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

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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. 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%-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 sensors. 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; Hedgcock 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 polyclonistropic 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 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 CO₂-sensing are distinct, AFD might be expected to respond to CO₂ 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 CO₂ 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 CO₂ and temperature-sensing pathways are at least partly distinct.

**The BAG O₂ Sensory Neurons**

**Sense CO₂**

Recent work has shown that the BAG neurons are transiently activated when O₂ 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% CO₂/10% O₂ mixture. We have previously shown that O₂ responses can modulate CO₂ avoidance (Bretscher et al., 2008). These data suggest that either BAG responds exclusively to O₂ but modulates neural circuits mediating CO₂ responses or that BAG is a primary sensor of both O₂ and CO₂.

To test BAG neuron CO₂ sensitivity, we created animals expressing cameleon YC3.60 in BAG from a pflp-17::YC3.60 transgene and imaged Ca²⁺ levels. The BAGL and BAGR neurons were exquisitely sensitive to a rise in CO₂ (Figures 3A–3C). Cameleon reported a rise in Ca²⁺ that peaked after ~30 s and then decayed (Figures 3A and 3B). The excitability threshold of BAG was below 0.25% CO₂. A plot of mean fluorescence ratio
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 (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).

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 Ca²⁺ responses to CO₂, using animals expressing the Ca²⁺ 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% CO₂ (Figures 3A–C). The BAG neurons exhibit a large “CO₂-ON” response. Mean BAG responses to 0%-x%-0%-x% CO₂ stimuli for x = 0.25%, 1%, 3%, 5%, or 10% CO₂. Shown are the full response (A), a 60 s interval across CO₂ introduction (B), and a 180 s interval across CO₂ removal (C) (n = 10 or more traces for all concentrations).

(D) Dose–response curve for the BAG CO₂ response. The mean fluorescence ratio change, ΔRF/R₀, is plotted as a percentage of the mean baseline fluorescence ratio, R₀. ΔRF = R₁ – R₀. R₁ was calculated from the peak of the BAG Ca²⁺ response at 200–260 s and R₀ from 120–180 s. Curve fit of the standard equation for a single-site binding process (Michaelis-Menten, y = Vmaxx/(Kd + x), where Vmax and Kd are constants with units of [% mean ratio change] and % CO₂, respectively) to the red data points using least-squares regression analysis. The blue data point (10% CO₂) was omitted from the curve fit because at 10% CO₂ the BAG fluorescence ratio (YFP/CFP) falls outside of the linear dynamic range of the Ca²⁺ sensor YC3.60. Curve fit gives Kd = 2.9% CO₂ and Vmax = 166% mean ΔRF/R₀, with a goodness of fit R² regression value of 0.995. Error bars indicate SEM.

(E) Mean BAG response to a 3% CO₂ stimulus lasting 18 min (n = 9 traces).

(F–H) BAG responses to a 0%-3%-0%-3%-0%-3%-0%-3%-0%-3%-0% CO₂ stimulus. (F) Mean fluorescence ratio (YFP/CFP), (G) mean percent (% ΔRF/R₀), and individual BAG Ca²⁺ traces plotted in a heat map (H) (n = 6 traces).

(I–K) Mean BAG Ca²⁺ responses in unc-13 mutants (I) and unc-31 mutants (J). (K) Mean percent (% ΔRF/R₀) values for (I) and (J). Asterisks indicate significance compared to wild-type.

Figure 3. The BAG Neurons Are Highly Sensitive to CO₂

(A–C) The BAG neurons exhibit a large “CO₂-ON” response. Mean BAG responses to 0%-x%-0%-x% CO₂ stimuli for x = 0.25%, 1%, 3%, 5%, or 10% CO₂. Shown are the full response (A), a 60 s interval across CO₂ introduction (B), and a 180 s interval across CO₂ removal (C) (n = 10 or more traces for all concentrations).

