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Report

Receptors and Neurons for Fly Odors in *Drosophila*

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Summary

Remarkably little is known about the molecular and cellular basis of mate recognition in *Drosophila* [1]. We systematically examined the trichoid sensilla, one of the three major types of sensilla that house olfactory receptor neurons (ORNs) on the *Drosophila* antenna, by electrophysiological analysis. We find that none respond strongly to food odors but that all respond to fly odors. Two subtypes of trichoid sensilla contain ORNs that respond to *cis*-vaccenyl acetate (cVA), an anti-aphrodisiac pheromone transferred from males to females during mating [2–4]. All trichoid sensilla yield responses to a male extract; a subset yield responses to a virgin-female extract as well. Thus, males can be distinguished from virgin females by the activity they elicit among the trichoid ORN population. We then systematically tested all members of the *Odor receptor* (*Or*) gene family [5–7] that are expressed in trichoid sensilla [8] by using an in vivo expression system [9]. Four receptors respond to fly odors in this system: Two respond to extracts of both males and virgin females, and two respond to cVA. We propose a model describing how these receptors might be used by a male to distinguish suitable from unsuitable mating partners through a simple logic.

Results and Discussion

ORNs in Trichoid Sensilla Respond to Fly Odors

We measured the responses of ORNs in trichoid sensilla of the antenna by single-unit electrophysiology. We tested all three trichoid-sensilla subtypes, T1, T2, and T3 [10], which contain one, two, and three ORNs, respectively (Figure 1A). These three subtypes occupy distinct but overlapping regions of the antennal surface and together comprise more than 20% of the sensilla in the antennae. Initially, we tested 86 compounds (see Supplemental Experimental Procedures available online), most of which are found in fruits or are fermentation products. These compounds were tested on 60 trichoid sensilla, 30 from males and 30 from females. The compounds were tested in mixtures, and no mixture elicited a response greater than 20 impulses/s (not shown), which represents less than 10% of the maximal response of these ORNs (see below). Some mixtures inhibited the spontaneous activity of T2 and T3 sensilla and produced decreases of 10–20 impulses/s in the action-potential rate. The three most inhibitory odors were subsequently determined to be 1-hexanol, hexyl acetate, and butyl acetate. The paucity of strong excitatory responses to food odors is consistent with the results of an earlier screen with a limited number of chemicals [11]; in this earlier screen, no strong responses were found, although modest responses were elicited by trans-2-hexenal and *cis*-vaccenyl acetate (cVA), an odorant that is considered below.

We next tested the odor of live flies. We placed 50 flies in a glass tube that was closed at both ends with a cotton mesh. Air was puffed through the tube toward the antenna of a fly mounted for electrophysiological recording (Figure 1B, top). We tested 75 individual trichoid sensilla, of all three subtypes, for responses to the odors of both males and virgin females. Air passing over male flies elicited a strong response from ORNs in a large group of trichoid sensilla (Figure 1B, top trace). These ORNs did not respond to the odor of virgin females (Figure 1B, bottom trace). These sensilla correspond to the T1 subtype [10], each of which houses a single ORN. T1 sensilla are found on both male and female antennae; in both cases they respond to the odor of males but not of virgin females. The T2 and T3 sensilla (n = 55) did not produce responses to fly odors when they were tested in this paradigm.

ORNs in Two Subtypes of Trichoid Sensilla Respond to cVA

These experiments showed that at least some trichoid sensilla respond to fly odors. However, we wished to know whether other trichoid sensilla might show responses to fly odors in a more sensitive assay. We therefore developed a new paradigm. Because flies approach each other closely during courtship, we reasoned that some pheromone-sensitive sensilla might be adapted for short-range information reception. Some of the chemical cues that influence courtship behavior in *Drosophila* are present in the cuticle, i.e., on the surface of the fly, and are long-chain unsaturated hydrocarbons [12, 13] of very limited volatility. Although some of these cues are believed to be detected via the taste system [14], it seemed possible that the olfactory system might also contribute to the reception of cuticular components at very close range during courtship.

