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A Chemosensory Gene Family Encoding Candidate Gustatory and Olfactory Receptors in *Drosophila*

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Summary

A novel family of candidate gustatory receptors (GRs) was recently identified in searches of the *Drosophila* genome. We have performed in situ hybridization and transgene experiments that reveal expression of these genes in both gustatory and olfactory neurons in adult flies and larvae. This gene family is likely to encode both odorant and taste receptors. We have visualized the projections of chemosensory neurons in the larval brain and observe that neurons expressing different GRs project to discrete loci in the antennal lobe and subesophageal ganglion. These data provide insight into the diversity of chemosensory recognition and an initial view of the representation of gustatory information in the fly brain.

Introduction

All animals have specialized mechanisms to recognize and respond to chemosensory information in the environment. Olfactory neurons recognize volatile cues that afford the organism the ability to detect food, predators, and mates. In contrast, gustatory neurons sense soluble chemical cues that elicit feeding behaviors. In insects, taste neurons also initiate innate sexual and reproductive responses. In *Drosophila*, for example, sweet compounds are recognized by chemosensory hairs on the proboscis and legs that activate proboscis extension and feeding (Dethier, 1976). Sexually dimorphic chemosensory bristles on the foreleg of males recognize cues from receptive females that are thought to elicit the embrace of mating (Tompkins et al., 1983; Possidente and Murphey, 1989). Females have yet a third set of specialized bristles on their genitalia that may cause oviposition in response to nutrients (Rice, 1977; Taylor, 1989). In this manner, gravid females will preferentially deposit their eggs on a rich environment that enhances survival of their offspring. These robust and innate gustatory responses provide the opportunity to understand how chemosensory information is recognized in the periphery and ultimately translated into specific behaviors.

Taste in *Drosophila* is mediated by sensory bristles that reside on the proboscis, legs, wing, and genitalia (Figure 1) (Stocker, 1994; Singh, 1997). Most chemosensory bristles are innervated by four bipolar gustatory neurons and a single mechanoreceptor cell (Falk et al., 1976). The dendrites of gustatory neurons extend into the shaft of the bristle and are the site of taste recognition that translates the binding of tastants into alterations in membrane potential. The sensory axons from the proboscis project to the brain where they synapse on projection neurons within the subesophageal ganglion (SOG), the first relay station for gustatory information in the fly brain (Stocker and Schorderet, 1981; Nayak and Singh, 1983; Shanbhag and Singh, 1992; Rajashekhar and Singh, 1994). Sensory axons from taste neurons at other sites along the body project locally to peripheral ganglia (Power, 1948). *Drosophila* larvae, whose predominant activity is eating, sense their chemical environment with gustatory neurons that reside in chemosensory organs on the head and are also distributed along the body surface (Figure 1) (Stocker, 1994). The pattern of projection of functionally distinct classes of taste cells and therefore the nature of the representation of gustatory information in the *Drosophila* brain remains unknown.

The identification of the genes encoding taste receptors and the analysis of the patterns of receptor expression may provide insight into the logic of taste discrimination in the fly. In *Drosophila*, the recognition of odorants is thought to be accomplished by about 60 seven-transmembrane domain proteins encoded by the *Drosophila* odorant receptor (DOR) gene family (Clyne et al., 1999; Gao and Chess, 1999; Vosshall et al., 1999, 2000). Recently, a large family of putative G protein–coupled receptors was identified by searching the *Drosophila* genome with an algorithm designed to detect seven-transmembrane domain proteins (Clyne et al., 2000). These genes were suggested to encode gustatory receptors (GRs) because members of this gene family were detected in the proboscis by RT-PCR experiments.

We have characterized and extended the family of putative G protein–coupled receptors originally identified by Clyne et al. (2000) and provide evidence that they encode both olfactory and gustatory receptors. In situ hybridization, along with transgene experiments, reveals that some receptors are expressed in topographically restricted sets of neurons in the proboscis, whereas other members are expressed in spatially fixed olfactory neurons in the antenna. Members of this gene family are also expressed in chemosensory bristles on

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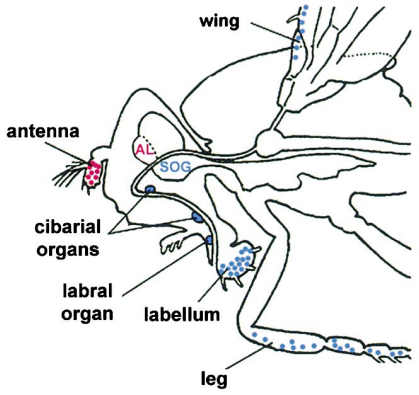

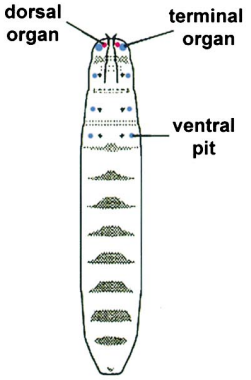

	ADULT						LARVA				
GR	In situ signal	labellum	antenna	cibarial organs	labral organ	leg	terminal organ	dorsal organ	mouth	gut	ventral pits
Gr2B1	–	–	–	–	+	–	+	+	–	+	+
Gr21D1	antenna	–	+	–	–	–	+	–	–	–	–
Gr22B1	–	+	–	–	–	–	–	–	–	–	–
Gr28A3	labellum	+	–	+	–	–	+	–	+	+	–
Gr32D1	labellum	+	–	+	–	+	+	–	–	–	–
Gr47A1	labellum	+	–	–	–	–	–	–	–	–	–
Gr66C1	labellum	+	–	+	–	–	+	–	+	–	–

Figure 1. Summary of *Drosophila* Chemosensory Tissues and GR Transgene Expression Patterns

The table summarizes the expression patterns of *GR promoter-Gal4* transgenes in adult and larval chemosensory tissues. Adult *Drosophila* sense gustatory cues with chemosensory bristles on the labellum of the proboscis, legs and wings, and with specialized structures of the internal mouthparts, the cibarial organs and the labral sense organ. Gustatory neurons on the proboscis send axonal projections to the subesophageal ganglion (SOG). Sensory neurons on the antenna recognize olfactory cues and project to the antennal lobe (AL). In *Drosophila* larvae, gustatory cues are recognized by neurons innervating the terminal organ and possibly the ventral pits, and olfactory cues are recognized by neurons innervating the dorsal organ and the terminal organ. Gustatory tissues are highlighted in blue and olfactory tissues are highlighted in pink. The schematic of the adult fly is adapted from Stocker, 1994. The schematic of the larva is adapted from Struhl, 1981.

the leg and in larval chemosensory organs. Finally, we have traced the projections of different subsets of larval chemosensory neurons to the subesophageal ganglion and the antennal lobe. These data provide insight into the diversity of chemosensory recognition in the periphery and afford an initial view of the representation of gustatory information in the fly brain.

Results

A Large Family of Candidate Chemoreceptors

A novel family of putative seven transmembrane domain proteins was recently identified in searches of the *Drosophila* genome (Clyne et al., 2000). Analysis of a database representing 60% of the *Drosophila* genome identified twenty-three full-length genes and 20 partial sequences. The expression of 19 genes was examined by RT-PCR analysis and revealed 18 transcripts in the proboscis labellum, suggesting that this novel gene family may encode the fly gustatory receptors (GRs). We have characterized the expression of these genes by in situ hybridization and transgene experiments and observe expression in both gustatory and olfactory chemosensory neurons in both larvae and adult flies.

We have extended the gene family by analyzing the recently completed euchromatic genome sequence of *Drosophila* (Adams et al., 2000) using reiterative BLAST searches (Altschul et al., 1990), transmembrane domain prediction programs (von Heijne, 1992), and hidden Markov model (HMM) analyses (Eddy, 1998). These searches have identified a total of 56 candidate GR genes in the *Drosophila* genome, including 23 GRs not previously described. Gene sequences are available at the URL http://cpmcnet.columbia.edu/dept/neurobeh/axel/gr.html. As originally reported, these genes encode putative seven transmembrane domain proteins of about 480 amino acids (Clyne et al., 2000). The family as a whole is extremely divergent and reveals an overall sequence identity ranging from 7%–50%. However, all genes share significant sequence similarity within a 33 amino signature motif in the putative seventh transmembrane domain in the C terminus (Figure 2). Analysis of the sequence of the 56 genes reveals the existence of four discrete subfamilies (containing ten, six, four, and three genes) whose members exhibit greater overall sequence identity ranging from 30%–50%. Twenty-two of the GR genes reside as individual sequences distributed throughout each of the *Drosophila* chromosomes, whereas the remaining genes are linked in the genome in small tandem arrays of two to five genes.

