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Molecular, Anatomical, and Functional Organization of the *Drosophila* Olfactory System

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Summary

Background: Olfactory receptor neurons (ORNs) convey chemical information into the brain, producing internal representations of odors detected in the periphery. A comprehensive understanding of the molecular and neural mechanisms of odor detection and processing requires complete maps of odorant receptor (Or) expression and ORN connectivity, preferably at single-cell resolution.
Results: We have constructed near-complete maps of Or expression and ORN targeting in the *Drosophila* olfactory system. These maps confirm the general validity of the "one neuron—one receptor" and "one glomerulus—one receptor" principles and reveal several additional features of olfactory organization. ORNs in distinct sensilla types project to distinct regions of the antennal lobe, but neighbor relations are not preserved. ORNs grouped in the same sensilla do not express similar receptors, but similar receptors tend to map to closely appositioned glomeruli in the antennal lobe. This organization may serve to ensure that odor representations are dispersed in the periphery but clustered centrally. Integrated with electrophysiological data, these maps also predict glomerular representations of specific odorants. Representations of aliphatic and aromatic compounds are spatially segregated, with those of aliphatic compounds arranged topographically according to carbon chain length.
Conclusions: These Or expression and ORN connectivity maps provide further insight into the molecular, anatomical, and functional organization of the *Drosophila* olfactory system. Our maps also provide an essential resource for investigating how internal odor representations are generated and how they are further processed and transmitted to higher brain centers.

Introduction

The sense of smell involves peripheral systems for odor recognition and central systems for odor discrimination. Odors are detected in the periphery by olfactory receptor neurons (ORNs), which project their axons centrally to synaptic modules in the brain called glomeruli [1]. Odors are recognized by odorant receptor (Or) molecules expressed in ORNs. These odorant receptors are predicted G protein-coupled receptors encoded by large and diverse gene families [2–4]. Activation of odorant receptors triggers ORNs to fire action potentials, which result in spatially defined patterns of glomerular activity in the brain [5, 6].

Two central principles of olfaction in both mammals and insects posit that each ORN expresses just a single odorant receptor and that each glomerulus receives input only from a single class of ORNs [1]. The evidence to support these two principles is substantial but not conclusive. Formal proof will only come with complete maps of Or expression and ORN connectivity. Complete olfactory maps should also shed light on other critical issues in olfaction. For example, what higher-order organizational principles underlie the specific arrangements of ORNs in the periphery and of glomeruli in the brain? And how might this anatomical organization relate to olfactory function? In addition, complete olfactory maps will also be necessary for a comprehensive understanding of the molecular and neural mechanisms of odor detection and processing.

For most species, the large numbers of odorant receptors, ORNs, and glomeruli make the construction of complete maps of receptor expression and ORN connectivity a formidable task. For example, in mice there are ~2 million ORNs, ~1000 odorant receptors, and ~2000 glomeruli. Fortunately, the olfactory system of the *Drosophila melanogaster* adult is organized according to similar principles but with vastly reduced numerical complexity: ~1300 ORNs, 62 odorant receptors, and ~50 glomeruli [7]. The construction of a complete olfactory map for *Drosophila* is therefore both a feasible and important goal.

Here, we report a systematic survey of *Drosophila* Or expression at cellular resolution and the construction of a near complete map of ORN connectivity. These expression and connectivity maps confirm the general validity of the "one neuron—one receptor" and "one glomerulus—one receptor" principles. They also reveal additional organization features of the *Drosophila* olfactory system, including a topographic arrangement of glomeruli in the antennal lobe and a tendency for ORNs expressing related receptors to cluster centrally but not in the periphery. These maps also allow us to predict internal representations of olfactory stimuli, revealing a chemotopic organization of the *Drosophila* antennal lobe.

Results

Adult Odorant Receptors

The 60 *Or* genes of *Drosophila* are predicted to encode a total of 62 odorant receptors, from transcripts originating from 62 distinct *Or* promoters [4, 8–10]. We constructed a set of *mCD8-GFP* reporter lines for all 62 promoters, as well as 59 *GAL4* reporter lines (see Table S1 in the Supplemental Data available with this article online). In parallel, we performed a set of in situ hybridization experiments to detect mRNA for 54 distinct *Or* genes in sections of the adult olfactory organs, the an-

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Table 1. Molecular and Connectivity Maps of the Adult Olfactory System

Or	Sensillum	Glomerulus	Or	Sensillum	Glomerulus
Or2a	at3	DA4m	Or59b	ab2	DM4
Or7a	ab4	DL5	Or59c	pb3	1
Or9a	ab8	VM3	Or65a	at4	DL3
Or10a	ab1	DL1	Or65b	at4	DL3
Or13a	ai1	DC2	Or65c	at4	DL3
Or19a	at3	DC1	Or67a	ab10	DM6
Or19b	at3	DC1	Or67b	ab9	VA3
Or22a	ab3	DM2	Or67c	ab7	VC4
Or22b	ab3	DM2	Or67d	at1	DA1
Or23a	at2	DA3	Or69aA	ab9	D
Or33c	pb2	VC1	Or69aB	ab9	D
Or35a	ac1	VC3	Or71a	pb1	VC2
Or42a	pb1	VM7	Or82a	ab5	VA6
Or42b	ab1	DM1	Or83c	at2	DC3
Or43a	at3	DA4l	Or85a	ab2	DM5
Or43b	ab8	VM2	Or85d	pb3	VA4
Or46aA	pb2	VA7l	Or85e	pb2	VC1
Or47a	ab5	DM3	Or85f	ab10	DL4
Or47b	at4	VA1v	Or88a	at4	VA1d
Or49a	ab10	DL4	Or92a	ab1	VA2
Or49b	ab6	VA5	Or98a	ab7	VM5v
Or56a	ab4	DA2	Gr21a	ab1	V

Sensillum type and target glomerulus for 44 odorant receptors for which *Or-mCD8-GFP* reporters were generated and validated by in situ hybridization. Reporters for *Or85b* and *Or98b* both target the glomerulus VM5d, but neither has been validated by in situ hybridization.

tennae and maxillary palps. For all *Or* genes for which we detected expression by both of these methods, we also probed sections simultaneously for the *Or* mRNA and the corresponding *Or* transgenic reporter. In all, we identified 42 *Or* genes for which the reporter line and in situ hybridization labeled identical subsets of ORNs. None of these *Or* genes is sex specific. In addition, our *Or83b* reporter is broadly expressed in ORNs, as is *Or83b* itself [8]. We failed to obtain a validated reporter for only five of the *Or* genes known to be expressed in the antenna or maxillary palp (*Or1a*, *Or33a*, *Or71a*, *Or85b*, and *Or98b*), for one of which (*Or71a*) a validated reporter has already been obtained by others [11, 12]. Thus, we can confidently map the expression of 44 *Or* genes in the antenna and maxillary palp (Table 1 and Figure 1).

A related family of 60 *Gr* genes encodes 68 predicted gustatory receptors [4, 13, 14], some of which may actually function as odorant receptors [15]. We therefore performed a systematic survey of *Gr* expression in the olfactory system, preparing *mCD8-GFP* reporter lines for 67 of 68 *Gr* promoters (B. Schlager, A. Kurtovic, A.C., and B.J.D., unpublished). Only *Gr21a* could be mapped to specific ORNs by both reporter expression and in situ hybridization, consistent with previous reports [13, 15]. Several other *Gr* reporters are also expressed in the antenna, but their expression is generally either weak or broad or could not be confirmed by in situ hybridization. We therefore conclude that few if any of the other *Gr* genes are likely to encode odorant receptors.

