Spatially restricted expression of candidate taste receptors in the *Drosophila* gustatory system

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Background: Taste is an important sensory modality in most animals. In *Drosophila*, taste is perceived by gustatory neurons located in sensilla distributed on several different appendages throughout the body of the animal. Here we show that the gustatory receptors are encoded by a family of at least 54 genes (*Gr* genes), most of which are expressed exclusively in a small subset of taste sensilla located in narrowly defined regions of the fly's body.

Results: BLAST searches with the predicted amino acid sequences of 6 7-transmembrane-receptor genes of unknown function and 20 previously identified, putative gustatory receptor genes led to the identification of a large gene family comprising at least 54 genes. We investigated the expression of eight genes by using a Gal4 reporter gene assay and found that five of them were expressed in the gustatory system of the fly. Four genes were expressed in 1%–4% of taste sensilla, located in well-defined regions of the proboscis, the legs, or both. The fifth gene was expressed in about 20% of taste sensilla in all major gustatory organs, including the taste bristles on the anterior wing margin. Axon-tracing experiments demonstrated that neurons expressing a given *Gr* gene project their axons to a spatially restricted domain of the subesophageal ganglion in the fly brain.

Conclusions: Our findings suggest that each taste sensillum represents a discrete, functional unit expressing at least one *Gr* receptor and that most *Gr* genes are expressed in spatially restricted domains of the gustatory system. These observations imply the potential for high taste discrimination of the *Drosophila* brain.

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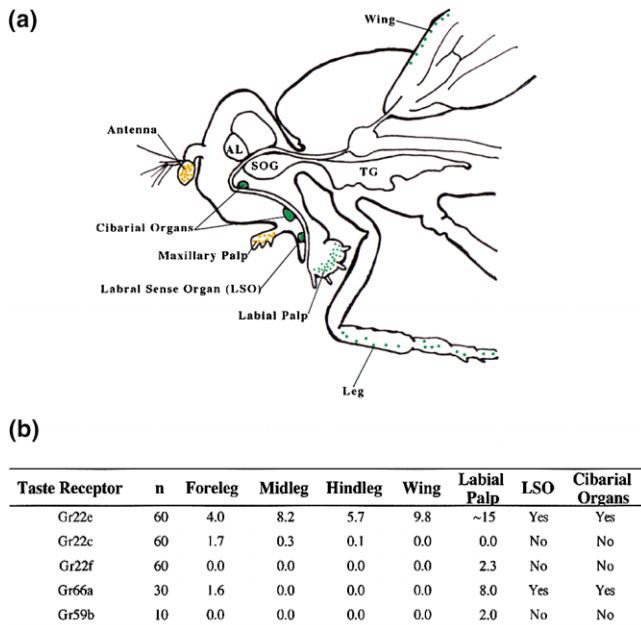
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Background

Chemoreception, the recognition of volatile and soluble chemicals, plays an important role in animal behavior such as feeding, avoidance of harmful substances, and recognition of mating partners, competitors, and predators. The complexity of behaviors elicited by chemosensory cues is well represented at the neuroanatomical and molecular-genetic levels in both vertebrates and invertebrates. For example, brain regions that receive and process chemosensory information are often larger than those processing other sensory information. Moreover, in both mammals and *C. elegans*, these receptors are encoded by very large and distinct families of seven-transmembrane receptors that are dedicated to olfactory, gustatory, or pheromonal sensory perception [1–11]

As in most vertebrates, chemoreception in *Drosophila* can be divided into two distinct modalities—smell (olfaction), the recognition of volatile molecules, and taste (gustation), the recognition of soluble molecules [12]. In *Drosophila*, olfactory sensilla are exclusively located on two distinct appendages of the head, the third segment of the antenna and the maxillary palp (Figure 1a). Each olfactory neuron

in these sense organs probably expresses one or a few olfactory receptor (*Or*) genes [13–15]. The *Or* genes form a large gene family encoding seven-transmembrane-receptor proteins, but these proteins share no similarity to the odorant receptors of vertebrates or chemoreceptors of *C. elegans*. The *Drosophila* taste sensilla are located on several different appendages and sense water and many water-soluble ligands, such as different sugars, amino acids, and salts [12, 16, 17]. The main taste organs are the labial palps, which are located at the distal end of the proboscis, and the labral and cibarial sense organs, which are located inside the pharynx (Figure 1a). Neurons in these sensilla project axons to the subesophageal ganglion (SOG) of the brain, where the processing of gustatory information first occurs. Additional chemosensory neurons are located in taste bristles on the tibiae and tarsi of all legs and on the anterior wing margin [12, 16]. In males, the tarsi of the forelegs have about 25% more sensilla, which might play a role during male courtship [16, 18]. Likewise, females contain a small number of poorly characterized taste sensilla on the vaginal plate of the abdomen, and these might have a function in oviposition site selection [19, 20].

Figure 1

(a)

(b)

Taste Receptor	n	Foreleg	Midleg	Hindleg	Wing	Labial Palp	LSO	Cibarial Organs
Gr22e	60	4.0	8.2	5.7	9.8	~15	Yes	Yes
Gr22c	60	1.7	0.3	0.1	0.0	0.0	No	No
Gr22f	60	0.0	0.0	0.0	0.0	2.3	No	No
Gr66a	30	1.6	0.0	0.0	0.0	8.0	Yes	Yes
Gr59b	10	0.0	0.0	0.0	0.0	2.0	No	No

Distribution of gustatory sensilla and summary of *Gr* gene expression. **(a)** Representation of chemosensory sensilla on the different appendages. The major gustatory organs are the paired labial palps on the distal end of the proboscis; each palp contains about 30 chemosensory sensilla. Additional chemosensory sensilla are located within the pharynx and comprise the labral and cibarial sense organs. Chemosensory sensilla are also found on all the legs as well as on the anterior wing margin. Not shown are a few chemosensory sensilla located on the female genitalia. The image is modified from Stocker [12]. Gustatory sensilla are shown in green; olfactory sensilla are shown in orange. "SOG" indicates the subesophageal ganglion, "AL" indicates the antennal lobe, and "TG" indicates the thoracic ganglia. **(b)** Spatial expression of different *Gr* genes in the adult gustatory system. The average number of LacZ-positive sensilla found in flies containing different Gal4-drivers is shown. Flies were hemizygous for both the indicated driver and the *UAS→lacZ* reporter. Numbers are per leg, per wing, and per palp. Total numbers of sensilla are approximately 37 female/50 male per foreleg, approximately 31 per mid leg, approximately 32 per hind leg, approximately 40 per anterior wing margin, and approximately 30 per labial palp. The total numbers on one side are approximately 170 and approximately 183 for females versus males, respectively [12]. N indicates the number of animals analyzed. The flies derived from two or three different lines of each construct, and the numbers were pooled. No differences in numbers of LacZ-positive cells among different lines of a construct were noticed. Some drivers produced stronger staining than others.

Most taste sensilla contain two to four chemosensory neurons and a single mechanosensory neuron [12, 21]. Electrophysiological and genetic studies on labial taste bristles in *Phormia* and *Drosophila* have shown that there are four different classes of chemosensory neurons [17]. The S (sugar) neuron is vigorously stimulated with a variety of different sugars and amino acids, the W (water) neuron responds to water, the L1 neuron (classical salt neuron) is most effectively stimulated by monovalent alkali halides, and the L2 neuron responds to anions [22–25]. Several

studies have indicated that at least S neurons show distinct specificities depending on the type of stimuli [22, 24].

Although gustatory neurons in *Drosophila* are well characterized, little is known about the molecular structure of the receptors they express. The large number of structurally distinct substrates recognized by flies argues for the presence of a large taste receptor gene repertoire. A gene, *tre1*, encoding a G protein-coupled receptor was identified recently by Ishimoto et al. [26]. These authors showed that a *Drosophila* strain with low trehalose sensitivity contained a mutation within *tre1* and that *tre1* mRNA was expressed in gustatory neurons of the labial palps. Homology searches have identified two additional genes closely related to *tre1*, but it is not known whether and where these genes are expressed. Regardless of whether these *tre1*-related genes encode functional taste receptors, their number is far too low to accommodate all soluble substrates flies can recognize. Clyne et al. recently reported the sequences of about twenty putative gustatory receptor genes encoding a new class of seven-transmembrane-receptor proteins [27]. The expression of these genes in gustatory sensilla was demonstrated by RT-PCR with RNA isolated from different appendages bearing taste sensory hairs. However, cellular expression and spatial distribution of these transcripts within these appendages has not been addressed.

