

Blocking Sensory Inputs to Identified Antennal Glomeruli Selectively Modifies Odorant Perception in *Drosophila*

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ABSTRACT: Neural coding of sensory input is a major unsolved issue in neuroscience. Current experimental methods rely on neural activity recording or visualization following sensory stimulation. Most of them, however, do not include behavioral correlates on the actual perception by the animal. We present a novel approach to address olfaction and coding in adult *Drosophila*. Sensory input was selectively blocked in two subsets of sensory neurons that project to different, albeit overlapping, groups of central targets, by means of tetanus toxin expressed under the control of the yeast transcription factor Gal4. Glomeruli DL1, DL2, VM1, and VM4 were tested following stimulation with benzaldehyde, ethyl acetate, propionic acid, butanol, or acetone at various concentrations. The behavioral response

was found to be modified in an odorant-specific and a concentration-dependent manner. Sensory input to DL2 and, to a minor extent, VM1 and/or VM4, appear to be required for benzaldehyde perception, while acetone is processed through DL1. None of these glomeruli, however, seem necessary for butanol perception. In addition, sexual differences were observed for some stimuli. These results demonstrate the behavioral relevance of odor representation as maps of glomerular activity generated in the antennal lobes following specific sensory input. The strategy used here should be useful to characterize olfactory coding, as new and selective Gal4 lines become available. © 2003 Wiley Periodicals, Inc. *J Neurobiol* 56: 1–12, 2003
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INTRODUCTION

Olfactory perception is mediated by strongly conserved neural mechanisms (Hildebrand and Shepherd, 1997). In mammalian olfactory bulbs as well as in insect antennal lobes (AL), glomeruli play key roles

in this process (Mori et al., 1999; Galizia and Menzel, 2000). These neuropilar subunits are targets for sensory axons whose response specificity is determined by their odorant receptor properties (Buck, 1996; Clyne et al., 1999). As a rule, sensory neurons sharing the same receptor project to only one or two glomeruli (Mombaerts et al., 1996; Gao et al., 2000; Vosshall et al., 2000; Scott et al., 2001). Thus, glomerular activity displays some degree of odor specificity that is further refined by local synaptic interactions (Linster et al., 1994; Yokoi et al., 1995). As a consequence, each odorant stimulation generates a map of glomerular activity that serves as an internal representation of the stimulus (Joerges et al., 1997; Rubin and Katz, 1999; Kauer and White, 2001; Meister and Bonhoffer, 2001; Sachse and Galizia, 2002). The quantitative compo-

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ment of odorant perception is thought to be encoded by the spiking frequency of sensory neurons (Rospars et al., 2000) as a result of interactions that are still not well understood between odorant and receptor molecules (Malnic et al., 1999; Lansky et al., 2001). In addition to spatial maps, a temporal representation is provided by synchronous firing of output neurons that link glomeruli to higher brain centers (Laurent, 1996a,b; Stopfer et al., 1997; Friedrich and Stopfer, 2001). It is widely accepted that spatial maps establish a code used by the animal for odor perception, although information about its relevance to behavior is still scarce (Laurent, 1997; Faber et al., 1999).

To better understand the implications of such neural processes, the behavioral output must be taken into account. In this regard, genetic approaches allow the manipulation of the olfactory pathway of intact animals, which are still suitable for behavioral studies. *Drosophila* offers a wide repertoire of methods to that purpose (Carlson, 1996) while its brain anatomy has been studied to the point of a reliable identification of glomeruli (Laissue et al., 1999). The Gal4/UAS system (Brand and Perrimon, 1993) allows one to intervene on identified cells by targeting the expression of desired genes, such as the one encoding tetanus toxin light chain (TNT) (Sweeney et al., 1995). By cleaving synaptobrevin, a key element of the presynaptic machinery, TNT impairs fusion of neurotransmitter vesicles, thus, synaptic transmission (Humeau et al., 2000). This approach has proved useful to unravel important aspects of several behaviors in *Drosophila* (Keller et al., 2002; Martin et al., 2002). Here, we address the consequences on olfactory behavior of suppressing selective sensory inputs to identified glomeruli of adult antennal lobes. We have chosen Gal4 drivers whose domains of expression overlap, as a strategy towards deciphering the functional code used by the animal during olfactory perception. As odorants, we have chosen those representing several chemical groups and for which previous behavioral data in *Drosophila* are available including synaptic changes following adaptation (Devaud et al., 2001).

METHODS

Fly Stocks and Crosses

The UAS-tetanus toxin lines (*UAS-TNTE* and *UAS-IMPTNT-VB*, insertions on the second and third chromosomes, respectively), and the *UAS-LacZ* strain were generously provided by Dr. Cahir O’Kane (Cambridge University, UK). The *IMPTNT-VB* construct encodes an inactive form of the toxin. The P[Gal4] lines 85 and 72 were generated by the groups of Drs. A. Brand (Wellcome Trust, Cambridge,

UK) and C. O’Kane, respectively, and here are referred to as *85B* and *72OK*. Line *85B* bears an inserted P element on the first chromosome, while line *72OK* (referred to as line C in Ferveur et al., 1997) bears the insertion on the second chromosome. The *ENG3* strain was obtained from Dr. M. Ramaswami (University of Arizona, Tucson, AZ). This transgenic line expresses the green fluorescent protein (GFP) associated with the presynaptic protein n-synaptobrevin, under the control of the panneural promoter *elav* (Estes et al., 2000). Crosses were set introducing the UAS–tetanus toxin constructs via males always. Experimental and control genotypes were obtained from crosses run in parallel. Also, males and females in each test were of the same age (1–4 days) and reared in the same culture vial. All flies were maintained at constant temperature (18°C) and humidity (50%). Culture density was kept similar for all crosses by allowing 30 females to lay eggs in each vial for 48 h. Offspring from at least five different and independent cultures were assayed several times along the year.

