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REVIEW ARTICLE

INSECT OLFACTORY RECEPTORS: CONTRIBUTIONS OF MOLECULAR BIOLOGY TO CHEMICAL ECOLOGY

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Abstract—Our understanding of the molecular basis of chemical signal recognition in insects has been greatly expanded by the recent discovery of olfactory receptors (Ors). Since the discovery of the complete repertoire of *Drosophila melanogaster* Ors, candidate Ors have been identified from at least 12 insect species from four orders (Coleoptera, Lepidoptera, Diptera, and Hymenoptera), including species of economic or medical importance. Although all Ors share the same G-protein coupled receptor structure with seven transmembrane domains, they present poor sequence homologies within and between species, and have been identified mainly through genomic data analyses. To date, *D. melanogaster* remains the only insect species where Ors have been extensively studied, from expression pattern establishment to functional investigations. These studies have confirmed several observations made in vertebrates: one Or type is selectively expressed in a subtype of olfactory receptor neurons, and one olfactory neuron expresses only one type of Or. In addition, all olfactory neurons expressing one Or type converge to the same glomerulus in the antennal lobe. The olfactory mechanism, thus, appears to be conserved between insects and vertebrates. Although Or functional studies are in their initial stages in insects (mainly *Drosophila*), insects appear to be good models to establish fundamental concepts of olfaction with the development of powerful genetic, imaging, and behavioral tools. This new field of study will greatly contribute to the understanding of insect chemical communication mechanisms, particularly with agricultural pests and disease vectors, and could result in future strategies to reduce their negative effects.

Key Words—Insects, olfaction, molecular biology, olfactory receptors, G-protein coupled receptor, SNMP, CD36, guanylyl cyclase, arrestin, *Caenorhabditis elegans*, *Drosophila melanogaster*, *Anopheles gambiae*.

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INTRODUCTION

Animals have developed highly specialized sensory organs to detect physiochemical characteristics in their environment. These signals are used in many aspects of their lives, including behavior, physiology, and metabolism. Vertebrates and insects share a common design in their olfactory system, with a similar cellular and molecular organization. Insects present particularly good models for basic studies on the sense of smell because they offer the possibility of using genetic engineering to alter their olfactory system, followed by fairly simple behavioral assessments. Moreover, among insects are found economically important agricultural pests and disease vectors, for which olfaction underlies behaviors that are critical for mate or host recognition and selection. Numerous studies have been conducted on chemical ecology of insects since the discovery of the first pheromone in the silkmoth, *Bombyx mori* (Butenandt et al., 1959), including identification and structural determination of chemical signals, and the corresponding electrophysiological and behavioral responses. Until 1980, however, little was known about the molecular elements involved in odorant detection at the level of the antennae. The development of biochemical and molecular studies then increased our knowledge of the molecular basis of chemical communication. In particular, our understanding of chemical senses was greatly expanded by the discovery of olfactory receptor (Or) gene families, first in the vertebrate *Rattus norvegicus* (Buck and Axel, 1991), and then in the nematode *Caenorhabditis elegans* (Troemel et al., 1995). The recent identification of Ors in insects is now providing new opportunities to understand the molecular basis of the general framework of olfaction.

This review focuses on the present knowledge of insect Ors, with some references to vertebrate Ors, in order to emphasize the striking evolutionary convergence towards a conserved organization of signaling pathways in olfactory systems. Olfactory receptors are implicated in one step of the olfactory cascade of events, which consist of combinatorial systems from stereochemical recognition to the generation of an odor code in the brain. Thus, Ors belong to a succession of interactions that, in concert, lead to the animal response. From the discovery of Ors to their first functional studies, we show what the molecular aspects of odor detection may bring to the understanding of chemical communication. Indeed, the discovery of Ors is one of the best examples of the application of the powerful tools of genetics and genomics to chemical ecology. Finally, we discuss the possible practical applications of this new field of study.

GENERAL OLFACTORY PROCESS

Insect chemical odorant messages are translated into neuronal electrical activities through specialized organs, principally the antennae, and processed by

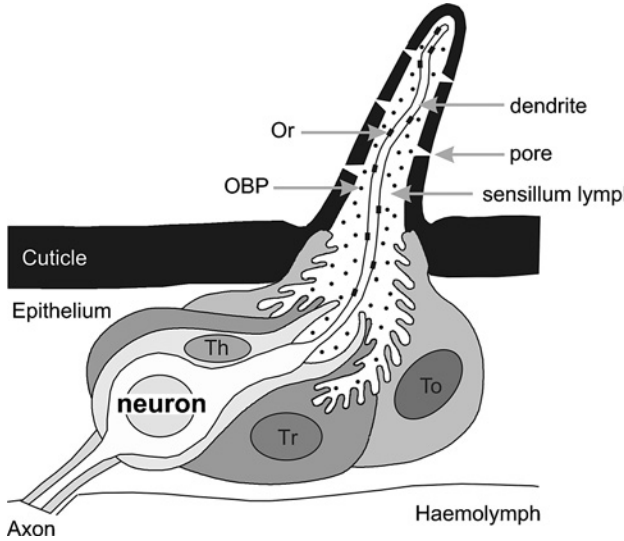

FIG. 1. General organization of an insect olfactory sensillum. Olfactory receptor neurons are bipolar cells: surrounded by accessory cells (To: tormogen, Th: thecogen, and Tr: trichogen cells). The dendrite is bathed in the sensillum lymph. Odorant binding proteins (OBPs) are soluble proteins present in the lymph, whereas olfactory receptors (Ors) are membrane proteins associated with the dendrite (modified from Steinbrecht et al., 1992).

brain centers to elicit behavioral–physiological responses. The antennae are composed of morphofunctional units, the sensilla, which contain one or several bipolar olfactory receptor neurons (ORNs) (Figure 1). The ORNs are specialized at the dendritic end for chemical detection and at the axonal end for neuronal signaling. These specialized sensory neurons transduce the chemical signal into an electrical response and bring the olfactory information from the periphery to the antennal lobes (ALs), which are the first relay stations in the brain (Hansson, 1995; Hildebrand, 1996; Mustaparta, 1996). The dendrites of olfactory neurons are bathed in an aqueous sensillar lymph that protects them from dehydration.

The incoming odor experiences several different extracellular steps, called the perireceptor events or early olfactory processing. These events range from odorant capture to the activation of a neuronal receptor. The generation of the corresponding electrical message, i.e., the intracellular events, is referred to as signal transduction *sensu stricto*. Three protein constituents are involved in the perireceptor events, the odorant-binding proteins (OBPs), the odor-degrading enzymes (ODEs), and the Ors of the sensory neurons (Vogt and Riddiford, 1981). After entering the sensillum through cuticular pores, the odorant molecule has to cross the aqueous lymph to reach the dendrite of the olfactory neuron. As most

odorant molecules are highly volatile and relatively hydrophobic compounds, they are bound by OBPs as they cross the lymph. More than functional carriers, these OBPs appear to act in solubilization and in first selection of olfactory information (reviewed in Leal, 2003). When the odorant–OBP complex arrives at the dendritic membrane, the odor reaches the transmembrane receptor, although it is not clearly understood if the complex dissociates near the receptor, which then binds to the odor alone, or if the complex itself docks with the receptor. Ors then play a dual role. First they allow discrimination among different odorants, as only cells possessing a suitable receptor type will respond to the odorant. Second, they transfer the chemical message from the extracellular to the intracellular face of the membrane upon binding with the ligand (or agonist). This phenomenon elicits a cascade of events leading to the nervous activity. Electrical signals are conveyed onto higher brain centers where they are integrated and contribute to elicit appropriate behavioral responses. Signal termination also plays a critical role in the olfactory process. It involves ODEs, soluble extracellular as well as intracellular membrane-bound and cytosolic enzymes, which are supposed to participate in ligand degradation after their interaction with receptors (Vogt, 2003).

EXPLORATORY INVESTIGATIONS OF OLFACTORY RECEPTORS IN INSECTS

Since the early 1980s, many investigations have been conducted to identify gene products that play a role in odor detection. Involvement of receptor molecules was suspected based on structure activity studies (e.g., Kafka and Neuwirth, 1975; Kikuchi, 1975), and their proteinaceous nature was suggested because chemicals that disrupt protein structure also disrupt odor response pathways (e.g., Villet, 1974; Frazier and Heitz, 1975). Moreover, other studies provided evidence for a second messenger pathway (e.g., Villet, 1978; Wieczorek and Schweikl, 1985) and a G-protein-mediated reaction cascade (Breer et al., 1994) elicited in olfactory sensory cells upon odorant stimulation. Functional approaches were developed to search for putative Ors in insect antennae. These studies led to the discovery of diverse proteins involved in the mechanism of insect olfaction. Vogt and Riddiford (1981) developed a pheromone binding test that allowed identification of proteins from a sensillar extract of the moth, *Antheraea polyphemus*. Using this assay, the first pheromone-binding protein (PBP) and pheromone-degrading esterase (PDE) were discovered. However, no Or was revealed using this assay system (Vogt and Riddiford, 1981).

