Odorant Reception in Insects: Roles of Receptors, Binding Proteins, and Degrading Enzymes

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Abstract

Our knowledge of the molecular basis of odorant reception in insects has grown exponentially over the past decade. Odorant receptors (ORs) from moths, fruit flies, mosquitoes, and the honey bees have been deorphanized, odorant-degrading enzymes (ODEs) have been isolated, and the functions of odorant-binding proteins (OBPs) have been unveiled. OBPs contribute to the sensitivity of the olfactory system by transporting odorants through the sensillar lymph, but there are competing hypotheses on how they act at the end of the journey. A few ODEs that have been demonstrated to degrade odorants rapidly may act in signal inactivation alone or in combination with other molecular traps. Although ORs in *Drosophila melanogaster* respond to multiple odorants and seem to work in combinatorial code involving both periphery and antennal lobes, reception of sex pheromones by moth ORs suggests that their labeled lines rely heavily on selectivity at the periphery.

INTRODUCTION

Periphery: the external sensory system that conveys information to the central nervous system

Semiochemical: a chemical that conveys information among organisms

Canonical receptor: receptor sensu stricto, one that interacts with odorants

ORN: olfactory receptor neuron

Insects are extremely successful animals whose lives intertwine with ours. They negatively affect human society when they become (*a*) agricultural pests that damage our crops and stored products or (*b*) vectors of diseases that cause tragic human suffering and death. By contrast, many other insect species are beneficial and some, like the honey bee, *Apis mellifera*, are essential for life on our planet as we know it. Insect prominence among other animals is due in part to a key physiological element for their survival and reproduction: a sophisticated olfactory system. Olfaction is orchestrated at various levels, starting with reception of semiochemicals at the periphery, processing of signals at the antennal lobes, integration of olfactory and other sensory modalities in the higher processing centers of the brain, and ultimately translation of olfactory signals into behavior. Thus, the cornerstone of a sophisticated olfactory system is the ability of the insect's peripheral system to selectively detect and rapidly inactivate minute amounts of odorants once they have conveyed information.

The entire olfactory system is heavily dependent on the types of receptors expressed on peripheral olfactory receptor neurons (ORNs; also called olfactory sensory neurons). This has been demonstrated unambiguously by expressing an allospecific pheromone receptor in the ORN that houses the bombykol receptor BmorOR1 in the silkworm moth, *Bombyx mori*. PxylOR1 is a receptor from the diamondback moth, *Plutella xylostella*, that is tuned to the major component of the sex pheromone (11*Z*)-hexadecenal (Z11-16Ald) (74). Electrophysiological and behavioral experiments showed that PxylOR1-expressing male silkworm moths responded equally to bombykol (=E10Z12-16OH) and Z11-16Ald. In brief, when the code is broken at the periphery, the entire olfactory system is tricked. After all, the olfactory code is reliant primarily on chemical language from the external environment than on the electrical language of the brain.

The main focus of this review is early olfactory processing, sometimes called perireceptor events (15), which involves the uptake, binding, transport, and inactivation of odorants, as well as receptor activation and signal transduction. Thus, the scope of this review is reception, not perception, of odorants, which should include further processing at various levels in the central nervous system. Odorants can be classified as chemical signals or chemical cues. They differ in that both emission and reception of a chemical evolved into a signal, whereas evolution acted only on reception, not emission, of a chemical cue (99). Throughout this review these two terms may be used interchangeably given the focus on reception, which evolved in the cases of chemical signals and cues. Here, I refer to semiochemicals or other molecules that activate odorant receptors as odorants, not odors, considering that "when referring to olfactory phenomena prior to and including reception of molecules at the sensory surface we are dealing with odorants and in the chain of subsequent events, whether consciously perceived or not, with odors" (24). The advent of the genome sequences of various insect species, coupled with functional genomics, sensory physiology, structural biology, and chemical ecology studies, triggered a recent exponential growth in our knowledge of the molecular basis of insect olfaction.

THE MOLECULAR MAKING OF THE INSECT OLFACTORY SYSTEM

Overview

The major peripheral olfactory proteins involved in the reception of odorants in insects are odorant-binding proteins (OBPs), odorant-degrading enzymes (ODEs), odorant receptors (ORs), ionotropic receptors (IRs), and sensory neuron membrane proteins (SNMPs). Functions of OBPs are being unraveled by biochemical, biophysical, structural biology, and kinetics studies (23, 49–51, 97, 98, 101–103), as well as RNA interference and electrophysiological studies (4, 68). ORs

from lepidopteran insects (17, 58–60, 62, 93, 94), *D. melanogaster* (20, 21), *Anopheles gambiae* and *Culex quinquefasciatus* (6, 25, 69, 92), and *Apis mellifera* (95) have been deorphanized, and ODEs have been isolated (12, 28, 29). The identification of a new family of IRs, complementary to the OR family and expressed in different olfactory neurons, provides new insights into the molecular mechanisms of odorant reception in insects (2, 78). Moreover, the roles of additional families of putative olfactory proteins, namely, SNMP (3, 30), and structural features of chemosensory proteins, C-minus (80) and plus-C OBPs (42), are emerging.

Deorphanize: to identify odorants that activate a test odorant receptor

Degradation: enzymatic breakdown of an odorant for catabolism, signal termination, or both

OBPs are the liaison between the external environment and ORs (47). Odorants reaching the port of entry, pore tubules, in a sensillum (Step 1, **Figure 1**) are bound (Step 2) and solubilized by OBPs, transported (Step 3) through the sensillar lymph that fills the cavity around the dendrites, and finally activate membrane-bound ORs. Regarding the mode of action of these OBPs, there are two standing, not necessarily mutually exclusive, but dichotomous hypotheses. There is convincing evidence in the literature supporting the notion that in moths, and possibly in other insect species, particularly mosquitoes, a ligand per se activates its OR (see below). By contrast, it has been hypothesized that an OBP from *Drosophila melanogaster*, LUSH (DmelOBP76a), forms an OBP·odorant complex that activates a receptor (44).

