LETTERS

Two chemosensory receptors together mediate carbon dioxide detection in *Drosophila*

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Blood-feeding insects, including the malaria mosquito *Anopheles gambiae*, use highly specialized and sensitive olfactory systems to locate their hosts. This is accomplished by detecting and following plumes of volatile host emissions, which include carbon dioxide (CO_2)[1]. CO_2 is sensed by a population of olfactory sensory neurons in the maxillary palps of mosquitoes[2,3] and in the antennae of the more genetically tractable fruitfly, *Drosophila melanogaster*[4]. The molecular identity of the chemosensory CO_2 receptor, however, remains unknown. Here we report that CO_2-responsive neurons in *Drosophila* co-express a pair of chemosensory receptors, *Gr21a* and *Gr63a*, at both larval and adult life stages. We identify mosquito homologues of *Gr21a* and *Gr63a*, *GPRGR22* and *GPRGR24*, and show that these are co-expressed in *A. gambiae* maxillary palps. We show that *Gr21a* and *Gr63a* together are sufficient for olfactory CO_2-chemosensation in *Drosophila*. Ectopic expression of *Gr21a* and *Gr63a* together confers CO_2 sensitivity on CO_2-insensitive olfactory neurons, but neither gustatory receptor alone has this function. Mutant flies lacking *Gr63a* lose both electrophysiological and behavioural responses to CO_2. Knowledge of the molecular identity of the insect olfactory CO_2 receptors may spur the development of novel mosquito control strategies designed to take advantage of this unique and critical olfactory pathway. This in turn could bolster the worldwide fight against malaria and other insect-borne diseases.

Carbon dioxide is a pervasive chemical stimulus that is important in the ecology of many insect species[5]. Interestingly, the ethological message conveyed by this gas is highly species- and context-specific. The hawkmoth, *Manduca sexta*, evaluates the quality of *Datura wrightii* flowers by measuring the amount of CO_2 that a given flower produces[6]; newly opened flowers emit more CO_2 and are preferred because they offer more nectar. In response to elevated CO_2 in their hives, honeybees show a stereotyped fanning response that ventilates the hive and reduces ambient CO_2 levels[7]. For blood-feeding female mosquitoes, CO_2 emitted in the breath of animal hosts (~4–5%) is an arousing stimulus that synergizes with host body odour to produce host-seeking behaviours[1,8]. The ecological relevance of CO_2 to fruitflies is less clear, but CO_2 is one component of the aversive *Drosophila* stress odorant (dSO)[9] and may also signal food source suitability[10].

The chemosensory neurons that are thought to underlie these CO_2-evoked behaviours have been functionally characterized on antennal, maxillary or labial appendages in a number of different insects[11]. *Drosophila* antennae have a small, CO_2-sensitive subpopulation of olfactory sensory neurons that have been designated ab1C (ref. 4). The antennal lobe of the fly brain has a single ventrally-situated glomerulus (V) that responds selectively to CO_2 (ref. 9). This V glomerulus is innervated by a population of olfactory neurons expressing the chemosensory receptor *Gr21a* (ref. 12); these neurons correspond to the ab1C neurons. *Gr21a* is a member of the gustatory receptor gene family, which includes bitter and sweet taste receptors necessary for taste recognition in the fly, along with a number of gustatory receptor genes expressed in the antenna that may function as odorant receptors[12]. Although clearly separable into two gene families, *Drosophila* gustatory receptors and odorant receptors are thought to have a common phylogenetic origin and were originally assigned to taste and smell modalities by gene homology and not function[13]. Genetic silencing[9] or ablation[10] of *Gr21a*-expressing neurons eliminates both adult[9] and larval[10] chemosensory responses to CO_2, confirming that these are the only CO_2-sensitive neurons in *Drosophila*.

We asked whether *Gr21a* is merely a marker for the CO_2-sensitive neurons in *Drosophila* or whether it is directly involved in CO_2 detection. As it has been previously reported that taste neurons can express multiple gustatory receptor genes[14], we began by screening for additional gustatory receptor genes expressed in ab1C neurons. Two other gustatory receptor genes are known to be expressed in the antenna[12], and fluorescent RNA *in situ* hybridization reveals that *Gr63a* is co-expressed with *Gr21a* (Fig. 1a), but that *Gr10a* is expressed in the adjacent ab1D neuron (Fig. 1b)[15]. Confirming these *in situ* hybridization results, neurons labelled with genetic markers under the control of *Gr21a* and *Gr63a* promoters co-converge upon the CO_2-sensitive V glomerulus (Fig. 1c). These chemosensory receptors are therefore co-expressed in the adult ab1C sensillum (Fig. 1d).

