Discussion

A gustatory gene in an olfactory neuron.

We have shown that *Gr21a*, a member of the gustatory receptor gene family is expressed in the olfactory receptor neuron ab1C that responds to CO_2. Although this neuron class has a very specific response to a very unusual odorant it is also similar to other ORNs. The cells are housed in a sensillum of the olfactory basiconic type, together with three other neurons that respond to odorants. For instance, the ab1D neuron responds to methyl salicyalate (Fig. 5D) and was recently shown to express a member of the olfactory receptor (OR) gene family (Hallem *et al.*, 2004). The axons of ab1C neurons converge onto a glomerulus in the antennal lobe as other ORNs do. Therefore, it would seem that members of the gustatory receptor gene family can be expressed in neurons with an olfactory function and that CO_2 perception is an integral part of the olfactory system.

Evidence from gene sequence comparisons suggests that the OR genes are in fact a subfamily of the GR gene family (Robertson *et al.*, 2003). This could mean that *Gr21a* is part of a group of olfactory genes that predates the formation of the OR gene family. In fact CO_2 sensitive neurons have been found in many insects (Stange, 1996) and the ability to detect it may be highly conserved. Among a comparison of GR and OR sequences in *Drosophila* and the mosquito *Anopheles gambiae,* very closely related sequences were found for *Gr21a* whereas many *Drosophila* OR genes do not have apparent orthologues in *Anopheles* (Hill *et al.*, 2002).

Our results do not provide direct evidence that CO_2 is the ligand for the *Gr21a* receptor, or indeed that *Gr21a* is involved in CO_2 transduction. However, there are several reasons to assume that *Gr21a* is involved in mediating the CO_2 response of the ab1C neurons. First of all we have no indication that ab1C neurons respond to any other odorant than CO_2 (de Bruyne *et al.,* 2001 and this study). This would preclude a role for *Gr21a* in the transduction of another odorant. Secondly, to date all evidence suggests that *Drosophila* ORNs express a single functional receptor gene that is responsible for all aspects of its odor responses properties (Dobritsa *et al*., 2003, Elmore *et al*., 2003, Hallem *et al*., 2004). One notable exception is the ubiquitously expressed *Or83b* gene, which seems not to be involved in olfactory transduction in the absence of another OR gene (Vosshall *et al.,* 1999, Dobritsa *et al.* 2003). In this context it is of some interest that *Or83b* is apparently not expressed in ab1C neurons, as the V glomerulus is not labeled in *Or83b-GFP* flies (Wang *et al*., 2003). Thus, an alternative explanation for the functional role of *Gr21a* is that it performs some other function in the ab1C neuron similar to *Or83b*. However, this would single out the ab1C neurons as the only ORNs where Gr21a performs this function.

Cell membranes are freely permeable to dissolved CO_2. Its conversion to H_2CO_3 and subsequent dissociation to raise intracellular pH may well provide an alternative mechanism for transduction similar to vertebrate acid taste (Lyall *et al*., 2001). In various vertebrate species non-olfactory receptor cells exist for water-dissolved CO_2. Such CO_2/H^+ sensitivity can be found in peripheral, arterial and pulmonary

chemoreceptors (Milsom, 2002). In addition a form of CO_2 chemoreception located in the brain is essential for regulating breathing in mammals but the cells mediating this have not been identified. The mechanisms that all these cells use for transducing the CO_2 signal have not been characterized but are thought to be varied (Milsom, 2002). However, the threshold concentrations that the ab1C neurons in *Drosophila* can detect are so low they are unlikely to have a significant effect on intracellular pH. Thus, the expression of a 7-transmembrane domain receptor in these neurons suggests a quite different transduction mechanism for sensing CO_2.

CO_2 perception; a submodality?

Because of the special nature of carbon dioxide as an odorant it is of some interest to see whether these ORNs behave like other ORNs. Though they are fully integrated in the olfactory system of the fly, do they constitute a separate part of it? In humans, CO_2 perception is fundamentally different from perceptions of odorants. CO_2 is an irritant to free nerve endings of the trigeminal nerve in the olfactory epithelium (Shusterman, 2002). Sensitivity differs considerably between insects and mammals. Typical psychophysical thresholds of detection in humans are around 25%, much higher than the 0.03% physiological threshold we found for *Drosophila*. This sensory threshold is in the same range as that of other insects such as the mosquito *Aedes aegypti* (Grant *et al.*, 1995). In some species of moth, a more accurate determination of thresholds for CO_2 receptor neurons has suggested sensitivity even a 100-fold lower (Stange and Wong, 1993). However, in terms of molecules per volume of air, odorants can often be detected at much lower levels (Kaissling, 1987). Thus, although

the ab1C neuron is much like any other olfactory neuron they are less sensitive, which probably is due to the fact that CO_2 is always present at relatively high concentrations. In addition, we did not find any other odorant that excites this neuron class whereas even the most narrowly tuned ORNs in the antenna show low level responses to at least one other odorant (de Bruyne *et al.*, 2001). This lower sensitivity and the fact that they are more narrowly tuned than other ORNs set them apart. Further studies need to be done to compare more of their physiological properties (*e.g.* adaptation rates, absolute sensitivity, temporal dynamics) to those of other ORNs to provide insight in the way they operate.