The kinetics of the olfactory system requires that stray odorant molecules are inactivated rapidly, i.e., on a millisecond timescale (28), particularly by insects whose navigation is guided by odorants. There are two competing hypotheses regarding signal termination (also called signal inactivation). The first hypothesis is that ODEs participate in pheromone degradation (Step 7, **Figure 1**) so rapidly that it leads to signal termination (28, 29). The second hypothesis is that signals are terminated by a hitherto unknown scavenger (34, 36) for which we coined the term molecular trap (26). A picture of how these olfactory proteins contribute to the making of the insect peripheral olfactory system is now emerging.

Olfactory Receptor Neurons and Odorant Receptors

It was not until the end of the past century that the first insect ORs from the fruit fly, *D. melanogaster*, were identified by a bioinformatics-based approach (7, 14) designed to fish out G-protein-coupled receptor (GPCR)-like olfactory genes, as well as by large-scale sequencing of antennal cDNA library (90).

The aftermath of the OR discovery, however, witnessed a spectacular expansion of our understanding of the molecular basis of insect olfaction. Although the genomes of insects of various taxa, including moths, honey bees, mosquitoes, ants, and beetles, have been sequenced, our current knowledge of insect olfaction is biased heavily toward olfaction in *D. melanogaster*. After all, this is a powerful model species for which a wealth of information and genetic tools are readily available; thus a great deal of research is focused on this species. The current knowledge of insect olfaction is reminiscent of yesteryear's textbook extrapolations in insect physiology that were made on the basis of a few model species investigated. It is therefore prudent to remind the reader that a one-size-fits-all approach is inconsistent with insect diversity. For example, it is unlikely that the selective pressures on the olfactory systems of moths that led to a remarkably sensitive, selective, and dynamic olfactory system have operated in the same fashion in *D. melanogaster*. The behavioral and ecological differences between flies and moths may have implications not only on the type of odorants they detect (general, small-molecule chemical cues versus species-specific, chemical signals, mainly long-chain, fatty-acid-derived pheromones), but also on how they process these semiochemicals.

Although *D. melanogaster* utilizes a fatty-acid-derived compound, (11*Z*)-octadecen-1-yl acetate (also known as *cis*-vaccenyl acetate or Z11-18OAc), as a multifaceted, short-range semiochemical

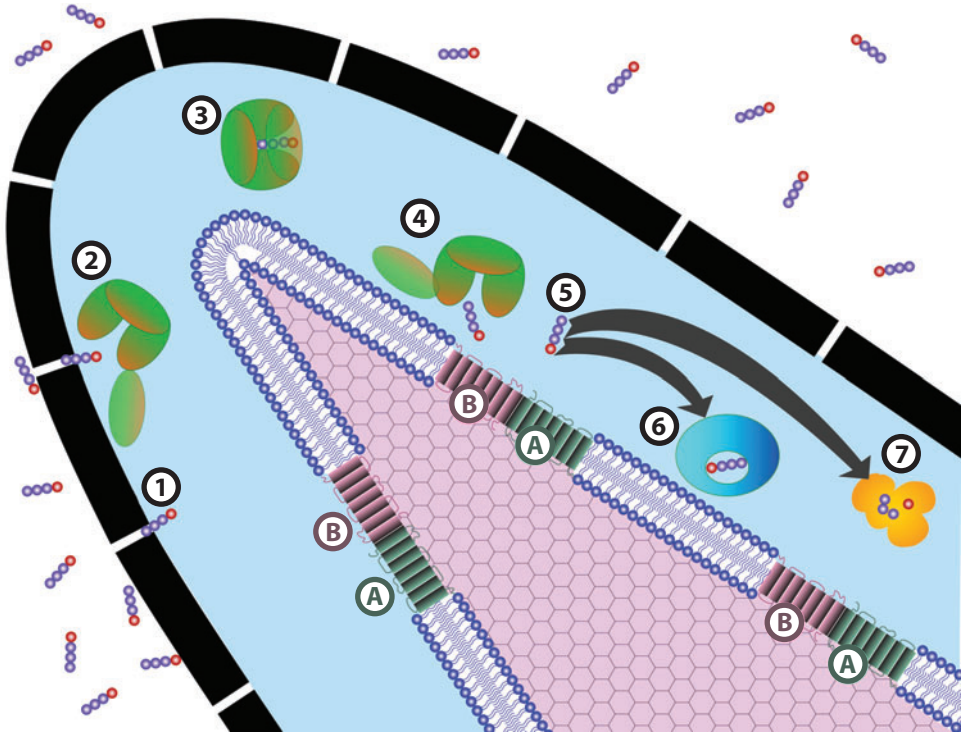

Figure 1

Schematic view of the mechanism of odorant binding, release, and inactivation highlighting three major olfactory proteins discussed in this review. (Step 1) Odorants reach the sensillar lymph through pore tubules and (Step 2) bind to odorant-binding proteins (OBPs). (Step 3) The odorant•OBP complex is transported through the sensillar lymph and activates receptors while bound (LUSH model) or it releases the ligand that directly activates receptors (moth and mosquito OBPs) (Step 4). Odorant receptors work as heteromers (A and B; stoichiometry unknown) with a binding unit (B) and a well-conserved coreceptor, Orco (A). (Step 5) Stray odorants are hypothesized to be inactivated by (Step 6) a hitherto unknown molecular trap and/or by (Step 7) the rapid action of odorant-degrading enzymes. **Figure 1** created by Steven Oerding (UC Davis, IET Mediaworks).

(reviewed in Reference 9), the majority of compounds in the chemical ecology of *D. melanogaster* are ubiquitous semiochemicals (20). These ecological differences are already reflected in the architecture of the peripheral olfactory system of moths and *D. melanogaster*. In *D. melanogaster*, 55 and 60 trichoid sensilla in male and female antennae, respectively, detect Z11-18OAc, whereas the other 360 sensilla are involved in the detection of general odorants (82). By contrast, each male antenna of the silkworm moth has 17,000 pheromone-detecting trichoid sensilla (and approximately 5,000 sensilla detecting general odorants) (77, 81). More strikingly, each male antennae of the polyphemus moth, *Antheraea polyphemus*, has 60,000 pheromone-sensitive trichoid sensilla and 10,000 basiconic sensilla, whereas each female antenna has 12,000 basiconic sensilla but lacks trichoid sensilla (5, 38, 55). Therefore, I argue that the way in which *D. melanogaster* detects general odorants should not be extrapolated to explain pheromone reception in moths and vice versa. There are, however, conserved mechanisms and analogous processing in the olfactory systems of *D. melanogaster* and other insects. The most striking example is the noncanonical OR coreceptor Or83b (43), which is highly conserved among all insect species analyzed thus far (61).

