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Report

A Taste Receptor Required for the Caffeine Response In Vivo

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Summary

Caffeine is a methylxanthine present in the coffee tree, tea plant, and other naturally occurring sources and is among the most commonly consumed drugs worldwide. Whereas the pharmacological action of caffeine has been studied extensively, relatively little is known concerning the molecular mechanism through which this substance is detected as a bitter compound. Unlike most tastants, which are detected through cell-surface G protein-coupled receptors, it has been proposed that caffeine and related methylxanthines activate taste-receptor cells through inhibition of a cyclic nucleotide phosphodiesterase (PDE) [1]. Here, we show that the gustatory receptor Gr66a is expressed in the dendrites of *Drosophila* gustatory receptor neurons and is essential for the caffeine response. In a behavioral assay, the aversion to caffeine was specifically disrupted in flies missing Gr66a. Caffeine-induced action potentials were also eliminated, as was the response to theophylline, the methylxanthine in tea. The *Gr66a* mutant exhibited normal tastant-induced action potentials upon presentation of theobromine, a methylxanthine in cocoa. Given that theobromine and caffeine inhibit PDEs with equal potencies [2, 3], these data further support the role of Gr66a rather than a PDE in mediating the caffeine response. Gr66a is the first gustatory receptor shown to be essential for caffeine-induced behavior and activity of gustatory receptor cells in vivo.

Results and Discussion

During the last few years, there has been considerable progress in defining the G protein-coupled receptors (GPCRs) that function as taste receptors in mammals and in *Drosophila*. Among the 68 predicted *Drosophila* gustatory receptors (GRs) [4–6], only the trehalose receptor, Gr5a, has been shown to be associated with a specific tastant [7–9]. Moreover, there is no report of a vertebrate or invertebrate taste receptor that is required for the responses to caffeine or other methylxanthines.

The *Drosophila* Gr66a receptor is an excellent candidate for being required for the bitter response because it is expressed in gustatory receptor neurons (GRNs) that respond to all aversive compounds [10]. *Gr66a* is expressed in small (S type) and intermediate (I type) sensilla [11], which contain GRNs that respond well to bitter compounds. Many of the I and S type sensilla also contain neurons that display sugar-induced activity. However, *Gr66a* is not expressed in large (L type) sensilla, which do not display a strong response to aversive tastants [12, 13]. Toxin-induced ablation or inhibition of synaptic transmission of *Gr66a*-expressing GRNs greatly reduces the avoidance behavior upon presentation of each of the bitter compounds tested [11, 14]. Because the bitter-responsive cells are eliminated in these flies, the requirement for the Gr66a receptor for the response to a specific aversive compound has not been defined.

To identify a bitter tastant that depends on Gr66a for the aversive behavioral response, we deleted the *Gr66a* locus. To do so, we took advantage of a P element transposon (GE20354, Genexel, Korea) that was situated 1.1 kb from the 3′ end of *Gr66a* and that inserted in the 5′ untranslated region of the flanking gene *CG7066* (Figure 1A). Flies homozygous for the GE20354 element exhibited normal gustatory responses (see below). Because mobilization of P elements is frequently associated with deletions that flank the initial insertion site, we genetically introduced transposase and used a PCR-based strategy [15] to identify a deletion that removed the *Gr66a* gene. After analyzing ~200 lines, we identified one (*ex83*) with a 3.3 kb deletion that disrupted *Gr66a* and two flanking genes (*CG7066* and *CG7188*; Figure 1A). The *ex83* flies were homozygous viable and fertile. In order to test whether any phenotype was due to disruption of *Gr66a* and not *CG7066* and *CG7188*, we introduced into *ex83* flies transgenes that restored either all three genes (Figure 1A; P[*8-Gr66*$^{+}$]) or just the two neighboring genes, *CG7066* and *CG7188*, but not *Gr66a* (Figure 1A; P[*7-Gr66a*$^{-}$]). Flies containing the GE20354 element were used and are referred to here as the wild-type control because these flies represented the parental background from which the *ex83* mutant was derived.