Accordingly, rather than adding odor stimuli to an air stream directed at the fly from a distance, we presented stimuli by approaching the antenna with the tip of a glass capillary carrying the odor (Figure 1C). This procedure was designed to simulate the proximity of two interacting flies. As an initial test of the feasibility of this paradigm, we drew into the capillary 500 pl of a solution of cVA, which has previously been shown to act as an anti-aphrodisiac pheromone in *Drosophila* [3, 4]; there is also evidence for its playing a role as an aggregation

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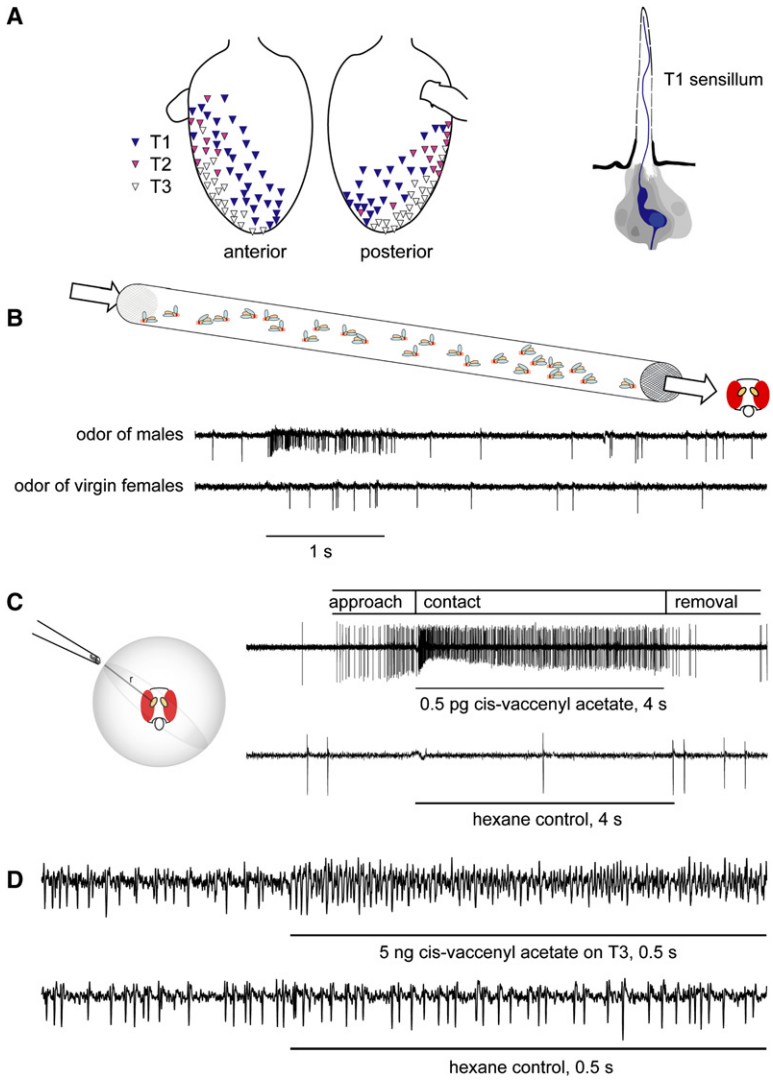

Figure 1. Responses of Trichoid Olfactory Neurons to Male and Female Odors and to *cis*-Vaccenyl Acetate

(A) Map of trichoid sensilla on the anterior and posterior surface of the male antenna; map is adapted from [10]. Female antennae show a similar distribution. T1, T2, and T3 sensilla are innervated by one, two, and three neurons, respectively. A schematic of a T1 sensillum is shown at right; the neuron is in blue, and the cuticle forming the porous hair is in black. Supporting cells are in gray.

(B) Responses to air blown over flies as a source of odors. Top, schematic of fly-odor-delivery system. The odor tube and fly head are not to scale. Bottom, action potentials elicited by air blown over male flies and virgin female flies.

