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Temperature, Oxygen, and Salt-Sensing Neurons in *C. elegans* Are Carbon Dioxide Sensors that Control Avoidance Behavior

Andrew Jonathan Bretscher,¹ Eiji Kodama-Namba,¹ Karl Emanuel Busch,¹ Robin Joseph Murphy,¹ Zoltan Soltesz,¹ Patrick Laurent,¹ and Mario de Bono¹,²

¹MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 0QH, UK
²Correspondence: debono@mrc-lmb.cam.ac.uk
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SUMMARY

Homeostatic control of body fluid CO₂ is essential in animals but is poorly understood. *C. elegans* relies on diffusion for gas exchange and avoids environments with elevated CO₂. We show that *C. elegans* temperature, O₂, and salt-sensing neurons are also CO₂ sensors mediating CO₂ avoidance. AFD thermosensors respond to increasing CO₂ by a fall and then rise in Ca²⁺ and show a Ca²⁺ spike when CO₂ decreases. BAG O₂ sensors and ASE salt sensors are both activated by CO₂ and remain tonically active while high CO₂ persists. CO₂-evoked Ca²⁺ responses in AFD and BAG neurons require cGMP-gated ion channels. Atypical soluble guanylate cyclases mediating O₂ responses also contribute to BAG CO₂ responses. AFD and BAG neurons together stimulate turning when CO₂ rises and inhibit turning when CO₂ falls. Our results show that *C. elegans* senses CO₂ using functionally diverse sensory neurons acting homeostatically to minimize exposure to elevated CO₂.

INTRODUCTION

As the major by-product of oxidative metabolism, CO₂ is ubiquitous in nature. Although CO₂ comprises only ~0.038% of Earth’s atmosphere, it can accumulate to higher levels in environments with high respiration rates (Lahiri and Forster, 2003). Organisms have evolved CO₂-sensing mechanisms to monitor both external and internal CO₂ concentrations, but how these systems function to control physiology and behavior remain poorly understood.

Mice can smell environmental CO₂ concentrations as low as 0.066% CO₂ using specialized olfactory neurons that express carbonic anhydrase II (Hu et al., 2007). Carbonic anhydrases catalyze hydration of CO₂ to generate H⁺ and HCO₃⁻. HCO₃⁻ is thought to stimulate the mouse olfactory neurons by activating a guanylate cyclase, GC-D (Hu et al., 2007; Sun et al., 2009). In humans the GC-D homolog is a pseudogene, and we cannot smell CO₂ (Young et al., 2007). However, we can taste CO₂ in carbonated solutions via sour-sensing cells on our tongues (Chandrashekar et al., 2009). In rodents, CO₂ levels of 10% or more elicit an innate fear response in which animals freeze and avoid open spaces (Ziemann et al., 2009). This response requires activation of the acid-sensing ion channel ASIC-1A in cells of the amygdala (Ziemann et al., 2009). High concentrations of inhaled CO₂ also modulate wakefulness by stimulating midbrain neurons (Williams et al., 2007; Richerson, 2004; Buchanan and Richerson, 2010).

Insects also sense and respond to environmental CO₂. *Drosophila* adults and larvae avoid CO₂ levels as low as 0.1% (Suh et al., 2004; Faucher et al., 2006). Like the CO₂-evoked fear behavior in mice, *Drosophila* CO₂ avoidance is innate (Suh et al., 2004) and may be part of an alarm response: stressed flies release 3- to 4-fold more CO₂ than unstressed flies (Suh et al., 2004). *Drosophila* senses gaseous CO₂ using two olfactory receptors, Gr21a and Gr63a, which are expressed in antennal sensory neurons (Jones et al., 2007; Kwon et al., 2007). Artificial activation of the Gr21a/Gr63a-expressing neurons elicits an avoidance response (Suh et al., 2007). Whether the Gr21a/Gr63a receptor binds molecular CO₂ or a CO₂ derivative is not known. Interestingly, some food-associated odorants inhibit Gr21a/Gr63a CO₂ receptor function, and the presence of food reduces CO₂ avoidance (Turner and Ray, 2009). Although *Drosophila* avoids gaseous CO₂, it is attracted to carbonated substrates, a response mediated by HCO₃⁻-sensitive neurons in the proboscis (Fischler et al., 2007).

Besides monitoring external CO₂, many animals also monitor internal CO₂. Internal CO₂ levels are regulated by respiratory gas exchange (Lahiri and Forster, 2003; Feldman et al., 2003; Bustami et al., 2002), but when left unregulated can lead to toxic changes in body fluid pH and death (Richerson, 2004). Mammalian respiratory CO₂ chemoreception occurs in the brain and carotid bodies (Lahiri and Forster, 2003). The molecular mechanisms are unclear, but CO₂-sensitive cells express carbonic anhydrases (Coates et al., 1998; Cammer and Brion, 2000), and changes in extracellular or intracellular pH modulate signaling via H⁺-sensitive ion channels (Lahiri and Forster, 2003; Richerson et al., 2005; Buckler et al., 2000; Feldman et al., 2003; Richerson, 2004; Jiang et al., 2005). Insects achieve respiratory gas exchange by opening and closing spiracles, but the control mechanisms involved are not known (Hetz and Bradley, 2005; Lehmann and Heymann, 2005).