OBP: odorant-binding protein

ODE: odorant-degrading enzyme

OR: odorant (or olfactory) receptor

IR: ionotropic receptor

SNMP: sensory neuron membrane protein

Odorant Receptor Coreceptor

Orco: odorant receptor coreceptor

First shown to be coexpressed with ORs in *D. melanogaster* (43), orthologs of the noncanonical OR Or83b had been identified earlier from moths (41) and mosquitoes (22) and named OR2 and OR7, respectively. Because they seem to perform the same function in all insects and given that the lack of a unified nomenclature was confusing, the old names were abandoned and they have now been renamed odorant receptor coreceptor (Orco) (91). Thus, DmelOrco is a synonym of Or83b and BmorOrco is a new name for BmorOR2. Given that insect ORs were then believed to be GPCRs, the discovery of Orco was a surprise—one of many recent surprises in insect olfaction (19). Orco, highly conserved among insect species (61), is involved in the localization of ORs to ORN dendrites, enhances odorant responsiveness without altering ligand specificity when coexpressed with ORs (1), and forms heteromeric complexes with other ORs (64).

In contrast to conventional GPCR topology, the N terminus of DmelOrco is intracellular, as initially demonstrated by fusing β-galactosidase to the N or C terminus and assaying for enzymatic activity or the lack of it in the cytosol and extracytosolic compartment, respectively (1). By using both endogenous and engineered acceptor sites for N-linked glycosylation as topological markers, the intracellular localization of the N terminus was corroborated, in addition to demonstrating for the first time that both the extracellular loop-2 and the C terminus are indeed extracellular (53). As discussed below, Orco is suggested to be a common subunit of odorant-gated ion channels in insects. By probing multiple *D. melanogaster* OR·DmelOrco complexes with the substitute cysteine accessibility method, it has been suggested that Orco is not involved in odorant binding (65). However, a nonodorant, *N*-(4-ethylphenyl)-2-[[4-ethyl-5-(3-pyridinyl)-4H-1,2,4-triazol-3-yl]thio]acetamide (VUAA1) evokes currents in HEK293 cells expressing either OR·Orco complexes or Orco alone (31). These results suggest that Orco alone can form a ligand-gated ion channel. However, there is no evidence in the literature refuting the hypothesis that odorants activate only the canonical unit of OR·Orco complexes.

OR·Orco complexes are suggested to form ligand-gated nonselective cation channels (see below). Taking into consideration that in various cation channels the negatively charged amino acid residues Glu, Asp, and Tyr form the inner wall of ionic pores and play a role in selective ion permeation, the potential role of these residues in pore formation has been examined extensively in Orco and two ORs, BmorOR1 and BmorOR3 (62). A large number of mutated receptors and mutated Orco have been examined by the *Xenopus laevis* oocyte voltage-clamp technique. Of the 83 tested, 13 point mutations affected ion selectivity, with 2 of them predicted to be located transmembrane domains of ORs (see below) and 1, residue Tyr-464 in BmorOrco, predicted to be located in transmembrane 7. Similar results were obtained with the corresponding Y478ADmelOrco in combination with *D. melanogaster* ORs. The effect of Orco mutation on receptor function was investigated further by expressing wild-type and mutated Orcos in transgenic flies, which were used for electrophysiological recordings. This was done by using the Gal4/UAS system under the control of Orco-Gal4 in *Orco* null mutant flies to express wild-type (DmelOrco and BmorOrco) and mutated coreceptor (Y478ADmelOrco and Y464ABmorOrco). The results were inconsistent with a stand-alone Orco channel, but demonstrated that the tested residues (Tyr-464 in BmorOrco and Tyr-478 in DmelOrco) were essential for receptor function in vivo (61), possibly by forming OR·Orco-specific complexes (see below).

Selectivity of Odorant Receptors

The identification of the first sex pheromones more than five decades ago triggered physiologists' interest in insect olfaction. Probing the system with early techniques such as electroantennogram

and single-sensillum recordings (also known as single-unit recordings) showed not only that the insect's olfactory system is strikingly sensitive, but also that minimal structural modifications render pheromone molecules inactive (33, 37).

This lock-and-key, tight selectivity remains somewhat enigmatic even in this postgenomic era. After all, the discovery and deorphanization of ORs revealed that in general these receptors are not the sole determinants of selectivity. That the glass is half full is due in part to our attempts to address questions not using the most suitable model.

Although moths and other insect species are under tremendous selective pressure to evolve species-specific chemical communication channels, plant- and yeast-derived kairomones play a more important role in chemical communication in *D. melanogaster*. Consequently, it is not entirely surprising that fruit flies and moths process odorants differently. *D. melanogaster* is certainly a wonderful model to address many fundamental questions regarding olfaction, and undeniably, studies using *D. melanogaster* generated a body of knowledge on reception of semiochemicals at the periphery, integration, and perception of odorants. However, I would argue that *D. melanogaster* is not the most suitable model to unravel the molecular mechanisms underpinning selectivity of the insect olfactory system. This is true particularly if one is interested in understanding how pheromone signals evolved to devise new strategies for insect pest control, or even to explore odorant reception for the design of biosensors requiring selective switches. After all, selectivity in *D. melanogaster* does not reside entirely in the periphery.

With the exception of a handful of ORNs on the trichoid sensilla tuned to Z11-18OAc, the majority of sensilla on the antennae and maxillary palps of *D. melanogaster* house ORNs, which broadly respond to odorants, and a single odorant stimulates multiple ORN classes in a peripheral combinatorial way (86). By contrast, a body of literature shows that pheromone-sensitive sensilla on the antennae of moths house ORNs specifically tuned to each constituent of a sex pheromone bouquet (33, 37). One of the most striking examples is the case of *B. mori*, in which sex-pheromone-sensitive ORNs have a very low threshold to bombykol but do not respond to bombykal even when challenged with very high doses (37). Likewise, bombykal-sensitive ORNs do not respond to bombykol at physiological doses.

