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Taste Perception and Coding in *Drosophila*

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Summary

Background: Discrimination between edible and contaminated foods is crucial for the survival of animals. In *Drosophila*, a family of gustatory receptors (GRs) expressed in taste neurons is thought to mediate the recognition of sugars and bitter compounds, thereby controlling feeding behavior.
Results: We have characterized in detail the expression of eight *Gr* genes in the labial palps, the fly's main taste organ. These genes fall into two distinct groups: seven of them, including *Gr66a*, are expressed in 22 or fewer taste neurons in each labial palp. Additional experiments show that many of these genes are coexpressed in partially overlapping sets of neurons. In contrast, *Gr5a*, which encodes a receptor for trehalose, is expressed in a distinct and larger set of taste neurons associated with most chemosensory sensilla, including taste pegs. Mapping the axonal targets of cells expressing *Gr66a* and *Gr5a* reveals distinct projection patterns for these two groups of neurons in the brain. Moreover, tetanus toxin-mediated inactivation of *Gr66a*- or *Gr5a*-expressing cells shows that these two sets of neurons mediate distinct taste modalities—the perception of bitter (caffeine) and sweet (trehalose) taste, respectively.
Conclusion: Discrimination between two taste modalities—sweet and bitter—requires specific sets of gustatory receptor neurons that express different *Gr* genes. Unlike the *Drosophila* olfactory system, where each neuron expresses a single olfactory receptor gene, taste neurons can express multiple receptors and do so in a complex *Gr* gene code that is unique for small sets of neurons.

Introduction

Taste is a vital sense for animals. Sensory cells located in the taste organs, such as the tongue of mammals or the labial palps of many insects, are dedicated to differentiating between a multitude of structurally diverse chemical compounds, some associated with nutrients and others with potentially harmful toxins. For example, humans and many other mammals are able to recognize hundreds of different soluble substrates, all of which are classified into the five basic taste qualities of bitter, sweet, umami, sour, and salty. Two distinct receptor families have been shown to mediate three of these taste qualities: the T2R protein family of about 30 to 40 G protein-coupled receptors (GPCRs) recognizes a diverse array of substrates that are perceived as bitter-tasting [1–3], and three distinct GPCRs, the T1Rs, recognize amino acids and sugars and provide the molecular basis for umami and sweet taste [4–10]. Many insects, including *Drosophila*, have taste preferences for these same ligands; for example, the fruit fly prefers foods that include sugars and amino acids, but avoids foods that contain compounds perceived as bitter [11–17].

In *Drosophila* only a single gene family—the gustatory receptor (*Gr*) genes—has been proposed to mediate many, if not all, taste qualities [18–22]. The *Gr* genes are almost exclusively expressed in taste neurons (called gustatory receptor neurons or GRNs), which are associated with taste bristles and pegs located at the tip of the labellum (the labial palps), in three clusters inside the pharynx (Figure 1), and numerous taste bristles on the legs and the anterior wing margin [18, 20, 22]. Expression analysis of a few *Gr* genes using the Gal4/UAS system revealed that each gene is expressed in a small subset of neurons, typically comprising less than 4% of GRNs, suggesting the possibility that each *Gr* gene is expressed in distinct, nonoverlapping populations of cells [18, 20]. Interestingly, one *Gr* gene (*Gr21a*) was found to be expressed exclusively in olfactory neurons of the antenna but in none of the adult gustatory receptor neurons [20].

Two of the 60 *Gr* genes, *Gr5a* and *Gr68a*, have been characterized and were shown to function as a sugar and a putative pheromone receptor, respectively [12, 13, 19]. *Gr5a* encodes a receptor for the sugar trehalose, a metabolic component of yeast and therefore a major food component of many *Drosophila* species [12, 13]. *Gr68a* encodes a male-specific receptor that is required for the tapping step during courtship and is likely to encode a pheromone receptor for a long-chain hydrocarbon [19]. Therefore, we propose that most fly GR proteins are chemosensory receptors for a wide range of soluble ligands that may mediate both pleasant (sweet) and repulsive (bitter) responses, as well as pheromone signals [19, 21].

To expand our understanding of taste coding in the peripheral taste organs and the brain, we carried out a more detailed expression analysis of the *Gr* gene family, determined the taste property of classes of GRNs expressing specific (sets of) *Gr* genes, and investigated the projection pattern of such neurons to the tritocerebrum and the subesophageal ganglion (SOG) —the first relay center of taste processing in the brain. Our results show that labellar *Gr* genes fall into two distinct groups. We found that seven of the *Gr* genes analyzed are partially coexpressed in overlapping sets of neurons. The projection patterns to the tritocerebrum/SOG of neurons expressing these receptors are therefore very similar, and behavioral studies suggest that these GRNs may mediate aversive taste response. An eighth *Gr* gene (*Gr5a*) is largely, and possible exclusively, expressed in a different set of neurons than the other seven *Gr* genes, has a very different projection pattern in the brain, and mediates trehalose sensitivity.

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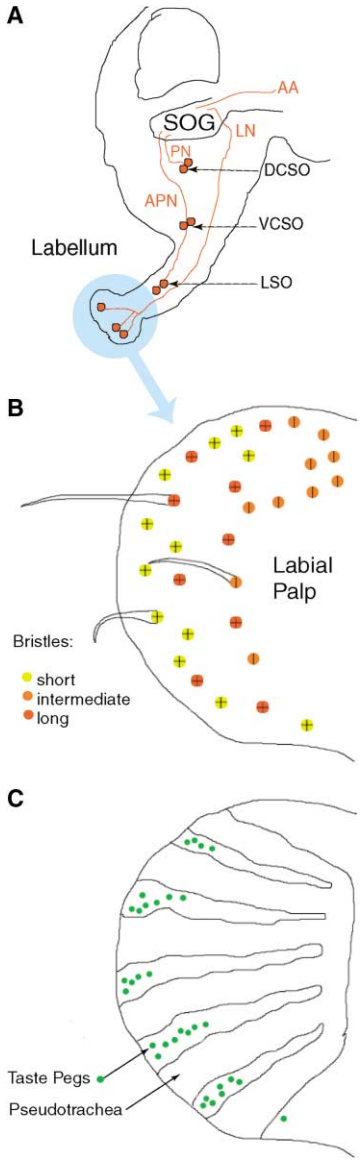

Figure 1. Organization of the Taste Sensory Organs and Processing Centers in the Head

(A) Side view of *Drosophila* head showing sensory neurons of the labellum projecting their axons to the SOG via the labral nerve (LN) and sensory neurons of the pharynx (LSO, VCSO, DCSO) via the accessory pharyngeal and pharyngeal nerves (APN, PN).

(B) Side view of the surface of one labial palp of the labellum. There are three morphologically different types of taste bristles—short (S, yellow), intermediate (I, orange), and long (L, red)—arranged in a stereotyped pattern. S and L bristles contain dendrites of four taste neurons and I bristles contain two.

(C) Side view of the inner surface of one labial palp depicting the taste peg sensilla that lie between the pseudotracheae. Each taste peg sensillum is thought to contain a single chemosensory neuron [23].

Results

The labellum, considered to be the main taste organ in *Drosophila*, has approximately 62 chemosensory bristles (sensilla) that are arranged in a stereotyped pattern. These sensilla are morphologically identified as short (S), intermediate (I), and long (L) [23] (Figure 1). S and L bristles house dendrites of four chemosensory neurons, whereas I bristles are associated with two chemosensory neurons [23]. To determine expression of *Gr* genes in these chemosensory neurons, we and others have employed the Gal4/UAS system [24]. This indirect method of expression analysis has proven far superior to RNA in situ hybridization due to low levels of *Gr* transcripts per cell and the wide distribution of taste neurons in tissues not amenable to sectioning procedures [18, 20, 25]. The Gal4/UAS analyses revealed that a given *Gr* gene is expressed in a small number of chemosensory neurons per labial palp (Table 1) and, in each case, in only one neuron per chemosensory bristle. Tanimura and coworkers [25] also demonstrated an association of specific *Gr* genes with certain bristles of the labellum. They found that the majority of receptors examined were expressed in one of the four neurons of S type sensilla. For example, several *Gr* genes were strongly expressed in a single neuron associated with three S type sensilla (S1, S3, and S6).

Several issues with broad implications for taste coding, however, remain to be elucidated. For example, it is still not known whether some *Gr* genes are coexpressed in the same neurons and, if so, to which extent. Similarly, it is not known what kind of taste properties are mediated by GRNs expressing these receptors. Finally, experiments to visualize axonal targets in the CNS of neurons expressing individual *Gr* genes have not been performed in any detail. To further advance our understanding of *Drosophila* taste perception, we addressed these questions: we determined the number of neurons expressing novel and previously characterized *Gr* genes, investigated their extent of coexpression, visualized the projection patterns of GRNs expressing these genes, and determined taste perception of flies lacking specific sets of GRNs.