Bitter compounds are aversive to flies, and given a choice between sucrose and a sucrose/bitter compound mixture, wild-type animals exhibit a very high propensity to consume the sucrose. Wild-type flies also favor 5 mM over 1 mM sucrose when given a choice between these concentrations of sugars. A preference index (PI) of 1.0 or 0 results if there is a complete feeding preference of one over the other of two testing tastants, while a PI of 0.5 indicates that there is no bias for either tastant. The assay is conducted by allowing 50 flies to feed in a microtiter dish containing wells alternating between the two test tastants, which have been mixed with either a red or blue dye. Feeding behavior is assessed by examining the colors of the abdomens. The color of the dye consumed reflects the intrinsic preference for one tastant over another, rather than a preference for one dye, because identical results are obtained regardless of the

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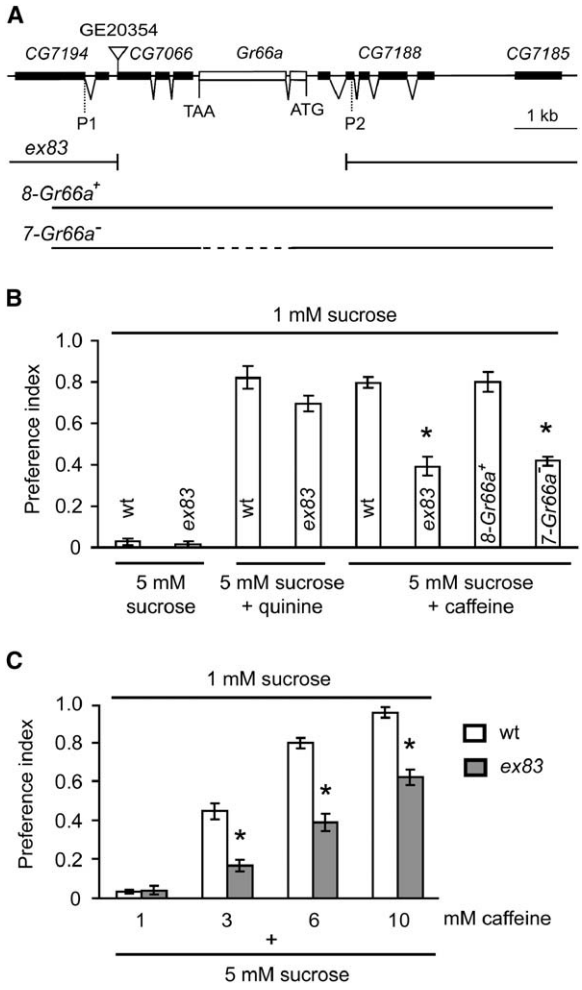

Figure 1. Deletion of *Gr66a* Causes a Defect in the Caffeine Avoidance Response

(A) Physical map of the *Gr66a* genomic region. *Gr66a* (*CG7189*) is located at 66C5 on the third chromosome. The inverted triangle indicates the insertion site of the P element transposon (GE20354). The PCR primers P1 and P2 were used to screen for deletions. The deletion in *ex83* flies, which removes *CG7066*, *Gr66a* (*CG7189*), and *CG7188*, is indicated. The genomic DNAs included in the P[*8-Gr66*$^+$] and P[*7-Gr66a*$^-$] transgenes are indicated. The *Gr66a* DNA missing in P[*7-Gr66a*$^-$] is indicated by the dashes.

(B and C) Defect in caffeine sensing in flies missing *Gr66a*.

