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

Eiji Kodama-Namba, Lorenz A. Fenk, Andrew J. Bretscher, Einav Gross, K. Emanuel Busch, Mario de Bono*

MRC Laboratory of Molecular Biology, Cambridge, United Kingdom

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, O₂ and CO₂. CO₂ 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 CO₂ changes. CO₂ evokes distinct AFD Ca²⁺ responses in animals acclimated at 15°C or 22°C. Mutants defective in synaptic transmission can reprogram AFD CO₂ responses according to temperature experience, suggesting reprogramming occurs cell autonomously. AFD is exquisitely sensitive to CO₂. Surprisingly, gradients of 0.01% CO₂/second evoke very different Ca²⁺ responses from gradients of 0.04% CO₂/second. Ambient O₂ provides further contextual modulation of CO₂ avoidance. At 21% O₂ tonic signalling from the O₂-sensing neuron URX inhibits CO₂ avoidance. This inhibition can be graded according to O₂ levels. In a natural wild isolate, a switch from 21% to 19% O₂ is sufficient to convert CO₂ from a neutral to an aversive cue. This sharp tuning is conferred partly by the neuroglobin GLB-5. The modulatory effects of O₂ on CO₂ avoidance involve the RIA interneurons, which are post-synaptic to URX and exhibit CO₂-evoked Ca²⁺ responses. Ambient O₂ and acclimation temperature act combinatorially to modulate CO₂ responsiveness. Our work highlights the integrated architecture of homeostatic responses in *C. elegans*.

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* E-mail: debono@mrc-lmb.cam.ac.uk

Introduction

To maintain a constant internal milieu animals use internal sensory receptors to monitor cues such as CO₂/pH [1], O₂ [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 O₂, CO₂/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 CO₂. This over-ride appears to be achieved by changing the set-point at which CO₂ sensors inhibit ventilation when [CO₂] 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 O₂ and a rise in CO₂ [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, O₂ and CO₂ 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 CO₂ 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 CO₂. The responses evoked in AFD by a change in CO₂ concentration are reprogrammed by acclimation temperature. This reprogramming does not appear to require synaptic input from other neurons. O₂ modulates CO₂ avoidance by setting the activity of the tonically signalling O₂ sensor URX. A switch from 21% to 19% O₂ is sufficient to convert CO₂ from a neutral stimulus to an aversive one in a *C. elegans* wild strain. Modulation of CO₂ responses by O₂ cues requires the interneuron RIA which itself responds to changes in CO₂ and is directly post-synaptic to URX. CO₂ 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 CO₂ as a sensory cue thus depends crucially on context. This may explain the remarkable complexity of CO₂ sensing in *C. elegans*.

evokes a Ca²⁺ 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 [O₂], and avoids both high and low O₂ [19]. Wild-caught *C. elegans* strongly avoids 21% O₂, both on and off food, and burrow to escape from the surface [20]. This avoidance response is sculpted by O₂-sensing neurons in the body cavity called AQR, PQR and URX [20,21,22]. When [O₂] 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 O₂ response is sharpened by a neuroglobin expressed in AQR, PQR and URX neurons, called GLB-5, that suppresses neuronal activity when ambient [O₂] falls just below 21% [20,23]. The AQR, PQR and URX neurons are all tonic receptors: they show sustained signalling as long as [O₂] is high [24]. This tonic activity stimulates sustained rapid movement until animals encounter a preferred lower [O₂] environment.

C. elegans also avoids elevated CO₂ [25,26]. As in vertebrates, high [CO₂] is harmful to *C. elegans*, reducing brood size and disrupting muscle structure [27]. An array of sensory neurons mediates CO₂ 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 O₂ levels [22].

Here we investigate how the temperature and O₂ sensing systems of *C. elegans* modulate the distributed circuit that mediates responses to CO₂.

Results

Previous temperature experience sets CO₂ avoidance in *C. elegans*

To examine if temperature can modify *C. elegans*' responses to CO₂ we grew N2(Bristol) animals at 22°C and compared their behavior in CO₂ gradients at 15°C and 22°C (Figure 1A, B) [25,28]. CO₂ avoidance at the two temperatures was similar when animals navigated 3%–0% and 5%–0% CO₂ gradients. However,

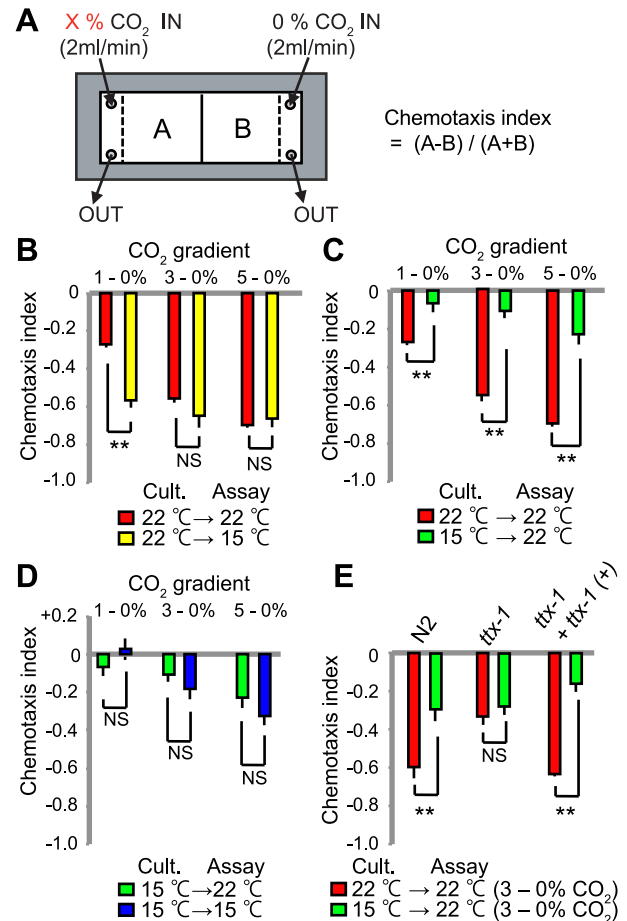


Figure 1. CO₂ avoidance is modulated by acclimation temperature. A. Assay for *C. elegans* CO₂ responses. Animals navigate a defined CO₂ gradient in a microfluidic device. The chemotaxis index is calculated by counting animals in two halves of the device, using the formula shown. B–D. Chemotaxis indices for animals cultivated at either 15°C or 22°C and assayed in different CO₂ 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 CO₂ avoidance by acclimation temperature. Assays were performed in 3%–0% CO₂ gradients. **, $p < 0.01$; n.s., not significant, Student's *t*-test.

