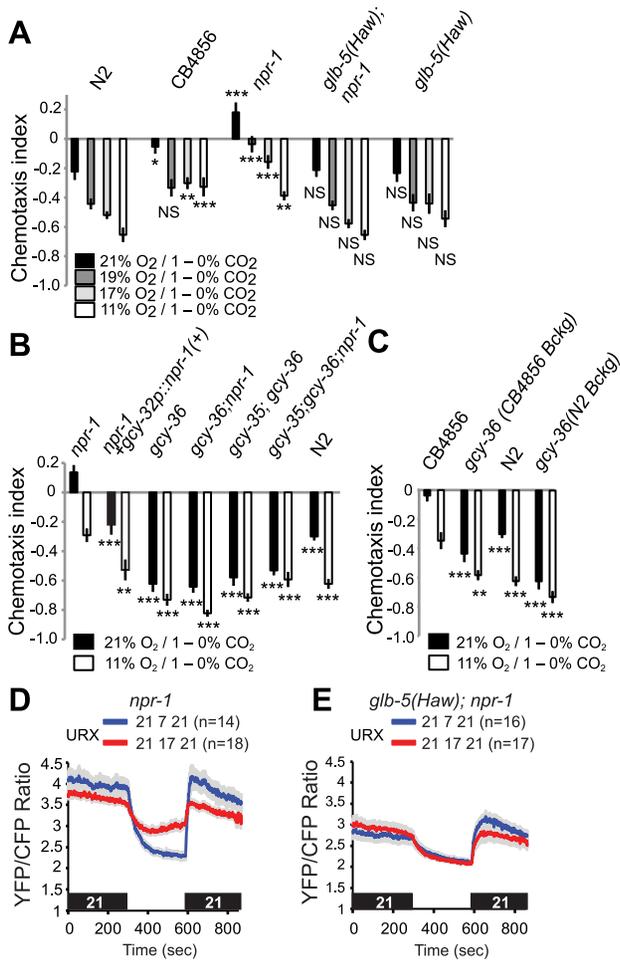


**Figure 5. Disrupting *gcy-35* or *gcy-36* confers strong CO<sub>2</sub> avoidance regardless of ambient O<sub>2</sub>.** A. *gcy-35* or *gcy-36* mutants strongly avoid the high CO<sub>2</sub> half of a 1–0% CO<sub>2</sub> gradient regardless of ambient O<sub>2</sub>. Statistics refer to comparisons to N2 at 21% O<sub>2</sub>. \*\*\*,  $p < 0.001$ , Anova, Bonferroni corrected *p*-value. None of the strains apart from N2 show significant differences between assays carried out at 21% and 11% O<sub>2</sub> (Student's *t*-test). B. The CO<sub>2</sub>-avoidance phenotype of *gcy-36* mutants can be rescued by expressing *gcy-36* cDNA in AQR, PQR and URX, using *gcy-32* or *gcy-36* promoters, or in URX alone, using the *flp-8* promoter. \*\*,  $p < 0.01$ , \*\*\*,  $p < 0.001$ , Anova, Bonferroni corrected *p*-value.  
doi:10.1371/journal.pgen.1004011.g005

at any O<sub>2</sub> tensions. Thus, variation at the *glb-5* and *npr-1* genes, which alter O<sub>2</sub> sensing circuits, changes the extent to which O<sub>2</sub> levels modifies CO<sub>2</sub> aversiveness.

To investigate how O<sub>2</sub> modified CO<sub>2</sub> avoidance in a non-domesticated *C. elegans* strain, we examined the responses of animals from the Hawaiian CB4856 isolate. As reported previously [23,25,26], the Hawaiian strain showed weaker CO<sub>2</sub> avoidance than N2 at 21% O<sub>2</sub>. Reducing O<sub>2</sub> levels to 19% was sufficient to strongly stimulate CO<sub>2</sub> avoidance in Hawaiian animals, and further decreases in [O<sub>2</sub>] had no significant effects (Figure 6A, C). Together, these data suggest that the Hawaiian animals do not avoid CO<sub>2</sub> when O<sub>2</sub> is at 21%, i.e. when animals are at the surface, and but that very small decreases in O<sub>2</sub> are sufficient to increase CO<sub>2</sub>-avoidance behavior. The sharp tuning of CB4856 responses to CO<sub>2</sub> by O<sub>2</sub> levels appears to involve the natural alleles of *npr-1*, *npr-1 215F*, the *glb-5*(*Haw*) alleles.

