Multiple gustatory receptors required for the caffeine response in *Drosophila*

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The ability of insects to detect and avoid ingesting naturally occurring repellents and insecticides is essential for their survival. Nevertheless, the gustatory receptors enabling them to sense toxic botanical compounds are largely unknown. The only insect gustatory receptor shown to be required for avoiding noxious compounds is the *Drosophila* caffeine receptor, Gr66a. However, this receptor is not sufficient for the caffeine response, suggesting that Gr66a may be a subunit of a larger receptor. Here, we report that mutations in the gene encoding the gustatory receptor, Gr93a, result in a phenotype identical to that caused by mutations in *Gr66a*. This includes an inability to avoid caffeine or the related methylxanthine present in tea, theophylline. Caffeine-induced action potentials were also eliminated in *Gr93a*-mutant animals, while the flies displayed normal responses to other aversive compounds or to sugars. The Gr93a protein was coexpressed with Gr66a in avoidance-gustatory receptor neurons (GRNs), and functioned in the same GRNs as Gr66a. However, misexpression of both receptors in GRNs that normally do not express either Gr93a or Gr66a does not confer caffeine sensitivity to these GRNs. Because Gr93a- and Gr66a-mutant animals exhibit the identical phenotypes and function in the same cells, we propose that they may be caffeine coreceptors. In contrast to mammalian and *Drosophila* olfactory receptors and mammalian taste receptors, which are monomeric or dimeric receptors, we propose that *Drosophila* taste receptors that function in avoidance of bitter compounds are more complex and require additional subunits that remain to be identified.

Gr93a | gustatory receptor neuron | taste | repellent | chemosensation

The sense of taste enables insects to sample their environment and identify nutrient-rich botanical sources. Consequently, as part of a protective mechanism, many plants produce toxic compounds to avoid consumption. In turn, insects have developed receptors to detect these noxious chemicals and prevent the deleterious effects resulting from ingestion.

In the fruit fly, *Drosophila melanogaster*, the detection of attractive and aversive tastants appears to be encoded primarily by a family of 68 7-transmembrane gustatory receptors (Grs) (1–4). The *Drosophila* Grs have virtually no sequence similarity with mammalian taste receptors. Rather, these receptors are conserved among distantly related insects, as related Grs are encoded in mosquitoes, such as *Anopheles gambiae* (5), which diverged from flies ≈250 million years ago (6). Thus, characterization of *Drosophila* Grs offers a genetically tractable model for dissecting the sense of taste common to a variety of insect pests.

Three receptors essential for sugar detection have been identified. The first is Gr5a, which is necessary for the response to trehalose (7–9). Gr5a and other *Drosophila* taste receptors are expressed in gustatory receptor neurons (GRNs) rather than neuroepithelial cells, as in mammals. The GRNs are housed in chemosensory bristles distributed in several body locations, including the main taste organ, the proboscis, which is the functional homolog of the mammalian tongue (10). Gr5a is expressed in most sugar-responsive GRNs (7–9, 11, 12). Gr64a is essential for the detection of multiple other sugars, including sucrose, glucose, and maltose (13, 14). A third receptor, Gr64f, is a coreceptor, which is broadly required for the detection of most sugars (15). Gr64f functions in combination with Gr5a for trehalose detection, and in concert with Gr64a for sensing sucrose, maltose, and glucose. However, it appears that the Gr64f/Gr5a and Gr64f/Gr64a receptor pairs are not sufficient for eliciting responses to sugars (15). Thus, detection of a single sugar receptor may require more than 2 receptors.

Many Grs are expressed in all or subsets of GRNs, which function in the detection of noxious compounds (11, 12); however, the only Gr shown to be required for the response to aversive compounds is Gr66a (16). This receptor, which is expressed in most avoidance GRNs, appears to be a subunit of a caffeine receptor, as mutation of Gr66a eliminates caffeine-avoidance behavior and caffeine-induced action potentials in the GRNs. However, misexpression of Gr66a is not sufficient to produce caffeine sensation, suggesting that a minimum of 2 Grs is required for caffeine detection.

Here, we show that mutations in *Gr93a* result in a phenotype identical to *Gr66a*-mutant animals. Both mutants are unable to respond to caffeine and the related methylxanthine, theophylline. In addition, Gr93a and Gr66a are expressed in the same GRNs. However, misexpression of these 2 receptors is not adequate to recapitulate caffeine sensation. These data indicate that the ability to sense caffeine requires at least 2 Grs in addition to other subunits.