One of our interests has been to obtain a more complete understanding of the *Drosophila* gene repertoire dedicated to chemosensory perception. The recent completion of the *Drosophila* genome sequence offers an opportunity to find these receptor genes [28]. Here we report that the genes described by Clyne et al. are members of a large gene family encoding at least 54 putative gustatory receptors (GRs) in *Drosophila* [27]. The members of this gene family share 15%–25% overall sequence similarity with each other. They encode seven transmembrane receptor proteins of 360–530 amino acids. We present an expression analysis of eight *Gr* genes by using a transgene reporter assay and show that they are expressed in taste sensilla of the gustatory sense organs in the adult fly (labial palp, labral, and cibarial sense organs, legs, and wings). Four *Gr* genes are expressed in narrowly defined sensory clusters in only some of these sense organs, while a fifth one is uniformly expressed in about 20% of all taste sensilla, as well as in the olfactory system. Four lines of evidence demonstrate that the cells in which the *Gr* genes are expressed are gustatory neurons. First, they are located at the base of taste bristles that are morphologically distinct from other sensory bristles. Second, they have a bipolar structure, projecting a dendrite into the cavity of the bristle and an axon toward the central nervous system. Third, cells in the labial palps expressing these receptors project their axons to the SOG, the primary relay station of gustatory-information processing in the brain. Last,

Figure 2

(a)

(b)

expression of the genes is not observed in *poxn* mutant flies in which gustatory sensilla are transformed into mechanosensory sensilla.

A spatial map of individual taste receptor gene expression and the maintenance of this map in the brain suggest that *Drosophila* and other insects might have a high discriminatory power for different taste qualities.

Results

A large family of putative gustatory-receptor (*Gr*) genes

In previous studies, we used BLAST searches with candidate *Or* gene sequences to identify the entire *Or* gene family [13] from the Berkeley *Drosophila* Genome Project (BDGP; [28, 29]). During these searches, we noticed that some of the retrieved sequences were genes for hydrophobic seven-transmembrane proteins with no apparent homology to the ORs [30]. Nevertheless, many of these genes were putative seven-transmembrane receptors, and we reasoned that some might be members of other large chemoreceptor gene families. Therefore, we scanned the BDGP database with each of these non-*Or* genes for closely related novel genes ([28, 29]; see Materials and methods). With one of these genes (referred to later as *Gr22f*), we identified five additional members (*Gr22a–e*) with extensive sequence similarity to each other. *Gr22f* was also identified by Clyne et al. ([27]; see Table 1). Reiterative BLAST searches with the *Gr22* genes and the putative *Gr* genes identified by Clyne and coworkers eventually lead to the identification of 54 related genes (see Materials and methods). Overall similarity within this family is low (15%–25%), but a 30 amino acid-long motif at the carboxy terminus is significantly more conserved (approximately 75%; Figure 2). The sequences of all genes are deposited at our web site at http://genetics. mc.duke.edu/faculty/amrein.htm.

We next investigated the genomic organization of the *Gr* genes. Thirty-six *Gr* genes are arranged in clusters of two to six members (Table 1). Genes within a cluster are more conserved (up to 50% identity/70% similarity; Figure 3). The intergenic distance between clustered genes is very short, in many cases between 150–300 base pairs from the end of one open reading frame to the beginning of the next. Two genes appear to be pseudogenes (*Gr22b* and *Gr22d*) since their coding sequence is interrupted by a stop codon and a frame shift mutation, respectively. The number of introns varies widely because some genes have five or more introns and others have none (Table 1). About half of the genes have a single conserved intron near the carboxy terminus. Clyne et al. [27] noted the presence of potentially alternative spliced genes at two loci, but in principle all genes that are arranged head to tail within a cluster and have conserved introns might be subjected to alternative splicing.

To further investigate the relationship between the *Gr* genes, we performed ClustalW alignments (MacVector; data not shown). Based on these alignments and gene tree prediction analysis, we subdivided the 54 putative receptor proteins into nine subfamilies, defined by members sharing a minimum 33% sequence similarity among themselves (Figure 3). Subfamilies consisted largely of genes derived from a single cluster, but in some cases they included genes from different genomic regions (Table 1 and Figure 3). Twelve genes were not assigned to any subfamily because they showed little overall homology to any of the other genes.

We also generated hydrophobicity profiles for all genes by using von Heijne algorithms [31] and found that the predicted amino acid sequences contained seven putative transmembrane segments. The highly conserved motif in the carboxy terminus likely falls within the last transmembrane segment, which is also highly conserved in many other seven-transmembrane-receptor proteins (Figures 2 and 3). Interestingly, *Or83b*, the gene for an odorant receptor with distinct structural features and a much wider expression pattern than all other *Or* genes shows significant sequence similarity to the *Gr* genes in this region. Moreover, *Or83b* also contains two introns found at corre-

(a) Physical map of the *Gr22* gene family. Gene positions were determined from genomic scaffolds obtained from the National Center for Biotechnology Information (NCBI) and Berkeley *Drosophila* Genome Project (BDGP) databases. Both scaffolds AE002940 and AE003585 contained *Gr* genes of the family 22, but because of inconsistencies in the alignment of the scaffolds due to long repeat sequences, it is possible to determine neither the exact physical distance between *Gr22e* and *Gr22f* nor the orientation of AE 002940 relative to other scaffolds (denoted by the break in the map). The lightly shaded bars depict the location of putative promoter regions that were joined to GAL-4 in the creation of the transgenes. The distances between the stop codon and the predicted translation initiation codon of neighboring genes are as follows: *Gr22a–Gr22b*, 402 nucleotides; *Gr22b–Gr22c*, 237 nucleotides; *Gr22c–Gr22d*, 263 nucleotides.
(b) Alignment of the sequences encompassing the last transmembrane segment from all identified *Gr* genes. Sequences are identified with the proposed names. Verticle lines denote intron-exon boundaries. Lowercase letters following the proposed names indicate alternate splicing of a gene. Query sequences were generated from sequences published in Clyne et al. [27] and from subsequent searches with both NCBI tBlastN and BlastP search engines. For a detailed description of search methods used, see Materials and methods. Residues identical in more than 50% of all sequences are darkly shaded, and residues conserved in more than 50% are lightly shaded.

Table 1

List of all identified putative gustatory-receptor genes.

