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Or83b Encodes a Broadly Expressed Odorant Receptor Essential for *Drosophila* Olfaction

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Summary

Fruit flies are attracted by a diversity of odors that signal the presence of food, potential mates, or attractive egg-laying sites. Most *Drosophila* olfactory neurons express two types of odorant receptor genes: *Or83b*, a broadly expressed receptor of unknown function, and one or more members of a family of 61 selectively expressed receptors. While the conventional odorant receptors are highly divergent, *Or83b* is remarkably conserved between insect species. Two models could account for *Or83b* function: it could interact with specific odor stimuli independent of conventional odorant receptors, or it could act in concert with these receptors to mediate responses to all odors. Our results support the second model. Dendritic localization of conventional odorant receptors is abolished in *Or83b* mutants. Consistent with this cellular defect, the *Or83b* mutation disrupts behavioral and electrophysiological responses to many odorants. *Or83b* therefore encodes an atypical odorant receptor that plays an essential general role in olfaction.

Introduction

The olfactory system has evolved the capacity to recognize and discriminate an inordinate number of chemically distinct odors that signal the presence of food, predators, or mating partners. The initial steps in odor detection involve the binding of a volatile odor to odorant receptor (OR) proteins displayed on ciliated dendrites of specialized olfactory sensory neurons (OSNs) that are exposed to the external environment. The OR genes that mediate odor detection in *Drosophila* fruit flies are expressed in subpopulations of OSNs (Clyne et al., 1999; Gao and Chess, 1999; Vosshall et al., 1999, 2000; Dobritsa et al., 2003; Elmore et al., 2003) and are members of a rapidly diverging superfamily of insect chemosensory genes that encode multitransmembrane domain receptors with no homology to nematode or vertebrate ORs (Hill et al., 2002). Genetic analysis coupled with electrophysiology has demonstrated that the characteristic odor response profile of a given OSN is governed by the selective expression of one or more members of the family of 61 OR genes in that neuron (Dobritsa et al., 2003; Elmore et al., 2003; Hallem et al., 2004a, 2004b). One member of the OR gene family, *Or83b*, is strikingly different from the other OR genes. Unlike the conventional ORs, it has clear homologs in other insect species that share nearly 70% amino acid identity with *Or83b* (Hill et al., 2002; Krieger et al., 2003; Pitts et al., 2004). *Or83b* and its homologs in other insects are coexpressed with conventional ORs in a large proportion of OSNs (Vosshall et al., 1999, 2000; Elmore et al., 2003; Krieger et al., 2003; Pitts et al., 2004).

Based on these observations, two models could account for *Or83b* function in insect olfaction. *Or83b* could bind distinct ligands independently of the other OR genes coexpressed with it in a given OSN, or it could act in concert with conventional ORs to recognize a wide variety of odors.

In the first model, *Or83b* might act in most OSNs to recognize and report the presence in the environment of an important odor signifying danger or a particularly rich food source. Insects have evolved a diversity of food preferences and inhabit many different ecological niches. However, the vast majority of insects have an important relationship to plants, either as food sources, sites for egg-laying, habitat for prey, or shelter. Green leaf volatiles such as E2-hexenal and linalool are produced by many different plants and elicit physiological and behavioral responses in insects as varied as moths, mosquitoes, and *Drosophila* (Rostelien et al., 2000; de Bruyne et al., 2001; Kline et al., 2003). Therefore, *Or83b* might interact selectively with various plant volatiles and transmit the same information to insects of diverse taxonomy: that they are in the presence of plants. Alternatively, *Or83b* could interact with a single odorant that has different meanings to different insects. For instance, isoamyl acetate is a key component of rotting fruit and signifies food to *Drosophila* (Stensmyr et al., 2003), while the same odorant is produced by honeybees as an alarm and aggregation pheromone (Galizia et al., 1998). In both cases, the stimulus is of great importance to these insects, and dedicating a broadly expressed receptor like *Or83b* to detecting it might be adaptive for each insect. In either variation of this first model, animals lacking *Or83b* would show essentially normal responses to general odors detected by conventional ORs but would be nonresponsive to the putative important odors of special meaning.

In the second model, *Or83b* would not function independently as a ligand binding OR but would play a more general role in concert with the conventional ORs with which it is coexpressed. Such alternative roles could include interacting with conventional ORs to produce a receptor complex competent for ligand binding, acting as a protein chaperone that directs ORs to the dendrite, serving as a link to the signal transduction cascade of the OSN, or a combination of all of these functions. In this scenario, severe deficits in olfactory function to a

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wide range of odors would be expected in animals lacking *Or83b*.

Despite the remarkable progress in recent years in elucidating the molecular logic of olfaction in *Drosophila* (Warr et al., 2001; Keller and Vosshall, 2003), existing data do not explicitly rule out either model. Electrophysiological analysis of the basiconic class OSNs revealed a diversity of responses to odorants that included activation and inhibition of a given OSN by different odorants. OSNs were identified with specialist and generalist properties, and a number of identified neurons did not respond to any of the approximately 50 odorants tested (de Bruyne et al., 1999, 2001; Stensmyr et al., 2003). Available data on the response properties of the remaining OSNs in the antenna associated with trichoid and coeloconic sensilla are less complete (Clyne et al., 1997). The hypothesized odorant of special importance that activates *Or83b* independent of the other ORs might lie outside of collection of odorants used in the basiconic studies or might emerge from analysis of the trichoid and coeloconic OSNs. Elegant genetic and functional analysis of *Drosophila* ORs produced the clear result that the response properties of an identified OSN require the expression of its cognate OR (Dobritsa et al., 2003; Elmore et al., 2003) and that replacing this OR with a novel OR alters the response properties of the neuron in an OR-dependent manner (Dobritsa et al., 2003; Hallem et al., 2004a, 2004b). While these results clearly implicate the conventional OR in the response properties of the OSN, they do not rule out that these ORs function in concert with *Or83b*, which is coexpressed with all of these ORs (Elmore et al., 2003). They also do not rule out an independent function for *Or83b*. *Or22a/b* and *Or43b* mutant neurons were not found to respond to any of the odorants tested, but the relevant odorant might not have been part of the stimulus panel.

To distinguish between these competing models for *Or83b* function, we used gene targeting in *Drosophila* to delete the *Or83b* gene and applied cell biological, electrophysiological, and behavioral techniques to characterize the mutant phenotype. We find clear support for the second model: in *Or83b* mutants, the normal localization of OR proteins in distal chemosensory dendrites is disrupted in both larval and adult olfactory systems; *Or83b* mutant larvae fail to chemotax to most odors tested, and adult flies show severe deficits in odor-evoked electrophysiology and behavior. Our data therefore imply that olfactory function in response to a broad range of odorants in *Drosophila* requires expression of a conventional OR along with *Or83b* in most olfactory neurons. These findings have important implications for control of medically and economically relevant insect pests, because clear homologs of *Or83b* exist in malaria mosquitoes and a variety of important agricultural pests (Krieger et al., 2003; Pitts et al., 2004). Our data suggest a strategy in which olfactory host-seeking behavior of pest insects could be disrupted by small molecule inhibitors of *Or83b* homologs.

Results

Expression of *Or83b* in Most *Drosophila* Olfactory Neurons

The expression of *Or83b* has been described only anecdotally (Vosshall et al., 1999, 2000), and we present here a detailed characterization of the distribution and subcellular localization of this protein. The fly has OSNs in three specialized structures: the dorsal organ of the larva and the maxillary palps and third antennal segments on the adult head. *Or83b* expression is restricted to OSNs and is not detected in other tissues, including gustatory neurons (Figure 1A; data not shown). We examined the expression of the *Or83b* mRNA throughout the four main developmental stages of *Drosophila*. *Or83b* is first detected late in embryonic development at stage 15, where its expression is limited to the antennal-maxillary complex, an anterior structure that contains the OSNs (Figure 1A). Later in development, it is expressed in all 21 OSNs of the larval dorsal organ located at the anterior tip of the larva (Figure 1A). During metamorphosis, all of these larval OSNs are destroyed and replaced by new OSNs that populate the antenna and maxillary palp (Stocker, 1994). In the pupa, *Or83b* is first detected in antennal OSNs at 80 hr after puparium formation (Figure 1A), which is late in pupal development and approximately coincident with the onset of expression of conventional OR genes (Clyne et al., 1999; Elmore and Smith, 2001). We detect *Or83b* expression in all 120 adult maxillary palp neurons (Figure 1A; data not shown) and ~70%–80% of antennal OSNs (Figure 1A). The exact proportion of neurons that express *Or83b* in the antenna is not known and is difficult to estimate because *Or83b* is expressed at different levels in different subpopulations of OSNs. Levels are highest at the dorsal-medial aspect of the antenna and are lowest at the lateral-distal region (Figure 1B, left).