DISCOVERY OF THE FIRST CANDIDATE Ors IN VERTEBRATES

The first Ors were discovered in the rat olfactory epithelium (Buck and Axel, 1991). These workers used an innovative approach that assumed that the Ors

would be members of the G-protein coupled receptor (GPCR) super family and encoded by genes expressed only in olfactory tissues. Indeed, GPCRs are involved in a variety of cellular processes, such as hormonal regulation, neurotransmission, and photoreception. Using degenerate oligonucleotide primers designed to anneal with conserved regions in the transmembrane domains (TMs) of the GPCR family, Buck and Axel (1991) used polymerase chain reaction (PCR) to amplify and identify complementary DNAs (cDNAs) encoding *Or* genes. Their homology-based approaches proved to be successful and led to the identification of several hundred *Or* genes selectively expressed in olfactory neurons.

Since then, *Or* genes have been identified from a variety of vertebrates, including humans (Ben-Arie et al., 1994), fish (Ngai et al., 1993), and birds (Nef et al., 1996), revealing strong conservation across the Chordata (Mombaerts, 1999). By analyzing genomic DNA sequences from the invertebrate nematode *C. elegans*, Troemel et al. (1995) identified a large family of genes that were expressed in chemosensory neurons and that encoded receptors with seven TMs. Robertson (2001) further characterized this gene family. These genes, named *sr* (serpentine receptor), are extremely divergent within *C. elegans*, and present poor primary sequence identity with vertebrate *Ors*, reflecting an apparently independent evolutionary origin. *Or* number varies widely among species, from approximatly 1000 genes in mammals and 800 in *C. elegans* to approximately 100 in fish and birds (Mombaerts, 1999).

Although thousands of putative Ors have been described in many species, up to now, few studies have reported that the corresponding proteins bind odorants (see "Or Functional Studies").

GENERAL PROPERTIES OF GPCRs

G-protein coupled receptors comprise a large membrane protein family whose members share many common features. They belong to a three-part complex that involves a membrane receptor for external signal reception, a heterotrimeric transducer (G protein), and one of several effector enzymes (e.g., phospholipase C or adenylyl cyclase) leading to the synthesis of second messengers such as inositol 1,4,5-triphosphate ($InsP_3$) or cyclic AMP (cAMP) (Krieger and Breer, 1999; Breer, 2003). These receptors have seven transmembrane regions (TMs) with the N-terminus of the protein situated outside the cell, defining an external domain, and the C-terminus situated inside the cell cytoplasm (Figure 2A) (Buck and Axel, 1991). The seven TMs are characterized by the presence of highly hydrophobic amino acids, and form a seven-α-helix bundle comprising the central core of the receptor. This global structure is common to all GPCRs (Figure 2B). The TMs are linked by six loops, three situated outside the cell and three in the cytoplasm (Figure 2A and B). This structure has been confirmed by the recent and

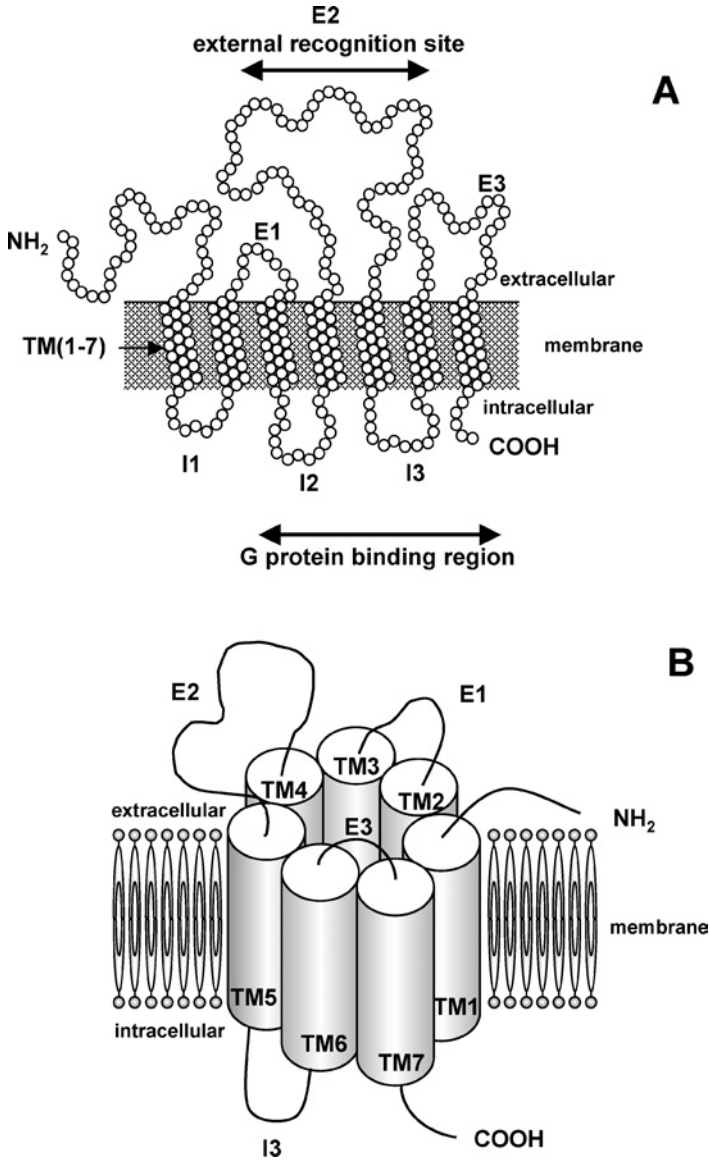

FIG. 2. Generalized olfactory receptor (Or) structure, a specific example of a G-protein coupled receptor. A: amino acid representation showing the seven transmembrane domains (TM), the three extracellular loops (E), and the three intracellular loops (I). Regions that may be involved in ligand binding or G-protein coupling are indicated with a double arrow. B: Or three-dimensional representation in the membrane.

first crystal structure determination of a GPCR, the bovine rhodopsin (Palczewski et al., 2000). To date, this remains the only structure determination of a GPCR.

GPCRs bind a wide number of ligands that range from small organic molecules, nucleotides, lipids, and peptides, to macromolecules such as proteins. Little is known about the exact sites of ligand binding. Candidate areas for ligand binding may include the pocket formed by the membrane spanning segments as well as the external loops (Figure 2B). Length variations in loops and terminal fragments could contribute to functional specificity for ligands and G-proteins (Palczewski et al., 2000). In particular, the second extracellular loop of rhodopsin makes extensive contacts with many extracellular regions as well as with the ligand retinal. Upon binding, the receptor undergoes conformational modifications such as changes in TM orientation and in the structure of the internal loops. These modifications lead to a more open conformation near the receptor G-protein binding site. G-protein activation then induces a cascade of intracellular reactions.

DISCOVERY OF *DROSOPHILA* ODORANT RECEPTOR GENES

Although known vertebrate Ors belong to the GPCR family, they present poor primary sequence identity between vertebrates and the invertebrate *C. elegans*. Perhaps due to these observations, identification of insect Ors has never been achieved by using homology strategies. This suggests that Ors in insects may represent a class of genes unrelated to vertebrates and other invertebrates.

With the beginning of the *D. melanogaster* genome project, which permitted assessment of genomic sequences, several research groups identified a large family of candidate *Drosophila* odorant receptor (*DOr*) genes (Clyne et al., 1999a; Gao and Chess, 1999; Vosshall et al., 1999). Based on the hypothesis that insect Ors should belong to the GPCR family, the three research groups used different algorithms to search for coding exons that encode seven TM proteins. Indeed, as TM domains mainly consist of hydrophobic amino acids, deduced proteins were selected based on their hydropathy profile. Since *Ors* should be expressed in the *Drosophila* olfactory organs (e.g., the antennae and maxillary palpi), candidate genes obtained were subjected to tissue-specific expression analyses. In particular, RNA *in situ* hybridization (ISH) allows specific transcript detection by using a labeled antisense RNA probe that hybridizes to the endogenous mRNA. The three independent approaches brought overlapping data resulting in the discovery of 19 candidate *Drosophila Or* genes, named *Or1–Or19*. These results were later extended with the completion of the *Drosophila* genome (Adams et al., 2000), resulting in the identification of 61 *DOr* genes, each containing seven TM GPCRs (Vosshall et al., 2000; Stocker, 2001). A recent analysis of the updated *Drosophila* genomic sequences (Release 3.1) predicted 62 Or proteins encoded by 60 *Or* genes (Robertson et al., 2003). A standardized nomenclature based on the chromosomal

location of the genes has been established for the *DOr* genes by the *Drosophila* Odorant Receptor Nomenclature Committee (2000).

