Role of G-Proteins in Odor-Sensing and CO₂-Sensing Neurons in *Drosophila*

C. Andrea Yao^{1,2} and John R. Carlson²

¹Interdepartmental Neuroscience Program, Yale University, New Haven, Connecticut, 06520-8074, and ²Department of Molecular, Cellular and Developmental Biology, Yale University, New Haven, Connecticut, 06520-8103

A central question in insect chemoreception is whether signaling occurs via G-proteins. Two families of seven-transmembrane-domain chemoreceptors, the odor (Or) and gustatory receptor (Gr) families, have been identified in *Drosophila* (Clyne et al., 1999, 2000; Vosshall et al., 1999). Ors mediate odor responses, whereas two Grs, Gr21a and Gr63a, mediate CO₂ response (Hallem et al., 2004; Jones et al., 2007; Kwon et al., 2007). Using single-sensillum recordings, we systematically investigate the role of G α proteins *in vivo*, initially with RNA interference constructs, competitive peptides, and constitutively active G α proteins. The results do not support a role for G α proteins in odor sensitivity. In parallel experiments, manipulations of G α_q , but not other G α proteins, affected CO₂ response. Transient, conditional, and ectopic expression analyses consistently supported a role for G α_q in the response of CO₂-sensing neurons, but not odor-sensing neurons. Genetic mosaic analysis confirmed that odor responses are normal in the absence of G α_q . G γ 30A is also required for normal CO₂ response. The simplest interpretation of these results is that G α_q and G γ 30A play a role in the response of CO₂-sensing neurons, but are not required for Or-mediated odor signaling.

Introduction

The role of G-proteins is a fundamental issue in insect chemosensory signaling. Chemoreception in vertebrates and *Caenorhabditis elegans* utilizes seven-transmembrane-domain receptors that signal via G-proteins. Insects also have large families of odor receptor (Or) and gustatory receptor (Gr) proteins that are predicted to contain seven transmembrane domains; however, Or and Gr proteins lack sequence similarity to G-protein-coupled receptors (GPCRs), and the topology of Ors differs from that of GPCRs (Neuhaus et al., 2005; Benton et al., 2006; Lundin et al., 2007).

The role of G-proteins in chemosensory signaling has been examined in *Drosophila*, but a clear consensus has not been reached. Studies suggest that G α_s and G γ_1 play a role in the response of Gr5a-expressing taste neurons (Ishimoto et al., 2005; Ueno et al., 2006). The requirement for G-proteins in olfaction is unclear. Two recent studies found that an Or and a ubiquitously expressed coreceptor, Or83b, form a ligand-gated ion channel (Sato et al., 2008; Wicher et al., 2008). However, the studies proposed different mechanisms for activation of the channel complex. One study found that G-protein-mediated signaling plays a negligible role in receptor activation (Sato et al., 2008), whereas the other found that the Or complex is activated by cyclic nucleotides and acts as a GPCR (Wicher et al., 2008). Other studies have reported that olfactory receptor neurons (ORNs) require G-proteins: mutations in G α_q were found to reduce the sensitiv-

ity of antennal neurons to several odors (Kain et al., 2008), and expression of G α_q RNA interference (RNAi) in ORNs led to decreased odor-evoked behavioral responses (Kalidas and Smith, 2002).

Whereas most antennal ORNs express Ors, which mediate responses to odors, one class of ORNs in the antenna expresses two Grs that mediate a response to CO₂ (Jones et al., 2007; Kwon et al., 2007). Many insect species have a similar class of ORNs dedicated to CO₂ sensation (Grant et al., 1995; Lu et al., 2007; Syed and Leal, 2007). However, the mechanism of CO₂ transduction in insects is unknown.

In this study, we investigate whether G-proteins are required for either Or- or Gr-mediated responses in the antenna. We disrupted signaling of each *Drosophila* G α and G γ protein individually *in vivo*, via several independent means, and generated null clones of G α_q , G α_o , and G α_s . We also investigated the G γ subunits. We found that individually disrupting each G α and G γ had little or no effect on the Or-mediated responses tested. However, disruption of G α_q or G γ 30A decreased the Gr-mediated CO₂ response. Using mosaic analysis, conditional and ectopic expression analyses, and transient expression of constitutively active G α proteins, we show that G α_q is necessary for both the development and function of the CO₂-sensing neuron. Our results support a model in which G α_q and G γ 30A play a role in CO₂ response but are not required for Or-mediated olfactory transduction.

Materials and Methods

Drosophila stocks and transgenes. To generate DG α_q -GAL4, 5.1 kb of DNA upstream of the ATG of G α_q -3 was inserted into the pGAL4 vector. The primers used were 5'-AGCGACAAACTGGGGAACAACTG and 5'-GCTAACTAGATTTATAGACGCTTTCGCGTG. To generate the UAS-G α peptide constructs, PAGE-purified, phosphorylated complementary primers were annealed and cloned into an upstream activation

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Correspondence should be addressed to Dr. John R. Carlson at the above address. E-mail: john.carlson@yale.edu.
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sequence (UAS) expression vector. Each peptide consists of a start codon, a glycine, which has been shown to stabilize the peptide (Gilchrist et al., 2002) and the last 11 amino acids of the specified $G\alpha$. Primer sequences were 5'-AATTCATGGGAGTCAATCGAACCTTAAGGAATATAATTTG-GTCTAAGC and 5'-GGCCGCTTAGACCAAATATATTCCTTAA-GTTTCGATTGCAAGTCCCATG ($G\alpha_q$), 5'-AATTCATGGGACAAAG-GATGCACCTTCGTCAATATGAATTGTTATAGGC and 5'-GGCCGCT-TATAACAATTCATATTGACGAAGGTGCATCCTTTGTCCCATG ($G\alpha_s$), 5'-AATTCATGGGACTCTCGGAGAACGTGTCCAGCATG-GGCCTATTCTAGGC and 5'-GGCCGCTTAGAATAGGCCCATGCTG-GACACGTTCTCCGAGAGTCCCATG ($G\alpha_{73B}$), 5'-AATTCATGG-GAATGACAAACACCTGCGCGGTGTGGACTGTACTAAGC and 5'-GGCCGCTTAGTACAGTCCACAGCCGCGCAGGTTGTTTGTCTAT-TCCCATG ($G\alpha_o$), 5'-AATTCATGGGAATCAAGAACAATCTGAAA-CAAATTGGCTTATTCTGAGC and 5'-GGCCGCTCAGAATAAGC-CAATTTGTTTCAGATTGTTCTTGATTCCCATG ($G\alpha_i$), and 5'-AAT-TTCATGGGAGTGATTAATATCTCTCTAAACCGGAAGTGGATATA-AGC and 5'-GGCCGCTTATATCCAGTTCGGGTTTAGAGAGATA-TTAATCACTCCCATG ($G\alpha_q$ scrambled).

