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An Increased Receptive Field of Olfactory Receptor Or43a in the Antennal Lobe of *Drosophila* Reduces Benzaldehyde-driven Avoidance Behavior

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Abstract

Most animals orient themselves in their environment through the perception of olfactory cues. In order to gain insight into the principles of olfactory processing in *Drosophila*, we misexpressed olfactory receptor Or43a in additional olfactory receptor neurons of the third antennal segment using enhancer trap line *GH320*. The behavioral response of *GH320/UAS-or43a* flies was changed upon benzaldehyde application. Using the T-maze assay, misexpressing flies performed a reduced avoidance reaction to benzaldehyde as compared with wild type. This reduction of avoidance could be mimicked in wild type flies by exposing them to a mixture of benzaldehyde and ethyl acetate. We therefore conclude that the application of benzaldehyde, an identified ligand of Or43a, resulted in activation of a number of glomeruli in transformed flies in addition to glomerulus DA4, which is the regular target of Or43a expressing neurons. Our results demonstrate the relevance of specific olfactory sensory input and subsequent processing in the antennal lobe for *Drosophila* behavior.

Key words: coding, ligand, neuron, olfaction, smell

Introduction

Insects have developed olfactory sensory systems with high sensitivity to volatile substances. These allow them to sense and respond to a variety of odorants over a wide range of concentrations. In response to olfactory signals, they perform an odor-guided behavior that is based on specific activation of neuronal circuits. In turn, behavioral response specificity is dependent on odorant receptor properties and olfactory coding. As a common rule, olfactory receptor neurons (ORN) express a given type of olfactory receptor (Or) and compute through parallel synaptic connections with subunits of the antennal lobe, the glomeruli (Gao *et al*., 2000; Vosshall *et al*., 2000). This specific neuronal network system encompasses the neuronal basis for olfactory coding and sets the ground for olfactory behavior.

Genetic analyses of mutants with defects in olfactory behavior have disclosed the mechanisms by which *Drosophila* responds to olfactory cues. Pioneering studies described the isolation of olfactory mutants with a defective response to airborne chemicals (Siddiqi, 1987; Helfand and Carlson, 1989; McKenna *et al*., 1989; Ayyub *et al*., 1990; Lilly and Carlson, 1990; Devaud, 2003). [^{3}H]2-Deoxyglucose uptake experiments enabled the identification of activated glomeruli in the antennal lobe of *Drosophila* after prolonged odorant application (Rodrigues and Buchner, 1984). Later, the development of the Gal4/UAS system (Brand and Perrimon, 1993) made it possible to manipulate the olfactory system in *Drosophila* in a noninvasive manner. Brain structures, including antennal glomeruli, were identified that underlie olfactory learning, memory and behavior (Ferveur *et al*., 1995; O'Dell *et al*., 1995; Connolly *et al*., 1996; Zars *et al*., 2000). Ferveur *et al*. (1995), for example, studied the neuronal basis of sexual orientation in male fruit flies with regionally feminized subsets of glomeruli in the antennal lobe by applying a courtship assay. Fiala *et al*. (2002) used transformed flies that expressed the Ca^{2+}-dependent color change of the chameleon to visualize representational intermediates in the antennal lobe upon application of odorants. Alternatively (Ng *et al*., 2002; Wang *et al*., 2003; Yu *et al*., 2004) derivatives of green fluorescent protein (GFP) were used to investigate antennal lobe activity. Both studies have in common that the activation of individual glomeruli is monitored merely as the result of a prolonged olfactory stimulation. Thus, the stimulation pattern can be regarded as a final activity representation that is derived from antennal lobe processing. The behaviorally relevant

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information associated with the individually activated sets of glomeruli has been investigated by blocking synaptic activity or increasing the number of synapses in individual glomeruli (Acebes and Ferrus, 2001; Devaud *et al.*, 2001; Keller *et al.*, 2002; Devaud, 2003). Moreover, inactivation of a single olfactory receptor did not reveal a defect in olfactory driven behavior most probably because alternative Ors can compensate for the olfactory function of the missing one (Elmore *et al.*, 2003). In spite of the growing knowledge about olfactory signal processing, the relevant properties of individual glomeruli in olfactory behavior are not known.