Next, we investigated the expression of *Gr21a* and *Gr63a* in the larval olfactory system. Larvae show robust avoidance of CO_2, which is mediated by *Gr21a*-expressing neurons[10]. Both *Gr21a-GAL4* and *Gr63a-GAL4* transgenes drive expression of a membrane-tethered green fluorescent protein (GFP) in the same neuron that innervates the larval terminal organ, which is thought to be primarily gustatory in function (Fig. 1e). This indicates that *Gr21a* and *Gr63a* are also co-expressed in the larval chemosensory system.

To generalize our results to other insects, we analysed *Gr21a* and *Gr63a* homologues in *A. gambiae*. Mosquitoes, in whom CO_2 plays an important role in human-host-seeking, have closely related homologues of both *Gr21a* and *Gr63a*, called *GPRGR22* and *GPRGR24*, respectively[16] (Fig. 1f). RNA *in situ* hybridization reveals co-expression of *GPRGR22* and *GPRGR24* in a subset of neurons in the maxillary palp, the CO_2-sensitive organ of the mosquito (Fig. 1g). No expression is detected in the antenna or proboscis (data not shown). As in the fly, these putative mosquito CO_2-responsive neurons do not express *GPROR7*, the *A. gambiae Or83b* orthologue (Fig. 1h). Thus these mosquito homologues share three key properties in common with fly *Gr21a*/*Gr63a*: they are co-expressed in the same sensory neurons; they are selectively expressed only in the olfactory appendage that responds to CO_2; and they do not express the olfactory co-receptor *Or83b*.

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To investigate the role of these gustatory receptor genes as putative CO_2 receptors, we ectopically expressed the antennal gustatory receptor genes both alone and in pairs in neurons normally unresponsive to CO_2 using the *GAL4*/UAS system (Fig. 2a). *Or22a-GAL4* drives expression in ~75% of the electrophysiologically accessible ab3A neurons that express *Or22a/b* and the co-receptor *Or83b* (ref. 17). No individual gustatory receptor gene was capable of conferring CO_2 responsiveness on the ab3A neurons (Fig. 2b), but as we previously demonstrated that fly odorant receptor genes are obligate OR/Or83b heterodimers[18], we asked whether a combination of two gustatory receptor genes could function as a CO_2 receptor. Neither *Gr21a* nor *Gr63a* confer responses to CO_2 when combined with *Gr10a*, but the combination of *Gr21a* and *Gr63a* produces a significant response to a stimulus of ~3% CO_2 (Fig. 2b). It is therefore the specific combination of these two gustatory receptor genes that is sufficient to induce CO_2 sensitivity rather than a generic requirement for the co-expression of any two antennal gustatory receptor genes. *Gr21a* and *Gr63a* together also increase the level of spontaneous activity in the ab3A neuron. We considered the possibility that this reflects activity in response to ambient CO_2 levels (0.035%), but we found that the activity of these neurons is not reduced in response to a CO_2-free air stream (data not shown). Prior results with odorant receptor genes indicate that some have substantial odour-independent activity[19], and this result suggests that gustatory receptor genes share this property. Further analysis of ectopically expressed *Gr21a*/*Gr63a* reveals a dose-dependent increase to stimuli of increasing CO_2 concentration, whereas animals expressing *Gr21a* alone do not respond to CO_2 at any concentration tested (Fig. 2c, compare blue and green curves).