Bombykol is normally a starting material in bombykal synthesis, so special care must be taken to make certain that chemical impurities do not obscure selectivity. In addition, bombykal is prone to isomerization and degradation, so sample quality is of paramount importance when addressing relevant questions regarding olfaction.

There are multiple steps for processing moth pheromone signals and translating them into behavior, but for selectivity and sensitivity, reception at the periphery is the first relay. In *D. melanogaster*, olfactory system sensitivity seems to be enhanced by a combinatorial code, with multiple ORs (and ORNs) detecting the same compounds, as opposed to a large number of highly tuned pheromone-detecting ORNs in moths. A combinatorial code operates in moths as they process centrally combined information received from various neurons regarding different pheromone components. However, the use of "combinatorial code" here may be misleading, as pheromones are not detected by multiple types of neurons/receptors at the periphery, but rather by specialized neurons in labeled lines (33). In brief, the olfactory systems of moths and flies differ regarding selectivity, which in the former is already manifested at the periphery, whereas in the latter selectivity is achieved by a two-step process—a combinatorial code sensu stricto. Broadly responding receptors and their ORNs are scattered at the periphery but clustered in the antennal lobe (8).

Excellent progress has been made to map ORs vis-à-vis ORNs and sensilla type (20, 21) as well as ORN projections in the antennal lobe (8). This body of knowledge at different levels of

the olfactory system of *D. melanogaster* led to elegant studies performed by lowering doses of stimuli to physiological relevant levels at which odorants were then classified as private stimuli, i.e., those that activate only one ORN type at that dosage, or public stimuli, i.e., odorants that selectively activate a population of other glomeruli (66). By contrast, moth sex pheromones are largely private odorants, unless the system is challenged with extremely high doses, well above physiological levels.

As pheromone receptors, mainly from moths, are deorphanized, a picture regarding selectivity is emerging. There are three distinct types of pheromone-sensitive ORs in moths: (*a*) ORs narrowly tuned to a constituent of the sex pheromone system, (*b*) ORs specifically responding to one of the pheromone components, but still sensitive to other components at higher doses, and (*c*) ORs broadly activated by multiple pheromone components.

The literature on *Bombyx mori* pheromone receptors places BmorOR1 and BmorOR3 in two different groups. Using *X. laevis* oocyte expression system, Nakagawa et al. (62) reported that when coexpressed with BmorOrco both BmorOR1 and BmorOR3 are narrowly tuned to bombykol and bombykal, respectively. Grosse-Wilde et al. (18) used HEK 293 cells, which do not require coexpression of Orco because a test receptor couples to a phospholipase C pathway, leading to a measurable increase of intracellular Ca^{2+} levels when the receptor is activated. In this system, BmorOR3 responded to bombykal in a dose-dependent manner but was not activated by bombykol. By contrast, BmorOR1 responded almost equally to bombykol and bombykal (18). Specificity was achieved with the addition of a silkworm moth's pheromone-binding protein, BmorPBP1 (see below).

A narrowly tuned OR from the European honey bee, *Apis mellifera*, AmelOR11, responded only to the main component of the queen retinue pheromone, 9-oxo-2-decenoic acid (95). Of five ORs from the European corn borer, *Ostrinia nubilalis*, OnubOR6 was narrowly tuned to Z11-16OAc, whereas the others, OnubOR1, OnubOR3, OnubOR4, and OnubOR5, responded broadly to isomers of the sex pheromone and a behavioral antagonist (94). An OR isolated from *Ostrinia scapulalis*, OscaOR1, was narrowly tuned to E11-14OH, a pheromone from a congeneric species, *O. latipennis* (59), whereas OscaOR4 was relatively specific to E11-14OAc, and OscaOR3 responded broadly to conspecific and allospecific pheromone constituents (60).

Receptor specificity has been examined with ORs from three moth species from different subfamilies that utilize somewhat related pheromone compounds. The sex pheromone for the diamondback moth, *P. xylostella*, is composed of Z11-16Ald, Z11-16OAc, and trace amounts of Z11-16OH, whereas the northern armyworm, *Mythimna separata*, utilizes Z11-16OAc and Z11-16OH, and the sex pheromone of the cucumber moth, *Diaphania indica*, is composed of E11-16Ald and E10E12-16Ald (58). While MsepOR1 was narrowly tuned to Z11-16OAc, the main constituent of the congeneric sex pheromone, PxylOR1 responded to Z11-16Ald but showed moderate response to its isomer, E11-16Ald; these receptors were not activated by the other compounds tested, including bombykol. In addition, DindOR1 responded with high specificity to its main pheromone compound, E11-16Ald, but showed a small to moderate response to Z11-16Ald (58). The authors concluded that these receptors showed "strict or high ligand specificity," but the most specific of all receptors tested, MsepOR1, was not challenged with Z11-16OAc isomer E11-16OAc. Apparently, these receptors show a high degree of specificity, but at least two of them cannot discriminate configurational (geometric) isomers. Moreover, it is not known whether ORs sensitive to the other constituents of the sex pheromones of these three species respond broadly or whether they are narrowly tuned. ORs play a significant role in selectivity, but other olfactory proteins may contribute to the overall specificity of the insect olfactory system.