Number	Subfamily	Names Proposed	Clyne et al., 2000	Predicted Gene	Genomic Clone	Map Position	Number of Introns	Size (amino acids)	Remarks
1	I	Gr2a		CG18531*	AE003420	2B1	3	410	
2	II	Gr5a		CG15779*	AE003435	5A13	5	420	
3		Gr8a		CG15371	AE003446	8D4	3	385	
4		Gr10a			AE003486	10B13	1	387	transgene created
5		Gr21a	21D1	CG13948	AE003588	21D2	3	364	
6	III	Gr22a			AE002940	22B	1	394	transgene created
7	III	Gr22b			AE002940	22B	1	386	pseudogene
8	III	Gr22c			AE002940	22B	1	383	transgene created
9	III	Gr22d			AE002940	22B	1	326	pseudogene
10	III	Gr22e			AE002940	22B	1	389	transgene created
11	III	Gr22f	22B1		AE003585	22B	1	376	transgene created
12	IV	Gr23a.a	23A1a	CG15396	AE003582	23A3	2	383	alternative splicing (1)
13	IV	Gr23a.b	23A1b	CG15396	AE003582	23A3	2	374	"
14	V	Gr28a		CG13787*	AE003617	28A1	2	450	
15	V	Gr28b		CG13788*	AE003617	28A1	3	513	
16	I	Gr32a	32D1	CG14916	AE003631	32D4	3	392	
17		Gr33a		CG17213*	AE003635	33C1	3	444	
18	VI	Gr36a			AE003654	36B3	2	391	
19	VI	Gr36b		CG15137*	AE003654	36B3	2	391	
20	VI	Gr36c		CG15137*	AE003654	36B3	2	390	
21	I	Gr39a.a	39D2a		AE003670	39D2	3	372	alternative splicing (1)
22	I	Gr39a.b	39D2b		AE003670	39D2	3	381	"
23	I	Gr39a.c	39D2c		AE003670	39D2	3	381	"
24	I	Gr39a.d	39D2d		AE003670	39D2	3	371	"
25		Gr39b	39D1		AE003670	39D3	3	348	
26		Gr43a	43C1	CG1712	AE003841	43B3	5	379	
27		Gr47a	47A1	CG12906	AE003830	47A2	1	361	
28		Gr57a		CG13441	AE003791	57B3	1	416	
29	VII	Gr58a	58A1		AE003455	58B5/6	1	395	
30	VII	Gr58b	58A2	CG13495	AE003455	58B5/6	1	408	
31	VII	Gr58c	58A3	CG13491	AE003455	58B6	1	412	
32	VII	Gr59a		CG13537*	AE003459	59C3/4	1	367	
33	VII	Gr59b		CG13537*	AE003459	59C3/4	0	387	transgene created
34	VI	Gr59c	59D2	CG13543	AE003459	59C4	1	394	
35	VI	Gr59d	59D1		AE003459	59C4/5	1	390	
36	VIII	Gr59e	59E2	CG15872	AE003461	59E3	2	399	
37	VIII	Gr59f	59E1	CG5365	AE003461	59E3	3	363	
38		Gr61a		CG13888	AE003469	61D4	7	436	
39		Gr63a		CG14979*	AE003479	63F5	2	508	transgene created
40	II	Gr64a		CG14986*	AE003480	64A4	6	410	
41	II	Gr64b		CG14986*	AE003480	64A4	6	406	exon
42	II	Gr64c		CG14986*	AE003480	64A4	4	419	structure
43	II	Gr64d		CG14987*	AE003480	64A4	4	417	uncertain
44	II	Gr64e		CG14988*	AE003480	64A4	6	409	
45	II	Gr64f		CG14988*	AE003480	64A4	6	419	
46	V	Gr66a		CG7189	AE003556	66C6	1	530	transgene created
47	I	Gr68a		CG7303*	AE003543	68D1	0	389	
48		Gr77a			AE003592	77E3/4	1	453	
49		Gr85a			AE003685	85F5	1	397	
50	IX	Gr92a			AE003729	92D1	1	386	
51	IX	Gr93a		CG13417*	AE003736	93F9	1	419	
52	IX	Gr93b		CG13411*	AE003736	93F9	1	401	
53	IX	Gr93c		CG13411*	AE003736	93F9	1	397	
54	IX	Gr93d			AE003736	93F9	1	381	

Nomenclature of *Gr* genes was according to the *Drosophila* odorant receptor nomenclature committee [53]. Asterisks denote incorrect predictions of amino acid sequences of putative gustatory receptors. Multiple CG number listings are the result of incorrect predictions due to gene fusion. Accession numbers of genomic clones listed are from BDGP database. The sequences of all genes can be viewed at our web site at http://genetics.mc.duke.edu/faculty/amrein.htm.

sponding positions in many *Gr* genes; this finding suggests a common ancestor for these genes (Figure 4).

We note that more than one third of all genes were not predicted/annotated by the BDGP (Release 2; Table 1). In addition, 20 genes were predicted but are significantly different than what we believe is their correct structure. For example, genes were frequently joined to upstream and downstream ORFs for the generation of "fusion proteins," and the most highly conserved exon encoding the carboxy terminus was excluded in several predicted proteins (Table 1).

The *Gr* genes are expressed in chemosensory neurons of the adult

To determine where the *Gr* genes are expressed in adult *Drosophila*, we first performed RNA in situ hybridization experiments. We amplified and cloned the predicted coding sequences of *Gr22a*, *Gr22c*, and *Gr22e* from genomic DNA by using the polymerase chain reaction (PCR). Antisense RNAs were generated by in vitro transcription and hybridized to sections from adult flies (see Materials and methods). No specific hybridization signals were detected in sections through the entire body and head. We carefully examined the organs containing chemosensory sensilla, but we were unable to detect clear hybridization signals in antenna, maxillary palp, labial palps, legs, wings, or female abdomens (data not shown). We performed in situ hybridization experiments with several odorant receptor probes and found them to be specifically expressed in subsets of antennal or maxillary palp neurons as previously described (data not shown; [13–15, 32]). The three most likely explanations for our findings are (1) very low levels of expression of these genes, such that in situ hybridization methods fail to identify their transcripts [27], (2) expression of these genes in extremely few cells, which might be lost in the sectioning procedure, or (3) the absence of these transcripts altogether in adult flies.

To further investigate the expression pattern of the *Gr* genes at 22B, we used an alternative, transgenic approach that allowed us to characterize gene expression patterns in whole animals. Importantly, transgene expression often accurately reflects expression of the endogenous gene, a feature well established in *Drosophila* in general and in the olfactory system in particular [32]. We cloned putative promoter fragments of the *Gr22a*, *Gr22c*, *Gr22e*, and *Gr22f* genes from genomic DNA by PCR and inserted them into the expression vector SM1 in front of the GAL4 gene (see Materials and methods). These "drivers," in combination with a *UAS→lacZ* reporter, allow visualization of transgene expression in vivo [33]. At least three independent transgenic lines of flies homozygous for each of these drivers were crossed to flies homozygous for a *UAS→lacZ* reporter, and we analyzed the double-hemizygous progeny by whole-mount staining to monitor

the activity of the promoter fragments (see Materials and methods). No β-gal activity was observed in the main body parts, such as the head, thorax, and abdomen. Analysis of the appendages of flies with the driver *P [22e]→Gal4*, however, demonstrated that this gene is expressed in many cells in the antenna, maxillary palps, proboscis, legs, and wings (Figure 5a,b). Specifically, *P [22e]→Gal4* is expressed in cells at the base of many chemosensory bristles of the labial palps and in cells located in the dorsal and ventral cibarial sense organs (Figure 5a). It is also expressed along the tibiae and tarsi of all legs and the anterior wing margin in cells that are located at the base of chemosensory bristles (Figure 5a). This highly specific, localized expression is particularly evident in the anterior wing margin, which contains two morphologically distinct types of bristles. Thinner and slightly bent chemosensory bristles occasionally interrupt thick and straight mechanosensory bristles [12, 16, 34]. Only cells at the base of chemosensory bristles expressed β-Gal (Figure 5a, top panel). Finally, a number of cells in the third antennal segment and the maxillary palp also express *Gr22e* (Figure 5b). Their expression pattern in olfactory organs is similar to the one observed for most *Or* genes, but it appears to cover a somewhat larger area [13–15, 32].

