Cellular/Molecular

Molecular and Cellular Organization of the Taste System in the *Drosophila* Larva

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We examine the molecular and cellular basis of taste perception in the *Drosophila* larva through a comprehensive analysis of the expression patterns of all 68 Gustatory receptors (Grs). *Gr-GAL4* lines representing each Gr are examined, and 39 show expression in taste organs of the larval head, including the terminal organ (TO), the dorsal organ (DO), and the pharyngeal organs. A receptor-to-neuron map is constructed. The map defines 10 neurons of the TO and DO, and it identifies 28 receptors that map to them. Each of these neurons expresses a unique subset of *Gr-GAL4* drivers, except for two neurons that express the same complement. All of these neurons express at least two drivers, and one neuron expresses 17. Many of the receptors map to only one of these cells, but some map to as many as six. Conspicuously absent from the roster of *Gr-GAL4* drivers expressed in larvae are those of the sugar receptor subfamily. Coexpression analysis suggests that most larval Grs act in bitter response and that there are distinct bitter-sensing neurons. A comprehensive analysis of central projections confirms that sensory information collected from different regions (e.g., the tip of the head vs the pharynx) is processed in different regions of the suboesophageal ganglion, the primary taste center of the CNS. Together, the results provide an extensive view of the molecular and cellular organization of the larval taste system.

Introduction

The *Drosophila* larva provides a numerically simple and genetically tractable model system in which to study the molecular and cellular basis of taste (Stocker, 2008). Three major external chemosensory organs lie on the anterior tip of the larval head: the dorsal organ (DO), the terminal organ (TO), and the ventral organ (VO) (see Fig. 1*A*). The multiporous dome of the DO has olfactory function (Oppliger et al., 2000), while six peripheral DO sensilla have terminal pores indicative of gustatory function, as do most sensilla in the TO and VO (Stocker, 1994). The cell bodies of neurons that innervate the DO lie in the DO ganglion (DOG). Neurons that innervate the TO fall into two groups, the dorsolateral group, which has cell bodies in the DOG, and the distal group, which has cell bodies in the TO ganglion (TOG) (Stocker, 1994; Vosshall and Stocker, 2007). The VO ganglion (VOG) contains the cell bodies of gustatory neurons innervating the VO. Three chemosensory organs lie in the pharynx: the dorsal, ventral, and posterior pharyngeal sense organs (DPS, VPS, and PPS, respectively) (see Fig. 1*A*) (Gendre et al., 2004).

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Neurons in the DOG project to the brain via the antennal nerve, and neurons from the TOG and VOG project to the brain via the maxillary nerve (Stocker, 1994; Python and Stocker, 2002; Vosshall and Stocker, 2007). Neurons in the DPS and PPS extend to the brain via the labral nerve, while the labial nerve carries projections from the VPS. The major taste center in the larval brain is the subesophageal ganglion (SOG) (Colomb et al., 2007; Vosshall and Stocker, 2007).

The *Gustatory receptor* (*Gr*) gene family contains 60 members that encode 68 proteins through alternative splicing (Clyne et al., 2000; Dunipace et al., 2001; Scott et al., 2001; Robertson et al., 2003). *Gr5a* and two closely related receptors encode sugar receptors (Dahanukar et al., 2007; Slone et al., 2007; Jiao et al., 2008); *Gr33a*, *Gr32a*, *Gr66a*, and *Gr93a* mutants are defective in bitter reception (Moon et al., 2006, 2009; Lee et al., 2009, 2010). In the adult, *Gr5a* and *Gr66a* are expressed in distinct subsets of gustatory receptor neurons to mediate sweet or bitter taste and acceptance or avoidance behavior (Thorne et al., 2004; Wang et al., 2004; Marella et al., 2006).

Among the 68 gustatory receptors, expression patterns of only 15 *Gr* genes have been examined in detail in the larva, using the *GAL4-UAS* system (Colomb et al., 2007; Thorne and Amrein, 2008). Ten other *Gr-GAL4* lines did not show larval expression. A comprehensive analysis, however, has not been performed, and the complete larval repertoire has not even been identified.

Here, we systematically examine the expression of all *Gr* genes in the *Drosophila* larva and the projections of expressing neurons in the CNS. We define 10 gustatory neurons and provide a receptor-to-neuron map. The results suggest that receptors are expressed combinatorially and that most detect bitter compounds.

Materials and Methods

Drosophila *stocks and transgenes.* Flies were cultured on standard cornmeal agar medium at room temperature (23 ± 2°C). *w;UAS-mCD8-GFP* was used as the GFP reporter (Lee and Luo, 1999).

The 5′ upstream regions used to construct the *Gr-GAL4* transgenes are described by Weiss et al. (2011). Briefly, 59 *Gr-GAL4* transgenes were constructed and 8 *Gr-GAL4* transgenes were kindly provided by H. Amrein (Texas A&M University, College Station, TX) and K. Scott (University of California, Berkeley, Berkeley, CA), for a total of 67 *Gr-GAL4* transgenes. To construct the *Orco-RFP* transgene, the *Orco* promoter (8.3 kb) was cloned into the *RFP-IRES-RFP-IRES-RFP* expression vector used in the study by Dahanukar et al. (2007), and injected into w^{1118}. *Orco-RFP* expression completely overlaps with the expression of the 21 larval olfactory receptor neurons (ORNs) in *Orco-GAL4; UAS-mCD8-GFP* flies (data not shown).

Quantitative analysis of expression and selection of representative lines. Different insertions of an individual *promoter-GAL4* transgene may exhibit variations in expression pattern due to positional effects. In an effort to identify *Gr-GAL4* lines that show expression patterns faithful to those of the endogenous *Gr* genes, we examined as many independent lines as possible for a given *Gr-GAL4* transgene. For each independent *Gr-GAL4* line, we crossed *Gr-GAL4* homozygotes and flies with *UAS-mCD8-GFP* homozygous on both chromosomes 2 and 3, and scored GFP reporter expression in at least 10 progeny of either sex at the third-instar larval stage. An effort was made to select larvae from within the culture medium, as opposed to older, third-instar larvae at the wandering stage. These progeny were heterozygous for the *Gr-GAL4* driver and contained two copies of the *UAS-mCD8-GFP* reporter.

To quantitate expression of each *Gr-GAL4* line, we counted the number of GFP-labeled cells on both the left and right sides of all larvae and calculated the average number of labeled cell pairs. For example, if we examined 10 larvae of a particular *Gr-GAL4* line and observed that seven animals expressed GFP in one TO-distal neuron on each side ($7 \times 2 = 14$), two animals expressed GFP in only one neuron on one side ($2 \times 1 = 2$), and one animal did not show GFP expression ($1 \times 0 = 0$), the maximum number of labeled TO-distal neurons is 1 (Table 1), and the average expression level is $(14 + 2 + 0)/20 = 0.8$ (data not shown). No *Gr-GAL4* drivers showed labeling in a pattern indicative of left/right asymmetry.

For each *Gr-GAL4* transgene, one pattern was observed in the majority of independent lines. Among lines showing this most common pattern, we selected as the representative line the one that showed the most penetrant expression, based on the calculations described above.

To examine whether two different *Gr-GAL4* transgenes labeled the same or different neurons, we crossed the representative lines for each *Gr-GAL4* transgene with a stock carrying *UAS-mCD8-GFP* to score progeny with one copy of each *Gr-GAL4* transgene and two copies of *mCD8-GFP*. The number of neuron pairs expressing GFP was determined by the same method described above. The number of labeled neurons was quantified in at least 10 animals for each double-driver combination. We note that expression of the *Gr66a-RFP* transgene that was used to analyze coexpression in the adult by Weiss et al. (2011) was too weak to be useful in the larva and that it is now possible to alleviate position effects via use of the phiC31 system (Groth et al., 2004; Fish et al., 2007).

