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A Spatial Map of Olfactory Receptor Expression in the *Drosophila* Antenna

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Summary

Insects provide an attractive system for the study of olfactory sensory perception. We have identified a novel family of seven transmembrane domain proteins, encoded by 100 to 200 genes, that is likely to represent the family of *Drosophila* odorant receptors. Members of this gene family are expressed in topographically defined subpopulations of olfactory sensory neurons in either the antenna or the maxillary palp. Sensory neurons express different complements of receptor genes, such that individual neurons are functionally distinct. The isolation of candidate odorant receptor genes along with a genetic analysis of olfactory-driven behavior in insects may ultimately afford a system to understand the mechanistic link between odor recognition and behavior.

Introduction

All animals possess a "nose," an olfactory sense organ that allows for the recognition and discrimination of chemosensory information in the environment. Humans, for example, are thought to recognize over 10,000 discrete odors with exquisite discriminatory power such that subtle differences in chemical structure can often lead to profound differences in perceived odor quality. What mechanisms have evolved to allow the recognition and discrimination of complex olfactory information, and how is olfactory perception ultimately translated into appropriate behavioral responses?

The recognition of odors is accomplished by odorant receptors that reside on olfactory cilia, a specialization of the dendrite of the olfactory sensory neuron. The odorant receptor genes encode novel serpentine receptors that traverse the membrane seven times. In several vertebrate species, and in the invertebrate *Caenorhabditis elegans*, as many as 1000 genes encode odorant receptors, suggesting that 1%–5% of the coding potential of the genome in these organisms is devoted to the recognition of olfactory sensory stimuli (Buck and Axel, 1991; Levy et al., 1991; Parmentier et al., 1992; Ben-Arie et al., 1994; Troemel et al., 1995; Sengupta et al., 1996; Robertson, 1998). Thus, unlike color vision, in which three photoreceptors can absorb light across the entire visible spectrum, these data suggest that a small number of odorant receptors are insufficient to recognize the full spectrum of distinct molecular structures perceived by the olfactory system. Rather, the olfactory sensory system employs an extremely large number of receptors, each capable of recognizing a small number of odorous ligands.

The discrimination of olfactory information requires that the brain discern which of the numerous receptors have been activated by an odorant. In mammals, individual olfactory sensory neurons express only 1 of 1000 receptor genes such that the neurons are functionally distinct (Ngai et al., 1993; Ressler et al., 1993; Vassar et al., 1993; Chess et al., 1994; C. Dulac and R. A., unpublished). The axons from olfactory neurons expressing a specific receptor converge upon two spatially invariant glomeruli among the 1800 glomeruli within the olfactory bulb (Ressler et al., 1994; Vassar et al., 1994; Mombaerts et al., 1996; Wang et al., 1998). The bulb therefore provides a spatial map that identifies which of the numerous receptors has been activated within the sensory epithelium. The quality of an olfactory stimulus would therefore be encoded by specific combinations of glomeruli activated by a given odorant.

The logic of olfactory discrimination is quite different in the nematode *C. elegans*. Despite the large size of the odorant receptor gene family, volatile odorants are recognized by only three pairs of chemosensory cells, each likely to express a large number of receptor genes (Bargmann and Horvitz, 1991; Colbert and Bargmann, 1995; Troemel et al., 1995). Activation of any one of the multiple receptors in one cell will lead to chemoattraction, whereas activation of receptors in a second cell will result in chemorepulsion (Troemel et al., 1997). The specific neural circuit activated by a given sensory neuron is therefore the determinant of the behavioral response. Thus, this invertebrate olfactory sensory system retains the ability to recognize a vast array of odorants but has only limited discriminatory power.

Vertebrates create an internal representation of the external olfactory world that must translate stimulus features into neural information. Despite the elucidation of a precise spatial map, it has been difficult in vertebrates to discern how this information is decoded to relate the recognition of odors to specific behavioral responses. Genetic analysis of olfactory-driven behavior in invertebrates may ultimately afford a system to understand the mechanistic link between odor recognition and behavior. Insects provide an attractive model system for studying the peripheral and central events in olfaction because they exhibit sophisticated olfactory-driven behaviors under the control of an olfactory sensory system that is significantly simpler anatomically than that of vertebrates (Siddiqi, 1987; Carlson, 1996). Olfactory-based associative learning, for example, is robust in insects and results in discernible modifications in the neural representation of odors in the brain (Faber et al.,

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1998). It may therefore be possible to associate modifications in defined olfactory connections with in vivo paradigms for learning and memory.

Olfactory recognition in the fruit fly *Drosophila* is accomplished by sensory hairs distributed over the surface of the third antennal segment and the maxillary palp. Olfactory neurons within sensory hairs send projections to 1 of 43 glomeruli within the antennal lobe of the brain (Stocker, 1994; Laissue et al., 1999). The glomeruli are innervated by dendrites of the projection neurons, the insect equivalent of the mitral cells in the vertebrate olfactory bulb, whose cell bodies surround the glomeruli. These antennal lobe neurons in turn project to the mushroom body and lateral horn of the protocerebrum (reviewed in Stocker, 1994). 2-deoxyglucose mapping in the fruit fly (Rodrigues, 1988) and calcium imaging in the honeybee (Joerges et al., 1997; Faber et al., 1998) demonstrate that different odorants elicit defined patterns of glomerular activity, suggesting that in insects, as in vertebrates, a topographic map of odor quality is represented in the antennal lobe. However, in the absence of the genes encoding the receptor molecules, it has not been possible to define a physical basis for this spatial map.

In this study, we identify a large family of genes that are likely to encode the odorant receptors of *Drosophila melanogaster*. Difference cloning, along with analysis of *Drosophila* genomic sequences, has led to the identification of a novel family of putative seven transmembrane domain receptors likely to be encoded by 100 to 200 genes within the *Drosophila* genome. Each receptor is expressed in a small subset of sensory cells (0.5%–1.5%) that is spatially defined within the antenna and maxillary palp. Moreover, different neurons express distinct complements of receptor genes such that individual neurons are functionally distinct. Identification of a large family of putative odorant receptors in insects indicates that, as in other species, the diversity and specificity of odor recognition is accommodated by a large family of receptor genes. The identification of the family of putative odorant receptor genes may afford insight into the logic of olfactory perception in *Drosophila*.

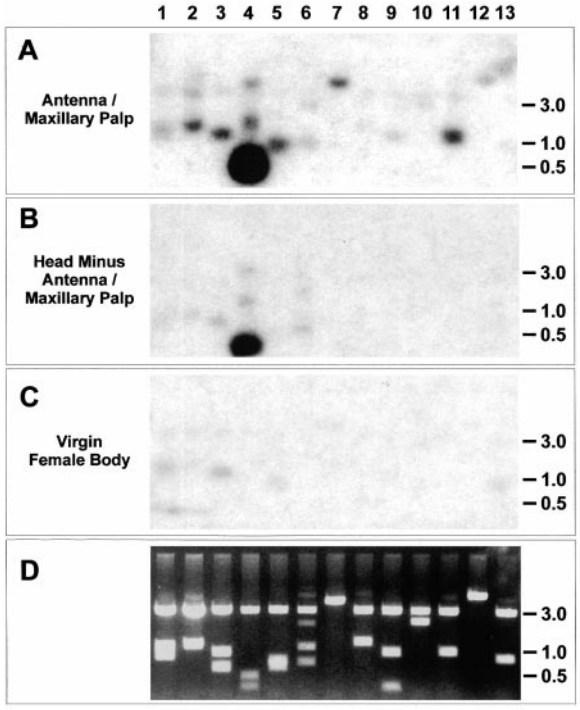

Figure 1. Identification of Rare Antennal- and Maxillary Palp–Specific Genes

Candidate antennal/maxillary palp–specific phage were subjected to in vivo excision, digestion of resulting pBLUESCRIPT plasmid DNAs with BamHI/Asp718, and electrophoresis on 1.5% agarose gels. Southern blots were hybridized with ^{32}P-labeled cDNA probes generated from antennal/maxillary palp mRNA (A), head minus antennal/maxillary palp mRNA (B), or virgin female body mRNA (C). The ethidium bromide–stained gel is shown in (D). Of the 13 clones displayed in this figure, 4 appear to be antennal/maxillary palp–specific (lanes 5, 7, 9, and 11). However, only two are selectively expressed in subsets of cells in chemosensory organs of the adult fly. *dor104*, a putative maxillary palp odorant receptor, is in lane 9. The clone in lane 11 (*RN106*) is homologous to lipoprotein and triglyceride lipases and is expressed in a restricted domain in the antenna (data not shown).