Larval Odorant Receptors

The larval olfactory system comprises just 21 ORNs [16, 17], which also expresses members of the *Or* gene family [18]. Since 14 of the *Or* reporters could not be detected in the adult, we suspected that these *Or* genes may in fact encode larval odorant receptors. Indeed, 11 of these reporters are expressed specifically in the larval olfactory system. We expanded this search to cover all *Or* reporters and identified another seven *Or* genes that are expressed in the larval as well as the adult olfactory system. This analysis confirmed 8 of the 10 larval *Or* genes previously identified by both RT-PCR and reporter experiments, but only 3 of the additional 13 receptors with RT-PCR evidence alone [18]. Thus, we have now identified a total of 20 larval *Or* genes (in addition to *Or83b*; Figure 1). This is a close match to the 21 ORNs, consistent with the notion that, in the larva, each ORN expresses a single and distinct odorant receptor [18]. Our analysis also confirms that the larval and adult odorant receptors are encoded by phylogenetically dispersed members of the same *Or* family, some of which are stage specific whereas others are common to both stages (Figure 1).

The Receptor-to-Neuron Map

In the adult, ORNs are housed in sensory sensilla of four distinct morphological types: basiconic, trichoid, coeloconic, and intermediate sensilla (in order of decreasing abundance) [19]. All four sensilla types are found on the antenna. The maxillary palp contains only basiconic sensilla. We mapped each adult *Or* gene to a specific sensillum type simply by noting the morphology of the sensilla innervated by GFP-positive dendrites in *Or-mCD8-GFP* or *Or-GAL4, UAS-mCD8-GFP* flies (Figure 1).

Most sensilla contain 2–4 ORNs [19], so we proceeded to identify pairs of *Or* genes expressed in distinct ORNs of the same sensillum. For this, we performed double labelings for most possible pairings of *Or* genes within both the basiconic and trichoid classes. This generally

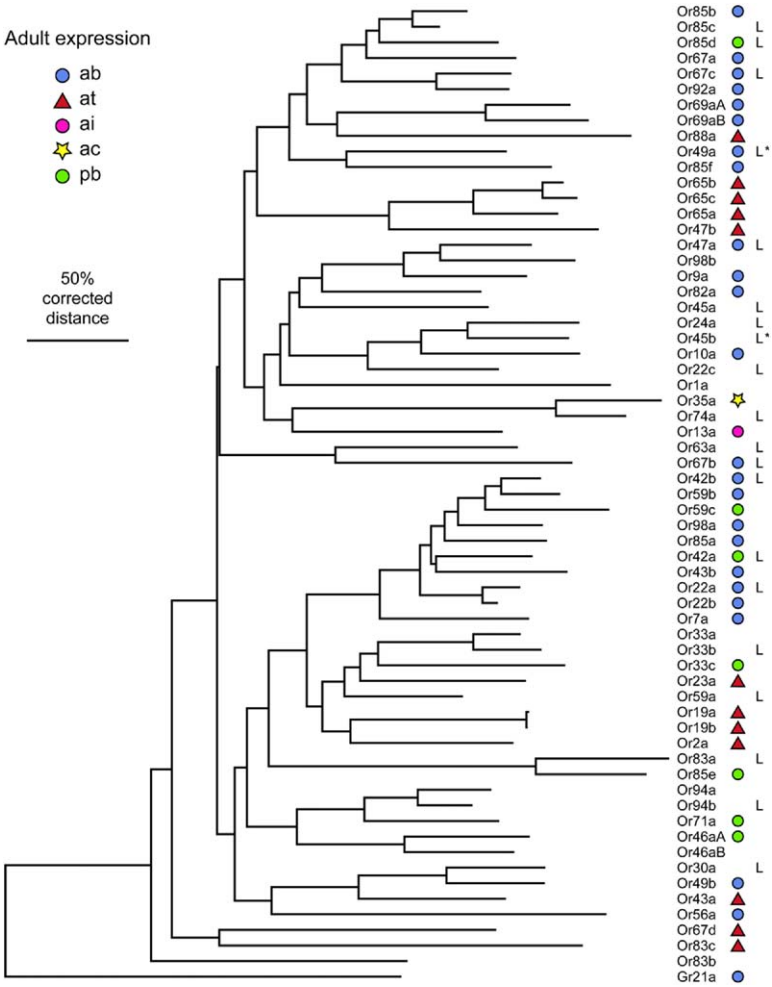

Figure 1. Phylogenetic Tree of Adult and Larval Odorant Receptors

Tree of all 62 predicted Ors and Gr21a, adapted from [4]. The sensillum type is indicated for each Or expressed in the adult. "L" indicates receptors expressed in ORNs of the larval dorsal organ (asterisk indicates larval *Or* genes identified by [18] but not confirmed by us). Or83b is broadly expressed in both adult and larval ORNs [8]. The scale bar indicates 50% divergence in corrected sequence, as defined by [4]. Sensilla types: ab, antennal basiconic; at, antennal trichoid; ai, antennal intermediate; ac, antennal coeloconic; pb, maxillary palp basiconic.

involved immunofluorescence detection of one *Or* transgenic reporter and in situ hybridization to detect the second *Or*, although in some cases we also performed either double in situ hybridization or double immunofluoresence (using the *mCD8-GFP* reporter for one *Or* and the *GAL4* reporter and *UAS-τlacZ* for the other). These methods allowed us to map 44 *Or* genes and *Gr21a* to 38 ORN classes that innervate 19 distinct and highly stereotyped sensilla types (Table 1 and Figure 2).

Basiconic Sensilla

The basiconic sensilla of the antenna comprise three large (ab1–3), three thin (ab4–6), and four small sensilla (ab7–10), following the nomenclature previously used for their morphological [19] and physiological [20] classification (Table 1 and Figure 2). The sensilla classes we define as ab9 and ab10 were not identified in the physiological studies, possibly because they are located more laterally on the antenna. We confirmed the expression of *Or* genes in 11 antennal basiconic ORN classes [7, 15, 21, 22], most of which had previously only been inferred from functional data [7], and we identified *Or* genes for another 11 ORN classes.

Our map of the maxillary palp comprises three thin basiconic sensilla (pb1–3), with *Or* genes assigned to all six ORN classes (Table 1 and Figure 2). This map concurs with that recently reported for this organ [12].

In summary, we identified 31 receptor genes expressed in the basiconic ORNs, defining a total of 28 distinct ORN classes and 13 sensilla classes. With the exception of one neuron in the ab6 sensillum, the receptor-to-neuron map of the basiconic sensilla of the antenna and palp is now most likely complete.