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animals in a 1–0% CO₂ gradient avoided the high CO₂ 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 CO₂ [28]. We therefore examined how previous temperature experience altered subsequent CO₂ responses. We grew animals at 15°C or 22°C, and assayed their CO₂ responses at each temperature. Strikingly, previous temperature experience altered CO₂ avoidance. Animals grown at 15°C avoided CO₂ 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 CO₂ avoidance even when exposed to relatively high CO₂ levels, 5% (Figure 1B–D). Thus, the temperature to which *C. elegans* has acclimated helps determine the aversiveness of CO₂.

Acclimation temperature does not reprogram CO₂ responses in AFD-defective mutants

We investigated if the AFD neurons helped to reprogram CO₂ 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 CO₂ avoidance in animals navigating CO₂ spatial gradients [28]. If AFD neurons were important for temperature regulation of CO₂ avoidance responses, then *ttx-1* mutants would display similar CO₂ avoidance regardless of cultivation temperature. As shown previously, *ttx-1* mutants grown at 22°C only avoided CO₂ 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 CO₂-avoidance behavior of animals cultivated at 15°C. These data suggest AFD is required for acclimation temperature to modify CO₂ aversive responses.

Acclimation temperature re-programs the CO₂ 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 CO₂ responsiveness of AFD. To measure CO₂-evoked Ca²⁺ responses in AFD we expressed the genetically encoded Ca²⁺ 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 CO₂ evoked in AFD the complex Ca²⁺ response described previously (Figure 2A) [28]. This typically consisted of an initial slight drop in Ca²⁺ when CO₂ levels rose, followed by a rise in Ca²⁺ to above pre-stimulus levels, and finally, when the CO₂ stimulus was removed, a Ca²⁺ spike that rapidly decayed back to baseline. By contrast, animals acclimated to 15°C exhibited a simple response: a rise in Ca²⁺ when CO₂ levels rose, and a fall when CO₂ was removed (Figure 2B). These data suggest that the previous temperature experience of *C. elegans* reconfigures the CO₂ 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 Ca²⁺ responses of AFD neurons to CO₂ in *snb-1* (*synaptobrevin-1*) mutants, which are defective in synaptic transmission [33]. CO₂-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 CO₂ 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 CO₂ that occurred within 1–2 s, and we always returned animals to 0% CO₂ between stimuli [29]. To examine AFD responses to rises in CO₂ from non-zero levels, we subjected animals acclimated to 22°C to a stimulus train involving multiple CO₂ switches, namely 0%–1%–3%–5%–3%–1%–0%. Whenever CO₂ levels increased, we observed an initial drop in Ca²⁺ followed by a rise in Ca²⁺ (Figure 2E). Whenever CO₂ levels decreased, we observed a spike of Ca²⁺ that rapidly returned to baseline. This pattern of CO₂ evoked Ca²⁺ response suggests that AFD can encode whether an animal is moving towards higher or lower CO₂.

Previous work has identified one potential molecular sensor for CO₂, the transmembrane guanylate cyclase *gcy-9* [34]. We compared CO₂-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 CO₂-responsiveness to AFD neurons (Figure S1).

AFD responses to CO₂ are reconfigured by the steepness of the CO₂ gradient

The ubiquity of CO₂ 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 CO₂ stimulus. Very rapid change in CO₂ levels may convey a different meaning from a very gradual change. In our behavioral experiments, animals navigated shallow CO₂ gradients and encountered changes in the order of 0.01% CO₂ per second (depending on speed and direction of travel in the gradient). To examine if AFD could respond to such shallow CO₂ gradients, we exposed animals cultivated at 22°C to gradual linear increases and decreases in CO₂ concentration at rates of 0.04% and 0.01% per second (Figure 3A,B). AFD responded to both these CO₂ gradients, but with very different response patterns. Gradients of 0.04% CO₂/second evoked AFD Ca²⁺ responses reminiscent of those elicited by sharp changes in CO₂ (>1% CO₂/second; see Figure 2): Ca²⁺ levels decreased while CO₂ was slowly rising to 5%, then rose sharply as CO₂ levels stabilized at 5%. When we gradually reduced CO₂ levels back to 0%, Ca²⁺ levels spiked, returning to baseline when animals were in 0% CO₂ (Figure 3A). By contrast, gradients of 0.01% CO₂/second evoked a series of Ca²⁺ spikes while CO₂ levels were rising (Figure 3B). Ca²⁺ levels tended to return to baseline when CO₂ levels stopped rising, but spiking resumed when CO₂ levels started falling. This spiking pattern disappeared when we imaged Ca²⁺ responses evoked by the same 0.01% CO₂/second gradient in animals acclimated to 15°C (Figure 3C). In these animals responses were more similar to those evoked by steeper CO₂ 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 CO₂, but with different response patterns. The also highlight complexity in how AFD encodes CO₂ stimuli.

Ambient O₂ levels regulate *C. elegans* CO₂ avoidance behaviour

To investigate further how different homeostatic responses are integrated, we examined if CO₂ avoidance behavior was modulated by different background ambient [O₂]. In body fluids and many ecological niches low [O₂] coincides with high [CO₂], and, conversely, 21% O₂ is associated with low CO₂. 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 CO₂ at different fixed concentrations of O₂. As expected, increasing [CO₂] elicited increasing avoidance behavior: *C. elegans* avoided 5% CO₂ more strongly than 3% or 1% CO₂ (Figure 4A) [25,26]. Moreover, CO₂ avoidance was influenced by the background ambient O₂ concentration. N2 animals navigated down CO₂ gradients more strongly when ambient O₂ concentration was 11%, than when it was 21%. Increased avoidance was particularly striking when animals navigated shallow gradients of 1–0% CO₂ (Figure 4A). Such shallow CO₂ gradients are likely to be ecologically relevant in the rotting habitats where *C. elegans* thrives.