Results

Generation of *Gr93a* Mutants. To characterize new requirements for Grs for *Drosophila* taste, we focused on *Gr93a*, because it was included in 1 of the 2 branches of the Gr phylogenetic tree (1) most distantly related to those that contain the Grs known to be required for the attractive (Gr5a, Gr64a, and Gr64f) and aversive (Gr66a) responses to tastants. To disrupt *Gr93a*, we obtained 2 transposable element lines, one of which had a Piggybac insertion (f01688) in the *Gr93a* intron (*Gr93a[1]*), while the other contained a P-element insertion (*EY11817*) in the 3′ noncoding exon (Fig. 1*A*). We mobilized the P-element, resulting in a 2.4-kilobase deletion, which removed the entire 419-aa coding region [see Fig. 1 *A* and *B*; *Gr93a[2]* and supporting information (SI) Fig. S1]. Thus, *Gr93a[2]* was a null allele. *Gr93a[1]* appeared to be a strong allele as the *Gr93a* RT-PCR product was greatly reduced relative to an internal control (*tubulin*) (Fig. 1*C*, *Upper*). However, *Gr93a[1]* may not be a null allele, as we detected a low-level of a *Gr93a* RT-PCR product after additional PCR

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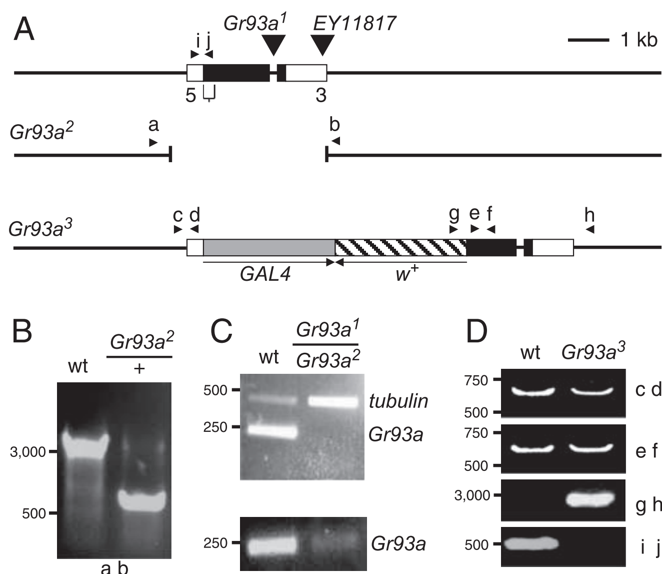

Fig. 1. Generation of *Gr93a* alleles. (*A*) Schematic of the *Gr93a* locus and alleles. The 2 *Gr93a* exons are indicated by rectangles. The insertion sites of the Piggybac transposon (*Gr93a*[1]) and the P-element, *EY11817* are indicated. The deletion in *Gr93a*[2] created by imprecise excision of *EY11817* is indicated. *Gr93a*[3] was generated by ends-out homologous recombination. The gray and striped boxes indicate the *GAL4* and *miniwhite* genes, respectively. The bracket below the representation of the complete *Gr93a* gene indicates the deletion in *Gr93a*[3], which removes the 5′ end of the protein-coding region. The arrowheads indicate the primers used for the PCR analyses in (*B*) and (*D*). The arrows indicate the orientation of the *GAL4* and *miniwhite* genes. (*B*) Confirmation of the deletion in *Gr93a*[2] by PCR (using primers a and b; see *A*) and by DNA sequencing. (*C*) Analyses of *Gr93a* and *tubulin* RT-PCR products from *Gr93a*[1]/*Gr93a*[2] and *w*[1118] flies. The *Gr93a* RT-PCR products in the top and bottom panels were obtained after 35 and 37 PCR cycles, respectively. (*D*) Confirmation of the *Gr93a*[3] mutation by PCR analyses using the indicated primer pairs.

amplification cycles (Fig. 1*C*, *Lower*) (see *Methods*). To create a third allele, we used ends-out homologous recombination (17). We deleted a 539 base-pair region encoding the N-terminal 180 residues and first 3 transmembrane segments, and inserted the *GAL4*-coding region at the site corresponding to the initiation codon in *Gr93a* (Fig. 1 *A* and *D*).

_Gr93a_ Is Required for Caffeine-Avoidance Behavior. To address whether *Gr93a* was required for the behavioral responses to either aversive or attractive tastants, we used a variation of the binary food-choice assay (18). We allowed starved flies to feed in a microtiter dish with wells alternating between agar mixed with sugar or agar alone. The 2 alternatives were mixed with either red or blue food dyes and the colors of the abdomens were assessed (red, blue, or purple). Preference indexes (PI) of 1 or 0 indicated that all of the flies consumed either the sugar or the agar alone, respectively, while a PI of 0.5 resulted if there was no bias. We found that the *Gr93a*-mutant flies displayed similar preferences for sugars as the wild-type controls (Fig. 2*A* and Table S1).

