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Diversity and expression of odorant receptors in *Drosophila*

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19.1 Introduction

Olfactory perception translates abstract chemical features of odorants into meaningful neural information to elicit appropriate behavioral responses (Shepherd, 1994; Buck, 1996). Specialized bipolar olfactory sensory neurons (OSNs) are responsible for the initial events in odor recognition. These have ciliated dendrites exposed to the environment, and a single axon that extends into the brain and forms synapses with second order projection neurons (PNs) (Shepherd, 1994; Buck, 1996). In arthropods and mammals, the first olfactory synapse is organized into glomeruli, spherical structures in which afferent olfactory neuron axons synapse with projection neuron dendrites (Hildebrand and Shepherd, 1997).

Molecular recognition of thousands of diverse odorants is mediated by a large family of odorant receptor (OR) genes, each encoding a different seven transmembrane domain G protein-coupled receptor (GPCR). OR genes were first identified in the rat by Buck and Axel (1991), using an innovative approach which assumed that the ORs would be members of the GPCR super family, encoded by an extremely large gene family expressed only in olfactory tissues. These assumptions proved to be correct and led to the identification of several hundred rat OR genes, selectively expressed in OSNs. Using degenerate oligonucleotide primers that would anneal with conserved regions in the transmembrane domains of the GPCR super family, Buck and Axel used the polymerase chain reaction (PCR) to identify OR genes in olfactory epithelium mRNA.

Since this initial report, OR genes have been identified in nematodes, bird, fish, and other mammals (Ngai *et al.*, 1993; Ben-Arie *et al.*, 1994; Troemel *et al.*, 1995; Nef *et al.*, 1996; Mombaerts, 1999). The number of ORs varies widely between species: nematodes and mammals have approximately 1000 OR genes, while fish and birds have approximately 100 ORs (Mombaerts, 1999). Despite the shared function and secondary structure of these ORs, there is no primary sequence identity between the OR genes of nematodes and vertebrates, reflecting an apparently independent evolutionary origin.

For insects, olfaction is a crucial sensory modality in locating food sources, identifying appropriate sites for oviposition, avoiding predators, and selecting mates. Enormous strides in our understanding of pheromonal communication and perception of general food odors have been made by basic research in insect olfaction, using large insects such as moths, locusts, and the honeybee (Hansson, 2002). Although the fruit fly *Drosophila melanogaster* was originally "domesticated" at the turn of the twentieth century as a useful organism for the study of genetics, it became a premier model organism for the new field of neurogenetics in the 1960s principally through the effort of Seymour Benzer. Benzer and his colleagues carried out large-scale genetic screens to isolate genes involved in such behaviors as courtship, learning and memory, circadian rhythms, phototaxis, and chemotaxis to odorants and tastants (Weiner, 1999). A large number of *Drosophila* mutants with defects in olfactory behavior have since been isolated using a variety of different behavior paradigms (Siddiqi, 1987; McKenna *et al.*, 1989; Lilly and Carlson, 1990, Carlson, 1991, 1996). Complementing the power of genetics to study olfaction is the recent completion of the sequencing of the euchromatic genome of *Drosophila*, the first insect genome to be completed (Adams *et al.*, 2000). This vastly simplifies the identification of genes mutated in particular mutant backgrounds and has made it feasible to annotate all predicted genes in the genome, including the OR genes which are the subject of this chapter.

The fruit fly olfactory system is anatomically simple (reviewed in Stocker, 1994). Larvae possess three chemosensory organs, the dorsal organs, terminal organ, and ventral organ, which together are responsible for detecting volatile and non-volatile stimuli (Heimbeck *et al.*, 1999; Oppliger *et al.*, 2000; Python and Stocker, 2002). A total of 100 neurons have been described in these three organs, which are located at the anterior tip of the larva. These neurons extend axons that synapse in either the larval antennal lobe or regions of the tritocerebrum and subesophageal ganglion of the larval brain (Python and Stocker, 2002). Emerging evidence suggests that the larval antennal lobe contains glomerulus-like structures. In the adult fly, all OSNs are contained in two chemosensory organs located on the head, the third segment of the antenna and the maxillary palp, while gustatory neurons and contact chemoreceptors are distributed over various body surfaces including the proboscis, the leg, wing, and female ovipositor

(Stocker, 1994). While mammals possess millions of OSNs that relay information to thousands of olfactory bulb glomeruli, adult *Drosophila* have a mere 1300 OSNs connected to 43 antennal lobe glomeruli. This vastly simplified olfactory system, that nevertheless retains many of the anatomical features found in the mammalian olfactory system, make the fly an excellent model system in which to study the sense of smell.

This chapter will discuss the isolation of *Drosophila* odorant receptor *(DOR)* genes, how these genes have expanded our understanding of the development and functional anatomy of the olfactory system, how the odor response profiles of OSNs respond to odorants, and the mechanisms by which odor-specific activity is relayed to the brain.

19.2 *Drosophila* odorant receptor genes

19.2.1 Using bioinformatics to identify the elusive fly or genes

In the decade that followed the original cloning of rat OR genes by Buck and Axel (1991), considerable effort was expended by many investigators to identify homologs of this gene family in other vertebrates and in invertebrates. This task proved to be straightforward in other mammals, birds, and even fish because the vertebrate OR gene family shows strong conservation across diverse species (Mombaerts, 1999). With the exception of one report in the honeybee, which likely represented contamination of honeybee with human genomic DNA (Danty *et al.*, 1994), no laboratory seemed to be able to identify insect homologs of vertebrate ORs using degenerate PCR primers designed against the vertebrate gene family. In the mid-1990s Cori Bargmann's group reported the first invertebrate chemosensory receptor genes from the nematode *C. elegans*. By analyzing partial genomic DNA sequences compiled by the consortium sequencing the *C. elegans* genome, Troemel *et al.* (1995) identified a large family of genes encoding seven transmembrane domain receptors selectively expressed in chemosensory neurons of the worm. Named *sr* (for serpentine receptor) genes, these are unrelated to any known protein family and are also extremely divergent within *C. elegans*. The first functional proof that these putative receptor genes indeed encode chemoreceptors came with the cloning of the *odr-10* gene the following year (Sengupta *et al.*, 1996). Animals deficient in *odr-10* function show severely reduced chemotaxis to the odorant diacetyl. Positional cloning of the gene defective in *odr-10* mutants identified a seven transmembrane domain protein related to the *sr* genes which is likely the diacetyl receptor.

