Pheromone reception in fruit flies expressing a moth's odorant receptor

Zainulabeuddin Syed*, Yuko Ishida*, Katherine Taylor†, Deborah A. Kimbrell†, and Walter S. Leal*‡

*Department of Entomology and †Section of Molecular and Cellular Biology, University of California, Davis, CA 95616

Communicated by Bruce D. Hammock, University of California, Davis, CA, September 12, 2006 (received for review June 29, 2006)

We have expressed a male-specific, pheromone-sensitive odorant receptor (OR), *BmorOR1*, from the silkworm moth *Bombyx mori* in an ''empty neuron'' housed in the ab3 sensilla of a *Drosophila* Δ*halo* mutant. Single-sensillum recordings showed that the *BmorOR1*-expressing neurons in the transgenic flies responded to the *B. mori* pheromone bombykol, albeit with low sensitivity. These transgenic flies responded to lower doses of bombykol in an altered stimulation method with direct delivery of pheromone into the sensillum milieu. We also expressed a *B. mori* pheromone-binding protein, *BmorPBP*, in the *BmorOR1*-expressing ab3 sensilla. Despite the low levels of *BmorPBP* expression, flies carrying both *BmorOR1* and *BmorPBP* showed significantly higher electrophysiological responses than *BmorOR1* flies. Both types of *BmorOR1*-expressing flies responded to bombykol, and to a lesser extent to a second compound, bombykal, even without the addition of organic solvents to the recording electrode buffer. When the semiochemicals were delivered by the conventional puffing of stimulus on the antennae, the receptor responded to bombykol but not to bombykal. The onset of response was remarkably slow, and neural activity extended for an unusually long time (>1 min) after the end of stimulus delivery. We hypothesize that *BmorOR1*-expressing ab3 sensilla lack a pheromone-degrading enzyme to rapidly inactivate bombykol and terminate the signal. We also found an endogenous receptor in one of the sensillum types on *Drosophila* antenna that responds to bombykol and bombykal with sensitivity comparable to the pheromone-detecting sensilla on *B. mori* male antennae.

BmorOR1 | BmorPBP | olfaction | signal termination | single-sensillum recordings

The exquisite olfactory system of insects has been intriguing to scientists, particularly since the observation early in the last century that male peacock moths were attracted to female moths and probably flew from several kilometers away to find mates (1). With the discovery of the first sex pheromone from the silkworm moth, *Bombyx mori* (2), it became evident that insects rely on semiochemicals for the recognition not only of potential mates but also, for example, of prey and of specific features of the environment. An array of 17,000 sensilla (3) on the antennae of the silkworm moth detect not only the major constituent of the sex pheromone, bombykol, but also a second compound, bombykal, that is released by the female pheromone gland (4). These pheromone-detecting sensilla house two olfactory receptor neurons (ORNs), one specifically tuned to bombykol and the other to bombykal (4). The selectivity and sensitivity of the system are so remarkable that minimal structural modifications to pheromone molecules render them inactive (5), whereas a single molecule of the natural product is estimated to be sufficient to activate neurons in male antennae (6). Although odorant receptors (ORs) from the silkworm moth have been isolated (7, 8), expressed in heterologous cell systems, and demonstrated to be activated by bombykol and bombykal (9, 10), the molecular basis underlying the extraordinary selectivity and sensitivity of the insect's ''nose'' is still *terra incognita*. Although the ligands (pheromones) are well defined in moths, these insects are not readily amenable to genetic manipulation. Thus, ORs mined from genomes normally have to be tested in *Xenopus* oocytes or other heterologous systems (7, 9, 10). On the other hand, the fruit fly, *Drosophila melanogaster*, is a model organism amenable to genetic manipulation and transgenesis. In these few years of the postgenomic era, we have gained considerable understanding of the molecular basis of insect olfaction because *Drosophila* has served as a model to allow identification and mapping of the ORs *vis-à-vis* types of sensilla/neurons, unveiling features of odor coding and enabling characterization of a mutant (Δ*halo*) that can serve as recipient of heterologous ORs (11–17). Nevertheless, chemical communication in the fruit fly seems to lack the long-range, species-specific sex pheromones commonly encountered in moths. Semiochemicals relevant to the fruit fly are more generic compounds, such as those associated with rotting and fermenting fruits, although some short-range sex pheromones are known (18). Taking advantage of the best of the two worlds, we have expressed a pheromone receptor, *BmorOR1*, and a pheromone-binding protein (PBP), *BmorPBP*, from the silkworm moth in transgenic flies to address issues of the sensitivity, selectivity, and dynamics of the insect olfactory system. Our data refute the hypothesis that a pheromone–PBP complex is essential for receptor activation in an insect system and support a direct pheromone–receptor interaction. We also present evidence suggesting that pheromone-degrading enzymes (PDEs) are *sine qua non* for signal termination and discuss possible roles of PBPs.

Results and Discussion

Innate Response of *Drosophila* ab4 Sensilla to the Silkworm Moth's Pheromone. To determine a possible background response of *Drosophila* antennae to the pheromone constituents of the silkworm moth, we recorded from all large and small basiconic sensilla while challenging with bombykol. Surprisingly, ab4 sensilla responded to the pheromone of the silkworm moth in a dose-dependent manner (Fig. 1; and see Fig. 8*A*, which is published as supporting information on the PNAS web site), whereas all other basiconic sensilla (ab1, ab2, ab3, ab5, ab6, and ab7) remained silent. The ab4 sensilla contain two cells (designated A and B), with (*E*)-2-hexenal being identified as the best stimulus for the A cell (Fig. 8*C*), whereas the B cell was silent to all tested compounds (19). The ab4A cell also responded to bombykal (Figs. 1 and 8*C*) with a profile (dose-dependence, threshold, and kinetics) similar to that observed with bombykol. Responses of the ab4A cells to bombykol and bombykal were recorded not only from wild-type flies (Oregon R), but also from

Author contributions: Z.S., Y.I., and K.T. contributed equally to this work; W.S.L. designed research; Z.S., Y.I., K.T., and D.A.K. performed research; and W.S.L. wrote the paper.