To assess whether *Gr93a* was required for avoiding bitter compounds, we assessed the ability of mutant flies to choose between either 1-mM sucrose or 5-mM sucrose combined with aversive compounds. The bitter tastants were mixed with a higher concentration of sucrose (5 mM) because some compounds that elicit avoidance responses, such as caffeine, suppress the attractive response to sugars (18). We found that the *Gr93a*[3] flies avoided quinine, strychnine, berberine, denatonium, lobeline, and papaverine to the same extent as wild-type (Fig. 2*B* and Table S2).

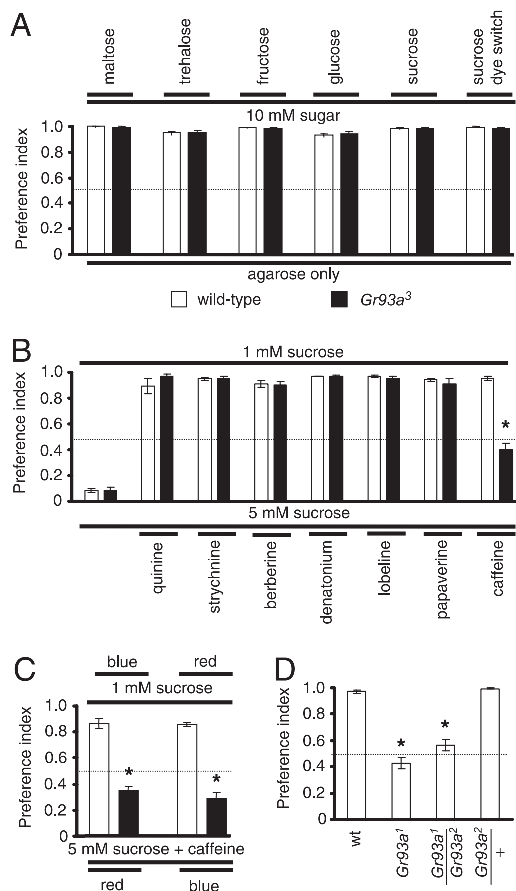

Fig. 2. Binary food-choice assays. (*A*–*C*) Wild-type (*white bars*) and *Gr93a*[3] (*black bars*). (*A*) Sugar preferences. Flies were allowed to choose between 1% agarose plus the indicated sugar (10 mM) or 1% agarose only. (*B*) Avoidance of noxious compounds. The 2 alternatives were 1-mM sucrose alone or 5-mM sucrose either alone or in combination with the following aversive compounds: 1-mM quinine, 0.5-mM strychnine, 0.1-mM berberine, 0.3-mM denatonium, 0.3-mM lobeline, 2-mM papaverine, or 10-mM caffeine. (*C*) The dye color did not affect caffeine avoidance. The red and blue dyes were added to 1-mM sucrose or 5-mM sucrose plus caffeine, as indicated. (*D*) Caffeine-avoidance behavior in *Gr93a*[1] and *Gr93a*[1]/*Gr93a*[2] flies. The error bars represent SEMs. The asterisks indicate significant differences from wild type ($P < 0.01$) using unpaired Student's *t* tests. See Tables S1–S4 for statistics.

In contrast to the results with other aversive compounds, caffeine avoidance was impaired in the *Gr93a*[3]-mutant animals (see Fig. 2*B* and Table S2). These results were surprising, as the only *Gr* expressed in avoidance GRNs (*Gr66a*) and previously characterized functionally was also required exclusively for the caffeine response (16). However, not every *Gr* expressed in avoidance GRNs is essential for the caffeine response because we have recently generated mutations in *Gr8a* and *Gr47a*, which are expressed in *Gr66a*-expressing GRNs but are not required for aversive behavior to caffeine (S.J.M. and C.M., unpublished observations). The wild-type avoidance of caffeine and the defect in the *Gr93a* mutants was not a consequence of different responses to the red or blue food coloring because the same phenotypes were observed upon switching the tastants/dye combinations (Fig. 2*C* and Table S3). To provide additional evidence that *Gr93a* was required for caffeine sensation, we examined additional alleles and found that *Gr93a*[1] and *Gr93a*[1]/*Gr93a*[2] transheterozygous flies displayed defects in caffeine avoidance similar to *Gr93a*[3] (Fig. 2*D* and Table S4). The caffeine response

