

Minireview

Olfactory Carbon Dioxide Detection by Insects and Other Animals

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Carbon dioxide is a small, relatively inert, but highly volatile gas that not only gives beer its bubbles, but that also acts as one of the primary driving forces of anthropogenic climate change. While beer brewers experiment with the effects of CO₂ on flavor and climate scientists are concerned with global changes to ambient CO₂ levels that take place over the course of decades, many animal species are keenly aware of changes in CO₂ concentration that occur much more rapidly and on a much more local scale. Although imperceptible to us, these small changes in CO₂ concentration can indicate imminent danger, signal overcrowding, and point the way to food. Here I review several of these CO₂-evoked behaviors and compare the systems insects, nematodes, and vertebrates use to detect environmental CO₂.

CO₂-EVOKED BEHAVIORS

Carbon dioxide is a byproduct of cellular respiration, which means animals constantly release it into the environment as waste. Plants, on the other hand, take up CO₂ from the environment and fix its carbon atoms as carbohydrates via photosynthesis, acting as CO₂ sinks. Thus, a given CO₂ concentration encountered in the environment can have vastly different behavioral relevance to animal species depending on their ecological niche and location.

Social insects like bees, ants, and termites encounter ambient CO₂ concentrations far above the atmospheric concentration of 0.038% (380 ppm) because their societies consist of many individuals living together in an enclosed space where CO₂ can accumulate. CO₂ concentrations inside bee hives have been measured at over 4% (40,000 ppm) (Seeley, 1974) and levels in termite nests range between 0.3–15% (3,000–150,000 ppm) (Ziesmann, 1996). Ants take advantage of this effect when they are outside their nests and use CO₂ seeping from the nest entrance to find their way home (Buehlmann et al., 2012). Inside the nest, however, the same stimulus means something very different. Elevated CO₂ levels can suffocate the nest's inhabitants and, in the case of the fungus-farming leaf cutter ants, threaten the food supply. Thus, to prevent CO₂ levels from getting dangerously high, social insects have evolved

sensory equipment to track ambient CO₂ levels and behaviors that prevent CO₂ accumulation in their nests. For both honeybees and bumblebees, rising CO₂ levels recruit worker bees to the entrances and exits of the hive to initiate a wing-fanning response that pushes fresh air through the hive until CO₂ levels return to normal (Seeley, 1974; Weidenmüller et al., 2002). This behavior is not evoked via simple oxygen depletion in the hive and the number of fanning bees correlates directly with the CO₂ concentration (Seeley, 1974). Ants and termites also detect ambient CO₂ levels in their nests, but because most are wingless, they cannot rely on a fanning response like bees. Instead, ants and termites must modify their nests by opening or closing entrances and exits to optimize ventilation (Kleineidam and Roces, 2000; Ziesmann, 1996).

Unlike the social insects, which are particularly concerned with the ambient CO₂ level, many solitary insects living in open environments pay special attention to gradients of CO₂ in the form of filamentous plumes arising from individual point sources. Although CO₂ is highly diffusible, the CO₂ plumes arising from a sleeping human remain intact long enough to activate upwind search behaviors in mosquitoes from several meters away (Gillies, 1980). Nearly every medically relevant blood-feeding insect that transmits a disease-causing pathogen to humans detects and follows CO₂ gradients as part of its host-seeking behavior. In addition to the malaria mosquito *Anopheles gambiae* and the dengue/yellow fever mosquito *Aedes aegypti*, other CO₂-loving hematophagous Diptera include tsetse flies (Voskamp et al., 1999) (sleeping sickness), black flies (Fallis and Raybould, 1975) (river blindness and filariasis), and sandflies (Pinto et al., 2001) (leishmaniasis). The reduviid bug *Triatoma infestans*, which transmits the trypanosome that causes Chagas' disease, also orients upwind to pulses of CO₂ (Barrozo and Lazzari, 2006). Even ticks (Steullet and Guerin, 1992) (Lyme disease) and fleas (Benton and Lee, 1965) (bubonic plague), which seem to have independently evolved blood-feeding behavior, are attracted to the CO₂ in our breath. Since CO₂ is such a ubiquitous respiratory waste product, however, a whiff of CO₂ alone is often not enough. Living, breathing humans also provide visual targets, produce thermal gradients, and release hundreds of compounds from the skin and in the breath that all contribute to a multimodal sensory stimulus that is species-specific and irresistible to hungry female mosquitoes

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and other biting insects (Gibson and Torr, 1999).