It has been suggested that individual glomeruli are multifunctional coding modules (Christensen *et al.*, 1998) in which spatiotemporal properties, as well as chemical characteristics, of odors are presented. Here, we investigate the contribution of glomeruli with input from ORNs that express benzaldehyde receptors in an escaping behavior paradigm using a T-maze assay. We set up a system with the functionally characterized olfactory receptor Or43a using wild type flies and Or43a misexpressing flies. Or43a detects at least four volatile substances including benzaldehyde (Stortkuhl and Kettler, 2001; Hallem *et al.*, 2004). A response to benzaldehyde has also been shown for Or7a, Or10a and weakly for Or85f (Hallem *et al.*, 2004). As known from a variety of behavior investigations, an escape reaction is observed in flies upon application of benzaldehyde in a T-maze behavior test (Devaud, 2003). We wanted to know if misexpression of Or43a in additional ORNs changes this repellent behavior. Our results show that a broadening of the receptive field by misexpression of Or43a changes behavior. It provides the information that benzaldehyde does not induce a dominant behavioral output.

Materials and methods

Fly stocks and construction of transformants

Flies were reared on a standard cornmeal-molasses-agar medium and kept at 24°C in an incubator with a 12 h/12 h day/night cycle. Wild type *Oregon R* and transformed flies (see below) with *yellow* (*y*) and *white* (w^{1118}) mutant backgrounds were used for *in situ* hybridization experiments and immunocytochemistry, as well as for behavior tests.

For misexpression experiments the Gal4 driver line *GH320* (G. Heimbeck University of Fribourg, Switzerland) and line *UAS-or43a* (Stortkuhl and Kettler, 2001) were crossed and the progeny was used in the behavior tests. We refer to it as *or43a-3c*. In order to identify the target glomerulus of Or43a expressing ORNs, line *or43a-gal4* containing a 3 kb *or43a*-promotor *gal4* fusion (S. Fischer, diploma thesis, Ruhr-University Bochum) was crossed to an *UAS-n-synaptobrevin-GFP* (*UAS-n-syb-GFP*) line (K. Ito, University of Tokyo, Japan).

In situ hybridization and immunocytochemistry

Expression of *or43a* in the third antennal segment was shown by *in situ* hybridization with a digoxigenin labeled RNA-probe as described by Vosshall *et al.* (1999) with modifications. Flies were mounted in OCT (Microm, Germany) and shock-frozen in liquid nitrogen. Next, 10 μm cryosections were fixed with 4% paraformaldehyde. Prehybridization and hybridization of sections was carried out at 58°C in a humid chamber. After blocking with 10% heat inactivated normal goat serum in Tris/Cl buffer, pH 7.5, immunocytochemical labeling was accomplished with an alkaline phosphatase coupled anti-digoxigenin antibody [1:1000 in Tris/Cl buffer, pH 7.5, 0.1% Triton X-100 (TBST); Roche, Mannheim, Germany]. Color development was allowed to proceed for 20–60 min.

Identification of the target glomeruli, both of Or43a expressing ORNs and of Or43a misexpressing ORNs, was carried out on whole mount preparations of dissected brains with minor modifications of published procedures (Laissue *et al.*, 1999). First, the head capsule was opened and heads were fixed in 4% paraformaldehyde. Then, isolated brains were incubated in blocking solution [3% donkey- and 3% goat serum in phosphate buffer saline, 0.1% Triton-X100 (PBST)]. In order to visualize the antennal lobe neuropile the monoclonal antibody (mab) nc82 (A. Hofbauer, University of Regensburg, Germany) was used in a 1:10 dilution in PBST. The secondary CY3 coupled goat-anti-mouse antibody (Jackson Immunoresearch, USA) was applied as 1:500 dilution in PBST. In order to visualize GFP expression, whole mounts were incubated with a primary monoclonal antibody against GFP (Molecular Probes, Eugene, OR) diluted 1:100 in PBST, followed by a CY2 coupled secondary donkey-anti-mouse antibody (Jackson Immunoresearch, USA), diluted 1:500 in PBST. Stained whole mounts were embedded in 75% glycerol and inspected under a confocal microscope (Leica, Bensheim, Germany).