Because there was no apparent evolutionary relationship between chemoreceptor genes in the nematode and in vertebrates, it seemed at least a possibility that the insect odorant receptors would represent yet a third class of genes, unrelated to ORs in either *C. elegans* or vertebrates. Following this logic, Vosshall *et al.*

(1999) pursued an approach that did not rely on any assumptions about the sequence of the *Drosophila* ORs. They used differential hybridization to identify genes selectively expressed in antennal and maxillary palp mRNA. Among the several dozen candidate genes, a single gene named *DOR104* [since renamed *Or85e*, by the *Drosophila* Odorant Receptor Nomenclature Committee (2000)] encoded a novel seven transmembrane domain receptor protein with no homology to any known proteins. *Or85e* was selectively expressed in a subset of OSNs in the maxillary palp and not detectably expressed anywhere else in the fly (Vosshall *et al.*, 1999) (Figure 19.1). Efforts by this group to identify additional *Or85e*-related genes by conventional molecular biology techniques failed.

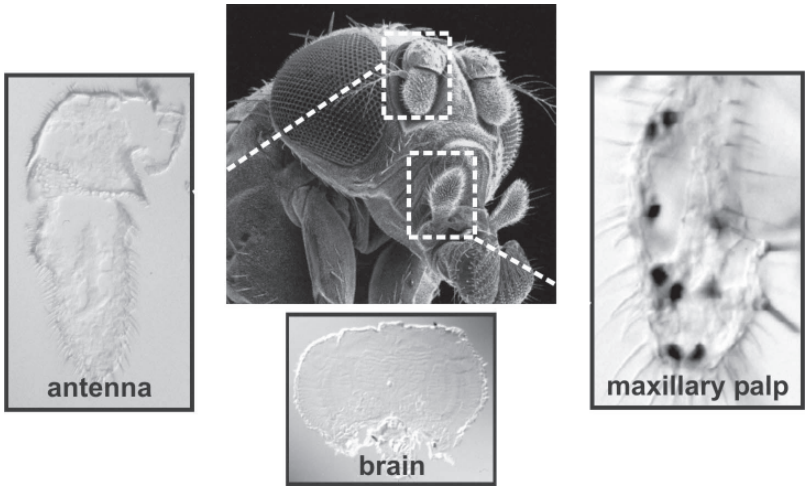

Figure 19.1 *Or85e* is selectively expressed in subsets of maxillary palp neurons. *In situ* hybridization with an antisense *Or85e* probe reveals no staining in antenna (left) or brain (center). Nine cells stain in this section of the maxillary palp (right). The relative positions of the two olfactory sensory organs on the head of the adult fly are indicated with white dashed lines.

By 1998, sequencing of the *Drosophila* genome was approximately 15 percent completed. Three research groups exploited these sequences to report the identification of candidate *Drosophila* odorant receptor (*DOR*) genes in 1999 (Clyne *et al.*, 1999; Gao and Chess, 1999; Vosshall *et al.*, 1999). Although the genomic sequences were highly fragmented and unannotated, all three groups used the logic that if the ORs constituted a multi-gene family of at least 100 members, then at least 10 genes should be represented in the partial genomic sequence then available. While the algorithms used to identify these genes in genomic sequence differed, all followed the same basic strategy: first, identify coding exons or genes, then identify those exons or genes that encode proteins containing

multiple membrane-spanning domains, and subject promising candidate genes to expression analysis (Figure 19.2). Nineteen candidate *DOR* genes were isolated by the combined efforts of the three groups. All of these were distant but clear homologs of *Or85e*, the maxillary palp receptor that originally emerged from a difference cloning approach (Figure 19.1; Table 19.1). None of the *DOR* genes showed any apparent sequence relatedness to any other known proteins, including the chemoreceptor proteins in other species.

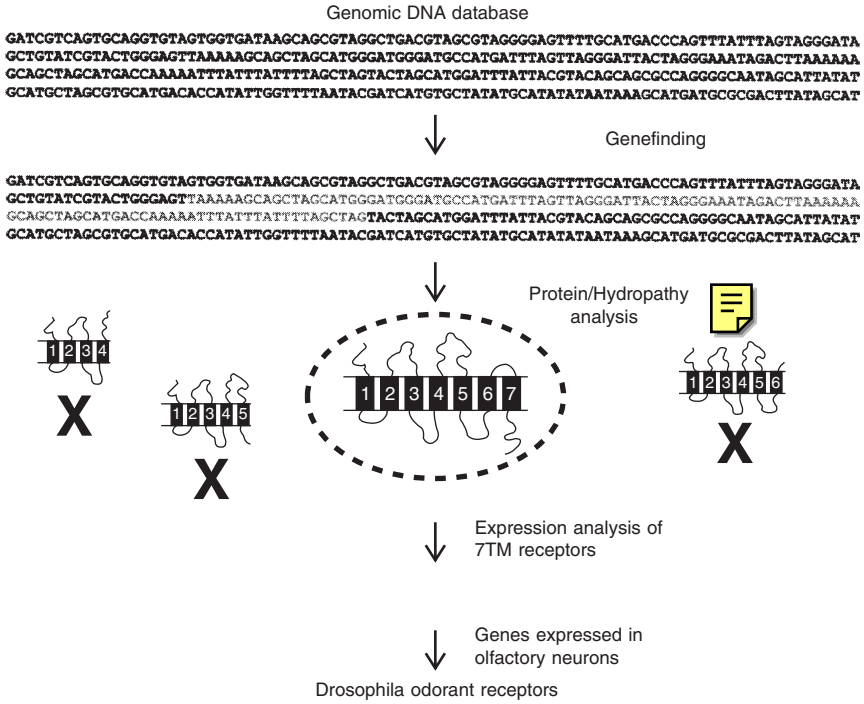

Figure 19.2 Mining *Drosophila* genome databases for OR genes. Flow chart illustrating the general steps used by three different groups to identify OR genes in raw genomic DNA (Clyne *et al.*, 1999; Gao and Chess, 1999; Vosshall *et al.*, 1999). Sequence files from *Drosophila* genome databases were downloaded and subjected to a variety of gene-finding programs. These programs parse the data into exons (black text) and introns (grey text) and generate protein-coding open reading frames. DNA sequences are for illustrative purposes only and represent a hypothetical gene fragment with two exons and one intron. The open reading frames were translated into protein and the resulting proteins were analyzed for hallmarks of GPCRs (Clyne *et al.*, 1999) or for transmembrane domains (Gao and Chess, 1999; Vosshall *et al.*, 1999). Proteins with seven transmembrane domains (7TM) were analyzed further by gene expression analysis (dashed line). Proteins with fewer transmembrane domains were discarded (X). Genes selectively expressed in OSNs were further analyzed as candidate *DOR* genes.