One of the most sensitive CO₂ detection systems yet studied belongs to a moth. Using a series of pressure modulations to mimic the effect of minute changes in CO₂ concentration, Gert Stange estimated that the labial palp organ of the moth *Heliothis armigera* may be able to detect deviations from the ambient CO₂ concentration as low as 0.5 parts per million (0.00005%) (Stange, 1992). The behavioral relevance of this information to moths depends largely on the ecology of the species. The hawkmoth *Manduca sexta* uses elevations in CO₂ to guide its choice of meals because the freshest blossoms release more CO₂ and provide better nectar rewards than older flowers (Thom et al., 2004). Another moth, *Cactoblastis cactorum*, looks for decreases in CO₂ to guide its feeding on the *Opuntia* cactus, because *Opuntia* opens its stomata at night to take up CO₂, acting as a CO₂ sink (Stange et al., 1995).

Rather than detecting CO₂ as an attractant directing host-seeking behaviors, adult *Drosophila* release CO₂ as a component of a highly aversive stress pheromone (Suh et al., 2004). *Drosophila* produce this stress odorant (dSO) in response to electric shock or violent shaking and presumably also to other more natural stressful stimuli in the wild. This novel use of CO₂ as a stress pheromone seems at odds with the favorite foods of *Drosophila*. Rotting fruits are covered with yeast and other microbes fermenting sugars in the fruit and producing CO₂ as a byproduct. As a behavioral aversion to one's own favored food source would be strongly selected against, the *Drosophila* aversion to CO₂ is probably contextual like that of ants inside and outside their nests. In support of this, Turner and Ray have identified specific odorants present in ripening fruit that modify the spiking of CO₂ neurons and block the behavioral avoidance of CO₂ (Turner and Ray, 2009).

Insects are not the only tiny invertebrates paying attention to small environmental gradients of CO₂. CO₂-evoked behaviors have also been identified in nematodes. Like in flies and ants, these behaviors are also contextual, as well-fed adult nematode worms (*C. elegans*) avoid 1% (10,000 ppm) CO₂, whereas starved worms do not (Bretscher et al., 2008; Hallem and Sternberg, 2008). This avoidance behavior may help the nematodes escape soil environments that are becoming inhospitable. Even with such a simple nervous system, nematodes need a way to judge whether staying in a less hospitable environment that definitely has food is better than moving to a new environment that may not. Interestingly, CO₂-evoked behaviors in worms are species-specific. In some parasitic nematode species, infective juveniles are attracted to CO₂ instead of being repelled by it, presumably because it is released by their insect hosts and can be used to direct host-seeking behavior. Despite species-specific differences in sensitivity and behavioral relevance of CO₂, all of the nematode species studied thus far seem to use the same set of sensory neurons and the same receptors to detect CO₂ (Dillman et al., 2012; Hallem et al., 2011a).

OLFACTORY CO₂ DETECTION MECHANISMS

The first hints at a molecular mechanism for the detection of environmental CO₂ came from a rather unlikely place. Although the human olfactory system is insensitive to CO₂, we do enjoy the taste of carbonated beverages. Mountaineers taking the carbonic anhydrase inhibitor acetazolamide to minimize the symptoms of altitude sickness often complain that the drug ruins the taste of their celebratory drinks upon reaching the summit (Graber and Kelleher, 1988). In more controlled tests,

when acetazolamide was applied to half of their tongue, human volunteers consistently reported that the untreated side had a stronger sensation of carbonation (Dessirier et al., 2000).

Carbonic anhydrases are enzymes that catalyze the hydration of water to form carbonic acid, which rapidly dissociates in solution to protons and bicarbonate. These enzymes are among the most catalytically efficient yet discovered, and are known to play important roles in pH maintenance in many plant and animal systems (Tashian, 1989). The fact that a carbonic anhydrase inhibitor alters the taste of carbonation indicates that we are not tasting CO₂ at all, but one of its metabolites: protons or bicarbonate.