Odorant-Binding Proteins and Their Controversial Roles

DMSO: dimethyl sulfoxide

The above-described experiments with the *X. laevis* oocyte expression system in which ORs·Orco complexes were activated by odorants in the absence of OBPs may be incorrectly interpreted to mean that OBPs are not essential for the function of the olfactory system. These naked receptor experiments are performed with hydrophobic odorants solubilized with dimethyl sulfoxide (DMSO), and the environment differs from the intact olfactory system in which other proteins, including ODEs, are at play. When pheromones were solubilized with cognate pheromone-binding proteins (PBPs), threshold responses from ORs from *Bombyx mori*, *Heliothis virescens*, and *Antheraea polyphemus* were two to three orders of magnitude lower than when pheromones were solubilized with DMSO (13, 17, 18). Similar results were obtained in vivo by expressing the bombykol receptor from *B. mori*, BmorOR1, in the "empty neuron" of *D. melanogaster* (21, 84). Sensitivity to bombykol was significantly enhanced when BmorOR1 was coexpressed with a silkworm PBP, BmorPBP1 (84). Several attempts in my laboratory to knock down BmorPBP1 and test phenotypes by electrophysiology have been unrewarding. It was possible, however, to demonstrate in the malaria mosquito *Anopheles gambiae* and the southern house mosquito, *Culex quinquefasciatus*, that knockdown of OBPs leads to reduced electroantennogram responses elicited by some of the odorants tested (4, 68). It is now accepted that OBPs solubilize ligands, help transport hydrophobic molecules through the aqueous environment of the sensillar lymph, and contribute to the sensitivity of the insect olfactory system. However, if these were the only roles for OBPs, why do the genomes of some species have as many as 50 *OBP* genes? Theoretically, these roles could be performed with just a handful of OBPs, if not with a single protein.

How OBPs deliver odorants to receptors and whether they play a role in selectivity are matters of considerable debate in the field of insect olfaction. Given the enormous diversity of insects, it is not entirely surprising that the literature seems dichotomous regarding the roles and modes of action of olfactory proteins. Two models have been proposed as the mechanisms for OR activation. The first model suggests that an odorant per se activates an OR, and the second suggests that an OR is activated by an OBP·odorant complex.

The mode of action of lepidopteran PBPs has been inferred from biochemical, biophysical, structural, and kinetics studies. Given that BmorPBP1 binds bombykol with high affinity at the sensillar lymph pH, but shows no affinity for the pheromone at low pH, it was postulated that a transition from the high or near-neutral pH (B-form, also called open form) to the low pH (A-form, also called closed form) of the protein is physiologically relevant (98). This pH-dependent conformational change was observed not only with recombinant BmorPBP1 but also with the native protein, which was purified from a large antennal sample (98). Thus, these comparative studies unambiguously demonstrated that periplasmic expression in transformed *Escherichia coli* leads to properly folded, functional PBPs (98) and that the observed pH-dependent conformational change is indeed physiologically relevant.

It was demonstrated previously that the surface of dendrites are negatively charged (39), which led to the notion of bulk and localized pH, with low pH at the surface of dendrites being the consequence of accumulation of protons that neutralize membrane charges (47). Subsequent structural studies confirmed the occurrence of two pH-dependent conformations of BmorPBP1 and showed that pheromone molecules and a α-helix in the C terminus of the protein compete for the binding cavity. A crystal structure of the BmorPBP1·bombykol complex showed that bombykol was bound in a large cavity in the core of the protein (75). A second conformation of BmorPBP1 obtained by NMR at low pH showed that the same binding site identified in the crystal structure at near-neutral pH was filled with a C-terminal α-helix (23), which was flexibly disordered in the BmorPBP1·bombykol complex (75).

When the C terminus of BmorPBP1 was truncated, binding affinity was retained at low pH, as demonstrated with BmorPBP1(1–128) (=BmorPBP1ΔP129-V142) (50). Structural studies with truncated BmorPBP1 led to the conclusions that for the ejection of bombykol the low pH form of BmorPBP1 must be locked by formation of the C-terminal α-helix and that the low pH form must have a higher affinity for intramolecular binding of this helix in the ligand-binding cavity than for bombykol (57). The C terminus of BmorPBP1 is composed mostly of nonpolar amino acid residues, except for three acid residues, Asp-132, Glu-137, and Glu-141, which lie on the surface of the helix and are well-conserved in moth PBPs (46). Of these, residues Asp-132 and Glu-141 form a molecular switch, which upon protonation at low pH triggers the formation of the C-terminal α-helix (101).

Structural studies with AtraPBP1, a pheromone-binding protein from the navel orangeworm, *Amyelois transitella*, showed that the C-terminal α-helix sequestered inside the ligand-binding cavity is attached to the protein core by salt bridges formed by His-80/Glu-132 and His-95/Glu-141 at each end of the helix (103). Mutations of His-80 and His-95 disabled salt bridges and allowed pheromone binding at low pH (102). Thus, deprotonation of His-80 and His-95 at neutral pH abolishes salt bridges that promote detachment and extrusion of the C-terminal helix from the core of the protein, exposing hydrophobic residues to interact with bound pheromone (102).

In summary, biophysical, biochemical, and structural evidence strongly supports that pheromones are released by a pH-dependent conformational change. With functional, properly expressed recombinant samples (98), this conformational change has been shown in PBPs from *B. mori* (23, 57, 75), *Amyelois transitella* (102, 103), and *Antheraea polyphemus* (10), thus suggesting that this is a common feature of lepidopteran PBPs. In addition, kinetics studies showed that release of bombykol from the BmorPBP1-pheromone complex is 10,000-fold faster at low pH and consistent with the dynamics of the insect olfactory system (50). These experimental observations support the hypothesis that, at least in moths, pheromones are transported through the sensillar lymph encapsulated in the binding cavity of PBPs, thus protecting them from aggressive pheromone-degrading enzymes. At the end of the journey, when PBP•pheromone complexes reach the vicinity of dendrites, a localized low pH triggers the release of pheromone to activate ORs. This model is consistent with the observation that naked receptors are activated directly by pheromones (see above). In light of insect diversity, which is strongly manifested in insect physiology, it would be naïve to assume that all OBPs function in the same manner.

There is evidence in the literature, however, that pH-dependent conformational changes might be physiologically relevant for other insect OBPs. For example, a classical OBP (for classification, see References 70 and 71) from *Cx. quinquefasciatus*, CquiOBP1 (27), binds a mosquito oviposition pheromone, (5*R*,6*S*)-6-acetoxy-5-hexadecanolide (MOP) (45), at neutral pH; is expressed in subtypes of sensilla, including one type that responds to MOP; and undergoes a pH-dependent conformational change, with loss of binding affinity at low pH (48). Gene knockdown experiments suggested that CquiOBP1 is involved in MOP detection in vivo (68), as reduced *CquiOBP1* transcripts were correlated with significant reduction of electroantennogram responses. Although these findings indicate that OBPs from moths and mosquitoes might have a common mode of action, the smaller size of mosquito OBPs suggests a different molecular mechanism.