Results

Cloning Candidate Odorant Receptors

In initial experiments, we isolated a cDNA encoding a putative odorant receptor by a difference cloning strategy designed to detect cDNA copies of mRNA present at extremely low frequencies in an mRNA population. In the antenna and maxillary palp, about 30% of the cells are olfactory neurons. If each neuron expressed only 1 of a possible 100 different odorant receptor genes at a level of 0.1% of the mRNA in a sensory neuron, then a given receptor mRNA would be encountered at a frequency of 1 in 300,000 in antennal mRNA. If 100 different receptor genes were expressed, then the entire family of receptor genes would be represented at a frequency of 1 in 3000 mRNAs. We therefore introduced experimental modifications into standard difference cloning to allow for the identification of extremely rare mRNAs whose expression is restricted to either the antenna or the maxillary palp.

Briefly, 5000 inserts from an antennal/maxillary palp cDNA library were prescreened (see Experimental Procedures) and then subjected to Southern blot hybridization with cDNA probes from antennal/maxillary palp, head minus antenna/maxillary palp, or virgin female body mRNA (see Figure 1). This Southern blot hybridization (or reverse Northern) to candidate cDNAs allows for the detection of sequences present at a frequency of 1 in 100,000 in the probe, a sensitivity about 100-fold greater than that of plaque screening (see Experimental Procedures). This procedure led to the identification of multiple antennal/maxillary palp–specific cDNAs that were analyzed by DNA sequencing and in situ hybridization. One cDNA, *dor104* (for *Drosophila odorant receptor*) (Figure 1, lane 9), encodes a putative seven transmembrane domain protein with no obvious sequence similarity to known serpentine receptors (Figure 3). In situ hybridization revealed that this cDNA anneals to

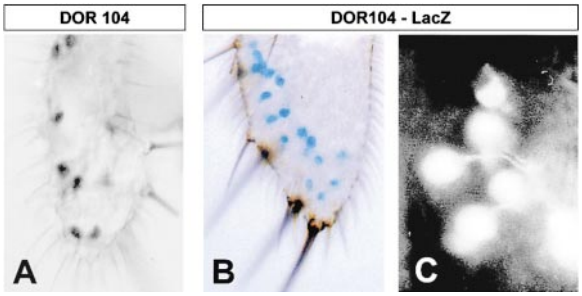

Figure 2. Expression of *dor104* in a Subset of Maxillary Palp Neurons

(A) A frontal section of an adult maxillary palp was hybridized with a digoxigenin-labeled antisense RNA probe and visualized with anti-digoxigenin conjugated to alkaline phosphatase. Seven cells expressing *dor104* are visible in this 15 μm section, which represents about one-third of the diameter of the maxillary palp. Serial sections of multiple maxillary palps were scored for *dor104* expression, and on average, 20 cells per maxillary palp are positive for this receptor.
(B) Transgenic flies carrying a *dor104-lacZ* reporter transgene were stained with X-gal in a whole-mount preparation. Maxillary palps were dissected from the head and viewed in a flattened coverslipped preparation under Nomarski optics, which allows the visualization of all 20 cells expressing *dor104-lacZ*.
(C) Dendrites and axons of neurons expressing *dor104-lacZ* are visible in this horizontal section of a maxillary palp. LacZ expression was visualized with a polyclonal anti-β-galactosidase primary antibody and a CY3-conjugated secondary antibody. Sections were viewed under epifluorescence and photographed on black and white film.

about 15% of the 120 sensory neurons within the maxillary palp but does not anneal with neurons in either the brain or antenna. Seven cells expressing *dor104* are shown in the frontal maxillary palp section in Figure 2A.

These observations suggested that *dor104* might be one member of a larger family of odorant receptor genes within the *Drosophila* genome. However, we were unable to identify additional genes homologous to *dor104* by low stringency hybridization to genomic DNA and cDNA libraries or upon analysis of linked genes in a genomic walk. We therefore analyzed the *Drosophila* genome database for families of multiple transmembrane domain proteins that share sequence similarity with *dor104*. Sequences representing about 10% of the *Drosophila* genome were downloaded (Berkeley Drosophila Genome Project) and subjected to GENSCAN analysis (Burge and Karlin, 1997) to predict the intron–exon structure of all sequences within the database. Open reading frames greater than 50 amino acids were searched for proteins with three or more predicted transmembrane-spanning regions using the dense alignment surface (DAS) and TMAP algorithms (Persson and Argos, 1994; Cserzo et al., 1997; also see Experimental Procedures). Of 229 candidate genes identified in this manner, 11 encoded proteins that define a novel divergent family of presumed seven transmembrane domain proteins with sequence similarity to the *dor104* sequence. This family of candidate odorant receptors does not share any conserved sequence motifs with previously identified families of seven transmembrane domain receptors. cDNA clones containing the coding regions for 5 of the 11 genes identified by GENSCAN analysis have been isolated from an antennal/maxillary palp cDNA library, and their sequences are provided in Figure 3. The remaining six protein sequences derive from GENSCAN predictions for intron–exon arrangement. Their organization conforms well to the actual structure determined from the cDNA sequences of other members of the gene family (Figure 3).

The receptors consist of a short extracellular N-terminal domain (usually less than 50 amino acids) and seven presumed membrane-spanning domains. Analysis of presumed transmembrane domains (Kyte and Doolittle, 1982; Persson and Argos, 1994; Cserzo et al., 1997) reveals multiple hydrophobic segments, but it is not possible from this analysis to determine unequivocally either the number or placement of the membrane-spanning domains. At present, our assignment of transmembrane domains is therefore tentative.

The individual family members are divergent, and most exhibit from 17%–26% amino acid identity. Two linked clusters of receptor genes constitute small subfamilies of genes with significantly greater sequence conservation. Two linked genes, *dor53* and *dor67*, exhibit 76% amino acid identity, whereas the three linked genes *dor71*, *dor72*, and *dor73* reveal 30%–55% identity (Figure 3; see below). Despite the divergence, each of the genes shares short, common motifs in fixed positions within the putative seven transmembrane domain structure that define these sequences as highly divergent members of a novel family of putative receptor molecules.

Expression of the *dor* Gene Family in Olfactory Neurons

If this gene family encodes putative odorant receptors in the fly, we might expect that other members of the family in addition to *dor104* would also be expressed in olfactory sensory neurons. We therefore performed in situ hybridization to examine the pattern of receptor expression of each of the 11 additional members of the gene family in adult and developing organisms. In *Drosophila*, olfactory sensory neurons are restricted to the maxillary palp and third antennal segment. The third antennal segment is covered with approximately 500 fine sensory bristles or sensilla (Stocker, 1994), each containing one to four neurons (Venkatesh and Singh, 1984). The maxillary palp is covered with approximately 60 sensilla, each of which is innervated by two or three neurons (Singh and Nayak, 1985). Thus, the third antennal segment and maxillary palp contain about 1500 and 120 sensory neurons, respectively.