For determination of the onset of *GH320* enhancer activity within pupal development *GH320* was crossed with a *UAS-lacZ* line. In the progeny β-Galactosidase expression was visualized on 10 μm cryosections with an anti β-Galactosidase antiserum (Rockland Immunochemicals, USA) diluted 1:1000 in TBST. For enhancement of sensitivity the ABC-technology (Vector Labs, USA) was used.

T-Maze assay

To test olfactory behavior a modified olfactory T-maze was used (Helfand and Carlson, 1989) in which flies can choose between two airstreams, one containing an odor and the other with air as control. At the control side, air was drawn through paraffin oil and at the test side air was drawn through a solution of odorant in paraffin oil at a rate of 1 l/min. Flies were separated by sex under anesthetization 24 h before the behavior tests. A population of ~35 either female or male flies was exposed to the odorant for only 30 s in order to avoid adaptation (Stortkuhl *et al.*, 1999). At the end of the test period flies were collected from each test tube of the T-maze and counted. The response index (RI)

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was calculated by subtracting the number of flies which had moved to the odor side from the number of flies at the control air side and dividing by the total number of flies. A response index above zero indicates a repellent reaction to the applied odorant. All behavior tests were performed in the dark. Odorants used were of the highest purity available. Response index values were calculated using the JUMP-program. Student's *t*-tests were calculated and means of RI-values were compared at a significance value of $P = 0.05$.

Results

Recent studies have shown that benzaldehyde is one of at least four ligands of the Or43a receptor (Stortkuhl and Kettler, 2001; Hallem *et al*., 2004). In electroantennogram (EAG) recordings, *or43a-3c* flies that misexpress the Or43a receptor in additional ORNs exhibited elevated electrophysiological responses on the third antennal segment upon application of benzaldehyde (Stortkuhl and Kettler, 2001). In behavior tests, wild type flies perform a robust avoidance reaction to this volatile substance (Siddiqi, 1983; Acebes and Ferrus, 2001; Anholt *et al*., 2001). We wanted to know if stimulation of *or43a-3c* flies with benzaldehyde leads to a change in behavior resulting in either an augmented repulsive behavior or in reduction of the avoidance reaction that is known for wild type flies.

Or43a expression does not interfere with ORN axon outgrowth

Studies on a variety of *Drosophila* Ors already provided evidence that they are neither expressed in the afferent portion of ORNs nor involved in development of the antennal system (Vosshall *et al*., 1999; Elmore and Smith, 2001; Dobritsa *et al*., 2003). It is therefore unlikely that Or43a expression could have an impact on development of the antennal system. However, to exclude that Or43a precedes or coincides with ORN outgrowth, we examined the onset of Or43a expression in pupal development of wild type flies by *in situ* hybridization. Cryosections of the antennal system at different stages of pupal development were probed with digoxigenin labeled *or43a* mRNA. A first labeling of cells was only visible 75 h after puparium formation (APF; Figure 1a). This result confirmed that wild type expression of Or43a mRNA starts late in development when connections of ORNs with the antennal lobe have already been established.

Before performing a behavior test on the *or43a-3c* line, we had to ascertain that *GH320* driven Or43a misexpression is also not activated before ORN outgrowth has started. Then any interference with axonal pathfinding can be excluded. According to recent findings of Jhaveri *et al*. (2000), the first axons arrive in the antennal lobe at 20 h APF and others follow subsequently. Since an antiserum, that recognizes Or43a in cryosections directly is not available, the onset of Gal4 activity in *GH320* was determined as β-galactosidase activity in a *GH320*/*UAS-lacZ* genotype. X-gal staining was

