The Molecular and Cellular Basis of Bitter Taste in Drosophila

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SUMMARY

The extent of diversity among bitter-sensing neurons is a fundamental issue in the field of taste. Data are limited and conflicting as to whether bitter neurons are broadly tuned and uniform, resulting in indiscriminate avoidance of bitter stimuli, or diverse, allowing a more discerning evaluation of food sources. We provide a systematic analysis of how bitter taste is encoded by the major taste organ of the Drosophila head, the labellum. Each of 16 bitter compounds is tested physiologically against all 31 taste hairs, revealing responses that are diverse in magnitude and dynamics. Four functional classes of bitter neurons are defined. Four corresponding classes are defined through expression analysis of all 68 gustatory taste receptors. A receptor-to-neuron-to-tastant map is constructed. Misexpression of one receptor confers bitter responses as predicted by the map. These results reveal a degree of complexity that greatly expands the capacity of the system to encode bitter taste.

INTRODUCTION

Understanding of a sensory system depends critically on the definition of the neuronal classes it comprises. Our understanding of human color vision, for example, rests on the classic definition of three classes of color-sensing cells, the determination of their spectral sensitivities, and the identification of the opsins that underlie the sensitivity of each (Nathans, 1989).

Animals rely on taste systems to detect toxins, which are often perceived as bitter. When taste organs make contact with a potential food source, the presence of bitter compounds is signaled by taste cells to the CNS. This input informs a decision that is critical to the animal’s survival: acceptance or rejection.

A central problem in the field of taste has been to define the bitter-sensitive neurons, their response spectra, and the receptors that impart their molecular specificity. Are bitter-sensitive cells tuned broadly and uniformly, leading to indiscriminate avoidance of potentially toxic substances, or are they diverse and more selectively tuned, providing the capacity for a more informative assessment of complex food sources? A comprehensive definition of the molecular and cellular basis of bitter taste across an entire taste organ is needed to allow basic principles of bitter coding to emerge. Such an analysis has not been performed in invertebrates and is difficult to perform in mammals because of the complexity of mammalian taste organs.

The labellum of Drosophila offers several advantages in the study of bitter taste. The organ is numerically simple. Each half of the labellum contains 31 prominent sensilla called taste hairs, most containing one bitter-sensitive neuron. The responses of all of these bitter-sensitive neurons can be measured in vivo by physiological recording. A large family of taste receptor genes, the Gr genes, has been defined. Behavioral responses to bitter tastants can be measured and interpreted in terms of cellular and molecular analyses.

The taste hairs of the labellum are arranged in a stereotyped pattern, with minor variation among flies. The hairs have been classified into three groups (Shanbhag et al., 2001) and named according to their morphology and position (Hiroi et al., 2002): long (L), intermediate (I), and short (S) (Figure 1A), with each individual sensillum of a class identified by a subscript, e.g., L1. Most hairs contain four taste neurons: one sensitive to sugars, one to water or low osmolarity; I type hairs contain just two taste neurons, one that responds to sugars and low concentrations of salt, and another that responds to bitter compounds and high concentrations of salt (Dethier, 1976; Falk et al., 1976; Fujishiro et al., 1984; Hiroi et al., 2004; Nayak and Singh, 1983; Rodrigues and Siddiqi, 1978).

The Gr family includes 60 members that are predicted to encode 68 seven-transmembrane receptors through alternative splicing (Clyne et al., 2000; Dunipace et al., 2001; Robertson et al., 2003; Scott et al., 2001). Genetic analysis has revealed that Gr5a and two closely related genes, all members of a clade of eight Gr genes, are required for responses to sugars (Dahanukar et al., 2007; Jiao et al., 2008; Slone et al., 2007). Gr32a, Gr33a, Gr66a, and Gr93a are required for responses to caffeine (CAF) and/or certain other bitter compounds (Lee et al., 2009, 2010; Moon et al., 2006, 2009). Analysis of Gr-GAL4 drivers has shown that Gr5a is expressed in sugar-sensitive neurons in each sensillum, while Gr66a is expressed in a distinct population of ~20 neurons that responds to a number of bitter compounds and that mediates aversion (Chyb et al., 2003; Marella et al., 2006; Thome et al., 2004; Wang et al., 2004). Two Gr5a-related genes map to Gr5a-expressing neurons, while...
a number of other Gr genes appear to be expressed in subsets of Gr66a-expressing neurons (Dahanukar et al., 2007; Lee et al., 2009; Moon et al., 2009; Thorne and Amrein, 2008; Thorne et al., 2004; Wang et al., 2004). The sensilla associated with these subsets have not been identified in most cases, however, and expression of the great majority of Gr genes has not been examined.

Historically, a critical question in the field has been whether all taste sensilla are functionally equivalent (Hiroi et al., 2002; Marella et al., 2006; Thorne et al., 2004; Wang et al., 2004). Previous physiological analysis of the labellum revealed that three sensilla, L7, L8, and L9 (Figure 1A), were similar in their responses to all of 50 tested compounds, mostly sugars (Dahanukar et al., 2007). A study of 21 sensilla and four sugars showed that all sensilla responded to all tested sugars, with some quantitative differences among sensilla of different morphology (Hiroi et al., 2002). A survey of a few bitter compounds revealed that none of the longer sensilla on the labellum responded, while all of the shorter hairs that were tested gave indistinguishable responses (Hiroi et al., 2004). An imaging study found that different subpopulations of bitter cells responded to most bitter compounds tested; striking differences in response profiles were not observed (Marella et al., 2006).