Both in the antenna and maxillary palp, each *Or83b*-positive OSN expresses *Or83b* along with at least one conventional OR gene (Figure 1B; data not shown; Dobritsa et al., 2003; Elmore et al., 2003). *Or22a/b* and *Or83b* mRNA overlap in a group of dorsal-medial neurons (Figure 1B). We raised an anti-Or83b anti-peptide antibody against loop residues between transmembrane domains three and four and used this antibody to determine the subcellular localization of Or83b. Or83b protein is found in ciliated OSN dendrites, the chemosensory specialization in which OR proteins are localized (Elmore and Smith, 2001; Dobritsa et al., 2003) and where olfactory signal transduction occurs (Figures 1C and 1D, left; see Figure 3A for a schematic; Ayer and Carlson, 1992). Double immunostaining with a rabbit anti-Or22a/b antibody and a mouse anti-Or83b antibody demonstrates that these two OR proteins colocalize in the distal portion of the dendrite inserted into the sensory hair (Figure 1C). Or83b is also detected in OSN cell bodies but is not seen in proximal axons in the antenna or at axonal termini in the antennal lobe of the brain (Figure 1D, center), suggesting that its function lies in the ligand-detecting region of these polarized sensory neurons.

To examine the connectivity of *Or83b*-expressing neurons, we used an *Or83b* promoter-enhancer transgene (Wang et al., 2003) in combination with the synaptic marker, n-synaptobrevin-Green Fluorescent Protein (nsyb-GFP) to visualize projections of these neurons in the antennal lobe (Estes et al., 2000). To do this, we took advantage of the two-component gene regulation system that uses the yeast Gal 4 transcription factor fused to the *Or83b* regulatory regions and crossed flies carrying this driver transgene to a UAS-nsyb-GFP re-

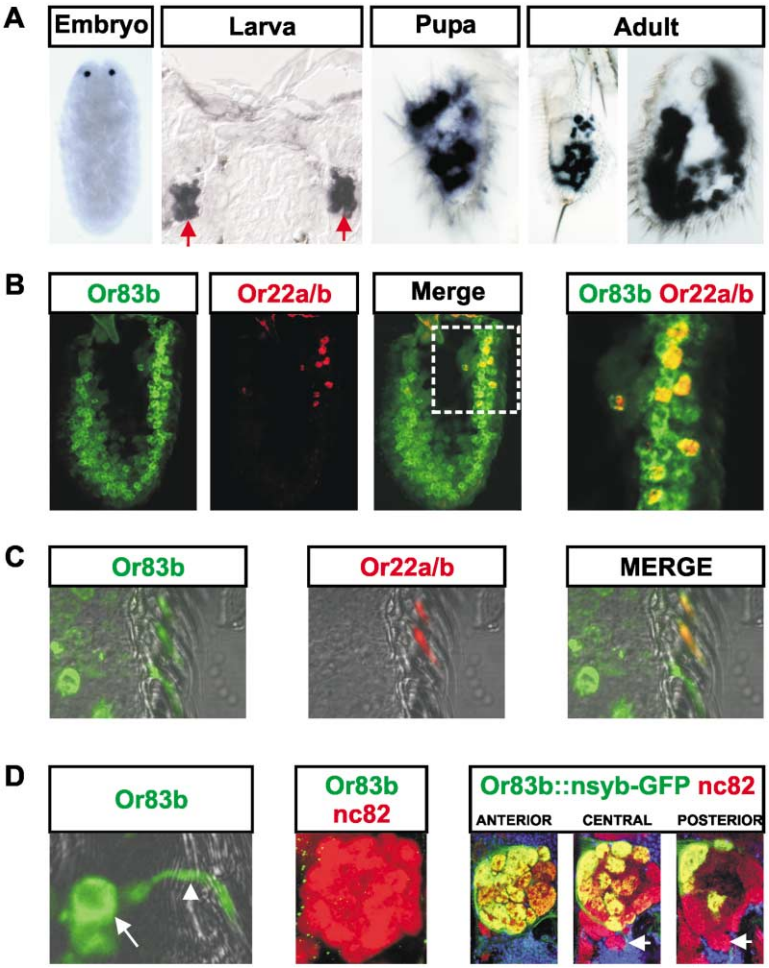

Figure 1. *Or83b* Is Expressed in Most Olfactory Sensory Neurons throughout Development

(A) RNA in situ hybridization with antisense *Or83b* probes (left to right): stage 16 embryo whole mount shows staining in the antennal-maxillary complex; frontal section of a third instar larva with staining in dorsal organ OSNs (red arrows); section of pupal antenna at 85 hr after puparium formation; section of adult maxillary palp; and adult antennal section.

(B) Two-color fluorescent in situ hybridization of adult antennal section shows coexpression of *Or83b* (green) and *Or22a/b* (red). White dashed box indicates area of inset shown at higher magnification on the right.

(C) *Or83b* (green) and *Or22a/b* (red) proteins are colocalized in the sensory dendrite of *Or22a/b*-positive OSNs in this confocal section from a wild-type antenna.

(D) *Or83b* protein is restricted to the OSN cell body (white arrow) and chemosensory dendrite (white arrowhead) (left). No *Or83b* protein is detected in the antennal lobe. Brain whole mounts stained with neuropil marker nc82 (Laissue et al., 1999) (red) and *Or83b* antibody (green). Detector gain in the green channel has been increased to reveal background staining (center). Projections of *Or83b*-expressing neurons visualized in *Or83b*-Gal4; UAS-nsyb-GFP animals show selective innervation of dorsal-medial glomeruli and absence of staining in the V glomerulus (white arrowhead). Three confocal sections of the same brain whole mounts stained with nc82 (red) and anti-GFP(green) (right).

sponder transgene (Brand and Perrimon, 1993). Consistent with the apparent medial to lateral gradient of *Or83b* expression in the antenna (Figures 1A and 1B), there is a similar gradient of fibers innervating the antennal lobe (Figure 1D, right). Dorsal and medial glomeruli, including those that receive input from *Or22a/b*-expressing OSNs, are brightly labeled. Lateral glomeruli receive considerably less innervation. Confocal sections through central and posterior aspects of the antennal lobe clearly show the sparsely innervated lateral and ventral glomeruli. Notably, the V glomerulus receives no apparent input from *Or83b*-expressing neurons (white arrow in Figure 1D, right).

Generation of an *Or83b* Null Mutant by Gene Targeting

To study the loss of function of *Or83b*, we generated null mutants in which the putative transcription start site and the first five transmembrane domains of this seven transmembrane domain receptor protein were deleted by gene-targeting techniques designed to replace these portions of the *Or83b* coding region with the *white* gene, a selectable eye color marker (Figure 2A; Gong and Golic, 2003). 14 homozygous mutants were obtained and confirmed to be null by Southern blotting with probes that lie both 5′ and 3′ of the targeted locus and by PCR directed against the *Or83b* coding region (Figure 2B). The homozygous mutants produce viable and fertile adult progeny with no gross developmental or physical defects. *Or83b* mutant larvae show normal locomotor and gustatory behavior (data not shown). Three mutant lines ($Or83b^1$, $Or83b^2$, $Or83b^3$) were selected for further characterization, in addition to a genetically matched control line in which the targeting construct integrated on the third chromosome, but left the *Or83b* region intact (referred to as $Or83b^{+/+}$ throughout).

No *Or83b* mRNA or Or83b protein is detectable in the homozygous $Or83b^2$ mutant strain (Figures 2C and 2D, center). Antibodies directed against the terminal cytoplasmic loop of Or83b, encoded by DNA sequences left intact by the targeted deletion, also show no detectable residual Or83b protein (data not shown). This rules out the possibility that partial expression of a truncated Or83b protein could interfere with our mutant analysis by causing dominant-negative effects. Similar expression results were obtained with the $Or83b^1$ and $Or83b^3$ strains (data not shown).