To shed further light on the genetic control of this cross-talk of CO<sub>2</sub> and O<sub>2</sub> responses, we examined how knocking out the soluble guanylate cyclases *gcy-35* and *gcy-36* altered CO<sub>2</sub> responses in different genetic backgrounds. Knocking out either soluble guanylate cyclase strongly stimulated CO<sub>2</sub> avoidance in *npr-1*



**Figure 6. Re-configuring O<sub>2</sub> sensing circuits by altering the *npr-1* and *glb-5* genes alters CO<sub>2</sub> avoidance behavior.** A. Tuning of CO<sub>2</sub> avoidance behavior by different O<sub>2</sub> concentrations in N2 (Bristol), CB4856 (Hawaiian), *npr-1(ad609)*, *glb-5(Haw); npr-1(ad609)*, and *glb-5(Haw)* animals. All assays used a 1–0% CO<sub>2</sub> gradient. Statistical comparisons are to the N2 response at the corresponding O<sub>2</sub> concentration, \*\*\*  $p < 0.001$ ; \*\*  $p < 0.01$ ; \*  $p < 0.05$  (Anova,  $p$  value protected Fisher's LSD). B. *gcy-35* and *gcy-36* mutants strongly avoid CO<sub>2</sub> regardless of genotype at the *npr-1* locus. Statistical comparisons are to the *npr-1* response at the corresponding O<sub>2</sub> concentration. \*\*\*,  $p < 0.001$ , \*\*,  $p < 0.01$ , Anova, Bonferroni corrected  $p$  value). C. CB4856 (Hawaii) animals defective in *gcy-36* strongly avoid CO<sub>2</sub> regardless of O<sub>2</sub> levels. Statistical comparisons are to the CB4856 response at the corresponding O<sub>2</sub> concentration. \*\*\*,  $p < 0.001$ , \*\*,  $p < 0.01$ , Anova, Bonferroni corrected  $p$  value). D, E. Tonic Ca<sup>2+</sup> levels in URX neurons of *glb-5(Haw); npr-1* animals kept at 21% O<sub>2</sub> and 17% O<sub>2</sub> is lower than Ca<sup>2+</sup> levels in URX in *npr-1* animals kept at the corresponding O<sub>2</sub> concentrations. Ca<sup>2+</sup> measurements were made using cameleon YC2.60. doi:10.1371/journal.pgen.1004011.g006

animals: the avoidance behaviour of *gcy-35; npr-1* or *gcy-36; npr-1* animals resembled that of *gcy-35* or *gcy-36* mutants, and of N2 animals at 11% O<sub>2</sub> (Figure 6B). We also examined the effect of disrupting *gcy-36* in the Hawaiian genetic background (Figure 6C). CB4856 animals defective in *gcy-36* avoided CO<sub>2</sub> much more strongly than CB4856 controls, and changing ambient O<sub>2</sub> had little effect on their CO<sub>2</sub> responses (Figure 6C). Thus, the modulation we describe in domesticated N2 also occurs in wild aggregating *C. elegans*. Expressing cDNA encoding the *npr-1* 215V allele found in N2 animals in the AQR, PQR and URX neurons, using the *gcy-32* promoter, restored N2-like behaviour to *npr-1*

mutants (Figure 6B). Thus, *npr-1* acts in the O<sub>2</sub>-sensing neurons themselves to counter the inhibitory effect of high O<sub>2</sub> on CO<sub>2</sub> avoidance.

To provide a neural explanation for why *npr-1* animals avoided CO<sub>2</sub> less than *glb-5(Haw); npr-1* animals at 17%, 19% and 21% O<sub>2</sub> (Figure 6A,  $p < 0.0001$ , Anova, Bonferroni-corrected  $p$  value at all three O<sub>2</sub> values), we compared tonic Ca<sup>2+</sup> signalling in URX at different O<sub>2</sub> concentrations. While URX Ca<sup>2+</sup> levels were similar in *npr-1* and *glb-5*; *npr-1* animals at 7% O<sub>2</sub>, Ca<sup>2+</sup> was higher in *npr-1* than in *glb-5*; *npr-1* animals at 21% and 17% O<sub>2</sub>, consistent with greater inhibition of CO<sub>2</sub> avoidance by URX signalling at these O<sub>2</sub> concentrations (Figure 6D, E).