(C) Recording responses with a capillary tip containing the stimulus. Left, schematic of stimulus presentation showing a capillary tip containing material; r indicates the radius at which the impulse rate exceeds the spontaneous rate by more than 20 impulses/s. The capillary tip and fly head are not to scale. Right top, response of the T1 neuron to an intermediate dose of *cis*-vaccenyl acetate. Approach of the stimulus to the sensillum, the time of sensillum contact, and stimulus removal are indicated. Right bottom, control stimulus.

(D) Response to *cis*-vaccenyl acetate in a T3 sensillum (top); response to a control stimulus in T3 (bottom).

pheromone [15, 16]. We found that as the capillary tip approached certain trichoid sensilla, the impulse rates of certain ORNs increased and reached a maximum of >200 impulses/s upon physical contact of the capillary tip with the sensillum shaft (Figure 1C, top trace). Control stimuli prepared with the hexane solvent alone gave no response (Figure 1C, bottom trace).

Having established a short-range delivery paradigm, we systematically examined the responses, initially to cVA, of trichoid sensilla across the entire antennal surface. Mature male flies contain approximately 1 μg of cVA, primarily in the ejaculatory bulb [15]. We loaded a capillary tip with 5 ng of cVA (0.005 fly equivalent) and approached 189 trichoid sensilla individually. We detected strong responses of >100 impulses/s in 169 of the 189 sensilla. Previous reports had shown that the ORN in T1 sensilla responds to cVA [11, 17, 18], and we confirmed this finding (Figure 2A). Responses to 5 ng of cVA exceeded 200 impulses/s in T1 sensilla. Also in agreement with the previous reports, some sensilla immediately adjacent to the zone containing T1 did not respond to cVA [18]. However, we determined that, in addition to the T1 subtype, a large number of sensilla more distolateral on the antennal surface also contained ORNs that are sensitive to cVA in our paradigm (Figures 1D and 2A). Neurons in the distolateral sensilla responded to the cVA stimulus with a rate increase of more than 100 impulses/s. Thus, there appear to be at least two populations of sensilla with ORNs that respond to this pheromone.

The Trichoid ORN Ensemble Distinguishes the Odors of Males and Virgin Females

To expand the scope of our analysis from a single defined pheromone, cVA, to a broad representation of the cuticular pheromone profile, we prepared hexane extracts of males and virgin females. Approximately 500 pl of extract was drawn into the capillary tip; this amount is equal to 0.25% of the material extracted from a single fly.

When a male extract was used as the odor source, all 147 trichoid sensilla tested, from all regions of the antennal surface, yielded responses (Figure 2B). Different ORNs began to respond to the approaching odor source at different distances. The T1 sensilla, which house a single ORN, appeared to be particularly sensitive; they showed responses greater than 20 impulses/s when the odor source came within a 1 cm radius. As the