Fluorescence imaging and immunostaining. GFP and RFP expression were imaged directly in analysis of peripheral expression in the larval head. Larval heads were removed from the body with microscissors and incubated in 50% glycerol in PBS for 1 h before direct observation of fluorescence.

Larval brains were dissected and immunostained as previously described (Python and Stocker, 2002). Anti-GFP (rabbit polyclonal; Invitrogen; 1:1000 dilution) was used to enhance the GFP signals of *UAS-mCD8-GFP*. *UAS-mCD8-GFP* allows visualization of the general morphology of GAL4-expressing cells. Synaptic areas were visualized with mouse monoclonal antibody nc82 (1:100) (a gift from Dr. Alois Hofbauer, University of Regensburg, Regensburg, Germany). nc82 recognizes the ELKS/CAST/ERC family protein Bruchpilot, which localizes to presynaptic active zones (Laissue et al., 1999; Wagh et al., 2006). The

Table 1. Summary of *Gr* expression patterns in the TO, DO, and pharyngeal organs

		TO			Pharyngeal organs		
	Lines[a]	Distal	Dorsolateral	DO	DPS	VPS	PPS
Gr2a	7/8	2	—	2	1	—	—
Gr5a	7/7	—	—	—	—	—	—
Gr8a	5/5	—	—	—	—	—	—
Gr9a	7/10	1	—	—	—	—	—
Gr10a	7/8	—	1	—	—	—	—
Gr10b	1/1	—	—	—	—	—	—
Gr21a	1/1[b]	1	—	—	—	—	—
Gr22a	9/11	1	—	—	—	—	—
Gr22b	14/15	—	—	—	1	—	1
Gr22c	1/1[b]	—	—	—	—	—	—
Gr22d	1/1	—	—	—	1	—	—
Gr22e	6/7	1	—	—	1	—	—
Gr22f	6/6	—	—	—	—	—	—
Gr23a	6/7	—	—	—	1	—	—
Gr28a	3/3[c]	2	—	2	—	2	2
Gr28b.a	15/15	1	—	—	1	—	—
Gr28b.b	9/12	—	—	—	—	—	—
Gr28b.c	4/4	—	—	—	—	—	—
Gr28b.d	1/1[c]	—	—	—	—	—	—
Gr28b.e	1/1[b]	1	—	—	—	—	—
Gr32a	2/2	1	1	—	2	—	2
Gr33a	8/12	4	2	—	2	2	2
Gr36a	7/8	—	—	—	—	—	—
Gr36b	8/8	1	—	—	—	—	—
Gr36c	5/5	1	—	—	—	—	—
Gr39a.a	1/1	2	—	—	1	—	2
Gr39a.b	3/3	2	—	—	1	—	—
Gr39a.c	1/1	—	—	—	—	—	—
Gr39a.d	1/1	—	—	—	—	—	2
Gr39b	5/5	—	—	—	1	—	2
Gr43a	17/23	—	—	—	1	—	—
Gr47a	1/1[b]	—	—	—	—	—	—
Gr47b	5/7	1	—	—	—	—	—
Gr57a	7/7	2	—	—	1	—	—
Gr58a	15/15	—	—	—	—	—	—
Gr58b	9/10	1	—	—	1	—	—
Gr58c	6/6	—	—	—	—	—	—
Gr59a	11/15	1	—	—	—	—	—
Gr59b	1/1[c]	—	—	—	—	—	—
Gr59c	12/13	1	—	—	—	—	—
Gr59d	3/3	3	—	—	1	—	—
Gr59e	13/16	1	—	—	—	—	—
Gr59f	6/8	1	—	—	—	—	—
Gr61a	7/10	—	—	—	—	—	—
Gr63a	5/13	1	—	—	—	—	—
Gr64a	9/9	—	—	—	—	—	—
Gr64b	8/9	—	—	—	—	—	—
Gr64c	4/4	—	—	—	—	—	—
Gr64d	7/8	—	—	—	—	—	—
Gr64e	3/3	—	—	—	—	—	—
Gr64f	4/5	—	—	—	—	—	—
Gr66a	6/6	4	2	—	2	2	2
Gr68a	3/3[c]	—	—	—	—	2	—
Gr77a	9/14	—	—	—	1	—	—
Gr85a	15/19	—	—	—	—	—	—
Gr89a	10/14	—	—	—	—	—	—
Gr92a	12/12	—	—	—	—	—	—
Gr93a	12/14	—	—	—	1	—	—
Gr93b	10/10	1	—	—	1	—	—
Gr93c	8/8	—	—	—	—	—	2
Gr93d	9/14	—	—	—	—	—	2
Gr94a	12/12	1	—	—	—	—	—
Gr97a	4/4	1	—	—	—	—	—
Gr98a	1/1	—	—	—	—	—	—
Gr98b	2/2	—	—	—	—	—	—
Gr98c	3/4	—	—	—	—	—	—
Gr98d	15/15	—	—	—	—	—	—

Numbers indicate the maximum number of neurons labeled on one side of the animal by the representative *Gr-GAL4* driver for each gene.

[a]Number of lines with expression consistent with the representative line/number of independent lines analyzed. For example, in the case of *Gr2a*, eight independent lines were analyzed; in seven of them, two was the maximum number of cells observed in one TO and in one DO. The line with the most penetrant expression was selected as the representative line. The one line that did not have expression consistent with the other lines had no expression at all, likely because of a positional effect. In the case of *Gr5a*, seven of seven lines had no expression in the larva. For *Gr36a*, seven of eight lines had no expression, while one line had ectopic expression in a large number of uncharacterized cells; thus, no expression is the representative expression pattern.

[b]Includes a line obtained from K. Scott (University of California, Berkeley, Berkeley, CA).

[c]Includes a line obtained from H. Amrein (Texas A&M University, College Station, TX).