### O<sub>2</sub> can modulate CO<sub>2</sub> avoidance in animals defective in AFD and BAG CO<sub>2</sub> sensors

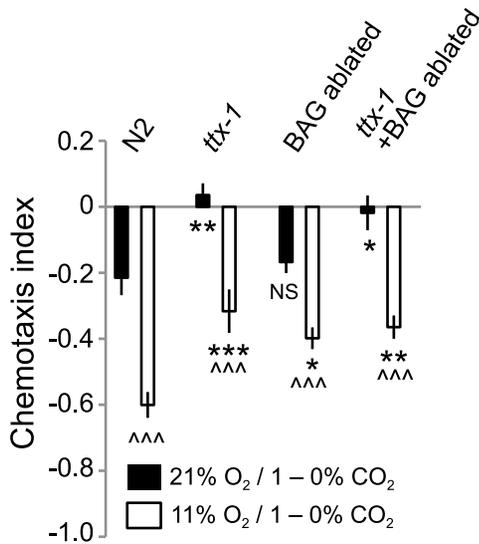
CO<sub>2</sub> avoidance in *C. elegans* is mediated by a distributed set of sensory neurons that includes the BAG O<sub>2</sub> sensor, the AFD temperature sensor, and the ASE gustatory neuron [28,34]. To examine if O<sub>2</sub> levels modified CO<sub>2</sub>-evoked Ca<sup>2+</sup> responses in any of these neurons we imaged their responses at 11% and 21% O<sub>2</sub> concentrations using the YC3.60 sensor (Figure S3A–C). We did not observe any differences between CO<sub>2</sub>-evoked responses at the two O<sub>2</sub> concentrations in any of the three neurons under our imaging conditions. This suggests either that O<sub>2</sub> modulation occurs downstream of these sensory neurons, or that our imaging conditions limit our ability to observe modulation by O<sub>2</sub>.

O<sub>2</sub> input could selectively modulate the CO<sub>2</sub> responses mediated by one CO<sub>2</sub>-sensing neuron, or it could modulate circuits involving multiple CO<sub>2</sub> sensors. To examine these possibilities, we specifically disrupted AFD and/or BAG function in N2 animals, and measured CO<sub>2</sub> avoidance at 21% and 11% O<sub>2</sub>. Genetically abating BAG neurons or disrupting AFD specification by mutating the *ttx-1* transcription factor, or doing both, reduced CO<sub>2</sub> avoidance at 11% O<sub>2</sub>, but did not abolish modulation by ambient O<sub>2</sub> levels (Figure 7). These data suggest that O<sub>2</sub> levels either modulate the output from several CO<sub>2</sub> sensors, or exert their effects on unidentified CO<sub>2</sub> sensors, or both.

### RIA interneurons are part of the circuit mediating O<sub>2</sub>-modulated CO<sub>2</sub> avoidance

To dissect further how O<sub>2</sub>-sensing neurons modulated CO<sub>2</sub> responses, we sought mutations that disrupted O<sub>2</sub> modulation without abrogating CO<sub>2</sub> responsiveness. One such mutation we identified was *ttx-7*, which disrupts a *myo-inositol-1-monophosphatase* [38]. *ttx-7* mutants showed only mild defects in CO<sub>2</sub> avoidance when assayed at 21% O<sub>2</sub> (Figure 8A–C). The chemotaxis index of *ttx-7* mutants was not significantly different from that of N2 controls when animals were assayed in 1–0% and 5–0% CO<sub>2</sub> gradients; we only observed a small but significant decrease in CO<sub>2</sub> avoidance when *ttx-7* mutants were assayed in 3–0% CO<sub>2</sub> gradients. However, *ttx-7* mutant animals did not increase their CO<sub>2</sub> avoidance when assayed at 11% O<sub>2</sub>, regardless of the CO<sub>2</sub> gradient we used (Figure 8A–C). *ttx-7* mutants behaved indistinguishably from N2 animals when assayed in O<sub>2</sub> gradients (Figure S4), suggesting they were not generally defective in O<sub>2</sub>-evoked responses.

To confirm that the defect in O<sub>2</sub>-dependent modulation of CO<sub>2</sub> avoidance was due to the *ttx-7* mutation, we showed we could restore strong CO<sub>2</sub> avoidance at 11% O<sub>2</sub> to *ttx-7* mutants by expressing *ttx-7* cDNA from the *ttx-7* promoter (Figure 8D). Together, these data suggest that *ttx-7* mutants can sense and respond to O<sub>2</sub> but cannot communicate information about