Expression of the driver *P [22c]→Gal4* is restricted to the tarsi of the foreleg at the base of bristles, which are gustatory based on morphological criteria (see above; Figure 5a, middle panel). No transgene expression was observed in the labial palps, the labral and cibarial sense organs, or the anterior wing margin. Yet another expression pattern was observed with the driver *P [22f]→Gal4* (Figure 5a), for which only four to eight positive cells are found on the labial palps in chemosensory sensilla. The cell bodies of these neurons are farther from the epithelial surface than are those of neurons in the wing margin, and it is therefore not always possible to correlate a LacZ-positive cell with a particular bristle. We noticed, however, that LacZ staining can be seen within the taste bristles themselves (Figure 5a, top panel, and Figure 6a). Only chemosensory, not mechanosensory, neurons extend their dendrite into the bristle cavity [16, 21], and this observation suggests that these LacZ-positive cells are gustatory neurons. The fourth transgene, *P [22a]→Gal4*, is not expressed in any of the four lines analyzed (data not shown). The promoter in this driver might not contain all the necessary elements for expression, the gene might be expressed in other tissues/stages of development, or it might not be expressed at all.
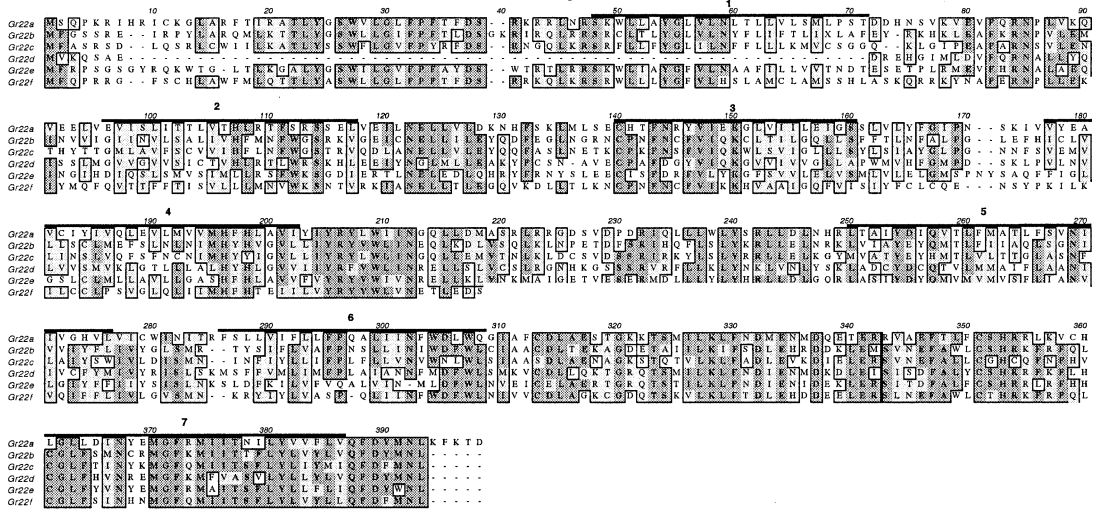
Our studies have shown that three of four *Gr* genes located at 22B are excellent candidates for encoding taste receptors. To generalize these findings for the entire *Gr* gene family, we created four additional transgenes by cloning the promoters of *Gr10a*, *Gr59b*, *Gr63a*, and *Gr66a* into
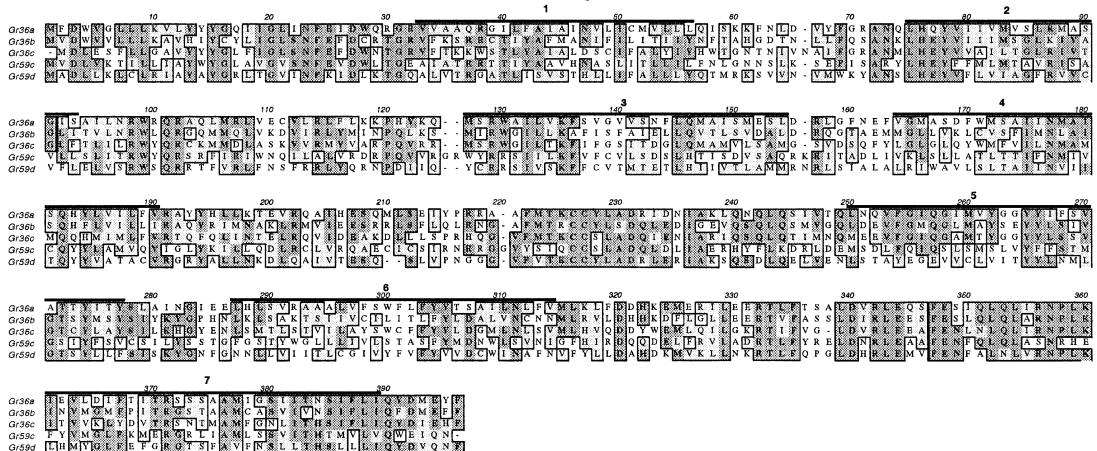
Figure 3

Subfamily II
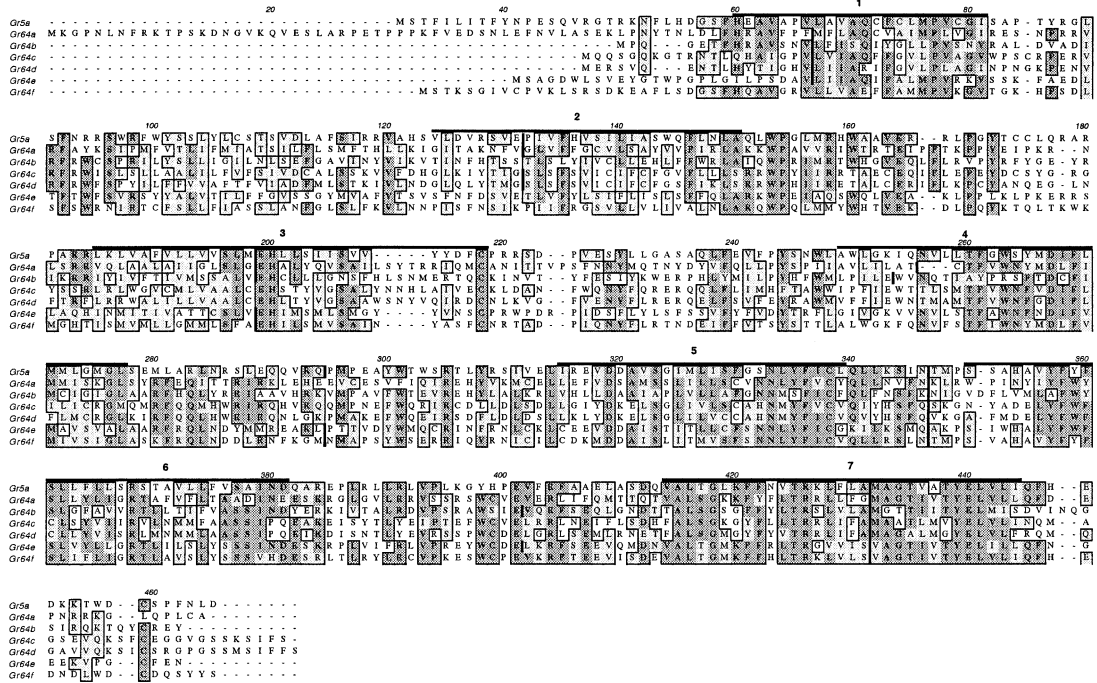
Subfamily III

Subfamily VI

the *GAL4* expression vector. Using these constructs, we generated and analyzed three independent lines of transgenic flies. Two of these drivers were expressed exclusively in the gustatory neurons of the adult. The driver *P [66a]→Gal4* is expressed in sensilla of the foreleg, the labial palps, and the labral and ventral cibarial sense organs but not in the mid leg, hind leg, or wing (Figure 5c). In the labial palps, the expression of *Gr66a* is not restricted to a single row of bristles, as is *Gr22f* expression, but extends more laterally through two rows of sensory bristles. The driver *P [59b]→Gal4* is expressed only in the labial palps and at weaker levels than either *Gr22f* or *Gr66a*.

Taken together, our analyses show that five of eight analyzed genes (62.5%) are expressed in distinct gustatory sensilla in all major taste organs of *Drosophila*. Most of the genes are expressed in a very small fraction (1%–4%) of gustatory sensilla in a spatially restricted region of the fly, whereas one gene is expressed in about 20% of sensilla distributed all over the fly (Figures 1b and 5a). Thus, they are excellent candidates for taste receptor genes.

The putative gustatory-receptor genes are expressed in gustatory neurons

To establish the neuronal identity of the *Gr*-expressing cells, we first performed immunolocalization experiments. Each sensillum contains not only gustatory neurons (and in most cases one mechanosensory neuron) but also three non-neuronal cells that form the hair, socket, and sheet surrounding the dendrites of the neurons [19]. We performed confocal microscopy and visualized all neurons with an anti-Elav antibody. We visualized the *Gr*-expressing cell with an anti-β-Gal antibody (Figure 6a). Both drivers, *P [22e]→Gal4* and *P [22f]→Gal4*, stain neurons that project a dendrite toward the epithelium and an axon toward the brain (Figure 6a, top right panel). Interestingly, none of the more than 50 sensilla we analyzed contained more than one β-Gal-positive cell.

