Cellular/Molecular

A Pheromone Receptor Mediates 11-*cis*-Vaccenyl Acetate-Induced Responses in *Drosophila*

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Insect pheromones elicit stereotypic behaviors that are critical for survival and reproduction. Defining the relevant molecular mechanisms mediating pheromone signaling is an important step to manipulate pheromone-induced behaviors in pathogenic or agriculturally important pests. The only volatile pheromone identified in *Drosophila* is 11-*cis*-vaccenyl acetate (VA), a male-specific lipid that mediates aggregation behavior. VA activates a few dozen olfactory neurons located in T1 sensilla on the antenna of both male and female flies. Here, we identify a neuronal receptor required for VA sensitivity. We identified two mutants lacking functional T1 sensilla and show that the expression of the VA receptor is dramatically reduced or eliminated. Importantly, we show misexpression of this receptor in non-T1 neurons, normally insensitive to VA, confers pheromone sensitivity at physiologic concentrations. Sensitivity of T1 neurons to VA requires LUSH, an extracellular odorant-binding protein (OBP76a) present in the sensillum lymph bathing trichoid olfactory neuron dendrites. Here, we show LUSH are also required in non-T1 neurons misexpressing the receptor to respond to VA. These data provide new insight into the molecular components and neuronal basis of volatile pheromone perception.

Key words: **olfaction; olfactory; sensory perception; behavior; pheromone; receptor**

Introduction

Chemicals released by individuals to influence the behavior of other members of the same species are defined as pheromones (for review, see Dahanukar et al., 2005). Pheromones are widely used in the animal kingdom, eliciting stereotypic behaviors in both invertebrates and vertebrates (Bigiani et al., 2005; Howard and Blomquist, 2005; Vosshall, 2005). Pheromone communication is used extensively in insects, in which a variety of chemicals trigger such diverse behaviors as mating, recruitment, aggregation, and dispersal (for review, see Vander Meer et al., 1998). However, the molecular components required for pheromone sensitivity are mostly unknown.

The only volatile pheromone identified to date in *Drosophila* is 11-*cis*-vaccenyl acetate (VA). This male-specific lipid induces aggregation behavior in both male and female flies (Bartelt et al., 1985; Xu et al., 2005). VA detection is mediated by olfactory neurons located in the trichoid sensilla on the ventral–lateral surface of the antenna (Clyne et al., 1997). Trichoid sensilla fall into multiple functional groups, including T1, and T2a and T2b (Clyne et al., 1997; Couto et al., 2005; Xu et al., 2005). For simplicity, the T2 types will be referred to as "non-T1" here. T1 sensilla typically contain a single olfactory neuron that is exquisitely tuned to VA, and have spontaneous action potential firing rates of ~1 spike per second (Clyne et al., 1997; Xu et al., 2005). T1 sensilla are clustered in the proximal portion of the trichoid sensilla expression zone (Xu et al., 2005) (see Fig. 1). Non-T1 sensilla as a group contain multiple neurons, have a combined spontaneous rate of ~35 spikes per second, and are not influenced by VA. Non-T1 neurons are inhibited by high concentrations of various alcohols (Clyne et al., 1997; Xu et al., 2005).

Recent work indicates the molecular mechanisms underlying pheromone perception are unique. LUSH, an extracellular odorant-binding protein (OBP76a), is required for VA-induced activation of T1 neurons and pheromone-triggered behaviors (Xu et al., 2005). LUSH is expressed exclusively in trichoid sensilla (Kim et al., 1998; Shanbhag et al., 2001, 2005). LUSH is secreted by non-neuronal support cells into the fluid bathing the trichoid olfactory neuron dendrites, but is not expressed by the neurons themselves (Kim et al., 1998; Shanbhag et al., 2001). Mutants defective for LUSH lack pheromone-induced behavioral responses and T1 sensitivity. Therefore, the binding protein is required for pheromone detection.

The LUSH protein itself appears to specifically trigger action potentials in T1 neurons, producing the normal spontaneous activity observed in wild-type animals. Spontaneous activity is reduced 400-fold in *lush* mutants, and is restored when recombinant LUSH protein is infused directly to mutant T1 sensilla (Xu et al., 2005). No effect is observed on the spontaneous activity of non-T1 neurons. Therefore, there is something in T1 sensilla lacking in non-T1 sensilla responsible for VA sensitivity and spontaneous activity in the presence of LUSH protein. A likely candidate for such a factor is a receptor expressed by T1 neurons lacking in non-T1 neurons. In this paper, we report the identification of a receptor candidate expressed by T1 neurons required for VA sensitivity.

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Materials and Methods

Identification of mutants lacking T1 sensilla. We screened a subset of the Zuker EMS mutant collection (Koundakjian et al., 2004) for abnormal responses to VA using single sensillum electrophysiological recordings (see below). We identified two mutants that lacked T1 sensilla, *tod*^{1} (T-one-deficient) mutants (Zuker no. 23-6129) and *tot*^{1} (T-one-transformed) mutants (Zuker no. 23-4626).

Single sensillum recordings. Extracellular electrophysiological recordings were performed according to de Bruyne et al. (2001). Flies (2–7 d of age; males and females) were under a constant stream of charcoal-filtered air (36 ml/min; 22–25°C) to prevent any potential environmental odors from inducing activity during these studies. 11-*cis*-Vaccenyl acetate was diluted in paraffin oil and 1 μl was applied to filter paper and inserted in a Pasteur pipette, and air was passed over the filter and presented as the stimulus. The purity of the VA was confirmed both by nuclear magnetic resonance and by mass spectroscopy. Signals were amplified 1000× (USB-IDAC System; Syntech, Hilversum, The Netherlands) and fed into a computer via a 16-bit analog-digital converter and analyzed off-line with AUTOSPIKE software (USB-IDAC System; Syntech). Low cutoff filter setting was 200 Hz, and the high cutoff was 3 kHz. Action potentials were recorded by inserting a glass electrode in the base of a sensillum. Data analysis was performed according to de Bruyne et al. (2001). Signals were recorded for 20 or 30 s, starting 10 s before VA stimulation. Action potentials were counted 1 s before VA stimulation and for 1 s after VA stimulation. All recordings were performed from separate sensilla with a maximum of two sensilla recorded from any single fly.