RNA in situ hybridization experiments were performed with digoxigenin-labeled RNA antisense probes to each of the 11 new members of the gene family under conditions of high stringency. One linked pair of homologous genes, *dor53* and *dor67*, cross-hybridizes, whereas the remaining ten genes exhibit no cross-hybridization under these conditions (see below). Eight of the 11 genes hybridize to a small subpopulation (0.5%–1.5%) of the 1500 olfactory sensory neurons in the third antennal segment (Figure 4). One gene, *dor71*, is expressed in about 10% of the sensory neurons in the maxillary palp but not in the antenna (Figure 4G). We have not detected

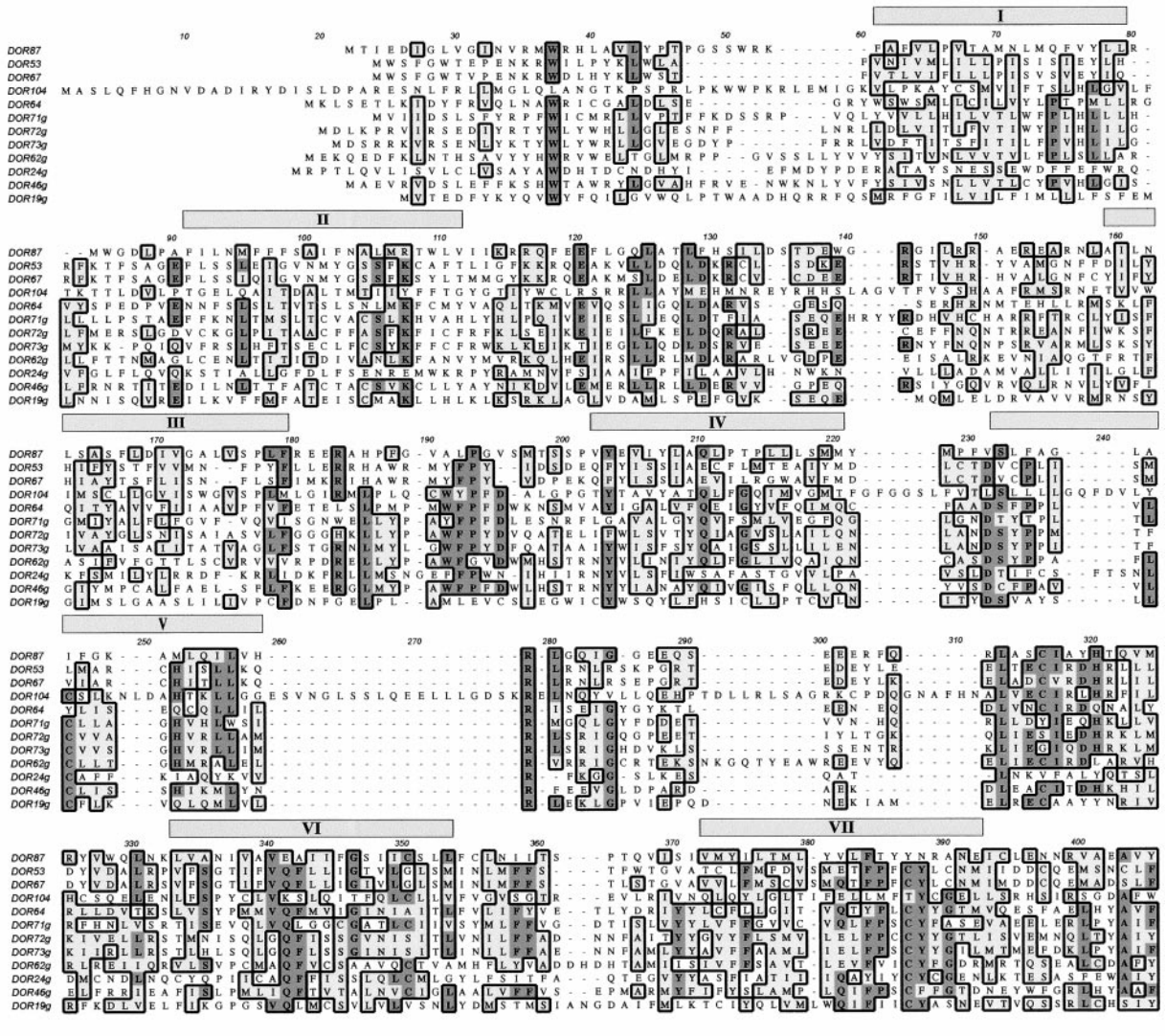

Figure 3. Predicted Amino Acid Sequences of *Drosophila* Odorant Receptor Genes

Deduced amino acid sequences of 12 *dor* genes are aligned using ClustalW (MacVector, Oxford Molecular). Predicted positions of transmembrane regions (I–VII) are indicated by bars above the alignment. Amino acid identities are marked with dark shading, and similarities are indicated with light shading. Protein sequences of *dor87*, *dor53*, *dor67*, *dor104*, and *dor64* were derived from cDNA clones. All others were derived from GENSCAN predictions of intron–exon arrangements in genomic DNA, as indicated by the letter "g" after the gene name. We obtained a partial cDNA clone for *dor62* and found it to be 100% identical to the GENSCAN protein in the region of amino acids 245–381. A 40-amino-acid extension for *dor19* was predicted by GENSCAN analysis. This has been replaced with an asterisk in the alignment, and isolation of cDNA clones for this receptor will resolve whether this extension is physically present in the protein.

expression of *dor46* or *dor19* in the antenna or the maxillary palp. Expression of this gene family is only observed in cells within the antenna and maxillary palp. No hybridization was observed in neurons of the brain, nor was hybridization observed in any section elsewhere in the adult fly or in any tissue at any stage during embryonic development. However, we do find hybridization to a small number of cells in the developing antennae in the late pupal stage (data not shown). We have not yet determined whether this family of receptors is expressed in the larval olfactory apparatus.

Only about one-third of the cells in the third antennal

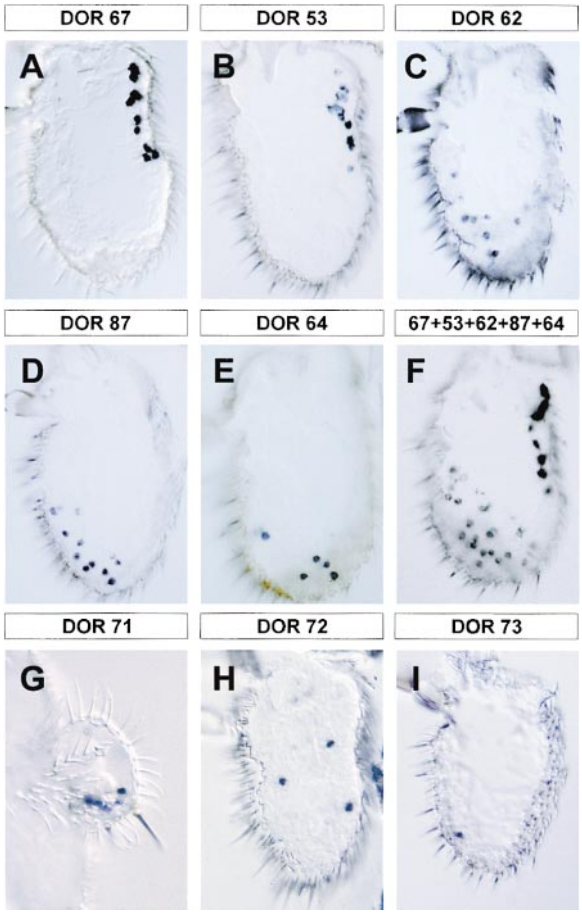

Figure 4. Receptor Gene Expression in Spatially Restricted Regions of the Antenna

Digoxigenin-labeled antisense RNA probes against eight *dor* genes each hybridize to a small number of cells distributed in distinct regions in the antenna. The total number of cells per antenna expressing a given receptor was obtained by counting positive cells in serial sections of multiple antennae. There are approximately 20 positive cells per antenna for *dor67* (A), *dor53* (B), and *dor24* (data not shown), 15 positive cells for *dor62* (C) and *dor87* (D), and 10 positive cells for *dor64* (E). The actual number of cells staining in these sections is a subset of this total number.