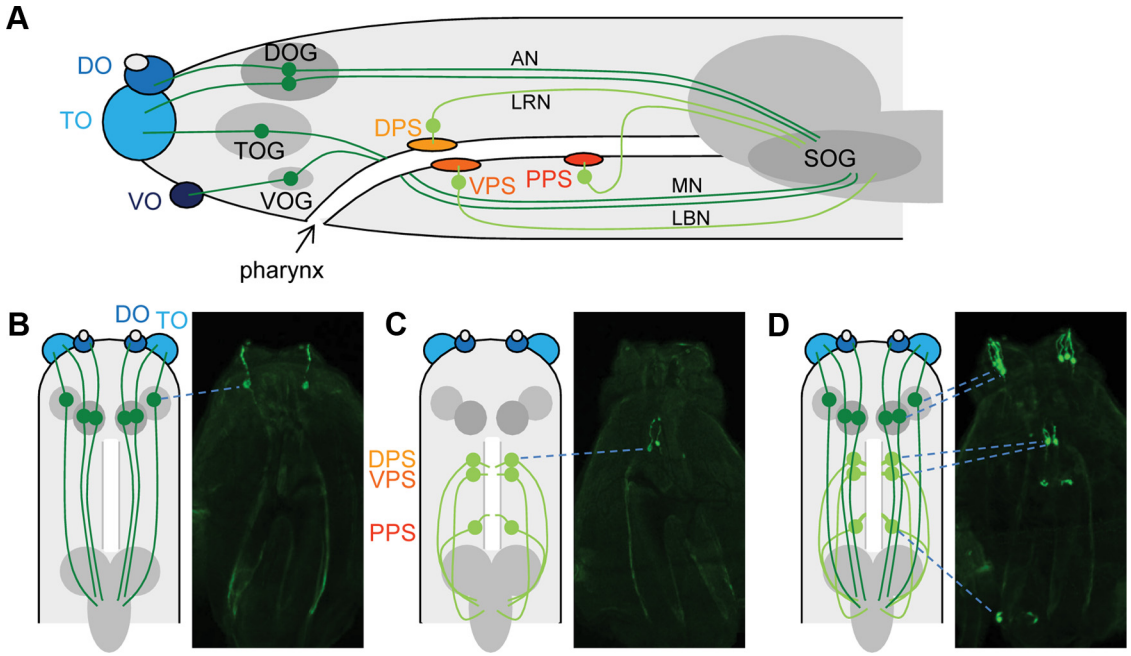

Figure 1. ***A***, The taste system of the larval head (lateral view) [adapted from the study by Stocker (2008) with permission from Landes Bioscience/Springer]. Anterior is to the left. The DOG, TOG, and VOG are represented by gray shaded ovals and contain the cell bodies of 36–37, 32, and 7 sensory neurons, respectively (Stocker, 2008). Three neurons innervating the TO have cell bodies in the DOG (TO-dorsolateral group; see Results and Fig. 2). Representative neuronal projections are depicted in green. The axons of all putative taste neurons extend to the SOG in the brain. Olfactory neurons are not represented here for simplicity. AN, Antennal nerve; LRN, labral nerve; LBN, labial nerve; MN, maxillary nerve. ***B–D***, Dorsal views of the taste system in the larval head. Anterior is at the top. ***B***, On the left is a schematic view of neurons (green) of the TO and DO. The VO is not shown. On the right is an example of a specific driver, *Gr59c-GAL4*, which drives expression in neurons that innervate the TO. ***C***, Left, Pharyngeal neurons (green). Right, *Gr77a-GAL4* drives expression in neurons that innervate the DPS. ***D***, Left, Neurons of the TO, DO, and pharyngeal organs are indicated in green. Right, *Gr33a-GAL4* drives peripheral expression in neurons that innervate the TO as well as the pharyngeal sense organs. The VPS is located ventral to the DPS, as shown in ***A***, and they are often superimposed in a dorsal or ventral view. The labeled cells between the DPS and PPS cells may be cells of the DPO (dorsal pharyngeal organ) (Gendre et al., 2004), but we could not identify them with confidence.

secondary antibodies used were goat anti-mouse and goat anti-rabbit IgG conjugated to either Alexa 568 or Alexa 488 (1:1000) (Invitrogen).

All images were collected on either a Zeiss LSM 510 laser-scanning confocal microscope or a Bio-Rad 1024 laser-scanning confocal microscope.

Results

To examine systematically the expression of the entire repertoire of Grs in the larva, we used the *GAL4-UAS* system. Most attempts to analyze expression of *Gr* genes by *in situ* hybridization have been unsuccessful, presumably due to low expression levels (Clyne et al., 2000; Dunipace et al., 2001; Scott et al., 2001; Dahanukar et al., 2007; Moon et al., 2009). The *GAL4-UAS* system has been a more successful approach (Brand and Perrimon, 1993; Dunipace et al., 2001; Scott et al., 2001; Chyb et al., 2003; Thorne and Amrein, 2008; Moon et al., 2009), and recently the expression of all Grs was examined in the labellum, the major taste organ of the adult head (Weiss et al., 2011). Results of this expression analysis of *Gr-GAL4* lines in the labellum agreed well with the results of functional analysis (Weiss et al., 2011), supporting the utility of the approach.

The *Gr-GAL4* transgenes we used are essentially those used in the study by Weiss et al. (2011), with minor differences in the number of lines examined for certain *Gr* genes and in the selection of representative lines for more detailed analysis. Collectively, the expression patterns of 67 *Gr-GAL4* drivers were examined. (One driver, *Gr23a-GAL4*, represents two Grs, Gr23a.a and Gr23a.b, which are encoded by alternative spliceforms that share a common 5′ region.) For some individual *Gr-GAL4* drivers, as many as 23 independent lines were examined in an effort to determine accurately the expression pattern of each gene (Table 1). For certain previously published *Gr* transgenes, a single line was analyzed (Dunipace et al., 2001; Scott et al., 2001; Weiss et al., 2011). In total, we analyzed 507 lines for the 67 *Gr-GAL4* drivers, a mean of 7.6 lines/driver. We examined third-instar animals that were heterozygous for the *Gr-GAL4* driver and that contained two copies of the *UAS-mCD8-GFP* reporter.

For each Gr, we selected one representative *Gr-GAL4* line on the basis of a quantitative analysis. To select a representative line for a particular Gr, at least 10 animals were scored for every line established for that Gr (i.e., a total of >5070 animals across all drivers). The mean number of labeled neurons in each line was calculated as a measure of expression pattern. For each Gr, one expression pattern was observed among the majority of lines and was defined as the representative pattern. Among lines showing the representative pattern for a Gr, one line that showed the most consistent and penetrant expression was selected as the representative line (Table 1; see also Materials and Methods).

Taste organs of the larval head express 39 *Gr-GAL4* drivers

Of the 67 *Gr-GAL4* drivers, 39 showed expression in the peripheral taste organs of the head (Table 1). Among the major cephalic taste organs, we focused on the TO, DO, DPS, VPS, and PPS; expression in the VO was difficult to discern due to a lack of easily distinguishable features or markers.

The 39 *Gr-GAL4* drivers fell into three categories. The first category contains 15 drivers that show expression in neurons innervating the sensory organs on the tip of the head (TO and/or DO), but not in the pharyngeal sense organs (DPS, VPS, or PPS) (Fig. 1*B*, Table 1). The second category consists of 11 drivers that show expression in the pharyngeal sense organs with no expres-