To test the dynamic range of O₂ regulation, we asked if increasing [O₂] to above 21% could further suppress CO₂

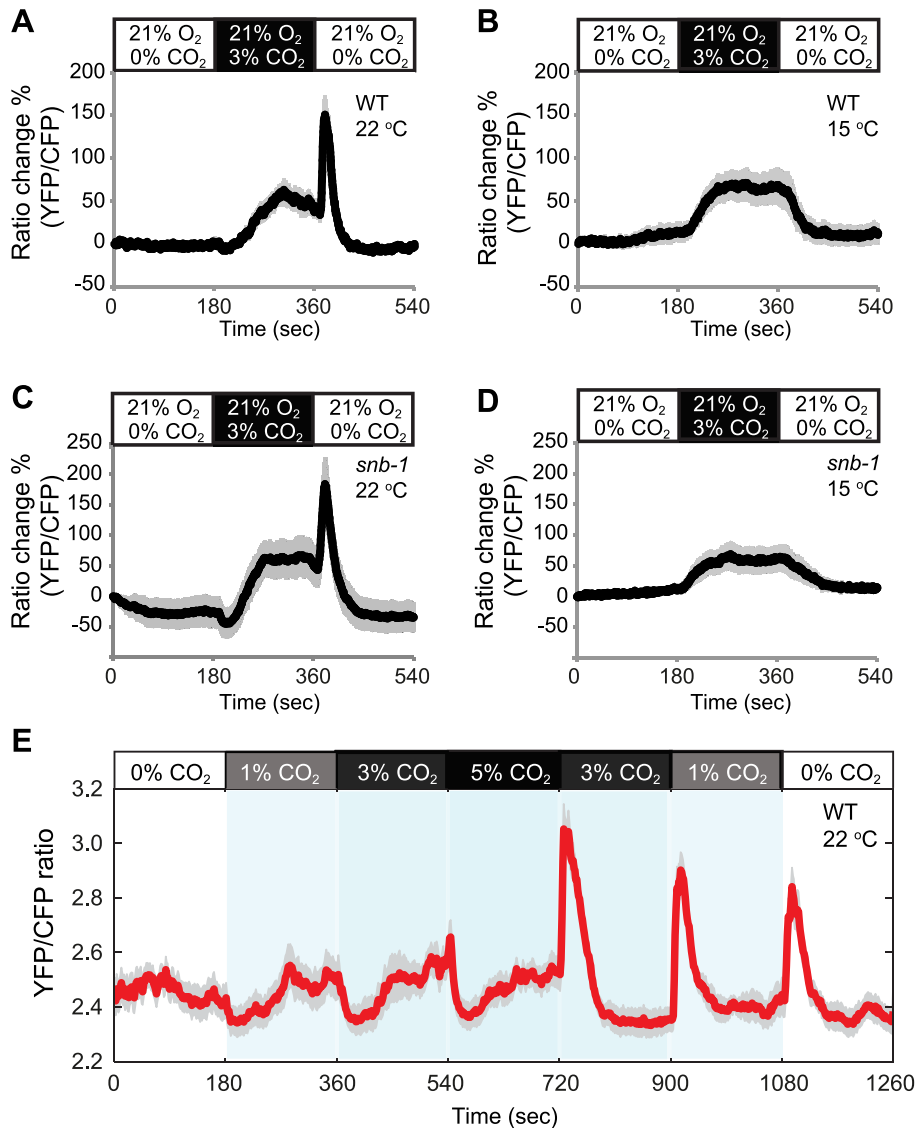


Figure 2. Acclimation temperature alters CO₂-evoked Ca²⁺ responses in AFD neurons. In animals cultivated at 22°C a rise and fall in CO₂ evokes a complex Ca²⁺ response in AFD neurons (A). Ca²⁺ initially falls when CO₂ begins to increase, then rises. When CO₂ levels fall, there is a Ca²⁺ spike. By contrast, animals cultivated at 15°C show a simple response to the same stimulus (B). C–D The effect of acclimation temperature on CO₂-evoked Ca²⁺ responses in AFD neurons is unaltered in *snb-1* mutants defective in synaptobrevin. E. Ca²⁺ responses evoked in AFD by a 0%–1%–3%–5%–3%–1%–0% CO₂ 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% O₂ without any apparent adverse effects [35]. Since *C. elegans* only weakly avoided 1% CO₂ in 21% O₂, we used a steeper 3–0% CO₂ gradient, to improve our dynamic range. Increasing ambient [O₂] to 50% significantly suppressed avoidance of 3% CO₂ (Figure 4B). These data suggest that ambient O₂ concentration provides a contextual cue to modulate *C. elegans* avoidance of CO₂.

Tonically signalling O₂ sensors inhibit CO₂ avoidance at high ambient [O₂]

Our results suggested that O₂-sensing neurons or neuroendocrine cells persistently signal O₂ concentration to modify the activity of CO₂ transducing circuits. Previous studies have shown

that the AQR, PQR and URX O₂ sensors signal tonically when ambient [O₂] is close to 21%, and become progressively less active as [O₂] falls [24]. The O₂-evoked Ca²⁺ responses of these neurons requires the atypical soluble guanylyl cyclases GCY-35 and GCY-36, which appear to be O₂ sensors [20,22,36]. In *gcy-35* or *gcy-36* loss-of-function mutants the Ca²⁺ levels in the O₂ sensing neurons reported byameleon YC3.60, are low, resembling those found in wild type animals kept at low [O₂] [24]. To test if tonic signalling by AQR, PQR and URX neurons persistently repressed CO₂ avoidance in high [O₂], we compared the CO₂ avoidance of wild type, *gcy-35*, and *gcy-36* mutants at 21% and 11% O₂. In 11% O₂ *gcy-35* and *gcy-36* mutants avoided CO₂ like N2 controls. However, whereas increasing background O₂ levels to 21% inhibited the CO₂ avoidance behavior of wild type animals, it had no effect on *gcy-35* or *gcy-36* mutant animals (Figure 5A). These