With the exception of *dor53* and *dor67*, which strongly cross-hybridize, the receptor genes likely identify different olfactory neurons, such that the number of cells staining with a mixed probe (F) is equal to the sum of those staining with the individual probes (A–E). The mixture of *dor53*, *dor67*, *dor62*, *dor87*, and *dor64* labels a total of about 60 cells per antenna. A total of 34 cells stain with the mixed probe in this 15 μm section.

Expression of the linked genes *dor71*, *dor72*, and *dor73* is shown in (G), (H), and (I), respectively. *dor71* is expressed in approximately ten cells in the maxillary palp. Five positive cells are seen in the horizontal section in (G). We also examined the expression of the other members of this linkage group and found *dor72* in approximately 15 cells (of which 3 label in this section) (H) and *dor73* in 1 to 2 cells per antenna (I).

segment and the maxillary palp are neurons (data not shown), which are interspersed with nonneuronal sensillar support cells and glia. We have performed two experiments to demonstrate that the family of seven transmembrane domain receptor genes is expressed in sensory neurons rather than support cells or glia within the antenna and maxillary palp. First, we developed two-color fluorescent antibody detection schemes to colocalize receptor expression in cells that express the neuron-specific RNA-binding protein ELAV (Robinow and White, 1988). An enhancer trap line carrying an insertion of GAL4 at the *elav* locus expresses high levels of LacZ in neurons when crossed to a transgenic *UAS-lacZ* responder line (Lin and Goodman, 1994). Fluorescent antibody detection of LacZ identifies the sensory neurons in a horizontal section of the maxillary palp (Figure 5B). Hybridization with the receptor probe *dor104* reveals expression in 5 of the 12 LacZ-positive cells in a horizontal section of the maxillary palp (Figure 5A). All cells that express *dor104* are also positive for LacZ (Figure 5C), indicating that this receptor is expressed only in neurons.

In a second experiment, we have demonstrated that the receptor genes are not expressed in nonneuronal cells. The support cells of the antenna express different members of a family of odorant-binding proteins (McKenna et al., 1994; Pikielny et al., 1994; Kim et al., 1998). These genes encode abundant low-molecular-weight proteins thought to transport odorants through the sensillar lymph (reviewed in Pelosi, 1994). Two-color in situ experiments with a probe for the odorant-binding protein PBPRP2 (Pikielny et al., 1994) reveal hybridization to a large number of cells broadly distributed throughout the antenna (Figure 5F). In the same section, however, the probe *dor53* anneals to a nonoverlapping subpopulation of neurons restricted to the medial–proximal domain of the antenna. In a similar experiment, in situ hybridization with the odorant-binding protein OS-F (McKenna et al., 1994) identifies a spatially restricted subpopulation of support cells in the antenna, whereas the *dor67* probe identifies a distinct subpopulation of neurons in a medial–proximal domain (Figure 5G). Thus, the putative odorant receptor genes are expressed in a subpopulation of sensory neurons distinct from the support cells that express the odorant-binding proteins. Taken together, these data demonstrate that 10 of the 12 family members we have identified are expressed in small subpopulations of olfactory sensory neurons in the antenna and maxillary palp.

Spatially Defined Patterns of Receptor Expression

The in situ hybridization experiments reveal that each receptor is expressed in a spatially restricted subpopulation of neurons in the antenna or maxillary palp (Figure 4). The total number of cells expressing each receptor per antenna was obtained by counting the positive cells in serial sections of antennae from multiple flies. These numbers are presented in the legend to Figure 4. *dor67* and *dor53*, for example, anneal to about 20 neurons on the medial–proximal edge of the antenna (Figures 4A and 4B), whereas *dor62* and *dor87* anneal to subpopulations of 20 cells at the distal edge of the antenna (Figures 4C and 4D). Approximately ten cells in the distal domain express *dor64* (Figure 4E). Each of the three linked genes *dor71*, *dor72*, and *dor73* is expressed in different neurons. *dor72* is expressed in approximately 15 antennal

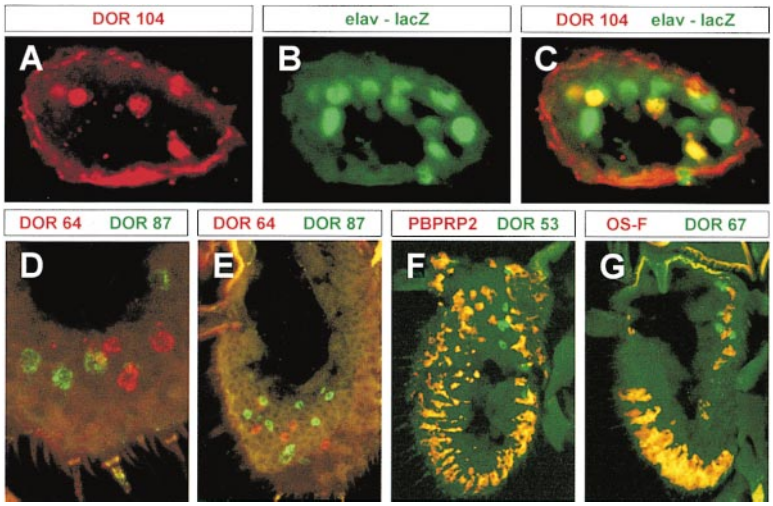

Figure 5. *Drosophila* Odorant Receptors Are Restricted to Distinct Populations of Olfactory Neurons

(A–C) Flies of the *C155 elav-GAL4; UAS-lacZ* genotype express cytoplasmic LacZ in all neuronal cells. (A–C) show confocal images of a horizontal maxillary palp section from such a fly incubated with an antisense RNA probe against *dor104* (red) and anti-β-galactosidase antibody (green). *dor104* recognizes five cells in this maxillary palp section (A), all of which also express *elav-lacZ* (B), as demonstrated by the yellow cells in the merged image in (C).

(D and E) *dor64* and *dor87* are expressed in nonoverlapping neurons at the tip of the antenna. Antisense RNA probes for *dor64* (digoxigenin-RNA; red) and *dor87* (FITC-RNA; green) were annealed to antennal sections and viewed by confocal microscopy. (D) is a digital superimposition of confocal images taken at 0.5 μm intervals through a 10 μm section of the antenna. Cells at different focal planes express both receptors, but no double-labeled cells are found.

(F and G) Two-color RNA in situ hybridization with odorant receptors and odorant-binding proteins demonstrates that these proteins are expressed in different populations of cells. *dor53* (FITC-RNA; green) labels a few cells internal to the cuticle at the proximal–medial edge, while PBPRP2 (digoxigenin-RNA; red) labels a large number of cells apposed to the cuticle throughout the antenna (F). The more restricted odorant-binding protein OS-F (digoxigenin-RNA; red) also stains cells distinct from those expressing *dor67* (FITC-RNA; green) (G).

cells (Figure 4H), while *dor73* is expressed in 1 to 2 cells at the distal edge of the antenna (Figure 4I). In contrast, *dor71* is expressed in approximately ten maxillary palp neurons but is not detected in the antenna (Figure 4G). The three sensillar types are represented in a coarse topographic map across the third antennal segment. The proximal–medial region, for example, contains largely basiconic sensilla. Receptors expressed in this region (*dor53* and *dor67*) are therefore likely to be restricted to the large basiconic sensilla. More distal regions contain a mixture of all three sensilla types, and it is therefore not possible from these data to assign specific receptors to specific sensillar types.

