Results

The gustatory receptor Gr21a is expressed in large basiconic sensilla of type ab1

The *Gr21a* gene is expressed in antennae (Scott *et al.*, 2001). We noted with interest that the expression pattern is reminiscent of the distribution of large basiconic sensilla (Shanbhag *et al.* 1999). In order to investigate in which type of cells this receptor is expressed we did several experiments. We exploited the Gal4-UAS binary expression system (Brand and Perrimon, 1993) to drive various transgenes under the control of the *Gr21a* promoter construct generated by Scott *et al.* (2001). The *Gr21a-Gal4* driver line was used to drive a UAS-reporter construct that carries an enhanced GFP protein linked to mouse CD8, which localizes to the cell membrane (Lee and Luo, 1999). This allows accurate assessment of the overall morphology of *Gr21a* expressing cells. Confocal analysis of GFP expression in whole antennae clearly showed GFP in sensory neurons in the third segment (Fig. 1A). No differences between males and females were observed (not shown). Labeled cells all exhibited a relatively small rounded cell body with a thin axon extending toward the proximal end of the segment, as well as a spindle shaped inner dendritic segment with a thin outer dendritic segment, which thickens upon entering blunt-tipped sensilla on the cuticle (Fig. 1B). The cellular structure clearly indicates these are olfactory receptor neurons. The sensilla innervated by these neurons are all shaped like basiconic

sensilla rather than the smaller coeloconic or the longer, pointed trichoid sensilla. The innervated sensilla are distributed on the proximo-medial side, the dorso-proximal edge and in two rows across the center of the posterior side, just distal to the sacculus (arrow in figure 1B). This distribution is typical for large basiconic sensilla (Fig. 1C-D, Shanbhag *et al.* 1999). These sensilla are further classified into three physiological types based on the response spectra of the ORNs contained in them (Fig. 1D, de Bruyne *et al.*, 2001). There are 45 ab1 sensilla, 27 ab2 sensilla and 18 ab3 sensilla. In a 3D reconstruction of the GFP signals in 4 preparations we counted 34-36 neurons (not shown). As this is more than 18 or 27, these results strongly suggest that *Gr21a* is expressed in ab1 sensilla and that our counts may underestimate the true number of cells expressing *Gr21a*. Some cells could have been closely apposed to another cell and not counted in our analysis or the Gal4 driver may not be strongly expressed in all cells.

Gr21a-driven expression of a calcium sensor shows ab1C neuron responses to CO_2

We have previously reported that ab1 sensilla contain four receptor neurons, each with quite different responses to a panel of odorants (Fig. 2G, de Bruyne *et al.*, 2001). The A neuron responds to several odorants, but is particularly sensitive to ethyl acetate. The B neuron responds mainly to 2,3-dibutanone. The C neuron responds exclusively to carbon dioxide. The D neuron shows strong responses to methyl salicylate and weaker responses to other aromatic compounds such as benzaldehyde.

In order to determine which of the four neurons present in ab1 sensilla expresses the *Gr21a* receptor we performed several experiments. As neuronal activation is reflected in elevated intracellular calcium levels, we used the *Gr21a-Gal4* driver to express the calcium sensitive reporter cameleon (Fiala *et al.,* 2001). Cameleon is a fusion of the calcium-binding protein calmodulin, its target peptide M13 and two modified GFPs with fluorescence in the yellow and cyan range of the spectrum, (EYFP and ECFP respectively, Miyawaki *et al.,* 1999). Calcium levels are reflected in the ratio between the two emitted wavelengths that shifts due to a conformational change in the cameleon protein.

Fluorescence of the *Gr21a-Gal4* driven cameleon protein can be viewed at the level of dendrites and somata in the antenna and in the converged axonal endings in the antennal lobe of the brain (Fig. 2A&B). We recorded changes in intracellular calcium in the *Drosophila* antennae and found clear increases that correlate in a dose-dependent manner with stimulation by CO_2 in air (Fig. 2C,E). Relatively strong responses were readily obtained to a concentration of just 0.1% CO_2, which is *ca*. 3x the concentration in ambient air (0.03%) and well below the threshold for anesthetic effects (ca. 30%). The fact that a neuronal response was observed to CO_2 suggests that *Gr21a* is expressed in ab1C neurons. To exclude the possibility that Gr21a is also expressed in other ORNs we tested other odorants (Fig2F&G) that include several chemical classes and are known to excite other ORNs in antennal basiconic sensilla, whether of the large category (ab1, 2 and 3) or others (ab4, 5, and 6). The odors were presented at concentrations that induce >100 spikes/s in single neurons (de Bruyne *et*

al., 2001). The odorants tested and the neurons they define were: ethyl acetate (ab1A and ab2A), pentyl acetate (ab3A and ab5B), ethyl butyrate (ab1A and ab3A), methyl salicylate (ab1D), benzaldehyde (ab4A), 1-octen-3-ol (ab6a), 2,3-dibutanone (ab1B and ab2A), 2-heptanone (ab3B) and geranyl acetate (ab5A). None of these odorants induced significant excitation in *Gr21a*-positive neurons (Fig2F) confirming that *Gr21a* is expressed in ab1C neurons.