These *Or* genes encode rather hydrophobic proteins of 370–400 amino acids. Or83b (see "The Particular Case of *Or83b* and its Orthologs"), which possesses 486 amino acids, is an exception. There is a low degree of sequence similarity among the *DOr* genes (17–26% identity), but there are some subfamilies with higher sequence similarity (40–60%). The strongest degree of sequence conservation among genes in the *DOr* family is observed on the 3′ regions that code for TM numbers 6 and 7. No sequence similarity was found between *DOr* genes and the vertebrate or *C. elegans Or* genes (Troemel et al., 1995; Mombaerts, 1999), which explains the failure of the homology-based cloning strategy. In fact, the invertebrate *Ors* form an independent gene family from the vertebrate family. However, their GPCR structure shows similarities with vertebrate Ors, suggesting their functional significance. In particular, DOrs exhibit a relatively large second extracellular loop, as revealed by statistical analyses of length of the receptors (Otaki and Yamamoto, 2003) (Figure 2A). This loop could assist in ligand binding, as suggested from the rhodopsin crystal structure (Palczewski et al., 2000), or alternatively could play a role in dimerization of receptors (Vosshall et al., 2000) (see "The Particular Case of *Or83b* and its Orthologs").

In parallel, the combined efforts of Clyne et al. (2000), Scott et al. (2001), Dunipace et al. (2001), and Robertson et al. (2003) led to the identification of a total of 68 gustatory receptor (Gr) proteins encoded by 60 genes (for a review on *Drosophila* gustatory receptors, see Chyb, 2004). In addition to phylogenetic interrelationship between Ors and Grs (see "Genetic Data, Molecular Evolution, and Developmental Aspects"), four *Gr* genes were found to be expressed in subsets of neurons in the antennae and/or maxillary palpi (Dunipace et al., 2001; Scott et al., 2001). Moreover, neurons expressing one of these *Grs* project axons to glomeruli in the antennal lobe (Scott et al., 2001), suggesting that these Grs may in fact function as Ors.

OR IDENTIFICATIONS IN OTHER INSECT SPECIES

With the discovery of *Drosophila* Or candidates that belong to the GPCR family, it could be postulated that the fundamental molecular nature of Ors, and in particular their GPCR structure, should be conserved across phyla. In addition to pharmacological and immunological evidence for GPCR activity upon odor stimulation in insect antennae (see "Exploratory Investigations of Olfactory Receptors in Insects"), a cDNA coding for a Gq α subunit has been cloned from the antennae of the noctuid moth, *Mamestra brassicae*, and its expression is associated with olfactory sensilla (Jacquin-Joly et al., 2002), suggesting the occurrence of functional GPCRs in moth antennae.

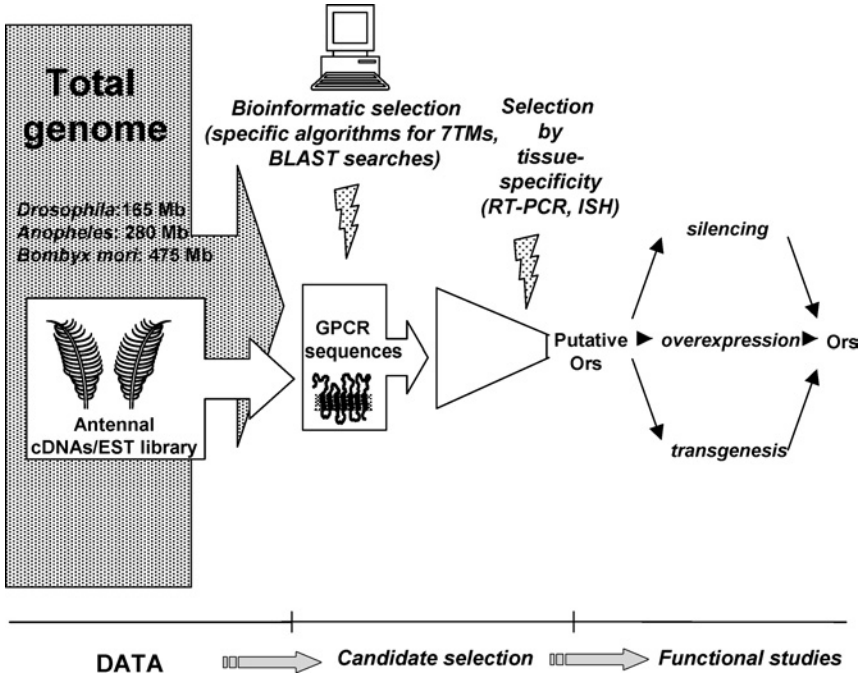

FIG. 3. General procedure used to identify potential *Ors* from insect genomic or cDNA sequence information. Bioinformatics is used to select potential expressed genes that code for proteins with seven TMs. Among these candidates, only genes expressed in olfactory organs are further considered. Functional studies are the final step, and serve to prove involvement in the olfactory process and allow ligand(s) determination. (EST: Expressed sequence tag; ISH: *in situ* hybridization; RT–PCR: Reverse transcription–polymerase chain reaction).

The same protocol as the one used for *Drosophila* (Figure 3), can then theoretically be applied to any insect species to isolate and characterize Ors.

1). *In silico* cloning of candidate GPCR sequences through bioinformatic analyses of sequence databases.
2). Selection among GPCR sequences via expression studies, e.g., reverse transcription–polymerase chain reaction (RT–PCR) on mRNA preparations of different tissues, *in situ* hybridization with olfactory tissues, etc.
3). Final determination through functional studies.

This general scheme, however, prerequires the availability of sequence data that could be achieved through international consortia for whole genome sequencing. Sequence data could also come from smaller-scale efforts such as EST

(expressed sequence tag) libraries developed from expressed genes in a particular tissue, or proteome analysis.

Up to now, the use of genomic data has allowed identification of Or candidates in several other insect species, such as the malaria vector mosquito, *Anopheles gambiae* (Fox et al., 2001, Hill et al., 2002; Pitts et al., 2004), the tobacco budworm, *Heliothis virescens* (Krieger et al., 2002, 2004), and the honey bee, *Apis mellifera* (Robertson, personal communication).

Taking advantage of the initiation of the *A. gambiae* genome sequencing by Genoscope (France), Fox et al. (2001) identified the first candidate odorant receptors from a nondrosophilan insect. Postulating that a similar family of seven TM receptors would also mediate odorant signaling in this species, they used a bioinformatic-homology-based approach to analyze the database for sequences similar to *Drosophila Ors*. Five genes were identified that were selectively expressed in olfactory organs (Fox et al., 2001, 2002; Pitts et al., 2004). After the *A. gambiae* genome sequencing project was completed, a total of 276 GPCRs were identified by using bioinformatics, mainly based on amino acid physico-chemical information (Hill et al., 2002). Among them, 79 candidate odorant receptors (named GPRors or AgOrs) were characterized for tissue expression and, along with 76 candidate gustatory receptors, for their molecular evolution relative to *Drosophila* (Hill et al., 2002). Eighty percent of the *AgOrs* show expression only in olfactory tissues, whereas four *AgOrs* show additional expression in legs. Scott et al. (2001) had previously reported that some *Drosophila Ors* are expressed in both antennae and legs. At least five of the characterized *AgOrs* display female-specific expression. As observed for *DOrs*, sequence similarity is low among the *AgOrs*.

A similar approach was used by Krieger et al. (2002, 2004) in the noctuid moth, *H. virescens*. Access to the nonpublic *H. virescens* genomic database from Genoptera (USA) allowed Krieger et al. (2002, 2004) to characterize a divergent gene family coding for the first candidate Ors in a lepidopteran crop pest. In total, 21 candidates were described in this species. Each receptor subtype appears to be expressed in a distinct population of sensory cells, as already observed for *Drosophila* as well as vertebrate *Ors* (see "Odor Coding: From Stereochemical Information to an Olfactory Sensory Map in the Brain"). A small group of three receptors share >40% identity and are expressed exclusively in male moths in pheromone-sensitive neurons, making them good candidate pheromone receptors.

Through a preliminary evaluation of the first draft of the *A. mellifera* genome, H. M. Robertson (personal communication) identified about 60 potential *Ors* in this species.