Humans cannot smell low concentrations of CO₂, but high levels of CO₂ (> 30%, 300,000 ppm) can activate trigeminal nociceptors to produce a burning sensation in our mucous membranes (Bensafi et al., 2008). Wang et al. (2010) recently reported that the CO₂-evoked responses of trigeminal sensory neurons of mutant mice lacking the TRP channel TRPA1 are much lower than those of wild-type mice. TRPA1-expressing HEK-293 cells, unlike controls, generate calcium responses to CO₂ and intracellular acidification. This suggests that CO₂ diffuses into the mucosal nociceptors and is converted to carbonic acid by an intracellular carbonic anhydrase. Then, the protons produced by dissociation of the carbonic acid activate the proton-sensitive TRPA1 channel to generate excitatory changes in membrane voltage. The action of carbonic anhydrase seems to be one of the few common themes running throughout the different systems animals have evolved to detect environmental CO₂.

In 1967, the Swedish scientist Holger Hansson described a histochemical staining technique using cobalt sulfate that deposits a black precipitate on sites of carbonic anhydrase (CA) enzymatic activity (Hansson, 1967). In 1984, Brown et al. used a modification of this technique to identify a population of ciliated CA-expressing neurons in the olfactory epithelia of rats. It took several more years, however, to identify these neurons as CO₂ sensors and to discover which of the many vertebrate CA isoforms they express.

The elucidation of the signal transduction pathway underlying olfactory CO₂ perception in vertebrates started in 1995 with the discovery of a population of cells in the rat olfactory epithelium that express a novel receptor guanylate cyclase gene called GC-D (Fülle et al., 1995). These neurons, which project to a group of specialized glomeruli on the caudal aspect of the olfactory bulb called the necklace glomeruli, express components of a cGMP-based signaling cascade instead of the typical olfactory cAMP-dependent cascade components: the rGC GC-D instead of the adenylyl cyclase ACIII (Fülle et al., 1995), the cGMP-sensitive phosphodiesterase PDE2A instead of the cAMP-sensitive PDE1C2 (Juilfs et al., 1997), and the cGMP-sensitive cyclic nucleotide gated ion channel CNGA3 (Han and Luo, 2010; Meyer et al., 2000). In 2007, Hu et al. (2007) added the final piece to the puzzle by confirming that these neurons innervating the necklace glomeruli respond to CO₂ at near atmospheric levels and their response requires both GC-D and the intra-cellular carbonic anhydrase CAII. When GC-D is expressed in a cell culture system, its cyclase activity is stimulated by bicarbonate (Guo et al., 2009; Sun et al., 2009), pointing to GC-D as the actual receptor underlying dose-dependent CO₂ olfactory responses. This may also explain why humans are unable to smell environmental CO₂, as it was recently discovered that the crucial GC-D gene has become a pseudogene in primate lineages (Young et al., 2007). The mechanism underlying olfactory detection of CO₂ by vertebrates is illustrated in