The authors declare no conflict of interest.

Freely available online through the PNAS open access option.

Abbreviations: BmorOR1, OR from the silkworm moth; BmorPBP, PBP from the silkworm moth; DCM, dichloromethane; OR, odorant receptor; ORN, olfactory receptor neuron; PBP, pheromone-binding protein; PDE, pheromone-degrading enzyme.

‡To whom correspondence should be addressed at: Honorary Maeda–Duffey Laboratory, Department of Entomology, University of California, Davis, CA 95616. E-mail: wsleal@ucdavis.edu.

www.pnas.org/cgi/doi/10.1073/pnas.0607874103

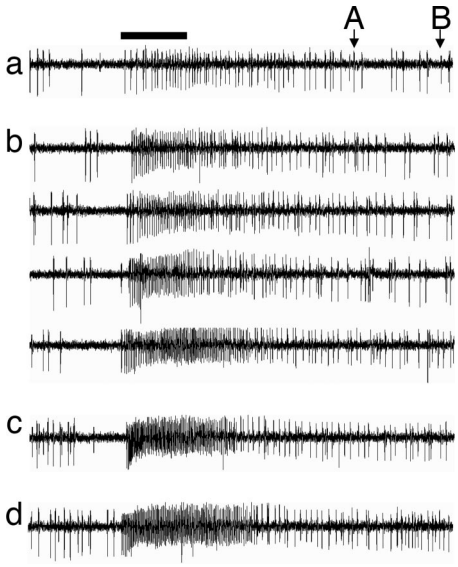

Fig. 1. Action potentials (spikes) from ORNs within an ab4 sensillum on the antenna of *Drosophila* (Oregon R). Individual action potentials (A and B) denote spikes from two ORNs based on their amplitudes. Traces show responses of ab4A to solvent (*a*); increasing doses of bombykol from 0.01 (top trace), 0.1, 1, and 10 μg (bottom trace) (*b*); bombykal (10 μg) (*c*); and (*E*)-2-hexenal (10 ng) (*d*) by the puffing method. (Scale bar, 0.5-s stimulation.)

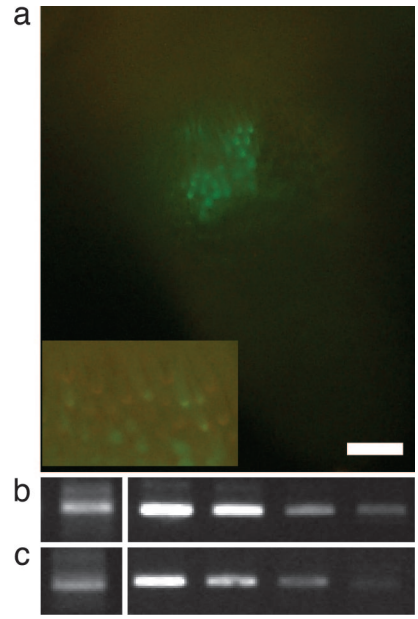

Fig. 2. Expression of GFP in ab3 sensilla and transcripts of *BmorOR1* and *BmorPBP* genes in the antennae of transgenic flies. (*a*) Proximomedial view of the antenna of an *Or22a-Gal4xUAS-GFP* fly under the fluorescent microscope, with GFP luminescence detected in the ab3 sensilla (*Inset*), which are brighter than the neighboring large basiconic sensilla ab1 and ab2. (Scale bar, 20 μm.) (*b*) Measurement of *BmorOR1* gene expression in BmorOR1+BmorPBP flies by RT-PCR. *Left*, 10-antennae equivalent; *Right*, standard PCR products representing $\approx 10^{-20}$, $\approx 10^{-21}$, $\approx 10^{-22}$, and $\approx 10^{-23}$ mol of standard, in order from left to right. (*c*) *BmorPBP* gene expression in the same flies as in *b*. *Left*, 10-antennae equivalent; *Right*, standard PCR products representing $\approx 10^{-20}$, $\approx 10^{-21}$, $\approx 10^{-22}$, and $\approx 10^{-23}$ mol of standard, in order from left to right.

the Δ*halo;Or22a-Gal4* mutants and from flies expressing the *BmorOR1* gene alone (hereafter referred to as BmorOR1 flies) or from flies expressing the *BmorOR1* and *BmorPBP* genes (hereafter referred to as BmorOR1+BmorPBP flies) (see Fig. 8). Interestingly, the onset of responses to both bombykol and bombykal in the ab4A neurons and the kinetics of signal termination (see below) were similar to the profiles obtained with (*E*)-2-hexenal and other stimuli. Despite the fact that ab4A neurons express an OR, *Drosophila* Or7a (15), with significantly low (17.2%) amino acid identity to BmorOR1, the native cell in the wild-type and mutant flies responded to both bombykol and bombykal. The sensitivity of these *Drosophila* sensilla were remarkably comparable to the sensitivity of pheromone-detecting sensilla in the silkworm moth (6), even considering possible differences in electrophysiological setups. These ab4 sensilla with innate response to bombykol and bombykal can be readily discriminated from ab3 (18, 19). This is an important feature for our tests with transgenic flies (see below), because the current *Drosophila* system focuses on ab3 sensilla. The Δ*halo* mutant has a deletion of the two ORs normally expressed in the ab3A neuron, thus producing an "empty neuron" in which an OR can be expressed by using the Gal4-UAS system (13).