Glomerular Analysis

First, the glomerular patterns of *85B* and *72OK* were observed with three different reporters (*LacZ*, τ -GFP, and n-synaptobrevin-GFP) to determine the approximate positions of the stained glomeruli, in optical (*LacZ*) and confocal (τ -GFP, n-syb-GFP) microscopy. Second, their positions were established precisely through a comparison between Gal4-nsybGFP and *ENG3* brains, in ALs reconstructed from 1- μ m optical sections taken at the confocal microscope and keeping track of the relative confocal plane along the z axis. *ENG3* brains were stained with propidium iodide to visualize the glial processes that delimit the glomeruli, following a protocol adapted from Baumann et al. (1996). Briefly, the dissected brains were incubated in propidium iodide (PI, Sigma; 20 μ g/mL in PBS) for 30 min without previous RNase treatment to stain cytoplasmic processes. After a brief 5-min washing in PBS, they were mounted for observation under confocal microscopy. The identity of the glomeruli was determined from their position, shape, and size, by reference to the three-dimensional atlas of the antennal lobe commonly used as standard (Laissue et al., 1999). The relative depth of each confocal plane was monitored to assist in the glomerular positioning as described in the figure legends. Observations were done on whole-mount brains laying flat on their posterior side over the microscope slide to ensure consistent orientation in all cases.

Behavioral Assay

The olfactory behavior test used is adapted from Tully and Quinn (1985), and has been described in detail elsewhere (Devaud et al., 2001). Briefly, groups of ~25 flies (either males or females) were placed in an olfactory T-maze, where they could choose during 30 s between two compartments, one containing the test odor and the other containing the solvent, paraffin oil. All tests were performed in the dark, at room temperature. The response index RI (Ayyub et

al., 1990) was calculated as the number of flies trapped in the odor compartment minus that of flies trapped in the oil compartment, and divided by the total. Flies trapped in the central compartment were scored, but not included in the calculation. RI values range between -1 (maximal repulsion) and 1 (maximal attraction). For statistical comparisons, RI values were averaged over 10 tests for each stimulus, and performed with flies from different culture vials. Each group of flies was tested only once. Tests in which more than one-third of the flies remained in the middle compartment were discarded. For each experimental condition (genotype, sex, odor, concentration), the values were averaged over several days at different times of the year. Possible circadian variations were avoided by performing all tests between 9 a.m. and 2 p.m. Each day of test included flies from different strains and sexes, tested for several odors and concentrations. Results are given as mean RI \pm S.E.M.

Odors

All chemicals were of the highest purity available from Fluka Chemicals (Steinheim, Germany), and were diluted in paraffin oil. They were chosen among those commonly used in previous works and representing different chemical groups. Two concentrations, low and high, were used for each odorant: 5×10^{-5} and 10^{-3} (v/v) (benzaldehyde and butanol), 5×10^{-5} and 10^{-2} (propionic acid), or 10^{-4} and 10^{-2} (ethyl acetate and acetone). These concentrations were chosen on the basis of their similar RI values in wild-type animals in previous tests, so that all low doses were moderately attractant, and high ones mildly repellent.

Histology

Brains, antennae, and maxillary palps of the *Gal4/UAS-LacZ* trans-heterozygotes were dissected in phosphate buffer and fixed in 1% glutaraldehyde (Sigma, St. Louis, MO) on ice, for 45 min. A 2.5% X-Gal solution (Sigma) was used for staining β -galactosidase activity, overnight at room temperature. After staining, the preparations were mounted and observed under transmission microscopy.

Statistical Analyses

Olfactory response index values (RI) were obtained from 10 replicates of 25 flies each, and are shown as histograms. The significance of RI differences was assessed with the non-parametric Mann-Whitney *U*-test. The effect of tetanus toxin expression was considered to be biologically relevant only when the responses of TNT-expressing flies were significantly different from those of three different control genotypes: *Gal4/UAS-IMPTNT*, *Gal4*, and *UAS-TNT-E*. If one of the pair wise comparisons yielded a nonsignificant difference, the behavior of toxin-expressing flies was not considered reliable. This conservative criterion was chosen to discard experimental values in the behavioral tests that

could result from different genetic backgrounds. For the sake of clarity, the figures display only the significance levels corresponding to the difference between flies expressing the active versus inactive forms of the toxin, driven by the corresponding *Gal4* (*Gal4/UAS-TNT* vs. *Gal4/UAS-IMPTNT*). The chi-square test was used to estimate the significance of olfactory responses with respect to the indifference index value (0.0). Sexual differences in the behavioral response were tested under the nonparametric Kruskal-Wallis test. All statistical tests were performed using the SPSS 8.0 software.

RESULTS

85B and 72OK Lines Show Specific Expression in the Olfactory Pathway

From a screening of more than 100 *Gal4* lines, two were chosen for detailed study and use for disturbing odorant perception. The selection criteria were the specificity and overlap of their expression patterns in the olfactory pathway. We characterized, to a first approximation, their expression patterns in whole-mount heads and brains of adults using the reporter construct *UAS-LacZ* (Fig. 1).