RT-PCR. RT-PCRs for Or gene products were performed using Trizol for RNA purification as described by the manufacturer (Invitrogen, Carlsbad, CA). The first strand of cDNA was synthesized from 1 μg of total RNA using reverse transcriptase (Ambion, Austin, TX) at 37°C for 1 h. Amplification was performed by PCR using Or67d specific primers, 5′-TGGTCACCTAATACTCACGGCTG-3′ and 5′-CGCAAGTTGTGGAAAGTTGAACG-3′. Or83b was amplified as a control for RNA for 40 cycles using specific primers of Or83b, 5′-GCCACCAAAATCAACGGAGTG-3′ and 5′-GAAGCAAACAAATCCAGGGAGAC-3′. RT-PCR products were separated and visualized on an agarose gel and ethidium bromide staining. Identical results were obtained in three independent experiments. Primer sequences and expected products for the complete set of Or genes we tested are presented in supplemental Table 1 (available at www.jneurosci.org as supplemental material).

Scanning electron microscopic analysis. Wild-type, *tot*^{1} and *tod*^{1} mutants were prepared for scanning electron microscopic analysis according to (Shanbhag et al., 1999). Briefly, heads of *w*^{1118}, *tod*^{1}, and *tot*^{1} flies were removed with a razor blade, and unfixed specimens were quickly coated with 20 nm gold in a sputter coater. Specimens were viewed with JSM-840A model (JEOL, Tokyo, Japan) for scanning electron microscope (10 kV). Photographs of the anterior surface of several antennas from each genotype were used to quantify the numbers of sensilla.

RNA in situ *hybridization.* Expression of Or67d in control, *tot*^{1}, and *tod*^{1} mutants were preformed as described previously (Smith et al., 1990), with modification for fluorescent probes as described by Couto et al. (2005). Antisense and sense riboprobes were transcribed from Or67d and Or83b cDNAs subcloned into PCR2.1-cloning vector (Invitrogen) using T7 RNA polymerase (Ambion). Or83b and Or67d antisense probes were incorporated with digoxigenin or fluorescein and detected with anti-DIG-POD (Roche, Indianapolis, IN) and anti-fluorescein-POD (Roche). Signals were amplified with the TSA-Plus Cyanine 5 system (PerkinElmer, Wellesley, MA) for Or83b or the TSA-Plus Fluorescence system (PerkinElmer) for Or67d (Fishilevich and Vosshall, 2005).

Misexpression of Or67d. A cDNA encoding Or67d was obtained by reverse transcription, PCR amplification of RNA isolated from antenna of *Drosophila w*^{1118}, a strain known to be responsive to VA pheromone (our unpublished observation) (see Figs. 1, 4). This strain has four amino acid polymorphisms compared with the predicted Or67d gene product in the genome sequence (Adams et al., 2000), specifically S98C, D238E, W252F, and S268P. These polymorphisms were confirmed as true genomic polymorphisms by sequencing clones from independent RT-PCRs. The cDNA was cloned into pUAST (Brand and Perrimon, 1993), and transgenic animals were generated using standard techniques. Or67d was misexpressed in non-T1 neurons by crossing the UAS Or67d transgenic flies to flies expressing Gal4 under control of the neuron-specific promoter for the embryonic lethal abnormal vision (ELAV) gene (Koushika et al., 1996).

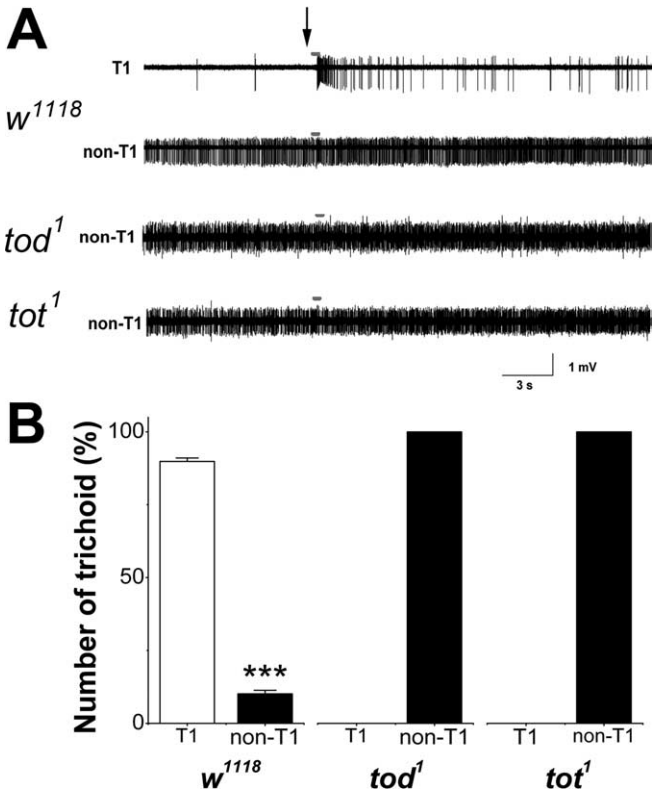

Figure 1. *tod*^{1} and *tot*^{1} mutants lack T1 sensilla. ***A***, Representative raw traces of neuronal activity from trichoid sensilla of *w*^{1118} (wild-type controls), *tod*^{1}, and *tot*^{1}. Single-unit recordings from the trichoid sensilla in the trichoid zone were obtained after stimulation with 11-*cis*-vaccenyl acetate (1%), known to selectively activate T1 neurons. The arrow represents onset, and the gray bars above each trace represent the stimulus duration (300 ms). ***B***, The number of identified T1 or non-T1 functional types in the trichoid zone in independent trials. For *tod*^{1}, $n = 69$; for *tot*^{1}, $n = 59$; for wild-type controls, $n \geq 2000$ recordings. T1 sensilla are lacking in the two mutants. Error bars represent SEM. For each data set, the statistical significance of the difference was tested using ANOVA for independent observations. ***$p < 0.01$ was considered significant between wild-type and two mutants animals for non-T1 sensilla in the trichoid zone.