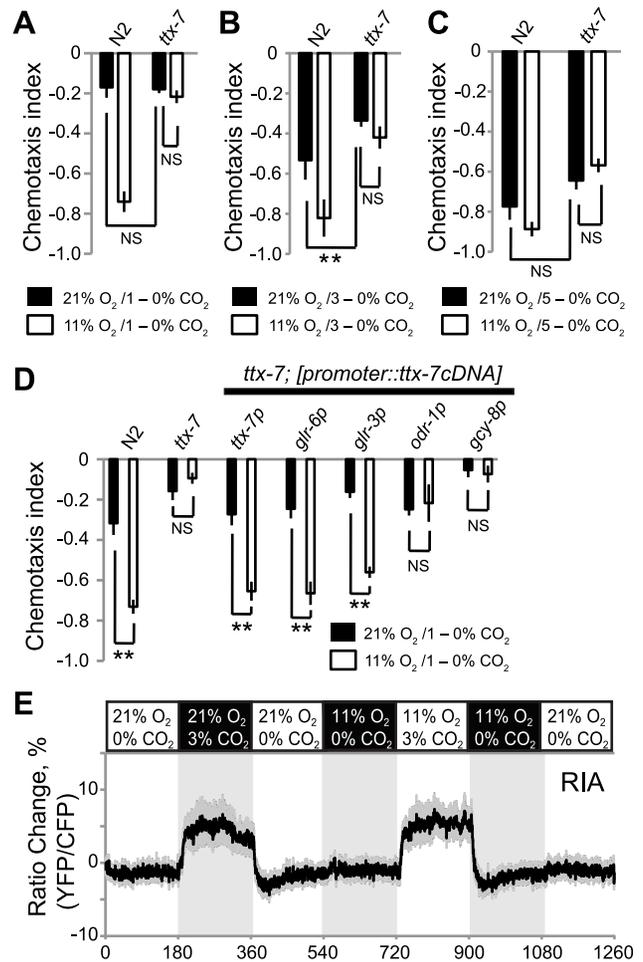


**Figure 7. Ambient O<sub>2</sub> can modulate CO<sub>2</sub> avoidance in animals lacking BAG and AFD CO<sub>2</sub> sensors.** Animals in which BAG neurons are ablated by specific expression of *egl-1* caspase, and AFD neurons are defective due to loss of *ttx-1*, retain O<sub>2</sub>-modulation of CO<sub>2</sub> avoidance. *egl-1* expression in BAG neurons is driven by the *flp-17* promoter.  $\Delta\Delta\Delta$ ,  $p < 0.001$ , Student's *t* test, comparing a strain's responses at 21% and 11% O<sub>2</sub>.  $\Delta\Delta\Delta$ ,  $p < 0.001$ ;  $\Delta\Delta$ ,  $p < 0.01$ ;  $\Delta$ ,  $p < 0.05$ , Anova, Bonferroni corrected *p* value, comparing responses to that of N2 at the same O<sub>2</sub> concentration. doi:10.1371/journal.pgen.1004011.g007

ambient [O<sub>2</sub>] to the appropriate circuits that mediate CO<sub>2</sub> responses.

To identify neurons where *ttx-7* acts to promote CO<sub>2</sub> avoidance at low [O<sub>2</sub>] we rescued the *ttx-7* CO<sub>2</sub> avoidance phenotype by driving *ttx-7* cDNA in small subsets of neurons. We focussed on neurons that receive synaptic input from the URX O<sub>2</sub> sensors, since our *gcy-36* rescue experiments implied that URX was sufficient for O<sub>2</sub> to modulate CO<sub>2</sub> avoidance (Figure 5B). URX neurons make several synapses onto the RIA interneurons [39]. In turn, RIA neurons receive direct or indirect inputs from many sensory neurons, and are connected to numerous downstream interneurons, making them good candidates for transmitting information about ambient O<sub>2</sub> to CO<sub>2</sub> circuits. Previous work has shown that *ttx-7* is required in the RIA neurons to promote appropriate synapse formation and to enable *C. elegans* to navigate temperature gradients [38]. Expressing *ttx-7* cDNA from the *glr-3* or *glr-6* promoters, which drive expression exclusively in RIA [40], restored strong CO<sub>2</sub> avoidance at 11% O<sub>2</sub> (Figure 8D). By contrast, *ttx-7* expression in AFD, using the *gcy-8* promoter, or in AWC and AWB olfactory neurons, using the *odr-1* promoter, did not. These data suggest that RIA interneurons are involved in communicating information from O<sub>2</sub>-sensing neurons and/or CO<sub>2</sub>-responsive circuits, to enable its integration.

We examined if CO<sub>2</sub> elicited a Ca<sup>2+</sup> response in RIA interneurons, and if this response was modulated by O<sub>2</sub> context. We exposed animals expressing cameleon YC3.60 in RIA to a stimulus train in which we sequentially altered O<sub>2</sub> and CO<sub>2</sub> levels, and measured fluorescence changes in the cell body. 3% CO<sub>2</sub> evoked a Ca<sup>2+</sup> response in RIA neurons that was not significantly altered by background O<sub>2</sub> (Figure 8E). These data suggest that RIA interneurons form part of a CO<sub>2</sub> responsive circuit. Our inability to detect modulation of CO<sub>2</sub>-evoked Ca<sup>2+</sup> responses in RIA by O<sub>2</sub> levels could reflect a limitation of our imaging