Ors from other insect species have also been identified through systematic sequencing of antennal expressed genes in EST library projects. Using this approach, Whitfield et al. (2002) discovered one potential *Or* from *A. mellifera* that appeared as an *Or83b* homolog (see "The Particular Case of *Or83b* and its

Orthologs"), Newcomb et al. (2003) identified sequences from the tortricid moth, *Epiphyas postvittana*, and Patch and Robertson (personal communication) discovered two others in the hawkmoth, *Manduca sexta*. However, since potential *Ors* are expressed at very low levels in the antennae, such a strategy often requires sequencing of several thousand ESTs with only several potential *Or* sequences being obtained. Another approach is to use normalized or subtractive libraries, in which rare transcripts are enriched.

To date, a complete list of *Or* genes has only been achieved in *Drosophila* and *Anopheles* through genomic data analyses. Even with these *Or* sequences in hand, the divergence of these receptors has not allowed *Or* identification in other species using homology-based PCR. Indeed, comparison of the *Or* sequences shows how divergent they are, even among species in the same insect order. From the literature, international databases, and personal communication, a nonexhaustive list of candidate Ors encompasses 4 insect orders, 10 families, and 12 species (Table 1).

THE PARTICULAR CASE OF *Or83b* AND ITS ORTHOLOGS

Although most homology-based strategies have failed to identify *Ors* in other species, Krieger et al. (2003) amplified one candidate *Or* subtype in several insect species from various orders. These included the honey bee, *A. mellifera* (Hymenoptera), the blowfly *Calliphora erythrocephala* (Diptera), and the yellow mealworm, *Tenebrio molitor* (Coleoptera). The candidate sequences showed high amino acid sequence conservation with *Drosophila* Or83b and AgOr7. Such PCR experiments have also allowed identification of homolog cDNAs in other species. For example, we identified a homolog *Or* cDNA in the noctuid *M. brassicae* (unpublished results), and Melo et al. (2004) cloned the *AaOr7* from the yellow fever mosquito, *Aedes aegypti*. These Ors share around 60–80% identity and could be considered as orthologs, defining an unusual receptor subtype highly conserved across insect orders (Figure 4). Dunipace et al. (2001) noted that among Ors, Or83b appears most similar to the Gr family proteins, and this conclusion is supported by phylogenetic analysis (Robertson et al., 2003). In addition to this unusually high degree of conservation across insect orders, the *Or83b* orthologs are all expressed in a large number of cells of the antennae, palpi, and proboscis (Clyne et al., 1999a; Vosshall et al., 2000; Krieger et al., 2003; Melo et al., 2004; Pitts et al., 2004). In *Drosophila*, *Or83b* is expressed in addition to the single specific *Or* typically expressed in each neuron. Therefore, this unique receptor subtype has been proposed to fulfill a special function common to all chemosensory neurons of insects. Although it may not function as a typical Or recognizing particular ligands, it could be involved in Or activation, for example, as a dimerization partner (Vosshall et al., 2000; Krieger et al., 2003). Heterodimerization is now well documented for nonolfactory GPCRs, such as GPCRs for transmitters and peptides where heterodimerization could modulate the binding specificity of

TABLE 1. INSECT OLFACTORY RECEPTOR CANDIDATES

Order Family	Species	Names	Database accession no.	References	Expression[a]	Data sources
Diptera	*Drosophila*	Or2a	NM_080307	Vosshall et al., 2000	A	Genome
Drosophilidae	*melanogaster*	Or7a	NM_078526	Vosshall et al., 2000	A	Genome
		Or9a	NM_078552	Vosshall et al., 2000	A	Genome
		Or10a	NM_078567	Vosshall et al., 2000	A	Genome
		Or13a	NM_078635	Vosshall et al., 2000	A	Genome
		Or19a	NM_080274	Vosshall et al., 2000	A	Genome
		Or22a	NM_078729	Vosshall et al., 2000	A	Genome
		Or22b	NM_058077	Vosshall et al., 2000	A	Genome
		Or23a	NM_078734	Vosshall et al., 2000	A	Genome
		Or33a	NM_078829	Vosshall et al., 2000	A	Genome
		Or33b	NM_078830	Vosshall et al., 2000	A	Genome
		Or35a	NM_165117	Vosshall et al., 2000	A	Genome
		Or42b	NM_078900	Vosshall et al., 2000	A	Genome
		Or43a	NM_078923	Vosshall et al., 2000	A	Genome
		Or43b	NM_078932	Vosshall et al., 2000	A	Genome
		Or47a	NM_078965	Vosshall et al., 2000	A	Genome
		Or47b	NM_078966	Vosshall et al., 2000	A	Genome
		Or49b	NM_078997	Vosshall et al., 2000	A	Genome
		Or56a	NM_079072	Vosshall et al., 2000	A	Genome
		Or59b	NM_079098	Vosshall et al., 2000	A	Genome
		Or65a	NA	Vosshall et al., 2000	A	Genome
		Or65b	NA	Vosshall et al., 2000	A	Genome
		Or65c	NA	Vosshall et al., 2000	A	Genome
		Or67a	NM_079281	Vosshall et al., 2000	A	Genome
		Or67c	NM_079294	Vosshall et al., 2000	A	Genome
		Or69a (=Or69aB)	NA	Vosshall et al., 2000 (modified names by Robertson et al., 2003)	A	Genome
		Or69b (=Or69aA)	NM_079326	Vosshall et al., 2000 (modified names by Robertson et al., 2003)	A	Genome
		Or82a	NA	Vosshall et al., 2000	A	Genome
		Or83c	NM_079520	Vosshall et al., 2000	A	Genome
		Or85a	NM_079553	Vosshall et al., 2000	A	Genome
		Or85b	NM_079555	Vosshall et al., 2000	A	Genome
		Or85f	NM_079565	Vosshall et al., 2000	A	Genome
		Or88a	NM_079624	Vosshall et al., 2000	A	Genome
		Or98a	NM_079812	Vosshall et al., 2000	A	Genome
		Or1a	NM_080290	Vosshall et al., 2000	MP	Genome
		Or33c	NM_078831	Vosshall et al., 2000	MP	Genome
		Or46a (=Or46aA)	NM_078953	Vosshall et al., 2000 (modified names by Robertson et al., 2003)	MP	Genome
		Or59c	NM_079099	Vosshall et al., 2000	MP	Genome
		Or71a	NM_168604	Vosshall et al., 2000	MP	Genome
		Or85d	NM_079557	Vosshall et al., 2000	MP	Genome
		Or85e	NM_079559	Vosshall et al., 2000	MP	Genome
		Or83b	NM_079511	Vosshall et al., 2000	O	Genome
		Or22c	NM_078730	Vosshall et al., 2000	Not expressed	Genome
		Or24a	NM_078746	Vosshall et al., 2000	Not expressed	Genome
		Or30a	NM_078796	Vosshall et al., 2000	Not expressed	Genome
		Or42a	NM_078898	Vosshall et al., 2000	Not expressed	Genome
		Or45a	NM_176115	Vosshall et al., 2000	Not expressed	Genome
		Or45b	NM_078943	Vosshall et al., 2000	Not expressed	Genome
		Or46b (=Or46aB)	NM_165752	Vosshall et al., 2000 (modified names by Robertson et al., 2003)	Not expressed	Genome
		Or49a	NM_078987	Vosshall et al., 2000	Not expressed	Genome
		Or59a	NM_079097	Vosshall et al., 2000	Not expressed	Genome
		Or63a	NM_079171	Vosshall et al., 2000	Not expressed	Genome
		Or74a	NW_047330	Vosshall et al., 2000	Not expressed	Genome
		Or88a	NM_079624	Vosshall et al., 2000	Not expressed	Genome
		Or85c	NM_079556	Vosshall et al., 2000	Not expressed	Genome
		Or92a	NM_079690	Vosshall et al., 2000	Not expressed	Genome
		Or94a	NM_079731	Vosshall et al., 2000	Not expressed	Genome
		Or94b	NM_079732	Vosshall et al., 2000	Not expressed	Genome
		Or98b	NM_079816	Vosshall et al., 2000	Not expressed	Genome
		Or67b	NM_079283	Vosshall et al., 2000	U	Genome
		Or67d	NM_140133	Vosshall et al., 2000	U	Genome
		Or19b	NM_167690	Robertson et al., 2003		

TABLE 1. CONTINUED.