Results

tod^1 and *tot*^{1} mutants lack T1 sensilla

With the goal of identifying all genetic loci required for VA pheromone detection, we undertook a screen to recover mutants with abnormal electrophysiological responses to VA pheromone (see Materials and Methods). We identified two mutants out of 1200 lines that lack functional T1 sensilla. *tod*^{1} and *tot*^{1} have normal basiconic and non-T1 sensilla, but no T1 sensilla. *tod*^{1} and *tot*^{1} are both recessive and fail to complement one another, revealing these mutants lack T1 sensilla as a result of lesions in independent genes. Figure 1*A* shows examples of single-unit recordings from the trichoid zone from *tod*^{1} mutants, *tot*^{1} mutants, and wild-type controls. In normal flies, there is a mixture of T1 and non-T1 subtypes in the trichoid zone, but the proximal part of this zone is enriched in T1 sensilla (Clyne et al., 1997; Xu et al., 2005). Morphologically, all trichoid sensilla are indistinguishable. However, T1 and non-T1 sensilla are clearly distinguishable by electrophysiology (Clyne et al., 1997; Xu et al., 2005). In a survey of over 2000 animals, recordings from random trichoid sensilla in the proxi-

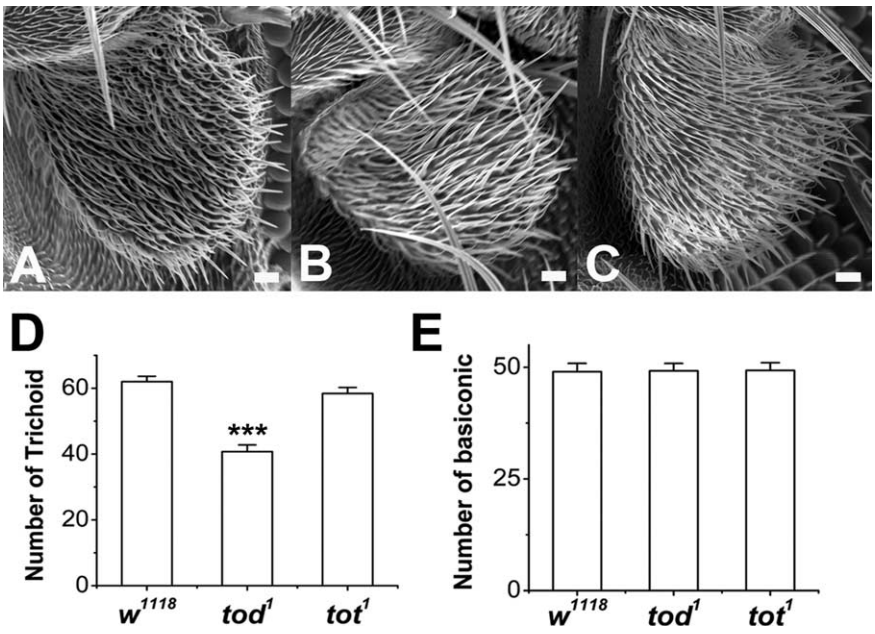

Figure 2. Scanning electron microscopy of trichoid sensilla from w^{1118}, tod^1, and tot^1. Trichoid sensilla, identified by their long slender morphology and sharp tips, are distributed on the anterior surface of the antenna regions in w^{1118} (***A***), tod^1 (***B***), and tot^1 (***C***) animals. Scale bar, 10 μm. Number of trichoid sensilla (***D***) and large basiconic (***E***) were quantified for each genotype. $n = 6$ antenna for each genotype. Error bars represent SEM. ***$p < 0.01$ was considered significant. There is a significant difference between wild type and tod^1, but not wild type and tot^1.

mal zone identify VA-sensitive T1 sensilla 89% of the time and non-T1 11% of the time (Fig. 1*B*) based on spontaneous activity rate and sensitivity to VA. VA could evoke activity in T1 neurons from 0.35 ± 0.14 spikes per second before stimulation to 36.89 ± 3.43 after stimulation. VA did not induce activity in non-T1 neurons (48.42 ± 3.93 before to 49.33 ± 4.13 after stimulation). Recordings from either tod^1 mutants or tot^1 mutants from the T1 zone always identified non-T1 sensilla. These sensilla have the classic characteristics of the non-T1 type, including lack of response to VA, more than one neuron present in the sensillum, and a high rate of spontaneous activity. We surveyed trichoid and large basiconic sensilla from across the antenna in tod^1 mutants and tot^1 mutants and could identify no VA-responsive neurons (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). Therefore, there does not appear to be a simple mislocalization of T1 sensilla to a different part of the antenna, but a complete loss of the T1 functional type.

tod^1 mutants and tot^1 mutants could lack T1 sensilla because the T1 sensilla are transformed into non-T1 sensilla during development. Alternatively, the T1 sensilla may simply no longer be present, perhaps because of death of T1 precursors. If the latter is true, there should be a reduction in the total number of trichoid sensilla in these mutants correlating with the number of T1 sensilla. Therefore, we performed scanning electron microscopic analysis of wild-type, tod^1, and tot^1 mutants to determine whether there are fewer trichoid sensilla compared with the wild-type controls. Figure 2 shows that tod^1 mutants have ~30% fewer (40.8 ± 2.1) trichoid sensilla compared with controls (62 ± 1.7), whereas tot^1 mutants (58.4 ± 1.9) have essentially the normal number of trichoids (Fig. 2*D*). One possibility is that T1 precursors fail to develop T1 sensilla in tod^1 mutants, resulting in a reduction in the total number of trichoid sensilla. In tot^1, in contrast, there is an overall increase in the non-T1 sensilla class at the expense of T1 class, whereas the total number of trichoid sensilla is equivalent to that of wild-type controls. This is consistent with a transformation of T1 into non-T1 sensilla instead of loss of T1 sensilla. The number of large basiconic sensilla is not different in any of the three genotypes (Fig. 2*E*). A survey of large basiconic sensilla revealed no functional abnormalities in tod^1 or tot^1 mutants (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). Additional characterization of these mutants will be reported elsewhere (S. Mistry, T. S. Ha, and D. P. Smith, manuscript in preparation).