**Figure 8. TTX-7 acts in RIA interneurons to promote CO<sub>2</sub> avoidance when ambient O<sub>2</sub> levels are low.** A–C. Mutations in *ttx-7* strongly reduce CO<sub>2</sub> avoidance at 11% O<sub>2</sub> but have relatively weak effects on CO<sub>2</sub> avoidance at 21% O<sub>2</sub>. ns, not significant,  $** p < 0.01$ , Student's *t* test. D. Expressing *ttx-7* specifically in RIA neurons, using the *glr-3* or *glr-6* promoters, restores strong CO<sub>2</sub> avoidance to *ttx-7* mutants assayed at 11% O<sub>2</sub>. Expressing *ttx-7* specifically in AFD, using the *gcy-8* promoter, or in AWC and AWB, using the *odr-1* promoter does not rescue the *ttx-7* phenotype. ns, not significant,  $** p < 0.01$ , Student's *t* test. E. CO<sub>2</sub> evokes a Ca<sup>2+</sup> response in RIA neurons. Ca<sup>2+</sup> responses were measured in immobilized animals cultivated at 22°C using a *pglr-6::YC3.60* Ca<sup>2+</sup> reporter. Shading highlights gas switch times. The CO<sub>2</sub>/O<sub>2</sub> stimulus train used is indicated above the plot. doi:10.1371/journal.pgen.1004011.g008

conditions. Alternatively, O<sub>2</sub> could regulate RIA independently of Ca<sup>2+</sup> entry, or could act on neurons downstream of RIA.

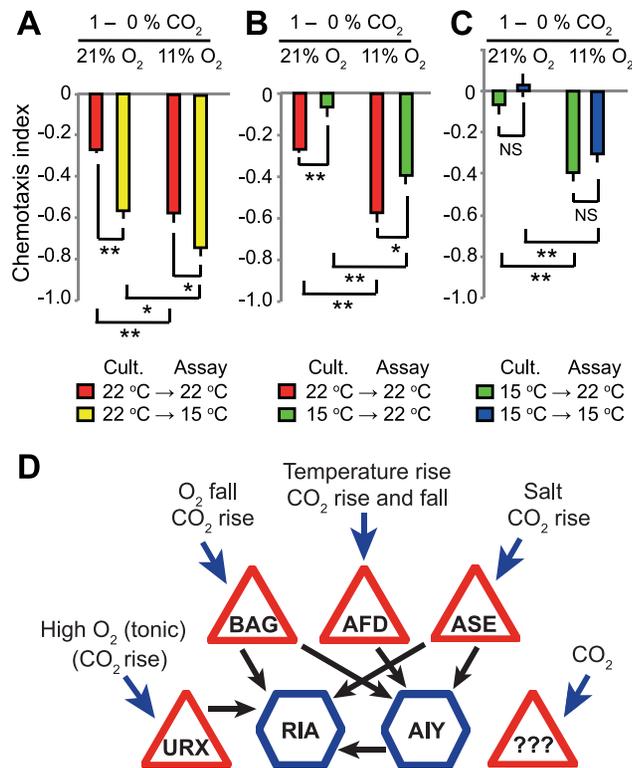
### Acclimation temperature and ambient O<sub>2</sub> act combinatorially to regulate CO<sub>2</sub> responsiveness

Both acclimation temperature and acute ambient O<sub>2</sub> concentrations altered *C. elegans*' responsiveness to CO<sub>2</sub>. We investigated how animals integrated information from all three homeostatic systems – temperature, O<sub>2</sub> and CO<sub>2</sub>. We grew animals at either 15°C or 22°C, and then assayed CO<sub>2</sub> responses at 15°C or 22°C in the presence of either 21% or 11% O<sub>2</sub>. Our results suggest that the temperature and O<sub>2</sub> sensing systems act additively to set CO<sub>2</sub> responsiveness. Decreasing O<sub>2</sub> from 21% to 11% enhanced avoidance of 1% CO<sub>2</sub> regardless of acclimation temperature and assay temperature (Figure 9A–C). Similarly, acclimating animals

to 15°C decreased avoidance of 1% CO<sub>2</sub> at both 21% and 11% O<sub>2</sub> (Figure 9A–C). As described previously (Figure 1A), animals acclimated to 22°C avoided a 1%–0% CO<sub>2</sub> gradient more strongly when assayed at 15°C rather than 22°C. Changing O<sub>2</sub> from 21% to 11% further stimulated CO<sub>2</sub> avoidance in these animals. These data highlight how *C. elegans* homeostatic responses are intertwined with each other.