Trichoid Sensilla

The trichoid sensilla are innervated by one, two, or three neurons (T1, T2, or T3 sensilla, respectively) [19]. There are only very limited physiological data available for the trichoid sensilla [23], and no receptor-to-neuron assignments have previously been made. We identified 12 *Or* genes expressed in 9 ORN classes in 4 distinct classes of trichoid sensilla (Table 1 and Figure 2): a single T1 sensillum (which we refer to as at1), a single T2 sensillum (at2), and two distinct classes of T3 sensillum (at3 and at4). The compositions of at2 and at3 seem to be strictly stereotyped, but at4 may be slightly variable (Figure 2). The total numbers of trichoid sensilla and

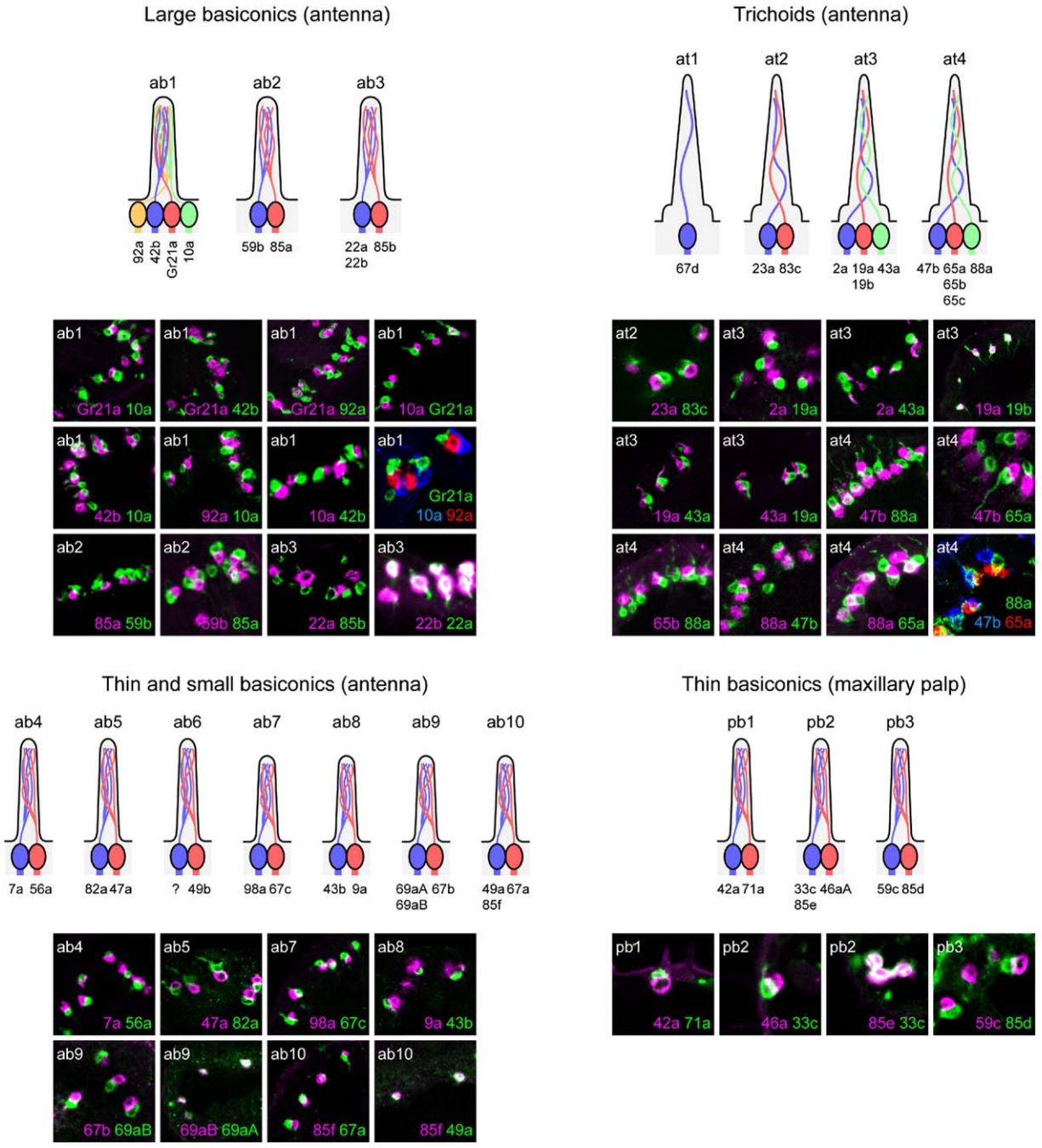

Figure 2. Sensillum and ORN Classes

Schematics showing the composition of 17 sensilla types on the antenna and maxillary palp and 36 ORN classes. The coeloconic sensillum ac1 and the intermediate sensillum ai1 are not shown. *Or* genes expressed in each ORN class are indicated (all are *Or* genes, except *Gr21a*). Selected double-labelings to support these classifications are shown. In each case the image shown is a section of an antenna or maxillary palp from an *Or-mCD8-GFP* line stained with anti-GFP (green), or an *OrA-mCD8-GFP*, *OrB-GAL4*, *UAS-τlacZ* fly stained with anti-GFP (green) and anti-β-galactosidase (blue). In situ hybridization was performed to detect mRNAs for another *Or* gene (magenta or red). The unassigned neuron in the ab6 sensillum may express both *Or85b* and *Or98b*, but the reporters for these genes have not yet been fully validated. The three neurons of at4 generally express distinct receptors—*Or47b*, *Or65a*/*Or65b*/*Or65c*, and *Or88a*—but we also found instances of two *Or47b*-positive neurons together with a single *Or88a*-positive neuron.

ORNs that we identified closely match the numbers reported in the morphological survey [19], and as we have assigned at least one *Or* to each ORN class, we anticipate that this receptor-to-neuron map of the trichoid sensilla is also likely to be complete.

Coeloconic and Intermediate Sensilla

Coeloconic sensilla house either two or three ORNs [19], and based on the number of glomeruli innervated by coeloconic ORNs (see below), we anticipate that there are eight distinct classes of coeloconic ORNs.

However, we could assign only a single *Or* gene, *Or35a*, to the coeloconic sensilla (Table 1 and Figure 1). *Or* or *Gr* genes might be expressed only at very low levels in the coeloconic ORNs, making them difficult to detect by these methods. Alternatively, these neurons may express some other type of chemoreceptor.

The intermediate sensilla number only about 20–30, and also contain either 2 or 3 ORNs [19]. We tentatively assigned *Or13a* to the intermediate sensilla, although it is also possible that one or another of the sensilla classes we identified as basiconic or trichoid by light microscopy may in fact correspond to sensilla described as intermediate by electron microscopy [19].

Odorant Receptors Expressed in the Same Sensillum Type or Class Are Unrelated

What logic, if any, guides the selection and pairing of *Or* genes in individual sensilla? With regard to sensillum type, one might predict that the different sensilla types would express different subfamilies of *Or* genes, as defined either by the sequences of the receptors they encode or their chromosomal locations. However, no obvious pattern emerged when we annotated either a phylogenetic tree or a genomic map of *Or* genes with the corresponding sensillum type (Figure 1 and data not shown). With regard to the specific combinations of receptors expressed in ORNs of the same sensilla, two extreme possibilities are that paired receptors might be closely related (because the odorants they detect must pass through a shared extracellular enviroment, and so may be chemically related) or maximally divergent (in order to minimize passive interference between ORNs [24]). To test these hypotheses, we determined the sequence distance between two receptors for each of the 990 possible pairs of the 45 odorant receptors on our map, and we binned each pair into one of four categories: pairs expressed in the same ORN class (e.g., both in ab3A), in distinct ORNs of the same sensillum (e.g., ab3A and ab3B), in different sensilla classes of the same type (e.g., ab3A and ab4A), or in different sensilla types (e.g., ab3A and at1A). We found that those receptors expressed in different neurons of the same sensillum are, on average, no more and no less closely related to each other than those expressed in different sensilla, different types of sensilla, or indeed any receptor pair chosen at random (Figure 3). Thus, although highly stereotyped, the selection and pairing of *Or* genes into distinct sensilla types and classes does not seem to follow any particular logic with regard to either the sequence of the receptor or the location of its gene.