tod^1 and tot^1 mutants are both defective for expression of one member of the odorant receptor family

tod^1 and tot^1 mutants lack functional T1 sensilla. Therefore, T1-neuron specific gene products should be absent in these mutants. We reasoned that a neuronal receptor mediating VA responses might be a member of the odorant receptor family expressed specifically by T1 neurons. Indeed, Ors have been shown to specify odor specificity to olfactory neurons in a number of systems, including *Drosophila* (Sengupta et al., 1996; Malnic et al., 1999; Dobritsa et al., 2003; Hallem et al., 2004). Therefore, we screened candidate members of the *Drosophila* Or gene family for reduced expression in tod^1 and tot^1 mutants.

PCR primers were designed to all members of the Or gene family (Robertson et al., 2003) whose function or odor specificity had not been established previously (Stortkuhl, 2001; Dobritsa et al., 2003; Elmore et al., 2003; Hallem et al., 2004). In addition, at least one gustatory receptor (Gr) is expressed in the antenna (Scott et al., 2001), and several others including Gr10a, Gr32a, and Gr63a may also be expressed (K. Scott, personal communication). We generated primers to assay expression of these orphan receptors by RT-PCR (supplemental Table 1, available at www.jneurosci.org as supplemental material). Each primer pair spanned an intron to allow us to distinguish cDNA products from potential genomic DNA contamination. We found that a single receptor gene, Or67d, was absent or drastically reduced in tod^1 and tot^1 mutants compared with wild-type controls. Figure 3 shows RT-PCR analysis of Or67d from wild-type, tod^1, and tot^1 mutant antenna. An Or67d spliced transcript is clearly present in wild-type antennas, but is reduced or absent in tod^1 and tot^1 mutants, even after 40 cycles of amplification (Fig. 3*B*). Or83b (Vosshall et al., 1999), expressed in most olfactory receptor neurons (Vosshall et al., 1999; Elmore et al., 2003; Larsson et al., 2004) was present in all three samples, as were all other Or genes tested (data not shown). These results suggest Or67d expression is specifically reduced or eliminated in tod^1 and tot^1 mutants, and correlates with the loss of the T1 functional class in these genetically distinct mutants.

Having identified a candidate receptor correlating with the presence of T1 neurons, we set out to establish whether the expression pattern of Or67d in the antenna was consistent with the known T1 neuron distribution. We performed *in situ* hybridization using fluorescently labeled antisense RNA probes to Or67d to characterize expression of this putative receptor. Figure 3*C*