In animals carrying an *Or83b* rescuing transgene under the control of the *Or83b*-Gal4 driver, *Or83b* expression is partially restored (Figures 2C and 2D, right). The medial to lateral gradient of *Or83b* expression is exaggerated in these animals, producing lower than normal levels of *Or83b* expression in the lateral-distal domains of the antenna. Rescue of *Or83b* expression in mutants

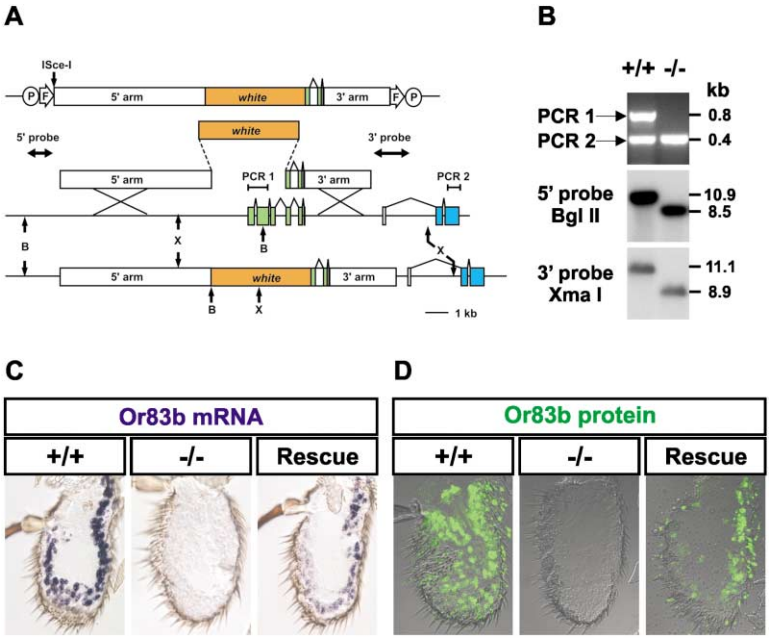

Figure 2. Disruption of the *Or83b* Locus by Gene Targeting

(A) Schematic of the *Or83b* targeting construct (top), *Or83b* gene region (middle), and targeted allele (bottom).

(B) PCR and Southern analysis of one *Or83b* mutant compared to wild-type control (right) using PCR primers and probes indicated in the schematic. Genomic DNA was digested with BglII (B; 5′ probe) and XmaI (X; 3′ probe).

(C) In situ hybridization of antennal sections with antisense *Or83b* probes in *Or83b*$^{+/+}$ (left), *Or83b*^{2} (middle), and *Or83b* transgenic rescue animals (*Or83b*-Gal4/UAS-*Or83b*;*Or83b*^{2}/*Or83b*^{2}) (right).

(D) Immunofluorescence of antennal sections stained with *Or83b* antibody in *Or83b*$^{+/+}$ (left), *Or83b*^{2} (middle), and *Or83b* transgenic rescue animals (*Or83b*-Gal4/UAS-*Or83b*;*Or83b*^{2}/*Or83b*^{2}) (right).

under control of this transgenic driver in larvae also shows lower than wild-type levels in certain dorsal organ neurons (data not shown).

Dendritic Localization Defects of Conventional Odorant Receptors in *Or83b* Mutants

Because *Or83b* is broadly coexpressed with other ORs, we asked whether the loss of *Or83b* function has an effect on the distribution or levels of expression of other coexpressed ORs. In adult olfactory neurons, both Or22a/b and Or43b proteins localize to OSN dendrites in wild-type antenna (Figures 3A and 3C; Elmore and Smith, 2001; Dobritsa et al., 2003). Or22a/b is particularly efficiently transported into the dendrite with little protein detected in the cell body, while Or43b is distributed in a manner similar to Or83b in both sensory dendrite and cell body (Figures 3A and 3C). In all three *Or83b* mutants, both Or22a/b and Or43b proteins are weakly detected in the cell body but no dendritic staining is seen (Figures 3A and 3C; data not shown). The available reagents do not permit us to determine whether residual Or22a/b and Or43b proteins are intracellular or on the surface of the cell body in *Or83b* mutants. In control experiments, no signal is detected with these antibodies in *Or22a/b* and *Or43b* null mutants (data not shown). Restoring *Or83b* gene function by transgenic rescue restores the subcellular localization of both Or22a/b and Or43b to the chemosensory dendrite (Figures 3A and 3C).

Because odorant binding and signal transduction occurs in the dendrite, the OR mislocalization defect found in *Or83b* mutants would be expected to disrupt odor-evoked physiology, as demonstrated below. It is of therefore of great importance to determine whether this failure of conventional OR proteins to be localized in the dendrite reflects a specific requirement of *Or83b* or is a nonspecific effect of a sick neuron. We therefore carried out a number of control experiments to determine whether other aspects of the biology of OSNs is normal in *Or83b* mutants. The stereotyped targeting of *Or22a*-expressing axons to the DM2 glomerulus in the antennal lobe is not affected in the mutant (Figure 3B). *Or22a*-nsyb-GFP-positive fibers are only found restricted to the DM2 glomerulus, and no ectopic innervation is seen. Levels of this synaptic marker are considerably lower in the mutant than in wild-type, which likely is a direct consequence of the disruption of neuronal activity in these neurons. We have obtained similar results on reduced trafficking of both membrane-targeted and synaptically targeted marker proteins in neurons that have been silenced genetically, perhaps because of a general effect on axonal and synaptic membrane protein transport in inactive neurons (S. Sachse and L.B.V., unpublished). This suggests that *Or83b* is not required for the establishment or maintenance of synaptic connections in glomeruli and that these connections persist in the absence of neuronal activity. The gross morphology of OSNs is normal in *Or83b* mutants, as judged by the levels and distribution of the neuron-specific microtubule associated protein, futsch, recognized by the 22c10 monoclonal antibody (Figure 3C; Hummel et al., 2000). In an additional control experiment, we hybridized a mix of antisense DOR gene probes to wild-type (data not shown) and mutant antennae and found that the numbers, distribution, and levels of gene expression of these OR genes are comparable in both genotypes (Figure 3D).

Finally, the subpopulation of *Or22a/b* neurons was electrically silenced in wild-type animals to determine if loss of neuronal activity per se affects the accumulation or maintenance of Or22a/b and Or83b protein in dendrites. Expression of the inward rectifying potassium channel Kir2.1 in *Or83b*-expressing neurons eliminates EAG responses to all odors tested (L. Giarratani and L.B.V., unpublished data; Baines et al., 2001). However, normal dendritic localization of Or22a/b (Figure 3E, top) and Or83b (Figure 3E, bottom) is maintained under conditions of prolonged electrical silencing that mimic other