The spatial pattern of neurons expressing a given receptor is conserved between individuals. In situ hybridization with two receptor probes to three individual flies reveals that both the frequency and spatial distributions of the hybridizing neurons are conserved in different individuals (Figure 6). At present, we cannot determine the precision of this topographic map and can only argue that given receptors are expressed in localized domains.

In preliminary experiments, we have demonstrated that the spatial pattern of expression of one receptor, *dor104*, can be recapitulated in transgenic flies with a promoter fragment flanking the *dor104* gene. The fusion of the presumed *dor104* promoter (consisting of 3 kb of 5′ DNA immediately adjacent to the coding region) to the *lacZ* reporter gene has allowed us to visualize a subpopulation of neurons expressing *dor104* within the maxillary palp. Whole-mount preparations of the heads of transgenic flies reveal a small subpopulation of sensory neurons within the maxillary palp whose cell bodies exhibit blue color after staining with X-gal (Figure 2B). The number of positive cells, approximately 20 per maxillary palp, corresponds well with that seen for *dor104* expression. Immunofluorescent staining of sections with antibodies directed against β-galactosidase more clearly reveals the dendrites and axons of these bipolar neurons in the maxillary palp (Figure 2C). Levels of LacZ expression in these transgenic lines are low, and further amplification will be necessary to allow us to trace the axons to glomeruli in the antennal lobe. Nonetheless, the data suggest that the information governing the spatial pattern of *dor104* expression in a restricted subpopulation of maxillary palp neurons resides within 3 kb of DNA 5′ to the *dor104* gene.

Individual Neurons Express Different Complements of Receptors

An understanding of the logic of olfactory discrimination in *Drosophila* will require a determination of the diversity and specificity of receptor expression in individual neurons. In the vertebrate olfactory epithelium, a given neuron is likely to express only one receptor from the family of 1000 genes (Ngai et al., 1993; Ressler et al., 1993; Vassar et al., 1993; Chess et al., 1994; C. Dulac and R. A., unpublished). In the nematode *C. elegans*, however, individual chemosensory neurons are thought to express multiple receptor genes (Troemel et al., 1995). Our observations with the putative *Drosophila* odorant receptors indicate that a given receptor probe anneals with 0.5%–1.5% of antennal neurons, suggesting that each cell expresses only a subset of receptor genes. If we demonstrate that each of the different receptor probes hybridizes with distinct, nonoverlapping subpopulations of neurons, this would provide evidence that neurons differ with respect to the receptors they express.

In situ hybridization was therefore performed with either a mix of five receptor probes (Figure 4F) or individually with each of the five probes (Figures 4A–4E). We observe that the number of olfactory neurons identified with the mixed probe (about 60 per antenna) approximates the sum of the positive neurons detected with the five individual probes. These results demonstrate

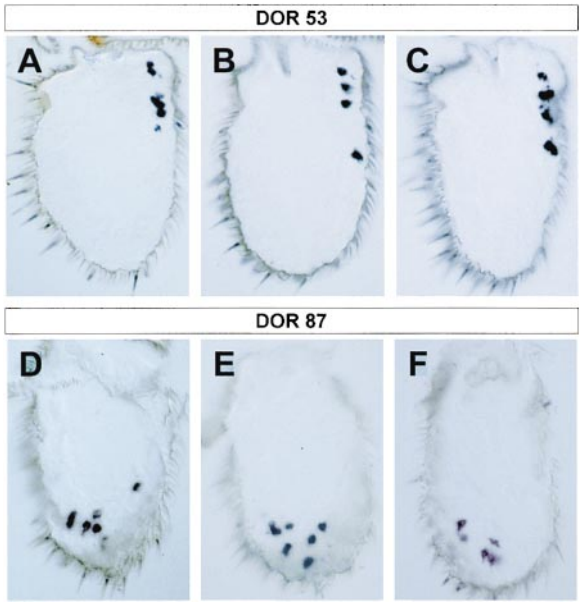

Figure 6. Receptor Expression Is Conserved between Individuals

Frontal sections of antennae from six different individuals were hybridized with digoxigenin-labeled antisense RNA probes against *dor53* (A–C) or *dor87* (D–F). *dor53* labels approximately 20 cells on the proximal–medial edge of the antenna, of which approximately 5 are shown labeling in these sections. *dor87* is expressed in about the same number of cells at the distal tip. Both the position and number of staining cells is conserved between different individuals and is not sexually dimorphic.

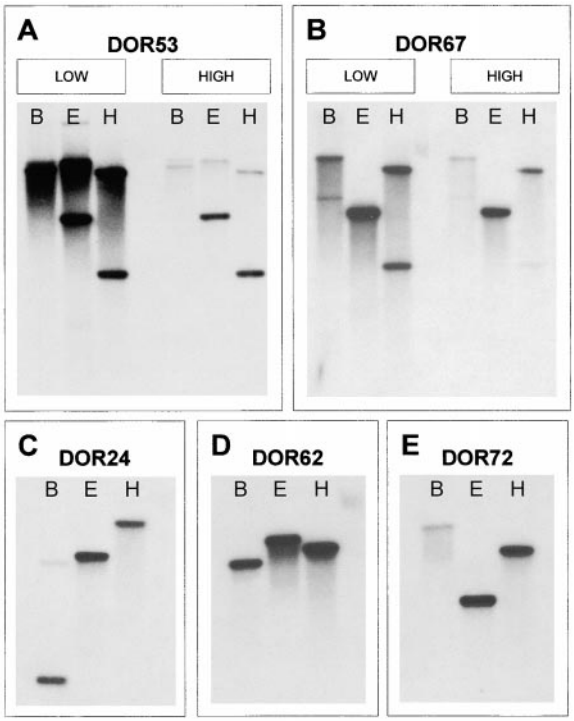

Figure 7. *Drosophila* Odorant Receptors Are Highly Divergent

Oregon R genomic DNA isolated from whole flies was digested with BamHI (B), EcoRI (E), or HindIII (H), electrophoresed on 0.8% agarose gels, and blotted to nitrocellulose membranes. Blots were annealed with ^{32}P-labeled probes derived from *dor53* cDNA (A), *dor67* cDNA (B), or DNA fragments generated by RT-PCR from antennal mRNA for *dor24* (C), *dor62* (D), and *dor72* (E). Strong cross-hybridization of *dor53* and *dor67* is seen at both high and low stringency (A and B), while *dor24*, *dor62*, and *dor72* reveal only a single hybridizing band in each lane at both low stringency (C–E) and high stringency (data not shown).

that individual receptors are expressed in distinct nonoverlapping populations of olfactory neurons.

We have performed an additional experiment using two-color RNA in situ hybridization to ask whether two receptor genes, *dor64* and *dor87*, expressed in interspersed cells in the distal antenna are expressed in different neurons. Antisense RNA probes for the two genes were labeled with either digoxigenin- or FITC-UTP and were used in pairwise combinations in in situ hybridization to sections through the *Drosophila* antenna. Although these two genes are expressed in overlapping lateral–distal domains, two-color in situ hybridization reveals that neurons expressing *dor64* do not express *dor87*; rather, each gene is expressed in distinct cell populations (Figures 5D and 5E). Taken together, these data suggest that olfactory sensory neurons within the antenna are functionally distinct and express different complements of odorant receptors. At the extreme, the experiments are consistent with a model in which individual neurons express only a single receptor gene.