In the first line, *85B*, reporter expression in the brain is consistently restricted to the AL, where three glomeruli could be observed [Figs. 1(A) and 2(E)]. Two are located in a ventromedian position, one next to the other, and appear to receive afferents from a median bundle. The third one, in a dorsolateral position, is innervated by dorsal fibers. Intensity of expression, most likely reflecting the number of incoming sensory afferents that express the driver, is lower in the latter. Outside the antennal lobe, a single bilateral neuron is stained [Fig. 1(A), arrowheads], whose projections do not seem to reach the antennal lobe. The cell bodies from which the stained processes originate are located in the olfactory appendages, the antenna, and maxillary palps. Although only 10–15 sensory cells are detected in the third antennal segment, funiculus, the second segment (nonolfactory) and maxillary palps show a more general expression [Fig. 1(B) and (C)]. No obvious sexual dimorphism in the distribution, or in the glomerular staining was observed.

The second line used here, *72OK* (Acebes and Ferrús, 2001), also displays specific expression in the AL, following a pattern similar to that of *85B*: two ventromedian glomeruli and one dorsolateral [Figs. 1(D) and 2(F)]. The former appear to share the same position as those in *85B*, although their shapes are slightly different, as outlined by the *LacZ*-stained fibers. Their innervation pattern also

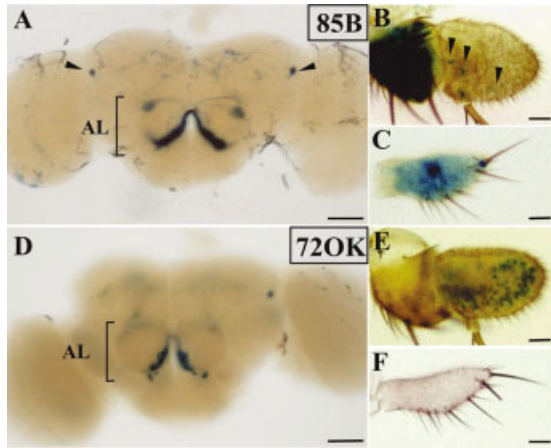


Figure 1 Expression patterns driven by Gal4-85B and Gal4-72OK inserts. Adult female brains from genotypes *85B/+; UAS-LacZ/+* (A–C) and *72OK/+; UAS-LacZ/+* (D–F) stained for β -galactosidase. (A) In *85B*, expression is mainly restricted to antennal lobes (AL) where three glomeruli are visible, two in a ventromedian position and one dorsolateral. A single cell body is also detected in each brain side (arrowheads), with protocerebral branches. (B) In the antenna, a small group of cells is shown (arrowheads) in the funiculus, while a larger group is detected in the second segment. (C) An additional group of sensory neurons is found in the palp. (D) In *72OK*, most of the staining is also restricted to the antennal lobes (AL), although some faint signal appears in the mushroom bodies. Three glomeruli are stained, two ventromedian and one, weakly visible, in a dorsolateral position. (E) The antennal funiculus shows a group of 50–70 cells expressing *72OK*. (F) The maxillary palp, by contrast, is devoid of expression. Bars in (A–D) = 20 μ m, (B–E) = 50 μ m, and (C–F) = 25 μ m.