BmorOR1 Flies Respond to Bombykol. We expressed in the ab3A neurons the OR BmorOR1, which has been previously isolated from *B. mori* (7, 8) and demonstrated to be sensitive to the pheromone constituents of the silkworm moth when expressed in heterologous noninsect cells (*Xenopus* oocytes and modified HEK cells) (7, 9, 10). Here, we tested by single-sensillum recordings the response of *BmorOR1* expressed in the olfactory system of another insect. Using the empty neuron in the Δ*halo* mutant (13) as the recipient and the construct *Or22a-Gal4* as the driver (13) in the Gal4-UAS system, *BmorOR1* was expressed specifically in the ab3 sensilla (*w/w or Y; Δhalo; UAS-BmorOR1/Or22a-Gal4*). We also used the Gal4 system to drive the expression of *BmorPBP*, a PBP gene from the silkworm moth, so as to generate a mutant coexpressing *BmorPBP* and *BmorOR1* in the ab3 sensilla (*UAS-BmorPBP/w or Y; Δhalo; UAS-BmorOR1/Or22a-Gal4*). Using GFP expression, we confirmed the previously demonstrated fidelity of this expression system (15) and ascertained that expression of receptor and binding protein genes was restricted to the ab3 sensilla (Fig. 2*a*). Transcription of the *BmorOR1* and *BmorPBP* genes was verified by RT-PCR (Fig. 2 *b* and *c*). Expression of *BmorOR1* restricted to the ab3 sensilla was also corroborated by the observation that no other cells, except the ab3A in transgenic flies and the endogenous ab4A (see above), responded to bombykol. Note that the *BmorPBP* gene was expressed at nearly the same level as *BmorOR1* (Fig. 2 *b* and *c*). In moths, PBPs are expressed at high concentrations in the pheromone-detecting sensilla. It has been estimated that in the wild silkmoth, *Antheraea polyphemus*, the concentration of a PBP is as high as 10 mM (20, 21). By comparing the amounts of protein extracted from male antennae of the silkworm moth with pure recombinant BmorPBP, we estimated that in the *B. mori* pheromone-detecting sensilla, BmorPBP is expressed at ≈3 mM (data not shown). Because of the small number of ab3 sensilla in *Drosophila*, it is technically difficult to determine the level of BmorPBP in our transgenic flies, but it was likely below micromolar levels. On the other hand, Kaissling (22) estimated that receptor molecules on the dendrites of the pheromone-detecting sensilla in moth antennae are expressed at levels (≈1.6 μM) at least three orders of magnitude lower than the levels of PBPs.

The Δab3A neuron in control flies (*w/w or y; Δhalo; Or22a-Gal4*) remained silent, showing no spontaneous activity ($n = 30$) (Fig. 3*a*). As observed in transgenic flies expressing other *Drosophila Or* genes (13, 15), a small population of ab3A neurons (6.7%) showed spontaneous activity with low firing (1.5 spikes per second) at irregular intervals. Spontaneous activity and response of ab3B upon heptan-2-one stimulation remained

NEUROSCIENCE

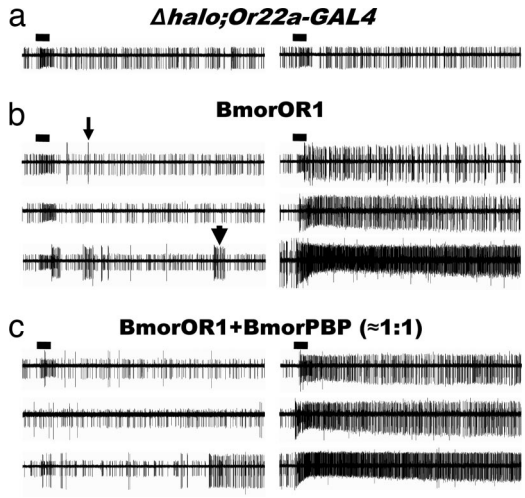

Fig. 3. Action potentials recorded from the ab3A neuron in transgenic flies in response to solvent (*Left*) and to 10 μg of bombykol (*Right*). (*a*) Control flies. (*b*) Typical recordings from flies expressing only the OR from *B. mori*, *BmorOR1*. (*c*) Typical recordings from flies expressing *BmorOR1* and *BmorPBP* in nearly equal molecular amounts. Note the irregular spontaneous spiking activity of the A cell (arrow). Representative traces from each BmorOR1-expressing genotype are shown to highlight variations in response intensity among sensilla and flies. (Scale bar, 2-s stimulus duration.) The irregular bursts (arrowhead) of the ab3A cells, as shown in solvent traces, are typical in flies with Δ*halo* background (13, 15).

unaltered. The Δab3A neuron expressing *BmorOR1* (Δab3A:BmorOR1) always showed spontaneous firing activity (large amplitude spikes), with bursts at irregular time intervals with or without the additional expression of *BmorPBP* in the ab3 sensilla (Fig. 3 *b* and *c*). By contrast, it has been reported that the neurons responding to (*Z*)-11-vaccenyl acetate in the T1 sensilla trichodea of the fruit fly require an odorant-binding protein (LUSH) to produce spontaneous activity (23).

The Δab3A:BmorOR1 neurons responded consistently (except for 1 ORN of 52 tested) to 2-s puffs of bombykol (Fig. 3) in a dose-dependent manner (see Fig. 9, which is published as supporting information on the PNAS web site), but there was no response to bombykal even up to the highest dose tested, 10 μg. For these experiments, we selected the dose of 10 μg, which elicited on average ≈25 spikes per second (Fig. 9). The response of the Δab3A:BmorOR1 neurons in flies expressing *BmorOR1* only [24.6 ± 2.1 spikes per second (mean ± SD); $n = 42$] was not significantly different (Wilcoxon–Mann-Whitney rank-sum test, $P = 0.1$) from the response of the Δab3A:BmorOR1 cells in flies expressing both *BmorOR1* and *BmorPBP* (22.8 ± 1.7 spikes per second; $n = 56$). As will be discussed below, once the receptors were activated they kept firing for at least 1 min.