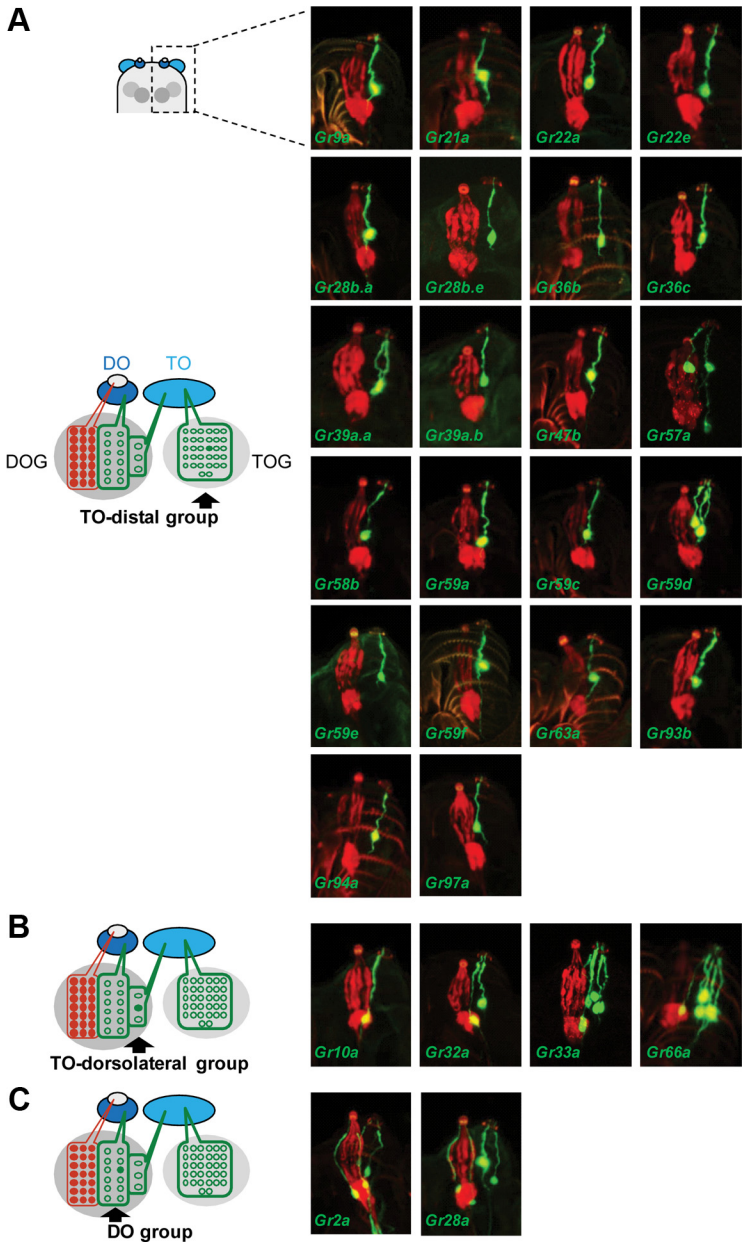

Figure 2. Division of *Gr-GAL4* drivers that show expression in TO- and/or DO-innervating neurons into three classes: the TO-distal, TO-dorsolateral, and DO classes. ***A***, The small diagram (above, left) shows a larval head, with magnified representations of the TO, DO, TOG, and DOG. The large diagram (below, left) depicts the cells of the TOG and DOG. The TOG contains the cell bodies of 32 sensory neurons that innervate the TO (the TO-distal group of neurons) (Stocker, 2008). The photographs at the right show the 22 *Gr-GAL4* drivers of the TO-distal class. *Orco-RFP* was used as an olfactory neuron-specific marker to indicate the position of the DOG; all 21 olfactory neurons of the DOG innervate the dome sensillum. *Gr39a.b-GAL4* does not exhibit full penetrance; the photograph shows only one labeled neuron, but in many cases two neurons are labeled. ***B***, Three neurons from the DOG innervate the TO (the TO-dorsolateral group). Four *Gr-GAL4* drivers fell into the TO-dorsolateral class. *Gr32a-GAL4*, *Gr33a-GAL4*, and *Gr66a-GAL4* also showed expression in the TO-distal neurons. In the case of *Gr33a-GAL4* and *Gr66a-GAL4*, two neurons (B1 and B2 in Fig. 4) are labeled in the TO-dorsolateral group but are very close to each other, making them difficult to distinguish in a two-dimensional photographic representation. ***C***, Twelve putative taste neurons from the DOG innervate the peripheral sensilla of the DO (the DO group). Two *Gr-GAL4* drivers fell into the DO class. Each also had expression in TO-distal neurons.

sion in the TO or DO (Fig. 1*C*, Table 1). The third category includes 13 drivers that express in both the TO/DO and the pharyngeal sense organs (Fig. 1*D*, Table 1).

All of the 15 drivers that express in neurons innervating the TO/DO, but not in the pharyngeal organs, express in only one TO neuron. All drivers that express in at least two neurons of the TO/DO also show expression in the pharyngeal sense organs (Table 1).

We note that, in addition to the 39 drivers that express in the cephalic chemosensory organs, drivers representing *Gr28b.b*, *Gr28b.c*, *Gr28b.d*, and *Gr89a* showed larval labeling, but elsewhere: we observed projections from the body to the ventral ganglion (data not shown). *Gr2a-*, *Gr28a-*, and *Gr33a-GAL4* also labeled projections from the body in addition to their expression in the head taste organs. *Gr28b.d* also expressed in head cells that we could not identify with confidence. Finally, we were interested to observe that of the 39 drivers that express in the cephalic chemosensory organs, 22 were recently found to be expressed in the labellum, the major organ of the adult head (Weiss et al., 2011), and at least four, *Gr32a-*, *Gr33a-*, *Gr39a-*, and *Gr68a-GAL4*, represent genes associated with courtship behavior (Bray and Amrein, 2003; Miyamoto and Amrein, 2008; Moon et al., 2009; Wang et al., 2011; Watanabe et al., 2011).

Classification of Gr expression into TO-distal, TO-dorsolateral, and DO classes

The 28 *Gr-GAL4* drivers expressed in neurons of the TO/DO (i.e., those of the first and third categories above; Fig. 1*B*,*D*) fell into three classes. These classes were distinguished based on the positions of the labeled cell bodies and the organs that they innervate on the tip of the head, regardless of whether they label pharyngeal organs. To classify the *Gr* genes, the representative *Gr-GAL4* driver for each *Gr* was crossed into an Orco-RFP-expressing line. Orco is a cation channel and chaperone required for odorant receptor (Or) function (Benton et al., 2006; Sato et al., 2008; Wicher et al., 2008). Orco is expressed in all 21 larval ORNs (Larsson et al., 2004) and thus marks the position of the DOG. Using Orco-RFP as a marker for the DOG allowed us to distinguish three partially overlapping classes of *Gr* drivers in the larval head: the TO-distal class, expressed in neurons with cell bodies in the TOG that innervate the TO; the TO-dorsolateral class, which shows expression in neurons with cell bodies in the DOG that innervate the TO; and the DO class, which shows expression in neurons with cell bodies in the DOG that innervate the DO (Fig. 2).

The TO-distal class contains 22 *Gr-GAL4*s (Fig. 2*A*); the TO-dorsolateral class contains 4 *Gr-GAL4*s (Fig. 2*B*); the DO class contains 2 *Gr-GAL4*s (Fig. 2*C*, Table 1). With the exception of *Gr10a-GAL4* in the TO-dorsolateral class, the drivers of the TO-dorsolateral and the DO classes also showed expression in neu-

rons that innervate the TO from the TOG (Fig. 2*B*,*C*). We note that penetrance of the *Gr28a-GAL4* driver was highly variable, but it showed somewhat more GFP-expressing neurons than previously reported (Thorne and Amrein, 2008), as well as expression in the DO neurons (Table 1, Fig. 2*C*).

A receptor-to-neuron map of the larval taste system

Of the 28 *Gr-GAL4* drivers expressed in neurons of the TO-distal, TO-dorsolateral, and DO groups, how many are coexpressed in individual neurons, and in how many neurons are they coexpressed? We performed a coexpression analysis designed to address fundamental questions regarding the functional organization of the larval taste system and its molecular and cellular complexity. The analysis is illustrated in Figure 3, and it has produced the receptor-to-neuron map shown in Figure 4. As described below, the analysis has identified 10 neurons, which we have named A1, A2, B1, B2, and C1–C6.

To identify coexpression, we systematically examined *Gr-Gal4* lines in pairwise combinations. Representative *Gr-GAL4* lines for two receptors were crossed, and progeny that carried one copy of each *Gr-GAL4* driver and two copies of *UAS-mCD8-GFP* were scored. When progeny containing both drivers contained the same number of labeled neurons as animals containing one copy of either parental driver alone, we inferred that the neurons labeled by one driver either overlapped precisely with the neurons labeled by the other driver or were a subset of them. When the double-driver progeny contained a greater number of neurons than animals containing either driver alone, the simplest interpretation is that some or all neurons labeled by one driver are distinct from the neurons labeled by the other.

Gr33a-GAL4 and *Gr66a-GAL4* drive expression in the greatest number of TO/DO neurons. Each driver labels four TO-distal neurons and two TO-dorsolateral neurons (Table 1, Fig. 2*B*; not all neurons can be observed clearly in a single, two-dimensional photographic representation). When *Gr33a-GAL4* was combined with *Gr66a-GAL4*, the number of neurons expressing GFP was unchanged, showing that *Gr33a-GAL4* and *Gr66a-GAL4* coexpress in the same neurons of the larval head sensory organs (Fig. 3*A*, top left). This finding is consistent with their coexpression in the adult labellum, and both genes are required in adults for the response to bitter compounds (Moon et al., 2009; Weiss et al., 2011).

