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Genetic and Functional Subdivision of the *Drosophila* Antennal Lobe

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Summary

Olfactory systems confer the recognition and discrimination of a large number of structurally distinct odor molecules. Recent molecular analysis of odorant receptor (OR) genes and circuits has led to a model of odor coding in which a population of olfactory sensory neurons (OSNs) expressing a single OR converges upon a unique olfactory glomerulus. Activation of the OR can thus be read out by the activation of its cognate glomerulus [1, 2]. *Drosophila* is a powerful system in which to test this model because the entire repertoire of 62 ORs [3–6] can be manipulated genetically. However, a complete understanding of how fly olfactory circuits are organized is lacking [6–13]. Here, we present a nearly complete map of OR projections from OSNs to the antennal lobe (AL) in the fly brain. Four populations of OSNs coexpress two ORs along with *Or83b*, and a fifth expresses one OR and one gustatory receptor (GR) along with *Or83b*. One glomerulus receives coconvergent input from two separate populations of OSNs. Three ORs label sexually dimorphic glomeruli implicated in sexual courtship [14–16] and are thus candidate *Drosophila* pheromone receptors. This olfactory sensory map provides an experimental framework for relating ORs to glomeruli and ultimately behavior.

Results and Discussion

Peripheral Organization of the *Drosophila* Olfactory System

We present the results of a large-scale genetic effort to label OSNs expressing each of the 62 known OR genes and map their projections to approximately 50 morphologically defined glomeruli in the adult AL [17]. Putative regulatory regions upstream of 49 ORs were cloned in front of the Gal4 transcription factor, and transgenic flies carrying these OR-Gal4 transgenes were crossed to cytoplasmic (UAS-lacZ) and synaptic (UAS-nsyb-GFP) reporters. Of these 49 OR-Gal4 transgenes, 30—comprising 25 antennal and five maxillary palp ORs—produce appropriate gene expression in subpopulations of adult OSNs and are presented here. Of the remaining 19 OR-Gal4 constructs, 11 are selectively expressed in the larval olfactory system (E.F., A.I. Domingos, K. Asahina, F. Naef, L.B.V., and M. Louis, unpublished data), one (*Or83b*) is broadly expressed in most OSNs, where it plays an essential role in olfaction [18], and seven either show no expression or are ectopically expressed (see legend to Figure 1). The remaining 13 OR-Gal4 lines were not generated as a result of design constraints imposed by the tight linkage of these ORs to unrelated genes or technical difficulties. Patterns of OR-Gal4:UAS-lacZ gene expression of 30 transgenes in the antenna and maxillary palp are similar to those obtained by an in situ hybridization screen (Figures 1A and 1B) [5, 6] and follow the same strict segregation of ORs expressed in the antenna and maxillary palp, with no OR-Gal4 lines expressed in both organs. These same lines were later used to determine the map of connectivity to the antennal lobe (Figure 2). Double-RNA in situ hybridization was performed to verify that OR-Gal4 lines reflect the expression of the endogenous OR mRNA (Figure 3C and data not shown). Although levels of staining varied, all cells appeared to have both endogenous OR and Gal4 transcripts. At least two OR-Gal4 lines were examined for each OR.

Glomerular Map of the Antenna

To determine how OSNs expressing different ORs connect to the brain, we traced genetically labeled axons to their termini in the adult-fly AL (Figure 2). Analysis of glomerular projections of the 25 antennal OR-Gal4:UAS-nsyb-GFP strains reveals that 23 populations of OSNs expressing different ORs target a single glomerulus, whereas two (*Or33b* and *Or67d*) project to two glomeruli. Both *Or33b*-Gal4 and *Or67d*-Gal4 are coexpressed with their respective endogenous ORs, suggesting that these OSN populations indeed innervate two glomeruli (Figure 3C). There is some apparent redundancy in the map, which is investigated further below: We find that six glomeruli are independently labeled by two different OR-Gal4 lines (Figure 2A).