The Receptor-to-Glomerulus Map

Anatomical studies using general synaptic markers have defined some 40–50 glomeruli in the *Drosophila* antennal lobe, with some minor discrepancies between different studies [25, 26]. Our *Or* transgenic reporter lines now provided a set of molecular markers for individual glomeruli, and so we could use these reporters individually or in combination to refine existing maps and establish an atlas of 49 glomeruli (Figure 4A).

To assign receptors to individual glomeruli, we examined ORN projections for each of the promoter-*mCD8-GFP* fusions. Previously, this approach had been used to map receptors to 13 glomeruli [11, 13, 27–29]. Our

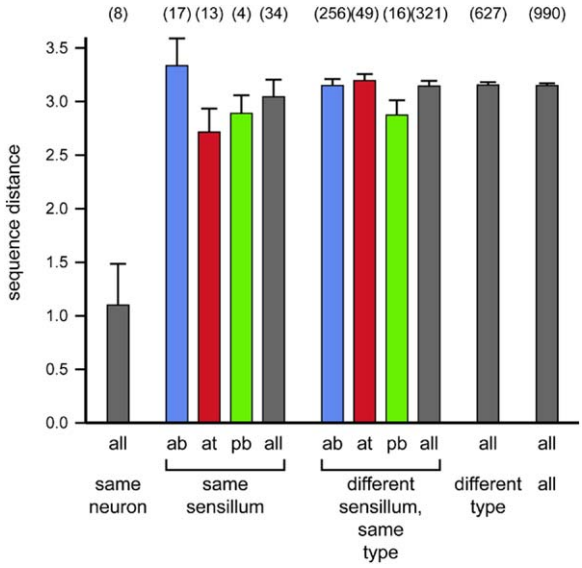

Figure 3. Ors in the Same Sensillum Are Unrelated

Sequence distances between all possible pairwise combinations of odorant receptors, binned according to whether the two receptors are expressed in the same neuron, same sensillum, different sensilla of the same type, or different types of sensilla. Sequence distances were calculated using the Jones-Taylor-Thornton method from the PHYLIP package [66]. Numbers in parentheses indicate the number of receptor pairs in each bin. Data are mean ± SEM. $p > 0.05$ for all pairwise comparisons, except those involving Ors expressed in the same neuron.

set of 44 verified adult *Or* reporters allowed us to correct 4 of these earlier assignments and to extend the coverage to a total of 37 glomeruli (Table 1 and Figure 4B). For each *Or*, the projections were identical in each animal examined (n = 8–10), with no differences between the sexes. Each *Or* reporter also labels just a single glomerulus (although a few reporters are weakly or ectopically expressed in additional ORN classes that target other glomeruli; Figure 4B). Through a series of unilateral deafferentation experiments [27], we determined that only the V glomerulus is innervated unilaterally; all other glomeruli on our map receive bilateral innervation.

We anticipated that many of the glomeruli that remained unassigned were likely to be innervated by coeloconic ORNs. To identify these glomeruli, we used an *ato-GAL4* reporter, which is expressed in all coeloconic ORNs of the antenna and the basiconic ORNs of the maxillary palp [30]. A total of 14 glomeruli are labeled in *ato-GAL4*, *UAS-mCD8-GFP* flies: the six glomeruli already assigned to the six ORN classes of the maxillary palp, the one glomerulus we had already assigned to an antennal coeloconic ORN (VC3), and seven still unassigned glomeruli (DC4, DL2, VL1, VL2a, VM1, VM4, and VM6). We therefore infer that VC3 and these seven additional glomeruli are the targets of the antennal coeloconic ORNs.

Segregation of Sensilla Types, but Not Sensilla Classes, in the Antennal Lobe

Our ORN connectivity map reveals a spatial organization in the antennal lobe that was not apparent from

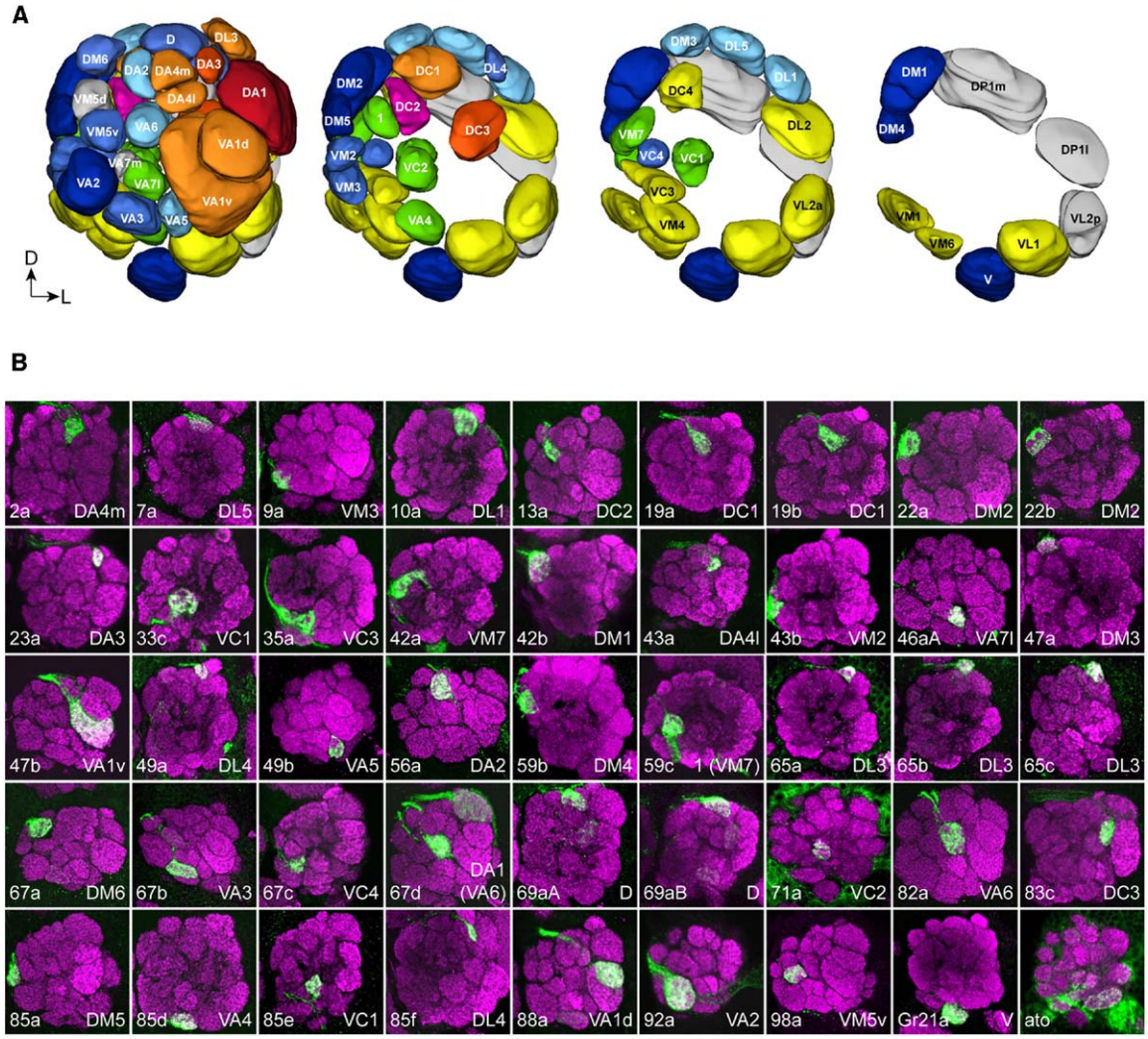

Figure 4. Map of ORN Projections in the Antennal Lobe

(A) 3D reconstruction of a male antennal lobe, showing the positions of 49 glomeruli. The view is anterior, with the labeled glomeruli removed in each successive panel to reveal the underlying glomeruli. Glomeruli are color coded as in Figure 5.