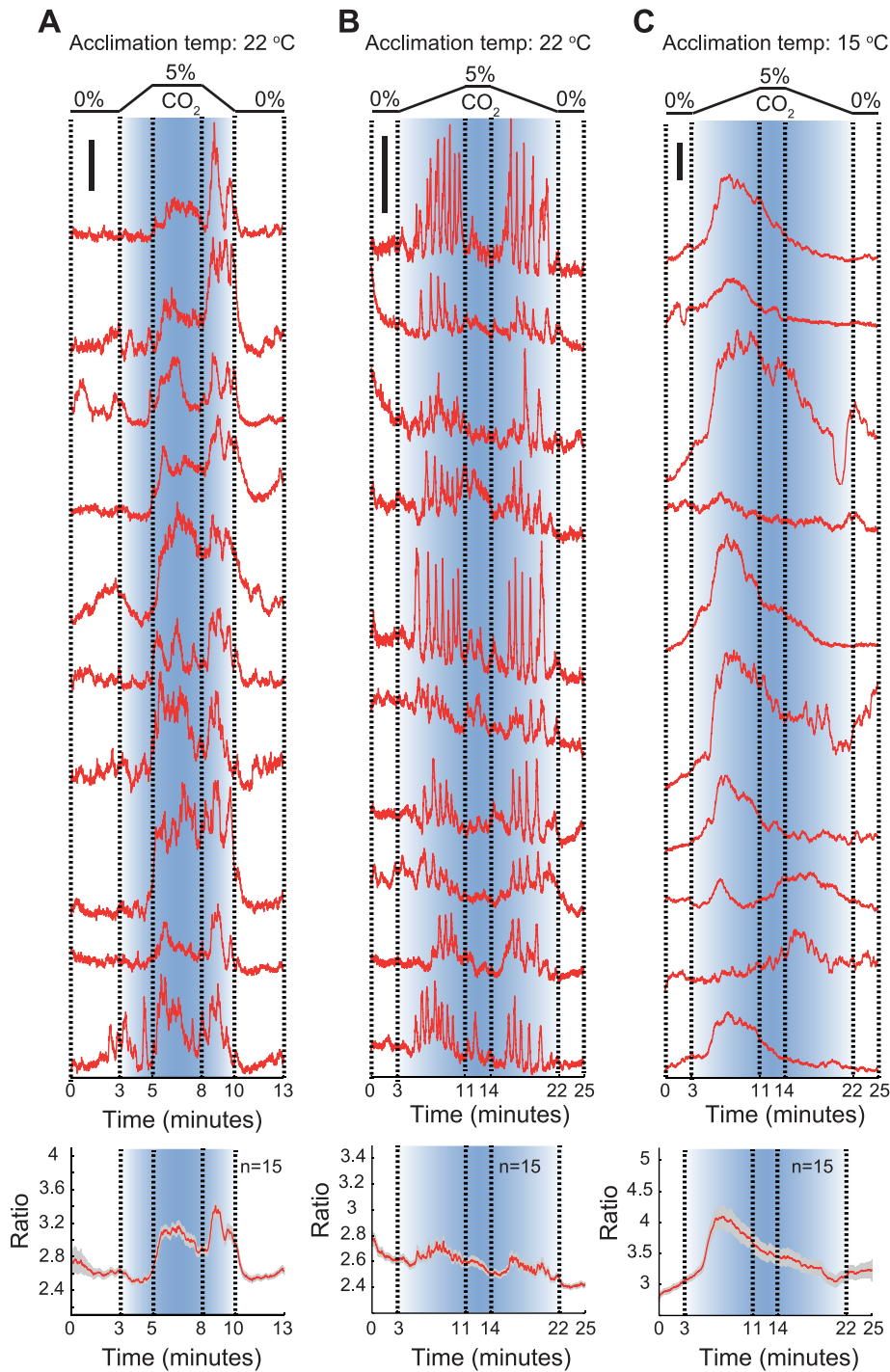


Figure 3. Shallow and steep CO₂ gradients evoke qualitatively different Ca²⁺ responses in AFD. A. Ca²⁺ responses evoked in AFD by CO₂ switches indicated at top, involving linear 0–5% and 5%–0% CO₂ gradients occurring over 2 minutes. This corresponds to a rate of change of 0.04% CO₂/second. The upper 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. Ca²⁺ responses evoked in AFD by CO₂ switches indicated at top, involving linear switches from 0–5% and 5%–0% CO₂ occurring over 8 minutes. This corresponds to a change of 0.01% CO₂/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 O₂ sensors represses CO₂ avoidance at high O₂ 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

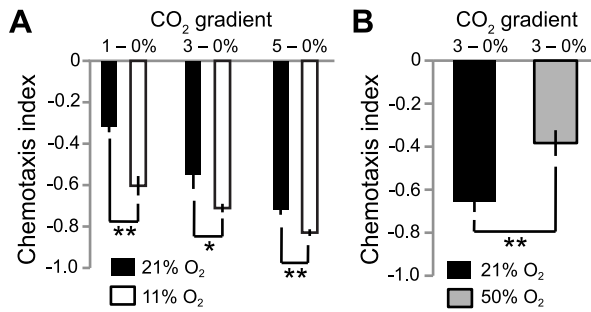


Figure 4. Ambient O₂ levels set CO₂ avoidance. A. *C. elegans* avoids shallow gradients of CO₂ more strongly when O₂ levels are low. The CO₂ gradients used are indicated above the graph. B. Artificially high O₂ levels can reduce CO₂ avoidance further. **, $p < 0.01$; *, $p < 0.05$, Student's *t*-test.

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and URX, restored to *gcy-36* mutants reduced CO₂ avoidance at 21% O₂ (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 O₂-regulated CO₂ avoidance phenotype of *gcy-36* mutants. These results suggest that tonic signalling by the URX O₂-sensing neuron can persistently suppress CO₂ avoidance while O₂ 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 O₂, but whose deletion only subtly alters O₂-evoked behaviors. We observed no effects of these deletions on O₂ regulation of CO₂ avoidance (Figure S2). We did however observe a slight decrease in CO₂ avoidance at 11% O₂ in mutants defective in *gcy-33*, an atypical soluble guanylate cyclase required for the BAG sensory neurons to respond to decreases in O₂ levels (Figure S2) [22]. BAG is also a major CO₂ sensor [28] [34].

The *npr-1* and *glb-5* genes modulate CO₂ avoidance by O₂

O₂ 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 O₂-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 O₂ sensors.

We investigated if variation at *npr-1* and *glb-5* altered O₂ modulation of CO₂ avoidance. In N2 animals, stepwise increases in O₂ from 11% to 21% caused stepwise decreases in CO₂ avoidance (Figure 6A). Animals defective in both the *npr-1* receptor and the *glb-5* neuroglobin (i.e. *npr-1* mutants) were attracted to CO₂ at 21% O₂, but became progressively more repelled by CO₂ as O₂ concentrations fell. A functional *glb-5* (*Hawaii*) allele made CO₂ more aversive to *npr-1* defective animals: decreasing [O₂] still stimulated CO₂ avoidance, but at each concentration tested *glb-5*; *npr-1* animals avoided CO₂ 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 CO₂ avoidance behaviour