Many small animals, including the nematode *C. elegans*, lack a specialized respiratory system and use diffusion for gas exchange for gas exchange and avoids environments with elevated CO₂. We show that *C. elegans* temperature, O₂, and salt-sensing neurons are also CO₂ sensors mediating CO₂ avoidance. AFD thermosensors respond to increasing CO₂ by a fall and then rise in Ca²⁺ and show a Ca²⁺ spike when CO₂ decreases. BAG O₂ sensors and ASE salt sensors are both activated by CO₂ and remain tonically active while high CO₂ persists. CO₂-evoked Ca²⁺ responses in AFD and BAG neurons require cGMP-gated ion channels. Atypical soluble guanylate cyclases mediating O₂ responses also contribute to BAG CO₂ responses. AFD and BAG neurons together stimulate turning when CO₂ rises and inhibit turning when CO₂ falls. Our results show that *C. elegans* senses CO₂ using functionally diverse sensory neurons acting homeostatically to minimize exposure to elevated CO₂.
exchange. As in other animals, high CO2 levels are toxic (Sharabi et al., 2009). C. elegans appears to control internal CO2 by avoiding environments where this gas exceeds ~0.5%. Avoidance requires cGMP-gated ion channels containing the TAX-2 and TAX-4 subunits (Bretscher et al., 2008; Hallem and Sternberg, 2008). Also implicated are the BAG sensory neurons, required for acute avoidance of a high CO2 and low O2 mixture (Hallem and Sternberg, 2008). Recent work indicates that the BAG neurons are transiently activated when ambient O2 levels fall below 10% (Zimmer et al., 2009).

Here, we show that the C. elegans head sensory neurons AFD, BAG, and ASE are primary CO2 sensors. AFD, BAG, and ASE were previously only known to detect changes in temperature, O2, and salt ion levels, respectively. Using Ca2+ imaging, we describe the CO2 responses of these neurons, which include ON, OFF, and perduring responses. We show that some, but not all, of the Ca2+ responses to CO2 depend on a cGMP-gated ion channel. Finally, we dissect how the C. elegans CO2 sensory system regulates CO2-evoked behavior. We find that the contribution of different sensors to behavior varies widely, depending on both context and stimulus dynamics.

RESULTS

Multiple Sensory Neurons Mediate C. elegans Avoidance of CO2

When placed in a 5%-0% CO2 gradient, C. elegans migrate away from high CO2 (Figures 1A and 1B) (Bretscher et al., 2008). We used this assay to identify potential CO2-sensing neurons. Mutants defective in either the TAX-4 or TAX-2 β cGMP-gated ion channel subunits show reduced CO2 avoidance, both in the presence and absence of E. coli food (Figure 1C) (Bretscher et al., 2008; Hallem and Sternberg, 2008). The defects of tax-2; tax-4 double mutants recapitulated those of single mutants (Figure 1C), consistent with α and β subunits functioning together. tax-2 and tax-4 are coexpressed in 14 of 40 C. elegans sensory neuron classes (White et al., 1986; Komatsu et al., 1996; Coburn and Bargmann, 1996), implicating a subset of these neurons in CO2 sensing. A tax-2 promoter mutation, tax-2(p694), also disrupted CO2 avoidance (Figure 1C). Previous work reported that this allele deletes exon 1 and ~1.6 kb of tax-2 upstream sequences (Coburn and Bargmann, 1996). However, our sequencing data suggest that it removes only 365 bp in this interval (details in Supplemental Experimental Procedures available online). tax-2(p694) mutants have deficits in behaviors mediated by the AFD, BAG, ASE, AQR, PQR, and URX neurons but appear wild-type for responses mediated by other tax-2 expressing neurons (Dusenberg et al., 1975; Hedgecock and Russell, 1975; Coburn and Bargmann, 1996; Coates and de Bono, 2002). Selectively expressing tax-2 cDNA in AFD, BAG, ASE, AQR, PQR, and URX in tax-2(p694) mutants restored CO2 avoidance to the same extent as a full-length tax-2 genomic fragment (Figures 1C and 1D). We next attempted to rescue the tax-2 (p694) defect by expressing tax-2 cDNA from neuron-specific promoters, confirming appropriate expression by polycistronic constructs that coexpress tax-2 and gfp (Coates and de Bono, 2002). Expressing tax-2 cDNA in the AFD thermosensory neurons strongly rescued CO2 avoidance, both on and off food (Figure 1D). In contrast, restoring tax-2 to the BAG O2-sensing neurons rescued CO2 avoidance on food, as shown previously (Hallem and Sternberg, 2008), but not off food. Expressing tax-2 cDNA in the ASE taste neurons or in the AQR, PQR, and URX O2-sensing neurons also partially rescued CO2 avoidance, both on food and off food (Figure 1D). These data implicate functionally diverse sensory neurons in CO2 avoidance.

The AFD Thermosensory Neurons Sense CO2

The AFD neurons are transiently activated when temperatures exceed cultivation levels (Kimura et al., 2004; Clark et al., 2006). To test whether AFD also responds to CO2, we monitored AFD intracellular Ca2+ levels during CO2 exposure using the ratiometric Ca2+ sensor cameleon YC3.60, expressed in AFD under control of the gcy-8 promoter (Yu et al., 1997). Animals expressing the Ca2+ sensor retained wild-type CO2 responses (Figure S1A; see Experimental Procedures). To deliver CO2 stimuli, we used a Y-shaped microfluidic chamber that enables the gas phase over an immobilized animal to be switched in less than 3 s (Persson et al., 2009). In all experiments, O2 was maintained at 21%, with nitrogen (N2) completing the balance. AFD Left and AFD Right neurons responded equally to CO2 (Figure 2A; data not shown). On CO2 exposure the AFD neurons exhibited a fall in intracellular Ca2+ that slowly reversed to rise above baseline levels (“CO2-ON” response) within 2 min of CO2 coming on (Figures 2A and 2C). Thus, the AFD CO2-ON response has two components to it, an “ON-minimum” and an “ON-maximum.” Strikingly, AFD also responded to removal of CO2 with a fast Ca2+ spike that peaked within 10 s (“CO2-OFF” response, Figures 2A and 2D). The OFF-maximum was the largest feature of the AFD Ca2+ pattern, being on average 3- to 4-fold greater than the ON-maximum (Figure 2B). All three components of the AFD CO2 response were concentration dependent (Figure 2B). To exclude the possibility that the observed activity could be due to AFD temperature sensing, we exposed animals to 0%-0%-0% CO2 mock switches. Under these conditions AFD gave no responses (first 9 min, Figure 2E).