The *Gr21a-mCD8-GFP* line was from Barry Dickson (Research Institute of Molecular Pathology, Vienna, Austria). The *UAS-DG α_q RNAi* line was from Michael Forte (Vollum Institute, Portland, OR). $G\alpha_q^{221C}$, *UAS-G α_q^{1F1} RNAi*, *UAS-G α_q* , *UAS-G α_q^{GTP}* , and *G α_q -GAL4* lines were from Gaiti Hasan (Tata Institute of Fundamental Research, Bangalore, India). The *UAS-G α_q^{GTP}* line was from Fumio Matsuzaki (RIKEN, Kobe, Japan). The *norpA²⁴* line was from William Pak (Purdue University, West Lafayette, IN). The *Gr21a-GAL4* line was from Kristin Scott (University of California, Berkeley, CA). *Or83b-GAL4* and *UAS-G α_q RNAi* lines were from Dean P. Smith (University of Texas Southwestern, Dallas, TX). The *P{GawB}NP1535* precise excision line was from Teiichi Tanimura (Kyushu University, Fukuoka, Japan). *UAS-G α_o^{GTP}* , $G\alpha_o^{007}$, and *UAS-ptx* lines were from Andrew Tomlinson (Columbia University, New York, NY). The *concertina^{RC10}* line was from Eric Wieschaus (Princeton University, Princeton, NJ). $G\alpha_q^1$ and *inaD* lines were from Charles Zuker (University of California, San Diego, La Jolla, CA). The *UAS-G α_o RNAi*, *UAS-G α_{73B} RNAi*, *UAS-G α_s RNAi*, *UAS-G γ_{30A} RNAi*, and *UAS-G γ_1 RNAi* lines were obtained from the National Institute of Genetics Fly Stock Center (Shizuoka, Japan). *UAS-G α_s* , *UAS-G α_s^{GTP}* , *yw*; *PNP1535/CyO*, and *yw*; *P{GawB}NP6216/CyO* lines were obtained from the *Drosophila* Genetic Resource Center (Kyoto, Japan). *G β 76C¹*, *tub-GAL80^{ts}*, *ey-FLP*, *FRT 42B*, $G\alpha_s^{R60}$, *FRT 42B*, *tubP-GAL80/CyO*, *FRT 42D*, *tubP-GAL80/CyO*, *UAS-DsRed*, *UAS-GFP*, *PBac{RB}Ggamma30Ae00834*, *hs-GAL4*, $G\alpha_s^{R60}$, and *Df(2L)ED678/CyO* lines were obtained from the Bloomington *Drosophila* Stock Center (Bloomington, IN). *Or22a-GAL4*, *UAS-Gr63a*, *UAS-Gr21a*, and *Δ halo* lines, in which *Or22a* and *Or22b* are deleted, were described previously (Dobritsa et al., 2003; Kwon et al., 2007).

Isolation of a $G\alpha_q$ mutant. The $G\alpha_q^{28}$ deletion line was generated by P-element excision of the *yw*; *P{GawB}NP6216/CyO* strain, which carries the *P{GawB}* insertion element in the 5' UTR of $G\alpha_q$. The P-element was mobilized using $\Delta 2-3$. Approximately 400 *w⁻* flies were screened by PCR for deletions in the $G\alpha_q$ coding region. Only one mutant, $G\alpha_q^{28}$, had a deletion that extended into the coding region. The mutation was mapped and confirmed by single-egg PCR analysis using the following primers: 5'-GCTGAACGTGCCAGGAA ATCCG and 5'-TTTAAATCTA-CAAACATTCGCAGT.

Electrophysiology. All single-unit recordings were performed as described previously (de Bruyne et al., 2001). All recordings were from female antennal sensilla with the exception of the mosaic analysis with a repressible cell marker (MARCM) and empty neuron experiment recordings, which were from males and females. Age- and gender-matched flies were used for the control recordings. All flies tested had constructs that were either injected or backcrossed into *w¹¹¹⁸* flies, ensuring a similar genetic background. Flies expressing the appropriate *GAL4* construct alone were used as the control in all experiments except for the antennal basiconic 1C (ab1C) $G\alpha$ peptide experiments. $G\alpha$ peptide constructs were injected into a wild-type strain that appears to have a low CO₂ response. Therefore, flies carrying the *UAS-G α* peptide alone were used as the control in these experiments. Methyl butyrate, ethyl acetate, and

butyric acid were obtained from Sigma. Methyl butyrate and ethyl acetate were dissolved in paraffin oil, whereas butyric acid was dissolved in water. A 500 ms pulse of odorant was puffed into a continuous air stream that was directed at the antenna. CO₂ recordings were performed essentially as described previously (Kwon et al., 2007). Briefly, a 25 ml/s air stream was replaced for 500 ms by a 25 ml/s flow of 0.1, 0.5, 1, 2, 5, or 20% CO₂ dissolved in N₂ (Airgas). The firing rates for both olfactory and CO₂ recordings were calculated by counting the number of spikes fired during the 500 ms stimulus period and multiplying by two. Neither background nor control pulse firing rates were subtracted. Statistical analysis of the methyl butyrate and butyric acid responses was performed using two-tailed Student's *t* test.

Immunohistochemistry. *w*; *Gq-GAL4/Gr21a-mCD8-GFP*; *UAS-DsRed* and *w*; *DGq-GAL4/Gr21a-mCD8-GFP*; *UAS-DsRed* flies were used to localize $G\alpha_q$. Sixteen micrometer sections of the antenna were fixed in 4% paraformaldehyde, washed extensively in PBS plus 0.3% Triton-X (PBS-T), and fixed in PBS-T plus 5% normal goat serum. The primary antibodies used were anti-DsRed (1:200; catalog #632496; Clontech) and anti-green fluorescent protein (GFP; 1:200; catalog #11814460001; Roche). Secondary antibodies used were anti-rabbit IgG conjugated to Alexa Fluor 568 (1:250; A10042; Invitrogen) and anti-rabbit IgG conjugated to Alexa Fluor 488 (1:500; A11001; Invitrogen). Samples were mounted in Vectashield (Vector Laboratories) and viewed on a Bio-Rad MRC 1024 confocal microscope.

Generation of null clones. $G\alpha_q$, $G\alpha_o$, and $G\alpha_s$ null clones were generated using the MARCM system (Lee and Luo, 2001). *ey-FLP* was used to generate clones in the antenna (Hummel et al., 2003). The *Gr21a-GAL4* reporter was used to identify clones including a CO₂ neuron, whereas the *Or22a-GAL4* reporter was used to identify clones including an ab3A neuron. The control recordings in the MARCM experiments were taken from GFP⁻ neurons on the same antennae as the GFP⁺ neurons as a precaution against genetic background and age effects.

Flies of the following genotypes were tested: (1) *w*; *FRT 42B*, $G\alpha_q^{28}/FRT 42B$, *tubP-GAL80*; *X-GAL4*, *UAS-GFP/ey-FLP* ($G\alpha_q$); (2) *w*; *FRT 42D*, $G\alpha_s^{R60}/FRT 42D$, *tubP-GAL80*; *X-GAL4*, *UAS-GFP/ey-FLP* ($G\alpha_s$); (3) *w*; *FRT 42D*, $G\alpha_o^{007}/FRT 42D$, *tubP-GAL80*; *X-GAL4*, *UAS-GFP/ey-FLP* ($G\alpha_o$); (4) *w*; *FRT 42B/FRT 42B*, *tubP-GAL80*; *X-GAL4*, *UAS-GFP/ey-FLP* (control).

Flies of the following genotype were used for the $G\alpha_s$ rescue experiment: *UAS-G α_s* ; *FRT 42D*, $G\alpha_s^{R60}/FRT 42D$, *tubP-GAL80*; *X-GAL4*, *UAS-GFP/ey-FLP*. These flies express $G\alpha_s$ only in ab1C or ab3A that are genotypically null for $G\alpha_s$.