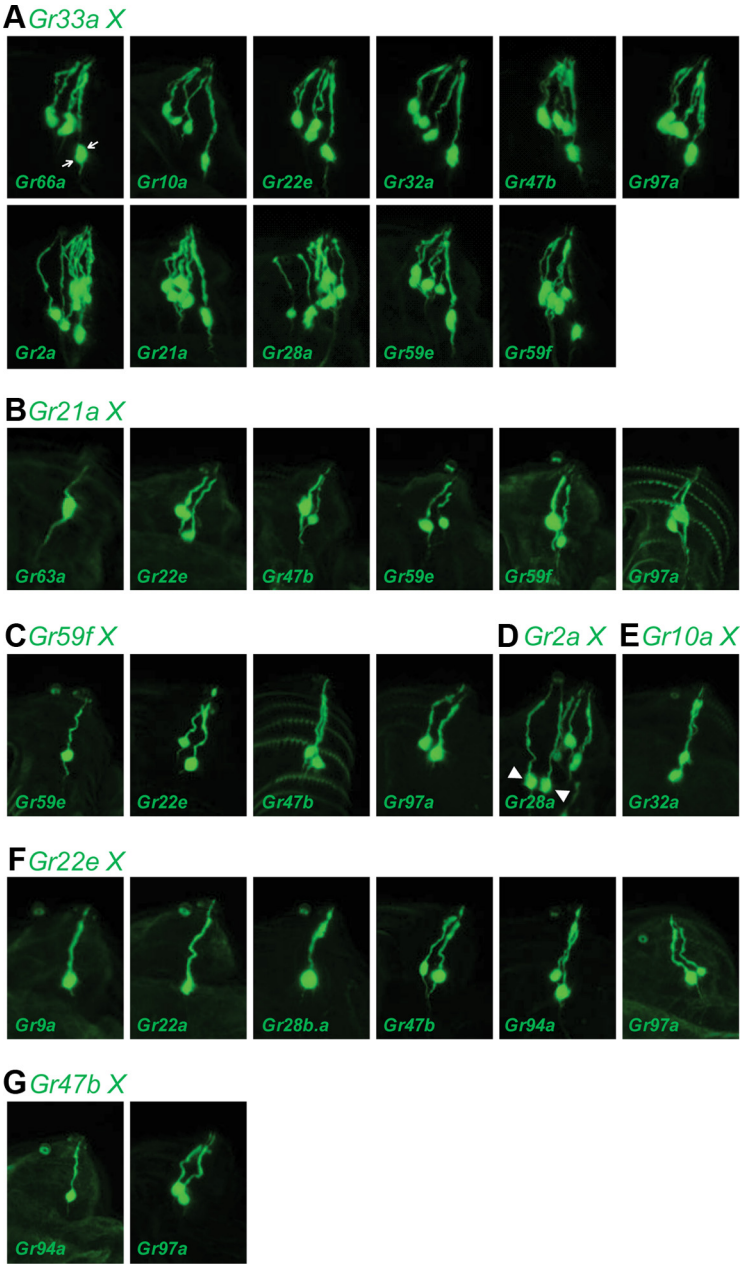

Figure 3. Determination of coexpression by double-driver analysis. The average number of neurons expressing GFP was determined from at least 10 animals of each genotype. ***A***, Top row, When *Gr33a-GAL4* was combined with *Gr66a-*, *Gr10a-*, *Gr22e-*, *Gr32a-*, *Gr47b-*, or *Gr97a-GAL4*, the number of labeled cells was the same as for *Gr33a-GAL4* alone (Fig. 2*B*), indicating that the neurons expressed in these lines are coincident with, or a subset of, the neurons expressed by *Gr33a-GAL4*. Five labeled cells can be observed easily, and in each case a sixth cell body and dendrite could be seen in another focal plane; two cell bodies at the bottom right (indicated by arrows in the first image; these cells are referred to as B1 and B2 in Fig. 4) are very close to each other. Bottom row, When *Gr33a-GAL4* was combined with *Gr2a-*, *Gr21a-*, *Gr28a-*, *Gr59e-*, or *Gr59f-GAL4*, a larger number of neurons was observed, indicating that these five drivers are expressed in at least some cells distinct from the *Gr33a-GAL4*-expressing neurons. ***B***, *Gr21a-GAL4* and *Gr63a-GAL4* are coexpressed in a neuron that does not express several other tested *Gr-GAL4* drivers. ***C***, Gr59e-GAL4 and *Gr59f-GAL4* are expressed in the same TO-distal neuron, in a neuron that does not coexpress several other tested drivers. ***D***, *Gr2a-GAL4* and *Gr28a-GAL4* are coexpressed in two neurons of the DO group (arrowheads). There is also labeling of some TO neurons. ***E***, When *Gr10a-GAL4* and *Gr32a-GAL4* were combined, the expression appeared identical with that of *Gr32a-GAL4* alone (Fig. 2*B*) (i.e., one TO-dorsolateral neuron and one TO-distal neuron are labeled). ***F***, *Gr22e-GAL4* is coexpressed in a TO-distal neuron with *Gr9a-GAL4*, *Gr22a-GAL4*, and *Gr28b.a-GAL4* but is not coexpressed with *Gr47b-GAL4*, *Gr94a-GAL4*, or *Gr97a-GAL4*. *Gr22e-GAL4* was also coexpressed with drivers representing *Gr28b.e*, *Gr32a*, *Gr36b*, *Gr36c*, *Gr58b*, *Gr59a*, *Gr59c*, *Gr93b* (data not shown). ***G***, *Gr47b-GAL4* and *Gr94a-GAL4* are coexpressed, in a neuron distinct from that expressing *Gr97a-GAL4*.

Gr33a-GAL4 was then crossed to *Gr10a-*, *Gr22e-*, *Gr32a-*, *Gr39a.a-*, *Gr47b-*, or *Gr97a-GAL4*, all of which label a smaller number of neurons than *Gr33a-GAL4*. The progeny showed labeling of the same number of neurons as *Gr33a-GAL4*, suggesting that these six