Expression Map of *Gr* Genes in the Labial Palps

Gal4 drivers (*p[Gr]-Gal4*) for eight *Gr* genes, *Gr5a*, *Gr22b*, *Gr22e*, *Gr22f*, *Gr28be*, *Gr32a*, *Gr59b*, and *Gr66a*, were combined with a *UAS-nucGFP* reporter gene encoding a green fluorescent protein tagged with a nuclear localization signal and images of optical sections through the entire labellum were collected by using confocal microscopy after anti-GFP antibody staining (for details, see Experimental Procedures). By using the map generated by Tanimura and coworkers as a guide [25], our detailed analysis of confocal stacks allowed us to more accurately determine the organization and number of neurons expressing each of these genes (Table 1 and Figure 2). The expression patterns fell into two broad groups: *Gr5a* (representing the first group and described later in greater detail) was expressed in a large number of neurons throughout the entire labial palp, whereas the other *Gr* genes had restricted expression to relatively few neurons.

Of the second group, *Gr66a* was expressed in the largest number ($n = 22 \pm 1$) of cells per palp. Significantly, only a single neuron per S and I type sensillum stained positive for this driver. The neurons associated with S type bristles, which are located more medially, appeared larger in size compared to more laterally located neurons of I type sensilla (Figures 2A and 2B).

Table 1. Peripheral Expression of *Gr*s

	Neurons Stained per Labial Palp				Other Peripheral Expression		Averages		
Receptor	This Study	Hiroi et al. [25]	Scott et al. [20]	Dunipace et al. [18]	LSO	VCSO	First Leg	Second Leg	Third Leg
Gr22b	10				N	Y	2	0	0
Gr22e	14	14 (10s)		15	Y	Y	2	8	10
Gr22f	3	15 (2s)	3	4–8	N	N	0	0	0
Gr28be	13		9		N	Y	2	0	0
Gr32a	8	10 (6s)	6		N	Y	5	3	3
Gr59b	4	18 (4s)		2	N	N	0	0	0
Gr66a	22	22 (8s)	9	8	Y	Y	8	7	6
Gr5a	71				N	N	10	4	4
Gr5a C.	55				N/D	N/D	N/D	N/D	N/DF

We determined the number of GFP-positive neurons in at least five to ten stained labella in two independent lines for each driver. In many cases, our average is comparable to the number of strongly staining neurons as determined by Hiroi et al. [25]. Also included are labellar neuron counts made by the groups that originally characterized the expression pattern of most of these receptors [18, 20]. Expression of *Gr*s in pharyngeal neurons was confirmed by X-Gal staining of *p[Gr]-Gal4; UAS-lacZ* flies. The expression of *Gr*s in the legs was determined by using *p[Gr]-Gal4; UAS-nucgpf* flies.

Gr22b, *Gr22e*, *Gr22f, Gr28be*, *Gr32a*, and *Gr59b* were expressed in fewer neurons than *Gr66a* (Figure 2 and Table 1). Expression of these receptors appears more restricted to larger neurons associated mostly with S type bristles. Taken together, our expression data are consistent with initial studies made by Axel and coworkers and by our group [18, 20] and confirm the more detailed analysis conducted by Hiroi and coworkers [25]. Thus, these expression studies provided the groundwork necessary to determine whether two or more *Gr* genes are actually coexpressed in the same neuron associated with an S type bristle.

Many *Gr* Genes Are Coexpressed in Some GRNs

Ideally, coexpression of *Gr* genes may be addressed by labeling individual *Gr* gene-specific probes with different markers. However, expression levels of these genes are too low for reliable detection of transcripts by RNA in situ hybridization. We attempted to use the Gal4/UAS system along with a second reporter system, the tetracycline transactivator/tet-O reporter system [26]. The sensitivity of this system, however, was too low to obtain reliable cell staining in taste neurons (S.B. and H.A., unpublished data). Therefore, we sought to address the issue of coexpression by quantification of labeled cells using the Gal4/UAS system, an approach that seemed feasible given the relatively low number of cells in which each receptor is expressed. We made transgenic fly lines expressing *UAS-nucGFP* under the control of two different Gal4 drivers and then counted and compared the number of labeled neurons to that of flies containing each driver alone (Figure 2 and Table 2). Surprisingly, in all cases where such double-driver experiments were carried out, the number of labeled cells expressing two drivers was close or equal to the number of labeled cells of flies containing the single driver with the higher cell count (Table 2). For example, in flies that express either the *p[Gr66a]-Gal4* or *p[Gr22e]-Gal4* driver, an average of 22 and 14 neurons/labial palp are labeled, respectively (Figures 2B and 2C, Table 2). In flies that express both drivers, again approximately 22 neurons are detected per palp, which indicates that most if not all cells that express *Gr22e* also express *Gr66a* (Figure 2K). Similar results were obtained when *Gr66a* was compared to *Gr32a* (Table 2).

We next asked how expression of *Gr22e* relates to expression of *Gr22b*, *Gr22f*, *Gr28be*, and *Gr59b*-receptors expressed in smaller numbers of neurons (Table 1 and Figures 2 and S1). The number of labeled cells in the corresponding double driver lines was approximately equal to the 14 labeled neurons observed in palps of flies containing only *Gr22e*, suggesting that these *Gr* genes are also all coexpressed with *Gr22e* and—by extension—with *Gr66a*. It is possible, however, that *Gr32a* and *Gr28be* are expressed in one or two neurons that do not express *Gr22e*.

The experiments above clearly indicate that the *Gr* genes examined thus far are expressed in overlapping sets of gustatory receptor neurons. The observed pattern of expression also shows that about eight to ten neurons express at least four of the receptors tested (*Gr66a*, *Gr22e*, *Gr28be*, and *Gr32a*) and that a few neurons might express up to six receptors (*Gr66a*, *Gr22e, Gr32a*, *Gr28be*, *Gr22b*, and *Gr22f* or *Gr59b*); thus, a given neuron may be identified by a *Gr* gene code. A second novel observation derived from our analysis is the finding that the receptors analyzed thus far are expressed only in one of the four neurons of S type sensilla and one of the two neurons of I type sensilla. This finding is supported by the observation that only a single dendrite extends from the cell body to the bristle shaft (Figures 2A–2H). Even in the few cases where two GFP-positive cells lay relatively close to each other, we have been able to track their dendrites into distinct bristles, ruling out the possibility that the corresponding neurons are associated with the same sensillum.

The Trehalose Receptor GR5a Defines a Largely Distinct Set of GRNs

The *Gr5a* gene encodes the only receptor with a known function in taste perception [11–13], but its expression has not been analyzed in detail [27]. Hence, it is of