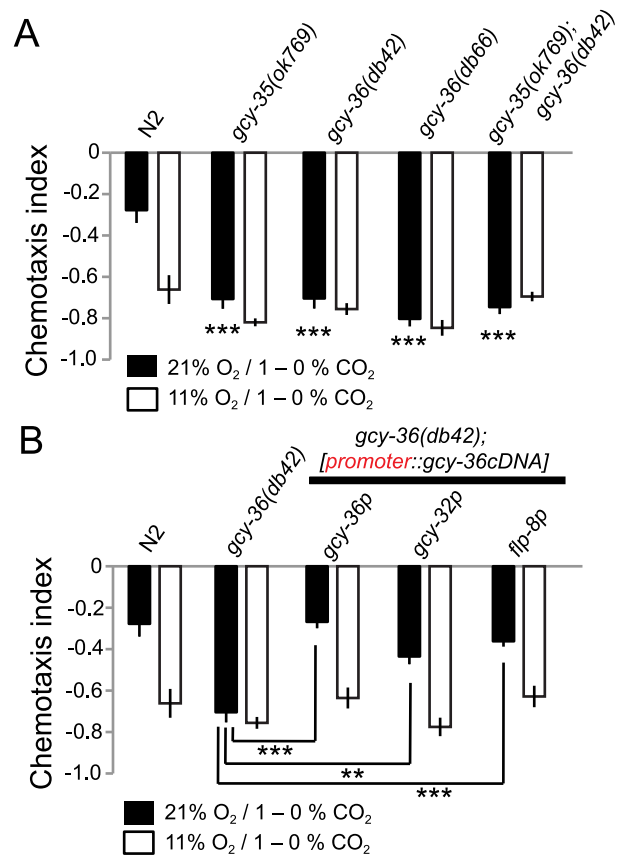


Figure 5. Disrupting *gcy-35* or *gcy-36* confers strong CO₂ avoidance regardless of ambient O₂. A. *gcy-35* or *gcy-36* mutants strongly avoid the high CO₂ half of a 1–0% CO₂ gradient regardless of ambient O₂. Statistics refer to comparisons to N2 at 21% O₂. ***, $p < 0.001$, Anova, Bonferroni corrected *p*-value. None of the strains apart from N2 show significant differences between assays carried out at 21% and 11% O₂ (Student's *t*-test). B. The CO₂-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.

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at any O₂ tensions. Thus, variation at the *glb-5* and *npr-1* genes, which alter O₂ sensing circuits, changes the extent to which O₂ levels modifies CO₂ aversiveness.

To investigate how O₂ modified CO₂ 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 CO₂ avoidance than N2 at 21% O₂. Reducing O₂ levels to 19% was sufficient to strongly stimulate CO₂ avoidance in Hawaiian animals, and further decreases in [O₂] had no significant effects (Figure 6A, C). Together, these data suggest that the Hawaiian animals do not avoid CO₂ when O₂ is at 21%, i.e. when animals are at the surface, and but that very small decreases in O₂ are sufficient to increase CO₂-avoidance behavior. The sharp tuning of CB4856 responses to CO₂ by O₂ 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 CO₂ and O₂ responses, we examined how knocking out the soluble guanylate cyclases *gcy-35* and *gcy-36* altered CO₂ responses in different genetic backgrounds. Knocking out either soluble guanylate cyclase strongly stimulated CO₂ avoidance in *npr-1*

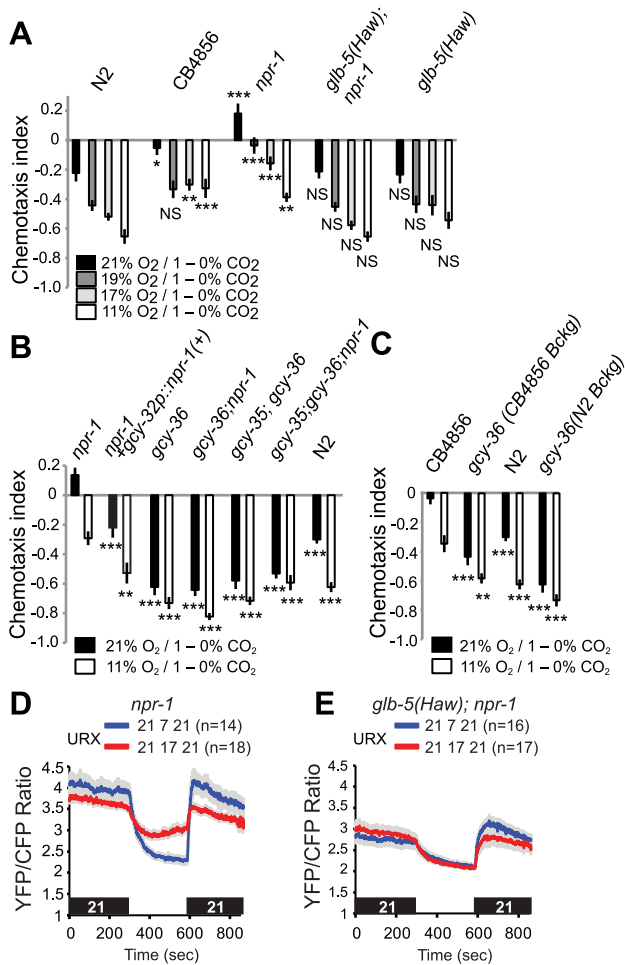


Figure 6. Re-configuring O₂ sensing circuits by altering the *npr-1* and *glb-5* genes alters CO₂ avoidance behavior. A. Tuning of CO₂ avoidance behavior by different O₂ concentrations in N2 (Bristol), CB4856 (Hawaiian), *npr-1(ad609)*, *glb-5(Haw); npr-1(ad609)*, and *glb-5(Haw)* animals. All assays used a 1–0% CO₂ gradient. Statistical comparisons are to the N2 response at the corresponding O₂ 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 CO₂ regardless of genotype at the *npr-1* locus. Statistical comparisons are to the *npr-1* response at the corresponding O₂ concentration. ***, $p < 0.001$, **, $p < 0.01$, Anova, Bonferroni corrected p value). C. CB4856 (Hawaii) animals defective in *gcy-36* strongly avoid CO₂ regardless of O₂ levels. Statistical comparisons are to the CB4856 response at the corresponding O₂ concentration. ***, $p < 0.001$, **, $p < 0.01$, Anova, Bonferroni corrected p value). D, E. Tonic Ca²⁺ levels in URX neurons of *glb-5(Haw); npr-1* animals kept at 21% O₂ and 17% O₂ is lower than Ca²⁺ levels in URX in *npr-1* animals kept at the corresponding O₂ concentrations. Ca²⁺ 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% O₂ (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 CO₂ much more strongly than CB4856 controls, and changing ambient O₂ had little effect on their CO₂ 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 O₂-sensing neurons themselves to counter the inhibitory effect of high O₂ on CO₂ avoidance.