Our differential cloning procedure identified one additional gene, *A45*, which shares weak identity (24%) with the *dor* gene family over a short region (93 amino acids). This gene, however, does not appear to be a classical member of the *dor* family: it is far more divergent and significantly larger than the other family members (486 amino acids). This gene is expressed in all olfactory sensory neurons (data not shown). If *A45* does encode a divergent odorant receptor, then it would be present in all sensory neurons along with different complements of the more classical members of the *dor* gene family.

The Size and Organization of the Odorant Receptor Gene Family

How large is the family of odorant receptor genes in *Drosophila*? Unlike vertebrate odorant receptors, which share 40%–98% sequence identity at the amino acid level, the fly receptors are extremely divergent. The extent of sequence similarity between receptor subfamilies ranges from 20%–30%. The maxillary palp receptor *dor104* is the most distantly related member of the family, with about 17% identity to the other receptor genes. Inspection of the receptor sequences suggests that Southern blot hybridizations, even those performed at low stringency, are unlikely to reveal multiple additional members of a gene family. In accord with this, Southern blot hybridization with receptor probes *dor24*, *dor62*, and *dor72*, performed at either high or low stringency, reveals only a single hybridizing band following cleavage of genomic DNA with three different restriction endonucleases (Figures 7C–7E). The two linked clusters of receptors contain genes with a greater degree of sequence conservation and define small subfamilies of receptor genes. A cluster of three receptors, *dor71*, *dor72*, and *dor73*, is located at map position 33B1-2. The antennal receptors *dor72* and *dor73* are 55% identical, and both exhibit about 30% identity to the third

gene at the locus, *dor71*, which is expressed in the maxillary palp. *dor67* and *dor53*, members of a second subfamily, reside within 1 kb of each other at map position 22A2-3 and exhibit 76% sequence identity. Not surprisingly, these two linked genes cross-hybridize at low stringency. Southern blots probed with either *dor67* or *dor53* reveal two hybridizing bands corresponding to the two genes within the subfamily but fail to detect additional subfamily members in the chromosome (Figures 7A and 7B).

The members of the receptor gene family described here are present on all but the small fourth chromosome. No bias is observed toward telomeric or centromeric regions. The map positions, as determined from P1 and cosmid clones (Berkeley Drosophila Genome Project; European Drosophila Genome Project) are provided in the Experimental Procedures. A comparatively large number of receptor genes map to chromosome 2 because the Berkeley Drosophila Genome Project has concentrated its efforts on this chromosome. Unlike the distribution of odorant receptors in nematodes and mammals (Ben-Arie et al., 1994; Troemel et al., 1995; Robertson, 1998), only small linked arrays have been identified, and the majority of the family members are isolated at multiple, scattered loci in the *Drosophila* genome.

The high degree of divergence among members of the *Drosophila* odorant receptor gene family is more reminiscent of the family of chemoreceptors in *C. elegans* than the more highly conserved odorant receptors of vertebrates. Estimates of the size of the *Drosophila* receptor gene family therefore cannot be obtained by either Southern blot hybridization or PCR analysis of genomic DNA. Rather, our estimates of the gene family derive from the statistics of small numbers. We identified 11 members of the odorant receptor gene family in a *Drosophila* genome database that includes roughly 10% of the genome. Recognizing a possible bias in our estimate, it seems reasonable at present to estimate that the odorant receptor family is likely to include 100 to 200 genes. This is in accord with independent estimates from in situ hybridization experiments that demonstrate that a given receptor probe hybridizes with 0.5%–1.5% of the neurons. If we assume that a given neuron expresses only a single receptor gene, these observations suggest that the gene family would include 100 to 200 members.

Discussion

The Size and Divergence of the Gene Family

We have identified a novel family of seven transmembrane domain proteins that is likely to encode the *Drosophila* odorant receptors. The number of different receptor genes expressed in the neurons of the antenna and maxillary palp will reflect the diversity and specificity of odor recognition in the fruit fly. How large is the *Drosophila* odorant receptor gene family? We have identified 11 members of this divergent gene family in the *Drosophila* genome database. The potential for bias notwithstanding, it seems reasonable to assume then, that since only 10% of the *Drosophila* genome has been sequenced, this gene family is likely to contain 100 to 200 genes. However, significant errors in our estimates could result from bias in the nature of the sequences represented in the 10% of the *Drosophila* genome analyzed to date. In situ hybridization experiments demonstrating that each of the receptor genes labels from 0.5%–1.5% of the olfactory sensory neurons are in accord with the estimate of 100 to 200 receptor genes.

Several divergent odorant receptor gene families, each encoding seven transmembrane proteins, have been identified in vertebrate and invertebrate species. In mammals, volatile odorants are detected by a family of as many as 1000 receptors, each expressed in the main olfactory epithelium (Buck and Axel, 1991; Levy et al., 1991; Parmentier et al., 1992; Ben-Arie et al., 1994). This gene family shares features with the serpentine neurotransmitter receptors and is conserved in all vertebrates examined. Terrestrial vertebrates have a second anatomically and functionally distinct olfactory system, the vomeronasal organ, dedicated to the detection of pheromones. Vomeronasal sensory neurons express two distinct families of receptors, each thought to contain 100 to 200 genes: one novel family of serpentine receptors (Dulac and Axel, 1995), and a second related to the metabotropic neurotransmitter receptors (Herrada and Dulac, 1997; Matsunami and Buck, 1997; Ryba and Tirindelli, 1997).

In the invertebrate *C. elegans*, chemosensory receptors are organized into four gene families that share 20%–40% sequence similarity within a family and essentially no sequence similarity between families (Troemel et al., 1995; Sengupta et al., 1996; Robertson, 1998). The four gene families in *C. elegans* together contain about 1000 genes engaged in the detection of odors. The nematode receptors exhibit no sequence conservation with the three distinct families of vertebrate odorant receptor genes. Our studies reveal that *Drosophila* has evolved an additional divergent gene family of serpentine receptors comprised of 100 to 200 genes. The observation that a similar function, chemosensory detection, is accomplished by at least eight highly divergent gene families sharing little or no sequence similarity is quite unusual.

Why is the evolutionary requirement for odorant receptors so often met by recruitment of novel gene families rather than exploiting preexisting odorant receptor families in ancestral genomes? The character of natural odorants along with their physical properties (e.g., aqueous or volatile) represent important selectors governing the evolution of receptor gene families. The use of common “anthropomorphic” odorant sets in the experimental analysis of olfactory specificity has led to the prevailing view that significant overlap exists in the repertoire of perceived odors between different species. Studies of odorant specificity in different species often employ odors at artificially high concentrations and may present an inaccurate image of the natural repertoire of odorants. We simply do not know the nature of the odors that initially led to the ancestral choice of receptor genes during the evolution of the nematode, insect, or vertebrate species. Clearly, vastly different properties in salient odors could dictate the recruitment of new gene families to effect an old function, olfaction. The character of the odor is not the only evolutionary selector. Odorant

receptors must interact with other components in the signal transduction pathway (G proteins [for review see Buck, 1996; Bargmann and Kaplan, 1998] and perhaps even RAMPs [McLatchie et al., 1998] and rho [Mitchell et al., 1998]) that may govern the choice of one family of serpentine receptors over another. Moreover, mammalian receptors not only recognize odorants in the environment but are likely to recognize guidance cues governing formation of a sensory map in the brain (Wang et al., 1998). Thus, the multiple properties required of the odorant receptors might change vastly over evolutionary time, and this might underlie the independent origins of the multiple chemosensory receptor gene families.