Table 19.1 Compiled expression data

#	OR name	Previous names	Entrez	SwissProt	Map	Antenna	Maxillary palp	Not detected
1	Or1a		CG17885	Q9W5G6	1A8		V	
2	Or2a	2F.1, DOR62, AN4	CG3206	O46077	2E1	V*C		
3	Or7a		CG10759	Q9W3I5	7D14	V		
4	Or9a		CG15302	Q9W2U9	9E.1	V		
5	Or10a		CG17867	Q9VYZ1	10B15	V		
6	Or13a		CG12697	Q9VXL0	13F16-18	V		
7	Or19a		CG18859	Q9I816	19B3-19C	V		
8	Or22a	22A.1, DOR53, AN11	CG12193	P81909	22A5	V*CG		
9	Or22b	22A.2, DOR67, AN12	CG4231	P81910	22A5	V*C		
10	Or22c	22C.1	CG15377	P81911	22C1			V*
11	Or23a	23A.1, DOR64, AN5	CG9880	P81912	23A3	V*G	G	C
12	Or24a	24D.1	CG11767	P81913	24E4			V*
13	Or30a		CG13106	Q9VLE5	30A3			V
14	Or33a	33B.1, DOR73, AN3	CG16960	P81914	33B10	V*		C
15	Or33b	33B.2, DOR72, AN1	CG16961	P81915	33B10	V*		C
16	Or33c	33B.3, DOR71, AN2	CG5006	P81916	33B10	*	V*CG	
17	Or35a		CG17868	Q9V3Q2	35D1	V		
18	Or42a		CG17250	Q9V9I2	42A2			V
19	Or42b		CG12754	Q9V9I4	42A2	V		
20	Or43a	43B.1, DOR87, AN14	CG1854	P81917	43A1	V*		
21	Or43b	25A.1, AN7	CG17853	P81918	43F5	V*CG	*	
22	Or45a		CG1978	Q9V568	45C5			V
23	Or45b		CG12931	Q9V589	45F1			V
24	Or46a	46F.1, AN9	CG17849	P81919	46E7-8		V*CG	
25	Or46b	46F.2, DOR19, AN8	CG17848	Q9V3N2	46E7-8	*		VC
26	Or47a	47E.1, DOR24, AN10	CG13225	P81921	47F1	V*C		
27	Or47b	47E.2	CG13206	P81922	47F6	V*		

28	Or49a		CG13158	Q9V6A9	49A5			V
29	Or49b	AN13	CG17584	Q9V6H2	49D1	V		
30	Or56a		CG12501	Q9V8Y7	56E1	V		
31	Or59a	59D.1, DOR46, AN6	CG9820	P81923	59E1	G		V *
32	Or59b		CG3569	Q9W1P8	59E1	V		
33	Or59c		CG17226	Q9W1P7	59E1		V	
34	Or63a		CG9969	Q9VZW8	63B1			V
35	Or65a		N.A.	P82982	65A7-11	V		
36	Or65b		N.A.	P82983	65A7-11	V		
37	Or65c		N.A.	P82984	65A7-11	V		
38	Or67a		CG12526	Q9VT08	67B2	V		
39	Or67b		CG14176	Q9VT20	67B10	V		
40	Or67c		CG14156	Q9VT90	67D2	V		
41	Or67d		CG14157	Q9VT92	67D5	(V)		
42	Or69a		N.A.	P82985	69E8-F1	V		
43	Or69b		CG17902	Q9VU27	69E8-F1	V		
44	Or71a		CG17871	Q9VUK5	71B1		V	
45	Or74a		CG13726	Q9VVF3	74A6			V
46	Or82a		N.A.	P82986	82A3-4	V		
47	Or83a		CG10612	Q9VNB3	83A6			V
48	Or83b	A45	CG10609	Q9VNB5	83A6	V	V	
49	Or83c		CG15581	Q9VNK9	83D4	V		
50	Or85a		CG7454	Q9VHS4	85A3	V		
51	Or85b		CG11735	Q9VHQ7	85A9	V		
52	Or85c		CG17911	Q9VHQ6	85A9			V
53	Or85d		CG11742	Q9VHQ2	85A11		V	
54	Or85e	DOR104	CG9700	P81924	85B2		V	
55	Or85f		CG16755	Q9VHE6	85D15	V		
56	Or88a		CG14360	Q9VFN2	88B1	V		
57	Or92a		CG17916	Q9VDM1	92E8			V
58	Or94a		CG17241	Q9VCS9	94D9			V

(Contd)

Table 19.1 *(Contd)*

#	OR name	Previous names	Entres	SwissProt	Map	Antenna	Maxillary palp	Not detected
59	Or94b		CG6679	Q9VCS8	94D9			V
60	Or98a		CG5540	Q9VAZ3	98B5	V		
61	Or98b		CG1867	Q9VAW0	98D4			V

N.A. = none assigned to date
* = Clyne *et al.*, 1999 (RT-PCR)
~ = Clyne *et al.*, 1999 (*in situ*)
G = Gao and Chess, 1999 (*in situ*)
V = Vosshall *et al.*, 1999 (*in situ*)
(V) = Fishilevich & Vosshall, unpublished)

19.2.2 The size and character of the *DOR* gene family

Mining the partial fly genome for OR genes yielded approximately 20 candidate odorant receptors. Each encodes a protein of approximately 370 to 400 amino acids. *Or83b* is significantly larger at 486 amino acids, with the additional amino acids lying in predicted intracellular loop regions of the protein. The *DOR* proteins are rather hydrophobic in character, but it is possible to assign putative membrane-spanning regions. While there is no consensus as to exact position of the transmembrane domains, there is general agreement that there are indeed seven membrane-spanning regions (Clyne *et al.*, 1999; Gao and Chess, 1999; Vosshall *et al.*, 1999). Further direct experimental proof will be required to determine which model of transmembrane segments is correct.

Upon the release of the complete euchromatic genome sequence of *Drosophila* (Adams *et al.*, 2000), it was possible to complete the annotation of this gene family and obtain a complete view of the number of *DOR* genes present in the database (Vosshall *et al.*, 2000). A total of 57 *DOR* genes were identified. More recent annotation of this gene family by Hugh Robertson revealed an additional four *DOR* genes for a current total of 61 members of the gene family (see Chapter 21 and Table 19.1). Because the *Drosophila* genome is undergoing continuous revision to fill gaps in euchromatic sequence, the total number of *DOR* genes may increase. However, the number is unlikely to increase substantially unless there is a large reservoir of undiscovered *DOR* genes in regions of heterochromatin, which is largely unsequenced.