The GR family shares little sequence similarity outside of the conserved C-terminal signature in the putative seventh transmembrane domain and therefore our searches of the genome database are unlikely to be exhaustive. Thus, this family of candidate gustatory receptors consists of a minimum of 56 genes. Moreover, our analysis would not detect alternatively spliced transcripts, a feature previously reported for some members of this gene family (Clyne et al., 2000). We have identified cDNAs or RT PCR products from only six genes and verification of the gene predictions therefore awaits the isolation and sequencing of additional cDNAs.

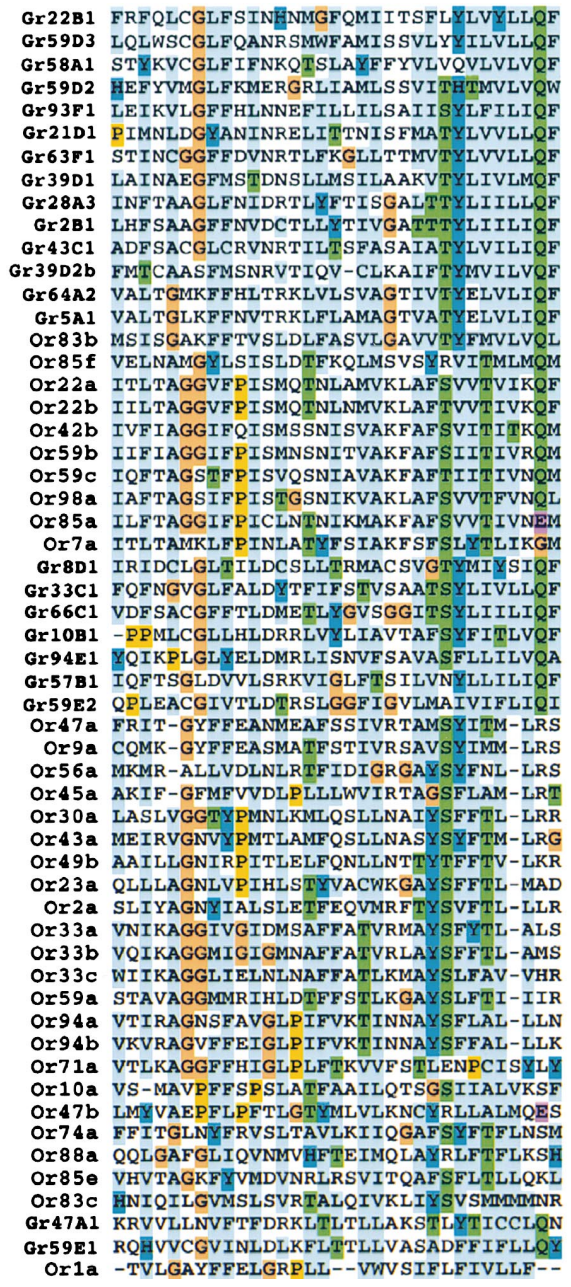

Figure 2. The Signature Motif of GRs in Present but Diverged in Members of the DOR Gene Family

Sequence alignments of the complete DOR and GR gene families reveal a common amino acid motif in the putative seventh transmembrane domain of the carboxyl terminus of all GRs and 33 DORs. Alignments are shown for 23 GRs and 33 DORs. The average identity in the C terminus is 29% for the GRs, 25% for the DORs, and 20% for the GRs plus DORs. Sequence relationships between the GR gene family and the DOR genes were analyzed with HMMs (Eddy, 1998), CLUSTAL alignments and neighbor joining trees (Saitou and Nei, 1987; Higgins and Sharp, 1988), and NxN BLASTP (Rubin et al., 2000) comparisons. The consensus alignment and coloring of conserved residues was assigned in ClustalX.

Interestingly, the 33 amino acid signature motif characteristic of the GR genes is present but somewhat diverged in 33 of the 60 members of the family of *Drosophila* odorant receptor (DOR) genes. (Figure 2). The DOR genes, however, possess additional conserved motifs not present in the GR genes and define a distinct family (Clyne et al., 1999; Gao and Chess, 1999; Vosshall et al., 1999, 2000). These observations suggest that the putative gustatory and olfactory receptor gene families may have evolved from a common ancestral gene.

GR Gene Expression in Olfactory and Gustatory Organs

Insight into the specific problem of the function of these candidate receptor genes and the more general question as to how tastants are recognized and discriminated by the fly brain initially requires an analysis of the patterns of expression of the individual GR genes in chemosensory cells. We have therefore performed in situ hybridization on sagittal sections of the adult fly head with RNA probes obtained from all 56 family members. Six of the genes are expressed in discrete, topographically restricted subpopulations of neurons within the proboscis (Figure 3A). Three of the genes revealed no hybridization to the proboscis but are expressed in spatially-defined sets of neurons within the third antennal segment, the major olfactory organ of the adult fly (Figure 3B). The remaining genes show no hybridization to adult head tissues.

Our analysis of the pattern of GR gene expression by in situ hybridization demonstrates that a small number of GR genes is transcribed in either the proboscis or the antenna, suggesting that this family encodes chemosensory receptors involved in smell as well as taste. However, we did not detect expression of over 80% of the family members using our in situ hybridization conditions. The sequence of these GR genes does not reveal nonsense or frameshift mutations that characterize pseudogenes. The inability to detect transcripts from the majority of the GR genes by in situ hybridization might result from low levels of expression of GR genes, expression in populations of chemosensory cells not amenable to analysis by in situ hybridization (e.g., leg, wing, or vulva), or expression at other developmental stages.

We therefore generated lines of flies expressing GR promoter transgenes to visualize the expression in a wider range of cell types with higher sensitivity. Transgenes were constructed in which putative GR promoter sequences (0.5–9.5 kb of DNA immediately upstream of the translational start) were fused to the Gal4 coding sequence (Brand and Perrimon, 1993). Flies bearing GR transgenes were mated to transgenic flies that contain either B-galactosidase (lacZ) or green fluorescent protein (GFP) under the control of the Gal4-responsive promoter, UAS. *GR promoter-Gal4* lines were constructed with upstream sequences from 15 chemoreceptor genes and transgene expression was detected for 7 lines (Figure 1). Five of the genes that were expressed by transgene analyses were also detected by in situ hybridization.

A Spatial Map of GR Expression in the Proboscis

Expression of the GR transgenes in the proboscis was initially visualized using the *UAS-lacZ* reporter. The labellum of the proboscis is formed from the fusion of two labial palps, each containing 31–36 bilaterally symmetric

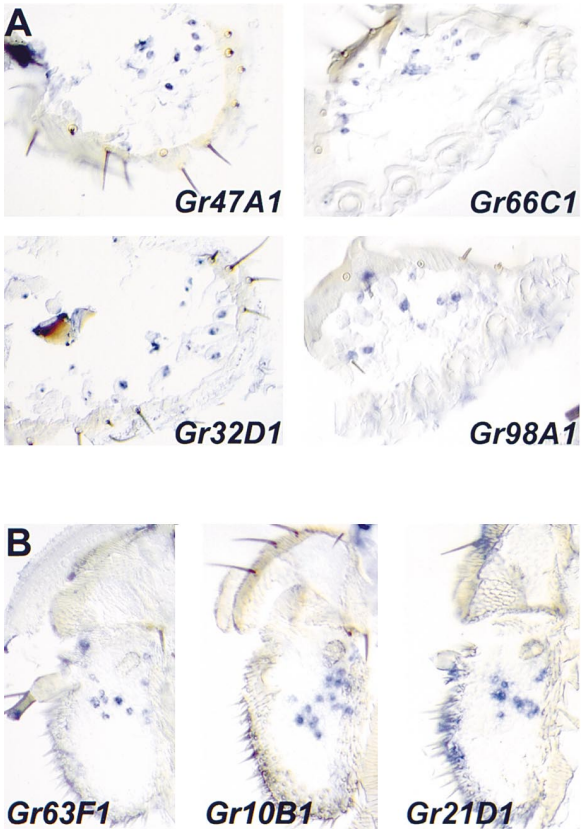

Figure 3. Expression of GR Genes in the Proboscis and Antenna

Digoxigenin-labeled antisense riboprobes derived from GR sequences hybridize to subsets of cells in adult chemosensory organs. (A) Six genes show specific hybridization to gustatory tissues. *Gr47A1*, *Gr66C1*, *Gr32D1*, *Gr98A1*, *Gr28A3*, and *Gr33C1* are expressed in single cells within chemosensory sensilla of the proboscis labellum (data not shown for *Gr28A3* and *Gr33C1*).