(B) Two-way choice tests were performed with the indicated flies in microtiter dishes containing wells alternating between one or the other compound mixed with blue or red dyes. All assays were performed with 5 mM sucrose (either alone or in combination with either 1 mM quinine or 6 mM caffeine as indicated) versus 1 mM sucrose alone. A value of 1.0 indicates a complete preference for 1 mM sucrose, whereas a value of 0 indicates a complete preference for the 5 mM sucrose (either alone or with quinine or caffeine). A value of 0.5 indicates no preference. See Experimental Procedures for the calculation of the preference index. Fifty flies were used per assay (n = 6). The error bars represent SEMs. The asterisks indicate significant differences from wild-type or *8-Gr66a*$^+$ flies ($p < 0.0001$) when ANOVA with the Scheffé post-hoc tests are used.

(C) Concentration-dependent behavioral responses when 1 mM sucrose and 5 mM sucrose are used in combination with different concentrations of caffeine as indicated (1–10 mM, n = 6). The error bars represent SEMs, and the asterisks indicate statistically significant differences between wild-type and *ex83* flies ($p < 0.0005$) when unpaired Student's t tests are used.

particular dye/tastant combination (see Experimental Procedures). As expected, given that *Gr66a* reporters are not expressed in sugar-responsive cells, wild-type and *ex83* flies both showed the same strong preferences for 5 mM sucrose versus 1 mM sucrose (Figure 1B; wild-type, PI = 0.03 ± 0.02, n = 6; *ex83*, PI = 0.01 ± 0.01, n = 6). The *ex83* flies exhibited an avoidance of quinine, demonstrating that there was not a broad defect in the aversion to bitter compounds (Figure 1B; wild-type, PI = 0.82 ± 0.06, n = 6; *ex83*, PI = 0.69 ± 0.04, n = 6).

Of significance here, we found that *ex83* flies displayed a strong defect in the avoidance of caffeine (Figure 1B; wild-type, PI = 0.80 ± 0.03, n = 6; *ex83*, PI = 0.39 ± 0.04, n = 6). This deficiency was rescued by the transgene encoding *Gr66a* and the two adjacent genes P[*8-Gr66*$^+$] (Figures 1A and 1B; PI = 0.80 ± 0.05, n = 6), but not by the transgene including just the two flanking genes P[*7-Gr66a*$^-$] (Figures 1A and 1B; PI = 0.42 ± 0.02, n = 6). The defect in the caffeine-induced avoidance response was observed over the range of caffeine concentrations (3, 6, and 10 mM) (Figure 1C). The caffeine-induced aversion to sucrose was reduced rather than eliminated in *ex83*. However, caffeine has dual effects on the taste response. Although there are separate neurons that are activated in response to caffeine and sucrose, it is known that caffeine also directly inhibits sugar-activated taste neurons [16].

To address whether Gr66a was required in the GRNs, rather than for central brain processing of the caffeine-initiated signal, we performed tip recordings from taste bristles (sensilla). Tastants at previously described concentrations were applied to a sensillum that is on the labellum (S6) and expresses *Gr66a* and displays responses to both sweet and bitter compounds [12, 13]. Consistent with the behavioral assays, the frequencies of action potentials induced upon application of sucrose were normal in *ex83* flies (Figures 2A and 2B; spikes/s: wild-type, 20.2 ± 3.7, n = 18; *ex83*, 23.1 ± 2.6, n = 13). In addition, the responses of *ex83* flies to two other sweet compounds tested were not reduced relative to wild-type flies (Figure 2B; spikes/s to trehalose: wild-type, 15.5 ± 2.8, n = 18; *ex83*, 14.6 ± 3.7, n = 14; spikes/s to glucose: wild-type, 18.8 ± 3.0, n = 15; *ex83*, 27.0 ± 3.8, n = 15). Application of the aversive compounds quinine, berberine, or denatonium also resulted in virtually the same frequencies of action potentials in wild-type (Figures 2C and 2D; spikes/s: 11.5 ± 1.9, 23.4 ± 5.4, and 15.6 ± 2.9, respectively, $n \geq 14$) and *ex83* flies (Figures 2C and 2D; spikes/s, 9.5 ± 2.1, 23.5 ± 5.3, and 14.8 ± 3.1, respectively, $n \geq 16$). Given that Gr66a-expressing cells respond to all bitter compounds tested [10] and toxin-induced ablation of these cells disrupts the responses to these bitter compounds [14], it appears that deletion of the *Gr66a* gene alone does not eliminate or impair the overall function of these cells.