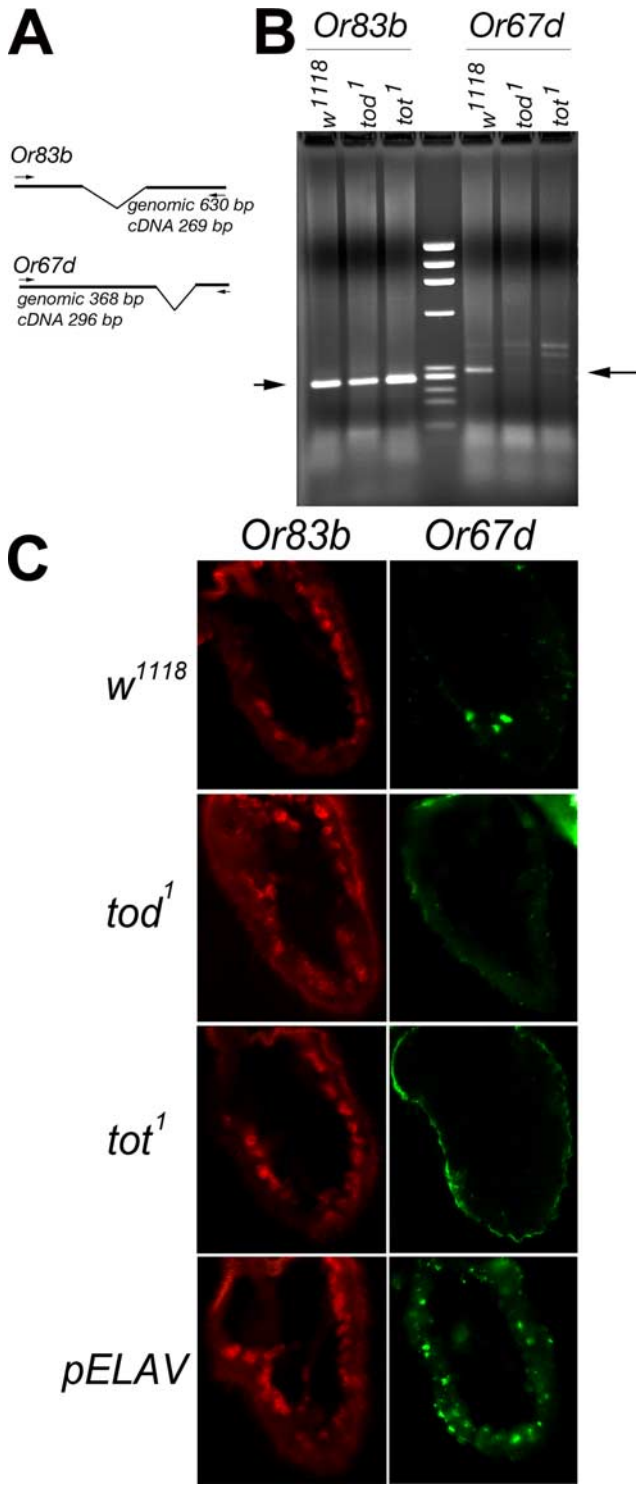

Figure 3. Expression of Or67d is defective in *tod*^{1} and *tot*^{1} mutants. ***A***, Schematic diagrams of Or83b and Or67d gene fragments and the relative location of the PCR primers. Primers were chosen to span an intron to discriminate genomic contamination from cDNA products. The thick line denotes the exon sequences, and the thin line denotes the intron region of each gene fragment. The expected cDNA size of the RT-PCR products was 296 bp for Or67d and 269 bp for Or83b. The expected genomic DNA size of the RT-PCR products was 368 bp for Or67d and 630 bp for Or83b. The arrows indicate priming sites for each specific primer sets. ***B***, Or67d in *tod*^{1} and *tot*^{1} mutants flies is absent or dramatically reduced (long arrow). Or83b was used as a positive control for RNA integrity and RT quality (short arrow). ***C***, RNA *in situ* hybridization with antisense probes to Or83b (left panels; red colors) and Or67d (right panels; green colors) in w^{1118}, *tod*^{1}, and *tot*^{1} mutants. The bottom panel shows antisense labeling in antennas misexpressing Or67d under control of the ELAV promoter. Or83 and Or67d signals were amplified with TSA-Plus Cy5 (red) or TSA-Plus Fluorescence systems (green).

shows that antisense probes to Or67d specifically label cells on the ventral–lateral surface of the third antennal segment. Serial sections reveal the labeled cells are concentrated in the proximal T1 zone (our unpublished observation). These probes failed to identify similar positive cells in antenna *tod*^{1} or *tot*^{1} (3C) consistent with the functional loss of T1 sensilla. Therefore, Or67d expression correlates well with the known distribution of T1 sensilla in wild-type antenna and the absence of T1 sensilla in the mutants.

Or67d confers VA sensitivity to non-T1 neurons

Expression of Or67d in the T1 zone and its absence in *tod*^{1} and *tot*^{1} mutants is consistent with Or67d being the T1 VA receptor, but does not prove this receptor is responsible for VA sensitivity. For example, Or67d may have some functional role specific to T1 neurons that is unrelated to VA sensitivity. Alternatively, a subset of non-T1 class olfactory neurons may also be absent in *tod*^{1} and *tot*^{1} that specifically express Or67d. Therefore, to definitively prove Or67d mediates VA sensitivity, we misexpressed Or67d in olfactory neurons that normally do not express this receptor and are VA insensitive. Previous work has shown that coexpression of an extra odorant receptor in a *Drosophila* olfactory neuron results in an odor sensitivity profile that is the combination of the sensitivity of the individual receptors (Goldman et al., 2005). Therefore, we misexpressed Or67d in all neurons by driving Or67d expression with the pan-neuron promoter, ELAV. ELAV is a neuron-specific splicing factor expressed in all *Drosophila* neurons (Campos et al., 1985; Koushika et al., 1996). We confirmed that flies expressing Or67d under control of the ELAV promoter misexpress the Or67d transgene in olfactory neurons in the antenna (Fig. 3*C*, bottom panel).

We compared the VA sensitivity of wild-type animals and those misexpressing Or67d in non-T1 sensilla. Neurons in wild-type non-T1 sensilla are insensitive to VA (Fig. 4, top left panel). However, animals misexpressing Or67d in all neurons have non-T1 neurons that are highly responsive to VA (Fig. 4, top right panel). Indeed, dose–response analysis reveals these neurons are nearly as sensitive to VA as wild-type T1 neurons (Fig. 4, graph). By all other criteria, these neurons are non-T1 and not T1 neurons. They display high spontaneous activity and contain multiple neurons, and their distribution is typical of the non-T1 functional class. The only difference we can observe in these neurons compared with wild-type controls is VA sensitivity. Conferring VA sensitivity on non-T1 neurons by expressing Or67d receptors demonstrates that this receptor is both necessary and sufficient to confer VA sensitivity on non-T1 neurons.

LUSH is also required for non-T1 neurons misexpressing Or67d to respond to VA

LUSH protein is required in the T1 sensillum lymph for T1 neurons to be sensitive to VA (Xu et al., 2005). Non-T1 sensilla also express LUSH protein in the sensillum lymph (Kim et al., 1998; Shanbhag et al., 2001, 2005). Is LUSH also required for sensitivity of non-T1 neurons misexpressing Or67d? We crossed the *lush*^{1} mutation into the stock misexpressing Or67d in all neurons. Figure 4 shows that LUSH protein is critical for non-T1 neurons to respond to VA as well, because when the *lush*^{1} mutation is crossed into the misexpressing flies, VA sensitivity is lost in non-T1 neurons (Fig. 4, bottom right panel). This clearly demonstrates that LUSH is required in the extracellular space in order for non-T1 neurons misexpressing Or67d to be responsive to VA. Therefore, both the receptor Or67d and a specific extracellular binding protein, LUSH, are required for VA sensitivity.