Based on these studies, it has been suggested that bitter-sensitive neurons of the labellum may generally recognize the same bitter compounds (Cobb et al., 2009; Marella et al., 2006). A similar model emphasizing functional homogeneity is often cited in mammals, in which multiple bitter receptors are coexpressed and taste receptor cells respond to a broad range of bitter compounds (Adler et al., 2000; Mueller et al., 2005; Yarmolinsky et al., 2009). However, a systematic analysis of the responses of the labellar taste sensilla to bitter compounds, such as those carried out with Drosophila olfactory sensilla and odorants (de Bruyne et al., 2001), has not been performed. Because of the limited scope of the extant studies, the basic principles of functional organization that underlie bitter coding in the fly remain unclear.

Here we investigate basic principles of bitter coding through a systematic behavioral, physiological, and molecular analysis. We first measure behavioral responses to a panel of diverse bitter compounds and find that the compounds vary greatly in the degree of aversion they elicit. We then test the physiological responses of all 31 labellar taste hairs to 16 diverse bitter tastants. The responses of different sensilla show extensive diversity both in magnitude and in response dynamics. We define four functional classes of bitter neurons and the results provide a functional map of the organ. We then examine the expression of all 68 members of the Gr family of taste receptors. Based on receptor expression, the bitter neurons fall into four classes that coincide closely with the four classes based on physiological responses. The results provide a receptor-to-neuron-to-tastant map of the organ. Misexpression of a receptor confers bitter responses that agree with predictions of the map. Together, the results reveal a degree of complexity that greatly expands the capacity of the system to encode bitter taste; it allows for combinatorial coding and may enable discrimination or adaptive responses to selected bitter stimuli.

Figure 1. The Drosophila Labellum and Its Physiological Responses
(A) A typical Drosophila labellum comprises two labellar palps, each of which has 31 sensilla that are categorized and numbered based on their position and morphology. We observe some variation in the number of sensilla; e.g., either S0 or S1 is missing in 54% of labella (n = 78) and the number of anterolateral I sensilla (I0–I5) ranges from 5 ≤ n ≤ 8 (n = 67). Sensilla are shaded according to their morphological classes. The numbering and classification of individual sensilla differ slightly from the previous literature (Hiroi et al., 2002; Shanbhag et al., 2001) in order to reflect observations in our laboratory strain. A, anterior; P, posterior; M, medial; L, lateral.
(B and C) Sample traces of physiological recordings from the S6 (B) and S9 (C) sensilla. Control traces with the diluent, TCC, are shown for both sensilla. (D) Sample traces of physiological recordings from I5 (left) and I9 (right) sensilla presented with DEN or TPH demonstrate functional heterogeneity among sensilla. The arrow indicates the contact artifact observed at the beginning of each trace. See Experimental Procedures for tastant abbreviations.
RESULTS

Bitter Compounds Elicit Differing Degrees of Aversive Behavior

We selected 14 compounds that have previously been described as bitter by virtue of their behavioral effects on various insect species (Koul, 2005; Schoonhoven et al., 2005). The 14 selected tastants include naturally occurring alkaloids, terpenoids, and phenolic compounds, as well as three synthetic compounds. Many of these compounds are toxic and many are perceived as bitter by humans. Some have been tested in Drosophila previously (Hiroi et al., 2004; Lee et al., 2010; Marella et al., 2006; Meunier et al., 2003; Thorne et al., 2004; Wang et al., 2004).

We used a modification of a two-choice behavioral paradigm (Tanimura et al., 1982) in which a population of flies is allowed to feed on a microtiter plate containing alternating wells of either 1 mM sucrose, labeled with a red dye, or a solution of 5 mM sucrose mixed with a bitter tastant, labeled with a blue dye (left). The abdomens are scored as red, blue, purple, or uncolored, indicating that the fly ingested the red solution, the blue solution, both solutions, or neither solution (right).

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In our experiments, a P.I. of 1.0 indicates a complete preference for the 5 mM sucrose solution; a P.I. of 0 indicates a complete preference for the 1 mM sucrose solution. We found that in control experiments, flies given a choice between 1 mM sucrose and 5 mM sucrose alone, with no added bitter compounds, showed a P.I. of 0.71, indicating a preference for the 5 mM concentration.

We tested a range of concentrations of the 14 tastants. Low concentrations of each tastant had little or no effect on the strong preference for 5 mM sucrose (Figure 2B and Figure S1, available online). However, with addition of increasing concentrations of each bitter tastant to the 5 mM solution, flies increasingly avoided the 5 mM sucrose-bitter mixture. For all compounds, we identified a concentration at which there was a near complete avoidance of the bitter compound, i.e., the P.I. approached 0. For some bitter tastants (e.g., azadirachtin [AZA] and umbelliferone [UMB]), testing was limited by the low solubility of the tastant, but near-maximal avoidance was observed at the highest concentrations available.

Some bitter compounds were more aversive than others (Figures 2B and 2C). To quantify the sensitivity of the fly to each compound we calculated the concentration of bitter tastant that is required to render 5 mM sucrose equally attractive, or “isoattractive,” to 1 mM sucrose. We defined the isoattractive concentration as the concentration at which the P.I. is 0.36, which is the arithmetic mean of the control P.I. (0.71) and the

![Figure 2. Drosophila Avoid Ingesting Bitter Tastants in a Two-Choice Assay](image-url)
minimal P.I. (0). Thus the isoattractive concentration for denatonium benzoate (DEN), illustrated in Figure 2B, lies between $10^{-4.5}$ M and $10^{-5}$ M.