Structural biology studies with OBPs from *An. gambiae*, AgamOBP1 (97), *Ae. aegypti*, AaegOBP1 (52), and *Cx. quinquefasciatus*, CquiOBP1 (54), showed that in contrast to moth PBPs there is no formation of an extended α-helix at the C terminus to compete with pheromone for the ligand-binding pocket. Here the short C terminus covers the binding cavity and is held in place by two hydrogen bonds of the C-terminal carboxylate oxygen, with one hydroxyl group of Tyr-54 and one hydrogen bonding with the δ-nitrogen of His-23 (or Arg-23). We hypothesized that this hydrogen bond triad is a pH-sensing lock that clamps the hinge, the C terminus, onto

a bound odorant, whereas the disruption of this hydrogen bond network at low pH would destabilize the C-terminal loop and unlock the bound ligands (54). Although operating differently at the molecular level, both types of pH-dependent conformational changes lead to loss of binding affinity at low pH with subsequent release of bound odorants. Here, too, OBP·odorant complexes are short-lived at low pH, thus supporting the hypothesis that an odorant per se activates an OR.

Contrary to the direct ligand hypothesis, a completely different mode of signal transduction has been suggested for an OBP from *D. melanogaster*, LUSH (100). Whereas T1 trichoid sensilla in wild-type flies responded to Z11-18OAc, T1 sensilla in a *lush* mutant defective for the expression of LUSH gave no response. Activity in a *lush* mutant was restored when LUSH expression was genetically rescued or added by injection of recombinant LUSH into T1 sensilla. By contrast, expression of ApolPBP1, then named APOL3, in *lush* mutant failed to restore activity (100), implying specificity of LUSH. This was the first and most elegant demonstration of the functional role of an OBP in vivo (100), which is in line with more recent findings by gene knockdown of mosquito (4, 68) and *D. melanogaster* (83) OBPs. Therefore, OBPs are essential for a functional olfactory system, but this is not the case for the hypothesis that OBP·odorant complexes activate ORs.

Structural studies suggested that Phe-121 plays an important role in a conformational change that LUSH undergoes upon binding Z11-18OAc (44). It was then hypothesized that this conformational change mediates activation of olfactory neurons in T1 sensilla. On the basis of the observation that infusion through recording electrodes of recombinant LUSH mutants led to a significant increase of action potential in the absence of pheromone and on the assumption that these mutants mimic pheromone-bound LUSH, it was concluded that LUSH bound to Z11-18OAc activates T1 neurons (44). It is intriguing, however, that the Z11-18OAc receptor, DmelOr67d, was activated by this pheromone (87) when expressed in the empty neuron system (11), which is devoid of LUSH. Ectopically expressed DmelOr67d also showed significant response to Z11-18OAc when the receptor was coexpressed in the empty neuron with a *Drosophila* SNMP (3). Although first discovered in moths (reviewed in Reference 88), the roles of SNMP have been better clarified in *Drosophila*.

An SNMP is essential for the olfactory ensemble of pheromone-detecting ORNs in T1 trichoid sensilla, but it is not required for functioning of basiconic sensilla (3). When the bombykol receptor BmorOR1 was expressed in the empty neuron (ab3A) thus devoid of an SNMP, the ab3A neurons responded to bombykol with low sensitivity (84). Ectopic expression in SNMP-endowed T1 neurons led to a sensitive detection of bombykol comparable to that of the native sensilla in the silkworm moth (85). Although heterologously expressed moth pheromone receptors work in the absence of SNMPs (62), reception of long-chain pheromones in *D. melanogaster* requires SNMP even for ORs transplanted from moths. The lack of an SNMP is manifested in lower sensitivity, as clearly demonstrated by ectopic expression of a moth OR in T1 neurons with or without SNMP. HvirOR13 expressed in DmelOR67-neurons conferred responsiveness to Z11-16Ald. The response was almost abolished in *snmp* mutants and restored by genetic rescue of SNMP (3). In summary, strong evidence in the literature shows that DmelOr67d and moth ORs respond to their cognate ligands in the absence of LUSH, although addition of LUSH does increase sensitivity. To reconcile this apparent conundrum, and support the notion that the LUSH·Z11-18OAc complex activates a receptor, we need experimental evidence to support that (*a*) DmelOR67d is activated by both Z11-18OAc and LUSH·Z11-18OAc, and (*b*) the receptor affinity for the complex is higher than the affinity for the pheromone alone.

LUSH is a unique type of OBP (71). For example, whereas the majority of insect OBPs are acidic proteins, the isoelectric point of LUSH is above 7. Therefore, the majority of olfactory proteins

in insects, including *D. melanogaster*, carry a net negative charge in the sensillar lymph, whereas LUSH carries a net positive charge. LUSH has orthologs in mosquitoes (also Diptera) (70), but not in Lepidoptera. Consequently, LUSH's putative role cannot be extrapolated even to the olfactory system of *D. melanogaster*, let alone insect species in distant taxa. When *Drosophila* ORs were expressed in the empty neuron system, the odorant profile matched those of the native environment (20, 21), suggesting that OBPs in the empty neuron replace those in original environment. The sensitivity of the system can be replicated with surrogated proteins in the empty neuron even for the detection of long-chain hydrophobic pheromones. The sensitivity of the DmelOr7a-neuron, which responds to bombykol in *D. melanogaster*, can be faithfully reproduced by expressing the receptor alone in the empty neuron (85), i.e., without LUSH.