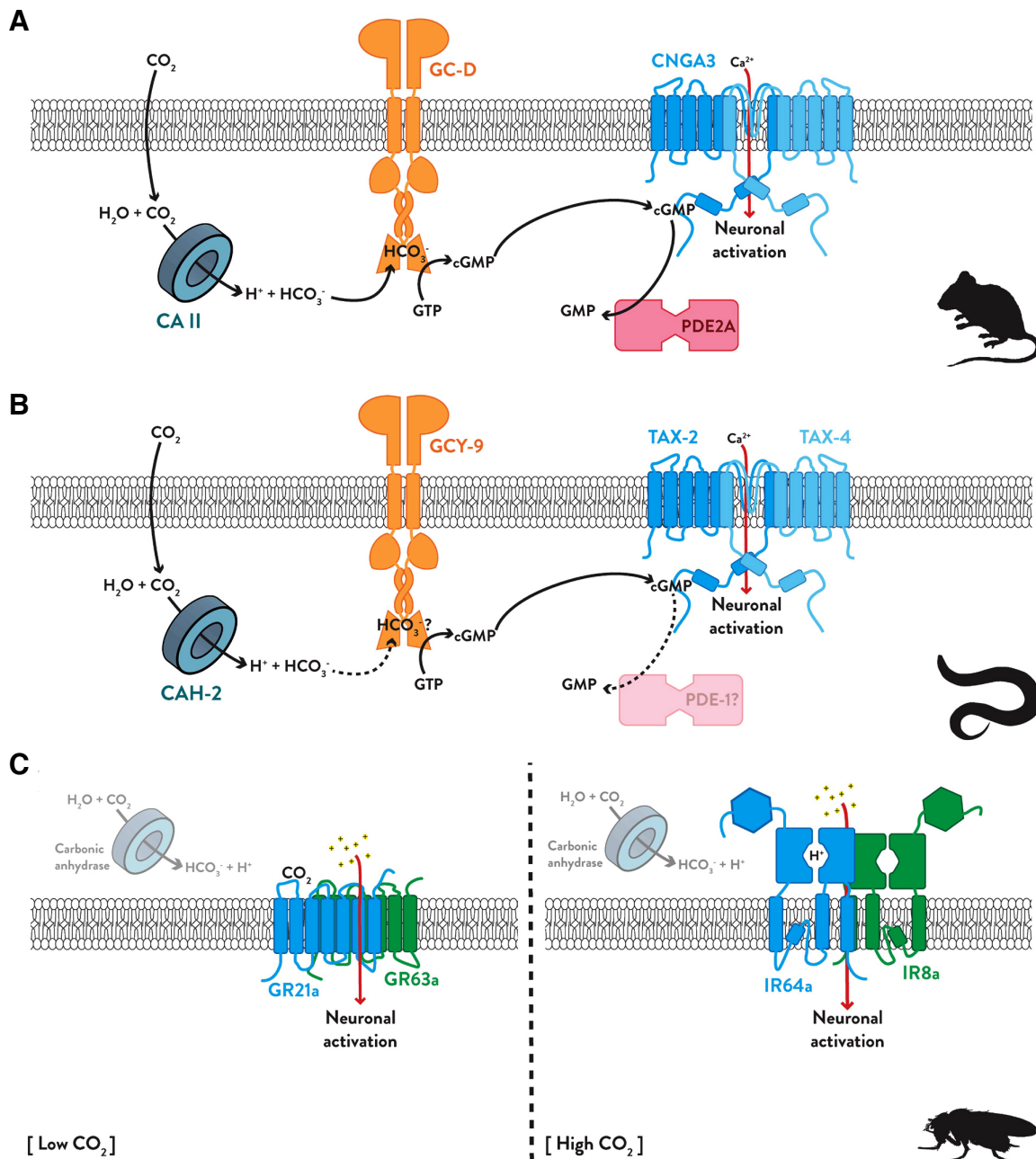


Fig. 1. (A) In the mouse system, CO₂ diffuses into the CO₂ receptor neurons. Its hydration is catalyzed by the carbonic anhydrase CAII to form carbonic acid, which immediately dissociates to form protons and bicarbonate. The bicarbonate activates the receptor guanylate cyclase GC-D, which converts bound GTP to cGMP. The cGMP then binds to the cyclic nucleotide gated channel CNGA3 and causes it to open and permit the entry of calcium ions that initiate action potentials that travel to the neck glomeruli in the olfactory bulb. The response to CO₂ is then terminated when PDE2A converts the cGMP into GMP. (B) Nematodes employ a remarkably similar system for CO₂ detection. The carbonic anhydrase CAH-2 produces the bicarbonate that is detected by GCY-9. GCY-9 produces the cGMP that activates the TAX-2/TAX-4 CNG channel. The role of a PDE in terminating the nematode response to CO₂ has not yet been confirmed. (C) In fruit flies, a role for carbonic anhydrase in CO₂ detection is likely but not yet confirmed. In low CO₂, the GR21a and GR63a gustatory receptors are required, but in high CO₂, the ionotropic receptors IR64a and IR8a work together to detect a drop in sensory lymph pH.

Fig. 1A.

Although they are only distantly related to rodents, the model organism *Caenorhabditis elegans* was recently found to employ a very similar system to detect gradients of CO₂ in the soil. In

fact, several neurons in nematodes including the AFD and ASE neurons can respond to CO₂, but the ciliated BAG neurons seem to be the most sensitive and important for behavioral responses to CO₂ (Bretscher et al., 2011). In the BAG neurons,

responses to CO₂ require expression of the CNG channel subunits TAX-2 and TAX-4 (Bretscher et al., 2008; Hallem and Sternberg, 2008) and the receptor guanylate cyclase GCY-9, which is surprisingly dissimilar to the vertebrate GC-D (Hallem et al., 2011b). Since the BAG neurons have also been found to express the carbonic anhydrase CAH-2 (Bretscher et al., 2011) and the cGMP-sensitive phosphodiesterase PDE-1 (Hallem et al., 2011b), nematodes seem to possess all the major players of the rodent CO₂ detection pathway (Fig. 1B). Still, it is not yet clear whether GCY-9 is sensitive to molecular CO₂ or one of its metabolites (likely bicarbonate). It also still remains to be determined whether CAH-2 or PDE-1 are actually required for CO₂ detection in the BAG neurons and whether the other CO₂-responsive neurons use the same mechanism.