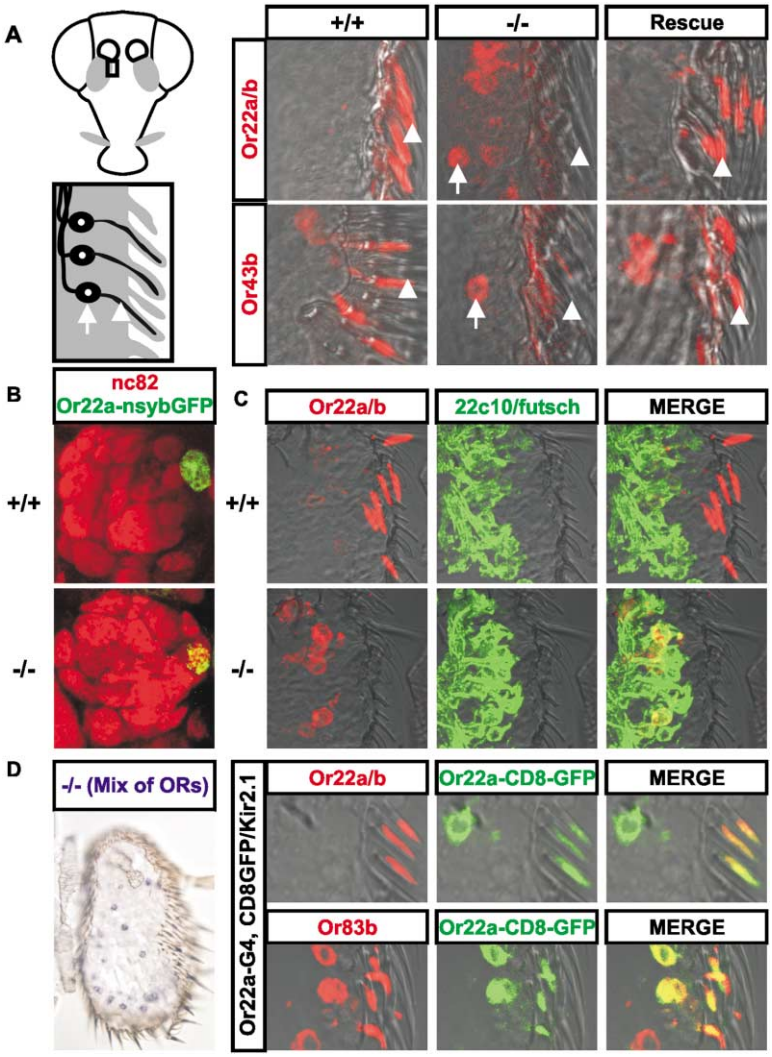

Figure 3. *Or83b* Is Required for Dendritic Localization of Conventional OR Proteins

(A) Schematic frontal view of fly head with antennae and maxillary palps shaded in gray. Inset represents antennal OSNs (black) with chemosensory dendrites inserted into olfactory sensilla (gray). Cell bodies are indicated with white arrow and dendrites with a white arrowhead. Top row: *Or22a/b* immunofluorescence in antennal sections from $Or83b^{+/+}$ (left), $Or83b^{2}$ (middle), *Or83b* transgenic rescue (*Or83b*-Gal4/UAS-$Or83b;Or83b^{2}/Or83b^{2}$) (right) animals. Bottom row: *Or43b* immunofluorescence in antennal sections from $Or83b^{+/+}$ (left), $Or83b^{2}$ (middle), *Or83b* transgenic rescue (*Or83b*-Gal4/UAS-$Or83b;Or83b^{2}/Or83b^{2}$) (right) animals. White arrowheads indicate position of dendrites; white arrows in middle panel indicate OR protein accumulation in cell bodies in $Or83b^{2}$ animals.

(B) Targeting of *Or22a*-expressing axons to the DM2 glomerulus is normal in $Or83b^{2}$ mutant antennal lobes. Whole-mount brain stained with nc82 (red) and GFP (green) antibodies. Genotypes of animals are: Or22a-nsyb-GFP;$Or83b^{2}$/TM6B (top) and Or22a-nsynaptobrevin-GFP; $Or83b^{2}/Or83b^{2}$ (bottom).

(C) Immunofluorescent staining with neuron-specific *futsch* antibody (22c10; green) of $Or83b^{+/+}$ (top) and $Or83b^{2}$ (bottom) antennae reveals normal morphology of mutant neurons. Or22a/b (red) shows the characteristic OR localization defect in $Or83b^{2}$ mutant antennae.

(D) OR gene expression is unaltered in *Or83b* mutants. $Or83b^{2}$ antenna hybridized with a mix of antisense DOR probes comprising *Or22a*, *Or23a*, *Or43a*, *Or43b*, *Or47a*, *Or47b*, and *Or88a*.

(E) Electrical silencing does not alter OR protein distribution. Two-color immunofluorescence staining of antennal sections with antibodies against Or22a/b (red; top left), Or83b (red; bottom left), GFP (green; middle); merged images (right). Genotype of stained animals is *Or22a*-Gal4, UAS-CD8-GFP/UAS-Kir2.1 aged 5 days after eclosion.

aspects of the *Or83b* phenotype, including the reduced levels of synaptic markers in OSN axonal termini (Figure 3B; S. Sachse and L.B.V., unpublished data). This suggests that the dendritic localization defect we see in *Or83b* animals reflects a specific role of this gene in OR protein localization and is not a secondary consequence of a loss of neuronal activity.

Dendritic Localization Defects of Conventional Odorant Receptors in *Or83b* Mutant Larvae

The effect of the *Or83b* mutation on OR localization in the 21 neurons of the larval dorsal organ was investigated. These neurons are clustered into a ganglion containing the cell bodies, each of which extends a dendrite that inserts into the dorsal organ dome. Cytoplasmic lacZ marker protein labels the cell bodies and basal portions of the dendrites but is excluded from the distal tip of the dendrite inserted in the dome (Figure 4A). We used the *Or83b*-Gal4 driver to misexpress an adult OR, *Or22a*, in all larval OSNs. Or22a protein is localized to the distal dendritic tips as seen in an exterior view of the dorsal organ dome (Figure 4B, middle). Lower levels of Or22a are detected in the cell bodies (data not shown). In *Or83b* mutant larvae, no Or22a protein is detected in dendrites and low levels are seen in the cell bodies (Figure 4B, middle; data not shown). In wild-type animals, *Or83b* protein is detected in distal dendritic tips inserted into the dorsal organ dome as well as the OSN cell bodies but is not detected in *Or83b* mutants (Figure 4B, left). The accumulation of the membrane bound marker CD8-GFP in the distal dorsal organ dendrites is maintained in *Or83b* mutant OSNs, ruling out a general defect in dendritic morphology or protein sorting in the mutants (Figure 4B, right). Therefore, larval OSNs have the same requirement for *Or83b* gene function in localizing ORs to the site of odor interaction in dendrites as adult OSNs.

Electrophysiological Characterization of *Or83b* Mutants

The dramatic and selective effect of the *Or83b* mutation on OR localization in olfactory dendrites would be ex-

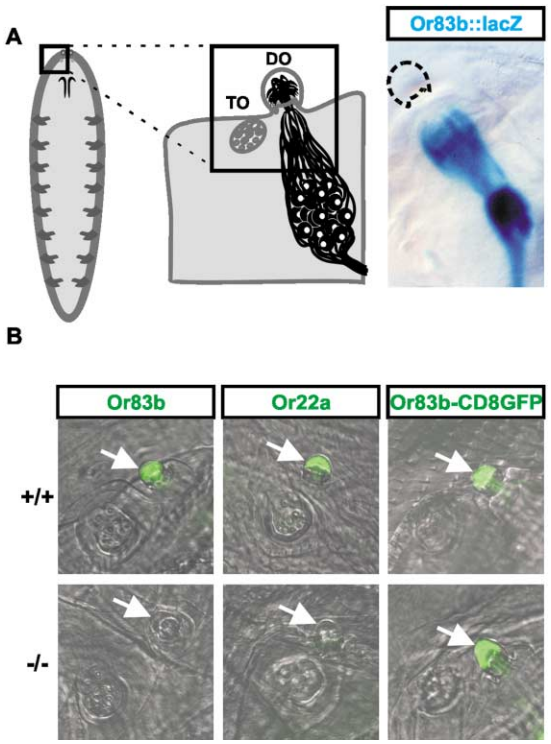

Figure 4. Odorant Receptor Localization Is Disrupted in *Or83b* Mutant Larvae

(A) Schematic dorsal view of third instar larva with anterior tip facing up. Inset shows cuticular surface of larva with terminal organ (TO) and dorsal organ (DO). Whole mount of an Or83b-Gal4;UAS-lacZ larva reveals lacZ staining in the dorsal organ (blue) (right). The dorsal organ dome is indicated by dashed black line.