As discussed above, BmorOR1 expressed in HEK 293 cells responded to both bombykol and bombykal (18), in marked contrast to the intact insect olfactory system, which has one type of ORN tuned to bombykol and another type of ORN tuned to bombykal (33, 37) without any cross-talk. Remarkable selectivity was achieved by using BmorPBP1 as a pheromone solubilizer (18). With odorants preincubated with BmorPBP1, BmorOR1 cells responded to bombykol in a dose-dependent fashion, with a threshold of 1 pM and signal saturation at 10^{-10}–10^{-9} M. Intriguingly, however, BmorOR3 cells, which responded to bombykal in DMSO, were not activated by bombykal preincubated with BmorPBP1 (18). It was then suggested that BmorPBP1 binds bombykol but not bombykal, thus augmenting the selectivity of the olfactory system. However, experimental and theoretical evidence in the literature suggests that BmorPBP1 cannot distinguish between these two ligands and binds bombykol and bombykal with apparently the same affinity (16). A general OBP from *B. mori*, BmorGOBP2, can discriminate between bombykol and bombykal, but this OBP is not expressed in the pheromone-detecting sensilla in the silkworm male antennae (105). PBP-enhanced selectivity has also been reported for two other moth species. HEK 293 cells expressing HvirOR13, a pheromone receptor from the tobacco budworm, *Heliothis virescens*, responded broadly to Z11-16Ald, Z11-16OAc, Z9-14Ald, and Z9-16OAc dissolved in DMSO (17). HvirOR13 did not respond or showed very small responses to the same ligands preincubated with HvirPBP1. When these odorants were preincubated with HvirPBP2, the HvirOR13 cells responded with three-orders-of-magnitude-lower threshold and enhanced selectivity toward Z11-16Ald (17). Similar selectivity was observed when HvirOR13 was coexpressed with HvirOrco in the *X. laevis* oocyte recording system devoid of OBPs (93).

The two systems differ in sensitivity. While signals recorded from HvirOR13 cells had a lower threshold and saturated at 10^{-8} M (when odorants were dissolved in DMSO) (17), the threshold of the *X. laevis* system was 10^{-8} M (93). Although performance of the same receptors in these two systems was evaluated at different doses, one would expect that lack of selectivity, not selectivity itself, would be manifested when a system is upset with high doses of ligands. Selectivity was demonstrated at higher doses in the *X. laevis* oocyte system than in the HEK 293 cell system. One obvious advantage of the HEK 293 cell system is the sensitivity provided by coupling a test receptor to an endogenous secondary messenger system that amplifies the signal.

However, the lack of Orco might explain these experimental differences, but dosage of test compounds cannot be completely ruled out. Indeed, it has been demonstrated that HEK 293 cells expressing the *Antheraea polyphemus* receptor ApolOR1 responded to the three constituents of the sex pheromone in DMSO, namely E6Z11-16OAc, E4Z9-14OAc, and E6Z11-16Ald (13). ApolOR1 cells responded also to the three compounds at 1 nM when they were preincubated with ApolPBP1, ApolPBP2, or ApolPBP3. However, at a dose of 1 pM, ApolOR1 cells responded selectively to E6Z11-16Ald only when it was preincubated with ApolPBP2 (13). It is, therefore, difficult to conclude at this point which role, if any, PBPs play in the selectivity of the insect olfactory system.

On the basis of the above-described experiments and given the body of evidence in the literature, it seems reasonable to conclude that at least in the case of moth pheromone receptors an odorant itself activates an OR, otherwise naked receptors would not respond owing to the lack of an OBP·odorant complex. How OBPs contribute to selectivity remains unknown, but it is highly unlikely that odorant·OBP complexes interact differently with ORs.

Odorant-Degrading Enzymes: Inactivation by Degradation?

To effectively utilize a pheromone for orientation while navigating toward a calling female, male moths must be endowed with an olfactory system that not only precisely detects a conspecific pheromone (see above), but also rapidly inactivates the chemical signal once the message is conveyed. That way the subsequent receptor firing (activation of the receptor) or the lack of it faithfully represents pheromone molecules encountered en route to the source of the signal or a detour from the pheromone plume, respectively. From an anthropomorphic perspective, signal reception during odorant-oriented navigation in insects somewhat resembles human sniffing (104), although the latter is a discontinuous process. During flight, the insect olfactory system is in continuous demand to inactivate signal molecules that are a few milliseconds old. Understanding the molecular mechanism(s) of signal inactivation is important in fundamental biology and may lead to novel molecular targets for insect pest control. Mounting evidence in the literature suggests that ODEs, particularly antennae-specific esterases, degrade pheromones so rapidly that odorants may be inactivated by degradation (12, 28, 29). However, we cannot rule out the possibility that insects employ a more generic molecular mechanism in addition to or instead of enzymatic degradation. Indeed, it has been suggested that the dynamics of the insect olfactory system requires a scavenger (34)—for which we coined the term molecular trap (26)—to inactivate pheromone/odorant signals. On the basis of our observation that the truncated form of BmorPBP1 retains binding affinity at low pH (50), it has also been suggested that enzymatic cleavage of the C terminus of BmorPBP1 in vivo is a possible molecular mechanism for signal inactivation (35). Although theoretically possible, this cleavage mechanism must await experimental evidence, including demonstration that endopeptidases in the sensillar lymph rapidly cleave the C terminus of pheromone-bound PBPs. By contrast, three decades of literature support the notion that antennal enzymes indeed degrade pheromones (reviewed in Reference 88).

To satisfy the criteria for an ODE, an enzyme must reside in the sensillar lymph and degrade an odorant (88). These criteria have been fulfilled by antennal esterases, which were also demonstrated to degrade pheromones, and were classified as pheromone-degrading enzymes (PDEs) (12, 28, 29). The most well-studied esterase is the PDE from *A. polyphemus* (28, 40, 89), which we renamed ApolPDE (28). As expected for an olfactory protein, ApolPDE is expressed in the pheromone-detecting sensilla starting at the late pupal developmental stage and peaks at 0.5 μM at day 2 adult stage (28). Consistent with a 17-amino-acid signal peptide (28), ApolPDE is a secreted protein. Kinetic studies with isolated native sensillar esterases (28, 88) and recombinant enzymes (28) showed that ApolPDE rapidly degrades the main component of the sex pheromone E6Z11-16OAc (28). It was estimated on the basis of degradation in vitro that under physiological conditions the half-life of stray pheromone molecules in the sensillar lymph is at most 15 ms (28, 89), which is consistent with the temporal resolution required for sustained odorant-mediated flight through a pheromone plume (28). Similar cases have been reported for other PDEs (12, 29), thus suggesting that antennal esterases evolved for pheromone signal inactivation in insects that utilize an ester as a sex pheromone.