### Discussion

Previous acclimation temperature and current ambient O<sub>2</sub> levels set the aversiveness of CO<sub>2</sub> to *C. elegans*. The temperature animals have experienced previously appears to modify CO<sub>2</sub> responsiveness by changing the CO<sub>2</sub> receptive properties of AFD. Acute ambient O<sub>2</sub> controls CO<sub>2</sub> preference by regulating tonic signaling from the O<sub>2</sub> sensing neuron URX. Changes in CO<sub>2</sub> responsiveness can be observed in shallow gradients with peak CO<sub>2</sub> levels of 1%. Such gradients are likely to be ecologically relevant for *C. elegans* in the rotting fruit habitats where they are commonly found [41].



**Figure 9. Acclimation temperature and ambient O<sub>2</sub> levels have additive effects on CO<sub>2</sub> avoidance.** A. Animals cultivated at 22°C but assayed at 15°C avoid CO<sub>2</sub> more strongly when ambient O<sub>2</sub> is low. B–C. Reducing O<sub>2</sub> levels from 21% to 11% increases CO<sub>2</sub> avoidance regardless of acclimation temperature or assay temperature. In A–C, \*\* *p*<0.01, \* *p*<0.05, ns, not significant, Student’s *t* test. D. Coalitions of CO<sub>2</sub> sensors elicit CO<sub>2</sub> escape responses according to O<sub>2</sub> environment, temperature experience, and CO<sub>2</sub> stimulus dynamics. Triangles represent sensory neurons and hexagons interneurons. Black arrows indicate synapses. Several neurons respond to CO<sub>2</sub> (blue arrows), each with distinct kinetics. Each of these neurons also responds to other sensory cues, as indicated. Three of the four identified CO<sub>2</sub> sensors synapse directly onto the RIA interneuron. The fourth, AFD, synapses onto AIY which in turn synapses on RIA. The URX O<sub>2</sub> sensor also synapses onto RIA. Note each neuron makes additional connections besides the ones highlighted here. doi:10.1371/journal.pgen.1004011.g009

*C. elegans* can thrive at temperatures that span ~15°C–25°C. Within this range, well-fed animals migrate to temperatures at which they were previously growing [13,29]. Temperature preference appears to be encoded in the AFD neurons: acclimation temperature changes the threshold at which rising temperature evokes Ca<sup>2+</sup> responses in this neuron [17,18]. We find that AFD neurons are required for temperature experience to change *C. elegans*’ CO<sub>2</sub> responsiveness. Acclimation temperature qualitatively reconfigures CO<sub>2</sub>-evoked Ca<sup>2+</sup> responses of AFD neurons. This re-configuration is retained in mutants defective in synaptic release, suggesting it can occur cell-autonomously. A speculative explanation of our observations is that AFD harbors multiple CO<sub>2</sub> sensors whose contribution to the CO<sub>2</sub>-evoked Ca<sup>2+</sup> response varies according to acclimation temperature.

AFD neurons are exquisitely sensitive to CO<sub>2</sub>. They respond robustly to changes in CO<sub>2</sub> that range from <0.01% CO<sub>2</sub>/sec to >1% CO<sub>2</sub>/sec. Remarkably, in animals acclimated to 22°C, the Ca<sup>2+</sup> responses evoked in AFD by slow (0.01% CO<sub>2</sub>/second) and faster (0.04% CO<sub>2</sub>/second) changes in CO<sub>2</sub> are qualitatively different. This may explain previous observations that AFD promotes CO<sub>2</sub> avoidance in shallow CO<sub>2</sub> gradients, but can inhibit CO<sub>2</sub> avoidance in steep ones [28].

*C. elegans* avoid CO<sub>2</sub> less strongly at high O<sub>2</sub> than at low O<sub>2</sub>. Ambient O<sub>2</sub> levels provide a contextual cue that modulates the aversiveness of CO<sub>2</sub>. We use the term ‘contextual’ because modulation can occur when O<sub>2</sub> levels are constant, and is sustained over many minutes. Contextual modulation by O<sub>2</sub> levels can be graded: as O<sub>2</sub> decreases from 21% to 11%, CO<sub>2</sub> avoidance rises. Modulation of CO<sub>2</sub> avoidance by O<sub>2</sub> requires the *gcy-35* and *gcy-36* soluble guanylate cyclases, which act in the O<sub>2</sub> sensing neurons AQR, PQR and URX to transduce O<sub>2</sub> levels. *gcy-35* or *gcy-36* mutants behave like animals kept at low O<sub>2</sub>, regardless of actual O<sub>2</sub> levels. The activity of the URX neurons alone appears sufficient to inhibit CO<sub>2</sub> avoidance at 21% O<sub>2</sub>. Previous work has shown that URX neurons are tonically activated by high O<sub>2</sub> [24], explaining the ability of these neurons to convey O<sub>2</sub> context persistently to CO<sub>2</sub> sensing circuits.