Among our panel of tastants, DEN elicits the strongest avoidance (Figure 2C). Interestingly, DEN has also been identified as the tastant that is perceived as most bitter by humans in psychophysical studies (Hansen et al., 1993; Keast et al., 2003). The isoattractive concentrations of our bitter panel ranged over more than two orders of magnitude, with the weakest avoidance elicited by escin (ESC) (Figure 2C).

These results confirmed that all members of the tastant panel are aversive or bitter to \textit{Drosophila} (Figure S1). The results also identified a concentration range over which each bitter compound is behaviorally active in this paradigm. Together these results established a foundation for a detailed physiological analysis of the cellular basis of bitter coding.

### Sensilla Are Diverse in Their Responses to Bitter Compounds

As a first step toward understanding the coding of bitter stimuli, we systematically examined the electrophysiological responses (Hodgson et al., 1955) elicited by all 14 bitter substances from all 31 labellar taste sensilla (Figure 1A). These tastants were tested at 1 mM or 10 mM, or 1% in one case, concentrations at which they were active in our behavioral paradigm. We also tested two additional compounds, aristolochic acid (ARI) and gossypol (GOS), described as bitter in other insect species, yielding a total of 16 \times 31 = 496 sensillum-tastant combinations, each tested $n \geq 10$ times.

All 16 compounds elicited action potentials from at least some sensilla. The action potentials were of a large amplitude characteristic of the bitter neuron (Figure 1B). In a few cases we observed a small number of additional action potentials of smaller amplitude, presumably generated by the water neuron, particularly in the initial period of the recording (e.g., see ARI trace in Figure 1B). Three of the 31 sensilla, S3, S5, and S9, generated a second, high-frequency and low-amplitude spike train of unknown source that appeared to be independent of stimulus identity and concentration (Figure 1C). However, in all cases the large-amplitude action potentials of the bitter neuron could easily be distinguished and are the basis of the analysis that follows.

We found that individual tastants elicit responses from subsets of sensilla, and that individual sensilla are activated by subsets of tastants (Figure 3 and Tables S1 and S2). Different sensilla responded to different subsets of stimuli. For example, I9 and I10 responded strongly to theophylline (TPH) but not DEN, whereas I3 and I1 responded strongly to DEN but not TPH (Figure 1D). Inspection of the response matrix (Figure 3) reveals extensive heterogeneity among the labellar sensilla, and by extension, among the bitter neurons that they contain.

### A Functional Map of Labellar Taste Sensilla

The L sensilla exhibited little or no physiological response to our panel of tastants, in agreement with a previous report (Hiroi et al., 2004). Two of the S sensilla, S3 and S5, also did not respond to any bitter tastants. All other S type sensilla were broadly tuned, responding to 9–15 of the 16 compounds with a spike frequency
of ≥ 10 spikes/s (Figure 3, Tables S1 and S2). I type sensilla were more narrowly tuned with respect to our panel of tastants, responding to 3–7 compounds. The strongest response was elicited by 10 mM CAF in the S5 sensillum (60.8 ± 3.3 spikes/s; n = 34).

A hierarchical clustering analysis identified five functional classes of labellar sensilla: two classes of broadly tuned sensilla (S-a and S-b), two classes of narrowly tuned sensilla (I-a and I-b), and a fifth class that did not display excitatory responses to any of our panel of tastants (L, S-c) (Figures 4A and 4B). The two classes of S sensilla are both broadly tuned, but the S-b sensilla exhibit greater mean responses to most tastants (Figure 4B). Notably, this class comprises the three sensilla that uniquely exhibited a second high-frequency action potential (Figure 1C).

Maps of the distribution of the sensilla of each class are shown in Figure 4C. The most broadly tuned sensilla (S-a and S-b classes) are located in the medial region of the labellum, while the narrowly tuned sensilla (I-a and I-b classes) are in lateral regions. The three classes of S sensilla are intermingled in the row of medial sensilla, while the I-a and I-b sensilla are restricted to the anterior and posterior portions of the labellum, respectively.

We note with interest that among the five bitter compounds that elicited responses >10 spikes/s from the I-a sensilla, three elicited the most aversive behavioral responses (DEN, sparteine sulfate salt [SPS], and (-)-lobeline hydrochloride [LOB]), and one elicited the fifth most aversive response (berberine chloride [BER]) (Figure 2C). The median isoattractive concentration for these five tastants was <0.1 mM; the median concentration for all the others was ~1 mM. Although gustatory input from other organs such as the legs probably influences this behavior, these results suggest the possibility that different classes of bitter-sensing neurons make different contributions to the behavior of the fly.

Temporal Coding of Bitter Stimuli

Some tastants elicited delayed responses. Four compounds (coumarin [COU], saponin [SAP], ESC, and GOS) exhibited delays of >100 ms in discharge (Figure 5A). We quantified these temporal dynamics by measuring the interval between the time at which electrical contact was registered (the contact artifact) and the onset of spike discharge. Different tastants elicited responses with delays of different lengths (Figure 5B). S-a and S-b sensilla showed comparable temporal dynamics for a given tastant. Differences among compounds in spike latency are not restricted to the labellum, but have also been noted in leg sensilla (Meunier et al., 2003).