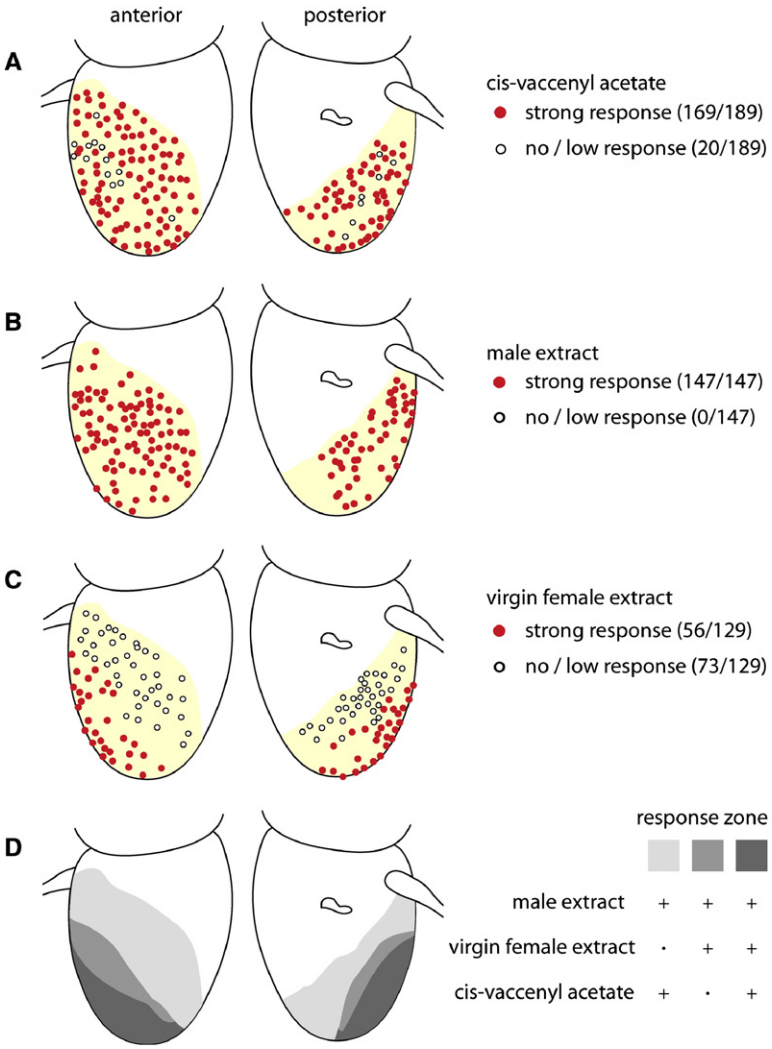

Figure 2. Anterior and Posterior View of the Right Antenna and the Locations of the Trichoid-Sensillum Recordings

Lateral is to the left in the anterior and to the right in the posterior view. (A) *cis*-vaccenyl acetate, (B) male extract, and (C) virgin-female extract. Neurons in sensilla responded with a combined firing rate of more than 100 impulses/s to the stimuli applied (strong response, red circles) or showed little or no response (less than 30 impulses/s, open circles). (D) Distribution of responses to extracts and to *cis*-vaccenyl acetate.

odor source became still closer, the impulse rates increased rapidly. ORNs in T2 and T3 sensilla appeared to be less sensitive and had impulse rates increasing only after the odor source approached a distance of 200 μm, as determined with an ocular micrometer. The responses were dose dependent; when we increased the dose from 0.25% fly equivalent to 5% fly equivalent, the response radius increased from 200 μm to 500 μm.

When an extract from virgin females was used as the stimulus, strong responses were observed in ORNs of all trichoid sensilla except T1 (Figure 2C). Thus, T1 sensilla appear to be tuned to male odor, whereas T2 and T3 sensilla yield strong responses to both males and virgin females. Sensitivity to male and virgin-female extracts was comparable in T2 and T3 sensilla.

The data in Figures 2A–2C include recordings from 405 trichoid sensilla on male antennae. Limited recordings from female antennae (60 sensilla) provided similar results, so the data from male and female antennae were pooled and are shown together in Figure 2. We also tested the extracts and cVA on large basiconic sensilla, a different morphological type of sensilla that house neurons sensitive to food-related odors, and found no responses to any of the fly-derived chemicals.

These in vivo recordings, taken together, demonstrate that trichoid sensilla respond to fly odors and that the odors of males and virgin females are registered differently across the ensemble of trichoid sensilla (Figure 2D). A limitation of the analysis is that it is difficult to ascribe responses to individual ORNs within trichoid sensilla. With the exception of T1, trichoid sensilla contain multiple ORNs. In recordings, this is evident from summation and cancellation events between impulses in the traces. In most cases we were unable to discriminate the activities of the individual ORNs because the action potentials, as recorded extracellularly, did not differ significantly in size or shape. Because of the inability to classify action potentials with confidence, we were unable to determine whether there is a functional subdivision among the ORNs sharing a sensillum. To address this limitation, we took advantage of another experimental system, the "empty neuron" system [9, 19], in an effort to analyze the responses of trichoid sensilla at a higher resolution.