Modulation of CO<sub>2</sub> avoidance by O<sub>2</sub> levels can be observed when N2 (Bristol), *npr-1*, *glb-5(Haw)*; *npr-1*, or CB4856 (Haw) animals navigate 1%–0% CO<sub>2</sub> gradients. However, the degree of inhibition varies across these genotypes. In N2 animals, the inhibitory effect of O<sub>2</sub> is limited by the action of the NPR-1 215V isoform in O<sub>2</sub>-sensing neurons. *npr-1 215V* does not appear to alter the excitability of O<sub>2</sub> sensors, since N2 and *npr-1* mutants show similar O<sub>2</sub>-evoked Ca<sup>2+</sup> responses in URX, AQR or PQR ([22] and data not shown). Instead, we speculate that NPR-1 215V inhibits neurotransmission from URX, for example through G<sub>o</sub> signaling [42,43], thus limiting the ability of URX to inhibit CO<sub>2</sub> responsiveness. Previous work has highlighted coupling of NPR-1 215V to G<sub>o</sub> in heterologous systems [44]. The potent O<sub>2</sub>-dependent inhibition of CO<sub>2</sub> avoidance found in *npr-1* mutants is suppressed by the *glb-5(Haw)* allele. This suppression appears to reflect a reduction in the excitability of URX. Tonic Ca<sup>2+</sup> levels in URX in *glb-5; npr-1* animals kept at 21% O<sub>2</sub> was only as high as that found in *npr-1* animals at 17% O<sub>2</sub>. In the CB4856 (Haw) strain the combination of the *npr-1 215F* and *glb-5(Haw)* alleles (potentially modified by other loci) enables a switch from 21% to 19% O<sub>2</sub> to convert CO<sub>2</sub> from a neutral to a strongly aversive stimulus. While this paper was in preparation independent work also highlighted modulation of CO<sub>2</sub> avoidance by O<sub>2</sub> in *npr-1* animals [45]. The assays used are different. Notably, in most of our work we used 1–0% CO<sub>2</sub> gradients, whereas Carrillo et al. used 10%–0% gradients.

CO<sub>2</sub> sensing in *C. elegans* is distributed across multiple sensory neurons, including the AFD and BAG neurons [28] (Figure 9D). Disrupting AFD and BAG abolishes CO<sub>2</sub> avoidance at 21% O<sub>2</sub>, but CO<sub>2</sub> avoidance at 11% O<sub>2</sub> is only partly reduced. Thus, CO<sub>2</sub> sensing neurons other than BAG and AFD can promote CO<sub>2</sub> avoidance at low O<sub>2</sub>. O<sub>2</sub> modulation of CO<sub>2</sub> responsiveness involves the RIA interneurons. *ttx-7* mutants disrupt O<sub>2</sub> modulation of CO<sub>2</sub> responsiveness, and expressing *ttx-7* cDNA selectively in RIA neurons rescues this phenotype. *ttx-7* encodes *myo*-inositol monophosphatase. In *ttx-7* mutants RIA neurons exhibit defects in localization of both pre- and post-synaptic components, including synaptobrevin, SYD-2 Liprin, and the glutamate receptor GLR-1 [38]. Synaptic communication via RIA is thus likely to be compromised in *ttx-7* mutants, and may explain the O<sub>2</sub>/CO<sub>2</sub> integration phenotype.

Previous studies of context-dependent changes in behavior in *C. elegans* have focused mainly on the effects of food or of food deprivation. *C. elegans*' migration in salt and odor gradients can switch from attraction to repulsion if animals are deprived of food in the presence of the chemical cue [46–49]. Food and food deprivation have also been shown to modulate *C. elegans* response to temperature gradients [50]. It remains to be seen if acclimation temperature and ambient O<sub>2</sub> levels have effects on other sensory modalities besides CO<sub>2</sub> sensing. Whether CO<sub>2</sub> itself can act as a contextual cue regulating other *C. elegans* sensory responses, including thermotaxis and O<sub>2</sub> sensing, is also unknown.