In contrast to the normal responses to other bitter compounds, we found that caffeine-induced action potentials were eliminated in *ex83* flies. Whereas caffeine gave rise to action potentials in wild-type (Figures 2E, 2F, and 2G, n = 17), virtually no response was detected in *ex83* flies, even at the highest caffeine concentration tested (Figures 2E, 2F, and 2G; n = 17). The deficit was due to absence of *Gr66a* and not the neighboring genes because the normal frequency of caffeine-initiated

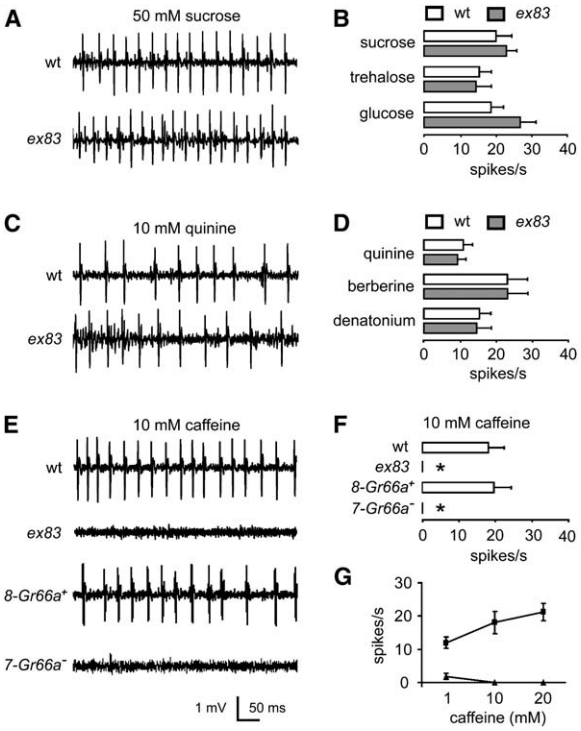

Figure 2. Elimination of Caffeine-Induced Action Potentials as a Result of Loss of *Gr66a*

The data were collected for 5 s after application of the indicated tastants to S6 sensilla on the labella. Representative traces showing the action potentials obtained with wild-type and *ex83* flies (A, C, and E) 50–550 ms after application of the indicated tastants. The mean number of spikes (B, D, F, and G) was based on quantitation of the nerve firings (± SEM) between 50 and 1050 ms after presentation of the compounds. The number of successful recordings (based on the appearance of tastant-induced spikes) and the total number of attempted recordings are indicated: n = successful recordings/total recordings. The means are based on all recordings, including unsuccessful recordings.

(A) Action potentials in response to 50 mM sucrose.

(B) Mean number of action potentials in response to sweet substances: 50 mM sucrose (wild-type, n = 14/18; *ex83*, n = 13/13), 50 mM trehalose (wild-type, n = 13/18; *ex83*, n = 10/14), and 50 mM glucose (wild-type, n = 14/15; *ex83*, n = 14/15).

(C) Action potentials in response to 10 mM quinine.

(D) Mean number of action potentials in response to bitter compounds: 10 mM quinine (wild-type, n = 16/20; *ex83*, n = 11/17), 10 mM berberine (wild-type, n = 14/17; *ex83*, n = 14/18), and 10 mM denatonium (wild-type, n = 12/14; *ex83*, n = 10/16).

(E) Action potentials in response to 10 mM caffeine.