(B) Antennal lobes of various *Or-mCD8-GFP* reporter lines were stained with anti-GFP to visualize the ORN axons (green), and counterstained with the synaptic marker mAb nc82 to visualize the glomerular structure of the antennal lobe (magenta). "ato" indicates *ato-GAL4*, *UAS-mCD8-GFP* animals. For Or71a, *Or71a-GAL4*, *UAS-mCD8-GFP* flies were examined, using the *Or71a-GAL4* line reported by [11]. All others are the direct fusions generated in this work.

In each panel, dorsal is up and lateral to the right. Images are projections of a few adjacent confocal sections. Note that the Or59c and Or67d reporters label two glomeruli each. In both cases, further experiments indicated that only one of these glomeruli is likely to be the target of the ORNs that express the endogenous receptor; the other (indicated in parentheses) is likely due to ectopic expression of the reporter in a second ORN class.

the few ORN classes previously examined. Specifically, afferents from ORNs in distinct sensilla types project to distinct regions of the antennal lobe: ORNs in antennal trichoid sensilla project to the lateral anterior region, antennal basiconic sensilla to the medial region, palp basiconic sensilla to the central-medial region, and antennal coeloconic sensilla to the posterior (Figure 5).

This segregation of sensory input according to sensillum type does not extend down to the level of individual sensilla classes. Specifically, ORNs that are neighbors in the same sensillum do not always project to neighboring glomeruli (Figure 6A). Nevertheless, ORNs in the same sensilla class might still innervate glomeruli that are generally close to each other within the antennal lobe. To test this possibility, we first determined the distances between all 666 pairs of the 37 assigned glomeruli and then asked whether glomeruli innervated by ORNs in the same sensilla are generally closer to each other than those innervated by ORNs in different sensilla. Distances were calculated in two different ways for each pair. An average physical distance between the geometric centers of the two glomeruli was determined from 3D reconstructions of four male antennal lobes (see Experimental Procedures). In addition, we

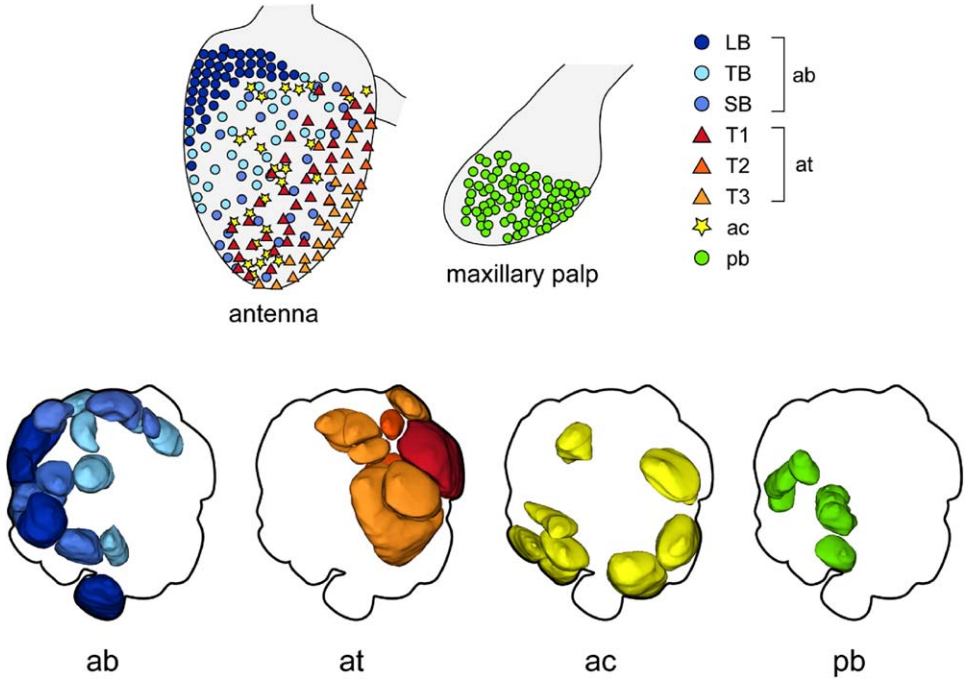

Figure 5. Segregation of Sensilla Types in the Antennal Lobe

Distribution of different sensilla types on the antenna or maxillary palp and their target glomeruli in the antennal lobe. Sensilla maps for the antenna and maxillary palp are adapted from [19] and [31], respectively. Glomeruli are colored according to sensillum type for the corresponding ORN class. LB, large basiconic; TB, thin basiconic; SB, small basiconic.

determined the "degrees of separation" between each pair: 1 for neighboring glomeruli, 2 for glomeruli that are not themselves neighbors but have a common neighbor, and so on. We found that, by either distance measure, pairs of glomeruli innervated by ORNs in the same sensilla are, on average, no closer together or further apart than those innervated by ORNs from different sensilla of the same type (Figures 6B and 6C; $p > 0.05$ for all comparisons; Mann-Whitney U test). Thus, sensory inputs from the different sensilla types are segregated into distinct regions of the antennal lobe, but within each of these regions the arrangement of glomeruli bears no obvious relationship to the location or pairing of ORNs in the periphery.

Similar Receptors Tend to Map to Proximate Glomeruli

We next asked whether receptors that are more closely related by sequence tend to map to glomeruli that are physically closer within the antennal lobe. To test this, we examined all 938 possible pairs of the 44 odorant receptors on our map (excluding the eight pairs that are coexpressed in the same neuron). For each pair, we compared the sequence divergence of the two receptors and the separation of the corresponding glomeruli in the antennal lobe. There is a strong positive correlation between the two, using either the actual distances between pairs of glomeruli ($r = 0.33$, $p < 0.0001$) or their degrees of separation. This correlation can be attributed entirely to the antennal basiconic sensilla ($r = 0.45$, $p < 0.0001$; Figures 7A and 7B), as it is not observed at all for the other two sensilla types ($r = 0.05$, $p = 0.49$ for at; $r = 0.20$, $p = 0.20$ for pb). Thus, among the antennal basiconic sensilla, pairs of ORNs that express more closely related receptors tend to map to more closely positioned glomeruli.

Functional Organization of the Antennal Lobe

By integrating our molecular and anatomical maps with existing electrophysiological data [20, 31, 32], we could predict odor-evoked activity patterns for a total of 29 glomeruli. We classified each of these 29 glomeruli according to whether the test odorants that elicited a strong response (above a threshold of 50 spikes/s) were linear aliphatic compounds or aromatic compounds containing a benzene ring. This analysis suggested a spatial separation of aliphatic and aromatic odor representations in the antennal lobe (Figure 8A). Glomeruli that respond primarily to aromatic odorants are clustered in a ventral-central region of the antennal lobe, whereas those that respond preferentially to aliphatic odorants are clustered in the medial region. This clustering does not bear any relationship to the clustering of inputs from the different sensilla types.

