Experimental procedures

Fly stocks and rearing

All flies were reared at 25°C, 60% RH and 12:12 LD. The wild type was *CS-5* and w^{1118} was the genetic background for P-element insertions. The *Gr21a* receptor driver line *w; P{Gr21a-GAL4}/CyO* was obtained from Kristin Scott. Gal4 driven apoptosis was induced by crosses with *w; ; P{UAS-rpr}/TM3 Sb* and membrane bound GFP was from *y w P{UAS-mCD8::GFP.L}; Pin/CyO* flies obtained from the Bloomington stock center. *CyO* and *TM3* are balancer chromosomes for the 2nd and 3rd chromosomes respectively.

Confocal microscopy

We looked at *y w P{UAS-mCD8::GFP.L}/+; P{Gr21a-GAL4}/CyO* females and *y w P{UAS-mCD8::GFP.L}/Y; P{Gr21a-GAL4}/CyO* males. To see the effect of *Gr21a*-driven expression of *rpr* on induced apoptosis we compared *y w P{UAS-mCD8::GFP.L}/+; P{Gr21a-GAL4}/+; P{UAS-rpr}/+* with *y w P{UAS-mCD8::GFP.L}/+; P{Gr21a-GAL4}/+; TM3 Sb/+*. Antennae were removed from anesthetized flies and mounted in slowfade (Molecular Probes, Eugene, OR) and directly viewed under a Leica DM RXE confocal laser-scanning microscope (Leica, Bensheim, Germany) with 40x or 63x objectives. GFP was excited with 488 nm and fluorescent emission collected in the 500-550 nm range. We used reflection of 633 nm laser-light in the 628-638 nm band as background. This visualizes mainly cuticular structures such as sensilla and tracheae

but also air trapped in the sacculus and under the second antennal segment. Stacks of optical sections (1 μm) were created, and off-line image analysis was done with AMIRA software (TGS, San Diego, CA).

Calcium imaging

Calcium signals from ORN cell bodies and dendrites were recorded from intact but restrained flies. Male flies, 10-16 days old, were immobilized in a plastic pipette tip (Ayer and Carlson, 1992, de Bruyne *et al.*, 2001). The antennae were held in position by a fine metal wire, such that the medio-posterior surface was facing upward. The preparation was covered with a cover slip allowing enough space for an air-stream to pass under it, over the antennae. For measuring calcium in the antennal lobe, female flies were prepared as described in Fiala *et al.*, 2002. Briefly, the fly was attached dorsally to a polyethylene foil. A hole was cut in the cuticle, trachea and muscle tissue covering the brain were removed and a drop of ringer added. Both preparations were imaged using two CCD cameras on an Olympus BX 50 microscope with a 20x W NA 0.5 objective as described in Fiala *et al.*, 2002. Optics and software were from TILL photonics. The cameleon 2.1 sensor is excited with 440 nm light and shifts emission between 480 nm at low calcium and 550 nm at high calcium. Binning on chip was set to give 2 μm/pixel for antennal preparations (76 x 53 pixels) and 1 μm/pixel for the antennal lobe (152 x 105 pixels). The fluorescence ratio, $F^R = 1000 \times F^{550nm} / F^{480nm}$, is used as a measure for calcium concentration. For each recording (10 sec, 3 frames/s, exposure time 60-100 ms) the baseline F^R was calculated

as a mean over the 4 frames before stimulation (-1 to 0 s). Response was quantified as the increase in fluorescence relative to this baseline as a percentage of the baseline ($\Delta F^R/F^R$). We did not observe qualitative differences between calcium signals in different parts of the antenna (not shown). The signal was calculated from a small region covering *ca.* 16% of total fluorescent area in the antenna or one entire glomerulus in the antennal lobe. The amplitude of a response was calculated as the mean of 4 frames during stimulation. Flies were stimulated three times with synthetic air, paraffin oil and four concentrations of CO_2. Odorants were tested in between, only once per fly and in random order.