(F) Mean number of action potentials in response to 10 mM caffeine (wild-type, n = 13/17 *ex83*, n = 0/17; *8-Gr66a*$^{+}$, n = 14/21; *7-Gr66a*$^{-}$, n = 0/18). Asterisks indicate significant differences from wild-type or *8-Gr66a*$^{+}$ ($p < 0.0001$) when ANOVA with the Scheffé post-hoc tests are used.

(G) Plot showing the mean number of action potentials in wild-type and *ex83* flies in response to 1, 10, and 20 mM caffeine (n = 17 for each caffeine concentration).

action potentials was restored upon introduction of the P[*8-Gr66*$^{+}$] transgene, which included *CG7066*, *Gr66a*, and *CG7188* (Figures 2E and 2F; 19.7 ± 4.6 spikes/s, n = 21), but not P[*7-Gr66a*$^{-}$], which encoded only *CG7066* and *CG7188* (Figures 2E and 2F; 0 ± 0 spikes/s, n = 18). Moreover, a Myc::Gr66a fusion protein, which was expressed under control of the *Gr66a* promoter by using the *Gal4/UAS* system [17], restored the caffeine-

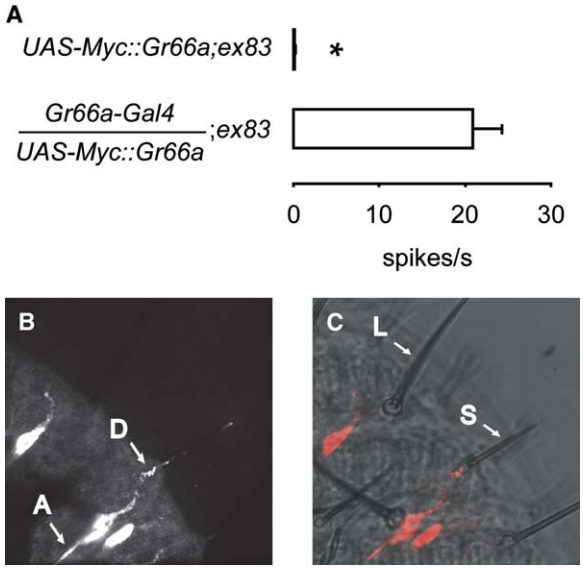

Figure 3. Subcellular Localization of the Gr66a Protein

(A) Expression of *UAS-Myc::Gr66a* under the control of *Gr66a-Gal4* rescues the ex83 phenotype. Shown are the action potentials in *UAS-Myc::Gr66a;ex83* flies and *Gr66a-Gal4/UAS-Myc::Gr66a;ex83* flies in response to 10 mM caffeine (± SEM; n = 2/12 and 12/13, respectively). Asterisk indicates a significant difference from between the two sets of data ($p < 0.00006$) when unpaired Student's t tests are used.

(B) Confocal fluorescent image of the Myc::Gr66a protein. The protein was detected in the labellum of flies containing P[*Gr66a-Gal4*] [11] and P[*UAS-Myc::Gr66a*] transgenes when anti-Myc antibodies were used. The axon (A) and dendrite (D) of a Myc::Gr66a positive GRN are indicated.

(C) Composite of the bright-field and fluorescent image of the Myc::Gr66a protein. L and S type sensilla are indicated. Anti-Myc staining was not detected in flies containing the P[*UAS-Myc::Gr66a*] but not the P[*Gr66a-Gal4*] transgene (data not shown).

induced action potentials in *ex83* flies (Figure 3A; 20.8 ± 3.4 spikes/s, n = 13).

Currently there are no reports that describe the subcellular localizations of *Drosophila* GR proteins in GRNs. Therefore, we examined the subcellular distribution of the Myc::Gr66a protein, which rescued the *ex83* phenotype. To spatially localize Myc::Gr66a, we stained whole mounts of labella with anti-Myc antibodies. Fly GRNs are bipolar neurons containing a single axon and a single dendrite. We found that in those GRNs expressing Myc::Gr66a, the protein was detected throughout the cells including cell bodies, axons, and the dendrites (Figures 3B and 3C), the latter of which are the sites of chemoreception in the GRNs. In most cases, staining was limited to the proximal region of the dendrites, presumably because of an inability of the primary and secondary antibodies to penetrate effectively the cuticle surrounding the sensilla. By following the stained dendrites through different focal planes, we traced at least 80% to the base of chemosensory sensilla (data not shown).