Each of the 61 known *DOR* genes encodes a different seven transmembrane domain GPCR. To date there is no evidence for alternative splicing of these genes to generate receptor protein diversity. The degree of sequence conservation of *DOR* genes within *Drosophila* is quite low, on the order of 17–26 percent. However, there are a number of subfamilies within the *DORs* with significantly higher degrees of sequence similarity (40–60 percent). Despite the very low degrees of sequence similarity between the *DORs*, they are classified as members of the same gene family based on certain strongly conserved amino acids which lie at fixed positions distributed throughout the protein. For instance, in the putative seventh transmembrane domain, the amino acid sequence Phe-Pro-X-Cys-Tyr-$(X)_{20}$-Trp (where X = any amino acid) is strongly conserved across the *DOR* family. In database searches for *DOR* genes using the BLAST algorithm (Altschul *et al.*, 1990), the strongest degree of sequence conservation across the gene family is in 3′ regions of the gene encompassing transmembrane domains 6 and 7. The significance of the divergence of *DOR* sequences in N-terminal regions of the protein and the comparative sequence similarity at C-terminal regions are currently unknown. In vertebrate OR genes, regions of sequence variability in the transmembrane regions has been proposed as a potential site for ligand interaction. It is possible that similar mechanisms operate in the ligand binding of the *DOR* genes.

Although the *DORs* have no apparent sequence similarity to chemosensory receptors in nematodes and vertebrates, they are clearly related to OR genes from the malaria mosquito, *Anopheles gambiae* (Fox *et al.*, 2001). As in the fly, sequence similarity among the cloned mosquito ORs is low, with 11 percent identity and 18 percent similarity across the four published genes. Nevertheless, the amino acid sequences that are signatures of this gene family are present in the mosquito ORs. The *DORs* are therefore founding members of an insect OR gene family and ORs from other insects are likely to resemble both mosquito and *Drosophila* receptors. This divergence across the gene family, with more closely related subfamilies, has also been seen for the chemosensory receptors of the nematode (Troemel *et al.*, 1995) and putative pheromone receptors in the mouse (Rodriguez *et al.*, 2002). In contrast, there tends to be more sequence conservation within the OR genes of vertebrates (Zozulya *et al.*, 2001; Zhang and Firestein, 2002).

Interestingly, because of the extremely low sequence similarly of ORs within *Drosophila* and between *Drosophila* and mosquito, it is likely that identification of OR genes in other insects will require access to genome sequences of these organisms. Conventional approaches that rely on degenerate PCR or low stringency hybridization across species are unlikely to succeed because the regions of *DOR* homology are extremely dispersed. A direct demonstration of this lies in the inability of Vosshall *et al*. (1999) to identify homologs of *Or85e* by conventional approaches. Although BLAST algorithms using *Or85e* as a query sequence routinely yield hits to a large number of annotated *DOR* genes, low stringency hybridization and degenerate PCR with *Or85e* sequences fail to cross-react with these *DORs*. Recent advances in increasing the throughput and reducing the cost of genomic sequencing should make the sequencing of additional interesting insects, such as moth, locust, honeybee, ant, and cockroach, feasible (Broder and Venter, 2000). A complementary approach of sequencing expressed genes has begun in the honeybee, and has already yielded an *Apis mellifera* homolog of *Or83b* (Whitfield *et al.*, 2002).

The lack of any apparent primary sequence identity between the OR genes of nematodes, insects, and vertebrates suggests that these arose through an independent mechanism in evolution (Vosshall *et al.*, 1999). Remarkably, this suggests that in order to recognize a very large number of different odorous ligands, an animal needs only to expand an ancestral GPCR into a multi-gene family and direct members of this gene family to express selectively in OSNs.

19.2.3 *DOR* gene expression patterns: formation of a peripheral olfactory sensory map

A role for *DOR* genes in olfaction requires that they are expressed in OSNs, where they can interact with odors and transmit odor-activated neuronal activity. To address this question Clyne *et al*. (1999) used reverse transcription-PCR (RT-

PCR) on antennal and maxillary palp mRNA preparations. Of the 16 *DOR* genes examined, ten were detected by RT-PCR in antenna, two in the antenna and maxillary palp, one in maxillary palp alone, and three in neither tissue (data marked * in Table 19.1). Analysis of *DOR* gene expression by RNA *in situ* hybridization to antenna and maxillary palp largely confirmed these RT-PCR data, with the exception that some *DORs* detected by RT-PCR were not detected by *in situ* hybridization (Clyne *et al.*, 1999; Gao and Chess, 1999; Vosshall *et al.*, 1999, 2000). Tissue localization of all 61 members has been determined: 39 *DOR* genes were found to be expressed in the antenna, nine in the maxillary palp, and 19 *DOR* genes were not detected. *Or83b* was unique in being expressed in both antenna and maxillary palp (Vosshall *et al.*, 1999, 2000). These data exceed the total number of 61 *DOR* genes because for certain members of the gene family, different groups obtained different results. For instance, *Or23a* was detected by Clyne *et al*. (1999) in antenna by RT-PCR, but was not detected in this tissue by *in situ* hybridization. Gao and Chess found *Or23a* in both antenna and maxillary palp by *in situ* hybridization (Gao and Chess, 1999), while Vosshall *et al*. found this receptor to be antennal specific (1999, 2000). The variability in these findings is most likely due to the variability inherent in the techniques of RT-PCR and *in situ* hybridization. Despite this variability, there is in general no overlap in antennal and maxillary palp *DOR* genes. This suggests that these two olfactory sensory organs respond to different odors. Alternatively, there may be some overlap in the odors perceived, but this is accomplished through different *DOR* proteins, perhaps with different ligand-binding affinities. Evidence for the latter model has been obtained by electrophysiological recordings from individual sensilla on both olfactory organs (de Bruyne *et al.*, 1999, 2001).

