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The Molecular Logic of Olfaction in *Drosophila*

Leslie B. Vosshall

Center for Neurobiology and Behavior, Columbia University, 701 West 168th Street, New York, NY 10032, USA

Correspondence to be sent to current address: Leslie B. Vosshall, Laboratory of Neurogenetics and Behavior, Rockefeller University, 1230 York Avenue, Box 63, New York, NY 10021, USA. e-mail: leslie@mail.rockefeller.edu

Abstract

Drosophila fruit flies display robust olfactory-driven behaviors with an olfactory system far simpler than that of vertebrates. Endowed with ~1300 olfactory receptor neurons, these insects are able to recognize and discriminate between a large number of distinct odorants. Candidate odorant receptor molecules were identified by complimentary approaches of differential cloning and genome analysis. The *Drosophila* odorant receptor (*DOR*) genes encode a novel family of proteins with seven predicted membrane-spanning domains, unrelated to vertebrate or nematode chemosensory receptors. There are on the order of 60 or more members of this gene family in the *Drosophila* genome, far fewer than the hundreds to thousands of receptors found in vertebrates or nematodes. *DOR* genes are selectively expressed in small subsets of olfactory neurons, in expression domains that are spatially conserved between individuals, bilaterally symmetric and not sexually dimorphic. Double *in situ* RNA hybridization with a number of pairwise combinations of *DOR* genes fails to reveal any overlap in gene expression, suggesting that each olfactory neuron expresses one or a small number of receptor genes and is therefore functionally distinct. How is activation of such a subpopulation of olfactory receptor neurons in the periphery sensed by the brain? In the mouse, all neurons expressing a given receptor project with precision to two of 1800 olfactory bulb glomeruli, creating a spatial map of odor quality in the brain. We have employed *DOR* promoter transgenes that recapitulate expression of endogenous receptor to visualize the projections of individual populations of receptor neurons to subsets of the 43 glomeruli in the *Drosophila* antennal lobe. The results suggest functional conservation in the logic of olfactory discrimination from insects to mammals.

Introduction

The olfactory sensory system permits animals to recognize and discriminate between a broad spectrum of volatile chemicals in the environment. Olfactory perception translates abstract chemical features of the odorous ligand into meaningful neural information, eliciting appropriate behavioral responses (Shepherd, 1994; Buck, 1996). The relative attractiveness or repulsiveness of an odorant is highly species-specific, and can be further modulated by experience. The initial events in odor recognition are mediated by specialized bipolar olfactory sensory neurons with ciliated or microvillar dendrites that are exposed to the exterior olfactory environment. The olfactory neuron extends a single axon to the central nervous system, where it forms synapses with second-order projection neurons (Shepherd, 1994; Stocker, 1994; Buck, 1996). In arthropods and mammals, the first olfactory synapse is organized into glomeruli, spherical synaptic specializations composed of afferent olfactory neuron axons and projection neuron dendrites (Hildebrand and Shepherd, 1997).

For insects, olfaction is an important sensory modality in locating food sources, identifying appropriate sites for oviposition, selecting mates and avoiding predators. The fruit fly, *Drosophila melanogaster*, is a powerful model organism for the investigation of mechanisms in odor coding because it has an olfactory system that is anatomically similar to but simpler than that of vertebrates. *Drosophila* has the additional advantage that it can be manipulated genetically, the sequence of its euchromatic genome has been completed, the development and anatomy of its olfactory system are well characterized, and simple behavior paradigms have been developed to test olfactory perception (Siddiqi, 1987; Tully, 1987; Carlson, 1996; Adams *et al.*, 2000).