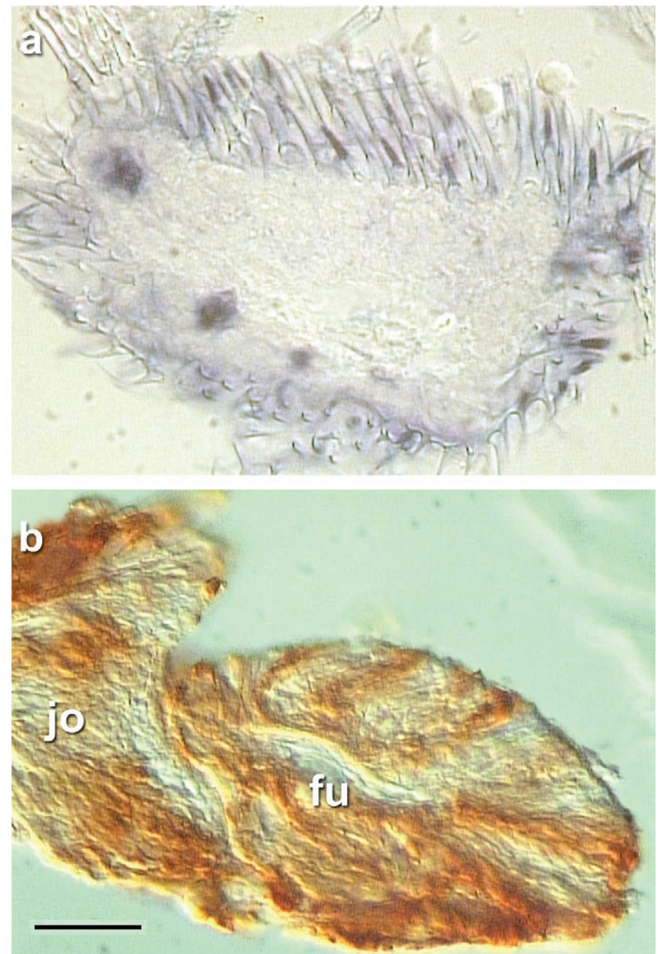

Figure 1 **(a)** *In situ* hybridization of a Dig-labeled *or43a* mRNA probe to a 10 μm pupal cryosection of wild type flies at 75 h APF. **(b)** β-Galactosidase immunodetection on a pupal cryosection of the *GH320/UAS-lacZ* line at 24 h APF. Fu, funiculus; jo, Johnston's organ. bar = 5 μm.

first detected in cryosections at 24 h APF when axonal connections with the antennal lobe are just established (Figure 1b and Tissot *et al*., 1997). We therefore conclude that *GH320* driven misexpression also starts late enough in olfactory organ development to exclude interference with ORN outgrowth (Tissot *et al*., 1998).

or43a-3c flies have an increased receptive field in the antennal lobe

After application of benzaldehyde, line *or43a-3c* exhibits an increased electrophysiological response in electroantennogram (EAG) recordings when compared with wild type (Stortkuhl and Kettler, 2001). According to the specific olfactory connectivity of ORNs with the antennal lobe, the application of benzaldehyde in *or34a-3c* flies should activate additional glomeruli (Gao *et al*., 2000; Vosshall *et al*., 2000). Subsequently, the receptive field in the antennal lobe should increase. To prove this hypothesis, the connectivity of ORNs with the antennal lobe was analyzed using the marker

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protein N-synaptobrevin-coupled GFP (n-syb-GFP) which labels synaptic terminals. n-syb-GFP expression was driven by a 3.0 kb promoter fragment containing *or43a-gal4* line (S. Fischer and B. Hovemann, unpublished results). Whole mounts of dissected brains of *or43a-gal4/UAS-n-syb-GFP* flies were examined under a confocal microscope. Fluorescence was detected only in glomerulus DA4 as previously also shown by Wang *et al*. (2003). When n-syb-GFP was driven by *GH320*, fluorescence was present in a large number of glomeruli, including DA4 (Figure 2), indicating that these glomeruli are targets of ORNs with Or43a misexpression.

or43a-3c flies exhibit a reduced avoidance behavior to benzaldehyde that can be mimicked in wild type flies