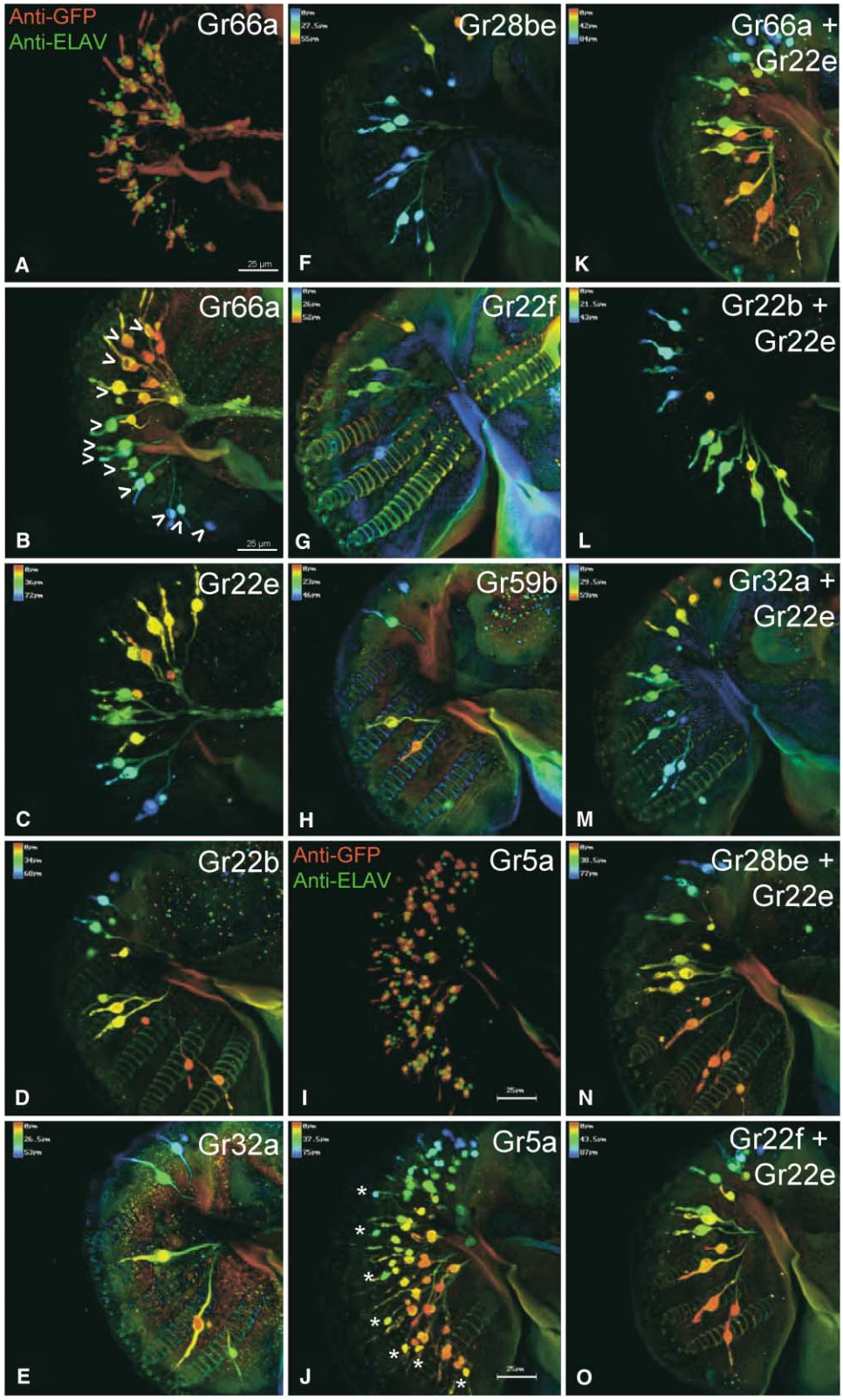

Figure 2. Expression of *Gr*s in the Labellum

Labella of *p[Gr]-Gal4; UAS-nucgpf* flies were dissected and stained with anti-GFP (red) and anti-ELAV (green) and visualized with a confocal microscope. ELAV staining (green) colocalizes with anti-nucGFP (red) demonstrating that the *Gr* genes are expressed in neurons of the labellum (A + I). All images are optical stacks, with multicolored images created by using the depth-coding option included with the confocal software. Depth coding was used to enhance the visualization of *Gr*-expressing (GFP-positive) neurons of the labellum and was often necessary

Table 2. Coexpression of Various *Gr* Genes

	Neurons/Palp			
Receptor	Single Driver	+Gr22e Driver	+Gr66a Driver	+Gr5a Driver
Gr22e	14 ± 1		23 ± 2	
Gr66a	22 ± 1	23 ± 2		
Gr22b	10 ± 2	13 ± 1		
Gr32a	8 ± 1	14 ± 1	23 ± 3	
Gr28be	13 ± 2	14 ± 2		
Gr22f	3 ± 1	13 ± 1		
Gr59b	4 ± 1	13 ± 1		
Gr5a	71 ± 11			
Gr5a C.	55 ± 5			64 ± 9

Average number of neurons expressing either of two *Gr* genes in the double-driver lines *p[Gr 1]-Gal4/UAS-nucgfp; p[Gr 2]-Gal4/TM3*. If the number of neurons stained in the double driver line is equal to that of either of the single *Gr* drivers, then the two *Gr*s are coexpressed in the same labellar neurons. Counts were derived from at least five to ten stained labella/genotype.

primary interest to determine the cellular expression profile of *Gr5a*. We created a *p[Gr5a]-Gal4* driver by cloning the putative promoter fragment of the *Gr5a* gene in front of the Gal4 coding sequence (see Experimental Procedures) and analyzed expression by using the *UAS-nucgfp* reporter. Several aspects of the observed *Gr5a* expression pattern are strikingly different from that of the previously analyzed *Gr* drivers.

Visual inspection of confocal z series unambiguously revealed that *Gr5a* is expressed in about three times as many neurons as the most abundant, previously analyzed driver (*p[Gr66a]-Gal4*; compare Figures 2A and 2B with 2I and 2J; see Figure S2 and Table 1). In contrast to neurons expressing the other receptors, *Gr5a*-expressing neurons were distributed over the entire surface of the palp and appeared all to be of the same (small) size (Figures 2I and 2J). Interestingly, *Gr5a*-expressing cells are often clustered in groups of two or three neurons, and rarely do we observe up to four neurons adjacent to one another (Figure 3E). When the dendrites of clustered neurons are visible, they converge into the same bristle, indicating that these neurons belong to the same sensillum (Figures 3E, S2C, and S2D). Finally, about seven *Gr5a*-expressing neurons per labial palp were located between the pseudotrachea (Figures 2J and 3F). These cells are not associated with bristles but coincide with the location of taste pegs, providing direct support that these sensilla may have a function in chemosensory perception as suggested by their morphology [23].

To investigate coexpression with the other *Gr* genes, we combined the *p[Gr5a]-Gal4* and *p[Gr22e]-Gal4* drivers within single flies. Due to the relatively large number and the high density of *Gr5a*-expressing cells (especially within clusters), cell counts were not sufficiently accurate, and we therefore used the difference in cell size as a criterion for coexpression. Analysis of each optical section from flies expressing *UAS-nucgfp* under the control of both these drivers revealed an average of nine large cells per palp (Figures 3A, 3D, and S3; and data not shown), whereas the same counting procedure applied to sections from flies with only the *p[Gr5a]-Gal4* revealed no large cells (Figures 2I, 2J, and 3B). Approximately nine large cells were also found in palps of flies with only the *p[Gr22e]-Gal4* driver (Figures 2C and 3C), strongly suggesting that at least these large cells do not express *Gr5a*. By extension, *Gr22b* and *Gr28be* expression is to a large extent also distinct from *Gr5a* expression, and *Gr32a*, *Gr22f*, and *Gr59b*, which appeared to be expressed exclusively in large neurons (Figures 2E, 2G, and 2H), may not be coexpressed with *Gr5a*. However, we cannot exclude the possibility that the few small cells that express some of these genes and about half of the (small) neurons expressing *Gr66a* also express *Gr5a*.

We note that the expression of another Gal4 driver for the *Gr5a* gene, which contained significantly more upstream sequence including a neighboring gene (*p[Gr5a_C]-Gal4*), was very similar to the expression of our *p[Gr5a]-Gal4* driver. This driver was shown to be expressed in cells of the labellum, as might be expected for a genuine sugar receptor [27]. Direct comparison of the two drivers showed that they exhibited similar expression profiles, the only difference being the somewhat lower cell count for *p[Gr5a_C]-Gal4* (Figures S2B, S2E, and Table 2). In summary, these studies show that *Gr5a* is expressed in a different pattern than any of the previously analyzed *Gr* genes.

Gr66a- and *Gr5a*-Expressing Neurons Mediate Different Taste Perceptions

The expression analysis presented above raises the question as to whether the neurons expressing the

to discriminate individual cells with similar locations along the X and Y axes. Note that ELAV staining is not revealed in these images due to single-channel imaging. *Gr66a* (A and B) is expressed in 22 taste neurons per labial palp, with *Gr22e* (C), *Gr22b* (D), *Gr32a* (E), *Gr28be* (F), *Gr22f* (G), and *Gr59b* (H) expressed in a decreasing number of neurons. *Gr5a* (I + J) is expressed in significantly more neurons than any of the other *Gr* genes examined—approximately 70 neurons per palp. Interestingly, *Gr5a* appears to be expressed in neurons associated with taste peg sensilla (*). *Gr22e* appears to be coexpressed in a subset of neurons expressing *Gr66a* (K). *Gr22b, Gr32a, Gr22f*, and *Gr28be* are coexpressed in subsets of neurons that express *Gr22e* and, hence, *Gr66a* (L–O). Arrowheads indicate larger neurons associated with S type bristles. Please note that nucGFP is found at significant levels outside the nucleus and is detected by anti-GFP antibody staining in the cytoplasm and dendrites of neurons at high magnification. All images are of similar magnification. For quantitative data, see Table 2.

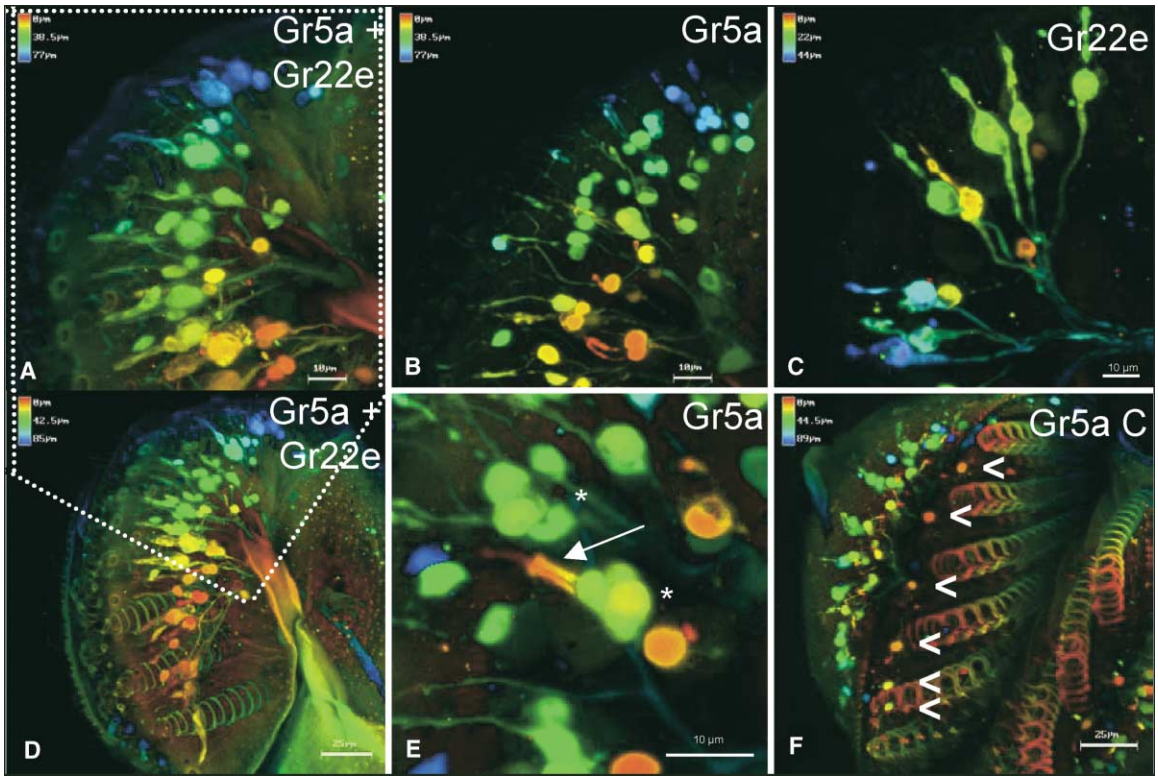

Figure 3. *Gr5a* Is Expressed in a Largely Nonoverlapping Set of Neurons in the Labellum