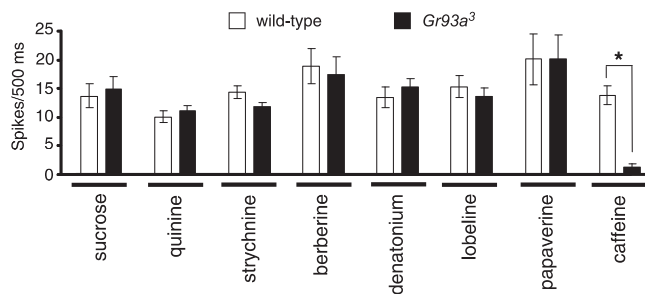

Fig. 3. *Gr93a* is required for caffeine-induced action potentials. Tip recordings were carried out on S6 bristles on the labella. Shown are average frequencies of action potentials (spikes/500 ms) after application of 50-mM sucrose, 1-mM quinine, 0.1-mM berberine, 1-mM denatonium, 1-mM lobeline, 5-mM papaverine, 1-mM strychnine, or 10-mM caffeine. The error bars represent SEMs. The asterisk indicates a significant difference from wild type ($P < 0.00001$) using the unpaired Student's *t* test. See Table S5 for statistics.

in *Gr93a*^{2}/+ was indistinguishable from wild-type, demonstrating that loss-of-function mutations in *Gr93a* were recessive (see Fig. 2*D* and Table S4).

Gr93a Functions in Aversive GRNs. Because both *Gr66a* and *Gr93a* are required for the aversive responses to caffeine, the question arises as to whether they function together in the same GRNs. As a first step in addressing this question, we performed tip recordings, which measure action potentials in the GRNs in response to tastants. Consistent with the behavioral assays, the frequencies of action potentials stimulated by application of sucrose and most bitter tastants were similar in *Gr93a*^{3} and wild-type (Fig. 3 and Table S5). However, caffeine-induced action potentials were virtually eliminated (see Fig. 3 and Table S5). These results indicate strongly that Gr93a is required for the caffeine response in GRNs. The combination of the *Gr93a*^{3} and *Gr66a*^{ex83} mutations does not appear to cause a more severe gustatory defect than the single mutations, as *Gr66a*^{ex83},*Gr93a*^{3} flies show wild-type electrophysiological responses to sucrose and quinine, in addition to the expected deficit in the caffeine-induced action potentials (Fig. S2).

Corequirements for Gr93a and Gr66a. Loss of *Gr66a* results in the same deficit in the caffeine response as in *Gr93a*-mutant animals, raising the possibility that the 2 Grs are functionally coexpressed. To detect Gr93a expression, we attempted to use the *GAL4*/*UAS* system to generate a *Gr93a* reporter. However, the *GAL4* reporter inserted in *Gr93a*^{3} was not expressed (data not shown). We also obtained 2 transgenes, 1 of which fused the *GAL4* gene to 9 kb of the *Gr93a* 5′ flanking sequence, and the other of which included 2 kb of 5′ flanking sequence and 7 kb of 3′ flanking sequence. Neither of these latter *GAL4* drivers was expressed (data not shown). Therefore, we attempted to raise anti-Gr93a polyclonal antibodies, although previous attempts to detect Grs with antibodies have not been successful. We found that the anti-Gr93a antibodies stained dendrites, axons, and cell bodies of a subset of GRNs in wild-type labella (Fig. 4*A* and Fig. S3*A*). Although the antibodies were not effective on Western blots, the immunostaining in the labellum appeared to be specific for Gr93a because the GRNs were not stained in *Gr93a*^{3}-mutant labella (see Fig. 4*A* and Fig. S3*C*). We did not detect anti-Gr93a expression in 2 other tissues examined: legs and antennae (data not shown). Because Gr66a antibodies were unavailable, we costained labella from *Gr66a-GAL4*;*UAS-mCD8*::*GFP* flies with anti-GFP and anti-Gr93a. The anti-Gr93a and anti-GFP signals overlapped extensively, if not completely (see Fig. 4*A* and Fig. S4). In addition, 2 internal taste neurons from the pharynx