The Molecular Basis of Pheromone Reception

Drosophila contains a family of 60 *Or* (*Odor receptor*) genes [5–7], and the following 12 family members have

been reported to map to individual ORNs of trichoid sensilla [8]: *Or2a*, *Or19a*, *Or19b*, *Or23a*, *Or43a*, *Or47b*, *Or65a*, *Or65b*, *Or65c*, *Or67d*, *Or83c*, and *Or88a*. We expressed each of these 12 *Or* genes in the "empty neuron" system, an in vivo expression system based on a mutant ORN, ab3A, that resides in a basiconic sensillum. The endogenous receptor genes of this ORN, *Or22a* and *Or22b*, are deleted, and the promoter of *Or22a* drives ectopic expression of another odor receptor in ab3A via the *UAS-GAL4* system. The odor responses conferred upon ab3A by the ectopically expressed receptor are then measured by single-unit electrophysiology [9, 19, 20].

We systematically tested the 12 trichoid receptors in the empty-neuron system with a panel of fly-derived chemicals: hexane extracts of males and virgin females, material from the genital regions of flies (males, virgin females, and mated females), and cVA. We obtained the genital odors by drawing a glass capillary, with a tip pulled to a diameter of 3 μm, across the genital region of a fly such that material visibly coated the tip. Preliminary experiments showed that the responses could be quantified most reproducibly not during the approach of a stimulus to the antenna but after the capillary tip contacted the sensillum. We therefore quantified responses mediated by the trichoid receptors by determining impulse rates of the ORN after contact. The 12 receptors were expressed and tested in both male and female recipients with all six stimuli, and no differences between the responses of male and female flies were identified.

Of the 12 receptors, four mediated responses to fly odors in this system (Table 1 and Figure 3). All four, Or47b, Or65a, Or67d, and Or88a, responded to male extract, and their action-potential frequencies increased by 50–200 impulses/s. Two of these receptors, Or65a and Or67d, did not respond to extract from virgin females. The sex specificity of Or65a and Or67d is consistent with a role for these receptors in the detection of male-specific pheromones. The other two receptors, Or47b and Or88a, responded to extract from virgin females; these responses were comparable to those they gave to male extracts. We note that both Or47b and Or88a were previously tested in the empty-neuron system with a panel of 110 odors, most of which were present in fruits and were of widely varying chemical structures, and no excitatory responses were recorded [21]. These results are consistent with the hypothesis that Or47b and Or88a detect a pheromone secreted by both males and females.

Male genital material elicited strong responses from Or65a, Or67d, and Or88a. Genital material from virgin females did not elicit a strong response from any of the 12 receptors. However, material from the genital region of females that were mated 1–4 hr previously produced responses from these three receptors, which, yielded firing rates comparable to those observed with male genital material. These results suggest that during copulation the male transfers compounds that activate these receptors.

One compound that the male transfers to the female during copulation is cVA [3, 4]. Or67d and Or65a both responded to cVA (Table 1; Figures 3C and 3D). The sensitivity of Or67d to cVA is consistent with previous observations; expression studies have shown that *Or67d* is expressed in T1 sensilla [8], which are sensitive to cVA, and ectopic expression of *Or67d* in other trichoid sensilla conferred sensitivity to cVA [18]. However, our results indicate that there are multiple receptors for cVA. Both Or67d and Or65a responded most strongly to cVA among a panel of six related compounds (Figures 3C and 3D; also Figure S1). The two receptors differed in their specificities, however; Or67d gave a relatively stronger response than did Or65a to *cis*-vaccenyl alcohol, for example. We note that our detection of a second cVA receptor, which has not been reported previously, may reflect the sensitivity of the short-range delivery paradigm we have designed.