The patterns of *DOR* gene expression revealed by *in situ* hybridization in antenna and maxillary palp allow us to make a number of important conclusions. Each *DOR* gene is expressed in a small subset of the ~1300 antennal and ~120 maxillary palp neurons. The number of OSNs expressing a given *DOR* gene varies from two (*Or49b*) to approximately 50 (*Or47b*), with an average of 25 OSNs expressing a given *DOR* gene. In the antenna, the neurons expressing a given *DOR* gene are located at discrete positions along the proximal–distal and medial–lateral axes. The relative position and number of neurons expressing a given gene is conserved between different animals. For instance, *Or22a* expression is limited to approximately 20 OSNs that lie at the medial–proximal aspect of the antenna. One technique to reveal gene expression that does not rely on RT-PCR or RNA *in situ* hybridization is the generation of transgenic flies in which putative *DOR* promoter sequences drive expression of a convenient marker protein. Such transgenic flies have been constructed and marker expression in these animals has been shown to overlap with the expression of the endogenous *DOR* gene (Vosshall *et al.*, 1999, 2000). Figure 19.3 shows an example of patterns of LacZ marker gene expression in flies carrying *Or43a*-Gal4 and *Or71a*-Gal4

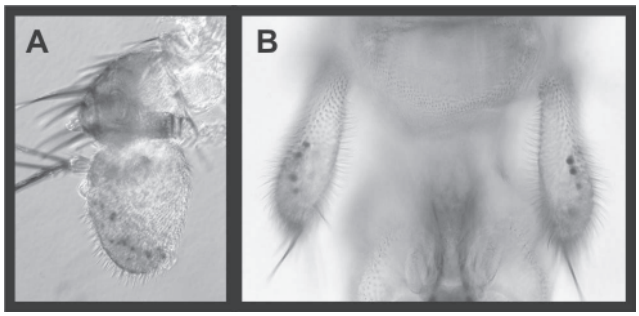

Figure 19.3 Spatial domains of *Drosophila* OR gene expression. A given OR gene is expressed in a small subset of OSNs, whose position is spatially conserved between animals and is bilaterally symmetric. A *Or43a* expression in the lateral distal domain of the antenna is revealed in an *Or43a*-Gal4:UAS-lacZ animal. B Expression of lacZ in maxillary palps of an *Or71a*-Gal4:UAS-lacZ animal demonstrates bilateral symmetry of neurons expressing *Or71a*. Whole mount preparations were stained with X-Gal to reveal OR gene expression as described (Vosshall *et al.*, 1999). LacZ-positive cells appear as dark circular spots on the surface of the sensory organs.

transgenes. By making use of the Gal4-UAS system, it is possible to cross these flies to animals carrying a UAS-LacZ transgene (Brand and Perrimon, 1993). The progeny of this cross reveal a small number of OSNs expressing LacZ in the lateral–distal domain of *Or43a*-Gal4:UAS-LacZ antenna (Figure 19.3a). No expression of this transgene is found outside of the antenna, mirroring the gene expression profile of the endogenous locus. The maxillary palps of *Or71a*-Gal4:UAS-LacZ animals contain a small number of LacZ-positive neurons whose position is conserved between different individuals and which is bilaterally symmetric in the two palps (Figure 19.3b). There is no apparent difference between the number and distribution of OSNs expressing a particular *DOR* gene in males and females, as revealed either by *in situ* hybridization or analysis of *DOR*-Gal4 transgenic flies. This finding is concordant with anatomical studies that have demonstrated little sexual dimorphism in the distribution and number of olfactory sensilla on the surface of the antenna and maxillary palp (reviewed in Stocker, 1994).

In vertebrates, OSNs are likely to express one or very few OR genes, leading to the model that the odor selectivity of an OSN is defined primarily by the OR gene it expresses (Malnic *et al.*, 1999). A similar logic is likely employed by the fly, because a number of studies have demonstrated that in the fly a neuron is likely to express only a single *DOR* gene. This was first demonstrated by performing *in situ* hybridization individually with five *DOR* probes and with a mixed probe containing five *DOR* genes. The number of neurons expressing these genes was additive, such that the total number of OSNs expressing the individual *DOR*

gene was equivalent to those obtained with the mixed probe (Vosshall *et al.*, 1999). A more direct demonstration of the non-overlapping expression of *DOR* genes in the antenna and maxillary palp was obtained by two-color *in situ* hybridization (Vosshall *et al.*, 1999, 2000). In these experiments, an antisense RNA probe against one receptor is labeled with digoxigenin and another *DOR* probe is labeled with FITC. These modified mRNAs are detected with antibodies that are labeled with different fluorophores. In comparing three palp genes (*Or46a*, *Or85e*, and *Or59c*), there was no apparent overlap among the OSNs expressing these *DORs*. Equivalent results were obtained with a series of *DOR* genes that label neurons either in the medial–proximal or lateral–distal domains of the antenna. Although these experiments sampled only a small subset of the total repertoire of *DOR* genes, they rule out any global coexpression of *DOR* genes within OSNs. Therefore, we hypothesize that in the fly the chemical specificity of a neuron is defined by the particular odorant receptor it expresses.

What is the function of the 19 or so *DOR* genes that are not detectably expressed by either RT-PCR or *in situ* hybridization? These might represent genes expressed at very low levels in OSNs, or alternatively genes expressed at developmental times or in tissues that have not been examined in experiments to date. Candidate larval OR genes have not been identified, nor have the receptors that interact with putative pheromones in *Drosophila*. It is conceivable that these "missing" receptors will be among the 19 non-detected *DORs*.

19.2.4 The enigma of Or83B

In situ hybridization with members of the *DOR* family suggests that each OSN expresses one or a few *DOR* genes. There is one striking exception: *Or83b* is expressed in a large proportion of olfactory neurons throughout the life cycle of *Drosophila*. Therefore each adult olfactory neuron expresses only one of the 42 conventional *DOR* genes along with the generally expressed odorant receptor, *Or83b*. The unusual expression profile of this odorant receptor complicates the problem of odorant discrimination in the fly. The olfactory system is designed to recognize a multitude of different volatile odorants and transmit information about the chemical structure and concentration of the odorant to the brain. Discrimination of a large number of odorous ligands is afforded by the segregation of different odorant receptors into functionally distinct neurons. Therefore an odorant receptor that is broadly expressed would appear to compromise this cellular specificity.

What is the function of this unusual odorant receptor? *Or83b* could play a role in olfaction independent of ligand binding. For instance, *Or83b* might be required for the targeting of conventional *DOR* proteins to dendritic membranes. Such a mechanism has been found for the metabotropic $GABA_B$ receptor, which is composed of a heterodimeric pair of seven transmembrane domain proteins. Surface expression and functional ligand binding of the $GABA_BR1$ subunit depends

on coexpression of a second $GABA_B$ receptor, $GABA_BR2$ (Jones *et al.*, 1998; Kaupmann *et al.*, 1998; White *et al.*, 1998). A similar mechanism has been suggested for the RAMPs, single transmembrane proteins that associate with seven transmembrane domain proteins and facilitate appropriate membrane insertion (McLatchie *et al.*, 1998).