In T-maze assays, wild type flies showed a robust concentration-dependent avoidance reaction to the application of benzaldehyde, as shown in Figure 3. The maximum response index of 0.8 was reached by applying benzaldehyde saturated in paraffin oil. In contrast, *or43a-3c* flies showed strongly reduced response indices to benzaldehyde when compared with wild type and parental flies (Figure 3). However, as expected, the response of *or43a-3c* flies to ethyl acetate, which is not a ligand of Or43a, remained comparable to wild type (Figure 4). Most olfactory behavioral studies have been performed by using single components, which stimulate a subset of ORNs on the third antennal segment. Thus, when we increase the subset of stimulated ORNs in wild type flies by applying a mixture of two odorants, the input into the antennal lobe that takes place by stimulation with benzaldehyde in *or43a-3c* should be mimicked. A mixture of an attractive odorant and benzaldehyde should reduce the avoidance reaction in wild type flies. First we tested ethyl acetate application to wild type flies at various concentrations and obtained values that complied with published results (Siddiqi, 1983). At high dilutions, ethyl acetate functioned as an attractant while at higher concentrations it

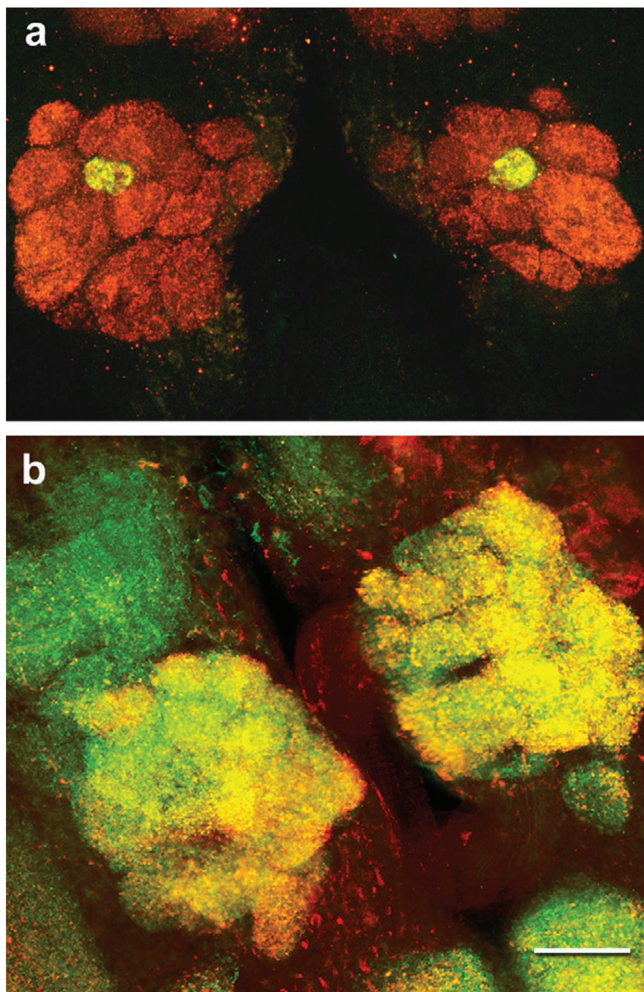

Figure 2 Double staining of a whole mount preparation of the antennal lobe with a monoclonal anti-GFP antibody (green) and the monoclonal antibody nc82 (red). **(a)** The control, n-syb-GFP expression of *or43a-gal4/UAS-n-syb-GFP,* reveals double staining (yellow) only in glomerulus DA4. **(b)** n-syb-GFP detection with anti-GFP antibody in line *or43a-3c* reveals double staining in most glomeruli (yellow). Bar = 10 μm.