Establishing a Topographic Map in the Antenna and the Brain

We observe that individual receptor genes in the fly are expressed in topographically conserved domains within the antenna. This highly ordered spatial distribution of receptor expression differs from that observed in the mammalian olfactory epithelium. In mammals, a given receptor can be expressed in one of four broad but circumscribed zones in the main olfactory epithelium (Ressler et al., 1993; Vassar et al., 1993). A given zone can express up to 250 different receptors, and neurons expressing a given receptor within a zone appear to be randomly dispersed (Ressler et al., 1993; Vassar et al., 1993). The highly ordered pattern of expression observed in the *Drosophila* antenna might have important implications for patterning the projections to the antennal lobe. In visual, somatosensory, and auditory systems, the peripheral receptor sheet is highly ordered and neighbor relations in the periphery are maintained in the projections to the brain. These observations suggest that the relative position of the sensory neuron in the periphery will determine the pattern of projections to the brain.

Our data on the spatial conservation of receptor expression in the antenna suggest that superimposed upon coarse spatial patterning of olfactory sensilla (Venkatesh and Singh, 1984; Ray and Rodrigues, 1995; Reddy et al., 1997) must be more precise positional information governing the choice of receptor expression. This spatial information might dictate the fixed topographic pattern of receptor expression in the peripheral receptor sheet and at the same time govern the ordered sensory projections to the brain. This relationship between positional identity and the pattern of neuronal projections has been suggested for both peripheral sensory neurons (Merritt and Whitington, 1995; Grillenzoni et al., 1998) and neurons in the embryonic central nervous system of *Drosophila* (Doe and Skeath, 1996).

Implications for Sensory Processing

In mammals, olfactory neurons express only 1 of the 1000 odorant receptor genes. Neurons expressing a given receptor project with precision to 2 of the 1800 glomeruli in the mouse olfactory bulb. Odorants will therefore elicit spatially defined patterns of glomerular activity such that the quality of an olfactory stimulus is encoded by the activation of a specific combination of glomeruli (Stewart et al., 1979; Lancet et al., 1982; Kauer et al., 1987; Imamura et al., 1992; Mori et al., 1992; Katoh et al., 1993; Friedrich and Korsching, 1997). Moreover, the ability of an odorant to activate a combination of glomeruli allows for the discrimination of a diverse array of odors far exceeding the number of receptors and their associated glomeruli. In the nematode, an equally large family of receptor genes is expressed in 16 pairs of chemosensory cells, only 3 of which respond to volatile odorants (Bargmann and Horvitz, 1991; Bargmann et al., 1993). This immediately implies that a given chemosensory neuron will express multiple receptors and that the diversity of odors recognized by the nematode might approach that of mammals, but the discriminatory power is necessarily dramatically reduced.

What does the character of the gene family we have identified in *Drosophila* tell us about the logic of olfactory processing in this organism? We estimate that the *Drosophila* odorant receptors comprise a family of 100 to 200 genes. Moreover, the pattern of expression of these genes in the third antennal segment suggests that individual sensory neurons express a different complement of receptors, and at the extreme, our data are consistent with the suggestion that individual neurons express one or a small number of receptors. As in the case of mammals, the problem of odor discrimination therefore reduces to a problem of the brain discerning which receptors have been activated by a given odorant. If the number of different types of neurons exceeds the number of glomeruli (43) (Stocker, 1994; Laissue et al., 1999), it immediately follows that a given glomerulus must receive input from more than one kind of sensory neuron. This implies that a single glomerulus will integrate multiple olfactory stimuli. One possible consequence of this model would be a loss of discriminatory power while maintaining the ability to recognize a vast array of odors. Alternatively, significant processing of sensory input may occur in the fly antennal lobe to afford discrimination commensurate with the large number of receptors.

This model of olfactory coding is in sharp contrast with the main olfactory system of vertebrates in which sensory neurons express only a single receptor and converge on only a single pair of spatially fixed glomeruli in the olfactory bulb. Moreover, each projection neuron in the mammalian bulb extends its dendrite to only a single glomerulus. Thus, the integration and decoding of spatial patterns of glomerular activity in vertebrates must occur largely in the olfactory cortex. In the fruit fly, the observation that the number of receptors may exceed the number of glomeruli suggests that individual glomeruli will receive input from more than one type of sensory neuron. A second level of integration in the antennal lobe is afforded by subsets of projection neurons that elaborate extensive dendritic arbors that synapse with multiple glomeruli. Thus, the *Drosophila* olfactory system reveals levels of processing and integration of sensory input in the antennal lobe that is likely to be restricted to higher cortical centers in the main olfactory system of vertebrates.

Experimental Procedures

Experimental Animals

Oregon R flies (*Drosophila melanogaster*) were raised on standard cornmeal-agar-molasses medium at 25°C. Transgenic constructs

were injected into *yw* embryos. *C155 elav-GAL4* flies were obtained from Corey Goodman (Lin and Goodman, 1994), and Gary Struhl provided the *UAS- (cytoplasmic) lacZ* stock.

Preparation and Differential Screening of a *Drosophila* Antennal/Maxillary Palp cDNA Library

Drosophila antennae and maxillary palps were obtained by manually decapitating and freezing 5000 adult flies and shaking antennae and maxillary palps through a fine metal sieve. mRNA was prepared using a polyA+ RNA Purification Kit (Stratagene). An antennal/maxillary palp cDNA library was made from 0.5 μg mRNA using the LambdaZAPIIXR kit from Stratagene.

Briefly, phage were plated at low density (500–1000 pfu/150 mm plate) and UV-cross-linked after lifting in triplicate to Hybond-N+ (Amersham). Complex probes were generated by random primed labeling (PrimeItII, Stratagene) of reverse transcribed mRNA (RT-PCR kit, Stratagene) from virgin adult female body mRNA and duplicate lifts hybridized at high stringency for 36 hr (65°C in 0.5 M sodium phosphate buffer [pH 7.3] containing 1% bovine serum albumin, 4% SDS, and 0.5 mg/ml herring sperm DNA). We prescreened the third lift with a mix of all previously cloned OBPs/PBPs (McKenna et al., 1994; Pikielny et al., 1994; Kim et al., 1998) to remove a source of abundant but undesired olfactory-specific clones. Approximately 5000 individual OBP/PBP and virgin female body negative phage clones were isolated and their inserts amplified by PCR with T3 and T7 primers, and approximately 3 μg of DNA was electrophoresed on 1.5% agarose gels. Gels were blotted to Hybond-N+ (Amersham), filters were UV-cross-linked, and the resulting Southern blots were subjected to reverse Northern analysis using complex probes generated from virgin female body mRNA. Approximately 500 clones not hybridizing with virgin female body probes were identified and consolidated onto secondary Southern blots in triplicate. These blots were probed with cDNA probes derived from antennal/maxillary palp, head minus antenna/maxillary palp, and virgin female body mRNA. A total of 210 clones negative with head minus antenna/maxillary palp and virgin female body probes and strongly positive, weakly positive, or negative with antennal/maxillary palp probes were further analyzed by sequencing and in situ hybridization.