Identification of the family of *Drosophila* odorant receptor (*DOR*) genes

In diverse species, a large family of odorant receptor genes, each encoding a different seven-transmembrane domain G protein-coupled receptor, mediates molecular recognition of thousands of distinct odorants. The first candidate odorant receptor genes were identified in the rat by a degenerate polymerase chain reaction approach using primers capable of identifying members of the G protein-coupled receptor superfamily (Buck and Axel, 1991). Mammalian odorant receptor genes are a distinct, extremely large subfamily of

this receptor superfamily which couple to G proteins and transduce signals across cellular membranes. Subsequently, odorant receptor genes were identified in other mammals, fish and birds using homology-based approaches with the rat sequences as a reference point (Ngai *et al.*, 1993; Ben-Arie *et al.*, 1994; Nef *et al.*, 1996; Mombaerts, 1999).

Caenorhabditis elegans chemosensory receptor genes also encode seven-transmembrane domain proteins, but these have no primary sequence identity with the vertebrate odorant receptors. Nematode odorant receptor genes were identified by molecular genetic analysis of chemosensory mutants and searching of *C. elegans* genome sequence databases for novel seven-transmembrane domain proteins (Troemel *et al.*, 1995; Sengupta *et al.*, 1996).

In both vertebrates and in nematodes, odorant receptor genes have the following properties: (i) they encode G protein-coupled seven-transmembrane domain receptors; (ii) they are selectively expressed in olfactory receptor neurons; and (iii) in each species they constitute a very large gene family of 100–1000 odorant receptors, and these are often linked in large arrays in the genome.

Candidate *DOR* genes were identified by a combination of difference cloning (Vosshall *et al.*, 1999) and analysis of *Drosophila* genomic sequence databases (Clyne *et al.*, 1999b; Gao and Chess, 1999; Vosshall *et al.*, 1999). An extensive screen for genes selectively expressed in adult olfactory sensory organs (the antenna and maxillary palp) yielded a single clone [*DOR104*; recently renamed *Or85e* (*Drosophila* Odorant Receptor Nomenclature Committee, 2000)] that encodes a putative odorant receptor, with seven transmembrane domains and no homology to any known gene. *Or85e* was found to be expressed in ~20 of the 120 olfactory neurons of the maxillary palp (Vosshall *et al.*, 1999). Attempts to identify additional members of the *DOR* gene family by conventional molecular biology techniques that would yield either physically linked or closely related genes to *Or85e* were unsuccessful. However, analysis of *Drosophila* genome databases by our group and others revealed 18 additional genes with significant sequence relatedness to *Or85e*, many of which are selectively expressed in olfactory receptor neurons in the adult fly (Clyne *et al.*, 1999b; Gao and Chess, 1999; Vosshall *et al.*, 1999). The completion of the euchromatic genome sequence has permitted an analysis of the complete repertoire of *DOR* genes. The fly genome has a total of 60 genes with homology to the *DOR* gene family (Table 1) (Adams *et al.*, 2000; *Drosophila* Odorant Receptor Nomenclature Committee, 2000; Rubin *et al.*, 2000; Vosshall *et al.*, 2000). Although these genes are extremely divergent, sharing on average only 20% amino acid identity, conserved sequences in the putative seventh transmembrane domain are a signature of this family and these are found in all 60 *DOR* genes.

Although odorant receptors in these diverse species share a common function and secondary structure, there is no primary sequence identity between the odorant receptor genes of nematodes, flies and vertebrates, reflecting an apparently independent evolutionary origin (Vosshall *et al.*, 1999). There is also a wide variation in the number of odorant receptor genes in a given species. Nematodes and mammals have a repertoire of 1000 receptor genes, while fish, birds and flies have ~100 genes (Mombaerts, 1999). The relatively small repertoire of odorant receptor genes in the fly may reflect the ecological specialization of this insect, which feeds largely on yeast that grows on rotting fruit. It will be of interest to examine the repertoire of odorant