We next compared the efficacy of the ectopic CO_2 response to that obtained in the native ab1C sensillum, and find that although the dose–response curves have a similar shape, the efficacy of the ectopic receptor is lower than the endogenous response (Fig. 2c, compare red and blue curves). A number of explanations may account for this difference in efficacy. There could be a requirement for a cell-type specific co-factor, which is missing in the ab3A cell. Alternatively, lower receptor expression levels or competition for trafficking factors with the resident odorant receptors in ab3A could lead to a reduced

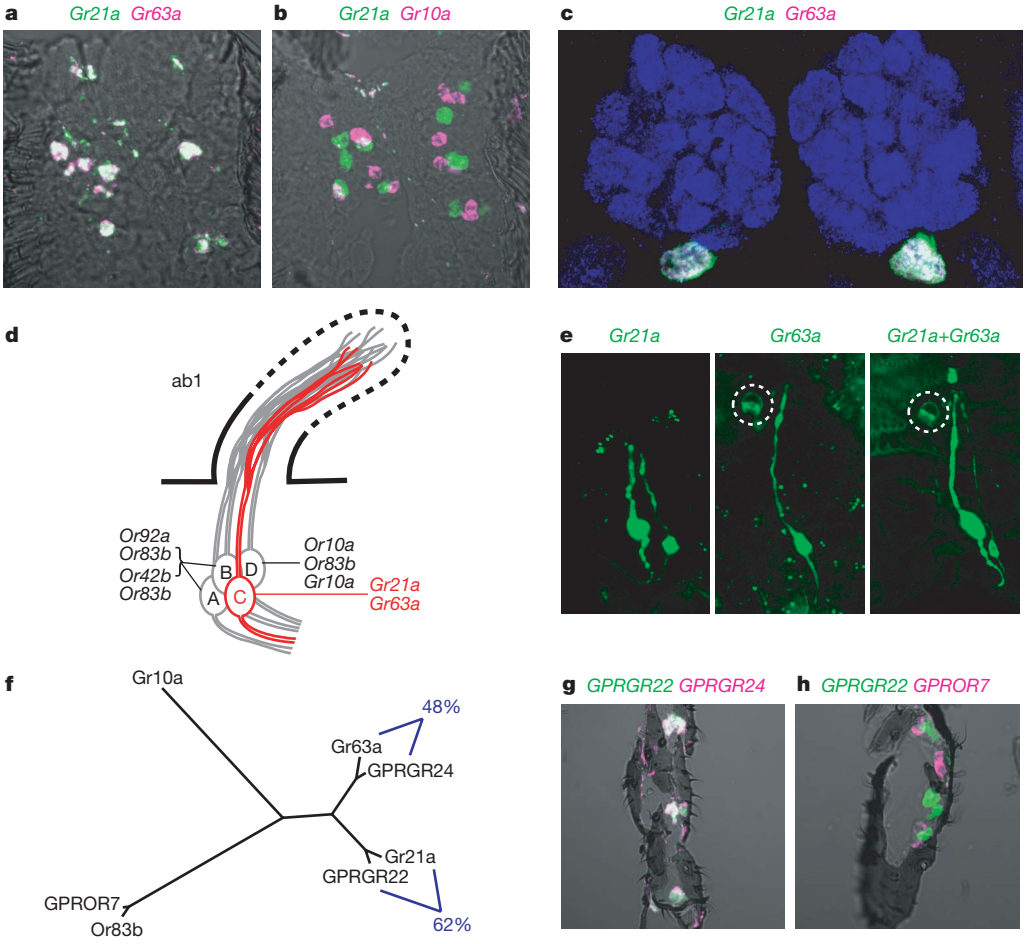

Figure 1 | *Gr21a* and *Gr63a* are co-expressed in the CO_2-responsive chemosensory neurons. **a**, Fluorescent double *in situ* hybridization on the third antennal segment of wild-type *Drosophila* (wild-type Berlin) reveals co-expression of *Gr21a* (green) and *Gr63a* (magenta) mRNA. **b**, *Gr21a* mRNA (green) is not co-expressed with the only other gustatory receptor gene expressed in the antenna, *Gr10a* (magenta). **c**, Olfactory neurons expressing *Gr21a-GAL4*; UAS-*CD8-GFP* (green) and *Gr63a-sytRFP* (magenta) co-converge upon the V glomerulus in the antennal lobe. Whole mount brain immunofluorescence preparation is counter-stained with the neuropil marker nc82 (blue)[28]. **d**, Diagram of the ab1 sensillum, with the CO_2-responsive ab1C neuron labelled in red. The receptor genes expressed in ab1D are indicated. The receptor pairs expressed in the two remaining neurons have not been conclusively assigned to either ab1A or ab1B. **e**, Larvae expressing a membrane-tethered GFP under control of *Gr21a-GAL4* (left) have two labelled neurons innervating the terminal organ, one of which probably represents ectopic *GAL4* expression as seen in other larval receptor *GAL4* lines[29]. This smaller cell of unknown function is visible in both the left and right panels. *Gr63a-GAL4* (middle) labels one neuron, and the combination of *Gr21a-GAL4* and *Gr63a-GAL4* (right) labels the same two neurons as *Gr21a-GAL4* alone, indicating co-expression. When visible, the adjacent olfactory dorsal organ is encircled by a white dotted line. **f**, Phylogenetic comparison of *Drosophila* CO_2 receptors and their nearest *A. gambiae* homologues, with percentage amino acid identity in blue. **g**, RNA *in situ* hybridization of *A. gambiae* maxillary palps reveals co-expression *GPRGR22* (green) and *GPRGR24* (magenta). **h**, mRNAs for *GPRGR22* (green) and *GPOR7* (magenta) are not co-expressed.