Other compounds elicited shorter delays in spike onset that differed among sensilla (Figures 5C and 5D). The length of the delay did not show a simple correlation with the magnitude of...
Figure 5. Sensillar Classes Exhibit Characteristic Latencies in Spike Generation

(A) Sample traces illustrating typical delays in spike onset. Recordings are from the S6 sensillum stimulated with CAF, COU, SAP, or GOS, and the S9 sensillum stimulated with ESC.

(B) The mean delay in spike onset is shown for S-a (represented by S2, S6, and S7) and S-b (represented by S3, S5, and S9) sensilla in response to the indicated tastants. For individual sensilla (not including CAF), 6 ≤ n ≤ 16, with a mean of 9.8 traces analyzed. * = no response.

(C) Sample traces of recordings from sensilla of the indicated functional classes stimulated with BER (left) or TPH (right). The time scales are expanded in order to illustrate clearly the delays in the onset of spike initiation. The spikes elicited from S3 by TPH have been marked with dots for clarity.

(D) The mean delay in spike onset is shown for sensilla of the indicated functional classes in response to BER (left) or TPH (right). Bars are grouped by sensillum class. 11 ≤ n ≤ 40, with a mean of 21 traces analyzed for each sensillum type.

(E) Bursting responses of S9 sensilla to the indicated tastants. Error bars are SEM (see also Figure S2).
the response: e.g., I-a and S-a sensilla yielded similar response magnitudes to BER (28 ± 3 and 27 ± 2 spikes/s, respectively; n = 24–47 sensilla of each individual type, with means for each type averaged across each class), but the delays in response differed by a factor of two (43 ± 2 and 81 ± 6 ms, respectively, n = 12–40). Taken together, these results suggest that such differences in spike onset may represent a salient feature of taste coding.

We note that erratic or “bursting” responses in S-b sensilla are occasionally observed in response to GOS and strychnine (STR) (Figure 5E) as well as BER, LOB, sucrose octaacetate (SOA), and ARI. Of the S5 sensilla that responded to BER, 63% of traces exhibited a bursting pattern (n = 19). Similar bursts of action potentials were reported for tarsal gustatory sensilla tested with high concentrations of bitter tastants (Meunier et al., 2003); we do not know whether such bursting responses contribute to taste coding.

Coding of Bitter Intensity

The intensity of bitter substances is a critical factor in evaluating the palatability of a food source. We examined the coding of bitter intensity, with a special interest in the sensitivity and dynamic range of neuronal responses, by systematically testing the responses of representative labellar sensilla to CAF, DEN, and LOB over a wide range of concentrations (Figure S2). All tested sensilla exhibited dose-dependent responses to each compound. In the case of most tantant-sensillum combinations the response threshold lay between 0.1 mM and 1 mM concentrations. While the limited solubility of some tastants precluded the analysis, corresponded closely to the five classes defined by in situ hybridization with Gr expression profiles and identified five classes

A Receptor-to-Neuron Map Reveals Distinct Classes of Bitter Neurons

Having analyzed first the behavior driven by bitter compounds and then the cellular basis of bitter response, we next examined its molecular basis. The expression of most Gr genes has not been examined and few have been mapped to individual sensilla (Dahanukar et al., 2007; Hiroi et al., 2002; Koganezawa et al., 2010). In situ hybridizations with Gr genes have been unsuccessful in most cases (Clyne et al., 2000; Dahanukar et al., 2007; Dunipace et al., 2001; Moon et al., 2009; Scott et al., 2001), perhaps because of low levels of Gr expression. However, there has been greater success in analyzing Gr expression patterns by using the GAL4/UAS system to drive reporter gene expression (Brand and Perrimon, 1993; Chyb et al., 2003; Dunipace et al., 2001; Moon et al., 2009; Scott et al., 2001; Thorne and Amrein, 2008).

We have analyzed the expression patterns of all 68 Gr family members by using Gr-GAL4 lines. We generated flies with Gr-GAL4 transgenes for 59 members of the Gr family and acquired previously published lines for eight receptors (Dunipace et al., 2001; Scott et al., 2001; Table S3). One line, Gr23a-GAL4, represents two receptors, Gr23a.a and Gr23a.b, which are encoded by alternatively spliced transcripts that share a common 5’ region. For most receptors, 2–6 independent Gr-GAL4 lines were examined (Table S3).

We found expression in labellar sensilla for 38 Gr-GAL4 drivers (Figure 6). Some drivers show expression in all labellar sensilla; most show expression in subsets of sensilla. The vast majority of the drivers are expressed in a single neuron of the sensilla in which they are expressed. To identify the neuron we carried out a series of double-label experiments.

Gr5a, a sugar receptor, is expressed in the sugar-sensitive neuron of all labellar sensilla, while Gr66a, a receptor required for CAF perception, is expressed in all bitter neurons (Thorne et al., 2004; Wang et al., 2004). To mark bitter-sensitive neurons we used a direct fusion of RFP to the Gr66a promoter (Gr66a-RFP), a construct whose expression pattern matches that of the Gr66a-GAL4 driver (Dahanukar et al., 2007). The RFP reporter is observed in each of the S and I sensilla, with the exceptions of S5 and S8.