Direct Stimulation of ab3 Sensilla. Despite consistent responses recorded from BmorOR1 and BmorOR1+BmorPBP flies, the neural activity of ab3A was rather low (≈25 spikes per second) compared with the best ligand for a given ORN in *Drosophila*, which can elicit up to 250 spikes per second (Fig. 8). We hypothesized that the low sensitivity of the ab3 sensilla to bombykol could be due to the low expression of *BmorPBP* in our transgenic flies (Fig. 2) and that higher pheromone doses might be necessary to elicit higher neural activity. Because only a very small fraction of a test stimulus delivered by the puffing method reaches the ORs (5), we tested the response of ab3 sensilla by direct stimulation, as is done in taste recording. The stimuli were incorporated in the saline solution used in the recording glass electrode, with 0.5% ethanol being added to improve the solubility of the hydrophobic pheromones. Other researchers have used DMSO to dissolve pheromones (10, 24), but we found that lower concentrations of ethanol (<5%) have no influence on the conformation of PBPs (data not shown). Firing activity of the ab3A cells in the control experiments (ethanol, but no pheromone) was very low but increased dramatically when the glass electrodes were filled with 160 μM (≈38 ppm) bombykol (Figs. 4 and 5). Response to bombykol in flies expressing both *BmorOR1* and *BmorPBP* was significantly higher than in flies devoid of *BmorPBP* ($n = 7$; Wilcoxon–Mann-Whitney rank-sum test, $P < 0.01$) (Figs. 4 and 5). The simplest explanation for this difference is that, despite the low-level expression, BmorPBP facilitated the diffusion of bombykol into the sensillar lymph. Thus, it is conceivable that high concentrations of PBP, as

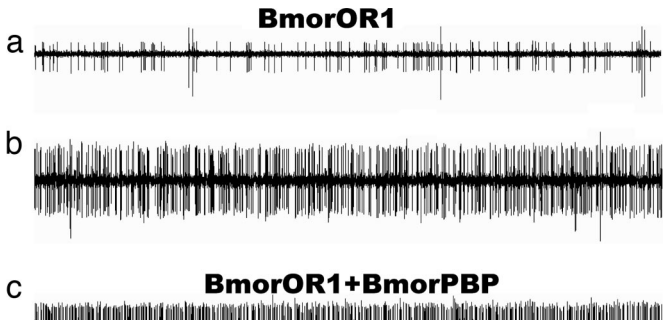

Fig. 4. Direct stimulation leads to increased response from ab3A. Action potentials from ab3A cells in response to (*a*) control containing 0.5% ethanol and (*b*) bombykol (≈38 ppm in 0.5% ethanol) in transgenic flies expressing *BmorOR1*. (*c*) Flies expressing both *BmorOR1* and *BmorPBP* showed stronger response to the same dose of bombykol. We did not observe any changes in response magnitude up to many minutes during recording, thus suggesting that there was no shortage of stimulus supply. Each trace shows a 10-s recording starting immediately after contact was established.

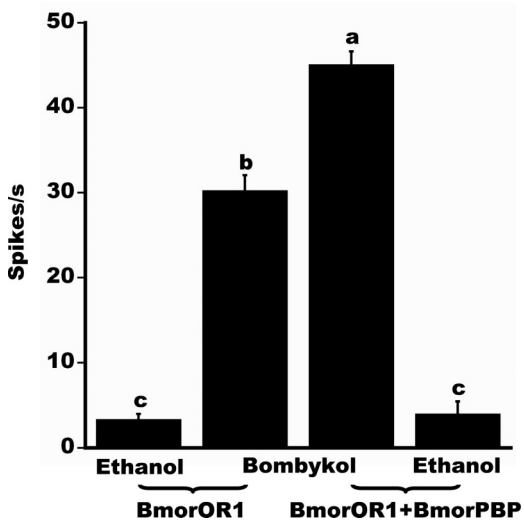

Fig. 5. Neural activity of ab3A cells in transgenic flies BmorOR1 and BmorOR1+BmorPBP in response to control containing 0.5% ethanol and to bombykol (≈38 ppm in 0.5% ethanol), recorded by the direct stimulation method. Treatments labeled with the same letter are not significantly different according to the Wilcoxon–Mann-Whitney rank-sum test. Spike counts were made for at least 100 s (length of each record) but are represented here for 1 s for consistency.

observed in moth sensilla (20, 21), may have an even more dramatic effect on the sensitivity of the insect olfactory system by increasing the uptake of pheromone molecules reaching the port of entry of the sensilla, the pore tubules. Indeed, earlier experiments with the wild silkmoth demonstrated that addition of a PBP to the pheromone-detecting sensilla decreased the threshold for pheromone response by a factor of 100 (25). Although the ab3A cells did not respond to puffs of bombykal (10 μg), we observed a consistent but weaker response to bombykal by direct stimulation at a much lower concentration ($\approx$38 ppm). Spike frequencies generated by bombykal were nearly half those recorded from bombykol. Interestingly, the responses to both semiochemicals were also observed when they were dissolved in the recording saline solution without ethanol, suggesting that despite low solubility there were enough semiochemicals in the recording electrodes to diffuse into the sensilla. When expressed in *Xenopus* oocytes, *BmorOR1* has been shown to respond to bombykol but not to bombykal (7, 9), whereas the receptor expressed in modified HEK cells responded to both bombykol and bombykal (10). In the latter studies, *BmorOR1*-expressing cells responded to both bombykol and bombykal only when the semiochemicals were dissolved in DMSO. In the presence of BmorPBP, however, the cells responded specifically to bombykol, leading to the hypothesis that BmorPBP contributes to the specificity of the olfactory system. By contrast, *BmorOR1* expressed in insect antennae (this work) responded to bombykol, and to a lesser extent to bombykal, in the presence of *BmorPBP* or when devoid of any solubilizers (BmorPBP or organic solvent). It is noteworthy that the empty neuron system seems to be devoid of a rapid bombykol-degrading enzyme, thus allowing delivery of the stimulus in the absence of PBPs, and consequently repeated stimulation (see below). In the natural system, PBP may be required not only for the sensitivity, and possibly selectivity, of the olfactory system but also for the protection of pheromone while it is being transported through the sensillar lymph to the pheromone receptors (26). Because of the low level of PBP expression (Fig. 2) in our transgenic flies, we cannot draw definitive conclusions about the role(s) of PBPs in pheromone detection. However, our data showing that flies expressing *BmorOR1* and devoid of *BmorPBP* responded to bombykol when stimulated directly or by puffing of the pheromone strongly suggest that the semiochemical alone, and not a PBP–pheromone complex, activates the OR.