number of functional CO_2 receptors. Such competition, leading to lower efficacy of ectopically expressed chemosensory receptors, has been noted by other investigators[17,18]. Finally, there is evidence that the ab1C neuron has a uniquely specialized dendritic architecture, with considerably more branching than other chemosensory neurons[20]. These special structural properties may be necessary for optimal Gr21a/Gr63a receptor function and may constrain its efficacy in other neurons. Taken together, we find that *Gr21a* and *Gr63a* together are sufficient to confer dose-dependent CO_2-responsivity on olfactory neurons normally unresponsive to CO_2.

To investigate the role of these gustatory receptor genes in the CO_2 responses of the native ab1C neuron, we screened for *Gr21a* and *Gr63a* null mutants by homologous recombination (Fig. 3a)[21,22]. *Gr21a* proved to be resistant to mutagenesis, but we obtained a single null mutant allele of *Gr63a*. PCR (polymerase chain reaction) analysis of $Gr63a^1$ indicates the selective loss of *Gr63a* without affecting a neighbouring gene, *CG1079* (Fig. 3a). $Gr63a^1$ flies lack the *Gr63a* transcript when compared with parental controls, but have normal levels of *Gr21a* (Fig. 3a). Electrophysiological recordings of ab1 sensilla in $Gr63a^1$ flies reveal a complete indifference to stimuli of ~2.25% CO_2, in stark contrast to wild-type parental control flies, whose ab1C neurons respond strongly. The $Gr63a^1$ allele is genetically recessive, because the sensilla of heterozygous individuals have an essentially wild-type CO_2 response. CO_2 responses in the $Gr63a^1$ are restored by rescuing *Gr63a* expression in the ab1C neurons using the *GAL4*/UAS system, while control $Gr63a^1$ flies bearing either the *Gr21a-GAL4* transgene or the UAS-*Gr63a* transgene alone fail to respond (Fig. 3b).

As genetic silencing of *Gr21a*-expressing neurons eliminates olfactory CO_2 avoidance in a T-maze[9], we asked whether $Gr63a^1$ flies have CO_2 avoidance defects. Whereas wild-type flies robustly avoid CO_2 in a T-maze, $Gr63a^1$ flies fail to distinguish room air from a ~2% CO_2 stimulus. Consistent with their electrophysiological responses, $Gr63a^1$ heterozygotes show a wild-type avoidance response, whereas $Gr63a^1$ flies bearing either *Gr21a-GAL4* or UAS-*Gr63a* transgenes fail to differentiate room air from 2% CO_2. When combined, however, these two transgenes rescue olfactory CO_2 avoidance in the mutant (Fig. 4). The failure of the rescue to reach wild-type levels in either the electrophysiological recordings or the behaviour is probably a consequence of the lower levels of *Gr63a* expression in rescued ab1C neurons when compared to wild-type ab1C neurons, as also discussed above (data not shown). These loss of function results prove that *Gr63a* is necessary for CO_2 chemoreception in *Drosophila* and strengthen our hypothesis that the *Drosophila* CO_2 receptor is composed of both Gr21a and Gr63a.