Five of the 38 drivers showed no coexpression with Gr66a-RFP (Figure S3, upper panel). These five receptors, which include Gr5a, are all known or predicted sugar receptors (Dahanukar et al., 2007; Jiao et al., 2008; Slone et al., 2007). The remaining 33 labellar Gr-GAL4 drivers labeled subsets of Gr66a-expressing neurons or all Gr66a-expressing neurons (Figure S3, lower panel) and thus may function in bitter taste perception. Our data are consistent with reports that Gr33a and Gr93a, in addition to Gr66a, contribute to the perception of CAF and other bitter tastants (Lee et al., 2009; Moon et al., 2006, 2009). None of the 33 bitter Gr-GAL4 drivers, with two exceptions (Table S3), was expressed in L, S8, or S9 sensilla, consistent with the lack of bitter physiological responses in these sensilla.

Some individual drivers are expressed broadly, e.g., Gr33a-GAL4 is expressed in all bitter-sensing neurons, whereas others are expressed only in a few, e.g., Gr22f-GAL4 is expressed only in S3, S5, and S9 (Figure 7). Likewise, an individual bitter neuron may express a large number of Gr-GAL4 lines (e.g., S5 expresses 28 drivers), whereas others express only a few (e.g., the bitter neuron of I6 expresses only 6 drivers).

We note with special interest that five drivers, Gr32a, Gr33a, Gr39a.a, Gr66a, and Gr89a, are expressed in all bitter neurons. This ubiquitous expression suggests a unique function for these receptors. In support of this suggestion, genetic analysis indicates that Gr33a is broadly required for responses to aversive cues important for both feeding and courtship behaviors (Moon et al., 2009).

We performed a hierarchical cluster analysis of sensilla based on their Gr-GAL4 expression profiles and identified five classes of sensilla (Figure 8A). These classes, defined by expression analysis, corresponded closely to the five classes defined by functional analysis (Figure 4A). The classifications agreed for 29 of the 31 sensilla.

These results establish a receptor-to-neuron map (Figure 8B). Taken together with the functional map (Figure 4) they provide a receptor-to-neuron-to-response map. The mapping reveals a correlation between the tuning breadth of a bitter-sensitive neuron and the number of Gr-GAL4 drivers it expresses. The broadly tuned S-a and S-b neurons express 29 and 16 Gr-GAL4 drivers, respectively, while the more narrowly tuned I-a and I-b neurons express 6 and 10 Gr-GAL4 drivers, respectively.
In summary, we have generated a receptor-to-neuron map of an entire family of chemosensory receptors and an entire ensemble of taste neurons in a major taste organ. Our data support a role for 33 Gr genes in the perception of bitter taste.

Misexpression of a Gr Confers Physiological Responses

The receptor-to-neuron map makes predictions about the functions of certain receptors. For example, according to the map only one receptor, Gr59c, is expressed by I-a but not I-b sensilla. I-a sensilla respond most strongly to BER, DEN, and LOB, whereas I-b sensilla show little or no response to these compounds. These results suggested the possibility that Gr59c might act in response to these compounds.

To test this possibility, we expressed UAS-Gr59c in I-b sensilla by using Gr66a-GAL4. We found that expression of Gr59c in fact conferred strong responses to BER, DEN, and LOB when expressed in each of three I-b sensilla, I10, I9, and I8 (Figure 9).

We also tested the effects of driving Gr59c expression in sensilla of the I-a, S-a, and S-b classes, which show moderate or strong responses to these compounds in wild-type. I-a and S-a sensilla express Gr59c in wild-type flies, but we reasoned that the use of the GAL4 system would increase the levels of its expression. We found that misexpression of Gr59c increased the responses to these compounds in all of these sensilla (Figure 9).

We also tested responses to AZA and CAF, which were not predicted by the receptor-to-neuron map to act via Gr59c. We found that expression of Gr59c did not increase the response to either tastant (Figure S4). Unexpectedly, responses were decreased by ectopic expression of Gr59c in many cases. One possible interpretation of these results is that misexpressed Gr59c titrates out other receptors or cofactors, thereby perturbing the formation of a receptor complex required for the endogenous response. This view is supported by observations that Gr gene dosage scales with physiological and/or behavioral responses (Kwon et al., 2007; Tanimura et al., 1988) and by genetic analysis indicating a role for a heteromeric complex of more than three Gr proteins in the detection of CAF (Lee et al., 2009; Moon et al., 2006, 2009).

We next drove Gr59c in sugar neurons, either singly or in combination with Gr66a or Gr33a, by using the Gr5a-GAL4 driver. Misexpression of Gr59c did not confer physiological responses to BER or other tested bitter compounds in sugar neurons (data not shown). These results suggest that Gr59c is not sufficient for the response to these compounds and probably acts in concert with other Gr proteins and/or cofactors that are specific to bitter neurons.

According to the receptor-to-neuron map, Gr59c is expressed in I-a sensilla along with five other Grs that are broadly expressed in all classes of bitter neurons. Taken together, our results support the hypothesis that Gr59c operates together with one
or more of these other Grs and our analysis confirms the prediction that Gr59c acts in response to at least three bitter tastants.

DISCUSSION

We have provided a systematic behavioral, cellular, and molecular analysis of bitter taste in Drosophila. The analysis has revealed extensive complexity in the coding of bitter taste.