We next examined whether repeated stimulation altered AFD Ca2+ responses. Some C. elegans sensory neurons, such as the ALM anterior touch neurons, habituate upon repeated stimulation (Kindt et al., 2007). The AFD OFF response remained undiminished upon repeated exposure to 3% CO2 (Figures 2E, 2F, and S1B). We also asked whether prolonged CO2 exposure affects AFD responses. After a 9 min exposure to 3% CO2, the ON-maximum had decayed to baseline levels, whereas the OFF-maximum was unaltered (Figure 2G).

CO2-evoked activity in AFD could be due to synaptic input to AFD. To test this, we imaged CO2 responses in unc-13 mutants, which have severe defects in synaptic release (Richardson et al., 1999). The AFD CO2 responses of unc-13 animals were indistinguishable from wild-type (Figures 2H and S1C). These data suggest that, as well as being a thermosensory neuron (Mori and Ohshima, 1995; Kimura et al., 2004; Clark et al., 2007), AFD is a CO2 sensor with both ON and OFF responses. The sensory endings of AFD have many finger-like projections, potentially providing a large surface for CO2 and temperature reception (Ward et al., 1975).
AFD only responds to a temperature rise above the cultivation temperature (Kimura et al., 2004; Clark et al., 2006). If AFD temperature and CO$_2$-sensing are distinct, AFD might be expected to respond to CO$_2$ at temperatures below the cultivation temperature. To test this, we built a temperature-controlled stage (see Supplemental Experimental Procedures). In animals grown at 22°C, AFD responded to CO$_2$ both at 15°C and at 22°C (Figures S1E and S1F). The shape of the response was similar at the two temperatures but smaller at 15°C than at 22°C. These data support the idea that AFD CO$_2$ and temperature-sensing pathways are at least partly distinct.

The BAG O$_2$ Sensory Neurons

Sensing CO$_2$

Recent work has shown that the BAG neurons are transiently activated when O$_2$ levels drop below 10% (Zimmer et al., 2009). Hallem and Sternberg (2008) showed that feeding animals lacking the BAG neurons have reduced avoidance of a 10% CO$_2$/10% O$_2$ mixture. We have previously shown that O$_2$ responses can modulate CO$_2$ avoidance (Bretscher et al., 2008). These data suggest that either BAG responds exclusively to O$_2$ but modulates neural circuits mediating CO$_2$ responses or that BAG is a primary sensor of both O$_2$ and CO$_2$.

To test BAG neuron CO$_2$ mediation, we created animals expressing cameleon YC3.60 in BAG from a pflp-17::YC3.60 transgene and imaged Ca$^{2+}$ levels. The BAGL and BAGR neurons were exquisitely sensitive to a rise in CO$_2$ (Figures 3A–3C). Cameleon reported a rise in Ca$^{2+}$ that peaked after ~30 s and then decayed (Figures 3A and 3B). The excitability threshold of BAG was below 0.25% CO$_2$. A plot of mean fluorescence ratio
prolonged high CO₂ the BAG Ca²⁺ spike decayed to a plateau feeding C can signal tonically in high CO₂ (Bretscher et al., 2008). During peak and a perduring Ca²⁺ plateau in response to elevated CO₂.

Thus, BAG exhibits both a transient change against percent (%) CO₂ suggests that BAG reaches half-maximal activity at ~2.9% CO₂ (Figure 3D). Thus, BAG neurons respond to both O₂ and CO₂.

Elevated CO₂ persistently stimulates locomotory activity in feeding C. elegans, suggesting that some CO₂-sensing circuits can signal tonically in high CO₂ (Bretscher et al., 2008). During prolonged high CO₂ the BAG Ca²⁺ spike decayed to a plateau that persisted until CO₂ removal, at which point Ca²⁺ returned to resting levels (Figure 3E). Thus, BAG exhibits both a transient peak and a perduring Ca²⁺ plateau in response to elevated CO₂.

As with AFD, we asked whether BAG neurons habituate. During five stimulus cycles of 3% CO₂, BAG showed a decrement in response amplitude after the first CO₂ stimulus, but no habituation thereafter (Figures 3F–3H).

To test if the BAG neurons are primary CO₂ sensors, we disrupted synaptic input to BAG using the unc-13 and unc-31 mutations. unc-31 mutants are defective in dense-core vesicle release, but not synaptic vesicle release (Speese et al., 2007). Neither the unc-13 nor the unc-31 mutations disrupted BAG Ca²⁺ responses, suggesting that BAG neurons are intrinsically CO₂ sensitive (Figures 3I–3K). However, the magnitude of Ca²⁺ responses in these mutants was significantly enhanced, particularly in unc-31 animals, suggesting that BAG activity is normally inhibited by neuromodulators.

The Asymmetric ASEL and ASER Taste Neurons Are Both Activated by CO₂

We next examined CO₂ responses in the ASE neurons that mediate chemotaxis to water-soluble cues, including salt ions such as Na⁺ and Cl⁻ (Bargmann and Horvitz, 1991; Ortiz et al., 2009). ASEL and ASER are functionally asymmetric (Hobert et al., 2002). ASEL is activated by a rise in the concentration of NaCl,
whereas ASER is activated by a drop (Suzuki et al., 2008). For NaCl responses, activation of ASEL inhibits animals from reversing, whereas activation of ASER increases reversal likelihood (Suzuki et al., 2008).