(B) Immunofluorescence showing exterior cuticle view stained with antibodies against *Or83b* (left), *Or22a/b* (middle), and GFP (right). Dorsal organ dome is marked with a white arrow. OSN cell bodies are located in a different focal plane and are not visible here. Genotypes of stained animals are: top (left to right) $Or83b^{+/+}$, *Or83b*-Gal4/UAS-Or22a;$Or83b^{1}$/TM6B, *Or83b*-Gal4/UAS-CD8-GFP; $Or83b^{1}$/TM6B. Bottom (left to right): $Or83b^{1}/Or83b^{1}$, *Or83b*-Gal4/UAS-Or22a; $Or83b^{1}/Or83b^{1}$, *Or83b*-Gal4/UAS-CD8-GFP; $Or83b^{1}/Or83b^{1}$.

pected to eliminate odor-evoked potentials in these neurons. To test this, we measured electroantennograms (EAGs), which are thought to reflect broad domains of receptor potentials summed around the site of the recording electrode in the fly antenna (Ayer and Carlson, 1992). Control preparations show strong negative potentials in response to all odorants tested (Figure 5A, top; Figure 5C). EAGs recorded in $Or83b^{2}$ mutant antennae produce no responses (Figure 5A, middle; Figure 5C), and normal EAG responses are restored by transgenic rescue of *Or83b* gene function (Figure 5A, bottom; Figure 5C). These results are consistent with a general requirement for *Or83b* in adult olfactory function to a broad range of odor stimuli.

EAGs provide a convenient measure of olfactory function across the entire antenna but suffer from a lack of single cell resolution and have low sensitivity. Therefore, to be certain that this mutation blocks odor-evoked activity at the single neuron level, we carried out extracellular single sensillum recordings on the identified ab1 sensillum, which contains three general odor-responsive neurons and a single neuron (ab1C) highly tuned to carbon dioxide (de Bruyne et al., 2001). In wild-type antennae, all four neurons in the ab1 sensillum show characteristic odor responsivity as previously described (Figure 5B, left; Figure 5D; de Bruyne et al., 2001; Stensmyr et al., 2003). *Or83b* mutant antennae show no odor-evoked action potentials to the general odorants ethyl acetate (ab1A), acetoin (ab1B), or methyl salicylate (ab1D), but responses to carbon dioxide (ab1C) are completely normal (Figure 5B, center; Figure 5D). Unlike the case of neurons with deficiencies in conventional odorant receptors (Dobritsa et al., 2003; Hallem et al., 2004a), *Or83b* mutant OSNs generally show very little spontaneous activity (Figure 5B, middle). We occasionally find evidence of spontaneous or damage-induced activity of the general odor neurons in these animals (data not shown), which may reflect low levels of spontaneous activity independent of receptor activation. Restoring *Or83b* gene function to antennal neurons by transgenic rescue also restores wild-type odor responses to the three affected neurons of the ab1 sensillum (Figure 5B, right; Figure 5D).

Larval Chemotaxis Phenotype of *Or83b* Mutants

The dramatic loss of odor-evoked potentials to a broad range of general odors at the level of the whole antenna and single neurons suggests a general and essential role for *Or83b* in odor detection. To determine the behavioral effects of this mutation on larval behavior, chemotaxis of larvae to a number of structurally distinct odorants was monitored with a single-animal video-tracking assay, modified from a previously described population assay (Monte et al., 1989). Tracks of animals responding to ethyl acetate are presented in Figure 6A. While the control *yw* animal chemotaxes toward the stimulus (Figure 6A, left), the $Or83b^{1}$ mutant larva does not respond to the odor and wanders at random around the plate (Figure 6A, middle). Expression of a rescuing UAS-*Or83b* transgene under control of the *Or83b*-Gal4 driver in the $Or83b^{1}$ mutant restores chemotaxis behavior (Figure 6A, right). We tested olfactory responses of animals with these three genotypes to 36 odors. Control *yw* larvae chemotax to all 36 odors (Figure 6B), while mutant larvae show no response to 34 of these odors and weak responses to 2 (Figure 6C). The *Or83b* rescuing transgene partially restores chemotaxis in $Or83b^{1}$ mutants, consistent with the nonuniform expression of the *Or83b*-Gal4 driver in the larval dorsal organ (Figure 6D; data not shown). Similar results were obtained with two additional mutant and control strains to a subset of these odors.

Perturbation of Adult Odor-Evoked Behavior in *Or83b* Mutants

To determine whether the electrophysiological defects seen in the antenna translate to behavioral phenotypes, we performed a modified trap assay (Figure 7A; Park et al., 2002; Zhu et al., 2003). Adult flies show strong behavioral responses to natural odors and synthetic odor blends but respond to only a subset of the single odors composing these blends (Stensmyr et al., 2003). The chemosensory behavior of adult *Or83b* mutant flies was tested with two of these behaviorally active single

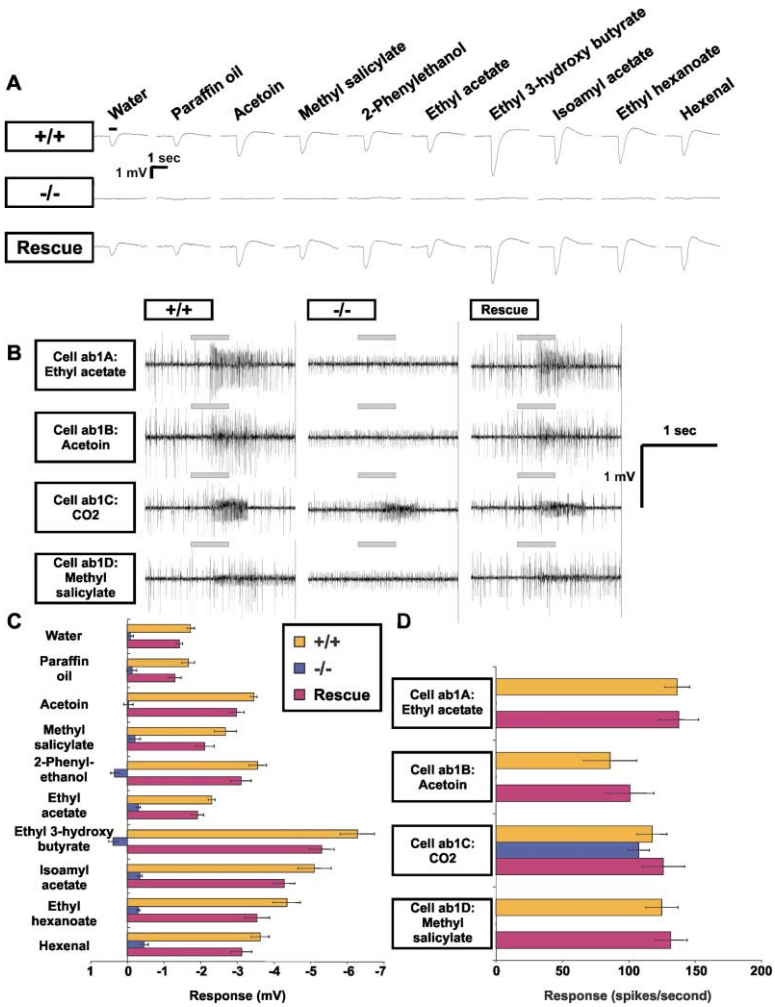

Figure 5. Adult *Or83b* Mutant Flies Show Severe Deficits in Antennal Odor-Evoked Electrophysiology

(A) Electroantennogram (EAG) plots recorded from $Or83b^2$ mutant antennae stimulated with eight different odors diluted 1/100 in paraffin oil or water, along with solvent controls. Stimulus onset and duration (0.5 s) is shown for the first trace as a horizontal bar. $Or83b^{+/+}$ control animals show robust potentials (top), $Or83b^2$ mutants show no detectable response to any stimulus (middle), and *Or83b* transgenic rescue (*Or83b*-Gal4/UAS-$Or83b;Or83b^2/Or83b^2$) animals have robust responses (bottom).

(B) Single unit recordings from the ab1 sensillum stimulated with odorants known to activate each of the four neurons selectively. Stimulus period is indicated by the gray bar on top of each trace (0.5 s).

(C) Summary data of EAG data plotted as mean ± SEM, n = 5 antennae per genotype.

(D) Summary of single sensillum recording data plotted as mean ± SEM, n = 5 neurons per genotype.

odors, acetoin and 2-phenylethanol (Figure 7B). As expected from the absence of odor-evoked physiological responses to these odors, *Or83b* mutants show severely impaired responses to acetoin and are anosmic to 2-phenylethanol (Figure 7B). Normal responses to acetoin and 2-phenylethanol are restored in $Or83b^2$ mutants carrying a UAS-*Or83b* rescuing transgene under control of the *Or83b*-Gal4 driver (Figure 7B).

