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.

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. doi:10.1371/journal.pgen.1004011.g001

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, 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 retime 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 reprograms 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 presynaptic input, we imaged the Ca²⁺ responses of AFD neurons to CO₂ in snb-1 (synaptobrevin-I) 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 decreased, 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 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.
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 are unaltered in snb-1 mutants defective in synaptobrevin. 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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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), while a rise and fall in CO2 evokes a simple 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.
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 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. 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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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(Hawai) 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(Hawai) allele to N2 animals bearing the npr-1 gain-of-function allele did not significantly change their CO2 avoidance behaviour 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 increase CO2 avoidance further. 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(Hawai) 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
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 *npy*-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
communicating information from O2-sensing neurons and/or not. These data suggest that RIA interneurons are involved in CO2-responsive circuits, to enable its integration.

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 *gy-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 *gy-8* promoter, or in AWB and AWC olfactory neurons, using the *adr-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 conditions. Alternatively, O2 could regulate RIA independently of Ca2+ entry, or could act on neurons downstream of RIA.

**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

**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 *gcg-8* promoter, or in AWB and AWC, using the *adr-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-C*::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
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.](image)

*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 [17,10]. 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 Gₛ isoform in O₂-sensing neurons. The potent O₂-evoked Ca²⁺ responses in URX, AQR or PQR are 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 [20] (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. *txt-7* mutants disrupt O₂ modulation of CO₂ responsiveness, and expressing *txt-7* cDNA selectively in RIA neurons rescues this phenotype. *txt-7* encodes myo-inositol monophosphatase. In *txt-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 *txt-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 chemo- sensory 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 [35], 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 [25,26]. Briefly, rectangular PDMS chambers with a 33 x 15 x 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-lin15 (+) 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 Gça12 transmembrane guanylate cyclase (A, B), whereas BAG responses do (C, D). For all experiments animals were grown at 22°C. (EPS)

Figure S2 Disrupting gcv-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 *txt-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 LAJ EB MG. Performed the experiments: EKN LAJ EB. Analyzed the data: EKN LAJ EB. Contributed reagents/materials/analysis tools: EKN LAJ EB KEK MG. Wrote the paper: EKN LAJ EB.

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