To provide a neural explanation for why *npr-1* animals avoided CO₂ less than *glb-5(Haw); npr-1* animals at 17%, 19% and 21% O₂ (Figure 6A, $p < 0.0001$, Anova, Bonferroni-corrected p value at all three O₂ values), we compared tonic Ca²⁺ signalling in URX at different O₂ concentrations. While URX Ca²⁺ levels were similar in *npr-1* and *glb-5*; *npr-1* animals at 7% O₂, Ca²⁺ was higher in *npr-1* than in *glb-5*; *npr-1* animals at 21% and 17% O₂, consistent with greater inhibition of CO₂ avoidance by URX signalling at these O₂ concentrations (Figure 6D, E).

O₂ can modulate CO₂ avoidance in animals defective in AFD and BAG CO₂ sensors

CO₂ avoidance in *C. elegans* is mediated by a distributed set of sensory neurons that includes the BAG O₂ sensor, the AFD temperature sensor, and the ASE gustatory neuron [28,34]. To examine if O₂ levels modified CO₂-evoked Ca²⁺ responses in any of these neurons we imaged their responses at 11% and 21% O₂ concentrations using the YC3.60 sensor (Figure S3A–C). We did not observe any differences between CO₂-evoked responses at the two O₂ concentrations in any of the three neurons under our imaging conditions. This suggests either that O₂ modulation occurs downstream of these sensory neurons, or that our imaging conditions limit our ability to observe modulation by O₂.

O₂ input could selectively modulate the CO₂ responses mediated by one CO₂-sensing neuron, or it could modulate circuits involving multiple CO₂ sensors. To examine these possibilities, we specifically disrupted AFD and/or BAG function in N2 animals, and measured CO₂ avoidance at 21% and 11% O₂. Genetically abating BAG neurons or disrupting AFD specification by mutating the *ttx-1* transcription factor, or doing both, reduced CO₂ avoidance at 11% O₂, but did not abolish modulation by ambient O₂ levels (Figure 7). These data suggest that O₂ levels either modulate the output from several CO₂ sensors, or exert their effects on unidentified CO₂ sensors, or both.

RIA interneurons are part of the circuit mediating O₂-modulated CO₂ avoidance

To dissect further how O₂-sensing neurons modulated CO₂ responses, we sought mutations that disrupted O₂ modulation without abrogating CO₂ 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 CO₂ avoidance when assayed at 21% O₂ (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% CO₂ gradients; we only observed a small but significant decrease in CO₂ avoidance when *ttx-7* mutants were assayed in 3–0% CO₂ gradients. However, *ttx-7* mutant animals did not increase their CO₂ avoidance when assayed at 11% O₂, regardless of the CO₂ gradient we used (Figure 8A–C). *ttx-7* mutants behaved indistinguishably from N2 animals when assayed in O₂ gradients (Figure S4), suggesting they were not generally defective in O₂-evoked responses.

To confirm that the defect in O₂-dependent modulation of CO₂ avoidance was due to the *ttx-7* mutation, we showed we could restore strong CO₂ avoidance at 11% O₂ 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 O₂ but cannot communicate information about

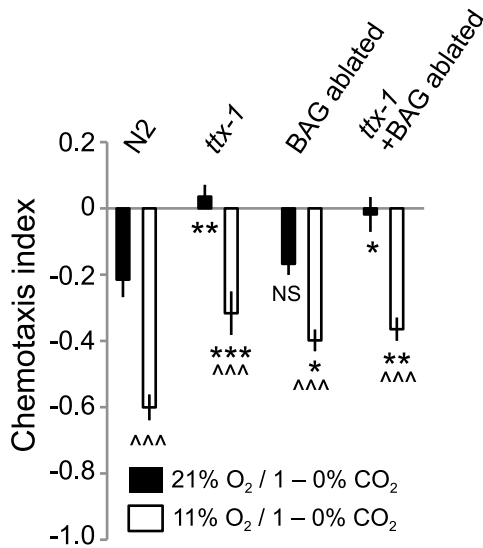


Figure 7. Ambient O₂ can modulate CO₂ avoidance in animals lacking BAG and AFD CO₂ sensors. Animals in which BAG neurons are ablated by specific expression of *egl-1* caspase, and AFD neurons are defective due to loss of *ttx-1*, retain O₂-modulation of CO₂ avoidance. *egl-1* expression in BAG neurons is driven by the *flp-17* promoter. ^{^^^}, $p < 0.001$, Student's *t* test, comparing a strain's responses at 21% and 11% O₂. ^{***} $p < 0.001$; ^{**} $p < 0.01$; ^{*} $p < 0.05$, Anova, Bonferroni corrected *p* value, comparing responses to that of N2 at the same O₂ concentration. doi:10.1371/journal.pgen.1004011.g007

ambient [O₂] to the appropriate circuits that mediate CO₂ responses.

To identify neurons where *ttx-7* acts to promote CO₂ avoidance at low [O₂] we rescued the *ttx-7* CO₂ avoidance phenotype by driving *ttx-7* cDNA in small subsets of neurons. We focussed on neurons that receive synaptic input from the URX O₂ sensors, since our *gcy-36* rescue experiments implied that URX was sufficient for O₂ to modulate CO₂ 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 O₂ to CO₂ 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 CO₂ avoidance at 11% O₂ (Figure 8D). By contrast, *ttx-7* expression in AFD, using the *gcy-8* promoter, or in AWB and AWC olfactory neurons, using the *odr-1* promoter, did not. These data suggest that RIA interneurons are involved in communicating information from O₂-sensing neurons and/or CO₂-responsive circuits, to enable its integration.