(B) Three genes, *Gr63F1*, *Gr10B1*, and *Gr21D1*, are specifically detected in the medial aspect of the third antennal segment, the adult olfactory organ. These expression patterns were maintained in more than 50 heads for each riboprobe. Probes were annealed to sagittal sections (15 μm) of the adult fly head to assay for expression in the proboscis and to frontal sections to examine expression in the antenna.

chemosensory bristles arranged in four rows (Figure 4) (Arora et al., 1987; Ray et al., 1993). The sensilla of the first three columns contains four chemosensory neurons and a single mechanoreceptor cell whereas the sensilla in the most peripheral row are composed of only two chemosensory neurons and one mechanoreceptor (Nayak and Singh, 1983; Ray et al., 1993). Each labial palp therefore contains approximately 120 chemosensory neurons.

The *GR promoter-Gal4* lines were crossed to *UAS-lacZ* flies and the progeny were examined for lacZ expression by staining of whole-mount preparations of the labial palp. Five transgenic lines exhibit lacZ expression in sensory neurons of the labial sensilla (Figure 4). The expression of each transgene is restricted to a single row of chemosensory bristles. *Gr47A1*, for example, is expressed in sensilla innervating the most peripheral row of bristles, whereas *Gr66C1* is expressed in sensilla that occupy a medial column (Figure 4). Flies bearing a *GR promoter-Gal4* gene were also crossed with *UAS-GFP* stocks. The expression of GFP allows greater cellular definition and reveals that each receptor is expressed in a single neuron within a sensillum (Figures 5A and 5B). The pattern of GR gene expression determined by GR promoter transgenes resembles that seen by in situ hybridization. However, we have been unable to directly demonstrate coexpression of the transgene reporter and the endogenous gene by dual label in situ hybridization due to low levels of GR gene expression. Nevertheless, this pattern of expression, in which a receptor is expressed in only one neuron in a sensillum and in one sensillar row, is maintained in over 50 individuals examined for each transgenic line and is also maintained in independent transformed lines for each GR transgene.

Receptor Expression in Other Chemosensory Neurons

Chemosensory bristles reside at multiple anatomic sites in the fly including the taste organs in the mouth, the legs and wings, as well as in the female genitalia (Figure 1) (Stocker, 1994). Three sensory organs reside deep in the mouth: the labral sense organ (comprised of 10 chemosensory neurons) and the ventral and dorsal cibarial organs (each containing six chemosensory neurons) (Stocker and Schorderet, 1981; Nayak and Singh, 1983). The function of these specialized sensory organs is unknown, but their anatomic position and CNS projection pattern suggests that they participate in taste recognition (Stocker and Schorderet, 1981; Nayak and Singh, 1983). Three of the five *GR promoter-Gal4* lines that are expressed in the proboscis are also expressed in the cibarial organs (Figure 5C and Figure 1). One gene, *Gr2B1*, is expressed solely in the labral sense organ and is not detected in the proboscis labellum or in the cibarial organs (Figure 5D).

Chemosensory bristles also decorate both the legs and wings of *Drosophila* with about 40 chemosensory hairs on each structure (Nayak and Singh, 1983; Hartenstein and Posakony, 1989). One gene, *Gr32D1*, expressed both in the proboscis and cibarial organ, is also expressed in two to three neurons in the most distal tarsal segments of all legs (Figure 5E). These results are consistent with the observation that exposure of the legs to tastants results in proboscis extension and feeding behavior (Dethier, 1976). The observation that members of this gene family are expressed in the proboscis and in chemosensory cells of the internal mouth organs and leg suggests that this gene family encodes gustatory receptors.

Expression of Gustatory Receptors in *Drosophila* Larvae

We have also examined the expression of GR transgenes in larvae. The detection of food in larvae is mediated by chemosensors that reside largely in the antennal-maxillary complex, a bilaterally symmetric anterior structure composed of the dorsal and terminal organs (Figure 6A and Figure 1) (Stocker, 1994; Campos-Ortega and Hartenstein, 1997; Heimbeck et al., 1999). Each of the two larval chemosensory organs comprises about 40 neurons. Neurons of the dorsal organ primarily detect

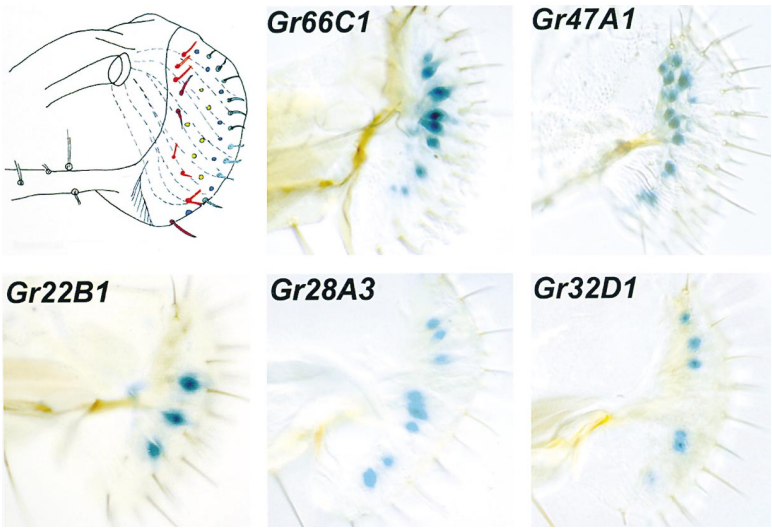

Figure 4. A Spatial Map of GR Expression in the Proboscis

GR promoter-Gal4 transgenes drive expression in subsets of cells in the proboscis. Flies containing *GR promoter-Gal4* and *UAS-lacZ* transgenes were examined for β-galactosidase activity staining on labial palp whole mounts. Each labial palp contains 31–36 chemosensory sensilla, arranged in approximately four rows. In the diagram of a labial palp, different rows of sensilla are depicted in different colors (adapted from Ray et al., 1993). Individual GRs show restricted expression in discrete subsets of chemosensilla. *Gr47A1* is expressed in 9–11 sensilla innervating the most peripheral row of bristles, *Gr32D1* is expressed in 6 sensilla innervating an intermediate row of bristles, *Gr22B1* is expressed in only 3–4 sensilla innervating small bristles, and *Gr66C1* and *Gr28A3* are expressed in 8–10 sensilla innervating small or medium bristles. The spatial patterns for the different receptors are identical in 2–5 independent transformant lines for each promoter construct, and are also fixed among over 20 different individuals within a line.

volatile odorants (Stocker, 1994), whereas the terminal organ is thought to detect both soluble and volatile chemical cues (Heimbeck et al., 1999).

We have asked whether members of the GR family are expressed in larval chemosensory cells by examining the larval progeny that result from crosses between *GR promoter-Gal4* and *UAS-GFP* flies. Examination of live larvae by direct fluorescent microscopy reveals that five of the seven GRs expressed in the adult are expressed in single neurons within the terminal organ (Figure 6 and Figure 1). GR-promoter fusions from each of the 5 genes show bilateral expression of GFP both in the neuronal cell body and in the dendrite. The dendrites extend anteriorly to terminate in the terminal organ, a dome-shaped structure that opens to the environment. In about 5% of the larvae, a second positive cell is observed in each of the lines.