The preceding results demonstrate that Gr66a is required for caffeine-induced response in vivo. To determine whether Gr66a was sufficient to confer caffeine responsiveness in a heterologous system, we expressed Gr66a in tissue-culture cells. However, we were unable to obtain cell-surface expression or Gr66a-dependent activity (Figure S1 in the Supplemental Data available

online), reminiscent of the situation with many olfactory receptors [18]. Therefore, we cannot exclude that Gr66a may be required as a coreceptor, rather than functioning independently as a caffeine-activated gustatory receptor.

It has been suggested that the caffeine response results from inhibition of a cyclic nucleotide phosphodiesterase (PDE) [1]. To investigate this possibility in *Drosophila*, we compared the caffeine-induced action potentials in wild-type and *ex83* flies, upon presentation of several methylxanthine inhibitors of PDE [2, 3]. In addition to caffeine (1,3,7-trimethylxanthine; Figure 4A), there are other naturally occurring methylxanthines such as theophylline (1,3-dimethylxanthine) and theobromine (3,7-dimethylxanthine), that are found in products such as tea and cocoa, respectively. We found that action potentials were induced in wild-type flies upon application of each of the methylxanthines tested (theophylline, 1,7-dimethylxanthine, and theobromine) (Figure 4B; 19.2 ± 4, 11.8 ± 2.4, 13.6 ± 3.2, respectively, $n \geq 18$). In *ex83* flies, the number of action potentials was eliminated or greatly reduced in response to theophylline or 1,7-dimethylxanthine (Figure 4B; 0 ± 0 and 1.0 ± 0.2 respectively, $n \geq 18$). However, the mutant flies exhibited normal nerve firings when exposed to theobromine (Figure 4B; 16.6 ± 0.5, n = 19).

In the current work, we demonstrate that the Gr66a gustatory receptor is expressed in dendrites and required for the caffeine response in *Drosophila* GRNs. In particular, we show that Gr66a is required for the caffeine-induced avoidance behavior and caffeine-induced action potentials in vivo. We conclude that caffeine is functioning through Gr66a in *Drosophila* GRNs, rather than through inhibition of a cyclic nucleotide PDE because the potencies of theobromine and caffeine for inhibition of cyclic nucleotide-PDEs are similar [2, 3] and deletion of *Gr66a* does not disrupt the response to theobromine. It is intriguing to speculate that the caffeine response in mammalian taste-receptor cells may also be mediated through a GPCR. Finally, it will also be of considerable interest to identify the nature of the signaling cascade that couples with Gr66a to give rise to the caffeine response in vivo.

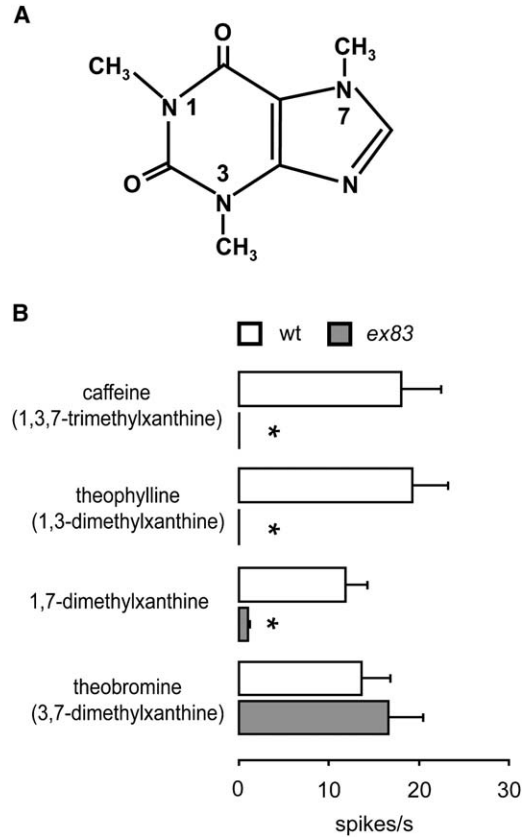

Figure 4. Differential Responses of *ex83* Flies to Related Methylxanthine Derivatives