We examined if CO₂ elicited a Ca²⁺ response in RIA interneurons, and if this response was modulated by O₂ context. We exposed animals expressing cameleon YC3.60 in RIA to a stimulus train in which we sequentially altered O₂ and CO₂ levels, and measured fluorescence changes in the cell body. 3% CO₂ evoked a Ca²⁺ response in RIA neurons that was not significantly altered by background O₂ (Figure 8E). These data suggest that RIA interneurons form part of a CO₂ responsive circuit. Our inability to detect modulation of CO₂-evoked Ca²⁺ responses in RIA by O₂ levels could reflect a limitation of our imaging

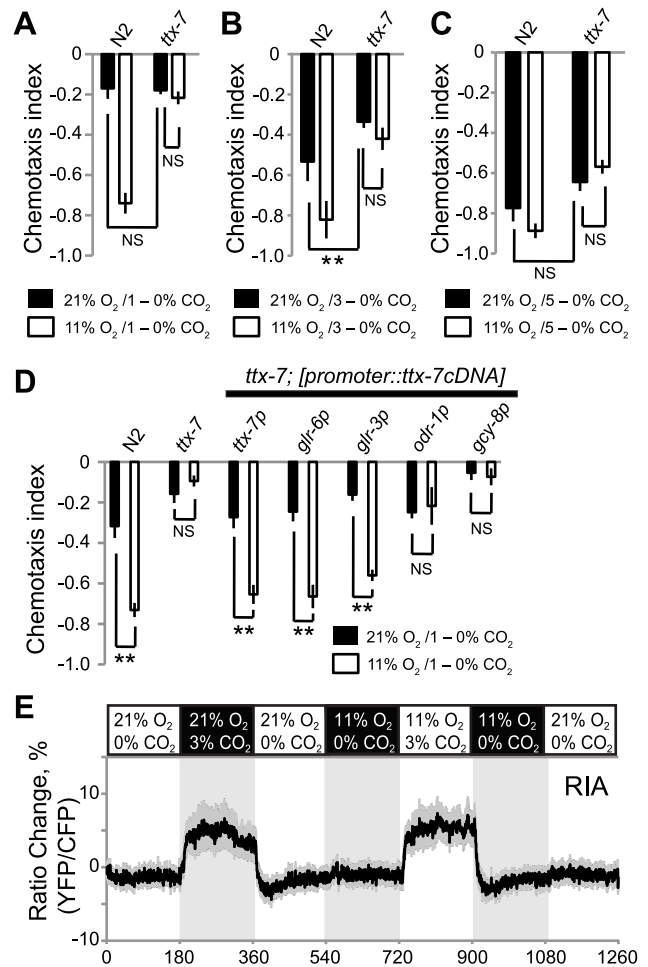


Figure 8. TTX-7 acts in RIA interneurons to promote CO₂ avoidance when ambient O₂ levels are low. A–C. Mutations in *ttx-7* strongly reduce CO₂ avoidance at 11% O₂ but have relatively weak effects on CO₂ avoidance at 21% O₂. ns, not significant, ^{**} $p < 0.01$, Student's *t* test. D. Expressing *ttx-7* specifically in RIA neurons, using the *glr-3* or *glr-6* promoters, restores strong CO₂ avoidance to *ttx-7* mutants assayed at 11% O₂. Expressing *ttx-7* specifically in AFD, using the *gcy-8* promoter, or in AWB and AWC, using the *odr-1* promoter does not rescue the *ttx-7* phenotype. ns, not significant, ^{**} $p < 0.01$, Student's *t* test. E. CO₂ evokes a Ca²⁺ response in RIA neurons. Ca²⁺ responses were measured in immobilized animals cultivated at 22°C using a *pglr-6::YC3.60* Ca²⁺ reporter. Shading highlights gas switch times. The CO₂/O₂ stimulus train used is indicated above the plot. doi:10.1371/journal.pgen.1004011.g008

conditions. Alternatively, O₂ could regulate RIA independently of Ca²⁺ entry, or could act on neurons downstream of RIA.

Acclimation temperature and ambient O₂ act combinatorially to regulate CO₂ responsiveness

Both acclimation temperature and acute ambient O₂ concentrations altered *C. elegans*' responsiveness to CO₂. We investigated how animals integrated information from all three homeostatic systems – temperature, O₂ and CO₂. We grew animals at either 15°C or 22°C, and then assayed CO₂ responses at 15°C or 22°C in the presence of either 21% or 11% O₂. Our results suggest that the temperature and O₂ sensing systems act additively to set CO₂ responsiveness. Decreasing O₂ from 21% to 11% enhanced avoidance of 1% CO₂ regardless of acclimation temperature and assay temperature (Figure 9A–C). Similarly, acclimating animals

to 15°C decreased avoidance of 1% CO₂ at both 21% and 11% O₂ (Figure 9A–C). As described previously (Figure 1A), animals acclimated to 22°C avoided a 1%–0% CO₂ gradient more strongly when assayed at 15°C rather than 22°C. Changing O₂ from 21% to 11% further stimulated CO₂ avoidance in these animals. These data highlight how *C. elegans* homeostatic responses are intertwined with each other.

Discussion

Previous acclimation temperature and current ambient O₂ levels set the aversiveness of CO₂ to *C. elegans*. The temperature animals have experienced previously appears to modify CO₂ responsiveness by changing the CO₂ receptive properties of AFD. Acute ambient O₂ controls CO₂ preference by regulating tonic signaling from the O₂ sensing neuron URX. Changes in CO₂ responsiveness can be observed in shallow gradients with peak CO₂ 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].