Discussion

A Highly Conserved, Broadly Expressed Odorant Receptor Essential for Olfaction

We show by cellular, physiological, and behavioral analysis that *Or83b* is essential for olfaction in *Drosophila*. *Or83b* is an atypical member of the OR gene family because it is highly conserved across insect species and is expressed in a large number of OSNs with different odor specificities (Dobritsa et al., 2003; Elmore et al., 2003; Hallem et al., 2004a). This receptor is selectively expressed only in OSNs throughout all four stages of *Drosophila* development, and no expression is detected in gustatory neurons or any other cell type. Fly *Or83b* is expressed in all OSNs of the larval dorsal organ and adult maxillary palp and in a large subset of adult antennal neurons. The onset of *Or83b* expression in both larval and adult olfactory systems is late, effectively ruling out any developmental role for this protein in patterning the axonal connections of these neurons.

An *Or83b* mutant was constructed by "ends-out" gene targeting and shown to be null for both mRNA and protein expression. Conventional OR proteins fail to accumulate in both adult and larval OSN dendrites in *Or83b* mutants and are restricted to the cell body. This suggests a role for *Or83b* in regulating the proper subcellular localization of the conventional ORs. The OR localization defect was shown to be specific to *Or83b* and not a secondary effect of a sick neuron. The distribution of a general membrane marker in larvae and the *futsch* microtubule marker is unaffected in the mutant, as is the stability of *Or22a*-expressing axonal connections to the DM2 glomerulus in the antennal lobe. Electrical silencing of wild-type neurons does not produce the same OR localization defect as the *Or83b* mutation.

Or83b mutant antennae show no odor-evoked potentials to a panel of eight odorants that elicit robust responses in wild-type antenna. Mutant ab1A, B, and D neurons fail to respond to their cognate stimuli and show little or no spontaneous electrical activity. In contrast, the carbon dioxide-sensitive ab1C neuron is normal in

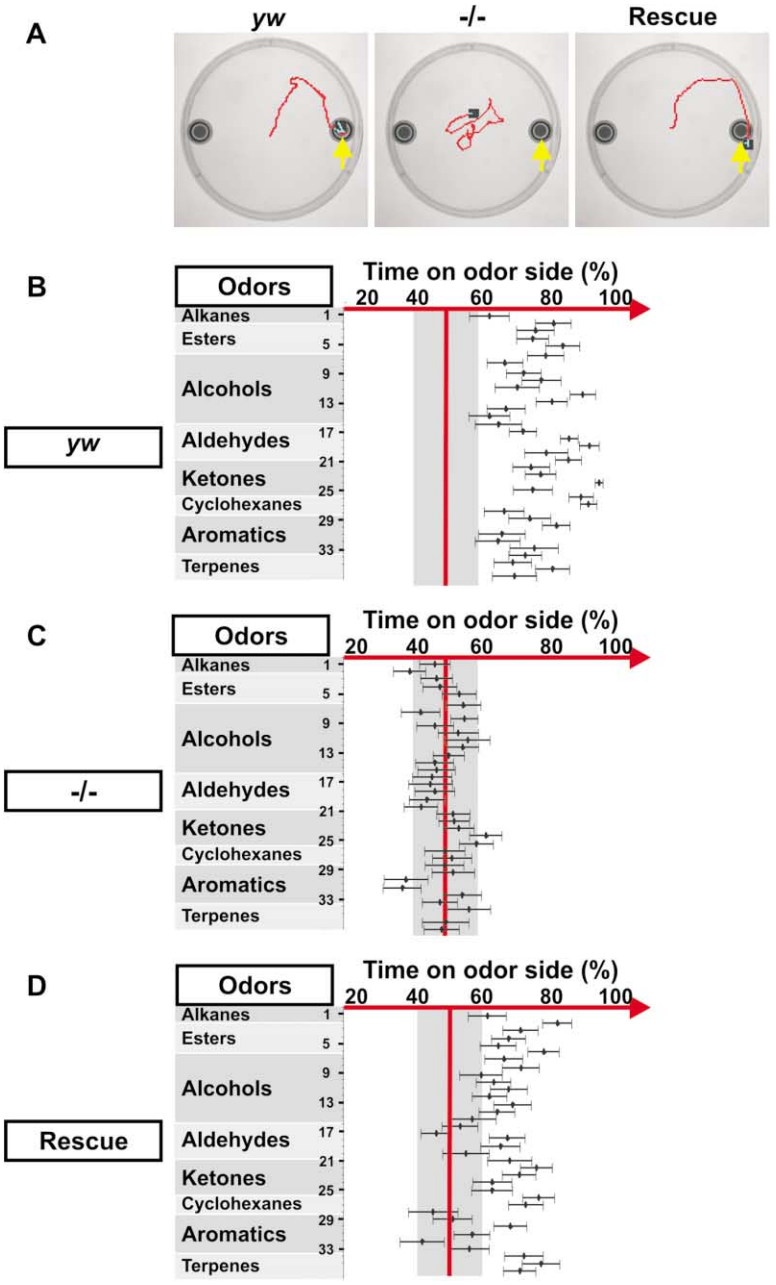

Figure 6. *Or83b* Mutant Larvae Are Anosmic

(A) Tracks of larvae recorded for 5 min in response to 2 μl undiluted ethyl acetate located on the right side of the plate (yellow arrow). The start position is the center of the plate and the end position of the larva is indicated by the black square. *yw* (left), *Or83b*^{1} (middle), *Or83b* transgenic rescue (*Or83b*-Gal4/UAS-*Or83b;Or83b*^{1}/*Or83b*^{1}) (right).

(B–D) Chemotaxis responses of control *yw* (B), *Or83b*^{1} (C), and *Or83b* transgenic rescue (*Or83b*-Gal4/UAS-*Or83b;Or83b*^{1}/*Or83b*^{1}) (D) animals to 36 odors are plotted as the percent of time the animal spends on the odor side, with the expected 50% value for anosmia indicated with the vertical red line and 95% confidence intervals represented by the shaded gray bar. Theoretical maximum chemotaxis is 100%. Confidence intervals are derived from an estimated standard deviation (34.6%) obtained by tracking control *yw* larvae ($n = 34$) in the absence of an odor stimulus. All plotted values are mean ± SEM, mean $n = 32$ (range $n = 18$–56) animals per odorant. Significance was established at $p < 0.05$ relative to anosmia with an unpaired two-tailed t test. *yw* control animals show statistically significant responses to all 36 odors. *Or83b*^{1} mutants did not chemotax to 34 odors and exhibited a weak aversive response to odor #2 and weak chemotaxis to odor #24. Partial transgenic rescue is obtained with an *Or83b* transgene (*Or83b*-Gal4/UAS-*Or83b;Or83b*^{1}/*Or83b*^{1}) such that these animals responded to 25/36 odors at a significance level of $p < 0.05$ relative to anosmia. Multiple comparisons between means across odors revealed statistically significant differences between the three genotypes ($p < 0.05$; Tukey's HSD Test). Odor numbers are indicated on the vertical axis along with categorization by functional class: (1) heptane, (2) methyl acetate, (3) ethyl acetate, (4) propyl acetate, (5) isoamyl acetate, (6) methyl hexanoate, (7) methan-1-ol, (8) propan-1-ol, (9) pentan-1-ol, (10) hexan-1-ol, (11) hexan-2-ol, (12) hexan-3-ol, (13) heptan-1-ol, (14) octan-3-ol, (15) 1-octen-3-ol, (16) pentanal, (17) hexanal, (18) heptanal, (19) octanal, (20) hex-2-enal, (21) acetone, (22) 2-butanone, (23) 2-heptanone, (24) 2-octanone, (25) 3-octanone, (26) methylcyclohexanol, (27) cyclohexanol, (28) cyclohexanone, (29) methylbenzoate, (30) acetophenone, (31) benzaldehyde, (32) toluene, (33) ethylbenzene, (34) anisole, (35) α-terpinene, (36) (-)-linalool.

the *Or83b* mutant. Finally, both larval and adult *Or83b* mutants have severe deficits in odor-evoked behavioral responses. Taken together, these data support a model in which *Or83b* acts in concert with conventional ORs to respond to many different odorants and argue against an independent function for *Or83b* in recognizing a particular odorant.