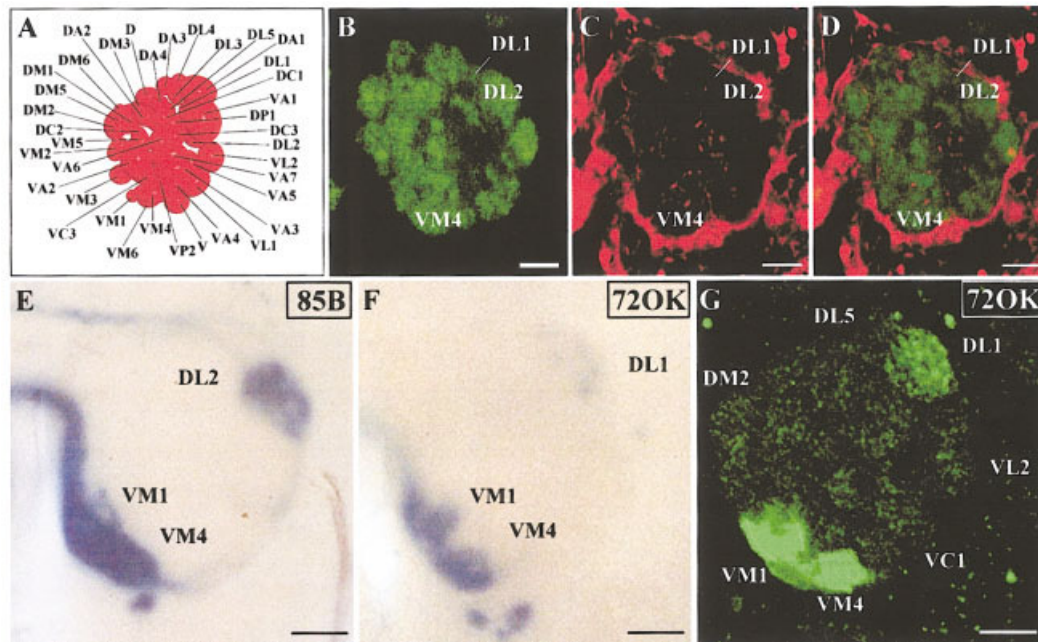


Figure 2 Glomerular identification in Gal4-85B and Gal4-72OK expression patterns. (A) Three-dimensional schematic representation of the glomerular organization of the *Drosophila* antennal lobe (anterior view), according to the atlas of Laissue et al. (1999). (B–D) Confocal section of an adult antennal lobe expressing the *ENG3* construct, a synaptobrevin-GFP reporter under the control of the panneural *elav* enhancer. (B) Visualization of individual glomeruli by n-syb-GFP. (C) Propidium iodide-stained glial processes, showing the outer limits of most glomeruli. (D) Merging of both images. (E–F) LacZ reporter expression in the AL of *85B* and *72OK* lines. (G) Confocal section showing glomerular expression in a *72OK/+; UAS-n-syb-GFP/+* antennal lobe. Setting the laser at high power allows to visualize very faintly stained glomeruli that help identify the three glomeruli stained in each Gal4 line. Some of them are outlined and labeled. Confocal images in (B) and (G) are taken between 75% and 100% of total depth of focus in the anterior-posterior axis of the antennal lobe. Glomeruli: DL1 = dorsolateral 1; DL2 = dorsolateral 2; DL5 = dorsolateral 5; DM2 = dorsomedian 2; VC1 = ventrocentral 1; VL2 = ventrolateral 2; VM1 = ventromedian 1; VM4 = ventromedian 4. In all images, dorsal is up and lateral is to the right. Bars = 20 μ m.

looks similar to that observed in *85B*. The third, dorsolateral, glomerulus appears very faintly stained, in a position roughly similar to the one observed in the line *85B*. Outside the AL, some weak staining was found in the mushroom bodies and in a few scattered cells, including a small group of two to three cell bodies visible at the base of the AL, without any detectable process. In olfactory appendages, line *72OK* expresses in a population of 50–70 cells in the funiculus [Fig. 1(E)], while no expression was evident in the maxillary palps [Fig. 1(F)]. As in the previous case, no obvious difference of expression between males and females was detected. Thus, the two populations of sensory neurons identified in both Gal4 lines are distinct in their location on the olfactory appendages. It is worth pointing out that stained cells in the funiculus could potentially be either neurons (which project to the antennal lobes) or support cells (which do not).

85B and 72OK Lines Are Expressed in Partially Overlapping Sets of Olfactory Glomeruli

The glomerular patterns of both Gal4 lines were investigated in more detail, to precisely identify the glomeruli innervated by the specific groups of sensory fibers, using the atlas of Laissue et al. (1999) as a reference [Fig. 2(A)]. Combining whole-mount optical images visualized by the reporter LacZ and confocal reconstructions from GFP fluorescence is a powerful tool to identify individual glomeruli (Devaud et al., 2001). Given the importance of glomeruli identification in this study, however, we made use of additional procedures to validate the expression patterns of these two lines. On the one hand, we compared each Gal-4 expression pattern against the whole glomerular array as revealed by the *ENG3* strain, which constitutively expresses a neural-synaptobrevin-GFP (n-syb-GFP) construct in neurons (Estes et al., 2000) [Fig. 2(B)]. On the other hand, we included the additional staining of glial cells by propidium iodide (IP), which helps to delimit the borders of many glomeruli, and thus further increases resolution [Figs. 2(C)–(D)].

Glomerular identification was especially critical for the dorsolateral glomerulus in *72OK*, for which the LacZ staining provided insufficient information. The analysis of adult brains from both Gal-4 lines expressing either LacZ, -GFP, or n-syb-GFP confirmed that the ventromedial glomeruli were identical in both lines. Following the current standard nomenclature, they were recognized as ventrome-

dian 1 (VM1) and 4 (VM4) glomeruli [Figs. 2(E)–(G)], two of the so-called “landmark” glomeruli because of their easy localization (Laissue et al., 1999). The dorsolateral glomerulus, however, was found to be different in each line. After close inspection of AL reconstructions, its position appears more ventral and slightly more posterior in *85B* than in *72OK*. This conclusion could be reached by making use of the n-syb-GFP signal driven by the *72OK* driver, which greatly improved the resolution of neuronal branching in that glomerulus, compared with the faint staining obtained with LacZ [compare Figs. 2(F) and (G)]. The same procedures allowed recognizing the dorsolateral 2 (DL2) glomerulus in *85B*, and the dorsolateral 1 (DL1) glomerulus in *72OK* [Figs. 2(E)–(G)]. This identification for *72OK* is consistent with a previous study (Acebes and Ferrús, 2001).

Even though some glomeruli are reported to exhibit positional variability, it must be pointed out that the glomeruli expressing these Gal-4 lines are among those of invariant position (Laissue et al., 1999). In our glomerular identification, the relative confocal plane was monitored in all cases along the anterior/posterior axis (see legend of Fig. 2), which confirmed the rather invariant localization of these glomeruli. Thus, in summary, although the antennal and maxillary cells expressing each line belong to distinct populations of sensory neurons, some of them share glomeruli VM1 and VM4 as projection targets while others are specific for DL1 or DL2. As in the antennae, these observations in the glomeruli yielded no sex differences.

Targeted Expression of Tetanus Toxin in Line 85B Selectively Impairs Olfactory Behavior

We made use of the two Gal4 lines to target TNT expression while monitoring the olfactory behavior response to specific odorants. Parallel crosses were set (see Material and Methods) to obtain experimental and control adults. The viability of each genotype class was measured and no deleterious effect of the toxin was detected. In addition to the parental lines (Gal4 drivers and UAS constructs separately), controls included animals with the same Gal4 lines driving the expression of an inactive form of the toxin (IMPTNT) (see genotypes in legends of Figs. 3–5). For each odorant tested (benzaldehyde, ethyl acetate, propionic acid, butanol, and acetone), two concentrations were tested: high and low (see Material and Methods). These concentrations were chosen because they were shown previously to induce repulsion and

attraction respectively, in wild-type (*Canton-S*) flies (Devaud, unpublished data).