The test odorants used in these physiological studies had been selected primarily in order to maximize their chemical diversity, rather than to systematically sample "odor space." Esters are, however, particularly well represented in these data sets and range in size from 4 to 12 carbons. Collectively, the odorant receptors or ORNs that are activated above a threshold of 50 spikes/s by these esters map to 16 of the 20 "aliphatic" glomeruli on our map. For each of these 16 glomeruli, we noted the carbon number of the ester that gave the maximum

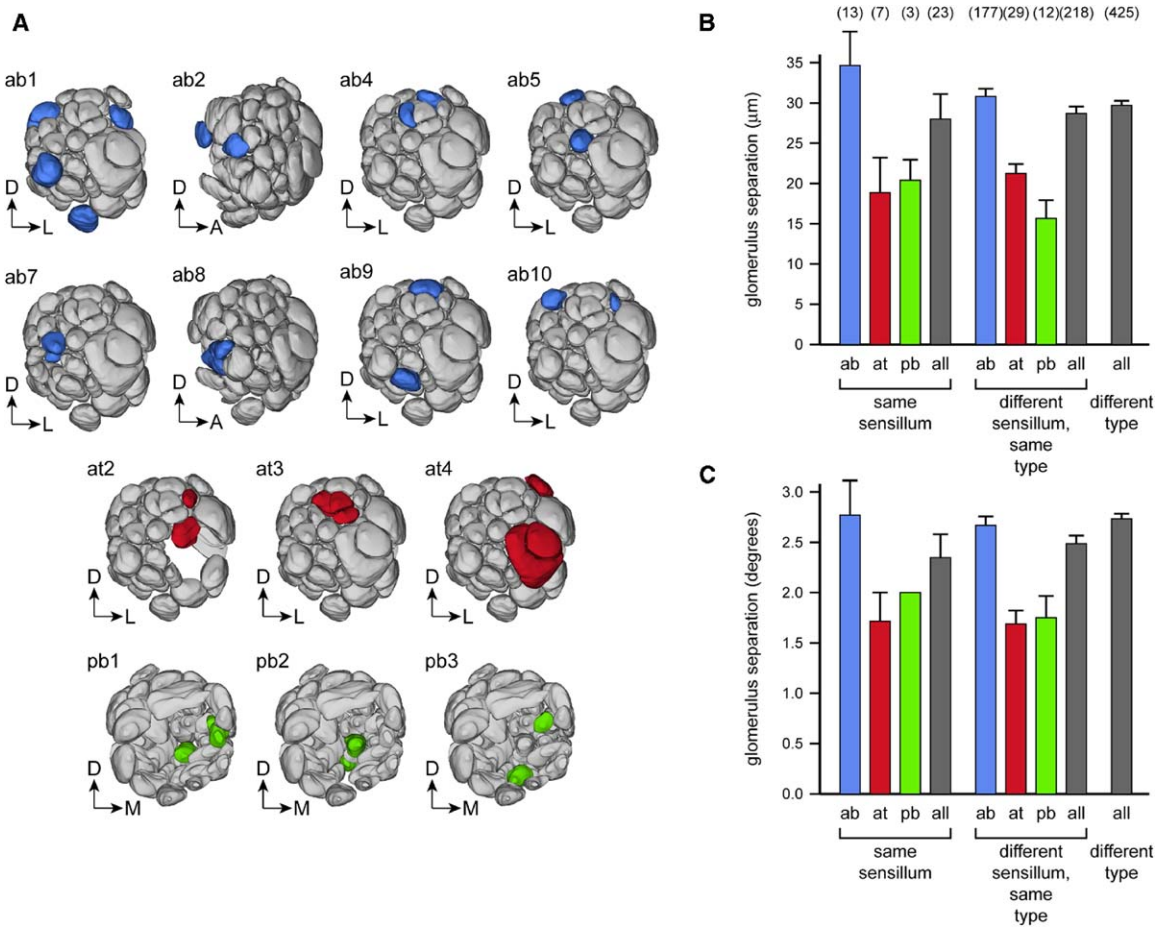

Figure 6. ORNs in the Same Sensilla Target Dispersed Glomeruli

(A) Maps of target glomeruli for ORN classes that are housed in the same sensilla. Antennal lobe reconstructions are viewed from various angles to aid visualization, and selected glomeruli have been removed where necessary to reveal underlying glomeruli.

(B and C) Distances between glomeruli for all pairwise combinations of all 37 assigned glomeruli, binned according to whether they are innervated by neurons housed in the same sensillum, different sensilla of the same type, or different types of sensilla. Numbers in parentheses indicate the number of glomerulus pairs in each bin. Data are mean ± SEM. Distances between glomeruli were calculated as either the physical distance between their respective centers (B) or as degrees of separation (C).

response. This revealed a broad ordering of glomeruli along the posterior to anterior axis, with more anterior glomeruli generally preferring larger esters (Figures 8B). A similar trend was also observed for alcohols and ketones, but for these compounds the data are too sparse to draw any strong conclusions.

Discussion

Odorant Receptor Choice

In both mammals and insects, individual ORNs are thought to express only a single functional odorant receptor [27, 33–35], although exceptions have been documented in both rats [36] and *Drosophila* [12]. The critical test of this hypothesis is to map, at single-cell resolution, the expression of the entire family of odorant receptor genes. We have almost completed this task for the adult olfactory system of *Drosophila melanogaster*, mapping 45 odorant receptors to 38 distinct ORN classes.

Only six ORN classes express more than one receptor (excluding the widely expressed Or83b [8], which heterodimerizes with other odorant receptors but is not functional by itself [37, 38]; as well as the low levels of some additional *Or* or *Gr* genes in some neurons). In four of these six cases, the coexpressed *Or* genes are closely linked and highly conserved, suggesting that they arose through a relatively recent gene duplication. These pairs of coexpressed receptors are likely to detect the same odorants, and so do not represent a meaningful exception to the one neuron—one receptor principle. The two cases of coexpressed but unrelated and unlinked *Or* genes are *Or33c* and *Or85e* in pb2A and *Or49a* and *Or85f* in an ab10 neuron. For the *Or33c*/*Or85e* pair, both receptors are functional when ectopically expressed, but the response profile of the pb2A neuron in which they are endogenously coexpressed can be attributed to *Or85e* alone [12]. Similar comparisons are not yet possible for the *Or49a*/*Or85f* pair, as there are no electrophysiological data available for *Or49a* or the ab10 sensillum.

In mammals, a negative feedback mechanism en-

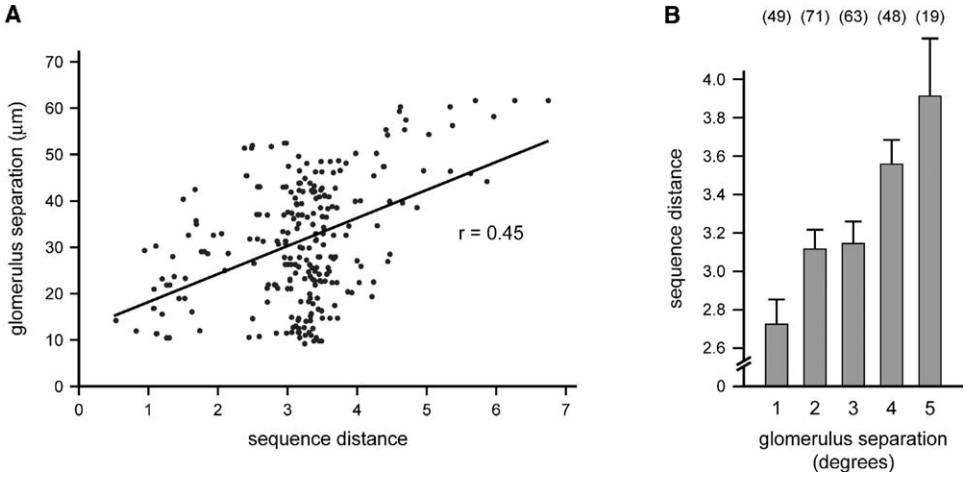

Figure 7. Correlation between Sequence and Glomerulus Distances

(A) Scatterplot of sequence distance versus glomerulus separation for all 250 pairwise combinations of odorant receptors expressed in antennal basiconic sensilla. Receptor pairs coexpressed in the same neuron (and hence targeting the same glomerulus) have been excluded. The receptor Gr21a is an outlier by sequence, and the corresponding V glomerulus is also a physical outlier in the antennal lobe. However, the correlation between receptor sequence distances and glomerulus distances remains significant even when pairs involving Gr21a are excluded ($r = 0.28$, $p < 0.0001$).