(A–D) *Gr5a* (B) is expressed in labellar neurons with cell bodies that appear significantly smaller than neurons that express *Gr22e* (C). This is especially obvious when comparing the single driver lines to the double driver line (*p[Gr5a]-Gal4/UAS-nucgfp; p[Gr22e]-Gal4/+*; [A] and [D]), which appears as a composite of the two single drivers and in which both large and small cell-bodied neurons stain. Note that [A] is an enlargement of an area in [D].

(E) *Gr5a* is often expressed in clusters of two to four neurons (indicated by an asterisk). The dendrites of these neurons can be seen converging to enter the shaft of the same bristle (arrow). Thus, unlike the other *Gr*s examined, *Gr5a* appears to be expressed in multiple neurons associated with the same bristle.

(F) *Gr5a* is also expressed in seven single neurons situated between the pseudotracheae (arrowheads), a location in which the taste peg sensilla are found. Shown is staining of the *Gr5a* driver from Chyb et al. [27].

Gr22e group of genes mediate a distinct taste modality as compared to that expected for neurons expressing *Gr5a*. Since *Gr5a* has been shown to encode a receptor for the sugar trehalose, we expect that inactivation of GRNs expressing this gene would result in reduction of sensitivity for trehalose. However, we would also predict that inactivation of GRNs expressing *Gr66a* or other *Gr* genes would not lead to any change in the perception for this sugar.

To investigate the function of these different subsets of neurons, we combined Gal4 drivers expressed in the two distinct sets of neurons with a UAS-tetanus toxin light chain (TNT) reporter. TNT specifically cleaves Synaptobrevin, a protein essential for neurotransmitter release [28], thereby rendering TNT-expressing neurons functionally inactive. In combination with the Gal4 system, TNT has been used for inactivation of neurons both in the peripheral and central nervous system of *Drosophila* [19, 29–33]. To control for genetic background and effects of potential *Gal4*-independent TNT expression, we used flies that contained the *UAS-tnt* reporter as controls. The effect on taste perception in these flies was measured using the two-choice feeding assay [11]. The feeding preference is represented by preference index (PI), whereby a PI of 0 and 1 indicate complete feeding preference for one or the other substrate, respectively, and a PI of 0.5 indicates no (or equal) preference for either of the substrates (for details, see Experimental Procedure and Figure 4).

As expected, flies expressing *UAS-tnt* under the control of *p[Gr5a]-Gal4* showed a significant reduction in their preference for 25 mM trehalose containing agarose when compared to control flies or flies with inactivated *Gr66a*-, *Gr22e*-, or *Gr32a*-expressing neurons (Figure 4). We also tested these flies for the perception of sucrose. At the minimal concentration where this sugar is reliably detected (2 mM), all flies, including those expressing TNT under the control of the *p[Gr5a]-Gal4* driver, continued to show a strong preference for the sucrose-containing substrate. Thus, our behavioral assays indicate that *Gr5a*-expressing neurons mediate high sensitivity to trehalose, but not sucrose. Second, neurons expressing *Gr66a*, *Gr22e*, and *Gr32a* (and probably all other receptors we characterized; see above) are not necessary for detection of either sugar at the concentrations tested.

What, then, is the role of *Gr66a*-expressing neurons? Based on the difference in expression of *Gr66a* and *Gr5a*

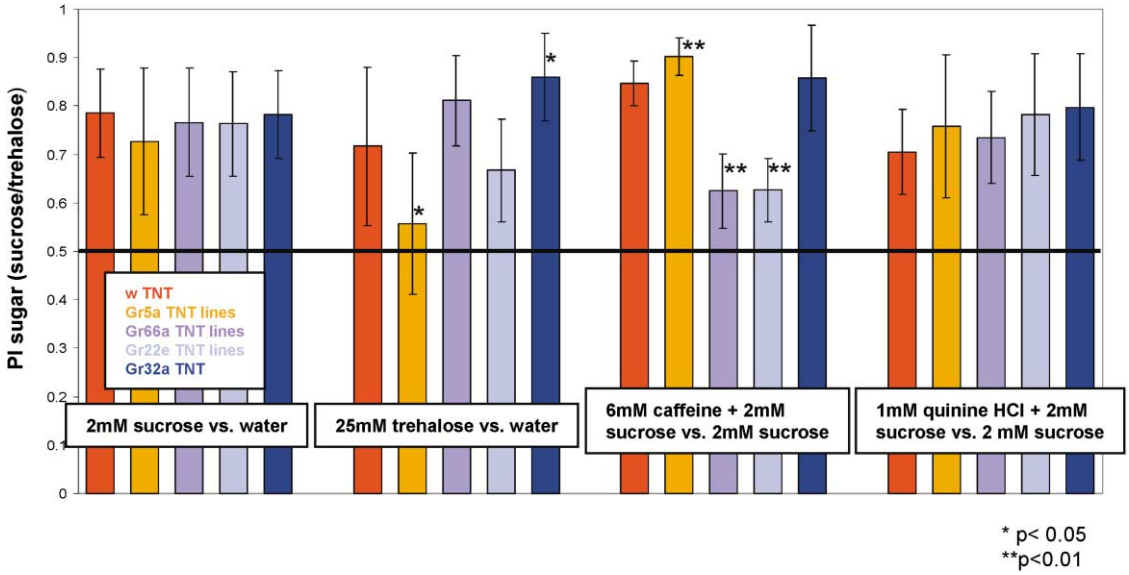

Figure 4. Trehalose and Caffeine Sensitivity Is Mediated by Neurons Expressing Different *Gr* Genes

A Preference index (PI) of 0.5 indicates no preference, whereas a PI of 1 indicates absolute preference for the tested substrate (for more details on feeding assays, see Experimental Procedures). Our *p[Gr5a]-Gal4;UAS-tnt* lines are significantly less sensitive to 25 mM trehalose compared to control flies (*w;UAS-tnt*) and flies with other *Gr* drivers. Both the *p[Gr66a]-* and *[Gr22e]-Gal4;UAS-tnt* lines have significantly reduced sensitivity to 6 mM caffeine. The *Gr5a* lines driving TNT show preference for sucrose alone over sucrose plus caffeine. No fly lines tested showed any decrease in sensitivity to 2 mM sucrose, 1 mM quinine hydrochloride (shown here), 0.25 mM denatonium benzoate, or 1 mM berberine (data not shown). With the exception of *Gr32a*, two lines from each driver construct were analyzed. No significant difference in any assay was observed between different lines from the same construct; therefore, these experiments were pooled. A total of at least five different feeding assays were carried out for each line, with the exception for the experiments tested for quinine sensitivity (at least four experiments/genotype).

and the distinct projection patterns of these neurons in the brain (see below), these two sets of neurons may be dedicated to the perception of distinct taste qualities. In order to investigate this possibility, we tested flies to chemicals known to deter feeding in insects, such as caffeine, denatonium benzoate, quinine, and berberine [14–17]. To promote feeding, we used an agarose-based medium that contained 2 mM sucrose and added the bitter compound to half of the feeding wells at the lowest possible concentration that provoked a reliable avoidance response (see Experimental Procedures). When control flies or flies lacking functional *Gr5a*- or *Gr32a*-expressing neurons were tested in this assay, profound avoidance of all bitter compounds was observed. When flies lacking functional *Gr66a*- or *Gr22e*-expressing neurons were tested with the same substrates, caffeine failed to be avoided by these flies (Figure 4, third panel). These flies, however, continued to respond normally to the other bitter compounds (Figure 4, fourth panel, and data not shown). Thus, these experiments suggest the 14 GRNs expressing *Gr22e* or a subset of neurons within this group are required for the detection of low concentrations of caffeine.

Axon Projections of GRNs Expressing Specific *Gr* Genes

The primary taste-processing centers in the fly brain are the subesophageal ganglion and the tritocerebrum, which receive direct input from all GRNs in the labellum, the pharynx, and some GRNs in the legs [34–40]. Retrograde labeling experiments of neurons associated with single labellar bristles have revealed a complex arborization pattern of axon termini in the *Drosophila* brain [34–40]. These investigations have also established that axons of chemosensory neurons located in the labellum project through the labral nerve (LN) into the SOG. In contrast, chemosensory neurons located in the pharynx send their axons to the tritocerebrum via the accessory pharyngeal nerve (APN) or the pharyngeal nerve (PN; Figure 1A).