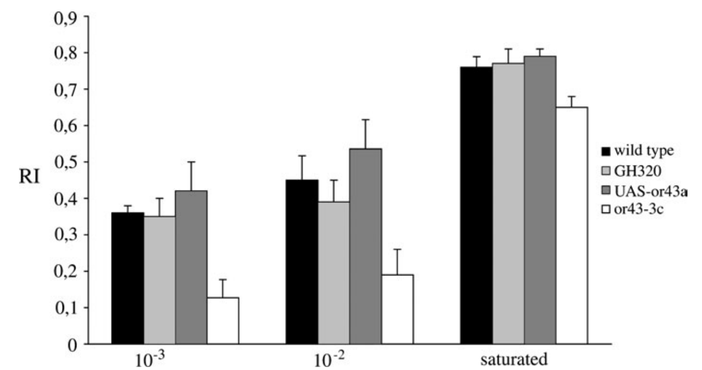

Figure 3 T-maze assay of wild type, parental lines *GH320* and *UAS-or43a* and misexpressing line *or43a-3c* with different concentrations of benzaldehyde. RI = response index (*n* = 30 for each measured value). Positive RI-values denote an avoidance response. Significance value *P* = 0.05.

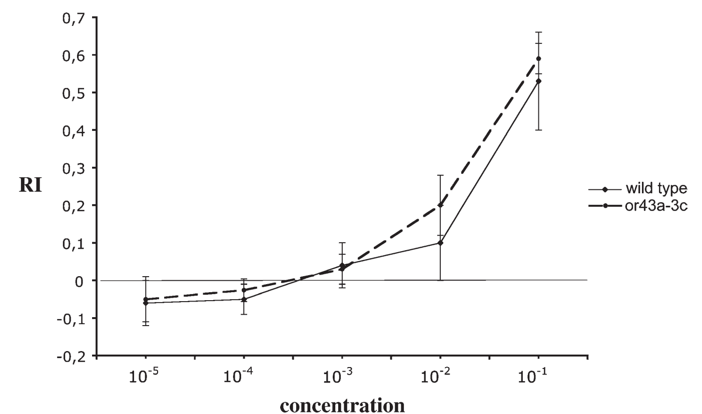

Figure 4 T-Maze assay of wild type and Or43a misexpressing line *or43a-3c* with different concentrations of ethyl acetate (n=35 for each measured value). Significance value *P* = 0.05.

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triggered a repellent reaction (Figure 4). Benzaldehyde was used in combination with a dilution of either 10^{-4} or 10^{-2} ethyl acetate. Both mixtures of benzaldehyde with ethyl acetate gave rise to a reduction of the avoidance reaction as shown by a reduced response index of 0.20 ± 0.06 (mean ± SEM) and 0.19 ± 0.05, respectively (Figure 5).

Discussion

Or43a misexpression does not precede ORNs outgrowth to the antennal lobe

We could not exclude changes of the synaptic connectivity of individual ORNs with single glomeruli a priori if Or43a expression started early enough to precede outgrowth of axonal connections of ORNs to the antennal lobe. Synaptic contact formation in the antennal lobe is reported to occur as early as 20 h APF (Tissot *et al*., 1998; Jhaveri *et al*., 2000). At this developmental stage *or43a* mRNA in its cognate ORNs has never been detected by *in situ* hybridization. Instead, this mRNA was first detected in olfactory neurons at 75 h APF. Here, we show that *GH320* driven expression begins at 24 h APF. Although there is an overlap of the beginning of antennal development and the onset of Or43a misexpression, we can exclude a detrimental effect of *GH320* driven Or43a expression on antennal lobe connectivity. Ors most likely are not required for axonal path finding. In a recent investigation, substitution of Or22a expression by Or67 did not alter synaptic connectivity in the antennal lobe (Dobritsa *et al*., 2003). In contrast to the vertebrate olfactory system, neither olfactory gene transcripts nor olfactory receptors have been detected in axon projections of ORNs in *Drosophila* (Vosshall *et al*., 1999; Elmore and Smith, 2001). Finally, the intimate interaction of ORNs and glia, as well as cell surface molecules, i.e. N-cadherin (N-cad) and the Down syndrome cell adhesion molecule (Dscam), and the signaling pathway molecules Dock and Pak, have to date been shown to be involved in ORN targeting in the antennal lobe