We imaged ASEL and ASER Ca$^{2+}$ responses to CO$_2$, using animals expressing the Ca$^{2+}$ sensor YC2.12 in ASE from a pflp-6::YC2.12 transgene (Suzuki et al., 2008). Both ASEL and ASER were activated by 1%, 3%, and 5% CO$_2$ (Figures 3A–D).
Although the responses of ASEL were generally ~2-fold larger than those of ASER (Figure 4E), ASE responses to CO₂ were slow, taking around 2 min for Ca²⁺ levels to peak (Figure 4F). Sustained elevated CO₂ led to sustained increases in Ca²⁺ (Figure 4F). As for AFD and BAG, ASE neurons appeared to be intrinsically CO₂ sensitive because Ca²⁺ responses were intact in unc-13 mutants (Figures 4G and S1D).

In summary, ASEL and ASER both respond to CO₂ by a slow rise in Ca²⁺ that persists while CO₂ is high and returns to baseline when CO₂ returns to baseline.

AQR, PQR, and URX O₂-Sensing Neurons Are Weakly CO₂ Responsive

We examined whether the AQR, PQR, and URX O₂-sensing neurons (Persson et al., 2009; Zimmer et al., 2009) respond to CO₂ because our tax-2 rescue data indicated that these neurons contribute, albeit weakly, to CO₂ avoidance. Average Ca²⁺ traces indicated that unlike AFD, BAG, and ASE, none of these neurons respond reliably to CO₂ (Figures S2A–S2D). URX most consistently showed CO₂-evoked activity, and this was retained in unc-13 mutants (Figures S2A, S2E, and S2F). AQR and PQR occasionally showed a Ca²⁺ rise associated with an increase in CO₂ but also showed apparent spontaneous activity that lay out of synchrony with the CO₂ stimulus (Figures S2B–S2D). The response of PQR to a 0%-3%-0%-3% CO₂ stimulus was dwarfed by its response to a 21%-11%-21%-11% O₂ stimulus (Figure S2C).

Having identified three C. elegans neuron classes that responded strongly to CO₂ and a further three that responded weakly to CO₂, we considered the possibility that all sensory neurons show some CO₂ responsiveness. Therefore, we imaged
**CO₂ Sensitivity in C. elegans**

Ca²⁺ responses to CO₂ in the ASH neurons that respond to various aversive stimuli (Hilliard et al., 2005). ASH showed no response to 3% CO₂ (Figure 4H). This suggests that AFD, BAG, and ASE are functionally specialized as CO₂ sensors.

**CO₂ Sensitivity in BAG and AFD Requires a cGMP-Gated Ion Channel**

Our tax-2 rescue data suggested that CO₂ sensing in BAG and AFD neurons involves cGMP signaling. To examine this further we imaged BAG responses to CO₂ in tax-2(p694) and tax-4 (null) mutants. Both mutations completely abolished CO₂-evoked Ca²⁺ responses in BAG (Figures 5A and 5C). This suggests that BAG CO₂ sensory transduction is mediated by TAX-2/TAX-4 cGMP-gated channels and by extension, upstream guanylate cyclases (gcys).

The only gcys genes known to be expressed in BAG are the atypical soluble guanylate cyclases gcy-31 and gcy-33 (Yu et al., 1997; Zimmer et al., 2009; Ortiz et al., 2006). These appear to be O₂ regulated (Gray et al., 2004; Boon and Marletta, 2005) because both are required for BAG O₂ responses (Zimmer et al., 2009). To examine if GCY-31, GCY-33, or both are required in CO₂ sensory transduction, we imaged BAG responses to 3% CO₂ in gcy-31; gcy-33 double-deletion mutants. Loss of gcy-31 and gcy-33 reduced the CO₂-evoked BAG Ca²⁺ response (Figures 5B and 5C). This suggests that GCY-31 and/or GCY-33 forms part of the CO₂ sensory system in BAG, although other molecules are likely to be involved.

We next imaged AFD responses in tax-2(null) and tax-2(p694) animals. Expression from the gcy-8 promoter is markedly reduced in tax-2 and tax-4 mutants (Satterlee et al., 2004), and YC3.60 expression was correspondingly low in AFD in tax-2(ot25null) animals. In contrast, expression in tax-2(p694) animals was similar to wild-type (data not shown). Both tax-2 mutations significantly reduced the AFD CO₂ response, but neither completely abolished it (Figures 5D–5F). The AFD ON-minimum appeared to be absent in both tax-2 mutants, whereas the AFD
mutants had a strong CO\textsubscript{2} avoidance defect off food, and been removed by laser ablation (Mori and Ohshima, 1995).