In some cases, we observe weak and variable labeling, in secondary glomeruli, that reflects either variability in the expression levels of ORs in different subpopulations of neurons or transgene variability, a phenomenon also noted by others [19]. Distinguishing between these possibilities is constrained by detection thresholds of in situ hybridization, which may not detect OR transcripts in cells weakly positive for the OR-Gal4 transgene. As previously reported, some *Or23a*-Gal4 lines mark a second, ventrally located glomerulus (possibly DP1m) [6, 7]. Other cases of weak and variable secondary innervation include the following: *Or65a*-Gal4, in the vicinity of D; *Or85a*-Gal4, in the vicinity of DM3; *Or56a*-Gal4, in the vicinity of DL4; *Or10a*-Gal4, in the vicinity of VA7m; and *Or33b*-Gal4, variable ectopic expression in multiple glomeruli. We base our general conclusions below on those glomeruli that show strong and reproducible labeling, although we recognize that the weakly labeled glomeruli may also contribute to the odor code.

Glomerular Map of the Maxillary Palp

Axonal projections of the five maxillary palp OR-Gal4 lines were examined in brain whole mounts (Figure 2B). All palp neurons target the same ventral-medial region in the AL that does not receive projections from anten-

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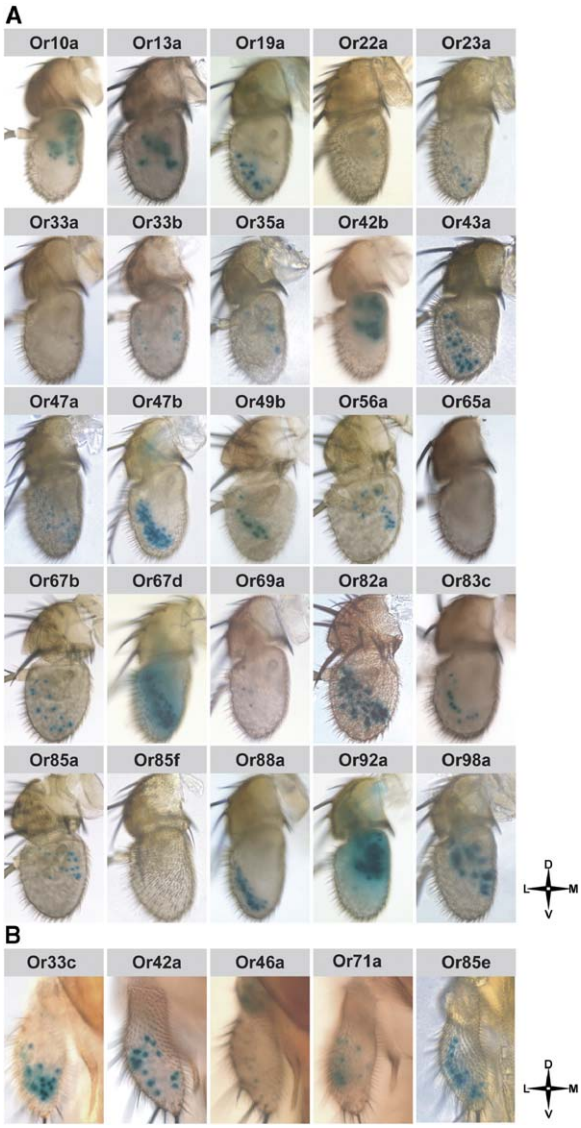

Figure 1. Peripheral Map of OR Gene Expression in *Drosophila* Chemosensory Organs Reveals Stereotyped and Interspersed Patterns of Gene Expression

(A) Expression of antennal OR-Gal4 transgenes revealed by lacZ activity staining (blue). *Or85f*-Gal4 is weak, and *Or65a*-Gal4 staining is not detectable under these staining conditions.