Functional Diversity of Bitter Neurons

We have defined five distinct classes of sensilla in the Drosophila labellum on the basis of their responses to bitter compounds. Four of these sensillar classes contain bitter-sensing neurons; other sensilla did not respond physiologically to any of our bitter tastants. This analysis, then, has defined four classes of bitter-sensing neurons that are diverse in their response profiles. Some are broadly tuned with respect to a panel of bitter compounds and some are more narrowly tuned. The neurons also vary in the temporal dynamics of their responses. Different neurons respond to the same tastant with different onset kinetics and an individual neuron responds to distinct tastants with diverse dynamics. The functional diversity of bitter-sensing neurons expands the coding capacity of the system: different tastants elicit responses from different subsets of neurons and distinct tastants elicit diverse temporal patterns of activity from these neurons.

Molecular Diversity of Bitter Neurons

The functional differences among neurons in the Drosophila labellum suggested underlying molecular differences. In particular, we wondered whether the four classes of bitter taste neurons defined by physiological analysis could be distinguished by molecular analysis. We constructed a receptor-to-neuron map of the entire Gr repertoire and found that four classes of bitter taste neurons emerged on the basis of receptor expression, classes that coincided closely with the four functional classes. Moreover, the neuronal classes that were more broadly tuned expressed more receptors.

Our systematic analysis does not support previous models that suggest functional uniformity among bitter neurons (Cobb et al., 2009; Marella et al., 2006). A previous physiological study of the labellum did not reveal functionally distinct neuronal classes but was limited in the number of sensilla and tastants that were examined (Hiroi et al., 2004). There are major technical challenges in recording from I and S sensilla; the S sensilla in particular are small, curved, and difficult to access because of their position on the labellar surface. Our finding of functional heterogeneity in labellar sensilla is consistent with the finding that two taste sensilla on the prothoracic leg responded to BER but not quinine, whereas another sensillum responded to quinine but not BER (Meunier et al., 2003). A recent study found that N,N-diethyl-m-toluamide (DEET) elicited different responses from several labellar sensilla tested (Lee et al., 2010). Functionally distinct bitter neurons have also been described in taste organs of caterpillars, and in the case of the Manduca larva, ARI and salicin activate spike trains that differ in dynamics (Glendinning et al., 2002, 2006).
diversity would become apparent among the bitter-sensing neurons.

There are also limitations to our receptor-to-neuron map. First, the map considers exclusively the 68 Grs. There are at least two additional receptors that can mediate bitter taste. DmXR, a G protein-coupled receptor, is expressed in bitter neurons of the labellum and is required for behavioral avoidance of L-canavanine, a naturally occurring insecticide (Mitri et al., 2009); the TRPA1 cation channel, also expressed in a subset of bitter neurons in the labellum, is required for behavioral and electrophysiological responses to ARI (Kim et al., 2010). Second, Gr-GAL4 drivers may not provide a fully accurate representation of Gr gene expression in every case. Genetic analysis has shown that Gr64a is required for the physiological responses of labellar sensilla to some sugars and is therefore expected to be expressed in labellar sugar neurons (Dahanukar et al., 2007). Our Gr64a-GAL4 driver, however, is not expressed in these neurons, suggesting the lack of a regulatory element. In light of the limitations to the use of the GAL4 system to assess receptor expression, we were encouraged that drivers representing almost all 68 Grs were expressed in chemosensory neurons, with very few exceptions (Figure 6, Table S3, and data not shown), and that the expression patterns in the labellum agreed well with the patterns of physiological responses (Figures 4 and 8). In addition, we were able to integrate the functional and expression data and predict a function for one Gr (Figure 9).

While our data support the hypothesis that Gr59c encodes a bitter receptor for BER, DEN, and LOB, Gr59c is not sufficient for responses to these compounds in sugar neurons. It is also apparently not necessary, in the sense that physiological responses to these tastants were observed in S-a sensilla that do not express the Gr59c driver. These observations suggest that there is another receptor for BER, DEN, and LOB that may recognize a different moiety of these tastants, providing multiple means of detecting some of the most behaviorally aversive bitter tastants in the panel.

We note that 38 of the Gr-GAL4 drivers, slightly more than half, showed expression in the labellum. The other Grs are probably expressed in other chemosensory neurons of the adult and larva (Dunipace et al., 2001; Jones et al., 2007; Kwon et al., 2007; Scott et al., 2001; Thorne and Amrein, 2008) (unpublished data, A.D., J.Y.K., L.A.W., F. Ling, and J.R.C.). Of the 38 labellar Gr-GAL4 drivers, 33 are expressed in bitter neurons, and only a few in sugar neurons. It seems probable that a high fraction of Grs are devoted to bitter perception because of the number and structural complexity of bitter compounds (Schoonhoven et al., 2005; Schwab, 2003). Sugars are simpler and more similar in structure. In order to detect the wide diversity of noxious bitter substances that an animal may encounter, a larger and more versatile
repertoire of receptors is likely needed. We note that in mice and rats, 36 bitter receptors have been identified (Wu et al., 2005), but fewer sugar receptors (Montmayeur et al., 2001; Nelson et al., 2001). Among the Grs mapped to bitter neurons, five map to all bitter neurons: Gr32a, Gr33a, Gr39a.a, Gr66a, and Gr89a. Some or all of these “core bitter Grs” may function as coreceptors, perhaps forming multimers with other Grs. These core Grs might play a role analogous to Or83b, an Or that is broadly expressed in olfactory receptor neurons and that functions in the transport of other Ors and as a channel, rather than conferring odor specificity per se (Benton et al., 2006; Sato et al., 2008; Wicher et al., 2008). If so, the core Grs may be useful in deorphanizing other Gs in heterologous expression systems. We note that in mammals, T1R3 functions as a common coreceptor with either T1R1 or T1R2 to mediate gustatory responses to amino acids or sugars, respectively (Zhao et al., 2003).