**C. elegans Carbonic Anhydrases Are Expressed in Several Neurons, Including BAG**

To further investigate molecular mechanisms of CO\textsubscript{2} sensing, we asked whether *C. elegans* CO\textsubscript{2} sensors express carbonic anhydrases, hallmarks of CO\textsubscript{2}-responsive neurons in other animals (Hu et al., 2007; Wang et al., 2002; Riederstrale and Hanson, 1985; Coates et al., 1998). Database searches indicate that the *C. elegans* genome encodes eight predicted carbonic anhydrases. Six, *cah-1* to *cah-6*, belong to the alpha family, and two, *bca-1* and *bca-2*, to the beta family. Because many members of the beta family are mitochondrial (Syrjänen et al., 2010; Faseas et al., 2010), we focused our studies on the alpha family. We fused upstream promoter regions of each gene to *gfp* and examined the resulting expression patterns. We found that *cah-1, 2, 3, and 6* show strong neuronal expression in adults (Figure S3A). *cah-4* was primarily expressed in the hypodermis (excluding the seam cells) and in the excretory cell, consistent with a kidney-like function for this cell. *cah-3* and *cah-5* show expression in intestinal cells, with *cah-3* expression being especially strong. Using a *pBAG::mCherry* marker, we showed that *cah-2*, but not apparently any of the other five *cah* genes, was expressed in BAG (Figure S3B). *cah-2* was also expressed in a set of four quadrant head neurons, other unidentified head neurons, the canal neurons CANL/R, whose processes run parallel to the tracts of the excretory cell, and a pair of tail neurons (Figure S5). Previous data suggest that *cah-2* is also expressed in AFD (Colosimo et al., 2004). These data suggest that BAG and AFD neurons are specialized CO\textsubscript{2} sensors that coexpress carbonic anhydrases and CO\textsubscript{2}-regulated cGMP pathways. They also raise the possibility that other *C. elegans* neurons and tissues respond to CO\textsubscript{2}.

**AFD and BAG Direct Avoidance Behavior in Spatial CO\textsubscript{2} Gradients**

To investigate how CO\textsubscript{2} sensors contribute to avoidance in spatial gradients, we genetically ablated neurons. We focused on AFD and BAG neurons because the Ca\textsuperscript{2+} responses of ASE to CO\textsubscript{2} stimuli were slow, and those of AQR, PQR, and URX, weak. Specification of the AFD neurons requires the *otd/Otx* homeodomain transcription factor *txt-1*, which is expressed only in AFD (Satterlee et al., 2001). *txt-1* mutants show thermotactic defects equivalent to those of animals in which AFD has been removed by laser ablation (Mori and Ohshima, 1995). *txt-1* mutants had a strong CO\textsubscript{2} avoidance defect off food, and a weaker defect on food (Figure 5G).

AFD and BAG correlated with suppression of reversals and omega turns. The timing of CO\textsubscript{2}-evoked Ca\textsuperscript{2+} responses in both AFD and BAG correlated with peaks in locomotory activity (Figure 6A). We investigated these correlations directly by ablating AFD and/or BAG and examining behavioral responses (Figure 6B). For statistical comparison, we chose time intervals before and after gas switches according to the occurrence of peaks in wild-type behavioral rates. In the absence of food, neither AFD nor BAG ablation abolished modulation of speed across shifts in CO\textsubscript{2} (Figures 6B and S4). Stronger phenotypes were observed for reversal and omega rates (Figure 6B). Unexpectedly, ablation of AFD increased reversal and omega rates following a sharp

**AFD and BAG Control Discrete Aspects of the *C. elegans* Response to CO\textsubscript{2}**

In 5%-0% CO\textsubscript{2} spatial gradients (Figure 1), a *C. elegans* moving at ~0.3 mm/s experiences a change of 0%-0.05% CO\textsubscript{2}/s, depending on bearing relative to the gradient. In our Ca\textsuperscript{2+}-imaging experiments, immobilized animals experienced much sharper temporal gradients of ~1% CO\textsubscript{2}/s. In the wild, animals are likely to encounter a variety of CO\textsubscript{2} gradients. To analyze behavioral responses to sharp CO\textsubscript{2} gradients, we designed a square-shaped microfluidic chamber that enables CO\textsubscript{2} levels over freely moving animals to be switched rapidly (Movie S1 available online). We recorded responses and used custom software to extract instantaneous speed, reversal rate, and rate of omega turns, in which an animal’s head and tail touch to form an “L” shape (N2, Figure 6B). In the absence of food, a rise in CO\textsubscript{2} from 0% to 5% elicited a brief slowing followed by a transient increase in reversals and omega turns (Figure 6B). A rapid drop in CO\textsubscript{2}, from 5% to 0%, elicited an acceleration that coincided with suppression of reversals and omega turns.

To investigate how CO\textsubscript{2} sensors contribute to avoidance in spatial gradients, we genetically ablated neurons. We focused on AFD and BAG neurons because the Ca\textsuperscript{2+} responses of ASE to CO\textsubscript{2} stimuli were slow, and those of AQR, PQR, and URX, weak. Specification of the AFD neurons requires the *otd/Otx* homeodomain transcription factor *txt-1*, which is expressed only in AFD (Satterlee et al., 2001). *txt-1* mutants show thermotactic defects equivalent to those of animals in which AFD has been removed by laser ablation (Mori and Ohshima, 1995). *txt-1* mutants had a strong CO\textsubscript{2} avoidance defect off food, and a weaker defect on food (Figure 5G). Wild-type avoidance was restored to *txt-1* mutants by a transgene containing *txt-1* genomic DNA (Figure 5G). These data suggest that the AFD neurons promote CO\textsubscript{2} avoidance in spatial CO\textsubscript{2} gradients.

To ablate BAG we expressed the *egl-1* programmed cell death activator from a BAG-specific *gcy-33* promoter (Conradt and Horvitz, 1998; Yu et al., 1997) (we thank M. Beverly and P. Sengupta for this line). Both BAGL and BAGR neurons were absent in greater than 90% of animals bearing this transgene (Table S1 available online). Surprisingly, the CO\textsubscript{2} avoidance of BAG-ablated animals was not significantly different from wild-type, both on and off food (Figure 5G). We asked if combined genetic ablation of AFD and BAG causes a synthetic CO\textsubscript{2} avoidance phenotype. Ablating the BAG neurons disrupted the residual CO\textsubscript{2} avoidance of *txt-1(p767)* mutants on food (Figure 5G). However, in the absence of food, *txt-1(p767); gpcy-33::egl-1* animals showed no greater defect than *txt-1(p767)* single mutants (Figure 5G). These data show that AFD and BAG promote CO\textsubscript{2} avoidance in spatial gradients on food, and that AFD and at least one other neuron that is not BAG promote avoidance when food is absent. Thus, the importance of different sensory neurons for CO\textsubscript{2} avoidance in spatial gradients depends on context.