(B) Expression of maxillary palp OR-Gal4 transgenes revealed by lacZ activity staining (blue). Whole mounts of heads from OR-Gal4: UAS-lacZ transgenic heterozygous animals were stained for β-galactosidase activity as described [5]. No staining was detected in structures other than antennae and palps (data not shown). All OR-Gal4 transgenes were constructed as described [6], with the following promoter lengths upstream of the initiating methionine: *Or10a*, 6.868 Kb; *Or13a*, 8.199 Kb; *Or19a*, 9.934 Kb; *Or33a*, 5.155; *Or33b*, 8.086 Kb; *Or33c*, 7.156 Kb; *Or35a*, 3.880 Kb; *Or42a*, 4.184 Kb; *Or42b*, 8.039 Kb; *Or49b*, 8.834 Kb; *Or56a*, 5.385 Kb; *Or65a*, 7.764 Kb; *Or67b*, 2.740 Kb; *Or67d*, 7.272 Kb; *Or69a*, 1.923 Kb; *Or82a*, 1.865 Kb; *Or83c*, 7.843 Kb; *Or85a*, 2.791 Kb; *Or85e*, 7.5 Kb; *Or85f*, 8.961 Kb; *Or88a*, 1.656 Kb; *Or92a*, 9.247 Kb; and *Or98a*, 9.538 Kb. *Or22a*, *Or23a*, *Or46a*, *Or47a*, and *Or47b* promoters were previously described [6]. The 11 OR-Gal4 expressed only in larval OSNs are described fully in a separate study (E.F., A.I. Domingos, K. Asahina, F. Naef, L.B.V., and M. Louis, unpublished data), as is *Or83b*-Gal4 [11, 18]. The following OR-Gal4 lines did not express or expressed too weakly to be analyzed: *Or2a*, 0.900 Kb; *Or7a*, 4.203 Kb; *Or22b*, 0.900 Kb; *Or65b*, 3.405 Kb; and *Or98b*, 5.120 Kb. *Or59c* (5.280 kb) and *Or85c* (7.588 Kb) showed ectopic expression that did not faithfully reproduce the expression of the endogenous OR. No PCR product could be obtained from the regions upstream of the following ORs, as a result of either annotation or technical issues: *Or9a*, *Or19b*, *Or43b*, *Or67a*, *Or67c*, *Or69b*, and *Or94a*. The following ORs were too closely linked to other genes to attempt an OR-Gal4 fusion construct: *Or46b*, *Or59b*, *Or65c*, *Or85b*, *Or85d*, and *Or94b*. Orientation is as specified at lower right: D denotes dorsal; V denotes ventral; L denotes lateral; and M denotes medial.

nal OSNs (Figure 4). *Or33c* and *Or85e* are coexpressed [13], and these OSNs target the VC1 glomerulus (Figure 2B). *Or46a*-expressing OSNs target an AL region with diffuse glomerular boundaries [17], and thus no glomerular identity could be assigned (Figures 2B, 3A, and 4). To clarify the position of *Or46a* relative to the assigned palp glomeruli, we examined simultaneously the projections of *Or46a*-expressing neurons compared to other palp OSNs. The *Or71a* glomerulus is located in the same anterior-posterior plane but medial to *Or46a*, whereas the *Or33c*/*Or85e* glomerulus is located posterior and slightly medial to *Or46a* (Figures 3C and 4). The segregation of antennal and maxillary palp projections in *Drosophila* was previously noted in anatomical tracing studies that preceded the advent of OR markers [20]. The functional significance of antennal and maxillary palp segregation remains obscure because no exclusive function has been ascribed to either olfactory organ.

Complexity in the Olfactory Circuit: Coexpression and Coconvergence

A complete list of ORs examined here and the glomeruli they target is presented in Figure 3; the list is sorted by ORs in Figure 3A and by glomeruli in Figure 3B. We find five cases in which two OR-Gal4 lines label the same glomerulus and one case in which a single glomerulus is marked by an OR-Gal4 and a GR-Gal4 line. We carried out two-color RNA in situ hybridization to distinguish between two possibilities that would lead to two different OR-Gal4 lines apparently labeling the same glomerulus—the same population of OSNs is labeled by different OR-Gal4 lines, or two separate OSN populations marked by two different OR-Gal4 lines coconverge to the same glomerulus. Endogenous mRNAs for *Or33a*/*Or56a*, *Or10a*/*Gr10a*, *Or33b*/*Or47a*, *Or33b*/*Or85a*, and *Or33c*/*Or85e* [13] are coexpressed (Figure 3B, left). Whereas *Or33b* is coexpressed with both *Or47a* and *Or85a*, *Or47a* and *Or85a* are expressed in different OSNs (data not shown). *Or67d* and *Or82a* are not coexpressed (Figure 3B, right). Therefore, glomerulus VA6 receives input from two separate populations of OSNs expressing different ORs (Figures 2A and 4).