We next investigated the projection patterns of neurons expressing the putative gustatory receptors. The gustatory neurons in the labial palps project their axons through the labial nerve to the SOG in the brain [35, 36]. We asked whether axons expressing a given receptor converge to a specific region within the SOG. Flies with the drivers *P [22e]→Gal4* and *P [66a]→Gal4* were crossed to a *UAS→lacZ* or *UAS→Tau-lacZ* line. Tau-LacZ is a Tau-β-Gal fusion protein that is preferentially localized in axons and

dendrites [37]. Flies with the driver *P [66a]→Gal4* show distinct staining of the labial and accessory pharyngeal nerves (Figure 6b, left panel), and this staining reflects the expression of this gene in neurons of the labial palps and the labral/cibarial sense organs (Figure 5c). As the nerves enter the brain, the axons terminate in two distinct regions, occupying only a fraction of the SOG. Axon projections of the driver *P [22e]→Gal4* were visualized with confocal microscopy (Figure 6b, right panel). These axons converge to a somewhat larger domain within the SOG, and this finding presumably reflects the wider expression domain of *Gr22e* when compared to *Gr66a* (Figure 5a,c). Nevertheless, the β-Gal-positive region occupies only a part of the SOG. Axonal convergence of gustatory receptor neurons, however, is not as defined as in the olfactory system, where axons expressing an individual *Or* gene project to one glomerulus in the antennal lobe [32, 38].

Finally, we used a genetic approach to show that the neurons expressing the *Gr* genes are indeed chemosensory neurons. POXN is a paired-box-containing transcription factor involved in several steps of neuron specification in all developmental stages [39–41]. Some *poxn* alleles do not interfere with the early steps in neurogenesis but affect the determination of chemosensory neurons in the adult [34]. For example, flies carrying the *poxn*[70-23] allele are viable but show complete transformation of gustatory neurons into mechanosensory neurons [34]. We generated *poxn* mutant flies carrying three Gal4 drivers and a single copy of a *UAS→lacZ* reporter and analyzed their expression (Figure 7; see also Materials and methods). None of the drivers was expressed in the gustatory organs in these flies, whereas siblings that carried one wild-type *poxn* allele expressed each driver in a normal pattern (Figure 7). Antenna and maxillary palp expression of *Gr22e* remained normal in *poxn* flies (data not shown).

Thus, by several different criteria, the *Gr* genes are expressed in gustatory neurons of the adult. Furthermore, our experiments also demonstrate that neurons in the labial palps and the labral/cibarial sense organs expressing individual taste receptors project their axon to a distinct region in the SOG of the brain.

Bilaterally symmetrical, spatially restricted expression of putative gustatory-receptor genes is maintained from animal to animal

The location of neurons expressing a given receptor is conserved between individuals. We determined this by

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Amino acid sequence alignment of three putative *Gr* subfamilies. Identical residues conserved in >50% of the sequences are darkly shaded. Similar residues conserved in >50% of the sequences are lightly shaded. Vertical lines indicate intron-exon boundaries, and horizontal lines show estimates of transmembrane domains. All

sequences were derived from the BDGP database. Genes were considered members of a subfamily when they shared at least 33% sequence similarity. X at position 68 indicates a stop codon in *Gr22b*. *Gr22d* is interrupted by a frame shift mutation within the first transmembrane segment.

Figure 4
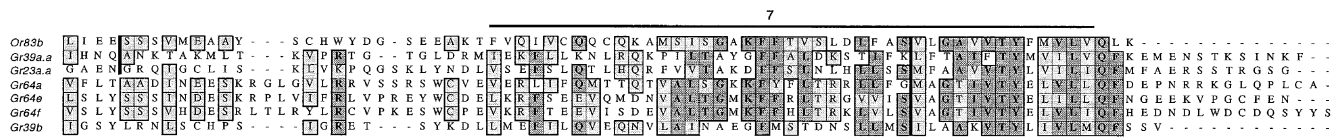
Amino acid sequence alignment of *Or83b* and *Gr* genes. The conserved seventh transmembrane segments of some *Gr* genes and *Or83b* are aligned. Identical residues conserved in >50% of the sequences are darkly shaded. Similar residues conserved in >50% of the sequences are lightly shaded. Vertical lines denote intron-exon boundaries, and a horizontal line gives the location of the transmembrane domain.

comparing the location and number of LacZ-positive sensilla of flies from three independent lines containing either the *P [22f]→Gal4* or the *P [22c]→Gal4* driver. The expression patterns of both transgenes were highly reproducible, but small variations were observed. For example, most flies with the *P [22c]→Gal4* driver had two positive cells at the tip of the foreleg; however, we found occasionally only one LacZ-positive cell at that location or one additional cell at a more proximal location in the foreleg or on the tarsi of the second and third leg (Figure 1b and data not shown). On the other hand, we have not recorded any positive cells on the labial palps, the labral and cibarial sense organs, or the wing. Flies with the *P [22f]→Gal4* driver had between two and four β-Gal-positive cells in a discrete row of taste bristles on the labial palps; again, this pattern is very restricted, as we have not encountered any β-Gal-positive cells in the legs or wings (Figure 1b).

Variations in receptor gene expression might reflect a stochastic mechanism underlying the transcriptional control of *Gr* gene expression. For example, a neuron within a given sensillum might have a certain probability of expressing *Gr X*, a lower probability of expressing *Gr Y*, and virtually no probability of expressing any of the remaining *Gr* genes. We cannot, however, exclude the possibility that these differences are transgene-dependent effects and that the endogenous gene is precisely expressed in the same cells in each animal. Whatever the reason for these modest variations, our experiments demonstrate that there exists a relatively precise topographic map for individual receptor gene expression. It should be noted that *Gr22e* is expressed in many more neurons than any other gene we have analyzed and that such a topographic map might not apply to this gene.

Discussion

Sophisticated chemosensory systems have evolved in animals to accommodate the needs of locating potential foods, identifying edible foods, and discerning them from contaminated ones. In addition, chemosensory cues play essential roles in specific mating behaviors in most animals. For example, the males of the moth *Manduca sexta* have highly developed olfactory sense organs that allow

them to find females over a distance of many miles [42]. In *Drosophila*, mating behavior is controlled at least to some extent by the gustatory system, as is the selection of sites for oviposition by the female [20, 43].

The recognition of hundreds of volatile and soluble substances requires a large number of specific receptor proteins expressed on the dendritic surface of chemosensory neurons. For example, the number of genes encoding odorant and gustatory receptors is estimated to be more than 1000 in the mouse and almost as many in *C. elegans* [1, 3–5]. About 60 olfactory-receptor genes were identified recently in *Drosophila* [32], and in this paper we show that a similar number of genes comprise a large taste-receptor gene family in this species. Knowledge of their structure and the characterization of their expression are significant steps toward a deeper understanding of how taste stimuli are perceived and processed in the brain.

The taste-receptor gene family

Searches of the entire genome of *Drosophila* identified at least 54 *Gr* genes. However, since conservation of this gene family is extremely low (15%–25%), this number might be an underestimate.