How, then, is the activation of sensory neurons expressing *Gr5a* or *Gr66a* translated into distinct behavioral responses? It is obvious that a better understanding of this problem requires the correlation of receptor activation in the sensory epithelium (for example, the labellum) with specific processing centers (SOG/tritocerebrum) that are activated by the encounter of a specific taste stimulus. Similar studies have greatly advanced our knowledge of olfactory perception both in mammalian and insect model systems [41–45]. The identification of neurons with a role in either promoting or preventing food intake allowed us to investigate this question in the fly taste system.

As a first step toward the creation of a functional taste map, we visualized axon targets of neurons expressing the *Gr* genes by combining the various Gal4 drivers with reporters encoding neuronal synaptobrevin-GFP fusion protein (nSYB-GFP), which preferentially localizes to the synapse [46]. The projection patterns of the eight *Gr* genes described here can be divided into three classes (I, II and III; Figure 5). The class I projection pattern

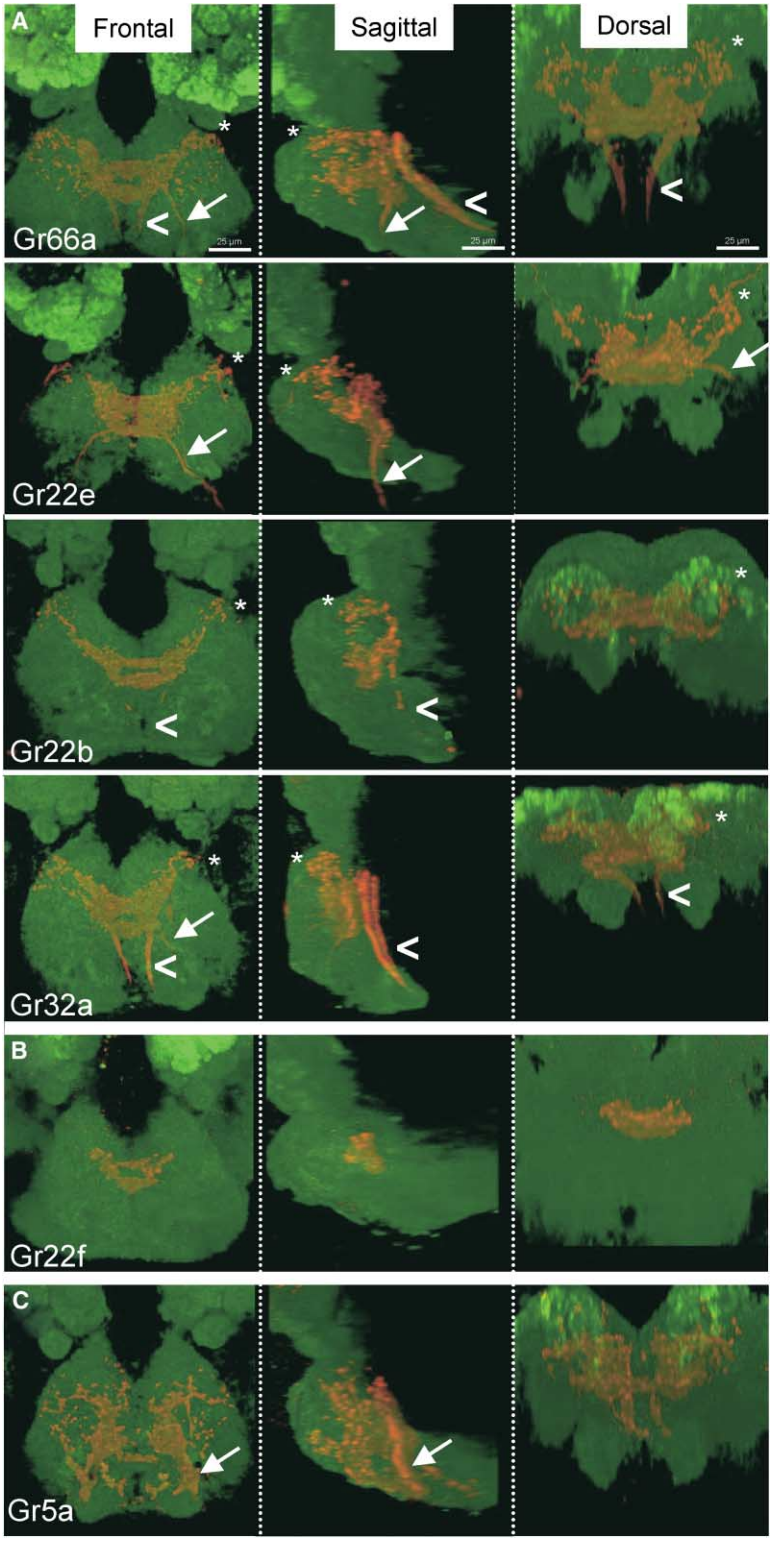

Figure 5. Distinct Axonal Projection Patterns in the Brain of GRNs Expressing Different *Gr* Genes

The axon termini of taste neurons from the labellum and pharynx expressing a given *Gr* were visualized by using a *n-Synaptobrevin-GFP* reporter. All images are confocal stacks of whole-mount brains stained with anti-GFP (red) to visualize axon termini and anti-nc82 (green) to visualize neuropil. All images are of identical magnification.

Since *Gr66a, Gr22e, Gr22b, Gr32a,* (A) and *Gr28be* (Figure S1) are coexpressed in neurons of the labellum and have additional peripheral expression in the pharynx and legs, the projection patterns produced in the tritocerebrum/SOG appear very similar (class I). The labellar input (arrow) and ascending afferent from the legs (arrowhead) is often strongly labeled. The pharyngeal input via the APN/PN is not visible, but terminations can be seen dorsolaterally (*). *Gr22f* and *Gr59b* ([B]; see also figure S1), while coexpressed with *Gr22e*, are expressed in fewer labellar neurons than the other *Gr*s and lack pharyngeal input. For these reasons their projection patterns in the brain (class II) are more restricted. Expression of *Gr5a* in a discrete set of labellar neurons produces a unique expression pattern in the SOG ([C]; class III). The labral nerve is indicated with an arrow.

(*Gr22e*, *Gr66a, Gr22b, Gr32a*, and *Gr28be*) is characterized by afferents of the pharyngeal nerves terminating in the dorsolateral region of the tritocerebrum, with additional fibers derived from the labral nerves extending their axons toward the adjacent region in the SOG. A significant number of fibers derived from the labral nerve cross the midline medially in the SOG (Figures 5A, S1C). Nayak and Singh [36] have characterized seven sensory neuron afferent types that enter the SOG. Using their descriptions as a guide, we believe that the class I projection pattern is composed of type I, type IV, and type VI fibers, along with input from the pharyngeal nerves.

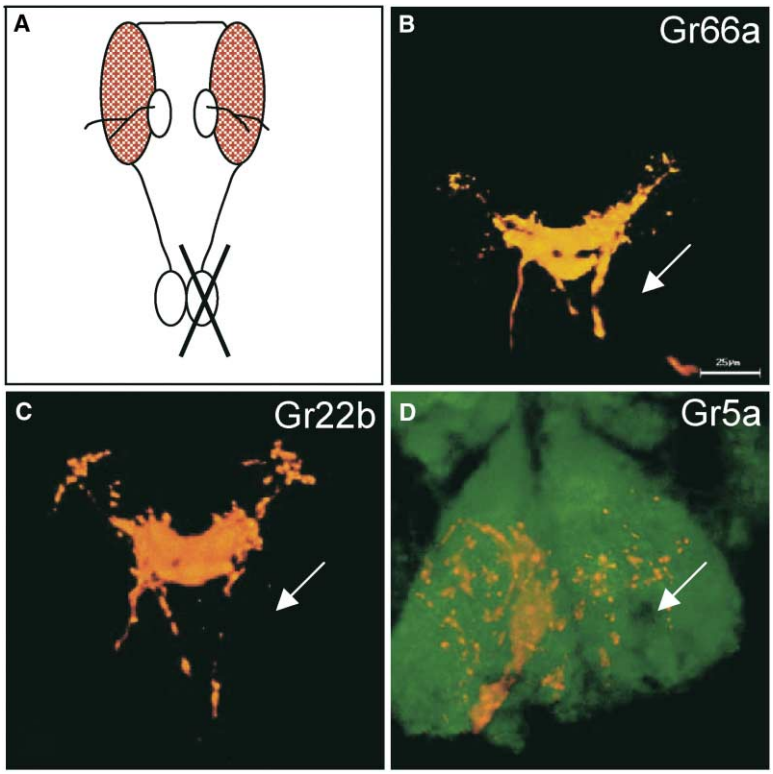

Figure 6. Distinct Ipsilateral and Contralateral Contribution of Gustatory Receptor Neurons Expressing *Gr5a*, *Gr66a*, or *Gr22b*

(A) Schematic of the fly head: the right labial palp was removed by mechanical ablation. Two weeks after ablation, the brains of flies [*p[Gr]-Gal4; UAS-nSyb-GFP]* were stained in order to determine what effect loss of the LN had on the projection pattern for a particular *Gr*. Loss of the LN was evident in all cases (arrows in [B]–[D]).