The shallow CO<sub>2</sub> gradients we study are likely to be common in the rotting fruit environments where *C. elegans* is frequently found. However, the ubiquitous production of CO<sub>2</sub> by aerobically respiring organisms means its value as a sensory cue likely depends crucially on context. Bacterial food, bacterial pathogens, predators, mates and conspecifics may all generate CO<sub>2</sub> gradients. Context-dependence of CO<sub>2</sub> responses has been observed previously. *C. elegans* CO<sub>2</sub> responses are modulated by food, exposure to hypoxia, and starvation [25]. Moreover, not only context, but also the rate of change in CO<sub>2</sub> concentration (whether it is slow or rapid), appears to modify the contribution of different CO<sub>2</sub>-sensing neurons to *C. elegans* CO<sub>2</sub> avoidance behaviors [28]. This complexity is mirrored in insects. For example in *Drosophila* airborne CO<sub>2</sub> is aversive [51], whereas dissolved CO<sub>2</sub> is attractive [52]. These properties are encoded by separate chemosensory neurons in the antenna (avoidance of gaseous CO<sub>2</sub>) and taste peg neurons (attraction to carbonation). Avoidance of airborne CO<sub>2</sub> is inhibited by olfactory odors, presumably to enable flies to approach fermenting fruit [53]. Together, these data suggest CO<sub>2</sub> sensing is remarkably sophisticated in both worms and flies. CO<sub>2</sub> has been implicated in ageing in *Drosophila* [54], whereas O<sub>2</sub>-sensing neurons modulate longevity in *Caenorhabditis* [55], consistent with neurons sensing these gases also modulating physiology.

## Materials and Methods

### Strains

Strains were maintained at 22°C with plentiful food using standard methods [56]. Strains used in this work are listed in Supplementary methods.

### Behavioral assays and analysis

Spatial carbon dioxide gradient assays were performed as described, with slight modifications [25,28]. Briefly, rectangular PDMS chambers with a 33×15×0.2 mm space connected to gas syringes were placed over 100–200 worms on a 9 cm NGM agar

plate. Assays ran for 20 minutes and the distribution of worms recorded by counting the number of animals in each of nine equal area divisions as well as in the two spaces at either end of the chamber. Animals were washed three times in a watch glass then transferred to the agar. A chemotaxis index was calculated by subtracting the number of animals in the low carbon dioxide half of the chamber from the number in the high carbon dioxide half and dividing by the total number of animals e.g. (A–B)/(A+B), as shown in Figure 1A. In chemotaxis assays, each data point represents the average of at least eight independent assays performed over three experimental days. Certified gases with indicated concentrations of O<sub>2</sub> and CO<sub>2</sub> were obtained from BOC UK Ltd. Assays marked 22°C were carried out at room temperature in a room in which temperature varied 22+/-1°C. Assays marked 15°C were carried out in a small thermostat-controlled room set to 15°C.

Statistical comparisons were carried out using the Student's *t* test or ANOVA, as indicated.

### Molecular biology and germline transformation

Standard methods for molecular biology were used [57]. Cosmid and cDNA subcloning were performed using the Invitrogen Multisite Gateway Three-Fragment Vector Construction Kit.

Germline transformation was by microinjection [58] using 2–20 ng/μl for the DNA to be tested, along with 50 ng/μl pJMZ-lin-15 (+) construct and carrier DNA, pBluescriptII SK (+).

### Ca<sup>2+</sup> imaging

Ca<sup>2+</sup> imaging was carried out as described previously [24,28], using an inverted microscope (Axiovert, Zeiss), a 40× C-Apochromat lens, and MetaVue acquisition software (Molecular Devices).

## Supporting Information

**Figure S1** CO<sub>2</sub>-evoked responses in AFD do not require the GCY-9 transmembrane guanylate cyclase (A, B), whereas BAG responses do (C, D). For all experiments animals were grown at 22°C. (EPS)

**Figure S2** Disrupting *gcy-33* reduces CO<sub>2</sub> avoidance at 11% O<sub>2</sub>, whereas disrupting *gcy-32* or *gcy-34* has no effect on CO<sub>2</sub> avoidance either at low or high O<sub>2</sub>. \*, *p*<0.05, \*\*, *p*<0.01, ns, not significant, Student's *t*-test. (EPS)

**Figure S3** CO<sub>2</sub>-evoked Ca<sup>2+</sup> responses in ASE (A), BAG (B) and AFD (C) neurons are not altered by background O<sub>2</sub> levels under our imaging conditions. CO<sub>2</sub> and O<sub>2</sub> stimuli are indicated above each plot. (EPS)

**Figure S4** *ttx-7* mutants behave like N2 animals in 21%–0% O<sub>2</sub> gradients. (EPS)

**Text S1** Strain list. (DOCX)

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## Author Contributions

Conceived and designed the experiments: EKN LAF AJB EG MdB. Performed the experiments: EKN LAF AJB EG. Analyzed the data:

EKN LAF AJB EG MdB. Contributed reagents/materials/analysis tools: EKN LAF AJB EG KEB MdB. Wrote the paper: EKN AJB MdB.

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