Table 1. Responses Mediated by Ectopically Expressed Receptors

	Or47b	Or88a	Or67d	Or65a
Male extract	++	+	++	+
Virgin-female extract	++	++	·	·
Male genital material	·	++	+++	++
Virgin-female genital material	·	·	·	·
Mated-female genital material	·	++	+++	++
cis-vaccenyl acetate	·	·	++	+

·, n < 50 impulses/s; +, 50 ≤ n < 100 impulses/s; ++, 100 ≤ n < 150 impulses/s; +++, 150 impulses/s ≤ n.

The response specificity of Or67d, as measured in the empty-neuron system, is nearly identical to that of the ORN in the T1 sensillum (Figure 3E). However, we note that the magnitude of the response to cVA in the expression system is approximately half that in T1 (Figures 3C and 3E). Dose-response curves show that the response threshold is also lower in the native T1 sensillum (Figure 3F); it appears as though the T1 neuron can detect a dose of approximately 10^{-4} ng, whereas the expressed Or67d receptor may require a dose of approximately 10^{-2} ng for detection. We also found slower rise and decay rates (not shown) and higher levels of spontaneous firing in the expression system (12 ± 1.21 impulses/s; SEM, n = 12, compared to, in T1 sensilla, 0.12 ± 0.04 impulses/s; SEM, n = 12). These results suggest that the expression system may lack a component that is present in the endogenous context [22]; for example, the odorant-binding protein LUSH was found to be required for normal response to cVA in T1 sensilla [17].

Whereas Or67d mediates responses to cVA in T1 sensilla, Or65a is expressed in the ORNs of trichoid sensilla that are more distolateral on the antenna and that also respond to cVA. We note that the *Or65a* gene is in close proximity to *Or65b* and *Or65c* and that the three genes are coexpressed in a single ORN [8]. Although neither Or65b nor Or65c mediated responses to any of the fly odors we tested in the empty-neuron system, we considered the possibility that they might contribute to the response of the ORN if they were coexpressed with Or65a, perhaps via heterodimer formation. Accordingly, we coexpressed all pairwise combinations of the three receptor genes and measured responses to all the stimuli indicated in Table 1. We found that coexpression of Or65b or Or65c with Or65a did not increase the response mediated by Or65a to any stimulus or change the level of spontaneous activity. Coexpression of