The Functional Organization of the *Drosophila* Antennal Lobe

The availability of a more complete map of AL projections allowed us to examine the organizational logic of this first olfactory synapse. There is a general trend for OSNs located in lateral/distal positions in the antenna to project to lateral AL glomeruli and for medial/proxi-

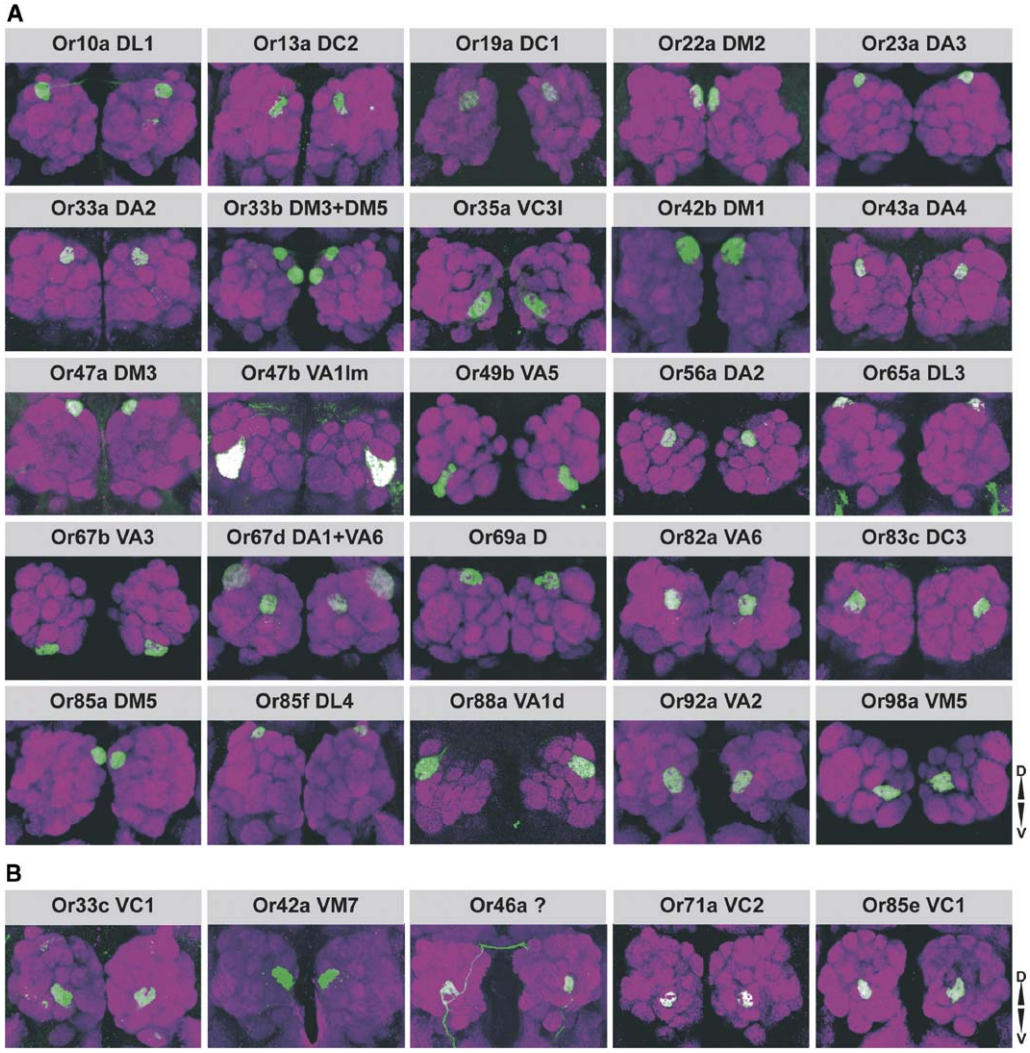

Figure 2. Genetic Map of Olfactory Sensory Axonal Projections Shows Stereotypy and Bilateral Symmetry in the *Drosophila* Antennal Lobe

(A) Whole-mount immunofluorescence of adult brains from antennal OR-Gal4:UAS-nsyb-GFP animals were stained with anti-GFP (green) and nc82 (magenta) as described [6]. Each data panel is labeled with the glomerulus labeled by a given OR-Gal4 line [17].