Pheromone Signal Termination in Mutant Flies. The kinetics of pheromone detection by the *BmorOR1*-expressing ab3A neurons was remarkably different from the dynamics of the natural olfactory systems of insects (Fig. 6). The long half-time (400 ms) for the rise of the receptor potential (Fig. 6*a*) and slow onset of spikes (see below) may be due to the lack of physiologically significant concentrations of BmorPBP in the sensillar lymph of the transgenic flies. The same transgenic flies with intact biochemical machinery for the ab3B neurons showed a fast rise (120 ms) of the receptor potential with a rapid onset of spikes (Fig. 6*b*) when responding to heptan-2-one. Also, the innate detectors for bombykol (ab4A) responded with fast rise (180 ms) of the receptor potential (Fig. 6*c*). Termination of bombykol signal was remarkably slow in transgenic flies expressing the *BmorOR1* gene. The $t_{1/2}$ of the fall of the receptor potential (Fig. 5 *Inset*) in Δab3A:BmorOR1 was as long as 8.8 s, whereas $t_{1/2}$ for the endogenous ab3B and ab4A responding to heptan-2-one and bombykol was 0.32 and 0.23 s, respectively (Fig. 6). Indeed, the electrophysiological profiles from the ab4A neuron in the fruit fly are similar to those shown by the olfactory system of the silkworm moth (4), suggesting that the biochemical milieu of the ab4 sensilla has odorant-binding proteins for the rapid uptake of bombykol (27) and odorant-degrading enzyme(s) (20) for the rapid inactivation of bombykol. In marked contrast, the silkworm moth pheromone receptor expressing ab3A ORN responded to bombykol but lacked the ability to terminate the rather low-strength signal (Fig. 6*a*). In general, even for high-strength signals, the half-time of the fall of the receptor potential is not longer than the duration of the stimulus. In *BmorOR1*-expressing flies, it takes more than four times the stimulus duration for the receptor potential to fall to one-half of its amplitude (Fig. 6).

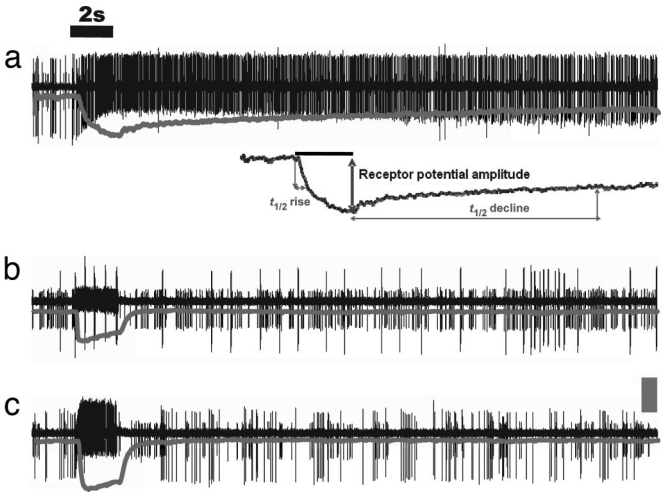

Fig. 6. Differences in response kinetics as observed in simultaneous recordings of action potentials (upper trace; highpass filter) and receptor potentials (lower trace; lowpass filter). (*a*) Bombykol (10 μg) elicited a slow rising response from ab3A that lasted beyond the stimulus duration. (*b*) Heptan-2-one (1 ng) elicited a response in ab3B cells that rose and fell quickly with stimulation. (*c*) Response of ab4A cell stimulated with bombykol (10 ng). Note that the kinetics of the innate neuron (ab4A) responding to bombykol (*c*) was comparable to that of the ab3B cell activated by heptan-2-one (*b*) but differed remarkably from that of the Δab3A:BmorOR1 neuron (*a*). (Vertical scale bar, 16 mV for all receptor potentials.)

Next, we compared the decay of the pheromone signal in transgenic flies (n = 10 for peristimulus time histograms expressing *BmorOR1* only vs. those expressing both *BmorOR1* and *BmorPBP*) (Fig. 7). In both types of flies, the peak of neural activity was reached approximately at the end of the stimulus, and the firing decayed monotonously (Fig. 7). We found no significant difference in the profiles from BmorOR1 and BmorOR1+BmorPBP flies, suggesting that BmorPBP, at least at the levels expressed here, has no influence on the (rise and) decay of the pheromone signal. The simplest explanation for these findings is that termination of these chemical signals is determined not by the ORs nor by PBPs, but rather by PDEs (20, 28, 29). When different *Drosophila Or* genes were expressed in the Δ*halo* mutant background (15), signal termination was similar to what had been observed in the native cells, probably because the ab3 sensilla contain multiple odorant-degrading enzymes, which can inactivate the somewhat ubiquitous compounds tested. Thus, it has been suggested that response termination is determined primarily by the OR rather than by the cellular environment in which the receptor operates (15). We would probably have been led to draw similar conclusions if *BmorOR1* were to be expressed in a hypothetical ab4A empty neuron. The biochemical milieu of the ab4 sensilla has all the olfactory proteins necessary for uptake, transport, delivery, and inactivation of bombykol. Fortunately, the ab3 sensilla in the Δ*halo* mutant seem to be devoid of odorant-degrading enzymes that can rapidly degrade bombykol. It is conceivable that enzymes evolved for the rapid degradation of the long-chain hydrophobic pheromones are more restricted to pheromone-detecting sensilla. For example, the PDE from the wild silk moth