Using MARCM, we were able to reliably produce $G\alpha_q^{-/-}$ ab3A neurons, $G\alpha_s^{-/-}$ ab3A neurons, and $G\alpha_s^{-/-}$ ab1C neurons at frequencies of approximately five GFP⁺ neurons per antenna. However, despite using two different $G\alpha_q$ deletion alleles, $G\alpha_q^{221C}$ and $G\alpha_q^{28}$, in combination with each of two different markers of ab1C neurons, *Gr21a-GAL4* and *Gr63a-GAL4*, few if any GFP⁺ ab1C neurons were generated. In two cases we observed no GFP⁺ ab1C neurons, in the third case we found a single GFP⁺ ab1C neuron in 74 antennae examined, and in the fourth case we observed a very low frequency of GFP⁺ ab1C neurons. Furthermore, the few GFP⁺ ab1C neurons generated do not resemble any of the GFP⁺ cells in the other experiments. These labeled ab1C neurons have much weaker GFP fluorescence, and the fluorescence could often be observed only in the cell body. Because of their low frequency and very weak GFP fluorescence, we are not confident that these GFP⁺ ab1C neurons are $G\alpha_q^{-/-}$.

Spatiotemporal expression of $G\alpha_q$ inhibitors. Temporal and regional gene expression targeting (TARGET) was used to express *UAS-G α_q^{GTP}* or *UAS-G α_q^{1F1} RNAi* selectively in adult flies (McGuire et al., 2003). *w*; *UAS-X*; *Gr21a-GAL4*, *tub-GAL80^{ts}* flies were raised at 18°C and transferred to 29°C 1 day after eclosion. Genetically identical, age-matched flies that were maintained at 18°C rather than transferred to 29°C were used as the control. As an additional guard against leaky $G\alpha_q^{GTP}$ or $G\alpha_q^{1F1}$ RNAi expression, we examined the antennae of 20-d-old adult *w*; *UAS-GFP*; *Gr21a-GAL4*, *tub-GAL80^{ts}* flies raised and maintained at 18°C. These flies did not show any GFP activity, confirming that *tub-GAL80^{ts}* suppresses *Gr21a-GAL4* at the permissive temperature (data not shown). Finally, we compared the responses of *Gr21a-GAL4* flies that

carried a UAS construct to those of controls that did not carry a UAS construct. Flies that do not have the UAS construct had similar CO₂ responses to the control flies that were not heat shocked.

hs-GAL4 and *UAS-Gα_q^{GTP}* or *UAS-Gα_s^{GTP}* were used to transiently express *Gα_q^{GTP}* or *Gα_s^{GTP}* in adult flies. Seven-day-old adult flies reared at 18°C were heat shocked for 30 min at 37°C and allowed to recover for 1 h at 25°C. Siblings that were placed at 25°C for 1.5 h were used as controls.

Statistical analysis. Each ORN was tested with multiple concentrations of each ligand. Therefore, we compared the responses from different genotypes using a two-way ANOVA with repeated measures in one factor followed by a Holm–Sidak *post hoc* test to compare individual concentrations. The between-group factor is the genotype of the fly, and the repeated measure is the concentration of the ligand. Two-tailed *t* tests were used to compare the spontaneous firing rates of neurons.

Results

Knockdown of *Gα_q* reduces CO₂ response but not odor response

As a first step toward testing the role of G-proteins in olfactory responses, we identified the *Gα* genes that are expressed in the antenna. The *Drosophila* genome contains eight *Gα* genes. We found evidence for antennal expression of six of them, *concertina*, *Gα_q*, *Gα_s*, *Gα73B*, *Gα_o*, and *Gα_i*, by reverse transcriptase (RT)-PCR analysis of adult antennal RNA (data not shown).

Drosophila antennae contain three functional types of large antennal basiconic (ab) sensilla, ab1, ab2, and ab3 (Fig. 1*a,b*). ab2 contains two ORNs, ab2A and ab2B, and ab3 contains ab3A and ab3B (de Bruyne et al., 2001). The odor response of each of these neurons is mediated by an Or unique to that ORN and Or83b, a broadly expressed coreceptor (Hallem et al., 2004; Larsson et al., 2004). ab1 contains four ORNs. Three of the neurons, ab1A, ab1B, and ab1D, have odor responses that are also mediated by a unique Or and Or83b. The other neuron, ab1C, responds uniquely to CO₂. This neuron does not express Or83b; instead, the CO₂ response is mediated by two Grs, Gr21a and Gr63a (Jones et al., 2007; Kwon et al., 2007).

To investigate the roles of the six *Gα* proteins in olfactory and CO₂ transduction in the antenna, we focused on responses from three Or-expressing ORNs, ab1A, ab2A and ab3A, and the Gr-expressing ORN, ab1C (Fig. 1*b*). To test whether disruption of *Gα* affects the function of the ORNs, we performed extracellular electrophysiological recordings (Clyne et al., 1997; de Bruyne et al., 2001) that allowed us to measure the response of a single ORN to either odors or CO₂. Among the six *Gα* genes expressed in the antenna, a homozygous viable mutant was available for one, *concertina*. We tested this mutant by electrophysiological recording and found that ab1A, ab2A, ab3A, and ab1C responded normally to broad concentrations of odorants and CO₂ (*n* = 6; *p* > 0.5 for each neuron class tested; data not shown). Therefore, *concertina* was not investigated further.

Figure 1. Experimental systems. *a*, *Drosophila* head. The arrowhead indicates the antenna. *b*, Schematic of antenna. Different colored dots represent different functional types of basiconic sensilla. *c*, Disruption of G-proteins in the endogenous CO₂ neuron. *d*, Heterologous expression of the CO₂ receptors with and without G-proteins. *e*, Sample recording traces from control and *Gα_q* RNAi-expressing ab1C. Panel *a* was adapted from Carlson (1996), *b* was adapted from van der Goes van Naters and Carlson (2006), and *c* and *d* were adapted from Kwon et al. (2007).

We then performed genetic manipulations that resulted in either loss of function or gain of function to investigate the roles of the five remaining *Gα* genes. We used either *Or83b-GAL4* (Kalidas and Smith, 2002) or *Gr21a-GAL4* (Scott et al., 2001) to drive expression of RNAi constructs or competitive peptides (loss of function), or constitutively active *Gα* proteins (gain of function) (Fig. 1*c*). Since *Or83b-GAL4* is expressed exclusively in Or-expressing ORNs and *Gr21a-GAL4* is expressed solely in ab1C, we could ask whether the different *Gα* proteins function in ORNs as opposed to other cell types.

First, we examined the requirement of G-proteins in ORNs by knocking down *Gα* activity with available *Gα* RNAi constructs. We found that RNAi against *Gα_q*, *Gα_s*, *Gα_o*, and *Gα73B* did not reduce the odor responses of ab1A, ab2A, or ab3A (Fig. 2*a*). Our results might seem to contradict a previous report showing that flies expressing this *Gα_q* RNAi construct had decreased behavioral responses to odors (Kalidas and Smith, 2002). One possible explanation is that *Gα_q* is required for behavioral response in a process that occurs downstream of ligand recognition and transduction, perhaps by coupling to other GPCRs. For instance, recent studies have found that ORNs express GABA_B receptors at the axon terminals and that expression of a *Gα_o* inhibitor alters

Figure 2. RNAi. *a*, RNAi against $G\alpha_q$, $G\alpha_s$, $G\alpha_o$, and $G\alpha_{73B}$ did not decrease odor responses of ab1A, ab2A, or ab3A ($p > 0.05$), ORNs whose responses are mediated by Ors (Dobritsa et al., 2003; Hallem et al., 2004; Larsson et al., 2004; Couto et al., 2005). *b*, $G\alpha_q$ RNAi decreased response to CO₂ ($p < 0.001$), which is mediated by Gr21a/Gr63a (Jones et al., 2007; Kwon et al., 2007). Overexpression of $G\alpha_q$ in a $G\alpha_q$ RNAi background rescued the CO₂ response. RNAi against $G\alpha_s$, $G\alpha_o$, and $G\alpha_{73B}$ did not affect CO₂ response ($p > 0.05$). $n = 6$ –17. Error bars indicate SEM.

synaptic transmission between the ORN and second-order neurons (Olsen and Wilson, 2008; Root et al., 2008).