(A) Chemical structure of caffeine (1,3,7-trimethylxanthine).

(B) Mean numbers of action potentials in response to methylxanthine derivatives. The number of successful recordings (based on the appearance of tastant-induced spikes) and the total number of attempted recordings are indicated: n = successful recordings/total recordings. The means are based on all recordings, including unsuccessful recordings. Recordings were performed on S6 sensilla stimulated with 10 mM theophylline (wild-type, n = 18/23; *ex83*, n = 0/20), 10 mM 1,7-dimethylxanthine (wild-type, n = 19/24; *ex83*, n = 3/18), and 10 mM theobromine (wild-type, n = 13/18; *ex83*, n = 14/19). The data obtained with caffeine are the same as shown in Figure 2F. The error bars indicate SEMs. Asterisks indicate significant differences between wild-type and *ex83* flies ($p < 0.0001$) when unpaired Student's t tests are used.

Experimental Procedures

Genetics, Fly Stocks, and Constructs

The P element in the GE20354 flies (purchased from Genexel, Korea) was mobilized by genetically introducing the transposase by using the Δ2-3 line [19]. We identified lines containing excisions of the P element, on the basis of reversion of w^+ to w^-, and screened for deletions that extended into Gr66a by using a PCR-based approach as described [15] in combination with the following primers: (P1) 5′-TCATCGGGCAAATTTAGCTTGACGCGATCC-3′ and (P2) 5′-TCGGATTCTGCTCCGGCCAGACGCTCGGAC-3′. Out of ~200 screened, one line was recovered that contained a deletion extending from the P element insertion site in *CG7066* to *CG7188* (Figure 1A). The size of the deletion, as determined by DNA sequencing, was 3389 base pairs. To create the P[*8-Gr66*$^+$] transgene, we subcloned an 8.0 kb KpnI fragment—which spanned *CG7066*, *Gr66a*, and *CG7188*—from the *Drosophila* genomic DNA clone, BAC RP 98-13E21, into the pCaSper4 vector [20]. The 7 kb P[*7-Gr66*$^-$] transgene differed from P[*8-Gr66*$^+$] because of removal of a HindIII/EcoRI fragment that included the *Gr66a* coding region. The P[*UAS-Myc::Gr66a*] transgene construct was generated by subcloning the full coding region of *Gr66a* into the pP[*UAS-Myc*] vector [21]. The P[*Gr66a-Gal4*] transgenic flies [11] were kindly provided by Dr. H. Amrein. All of the transgenic lines were genetically introduced into the *ex83* deletion-mutant background.

Chemicals

Sucrose, glucose, caffeine, theophylline, theobromine, 1,7-dimethylxanthine, quinine, denatonium, KCl, and tricholine citrate were purchased from Sigma-Aldrich, trehalose was obtained from Fluka, and berberine sulfate trihydrate was obtained from Wako Pure Chemical Industries.