Although the *Or* and *Gr* gene families share no apparent sequence homology to each other, one odorant receptor gene, *Or83b*, was identified numerous times in our BLAST searches for novel *Gr* genes. Alignment of the predicted carboxy terminal domains of OR83B and some GR proteins as well as the conserved positions of two introns reveals that the *Or83b* and *Gr* genes have a common ancestor (Figure 4). *Or83b* is an unusual olfactory receptor gene. In contrast to the other *Or* genes, which are expressed in distinct domains within the third antennal segment or the maxillary palp, *Or83b* is the only *Or* gene that is expressed in all olfactory neurons [13, 32]. Moreover, the OR83b protein is structurally distinct from the other ORs, both in size and sequence (461 amino acids versus 360–410 amino acids for most other ORs). Even though the function of OR83b is unknown, Vosshall et al. speculated that it might function as a coreceptor by forming a dimer with one of the more specifically ex-

pressed OR proteins [32]. Such a dimer could accommodate ligand binding specificity through the specifically expressed receptor and signaling function through the ubiquitously expressed OR83b receptor. Precedents for dimeric seven-transmembrane receptors are the metabotrophic GABA$_B$ receptors, in which surface expression and ligand binding of one subunit, GABA$_B$R1, is only observed in the presence of a second subunit, GABA$_B$R2 [44–46]. A ubiquitously expressed GR protein with a similar function in taste signaling analogous to that proposed for OR83b in olfactory signaling has yet to be identified. It is also possible that individual GR proteins provide both ligand binding and signaling function. In any case, the structural similarity of *Or83b* and the *Gr* genes suggests a common ancestor. Taste is an evolutionarily "older" sense than smell, and the *Or83b* gene may have directly evolved from gustatory-receptor genes. Thus, *Or83b* might provide a molecular link between the two senses. Alternatively, *Or83b* might encode an ancestral GR protein "recruited" by the olfactory system for a peculiar function. It is interesting to note that one *Gr* gene (*Gr22e*) is also expressed in the olfactory system, and this finding suggests a lack of clear-cut boundaries between the two senses (Figure 5b).

Five out of the eight *Gr* genes we analyzed are almost exclusively expressed in the gustatory system of the adult fly (Figure 5). The expression of four of these genes is restricted to a few sensilla within a spatially defined domain in one or several gustatory organs. By morphological, genetic, and neuro-anatomical criteria, the cells expressing these genes are gustatory neurons (Figures 6 and 7). One transgene, driver *P [22e]→Gal4*, is expressed in both gustatory and olfactory sensilla, which raises the possibility that some Gr proteins might function as olfactory receptors. However, we have not found a single cDNA clone for *Gr22e* among 10^6 clones screened from an antennal cDNA library, a number that yields 5–20 cDNA clones when individual *Or* genes are used as probes (data not shown). It remains to be seen whether *Gr22e* is indeed expressed in the olfactory system or whether the promoter fragment used in our constructs lacks a negative regulatory element. If that element were present, expression would be restricted to the gustatory system. The expression of *Gr22e* and perhaps additional *Gr* genes in both the olfactory and gustatory systems would raise interesting questions regarding the function of these receptors and the ligands interacting with them.

Three of the eight transgenes investigated showed no expression in the adult. One interpretation is that the corresponding *Gr* genes have other functions. In rodents, a significant number of olfactory receptors are expressed in sperm, and other olfactory receptors are expressed in the developing heart and erythroid cells [2, 47, 48]. It should also be noted that 33% of all *Or* genes are not expressed in the olfactory system in *Drosophila* [32]. It will therefore be interesting to see whether some of the *Or* and *Gr* genes not expressed in the chemosensory system have acquired other functions as well. Alternatively, these genes might lack functional promoters and be pseudogenes. Finally, we cannot rule out the possibility that the absence of transgene expression is caused by a lack of essential regulatory promoter elements in our constructs.

Molecular logic of taste perception

An important factor that influences the discriminatory power of chemosensory systems is the number of different receptor genes expressed in a single neuron. In theory, the expression of a single receptor per sensory neuron in the olfactory system of *Drosophila* and mammals provides a tool for specific activation of one type of neuron by a specific stimulus in the entire olfactory epithelium (the ubiquitously expressed *Or83b* gene in *Drosophila* might be viewed as a coreceptor without ligand specificity). In these systems, neurons expressing a specific receptor project their axons to one (two for mammals) of about 40 (2000) glomeruli of the antenal lobe (olfactory bulb), the functional units in the first relay station of the brain [32, 38, 49, 50]. In this way, stimulation of one neuron type can generate a highly specific activation pattern in the brain. The large number of different activation patterns is a prerequisite for the generation of the discriminatory prowess observed in these systems (it is believed that humans can distinguish between thousands of different odors). The expression of multiple receptors in a neuron, as observed in the chemosensory system of *C. elegans* or the gustatory system in mammals, will unavoidably lead to the activation of processing centers by many different stimuli and reduce discriminatory potential [3, 10, 11].

What is the neuron-to-receptor ratio in the *Drosophila* gustatory system? Based on our analysis of about 15% (8/54) of all *Gr* genes, we are able to predict a global expression profile of this gene family. The ratio of expressed genes to total number of genes appears to be similar in the gustatory (62.5%; our studies) and olfactory systems (66.7%; [32]). Thus, about 30–35 *Gr* genes might be expressed in the gustatory system. Our studies revealed two distinct expression profiles. One gene, *Gr22e*, is expressed in about 20% of the taste sensilla throughout the gustatory system, whereas the remaining genes are expressed in more defined regions that occupy only about 1%–4% of all taste sensilla. If the remaining *Gr* genes are expressed in a similar profile, we would expect to find about 6–7 genes expressed in 20% of sensilla and 27–28 genes expressed in 1%–4% of sensilla (about 20 *Gr* genes would not be expressed at all). Such a breakdown would ultimately require that a given sensilla express about two *Gr* genes. However, since each sensillum contains on average three gustatory neurons, the one-neuron-to-one-*Gr*-gene rule would still apply. In fact, the remaining neuron in each