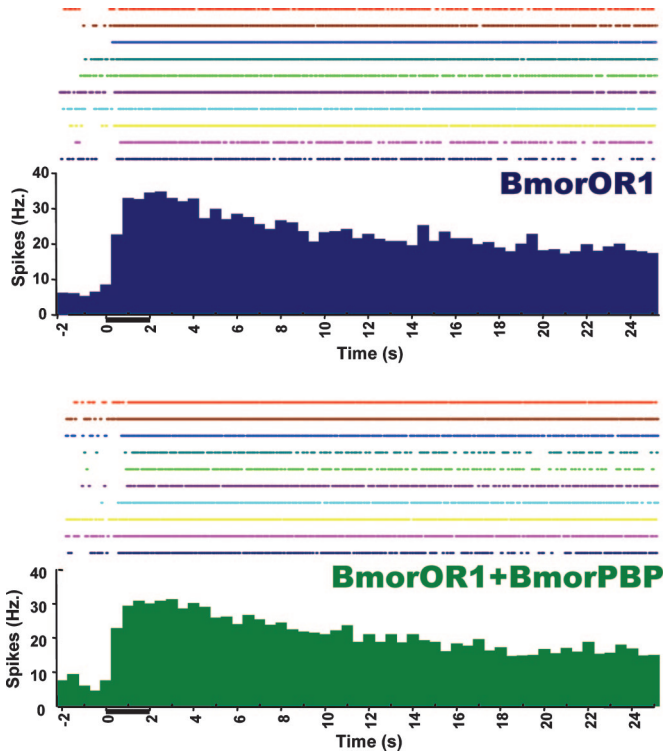

Fig. 7. Responses of 10 different ab3A neurons to bombykol (10 μg) in each type of specified transgenic fly. Dots in each row depict individual spikes, whereas multiples rows show responses from different ORNs. Average firing rates in 500-ms bins for each genotype are shown in peristimulus time histograms. Note that the responses peaked toward the end of the 2-s stimulus period and remained higher than the levels of spontaneous firing activity at the prestimulus.

is expressed specifically in the male antennae, whereas other esterases, including those that might be involved in the degradation of general compounds like plant-derived esters, are more widely distributed (29).

In summary, we have presented evidence that the male-specific OR from the silkworm moth, *BmorOR1* (7–9), can be functionally expressed in the insect system of *Drosophila*. When stimuli were applied directly to the sensillar lymph (through a recording glass electrode), *BmorOR1* responded to both bombykol and bombykal, although the latter generated nearly half of the frequency of the former. Therefore, the native specificity in the silkworm moth antennae with one receptor cell tuned to bombykol and the other neuron responding specifically to bombykal (4) might be derived from some other complementary process(es). Despite the low levels of *BmorPBP* expression, responses of the ab3 cells expressing both receptor and binding protein were significantly higher than responses from flies equipped only with *BmorOR1*, indicating that large concentrations of PBP in the natural system may enhance sensitivity. BmorOR1 responded to both bombykol and bombykal, even when BmorPBP was not present in the sensillar lymph, thus indicating that bombykol alone, not the bombykol–BmorPBP complex, activates the receptor. In the surrogate sensilla of transgenic flies, the pheromone signal could not be terminated rapidly and the receptor responded for at least 1 min after the end of stimulus. Although the ab3 sensilla seem to lack an efficient bombykol-degrading enzyme, the ab4 sensilla appear to be equipped with olfactory proteins required for the uptake, transport, delivery, reception, and inactivation of bombykol. These ab4 sensilla detect bombykol (and bombykal) with sensitivity that rivals the pheromone-detecting sensilla on the silkworm moth antennae and terminate the signals rapidly at the end of the stimulus.

Materials and Methods

***Drosophila* Stocks.** Oregon R flies were used as the standard wild type strain. Strains carrying Δ*halo* and *Or22a-Gal4* (13) were provided by John Carlson (Yale University, New Haven CT). *UAS-GFP* flies were obtained from the Bloomington Drosophila Stock Center (Bloomington, IN; http://flystocks.bio.indiana.edu). Transgenic strains were made that carry the *B. mori* genes for an OR, *BmorOR1*, and for a pheromone-binding protein, *BmorPBP*, driven by a *UAS* promoter. These strains are thus designated as carrying *UAS-BmorOR1* and *UAS-BmorPBP*.

Insect Transgenesis. cDNA was synthesized from day-0 adult antennae of the silkworm moth (Daizo Matsumura strain) by using the SMART RACE cDNA amplification kit (Takara, Kyoto, Japan) and SuperScript II (Invitrogen, Carlsbad, CA) as reverse transcriptase, and subsequently treated with RNase H (New England Biolabs, Ipswitch, MA). On the basis of the published cDNA sequences for BmorPBP (30) and BmorOR1 (7), each ORF region was PCR-cloned and inserted into the pUAST vector multicloning site (31). Each insert in the vector was verified by DNA sequencing, and the P-element vectors were purified using the Plasmid mini kit (Qiagen, Valencia, CA). Transformations of these pUAST constructs into w^{1118} embryos were done by Genetic Services (Cambridge, MA). Single insertion lines of *UAS-BmorOR1* or *UAS-BmorPBP* were established for each of chromosomes X, 2, and 3. Selected lines were further established with Δ*halo* backgrounds and crossed to produce flies expressing both *BmorOR1* and *BmorPBP* using the *Or22a-Gal4* driver.