(B) Analysis of maxillary palp OR-Gal4 lines performed as in (A) above, except that *Or46a*-Gal4 was crossed to UAS-CD8-GFP. We were unable to assign *Or46a*-Gal4 OSN projections to a known glomerulus, in contrast to previous reports that mapped this to VA5 [12]. All images are projections of confocal Z-series, available as raw confocal stacks at http://www.rockefeller.edu/labheads/vosshall/reprints.php. Multiple independent samples from at least two OR-Gal4 lines per transgene were examined to make glomerular assignments. Orientation is as specified at lower right.

mal OSNs to target medial AL glomeruli (Figures 1 and 2). This segregation is most likely related to the topographic segregation of trichoid and basiconic classes of sensilla on the surface of the antenna [20]. *Or47b*, *Or88a*, and *Or67d* are examples of the former, and *Or42b*, *Or33b*, and *Or22a* are examples of the latter. There are exceptions, notably *Or19a*, which targets a dorsal/medial glomerulus, although *Or19a* OSNs are located in the lateral/distal domain in the antenna. Of the 47 distinct glomerular compartments described [17], this study assigns a genetic OR identity to 26. We are unable to assign a name to *Or46a*, which may be a previously unnamed glomerulus. Other studies mapped *Gr21a* OSNs to V [8] and *Or59c* OSNs to 1 [12], bringing the total known number of OR assignments to AL glomeruli in the present literature to 29. Eighteen glomeruli remain to be associated with chemosensory receptors, and 20 ORs were not included in our mapping here. Thus, it is likely that projections of distinct OSNs can account for the remaining uncharacterized glomeruli.

We went on to synthesize available knowledge of OSN odor-response profiles with their glomerular identity to determine whether there is any obvious chemotopic organization in the fly AL (Figures 3A and 4). This analysis was constrained by the limited and nonoverlapping collections of odorants used by different groups as well as differences in experimental techniques that make it difficult to compare across studies [11, 13, 21–24]. Each OR/glomerulus was screened with a small subset of the 76 odors used across these six

A

OR	Glomerulus	Ligands
Or10a	DL1	3/28 [11, 21]: isoamyl acetate, acetophenone, methyl salicylate
Or13a	DC2	2/16 [11]: 1-octen-3-ol, 3-octanol
Or19a	DC1	11/30 [11, 21]: cineole, hexane, isoamyl acetate, pentyl acetate, ethyl butyrate, 1-hexanol, 1-octen-3-ol, 2-heptanone, 2-octanone, 1-octanol, butyl acetate
Or22a	DM2	11/29 [11, 21]: r-carvone, octanal, 3-octanone, caproic acid, hexane, isoamyl acetate, pentyl acetate, 1-octen-3-ol, ethyl butyrate, ethyl proprionate, butyl acetate
Or23a	DA3	0/16 [21]
Or33a	DA2	0/16 [11]
Or33b	DM3+DM5	0/28 [11, 21]
Or33c	VC1	1/11 [13]: E2-hexenal
Or35a	VC3l	11/17 [21]: pentyl acetate, ethyl butyrate, 1-hexanol, 1-octen-3-ol, E2-hexenal, cyclohexanone, 2-heptanone, 1-butanol, 1-octanal, ethyl 3-hydroxy butyrate, 1-nonanal, 2-pentanone
Or42a	VM7	9/30 [13, 22]: ethyl acetate, isoamyl acetate, E2-hexenal, 2-heptanone, ethyl butyrate, 1-hexanol, 2,3-butanedione, propyl acetate, 1-butanol
Or42b	DM1	11/16 [11]: cyclohexanone, r-carvone, s-carvone, linalool, 3-octanone, 3-octanol, 1-octen-3-ol, benzaldehyde, caproic acid, hexane, isoamyl acetate
Or43a	DA4	9/39 [11, 21, 23, 24]: cineole, hexane, isoamyl acetate, hexanol, 1-octen-3-ol, cyclohexanol , cyclohexanone, benzaldehyde, benzyl alcohol
Or46a	?	1/11 [13]: 4-methylphenol
Or47a	DM3	6/28 [11, 21]: hexane, isoamyl acetate, ethyl acetate, pentyl acetate, 2-heptanone, 3-(methylthio)-1-propanol
Or47b	VA1l/m	0/28 [11, 21]
Or49b	VA5	2/17 [21]: 3-methyl phenol, 2-methyl phenol
Or56a	DA2	0/16 [11]
Or65a	DL3	0/17 [21]
Or67b	VA3	13/39 [11, 22]: cyclohexanone, 3-octanone, 1-octen-3-ol, hexane, pyridine, pentyl acetate, 1-hexanol, E2-hexenal, 2-heptanone, 1-butanol, 1-heptanol, benzaldehyde, acetophenone
Or67d	DA1+VA6	N.D.
Or69a	D	1/16 [11]: isoamyl acetate
Or71a	VC2	1/23 [11, 13]: 4-methylphenol
Or82a	VA6	1/17 [21]: geranyl acetate
Or83c	DC3	N.D.
Or85a	DM5	4/30 [11, 21]: ethyl butyrate, 1-hexanol, ethyl 3-hydroxybutyrate, E2-hexen-1-ol
Or85e	VC1	3/11 [13]: ethyl acetate, cyclohexanone, (-) fenchone
Or85f	DL4	0/16 [21]
Or88a	VA1d	2/29 [11, 21]: hexane, isoamyl acetate
Or92a	VA2	3/16 [11]: r-carvone, s-carvone, octanal
Or98a	VM5	7/15 [21]: pentyl acetate, ethyl butyrate, 1-hexanol, 1-octen-3-ol, 2-heptanone, E2-hexen-1-ol, linalool