We note finally that the receptor-to-neuron map defines intriguing developmental problems. How do the five classes of sensilla acquire their diverse functional identities? How does an individual taste neuron select, from among a large Gr repertoire, which receptor genes to express? In the olfactory system of the fly, the expression of each receptor gene is dictated by a combinatorial code of cis-regulatory elements and by a combinatorial code of transcription factors (Bai and Carlson, 2010; Bai et al., 2009; Clyne et al., 1999; Miller and Carlson, 2010; Ray et al., 2007, 2008; Tichy et al., 2008). Mechanisms of receptor gene choice were elucidated in part by identifying upstream-regulatory elements that were common to coexpressed Or genes. The receptor-to-neuron map that we have established for the taste system lays a foundation for identifying regulatory elements shared by coexpressed Gr genes, which in turn may elucidate mechanisms of receptor gene choice in the taste system. It will be interesting to determine whether the mechanisms used in the olfactory and taste systems are similar.

**Taste Coding in the Labellum**

In principle the design of the *Drosophila* taste system could have been extremely simple. Every sensillum could be identical, and all sensilla could report uniformly the valence of each tastant, e.g., positive for most sugars and negative for bitter compounds. Such a design would be economical to encode in the genome and to execute during development.

The design of the *Drosophila* olfactory system is not so simple. Physiological analysis of the fly has identified 17 functionally distinct types of olfactory sensilla (Clyne et al., 1997; de Bruyne et al., 1999, 2001; Elmore et al., 2003; van der Goes van Naters and Carlson, 2007; Yao et al., 2005). This design allows for a combinatorial coding of odors. A recent study of the *Drosophila* larva defined an odor space in which each dimension represents the response of each component of olfactory input (Kreher et al., 2008). The distance between two odors in this space was proportional to the perceptual relationship between them. In principle, a coding space of high dimension may enhance sensory discrimination and allow for a more adaptive behavioral response to a sensory stimulus.

Here we have found that the fly’s taste system is similar to its olfactory system in that its sensilla fall into at least five functionally distinct types, four of which respond to bitter stimuli. This heterogeneity provides the basis for a combinatorial code for tastes and for a multidimensional taste space. A recent report has suggested that flies cannot discriminate between pairs of bitter stimuli when applied to leg sensilla (Masek and Scott, 2010); it will be interesting to extend such analysis to the labellum and especially to examine pairs of stimuli that have been shown to activate distinct populations of neurons. Our physiological analysis thus invites an extensive behavioral analysis, beyond the scope of the current study, which explores the extent to which such a taste space supports taste discrimination in the fly.

Why might there be selective pressure to enhance the coding of bitter taste? Why not simply coexpress all bitter receptors in one type of neuron that activates a single circuit, thereby
In summary, it is difficult to draw definitive conclusions about the temporal dynamics elicited by different tastants (Figure 5). The virtue of differences in the temporal dynamics of their firing receptors yet the worm is able to discriminate odors (Bargmann, 2007). Taste neurons that project to similar locations in the SOG could also activate different circuits with distinguishable behavioral consequences. Like the fly taste system, the Caenorhabditis elegans olfactory system does not contain glomeruli and its sensory neurons coexpress many receptors yet the worm is able to discriminate odors (Bargmann, 2006). Finally, we note that different sensory neurons that project to similar positions may carry distinguishable information by virtue of differences in the temporal dynamics of their firing (Wilson and Mainen, 2006). We have in fact identified differences in the temporal dynamics elicited by different tastants (Figure 5). In summary, it is difficult to draw definitive conclusions about the functional roles of taste neurons from the currently available anatomical analysis.

A final consideration raised by our analysis is how the responses of the different functional classes of taste sensilla are temporally integrated to control feeding behavior. The different functional classes of sensilla differ in length and are located in different regions of the labellar surface. Moreover, during the course of feeding the labellum expands, changing the positions of the various sensilla with respect to the food source. It seems probable that there is a temporal order in which labellar taste sensilla send information to the CNS.

In summary, we have provided a systematic behavioral, physiological, and molecular analysis of the primary representation of bitter compounds in a major taste organ. We have defined the molecular and cellular organization of the bitter-sensitive neurons, and we have found extensive functional diversity in their responses. The results provide a foundation for investigating how this primary taste representation is transformed into successive representations in the CNS and ultimately into behavior.

**EXPERIMENTAL PROCEDURES**

**Drosophila Stocks**

Flies were grown on standard cornmeal agar medium. Canton-S flies that were used for electrophysiological recordings and behavior experiments were raised at room temperature (23°C ± 2°C), while transgenic flies used for both recordings and GFP visualization were raised at 25°C. For electrophysiological recordings, freshly eclosed flies were transferred to fresh food and allowed to age for 5–7 days prior to experimentation. For GFP visualization, most lines (72%) were doubly homoyzogous for the Gr-GAL4 driver and for the UAS-mCD8:GFP reporter; the remaining lines were homozygous lethal. Flies were aged 5–15 days and maintained at 25°C until dissection. Only males were used for all electrophysiological, expression, and behavioral studies. All transgenic constructs were injected into w^1118 flies.

**Transgenic Flies**

w; UAS-mCD8:GFP was used as the GFP reporter and Gr66a-RFP was from Dahanukar et al. (2007).