Order Family	Species	Names	Database accession no.	References	Expression[a]	Data sources
Diptera Culicidae	*Anopheles gambiae*	AgOr1	AF364130	Fox et al., 2001	O	Genome
		AgOr2	AF364131	Fox et al., 2001	O	Genome
		AgOr3 & 4	AF364132	Fox et al., 2001	O	Genome
		AgOr5	AY062432	Fox et al., 2002	O	Genome
		79 GPRors		Hill et al., 2002		Genome
		AgOr7	AY363725	Pitts et al., 2004	A, MP, p	Genome
	Aedes aegypti	AaOr7	AY582943	Melo et al., 2004	A,MP,L,p	Homology cloning
Diptera Calliphoridae	*Calliphora erythrocephala*	CeryR2	AJ555538	Krieger et al., 2003	A	Homology cloning
Lepidoptera Noctuidae	*Heliothis virescens*	HR1	AJ487476	Krieger et al., 2002	A,P	Genome
		HR2	AJ487477	Krieger et al., 2002	A,P	Genome
		HR3	AJ487478	Krieger et al., 2002	A,P	Genome
		HR4	AJ487479	Krieger et al., 2002	A,P,w,ab	Genome
		HR5	AJ487480	Krieger et al., 2002	A,P,l	Genome
		HR6	AJ487481	Krieger et al., 2002	A,P,l,w,t ab	Genome
		HR7	AJ487482	Krieger et al., 2002	A	Genome
		HR9	AJ487484	Krieger et al., 2002	A	Genome
		HR8	AJ487483	Krieger et al., 2002	A,P, T	Genome
		HR10	AJ748325	Krieger et al., 2004		Genome
		HR11	AJ748326	Krieger et al., 2004	Am,Af	Genome
		HR12	AJ748327	Krieger et al., 2004		Genome
		HR13	AJ748328	Krieger et al., 2004	Am,p,l,w,ab	Genome
		HR14	AJ748329	Krieger et al., 2004	Am	Genome
		HR15	AJ748330	Krieger et al., 2004	Am	Genome
		HR16	AJ748331	Krieger et al., 2004	Am	Genome
		HR17	AJ748332	Krieger et al., 2004		Genome
		HR18	AJ748333	Krieger et al., 2004		Genome
		HR19	AJ748334	Krieger et al., 2004		Genome
		HR20	AJ748335	Krieger et al., 2004		Genome
		HR21	AJ748336	Krieger et al., 2004		Genome
Lepidoptera Noctuidae	*Mamestra brassicae*	MbraR2	AY485222	Jacquin-Joly et al., 2003, direct submission	A, p	Homology cloning
Lepidoptera Bombycidae	*Bombyx mori*	BmorR2	AJ555487	Krieger et al., 2003	A	Homology cloning
Lepidoptera Saturniidae	*Antheraea pernyi*	AperR2	AJ555486	Krieger et al., 2003		Homology cloning
Lepidoptera Sphingidae	*Manduca sexta*	2 Ors	NA	Patch and Robertson, personal communication		EST[b]
Lepidoptera Tortricidae	*Epiphyas postvittana*	3 Ors	NA	Newcomb et al., 2003		EST
Hymenoptera Apidae	*Apis mellifera*	AmelR2	AJ555537	Krieger et al., 2003		Homology cloning
		= *Or83b* ortholog ~60 Ors		Whitfield et al., 2002 Robertson, personal communication		EST genome
Coleoptera Tenebrionidae	*Tenebrio molitor*	TmolR2	AJ555539	Krieger et al., 2003		Homology cloning

[a] Abbreviations—O: olfactory tissues (A: antennae and M.P.: maxillary palpi); P: proboscis; L: legs; W: wings; T: thorax; Ab: abdomens; m: male; f: female; U: unknown expression pattern. Capital letters: high expression, small letters: low expression. NA: not available.

[b] EST: expressed sequence tag project.

the conventional receptor (Jordan and Devi, 1999; Pfeiffer et al., 2001), or as described for the GABA BN1/B2 receptor dimer (Robbins et al., 2001) (for a review on GPCR dimerization, see Terrillon and Bouvier, 2004). However, the *Drosophila* Or43a appeared to respond to odorants when expressed in *Xenopus laevis* oocytes where there is no Or83b expression (Wetzel et al., 2001), leaving

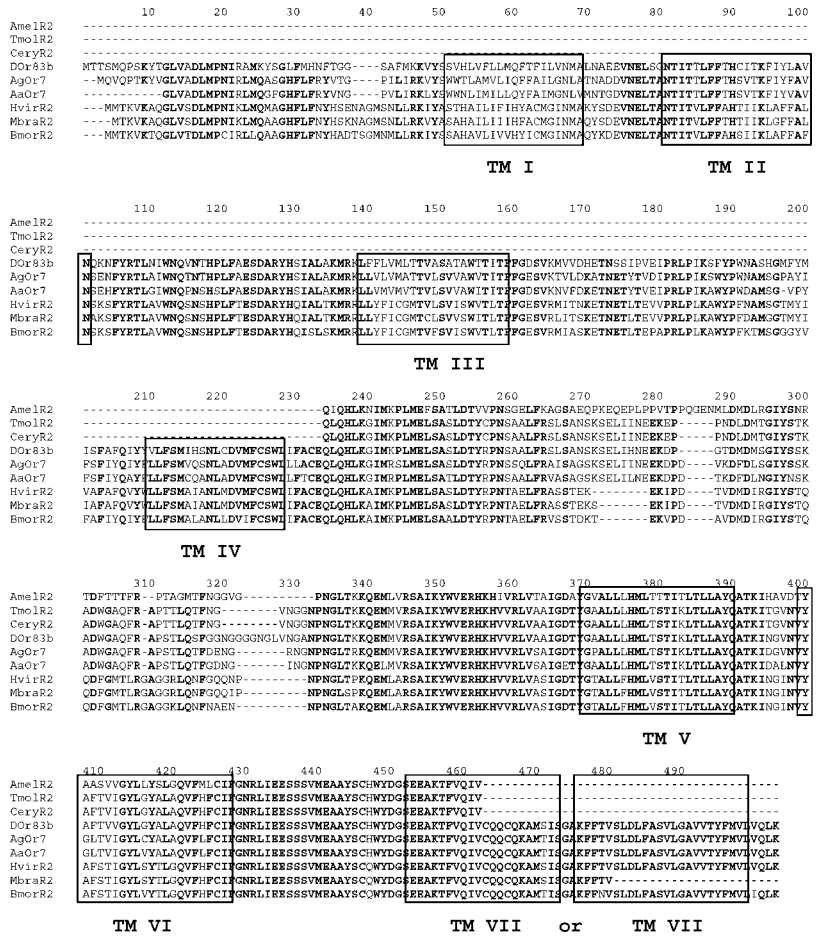

FIG. 4. Amino acid alignment of the DOr83b ortholog receptors from nine insect species: a unique example of a conserved insect Or family. Conserved residues shared by all or all but one species are bold. The seven transmembrane domains (TM) are boxed. Two possible TM VII positions have been proposed (Fox et al., 2001; Pitts et al., 2004). Accession numbers and names of the sequences used in this figure are listed in Table 1.

the question of Or83b heterodimerization partner unresolved (see "Or Functional Studies").

DISCOVERY OF OTHER NEURONAL RECEPTOR TYPES: SNMPs AND GCs

The Sensory Neuron Membrane Proteins (SNMPs). Before the discovery of the *DOr* genes, Rogers et al. (1997) purified and cloned an abundant protein in

A. polyphemus ORN membrane extracts, during a search for membrane proteins that might support olfactory mechanisms in Lepidoptera. Molecular and immunological analyses suggest that this protein, named SNMP-1 for sensory neuron membrane protein, is uniquely expressed in cilia, dendrite, and cytosolic granules of the soma of ORNs (Rogers et al., 1997, 2001a). Sequence analysis has revealed the presence of two TMs with a large extracellular loop, and homologies with vertebrate proteins from the CD36 family, a phylogenetically diverse family of receptor-like membrane proteins implicated in diverse functions, such as endocytosis or binding to apoptotic cells (e.g., Ohgami et al., 2001). SNMP-1 homolog proteins were then found in diverse lepidopteran species, including *M. sexta* (Rogers et al., 2001b). In the latter species, these workers also identified a second SNMP type (SNMP-2) that shared the same characteristics as SNMP-1 (two TMs and localization restricted to ORNs), although SNMP-2 was quite divergent in sequence, with only 27% identity with SNMP-1. These results highlighted SNMP diversity within species.

Although the function of SNMP in the olfactory process is not yet understood, several hypotheses have been proposed (Rogers et al., 2001a) (Figure 5). A first hypothesis is that SNMPs could act as Ors in Lepidoptera, since a previous study that used a photoaffinity analog of the pheromone identified a pheromone-binding membrane protein of similar size and tissue distribution (Vogt et al., 1988). SNMP could bind either the pheromone directly or the pheromone–PBP complex. However, since all the Ors known to date belong to the GPCR family, this hypothesis was not further extended. The observed interaction with pheromone analogs could have resulted from an alternative or complementary process to GPCR binding.