Olfactory responses of control lines show the regular attraction or repulsion to each odorant concentration (Fig. 3). Low concentrations yield positive RI values in the range 0.2/0.4, while high concentrations yield negative RI values around $-0.4/-0.6$. These values are consistent with previous observations on wild-type flies (Acebes and Ferrús, 2001). Thus, neither the P-element insertion nor the expression of the inactive toxin had a noticeable effect on olfactory behavior, at least for the tested stimuli.

Animals expressing the active toxin, however, exhibit a significantly different behavior, in an odorant- and concentration-dependent manner, compared to controls. Throughout this report, statistical significance is always given between adults expressing the active and inactive forms of the toxin. Data, however, were considered as acceptable for further analysis only if the statistical significance was reproduced when comparing TNT-expressing animals with the two parental strains. Under these criteria, benzaldehyde evokes a markedly reduced response in females at both high and low concentrations (Fig. 3). These differences can be attributed to the activity of the toxin because they were maintained with respect to animals that expressed the inactive toxin (*85B/IMPTNT-VB*). In addition to benzaldehyde, TNT-expression in *85B* neurons impairs propionic acid perception at low concentration in males. Perception to all other odorants tested does not seem to be affected in this genotype.

Males and females from the experimental group show different tendencies in their respective responses to benzaldehyde and propionic acid. Although a Kruskal-Wallis statistical test does not reveal a significant effect for sex, inactivating inputs from *85B* neurons appears to cause a stronger effect in females than in males under benzaldehyde stimulation, while the opposite is observed for propionic acid perception. The reduction in the behavioral response to benzaldehyde and its apparent sexual dimorphism was further measured by analyzing a dose-response curve in sex-sorted adults (Fig. 4). The response profile to benzaldehyde in the control group is similar to that obtained for this and other odors using the same test with various genetic backgrounds (Charro and Alcorta, 1994; Acebes and Ferrús, 2001; Devaud et al., 2001). That is, indifference to very low concentrations, attraction to moderate ones, and repulsion to high doses. The general trend of the experimental genotype is a reduced behavioral response that could result from a general loss of sensitivity. Rather than a shift of the curve towards the high concentration

range, however, the data show significant differences at specific odorant concentrations only. Comparing the profiles of the experimental animals (continuous line) with that of controls (dotted line), it is noticeable that the drop between 10^{-4} and 10^{-3} v/v concentrations is steeper in controls. These observations reveal a concentration-dependent component of the olfactory code (see Discussion). In this experiment, the repellent reaction is clearly maintained at high concentrations, demonstrating that the odorant is perceived and that the functional inactivation of *85B* inputs does not cause anosmia to benzaldehyde. Sex differences, on the other hand, are observed at certain concentrations only—namely, 10^{-5} and 10^{-2} ($p < .05$ in both cases, Kruskal-Wallis test)—and can be attributed to the toxin, because no sex effect was observed in *85B/IMPTNT-VB* flies.

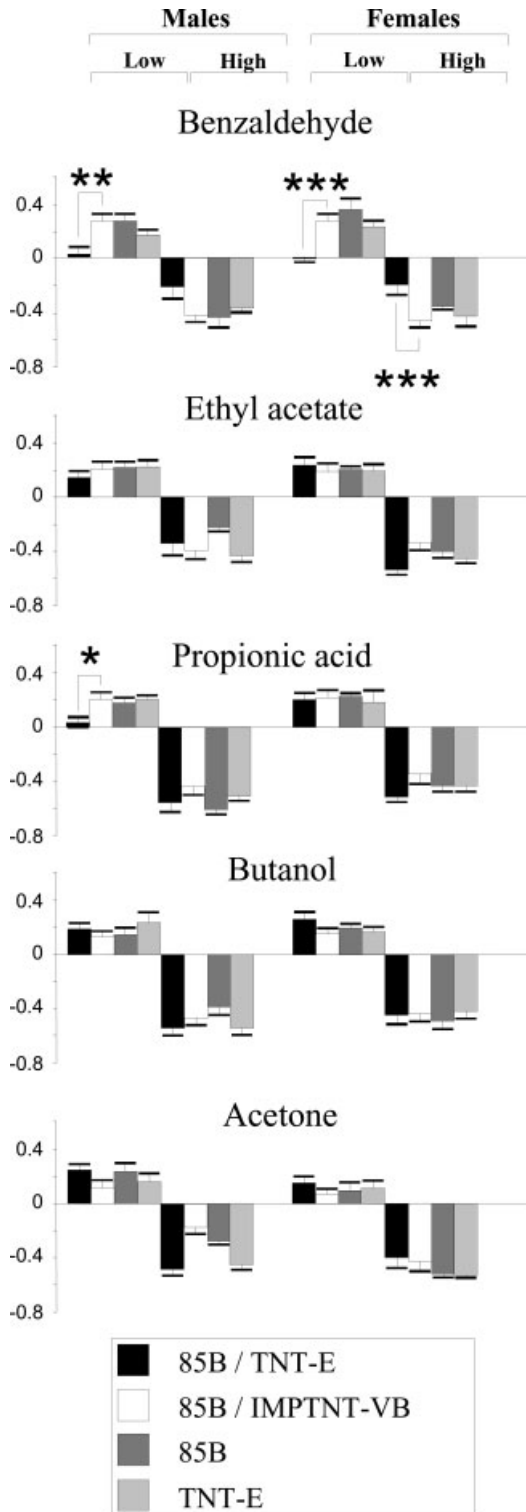
Targeted TNT Expression by *72OK* Reveals a Different Set of Olfactory Impairments

The same battery of olfactory tests was carried out in flies in which TNT was under the control of the *72OK* driver (Fig. 5). As observed in the case of *85B*, the general effect here is a reduction of the response, either attraction or repulsion, as indicated by the shift of RI values towards 0. Also, as in the previous case, differences in response index values are odorant- and concentration-specific, as well as sexually dimorphic. They are not, however, registered for the same repertoire of stimuli. *72OK/TNT-E* males respond abnormally to the low benzaldehyde and propionic acid concentrations, and to the high ethyl acetate concentration. Females of the same genotype show defective responses to concentrated propionic acid and acetone. Butanol perception, as with the previous driver, is not affected by silencing *72OK* synapses. A significant sex effect was found for the low concentrations of benzaldehyde, propionic acid, and acetone, as well as for concentrated acetone ($p < .05$ in all cases, Kruskal-Wallis test). Thus this set of data reveals, even more clearly than in the case of driver *85B*, the relevance of stimulus concentration in the establishment of a perception code.