(D) Dose–response curve for the BAG CO₂ response. The mean fluorescence ratio change, ΔRF, is plotted as a percentage of the mean baseline fluorescence ratio, R₀. ΔRF = RF₁ – RF₀. RF₁ was calculated from the peak of the BAG Ca²⁺ response at 200–260 s and RF₀ from 120–180 s. Curve fit of the standard equation for a single-site binding process (Michaelis-Menten, y = Vmaxx/(Kd + x), where Vmax and Kd are constants with units of [% mean ratio change] and % CO₂, respectively) to the red data points using least-squares regression analysis. The blue data point (10% CO₂) was omitted from the curve fit because at 10% CO₂ the BAG fluorescence ratio (YFP/CFP) falls outside of the linear dynamic range of the Ca²⁺ sensor YC3.60. Curve fit gives Kd = 2.9% CO₂ and Vmax = 166% mean ΔRF/R₀, with a goodness of fit R² regression value of 0.995. Error bars indicate SEM.

(E) Mean BAG response to a 3% CO₂ stimulus lasting 18 min (n = 9 traces).

(F–H) BAG responses to a 0%-3%-0%-3%-0%-3%-0%-3%-0%-3%-0% CO₂ stimulus. (F) Mean fluorescence ratio (YFP/CFP), (G) mean percent (% ΔRF/R₀), and individual BAG Ca²⁺ traces plotted in a heat map (H) (n = 6 traces).

(I–K) Mean BAG Ca²⁺ responses in unc-13 mutants (I) and unc-31 mutants (J). (K) Mean percent (% ΔRF/R₀) values for (I) and (J). Asterisks indicate significance compared to wild-type.
Although the responses of ASEL were generally ~2-fold larger than those of ASER (Figure 4E), ASE responses to CO\textsubscript{2} were slow, taking around 2 min for Ca\textsuperscript{2+} levels to peak (Figure 4F). Sustained elevated CO\textsubscript{2} led to sustained increases in Ca\textsuperscript{2+} (Figure 4F). As for AFD and BAG, ASE neurons appeared to be intrinsically CO\textsubscript{2} sensitive because Ca\textsuperscript{2+} responses were intact in unc-13 mutants (Figures 4G and S1D).

In summary, ASEL and ASER both respond to CO\textsubscript{2} by a slow rise in Ca\textsuperscript{2+} that persists while CO\textsubscript{2} is high and returns to baseline when CO\textsubscript{2} returns to baseline.

**AQR, PQR, and URX O\textsubscript{2}-Sensing Neurons Are Weakly CO\textsubscript{2} Responsive**

We examined whether the AQR, PQR, and URX O\textsubscript{2}-sensing neurons (Persson et al., 2009; Zimmer et al., 2009) respond to CO\textsubscript{2} because our tax-2 rescue data indicated that these neurons contribute, albeit weakly, to CO\textsubscript{2} avoidance. Average Ca\textsuperscript{2+} traces indicated that unlike AFD, BAG, and ASE, none of these neurons respond reliably to CO\textsubscript{2} (Figures S2 A–S2D). URX most consistently showed CO\textsubscript{2}-evoked activity, and this was retained in unc-13 mutants (Figures S2 A, S2E, and S2F). AQR and PQR occasionally showed a Ca\textsuperscript{2+} rise associated with an increase in CO\textsubscript{2} but also showed apparent spontaneous activity that lay out of synchrony with the CO\textsubscript{2} stimulus (Figures S2 B–S2D). The response of PQR to a 0%-3%-0%-3% CO\textsubscript{2} stimulus was dwarfed by its response to a 21%-11%-21%-11% O\textsubscript{2} stimulus (Figure S2C).