Implications for the Molecular Logic of the Fly Olfactory System

The number of ORs expressed in a given OSN is an important determinant of the coding logic of the olfactory system. In the nematode, *C. elegans*, 16 pairs of chemosensory neurons express an estimated 500 different chemosensory receptor genes. Of necessity, each chemosensory neuron expresses a large number of receptors (Troemel et al., 1995; L'Etoile and Bargmann, 2000; Wes and Bargmann, 2001), but each receptor functions independently within a neuron to recognize ligands that activate the chemosensory neuron to elicit either attractive or aversive behavioral responses (Troemel et al., 1997). By organizing receptor expression according to behavioral output, which is regulated by the activation of the sensory neuron, the animal retains great discriminatory power in the face of a severely constricted number of sensory neurons and no central chemosensory processing circuits.

In contrast, each mammalian olfactory neuron ex-

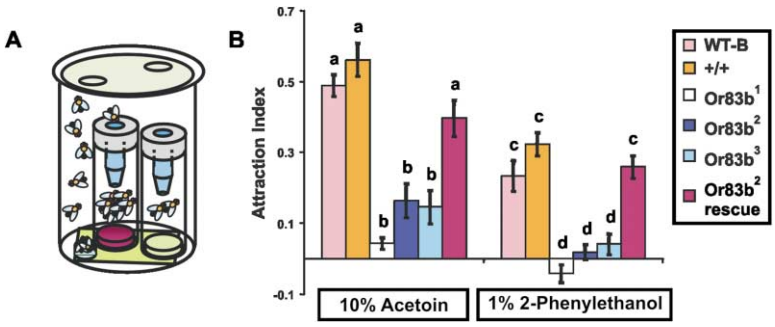

Figure 7. Adult *Or83b* Mutant Flies Have Severe Deficits in Odor-Evoked Behavior

(A) Schematic drawing of modified trap assay, with the odor-baited trap indicated in red and control trap in green.

(B) Attraction index plots of wild-type Berlin (WT-B), $Or83b^{+/+}$, $Or83b^{1}$, $Or83b^{2}$, $Or83b^{3}$, and *Or83b* transgenic rescue (*Or83b*-Gal4/UAS-*Or83b*;$Or83b^{2}$/$Or83b^{2}$) flies in response to 10% acetoin and 1% 2-phenylethanol. Values are mean ± SEM, n = 8–16 separate trap experiments, each containing approximately 100 flies. Bars labeled with different letters are statistically different ($p < 0.05$; Tukey's HSD test). Mutant responses to acetoin but not to 2-phenylethanol are statistically significantly different from anosmia (attraction index = 0; $p < 0.05$; two-tailed t tests).

presses a single odorant receptor gene that bestows upon that neuron a restricted receptive range for odors (Araneda et al., 2000). In part because the odorant receptor itself is an important determinant in glomerular target selection (Feinstein and Mombaerts, 2004), mammalian olfactory neurons have evolved elaborate regulatory mechanisms to suppress the expression of more than one functional odorant receptor gene per neuron (Chess et al., 1994; Serizawa et al., 2003; Lewcock and Reed, 2004; Shykind et al., 2004). Unlike the situation in *C. elegans*, each sensory neuron contributes information about a small fraction of the odor universe, and significant olfactory processing must occur in higher order olfactory cortical regions to decode the salience of the odor stimulus and produce an appropriate behavioral response.

Initial analysis of the *Drosophila* OR gene family suggested that the fly olfactory system was organized according to the vertebrate one receptor:one neuron:one glomerulus model. OR genes were found in nonoverlapping subpopulations of OSNs, and all OSNs expressing a given OR were found to converge upon a distinct and dedicated antennal lobe glomerulus (Clyne et al., 1999; Gao and Chess, 1999; Vosshall et al., 1999, 2000; Gao et al., 2000). However, further analysis has somewhat complicated this initial view. Dobritsa et al. (2003) showed that *Or22a* and *Or22b* are coexpressed in the ab3a neuron, that *Or22a* functions independently of *Or22b*, and that *Or22b* does not discernibly contribute to the odor code of this neuron. We show here that this same neuron expresses *Or83b* in addition to these two conventional odorant receptors. If each of these three ORs hypothetically interacts with distinct ligands, this would substantially alter our view of how the fly olfactory system is organized. In such a multireceptor OSN model, diverse stimuli would activate the same neuron, but all would lead to activation of the DM2 glomerulus to which the neuron projects. The animal would thus have no means to determine which of the three receptors was activated, resulting in a possible loss of odor discrimination. Our data strongly argue against an independent function for *Or83b* in odor detection and instead suggest that it acts as an essential cofactor for localizing conventional ORs in chemosensory dendrites. Therefore, the fly is likely to retain an organizational logic similar to that employed in vertebrates, despite expressing more than one OR in each olfactory neuron.

A Universal Factor for Odorant Receptor Function in *Drosophila*

This study demonstrates a genetic requirement for *Or83b* in dendritic localization of conventional ORs in vivo and would be consistent with a model in which *Or83b* interacts with the conventional odorant receptors to form a heteromeric receptor complex. Conclusive evidence for direct association between *Or83b* and conventional ORs, as well as more detailed biochemical insights into the function of *Or83b*, awaits the development of an expression system that can be used to examine the interactions of biologically active *Drosophila* ORs.

Functional interaction between chemosensory receptors has been described in mammalian taste, where the assembly of different heterodimeric receptors determines whether a neuron responds to sweet or amino acid stimuli: the T1R2/3 heterodimer encodes a sweet taste receptor, while T1R1/2 encodes the umami or amino acid receptor (Nelson et al., 2001, 2002; Zhao et al., 2003). Homodimeric T1R3 receptors detect only high concentrations of natural sweeteners such as sucrose (Zhao et al., 2003). Therefore, in the mouse three different classes of gustatory receptor cells express T1R3, but the functional specificity of the cell is determined by whether a given cell expresses T1R2 (regular sweet), T1R1 (umami), or neither (low-affinity sweet).

Broad expression of atypical chemosensory receptor genes, such as described here for *Or83b*, has also been seen. In the rodent vomeronasal organ, members of the V2R2 pheromone receptor subfamily are broadly expressed along with other more selectively expressed receptors (Martini et al., 2001), but biochemical evidence that they interact functionally is lacking. Amino acid detection in fish relies in part on a broadly expressed receptor of the same structural class as the T1R taste receptors and V2R pheromone receptors, but it is not known whether this 5.24 receptor functions in concert with other fish receptors with which it is coexpressed (Speca et al., 1999).

Our data on *Or83b* point to a unique and unprecedented requirement of a single receptor protein for the functionality of an extremely diverse family of receptors. These results are unexpected because the *Drosophila* odorant receptor genes are unrelated to other receptor superfamilies that have been shown to heterodimerize through conserved protein-protein interaction domains

(Margeta-Mitrovic et al., 2000). Further, this receptor family is undergoing rapid evolutionary change with virtually no direct homologs recognized between the fly and mosquito genomes (Hill et al., 2002; Robertson et al., 2003). In contrast, *Or83b* is extremely conserved and has clear homologs in distant insect species. If these homologs are performing the same function in these divergent insect species, it would follow that *Or83b* must recognize a conserved feature of all OR proteins with which it is expressed, either directly by heterodimerizing with the ORs or through the action of accessory proteins. The investment of *Drosophila* in a single protein for odor detection suggests that *Or83b* performs a key function in all insects that cannot be diversified or made redundant. The alternative to maintaining strong selection pressure on *Or83b* would be to require parallel selection on a diversity of other genes that each interacts with a partner OR protein. Such a coevolution of two large gene families is suggested by the work on the M10 MHC family that associates with a family of V2R receptors in the vomeronasal organ (Ishii et al., 2003; Loconto et al., 2003). There may be barriers to the generation of large numbers of new genes in *Drosophila*, which maintains a compact genome with few pseudogenes.