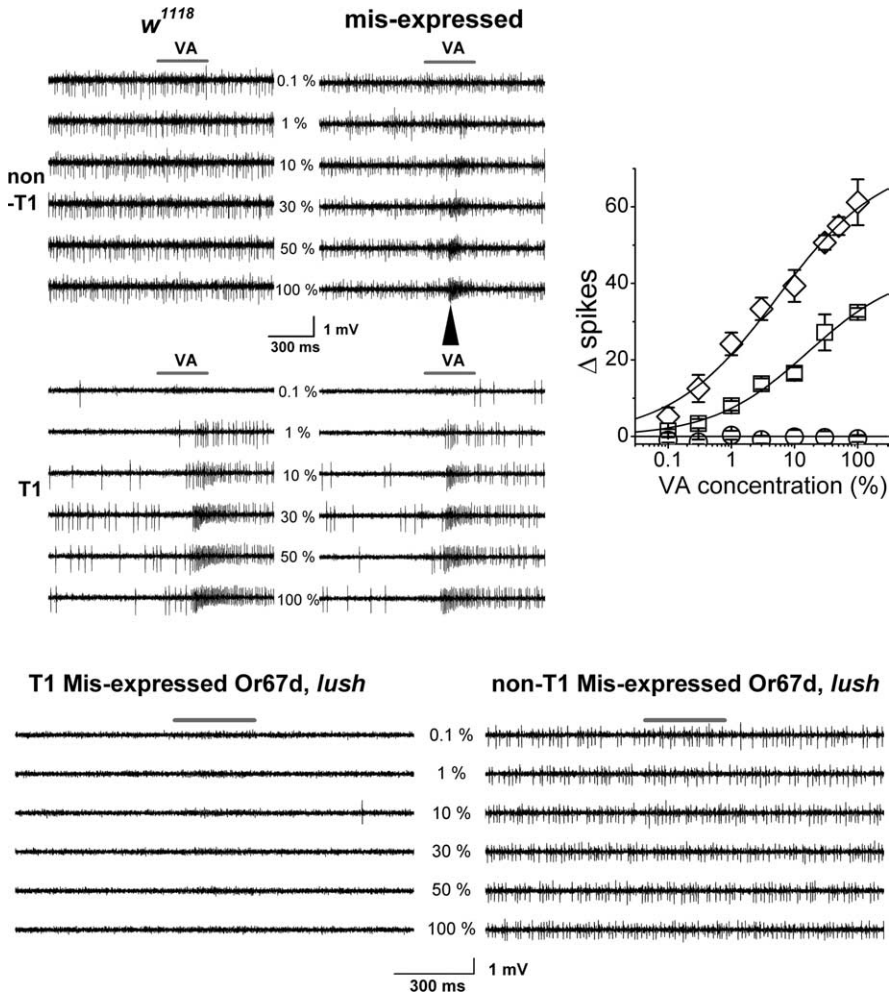

Figure 4. Non-T1 neurons misexpressing Or67d display VA-induced, dose-dependent activity. Typical single-unit recordings of non-T1 and T1 sensillum at different concentrations of VA in w^{1118} controls and flies misexpressing Or67d. Action potentials from single sensilla were recorded with different stimulus concentrations of VA from 0.1 to 100%. The gray bars above each group represent duration of VA application (300 ms). The graph shows dose–response curves for wild-type T1 neurons (diamonds), non-T1 neurons misexpressing Or67d (squares), and w^{1118} non-T1 (circles). Note dose-dependent VA activation in non-T1 neurons from flies misexpressing Or67d, but not in the wild-type non-T1 neurons. The relative overexpression of Or67d in the T1 sensilla of the pELAV-Gal4; UAS Or67d animals did not alter VA sensitivity compared with wild-type T1 neurons. The experimental data were fitted with a logistic function, derived from the Hill equation (Hill, 1910). The statistical means and SEs were calculated using the values obtained from at least six independent experiments. Bottom panels, Representative raw traces from trichoid sensilla of flies misexpressing Or67d in the *lush*^{1} mutant background. VA responses are absent in both T1 and non-T1 sensilla. Each data point represents the mean ± SEM.

Discussion

Pheromones drive innate behaviors in insects. VA pheromone functions to attract other individuals to a location, most likely at food sources (Vander Meer et al., 1986). We previously showed the extracellular protein LUSH is required for VA pheromone sensitivity, both for T1 neuron responses and for behavioral responses to pheromone (Xu et al., 2005). In this paper, we identified an odorant receptor expressed by trichoid neurons that mediates VA pheromone sensitivity. Or67d expression is defective in two independent mutants that lack functional T1 sensilla, and this receptor is necessary and sufficient to confer VA sensitivity on non-T1 neurons that are normally insensitive to this pheromone. Together, these data provide strong proof that Or67 is the receptor, or a component of the receptor, that mediates VA sensitivity in olfactory neurons in T1 sensilla.

Although there are no Or67d mutants available to show directly that Or67d mediates VA behavior, this is almost certainly true. LUSH OBP is expressed exclusively in trichoid sensilla (Kim et al., 1998; Shanbhag et al., 2001, 2005), and *lush*^{1} mutants are completely defective for behavioral responses to VA pheromone (Xu et al., 2005). Therefore, VA-induced behavior is mediated through trichoid sensilla. Because T1 neurons are the only trichoid neurons that respond to VA (Clyne et al., 1997; Xu et al., 2005), and Or67d mediates VA sensitivity in these trichoid neurons, it is reasonable to deduce that the behavioral responses to VA pheromone are mediated by Or67d receptors. The responses of *tod*^{1} or *tot*^{1} mutants to VA should also be defective, because they both lack T1 sensilla. Unfortunately, these mutants are heavily mutagenized, and loss of VA-induced behavior is masked by the general locomotor defects of these animals.

What are the neuronal circuits that mediate VA-induced behaviors? Our identification of Or67d as a component of the neuronal VA receptor is consistent with expression studies identifying Or67d receptor expression in one class of trichoid sensillum (Couto et al., 2005). A class of trichoid sensilla was identified that expressed Or67d and contained a single neuron (Couto et al., 2005). Or67d-expressing neurons have been shown to innervate two glomeruli in the antennal lobes, DA1 and VA6 (Couto et al., 2005; Fishilevich and Vosshall, 2005). We speculate, therefore, that higher brain functions are alerted to of the presence of VA pheromone through activity in these glomeruli. Using genetically encoded fluorescent monitors of neuronal activation (Nagai et al., 2001; Pologruto et al., 2004), it should now be possible to elucidate the neuronal circuits in higher brain centers that are triggered by VA pheromone.