(B and C) For *Gr66a* and *Gr22b*, no decrease in axon terminations was observed on the side of the SOG no longer directly innervated by the LN.

(D) In the case of *Gr5a*, ablation of one LN resulted in a significant reduction in the number of termini seen in the corresponding half of the SOG.

Interestingly, we found that many of these class I projection patterns in the SOG/tritocerebrum received significant input from ascending afferents from sensory neurons in the legs.

Gr22f and *Gr59b* are expressed in a subset of *Gr22e*-expressing neurons of the labellum (Figures 2 and S1, and Table 2) and are not expressed in pharyngeal sensilla, producing a "class II" projection pattern (Figures 5B and S1). The main difference between class I and class II projection patterns is a complete lack of anterolateral terminals derived from the pharyngeal input. In addition, fewer terminations are found in the SOG, which is expected based on the lower number of labellar neurons expressing these two genes. The class II projection pattern is composed of the same labellum-derived afferent types as class I—types I, IV, and VI—but lack the pharyngeal input.

The last projection pattern type, class III, is seen for only a single receptor, *Gr5a* (Figure 5C) and is entirely different from the class I and II patterns. This observation further supports the conclusion from our coexpression analysis, which suggested that *Gr5a* is expressed in a largely unique set of neurons. With peripheral head expression restricted to the labial palps, there is no pharyngeal component to the projection pattern. Labral nerve input appears to consist mainly of type II fibers.

Because the projection patterns are a composite of axon terminations from neurons of the labellum, pharynx, and forelegs that express a given receptor, it is not possible in these experiments to unequivocally determine which terminations arise from which neurons. Ablation experiments of labial palps (Figure 6) and legs (data not shown) were performed to clarify this issue, yet some ambiguity remains.

Unilateral versus Bilateral Projections of Labellar GRNs

Class I and II patterns originate from gustatory neurons of the labellum and have a significant number of medial fibers that project contralaterally (Figures 5A and 5B). In comparison, class III patterns, which also originate exclusively from labellar neurons, lack this dense medial stain (Figure 5C). This observation suggests that projections from *Gr5a*-expressing neurons of the left or right labial palp terminate preferentially on the left or right side in the SOG, respectively, whereas labellar neurons expressing any of the other genes examined bifurcate and send axons both ipsilaterally and contralaterally, resulting in the intense medial stain.

To test this hypothesis, we surgically ablated the right labial palp, effectively killing sensory neurons of this palp (for details, see Experimental Procedure). Two weeks after surgery, anti-GFP antibody staining of flies expressing *UAS-nSyb-GFP* under the control of Gal4 drivers of *Gr66a*, *Gr22b*, and *Gr5a* revealed the absence of staining of the right, but not left, labral nerve, indicating that the majority of sensory neurons of the right palp were killed (Figure 6). In *p[Gr66a]-Gal4* and *p[Gr22b]-Gal4* flies, no obvious difference in density of terminations was seen in the two halves of the SOG (Figures 6B and 6C). This finding supports the idea that labellar neurons generating class I staining patterns in the SOG have similar numbers of termini in the ipsilateral and contralateral side of the SOG [36].

Labial palp ablation in *p[Gr5a]-Gal4* flies revealed a different result. Lack of innervation by the right LN resulted in a significant reduction in terminations of *Gr5a*-expressing neurons in the right half of the SOG (Figure 6D). The simplest interpretation of this result is that

axons of *Gr5a*-expressing neurons from the left labial palp extend branches that terminate mostly in the left half of the SOG, a notion that is consistent with the comparatively small number of medial fibers (Figure 5C).

Discussion

A crucial determinant for discerning chemical cues present in the environment is embedded in the peripheral expression pattern of cell surface receptors in sensory epithelia. In the olfactory systems of *Drosophila* and mice, each olfactory receptor neuron expresses only one of 60 or one of approximately 1000 *Or* genes, respectively, enabling these animals to discriminate between hundreds or thousands of different odors [47–49]. In contrast, taste cells of the tongue allow mammals to distinguish only a few taste qualities: bitter, sweet, umami, salty, and acidic taste [50]. Lack of discrimination between the hundreds of diverse chemical compounds—all perceived as bitter—is thought to be caused by coexpression of the approximately 40 T2R receptors in a single set of taste cells [3, 4]. Therefore, activation of the bitter taste cells by any one of the T2Rs is likely to generate a single activation pattern in taste centers of the brain, leading to a similar, repulsive behavioral output. Associating primary taste centers in the mammalian brain with specific taste modalities has, as of yet, proved challenging.

Insect taste is still rather poorly understood, especially at the molecular level. *Drosophila melanogaster*, which exhibits remarkably similar taste preferences with humans, is the only insect for which candidate receptors have been characterized experimentally. The investigations presented here provide significant new insights into insect taste perception.

Gustatory Neurons Mediating Avoidance Behavior Are Defined by Subtle Differences in the *Gr* Gene Code

Initial expression studies [18, 20] suggested that the fly gustatory receptors are not simply coexpressed in three sets of cells dedicated to bitter, sweet, and umami taste like the T2Rs, T1R2/T1R3, and T1R1/T1R3 receptors of mammals. Instead, these experiments suggested that they either are expressed according to the one receptor: one neuron hypothesis well established for insect and mammalian olfactory systems or they are expressed in partially overlapping sets of neurons. Our analysis supports the latter of these possibilities. We found that most labellar *Gr* genes (seven out of eight) are expressed in a single neuron of mostly S and some I type bristles. Most interestingly, our coexpression studies provide evidence that individual neurons express anywhere from one to six receptors (Figures 2K to 2O and Table 2). In this way, S bristle-associated neurons are defined by unique receptor gene codes, thereby outfitting the labellum with an array of sensory assemblies that may exhibit distinct—albeit overlapping—ligand specificities (Figure 7A).

The functional implications of distinct neuronal receptor codes on taste perception are currently unclear and will require analysis of mutations of individual *Gr* genes. However, a general role for these neurons in feeding inhibition ("avoidance neurons") can be inferred from experiments presented in this paper and supported through analogy with the mammalian taste system/receptors. First, avoidance neurons express the majority of analyzed *Gr* genes—and by extension—the majority

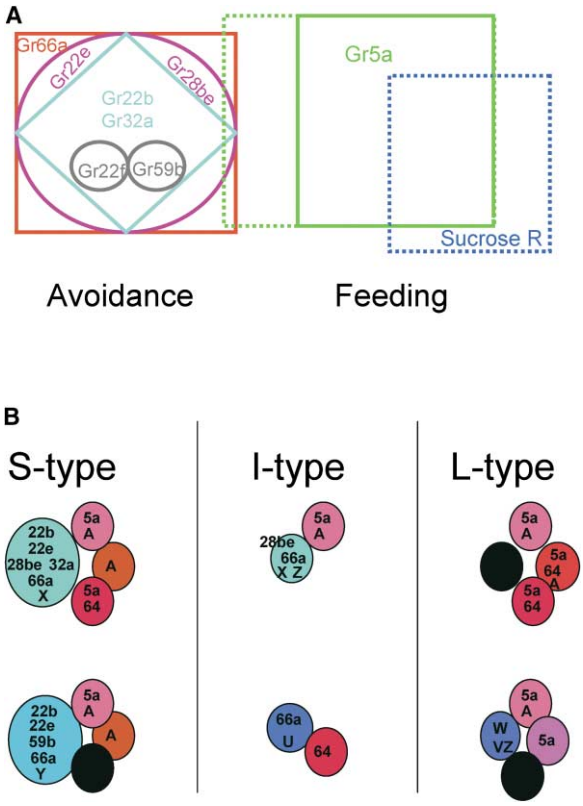

Figure 7. Model of Taste Coding in the Labellum

(A) Representation of coexpression and exclusive expression of *Gr* genes analyzed in this study. *Gr66a* is expressed in 22 neurons per labial palp and is coexpressed with *Gr66e* in 14 of these neurons. Most neurons that express *Gr22b, Gr32a, Gr28be, Gr22f*, and *Gr59b* also express *Gr66a* and *Gr22e*. We propose that these and additional not-yet-characterized receptors are expressed in avoidance neurons and inhibit feeding. From our analysis it is not possible to predict with certainty which combinations of *Gr* genes are expressed in a given neuron; however, the restricted expression of most *Gr* genes to S bristles indicates that some neurons express up to six *Gr* genes. *Gr22f* and *Gr59b* expression may be mutually exclusive, based on location of the corresponding neurons [25]. *Gr5a* is expressed in the greatest number of labellar neurons—approximately 70 per palp. The vast majority (and possibly all) of these neurons may not express any of the other *Gr*s examined, and we propose that *Gr5a* defines a discrete subset of taste neurons of the labellum that are sensitive to sugar. However, partially overlapping set(s) of neurons may express other sugar receptors (for example, a receptor for sucrose). Note that our analysis cannot exclude the possibility that a few (small) neurons expressing the other *Gr* genes also express *Gr5a* (dotted line).