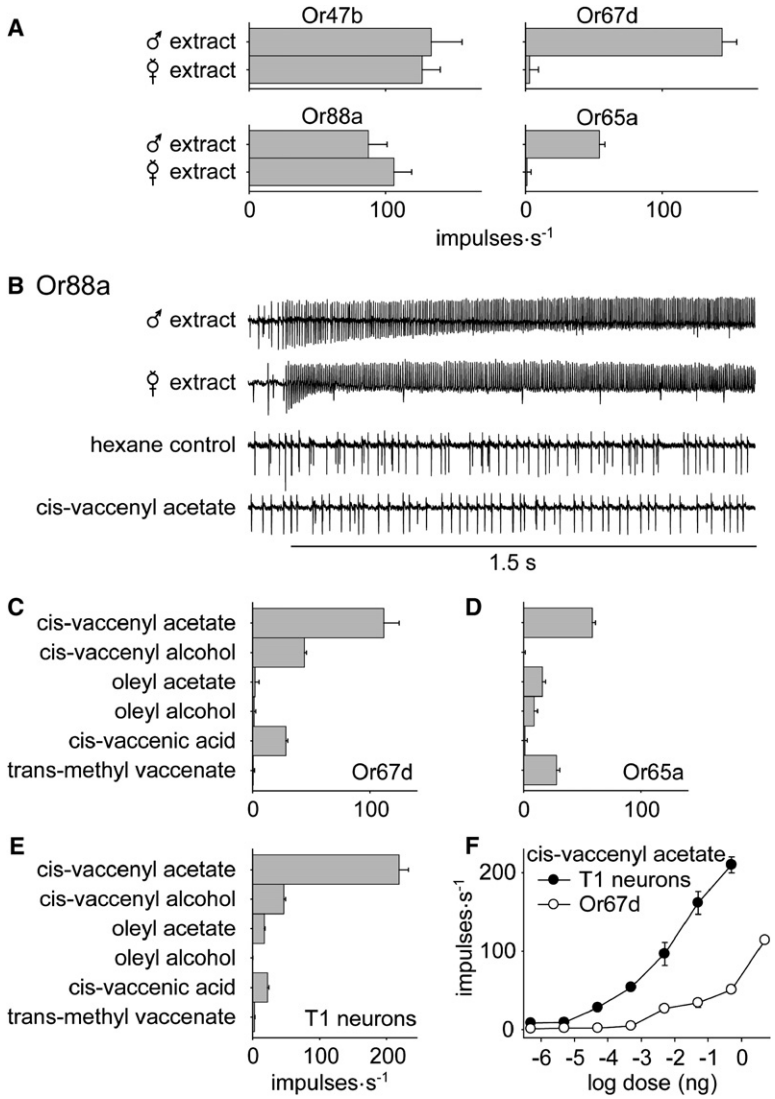

Figure 3. *Or*-Mediated Responses to Male and Virgin-Female Extracts and to cVA and Related Compounds in the In Vivo Expression System

(A) Responses to extracts. Error bars indicate SEM; n = 10–12. (B) Representative traces for Or88a. The shapes and sizes of action potentials vary over the course of stimulation, as observed with other classes of sensilla [31]; there is no consistent difference in the traces obtained with male and female extracts. Responses to cVA and related compounds for (C) Or67d, (D) Or65a, and (E) the native T1 neurons. In (C)–(E), odors were used at a dose of 5 ng. (F) Responses to cVA in the T1 neurons and responses mediated by Or67d in the expression system. In (C)–(F), error bars indicate SEM; n = 10–12.

Or65b and Or65c yielded little, if any, response to any stimulus.

Finally, we note with interest that although Or88a conferred responses to male genital material, it did not mediate responses to cVA (Table 1 and Figure 3B), suggesting that it detects an additional pheromone that is also transferred from males to females upon mating.

Model of the Olfactory Basis of Mate Recognition

We have identified four receptors that mediate responses to fly odors. Or47b and Or88a mediate responses to the odors of both males and virgin females. Or65a and Or67d mediate responses to cVA, a male-specific lipid that is present in male genital material, is presumably extracted in our hexane extracts, and is transferred to females upon mating. Or88a also responds to a compound in male genitalia, but this compound is distinct from cVA.

The responses of these receptors suggest a working model of the olfactory basis of mate recognition by males (Figure 4). In this model, neural activity mediated by Or47b and Or88a reports the proximity of a fly, either male or female. This olfactory recognition may contribute to the recognition mediated by other sensory modalities; recognition of conspecifics is a prerequisite to successful courtship. The activity of Or65a, Or67d, or both would indicate that the partner is a male or a recently mated female; thus, when the antenna of a male is in close proximity to another fly, the activation of Or65a and/or Or67d would report that the other fly is unsuitable as a mate. The lack of a signal from these receptors would permit continued courtship activity by the male.

A well-documented phenomenon can be interpreted in terms of this model. Mature males not only court virgin females but also vigorously court newly eclosed males [23, 24]. Young males, like virgin females, lack cVA [15] and would not be expected to activate Or65a and Or67d, allowing courtship to proceed.