Taken together, our results suggest that two chemosensory receptors, *Gr21a* and *Gr63a*, are necessary and sufficient for detection of CO_2 in *Drosophila*. Despite the fact both *Gr21a* and *Gr63a* are required for CO_2 responses, our data at present do not allow us to resolve whether one subunit acts as a chaperoning co-receptor while the other subunit confers ligand specificity (as is the case for odorant receptors and Or83b), or whether both subunits are required for both functions. This is because, unfortunately, our attempts to tag these proteins while retaining function have failed. Previous work in other biological systems has implicated several cytosolic proteins as gas sensors. Atypical soluble guanylate cyclases are candidate oxygen sensors in *Caenorhabditis elegans*[23], while conventional soluble guanylate cyclases are cytosolic receptors for nitric oxide and carbon monoxide[24,25]. A nuclear receptor in *Drosophila* has been suggested as an additional receptor for nitric oxide and carbon monoxide[26]. Bacteria utilize haem-containing myoglobin in chemotaxis towards oxygen[27]. Although our genetic evidence strongly suggests that *Gr21a*/*Gr63a* encodes the first example of a membrane-associated gas sensor, we cannot exclude the possibility that additional secreted or cytosolic proteins, such as those described previously, are essential co-factors for CO_2 detection. Further biochemical investigation—most effectively carried out in a cell-based heterologous expression system—will be required to address this question.

It remains to be resolved whether the Gr21a/Gr63a receptor binds gaseous CO_2 or a metabolite, such as bicarbonate (HCO_3^-). It will also be of interest to elucidate the signal transduction cascade to which these gustatory receptors couple in order to transform the absolute concentration of environmental CO_2 into precise trains of neuronal action potentials. As CO_2 is an important stimulus for a large number of insect pests, the identification of the CO_2 receptor provides a potential target for the design of inhibitors that might be useful as insect repellents. These would be important weapons in the fight against global infectious disease by reducing the attraction of blood-feeding insects to human hosts.