Neurons from the antenna and maxillary palp extend axons that synapse specifically in the antennal lobe of the brain. It is conceivable that this broadly expressed *DOR* gene plays a role in the differentiation and development of the olfactory system, perhaps targeting axons to olfactory glomeruli in the brain. In the mouse, the olfactory receptor itself influences target selection in the olfactory bulb (Mombaerts *et al.*, 1996; Wang *et al.*, 1998). Because *Or83b* is expressed in most olfactory neurons, it is unlikely to be involved in the mechanism that targets axons to specific glomeruli, but might be a general factor required for guidance of all olfactory neuron axons to the antennal lobe.

A second, conceptually different model for *Or83b* function is that this ubiquitous odorant receptor functions in ligand binding. If *Or83b* were a ligand-binding odorant receptor, its cognate ligand would activate most olfactory neurons and therefore many glomeruli in the antennal lobe. This would pose a formidable problem in odor recognition for the brain. Alternatively, *Or83b* may act as a receptor independent of ligand, constitutively activating signaling pathways in the cell to increase levels of second messengers and therefore sensitizing the cell to activation by conventional *DOR* proteins. An analogous function has been proposed for the receptor guanylate cyclases expressed in *C. elegans* chemosensory neurons (L'Etoile and Bargmann, 2000). Finally, *Or83b* might associate with conventional *DOR* proteins and modify their ligand specificity. Heterodimerization of kappa and mu opioid receptors, as well as dopamine and somatostatin receptors has been documented and produces receptors with altered ligand specificities (Jordan and Devi, 1999; Rocheville *et al.*, 2000).

Conceptually similar models have been proposed for chemosensory receptors in two vertebrate model systems. In the goldfish, two members of the V2R family of putative pheromone receptors, 5.3 and 5.24, are very broadly expressed in OSNs (Speca *et al.*, 1999). It is likely that neurons expressing 5.3 and 5.24 express additional, more selectively distributed members of the V2R family. In rodents, a subfamily of V2R receptors, called V2R2, shows very broad expression in the vomeronasal organ (Martini *et al.*, 2001). Vomeronasal organ neurons express two receptors: broadly distributed V2R2 and a more selectively distributed member of the V2R family. Understanding the function of these broadly expressed receptors will require either modeling receptor function in heterologous cells or the generation of mutant animals that lack the broadly expressed receptor. It will then be possible, through a genetic approach, to dissect the various proposed models for the function of *Or83b* and other receptors with a similarly broad expression profile.

19.2.5 Relating *DOR* gene expression to olfactory function

OSNs in insects are bipolar neurons that insert dendrites into sensory hairs called sensilla. The number and distribution of different sensilla are strongly conserved across individuals (Shanbhag *et al.*, 1999). Elegant electrophysiological experiments have probed the odor responses of a large number of antennal and maxillary palp sensilla (de Bruyne *et al.*, 1999, 2001) (see Chapter 18 for a full discussion of this topic). It is now possible to begin to superimpose the maps of sensillar type on the antenna with the type of *DOR* gene expressed by the underlying OSN, and to relate this to the response properties of the neuron and the sensillum. There is a striking overlap in the patterns of *DOR* gene expression and the electrophysiological response maps, giving further strength to the argument that the *DOR* genes indeed are ligand-binding odorant receptors. With the advent of *DOR*-Gal4 transgenic reagents, it will be possible to label individual dendrites of neurons expressing that *DOR* gene and directly assess the ligand-binding properties of a given neuron.

19.2.6 Approaches to identifying ligand–receptor relationships of the *DOR* genes

The evidence that the *DOR* genes are ligand-binding odorant receptors is suggestive, if not fully conclusive: the *DOR* genes encode seven transmembrane domain GPCRs; the size of the *DOR* gene family is consistent with a role in recognizing a large number of odorants; each *DOR* gene (with the exception of *Or83b*) is expressed in a small subset of OSNs that may overlap with functional subtypes defined by electrophysiology. What has not been demonstrated in the publications reporting *DOR* gene isolation is that a given *DOR* interacts with a particular odorant. In other olfactory systems, evidence of direct ligand–receptor relationships has been obtained by a variety of genetic and electrophysiological techniques.

In the nematode, *C. elegans*, *odr-10* mutants show dramatically reduced attraction to the odorant diacetyl. Molecular cloning of the gene defective in *odr-10* mutants revealed it to be a seven transmembrane domain GPCR (Sengupta *et al.*, 1996). Direct demonstration that the *odr-10* protein is a diacetyl receptor came from experiments in which neurons normally non-responsive to diacetyl were reprogrammed to express *odr-10*, producing animals that were repelled by this normally attractive stimulus (Troemel *et al.*, 1997). The availability of genetic approaches in *Drosophila* makes the generation of *DOR* mutants feasible and is likely to generate compelling data on how the complement of chemosensory receptors in the fly recognizes odors.

An alternative to genetics has been heterologous expression of ORs, either *in vivo* (Zhao *et al.*, 1998; Araneda *et al.*, 2000) or in tissue culture cells (Krautwurst *et al.*, 1998; Touhara *et al.*, 1999), and the use of functional imaging of isolated OSNs to determine their response profiles (Malnic *et al.*, 1999; Leinders-Zufall *et al.*, 2000). These studies of vertebrate chemosensory receptors have led to the

conclusion that a given vertebrate OR recognizes multiple odorants and that a given odorant activates neurons expressing a number of different ORs. These types of odorant responses will give rise to a combinatorial code in which a particular odorant activates a diverse array of OSNs expressing different receptors.

Because *Drosophila* has considerably fewer ORs than vertebrates, it was of interest to determine if this type of combinatorial coding operates in the fly or if fly receptors are significantly narrower in their response properties. Recent studies have addressed this question for a single receptor using both *in vivo* overexpression and heterologous expression. *Or43* was found to respond to benzaldehyde, as well as benzyl alcohol, cyclohexanol, and cyclohexanone (Störtkuhl and Kettler, 2001; Wetzel *et al.*, 2001). *In vivo* overexpression was performed with the Gal4-UAS system to expand the expression domain of *Or43* artificially from ~20 neurons to >1000 neurons in the antenna. In these animals, most neurons will express three receptors: a specific *DOR* gene that is naturally expressed in this OSN, *Or83b*, and ectopic *Or43a*. These animals are then exposed to odorants and odor-evoked extracellular receptor potentials are recorded in the antenna using the electroantennogram (EAG) technique. A given odor will give a reproducible EAG response in wild-type animals. In animals overexpressing *Or43a*, there is a significant potentiation in the amplitude of the EAG response which is selective for a few structurally related odorants. In a companion study, the same *DOR* was heterologously expressed in Xenopus oocytes along with a promiscuous G protein ($G_q\alpha 15$) and odor-evoked currents were only obtained with the same odorants that proved to be successful in the *in vivo* experiments (Wetzel *et al.*, 2001). Taken together, these studies suggest that *Or43a* is a receptor for benzaldehyde and a small number of structurally related compounds. With the relatively small number of *DOR* genes in the fly, it is feasible to perform these types of experiments for the entire gene family and obtain a complete picture of the odor-responsive properties of this gene family. The very large number of chemosensory receptors in vertebrates and in the nematode likely preclude such a complete description of the odor selectivity of an entire OR gene family.