For Gr-GAL4 constructs, primers were used to amplify DNA sequences upstream of the translation initiation codon of Gr genes with Canton-S genomic DNA as a template. Constructs were cloned into pG4PN (Brand and Perrimon, 1993). The size of the promoters varied (Table S3) but was generally dictated by the distance between the translation initiation codon of the Gr gene and the coding region of the next 5′ gene. The average promoter size was 3.9 kb. Additional lines were kindly provided by H. Amrein (Gr28a-GAL4, Gr28b-d-GAL4, Gr59b-GAL4, and Gr66a-GAL4) and K. Scott (Gr21a-GAL4, Gr22c-GAL4, Gr28b.e-GAL4, and Gr47a-GAL4). Samples were analyzed by using a Bio-Rad 1024 laser-scanning confocal microscope.

The coding region of Gr59c was amplified from Canton-S cDNA prepared from labelia and was inserted into the pUAST expression vector (Brand and Perrimon, 1993). Two independent lines were tested physiologically.

**Tastants**

For electrophysiological recordings, tastants were dissolved in 30 mM tricholine citrate (TCC; Sigma-Aldrich, St. Louis, MO), an electrolyte that inhibits the activity of the water cell (Wieczorek and Wolff, 1989); for the behavioral assay, tastants were dissolved in water. All tastants were stored at −20°C, and aliquots were kept at 4°C and used for no more than one week. Tastants of the highest available purity were obtained from Sigma-Aldrich and stored as recommended. All tastants were tested at the following concentrations unless otherwise indicated: arsilotic acid (AR), 1 mM; azadiractin (AZA), 1 mM; berberine chloride (BER), 1 mM; caffeine (CAF), 10 mM; coumarin (COU), etc.
10 mM; N-Diethyl-m-toluamide (DEET), 10 mM; denatonium benzoate (DEN), 10 mM; escin (ESC), 10 mM; gossypol from cotton seeds (GOS), 1 mM; (-)-lobeoline hydrochloride (LOB), 1 mM; saponin from quillaja bark (SAP), 1%; D-(+)-sucrose octaacetate (SOA), 1 mM; spartane sulfate salt (SPS), 10 mM; strychnine nitrate salt (STR), 10 mM; theophylline (TPH), 10 mM; and umbelliferone (UMB), 10 mM. Additional tastants that did not elicit physiological responses >10 spikes/s in limited testing included gibererelic acid, 10 Mm; (−)-catechin, 1 mM; cucubertacin I hydrate, 1 mM; atropine, 1 mM; N-phenylthiourea, 1 mM; harmane, 1 mM; (−)-nicotine, 10 mM; gallic acid, 10 mM; (−)-sinigrin hydrate, 10 mM; theobromine, 10 mM; α-(methylamino)methylbenzyl alcohol, 10 mM; and naringen, 1 mM.

Electrophysiology
Extracellular single-unit recordings were performed by using the tip-recording method (Hodgson et al., 1955). Flies were immobilized via a reference electrode containing Drosophila Ringer’s solution which was threaded through the thorax and head to the tip of the labellum. This electrode served as the indifferent electrode. Tastants were introduced to individual sensilla via a glass recording electrode (10–15 μm tip diameter) filled with tastant solution. Traces of action potentials were recorded by using TasteProbe (Syntech, The Netherlands) and analyzed with Autospike 3.2 software (Syntech). Responses were quantified by counting the number of spikes generated over a 500 ms period beginning 200 ms after contact. When measuring latencies in spike generation, only traces in which the first contact was successful were used for our calculations. In some recordings, sensilla or groups of sensilla were anomalously unresponsive, presumably because of damage resulting from the insertion of the reference electrode. We therefore tested the viability of labellar sensilla with a positive control (for example, BER was used to test I-a sensilla and CAF to test I-b sensilla). A maximum of eight tastants were tested on a single sensillum with a minimum of 5 min between presentations.

Behavioral Assays
The two-choice assay was performed with minor modifications of the original protocol (Taninura et al., 1982). Fifty flies (3–5 days old) were transferred to a vial containing moistened Kimwipes and starved at room temperature for 22 hr. Flies were introduced to a 60-well plate containing alternating wells of 1 mM sucrose (containing 0.5 mg/ml surahodamine B, Sigma) or 5 mM sucrose plus bitter taste (containing 0.25 mg/ml indigo carmine, Sigma) and allowed to feed for 2 hr in the dark at 25°C. Flies were anesthetized by freezing the plates at −20°C and the abdomens were scored blind to experimental condition as red, blue, purple, or white. In most trials more than 50% of flies participated, i.e., were scored as red, blue, or purple, and only trials in which more than 33% of flies participated were included in our analysis. A minimum of six independent trials were performed for each tastant and for each concentration. The P.I. were calculated as follows: P.I. = (N_red + 0.5 N_azure)/N_red + N_purple + N_azure, where N_red, N_azure, and N_purple represent the number of flies with red, blue, and purple abdomens. Control experiments showed that the dyes did not affect preference.

Statistical Analyses
Hierarchical cluster analyses with Ward’s method were performed by using the statistics program PAST (http://folk.uio.no/ohammer/past) (Hammer et al., 2001). All error bars are standard errors of the mean (SEM).

SUPPLEMENTAL INFORMATION
Supplemental Information includes four figures and three tables and can be found with this article online at doi:10.1016/j.neuron.2011.01.001.

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