In addition to the mechanistic similarities in CO₂ detection in nematodes and rodents, the CO₂ neurons of both species are multimodal. Not only do the nematode BAG neurons respond to CO₂, they are also sensitive to oxygen and contribute to oxygen-evoked behaviors. Decreases in environmental oxygen activate the soluble guanylate cyclases GCY-31 and GCY-33, which then produce cGMP to open the same TAX-2/TAX-4 CNG channel (Zimmer et al., 2009). This means that increases in CO₂ and decreases in O₂ cause a similar increase in BAG neuron firing, but since increases in CO₂ and decreases in O₂ both indicate that a worm's current habitat is becoming less desirable, detecting both stimuli with the same multimodal neurons makes a certain amount of sense. In mice, the CO₂-sensitive GC-D neurons also respond to urinary peptides that are thought to play a role in fluid and salt balance (Leinders-Zufall et al., 2007). But the GC-D neurons respond even more sensitively to CS₂, which is a component of mouse breath that has been implicated in the social transfer of acquired food preferences from experienced mice to naïve mice (Munger et al., 2010). Hopefully, further studies will clarify how the rodent brain can make sense of the output of the GC-D neurons as they respond to such seemingly disparate stimuli.

Despite lying somewhere between nematodes and rodents in terms of organismal complexity, insects appear to employ very different CO₂ detection systems. CO₂-sensitive neurons have been identified in many insects and studied both electrophysiologically and ultra-structurally (Stange and Stowe, 1999), but the underlying receptors responsible for detecting CO₂ have only been identified recently in the model insect *Drosophila melanogaster* (Jones et al., 2007; Kwon et al., 2007). Unlike mosquitoes, which keep their CO₂ neurons in structures above the mouthparts called maxillary palps (Omer and Gillies, 1971), *Drosophila* CO₂ neurons are located in the antennae (de Bruyne et al., 2001). Suh et al. (2004) used calcium imaging in the fly brain to identify a single antennal lobe glomerulus that responds to small increases in CO₂. This glomerulus, named the V glomerulus because of its ventral-most position, was earlier identified as being innervated by neurons expressing the gustatory receptor Gr21a (Scott et al., 2001). Suh et al. (2004) were able to confirm the identity of the CO₂ neurons by showing that although wild-type flies innately avoid even small increases of CO₂ in a t-maze, flies with genetically silenced Gr21a neurons fail to detect and avoid CO₂.

Soon, a second gustatory receptor, Gr63a, was found to be co-expressed with Gr21a in the CO₂ neurons. Gr63a null mutant flies fail to respond to elevated CO₂ and ectopic expression of both Gr21a and Gr63a together, but not either one alone, confers CO₂-sensitivity on non-CO₂ neurons (Jones et al., 2007). The malaria mosquito *Anopheles gambiae* has clear homologues of Gr21a and Gr63a, which are co-expressed in

the CO₂ neurons of its maxillary palps (Jones et al., 2007) and which function as CO₂ receptors when ectopically expressed in *Drosophila* (Lu et al., 2007). Phylogenetic analysis of the insect gustatory receptors reveals that Gr21a and Gr63a are more related to each other than any of the other Grs and that mosquitoes, moths, and beetles actually have three CO₂ receptor genes (i.e., Gr1, Gr2, and Gr3) (Robertson and Kent, 2009). Thus, the primary olfactory CO₂ receptor in insects is a heteromeric complex of unknown stoichiometry consisting of members of a highly conserved gustatory receptor subfamily (Fig. 1C).

While most insect gustatory receptors are expressed in taste organs (i.e., the mouth parts, forelegs, and wing margins) and olfactory receptors are expressed in olfactory organs (i.e., the antennae and maxillary palps), the fact that the insect CO₂ receptors are gustatory receptors expressed in olfactory organs begs the question of their true ligand. Is it the volatile CO₂ or a more soluble CO₂ metabolite like bicarbonate, which is the ligand for the CO₂-responsive guanylate cyclases in nematodes and rodents? If the GR21a/GR63a receptor responds to bicarbonate rather than CO₂ itself, it would suggest the involvement of a carbonic anhydrase like the ones required in the nematode and vertebrate systems.