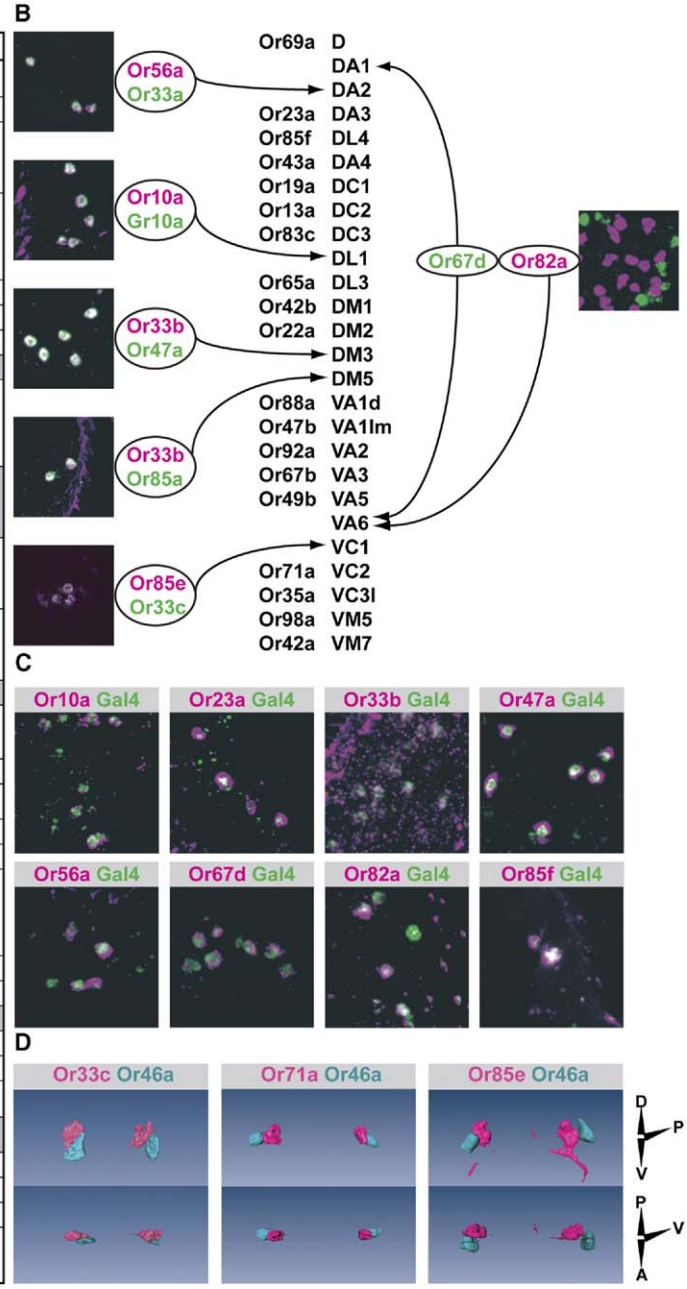

Figure 3. The Glomerular Map Reveals Unexpected Complexity of OR Coexpression and Coconvergence

(A) Summary of OR glomerular assignments and ligand specificity. Maxillary palp genes are highlighted in light gray. Assignments for *Or46a* and *Or83c* differ from a previous study [12]. Odor ligands that activate each OR or its corresponding glomerulus are listed in the right column and are drawn from previous studies [11, 13, 21–24]. Listed are the number of strong ligands/total ligands tested in all studies that examined the OR/glomerulus, the references, and a list of strong ligands. Criteria for inclusion were odorants classified as "strong ligands" in heterologous expression systems [23, 24], odorants that induced activity at 20% saturated vapor in imaging experiments [11], or those that produced more than 100 spikes/s in electrophysiological experiments [13, 21, 22]. N.D. denotes not done.