(A) AFD and BAG CO\textsubscript{2}-evoked neuronal events correlate with CO\textsubscript{2}-evoked behavioral events. Behavioral plots reproduced from (B). (B) Average speed, reversal, and omega rates of wild-type (N2). AFD-ablated (*txt-1*), BAG-ablated (pgcy-33::egl-1), and AFD-ablated BAG-ablated (*txt-1; pgcy-33::egl-1*) animals off food across a 0%-5%-0% CO\textsubscript{2} stimulus. Stimulus bar and light blue shading indicate the timing of gas switches. Gray shading indicates SEM. Speed (μm/s, black line) calculated in 3 s bins. Reversal (orange line) and omega rates (maroon line) are in event initiations per animal per minute calculated in 6 s bins. N2, n = 59 movies; *txt-1(p767), n = 20 movies; pgcy-33::egl-1, n = 15 movies; *txt-1, pgcy-33::egl-1*, n = 16 movies.
CO₂ rise (ttx-1, Figures 6B, 7B, 7C, 7H, and 7I) and reduced suppression of omega turns following a CO₂ fall (ttx-1, Figures 6B, 7K, and 7L), suggesting that AFD acts to suppress reversals and omega turns at these two time points. Ablation of BAG abolished reversal and omega responses to a rise in CO₂ (pBAG::egl-1, Figures 6B, 7B, 7C, 7H, and 7I) and reduced the suppression of omega turns following a CO₂ fall (pBAG::egl-1, Figures 6B, 7B, 7C, 7H, and 7I), consistent with BAG excitation promoting reversals and omega turns. Coablation of AFD and BAG abolished the suppression of reversals and omega turns following a fall in CO₂ (ttx-1; pBAG::egl-1, Figures 7F and 7L). This effect was due to reduced reversal and omega rates under prolonged

Figure 7. AFD and BAG Together Promote Turning When CO₂ Levels Rise and Inhibit Turning When CO₂ Levels Fall

(A–L) Statistical analysis of reversal and omega turns of wild-type and ablated animals during 0%-5% CO₂ increases and 5%-0% CO₂ decreases. Average behavioral traces are shown at left, time-averaged behavioral rates before and after gas switches are shown at middle, and average changes in behavioral rates are shown at right. Rates are in initiations of reversals or omega events per animal per minute. (A, D, G, and J) Average reversal and omega rates during 0%-5% CO₂ and 5%-0% CO₂ gas switches. Error bars omitted for clarity. (B, E, H, and K) Time-averaged reversal (B and E) and omega (H and K) rates before (red bars) and after (dark gray bars) an increase (B and H) or a decrease (E and K) in percent (%) CO₂. Intervals for comparison coincide with stationary points in wild-type behavioral rates. Error bars indicate SEM. (C, F, I, and L) Average change in reversal (C and F) and omega (I and L) rates across an increase (C and I) or a decrease (F and L) in percent (%) CO₂. Difference calculations based on data in (B), (E), (H), and (K), and error bars calculated from SEM values in (B), (E), (H), and (K) using error propagation formulae. Significance markers indicate comparisons against wild-type, unless otherwise indicated.
DISCUSSION

The AFD, BAG, and ASE Sensory Neurons Exhibit Distinct CO2 Responses

C. elegans, like mammals, monitors CO2 using multiple neuron types. CO2 sensors include the ASE neurons with sensory endings directly exposed to the external environment and AFD and BAG neurons whose dendrites lie within the animal. All three neuron types are primary CO2 sensors: their CO2 responses are unimpaired in unc-13 mutants defective in synaptic release. Each neuron type has a unique CO2 response. In AFD, a rise in CO2 triggers an initial drop in intracellular Ca2+ levels (AFD ON-minimum), then a rise above baseline (AFD ON-maximum), and when CO2 is removed, a spike (AFD OFF-maximum). This complexity may reflect multiple CO2-transduction mechanisms. In contrast, BAG and ASE neurons are activated by a rise, but not a fall, in CO2. In BAG, Ca2+ peaks within 60 s of a rise in CO2, then decays to a plateau that persists as long as CO2 remains high; Ca2+ drops back to baseline upon CO2 removal. ASE responds slowly to CO2 exposure: Ca2+ takes 2 min to peak but remains elevated while CO2 is high. The tonic activity of BAG and ASE neurons in high CO2 may allow C. elegans to modify responses to other cues, perhaps by affecting sensory pathways or inter-neuron networks.