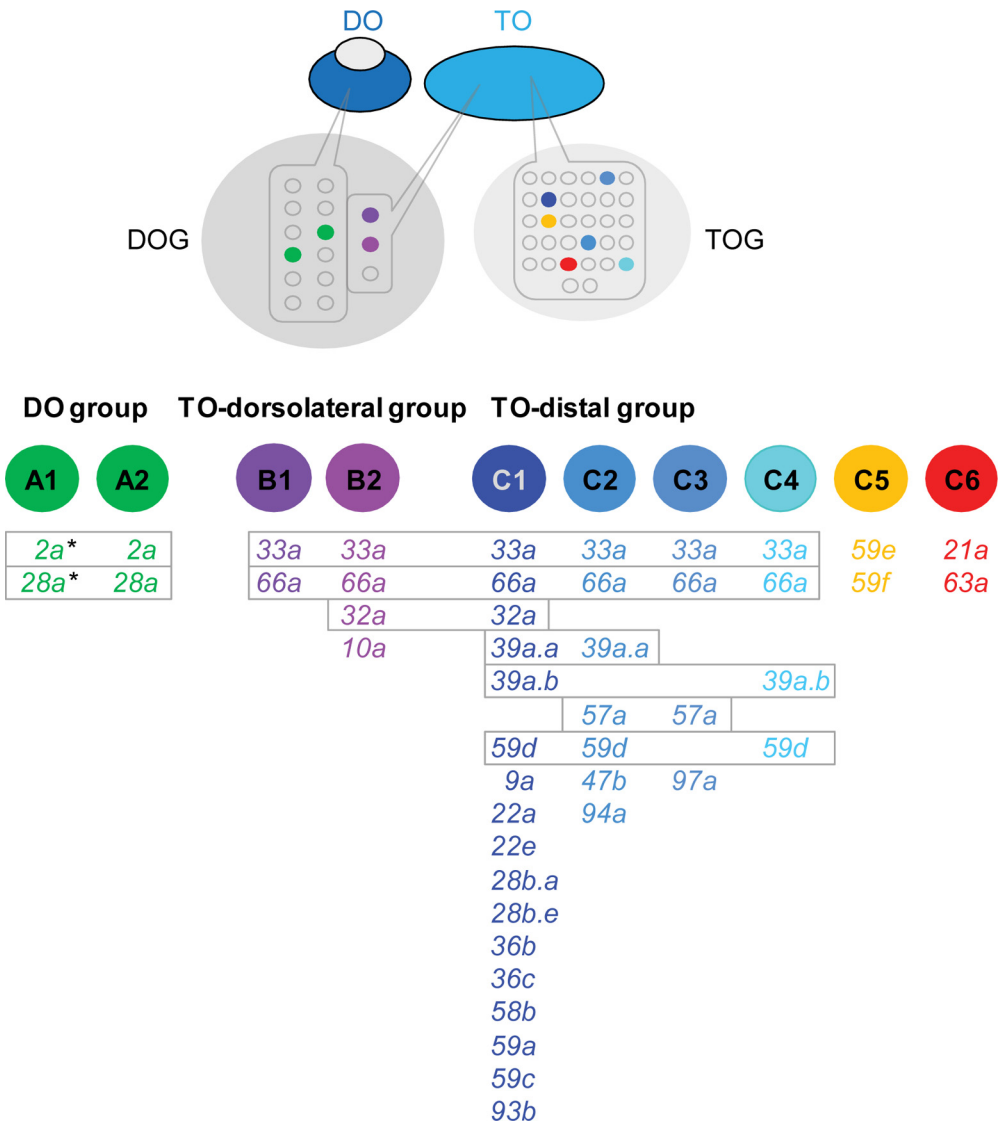

Figure 4. A receptor-to-neuron map. Coexpressed *Gr-GAL4* drivers are listed under each neuron, and receptors that map to multiple neurons are boxed in gray. The asterisk (*) indicates that *Gr2a-GAL4* and *Gr28a-GAL4* are also expressed in up to three TO-distal neurons, but we were unable to identify them with confidence; although penetrance of expression in the DO group was high, it was lower in TO-distal neurons. Positions of cell bodies in the diagram of the DOG and TOG are arbitrary.

receptors are expressed in a subset of *Gr33a*-expressing neurons (Fig. 3*A*, top row) (data not shown).

By contrast, when *Gr2a-*, *Gr21a-*, *Gr28a-*, *Gr59e-*, *Gr59f-*, and *Gr63a-GAL4* were combined with *Gr33a-GAL4*, the number of labeled neurons was increased, showing that some or all of the cells in which these six drivers are expressed are distinct from the *Gr33a-GAL4*-expressing neurons (Fig. 3*A*, bottom row) (data not shown). Of the 28 *Gr-GAL4* drivers expressed in neurons of the TO/DO, these were the only six drivers that labeled an increased number of neurons when combined with *Gr33a-GAL4*. We considered each of these six drivers in turn.

Gr21a-GAL4 and *Gr63a-GAL4* are coexpressed in one larval neuron (Fig. 3*B*), as has been shown previously; they are also coexpressed in the adult. Coexpression in an *in vivo* expression system was sufficient to confer a response to CO_2 (Jones et al., 2007; Kwon et al., 2007). We performed a series of crosses with additional drivers, none of which was coexpressed with *Gr21a-GAL4* and *Gr63a-GAL4* (Fig. 3*B*) (data not shown). This direct evidence, together with inference from other crosses described below, support the conclusion that this neuron expresses only *Gr21a* and *Gr63a*. We have termed the neuron C6 (Fig. 4).

Gr59e-GAL4 and *Gr59f-GAL4* are also coexpressed, in one TO-distal neuron (Fig. 3*C*). This neuron was confirmed directly to be distinct from the TO-distal neuron that coexpresses *Gr21a-GAL4* and *Gr63a-GAL4* (Fig. 3*B*), as well as from neurons expressing *Gr22e-*, *Gr47b-*, *Gr97a-* (Fig. 3*B*) and *Gr33a-GAL4* (Fig. 3*A*). We have denoted this neuron C5 (Fig. 4).

Gr2a-GAL4 and *Gr28a-GAL4* express in two neurons of the DO group (Table 1, Fig. 2*C*), and their coexpression in DO neurons was demonstrated by crossing the two lines (Fig. 3*D*, arrowheads). These DO neurons are termed A1 and A2 (Fig. 4). *Gr2a-GAL4* and *Gr28a-GAL4* are the only drivers expressed in DO neurons. We note that *Gr2a-GAL4* and *Gr28a-GAL4* are also expressed, but appear not to be coexpressed, in TO-distal neurons; however, expression was variable in TO-distal neurons, it is not depicted in Figure 4, and definitive mapping will require further