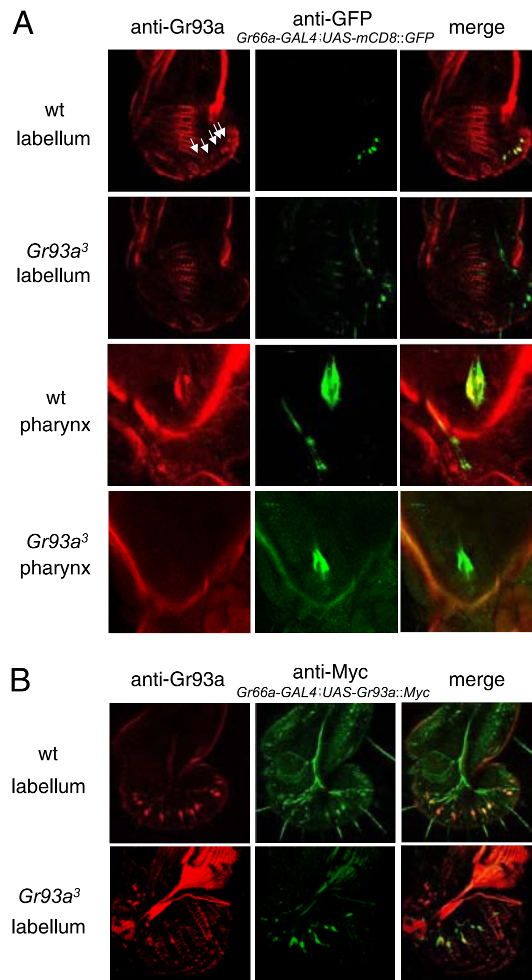

Fig. 4. Expression of the Gr93a protein in the labellum and pharynx. (*A*) Coexpresson of Gr93a in *Gr66a*-expressing GRNs. GFP was expressed using the *UAS-mCD8*::*GFP* and the *Gr66a-GAL4* transgenes (anti-Gr93a antibodies, *red*; anti-GFP antibodies, *green*). The right panels show the merged anti-Gr93a and anti-Gr66a signals. The genotypes and tissues are indicated to the left side. Examination of multiple stacks of confocal optical sections indicates that the Gr93a protein and the *Gr66a* reporter are coexpressed (Fig. S4). Expression of the *Gr66a* reporter in *Gr93a*^{3} labella (*Second row*) indicates that the Gr66a- and Gr93a-expressing GRNs are present in *Gr93a*^{3}. Gr93a is expressed in the pharynx in *Gr66a*-expressing GRNs (*Third row, left*), but not in *Gr93a*^{3} pharynx (*Fourth row, left*). (*B*) The Gr93a::Myc protein was expressed in flies harboring the *Gr66a-GAL4* and *UAS-Gr93a*::*Myc* transgenes. Labella were stained (anti-Gr93a, *red*; anti-Myc, *green*) and the signals were detected by confocal microscopy. The merged images are shown to the right.

expressed the Gr93a protein and the *Gr66a* reporter (see Fig. 4*A*). The Gr expression in these latter neurons, which are situated between the mouth and digestive system, suggest that flies may evaluate food quality after it is ingested and before it is transmitted to the gut. We introduced the *Gr66a-GAL4*;*UAS-mCD8*::*GFP* transgenes into the *Gr93a*^{3} background and found that the anti-GFP signals were indistinguishable between wild-type and *Gr93a*^{3} flies (see Fig. 4*A*). These data, combined with the observation that the anti-GFP signal was present in *Gr93a*^{3} labella expressing the *mCD8*::*GFP* reporter (see Fig. 4*A*), indicate that the *Gr93a*^{3} mutation did not result in loss of the GRNs that express Gr93a and Gr66a.

To address whether Gr66a and Gr93a both function in the same GRNs, we first tested whether the behavioral and electro-

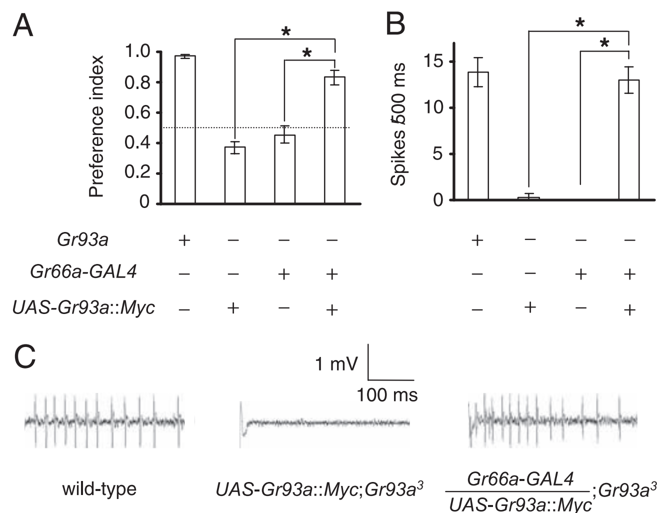

Fig. 5. Rescue of the caffeine sensation defect in *Gr93a*^{3} using the *Gr66a-GAL4* and *UAS-Gr93a::Myc* transgenes. (*A*) Binary food-choice assay using either *Gr93a*$^{+}$ flies or *Gr93a*^{3} mutant flies harboring the *Gr66a-GAL4* and/or *UAS-Gr93a::Myc* transgenes. (*B*) Average frequencies of action potentials (spikes/500 ms) induced by presentation of 10-mM caffeine using the indicated fly lines. (*C*) Sample tip recordings. The asterisks indicate significant differences ($P < 0.01$) from wild-type. See Tables S6 and S7 for statistics.