AFD, BAG, and ASE also sense other stimuli. AFD senses temperature (Kimura et al., 2004), BAG senses ambient O2 (Zimmer et al., 2009), and ASE senses salt (Suzuki et al., 2008). This may enable sensory integration within sensory neurons. For each of the three neurons, CO2 and non-CO2 stimuli evoke distinct Ca2+ responses. When temperature rises above the cultivation level, AFD responds with a monophasic Ca2+ spike that lasts a few seconds (Kimura et al., 2004; Clark et al., 2007). The dissimilar Ca2+ and temperature responses suggest that the two stimuli are sensed differently. Supporting this, AFD responds to CO2 below the cultivation temperature. The Ca2+ responses of BAG to high CO2 and low O2 are more similar in shape (Figure 3) (Zimmer et al., 2009). In contrast, the responses of ASE to CO2 and NaCl differ markedly (Figure 4) (Suzuki et al., 2008). First, unlike CO2, NaCl evokes an asymmetric response in ASE and ASER: a rise in NaCl triggers a Ca2+ spike in ASE but a drop in Ca2+ in ASER. Second, ASE/L/R Ca2+ responses to NaCl adapt rapidly, whereas sustained CO2 stimulation leads to sustained high Ca2+ in ASE (Figure 4F). Third, whereas ASE responses to CO2 are slow, taking around 2 min for Ca2+ to peak, responses to NaCl peak within 30 s of stimulus exposure. The slowness of ASE CO2 responses could reflect rate-limiting hydration of environmental CO2.

cGMP Signaling Mediates CO2 Responses

CO2 sensing in AFD, BAG, and ASE involves cGMP signaling. Mutating the cGMP-gated channel subunit tax-2 partially abolishes the AFD Ca2+ response to CO2 and completely abolishes CO2-evoked activity in BAG (Figure 5). CO2-evoked Ca2+ responses in ASE likely also depend on cGMP-gated channels because expression of tax-2 cDNA in ASE in tax-2 mutants partially restores CO2 avoidance (Figure 1). In mouse olfactory epithelia, CO2 sensing requires the transmembrane guanylate cyclase GC-D, which is activated by HCO3- (Hu et al., 2007;
Sun et al., 2009). The hallmarks that make GC-D HCO$_3^-$ regulated are unknown, but the *C. elegans* genome encodes 27 transmembrane guanylate cyclase (gcy), a subset of which could be similarly regulated (Yu et al., 1997; Ortiz et al., 2006). The AFD neurons express gcy-8, gcy-18, gcy-23, and gcy-29. gcy-18 gcy-23 triple mutants have a thermotaxis defect similar to that of the AFD specification mutant tax-1 (Inada et al., 2006), but have no defect in CO$_2$ avoidance in a 5%-0% CO$_2$ gradient (data not shown). ASE neurons express 11 transmembrane guanylate cyclases, nine of which are expressed asymmetrically either in ASEL or ASER (Ortiz et al., 2006).

Transmembrane guanylate cyclase expression has not been reported in BAG. However, BAG expresses the atypical soluble guanylate cyclases GCY-31 and GCY-33 (Yu et al., 1997). Simultaneously disrupting gcy-31 and gcy-33 reduced the CO$_2$-evoked Ca$^{2+}$ response amplitudes in BAG, suggesting that GCY-31 and/or GCY-33 contribute to CO$_2$ sensing. GCY-31 and GCY-33 are thought to function as heterodimers that have an O$_2$-binding heme cofactor (Boon and Marletta, 2005) and are required for BAG O$_2$-evoked Ca$^{2+}$ responses when O$_2$ drops below 10% (Zimmer et al., 2009). An intriguing possibility is that the GCY-31/GCY-33 heterodimer is inhibited by O$_2$ and activated by CO$_2$, making it a sensory integrator of CO$_2$ and O$_2$ signals in BAG (Figure 8A); however, we cannot rule out the possibility of a linked mutation disrupting BAG responses.

AFD, BAG, and ASE are unlikely to be the only CO$_2$-responsive neurons in *C. elegans*. The AQR, POR, and URX O$_2$-sensing neurons showed sporadic responses to CO$_2$ (Figure S2), and selective expression of tax-2 cDNA in these neurons partially restored CO$_2$ avoidance to tax-2(p694) mutants, suggesting that they are CO$_2$ sensitive. Moreover, more than ten *C. elegans* neurons express carbonic anhydrases, some of which may be unidentified CO$_2$ sensors.

**The Contribution of Different Sensors to CO$_2$ Avoidance Varies with Stimulus Dynamics and Context**

Why does *C. elegans* have multiple CO$_2$ sensors? One reason is that sensors are deployed differently according to the dynamics of the CO$_2$ stimulus. For example, when food is absent, BAG mediates responses to sharp CO$_2$ gradients but is less important for navigating shallow gradients (compare Figures 5G and 6B). A second reason is that context modifies the behavioral changes needed to escape CO$_2$. For example, when food is present, *C. elegans* move slowly and reverse frequently. To efficiently escape high CO$_2$ in a food-containing environment, *C. elegans* increase speed and suppress reversals relative to the “on food” ground state. By contrast when food is absent, animals are already moving quickly and reversing less frequently. Correspondingly, the importance of BAG for CO$_2$ avoidance depends on both stimulus shape and food context. Whereas BAG-ablated animals respond poorly to rapid CO$_2$ changes when food is present, they respond like wild-type animals when food is absent (pBAG:egl-1, Figures 6 and S6). Conversely, in shallow gradients BAG acts redundantly with AFD to promote CO$_2$ avoidance when food is present but is not important when food is absent, even when AFD is ablated (Figure 5G).

How do the Ca$^{2+}$ responses of CO$_2$ sensory neurons encode behavior? CO$_2$-evoked neuronal events in AFD and BAG correlate with peaks and troughs in locomotory rates (Figure 6A). To investigate these relationships, we ablated CO$_2$ sensors. One caveat of neuronal ablation is that it can only remove a neuron in its entirety, and not individual components of its responses. Ablation of AFD and BAG neurons one at a time and together suggests that: (1) BAG activation and the AFD ON-maximum act antagonistically, promoting and suppressing reversal and omega rates, respectively (Figures 7C and 7I); (2) BAG plateau activity and the AFD ON-maximum both act to promote reversal and omega rates during maintained high CO$_2$ (ttx-1; BAG(-), Figures 7E and 7K); and (3) decay of BAG activity and the AFD OFF-maximum act together to suppress reversals and omega turns following CO$_2$ removal (ttx-1; BAG(-), Figures 7F and 7L). Together our data suggest that when an animal is migrating up a CO$_2$ gradient, BAG and AFD trigger turning, whereas when
an animal is migrating down a CO2 gradient, AFD and BAG suppress turning (Figure 8B). Therefore, it appears that the three different components of the AFD CO2 response may differentially regulate behavior (1, 2, 3, AFD, Figure 8B). Because AFD(−) BAG (−) animals still respond to CO2, we also infer the existence of an additional sensory neuron, XYZ, that is neither ASE nor AQR, PQR, URX, that promotes turning when CO2 rises (Figure 8B).