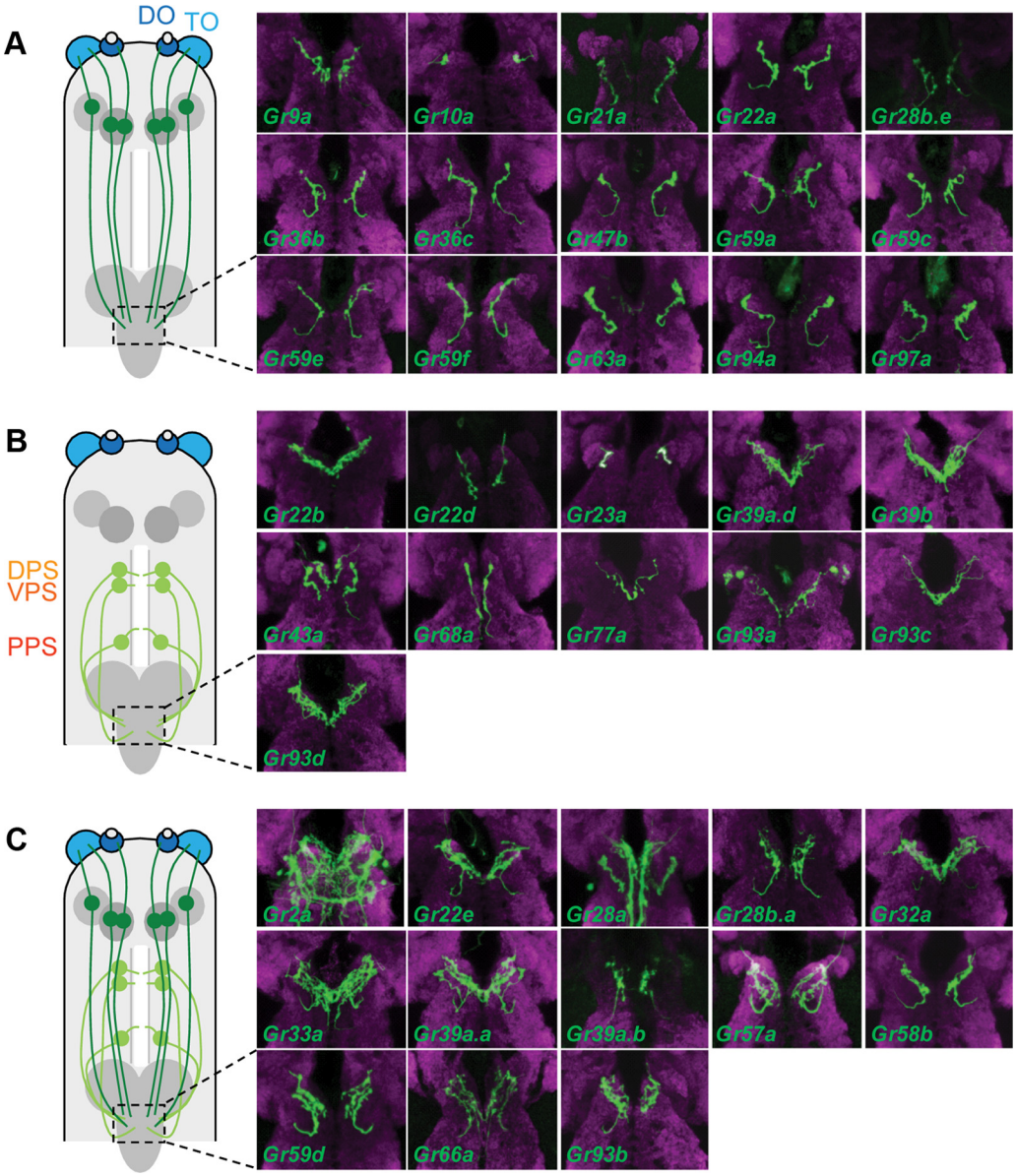

Figure 5. Projection patterns in the SOG of the brain, shown for each of the 39 *Gr-GAL4*s expressed in head taste organs. ***A***, *Gr-GAL4* drivers that express exclusively in neurons that innervate the TO and/or DO. ***B***, *Gr-GAL4* drivers that express exclusively in neurons that innervate the pharyngeal sense organs. ***C***, *Gr-GAL4* drivers that express both in TO/DO neurons and pharyngeal neurons.

analysis. *Gr28a* has previously been found to be expressed in various adult and larval sensory organs (Thorne and Amrein, 2008).

We analyzed in more detail the TO-dorsolateral neurons, two of which are labeled by *Gr33-GAL4* and *Gr66a-GAL4;* they are closely adjacent and are often superimposed in dorsal views (Fig. 2*B*). *Gr10a-GAL4* and *Gr32a-GAL4* are each expressed in one of these neurons; *Gr32a-GAL4* is also expressed in one TO-distal neuron (Fig. 2*B*). When the *Gr10a-GAL4* and *Gr32a-GAL4* drivers were combined, expression was observed in two cells (Fig. 3*E*) and appeared identical to that of *Gr32a-GAL4* alone (Fig. 2*B*). These results indicate that *Gr10a-GAL4* and *Gr32a-GAL4* are coexpressed in one TO-dorsolateral neuron, termed B2, along with *Gr33a* and *Gr66a* (Fig. 4).

Next, we examined in more detail the TO-distal neurons. Many of the *Gr-GAL4* drivers are expressed in a single TO-distal neuron (Fig. 2*A*). We found that 12 of them, including *Gr9a-*, *Gr22a-*, and *Gr28b.a-GAL4*, when combined with *Gr22e-GAL4*, showed labeling of a single neuron, indicating that all of these drivers are expressed in a single neuron, C1 (Fig. 3*F*) (data not shown). By contrast, *Gr47b-*, *Gr94a-*, and *Gr97a-GAL4*, combined with *Gr22e-GAL4*, labeled two neurons (Fig. 3*F*). Additional crosses showed that *Gr47b-* and *Gr94a-GAL4* are coexpressed in a neuron, C2, distinct from that expressing *Gr97a-GAL4*, C3 (Fig. 3*G*). Using *Gr22e-GAL4*, *Gr47b-GAL4*, and *Gr97a-GAL4* as markers, we performed similar double-driver experiments with *Gr39a.a*, *Gr39a.b*, *Gr57a*, and *Gr59d* (data not shown), which defined a fourth TO-distal neuron, C4 (Fig. 4).

Correlations between receptor expression and central projection patterns

We examined projection patterns in the brain of all 39 larval *Gr-GAL4* lines that drive expression in the taste organs of the head. We compared the projections of three categories of drivers: the 15 drivers that show expression in the TO/DO but not in the pharyngeal organs (Figs. 1*B*, 5*A*); the 11 drivers that show expression in the pharyngeal sense organs but not the TO/DO (Figs. 1*C*,

5*B*); and the 13 drivers that express in both the TO/DO and the pharyngeal sense organs (Figs. 1*D*, 5*C*).

With a few exceptions, patterns within a category were more similar than patterns between categories. Among the patterns of the TO/DO category, most are similar, consistent with the conclusion from the double-driver analysis that many drivers are coexpressed in the same cell, C1. A distinct pattern is shown by *Gr10-GAL4*, which is the only driver in this category expressed in the TO-dorsolateral neurons, in B2. The neurons in this group project to the brain via the antennal nerve, whereas the TO-distal neurons such as C1 project via the maxillary nerve. We note that *Gr21-GAL4* and *Gr63a-GAL4*, coexpressed in C6, also appear similar to each other and distinct from the others. This finding is consistent with the earlier finding that the *Gr21a-GAL4* projection was distinct from that of others (Colomb et al., 2007).

Many patterns of the pharyngeal-only class show similarities; for example, most of these afferents can be seen to extend to, or very near, the midline. There are some differences; for example, Gr68a-GAL4 shows a distinct pattern from the others; it is also the only driver in this category that is expressed in the VPS (Table 1). Overall, however, a comparison of all members of the TO/DO and pharyngeal-only categories suggests that sensory information collected from different regions (i.e., the tip of the head vs the pharynx) is processed in different subregions of the SOG, consistent with previous studies performed with limited numbers of *Gr-GAL4* drivers (Colomb et al., 2007).

Gr-GAL4 drivers with expression in both the TO/DO and pharyngeal sense organs showed more complex brain projection patterns (Fig. 5*C*). In a number of cases, the projections can be interpreted as a composite of the projection patterns observed in the TO-specific and pharyngeal-specific categories (Fig. 5*A*,*B*).

Discussion

A comprehensive view of *Gr* expression in larvae

Of the 67 *Gr-GAL4* transgenes, 43 showed expression in the larva, of which 39 were expressed in the major taste organs of the head. The 39 *Gr-GAL4* drivers are expressed in combinatorial fashion (Table 1, Fig. 4). Individual *Gr-GAL4* drivers are expressed in up to 12 cells, in the case of *Gr33a-* and *Gr66a-GAL4;* approximately one-half, however, are expressed in only one cell.

We acknowledge that for some *Gr-GAL4* drivers the observed pattern of expression may not be identical with that of the endogenous *Gr* gene. It was precisely with this concern in mind that we analyzed a mean of 7.6 independent lines for each of the 67 *Gr* drivers, and that we established a rigorous, quantitative protocol for identifying a representative line for each gene. In the absence of an effective *in situ* hybridization protocol, the approach used here seemed likely to be the most informative in providing a comprehensive systems-level view of larval taste reception.

Features of the receptor-to-neuron map

The Gr receptor-to-neuron map of the dorsal and terminal organs identified 10 neurons. Two neurons have cell bodies in the DOG and innervate the DO, two have cell bodies in the DOG and innervate the TO, and six have cell bodies in the TOG and innervate the TO.