According to early electrophysiological experiments, treatment with acetazolamide dramatically reduces the response of honeybee CO₂ neurons (Stange, 1974), strongly suggesting a role for carbonic anhydrase in the olfactory CO₂ detection mechanism of at least some insects. The mechanism of CO₂ detection in bees, however, remains a mystery because the honeybee genome does not contain Gr21a or Gr63a orthologues (Robertson and Kent, 2009). Although bees are sensitive to a wide range of CO₂ concentrations (Stange and Diesendorf, 1973), it is possible that they instead employ variants of a second class of receptors recently implicated in the detection of high concentrations of CO₂ by the *Drosophila* antenna.

Ai et al. found that flies with silenced Gr63a neurons fail to avoid low concentrations of CO₂, which is consistent with previous reports, but that they are still capable of avoiding environments with CO₂ concentrations above 5% (50,000 ppm). In addition to activating the V glomerulus, which is sensitive to much lower concentrations of CO₂, odor streams containing high concentrations of CO₂ also activate the more central DC4 glomerulus. The sensory neurons that innervate DC4 express a pair of variant ionotropic glutamate receptors, Ir64a and Ir8a, which mediate a response to acid. The fact that CO₂ activates these neurons strongly suggests the involvement of a carbonic anhydrase catalyzing the conversion of CO₂ to carbonic acid (Fig. 1C). Relatively few remaining experiments should suffice to identify the true ligand of the GR21a/GR63a heteromeric receptor complex and to confirm a role for a carbonic anhydrase in insect CO₂ detection, but many questions remain to be answered regarding the relevant signal transduction mechanisms.

Bioinformatic sequence analysis has revealed enough similarity between the insect gustatory receptors and the insect olfactory receptors to place both subfamilies together in a larger superfamily of insect chemoreceptors (Robertson et al., 2003). Since most family members are predicted to have seven transmembrane domains, the insect chemoreceptors were long assumed to be canonical GPCRs like the vertebrate odorant receptors. Surprisingly, though, both the insect gustatory and olfactory receptors act as ligand-gated cation channels when expressed in heterologous cell culture systems (Sato et al., 2008; 2011; Wicher et al., 2008). Perhaps even more surpris-

ingly, several studies have reported conflicting results using different expression systems and experimental approaches and concluded that at least some members of the superfamily couple to G proteins (Kain et al., 2008; 2009; Wicher et al., 2008).

In the CO₂-responsive Gr21a/Gr63a neurons specifically, both the knockdown and constitutive activation of G α q reduce CO₂ sensitivity (Yao and Carlson, 2010). The loss of function of another group of receptors, the TRPC class of Transient Receptor Potential cation channels, along with their canonical signaling partner phospholipase C (PLC21C) have also recently been found to reduce the sensitivity of the Gr21a neurons in *Drosophila* (Badsha et al., 2012). The most attractive hypothesis that attempts to reconcile all of these results is that all of the insect ORs and GRs, including the CO₂ receptors, are ligand-gated cation channels that are modulated by other signaling pathways, perhaps via phosphorylation or some other post-translational modifications (Nakagawa and Vosshall, 2009). Lastly, the full details of signal transduction via the newly discovered but evolutionarily ancient family of variant ionotropic glutamate receptors or IRs that also plays an important, but independent role in insect olfaction and CO₂ detection have yet to be determined (Benton et al., 2009; Croset et al., 2010). Many further experiments will be necessary to fully understand all the relevant players in insect olfactory and gustatory signal transduction such that the entire pathway of environmental CO₂ detection comes to light.

CONCLUSION

Although CO₂ is a ubiquitous environmental stimulus, a given CO₂ concentration can mean different things to different species in different ecological niches. For this reason, it is unsurprising that different animals respond to environmental CO₂ with unique sets of behaviors. What is surprising is that animals as distantly related as nematodes and rodents can share such a similar CO₂ detection mechanism, while evolution has clearly put forth multiple unique solutions to the problem of CO₂ detection in insects. Once the molecular details of all the signal transduction mechanisms responsible for CO₂ detection in more diverse species have been identified, it will be very interesting to trace the path evolution has taken in gaining and losing CO₂ receptors. It is also my hope that a better understanding of CO₂ detection mechanisms will not only help mountaineers avoid the “Champagne Blues”, but also reduce the transmission of infectious parasites by CO₂-tracking, blood-feeding insects.

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