The sensilla that house the CO_2-sensitive neurons in several species of mosquitoes are found on the maxillary palp, not the antenna (Grant *et al.,* 1995). However, like in *Drosophila,* they also contain neurons that respond to other odorants (Grant and O'Connell, 1996). In several moth species CO_2-sensitive neurons are in the labial palps (Kent *et al.,* 1986). Although the location of the receptor neurons varies, the axons project to a prominent ventrally located glomerulus in *Drosophila*, mosquitoes and moths (Scott *et al.,* 2001, Distler *et al.* 1998, Kent *et al.,* 1986). Is the ventral location of a glomerulus for CO_2 conserved? In *Drosophila* the V-glomerulus is special because it is one of only 4 glomeruli receiving unilateral input, as opposed to bilateral input to all others (Stocker, 1994). This may be indicative of a separate functional circuit that is wired for a different mode of computation with respect to left-right comparisons. This would make sense because diffusion speed of CO_2 is higher than for other odorants, quickly eliminating any small-scale temporal or spatial gradients.

Calibrating olfactory responses: Spikes, calcium and behavior

One distinct advantage of studying stimulation with CO_2 as opposed to other odorants is the fact that its concentration can be unequivocally determined in different behavioral and physiological paradigms. In addition, it can be measured accurately with infrared CO_2 sensors. We have shown that it is the only odorant that excites a single receptor neuron class over a wide range of concentrations. Thus, we can draw conclusions about the relation between behavior and the neural activity in the ab1C neurons. Moreover, because we used identical stimulation methods in single sensillum recordings and calcium imaging, we were able to calibrate intracellular calcium concentration changes to neural activity indicated by action potential firing rates.

To our knowledge, our study is the first to compare calcium-imaging data with spike rates for a single cell type in *Drosophila*. A neural response constitutes various biochemical and bioelectrical events. Any one of these can be measured and used to monitor neural activity. Calcium is an important second messenger in neurons (Berridge *et al.,* 1998, Augustine *et al.,* 2003). Its intracellular concentration is affected by entry through calcium channels during membrane depolarization, release from and re-absorption into internal stores, and buffered by binding to effector-proteins such as calmodulin. It is thought that calcium concentration is regulated very locally in the sub-compartments of a neuron (Augustine *et al.,* 2003). At the pre-synaptic end of a neuron, calcium is the link between membrane depolarization and transmitter release and its concentration here should reflect the rate of firing. However, it also

plays a role in mediating adaptation of olfactory receptor neurons (Zufall and Leinder-Zufall, 2000) and this could mean that its concentration is not necessarily correlated with action potential firing rate. Here, we have established that, at least for this neuron type, calcium is a reliable indicator of action potential firing rate, because the relation between neural activity, as measured in action potential firing and calcium signals, is linear. We compared the temporal features of the calcium signals in the antenna, where dendrites and somata of the ab1C neurons are located, with those in the presynaptic terminals in the antennal lobe of the brain. Our results indicate that in both compartments of the neurons calcium concentration follow similar time courses. Transmembrane calcium currents have been observed in insect ORNs (Lucas and Shimara, 2002) that probably contribute to the receptor potential generated in response to odorants. This receptor potential is tightly correlated with action potential firing rates (Kaissling, 1987), which could explain the consistency between the two signals.

Drosophila offers unique possibilities to investigate coding of behaviorally relevant odor stimuli, since actual spike counts can be compared to easily manipulated concentration changes and linked to behavioral output in simple paradigms. To mosquitoes, CO_2 is an attractive stimulus (Geier *et al*., 1999). In these bloodsucking insects the role of CO_2 as an important cue in host-finding behavior is well described (Bowen, 1991). By contrast the behavioral role of CO_2 perception in moths is not well understood (Stange, 1996). We observed that flies avoid CO_2 when it is presented on its own. The main resources for food, oviposition sites and social interactions with

other flies are sugar-containing media with a certain level of yeast fermentation, such as ripe fruits. It may be advantageous for the flies to balance the occurrence of attractive odorants with presence of CO_2. A dominance of input from ab1C neurons could signal fermentation rates that are too high, and indicate the medium should be avoided for fear of intoxication. We have used the strong behavioral response resulting from activity in these specialized neurons, to determine what firing rates in ORNs can be reasonably assumed to drive behavioral responses. Our results demonstrate that rates above 50 spikes/s lead to a saturation of the behavioral response whereas relatively low rates already induce a robust response. It is important to take the intensity of stimuli and the neural activity they induce in sensory neurons into account, when drawing conclusions about neural coding of odorants. The genetic model species *Drosophila* offers an excellent opportunity to give accurate descriptions of the relations between odor processing in ORNs (de Bruyne *et al.,* 1999, 2001, Wilson *et al.,* 2003), processing in neural networks of the antennal lobe (Wilson *et al.,* 2003, Wang *et al.,* 2003, Ng *et al.,* 2003) and behavior.