(B) The same receptor pairs as in (A) were grouped according to the degrees of separation of their target glomeruli. Data are the mean sequence distance ± SEM (n) for each category.

sures that only one functional receptor is expressed [39–41]. The choice of a specific receptor is largely stochastic, although each ORN is somehow restricted to selecting from a large subset of "available" receptor genes according to its position in the olfactory epithelium [33, 34, 42]. In contrast, receptor choice in *Drosophila* appears to be entirely deterministic, as indicated by the highly stereotyped patterns of *Or* expression in olfactory sensilla. There is also no evidence for any negative-feedback mechanism in *Drosophila*, as the loss of an endogenous receptor does not lead to the expression of an alternative receptor [21], nor does ectopic expression of a second receptor block the expression of an endogenous receptor [43].

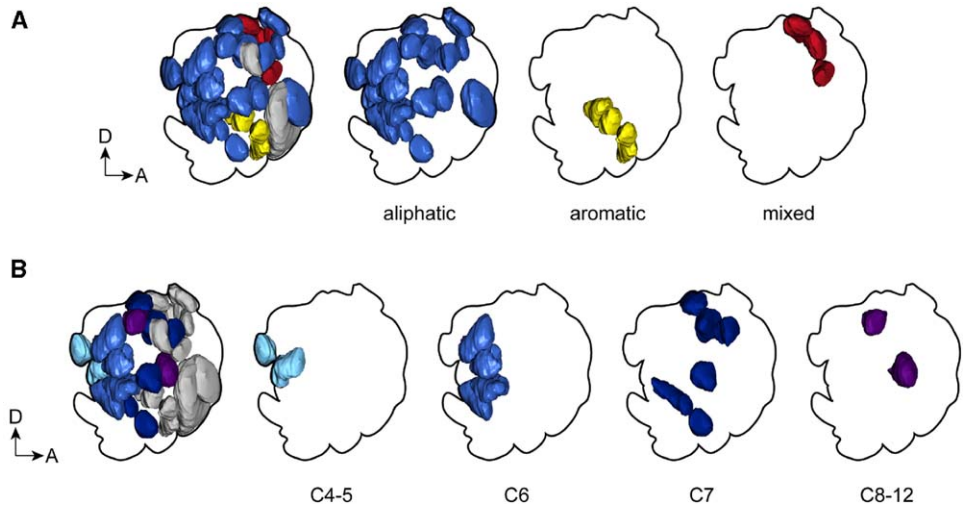

Figure 8. Chemotopic Organization of the Antennal Lobe

(A) Odor preferences for 29 glomeruli, based on existing physiological data [20, 31, 32]. Glomerular targets of ORNs preferentially activated by aliphatic and aromatic compounds are colored blue and yellow, respectively, and those responding to both classes are colored red. Glomeruli activated by aliphatic compounds are DC1, DL3, DM2, DM3, DM4, DM5, DM6, VA1d, VA4, VA6, VC3, VC4, VM2, VM3, VM5v, VM7, and 1. Glomeruli activated by aromatic compounds are VA5, VA7l, VC1, and VC2. Glomeruli activated by both aliphatic and aromatic compounds are DA4l, DL1, and DL5. The VA1v and DA2 glomeruli are not predicted to respond to any of the test odorants and are colored gray.

(B) Carbon chain length preferences for 16 glomeruli with strong responses to esters. C4-5 (ethyl acetate and ethyl proprionate): DM4 and VM7; C6 (ethyl butyrate): DM2, VM2, DM5, VM3, VC4, and 1; C7 (pentyl acetate, isoamyl acetate): VC3, DM3, VM5v, DA4m, DC1, and VA4; C8-12 (ethyl hexanoate, geranyl acetate): VA6 and DM6.

The 45 receptors we have mapped can be paired in nearly 1000 different ways, yet less than 20 distinct combinations are actually deployed in olfactory sensilla. Why have these specific combinations been selected? One possibility is that ORNs compartmentalized into the same sensillum might express closely related receptors, as the odorants they detect are transported and processed by the same set of molecules in their common sensillar lymph, and so may be chemically related. However, we found that pairs of *Or* genes expressed in the same sensillum are no more closely related to each other than any randomly selected pair. Similarly, electrophysiological surveys of a more limited set of basiconic sensilla have shown that ORNs housed in the same sensilla tend to have distinct rather than similar response spectra [20, 31, 44]. These observations are more readily explained by a model in which ORNs housed in the same sensilla instead express divergent receptors, so as to minimize their functional overlap and afford each ORN a greater dynamic range [24].

Nevertheless, there are still many different ways in which pairs of divergent odorant receptors could be combined, so this consideration alone cannot completely explain the specific combinations deployed. An additional factor may be that two odorants could be discriminated with a higher spatial and temporal resolution if the ORNs that detect them are placed in the same rather than distinct sensilla, possibly even allowing the insect to discern whether two odorants are present in the same or different filaments of an odor plume [45]. This might be particularly relevant for odorants such as pheromones, for which it may be critically important to distinguish whether the individual components of a blend originate from a single source (a potential mate) or from two closely spaced sources.

Whatever the logic behind these pairings, it will be of great interest to determine how they are programmed developmentally. At present, little is known of these mechanisms [7]. ORNs in the same sensillum are likely to be related by lineage, and so selection of a specific *Or* gene might be part of the instrinsic mechanisms that generate diverse cell fates within each lineage. Alternatively, by analogy to the signaling mechanisms that coordinate rhodopsin gene selection between R7 and R8 cells in the same ommatidium in the eye [46], one ORN in each sensillum might choose its *Or* first and then instruct the *Or* choice of its neighbor(s). The promoter regions we have defined will be a valuable guide in computational and experimental approaches aimed at defining the *cis*-acting determinants of *Or* choice, while the transgenic reporters should facilitate genetic screens to identify the *trans*-acting factors.

Olfactory Wiring

In *Drosophila*, axons of ORNs that express the same odorant receptor are thought to converge upon a single glomerulus, with each glomerulus receiving input from just a single class of ORN. The *Or* axonal reporters generated prior to this study all label a single glomerulus per antennal lobe [27, 28], as does each of our 45 verified reporters. The few cases in which we and others [27, 29] have observed *Or* reporters targeting multiple glomeruli can most likely be explained by low levels of "ectopic" *Or* expression or by reporters that do not faithfully mimic the endogenous *Or* expression. Innervation of a single glomerulus thus appears to be a strict rule.