The most convincing evidence that PDEs are involved in pheromone signal inactivation is for esterases, but in addition to acetates (esters), female-produced sex pheromones in moths include

fatty acid–derived straight-chain alcohols and aldehydes, unsaturated hydrocarbons, epoxides, and other compounds. As reviewed elsewhere (88), in vitro experiments have demonstrated that various types of pheromones are degraded by antennal aldehyde oxidases, aldehyde dehydrogenases, epoxide hydrolases, glutathione-*S*-transferases, and cytochrome P450s. However, unambiguous evidence in support of their role in signal termination must await further experiments. First and foremost is the need to demonstrate that these enzymes are part of the sensillar lymph, particularly given that most of them are well-known cytosolic enzymes. In addition, most of these genes have not been cloned, and as a result, there is no evidence to indicate that recombinant enzymes rapidly degrade pheromones.

Earlier biochemical studies put forth convincing evidence that an aldehyde oxidase from *Manduca sexta* is antennae specific and rapidly degrades a pheromone constituent, bombykal (72). On the basis of the extraction procedure it was also suggested that the bombykal-degrading aldehyde oxidases are components of the sensillar lymph (72, 73). Last, it was confirmed that these antennal aldehyde oxidases do not require a soluble cofactor to oxidize aldehydes (72), implying they degrade pheromone molecules in the sensillar lymph. It is now known that AOXs carry molybdenum cofactor, nonheme iron, and flavin adenine dinucleotide as prosthetic groups. The first aldehyde oxidases have been cloned, including an antennae-specific enzyme from *B. mori* (56, 67). As expected for these types of enzymes, they do not have a signal peptide and are likely cytosolic, unless they are translocated by a hitherto unknown mechanism. These inconsistencies must be resolved before we can conclude that in general pheromones are inactivated in the sensillar lymph by the degrading enzymes, as shown for *A. polyphemus* (28).

Given the amphipathic nature of lepidopteran sex pheromones, it is not unconceivable that pheromone molecules diffuse across the dendritic membrane, but this is expected to be a slow process on the timescale of signal inactivation. The question thus remains whether OBPs or other olfactory proteins rapidly trap stray pheromone molecules for subsequent slow degradation.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

The progress in the field of insect olfaction over the past decade has been remarkable. We now know that insect ORs are not GPCRs, as initially envisioned. They are either IRs or seven-transmembrane proteins (ORs) with an inverse topology that form heteromers with a well-conserved coreceptor, Orco. At least for the reception of a long-chain pheromone in trichoid sensilla in *D. melanogaster* antennae an SNMP is required for OR·Orco function, whereas general odorants are detected by basiconic sensilla that do not express SNMPs. In marked contrast to pheromone reception in moths, various neurons housing different types of ORs in *D. melanogaster* and converging onto different glomeruli are activated by the same odorant, generating a combinatorial code in the antennal lobe. In moths, ORNs responding specifically to components of the sex pheromone and converging onto specialized glomeruli form labeled lines. Although they ultimately utilize a combinatorial code at the antennal lobe level (for integration of signals from various pheromone constituents and behavioral antagonists), the two systems differ at the periphery. Highly specific neurons are very important for moths so that they keep isolated chemical communication channels. By contrast, sloppy neurons at the periphery that can thus detect a large array of plant- and yeast-derived compounds and other semiochemicals with a handful of receptors are important for *D. melanogaster*. Specific reception of sex pheromones in moths is achieved in part by ORs that seem to have evolved for specific interaction with pheromones. It is clear, however, that the specificity profile of ORNs is not faithfully reproduced when the ORs housed in these neurons are expressed and examined in heterologous systems devoid of other olfactory proteins.

Challenges remain for unraveling not only the molecular specificity determinants, but also the molecular processes that contribute to the inordinate sensitivity of the insect olfactory system. Though the notion that OBP·odorant complexes activate ORs is appealing as a possible mechanism to enhance specificity of the olfactory system, a body of evidence suggests that odorants alone activate ORs. It is clear, however, that OBPs are important for transporting odorants through the sensillar lymph and increase the sensitivity of the olfactory system. Whether they work differently in different species, by releasing odorants at the end of the journey or retaining ligands to activate ORs, awaits further investigation.

We soon should learn how OR structures contribute to signal amplification and, consequently, to the sensitivity of the insect olfactory system. The now defunct GPCR hypothesis (79) was appealing because it explained the inordinate sensitivity of the insect olfactory system given that the signal would be amplified by secondary messengers. The evidence that ORs form stand-alone channels with Orco is solid (19, 63, 76, 96), but it does not offer a complete explanation for the remarkably low threshold of pheromone detection in vivo (32, 33). In that regard the evidence that they are both ligand-gated and cyclic-nucleotide-activated channels (96) seems more plausible. This along with OR specificity determinants remain exciting areas for future research. Although considerable progress has been made in our understanding of the molecular making of odorant ion channels (61), the molecular basis of OR-odorant interactions remains terra incognita.

SUMMARY POINTS

1. Despite their commonalities, reception of general odorants by *D. melanogaster* and sex pheromones by male moths is strikingly different, and neither should be generalized in toto to describe how insects perceive the chemical world.
2. In *D. melanogaster*, sloppy ORs at the periphery allow the detection of a repertoire of odorants far larger than the numbers of receptors.
3. Male moths detect sex pheromones with tuned ORs, which confer specificity to peripheral ORNs, possibly with the assistance of other olfactory proteins.
4. Odorants are transported to ORs by OBPs, which contribute to the sensitivity and possibly the selectivity of the insect olfactory system.
5. These binding proteins are hypothesized to release odorants that directly activate their receptors, but one specific type, LUSH, is suggested to activate a receptor while bound to a ligand.
6. In moths, signal termination is mediated by PDEs, but solid experimental evidence is limited to esterases, thus raising questions whether other more generic molecular processes are also involved.
7. An SNMP is essential for reception of fatty acid–derived odorants in trichoid sensilla of *D. melanogaster*, but its role in moths remains elusive.
8. ORs and a well-conserved Orco form heteromeric ion channels, but their structures and how they contribute to signal amplification remain to be determined.

DISCLOSURE STATEMENT

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102. Characterizes molecular elements in PBPs leading to reduced pheromone binding at low pH.

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