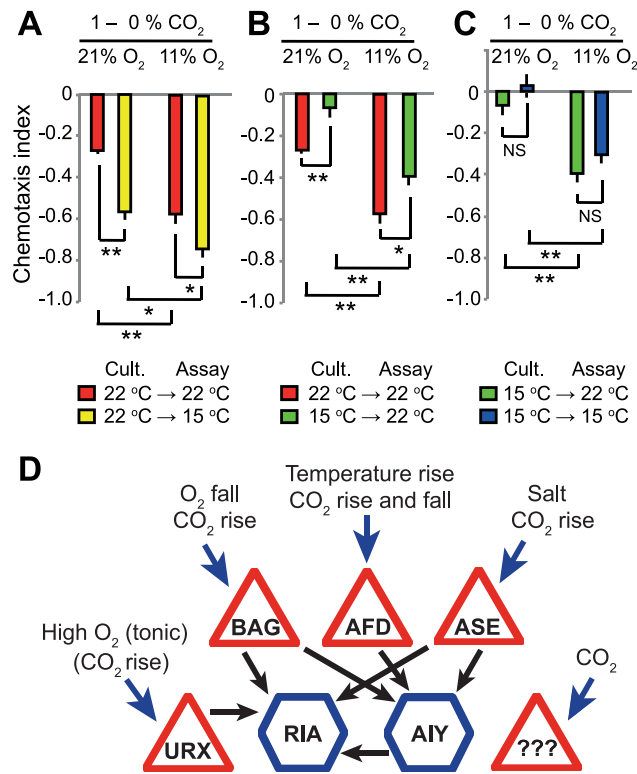


Figure 9. Acclimation temperature and ambient O₂ levels have additive effects on CO₂ avoidance. A. Animals cultivated at 22°C but assayed at 15°C avoid CO₂ more strongly when ambient O₂ is low. B–C. Reducing O₂ levels from 21% to 11% increases CO₂ avoidance regardless of acclimation temperature or assay temperature. In A–C, ** *p*<0.01, * *p*<0.05, ns, not significant, Student’s *t* test. D. Coalitions of CO₂ sensors elicit CO₂ escape responses according to O₂ environment, temperature experience, and CO₂ stimulus dynamics. Triangles represent sensory neurons and hexagons interneurons. Black arrows indicate synapses. Several neurons respond to CO₂ (blue arrows), each with distinct kinetics. Each of these neurons also responds to other sensory cues, as indicated. Three of the four identified CO₂ sensors synapse directly onto the RIA interneuron. The fourth, AFD, synapses onto AIY which in turn synapses on RIA. The URX O₂ 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 Ca²⁺ responses in this neuron [17,18]. We find that AFD neurons are required for temperature experience to change *C. elegans*’ CO₂ responsiveness. Acclimation temperature qualitatively reconfigures CO₂-evoked Ca²⁺ 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 CO₂ sensors whose contribution to the CO₂-evoked Ca²⁺ response varies according to acclimation temperature.

AFD neurons are exquisitely sensitive to CO₂. They respond robustly to changes in CO₂ that range from <0.01% CO₂/sec to >1% CO₂/sec. Remarkably, in animals acclimated to 22°C, the Ca²⁺ responses evoked in AFD by slow (0.01% CO₂/second) and faster (0.04% CO₂/second) changes in CO₂ are qualitatively different. This may explain previous observations that AFD promotes CO₂ avoidance in shallow CO₂ gradients, but can inhibit CO₂ avoidance in steep ones [28].

C. elegans avoid CO₂ less strongly at high O₂ than at low O₂. Ambient O₂ levels provide a contextual cue that modulates the aversiveness of CO₂. We use the term ‘contextual’ because modulation can occur when O₂ levels are constant, and is sustained over many minutes. Contextual modulation by O₂ levels can be graded: as O₂ decreases from 21% to 11%, CO₂ avoidance rises. Modulation of CO₂ avoidance by O₂ requires the *gcy-35* and *gcy-36* soluble guanylate cyclases, which act in the O₂ sensing neurons AQR, PQR and URX to transduce O₂ levels. *gcy-35* or *gcy-36* mutants behave like animals kept at low O₂, regardless of actual O₂ levels. The activity of the URX neurons alone appears sufficient to inhibit CO₂ avoidance at 21% O₂. Previous work has shown that URX neurons are tonically activated by high O₂ [24], explaining the ability of these neurons to convey O₂ context persistently to CO₂ sensing circuits.

Modulation of CO₂ avoidance by O₂ levels can be observed when N2 (Bristol), *npr-1*, *glb-5(Haw)*; *npr-1*, or CB4856 (Haw) animals navigate 1%–0% CO₂ gradients. However, the degree of inhibition varies across these genotypes. In N2 animals, the inhibitory effect of O₂ is limited by the action of the NPR-1 215V isoform in O₂-sensing neurons. *npr-1 215V* does not appear to alter the excitability of O₂ sensors, since N2 and *npr-1* mutants show similar O₂-evoked Ca²⁺ 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_o signaling [42,43], thus limiting the ability of URX to inhibit CO₂ responsiveness. Previous work has highlighted coupling of NPR-1 215V to G_o in heterologous systems [44]. The potent O₂-dependent inhibition of CO₂ 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 Ca²⁺ levels in URX in *glb-5*; *npr-1* animals kept at 21% O₂ was only as high as that found in *npr-1* animals at 17% O₂. 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% O₂ to convert CO₂ from a neutral to a strongly aversive stimulus. While this paper was in preparation independent work also highlighted modulation of CO₂ avoidance by O₂ in *npr-1* animals [45]. The assays used are different. Notably, in most of our work we used 1–0% CO₂ gradients, whereas Carrillo et al. used 10%–0% gradients.

CO₂ sensing in *C. elegans* is distributed across multiple sensory neurons, including the AFD and BAG neurons [28] (Figure 9D). Disrupting AFD and BAG abolishes CO₂ avoidance at 21% O₂, but CO₂ avoidance at 11% O₂ is only partly reduced. Thus, CO₂ sensing neurons other than BAG and AFD can promote CO₂ avoidance at low O₂. O₂ modulation of CO₂ responsiveness involves the RIA interneurons. *ttx-7* mutants disrupt O₂ modulation of CO₂ 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 O₂/CO₂ 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 O₂ levels have effects on other sensory modalities besides CO₂ sensing. Whether CO₂ itself can act as a contextual cue regulating other *C. elegans* sensory responses, including thermotaxis and O₂ sensing, is also unknown.

The shallow CO₂ gradients we study are likely to be common in the rotting fruit environments where *C. elegans* is frequently found. However, the ubiquitous production of CO₂ 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 CO₂ gradients. Context-dependence of CO₂ responses has been observed previously. *C. elegans* CO₂ responses are modulated by food, exposure to hypoxia, and starvation [25]. Moreover, not only context, but also the rate of change in CO₂ concentration (whether it is slow or rapid), appears to modify the contribution of different CO₂-sensing neurons to *C. elegans* CO₂ avoidance behaviors [28]. This complexity is mirrored in insects. For example in *Drosophila* airborne CO₂ is aversive [51], whereas dissolved CO₂ is attractive [52]. These properties are encoded by separate chemosensory neurons in the antenna (avoidance of gaseous CO₂) and taste peg neurons (attraction to carbonation). Avoidance of airborne CO₂ is inhibited by olfactory odors, presumably to enable flies to approach fermenting fruit [53]. Together, these data suggest CO₂ sensing is remarkably sophisticated in both worms and flies. CO₂ has been implicated in ageing in *Drosophila* [54], whereas O₂-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 O₂ and CO₂ 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 (+).

Ca²⁺ imaging

Ca²⁺ 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 CO₂-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 CO₂ avoidance at 11% O₂, whereas disrupting *gcy-32* or *gcy-34* has no effect on CO₂ avoidance either at low or high O₂. *, *p*<0.05, **, *p*<0.01, ns, not significant, Student's *t*-test. (EPS)

Figure S3 CO₂-evoked Ca²⁺ responses in ASE (A), BAG (B) and AFD (C) neurons are not altered by background O₂ levels under our imaging conditions. CO₂ and O₂ stimuli are indicated above each plot. (EPS)

Figure S4 *ttx-7* mutants behave like N2 animals in 21%–0% O₂ 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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