DISCUSSION

This report describes an original strategy to address the neural basis of olfactory perception in adult *Drosophila*. It is based on the comparison between two sets of perceptual changes when the inputs to two overlapping arrays of glomeruli are silenced using the

targeted expression of tetanus toxin (Figs. 3–5). Inactivating glomerular input from *85B* and *72OK* neurons elicited perception changes that were odorant-specific, concentration-dependent, and sexually dimorphic.



The Experimental Manipulation

Upon examination of the expression patterns in both Gal4 lines, it is unlikely that the inputs blocked by each driver represent the complete afferents to the corresponding glomeruli. Still, we reasoned that their inactivation should be sufficient to disturb behavior, as proven by the results.

Establishing a link between modified responses to given odorants and individual glomeruli obviously depends on a reliable identification of those glomeruli. This is feasible in *Drosophila* because a reference three-dimensional map of glomeruli is available (Laissue et al., 1999), as in some other insect species (e.g., Chambille and Rospars, 1985; Flanagan and Mercer, 1988; Rospars and Hildebrand, 1992; Galizia et al., 1999). Instead of the immunohistochemistry method employed in that pioneering work, however, we have used here the *ENG3* line to visualize the entire glomerular population in confocal microscopy. This approach had proven useful for glomerular identification in a previous work (Devaud et al., 2001), and has been now complemented by additional staining of the glia. The reliability of glomerular identification is dependent on their invariance in position and at least size or shape (Jefferis et al., 2001). The choice of Gal4 lines for this study took this into consideration as well, and the target glomeruli are invariant enough as to be considered “landmark” glomeruli in the standard map (Laissue et al., 1999).

Expressing the tetanus toxin light chain is known to prevent synaptobrevin-dependent release (Humeau et al., 2000), so olfactory transduction should not be affected in the manipulated sensory neurons. Because the toxin light chain does not go across cell membranes, release from neighboring terminals should not be affected. Thus, the experimental manipulation should be considered as the selective inactivation of glomerular input from sensory synapses only. Recently, Park et al. (2002) proposed a different method, based on expression of the cell-death gene *reaper* in a

Figure 3 Adult olfactory response expressing 85B-driven tetanus toxin. Results indicate mean RI values obtained on 10 replicates, ~25 flies each. Statistical differences were considered to be relevant when detected between *85B/TNT-E* flies and their three respective controls. Levels of significance are indicated only for the comparison between the responses from *85B/TNT-E* and *85B/IMPTNT-VB* flies (see Materials and Methods). Note the effects on certain odorants (benzaldehyde and propionic acid) but not others (ethyl acetate, butanol and acetone). Mann-Whitney test *: $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

specific population of olfactory neurons. This proved also to be efficient in terms of behavioral impairments, although this approach was informative regarding peripheral, not central, odorant processing. The chronic blockade by tetanus toxin appears innocuous for the animal, because no marked increase in lethality was observed during development of tetanus-expressing flies using as reference the viability of sibling genotypes. We also looked for possible locomotion defects in these flies. Such defects would increase the proportion of animals remaining in the central compartment of the T-maze, which was not the case ($p > .05$ for all pairwise comparisons).

Regarding the interpretation of the behavioral data, it should be noted that the criteria to accept the behavioral data have been particularly stringent: a response index value was accepted as abnormal only if the change was statistically significant with respect to three different control genotypes. The application of this strict criterion is justified to minimize the intrinsic problem of data reproducibility commonly seen in behavioral studies in all species. In *Drosophila*, however, we can take advantage of the large number of individuals used and the averaging over different cultures and seasonal periods. It is worth pointing out, however, that there is an intrinsic factor of variability that affects the number of neurons. In the antennal nerve, for instance, the left versus the right side of each animal shows 5% difference in the number of sensory axons, on average (Acebes and Ferrús, 2001). Interindividual variability in this nerve is of the same order also, but it can be much larger in the mushroom bodies (Heisenberg et al., 1995). Under these conditions and validation criteria, it is likely that some biologically meaningful information may be lost in this, as well as many others, set of experiments, albeit to the benefit of the drawn conclusions.