19.3 *Drosophila* gustatory receptor (*GR*) genes: a gene family that subserves both gustatory and olfactory modalities

The *DOR* genes are not the only chemosensory receptors in the fly genome. Using the same algorithms that yielded the *DOR* gene family, Clyne *et al*. (2000) isolated a second large family of 43 genes encoding seven transmembrane GPCRs. These were named gustatory receptors (*GR*), based on RT-PCR data indicating selective expression in taste tissues. Other researchers have expanded this gene family to at least 55 members (Dunipace *et al.*, 2001; Scott *et al.*, 2001). Unlike

the *DORs*, the *GRs* do exhibit some alternative splicing, which has the potential to produce multiple proteins from a given *GR* gene (Clyne *et al.*, 2000). The *GR* genes encode seven transmembrane domain proteins of approximately the same size as the *DOR* genes. As was seen for the *DORs*, the sequence similarity of the entire *GR* gene family is quite low, but there are a number of subfamilies with higher degrees of sequence relatedness. Interestingly, amino acid motifs at the C-termini of both gene families appear to be related, suggesting that these two chemosensory families may have a common evolutionary origin. The broadly expressed *DOR* gene, *Or83b*, is the most similar to the *GRs* and may represent the most ancient linkage between these two families of receptors.

Confirming the initial expression data obtained by RT-PCR Scott *et al*. (2001) obtained *in situ* hybridization evidence that four of the *GRs* are expressed in the adult labellum, the site of gustatory neurons. A fifth *GR* gene was found to be selectively expressed in the third antennal segment. The expression of many other *GRs* was not detected by *in situ* hybridization. To obtain tissue-specific expression data, two groups generated *GR*-Gal4 transgenic fly lines that would permit them to examine the distribution of members of the *GR* gene family (Dunipace *et al.*, 2001; Scott *et al.*, 2001). Characterization of a large number of *GR*-Gal4 transgenic lines yielded these important conclusions: *GR* genes are expressed in both gustatory and olfactory neurons; *GR* genes are detected in both adult and larval chemosensory organs; a given neuron is likely to express only a single *GR* gene; a given *GR* gene can be expressed in both gustatory and olfactory neurons. The neurons expressing *GRs* in the antenna are located in a distinctive medial location on the exterior face of the antenna, in a region in which *DOR* gene expression has not been detected. Because anatomical studies have not identified any sensilla that are likely to be gustatory in nature, the hypothesis is that *GR* genes expressed in antenna are in fact odorant receptors. It will be of interest to determine whether this hypothesis is correct, and if so, what odorants activate the *GRs*.

Although we do not know the ligand-binding properties of the *GR* genes expressed in olfactory neurons, recent genetic evidence suggests that at least one *GR* gene, *Gr5a*, is a molecular sweet taste receptor for the sugar trehalose (Dahanukar *et al.*, 2001; Ueno *et al.*, 2001). This conclusion must be tempered by an earlier report that a putative peptide GPCR linked to *Gr5a*, named *Tre1*, is in fact the trehalose receptor (Ishimoto *et al.*, 2000). In these experiments, a P-element located between *Tre1* and *Gr5a* was mobilized to generate local deletions around the site of insertion. To determine which gene is responsible for the behavior defect, Dahanukar *et al*. (2001) generated rescuing transgenes which contained either a functional *Gr5a* or *Tre1* gene. Rescue of the trehalose taste sensitivity mapped to the *Gr5a* gene, which is now considered to be the true trehalose receptor. Therefore, this constitutes the first functional proof that *GR* genes are receptors for non-volatile stimuli such as sugars. More work will be

required to determine if *GR* genes are exclusively sweet taste receptors, or if *GRs* expressed in olfactory tissues are specific for volatile olfactory stimuli.

19.4 Representations of chemosensory stimuli in the fly brain

How does the brain sense the activation of a defined subset of olfactory receptor neurons? In the mouse, all neurons expressing a given OR extend axons that synapse in two glomeruli in each olfactory bulb (Ressler *et al.*, 1994; Vassar *et al.*, 1994; Mombaerts *et al.*, 1996). Therefore activation of a given complement of ORs in the periphery will be represented in the olfactory bulb by the specific activation of a subset of glomeruli. This spatial map may be interpreted by higher brain centers to yield information about the nature and concentration of the odorous stimulus.

The fly antennal lobe contains at least 43 glomeruli which are likely to be the functional homologs of glomeruli in the vertebrate olfactory bulb (Laissue *et al.*, 1999). To ask how the projections of fly OSNs are organized in the antennal lobe, genetic tracing techniques that permit selective labeling of all neurons expressing a given receptor as they form synapses in the brain were developed. Genetic tracing techniques were required because OSNs expressing a given OR gene are interspersed with other OSNs, so it is not possible to use conventional dye-filling techniques to trace the circuitry of these neurons. The Gal4-UAS binary transcription system (Brand and Perrimon, 1993) was used to express Gal4 in all neurons expressing a given receptor, and then reveal this expression with a number of different marker proteins (Gao *et al.*, 2000; Vosshall *et al.*, 2000). A powerful tool to visualize axonal projections is the nsyb-GFP marker protein, an N-terminal fusion of neuronal synaptobrevin to the green fluorescent protein (Estes *et al.*, 2000) which accumulates in synaptic terminals. Promoter regions from a number of different *DOR* genes were placed upstream of Gal4, transgenic flies were generated, and these were crossed to flies carrying the UAS-nsyb-GFP transgene. Analysis of nsyb-GFP-labeled fibers in these animals produced the following important conclusions: all neurons expressing a given *DOR* gene extend axons that synapse with one or two unique, bilaterally symmetric antennal lobe glomeruli; these patterns of innervation are invariant between individuals of different ages and are not sexually dimorphic; all neurons examined form synapses in identified glomeruli in the ipsilateral antennal lobe, then branch and innervate the corresponding glomerulus in the contralateral antennal lobe; there is no overlap in the projections of *DOR* gene-expressing neurons examined to date, suggesting that at the extreme, every population of *DOR*-expressing neurons occupies a unique glomerulus (Gao *et al.*, 2000; Vosshall *et al.*, 2000). An example of such an experiment is shown in Figure 19.4. Depicted is a whole mount preparation of an adult brain stained with nc82 to reveal the architecture