A second report showed that a different $G\alpha_q$ RNAi construct, $G\alpha_q^{1F1}$, decreased the electrophysiological odor response of ab2A (Kain et al., 2008). We also tested $G\alpha_q^{1F1}$ RNAi using the same broad odorant concentration range used to test the previous $G\alpha_q$ RNAi construct and, consistent with our previous results, we did not observe a decrease in odor response in ab1A, ab2A, or ab3A ($n = 6$; $p > 0.1$ for each neuron class; data not shown). The discrepancy with the other study may be attributable to the use of different drivers: we used *Or83b-GAL4*, whereas the previous analysis of ab2A used $G\alpha_q$ -*GAL4*, which expresses in non-neuronal support cells as well as in ORNs (Talluri et al., 1995; Kain et al., 2008). Therefore, the reduction in olfactory sensitivity in the previous study could have been caused by effects on cells other than ORNs.

In contrast, $G\alpha_q$ RNAi, but not other $G\alpha$ RNAi constructs, produced a major decrease in CO₂ response (Figs. 1*e*, 2*b*). The CO₂ response was significantly reduced across a wide response range (Fig. 2*b*). If this reduction in CO₂ response is in fact caused by an RNAi-mediated reduction in levels of $G\alpha_q$, then overexpression of $G\alpha_q$ RNA should rescue the response. Indeed, overexpression of $G\alpha_q$ -3 RNA, the antennal $G\alpha_q$ splice variant (Talluri et al., 1995; Ratnaparkhi et al., 2002; Kain et al., 2008),

restored the CO₂ response in flies expressing $G\alpha_q$ RNAi (Fig. 2*b*). To test whether CO₂ response depends specifically on the antennal $G\alpha_q$ splice variant, we tested flies with a missense mutation that only affects the other major $G\alpha_q$ splice variant, $G\alpha_q$ -1 (Scott et al., 1995), and found no defect in response to concentrations of CO₂ ranging from 0.1 to 20% ($n = 9$; $p > 0.1$; data not shown). We also tested the second $G\alpha_q$ RNAi construct, $G\alpha_q^{1F1}$, for its effects on CO₂ response and obtained results similar to those with the first $G\alpha_q$ RNAi construct. $G\alpha_q^{1F1}$ RNAi decreased CO₂ response across a wide concentration range; at concentrations $>0.1\%$ there was a 25–30% decrease in CO₂ response ($n = 12$; $p < 0.001$; data not shown). Again, expression of $G\alpha_q$ -3 RNA rescued the defect ($n = 9$; data not shown).

Recent studies have suggested that $G\alpha_s$ may play a role in chemosensory transduction (Wicher et al., 2008). Therefore, we tested an additional $G\alpha_s$ RNAi construct, *DG* α_s RNAi, with the same broad concentration range of odorants. Expression of *DG* α_s RNAi in *Gr5a*-expressing taste neurons reduced $G\alpha_s$ expression levels and reduced electrophysiological responses to sugar (Ueno et al., 2006). However, consistent with our results with the other $G\alpha_s$ RNAi construct, *DG* α_s RNAi did not decrease the odor or CO₂ responses of ab1A, ab2A, ab3A, or ab1C ($n = 6$ –12; data not shown). Taken together, our RNAi data suggest that $G\alpha_q$ is re-

Figure 3. Constitutively active $G\alpha$ proteins. **a**, $G\alpha_q^{GTP}$ decreased the odor responses of ab1A, ab2A, and ab3A ($p \leq 0.01$), whereas $G\alpha_s^{GTP}$, $G\alpha_o^{GTP}$, and $G\alpha_i^{GTP}$ did not ($p > 0.05$). **b**, $G\alpha_q^{GTP}$ dramatically decreased CO₂ response ($p < 0.001$), whereas $G\alpha_s^{GTP}$, $G\alpha_o^{GTP}$, and $G\alpha_i^{GTP}$ had no effect ($p > 0.05$). $n = 6$ –15. Error bars indicate SEM.

quired for CO₂ response, whereas no $G\alpha$ proteins are required for Or-mediated responses.

Competitive $G\alpha_q$ peptide reduces CO₂ response but not odor response

As a complementary approach to RNAi, we next used competitive peptides to knock down $G\alpha$ protein activity. Peptides consisting of the 11 C-terminal amino acids of a $G\alpha$ protein have been shown to bind to GPCRs and compete with the endogenous $G\alpha$ (Hamm et al., 1988; Gilchrist et al., 1999). Such peptides do not activate downstream signaling pathways and have been shown to decrease the receptor-mediated response to ligands in cell culture and in transgenic mice (Rasenick et al., 1994; Akhter et al., 1998).

We generated *UAS* lines that express the 11 C-terminal amino acids of $G\alpha_q$, $G\alpha_s$, $G\alpha_o$, $G\alpha_{73B}$, and $G\alpha_i$, and expressed each using *Or83b-GAL4* or *Gr21a-GAL4*. Consistent with the RNAi results, none of the peptides affected the Or-mediated ab1A, ab2A, and ab3A responses ($n = 6$; $p > 0.05$ for each neuron class; data not shown). However, the $G\alpha_q$ peptide decreased ab1C response to concentrations of CO₂ ranging from 0.5 to 20% ($n = 24$; $p < 0.001$; data not shown). Also consistent with the RNAi results, $G\alpha_s$, $G\alpha_o$, $G\alpha_{73B}$, and $G\alpha_i$ peptides did not affect the ab1C response to any of the five CO₂ concentrations tested ($n = 9$ –21; $p > 0.05$; data not shown). As an additional control for the

specificity of the $G\alpha_q$ peptide, we generated a scrambled $G\alpha_q$ peptide that has the same amino acid composition as the $G\alpha_q$ peptide, but in a random order. Flies expressing the $G\alpha_q$ scrambled peptide responded to CO₂ at levels comparable to control flies at all concentrations tested ($n = 15$; $p > 0.05$; data not shown).

Constitutively active $G\alpha_q$ reduces CO₂ response

The previous two methods sought to knock down $G\alpha$ protein signaling. We next used a gain-of-function manipulation: expression of a constitutively active form of a $G\alpha$ protein, $G\alpha^{GTP}$. $G\alpha^{GTP}$ proteins contain a single point mutation that renders them unable to hydrolyze GTP and, therefore, constitutively active (Bourne et al., 1991). If a certain $G\alpha$ protein mediates olfactory or CO₂ transduction, constitutive activation of the G-protein should mimic prolonged exposure to odors or CO₂, resulting in ORN adaptation and subsequent decrease in sensitivity.

$G\alpha_q^{GTP}$ nearly abolished CO₂ response at all tested doses (Fig. 3b). The decreased CO₂ response is not attributable to cell death: ab1C expressing $G\alpha_q^{GTP}$ and *GFP* appear morphologically normal, as observed in confocal images (data not shown). The severe reduction of CO₂ sensitivity after long-term expression of $G\alpha_q^{GTP}$ is consistent with the notion that $G\alpha_q$ mediates CO₂ transduction. Mutations in human rhodopsin that result in a constitutively active opsin protein lead to photoreceptor adapta-

tion and loss of cell sensitivity (Jin et al., 2003). Constitutive activation of ab1C may result in a similar loss of CO₂ sensitivity.

The effect of $G\alpha_q^{GTP}$ is specific: expression of $G\alpha_s^{GTP}$, $G\alpha_o^{GTP}$, and $G\alpha_i^{GTP}$ did not affect CO₂ response (Fig. 3*b*). Thus, the finding that CO₂ response is specifically affected by $G\alpha_q^{GTP}$ is in agreement with previous results.