(B) Summary of OR/glomerular assignments sorted by glomerulus, with cases of coexpression at the left and coconvergence at the right. In situ hybridization was performed as previously described [5] with digoxigenin- (magenta) and fluorescein-labeled (green) riboprobes, detected first with TSA-Plus Fluorescein System (fluorescein; Perkin Elmer) and then with TSA-Plus Cyanine 5 System (digoxigenin; Perkin Elmer), after the fluorescein reaction was quenched for 1 hr with 3% hydrogen peroxide. Anti-digoxigenin-POD and anti-fluorescein-POD were diluted 1:500 (Roche).

(C) Two-color RNA in situ hybridization demonstrates faithful expression of OR-Gal4 transgenes. Frozen sections of OR-Gal4 animals were hybridized with OR-digoxigenin (magenta) probes and Gal4-fluorescein (green) probes.

(D) Three-dimensional reconstructions of maxillary palp projections from brains of *Or46a*-nsyb-GFP:OrX-Gal4:UAS-IMPTNT [30] animals visualized with anti-GFP (cyan) and anti-tetanus toxin (magenta) to clarify the positions of known palp glomeruli relative to *Or46a*. The segmentation software Amira (Mercury Computer Systems, Berlin) was used to examine and rotate confocal Z-stacks. Orientation is as specified at lower right: A denotes anterior; P denotes posterior.

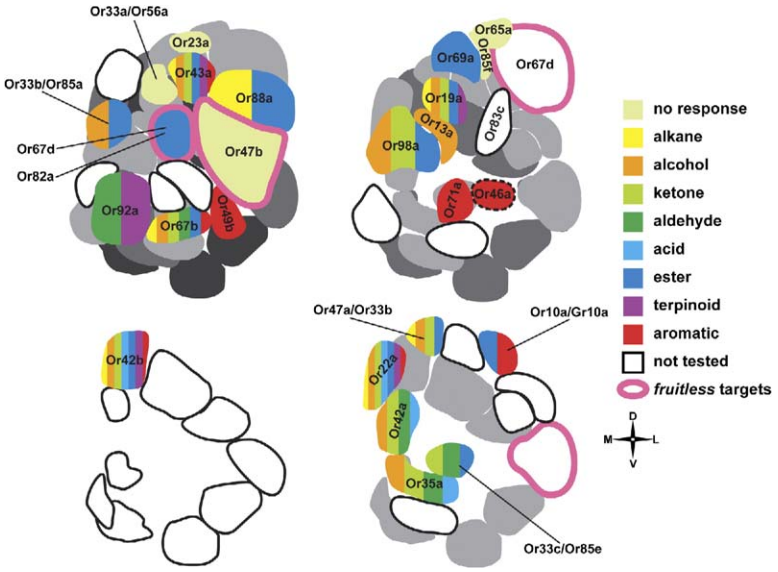

Figure 4. The Genetic Map of Olfactory Sensory Projections Reveals Functional Subdomains in the *Drosophila* Antennal Lobe

Schematic of mapping results coded for functional properties where ORs and glomeruli are colored according to the functional groups of odorants that activate them. The odorants are classified by the functional group with the highest priority (IUPAC nomenclature), except for aromatic ring compounds and terpene derivatives, which are placed in separate categories (see legend to Figure 3A); *fru* target glomeruli are outlined in pink [15, 16]; the estimated position of *Or46a* maxillary palp glomerulus is indicated by dashed line. Antennal-lobe model adapted from Figure 2 of Laissue et al. [17] with antennal-lobe sections presented from anterior to posterior, clockwise from top left, with depth-coding of black for deep, gray for intermediate, and white for superficial sections. Forty-seven individual glomeruli are represented, comprising 43 glomeruli with subcompartments previously identified [17], along with *Or46a*, which could not be assigned.

studies, and thus no comprehensive survey of the ligand specificity of a given OR/glomerulus exists. Nevertheless, we find a greater tendency for OSNs expressing broadly responsive ORs to project to dorsal/medial glomeruli, whereas the more selective glomeruli are located at ventral/lateral positions (Figure 4). However, there are many exceptions to this rule, and the ordered chemotopy described in the mouse olfactory bulb [25] is not obvious in the fly AL.