Table 1 Expression patterns of 60 *DOR* genes

Antenna (35)	Maxillary palp (7)	Antenna & palp (1)	Not detected (17)
Or2a (2E1)	Or1a (1A8)	Or83b (83A6)	Or22c (22C1)
Or7a (7D14)	*Or33c* (33B10)		*Or24a* (24E4)
Or9a (9E1)	Or46a (46E7–8)		Or30a (30A3)
Or10a (10B15)	Or59c (59E1)		Or42a (42A2)
Or13a (13F16–18)	Or71a (71B1)		Or45a (45C5)
Or19a (19B3–19C)	Or85d (85A11)		Or45b (45F1)
Or22a (22A5)	Or85e (85B2)		Or46b (46E7–8)
Or22b (22A5)			*Or49a* (49A5)
Or23a (23A3)			*Or59a* (59E1)
Or33a (33B10)			*Or63a* (63B1)
Or33b (33B10)			*Or74a* (74A6)
Or35a (35D1)			*Or83a* (83A6)
Or42b (42A2)			*Or85c* (85A9)
Or43a (43A1)			*Or92a* (92E8)
Or43b (43F5)			*Or94a* (94D9)
Or47a (47F1)			*Or94b* (94D9)
Or47b (47F6)			*Or98b* (98D4)
Or49b (49D1)			
Or56a (56E1)			
Or59b (59E1)			
Or65a (65A7–11)			
Or65b (65A7–11)			
Or65c (65A7–11)			
Or67a (67B2)			
Or67b (67B10)			
Or67c (67D2)			
Or69a (69E8-F1)			
Or69b (69E8-F1)			
Or82a (82A3–4)			
Or83c (83D5)			
Or85a (85A3)			
Or85b (85A9)			
Or85f (85D15)			
Or88a (88B1)			
Or98a (98B5)			

The approximate chromosomal positions of the 60 *DOR* genes, inferred from the genome sequence by Celera Genomics, are indicated in brackets following the name of each receptor. Receptor names reflect a recent standardization of nomenclature (*Drosophila* Odorant Receptor Nomenclature Committee, 2000).

receptor genes in an insect, such as the honeybee, which possesses a more elaborate olfactory sensory system and perceives a wider variety of odorants.

The spatial organization of *DOR* gene expression in the antenna and maxillary palp

Adult fruit flies have ~1300 olfactory receptor neurons, distributed between the third antennal segment (~1180 neurons) and the more ventrally located maxillary palp (~120 neurons). Each *DOR* gene is expressed in a small subset of olfactory neurons in either the antenna or the maxillary palp (Table 1; Figure 1) (Clyne *et al.*, 1999b; Gao and Chess, 1999; Vosshall *et al.*, 1999, 2000). Double labeling with markers that label *Drosophila* neurons confirmed that the cells expressing *DOR* genes are indeed olfactory receptor neurons (Vosshall *et al.*, 1999). While the average *DOR* gene is expressed in 20 olfactory neurons, some receptors are expressed in only 2–3 neurons (*Or33a*, *Or49b*), while *Or47b* is expressed in >50 neurons. Seven *DOR* genes are found solely in the maxillary palp, where they appear randomly interspersed but non-overlapping in expression (Vosshall *et al.*, 2000).

In the antenna, the expression of *DOR* genes is highly stereotyped, with each gene labeling a conserved subset of neurons. *In situ* hybridization with the linked genes *Or65b* and *Or65c* (Figure 1) reveals that these genes are expressed in non-overlapping domains in the ventral-lateral and ventral-medial antenna, respectively. The relative position of neurons expressing a given receptor is conserved between individual flies and is not sexually dimorphic. A total of 35 *DOR* genes are expressed in the adult antenna (Table 1) (Vosshall *et al.*, 2000).

The expression of 17 *DOR* genes is not detected in either organ, or in any other tissue in the adult fly. These may be expressed at low levels in the olfactory system and are therefore not detectable by conventional *in situ* hybridization methods. Alternatively, they may be expressed in a tissue or at a developmental time point that has not been examined in these studies. Neither these 17 nor any other *DOR* genes were found to be expressed in the larval chemosensory system, nor in the gustatory system of the larva or adult (Vosshall *et al.*, 1999, 2000). *DOR* genes are therefore likely to mediate recognition of volatile olfactory stimuli. A family of genes encoding novel seven-transmembrane domain G protein-coupled receptors distinct from the *DOR* genes has been proposed as candidate taste receptors in the fly (Clyne *et al.*, 2000).