Odor responses of ab1A, ab2A, and ab3A were not reduced by expression of $G\alpha_s^{GTP}$, $G\alpha_o^{GTP}$, or $G\alpha_i^{GTP}$ (Fig. 3*a*), consistent with our previous data. However, $G\alpha_q^{GTP}$ did reduce the odor responses of ab1A, ab2A, and ab3A (Fig. 3*a*). Notably, the effect of $G\alpha_q^{GTP}$ on CO₂ response is much more severe than on odor responses. To better understand the different effects of $G\alpha_q^{GTP}$ on the Or- and Gr-mediated responses in these long-term expression studies, we next examined how transient expression of $G\alpha_q^{GTP}$ affects the neurons.

Transient expression of $G\alpha_q^{GTP}$ increases the spontaneous firing of CO₂-sensing neurons but not odor-sensing neurons

As shown above, long-term expression of $G\alpha_q^{GTP}$ resulted in decreased responses from both odor-sensitive and CO₂-sensitive neurons (Fig. 3*a,b*). Based on our previous data, which showed that loss of $G\alpha_q$ function affected Gr- and Or-expressing neurons differently, long-term expression of $G\alpha_q^{GTP}$ may be decreasing the odor and CO₂ responses via different mechanisms. For instance, $G\alpha_q^{GTP}$ could decrease responses by causing either adaptation or deterioration in health. To distinguish between these two possibilities and to investigate whether Grs or Ors signal through $G\alpha_q$, we transiently expressed $G\alpha_q^{GTP}$ in adult flies using *hs-GAL4* and tested the flies immediately. Non-heat-shocked siblings were used as controls. If $G\alpha_q$ mediates odor or CO₂ responses, transient expression of $G\alpha_q^{GTP}$ should mimic constitutive receptor activation, even in the absence of a ligand. Consistent with this prediction, we found that transient expression of $G\alpha_q^{GTP}$ produced a threefold increase in the spontaneous firing rate of ab1C (Fig. 4*a,b*). This effect is specific to ab1C; $G\alpha_q^{GTP}$ expression in ab3A does not affect its spontaneous firing rate (Fig. 4*c,d*). The effect is also specific to $G\alpha_q^{GTP}$, as transient $G\alpha_s^{GTP}$ expression in neither ab1C nor ab3A affects its spontaneous firing rate ($n = 12$; $p > 0.4$ for each neuron class; data not shown).

The increase in spontaneous firing observed in ab1C is consistent with the direct involvement of $G\alpha_q$ in CO₂ signaling. Conversely, short-term expression of $G\alpha_q^{GTP}$ in ab3A did not affect spontaneous firing. Consistent with our $G\alpha_q$ loss-of-function results, this result suggests that $G\alpha_q$ does not act directly in ab3A signaling. An indirect role, such as a role in neuronal maintenance, may thus account for the effect of long-term $G\alpha_q^{GTP}$ expression in ab1A, ab2A, and ab3A (Fig. 3*a*). The finding that transient expression of $G\alpha_q^{GTP}$ increases the spontaneous firing of ab1C but not ab3A further suggests that $G\alpha_q$ is involved directly in CO₂ signaling but not odor signaling.

Next, we examined the odor and CO₂ responses of ab3A and ab1C neurons that transiently express $G\alpha_q^{GTP}$. Our results thus far suggest that long-term expression of $G\alpha_q^{GTP}$ decreases ab1C responses via adaptation of the neuron, whereas $G\alpha_q^{GTP}$ may affect the health of ab3A. If this interpretation is correct, we would predict that short-term expression of $G\alpha_q^{GTP}$ should affect their responses differently. In accordance with this prediction, ab3A neurons that transiently express $G\alpha_q^{GTP}$ have normal responses to odorant dilutions ranging from 10^{-8} to 10^{-2} ($n = 9$; $p > 0.8$; data not shown). Conversely, ab1C neurons that transiently express $G\alpha_q^{GTP}$ have a reduced response to CO₂ ($n = 12$; $p < 0.001$; data not shown). Moreover, the reduction in CO₂ response is much less pronounced than in flies that expressed

$G\alpha_q^{GTP}$ for longer. In heat shocked flies, the response to 1% CO₂ is reduced by 33% compared to 88% for long-term expression (Fig. 3*b*) (data not shown). The most likely explanation for these results is that the longer expression period of $G\alpha_q^{GTP}$ produces a greater degree of adaptation and results in a greater loss of sensitivity.

The results, taken together, are consistent with a model in which G-proteins act directly in Gr21a/Gr63a signaling but not in Or signaling.

Verification of $G\alpha_q$ expression in CO₂-sensitive neurons

Above we used three independent means of altering levels of $G\alpha_q$ signaling in ab1C, and all three affected CO₂ response. Others have shown that $G\alpha_q$ is expressed in a majority of ORNs, including the large basiconic ORNs tested in this study (Talluri et al., 1995; Kain et al., 2008). However, to confirm that ab1C expresses $G\alpha_q$, we performed a double-label analysis. To visualize ab1C, we used a *Gr21a-GFP* direct fusion line (Couto et al., 2005) and observed GFP in a subset of large basiconic sensilla on the proximomedial region of the antenna, consistent with the location of ab1C neurons (de Bruyne et al., 2001).

To determine whether $G\alpha_q$ is expressed in these GFP-expressing cells, we first used an anti- $G\alpha_q$ antibody in a double-label experiment. However, the antibody showed a broad labeling pattern with a signal-to-noise ratio that was not sufficiently high for us to draw confident conclusions about colocalization. As a second means of determining whether the GFP-expressing ab1C cells express $G\alpha_q$, we used two independently generated *GAL4* lines driven by the $G\alpha_q$ promoter, $G\alpha_q$ -*GAL4* (Kain et al., 2008) and *DGαq*-*GAL4*, which we generated. Flies carrying $G\alpha_q$ -*GAL4*; *UAS-DsRed* or *DGαq*-*GAL4*; *UAS-DsRed* both showed expression of *DsRed* in many cells broadly distributed across the antenna (data not shown), as reported previously in *Drosophila* (Talluri et al., 1995; Ratnaparkhi et al., 2002; Kain et al., 2008), and consistent with results found with an *Anopheles* ortholog (Rutzler et al., 2006). Previous reports have shown that $G\alpha_q$ is expressed in a majority of ORNs as well as in non-neuronal support cells (Talluri et al., 1995; Kain et al., 2008). All GFP-positive cells were also positive for *DsRed*, indicating that all *Gr21a-GFP*-expressing neurons also express $G\alpha_q$ -*GAL4* (data not shown). Thus, these results support the conclusion that the CO₂-sensing neurons express $G\alpha_q$.

Analysis of odor and CO₂ responses in $G\alpha_q$, $G\alpha_s$, and $G\alpha_o$ null clones

Next, we examined ORNs that are null for $G\alpha$ proteins. We examined $G\alpha_q$, $G\alpha_s$, and $G\alpha_o$ because these three $G\alpha$ genes have been identified in ORNs in many different species of insects (Talluri et al., 1995; Miura et al., 2005; Rutzler et al., 2006). Null alleles for $G\alpha_q$ (Banerjee et al., 2006), $G\alpha_s$ (Wolfgang et al., 2001), and $G\alpha_o$ (Katanaev et al., 2005) are homozygous lethal. Therefore, we used the MARCM system (Lee and Luo, 2001) to generate homozygous null mutant clones of these $G\alpha$ genes in the antenna. We used *Gr21a-GAL4*, *UAS-GFP* to identify mutant ab1C neurons and *Or22a-GAL4*, *UAS-GFP* to identify mutant ab3A neurons.