Odorant Specificity

The behavioral data on the five odorants tested show clear specificity in each line rather than general effects on olfactory perception. For instance, butanol perception is not modified at all in either experiment. Still, it is noteworthy that the TNT-expressing flies are affected in their response to a significant proportion of the tested sample of stimuli. This is consistent with the Gal4 expression patterns, which are unlikely to reflect those of only two different olfactory receptor genes. Populations of sensory neurons sharing a single olfactory receptor are known to project on a specific glomerulus, more rarely two (Gao et al., 2000; Vosshall et al., 2000). Here, the number of glomerular targets revealed by each Gal4 line and their overlap,

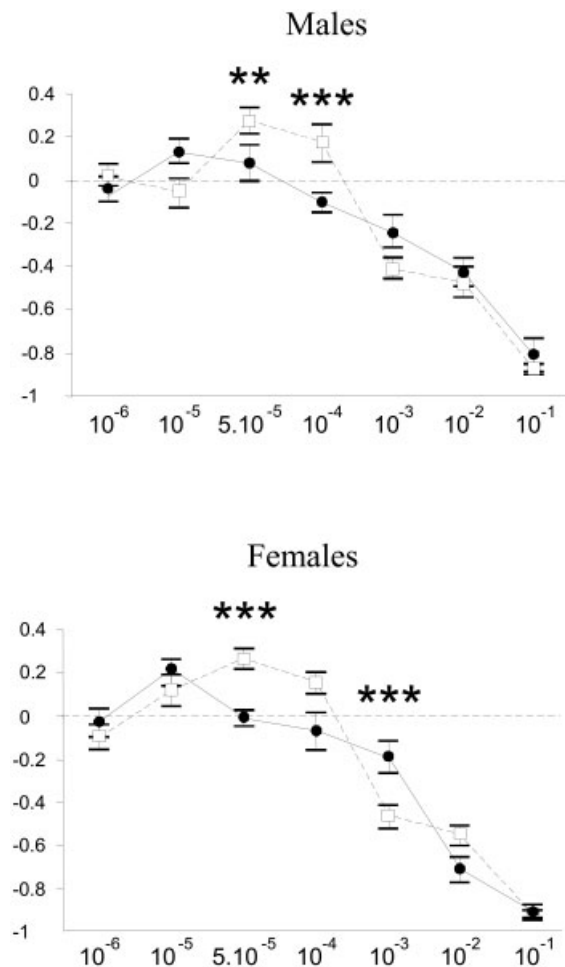


Figure 4 Dose–response curve to benzaldehyde in adults expressing 85B driven tetanus toxin. The mean RI values in response to various odorant concentrations are shown for experimental 85B/TNT-E (filled circles, continuous line) and control 85B/IMPTNT-VB (open squares, dotted line) flies. Note the significant loss of sensitivity at certain concentrations only, and the sexual dimorphism in the effect. ** $p < 0.01$, *** $p < 0.005$.

together with the distinct locations of somata at the periphery, suggest that the neurons in either 85B or 72OK line express diverse odorant receptors. This is consistent with data obtained by inactivating an entire group of olfactory sensillae, believed to house sensory neurons with different receptors (Park et al., 2002).

Although the perception of four odorants out of the five tested is affected, the way in which they are affected depends on the input that is inactivated. This result is a strong argument in favor of the relevance to behavior of the combinatorial information provided by the AL. Also, this is consistent with recent studies (Keller et al. 2002; Park et al., 2002) where, using the same experimental approach, inactivating a subset of

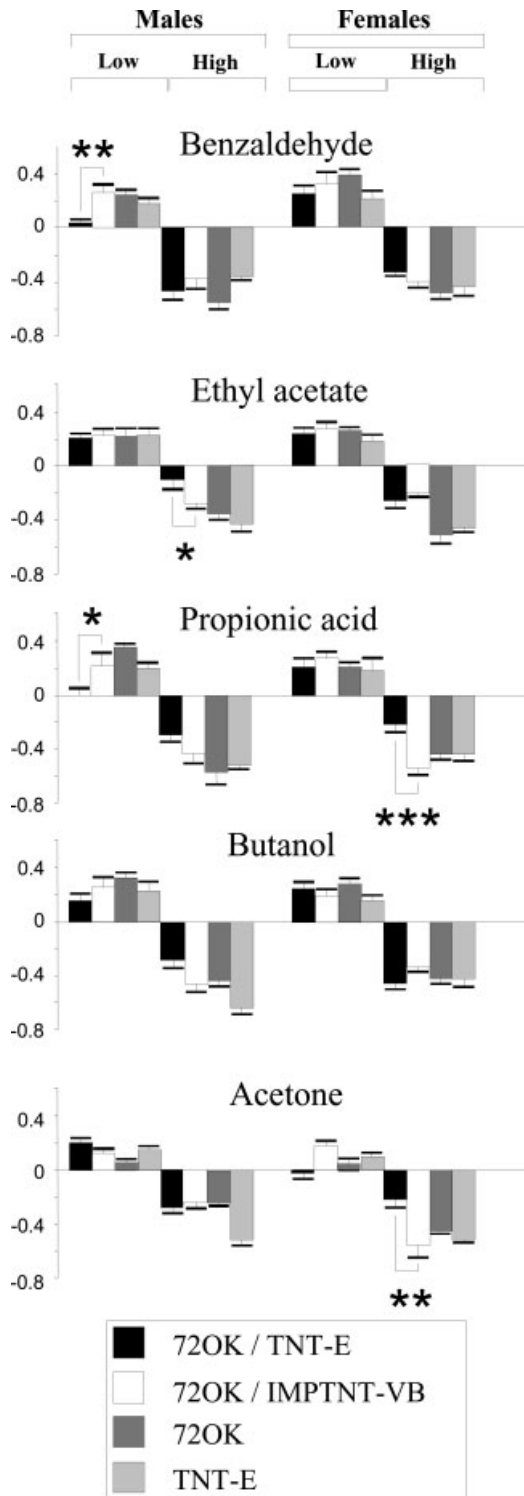


Figure 5 Olfactory responses in adults expressing 72OK-driven tetanus toxin. Data are shown as mean RI values obtained on 10 replicates, ~25 flies each. Significant differences (asterisks) are indicated only for the comparison between the 72OK/TNT-E and 72OK/IMPTNT-VB flies. Mann-Whitney test * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

antennal neurons resulted in a specific behavioral phenotype. In these studies, however, no attempt was made to identify the glomerular targets. Visualization of glomerular activity in other animals showed that odorants are represented as spatial maps (Joerges et al., 1997; Rubin and Katz, 1999; Sachse et al., 1999), according to the selectivity of their afferents and synaptic interactions between glomeruli (Linster et al., 1994; Yokoi et al., 1995). As expected, expressing the toxin in the described neurons modifies the glomerular representations of several odorants, and the sample used here illustrates this feature for benzaldehyde and propionic acid in the case of 85B and 72OK neurons, and for ethyl acetate and acetone in the case of 72OK neurons. In the same context, we have shown in a previous study that selective adaptation to a given odorant, benzaldehyde, correlated with synapse elimination in the V glomerulus (Devaud et al., 2001).