RT-PCR. To verify and quantify expression, cDNA for 3′-RACE was synthesized from day-1 adult male antennae (three groups of 10) from transgenic flies. *PfuUltra* II Fusion HS DNA polymerase (Stratagene, La Jolla, CA) and Advantage GC-2 polymerase mix (Takara) were used for PCR amplification of BmorPBP and BmorOR1 cDNA fragments, respectively. Gene-specific primers were 5′-CATGGCTGTGGGCTCAGTGGATGCGTCTC-3′ (forward primer for BmorPBP), 5′-CGGGAGCGTGGCGGATAGAATACCAGACGC-3′ (forward primer for BmorOR1), and the long UPM reverse primer in the SMART RACE cDNA amplification kit. The PCR products were confirmed by sequencing after they were subcloned into the EcoRV recognition site of pBluescript SK (+) (Stratagene). The molecular weight of DNA fragments was calculated with the DNA/RNA/Protein/Chemical Molecular Weight Calculator (www.changbioscience.com/genetics/mw.html). After linearization of these sequenced plasmid DNAs by digestion with NotI (New England Biolabs), each insert DNA sequence was reamplified by PCR and gel-purified, and the amount of DNA was measured by UV (OD, 260 nm). Serial dilutions ($\approx 10^{-20}$ to $\approx 10^{-23}$ mol) were prepared as DNA template standards for calibration curves. The following PCR stepwise programs were carried out: 94°C for 3 min; 40 (for *BmorPBP* gene transcript) or 55 (for *BmorOR1* gene transcript) cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min. PCRs were carried out under the same conditions and using a 10-antennae equivalent and the standard serial dilutions as templates. PCR products were separated on 0.8% agarose gels, recorded with a digital camera (Gel Doc EQ; Bio-Rad, Hercules, CA), and quantified with Image SXM software (www.ImageSXM.org.uk).

Stimulus. (*E,Z*)-10,12-hexadecadien-1-ol (bombykol) and (*E,Z*)-10,12-hexadecadien-1-al (bombykal) were purchased from Plant Research International (Wageningen, The Netherlands) and were dissolved in either hexane or dichloromethane (DCM) to

make solutions of 10, 1, 0.1, and 0.01 μg/μl. Sensillum identity was verified by specific ligands identified as best stimuli for each sensillum type (18, 19). We used ethyl acetate 99.8% (Sigma-Aldrich, St. Louis, MO) and pure CO_2 (compressed cylinder; Airgas, Radnor, PA) for ab1; ethyl acetate and ethyl 3-hydroxybutyrate 97% (Fluka, St. Louis, MO) for ab2; ethyl hexanoate 99+% (Fluka) and heptan-2-one 99% (Sigma-Aldrich) for ab3; (*E*)-2-hexenal 98% (Sigma-Aldrich) for ab4; pentyl acetate 99% (Sigma-Aldrich) for ab5; and 1-octen-3-ol 98% (Fluka) and pentyl acetate for ab6 and ab7, respectively. The activity of bombykol was tested in all basiconic sensilla types. All dilutions were made either wt/vol or vol/vol in DCM. An aliquot of a stimulus chemical dissolved in DCM was loaded on a filter paper strip, the solvent was evaporated (30 s), and the strip was placed in a 5-ml polypropylene syringe from which various volumes were ejected (see below). DCM alone and an empty syringe were used as a control. For direct stimulation (see below), 0.5 μl of 32 mM bombykol or bombykal in ethanol was diluted in 99.5 μl of the recording electrode buffer, 0.1 M KCl, whereas the control was prepared by diluting 0.5 μl of ethanol with 0.1 M KCl. For direct stimulation without solvent, 4 μl of a bombykol (or bombykal) solution (1 μg/μl) was transferred to 100-μl V-vials (Wheaton Science Products, Millville, NJ), and the solvent was evaporated with a gentle stream of helium. After addition of 100 μl of 0.1 M KCl, the vial was capped and vortexed for 30 s, and the solutions were used to fill the recording electrodes.

Single-Sensillum Recordings. Electrophysiological recordings were performed on 1- to 10-day-old flies. A fly was mounted on a platform wedged into the narrow end of a truncated plastic pipette tip and placed on a slide. A glass micropipette was used to lift and hold the antenna onto a coverslip in a stable position (19, 32). Chloridized silver wires in drawn-out glass capillaries filled with 0.1 M KCl and 0.5% polyvinylpyrrolidone (PVP) were used as reference and recording electrodes, respectively, except in the direct-stimulation method (below), in which the recording electrode was filled with 0.1 M KCl saline without PVP. The reference electrode was placed in the eye, and under the microscope (BX51WI, ×800 magnification; Olympus, Melville, NY), the recording electrode was brought into contact with the base of the sensillum. Recorded extracellular action potentials were amplified ×1,000 and fed into an IDAC4-USB box (Syntech, Hilversum, The Netherlands) via a high-impedance ($>10^{12}$ Ω) preamplifier, recorded on the hard disk of a PC via a 16-bit analog/digital IDAC4-USB box, and analyzed with Auto Spike version 3.7 software (Syntech). AC signals (action potentials or spikes) were bandpass-filtered between 100 and 10,000 Hz. For the DC signals (receptor potentials/sensillum potentials) a high filter of 3 kHz was used, whereas the lowpass filter was set at DC. The activity of colocated ORNs in single sensilla was assessed based on differences in spike amplitudes. The ORN with the largest spike amplitude was termed A, the second largest B, and so forth. Identity of an ab3 or ab4 sensillum was confirmed by stimulating with heptan-2-one or (*E*)-2-hexenal. These two semiochemicals rather specifically elicit high response from ab3B and ab4A cells. The preparation was held in a humidified air stream delivered at 20 ml/sec via a glass tube (6 mm i.d.), the outlet of which was ≈10 mm from the preparation. This setup resulted in a delay of ≈70 ms due to the travel time of odor from the stimulus source to the antenna. At least five flies of each genotype were recorded, and up to seven sensilla from each fly were tested. Data were pooled because we saw no significant differences between sexes or age groups.