Why would Or65a and Or67d not be activated in the antenna of a male by material in its own genital region? Perhaps very little of the internal genital material is released to the air unless the region is manipulated by a capillary tip or washed in hexane, and perhaps what little is released under natural conditions can normally be detected only at very close range; if cVA were released in large amounts and inhibited mating over a long range,

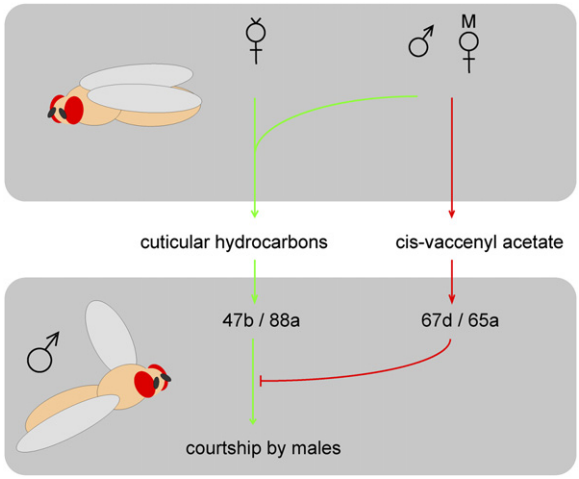

Figure 4. Model of the Olfactory Basis of Mate Recognition by Males

Odors of virgin females, males, and mated females are mediated through the indicated receptors. The "/" between receptors indicates the formal possibility that only one member of each receptor pair may act in mate recognition; moreover, the model does not imply that these are the only receptors acting in the process of male mate recognition.

then mating might be inhibited at sites where flies congregate and often mate, such as rich food sources. It is also possible that the fly adapts to the ambient level of cVA, produced by its own genital region, and is sensitive to increases above that level.

Why are there two cVA receptors, expressed in two distinct ORNs, in different subtypes of trichoid sensilla? There is evidence that cVA serves two functions as a pheromone in *Drosophila*. First, cVA has been shown to act as an anti-aphrodisiac, detering males from courting with a recently mated female [3, 4]. Second, cVA is deposited by females during egg laying, and there is evidence that it enhances the attractiveness of the oviposition substrate to other flies [15, 16]. Perhaps Or65a and Or67d activate two distinct behavioral circuits and thereby separately mediate two functions of cVA in conjunction with other cues.

Interestingly, we did not identify a receptor for female-specific odors, although there is evidence that 7,11-heptacosadiene and 7,11-nonacosadiene, two female-specific hydrocarbons [12, 13], act as aphrodisiacs. It is possible that some of the trichoid receptors respond to these compounds, which we have not tested individually, or other female-specific compounds but do not function efficiently in our expression system. It is also possible that these compounds are detected by gustatory receptors, perhaps members of the Gr family [25]. One class of gustatory neuron, which expresses *Gr68a*, has been shown to be required for normal courtship [14, 26]. Finally, we note the possibility that some of the receptors that did not respond to the tested stimuli might detect pheromones of other *Drosophila* species.

It is striking that we observed no differences between males' and females' antennal responses to any of the fly odors tested. This similarity is in stark contrast to the extreme sexual dimorphism in antennal responses to pheromones in moths, such as *Bombyx mori* [27, 28] and *Manduca sexta* [29, 30]. The similarity in *Drosophila* peripheral olfactory responses suggests that in the fly, differences in male and female behavioral responses may be determined by differences in reception of other classes of sensory input, such as taste information, or by differences in the transmission or processing of olfactory information. It is possible that cVA, for example, is sensed through the same peripheral mechanisms in males and females but that only in males is the primary representation transformed in a way that accords it a negative valence.

In summary, we have carried out a systematic analysis of the trichoid sensilla, one of the three major types of sensilla on the *Drosophila* antenna. We have shown that these sensilla appear to be specialized for sensing fly odors, as opposed to food odors. The differential activity of ORNs in trichoid sensilla provides an olfactory basis for a male's ability to discriminate suitable from unsuitable mating partners. We have further explored the molecular basis of these responses and have identified four odor receptors that mediate responses to fly odors. We have proposed a model in which olfactory information flows through these receptors according to a simple logic. Although the full repertoire of pheromones and receptors has yet to be characterized, it is possible that the model may be richly elaborated without undergoing an alteration in its fundamental logic.

Supplemental Data

Supplemental Data include Experimental Procedures and one figure and are available online at http://www.current-biology.com/cgi/content/full/17/7/606/DC1/.

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