As a control for efficiency of recombination, we first generated clones in a wild-type background that contained no mutations of G-protein genes. We found that GFP⁺ ab3A and ab1C were generated consistently: 5.5 ± 1.1 and 4.5 ± 0.8 GFP⁺ neurons were detected per antenna, respectively ($n = 22$ antennae; $n = 20$ antennae), with ~80% of all antennae examined having at least one GFP⁺ neuron. This control experiment established criteria by which to evaluate the frequency and appearance of GFP⁺ cells mutant for G-protein genes.

We then generated null clones of $G\alpha_q$. The frequencies at which GFP⁺ ab3A and ab1C were generated (7.0 ± 1.2 neurons/antenna; 5.7 ± 0.7 GFP⁺ neurons/antenna) were similar to the frequencies in the control experiment. $G\alpha_q^{-/-}$ ab1C showed normal responses to CO₂ in 6-d-old flies, consistent with our previous analyses showing that $G\alpha_q$ is not required for CO₂ signaling (Fig. 5*a*). When older flies (~ 20 d) were examined, a decrease in CO₂ response was observed in $G\alpha_q^{-/-}$ ab1C neurons at all concentrations tested, with a 33% decrease in response at 1% CO₂ ($n = 16$ – 18 ; $p < 0.001$). Since the decrease was observed only in older flies, it may reflect a progressive deterioration of neuronal health, rather than a specific defect in the forward pathway of CO₂ signaling. The simplest interpretation of these results is that $G\alpha_q$ is not required for CO₂ signaling.

The absence of $G\alpha_q$ in ab3A did not affect its response to low concentrations of methyl butyrate (0.05% and lower) or butyric acid (1% and lower) (Fig. 5*b,c*). However, at near-saturating concentrations of both odorants tested, there was a small ($\leq 17.0\%$) decrease in the odor response regardless of the age of the fly. These abnormalities were rescued by driving a copy of the $G\alpha_q$ gene specifically in ab3A, suggesting that the role of $G\alpha_q$ is cell autonomous (Fig. 5*d,e*). Thus, $G\alpha_q$ may contribute to ab3A odor responses, but its contribution is very modest.

For $G\alpha_q$, we generated a null allele, $G\alpha_q^{28}$. This allele was isolated by imprecise excision of a P-element in the 5' UTR of $G\alpha_q$ -3. $G\alpha_q^{28}$ contains a 5428 bp deletion that removes the first eight exons of $G\alpha_q$ -3. $G\alpha_q^{28}$ yielded GFP⁺ ab3A at a frequency similar to that of the control experiment (5.3 ± 1.3 vs 5.5 ± 1.1 GFP⁺ neurons/antenna; $n = 14$ and $n = 22$ antennae, respectively). Consistent with our previous results, the absence of $G\alpha_q$ in ab3A did not affect olfactory responses to the two odors tested (Fig. 5*f,g*). A previous report showed that mosaic analysis with another $G\alpha_q$ null allele, $G\alpha_q^{221C}$, resulted in decreased electroantennogram responses, which represent a decrease in the summed receptor potentials of populations of antennal neurons (Kain et al., 2008). To address this discrepancy, we tested the null allele used in this report ($G\alpha_q^{221C}$) and obtained results indistinguishable from those with $G\alpha_q^{28}$ both in terms of the frequency of clones generated and the phenotype of the $G\alpha_q^{221C}$ clones (Fig. 5*h,i*). Taken together, the simplest interpretation of our results is that $G\alpha_q$ is not required for olfactory transduction in ab3A.

Efforts to produce $G\alpha_q^{-/-}$ ab1C yielded very different results from those designed to produce $G\alpha_q^{-/-}$ ab3A or control clones with marked ab1C neurons. First, GFP⁺ ab1C neurons were either not generated or generated at a very low frequency in the $G\alpha_q$ MARCM experiments (see Materials and Methods). Despite using two different $G\alpha_q$ deletion alleles, $G\alpha_q^{28}$ and $G\alpha_q^{221C}$, and two different markers of ab1C neurons, *Gr21a-GAL4* and *Gr63a-GAL4*, few if any GFP⁺ ab1C neurons were generated, implicating a crucial role for $G\alpha_q$ in ab1C development and/or survival.

No $G\alpha_q^{-/-}$ ab1C or ab3A neurons were identified. This is consistent with findings by others that $G\alpha_o$ is required for asym-

Figure 4. Transient expression of $G\alpha_q^{GTP}$. *a*, Traces of ab1C spontaneous firing from control flies and flies transiently expressing $G\alpha_q^{GTP}$. Dots mark spikes from ab1C, which can be distinguished by their relative amplitudes; other spikes derive from other neurons in the same sensillum. *b*, Heat-shock-induced expression of $G\alpha_q^{GTP}$ increased the spontaneous firing rate of ab1C threefold. *c*, Traces of ab3A spontaneous firing from $G\alpha_q^{GTP}$ -expressing and control flies. Dots mark spikes from the ab3A neuron. *d*, The spontaneous firing rate of ab3A was unaffected by $G\alpha_q^{GTP}$ expression ($p = 0.4$). $n = 9$ – 17 . Error bars indicate SEM.

metrical cell division and nervous system development (Katanaev et al., 2005; Katanaev and Tomlinson, 2006).

In summary, $G\alpha_q^{-/-}$ ab1C had normal responses to CO₂, $G\alpha_q^{-/-}$ ab3A had small reductions in odor response, $G\alpha_q^{-/-}$ ab3A was normal in response to two odorants tested, and efforts to determine the physiological effect of a complete loss of $G\alpha_q$ function in ab1C were inconclusive. The simplest interpretation of the repeated difficulty in obtaining $G\alpha_q^{-/-}$ ab1C is that $G\alpha_q$, like $G\alpha_o$, plays a major role in ab1C development or survival.

Pertussis toxin, an inhibitor of $G\alpha_o$, does not affect CO₂ or odor responses

The mosaic analysis described above supports a role for $G\alpha_o$ in olfactory system development. To test the possibility of a role for $G\alpha_o$ in ORN function, we analyzed the effect of pertussis toxin (PTX) on odor and CO₂ response. PTX is a selective inhibitor of $G\alpha_o$, since $G\alpha_o$ is the only G-protein in *Drosophila* that contains the PTX recognition site (Ferris et al., 2006). We found that expression of *ptx* in ab1A, ab2A, ab3A, and ab1C did not affect odor or CO₂ responses across the broad concentration range tested ($n = 6$; $p > 0.2$ for each neuron class; data not shown).

These results argue against a role for $G\alpha_o$ in the physiology of odor or CO₂ signaling, consistent with our results from experiments with RNAi, competitive peptides, and $G\alpha_q^{GTP}$.

Disruption of $G\alpha_q$ signaling in adults decreases CO₂ response

Our results thus far suggest that although $G\alpha_q$ is not required for Or-mediated responses, it is required for the Gr-mediated CO₂ response. To test whether $G\alpha_q$ plays a role in the physiology of the CO₂ neuron, as opposed to an exclusively developmental role, we used the TARGET system (McGuire et al., 2003). TARGET allowed us to interfere with $G\alpha_q$ signaling selectively in adult flies: thus, expression of $G\alpha_q^{1F1}$ RNAi or $G\alpha_q^{GTP}$ in ab1C was repressed during development and expressed only after eclosion.