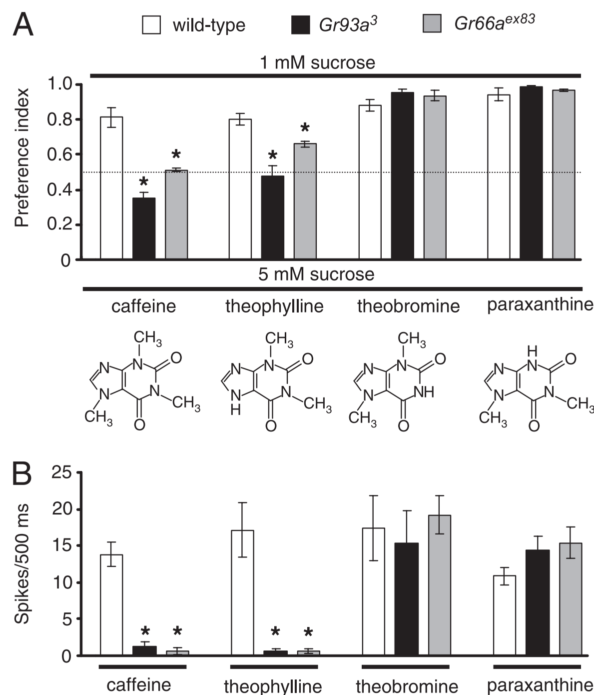

Fig. 6. *Gr93a* and *Gr66a* are required for detection of the same methylxanthines. (*A*) Binary food-choice assays were conducted with 1-mM sucrose versus 5-mM sucrose plus 6-mM of each methylxanthine. (*B*) The average frequencies of action potentials (spikes/500 ms) were collected upon application of 10-mM of each methylxanthine. Error bars represent SEMs. The asterisks indicate significant differences from wild type using the unpaired Student's *t* test. See Tables S8 and S9 for statistics.

physiological deficits in *Gr93a*-mutant animals were rescued by expression of a wild-type *Gr93a* transgene (*UAS-Gr93a::Myc*) under the control of the *Gr66a* promoter (*Gr66a-GAL4*). We found that introduction of these transgenes into *Gr93a* mutant flies (see Fig. 4*B*) significantly suppressed the impairment in caffeine avoidance (Fig. 5*A*; Table S6) and caffeine-induced action potentials (Fig. 5 *B* and *C*; Table S7). Because *Gr66a* and *Gr93a* are both required for caffeine avoidance, it is possible that they are sufficient for detecting caffeine in GRNs. However, coexpression of the 2 Grs in Gr5a-expressing GRNs (Fig. S5) did not elicit behavioral or electrophysiological responses to caffeine (Fig. S6). The Gr93a and Gr66a::Myc proteins localized normally in Gr66a and Gr93a mutants, respectively, indicating that 1 receptor did not affect trafficking of the other receptor (see Fig. S3).

To test further whether there are identical requirements for *Gr66a* and *Gr93a* for *Drosophila* taste, we assayed the responses of the mutant flies to a variety of methylxanthines related to caffeine. Wild-type flies avoid all methylxanthines tested. In addition to caffeine (1,3,7-trimenthylxanthine), this includes theophylline (1,3-dimethylxanthine) and theobromine (3,7-dimethyxanthine), which are produced in tea and cocoa, respectively, and paraxanthine (1,7-dimethyxanthine) (Fig. 6*A*). Presentation of these methylxanthines also produced action potentials in the GRNs (Fig. 6*B*). As we have reported previously, *Gr66a*^{ex83} mutant flies displayed deficits in the behavioral and electrophysiological responses to caffeine and theophylline, but not theobromine (see Fig. 6) (16). However, in the present study, we found that paraxanthine induced action potentials and aversive behavior in *Gr66a*^{ex83} (see Fig. 6). Of significance here, we found that these 4 methyxanthines elicited the same behavioral and electrophysiological profiles in the *Gr93a* and *Gr66a* mutants (see Fig. 6; Tables S8 and S9).

Discussion

The ability to avoid ingestion of noxious botanical compounds is crucial for insect survival. However, before the current study, only 1 receptor, *Gr66a*, which is essential for the caffeine response, was associated with a specific bitter tastant (16). Because Gr66a appears to be insufficient for generating a caffeine response, a critical question concerns the molecular complexity of the receptors that operate in detecting deleterious nonvolatile substances.