Alternatively, due to the SNMP homologies with CD36 and the presence of a big extracellular loop, they may interact with proteinaceous ligands (like OBPs) and act as docking proteins for the odorant–OBP complex. This may facilitate access of the odorant to the Or (Figure 5A).

A third possibility is that SNMPs interact with other dendritic proteins, such as the Ors, to form active heterodimer complexes (Figure 5B). Finally, SNMPs may act as scavengers, allowing internalization of odorants, OBPs or odorant–OBP complexes. This would lead to metabolism of OBPs or the complexes by intracellular enzymes (Figure 5C) (Vogt, 2003). Other possible functions in the neuron signaling process may occur and have yet to be tested by functional studies.

The Guanylyl Cyclases (GCs). This family of one TM protein hydrolyzes guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP). GCs have been identified in vertebrate as well as invertebrate species, including *C. elegans* and insects. Some have been proposed to be implicated in odorant–pheromone reception or its regulation. Indeed, *C. elegans* GCs are specifically expressed in the chemosensory neurons (Yu et al., 1997), and overexpression of one of them, ODR-1, modifies odorant discrimination and olfactory adaptation

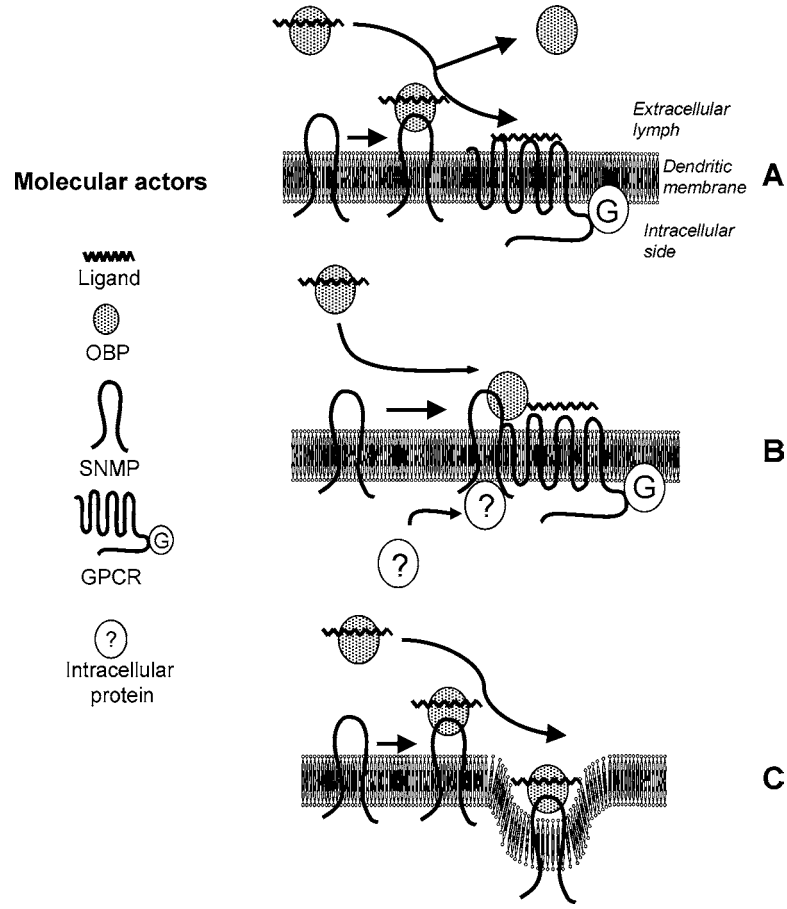

FIG. 5. Proposed functions for SNMP (from Rogers et al., 2001a,b). A: SNMP may facilitate access of the odor–OBP complex to a classical GPCR. B: SNMP may complex with Or and/or other intracellular proteins, contributing to odor or odor–OBP complex reception. C: SNMP may have scavenger properties, related to odor or odor–OBP complex internalization.

(L'Etoile and Bargmann, 2000). In the moth, *M. sexta*, cGMP immunoreactivity and soluble GC have been localized in the antennal sensilla (Stengl et al., 2001), suggesting that the intracellular messenger cGMP plays a role in olfactory transduction. In addition, Tanoue et al. (2001) reported the isolation of a GC receptor type cDNA from male *B. mori* antennae. Immunohistochemical study revealed that the protein localizes in the antennal lobe glomerulus and in the soma and axon of sensory neurons (Tanoue et al., 2001). A novel type of GC, cloned in *M.*

sexta, appears to be expressed in the cell bodies and dendrites, but not axons, of ORNs (Nighorn et al., 2001). So far, odorant binding on the extracellular portion of GC receptors has not been observed, and their role in the olfactory process is not yet understood.

GENETIC DATA, MOLECULAR EVOLUTION, AND DEVELOPMENTAL ASPECTS

Gene Structures and Genome Localization. Contrary to monoexonic mammalian *Ors*, insect and *C. elegans Or* genes (Troemel et al., 1995) carry introns. Some *Drosophila Or* genes may undergo alternative splicing (Robertson et al., 2003), as noted for several *Gr* genes in *Drosophila* (Clyne et al., 2000) and *A. gambiae* (Hill et al., 2002).

In *Drosophila*, the majority of *Or* genes are spread widely through the genome, indicating that they are old members of a gene family that have been distributed around the genome by the processes of genome flux (Robertson et al., 2003). This ancient origin of the chemoreceptor family is also supported by intron evolution analysis, as well as the observation of an extreme divergence within the family (Robertson et al., 2003). Some genes are still clustered, indicating relatively recent gene duplication. The *Drosophila Or* genomic distribution is in contrast with the pattern observed with the mammalian *Ors* (Zhang and Firestein, 2002) and the *C. elegans* chemoreceptor genes (Robertson, 2001), which are highly clustered on particular chromosomes, reflecting the relatively recent expansions of these chemoreceptor families.

Comparing the molecular evolution of *Drosophila* and *A. gambiae Or* genes has revealed the expansion of gene subfamilies unique to each dipteran lineage. In *A. gambiae*, the *Or* family members are dispersed among the three chromosomes, with most *AgOrs* tightly linked as pairs, triplets, or larger clusters of up to nine genes, whereas 17 *AgOrs* exist as single genes (Hill et al., 2002). The large clusters of *AgOr* genes are exclusively made up of recently duplicated genes. This pattern of lineage-specific gene subfamily expansion may reflect the ecological and physiological relevance of these receptors: they may be responsible for detection of signals uniquely important to each species, like fruit odors for *D. melanogaster* and human host odors for *A. gambiae* (Hill et al., 2002). The high divergence between *Or* sequences from insects, nematodes, and vertebrates suggests that they have an independent origin, arising from an independent mechanism of evolution (Vosshall et al., 1999).

Scott et al. (2001) introduced the notion that the *Drosophila Or* and *Gr* families are evolutionarily related in a chemoreceptor superfamily, and this is supported by phylogenetic analysis (Robertson et al., 2003). In addition, the presence of antennal Grs that may function as Ors (see "Discovery of *Drosophila* Odorant Receptor Genes") suggests that *Or* function has evolved separately several times within the superfamily.

Regulation of Or Gene Expression. A common feature between vertebrate and invertebrate *Ors* is the hypothesis that one neuron expresses only one *Or* type (see "Odor Coding: From Stereochemical Information to an Olfactory Sensory Map in the Brain"). Although this hypothesis has been confirmed in mammals and in *Drosophila* (Malnic et al., 1999; Vosshall et al., 2000), *C. elegans* appears to be an exception, since more than one type of *Ors* is expressed in a given sensory neuron (Troemel et al., 1995).

Little is known about the regulatory processes that underly expression of only one *Or* type per neuron. The mechanism of segregation could involve regulatory elements situated near the transcription initiation sites of *Or* genes, which would receive specific information about the *Or* expression area (Qasba and Reed, 1998).

In *Drosophila*, expression of abnormal chemosensory jump 6 (Acj6), a transcription factor, determines the receptive odorant profiles of ORNs, and then may contribute to the choice of the receptor gene to be expressed (Clyne et al., 1999b). An unusual mode of gene expression, that involves a mutually exclusive expression of odorant receptors, has been recently demonstrated in mice (Serizawa et al., 2000).

Developmental Aspects and Sexual Dimorphism. Few studies have investigated the temporal expression pattern of *Or* genes from embryonic development to adult stage. In *Drosophila*, Vosshall et al. (1999) reported an absence of *DOr* gene expression at any stage during embryonic development. To our knowledge, *Drosophila* larval stages were not studied. Some studies have investigated *DOr* expression during pupal development, hypothesizing that *Or* genes might have a role in guiding the axons of the olfactory neurons to the correct glomeruli. Indeed (see "Other Possible Functions for Ors"), vertebrate *Or* genes have been proposed to play such a role in development (Mombaerts et al., 1996; Wang et al., 1998). Although different members of the *DOrs* family initiate expression at different times during antennal development (Clyne et al., 1999a), *DOrs* seem to be expressed only after the establishment of synaptic connections (Vosshall et al., 2000).