Gr2B1 is expressed in only a single neuron in the labral sense organ of the adult, but is expressed in an extensive population of chemosensory cells in larvae. This gene is expressed in two neurons innervating the dorsal organ, one neuron innervating the terminal organ, and a single bilaterally symmetric neuron innervating the ventral pit in each thoracic hemisegment (Figure 6C). The ventral pit contains a single sensory neuron that may be involved in contact chemosensation. The GR genes are therefore likely to play a significant role in chemosensory recognition in larvae as well as adults.

The Diversity of GR Expression in Individual Neurons

Olfactory neurons of mammals as well as *Drosophila* express a single odorant receptor such that the brain can discriminate odor by determining which neurons have been activated (Ngai et al., 1993; Ressler et al., 1993; Vassar et al., 1993; Chess et al., 1994; Gao et al., 2000; Vosshall et al., 2000). In contrast, nematode olfactory neurons and mammalian gustatory cells coexpress multiple receptor genes (Bargmann and Horvitz, 1991; Troemel et al., 1995; Hoon et al., 1999; Adler et al., 2000). We have therefore examined the diversity of GR gene expression in individual larval taste neurons. In larvae, most receptors are expressed in only one neuron in the terminal organ. Crosses between five *GR promoter-Gal4* lines and flies bearing *UAS-GFP* reveal a single intensely stained neuron within each terminal organ. We then generated 7 lines bearing two different *GR promoter-Gal4* transgenes along with the *UAS-GFP* reporter. In every line bearing two *GR promoter-Gal4* fusions, we observed two GFP positive cells per terminal organ (Figures 6F and 6G). These experiments demonstrate that individual gustatory neurons of larvae express different complements of receptors and are likely to respond to different chemosensory cues.

The Projections of Larval Chemosensory Neurons to the Brain

In other sensory systems, a spatial map of receptor activation in the periphery is maintained in the brain such that the quality of a sensory stimulus may be encoded in spatially defined patterns of neural activity. We have therefore used *GR promoter-Gal4* transgenes to drive the expression of *UAS-nSyb-GFP* to visualize the projections of sensory neurons expressing different GR genes. nSyb-GFP is a C-terminal fusion of green fluorescent protein to neuronal synaptobrevin that selectively labels synaptic vesicles, allowing the visualization of terminal axonal projections (Estes et al., 2000). Whole-mount brain preparations from transgenic flies were examined by immunofluorescence with an antibody against GFP and a monoclonal antibody, nc82, which labels neuropil and identifies the individual glomeruli in the antennal lobe (Laissue et al., 1999). These experiments were initially performed with larvae because of the relative simplicity of the larval brain and the observation that a given GR is expressed in only a small number of gustatory neurons.

The *Drosophila* larval brain is composed of two dorsal

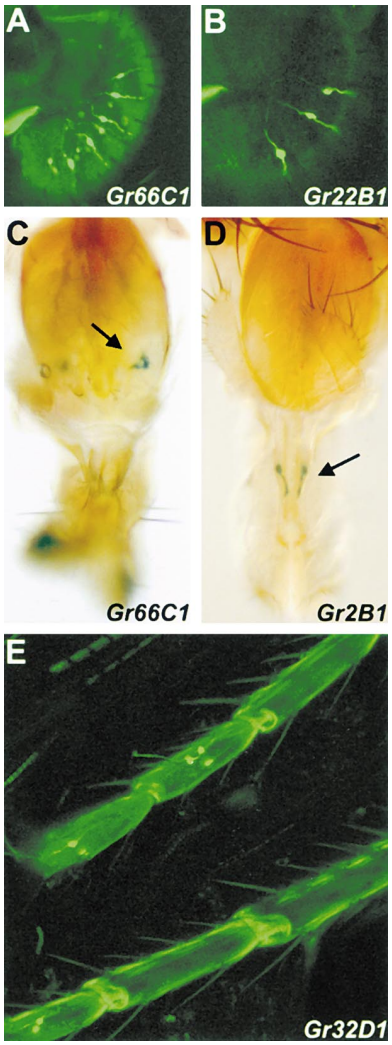

Figure 5. GRs Are Expressed in a Variety of Chemosensory Neurons

(A and B) Expression of GFP allows visualization of dendrites and axons of neurons in the proboscis. GFP was detected in labial palp whole mounts of *GR promoter-Gal4: UAS-GFP* flies by direct fluorescence microscopy. Each transgene drives expression of GFP in a single bipolar neuron within a sensillum. *Gr66C1* is expressed in 9 neurons (6–7 in focus) (A) and *Gr22B1* is expressed in 3 neurons (B) innervating different rows of chemosensory bristles.

(C–E) GRs are expressed in chemosensory sensilla that reside on the internal mouthparts of the proboscis and on tarsal segments of legs. In addition to expression in the proboscis labellum, *Gr32D1*, *Gr66C1*, and *Gr28A3* are also detected in the cibarial organs of the mouth. (C) LacZ expression in a whole-mount proboscis is illustrated for the *Gr66C1-Gal4: UAS-lacZ* line. The arrow denotes the cibarial organ. (D) One transgenic line, *Gr2B1-Gal4*, drives expression exclusively in the labral sense organ of the mouth, and not in the cibarial organs or in the labellum of the proboscis. The arrow denotes the labral sense organ. (E) *Gr32D1* is expressed in the proboscis labellum and in the cibarial organs. In addition, *Gr32D1-Gal4* drives expression of GFP in 2–3 neurons in the fourth and fifth tarsal segments of all legs. Receptor expression was examined by β-galactosidase activity staining of *GR promoter-Gal4: UAS-lacZ* flies (C and D) or by fluorescent visualization of *GR promoter-Gal4: UAS-GFP* flies (E).

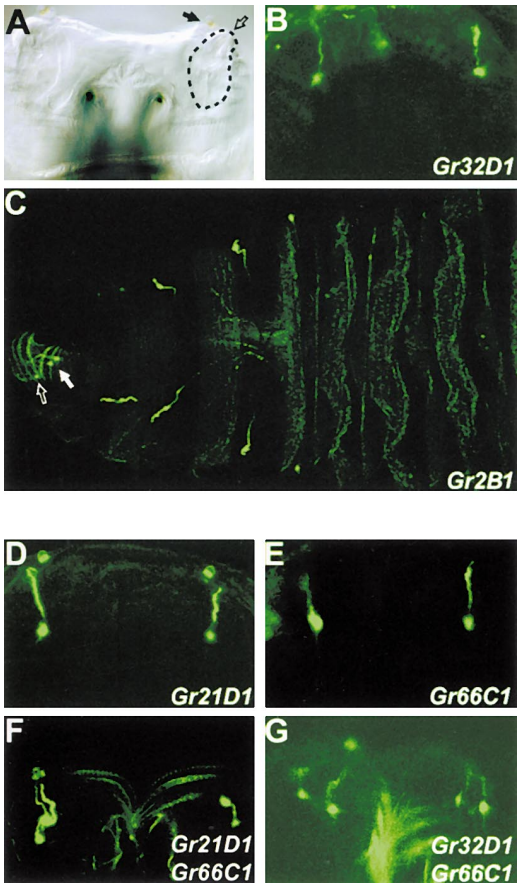

Figure 6. GRs Are Expressed in Larval Chemosensory Neurons

(A) The antenno-maxillary complex of larvae is a bilaterally symmetric structure containing the dorsal organ mediating smell and the terminal organ involved in both taste and smell. Shown is the anterior ventral region of a larva viewed by differential interference contrast. On one-half of the larval head, the sensilla of the terminal organ is outlined with black dotted lines, and the pore of the terminal organ is denoted by an outlined arrow. The dome of the dorsal organ is denoted by a filled arrowhead.