Implications of Coexpression for Odor Coding

We report five subpopulations of OSNs that express multiple receptors along with the universal coreceptor *Or83b* [18]. What might be the function of such OR coexpression? On the basis of previously published odor-response profiles for *Or33b*, *Or47a*, and *Or85a* [21], we suggest that OR coexpression could modulate ligand-response profiles. Both *Or47a* and *Or85a* respond to more odors when ectopically expressed in the ab3A "empty" neuron than the native neurons, which coexpress *Or33b*/*Or47a* or *Or33b*/*Or85a* [21]. *Or33b* expressed alone in the "empty" neuron responds weakly and with inhibition to most odors, giving a weak excitatory response only to ethyl propionate [21]. Thus, *Or33b* coexpression in the native OSN could function to temper the relatively broad tuning of *Or47a* and *Or85a*. Confirmation of such a role awaits further genetic analysis of these ORs in vivo.

We identify an intriguing OSN population that coexpresses members of the OR and GR families, along with *Or83b*. The role of GRs in the antenna is poorly understood, although *Gr21a* is expressed in neurons that respond to carbon dioxide [8, 26, 27]. It will be interesting to determine whether *Gr10a* contributes to the detection of odors along with *Or10a* and subserves an olfactory instead of gustatory function.

Candidate *Drosophila* Pheromone Receptors

Recent work examining the expression of the male-specific isoform of the *fruitless* (*fru*) transcription factor implicates two large, sexually dimorphic glomeruli (VA1lm and DA1) in male courtship behavior [14–16]. Our study has revealed the molecular identity of the OSNs projecting to these glomeruli as *Or47b* and *Or67d*, respectively. Other glomeruli that receive input from *fru*-expressing OSNs are VL2a [15, 16] and, occasionally, VA6 [15], which we identify here as receiving coconvergent input from Or82a- and Or67d-expressing OSNs. The identity of the VL2a-projecting OSNs remains obscure. Interestingly, these OSNs and the glomeruli to which they project show little or no activation in response to general odors (Figures 3A and 4) [11, 21]. The exception is *Or82a*, which responds very selectively to geranyl acetate, a green-leaf volatile that is also a major component of medfly male sex pheromone [28]. On the basis of anatomical tracing studies presented here, we suggest that *Or82a*- and *Or67d*-expressing OSNs target the same glomerulus. Whether they synapse uniformly upon the same population of postsynaptic projection neurons or whether the glomerulus has functional subcompartments is not known. In either scenario, the glomerulus might act as a coincidence detector that would require both the *Or82a* ligand, geranyl acetate, and the unknown *Or67d* ligand for activation.

Courtship behavior in *Drosophila* involves multimodal input from visual, gustatory, auditory, and olfactory cues [29]. The involvement of volatile pheromones in *Drosophila* sexual behavior has long been inferred, but neither the putative pheromones nor the receptors that detect them are known. We suggest that these ORs are pheromone receptors that respond to volatile pheromones. In support of this, silencing or reprogramming these OSNs leads to selective disruption in male sexual behavior [15, 16] (J.E. Mehren, L. Giarratani, and L.B.V., unpublished data).

Conclusions

In this paper, we present a nearly complete map of olfactory projections to the fly AL. From this map, we

identify five populations of OSNs that express multiple receptors and two populations of OSNs expressing different ORs that coconverge upon a common glomerulus. An analysis of published odor-response profiles for these ORs and their glomeruli suggests that more broadly tuned neurons map to the dorsal/medial domain, whereas more restricted OSNs map to ventral/lateral glomeruli. We also identify candidate *Drosophila* pheromone receptors by virtue of their innervation of sexually dimorphic *fru*-positive glomeruli. A number of intriguing questions follow from our study. First, what is the genetic identity of the projections that target the posterior face of the antennal lobe? These glomeruli may receive input from OSNs expressing OR or GR genes we did not examine in this study. Second, it will be of interest to understand in greater detail what effects receptor coexpression and OSN coconvergence have on the capacity of the fly to detect and discriminate odors. Finally, the availability of candidate pheromone receptors in *Drosophila* will make it possible to study sex pheromones in a genetically tractable organism from the circuits they activate to the stereotyped behaviors they elicit.

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