Why does VA pheromone detection require both a specific receptor and a binding protein? One possible explanation is specificity. Most odorant receptors in *Drosophila* appear to be broadly tuned (Clyne et al., 1999; Dobritsa et al., 2003; Hallem et al., 2004). Broad tuning of receptors presumably allows *Drosophila* to detect a wide range of biologically relevant chemicals in the environment with a limited repertoire of 62 different receptor proteins. In contrast, pheromone detection should be highly specific so that inappropriate cues are not misconstrued as pheromones. Perhaps Or67d is specifically tuned to VA but requires LUSH to transfer VA through the aqueous lymph. Alternatively, Or67d may be more broadly tuned, and LUSH OBP restricts the diversity of ligands that are presented to the receptor by only interacting with VA. A third possibility is that LUSH protein undergoes a unique conformational change after VA binding and this conformation of LUSH triggers Or67d activation. Consistent with this last model, folded, but not unfolded LUSH protein alone appears to be a partial

agonist of T1 neurons (Xu et al., 2005). Additionally, x-ray crystal structures show unique conformational changes in LUSH after binding alcohols (Kruse et al., 2003) and VA (J. D. Laughlin, T. S. Ha, D. P. Smith, and D. N. M. Jones, manuscript in preparation). Now that these components have been identified, future work will focus on defining the biochemical relationship between LUSH, VA, and Or67d.

This work extends the evidence that extracellular LUSH protein is critical for VA detection. Non-T1 neurons misexpressing Or67d are only sensitive to VA when LUSH protein is present. Therefore, LUSH is required for VA detection by Or67d-expressing neurons. Detection of an unknown contact pheromone involved in courtship behavior in *Drosophila* is mediated by taste receptor Gr68a (Bray and Amrein, 2003). It will be of interest to determine whether a binding protein mediates this behavior as well. Artificial stress results in release of CO_2 from adult fruit flies and may act like a pheromone (Suh et al., 2004). Recent work in the moth *Bombyx mori* has uncovered several putative sex pheromone receptors for the female sex pheromone bombykol (Krieger et al., 2005; Nakagawa et al., 2005). *B. mori* Or1 (BmOR1) and BmOR3 are expressed preferentially in male antenna, in neurons known to respond to female pheromone (Krieger et al., 2005). Heterologous expression of BmOR1 in *Xenopus laevis* oocytes confers sensitivity to bombykol at high pheromone concentrations (Nakagawa et al., 2005). The poor sensitivity of moth pheromone receptors expressed in oocytes may result from relatively poor expression or from missing factors that contribute to sensitivity (like extracellular pheromone binding proteins). Misexpression of *Drosophila* Ors in "empty" olfactory receptor neurons (lacking endogenous receptors) has been shown to confer odor response profiles that are very similar to the endogenous neurons expressing those receptors, indicating this is a more physiologic approach (Hallem et al., 2004). Using a similar approach, we conferred VA sensitivity on non-T1 neurons at pheromone concentrations that are close to the threshold levels that activate T1 neurons. This supports the idea that Or67d in combination with LUSH protein confers sensitivity to physiological relevant concentrations of VA *in vivo*.

Defining the molecular basis for VA pheromone perception in *Drosophila* provides an important step in understanding the molecular basis of pheromone-induced behavior in an animal model system amenable to genetic analysis. If pheromone detection mechanisms in *Drosophila* prove similar to those in other insects, this information may have practical application to control of insect pests and disease vectors in the future.

References

Adams MD, Celniker SE, Holt RA, Evans CA, Gocayne JD, Amanatides PG, Scherer SE, Li PW, Hoskins RA, Galle RF, George RA, Lewis SE, Richards S, Ashburner M, Henderson SN, Sutton GG, Wortman JR, Yandell MD, Zhang Q, Chen LX, et al. (2000) The genome sequence of *Drosophila melanogaster.* Science 287:2185–2195.

Bartelt RJ, Schaner AM, Jackson LL (1985) *cis*-Vaccenyl acetate as an aggregation pheromone in *Drosophila melanogaster.* J Chem Ecol 11:1747–1756.

Bigiani A, Mucignat-Caretta C, Montani G, Tirindelli R (2005) Pheromone reception in mammals. Rev Physiol Biochem Pharmacol 154:1–35.

Brand A, Perrimon N (1993) Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development 118:401–415.

Bray S, Amrein H (2003) A putative *Drosophila* pheromone receptor expressed in male-specific taste neurons is required for efficient courtship. Neuron 39:1019–1029.

Campos AR, Grossman D, White K (1985) Mutant alleles at the locus *elav* in *Drosophila melanogaster* lead to nervous system defects. A developmental-genetic analysis. J Neurogenet 2:197–218.

Clyne P, Grant A, O'Connell R, Carlson JR (1997) Odorant response of individual sensilla on the *Drosophila* antenna. Invert Neurosci 3:127–135.

Clyne PJ, Certel SJ, deBryne M, Zaslavsky L, Johnson WA, Carlson JR (1999) The odor-specificities of a subset of olfactory neurons are goverend by acj6, a POU-domain transcription factor. Neuron 22:339–347.

Couto A, Alenius M, Dickson BJ (2005) Molecular, anatomical, and functional organization of the *Drosophila* olfactory system. Curr Biol 15:1535–1547.

Dahanukar A, Hallem EA, Carlson JR (2005) Insect chemoreception. Curr Opin Neurobiol 15:423–430.

de Bruyne M, Foster K, Carlson JR (2001) Odor coding in the *Drosophila* antenna. Neuron 30:537–552.

Dobritsa AA, van der Goes van Naters W, Warr CG, Steinbrecht RA, Carlson JR (2003) Integrating the molecular and cellular basis of odor coding in the *Drosophila* antenna. Neuron 37:827–841.