CO2 Avoidance Behavior in C. elegans Appears to Be a Homeostatic Mechanism
Elevated tissue CO2 is toxic (Richerson, 2004). In C. elegans, CO2 levels exceeding 9% disrupt body muscle organization and general development and reduce fertility (Sharabi et al., 2009). The CO2 responses of AFD, BAG, and ASE neurons do not habituate upon multiple exposures to CO2 (Figures 2 and 3; data not shown). C. elegans CO2 avoidance in spatial gradients is also nonhabituating over a similar period (data not shown). By contrast, C. elegans attraction to benzaldehyde (L’Etoile et al., 2002), response to noxious Cu2+ ion stimuli (Hilliard et al., 2005), and response to nose touch (Kindt et al., 2007) all habituate. Moreover, BAG and ASE neurons show tonic signaling while CO2 levels are high, at least over 20 min. We speculate that C. elegans CO2 avoidance habituates slowly and performs a homeostatic function by preventing CO2 poisoning of body tissues. C. elegans CO2 avoidance provides an opportunity for detailed examination of a CO2 homeostatic system with comparative ease relative to the systems of more complex animals.

EXPERIMENTAL PROCEDURES

Strains
Strains were grown at 22°C under standard conditions (Brenner, 1974). Mutant combinations were made by following visible phenotypes or using PCR to confirm genotype. A full list of strains can be found in Supplemental Experimental Procedures.

Behavioral Assays
Spatial CO2 gradient assays were as described (Bretscher et al., 2008). Briefly, polydimethylsiloxane (PDMS) chambers connected to gas syringe pumps were placed over adult worms on a 9 cm agar plate. After 10 min the distribution of worms was used to calculate a chemotaxis index (Figure 1). Chemotaxis bar graphs represent the average of nine independent assays performed over 3 days.

For temporal gradient assays a square 11 x 11 x 0.2 mm PDMS chamber was placed over adult worms on 6 cm agar plates. For off-food assays, ~40 animals were picked after washing in M9 Buffer to remove adhering E. coli. For on-food assays, a 2-day-old 20 µl E. coli lawn was used. Worms were allowed to crawl on food for 1 hr. After placing the chamber, animals were left for 4 min before exposure to a 0%-5%-0% CO2 stimulus. Behavior was captured using a Grasshopper CCD camera (Point Grey Research). A TTL-output from a frame counter (custom built) controlled opening and closing of Teflon™ pinch valves (Automate Scientific) at defined time points, controlling the switching of gases. Worms were tracked using DIAS Software (Solltech), and worm object paths were created. The centroid X and Y coordinates, maximum length, mean width, perimeter, and roundness were extracted for each worm object across frames. From these parameters, speed, omega initiation rate, and reversal initiation rate were calculated using a custom-written program in MATLAB (The MathWorks). Omega turns were detected by circular object topologies. This method gave 90.9% success using the stringent criterion that worm head touches worm tail. Reversal events were defined as forward movement (F), followed by backward movement (B), followed by return to forward movement (F). Using the criterion of an F-B-F event and optimized parameters minimum allowable reversal angle (150°), maximum reversal duration (7.5 s), and minimum reversal distance (0.3 mm, life size), reversal detection success rate ran at 81.25%. Detection parameters were optimized by minimizing the sum of the squared differences between detection outputs of computer and a human observer for Movie S1. Behavior occurring during merger of worm objects was discarded. Temporal gradient assay data represent the average of 16 or more movies for off food and nine or more for on food.

In all experiments, percent (%) CO2 was balanced by percent (%) N2 while 21% O2 was maintained. In rescue experiments, transgenic animals were preselected by following conjection markers. In all figures, statistical significance was determined using the two-tailed Student’s t test.

Calcium Imaging
Ca2+ imaging was on an inverted microscope (Axiovert; Zeiss), using a 40x C-Apochromat lens and MetaMorph acquisition software (Molecular Devices). Agarose pads were made in M9 Buffer (pH 6.8) and 1 mM CaCl2, mimicking an NGM substrate. Worms expressing the Ca2+ sensor YC3.60 showed wild-type avoidance in 5%-0% CO2 gradients (Figure S1). Worms were glued to pads using Nexaband glue (WPI Inc.) and placed under the stem of the Y-chamber microfluidic device. Photobleaching was minimized using a 2.0 optical density filter and a shutter to limit exposure time to 100 ms per frame. An excitation filter (Chroma) restricted illumination to the cyan channel. A beam splitter (Optical Insights) was used to separate the cyan and yellow emission light. The ratio of the background-subtracted fluorescence in the YFP and CFP channels was calculated with Jmalyze (Kerr and Schaffer, 2008). Fluorescence ratio (YFP/CFP) plots were made in MATLAB. Movies were captured at 2 fps. Average Ca2+ traces were compiled from at least six recordings made on 2 or more days.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, one table, and two movies and can be found with this article online at doi:10.1016/j.neuron.2011.02.023.

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REFERENCES