Olfactory neurons express one *DOR* gene and the ubiquitous odorant receptor *Or83b*

In the mouse, each olfactory receptor neuron is likely to express only a single odorant receptor gene of the 1000 possible odorant receptors in the mouse genome, making that neuron functionally distinct (Chess *et al.*, 1994; Malnic

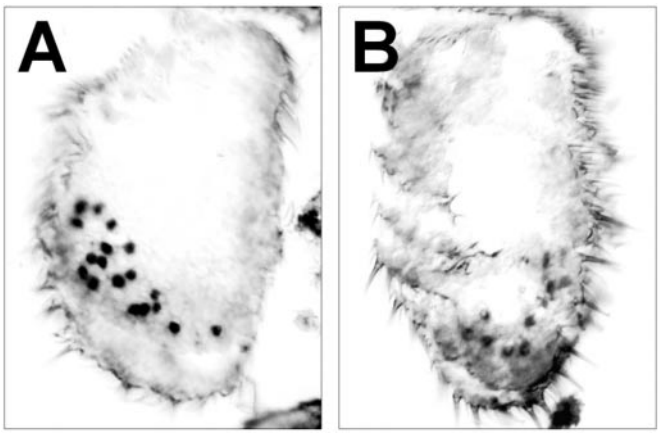

Figure 1 Expression of two antennal *DOR* genes in subsets of olfactory neurons. **(A)** *Or65b*; **(B)** *Or65c*. Frozen adult antennal frontal sections were hybridized with digoxigenin-labeled antisense RNA probes for these two receptor genes. Expression was visualized with anti-digoxigenin antibodies coupled to alkaline phosphatase. Sections are oriented with dorsal up and medial right. Each gene is expressed in a small subset of olfactory receptor neurons, with *Or65b* restricted to the ventral-lateral region and *Or65c* to the ventral-medial domain.

et al., 1999). Neurons expressing a given receptor are scattered at random across the olfactory sensory epithelium, but converge to a single glomerulus on each side of the olfactory bulb in the brain (Ressler *et al.*, 1994; Vassar *et al.*, 1994; Mombaerts *et al.*, 1996). Therefore activation of a given odorant receptor is sensed in the brain by the activation of all cells expressing this receptor.

In contrast, the nematode *C. elegans* uses a different logic of odor coding. Each of the 16 pairs of chemosensory neurons probably expresses up to 20 odorant receptors (Bargmann and Horvitz, 1991; Troemel *et al.*, 1995). Activation of any one of the odorant receptors expressed in a neuron will produce a stereotyped behavior that is the property of the neuron that is activated, not the identity of the particular activated odorant receptor (Troemel *et al.*, 1997).

Examination of the number of odorant receptor genes expressed per neuron in *Drosophila* suggests that the fruit fly olfactory system more closely resembles that of the mouse. Olfactory neurons in *Drosophila* are likely to express only a single *DOR* gene. There are 120 olfactory neurons in the maxillary palp, and each of seven receptors expressed in this organ labels ~20 neurons. Double-label *in situ* hybridization experiments with three of these seven genes (*Or46a*, *Or59c* and *Or85e*) demonstrated that each receptor is expressed in distinct, non-overlapping subset of neurons in the maxillary palp (Vosshall *et al.*, 2000). Similar experiments that examined the expression of 15 antennal *DOR* genes failed to show co-expression of any two *DOR* genes in the same neuron (Vosshall *et al.*, 1999, 2000). It is therefore likely that a given olfactory neuron in *Drosophila* expresses only a single *DOR* gene.