One *A. gambiae* putative *Or* (*AgOr7*) has been extensively studied from a developmental point of view (Pitts et al., 2004). Although the most robust expression was observed in adult olfactory organs, this gene appeared to be expressed during preimago stages, including early stage larvae, late stage larvae, and pupae, but not in the embryos. In *A. polyphemus* and *M. sexta*, another class of membrane proteins (the SNMPs) are expressed late in adult development and into adult life (Rogers et al., 1997, 2001b). At this point, morphogenesis has been completed and olfactory neurons are functional.

Differential expression of *Or* in female or male olfactory organs may be correlated with functional differences between the sexes. Although *DOrs* do not appear to be sexually dimorphic in *Drosophila* organs, at least five female-specific *Ors* have been identified in *A. gambiae* (Fox et al., 2001; Zwiebel and Takken, 2004). In mosquitoes, host selection and blood feeding are restricted

to females. Thus, female-specific expression of *Ors* may be indicative of a role in establishing host preference (Fox et al., 2001). In *Drosophila*, a putative gustatory receptor, Gr68a, is expressed in chemosensory neurons of male-specific gustatory bristles in the forelegs, with no expression in females (Bray and Amrein, 2003). In addition, Gr68a expression is dependant on the sex determination gene, *doublesex*. These considerations are consistent with a function in pheromone recognition in *Drosophila* since males perceive the female nonvolatile pheromone during courtship (Coyne et al., 1994). Indeed, molecular analyses of *Gr68a* proved that it is required for normal male courtship (Bray and Amrein, 2003) (see "Future Perspectives, Opportunities, and Challenges of Insect or Studies").

Very recently, a male-exclusive expression of three putative Ors from the moth *H. virescens* lead to the identification of the first good lepidopteran pheromone receptor candidates (Krieger et al., 2004).

SIGNAL TRANSDUCTION IN INSECT SENSORY CELLS

Olfactory transduction has been reviewed in great depth by Krieger and Breer (1999). Here, we will only give a brief overview of how Or activation could lead to the generation of an electrical signal. The different elements involved in this transduction cascade are also involved in other diverse phenomena, such as adaptation of the ORNs (as reviewed for vertebrates by Zufall and Leinders-Zufall, 2000).

The binding of the odorant to its specific receptor situated in the dendritic membrane leads to the activation of a G-protein, which in turn mediates the response via intraneuronal second messengers that trigger the opening of ion channels and local depolarization. Ultimately, this elicits an action potential in the neuron (Figure 6). G-proteins are heterotrimers, comprising α, β, and γ subunits. The α subunit is responsible for GTP binding and hydrolysis to GDP. G-proteins are generally referred to by this α subunit, since it is hypothesized that it confers the G-protein specificity: for example Gq proteins activate phospholipase C (PLC), whereas Gs activate adenylyl cyclase. From biochemical, electrophysiological, and molecular data, the current hypothesis is that the PLC–InsP3 reaction cascade may be the major pathway for signal transduction in insect olfactory neurons. Indeed, Gqα3 mediates odor responses in *Drosophila*, as recently demonstrated by Kalidas and Smith (2002), who targeted Gqα3 silencing in ORNs by RNA-mediated interference (RNAi) (see "Future Perspectives, Opportunities, and Challenges of Insect or Studies"). Using the *Or83b* promoter, they expressed the silencing construct in a large fraction of ORNs leading to olfactory defects *in vivo* at both the physiological and the behavioral levels.

PLC mediates the hydrolysis of inositol 4,5-bisphosphate (PIP_2) into inositol 1,4,5-triphosphate ($InsP_3$) and diacyl glycerol (DAG) (Krieger and Breer, 1999).

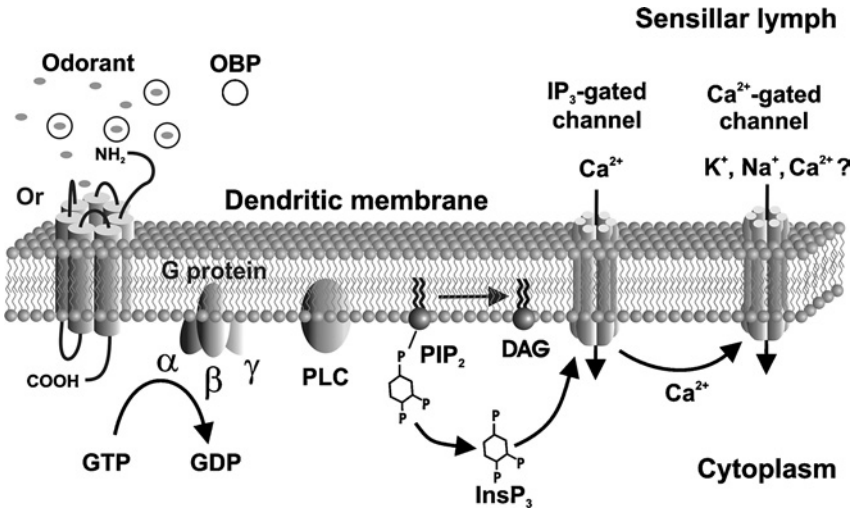

FIG. 6. Hypothetical olfactory transduction cascade in insect olfactory neurons. Ligand binding to the olfactory receptor (Or) activates a G-protein, which in turn activates a phospholipase C (PLC). PLC mediates the hydrolysis of PIP_2 (inositol 4,5-biphosphate) into $InsP_3$ (inositol 1,4,5-triphosphate) and DAG (diacyl glycerol). Downstream events then lead to ion channel opening and membrane depolarization.

The downstream events that lead to membrane depolarization are not yet well understood. $InsP_3$ could act on IP_3-gated ion channels leading to Ca^{2+} entry in the cell, which in turn act on Ca^{2+}-gated cationic ion channels (Figure 6). These inward currents form a receptor potential that elicits a discharge of action potentials that travel down ORN axons to the first relay in the brain, the AL, encoding odor quantity and quality.

ODOR CODING: FROM STEREOCHEMICAL INFORMATION TO AN OLFACTORY SENSORY MAP IN THE BRAIN

Olfactory messages are generally composed of blends of molecules. Odorants are perceived as a precise mixture, which is particularly exemplified by pheromonal blends used by insects for intraspecific communication (see the Pherolist: Witzgall et al., 2004).

With approximately 1,300 antennal ORNs, 120 maxillary palp ORNs, and 62 Ors, *Drosophila* is able to recognize and discriminate among a large number of distinct odorants. How is the combination of the stereochemical information detected by ORNs transferred to the higher centers of the nervous system? In mammals, individual ORNs express only 1 of 1,000 receptor genes, and the axons from ORNs expressing a specific receptor converge upon two

spatially invariant glomeruli within the olfactory bulb (reviewed and discussed in Mombaerts, 2004). The quality of the stimulus is, therefore, encoded by specific combinations of activated glomeruli. An olfactory sensory map in the mammalian olfactory bulb corresponds to a spatial map of Or expression in the sensory epithelium.

Although such a logic of olfactory discrimination is not observed in the invertebrate *C. elegans*, where each chemosensory cell expresses a large number of *Or* genes (Troemel et al., 1995), this functional correspondence appears to be conserved between insects and mammals, as illustrated below for *Drosophila*.

Maps of Drosophila Olfactory Receptors in the Antennae. DOr gene expression patterns were analyzed by RT–PCR and RNA ISH in the two olfactory sensory organs of the adult fly (e.g., the third antennal segment and the maxillary palpus) (Gao and Chess, 1999; Clyne et al., 1999a; Vosshall et al., 1999, 2000). Expression of *DOr* genes is specifically localized either in the antenna (39 *DOr* genes), or in the maxillary palpus (9 *DOr* genes), or in both organs (in the case of *Or83b*), with little variation according to the techniques used by different research groups (Gao and Chess, 1999; Clyne et al., 1999a; Vosshall et al., 1999, 2000) (Table 1). Some *Ors*, however, were not detected, perhaps due to their low level of expression or, alternatively, they may be expressed in other tissues or during different times during development. Within the olfactory organs, *DOr* genes are selectively expressed in small subsets of ORNs, which appear bilaterally symmetric and spatially conserved between individuals (Vosshall et al., 1999, 2000). Furthermore, Vosshall et al. (1999, 2000) demonstrated that there are nonoverlapping expression patterns between the *DOr* genes in the antenna or in the maxillary palpus. This suggests that each *DOr* is expressed selectively in a small subset of sensory cells that is spatially defined within the antenna and maxillary palpus. Each ORN expresses one of the *DOr* genes and is, therefore, functionally distinct (Vosshall et al., 1999, 2000). The number of ORNs expressing a given *DOr* type varies from 2 to 50, with an average of 25. The ubiquitous *Or83b* gene appears as an exception since it is expressed in approximately two-thirds of all ORNs and co-expressed in these ORNs with another *Or* type (Vosshall et al., 1999; Kalidas and Smith, 2002) (see "The Particular Case of *Or83b* and its Orthologs").