(B–E) *Gr32D1*, *Gr66C1*, and *Gr28A3* are expressed in the proboscis labellum in the adult (Figure 4), and are expressed in a single, bilaterally symmetric neuron in the terminal organ of larvae (B and E, and data not shown). *Gr2B1* is expressed in the labral sense organ of the adult proboscis, and is expressed in two neurons innervating the dorsal organ (filled arrow), one neuron innervating the terminal organ (outlined arrow), and one neuron innervating the ventral pits in each of the thoracic segments in larvae (C). *Gr21D1* is expressed in the adult antenna and in a single larval neuron innervating the terminal organ (D). The dome of the dorsal organ is autofloursecent.

(F and G) Different GRs are expressed in distinct chemosensory neurons. In larvae bearing two *GR promoter-Gal4* fusions and *UAS-GFP*, two GFP positive cells per terminal organ are observed. The different promoter combinations illustrated are *Gr21D1-Gal4* plus *Gr66C1-Gal4* (F) and *Gr32D1-Gal4* plus *Gr66C1-Gal4* (G). The pseudotracheae of the larval mouth shows autoflourescence.

brain hemispheres fused to the ventral hindbrain (Figure 7A). The brain hemispheres and the hindbrain contain an outer shell of neuronal cell bodies and a central fibrous neuropil. Determination of the number of neuroblasts and the number of cell divisions suggest that there are ~10,000–15,000 neurons in the larval brain, a value 10- to 20-fold lower than in the adult (Hartenstein and Campos-Ortega, 1984; Hartenstein et al., 1987; Truman et al., 1993). Chemosensory neurons send axonal projections to two distinct regions of the larval brain, the

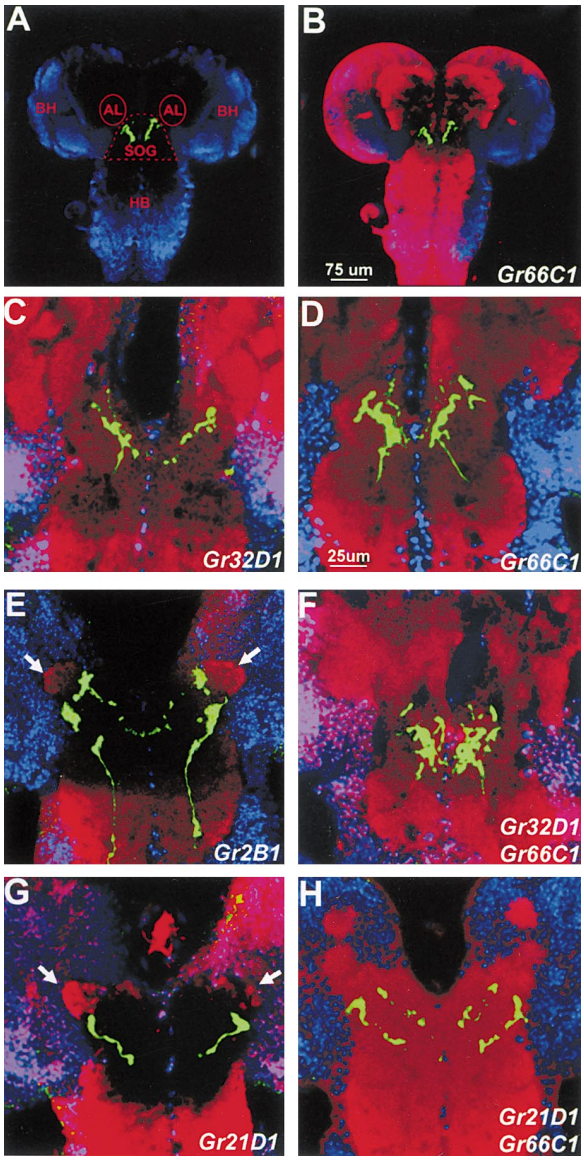

Figure 7. Axonal Projections of Larval Chemosensory Neurons

Projections of neurons bearing different GRs are spatially segregated in the larval brain. In all panels, whole-mount larval brains from *GR promoter-Gal4: UAS-nSyb-GFP* flies were stained with anti-GFP to label axonal termini (green), mAb nc82 to label neuropil (red), and TOTO-3 to counterstain nuclei (blue). Each image represents a composite of 1 μm optical sections through the larval brain, encompassing the terminal projections. Projections extend 5–10 μm in depth for (B), (C), (D), and (G) and 10–20 μm in depth for (E), (F), and (G).

(A) The larval brain is composed of the two dorsal brain hemispheres (BH) and the ventral hindbrain (HB). The subesophageal ganglion (SOG) resides in the hindbrain, at the juncture of the hindbrain with the brain hemispheres. The antennal lobe (AL) is a small neuropil on the anterior edge of the brain hemisphere (denoted with an arrow in [G]).

(B–D) GR-bearing neurons project to discrete locations in the larval brain. *Gr32D1* is expressed in the proboscis in the adult and in one neuron in the terminal organ in larvae. In *Gr32D1-Gal4:UAS-nSyb-GFP* larval brains, a single terminal arborization is observed in the SOG (C). A similar pattern is observed for neurons expressing *Gr66C1*, a gene expressed in the adult proboscis and in a single neuron in the terminal organ and two in the mouth of larvae (B and D). (D) is a higher magnification (3×) of (B).

antennal lobe and the subesophageal ganglion (SOG) (Stocker, 1994; Heimbeck, et al., 1999). The antennal lobe is a small neuropil in the medial aspect of the deuterocerebrum within each brain hemisphere. The antennal lobe receives input from neurons of the dorsal and terminal organ and presumably participates in processing olfactory information. The SOG resides in the most anterior aspect of the hindbrain, at the juncture of the hindbrain with the brain hemispheres. The SOG receives input from the terminal organ and mouthparts and is thought to process gustatory information. Whereas the projections of populations of chemosensory cells have been traced to the antennal lobe and the SOG, the patterns of axonal projections for individual sensory cells have not been described. Moreover, the connections of chemosensory axons with second order brain neurons is unknown for the larval brain.

Gr32D1-Gal4 is expressed in multiple neurons in the proboscis of the adult, but it is expressed in only a single neuron in the terminal organ of larvae (Figure 6B). In larvae containing the *Gr32D1-Gal4* and *UAS-nSyb-GFP* transgenes, it is possible to visualize the axons of *Gr32D1*-expressing cells as they course posteriorly to enter the subesophageal ganglion (data not shown). The axons then turn dorsally and intensely stained fibers terminate in the medial aspect of the SOG (Figure 7C). A similar pattern is observed for neurons expressing *Gr66C1* (Figure 7B,D), a gene expressed in the proboscis in the adult and in a single neuron in the terminal organ and two in the mouth of larvae (Figure 6E). However, the terminal arbors of *Gr66C1* neurons are consistently thicker than that observed for *Gr32D1*, perhaps reflecting the increased number of *Gr66C1*-bearing neurons. The reporter nSyb-GFP stains axons only weakly but shows intense staining of what is likely to be terminal projections of sensory neurons that synapse on second order neurons in the neuropil of the SOG. This terminal arbor extends for about 40 μm and reveals a looser, more distributed pattern that the tight neuropil of the olfactory glomerulus. The position and pattern of the terminal projections from individual chemosensory cells in the terminal organ show bilateral symmetry and are maintained in over 20 larvae examined.

A more complex pattern of projections is observed for *Gr2B1*, a gene expressed in one neuron in the terminal

(E) Projections of gustatory neurons from different body regions are spatially segregated in the fly brain. *Gr2B1* is expressed in two neurons innervating the dorsal organ, one neuron innervating the terminal organ, and one neuron innervating the ventral pits. Axons from ventral pit neurons enter the hindbrain via thoracic nerves and terminate in the antennal lobe (arrows), in a location that is distinct from the termini of other *Gr2B1*-bearing neurons.

(F) Segregation is less apparent in the terminal projections of two different taste receptors. Larvae that contain *Gr66C1-Gal4* and *Gr32D1-Gal4* along with *UAS-nSyb-GFP* reveal two partially overlapping projection patterns.