Having identified three C. elegans neuron classes that responded strongly to CO\textsubscript{2} and a further three that responded weakly to CO\textsubscript{2}, we considered the possibility that all sensory neurons show some CO\textsubscript{2} responsiveness. Therefore, we imaged
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 known to be expressed in BAG are the atypical soluble guanylate cyclases gcyl-31 and gcyl-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 gcyl-31; gcyl-33 double-deletion mutants. Loss of gcyl-31 and gcyl-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 gcyl-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 mutaints (Satterlee et al., 2004), and YC3.60 expression was correspondingly low in AFD in tax-2 mutaints. This suggests that BAG CO₂ sensory transduction is mediated by TAX-2/TAX-4 cGMP-gated channels and by extension, upstream guanylate cyclases (gcys).

Ca²⁺ responses to CO₂ in the ASH neurons that respond to various aversive stimuli (Hillard 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 Sensing in C. elegans

A

- Food

B

\[ \text{Speed (} \mu \text{m/s)} \]

\[ \text{Time (s)} \]

\[ \text{Reversals / min} \]

\[ \text{Omegas / min} \]

\[ \text{BAG} \]

\[ \text{AFD} \]

\[ \text{N2} \]

\[ \text{tk-1} \]

\[ \text{pBAG:sgl-1} \]

\[ \text{tk-1; pBAG:sgl-1} \]
ON-maximum was absent in tax-2(null) animals but enhanced in tax-2(p694) animals (Figures 5D–5F). Our data suggest that all three components of the AFD CO2 response involve TAX-2 mediated cGMP pathways but that other pathways also contribute.

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

To further investigate molecular mechanisms of CO2 sensing, we asked whether C. elegans CO2 sensors express carbonic anhydrases, hallmarks of CO2-responsive neurons in other animals (Hu et al., 2007; Wang et al., 2002; Riddlerstrale 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 (Syriänen et al., 2010; Fasseas 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 CO2 sensors that coexpress carbonic anhydrases and CO2-regulated cGMP pathways. They also raise the possibility that other C. elegans neurons and tissues respond to CO2.

AFD and BAG Direct Avoidance Behavior in Spatial CO2 Gradients

To investigate how CO2 sensors contribute to avoidance in spatial gradients, we genetically ablated neurons. We focused on AFD and BAG neurons because the Ca2+ responses of ASE to CO2 stimuli were slow, and those of AQR, PQR, and URX, weak. Specification of the AFD neurons requires the otd/Obx homeodomain transcription factor ttx-1, which is expressed only in AFD (Satterlee et al., 2001). ttx-1 mutants show thermotactic defects equivalent to those of animals in which AFD has been removed by laser ablation (Mori and Ohshima, 1995). ttx-1 mutants had a strong CO2 avoidance defect off food, and a weaker defect on food (Figure 5G). Wild-type avoidance was restored to ttx-1 mutants by a transgene containing ttx-1 genomic DNA (Figure 5G). These data suggest that the AFD neurons promote CO2 avoidance in spatial CO2 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 CO2 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 CO2 avoidance phenotype. Ablating the BAG neurons disrupted the residual CO2 avoidance of ttx-1(p767) mutants on food (Figure 5G). However, in the absence of food, ttx-1(p767); pgcy-33::egl-1 animals showed no greater defect than ttx-1(p767) single mutants (Figure 5G). These data show that AFD and BAG promote CO2 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 CO2 avoidance in spatial gradients depends on context.

AFD and BAG Control Discrete Aspects of the C. elegans Response to CO2

In 5%-0% CO2 spatial gradients (Figure 1), a C. elegans moving at ~0.3 mm/s experiences a change of 0%-0.05% CO2/s, depending on bearing relative to the gradient. In our Ca2+-imaging experiments, immobilized animals experienced much sharper temporal gradients of ~1% CO2/s. In the wild, animals are likely to encounter a variety of CO2 gradients. To analyze behavioral responses to sharp CO2 gradients, we designed a square-shaped microfluidic chamber that enables CO2 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, turns in which an animal’s head and tail touch to form an “Ω” shape (N2, Figure 6B). In the absence of food, a rise in CO2 from 0% to 5% elicited a brief slowing followed by a transient increase in reversals and omega turns (Figure 6B). A rapid drop in CO2, from 5% to 0%, elicited an acceleration that coincided with suppression of reversals and omega turns.