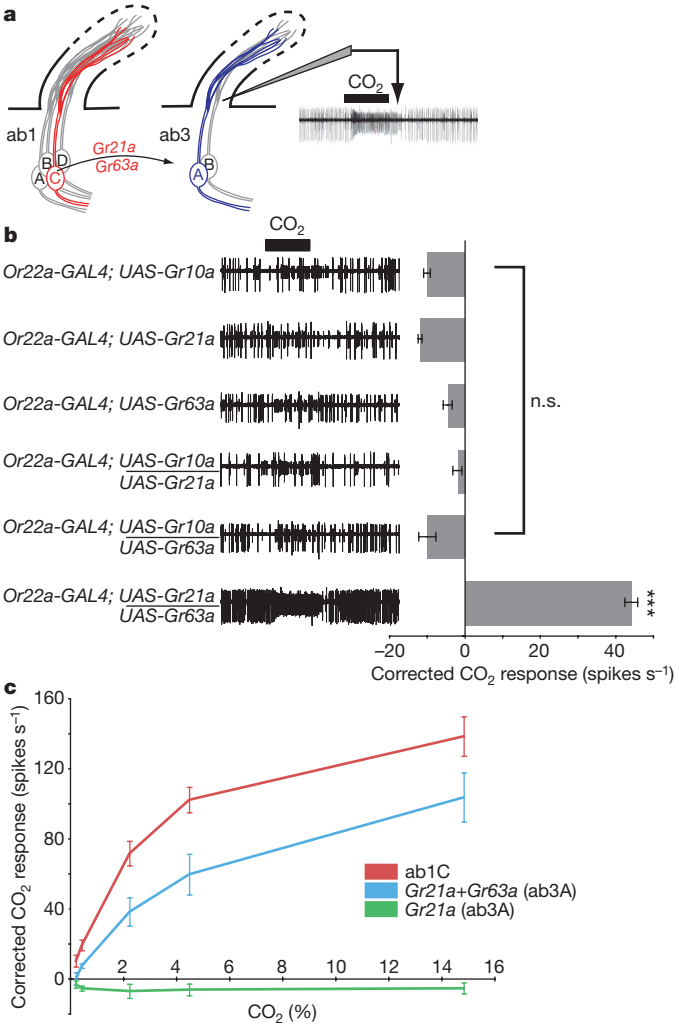

Figure 2 | Expression of both *Gr21a* and *Gr63a* confers CO_2 sensitivity on normally CO_2-insensitive neurons. **a**, Diagram outlining the methodology for the transfer of the CO_2 receptor to the ab3A neuron. Extracellular recording of spikes emitted by CO_2 is at the right. **b**, The indicated combinations of antennal gustatory receptor genes were ectopically expressed in ab3A neurons using the *Or22a-GAL4* driver. Single-sensillum electrophysiological recordings on transgenic ab3 sensilla, recognized by their characteristic response to ethyl hexanoate (ab3A) and 2-heptanone (ab3B)[17], were made for both room air (~0.035% CO_2) and ~3% CO_2. Representative traces (stimulus bar, 1 s) are on the left, and mean responses (±s.e.m.) are on the right ($n = 15$–18 sensilla per genotype). Significant responses to CO_2 are only found with the combination of *Gr21a* and *Gr63a* (Tukey HSD test; $P < 10^{-6}$). n.s., not significant. **c**, Dose–response curves for the combination of *Gr21a* and *Gr63a* (blue curve) versus expression of *Gr21a* alone (green curve) in ab3A neurons as compared to the CO_2 response in native ab1C neurons (red curve). Mean responses (s.e.m.) are plotted ($n = 10$ sensilla per genotype).

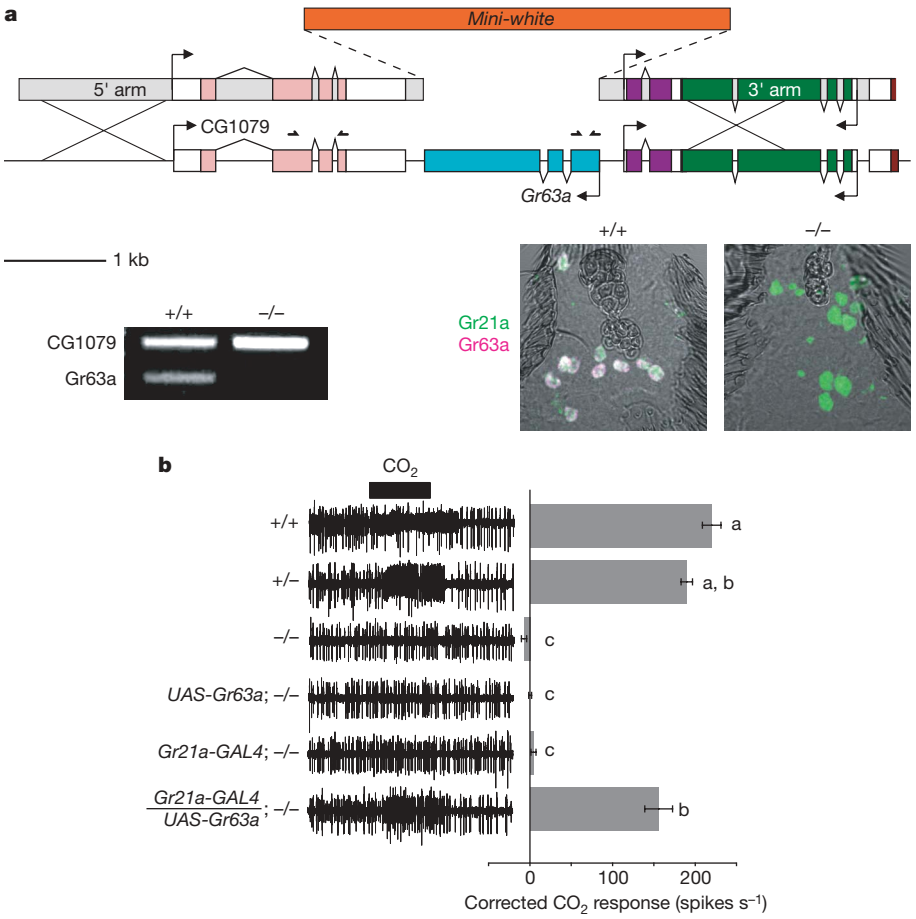

Figure 3 | *Gr63a*^{1} mutants are electrophysiologically and behaviourally insensitive to CO_2. **a**, Diagram of the *Gr63a* gene targeting construct and the *Gr63a* genomic locus. PCR using primers denoted by small arrows above the locus diagram indicates the absence of *Gr63a* product in *Gr63a*^{1} mutants. *Gr63a*- flies lack *Gr63a* mRNA (magenta) compared to wild-type controls, while *Gr21a* mRNA (green) levels remain unchanged. **b**, *Gr63a*^{1} mutant ab1 sensilla (−/−) do not respond to ~2.25% CO_2 when compared to parental wild-type or heterozygous (+/−) flies. Responses are rescued by the combination of *Gr21a-GAL4* and UAS-*Gr63a* in the mutant *Gr63a*^{1} background but not by either transgene alone. Representative traces (stimulus bar, 1 s) at left and mean responses (±s.e.m.; $n = 12$ for all genotypes) on the right were quantified as in Fig. 2. Statistical significance was calculated using a Tukey HSD test comparing all pairs of means ($P < 0.001$). Bars labelled with different letters are significantly different.