More difficult to verify, in any species, has been the postulate that each glomerulus receives input only from a single class of ORNs (the one glomerulus—one receptor hypothesis). No exceptions have yet been reported in the main olfactory systems of mice or *Drosophila*. However, with only a small fraction of odorant receptors examined in each case, the chances of detecting a glomerulus with multiple inputs had, until now, been vanishingly small. With the map of ORN connectivity now almost complete for *Drosophila*, we can confirm that most, and probably all, glomeruli do indeed receive input from just a single class of ORN. Specifically, we could assign each of 38 glomeruli to a single and distinct ORN class and most of the remaining glomeruli to a nonoverlapping set of unidentified coeloconic ORNs.

Our connectivity map also reveals an unanticipated topographic organization of the antennal lobe, with ORNs in distinct sensilla types projecting into distinct regions of the antennal lobe. A similar topographic organization may apply in the vertebrate main olfactory system [6, 42]. Within these regions, however, neighbor relationships are not preserved—ORNs that are neighbors in the same sensillum do not target neighboring glomeruli, or indeed even closely positioned glomeruli.

For the antennal basiconic sensilla, the distance between glomeruli does, however, correlate with the sequence distance between the corresponding odorant receptors. Thus, whereas ORNs are grouped into sensilla in ways that appear to favor combinations of divergent receptors, their target glomeruli may be arranged in part in ways that tend to juxtapose ORNs that express similar receptors. This redistribution of ORNs between the periphery and the antennal lobe may contribute to the formation of chemotopic maps in the brain.

A Chemotopic Map in the Antennal Lobe

The peripheral [7, 12, 20, 31] and central [43, 47–49] mechanisms of *Drosophila* olfaction have been well described. What has been missing until now is the causal link between the two. Our expression and connectivity maps provide this link. The internal representations of specific odorants can now be explained and predicted from the knowledge of the receptors they activate, the ORNs that express these receptors, and the glomeruli that these ORNs target.

We have predicted odor respresentations covering 29 glomeruli for the diverse set of odorants used in the physiological studies [20, 31, 32]. These odor maps revealed a functional organization of the antennal lobe that was not apparent from imaging studies in *Drosophila* [43, 47, 48] but is consistent with imaging and electrophysiological data from other insects [50–52]. Specifically, aromatic and aliphatic compounds are predicted to activate spatially distinct regions of the antennal lobe. A similar segregation of aromatic and aliphatic representations has also been suggested for the larval olfactory system [18]. We also find that, within the "aliphatic cluster" of the adult antennal lobe, compounds of increasing carbon chain length are predicted to successively shift the activity pattern in an anterior direction.

These features are also not unique to insect olfactory systems. Accumulating evidence points to a similar functional organization of the mammalian olfactory bulb, with distinct chemical classes activating distinct glomerular clusters [53–55] and carbon chain length represented topographically within each cluster [53, 56–60]. Thus, the chemotopic organization of the antennal lobe that emerges from our map of the *Drosophila* olfactory system appears to be a common feature of both insect and mammalian olfactory systems.

Odor Processing in the Antennal Lobe

In the antennal lobe, ORN axons synapse with second order projection neurons (PNs), which extend axons to the protocerebrum. As high-resolution anatomical maps are beginning to emerge for PN axons [61, 62], it may soon be possible to predict odor representations at higher brain levels as well. It will be fascinating to learn to what extent this chemotopic map is retained or transformed at higher levels. Before doing so, however, it will be essential to understand the transformations that take place within the antennal lobe itself. Imaging studies have indicated a high degree of correlation between the ORN and PN responses for individual glomeruli [43, 47], suggesting that the antennal lobe is primarily a relay station with little transformation of olfactory information. In contrast, electrophysiological data suggest that PNs are more broadly tuned and dynamic in their responses than the corresponding ORNs [49]. A caveat to this result, however, was that ORN and PN responses could be compared only for a single glomerulus, DM2.

Our connectivity map will now allow more systematic comparisons of ORN input and PN output in the antennal lobe. The electrophysiological data for PNs are still too limited for us to significantly extend this analysis here. Nevertheless, we can make ORN versus PN comparisons for two additional glomeruli: VA7l and DM1. The VA7l PNs appear to be more broadly tuned than their presynaptic pb2B ORNs [31, 49], whereas the DM1 PNs seem to be a much closer match to the corresponding ORNs, most likely of the ab1B class [20, 49]. Thus, some odors may undergo complex transformations in the antennal lobe, whereas others may be transmitted to higher brain centers with little further processing.

In conclusion, our detailed maps of *Or* expression and ORN connectivity have not only confirmed and extended our understanding of the basic molecular and anatomical principles of the olfactory system, they also provide a framework for understanding its functional organization. With these maps, we can now explain and predict how the peripheral activation of odorant receptors produces a chemotopic represention in the antennal lobe. In future, these maps can also be used to determine precisely how olfactory information is further processed in the antennal lobe and transmitted to higher brain centers.

Experimental Procedures

Reporters and Flies

Predicted promoter regions were amplified by genomic PCR (Table S1) and cloned into a transformation vector containing a single open reading frame encoding for a fusion protein consisting of the mCD8 extracellular and transmembrane domains, four tandem copies of GFP, and two c-*myc* epitope tags. Many promoter fragments were also cloned into a GAL4 transformation vector. For double-reporter stainings, flies carrying *OrA-mCD8-GFP*, *OrB-GAL4*, and *UAS-τlacZ* were examined. The *ato-GAL4* line is *NP6558* [63]. All flies were raised at 25°C on standard cornmeal-yeast-agar medium. For deafferentation experiments, the left antenna or maxillary palp was removed from freshly eclosed flies, which were then aged 15 days before dissection and staining. For all other experiments, 3- to 5-day-old flies were examined.

Immunohistochemistry

Whole-mount adult brains and head cryosections were stained and imaged as described in [64]. To measure glomeruli volumes, whole-mount brains of 4-day-old adult males were stained with mAb nc82 and imaged at 512 × 512 pixels in 0.5 μm confocal sections on a Zeiss LSM510 Meta confocal microscope. Glomeruli contours were traced in individual sections using the Contour Surface option of Imaris 4.0 (Bitplane AG).

In Situ Hybridization

10 μm cryosections of adult heads were pretreated, hybridized with digoxygenin-labeled probes, and washed according to [65], with some modifications. In brief, sections were fixed in 4% paraformaldehyde for 10 min, acetylated for 10 min, treated with 5 μg/ml proteinase K (Roche) in PBS for 5 min, washed, and prehybridized for 4 hr at room temperature. RNA probes were prepared by in vitro transcription of 1–1.5 kb genomic regions for each *Or*, which had been PCR amplified and cloned into pBluescript. Hybridization was performed at 65°C overnight. Slides were then washed and incubated with rabbit anti-GFP (Molecular Probes, 1:1000) and alkaline phosphatase (AP)-conjugated goat anti-DIG Fab fragments (Roche, 1:2000) at 4°C overnight. For triple stainings, mouse anti-β-galactosidase (Promega, 1:1000) was also used. Fluorescent detection was performed using 1 hr incubations with Alexa Fluor 488 goat anti-rabbit IgG (Molecular Probes, 1:500) and, when necessary, Alexa Fluor 633 goat anti-mouse IgG (Molecular Probes, 1:500), followed by Fast Red (Dako Cytomation) for 3 hr at room temperature.

Supplemental Data

Supplemental Data include one table and can be found with this article online at http://www.current-biology.com/cgi/content/full/15/17/1535/DC1/.

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