A notable exception to this conclusion is a single odorant

receptor, *Or83b*, which is expressed in all olfactory neurons throughout the life cycle of *Drosophila* (Vosshall *et al.*, 1999, 2000). The function of this non-canonical odorant receptor gene is unknown. Similarly broad expression has been observed for two goldfish odorant receptors, which are expressed in >50% of olfactory neurons in this animal (Speca *et al.*, 1999). In the fly, the ubiquitous odorant receptor may act as a co-receptor by heterodimerizing and altering the ligand specificity of conventional *DOR* genes, as has been suggested for other G protein-coupled receptors (Jordan and Devi, 1999; Rocheville *et al.*, 2000). Alternatively, it may be required for functional assembly and dendritic targeting of conventional odorant receptors, as seen with the association of two $GABA_B$ subunits into a functional receptor (Jones *et al.*, 1998; Kaupmann *et al.*, 1998; White *et al.*, 1998). Analysis of mutations in the *Or83b* gene will provide insights into its function in the olfactory system of *Drosophila*.

An olfactory sensory map in the antennal lobe

How does the brain sense the activation of a distinct subset of olfactory receptor neurons? In the mouse, neurons expressing a given receptor extend axons that synapse with one or two glomeruli in the olfactory bulb of the brain (Ressler *et al.*, 1994; Vassar, *et al.*, 1994; Mombaerts *et al.*, 1996). Therefore neurons expressing one of the 1000 possible odorant receptors are precisely wired to two of 1800 glomeruli in each olfactory bulb. This convergent wiring scheme simplifies the discrimination of olfactory stimuli: the activation of neurons expressing a given receptor is sensed in the brain by the activation of a specific glomerulus. This may form the basis of an olfactory code that is interpreted by higher brain centers to produce the qualitative experience of the odor.

The antennal lobe, the olfactory bulb equivalent of the fly, possesses 43 glomeruli and receives input from the ~1300 olfactory neurons of the antenna and maxillary palp (Stocker, 1994; Laissue *et al.*, 1999). The number of *DOR* genes detectably expressed in adult olfactory neurons (42) approximates the number of glomeruli (43). This would suggest a convergent organization of projections similar to that seen in the mouse olfactory system. We and others have used genetic labeling techniques to show that all neurons expressing a given receptor indeed converge upon one or two antennal lobe glomeruli (Gao *et al.*, 2000; Vosshall *et al.*, 2000) (Figures 2 and 3). In these experiments, 2–8 kb of DNA upstream of the coding region of five *DOR* genes were fused to the yeast transcription factor Gal4 (Vosshall *et al.*, 1999, 2000). These promoter fusion constructs give robust expression that recapitulates the number and spatial distribution of neurons expressing the endogenous *DOR* gene (Vosshall *et al.*, 1999, 2000). Olfactory neuron projections were examined in animals transgenic for both

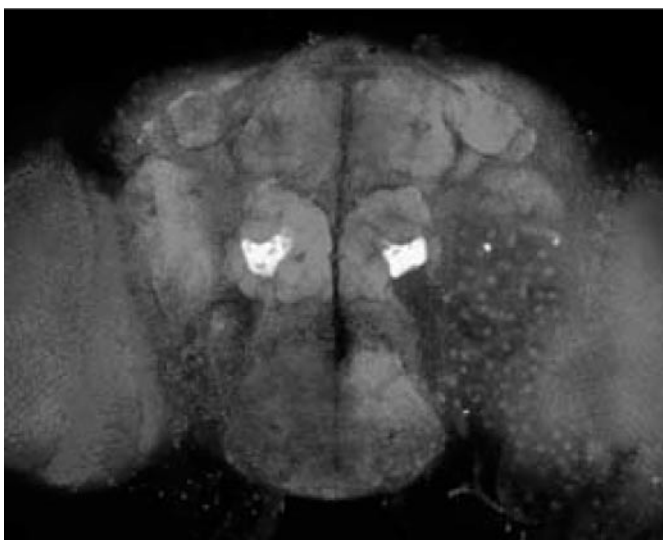

Figure 2 Convergence of neurons expressing *Or47b* to the VA1 glomerulus. Brain whole mount antibody staining of *Or47b*–Gal4:UAS–*nsyb*–GFP animals reveals that the large ventral-lateral glomerulus, VA1, receives input from all olfactory receptor neurons expressing *Or47b*. Olfactory neurons expressing the synaptic marker *nsyb*–GFP are visualized with an anti-GFP antibody (white), and the morphology of the brain is revealed by staining with the central neuropil-specific nc82 monoclonal antibody (gray). The brain whole mount is oriented with dorsal up and is viewed frontally. The antennal lobes are located at the center of the brain, the optic lobes are visible at the left and right, and the mushroom body is visible in regions dorsal to the antennal lobe.