Increases in intracellular calcium concentration at the level of dendrites and cell bodies of ORNs are most likely due to increased membrane conductance, caused by sensory transduction of the CO_2 stimulus (receptor potentials). By contrast, a calcium sensor present in axonal endings of ORNs in the antennal lobe is more likely to respond to action potential-induced changes in calcium concentrations. How do these two signals compare? We used the method developed by Fiala *et al.* (2002) to record calcium changes in the antennal lobe. First, we note that cameleon protein can only be seen in a single glomerulus, positioned rather ventrally in the antennal lobe (Fig. 2B), the V glomerulus. Secondly, calcium increases can be measured during stimulation with CO_2 that show time courses and amplitudes very similar to those observed in the antenna (Fig. 2D). Like the cell bodies and dendrites, ORN axonal endings in the V glomerulus do not respond to other odorants (data not shown).

In dipteran insects, single ORNs generally innervate a single glomerulus on the ipsilateral side of the brain but also send a branch to the same glomerulus of the contralateral side of the brain. However, the V-glomerulus is innervated by neurons from the ipsilateral side only (Stocker *et al.*, 1994). Scott *et al*. (2001) confirmed that

Gr21a expressing neurons do not cross the midline. We ablated the antenna on one side of the fly and found that calcium increase can only be recorded from the V glomerulus on the undamaged side (Fig. 2H&I). We conclude that CO_2 sensitive ab1C neurons differ from other ORNs studied in *Drosophila* in that they project only ipsilaterally to the antennal lobe.

The ab1C neuron is sensitive around ambient CO_2 concentrations

How sensitive are these ab1C neurons to changes in levels of CO_2? Action potentials can be recorded from the four neurons in ab1C sensilla and attributed to each cell based on their characteristic amplitude and shape (de Bruyne *et al.*, 2001). Figure 3A shows a typical recording from an ab1 sensillum. The C neuron is the only one of the four cells rapidly increasing its firing upon receipt of the CO_2 stimulus. The ab1C neuron responds in a phasic-tonic way, typical for most ORNs in *Drosophila,* and ends its activity immediately after the stimulus is removed. The end of stimulation induces a short period of quiescence where spiking is below the spontaneous rates seen prior to stimulation. Although many ORNs on the antenna continue firing after stimulus removal; the short quiescence period seen in ab1C is found in several other ORN classes as well (de Bruyne *et al.*, 2001). It is in fact noteworthy that spontaneous action potentials are fired at all before stimulation. As the preparation was bathed in synthetic air free of CO_2, this activity occurs in a truly non-stimulated state. This indicates that ab1C neurons are spontaneously active and that this activity is not due

to low levels of stimulation by contaminants from the stimulus delivery, as can theoretically be the case for other ORNs.

We then presented a range of CO_2 concentrations that the fly is likely to encounter in its natural environment (Nicolas and Sillans, 1989, Stange, 1996). While 0.025% induced a small but noticeable increase in firing, 0.05% clearly raised activity to 25 spikes/s above spontaneous firing (Fig. 3B) indicating that sensory threshold is somewhere near ambient concentrations. Firing rates rose very rapidly at higher doses. When plotted on a normal non-logarithmic scale the relation for this dose range is linear (Fig, 3C). Most other ORNs in *Drosophila* do not show this linear relation but instead have typical sigmoid shaped dose response curves (de Bruyne *et al.*, 2001). A similar linear relationship has also been observed in mosquito CO_2 receptor cells (Grant *et al.*, 1995). Since we used the same CO_2 stimulation system for calcium imaging, we can compare excitation levels in ab1C neurons as measured in spikes with calcium signals, by calculating the spike rates for the concentrations used in the imaging data (Fig. 3D). This clearly demonstrates that, at least for the range of concentrations we tested with both methods, calcium concentration is linearly dependent on average firing rate.

However, even though both neuronal firing rate and cameleon fluorescence show a sharp rise and fall associated with start and end of stimulation the temporal structures of their signals differ. Whereas spike rates reach a maximum within the first 100 ms of a response (not shown) and then decline, cameleon fluorescence takes *ca.* 1500 ms to reach its maximum (Figure 2C). It remains to be seen whether this is

due to slow dynamics of the cameleon sensor or truly reflects certain changes in calcium levels 'lagging behind' spiking activity.

CO_2 is repellent at a range of concentrations.