We mapped 28 receptors to these 10 neurons. All of these neurons express at least two *Gr-GAL4* drivers. Two receptors, Gr21a and Gr63a, are coreceptors for CO_2; neither is sufficient to confer chemosensory function alone (Faucher et al., 2006; Jones et al., 2007; Kwon et al., 2007). It is conceivable that many other Grs may also require a coreceptor, which may explain the lack of neurons expressing a single *Gr-GAL4.* The number of receptors per neuron ranges up to 17, in the case of C1. This number is comparable with the maximum number of *Gr-GAL4*s observed in a labellar neuron (29), and much greater than the number of Ors observed in individual neurons of either the larval or adult olfactory system (Couto et al., 2005; Fishilevich and Vosshall, 2005; Goldman et al., 2005; Kreher et al., 2005).

Among the 10 identified cells, individual *Gr-GAL4* drivers are expressed in as few as one cell and as many as six cells. Most of the drivers are expressed in only one of these 10 cells. The drivers expressed in six cells, *Gr33a-GAL4* and *Gr66a-GAL4*, are expressed in all bitter neurons of the adult labellum. We note that *Gr33a-GAL4* and *Gr66a-GAL4* are the only drivers expressed in B1, arguing against the possibility that both of these receptors function exclusively as chaperones or as coreceptors that require another Gr for ligand specificity.

There is little cellular redundancy. Only two neurons, A1 and A2, express the same complement of receptors. All other neurons contain a unique subset of the Gr repertoire. In this respect, the larval taste system differs from the adult taste system (Weiss et al., 2011) but is similar to the larval olfactory system, which also contains little if any cellular redundancy (Fishilevich et al., 2005; Kreher et al., 2005).

Analysis of the central projections of all 39 *Gr-GAL4* drivers provided evidence for a systematic difference among projection patterns between TO/DO neurons and pharyngeal neurons (Fig. 5, compare *A*, *B*). These results support the conclusion that sensory information collected from the tip of the head is processed in different regions of the SOG than information collected in the pharynx, i.e., that evaluation of a potential food source before ingestion and the testing of food quality during ingestion are functionally partitioned in the brain. Similar inferences were drawn in an elegant study of a limited number of *Gr-GAL4* transgenes (Colomb et al., 2007).

Sugar receptors

Conspicuously absent from the list of *Gr-GAL4* drivers expressed in the larval taste system are those representing the eight members of the sugar receptor subfamily (Gr5a, Gr61a, Gr64a–f) (Robertson et al., 2003). The founding member of this family, *Gr5a*, mediates response to the sugar trehalose (Dahanukar et al., 2001), and two other members of the subfamily have been shown to encode sugar receptors as well (Dahanukar et al., 2007; Slone et al., 2007; Jiao et al., 2008). We did not observe GFP expression for these genes in cells of the taste organs or in neural fibers in the brain or ventral ganglion. Most of these *Gr-GAL4* transgenes drive expression in the adult (Dahanukar et al., 2007) (A. Dahanukar, J. Y. Kwon, L. A. Weiss, F. Ling, and J. R. Carlson, unpublished results), but we acknowledge that these transgenes may not faithfully reflect expression in the larva.

Given that *Drosophila* larvae respond to sugars (Miyakawa, 1982; Schipanski et al., 2008), as do larvae of other insect species (Dethier and Kuch, 1971; Glendinning et al., 2000; Schoonhoven and van Loon, 2002), how do they detect them without members of the sugar receptor subfamily? Other Grs, including the recently identified fructose receptor Gr43a (Sato et al., 2011), may underlie sugar detection in the larva. We note that *Gr59e-GAL4* and *Gr59f-GAL4* are coexpressed in a cell that does not express the bitter cell markers *Gr33a-GAL4* or *Gr66a-GAL4*. Sugar reception may also be mediated by other kinds of receptors, such as those of the TRPA family (Xu et al., 2008).

Bitter receptors

In adult *Drosophila*, *Gr33a-GAL4* and *Gr66a-GAL4* are coexpressed with other *Gr-GAL4*s in bitter neurons; the simplest interpretation of expression and functional analysis is that multiple bitter receptors are coexpressed (Thorne et al., 2004; Wang et al., 2004; Lee et al., 2009; Moon et al., 2009; Weiss et al., 2011).

In the larva, we found that most larval *Gr-GAL4*s are coexpressed with *Gr33a-* and *Gr66a-GAL4*, suggesting the possibility that most larval Grs act in bitter response. We note that, of the 17 *Gr-GAL4*s coexpressed in the C1 neuron, 15 are coexpressed in a bitter neuron of the labellum (Weiss et al., 2011). We also establish that there are distinct molecular classes of *Gr33a-GAL4*, *Gr66a-GAL4*-expressing neurons. The simplest interpretation of these results is that there are distinct bitter-sensing neurons in larvae.

Larvae must determine whether to accept or reject a food source (Tompkins, 1979; Schoonhoven and van Loon, 2002), and in principle this determination could be made by a simple binary decision-making circuit. However, the existence of six *Gr33a-GAL4*, *Gr66a-GAL4*-expressing neurons expressing distinct subsets of *Gr-GAL4*s suggests a greater level of complexity in the processing of gustatory information. One possibility is that C1, which expresses the largest subset of drivers among the TO/DO neurons, may activate an aversive behavior in response to many of the bitter compounds that the larva encounters, while C2, C3, C4, or B2 either potentiates the response or activates a different motor program in response to chemical cues of particular biological significance or exceptional toxicity. The existence of heterogenous bitter-sensing cells, some more specialized than others, is a common theme in insect larvae (van Loon and Schoonhoven, 1999; Glendinning et al., 2002; Marion-Poll and Descoins, 2002; Schoonhoven and van Loon, 2002). In particular, many species contain a taste cell that responds physiologically to many aversive compounds and whose activity deters feeding. C1 could be such a cell, and its coexpression of many receptors may provide the molecular basis of a broad response spectrum.

It is striking that the number of TO/DO neurons that express *Gr-GAL4*s is small compared with the total number of TO/DO neurons. We mapped *Gr-GAL4* expression to only 10 cells in the TO/DO (although *Gr2a-GAL4* and *Gr28a-GAL4* were each expressed in two TO neurons that we were unable to map). The DOG and TOG contain 36–37 and 32 sensory neurons, respectively, among which 21 in the DOG are olfactory (Stocker, 2008). Thus, of the nonolfactory cells, on the order of 20–30% express *Gr-GAL4* drivers. It will be interesting to determine how many of the other DOG/TOG cells express other chemoreceptor genes, such as *Ppk*, *Trp*, or *IR* genes, and how many of the other neurons have mechanosensory, thermoreceptive, hygrosensory, or other sensory functions (Stocker, 2008).

The role of *Gr* genes in the larval pharyngeal organs is unknown. In adult pharyngeal sensilla, the TRPA1 channel, which detects irritating compounds, regulates proboscis extension (Kang et al., 2010). It is possible that Grs expressed in larval pharyngeal organs may also play a role in modulating feeding behavior. Of the 24 *Gr-GAL4* drivers expressed in the larval pharyngeal organs, 9 are coexpressed with *Gr33a-GAL4* and *Gr66a-GAL4* in the TO/DO (Table 1, Fig. 4); it seems plausible that they may monitor ingested food for the presence of aversive compounds.

In summary, we have analyzed essential features of the molecular and cellular organization of a numerically simple taste system in a genetic model organism. We have defined 10 gustatory receptor neurons and have provided evidence that they express Grs in combinatorial fashion, with most of these neurons and receptors acting in the perception of bitter compounds. The results lay a foundation for a molecular and genetic analysis of how these receptors and neurons, and the downstream circuitry, underlie a critical decision: whether to accept or reject a food source.

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