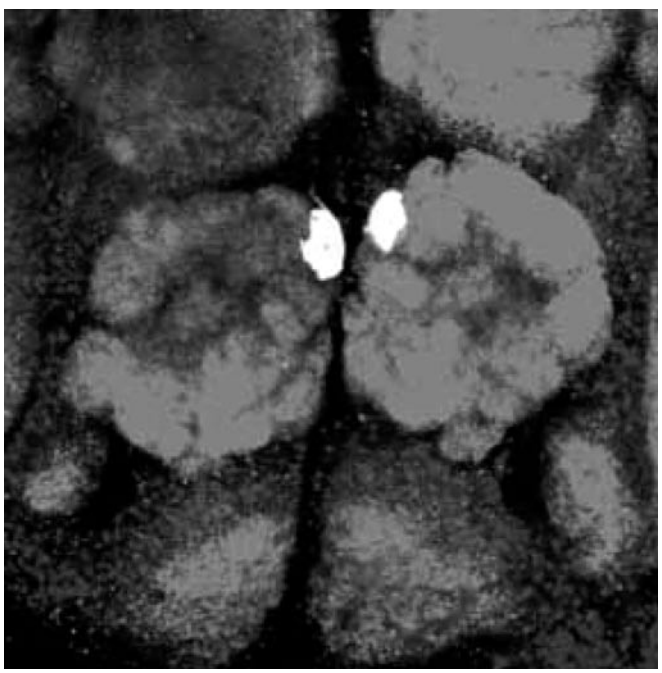

Figure 3 Convergence of neurons expressing *Or22a* to the DM2 glomerulus. All neurons expressing *Or22a* extend axons that synapse with the dorsal-medial DM2 glomerulus. Antennal lobe glomeruli are visualized by staining with the monoclonal antibody nc82 (dark gray), which stains all central neuropil in the adult *Drosophila* brain. Neurons expressing *nsyb*–GFP under control of the *Or22a*–Gal4 transgene are visualized with an anti-GFP antibody (white). This brain whole mount is oriented with the dorsal side up and is viewed frontally.

the *DOR*–Gal4 transgene and a transgene expressing a C-terminal fusion of Green Fluorescent Protein (GFP) to the synaptic vesicle protein, *n*-synaptobrevin (UAS–*nsyb*–GFP) (Estes *et al.*, 2000). This fusion protein accumulates at axon terminals and permits the resolution of single axons as they enter the antennal lobe (Estes *et al.*, 1997; Ito *et al.*, 1998). All neurons expressing either *Or47b* (Figure 2), *Or22a* (Figure 3) or *Or47a* (data not shown) each converge upon a single glomerulus in the antennal lobe (Vosshall *et al.*, 2000). The position and size of these glomeruli is invariant between individuals and not sexually dimorphic. Convergence to a single ventral antennal lobe glomerulus is seen for all cells expressing *Or46a*, a maxillary palp receptor (Vosshall *et al.*, 2000). In contrast, *Or23a*-expressing neurons synapse with two glomeruli in the antennal lobe (Gao *et al.*, 2000; Vosshall *et al.*, 2000).

These results suggest that there is a spatial map encoding odor quality in the antennal lobe of the *Drosophila* brain. Functional imaging of glomerular activation in diverse species is in accord with this model of convergent olfactory coding. In the honeybee, moth, fish, rat and salamander, a given odorant elicits activation of a small, spatially invariant subset of glomeruli that is conserved between different individuals (Kauer *et al.*, 1987; Hansson *et al.*, 1992; Friedrich and Korsching, 1997; Galizia *et al.*, 1999; Rubin and Katz, 1999; Sachse *et al.*, 1999). Although both molecular convergence and spatial patterns of glomerular activation are well described, whether the brain uses this convergence in odor coding is controversial (Laurent, 1997, 1999). The genetic manipulability of *Drosophila*, coupled with the small number and unambiguously identifiable glomeruli, should allow a functional test of the relevance of spatial coding in the antennal lobe for olfactory perception.