(B) Representation of avoidance (blue hues) and feeding (red hues) neurons in S, I, and L type taste bristles in the labellum. S and L type bristles contain one avoidance and three feeding neurons, whereas I type bristles contain one of each. Different (sets of) avoidance and feeding neurons express different combinations of cognate receptors (see above). Receptors for additional sugars and possible amino acids (Gr64, A) in feeding neurons and other bitter receptors (U–Z) expressed in avoidance neurons of all bristle types are proposed but have not been identified. Some neurons (black) might be dedicated to salt and/or sour perception.

of the genes in the entire *Gr* gene family. In mammals, bitter taste receptors far outnumber the sweet taste receptors (40:3). Second, avoidance neurons associated with S type bristles do not express the receptors for the sugar trehalose encoded by the *Gr5a* gene. In fact, avoidance neurons associated with S type bristles have a distinct appearance compared to neurons expressing the *Gr5a* gene. In mammals, the sweet/umami taste receptors and the bitter taste receptors are expressed in distinct group of cells [3, 4]. Third, inactivation of avoidance neurons has no effect on sucrose or trehalose sensitivity in flies but significantly reduces their sensitivity to caffeine. Fourth, avoidance neurons and *Gr5a*-expressing neurons have distinct targets in the SOG, a feature consistent with the detection of different taste qualities by these neurons.

If the avoidance neurons have a general function in the detection of toxic or otherwise undesirable chemicals, what is the rationale for a complex and distinct *Gr* gene code among different groups of such neurons? We propose that the receptor code allows a fly to discriminate among different chemicals, which are in general avoided but might have distinct consequences on their health if ingested. According to such a proposal, a fly encountering a food source rich in nutrients (sugars) but contaminated with toxic chemicals may choose between feeding and avoidance, depending on the impact the particular toxic compound may have on its health. There is indirect evidence from feeding studies in *Maduca sexta* larvae that discrimination between the bitter substrates caffeine and aristolochic acid does occur in insects, even though actual taste preference, adaptation, or both may contribute to this phenomenon [15]. Thus, discrimination among toxic/bitter-tasting compounds might be possible in insects including *Drosophila*.

It was somewhat surprising that the sensitivity to other compounds known to be avoided by insects—denatonium benzoate, quinine hydrochloride, and berberine—was not affected in animals lacking *Gr66a*-expressing neurons. This may simply be explained by the presence of additional neurons expressing receptors that recognize these particular substrates. Alternatively, one or a few neurons coexpressing *Gr66a* along with a receptor for one (of these) ligand(s) might not have been completely inactivated by TNT. Finally, studies in rodents indicate that caffeine may directly affect neurons in the brain, circumventing activation of taste cells altogether [51–54]. This is not likely to be the case in our experiments, because none of the *Gr* genes examined is expressed in the CNS.

Relatively few studies have investigated bitter taste sensitivity in insects, particularly *Drosophila* [14, 15, 17]. Electrophysiological studies have identified bristles in the legs, but not the labellum of *Drosophila*, that respond to bitter-tasting chemicals [16]. However, S type sensilla are notoriously difficult to record from, because their bristles are extremely difficult to access for this type of experiment [25].

Gr5a-Expressing GRNs Mediate Sweet Taste Perception

Gr5a-expressing neurons represent more than half of chemosensory cells in the labellum and appear to be associated with all sensilla types, including the taste pegs. In fact, association of *Gr5a* with taste pegs provides the best evidence yet that these sensilla have a specific chemosensory function in the detection of trehalose. Significantly, *Gr5a*-expressing neurons define a largely distinct set of neurons from the avoidance neurons (Compare Figures 2A–2H with Figures 2I and 2J; see Figures 3A and 3D). This observation is consistent with our results from behavioral investigations of flies lacking the function of specific sets of neurons. Specifically, inactivation of *Gr5a*-expressing neurons leads to a reduction in trehalose sensitivity, but the sensitivity to any bitter substrate tested was unaffected (Figure 4). These flies did not exhibit reduced sucrose sensitivity, another nutrient-relevant sugar for *Drosophila* (Figure 4). This result is somewhat unexpected, as electrophysiological investigations have led to the proposal that a single neuron in L, I, and S bristles is responsive to several sugars including trehalose and sucrose (the "sugar" neuron) [13, 25, 55, 56]. According to these studies, sugar neurons may express a single, broadly tuned sugar receptor, or more likely, they may coexpress several distinct sugar receptors, each of which recognizes a specific sugar (i.e., sucrose, trehalose, fructose, etc.). This latter possibility is favored from genetic studies, which have shown that mutation in the *Gr5a* gene reduces the sensitivity of flies to trehalose, but not to sucrose [11–13]. However, the proposition of a single sugar neuron per bristle is also not consistent with our expression studies, which show that two to three neurons within a bristle can express *Gr5a* (Figures 3E and S2). We cannot exclude the possibility that the *p[Gr5a]-Gal4* drivers do not represent endogenous *Gr5a* expression, but we think this is unlikely to be the case for two reasons. First, several lines with our *p[Gr5a]-Gal4* show the same expression, and second, the *p[Gr5a_C]-Gal4* driver containing a much larger promoter fragment produces a similar expression profile, with many clusters of *Gr5a*-expressing neurons associated with the same bristle (Figure S2).

In order to realign the electrophysiological data with our expression analysis, we propose another explanation: the "sugar neuron" identified in electrophysiological studies expresses many (possibly all) distinct sugar receptors, including GR5a. However, one or two additional neurons per bristle express only a fraction, or possibly just one, of the sugar receptors present in the sugar neuron (Figure 7B). Worth noting in this context is the fact that electrophysiological recordings are carried out at significantly higher substrate concentrations (up to 100 mM for sucrose and trehalose) than our behavioral experiments (2 mM for sucrose and 25 mM for trehalose). Our model is also more consistent with recent experiments that noted different electrophysiological sugar responses among labellar sensilla [25].

Approximately 45 labellar neurons have not yet been associated with any *Gr* gene, and some of these neurons might express putative candidate receptors for sucrose or additional sugars. These genes are likely to be encoded by members of the *Gr64* gene cluster, which share much higher sequence similarity with *Gr5a* than any other *Gr* genes [12, 13, 18, 20, 57]. *Gal4* drivers for two of these genes (*Gr64a* and *Gr64e*) were analyzed and found to be expressed in the pharyngeal taste or-

gans, but not in the labellum (N.T. and H.A., unpublished data). Whether these two receptors are indeed involved in sugar detection remains to be seen, but we would predict that other *Gr* genes for sugars like sucrose and fructose would be expressed more broadly and in taste neurons of labellar bristles and pegs.

In summary, our expression and behavioral studies suggest two fundamentally different roles for neurons expressing nonoverlapping groups of *Gr* genes (Figure 7B) in the detection of substrates that lead to feeding or avoidance behavior. According to this new model, S and I bristles on the labial palps contain one avoidance neuron and one or more feeding neurons (depending on the number of neurons associated with the particular bristle). The avoidance neuron expresses multiple *Gr* genes, and avoidance neurons of different bristles express these *Gr* genes in different combinations (the *Gr* gene code). The feeding neurons, which appear morphologically smaller than the avoidance neurons, express an entirely different set of receptors that includes *Gr5a* and possibly *Gr* genes encoding receptors for other sugars, amino acids, and peptides.

A Taste Map in the Brain

The different functions for GRNs expressing *Gr66a* and *Gr5a* are also supported by their different projection patterns in the brain. Neurons expressing *Gr66a* or any of the partially coexpressed receptors target similar regions in the SOG/tritocerebrum, though the number of termini differs significantly depending on the number of peripheral sensory neurons the *Gr* is expressed in (Figures 5A and 5B). For example, *Gr66a*-expressing neurons show a robust array of termini in the SOG/tritocerebrum, whereas the termini of *Gr59b*- and *Gr22f*-expressing neurons are significantly less numerous. In all cases, dense, contralaterally projecting fibers provide extensive innervation of both halves of the SOG by labellar neurons, as demonstrated by labial palp ablation experiments (Figure 6).

An entirely different projection pattern is observed for feeding neurons that express *Gr5a*. Most strikingly, the axon termini of these neurons are distributed over a very large area of the SOG and extend into regions not innervated by avoidance neurons. A second striking difference is the poorly established contralateral connective between the two halves of the SOG, suggesting that neurons located in the right labial palp preferentially terminate in the right half of the SOG. This idea was tested and confirmed through ablation studies (Figure 6) and has interesting implications, namely that spatially restricted activation of neurons in one palp will preferentially stimulate the same side of the SOG; this could potentially allow for spatial discrimination of taste input in the brain. This feature might allow the fly to orient its labellum in the direction of a food source, identifying regions with high concentrations of trehalose or other sugars [58].

The distinct pattern of axon termini in the SOG of neurons required for feeding and avoidance suggests that these behaviors are mediated through different neuronal pathways. Anatomical studies in honeybees have identified second-order neurons that mediate synaptic activity of primary taste neurons to higher brain centers [59]. It will be interesting to see whether second-order neurons contacting synapses of avoidance and feeding neurons define different target regions in these higher brain centers.