(G and H) Distinct projection patterns are observed for the two different chemosensory modalities, taste and smell. *Gr21D1* is expressed in the adult antenna and in a single neuron in the terminal organ of larvae. *Gr21D1* axons enter the antennal lobe (arrows) (G). In larvae that contain *Gr21D1-Gal4* and *Gr66C1-Gal4* along with *UAS-nSyb-GFP*, two discrete termini are apparent, one entering the SOG, and a second entering the antennal lobe (H).

organ, two in the dorsal organ, and a single bilaterally symmetric neuron in each thoracic hemisegment (Figure 6C). One set of fibers appears to terminate in the antennal lobe (Figure 7E). A second more posterior set of fibers can be traced from the thorax into the hindbrain, with fibers terminating posterior to the antennal lobe (Figure 7E). This pattern of projections is of interest for it implies that neurons in different locations in larvae that express the same receptor project to discrete locations in the larval brain, suggesting the possibility that the same chemosensory stimulus can elicit distinct behavioral outputs.

We have attempted to determine whether neurons in the terminal organ that express different GRs project to discrete loci within the SOG. We therefore generated larvae that express two promoter fusions, *Gr66C1-Gal4* and *Gr32D1-Gal4*, along with a *UAS-nSyb-GFP* transgene. The projections in these flies are broadened, suggesting that these sets of neurons terminate in overlapping but nonidentical regions of the SOG (Figure 7F). More definitive data to support the existence of a topographic map of taste quality will require two-color labeling of the different fibers to discern whether the projections from neurons expressing different GRs are spatially segregated in the SOG.

Are GRs also Odorant Receptors?

A large family of presumed olfactory receptor genes in *Drosophila* (the DOR genes) has been identified that is distinct from the GR gene family (Clyne et al., 1999; Gao and Chess, 1999; Vosshall et al., 1999, 2000). Expression of the DOR genes is only observed in olfactory sensory neurons within the antenna and maxillary palp, where a given DOR gene is expressed in a spatially invariant subpopulation of cells (Clyne et al., 1999; Gao and Chess, 1999; Vosshall et al., 1999, 2000). In situ hybridization experiments demonstrate that three members of the GR gene family are also expressed in subpopulations of antennal neurons (Figure 3B). These observations suggest either that the odorant receptors in *Drosophila* are encoded by at least two different gene families or that previously unidentified taste responsive neurons reside within the antenna.

In *Drosophila*, olfactory information is transmitted to the antennal lobe, whereas gustatory neurons in the proboscis and mouth relay sensory information to the subesophageal ganglion (Stocker, 1994). We therefore examined the spatial pattern of expression of GRs in the antenna and the pattern of projections of their sensory axons in the brain. In situ hybridization with the three GR genes reveals that each gene is expressed in about 20–30 cells/gene in the antenna (Figure 3B). Similar results are obtained in a cross between an antennal *GR promoter-Gal4* line, *Gr21D1-Gal4*, and *UAS-LacZ* or *UAS-GFP* lines (Figures 8A and 8B). This pattern of GR gene expression is maintained in over 50 antennae that we have analyzed. The GR-positive cells occupy regions of the antenna that do not express identified members of the DOR gene family (Vosshall et al., 2000), suggesting that there is spatial seggregation of these two receptor families.

We next asked whether antennal neurons expressing a GR gene project to the antennal lobe in a manner

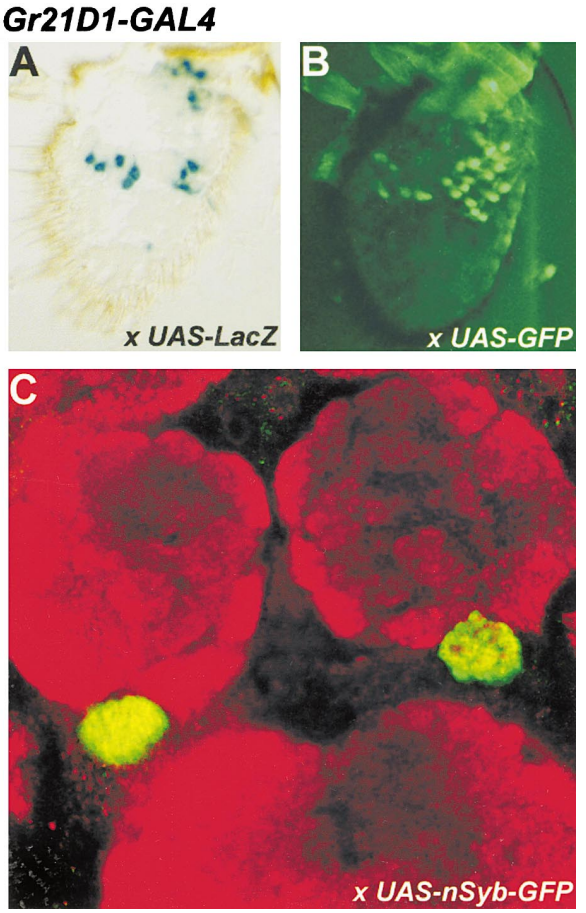

Figure 8. A Subset of GRs Encode Olfactory Receptors

GR-bearing neurons in the antenna project to discrete glomeruli in the antennal lobe. Adult transgenic flies in which *Gr21D1 promoter-Gal4* drives expression of *UAS-lacZ* (A) or *UAS-GFP* (B) show specific labeling in subsets of cells in the medial aspect of the antenna. This expression pattern resembles that determined for the endogenous gene. LacZ expression was detected in 15 μm frontal sections of the antenna (A); GFP expression was examined in whole antennae (B). (C) *Gr21D1*-bearing neurons project to a single bilaterally symmetric glomerulus on the ventral-most region of the antennal lobe. Whole-mount brains of *Gr21D1-Gal4: UAS-nSyb-GFP* flies were examined by fluorescent immunohistochemistry, with anti-GFP to visualize axonal termini of *Gr21D1*-bearing neurons (green), mAb nc82 to label brain neuropil (red), and TOTO-3 to counterstain nuclei (blue). *Gr21D1*-bearing neurons send projections to the V glomerus in the antennal lobe (Stocker et al., 1990; Laissue et al., 1999) and do not project to the subesophageal ganglion (located in the bottom part of C).

analogous to that observed for cells expressing the DOR genes. Transgenic flies expressing a *Gr21D1 promoter-Gal4* fusion were crossed to animals bearing the *UAS-nSyb-GFP* transgene. These studies demonstrate that neurons expressing the *Gr21D1* transgene project to a single, bilaterally symmetric glomerulus in the ventral-most region of the antennal lobe (the V glomerulus) (Figure 8C) (Stocker et al., 1990; Laissue et al., 1999) and do not project to the SOG. Thus, as in the case of the family of DOR genes (Gao et al., 2000; Vosshall et al., 2000), neurons expressing the same receptor project to a single spatially invariant glomerulus.

Gr21D1 is also expressed in one cell of the terminal organ of larvae (Figure 6D). We have therefore traced the projections of Gr21D1-bearing neurons to the larval brain. *Gr21D1* axons enter the larval brain and terminate in the antennal lobe rather than the SOG (Figure 7G). The segregation of projections from presumed olfactory and gustatory neurons is apparent in larvae that contain *Gr21D1-Gal4* and *Gr66C1-Gal4* along with *UAS-nSyb-GFP*. In these transgenic flies, two distinct sets of termini are observed, one entering the SOG, and a second entering the antennal lobe (Figure 7H).

Thus, a member of the GR gene family is expressed in sensory neurons of the antenna and the terminal organ of larvae, and GR-bearing neurons project to the antennal lobe. These data suggest that at least two independent gene families, the DORs and the GRs, recognize olfactory information. The GR gene family is therefore likely to encode both olfactory and gustatory receptors, and neurons expressing distinct classes of GR receptors project to different regions of the fly brain.