Immunohistochemistry

To determine the subcellular localization of Myc::Gr66a, we expressed the fusion protein by genetically combining the P[*Gr66a-Gal4*] [11] and P[*UAS-Myc::Gr66a*] transgenes. To visualize Myc::Gr66a protein expression, we dissected the labella from P[*Gr66a-Gal4*]/P[*UAS-Myc::Gr66a*] flies from adult heads, fixed them in 4% paraformaldehyde with 0.2% Triton X-100 for 30 min on ice, and rinsed them three times with 1× PBS, 0.2% Triton X-100 (PBS-T). The samples were blocked for 30 min with 5% heat-inactivated goat serum in PBS-T and incubated at 4°C overnight in a 1:200

dilution of mouse anti-Myc antibodies (Santa Cruz) in blocking buffer. The tissues were washed three times with PBS-T, blocked as above for 30 min, and incubated with the secondary antibodies (goat anti-mouse Alexa568; Molecular Probe) at a dilution of 1:200 in PBS-T with 5% heat-inactivated goat serum for 2 hr at room temperature. After a final wash, the labella were cut in half, mounted in 70% glycerol, and visualized with an Ultraview confocal microscope (PerkinElmer).

Electrophysiology

Recordings of action potentials on labellar hairs were performed with modifications of previously described methods [22, 23]. In brief, newly eclosed flies were immobilized by inserting a glass capillary filled with Ringer's solution into the abdomen all the way through to the head. This electrode also served as the indifferent electrode. The labellar hairs were stimulated with a recording electrode (10–20 μm tip diameter). For recording the sweet responses, the sugar solution used for stimulation contained 30 mM tricholine citrate (TCC) as the electrolyte [23]. One millimolar KCl was used as the electrolyte for the recordings of bitter compounds.

The recordings were performed on S6 sensilla on the labial palp. The recording electrode was connected to a preamplifier (TastePROBE, Syntech, Hilversum, The Netherlands), and the signals were collected and amplified (10×) by using a signal-connection interface box (Syntech) in conjunction with a 100–3000 Hz band-pass filter. Recordings of action potentials were acquired with a 9.6 kHz sampling rate and analyzed with Autospike 3.1 software (Syntech).

Behavioral Assays

The two-way choice assay was carried out as described previously [16]. In brief, 50 flies (3–6 days old) were starved overnight and introduced into a 72-well microtiter dish filled with 1% agarose combined with one of two types of test mixtures in alternating wells. For the sucrose test, this consisted of wells with either 5 mM or 1 mM sucrose. The aversion to quinine and caffeine was assayed with 1 mM sucrose versus 5 mM sucrose plus 1 mM quinine or 5 mM sucrose plus 1–10 mM caffeine, respectively. For monitoring food intake, one test mixture contained a blue dye (brilliant blue FCF, 0.125 mg/ml; Wako Chemical; 027-12842), and the other contained a red dye (sulforhodamine B, 0.2 mg/ml; Sigma-Aldrich; S9012). After the flies were allowed to feed for 90 min at room temperature in the dark, the animals were frozen at −20°C. The numbers of flies that were blue (N^B), red (N^R), or purple (N^{MIX}) were determined on the basis of the colors of the abdomen, and the preference index (PI) values were calculated according to the following equation: $(N^B + 0.5N^{mix})/(N^R + N^B + N^{mix})$ or $(N^R + 0.5N^{mix})/(N^R + N^B + N^{mix})$, depending on the dye/tastant combinations. In some experiments, the tastant and red/blue food dye mixtures were switched to ascertain that the dyes did not cause a preference. No preference was observed for the red and blue food intake dyes obtained from Sigma-Aldrich and from Wako Chemical, respectively. For example, in those assays in which wild-type flies were allowed to choose between 1 mM sucrose versus 5 mM sucrose plus 6 mM caffeine, the PI values were 0.78 ± 0.04 when blue and red dyes were used for the sucrose only or sucrose plus caffeine, respectively, and 0.81 ± 0.03 when the dyes were switched.

Data Analyses

All error bars represent standard errors of the means (SEMs). Unpaired Student's t tests were used to compare two sets of data. ANOVA with the Scheffé post-hoc tests were used to compare multiple sets of data. p values < 0.05 were considered to be statistically significant.

Supplemental Data

Supplemental Data include Experimental Procedures and one figure and are available with this article online at: http://www.current-biology.com/cgi/content/full/16/18/1812/DC1/.

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