We propose that Gr93a might be a coreceptor required in concert with Gr66a for sensing caffeine. In support of this conclusion, mutation of Gr93a and Gr66a results in identical phenotypes. Both Grs are required for avoiding caffeine and for caffeine-induced action potentials in GRNs, but not for the responses to any other unrelated compound tested. Moreover, the 2 Grs are expressed in the same GRNs and function in the same cells, as introduction of a wild-type *Gr93a* transgene under control of the *Gr66a* transcriptional control rescued the *Gr93a*-mutant phenotype. However, unlike the odorant receptor (Or), Or83b, which is required for the spatial localization of other Ors (19), neither Gr66a nor Gr93a appeared to impact of the cellular distribution of the other receptor.

We suggest that the composition of the *Drosophila* taste receptors is more complex than for other types of chemosensory receptors in flies or in mammals. In mammals, taste receptors are either homo- or heterodimers, while olfactory receptors are homomeric proteins (20, 21). *Drosophila* Ors appear to heterodimers comprised of Or83b in combination with one additional Or (19, 22). The *Drosophila* CO_2 receptor is a heterodimer consisting of Gr21a and Gr63a (23, 24). Misexpression of these 2 receptors is sufficient to confer CO_2 sensitivity to neurons that do not normally respond to CO_2 (23, 24). In contrast, cointroduction of the 2 taste receptors Gr66a and Gr93a in Gr5a-expressing GRNs does not confer caffeine sensitivity to these cells. Although we cannot exclude that there are critical downstream-signaling molecules missing in Gr5a-expressing GRNs, we suggest that functional bitter taste receptors are not het-

erodimers, but are higher order assemblies consisting of additional subunits. Because caffeine and many other botanical compounds function as naturally occurring insect repellents and pesticides, the identification of the composition of the Grs that are sufficient for avoiding toxic chemicals offers potential for devising mechanisms for reducing interactions between insect pests and plants.

Methods

Genetics, Fly Stocks, and Constructs. The *f01688*, *EY11817*, and *UAS-mCD8*::*GFP* fly lines were from the Exelixis Collection at the Harvard Medical School or from the Bloomington Stock Center. The *Gr66a-GAL4* line was provided by H. Amrein (11). The *f01688* Piggybac line was semi-lethal; however, after 5 out-crossings to w^{1118}, the line ($Gr93a^1$) was fertile and viable. *EY11817* inserted in the 3′ untranslated region 670 base pairs 3′ to the Gr93a stop codon. Caffeine avoidance in *EY11817* was similar to wild-type: PI was 0.86 ± 0.04 and 0.91 ± 0.02, respectively. We mobilized the *EY11817* P element by crossing them to a genetic source of transposase: *w*; *Sp/Cyo; ry* Sb^1 P{*ry*[+*t*7.2] = Δ2–3}99B/TM6B. The imprecise excision deleted the entire *Gr93a* coding region ($Gr93a^2$). To identify the $Gr93a^2$ deletion, we screened ≈200 lines by PCR using the following primers: *a*, 5′-AAATTTAATGGCGATACTTGTT-3′; and *b*, 5′-ACATATTGTAGCTACCT-CACCC-3′. $Gr93a^2$ had a 2,357 base-pair deletion extending from 365 nucleotides upstream of the *Gr93a* start codon to the P-element-inserted site. This deletion removed the entire *Gr93a* coding region. The wild-type control for all experiments was w^{1118}.

Homologous Recombination and Generation of Transgenic Flies. We generated $Gr93a^3$ by ends-out homologous recombination (17). We subcloned 2 PCR-amplified genomic fragments (3.16 and 3.03 kb) into a modified pw35 vector containing the yeast *GAL4* gene, obtained nonhomologous insertions by germline transformation, and generated homologous recombinants as described (17). The $Gr93a^3$ mutation was verified by PCR and out-crossed 5 times to w^{1118}.

To obtain the *UAS-Gr93a*::*Myc* transgene, we amplified the full-length *Gr93a* cDNA by RT-PCR using fly head mRNA (Stratagene), and inserted the DNA coding for a Myc tag in place of the *Gr93a* stop codon. The DNA sequence of the construct was verified by sequencing and subcloned into the pUAST vector.

RT-PCR Analyses of $Gr93a^1/Gr93a^2$. Whole-fly mRNA from wild-type and $Gr93a^1/Gr93a^2$ flies were extracted (Stratagene) and AMV reverse transcriptase was used to generate cDNAs (Promega). For quantitative RT-PCR, we used the following primers: 5′-TGGGATAAGAGTGTTGAAA-3′ and 5′-CTGTAAG-TAGCTTAATCA-3′ with tubulin primers as an internal control.