Electrophysiology of single sensilla and electroantennograms

The single sensillum recording technique was described in detail elsewhere (de Bruyne *et al*. 1999, 2001). Male flies, 4-10 days old, were immobilized in a plastic pipette tip. One antenna was held in position by a glass microcapillary. A sharp (< 1μm) electrode was inserted through the wall of a single olfactory sensillum to contact the lymph surrounding the dendrites of the set of ORNs. Recordings were made with electrolytically sharpened tungsten wires or, alternatively, with AgCl-coated silver wire inserted in saline filled glass capillaries (0.015M KCl, 1 mm dia). The reference electrode was inserted in the eye or at the base of the proboscis. Recordings can be stable up to 2 hrs. Signals were amplified 1000x either with an IsoDam amplifier (WPI, Sarasota, FL) or digitally via a 10x active probe fed into an AD converter (USB-IDAC, Syntech, Hilversum, the Netherlands), Responses were

analyzed off-line using Autospike software (Syntech, Hilversum, the Netherlands). Action potentials were counted during stimulation (500ms), starting 75 ms after odor injection. We analyzed only recordings in which action potentials could be reliably attributed to the activity of a single neuron, based on amplitude differences (see de Bruyne *et al*. 2001).

Electroantennogram recordings in *Drosophila* are described in detail in Ayer and Carlson (1992). Flies were immobilized in a plastic pipette tip and a glass electrode placed on the proximo-medial side of the antenna. The reference electrode was inserted at the base of the proboscis. We used a 10x DC amplifier, AD converter and analysis software from Syntech, Hilversum, the Netherlands.

Odor stimulation

Stimulation with odorants or CO_2 was essentially as described in de Bruyne *et al*. (1999, 2001). The same method was used for single sensillum recordings, electroantennograms and calcium imaging. Briefly, a glass tube held 3-5 mm from the preparation supplied continuous humidified synthetic air (160 cm/s). Odors were injected into the air for a brief period (0.5 or 1, or 3 seconds) from filter papers placed in 5ml disposable syringes, reaching a headspace dilution factor of 6-10%. All odors were from Aldrich (Milwaukee, WI) at highest available purity (>97%), dissolved at 1% v/v in paraffin oil (Fluka, Buchs, Switzerland) and pipetted (20 µl) onto filter paper. CO_2 was obtained from ultra pure grade pressurized gas tanks (Airgas, Cheshire, CT or Messer Griesheim, Krefeld, Germany). A range of concentration was

made up in two ways. For the range of 9 concentrations in figure 3B we used tanks with 0.17%, 0.48%, 1,6% and 5% of CO_2 in N_2, then diluted dynamically via calibrated flow meters (Aalborg, Orangeburg, NY). For all other stimulations we diluted 100% or 5% CO_2 manually in 5 ml syringes with synthetic air (80% N_2 + 20% O_2). Comparing the two methods for the overlapping concentration range did not show significant differences in neuronal response (data not shown).

Behavior

The behavior of wild type *CS-5* and white-eyed w^{1118} flies in response to various concentrations of CO_2 was tested in a T-maze, a well-established olfactory choice test for *Drosophila* (Tully and Quinn, 1985, Beck *et al.,* 2000, Heimbeck *et al.,* 2001). We used a tube diameter of 2 cm, sucking 300 ml/min humidified room air through each arm. The central sliding compartment (2 cm dia) was 11 % of the total internal volume. Flies (males and females) were starved overnight on agar and humidified filter paper, loaded in groups of 30-50 in the central sliding compartment, moved down and left to choose during 3 min. CO_2 was added to one of the arms at one of the water bubblers. Concentrations were made up volumetrically with either 5% or 100% CO_2 from a gas tank. We changed the direction from which the flies were loaded as well as the orientation of the two arms so that experiments were balanced for any orientation bias other than related to the odor stimulus. Under these conditions flies were always repelled by CO_2 and an avoidance index was calculated from their number (flies in control - flies in CO_2) / (flies in CO_2 and control). The

number of flies remaining in the center was relatively constant in all experiments at 28 ± 5% (SEM). To test the effect of ablation of the ab1A neurons on CO_2 avoidance behavior we compared *w; P{Gr21a-GAL4}/+; P{UAS-rpr}/+* flies with their siblings *w; P{Gr21a-GAL4}/+; TM3 Sb/+* to check for genetic background and with *w; P{Gr21a-GAL4}/+; +/+* and *w; +/+; P{UAS-rpr}/+* to check for effects of the two P-element insertions. The flies were sorted for genotype, while under CO_2 anesthesia, two days before testing them in the T-maze.