Dose Dependence

Another aspect relevant to olfactory perception is the importance of odorant concentration: the selective blockade of sensory inputs affects the perception of odorants in a dose-dependent manner. The activity of a given subset of neurons (and probably glomeruli) must then be required for a limited range of concentrations for the considered odorant, consistently with available data on *Drosophila* (Park et al., 2002). For example, perception of benzaldehyde is significantly impaired at low and high doses in 85B/TNTE flies, but only at low doses in 72OK/TNTE flies. Conversely, the inactivation of 72OK, but not 85B, neurons affects the response to the high concentration of ethyl acetate. Remarkably, this dose dependence is nonlinear, as shown by the dose-response curve obtained for benzaldehyde. The stronger repellent effect of the 10^{-3} concentration on TNT-expressing flies suggests that toxin expression does not produce a mere loss of sensitivity.

These results are in keeping with physiological data available from other species, showing that additional sensory neurons and glomeruli are activated by an odorant when its concentration increases (e.g., De Bruyne et al., 1999, 2001; Friedrich and Korsching, 1997; Rubin and Katz, 1999). For example, according to this view the 85B neurons projecting onto the DL2 glomerulus would be sensitive to only high doses of benzaldehyde. In parallel, VM1 and/or VM4 would be involved in processing lower doses of this odorant because both lines are impaired. Because a previous report (Devaud et al., 2001) showed that DM2 and V glomeruli were affected by exposure to concentrated benzaldehyde, it seems that benzaldehyde perception

involves activity in glomeruli DL2, DM2, and V at least. Interestingly, *85B/TNTE* flies still respond normally to very high concentrations of that odorant (see the IR values for 10^{-2} and 10^{-1} benzaldehyde in Fig. 5). A possible explanation to this would be that such doses lead the system close to saturation, so that with a great number of sensory neurons and glomeruli being activated the loss of some input has no behavioral effect. This hypothesis is in accordance with the view of a progressive recruitment of sensory neurons and glomeruli resulting in perceptual changes of increasing concentrations of an odor (Johnson and Leon, 2000; Kajiya et al., 2001). Such recruitment could account for shifts from attraction to avoidance observed in *Drosophila* as in other species.

Comparisons between these laboratory experimental conditions and those in a natural environment are difficult to draw, mainly due to the dearth of *Drosophila* open field data on olfaction. It should be noted, however, that larvae and adults expend most of the time inside, or around of, degrading fruits where the odorant concentrations are very high, certainly within the range of those used here. Foraging and search for suitable egg laying substrate, however, involve wide odorant concentration changes. In this context, the nonlinearity of the dose dependence (e.g., benzaldehyde) seen here under laboratory conditions, might suggest that odor perception is processed differently during exploratory flights than when on feeding grounds.

Sexual Dimorphism

More surprisingly, we observe important differences in the behavioral impairments of toxin-expressing males and females, in the absence of overt sexual dimorphism in the Gal4 expression patterns. Differences in the number or identity of inactivated sensory neurons in each sex are then unlikely to intervene. Rather, the data suggest that sex-specific odorant processing may result from differences in the AL circuitry. In this context, Ferveur et al. (1995) showed that genetic feminization of glomeruli, but not antennae, correlates with changes in sexual behavior. Thus, beside the reported dimorphism of sensory organs (Stocker, 1994), some more subtle differences must take place in the *Drosophila* AL, despite its similar glomerular organization in both sexes (Laissue et al., 1999). In the present set of data, the effect of TNT in *85B* and *72OK* domains is more noticeable in females than in males. Males exhibit similar patterns of behavioral impairment in both Gal4 lines (with the exception of ethyl acetate at high concentration). Females, by contrast, do not show any overlap in the

stimuli to which their response is affected by toxin expression: benzaldehyde at both concentrations in *85B* versus propionic acid and acetone at high concentrations in *72OK*.

Thus, females appear to be behaviorally more sensitive to functional alterations of their olfactory pathway. It is plausible that this effect could result from subtle differences in connectivity or synaptic modulation in the AL of each sex. These presumed differences could account also for the reported higher sensitivity of females to P-element perturbations in a paradigm of benzaldehyde avoidance (Anholt et al., 1996) or the very low intersexual correlation of genetic polymorphisms for the same behavior (Anholt et al., 2001). Interestingly, humans show sexual dimorphism for benzaldehyde perception also in the direction of enhanced sensitivity in females (Dalton et al., 2002).

Taken together, these results prove the behavioral relevance of olfactory information processing by sensory neurons and their glomerular targets. Analyzing the effects of tetanus toxin expression on the response to various stimuli allows to make predictions on some key aspects of olfactory coding, such as concentration dependence and sexual dimorphism. Independent evidence to validate these predictions are still to be obtained through visualization methods (Galizia and Menzel, 2000) when adapted to *Drosophila*.

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