In order to further examine the function of ab1C neurons we studied behavioral responses to establish the perceptual qualities of CO_2 for the fly. We used a version of the T-maze assay that is widely used in studies of *Drosophila* learning and memory (Tully and Quinn, 1985, Beck *et al*., 2000, Schwaerzel *et al.,* 2003) as well as innate olfactory behavior (Heimbeck *et al*., 2001, Stensmyr *et al.,* 2003). Flies were loaded in the central chamber of the device and given 3 minutes to distribute themselves between two arms. An Index was calculated (see experimental methods) in which 0 means flies are distributed equally between the stimulus and control arm of the maze. An index of 1 means all flies are in the control arm and at -1 all are in the stimulus side. When no odor was mixed into the airflow the flies were equally distributed between the two arms (Fig. 4A) but when a relative high concentration of CO_2 (8%) was added to one side the flies moved quickly away from it and collected in the other arm. To make sure there was no other stimulus associated with adding gas from a pressurized tank (noises, turbulence, cooling etc.), we also tested synthetic air at the same rate, with no effect on the distribution of flies. We again tested different concentrations of CO_2. Our results show that wild-type flies respond to a rise in CO_2 concentration of as little as 3x ambient (0.1%) with avoidance behavior (Fig. 4B). We could not detect any significant effect of additions of 0.03% CO_2 and below. The

dose-response relation also shows that there is no increase in performance above 0.3% CO_2, indicating that around this concentration excitation of central neurons mediating the behavioral response is already maximal. From the linear relation between ab1C neuron firing rate and CO_2 concentration (Fig. 3D) we can derive the relationship between the firing rate of ab1C neurons and behavioral output (Fig. 4C). Maximum output is reached at a rate of 50 spikes/s input. The relationship shown in figure 4C demonstrates that the strength of CO_2 avoidance behavior is encoded in rate increases between 15 and 50 spikes/s.

Specific ablation of ab1C neurons eliminates CO_2 responses.

Can we be sure that the behavior we observe is completely driven by input from the ab1C neurons? Previous studies have shown that at least in antennal basiconic sensilla there are no ORNs other than ab1C neurons that respond to high doses of CO_2 (de Bruyne *et al.,* 2001). Similarly, palpal basiconic sensilla do not contain CO_2 sensitive ORNs (our own unpublished data). To determine if ab1C neurons are mediating avoidance behavior we genetically ablated these cells by using the Gr21a-Gal4 to drive expression of the pre-apoptotic gene *reaper (rpr).* This has previously been shown to induce cell death in neurons and accessory cells of *Drosophila* olfactory sensilla (Park *et al.*, 2002, Dobritsa *et al.*, 2003). When we examined the *Gr21a-Gal4; UAS-GFP; UAS-rpr* flies most of the antennae showed no GFP positive cells at all (Fig. 5A-B). However, we did find some flies that had a few GFP-positive cells, some of which displayed the small soma typical for cells undergoing apoptosis (not

shown). In order to determine the effect of removing ab1C cells on other ORNs of the antenna we recorded electroantennograms (Fig. 5C). These are glass electrode recordings from the surface of the antennal cuticle that capture the summation of receptor potential events from an unknown number of ORNs (Ayer and Carlson, 1992). To avoid effects of genetic background we used as controls siblings that did not receive the chromosome with the *Gr21a-Gal4* P-element. The CO_2 response was absent in *Gr21a-rpr* flies, whereas EAG response to other odorants, detected by other ORNs, were unaffected (Fig. 5C). This demonstrates there is no residual CO_2 sensitivity in other ORNs. We then examined individual ab1 sensilla to see the effect of *Gr21a*-driven apoptosis on electrical activity. Most recordings from ab1 sensilla in *Gr21a-rpr* flies lacked spontaneous spikes from the C neuron and also showed no response to stimulation with CO_2, whereas the sibling controls had normal responses to CO_2 (Fig. 5D). In 25 out of 28 sensilla on five flies we found no evidence for a functional C neuron. However, in one fly we recorded from 8 sensilla with a functional C neuron. It would seem therefore that induction of apoptosis occurs in most sensilla of most flies but some flies escape the treatment.

How does the removal of ab1C neurons affect CO_2 avoidance behavior? We compared the responses from *Gr21a-rpr* flies with siblings that did not receive the *UAS-rpr* P-element and found a drastic reduction in the normally robust avoidance at 0.3% CO_2 (Fig. 5E). We chose this concentration because avoidance behavior is maximal but not yet saturated (Fig. 4B) so that a reduction in sensitivity would be easily detected. Two further control lines, homozygous for either *Gr21a-Gal4* or *UAS-*

rpr, also showed normal avoidance behavior. In further experiments we confirmed that the w^{1118} background, heterozygous *Gr21a-gal4* or *UAS-rpr* flies also do not differ from wild type (CS-5) flies for this concentration of CO_2 (not shown). Therefore the loss of behavioral response to CO_2 can only be caused by the lack of ab1C neurons and indicates they are responsible for driving avoidance behavior.