Discussion

A Family of Gustatory and Olfactory Receptors

Specialized sense organs have evolved to recognize chemosensory information in the environment. The antennae in insects, the amphid in nematodes, and the nose of mammals allow the recognition of a vast repertoire of volatile odorants often over long distances. Taste organs have evolved to accommodate a distinct function, the recognition of soluble chemical cues over shorter distances. In vertebrates, taste is largely restricted to the tongue and palate, whereas in insects, gustatory neurons are more broadly distributed along the body plan and reside not only in the proboscis and pharynx but also on the wings, legs, and female genitalia. Anatomic and functional segregation of the gustatory and olfactory systems is not only apparent in the peripheral receptor field but in the projections to the brain. In the fly, for example, olfactory neurons project to the antennal lobe, whereas most gustatory neurons ultimately synapse within the subesophageal ganglion. This separation is also observed in vertebrates where taste and smell are accommodated by distinct sense organs and conveyed to different brain regions by different cranial nerves. Thus, a common sensory function, the recognition of chemical cues, has undergone specialization to allow for the recognition of at least two distinct categories of chemosensory information, each eliciting distinct behavioral responses.

In this study, we have characterized the patterns of expression of a large family of genes in *Drosophila* that are likely to encode both odorant and gustatory receptors. A family of candidate taste receptors was identified by searching the *Drosophila* genome with an algorithm designed to detect genes encoding seven transmembrane domain proteins (Clyne et al., 2000). We have extended this analysis through a search of the complete euchromatic genome of *Drosophila* and identify 56 genes within the family. All of the GR genes contain a signature motif in the carboxyl terminus that is also present within some members of the DOR gene family, suggesting that these two families share a common origin.

The GR family of proteins was tentatively identified as gustatory receptors solely on the basis of PCR analysis of proboscis RNA (Clyne et al., 2000). We have performed both in situ hybridization and transgene experiments that demonstrate that members of this gene family are expressed in the antennae, proboscis, pharynx, leg, and larval chemosensory organs. Thus, a single gene family encodes chemosensory receptors containing both olfactory and gustatory receptors. We have generated flies bearing GR promoter transgenes from 15 GR genes. Expression is observed in seven lines and is restricted to chemosensory cells. No expression is detected in other neurons or in nonneuronal cells. These data suggest that the expression of this family is limited to gustatory and olfactory neurons, and that the inability to observe expression in eight transgenic lines perhaps reflects the structural inadequacy of the promoters.

A common gene family encoding both olfactory and taste receptors is not present in vertebrates, where the main olfactory epithelium, the vomeronasal organ, and the tongue express receptors encoded by independent gene families (Buck and Axel, 1991; Dulac and Axel, 1995; Herrada and Dulac, 1997; Matsunami and Buck, 1997; Ryba and Tirindelli, 1997; Hoon et al., 1999; Adler et al., 2000; Matsunami et al., 2000). Our observations are more reminiscent of the chemosensory receptor families in *C. elegans* that encode odorant receptors expressed in the amphid neurons and taste receptors in sensory neurons responsive to soluble chemicals (Troemel et al., 1995, 1999; C. I. Bargmann, personal communication).

Patterns of GR Gene Expression and Taste Modalities

The size of the family of candidate taste receptors and the pattern of expression in chemosensory cells provides insight into the problem of the recognition and discrimination of gustatory cues. On average, each GR is expressed in 5% of the cells in the proboscis labellum, suggesting that the proboscis alone will contain at least 20 distinct taste cells expressing about 20 different GR receptors. Moreover, a given receptor is expressed in one of the four rows of sensilla such that the sensilla in different rows are likely to be functionally distinct. Electrophysiologic studies have suggested that all sensilla are identical and contain four distinct cells, each responsive to a different category of taste (Dethier, 1976; Rodriques and Siddiqi, 1978; Fujishiro et al., 1984). Our data are not consistent with these conclusions and argue that different rows of sensilla are likely to contain cells with different taste specificities.

At present, we do not know the nature of the ligands recognized by these GR receptors, nor do we know whether all taste modalities are recognized by this gene family. In mammals, gustatory cues have classically been grouped into five categories: sweet, bitter, salt, sour, and glutamate (*umami*) (Kinnamon and Margolskee, 1996; Lindemann, 1996; Gilbertson et al., 2000). Sugar and bitter taste are likely to be mediated by G protein–coupled receptors since these modalities require the function of a taste cell–specific G_a subunit, gustducin (McLaughlin et al., 1992; Wong et al., 1996). Recently, two novel families of seven transmembrane

proteins (the T1Rs and T2Rs) were shown to be selectively expressed in taste cells in the tongue and palate epithelium (Hoon et al., 1999; Adler et al., 2000; Matsunami et al., 2000). Genetic experiments implicated members of the T2R family in the recognition of bitter tastants (Adler et al., 2000; Matsunami et al., 2000) and functional studies directly demonstrated that members of the T2R family serve as gustducin-linked bitter taste receptors. (Chandrashekar et al., 2000). A large number of candidate genes have been suggested to encode receptors for other taste modalities, but in only a few instances have functional data and expression patterns supported these assumptions. In mammals, an amiloride-sensitive sodium channel has been suggested as the salt receptor (Heck et al., 1984), a degenerin homolog (MDEG-1) (Ugawa et al., 1998) and a potassium channel (Kinnamon et al., 1988) as sour or pH sensors, and a rare splice form of the metabotropic glutamate receptor as the *umami* sensor (Chaudhari et al., 2000). In *Drosophila*, genetic analysis of mutant flies defective in the recognition of the sugar trehalose has led to the identification of a transmembrane receptor distinct from GRs that reduces the sensitivity to one class of sugars (Ishimoto et al., 2000). The interpretation of the role of these putative taste receptors in taste perception awaits a more definitive association between tastant and gene product.

The Logic of Taste Discrimination

How does the fly discriminate among multiple tastants? One mechanism of chemosensory discrimination, thought to operate in the olfactory system of insects and vertebrates, requires that individual sensory neurons express only one of multiple receptor genes (Buck and Axel, 1991; Ngai et al., 1993; Ressler et al., 1993; Vassar et al., 1993; Chess et al., 1994; Clyne et al., 1999; Gao and Chess, 1999; Vosshall et al., 1999). Neurons expressing a given receptor project axons that converge on topographically invariant glomeruli such that different odors elicit different patterns of spatial activity in the brain (Ressler et al., 1994; Vassar et al., 1994; Mombaerts et al., 1996; Wang et al., 1998; Gao et al., 2000; Vosshall et al., 2000). The nematode *C. elegans* uses a rather different logic, in which a given sensory neuron dictates a specific behavior but expresses multiple receptors (Bargmann and Horvitz, 1991; Troemel et al., 1995, 1997). In the worm olfactory system, discrimination is necessarily more limited and exploits mechanisms to diversify the limited number of sensory cells (Colbert and Bargmann, 1995; Troemel et al., 1999; L'Etoile and Bargmann, 2000). A similar logic has been suggested for mammalian taste. Several members of the T2R family of about 50 receptor genes, each thought to encode bitter sensors, are coexpressed in sensory cells within the tongue (Adler et al., 2000). This organization allows the organism to recognize a diverse repertoire of aversive tastants but limits the ability to discriminate among them.

What can we discern about the logic of taste discrimination from the pattern of GR gene expression in *Drosophila*? First, the number of GR genes, 56, approximates the number of DOR genes, suggesting that the fly recognizes diverse repertoires of both soluble and volatile chemical cues. Moreover, our data argue that individual sensory neurons differ with respect to receptor gene expression and are therefore functionally distinct. Experiments with *Drosophila* larvae demonstrate that a given GR gene is expressed in one neuron in the larval terminal organ. Strains bearing two different GR-promoter fusions reveal twice the number of expressing cells. Similar results are obtained in adult gustatory organs (data not shown). More definitive experiments to examine the diversity of receptor expression in a single neuron, employed successfully in the olfactory system, have been difficult since the levels of GR RNA are 10- to 20-fold lower than odorant receptor RNA levels. Nevertheless, our experiments demonstrate that different gustatory neurons express different complements of GR genes and at the extreme are consistent with a model in which gustatory neurons express only a single receptor gene.