Elmore T, Ignell R, Carlson JR, Smith DP (2003) Targeted mutation of a *Drosophila* odor receptor defines receptor requirement in a novel class of sensillum. J Neurosci 23:9906–9912.

Fishilevich E, Vosshall LB (2005) Genetic and functional subdivision of the *Drosophila* antennal lobe. Curr Biol 15:1548–1553.

Goldman AL, Van der Goes van Naters W, Lessing D, Warr CG, Carlson JR (2005) Coexpression of two functional odor receptors in one neuron. Neuron 45:661–666.

Hallem EA, Ho MG, Carlson JR (2004) The molecular basis of odor coding in the *Drosophila* antenna. Cell 117:965–979.

Hill AV (1910) The possible effects of the aggregation of the molecules of haemoglobin on its oxygen dissociation curve. J Physiol (Lond) 40:4–7.

Howard RW, Blomquist GJ (2005) Ecological, behavioral, and biochemical aspects of insect hydrocarbons. Annu Rev Entomol 50:371–393.

Kim M-S, Repp A, Smith DP (1998) LUSH odorant binding protein mediates chemosensory responses to alcohols in *Drosophila melanogaster.* Genetics 150:711–721.

Koundakjian EJ, Cowan DM, Hardy RW, Becker AH (2004) The Zuker collection: a resource for the analysis of autosomal gene function in *Drosophila melanogaster.* Genetics 167:203–206.

Koushika SP, Lisbin MJ, White K (1996) ELAV, a *Drosophila* neuron-specific protein, mediates the generation of an alternatively spliced neural protein isoform. Curr Biol 6:1634–1641.

Krieger J, Grosse-Wilde E, Gohl T, Breer H (2005) Candidate pheromone receptors of the silkmoth *Bombyx mori.* Eur J Neurosci 21:2167–2176.

Kruse SW, Zhao R, Smith DP, Jones DNM (2003) Structure of a specific alcohol-binding site defined by the odorant binding protein LUSH from *Drosophila melanogaster.* Nat Struct Biol 10:694–700.

Larsson MC, Dominguos AI, Jones WD, Chiappe ME, Amrein H, Vosshall LB (2004) Or83b encodes a broadly expressed odorant receptor essential for *Drosophila* olfaction. Neuron 43:703–714.

Malnic B, Hirono J, Sato T, Buck LB (1999) Combinatorial receptor codes for odors. Cell 96:713–723.

Nagai T, Sawano A, Park ES, Miyawaki A (2001) Circularly permuted green fluorescent proteins engineered to sense Ca^{2+}. Proc Natl Acad Sci USA 98:3197–3202.

Nakagawa T, Sakurai T, Nishioka T, Touhara K (2005) Insect sex-pheromone signals mediated by specific combinations of olfactory receptors. Science 307:1638–1642.

Pologruto TA, Yasuda R, Svoboda K (2004) Monitoring neural activity and Ca^{2+} with genetically encoded Ca^{2+} indicators. J Neurosci 24:9572–9579.

Robertson HM, Warr CG, Carlson JR (2003) Molecular evolution of the insect chemoreceptor gene superfamily in *Drosophila melanogaster.* Proc Natl Acad Sci USA 100 [Suppl 2]:14537–14542.

Scott K, Brady R, Cravchik A, Morozov P, Rzhetsky A, Zuker C, Axel R (2001) A chemosensory gene family encoding candidate gustatory and olfactory receptors in *Drosophila.* Cell 104:661–673.

Sengupta P, Chou JH, Bargmann CI (1996) odr-10 encodes a seven transmembrane domain receptor required for responses to the odorant diacetyl. Cell 84:899–909.

Shanbhag SR, Müller B, Steinbrecht RA (1999) Atlas of olfactory organs of *Drosophila melanogaster.* 1. Types, external organization, innervation and distribution of olfactory sensilla. Int J Insect Morphol Embryol 28:377–397.

Shanbhag SR, Hekmat-Scafe DS, Kim M-S, Carlson JR, Pikielny C, Smith DP,

Steinbrecht RA (2001) Expression of odorant-binding proteins in *Drosophila* olfactory organs. Microsc Res Tech 55:297–306.

Shanbhag SR, Smith DP, Steinbrecht RA (2005) Three odorant-binding proteins are co-expressed in the sensilla trichodea of *Drosophila melanogaster.* Arthropod Struct Dev 34:153–165.

Smith DP, Shieh B-H, Zuker CS (1990) Isolation and structure of an arrestin gene from *Drosophila.* Proc Natl Acad Sci USA 87:1003–1007.

Stortkuhl KF (2001) Functional analysis of an olfactory receptor in *Drosophila melanogaster.* Proc Natl Acad Sci USA 98:9381–9387.

Suh GS, Wong AM, Hergarden AC, Wang JW, Simon AF, Benzer S, Axel R, Anderson DJ (2004) A single population of olfactory sensory neurons mediates an innate avoidance behaviour in *Drosophila.* Nature 431:854–859.

Vander Meer RK, Obin MS, Zawistowski S, Sheehan KB, Richmond RC (1986) A reevaluation of the role of *cis*-vaccenyl acetate, *cis*-vaccinol, and esterase 6 in the regulation of mated female sexual attractiveness in *Drosophila melanogaster.* J Inect Physiol 32:681–686.

Vander Meer RK, Breed MD, Espelie KE, Winston ML (1998) Pheromone communication in social insects: ants, wasps, bees, and termites. Boulder, CO: Westview Press.

Vosshall LB (2005) Social signals: the secret language of mice. Curr Biol 15:R255–R257.

Vosshall LB, Amrein H, Morozov PS, Rzhetsky A, Axel R (1999) A spatial map of olfactory receptor expression in the *Drosophila* antenna. Cell 96:725–736.

Xu P-X, Atkinson R, Jones DNM, Smith DP (2005) *Drosophila* OBP LUSH is required for activity of pheromone-sensitive neurons. Neuron 45:193–200.