Prospects for Exploiting *Or83b* for Behavior-Based Insect Control Strategies

Insects are the primary vectors for the infectious diseases malaria, dengue fever, yellow fever, and West Nile encephalitis, and they locate human hosts largely through their exquisitely sensitive olfactory systems (Takken and Knols, 1999). Host-seeking behavior is thought to require a number of different sensory stimuli to provide the maximum likelihood that the human host is near. For instance, mosquitoes are attracted to general odors along with carbon dioxide and body heat (Takken and Kline, 1989; Mukabana et al., 2004). Our finding that the *Or83b* mutation disrupts *Drosophila* behavioral responses to many odors suggests a potential chemical strategy to disrupt the function of homologous genes in pest insects. Small molecule inhibitors that mimic the effects of this mutation may blunt or eliminate olfactory responses in pest insects, ultimately controlling the damaging olfactory-mediated behaviors that result in the spread of disease.

Experimental Procedures

Drosophila Stocks

All fly stocks were maintained on conventional cornmeal-agar-molasses medium under a 12 hr light:12 hr dark cycle at 18°C or 25°C. Fly stocks were kindly provided as indicated: w1118; 70FLP, 70I-SceI, Sco/CyO and w1118;70FLP (K. Golic); WT-B flies (M. Heisenberg); *Or22a/b* deficient Delta-Halo (M. Welte) (Gross et al., 2003); *Or43b* mutants (D. Smith).

Transgenic Fly Constructs and Strains

A partial clone encoding *Or83b* was isolated from an antennal/maxillary palp-cDNA library in a differential hybridization screen (Vosshall et al., 1999). Subsequently, a putative full-length cDNA clone (1917 nucleotides, GenBank accession AY567998) encoding a protein of 486 amino acids was isolated and sequenced by standard methods. This full-length clone contains a polymorphism at nucleotide 595, which results in an amino acid change of I to T. This full-length cDNA was cloned via 5′NotI and 3′XhoI sites into the pUAST vector (Brand and Perrimon, 1993) and transformants generated by standard methods. Full-length *Or22a* cDNA was cloned into pUAST via 5′ EcoRI and 3′ XhoI restriction sites. The *Or83b*-Gal4 transgene was constructed as previously described (Wang et al., 2003). 20 independent transgenic lines were obtained and a single line resembled the expression of endogenous *Or83b* mRNA, although the onset of expression is significantly delayed and the levels of expression are considerably lower.

Or83b Targeting Construct and Mutant Screen

5′ and 3′ homology regions were amplified by Expand High Fidelity PCR (Roche) from Oregon-R genomic DNA with primers corresponding to nucleotides 266,110–266,128/272,596–272,617 (5′arm = 6.507 kb fragment; primers contained terminal AvrII sites) and 259,153–259,175/262,788/262,809 (3′arm = 3.656 kb fragment; primers contained terminal NotI sites) on GenBank entry AE003603. Fragments were subcloned into the AvrII and NotI sites, respectively, of CMC105 (C.-M. Chen and G. Struhl, personal communication). This vector contains two polylinkers flanking the mini-white gene with a unique I-SceI site 5′ of the white gene, flanked by FRT sites and containing conventional P element repeats. This construct was designed to delete the putative transcription start site and sequences from exon 1 to the middle of exon 5, comprising the N terminus and transmembrane domains 1–5 of the protein, replacing them with the eye color marker, white (Figure 2A).

Virgin female flies carrying one of three independently generated targeting construct insertions on either X or 2 were crossed to w1118;70FLP, 70I-SceI, Sco/CyO, and 3-day-old progeny were heat shocked at 38°C for 60 min. Approximately 15,000 non-CyO, mosaic, or white-eyed adult virgin females were crossed to w1118;70FLP males and progeny were heat shocked as described above. Red-eyed flies were individually balanced to establish stable lines. Of 49 mutant alleles obtained, 14 were simple gene replacements and 35 had insertions of the white gene either 5′ or 3′ of the coding region. Genomic DNA prepared from homozygous lines was subjected to PCR with primers that lie within the *Or83b* coding region, corresponding to nucleotides 263,704–263,715/264,501–264,532 on AE003603. Internal control PCR primers lie within the adjacent gene (CG2663), corresponding to nucleotides 255,380–255,397/255,821–255,838 on AE003603. We confirmed PCR results with genomic Southern blots, generated by digesting genomic DNA with BglII or XmaI and probing blots with regions upstream of the 5′ arm or downstream of the 3′ arm (corresponding to nucleotides 273,968–273,010 and 257,473–258,940 on AE003603, respectively).

Histology

In situ hybridization was carried out as described, using an antisense digoxigenin-labeled RNA probe corresponding to the entire *Or83b* cDNA or previously described probes (Vosshall et al., 1999). Two-color in situ hybridization and whole-mount brain immunofluorescence were carried out as described (Vosshall et al., 2000). Anti-*Or43b* and anti-*Or22a/b* antibody staining was carried out as described (Elmore and Smith, 2001; Dobritsa et al., 2003). An *Or83b*-specific rabbit polyclonal anti-peptide antibody against synthetic peptide SSIP VEIPRLPIKS was raised and affinity-purified by Bethyl Laboratories (Montgomery, TX) and used at 1:5000. Monoclonal antibody 22c10 was used at 1:100. Mouse anti-*Or83b* antibody was provided by the UT Southwestern Medical Center PGA and used at 1:100.

Electroantennography

Electroanntennograms were recorded with saline-filled capillary glass electrodes as previously described (Ayer and Carlson, 1992) from 2- to 4-day-old adult flies. The third segment of one antenna was removed and a ground electrode slipped over the remaining two segments, while the recording electrode was placed at the tip of the other intact antenna. Instrumentation and EAG software were supplied by Syntech (Hilversum, NL). Odorants were diluted 1/100 (v/v) in paraffin oil or water and delivered from odor cartridges made from Pasteur pipettes containing 30 μl of diluted odorant on strips of filter paper.

Single Sensillum Recordings

Single-neuron responses were recorded with electrolytically sharpened tungsten microelectrodes, inserted at the base of basiconic

sensilla as previously described (de Bruyne et al., 2001; Stensmyr et al., 2003) Instrumentation and SSR software were supplied by Syntech (Hilversum, Netherlands). Odor delivery was performed from odor cartridges according to Stensmyr et al. (2003). Odorants were diluted in acetone to a concentration of 1 μg/μl, from which 10 μl aliquots were pipetted onto strips of filter paper in Pasteur pipettes and the acetone allowed to evaporate. Carbon dioxide was delivered from a 1 ml syringe, into which carbon dioxide was drawn from a container and diluted to $\sim$5% before delivery. Responses (spikes/s) were quantified by counting the number of spikes 500 ms after the response and subtracting the number 500 ms before, and then doubling this value.

Behavioral Analysis

Single larval chemotaxis assays were adapted from a previously described population assay (Monte et al., 1989). The locomotor behavior of each larva was tracked for 5 min on a 100 mm Petri dish coated with agarose with Noldus Ethovision Pro software and chemotaxis scored as the proportion of time spent on the semicircle containing the odor source. All odors were delivered full-strength at a stimulus dose of 2 μl on filter circles placed inside inverted plastic caps to prevent diffusion of the odorant into the agarose.

Adult behavior was measured using a trap assay modified from previously described assays (Park et al., 2002; Zhu et al., 2003). 2- to 4-day-old adult flies were starved for 48 hr in humidified chambers. Approximately 100 flies per assay were narcotized by hypothermia and introduced in an inverted plastic lid (25 mm) into the bottom of a humidified 10 $\times$ 18.5 cm 1 l glass beaker covered by ventilated lids made from plastic Petri dishes. Odor-baited traps were constructed from glass vials (2.5 $\times$ 10 cm) sealed with a cotton plug perforated with a pipet tip. Traps were baited with 2 ml of odor diluted in water and absorbed in a piece of cotton plug at the bottom of the trap. Control traps contained an equivalent volume of water. Flies were permitted 4 hr to choose a trap and an attraction index was calculated:

$$\text{Attraction index} = \frac{\#\text{ flies [ODOR TRAP]} - \#\text{ flies [CONTROL TRAP]}}{\text{Total \# flies [BEAKER]}}$$

where attraction index of 1 corresponds to complete attraction of all flies into baited odor trap and attraction index of 0 corresponds to anosmic flies. Flies that did not emerge from the plastic lid were excluded, but their numbers did not exceed 10 individuals per experiment.

Data were analyzed for statistical significance using two-tailed Student t tests, ANOVA, and Tukey's HSD test with SPSS. All odorants were obtained from Sigma-Aldrich and were of the highest purity available.

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