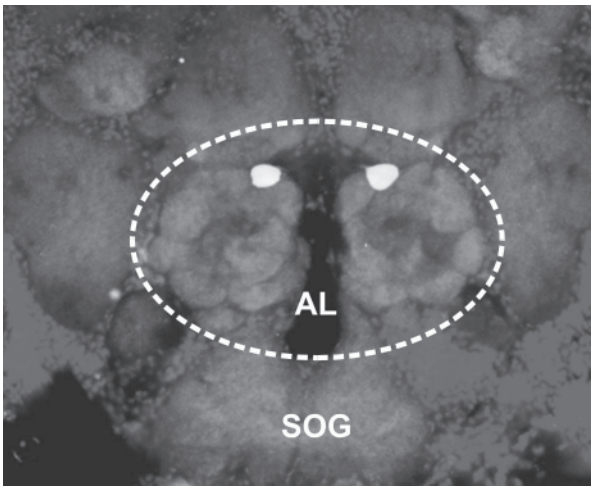

Figure 19.4 Convergence of olfactory axons in the brain. All neurons expressing *Or47a* converge upon a single dorsal medial glomerulus named DM3, which is bilaterally symmetric in the left and right antennal lobe (Vosshall *et al.*, 2000). Glomerular assignments were derived from a three-dimensional map of the antennal lobe (Laissue *et al.*, 1999). Frontal view of an adult brain whole mount preparation counterstained with nc82 antibody to reveal architecture of the brain. *Or47a* neurons were labeled by expression of synaptically targeted GFP under control of *Or47a* regulatory regions. GFP appear bright white in this micrograph. The antennal lobe (AL) is indicated with the dashed line. The subesophageal ganglion (SOG), which receives input from gustatory neurons, lies ventral to the AL.

of the brain. Axons of neurons expressing nsyb-GFP under control of the *Or47a* promoter are labeled in green (white in the figure). These neurons converge upon a single dorsal–medial glomerulus corresponding to DM3 in the three-dimensional map of the antennal lobe (Laissue *et al.*, 1999).

While the *DOR* genes appear to use a labeled-line system to relay information from the periphery to the brain, *GR*-expressing gustatory neurons have much more dispersed central projections. The same technique described above was used to trace projections of *GR*-expressing gustatory neurons in the adult and larva. Rather than clear convergence upon a restricted portion of the neuropil, *GR*-expressing neurons extend axons that occupy substantial space in the target region, the subesophageal ganglion (Dunipace *et al.*, 2001; Scott *et al.*, 2001). Further experiments are required to determine if each *GR* gene occupies a unique position in the subesophageal ganglion or whether there is broad overlap in the projections of gustatory neurons in the brain. The conceptual implication of overlap is that the processing of gustatory cues will of necessity be subject to a loss of discrimination. Olfactory information in contrast is more likely to retain

information about the nature of the stimulus because glomeruli acts as points of convergence that relay ligand information faithfully.

How are odorant-evoked patterns of activity in the antennal lobe represented in higher brain centers? To answer this question, two groups performed sophisticated anatomical tracing experiments to trace the projections of single projection neurons whose dendrites innervate a given glomerulus (Marin *et al.*, 2002; Wong *et al.*, 2002). To accomplish this, they made us of an enhancer trap line that expresses Gal4 in a large number of the projections neurons that surround the antennal lobe, as well as some unrelated neurons (Stocker *et al.*, 1997). Using various techniques to generate somatic mosaic clones in which only a single of these projections neurons now expresses Gal4, both groups were able to label these neurons with GFP variants that revealed the morphology of the entire neuron from dendrite to axon. Analysis of these animals revealed remarkable stereotypy in the axons arborization of all projection neurons whose neurons innervated a given glomerulus. Cluster analysis revealed that the glomerular identity of a given projection neuron could be predicted based solely on its characteristic axonal morphology in the mushroom body and lateral horn of the protocerebrum, the two target regions of antennal lobe neurons. This result suggests that an intricate and highly conserved genetic program patterns not only convergent glomerular projections of OSN axons and projection neuron dendrites, but also controls the patterns of innervation of projection neuron axons in higher brain centers. However, the patterns of axonal innervation of the lateral horn of the protocerebrum are much more diffuse than the spatially restricted glomeruli. There is likely to be extensive overlap in the projections of projections neurons receiving input from different glomeruli. The implications of this stereotyped wiring for olfactory processing are that odors are represented first by the specific activation of subsets of glomeruli and this information is then relayed and refined and interpreted by the summed action of a large network of second- and third-order neurons in the mushroom body and lateral horn of the protocerebrum. Direct functional proof of this hypothesis of odor coding will require the evolution of techniques that permit functional imaging of brain activity in living animals perceiving and interpreting odor cues.

19.5 Conclusion and future prospects

The identification of *DOR* genes has permitted analysis of many different aspects of olfactory biology in the fruit fly. We now know the complete repertoire of genes that are likely to recognize odorants. Techniques exist to measure the response properties of a given OR and to examine the function of the neuron *in vivo*. The first- and second-order olfactory projections have been mapped anatomically. Although the fly is generally deemed to be too small for

electrophysiology, recent advances in functional imaging promise to make the analysis of olfactory processing in living animals an experimental reality (Zemelman and Miesenbock, 2001).

One fundamental unsolved question in sensory biology is how the olfactory system processes distinct olfactory cues to elicit appropriate behavioral responses. With the complete repertoire of odorant receptor genes in hand, along with a growing understanding of the neuroanatomy of the system, it has now become possible to address this question in *Drosophila*. There are a number of behavior genetic approaches to matching specific odorous ligands with identified odorant receptor genes, the neurons that express these receptor genes, and the circuits that lead to stereotyped behaviors. *Drosophila* therefore promises to be a useful genetic model system of olfaction for the foreseeable future.

Abbreviations

7TM, seven transmembrane domain; *DOR*, *Drosophila* odorant receptor; EAG, electroantennogram ; GFP, green fluorescent protein; GPCR, G-protein coupled receptor; *GR*, gustatory receptor; nsyb, neuronal synaptobrevin; OR, odorant receptor; OSN, olfactory sensory neuron; PCR, polymerase chain reaction; PN, projection neuron; RT-PCR, reverse transcription-polymerase chain reaction; SOG, subesophageal ganglion.

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