Expression of $G\alpha_q^{1F1}$ RNAi or $G\alpha_q^{GTP}$ selectively in adulthood was sufficient to decrease CO₂ response ($n = 9$ – 15 ; $p < 0.001$). In

Figure 5. MARCM analysis. *a*, *Gαs*^{−/−} ab1C had normal CO₂ responses ($p = 0.19$). *b*, *c*, *Gαs*^{−/−} ab3A had a decreased response to near-saturating concentrations of methyl butyrate (*b*) and butyric acid (*c*; $p < 0.01$ for responses to 0.1% and higher concentrations of methyl butyrate and 5% and 10% butyric acid). *d*, *e*, Expression of *Gαs* selectively in *Gαs*^{−/−} ab3A rescues the responses to all concentrations of methyl butyrate (*d*) and butyric acid (*e*). *f*–*i*, *Gαq*^{−/−} ab3A neurons generated using either the *Gαq²⁸* (*f*, *g*) or *Gαq^{221C}* (*h*, *i*) allele had normal responses to methyl butyrate and butyric acid ($p > 0.5$ for both alleles). $n = 8$ –24. Error bars indicate SEM.

fact, for both constructs, a 30–83% decrease in response was observed at all CO₂ concentrations tested, which ranged from 0.1 to 5%. The level of reduction observed in these flies was similar to that observed when the constructs were expressed with *Gr21a-GAL4* alone (Fig. 3*b*) (data not shown). This similarity is not surprising given that the *Gr21a-GAL4* driver is likely expressed late in development: although the developmental time course of *Gr21a* expression is unknown, *Or* genes in the antenna are first detected during the late pupal stage (Clyne et al., 1999; Elmore and Smith, 2001; Larsson et al., 2004).

Gαq increases CO₂ response but not odor response in an *in vivo* expression system

Coexpression of *Gr21a* and *Gr63a* in an *in vivo* expression system, the “empty neuron system” (Dobritsa et al., 2003), conferred a

Figure 6. Ectopic expression of the CO₂ receptors in the empty neuron. *a*, Sample traces of responses to 5% CO₂ from neurons expressing *Gr21a* and *Gr63a* alone or with *Gαq* or *Gαs*. The bar indicates a 500 ms pulse of 5% CO₂. *b*, Coexpression of *Gαq* with *Gr21a* and *Gr63a* increased CO₂ response. Coexpression with *Gαs* had no effect ($p = 0.6$). $n = 17$ –27. Error bars indicate SEM.

CO₂ response to ab3A, which normally does not respond to CO₂ (Fig. 1*d*) (Kwon et al., 2007). However, the response was modest compared to the endogenous ab1C response, perhaps because of limiting quantities of another component in ab3A essential for a full CO₂ response. We coexpressed *Gαq* and found that it dramatically increased the CO₂ response (Fig. 6). The response increased by a factor of five, to a level comparable to that of the endogenous neuron, at 0.1, 0.5, and 1% CO₂ ($n = 17$ –21; $p > 0.1$; data not shown). This effect is specific to *Gαq*: coexpression of *Gαs* with *Gr21a* and *Gr63a* did not increase the CO₂ response (Fig. 6). The effect of *Gαq* is also specific to the CO₂ response: as a control, we expressed *Gαq* in wild-type ab3A neurons and found that overexpression of *Gαq* did not increase responses to a broad concentration range of the two odorants tested, arguing against the possibility that *Gαq* overexpression causes a general increase in ab3A excitability ($n = 12$; $p > 0.05$; data not shown).

Gγ30A is also required for CO₂ response

Our data support a role for *Gαq* in CO₂ response in ab1C. In *Drosophila* phototransduction, *Gγ30A* is the binding partner of *Gαq*-1 (Schulz et al., 1999). Given that *Gγ30A* RNA was also detected using RT-PCR from adult antennal RNA (data not shown), we wondered whether *Gγ30A* is also the binding partner of *Gαq* in ab1C.

In functional tests we found that *Gγ30A* RNAi decreased CO₂ response (Fig. 7*a*). Consistent with this result, flies homozygous for a 6 kb insertion in *Gγ30A* have a reduced CO₂ response (Fig. 7*a*). Flies with one copy of the insertion in heterozygous combination with a deletion spanning the *Gγ30A* gene region had a response to CO₂ that was even further reduced (Fig. 7*a*). In contrast, most *Or*-mediated responses appeared normal in flies with this insertion (Fig. 7*b*). ab1A had a slight decrease in odor re-

Figure 7. *Gγ*. *a*, RNAi against *Gγ30A* decreased the CO₂ response ($p < 0.001$). Flies homozygous for an insertion in *Gγ30A* had a decreased CO₂ response relative to heterozygotes used as a control for the genetic background ($p < 0.001$). Flies with one copy of the insertion allele and one copy of a deletion allele spanning the *Gγ30A* region had a further decreased response to CO₂ ($p < 0.001$). RNAi against *Gγ1* did not affect the CO₂ response ($p = 0.8$). *Gγ1* mutant flies also had normal CO₂ responses ($p = 0.6$). *b*, Insertions in *Gγ30A* or *Gγ1* had little or no effect on odor responses ($p > 0.1$ for all combinations tested except for the ab1A response of *Gγ30A* insertion flies; for details, see Results). $n = 6–18$. Error bars indicate SEM.

sponse. However, the effect was modest relative to the decrease in CO₂ response.

There is only one other *Gγ* gene in the *Drosophila* genome, *Gγ1*. *Gγ1* RNA was also detected using RT-PCR from adult antennal RNA (data not shown). *Gγ1* has been found to play a role in sugar sensing by *Gr5a*-expressing gustatory neurons in the labelum (Ishimoto et al., 2005). A *Gγ1* RNAi construct (Ishimoto et al., 2005) that decreased responses of gustatory neurons to sugar did not affect the CO₂ response (Fig. 7*a*). An insertion in the 5' UTR of *Gγ1*, which decreases *Gγ1* expression by ~90% (Ishimoto et al., 2005), also did not affect CO₂ response (Fig. 7*a*) or the odor responses of ab1A, ab2A, or ab3A (Fig. 7*b*).

In summary, these results suggest that *Gγ30A* is required along with *Gα_q* in the response of ab1C to CO₂. The results also suggest that Or-mediated odor responses do not require *Gγ30A* or *Gγ1*.

Other visual transduction genes are not required for CO₂ response

The involvement of *Gα_q* and *Gγ30A* in both vision and CO₂ sensing suggested that these two processes might share other signaling elements. We tested the phototransduction mutants *GB76C* (*Gβ*), *inaD* (a scaffolding protein), and *norpA* (phospholipase C). Null mutant flies of each of these three genes had normal responses to CO₂ (data not shown). This finding raises the possibility that although CO₂ response requires *Gα_q* and *Gγ30A*, these G-proteins may not signal through canonical G-protein-signaling pathways. A recent study proposed that Ors are both GPCRs and ligand-gated ion channels, and that G-protein activation results in current through the Or channel (Wicher et al., 2008). Our data suggest that *Gα_q* plays a direct role in CO₂ signaling. However, we cannot determine whether *Gα_q* activates conventional downstream signaling components or if it acts on a channel formed from Gr21a and Gr63a.

Discussion

Recent studies in heterologous systems have provided evidence that insect odor receptors can act as ligand-gated ion channels (Sato et al., 2008; Wicher et al., 2008). Canonical members of the Or family form a complex with the coreceptor Or83b (Larsson et al., 2004; Neuhaus et al., 2005; Benton et al., 2006), and the heteromeric receptor appears to play a direct role in olfactory signaling as an ion channel. Conversely, for Grs, there is evidence that taste neurons expressing *Gr5a* require G-proteins (Ishimoto et al., 2005; Ueno et al., 2006). These findings have raised an intriguing question: to what extent does signaling by the Or–Gr superfamily of insect chemoreceptors differ from that of chemoreceptors in vertebrates and *C. elegans*? Odor and taste receptors in these organisms are believed to signal directly through G-proteins. We have systematically examined the G-proteins of *Drosophila* using both loss-of-function and gain-of-function experiments for roles in chemosensory response. All experiments in this study were performed *in vivo* in *Drosophila* ORNs, as opposed to in heterologous systems.