Stimulation. In the puffing method, the preparation was stimulated with a 2-s pulse during which 4 ml of charcoal-filtered air from a 5-ml polypropylene syringe containing the stimulus was added to the main air stream (except in the case depicted in Fig. 1, in which the stimulation was for 500 ms, resulting in 1-ml expulsion). To prevent changes in air flow during stimulation, a charcoal-filtered air flow of 2 ml/sec was delivered via another solenoid valve through a blank syringe into the glass tube and at the same distance from the preparation. During stimulation, the compensatory flow was switched off. For direct stimulation, we essentially used the method commonly used in insect taste recordings, except that we made the contact by penetrating an olfactory sensillum at the base. Data collection and all other methods remained as in the puffing method, except for data recordings that started upon contact with the sensillum.

We thank Dr. John R. Carlson for kindly providing the Δ*halo* and *Or22a-Gal4* stocks, Dr. Marien de Bruyne (Monash University, Victoria, Australia) for the macros used to generate peristimulus time histograms, Dr. Jane-Ling Wang for advice on statistical analysis, and Drs. Shizuo George Kamita and Karl-Ernst Kaissling for suggestions to improve an early draft of the manuscript. This work was supported by National Science Foundation Grant 0234769, National Research Initiative/U.S. Department of Agriculture/Cooperative State Research, Education, and Extension Service Grant 2003–35302-13648, and National Institutes of Health Grant 1U01AI058267–01 (all to W.S.L.) and by National Aeronautics and Space Administration Grant NNA04CC76A (to D.A.K.).

1. Fabre J-H (1916) *Souvenirs Entomologique*; trans de Mattos AT *The Life of the Caterpillar* (Dodd, Mead, New York).
2. Butenandt A, Beckmann R, Stamm D, Hecker E (1959) *Z Naturforsch* 14b:283–284.
3. Steinbrecht RA (1970) *Z Morph Tiere* 68:93–126.
4. Kaissling K-E, Kasang G, Bestmann HJ, Stransky W, Vostrowsky O (1978) *Naturwissenschaften* 65:382–384.
5. Kaissling K-E (1987) *R. H. Wright Lectures on Insect Olfaction*, ed Colbow K (Simon Fraser Univ, Burnaby, BC, Canada).
6. Kaissling K-E, Priesner E (1970) *Naturwissenschaften* 57:23–28.
7. Sakurai T, Nakagawa T, Mitsuno H, Mori H, Endo Y, Tanoue S, Yasukochi Y, Touhara K, Nishioka T (2004) *Proc Natl Acad Sci USA* 101:16653–16658.
8. Krieger J, Grosse-Wilde E, Gohl T, Breer H (2005) *Eur J Neurosci* 21:2167–2176.
9. Nakagawa T, Sakurai T, Nishioka T, Touhara K (2005) *Science* 307:1638–1642.
10. Grosse-Wilde E, Svatos A, Krieger J (2006) *Chem Senses*, in press.
11. Clyne PJ, Warr CG, Freeman MR, Lessing D, Kim J, Carlson JR (1999) *Neuron* 22:327–338.
12. Vosshall LB, Amrein H, Morozov PS, Rzhetsky A, Axel R (1999) *Cell* 96:725–736.
13. Dobritsa AA, van der Goes van Naters W, Warr CG, Steinbrecht RA, Carlson JR (2003) *Neuron* 37:827–841.
14. Larsson MC, Domingos AI, Jones WD, Chiappe ME, Amrein H, Vosshall LB (2004) *Neuron* 43:703–714.
15. Hallem EA, Ho MG, Carlson JR (2004) *Cell* 117:965–979.
16. Hallem EA, Fox AN, Zwiebel LJ, Carlson JR (2004) *Nature* 427:212–213.
17. Benton R, Sachse S, Michnick SW, Vosshall LB (2006) *PLOS Biol* 4:e20.
18. Stensmyr MC, Giordano E, Balloi A, Angioy A-M, Hansson BS (2003) *J Exp Biol* 206:715–724.
19. de Bruyne M, Foster K, Carlson JR (2001) *Neuron* 30:537–552.
20. Vogt RG, Riddiford LM, Prestwich GD (1985) *Proc Natl Acad Sci USA* 82:8827–8831.
21. Klein U (1987) *Insect Biochem* 17:1193–1204.
22. Kaissling K-E (2001) *Chem Senses* 26:125–150.
23. Xu P, Atkinson R, Jones DNM, Smith DP (2005) *Neuron* 45:193–200.
24. Pophof B (2002) *Naturwissenschaften* 89:515–518.
25. van den Berg MJ, Zielgelberger G (1991) *J Insect Physiol* 37:79–85.
26. Vogt RG, Riddiford LM (1986) in *Mechanisms in Insect Olfaction*, ed Kennedy CEJ (Oxford Univ Press, New York), pp 201–208.
27. Leal WS, Chen AM, Ishida Y, Chiang VP, Erickson ML, Morgan TI, Tsuruda JM (2005) *Proc Natl Acad Sci USA* 102:5386–5391.
28. Maïbèche-Coisne M, Nikonov AA, Ishida Y, Jacquin-Joly E, Leal WS (2004) *Proc Natl Acad Sci USA* 101:11459–11464.
29. Ishida Y, Leal WS (2005) *Proc Natl Acad Sci USA* 102:14075–14079.
30. Krieger J, von Nickisch-Rosenegk E, Mameli M, Pelosi P, Breer H (1996) *Insect Biochem Mol Biol* 26:297–307.
31. Brand AH, Perrimon N (1993) *Development (Cambridge, UK)* 118:401–415.
32. Clyne PJ, Grant A, O'Connel R, Carlson JR (1997) *Invert Neurosci* 3:127–135.