Chemicals. Caffeine, quinine hydrochloride, denatonium benzoate, papaverine hydrochloride, strychnine nitrate salt, sucrose, glucose, maltose, fructose, tricholine citrate, and sulforhodamine B were from Sigma-Aldrich. Lobeline hydrochloride, and trehalose were from Fluka, and berberine sulfate trihydrate, and brilliant blue FCF were from Wako Chemical.

Immunohistochemistry and Generation of Anti-Gr93a Antibodies. Antibody stainings were performed as described (25). Briefly, we placed freshly dissected tissue (e.g., labella) from 3- to 7-day-old flies into wells of 48-well cell-culture cluster plates (Costar Corp.) maintained on ice and containing 940 μl of Fix Buffer (0.1M Pipes pH6.9, 1 mM EGTA, 1% TritonX-100, 2 mM $MgSO_4$, 150 mM NaCl) and 60 μl of 37% formaldehyde. The formaldehyde was added to the wells containing the Fix Buffer and mixed immediately before adding the tissue. We transferred as many dissected tissues into the Fix Buffer-containing formaldehyde as we could dissect in 30 min. The tissues were incubated for another 30 min, washed with Wash Buffer (1× PBS, 0.2% saponin) and blocked for 4 to 8 h at 4 °C with 1 ml of Blocking Buffer (1× PBS, 0.1% saponin, 5 mg/ml BSA). The tissues were transferred and incubated into the primary antibody mixture overnight at 4 °C, washed 3 times with Wash Buffer for 15 min each, incubated in the secondary antibody mixture for 4 h at 4 °C, and washed 3 times with Wash Buffer for 15 min each on ice. The tissues were transferred into 1.25× PDA Dilution Buffer (37.5% Glycerol, 187.5 mM NaCl, 62.5 mM Tris pH8.8), incubated >1 h at 4 °C, and mounted and analyzed using a Carl Zeiss confocal microscope.

We generated the polyclonal rabbit anti-Gr93a antibodies using the following peptide: [KLH]-CIESQDERYRNTKYRR-NH_2 (Peptron). The antibodies were preabsorbed using fly embryos and used for staining at a 1:1,000 dilution. Other antibodies were used at the following dilutions: mouse anti-Myc (1:200, Santa Cruz), rabbit anti-GFP (1:500, Santa Cruz), mouse anti-GFP (1:1,000, Invitrogen–Molecular Probes), goat anti-mouse secondary antibodies (Alexa 488; 1:200, Invitrogen–Molecular Probes) and goat anti-rabbit secondary antibodies (Alexa 568; 1:200, Invitrogen–Molecular Probes).

Behavioral Assays. The binary food-choice assays were performed as described (16). In short, we starved the flies (3–7 days old) for 18 h on 1% agarose. We then placed the flies into 72-well microtiter dishes, which contained wells filled with 1% agarose plus either red dye (sulforhodamine B, 0.2 mg/ml, Sigma-Aldrich) or blue dye (brilliant blue FCF, 0.125 mg/ml, Wako Chemical). Either the red or blue dye mixtures contained aversive compounds (1 mM quinine, 0.3 mM denatonium, 0.1 mM berberine, 0.3 mM lobeline, 2 mM papaverine, or 0.5 mM strychnine) or sugars at the indicated concentrations. The flies were placed in the microtiter dishes for 90 min at room temperature (in the dark and in a humidified chamber), and the numbers of the flies that were blue (N^B), red (N^R), or purple (N^P) were ascertained by inspection of the abdomen color. The PIs were determined using the following equation: $PI = (N^B + 0.5\, N^P)/N^{Total}$ or $(N^R + 0.5\, N^P)/N^{Total}$. The dyes did not influence the preferences. Every experiment was conducted ≥4 times.

Electrophysiology. To determine the frequencies of action potentials in response to tastants, we performed tip recordings on taste sensilla as described (16). Briefly, we immobilized 3-day-old flies by puncturing the thorax with a glass capillary filled with Ringer's solution and sliding it into the head. This electrode also served as a reference electrode. We stimulated the labellar bristles with the recording electrode (10–20 μm tip diameter) using the indicated concentrations of aversive compounds and either 1-mM KCl or 50-mM sucrose plus 30-mM tricholine citrate as the electrolyte. We performed the recordings on S6 sensilla on the labial palp. We connected the recording electrode to a preamplifier (Taste PROBE, Syntech), amplified the signals 10× using a signal connection interface box (Syntech) in conjunction with a 100 to 3,000 Hz band pass filter, and recorded action potentials at a 12 kHz sampling rate. We performed all recordings 6 to 12 times.

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