Figure 5

Expression of the *Gr22* genes in the chemosensory system of the adult fly. **(a)** Expression of the *Gr22* genes in the *Drosophila* gustatory system. Expression of the three *GAL4* constructs driven by the promoters of *Gr22e*, *Gr22c*, and *Gr22f* in flies hemizygous for one of these transgenes and hemizygous for a *UAS→lacZ* reporter transgene. LacZ expression of *Gr22e* is found in taste bristles on the labial palp, labral and cibarial sense organs, legs, and anterior wing margin. *Gr* expression is only observed in leg and wing chemosensory bristles, which are thin and slightly bent, but not in mechanosensory bristles, which are straight and thick. Expression of *Gr22c* and *Gr22f* is observed in taste bristles in the foreleg and the labial palps, respectively, within a spatially restricted region. **(b)** Expression of *Gr22e* in the *Drosophila* olfactory system. LacZ staining of the third antennal segment and the maxillary palp reveals many β-Gal-positive cells, similar to those observed with certain olfactory receptor genes, in a broad expression zone. **(c)** LacZ staining of flies expressing the driver *P [Gr66a]* reveals spatially restricted expression in taste hairs in the labial palps, labral and cibarial sense organs, and forelegs. The expression of *P [Gr59b]* is much weaker and is restricted to taste hairs in the labial palps.
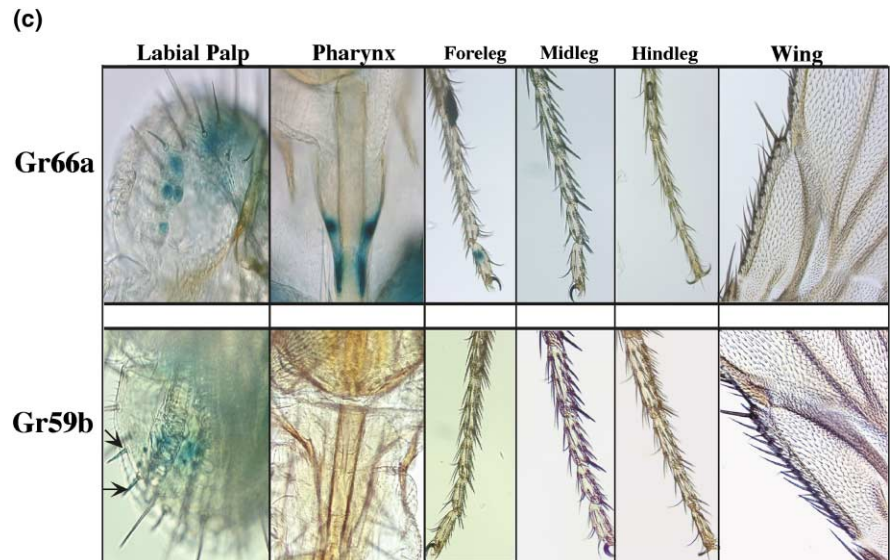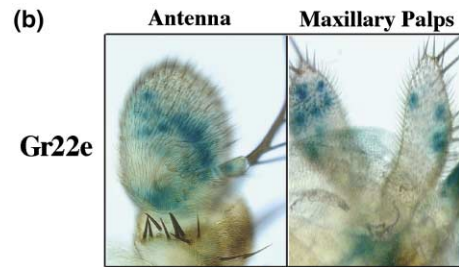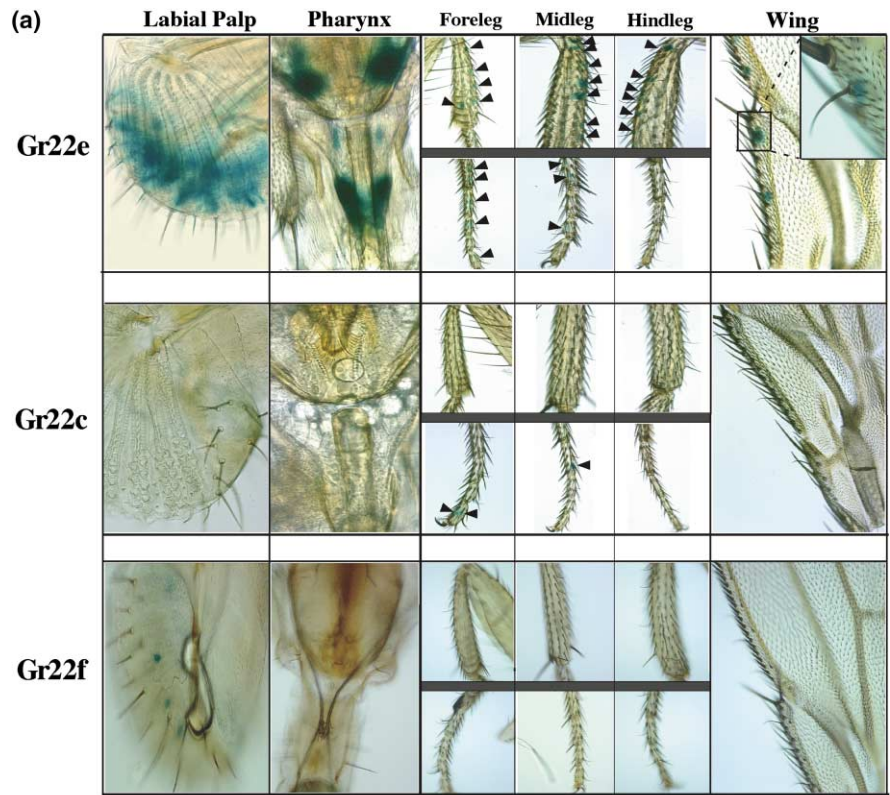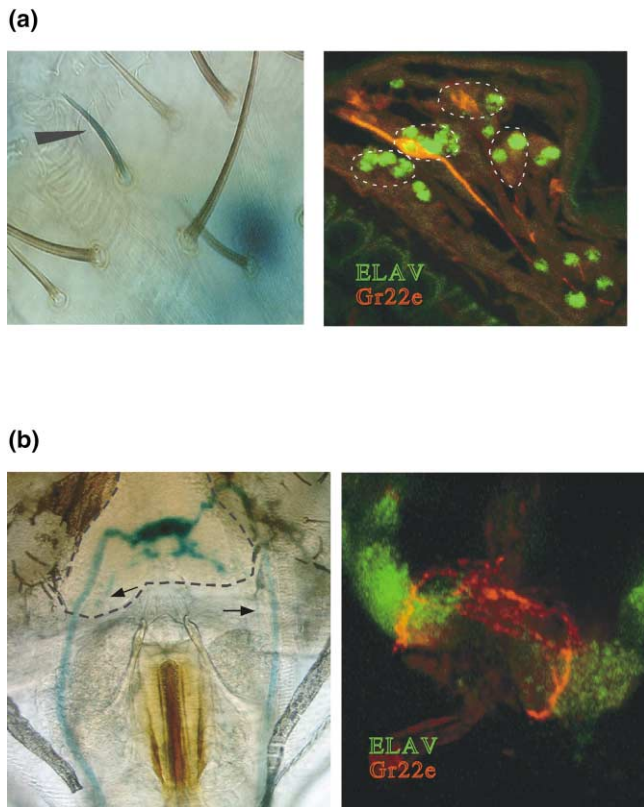
Figure 6

(a)

(b)

Gr genes are expressed in labial-palp neurons, which project axons to the subesophageal ganglion in the brain. The left panels show whole-mount LacZ staining, and the right panels show confocal images captured with a Zeiss LSM-410 confocal microscope. Neurons were identified and visualized with mouse monoclonal Elav antibody 9F8A9 and an anti-mouse Alexa secondary antibody. β-Gal-expressing cells were identified with a polyclonal anti-β-Gal rat antibody and Cy-3 labeled secondary anti-rabbit antibodies. Elav staining is nuclear, and β-Gal staining is cytoplasmic. **(a)** Expression of *Gr* genes in gustatory neurons is shown for the *Gr22e* gene by LacZ staining and β-gal/Elav antibody staining. The left panel shows LacZ staining of the dendrite, which extends into the hair cavity (arrowhead). The cell body is visible at the right border of the image. In the right panel, tissue sections of gustatory sensilla (dotted circles) from a fly with the driver *P [22e]→Gal4* were stained with both a pan-neuronal marker (anti-Elav antibody) and a β-gal antibody. Two sensilla contain a single neuron expressing β-Gal (only part of the neuron on the right is present in the section). Dendritic (toward top) and axonal (toward bottom) projections can be readily identified. **(b)** Axonal projections of flies expressing *Gr66a* and *Gr22e* were visualized in whole-mount LacZ staining (left) and whole-mount antibody staining (right), respectively. In the left panel, the labial nerve of a fly with the driver *P [66a]→Gal4* can be identified entering the subesophageal ganglion of the brain from below. Also visible, although more weakly stained, is the accessory nerve carrying axons from neurons of the labral sense organ (arrows). The axons terminate within the SOG as two discrete domains per hemisphere. The broken line outlines the subesophageal ganglion. The right panel shows a whole-mount antibody staining of a dissected fly brain expressing the *P [Gr22e]→Gal4* driver. Here, the axon termini are somewhat less converged, and individual axons can be identified. Note that the reporter for *P [66a]* was *UAS→lacZ* and the reporter for *P [22e]* was *UAS→Tau-lacZ*.

Figure 7
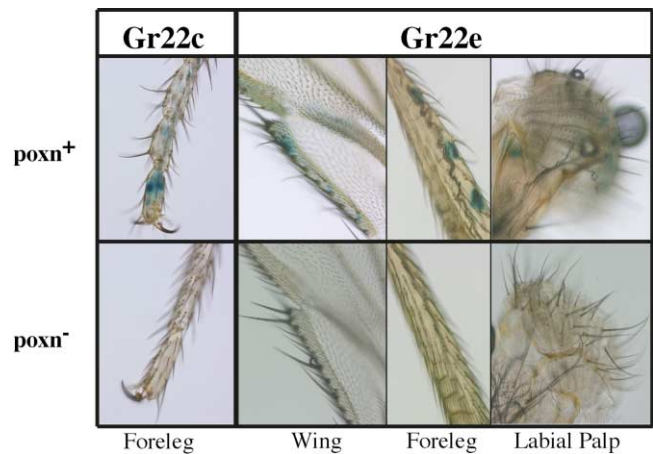
Gr22c and *Gr22e* expression is absent in *poxn* flies lacking chemosensory neurons. The flies shown on the top panel had one wild-type copy of the *poxn* gene and either the *poxn*[70-23] allele or a deletion for the *poxn* locus (42WMG) and were hemizygous for both the driver and the *UAS→lacZ* reporter gene. The flies shown on the bottom panel were siblings with a *poxn*[70-23] allele-carrying chromosome and a chromosome bearing the 42WMG deletion; they were also hemizygous for both the driver and the *UAS→lacZ* reporter. LacZ-positive cells were absent in the homozygous *poxn* flies, whereas the heterozygous flies exhibited staining that is indistinguishable from that of wild-type flies.

sensillum might express another type of receptor, such as the trehalose receptor [26]. Therefore, our data are consistent with a model found in the olfactory system of both *Drosophila* and mammals in which each sensory neuron expresses only one receptor gene. We also find convergence of axons expressing a specific *Gr* gene to a specific domain within the SOG. This situation is similar to that found in the olfactory system in which neurons expressing a specific *Or* project their axons to a single glomerulus in the antennal lobe [32, 38].