How does the brain discern which of the different gustatory neurons is activated by a given tastant? As in other sensory systems, it is possible that axons from different taste neurons segregate to spatially distinct loci in the subesophageal ganglion. In such a model, taste quality would be represented by different spatial patterns of activity in the brain. Preliminary experiments suggest that neurons expressing different GRs project to spatially segregated loci within the brain. Clear segregation of axonal termini is observed for presumed taste neurons that project to the SOG and olfactory neurons that project to the antennal lobe. A second interesting pattern of projections is observed for the presumed gustatory receptor *Gr2B1*, a gene expressed in neurons in the terminal and dorsal organs and in a single neuron in the ventral pit present bilaterally in each thoracic segment. At least two spatially segregated targets are observed for these neurons in the larval brain: one set of fibers terminates in glomeruli of the antennal lobe and a second set of fibers (from the ventral pits) project to the SOG. Thus, neurons expressing the same receptor in different chemosensory organs project to distinct brain regions. In this manner, the same chemosensory cue could elicit distinct behaviors depending upon the cell it activates. Sucrose, for example, could elicit chemoattraction upon exposure to the thoracic neurons and eating behavior upon activation of neurons in the terminal and dorsal organ.

These data establish that presumed olfactory neurons and gustatory neurons expressing GR genes project to different regions of the larval brain. Taste neurons expressing different GR genes, however, all project to the SOG. The current data do not permit us to discern whether axons from neurons expressing different GR genes project to spatially distinct loci within the SOG. The axon termini of gustatory neurons terminate in more diffuse, elongated structures than the tightly compacted glomeruli formed by olfactory sensory axons, rendering it difficult at present to discern a topographic map of gustatory projections in the larval brain.

Sensory Perception in Larvae

Insects provide an attractive model system for the study of chemosensory perception because they exhibit sophisticated taste and olfactory driven behaviors that are controlled by a chemosensory system that is anatomi-

cally and genetically simpler than vertebrates (Nassif et al., 1998). *Drosophila* larvae afford a particularly facile organism because much of their behavior surrounds eating. Gustatory neurons in the terminal organ and along the body plan, together with olfactory sensory cells in the dorsal and terminal organs, combine to identify food sources and elicit eating behaviors (Stocker, 1994).

Members of the *Drosophila* odorant receptor (DOR) family are expressed in the adult olfactory system but cannot be detected in larval chemosensory organs (L. B. Vosshall, personal communication). We have demonstrated that the GR genes are expressed in larval olfactory and gustatory neurons and may encode the entire repertoire of larval chemosensory receptors. The simplicity of the *Drosophila* larvae, coupled with the ease of behavioral studies, suggests that it may be possible to relate the recognition of chemosensory information to specific behavioral responses and ultimately to associate changes in behavior with modifications in specific connections.

Experimental Procedures

Experimental Animals

Drosophila stocks were reared on standard cornmeal-agar-molasses medium at 25°C. *Oregon R* strains were used for in situ hybridization experiments, and *yw* or *W1118* strains were used for transgene injections. P element–mediated germline transformations and all subsequent fly manipulations were performed using standard techniques (Rubin et al., 1985). In some cases, transgenic constructs were injected as mixtures of two constructs, and progeny of individual transformants were analyzed by polymerase chain reaction (PCR) to determine their genotype. All analyses were performed on two to five independent transgenic lines for each construct.

Identification of Additional GR genes

A search for novel seven transmembrane domain receptors was performed among 5660 predicted *Drosophila* proteins of "unknown function" (Adams et al., 2000) using a transmembrane prediction program (TopPred) (von Heijne, 1992). We selected 310 *Drosophila* genes for in situ hybridization analysis, 20 of which were novel members of the GR gene family previously described (Clyne et al., 2000). Additional members of the GR gene family were identified using BLAST (Altschul et al., 1990) and hidden Markov model (Eddy, 1998) searches of *Drosophila* genome databases with existing GR members as templates. The predicted protein sequences of the 23 novel GR genes and the previously identified genes are available at the URL: http://cpmcnet.columbia.edu/dept/neurobeh/axel/gr.html. GRs were grouped into subfamilies by BLASTP comparisons (Altschul, et al., 1997) with an e value cutoff of 10^{-5}. Sequence relationships between the GR gene family and the DOR genes were analyzed with HMMs (Eddy, 1998), CLUSTAL alignments and neighbor joining trees (Saitou and Nei, 1987; Higgins and Sharp, 1988), and NxN BLASTP (Rubin et al., 2000) comparisons.

Five GR genes were isolated by PCR from proboscis cDNA using primers corresponding to the extent of the predicted coding region. Proboscis cDNA was obtained from one thousand microdissected probosces, using Dynal mRNA Direct (610.11) and Perkin-Elmer GeneAmp (N808–0017) kits. PCR products were cloned into pGEM-T (Promega) and sequenced in their entirety, using ABI 310 or 377 sequencing systems. An antennal cDNA library (kindly provided by Dr. Leslie Vosshall) was screened (3×10^6 inserts) with PCR probes for *Gr63F1*, *Gr10B1*, and *Gr21D1*, and 6 independent cDNAS of Gr63F1 were isolated and sequenced. Sequences of *Gr43C1*, *Gr47A1*, *Gr58A3*, and *Gr59E1* matched the previously reported sequences (Clyne et al., 2000), and sequences of *Gr10B1* and *Gr63F1* are included in the list above.

In Situ Hybridization

RNA in situ hybridization was performed as previously described (Vosshall et al., 1999). Riboprobes for the 56 GR genes were generated from PCR products corresponding to predicted exons and ranged from 300–800 bp in length. Newly eclosed flies were used for in situ hybridization experiments because hybridization signals were found to be more robust at this stage.

Construction of GR Transgenes

Generation of 15 *GR promoter-Gal4* transgenes was performed as previously described (Vosshall et al., 2000). Briefly, sequences immediately adjacent to the predicted ATG initiation codon and a variable distance upstream were isolated by long-range PCR with genomic DNA as template, and upstream elements were cloned into a modified CaSpeR-AUG-Gal4 vector (Vosshall et al., 2000). Regulatory element lengths for each of the GR transgenes are as follows: *Gr2B1*, 2.240 kB; *G21D1*, 9.323 kB; *Gr22B1*, 8.249 kB; *Gr28A3*, 4.245 kB; *Gr32D1*, 3.776 kB; *Gr47A1*, 7.321 kB; *Gr66C1*, 3.153 kB and *Gr5A1*, 5.156 kB; *Gr10B1*, 0.656 kB; *Gr33C1*, 3.315 kB; *Gr39D2A*, 8.227 kB; *Gr59E2*, 2.586 kB; *Gr77E1*, 9.502 kB; *Gr93F1*, 9.368 kB; and *Gr98A1*, 1.086 kB. The first 7 transgenes drive reporter expression in chemosensory tissues; the remaining 8 transgenes were not detectably expressed in adults or larvae.

Visualization of lacZ, GFP, and nSyb-GFP Reporters

GR promoter-Gal4 lines were crossed to *UAS-LacZ* stocks, and whole-mount heads of progeny were examined for β-galactosidase activity, following existing staining procedures (Wang et al., 1998). To enhance visualization of sensilla in the proboscis labellum, probosces were bisected and pseudotracheae were removed by microdissection. Images were recorded using a Nikon SPOT-RT digital microscope system equipped with differential interference contrast.

Progeny resulting from crosses of *GR promoter-Gal4* to *UAS-GFP* were examined for GFP expression by direct flourescence microscopy. Adult organs and live larvae were mounted in glycerol using small coverslips as spacers and GFP flourescence was recorded with a BioRad 1024 confocal microscope.

To visualize axonal projections of GR-bearing neurons, *GR promoter-Gal4* flies were mated with *UAS-nSyb-GFP*, and brains of F1 progeny were examined by flourescent immunohistochemistry. Larval brains were dissected and antibody staining was carried out as described in (Vosshall et al., 2000). Expression of nSyb-GFP was visualized with a rabbit anti-GFP antibody (Molecular Probes) and a goat anti-rabbit secondary antibody coupled to Alexa Fluor 488 (Molecular Probes). The nc82 monoclonal antibody (Laissue et al., 1999) was used to label brain neuropil and was visualized with goat anti-mouse IgG coupled to CY3 (Jackson ImmunoResearch). Cell nuclei were counterstained with TOTO-3 (Molecular Probes). Images were analyzed with a BioRad 1024 confocal microscope.

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