Conclusion and future directions

The identification of insect odorant receptors has provided initial insight into the molecular logic of olfaction in *Drosophila*. There are 60 *DOR* genes, 42 of which are detectably expressed in subsets of neurons in the antenna or maxillary palp and one of which is expressed in all olfactory neurons. Olfactory neurons are likely to express only one of the conventional *DOR* genes in addition to the ubiquitous odorant receptor *Or83b*. All neurons expressing a given receptor extend axons that form synapses with one or two of the 43 antennal lobe glomeruli. A similar convergence is observed in the mouse olfactory system (Ressler *et al.*, 1994; Vassar *et al.*, 1994; Mombaerts *et al.*, 1996). Therefore in both the fly and the mouse, odorant receptor gene expression is correlated with neuronal functional specificity.

What is the evidence that the *DOR* genes indeed encode the ligand-binding odorant receptors in *Drosophila*? *DOR* genes, like odorant receptors in other species (Mombaerts, 1999), are predicted to encode G protein-coupled receptors with seven transmembrane domains (Clyne *et al.*, 1999b; Gao and Chess, 1999; Vosshall *et al.*, 1999, 2000). The expression of *DOR* genes is restricted to olfactory neurons of the adult fly (Clyne *et al.*, 1999b; Gao and Chess, 1999; Vosshall *et al.*, 1999, 2000), and the spatial patterns of expression approximate the number and distribution of different functional types of neurons in the antenna and maxillary palp defined by electrophysiology (Siddiqi, 1987; de Bruyne *et al.*, 1999). The *acj6* mutant displays both altered chemical specificity of individual identified neurons and alterations in *DOR* gene expression (Clyne *et al.*, 1999a). *DOR*–GFP fusion proteins are localized to dendrites, the site of interaction of odorous ligand with the odorant receptor (L.B. Vosshall and R. Axel, unpublished data). The current evidence is largely circumstantial and definitive proof that these are functional odorant receptors awaits experiments that demonstrate binding of odorous ligands to this family of receptors.

Further understanding of the molecular basis of olfactory coding in insects will require associating receptors with specific ligands. Electrophysiological experiments have provided an initial glimpse into the peripheral odor code in adult flies. Single sensillum recordings have defined six functional classes of neurons in the maxillary palp, whose ligand specificity is narrowly defined (Clyne *et al.*, 1999a; de Bruyne *et al.*, 1999). The number of functional classes in the antenna is considerably larger and the spatial distribution of these sensilla corresponds well to the map of odorant receptor gene expression (Siddiqi, 1987; Clyne *et al.*, 1997). The next step will be to match these functional classes of neurons with the particular *DOR* genes that they express, and the glomerulus in the brain which these neurons innervate. Combining this analysis with functional imaging that can resolve the activation of individual glomeruli in response to odorous ligands will be of interest (Friedrich and Korsching, 1997; Galizia *et al.*, 1999; Rubin and Katz, 1999; Sachse *et al.*, 1999).

These spatial maps in the peripheral olfactory system and in the antennal lobe are ultimately decoded by higher brain centers, allowing the animal to experience the quality of the odor. Olfactory information is relayed via antennal lobe projection neurons to both the mushroom body and the lateral horn of the protocerebrum (Stocker, 1994; Ito *et al.*, 1998). The circuits involved in this process of decoding are poorly understand in all animals but the prospects for understanding higher level olfactory coding in the fruit fly are good: these insects respond to a restricted subset of odorants, and have a limited olfactory receptor repertoire and a relatively small number of glomeruli. The application of genetics, electrophysiology and imaging to this problem promise to yield answers in the short term.

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