We found that disruption, knockdown, or even complete elimination of G-proteins had little or no effect on Or-mediated signaling *in vivo*. Our results indicate that CO₂ response, mediated by two Grs, requires *Gα_q* and *Gγ30A*. Further investigation using constitutively active *Gα_q*, mosaic analysis, and conditional expression showed that *Gα_q* is required for both the survival and function of ab1C.

Reduction of G-protein function has little or no effect on Or-mediated odor response

Recent studies have proposed conflicting models for Or signaling. One study suggested in particular that *Gα_q* is required by ab2A for full sensitivity to low concentrations of odors (Kain et al., 2008). However, the study used a pan-neuronal driver to mark *Gα_q^{-/-}* cells and examined a narrow response range. Using ORN class-specific GAL4 drivers and examining a broad response range of odor concentrations, we tested three ORN classes, including ab2A, that had been manipulated in multiple ways to cause a loss of *Gα* function. We found that knockdown of *Gα_q* in ab1A, ab2A, and ab3A did not decrease odor responses. Most strikingly, ab3A neurons that were genotypically *Gα_q^{-/-}*, as determined with an ab3A-specific driver, showed normal responses across a broad response range for the odors tested. Thus, our findings do not support the model that *Gα_q* is required for Or signaling.

A second recent study proposed that Or83b can act as a cyclic-nucleotide-gated ion channel that depends on *Gα_s* (Wicher et al.,

2008). Our results suggest that $G\alpha_s$ is not required for the primary response to odors. $G\alpha_s^{-/-}$ ab3A has a decreased response to higher concentrations of odors; however, this effect is modest and suggests that the primary pathway of olfactory transduction in ab3A neurons does not require $G\alpha_s$.

The third study proposed that the Or–Or83b complex is a ligand-gated ion channel whose activation does not require G-proteins (Sato et al., 2008). Our results support this proposition that G-proteins are not required for primary Or signaling. A dual-activation model was recently proposed to reconcile the different models of Or signaling (Nakagawa and Vosshall, 2009). In this model, Or activation results in a primary ionotropic response as well as a slow metabotropic component that modulates the Or complex. Our results could be interpreted to support this model, with the $G\alpha_s$ pathway potentiating the main response. However, we note that manipulation of $G\alpha_s$ signaling with RNAi, competitive peptides, and $G\alpha_s^{GTP}$ had no effect on the response of ab1A, ab2A, or ab3A. Deletion of the $G\alpha_s$ gene produced at most a 17.1% reduction in response. Furthermore, the decreased response was only seen at the highest odor concentrations, at which the ORN is near or at its maximum response rate for the given odor. If the role of $G\alpha_s$ were to potentiate receptor function, one might expect that it would boost low signals or signals across multiple concentrations, rather than only at the highest concentrations.

Gr-mediated CO₂ response requires G-proteins

In contrast to our results with Ors, we found that multiple, complementary approaches consistently revealed a role for G-proteins— $G\alpha_q$ and $G\gamma 30A$ —in Gr-mediated CO₂ response. Knockdown of $G\alpha_q$ via various genetic manipulations all resulted in reduced CO₂ response in ab1C. One might hypothesize that $G\alpha_q$ plays an indirect role, such as in neuronal maintenance or in modulation of the response. However, this hypothesis is difficult to reconcile with the finding that transient expression of $G\alpha_q^{GTP}$ increases the spontaneous firing rate of ab1C. The simplest interpretation of the transient expression analysis is that $G\alpha_q$ plays a direct role in CO₂ signaling, although the mechanism by which it acts is not revealed by our analysis.

Different requirements for G-proteins in different chemosensory neurons

Signaling in ab1C differs from that in ab1A, ab2A, and ab3A in its requirements for G-proteins. This difference is consistent with other differences between ab1C and the other ORNs. ab1C expresses Gr members of the Or–Gr insect chemoreceptor superfamily, whereas most other ORNs express Or members. ab1A, ab2A, and ab3A express the coreceptor *Or83b*; ab1C does not express *Or83b*, and a Gr gene analogous to *Or83b* has not been identified. *Or83b*-expressing ORNs have GABA_B receptors, which signal through $G\alpha_o$, at their axon terminals, thereby allowing for inhibitory modulation of synaptic transmission (Olsen and Wilson, 2008; Root et al., 2008). ab1C does not express GABA_B receptors and does not exhibit presynaptic inhibition (Root et al., 2008).

Different ORNs also differ in their developmental requirements for G-proteins. We found that $G\alpha_q$ plays a role in the development of ab1C but not ab3A. These results are consistent with differences in morphology between ab1C and the other ORNs tested (Shanbhag et al., 1999). Moreover, a microRNA, *miR-279*, and its target, *Nerfin-1*, specifically regulate the development of CO₂-sensitive neurons (Cayirlioglu et al., 2008).

There may be additional differences in the requirements for G-proteins among chemosensory neurons that express Gr genes.

There is evidence that $G\alpha_s$ and $G\gamma_1$ each play a role in the sugar response of *Gr5a*-expressing taste neurons, but not in the bitter response of *Gr66a*-expressing taste neurons (Ishimoto et al., 2005; Ueno et al., 2006). The functional significance of the heterogeneity in G-protein requirements merits investigation.

Receptor promiscuity and the evolution of insect olfactory transduction: a unifying model

It is intriguing and perhaps revealing that despite the different requirements for G-proteins, receptors of the Or–Gr family are able to function in quite distinct contexts. The ab1C receptors *Gr21a* and *Gr63a* can also function in ab3A, although they are limited by the availability of $G\alpha_q$ (Fig. 6), which is not required for the function of the endogenous ab3A receptor, *Or22a*. Reciprocally, the Ors *Or43a* and *Or83b* can function when coexpressed in ab1C (Benton et al., 2006). Moreover, coexpression of *Or22a* and *Or83b* in either *Gr66a*- or *Gr5a*-expressing taste neurons confers odor responses to both (Hiroi et al., 2008).

Do receptors signal through alternate pathways when expressed in alternate contexts? Coexpression of *Or43a* and a promiscuous $G\alpha$, without *Or83b*, was found to confer an odor response to *Xenopus* oocytes (Wetzel et al., 2001). The *Bombyx mori* receptor BmOR1 did not respond to its ligand when expressed alone in *Xenopus* oocytes, but was capable of responding when coexpressed with either the *Or83b* ortholog, *BmOR2*, or with *BmGaq* (Nakagawa et al., 2005). The response was more robust with *BmOR2*, which is reminiscent of our finding that the CO₂ response is greater when *Gr21a* and *Gr63a* are coexpressed with $G\alpha_q$, a gene required for the endogenous CO₂ response.

One simple, unifying model consistent with all of these results taken together is that Ors and Grs are capable of acting via multiple mechanisms, one requiring *Or83b* and one requiring G-proteins, efficiently in one case and inefficiently in the other. Ors are believed to represent a relatively recent branch of an ancient Gr family (Robertson et al., 2003). Perhaps Ors evolved to rely exclusively on a ligand-gated ionotropic signaling mechanism, whereas Grs maintained the ability to signal via a G-protein-dependent mechanism. If so, a limited ability of Ors and Grs to each signal through another mechanism might reflect this evolutionary transition.

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