Analysis of *Drosophila* Genome Project Sequences for Transmembrane Proteins

All *Drosophila* genomic sequences were batch downloaded in April 1998 from the Berkeley Drosophila Genome Project (Berkeley Drosophila Genome Project, unpublished). Genomic P1 sequences were first analyzed with the GENSCAN program (Burge and Karlin, 1997; http://CCR-081.mit.edu/GENSCAN.html), which predicts intron–exon structures and generates hypothetical coding sequences (CDS) and open reading frames. GENSCAN-predicted proteins shorter than 50 amino acids were discarded. The remaining open reading frames were used to search for putative transmembrane regions greater than 15 amino acids with two programs that were obtained from the authors and used in stand-alone mode locally (see Persson and Argos, 1994; Cserzo et al., 1997). The Dense Surface Alignment (DAS) program is available at http://www.biokemi.su.se/~server/DAS/ or from M. Cserzo (miklos@pugh.bip.bham.ac.uk). TMAP is available at ftp://ftp.ebi.ac.uk/pub/software/unix/ or by contacting the author, Bengt Persson (bpn@mbb.ki.se). Scripts were written to apply the DAS and TMAP programs repeatedly to genome scale sequence sets. Genes showing significant sequence similarity to the NCBI nonredundant protein database using BLAST analysis (Altschul et al., 1990, 1997) were eliminated. All scripts required for these computations were written in standard ANSI C and run on a SUN Enterprise 3000.

Of 229 novel *Drosophila* proteins with three or more predicted transmembrane-spanning regions, 35 showed no clear sequence similarity to any known protein and were selected for further analysis by in situ hybridization. Probes for in situ hybridization were generated by RT-PCR using antennal/maxillary palp mRNA as a template.

Map Positions of *dor* Genes

The chromosome position of *dor104* was determined by in situ hybridization of a biotin-labeled probe to salivary gland polytene chromosome squashes as described (Amrein et al., 1988).

Chromosomal positions of all other *dor* genes were based on chromosome assignments of the P1 clones to which they map, as determined by the Berkeley Drosophila Genome Project (personal communication; http://www.fruitfly.org; see also Hartl et al., 1994; Kimmerly et al., 1996). *dor62* maps to a cosmid sequenced by the European Drosophila Genome Project (unpublished; http://edgp.ebi.ac.uk/; Siden-Kiamos et al., 1990). The following are the map positions and P1/cosmid clone accession numbers of the *dor* genes: *dor62* [(X)2F; 62D9 (EDGP cosmid)]; *dor67* [(2L)22A3, DS00676]; *dor53* [(2L)22A2-3; DS05342]; *dor64* [(2L)23A1-2; DS06400]; *dor71* [(2L)33B1-2; DS07071]; *dor72* [(2L)33B1-2; DS07071]; *dor73* [(2L)33B1-2; DS07071]; *dor87* [(2R)43B1-2; DS08779]; *dor19* [(2R)46F5-6; DS01913]; *dor24* [(2R)47D6-E2; DS00724]; *dor46* [(2R)59D5-7; DS07462]; and *dor104* [(3L)85B].

The Isolation of *dor* cDNA Clones and Southern Blotting

We screened 3×10^6 clones of the antennal/maxillary palp library described above with PCR probes for the genes *dor87*, *dor53*, *dor67*, *dor64*, and *dor62*. cDNAs were present at a frequency ranging from 1:200,000 (*dor67*) to 1:1,000,000 (*dor62*) in the library, and their sequences were remarkably similar to the hypothetical CDS predicted by the GENSCAN program. The frequency of these genes is similar to that of *dor104*, which is present at 1:125,000 in the antennal/maxillary palp library. All sequencing was with ABI cycle sequencing kits, and reactions were run on ABI 310 or 377 sequencing systems.

Five micrograms of Oregon R genomic DNA isolated from whole flies were digested with BamHI, EcoRI, or HindIII, electrophoresed on 0.8% agarose gels, and blotted to Nitropure nitrocellulose membranes (Micron Separations Inc.). Blots were baked and annealed with ^{32}P-labeled probes derived from cDNA probes of *dor53* and *dor67* or PCR fragments from *dor24*, *dor62*, and *dor72*. Hybridization was at 42°C for 36 hr in 5×SSCP, 10× Denhardts, 500 μg/ml herring sperm DNA, and either 50% (high stringency) or 25% (low stringency) formamide (Sambrook et al., 1989). Blots were washed for 1 hr in 0.2×SSC, 0.5% SDS at 65°C (high stringency), or 1×SSC, 0.5% SDS at 42°C (low stringency).

In Situ Hybridization

RNA in situ hybridization was carried out essentially as described (Schaeren-Wiemers and Gerfin-Moser, 1993). This protocol was modified to include detergents in most steps to increase sensitivity and reduce background. The hybridization buffer contained 50% formamide, 5×SSC, 5× Denhardts, 250 μg/ml yeast tRNA, 500 μg/ml herring sperm DNA, 50 μg/ml heparin, 2.5 mM EDTA, 0.1% Tween-20, and 0.25% CHAPS. All antibody steps were in the presence of 0.1% Triton X-100, and the reaction was developed in buffer containing 0.1% Tween-20. Slides were mounted in Glycergel (DAKO) and viewed with Nomarski optics.

Fluorescent in situ hybridization was carried out as above with either digoxigenin- or FITC-labeled RNA probes. The digoxigenin probe was visualized with sheep anti-digoxigenin (Boehringer) followed by donkey anti-sheep CY3 (Jackson). FITC probes were visualized with mouse anti-FITC (Boehringer) and goat anti-mouse Alexa 488 (Molecular Probes) following preincubation with normal goat serum. Sections were mounted in Vectashield reagent (Vector Labs) and viewed on a Biorad 1024 confocal microscope.

For double labeling with a neuronal marker, animals of the genotype *C155 elav-Gal4; UAS-lacZ* were sectioned and first hybridized with a digoxigenin-labeled antisense *dor104* RNA probe and developed as described above. Neuron-specific expression of LacZ driven by the *elav-Gal4* enhancer trap was visualized with a polyclonal rabbit anti-β-galactosidase antibody (Organon-Technika/Cappel), visualized by a goat anti-rabbit Alexa 488–conjugated secondary antibody (Molecular Probes), following preincubation with normal goat serum.

The proportion of neurons in the third antennal segment was calculated by comparing the number of nuclei staining with the 44C11 ELAV monoclonal antibody (kindly provided by Lily Jan) and those staining with TOTO-3 (Molecular Probes), a nucleic acid counterstain, in several confocal sections of multiple antennae. On average, 36% of the nuclei in the antenna were ELAV positive.

dor104-lacZ Transgene Construction and Histochemical Staining

A genomic clone containing the *dor104* coding region and several kilobases of upstream sequence was isolated from a genomic library prepared from flies isogenic for the third chromosome (a gift of Kevin Moses and Gerry Rubin). Approximately 3 kb of DNA immediately upstream of the putative translation start site of *dor104* was isolated by PCR and subcloned into the pCasperAUGβGal vector (Thummel et al., 1988). β-galactosidase activity staining was carried out with whole-mount head preparations essentially as described in Wang et al. (1998). Frozen sections of *dor104-lacZ* maxillary palps were incubated with a polyclonal rabbit anti-β-galactosidase antibody and visualized as described above.

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GenBank Accession Numbers

The accession numbers for the cDNA sequences reported in this paper are AF127921 (*dor62*), AF127922 (*dor104*), AF127923 (*dor53*), AF127924 (*dor67*), AF127925 (*dor64*), and AF127926 (*dor87*). Receptor sequences derived solely from GENSCAN analysis are available at http://cpmcnet.columbia.edu/dept/neurobeh/axel/dorg.html.

Note Added in Proof

While this paper was in press, Clyne et al. (1999) reported a gene family identical to that described in this paper, whose members are expressed in olfactory sensory neurons: Clyne, P.J., Warr, C.G., Freeman, M.R., Lessing, D., Kim, J., and Carlson, J.R. (1999). A novel family of divergent seven-transmembrane proteins: candidate odorant receptors in *Drosophila*. Neuron *22*, 327–338.