Similar Logic of Taste Perception in Insects and Mammals

Taste is an ancient sense, which exists in bacteria in the form of chemotaxis. Neuroanatomical and molecular comparison of taste systems between mammals and insects imply that this sense has evolved independently in these phyla [21]. In mammals, for example, taste ligands are perceived through sensory epithelial cells in the lingual epithelium of the tongue. These cells then activate secondary neurons that innervate taste centers in the brain. In insects, tastants are detected by primary sensory neurons that directly innervate the CNS. Moreover, insects have multiple taste organs (legs, wings, and in some cases, the female genitalia) for which no counterparts exist in mammals. Finally, sequence comparison of the *Gr* and *T1R*/*T2R* genes has failed to reveal any direct kinship between mammalian and insect taste receptors [18, 20, 22].

However, a remarkable convergence of anatomical as well as molecular features of gustatory systems between mammals and insects (*Drosophila*) appears to emerge from our studies. The functional taste units, the taste buds in the tongue and the taste bristles of the labellum, are composed of 30 to 100 taste cells and two to four chemosensory neurons, respectively. Individual taste cells in each taste bud are dedicated to the perception of sweet, umami, or bitter taste sensation based on the T1R or T2R receptors they express. Similarly, our data indicate that taste bristles of the labellum contain neurons that either respond to repulsive or attractive stimuli, properties that are likely determined by the specific (set of) taste receptors they express.

Despite the sequence divergence of mammalian and insect taste receptors, we believe there are intriguing similarities at the molecular level as well. First, the number of taste receptors in mammals and *Drosophila* is very similar. The eight genes described here probably encode a significant number of the functional labellar taste receptors. Some of the 60 *Gr* genes are likely to encode taste receptors only expressed in the pharynx, legs, and wings or might only be expressed in the larva [18, 20]. Other *Gr* genes are likely to function as pheromone receptors [19], or might recognize internal ligands based on their restricted expression in the CNS (N.T. and H.A., unpublished data). Considering these alternative functions for some *Gr* genes, we estimate that the fly has about 30 to 45 labellar taste receptors, a number close to the total number of T1Rs and T2Rs ($\sim$30 in humans and 45 in mice).

In addition to the similar size of the *Gr* and *T1R*/*T2R* gene families, taste receptors of mammals and *Drosophila* fall into similar functional groups. Only three mammalian T1R receptors are thought to be dedicated to the detection of attractive stimuli (sugars and amino acids/proteins), whereas the large majority—the T2Rs—are thought to be exclusively involved in the detection

of repulsive (bitter) ligands. If our expression analysis is more or less representative of the entire *Gr* gene family, we might expect that 25 to 40 *Gr* genes will be expressed in the avoidance neurons, whereas just three to six are expected to be expressed in feeding neurons. Identification and analysis of *Gr* genes encoding receptors for known ligands, combined with biochemical analyses, should reveal whether additional molecular features are shared between the GRs and T1Rs and T2Rs, such as whether *Drosophila* also possess a specific receptor for amino acids and whether some receptors also function as multimers, as is proposed for mammalian T1Rs [4, 10].

Experimental Procedures

Genetics/Fly Strains

Drosophila stocks were raised on standard cornmeal-agar-molasses medium at 25°C. Transgenic Gal4-driver lines for *Gr66a, Gr22e, Gr22f*, and *Gr59b* were generated in a previous study by this lab [18]. The transgenic driver lines for *Gr32a* and *Gr28be* were kindly provided by Kristin Scott [20]. A *p[Gr5aC]-Gal4* line, with a 8.5 kb upstream sequence to *Gr5a*, was generously supplied by John Carlson and previously published in Chyb et al. [27]. A second *p[Gr5a]-Gal4* line, which used a 1 kb upstream fragment of *Gr5a*, was generated by PCR from genomic DNA by using standard PCR protocol with primers 5′-TGGTACCAAATGCAATAACAATAAAAACGCGC-3′ and 5′-GGGATTCTAACGATTTGGATAGATTCACCTCG-3′. The PCR fragment was cloned directly into pGEMT and then excised with ACC65I/NOT I and cloned into SM1 vector upstream of Gal4.

Lines homozygous for a driver and reporter (for example, *UAS-nucgfp; p[Gr22e]-Gal4)* were made and crossed to a driver line with Gal4 under the control of a different *Gr* promoter to produce double driver lines of genotype *p[Gr5a]-Gal4/UAS-nucgfp; p[Gr22e]-Gal4/+*. *UAS-tnt* lines were obtained from S. Sweeney.

Immunofluorescence

For all immunofluorescence experiments, expression of two lines for each *Gr* driver was examined. In all cases, identical expression profiles were seen for both drivers of the same *Gr*.

Labellum

P[Gr-promoter]-Gal4 lines were crossed to *UAS-nucgfp* stocks. Progeny were aged for at least 4 days before dissection. In order to allow sufficient antibody penetration to the tissue, labella were dissected from the rest of the proboscis prior to incubation with primary antibody. Antibody staining was conducted as described by Laissue et al. [60], except that 5% heat-inactivated goat serum was added to the blocking solution (protocol provided by Leslie Vosshall). Primary antibodies used were rabbit anti-GFP (Molecular Probes; A-6455) at a 1:1000 dilution and mouse anti-ELAV as a neuronal marker (provided by Leslie Vosshall) at a 1:10 dilution. Secondary antibodies used for visualization were goat anti-rabbit Cy3 (Jackson Immunoresearch Laboratories; 111-165-144) at a 1:500 dilution and goat anti-mouse ALEXA 488 (Molecular Probes; A-11017) at a 1:100 dilution.

Brains and Thoracic-Abdominal Ganglia

P[Gr-promoter]-Gal4 lines were crossed to either *UAS-n-Synaptobrevin-GFP* flies [46] to visualize axon termini or *UAS-mCD8::GFP* flies (obtained from Bloomington *Drosophila* stock center; 5137) to visualize axonal projections of GRNs. The immunofluorescence protocol followed is similar to that for labellum staining. Primary antibodies used were rabbit anti-GFP (1:1000) and mouse anti-nc82 (kindly provided by R. Stocker), a neuropil marker, at a 1:10 dilution.

Confocal Microscopy

A Zeiss LSM 410 confocal microscope with a KrAr laser was used for all immunofluorescence imaging. For all optical stacks produced, optical sections were taken at intervals of 1 μM. Multicolored, depth-coded images from a single channel, which revealed only GFP-expressing neurons, were produced with Zeiss LSM software. Adobe Photoshop 6.0 was used to adjust the contrast in images when required. Color spectra were used for depth coding, with red indicating the first optical section (surface) and blue indicating the last (deepest) optical section or vice versa.

Labellar Cell Counting

Hard copies of all optical stack images were used to obtain reliable cell counts. In the case of *Gr5a*, in which a significant number of small neurons stained, it was necessary to divide each labellum into sections of higher magnification, and a count made for each section. In many cases, we found that the multicolored, depth-coded stacks aided in the visualization of stained neurons, and often these images were used in conjunction with the unaltered red/green images, which allowed us to identify stained cells as neurons due to anti-ELAV staining (green). In all cases at least five to ten well-stained labella were used to determine the average cell count. Additional labella (up to 30) were also viewed to confirm the staining pattern seen. Two driver lines for each *Gr* were always analyzed.

Feeding-Preference Assays

In all feeding preference assays (FPAs), 50–70 male flies aged between 6–9 days were used. Again, for all assays, two *Gr* driver lines were tested. Flies were starved for 28 hr at 25°C in vials containing dampened Whatman paper. Flies were allowed to feed in the dark at 25°C for 2 hours. FPAs were always carried out at the same time of day. Cages with 6 × 6 well micro plates (Falcon; 353911) were used for the FPA. Wells contained alternating test solutions with red (20 mg/mL sulforhodamine B; Sigma, S-9012) or blue (5 mg/mL erioglaucine; Sigma, A-86,114-6) dye in agarose (Promega; V3121). After the assay, flies were immediately frozen, then sorted and counted based on the color dye witnessed in their abdomen. The preference index (PI) for a red-colored substrate, for example, is calculated as $(n_{red} + 1/2\ n_{purple})/n_{total}$. The closer the PI is to one, the higher the preference for the red-colored substrate; the closer the PI to zero, the higher the preference for the blue-colored substrate. A PI of 0.5 indicates no (or equal) preference for the two substrates. Substrate color alone did not appear to affect feeding preference (data not shown). The following substrate combinations were tested: 2 mM sucrose versus dH_2O, 25 mM trehalose versus dH_2O, 6 mM caffeine + 2 mM sucrose versus 2 mM sucrose, 0.25 mM denatonium benzoate + 2 mM sucrose versus 2 mM sucrose, 1 mM quinine hydrochloride + 2 mM sucrose versus 2 mM sucrose, 1 mM berberine + 2 mM sucrose versus 2 mM sucrose, and 100 mM NaCl + 2 mM sucrose versus 2mM sucrose. All chemical compounds were obtained from Sigma.

Averages and standard deviations, as well as basic Student's t tests to determine significance were used to analyze numerical data and to produce graphs (Microsoft Excel 2000).

Ablation of Labial Palps

The right labial palp of 1- to 3-day-old flies was surgically ablated with a wolfram needle under a dissection microscope. Flies were aged for 2 weeks before their brain was dissected and stained.

Supplemental Data

Supplemental Data including three figures are available at http://www.current-biology.com/cgi/content/full/14/12/1065/DC1/.

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