The timing of CO2-evoked Ca2+ 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 CO2 (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

Figure 6. AFD and BAG Control Behavioral Responses to Changes in Percent (%) CO2

(A) AFD and BAG CO2-evoked neuronal events correlate with CO2-evoked behavioral events. Behavioral plots reproduced from (B). (B) Average speed, reversal, and omega rates of wild-type (N2). AFD-ablated (ttx-1), BAG-ablated (pgcy-33::egl-1), and AFD-ablated BAG-ablated (ttx-1; pgcy-33::egl-1) animals off food across a 0%-5%-0% CO2 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; ttx-1(p767), n = 20 movies; pgcy-33::egl-1, n = 15 movies; ttx-1, pgcy-33::egl-1, n = 16 movies.
CO₂ rise (\textit{ttx-1}, Figures 6B, 7C, 7H, and 7I) and reduced suppression of omega turns following a CO₂ fall (\textit{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 (\textit{pBAG::egl-1}, Figures 6B, 7C, 7H, and 7I) and reduced the suppression of omega turns following a CO₂ fall (\textit{pBAG::egl-1}, Figures 6B, 7K, and 7L), 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 (\textit{ttx-1}; \textit{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 I) 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.
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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 CO₂ 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/ASER 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;
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Sun et al., 2009). The hallmarks that make GC-D HCO3− 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 ttx-1 (Inada et al., 2006), but have no defect in CO2 avoidance in a 5%-0% CO2 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 CO2-evoked Ca2+ response amplitudes in BAG, suggesting that GCY-31 and/or GCY-33 contribute to CO2 sensing. GCY-31 and GCY-33 are thought to function as heterodimers that have an O2-binding heme cofactor (Boon and Marletta, 2005) and are required for BAG O2-evoked Ca2+ responses when O2 drops below 10% (Zimmer et al., 2009). An intriguing possibility is that the GCY-31-GCY-33 heterodimer is inhibited by O2 and activated by CO2, making it a sensory integrator of CO2 and O2 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 CO2-responsive neurons in C. elegans. The AQR, PQR, and URX O2-sensing neurons showed sporadic responses to CO2 (Figure S2), and selective expression of tax-2 cDNA in these neurons partially restored CO2 avoidance to tax-2(p694) mutants, suggesting that they are CO2 sensitive. Moreover, more than ten C. elegans neurons express carbonic anhydrases, some of which may be unidentified CO2 sensors.

The Contribution of Different Sensors to CO2 Avoidance Varies with Stimulus Dynamics and Context

Why does C. elegans have multiple CO2 sensors? One reason is that sensors are deployed differently according to the dynamics of the CO2 stimulus. For example, when food is absent, BAG mediates responses to sharp CO2 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 CO2. For example, when food is present, C. elegans moves slowly and reverses frequently. To efficiently escape high CO2 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 CO2 avoidance depends on both stimulus shape and food context. Whereas BAG-ablated animals respond poorly to rapid CO2 changes when food is present (pBAG::egl-1, Figures 6 and 56). Conversely, in shallow gradients BAG acts redundantly with AFD to promote CO2 avoidance when food is present but is not important when food is absent, even when AFD is ablated (Figure 5G).

How do the Ca2+ responses of CO2 sensory neurons encode behavior? CO2-evoked neuronal events in AFD and BAG correlate with peaks and troughs in locomotory rates (Figure 6A). To investigate these relationships, we ablated CO2 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 CO2 (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 CO2 removal (ttx-1; BAG(-), Figures 7F and 7L). Together our data suggest that when an animal is migrating up a CO2 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 × 11 × 0.2 mm PDMS chamber was placed over adult worms on 6 cm agar plates. For off-foad assays, ~40 animals were picked after washing in M9 Buffer to remove adhering E. coli. For on-foad 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 (Soltech), 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 pre-selected by following coinjection 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 Schafer, 2006). 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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