Conclusion

What is the discriminatory power of the insect gustatory system? Humans and, presumably, many other mammals can distinguish only a handful of different taste qualities: sweet, bitter, sour, salty, and umami (the taste of monosodium glutamate). This relatively modest discriminatory power is consistent with the observation that neurons within the taste buds of the tongue express many members of a recently identified bitter taste-receptor gene family [10, 11].

Our analysis of the *Gr* genes in *Drosophila* indicates that the insect gustatory system resembles the olfactory systems of both insects and mammals, and this finding suggests the potential for high taste discrimination. However, behavioral studies are necessary to determine whether the observed spatial segregation of specific types of taste

neurons correlates with the acquisition of many different taste qualities distinguished by the fly brain.

While this paper was being reviewed, a study describing the expression of seven members of the *Gr* gene family was published by another research group [51]. The authors of that work also used a transgenic approach and found that the *Gr* genes are expressed in the gustatory system of both larvae and adults.

Materials and methods

Identification of Gr gene family

All non-*Or* genes obtained from blast searches with known *Or* genes were used as query sequences. One of them (later to be known as *Gr22f*) retrieved five additional sequences when used as a query sequence (*Gr22a−e*). None of them, however, represents a predicted gene according to the Celera/Berkeley *Drosophila* genome database (release 2). These genes were closely linked to each other at 22B. *Gr22f* was also listed as one of the 19 proposed gustatory receptor genes identified by Clyne et al. [27]. We used the highly conserved carboxy terminus of the genes at 22B and the ones identified by Clyne et al. in additional blast searches to identify new putative *Gr* genes and we repeated this procedure until no new sequences were identified. This led to a total of 50 genes. We performed BLAST searches with each complete, putative protein sequence and recovered an additional four genes, resulting in a total of 54 genes. An alignment of the conserved carboxy terminus of all 54 proteins is shown in Figure 2b, and their entire predicted sequences are deposited on our web site.

In situ hybridization

Antisense digoxigenin-labeled RNA probes were generated by standard methods, and in situ hybridizations were performed according to the protocol previously described in Vosshall et al. [13].

lac Z staining

For whole-mount staining, flies were fixed for 45 min at room temperature in 1% glutaraldehyde in 1× PBS and then washed briefly in 1× PBS. All appendages were separated from the abdomen and thorax, and the entire fly was placed in X-gal buffer (0.1 M sodium phosphate [pH 7.4], 2 mM magnesium chloride, 0.01% sodium deoxycholate, 0.02% Nonidet p40) for 1 hr. The blue stain was generated by exposure in the dark at 37°C to the staining solution (X-gal buffer with 1 mg/ml X-Gal, 1 mM spermidine HCl, 5 mM potassium-ferricyanide, and 5 mM potassium-ferrocyanide) for 16–18 hr. Tissue was washed with 1× PBS for 30 min and then stored in 70% glycerol in 1× PBS until viewing. Stained tissue was mounted with the same glycerol solution and analyzed with a Zeiss Axioskop 2 microscope.

Antibody staining

Fly heads were embedded in OCT, and 12–15 μm sections were cut with a Reichert-Jung cryostat. Tissue was then fixed for 7 min in 2% paraformaldehyde in 1× PBS, washed briefly in 1× PBS, and permeabilized for 30 min in 1× PBS, 0.1% Triton X-100 (PT). Sections were blocked for 30 min in 1× PBS, 0.1% Triton X-100, 5% heat-inactivated goat serum (PTS), following which the slides were incubated at 4°C overnight in a 1:100 dilution of anti-β-galactosidase antibody (Cappel/ICN) and a 1:10 dilution of Elav antibody in PTS. After the tissue was washed with PT, it was blocked again in PTS for 30 min before incubation with the secondary antibodies, goat anti-rabbit CY3 (Jackson Laboratories) at a dilution of 1:250 and goat anti-mouse ALEXA 488 (Molecular Probes) at a dilution of 1:100 in PTS for 2 hr at room temperature. After a final wash, the slides were mounted with ProLong Antifade Kit (Molecular Probes) and analyzed with a Zeiss LSM 410 confocal microscope.

Molecular biology

For the generation of promoter-Gal4 drivers, putative promoters, varying from 0.8 to 3.0 kilo bases in length, were defined by the beginning of the coding sequence of the gene under investigation and the end or beginning of the coding sequence 5′ of that gene. In cases in which no gene within 3 kb upstream was located, we amplified a 3 kb fragment and assumed it was likely to contain the entire promoter. Promoter fragments were cloned by PCR from 1 μg of genomic DNA from an Ore-R wild-type strain. PCR conditions were as follows: 2 min 94°C, 30 s 94°C, 30 s 52°C–58°C, 3 min 68°C; 35 cycles, 7 min 68°C. PCR fragments were purified with a Geneclean DNA purification kit and cloned into a pGEMT easy vector (Promega). Correct clones were identified by DNA sequencing or restriction digestion. The promoter fragments were liberated by Acc65I/NotI digestion and cloned into the Gal4 transformation vector SM1. Promoter fragments were defined at the 3′ end by the translation initiation codon of the predicted open reading frame of the *Gr* gene and the next upstream gene at the 5′ end. If the next upstream gene was less than 1 kb or more than 3 kb upstream of the translation initiation codon of the *Gr* gene, a 1 kb or 3 kb DNA fragment was amplified. Primers for the different promoters were as follows: pGr22c.1, 5′-TGGCTACCCGTACTTTCCACGGT-3′; pGr22c.2, 5′-CAACGAAAATTAAATGGTCAATTCC-3′; pGr22e.1, 5′-TGGGTACCCTAATTGCGAACGACTG-3′; pGr22e.2, 5′-GAGTCAGCCTCGAATGGTCACG-3′; pGr22f.1, 5′-GGGTACCGCGAGATAATCGCGAGCATTC-3′; pGr22f.2, 5′-CCCTGGGTAAAACTAATGTCAACA-3′; pGr10a.1, 5′-TGGGTACCCCCTTATCAATGTGACTC-3′; pGr10a.2, 5′-CCGCTTTACCCAGCAAACTGAG-3′; pGr59b.1, 5′-TCGGGTACCAACAATGAAGCGGATA-3′; pGr59b.2, 5′-TCCTTTGTGCACTAGTTTGTAG-3′; pGr63a.1, 5′-AGGTACCAGGAGATGAACATGACGC-3′; pGr63a.2, 5′-GCACCTGCACACGCCCCCTTAT-3′; pGr66a.1, 5′-TGGTACCTAGCAAGAGCAAGTGCGAA-3′; pGr66a.2, 5′-ACTACAGCTTTGAATAACCGATTC-3′.

Genetics

Flies were raised on standard cornmeal-agar-molasses medium at 25°C. The generation of transgenic flies was performed as described in Amrein and Axel [52].

Flies with driver transgenes integrated on the third chromosome were used for the generation of strains that were *w/w*; *CyO/poxn⁷⁰⁻²³;P [Gr22e]→Gal4/P [Gr22e]→Gal4* and *w/w*; *CyO/poxn⁷⁰⁻²³;P [Gr22c]→Gal4/P [Gr22c]→Gal4*. A UAS→lacZ reporter strain with the reporter construct integrated on the third chromosome was used for the generation of the strain *w/w*; *CyO/Df[2R]42WMG;UAS→lacZ/ UAS→lacZ*.

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