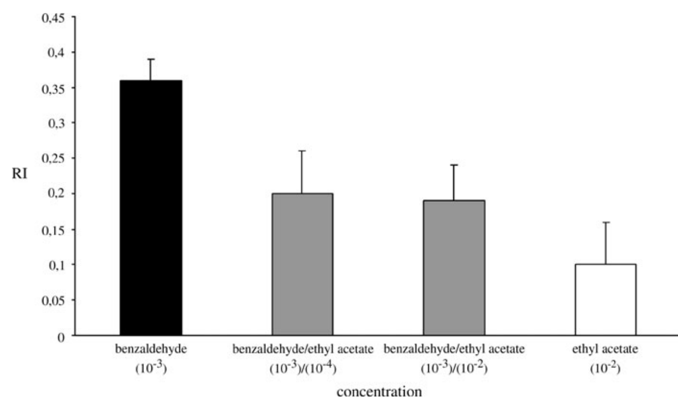

Figure 5 T-maze of wild type flies to mixtures of benzaldehyde and ethyl acetate at the indicated concentrations. (n=18 for each measured value). Significance value *P*= 0.05.

(Hummel *et al*., 2003; Hummel and Zipursky, 2004; Jhaveri and Rodrigues, 2002; Ang *et al*., 2003).

Misexpression of Or43a leads to changed olfactory behavior

Recent studies, including our own, have demonstrated that olfactory receptor proteins are receptive to a heterogeneous but overlapping spectrum of ligands. This functional redundancy might allow the animal to compensate for the loss of a single olfactory receptor function. Consequently, in a recent study the inactivation of a single olfactory receptor function failed to result in a defect in olfactory driven behavior (Elmore *et al*., 2003).

Overexpression of an Or in additional ORNs, however, will not only allow ligand determination by EAG measurements but might also modify olfactory-driven behavior. In this study, we observed that misexpression of Or43a, one of the benzaldehyde receptors, reduces the behavioral escape reaction in a non-sexually dimorphic way. This alteration in behavior was dependent on the application of benzaldehyde, one of the genuine Or43a olfactory receptor ligands. Application of ethyl acetate, which is not a ligand of Or43a, gave rise to a behavior reaction that was indistinguishable from the wild type. It indicated to us that Or-function in *or43a-c3* flies in general was not disturbed by misexpresssion of Or43a. Instead, the change in behavior must have been due to Or43a function in those neurons where it had been ectopically expressed. Troemel *et al*. (1997) demonstrated quite clearly in *Caenorhabditis elegans* that the olfactory behavior is dependent on activation of a specific neuronal circuit and not on activation of a particular receptor. By misexpressing Or43a in additional ORNs, we were able to stimulate simultaneously different neuronal circuits of the antennal system. Thus, the application of benzaldehyde could have activated different circuits that mediate either an escape reaction or attraction, leadign to a reduced escape reaction. This idea was corroborated by the experiment using wild type flies where simultaneous application of ethyl acetate and benzaldehyde also reduced the escape reaction.

The glomerulus DA4-dependent escape reaction is not a dominant behavior

There is a number of benzaldehyde binding olfactory receptors in *Drosophila* (Hallem *et al*., 2004). Here, we investigated whether benzaldehyde activated neuronal circuits are dominating olfactory processing by misexpressing Or43a in additional ORNs and subsequently increasing the receptive field in the antennal lobe. Surprisingly, flies exhibited a decreased avoidance response to benzaldehyde. How can an escape reaction become reduced? Lateral inhibition has been shown in calcium imaging studies of the antennal lobe of bees to occur in a dose-dependent manner (Sachse and Galizia, 2002, 2003), in parallel with recruitment of activated

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glomeruli in the antennal lobe (Sachse and Galizia, 2003). By increasing the receptive field, our experiments could have induced an inhibition of glomeruli, including DA4, that are connected with benzaldehyde receptor-expressing ORNs. As a result of this inhibition, the specific escape reaction might have been suppressed. Support for this hypothesis comes from patch-clamp recordings using single identified glomeruli, which revealed lateral interaction of glomeruli in the antennal lobe (Sachse and Galizia, 2003; Wilson *et al*., 2004). Taken together, our data demonstrate that a broadening of the receptive field in the antennal lobe by either overexpression of the Or43a receptor or application of a mixture of attractive and repellent components reduces the avoidance reaction. Simultaneous activation of different neuronal circuits at the level of the antennal lobe can thus modulate olfactory behavior.

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