METHODS

RNA *in situ* hybridization and immunofluorescence. Double fluorescent RNA *in situ* hybridization was performed on fly antennae as described previously[15], and on mosquito maxillary palps without protocol modification. Adult mosquitoes (*A. gambiae* strain G3; MRA-112) were obtained from MR4. Whole mount brain and larval immunostaining was performed as previously described[28,29]. Details can be found in Supplementary Methods.

Gustatory receptor transgene generation. *Gr10a* was amplified from Oregon-R antennal complementary DNA, and *Gr63a* was amplified from *yw* genomic DNA. *GPRGR22* and *GPRGR24* were amplified from *A. gambiae* G3 antennal cDNA. Construct details can be found in Supplementary Methods.

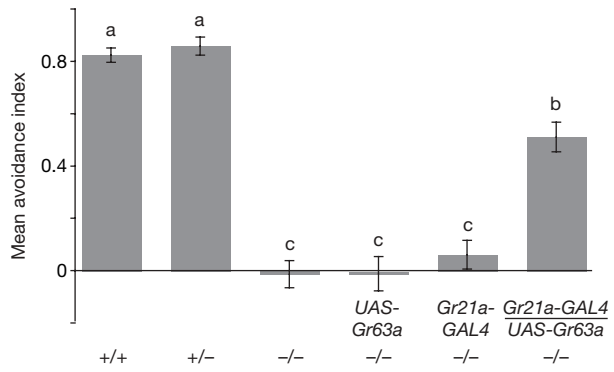

Figure 4 | *Gr63a*^{1} mutants and the *GAL4* and UAS controls are all indifferent to CO_2 in a T-maze, whereas wild-type and heterozygous *Gr63a*^{1} flies show robust avoidance. This deficit is rescued in *Gr21a-GAL4*/UAS-Gr63a; *Gr63a*^{1} flies. Mean avoidance ± s.e.m. is indicated ($n = 15$). Statistical significance was calculated using a Tukey HSD test comparing all pairs of means ($P < 0.01$). Bars labelled with different letters are significantly different.

Single sensillum electrophysiology. Extracellular recordings of ab1 and ab3 sensilla from individual flies (2–10 days old) were made as described[4,22] and as in the Supplementary Methods.

***Gr63a* targeting construct and mutant screen.** Genomic DNA both 5′ and 3′ of the *Gr63a* coding sequence was amplified from *yw* flies and cloned into the CMC105 gene targeting vector[22] (Supplementary Methods). Four independent insertions of the targeting construct were screened as described[22]. The progeny of approximately 16,500 virgin mosaic or white-eyed females (~330,000 flies) were screened for re-insertion on the third chromosome, and we recovered a single mutant allele, *Gr63a*^{1}. PCR confirmation of *Gr63a*^{1} was performed on genomic DNA preparations of the mutant line and its corresponding wild-type parental targeting construct insertion with primers within *Gr63a* itself and within the neighbouring gene CG1079 (Supplementary Methods). A similar screen for a *Gr21a* mutant produced no mutants among ~350,000 progeny derived from five independent targeting construct insertions.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions W.D.J. carried out all the experiments and analysed the data. P.C. and I.G.K. generated and characterized the *Gr63a-sytRFP* transgene in the laboratory of S. L. Zipursky at UCLA. W.D.J. and L.B.V. together designed the experiments, interpreted the results, produced the figures, and wrote the paper.

Author Information Genbank accession numbers for *A. gambiae* genes in this paper are: *GPROR7* (AY843205), *GPRGR22* (DQ989011) and *GPRGR24* (DQ989013). Genbank accession numbers for *D. melanogaster* genes in this paper are: *Gr10a* (DQ989010), *Gr21a* (DQ989014) and *Gr63a* (DQ989012). Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to L.B.V. (leslie@mail.rockefeller.edu).