The spatial map of *Or* expression in olfactory organs approximates the number and distribution of different functional types of neurons in the antennae and maxillary palpi defined by electrophysiology (de Bruyne et al., 1999, 2001), suggesting that the *DOr* genes indeed encode the ligand-binding odorant receptors in *Drosophila* (Vosshall, 2001).

A Sensory Map in the Brain. The anatomy of the insect olfactory system resembles that of vertebrates. Insect ORN axons project to the ALs, the equivalent of the vertebrate olfactory bulb. The ALs are organized in synaptic regions called

glomeruli, which are the first odorant processing areas. Olfactory information is then relayed via antennal lobe projection neurons, the equivalent of the vertebrate mitral cells, to both the mushroom bodies, and the lateral horn of the protocerebrum (Stocker, 1994; Ito et al., 1998). The coding of sensory stimuli into specific patterns of neuronal activity generates an internal representation of the external world that is processed by brain centers to elicit complex sensory responses. Many studies have provided evidence that each individual ORN projects to a single glomerulus within the AL. In *Drosophila*, the availability of *Or* genes has allowed the use of genetic labeling techniques that have revealed that all ORNs expressing a given receptor converge upon one or two AL glomeruli (Gao et al., 2000; Vosshall et al., 2000), whose position and size are invariant among individuals. This conserved topographic map of DOr projections is consistent with previous studies in insects that mapped AL activities after specific olfactory stimulation (Rodrigues, 1988; Galizia et al., 1999). These studies demonstrated that different odorants elicit distinct patterns of glomerular activity in *Drosophila* as well as *Apis mellifera*. The quality of the olfactory stimulus would, therefore, be encoded by the specific combination of glomeruli activated by a given odorant. Activated glomeruli define an odor-specific map that may then be decoded in higher brain centers.

OR FUNCTIONAL STUDIES

Since *DOrs* have been discovered only recently, few studies have reported functional assays of insect Ors. Here, we will first briefly describe the development of functional studies of Ors from vertebrates and the nematode, *C. elegans*, that will allow us to describe the different functional techniques and highlight their difficulties. The major impediment to functional studies is that membrane bound Ors are not water soluble, which makes them difficult to study by using the classical techniques typically used to elucidate structure–function relationships. Then, we will review the first functional studies of Ors in insects.

*O*r Functional Studies Developed in Vertebrates and *C. elegans*

Functional Expression in Heterologous Systems. Vertebrate Ors have been studied extensively by using functional expression in various heterologous systems that do not normally express these proteins. These systems have included mRNA injection in *Xenopus laevis* oocytes, transfection of *Escherichia coli* bacterial cells or HEK eukaryotic cells, and recombinant baculovirus infection of insect cells (e.g., Raming et al., 1993a; Zhang et al., 1997; Krautwurst et al., 1998; Wetzel et al., 1999). However, such experiments have had to overcome obstacles. Since Ors naturally function in the cell plasma membrane, the most difficult obstacle has been to target the heterologous protein to the membrane. Thus, *Or* genes are often fused to a signal sequence that will target the protein to

the cell membrane after expression. In addition, a cDNA encoding a G-protein has to be co-transfected in the cell system to allow coupling with the Or and signal transduction for a chosen pathway. Activation of Or protein upon odorant stimulation then has to be visualized and quantified. Calcium imaging has been used extensively for functional vertebrate Or studies (see the review from Touhara, 2002). Or–ligand interaction leads to intracellular calcium increase (see "Signal Transduction in Insect Sensory Cells"). This calcium increase, an indirect measure of Or activation, is assayed by fluorescence intensity of a probe injected in the cell. Such functional studies, in addition to being difficult, are then performed by using an artificial reconstituted system that may not reflect the natural one. Second, although odorants are volatile airborne molecules, these reconstituted systems require delivery of the odorant in solution, and many functional studies have used odorant concentrations that are higher than physiological concentrations to obtain responding cells (e.g., Wetzel et al., 1999). In addition, the quality of some odorants is perceived differently at different concentrations (Touhara, 2002).

Combination of Calcium Imaging and Single Cell RT–PCR. Touhara et al. (1999) and Touhara (2002) have reported experiments aimed at deciphering odorant–Or pairing by a two-step process. The response profile of an isolated olfactory neuron to a panel of odorants was first examined through calcium imaging. Then, the *Or* expressed in the individual neuron was identified by single cell RT–PCR. In this way, odorant–Or couples were identified in mice (Malnic et al., 1999). Neurons expressing the same Or appear to recognize several odorant molecules with a selectivity that is a function of the size of the chain or the functional group that they are carrying. On the other hand, Malnic et al. (1999) showed that one odorant could be recognized by multiple Ors. Based on the limited experimental information, one might speculate that odorant discrimination may be the result of a combinatory code where different odorants stimulate unique sets of Ors and ORNs.

Functional Expression In Vivo. This strategy involves targeted gene expression in the sensory neurons of the olfactory epithelium followed by functional studies *in vivo*. Zhao et al. (1998) and Araneda et al. (2000) used a recombinant adenovirus to drive expression of a defined *Or* gene in an increased number of rat olfactory neurons. Electrophysiological recording showed that overexpression of a single gene led to greater sensitivity for a small subset of odorants (C7–C10 saturated aliphatic aldehydes). Zhao et al. (1998) demonstrated that Ors mediate a physiological response with some specificity. However, the window of specificity is somewhat broad because several compounds that possess some chemical similarities could activate the same receptor.

Functional studies have also been conducted in the nematode *C. elegans*, which offers a simple behavioral test based on attraction–repulsion according to the neuron were the Or is targeted. Such analysis led to the identification of the diacetyl receptor ODR-10 in *C. elegans* (Sengupta et al., 1996). Functional

expression of a mammalian *Or* using *C. elegans* as a reporter system has also been achieved (Milani et al., 2002). Transgenic nematodes expressing rat receptor I7 in targeted neurons showed modified odorant responsiveness during volatile attraction or avoidance behaviors. Thus, *C. elegans* appears to be a good *in vivo* system to test functional properties of Ors from different origins.

In addition, a new membrane protein was discovered in *C. elegans* (ODR-4), and this protein is thought to participate in membrane targeting of Or (Dwyer et al., 1998). Indeed, co-expression of ODR-4 with a rat Or in nonmature olfactory cells displayed perfect membrane targeting (Gimelbrant et al., 2001). ODR-4 homologs in vertebrates or insects have not yet been discovered.

Structure and Modeling. Our knowledge of Or structure–function relationships could be enhanced through the elucidation of Or three-dimensional structure. However, X-ray diffraction analyses of crystallized Or protein, and GPCR in general, is challenging because of the difficulties in obtaining large quantities of purified protein for crystallization. Combination of computer modeling and site-directed mutagenesis in Or could help in the identification of potential ligands and exploration of odorant binding sites. Currently, vertebrate protein modeling has been used to successfully identify receptor agonists and antagonists by virtual screening of compound libraries (Bissantz et al., 2003).

Initial Or Functional Studies in Insects

Clyne et al. (1999b) first showed that a mutation that alters the expression of a subset of *Or* genes alters the odorant specificity of a subset of *Drosophila* ORNs. Direct evidence for the involvement of one *Or* gene in olfaction came from two complementary and concomitant studies with *Drosophila* (Störtkuhl and Kettler, 2001; Wetzel et al., 2001). Using the GAL4/UAS system, an inducible targeted gene expression system widely used in *Drosophila*, Störtkhul and Kettler (2001) overexpressed the *Or43a* gene in the third antennal segment and tested for an increase in odor response *in vivo* by using electroantennography (EAG). Whereas *Or43a* is expressed in 15 neurons of wild-type fly antennae, overexpression led to approximately 1,200 neurons expressing *Or43a*. Overexpression conferred increased EAG responses to several structurally related compounds: cyclohexanol, cyclohexanone, benzaldehyde, and benzyl alcohol, all sharing a six carbon ring with a single polar group. Wetzel et al. (2001) expressed *Or43a* in *Xenopus* oocytes, a heterologous system. Using two-electrode voltage-clamp recording, they showed that the same four odorants from the *in vivo* study also activated the expressed receptor in the *Xenopus* system.

Although these studies provide direct evidence for Or43a function, they raise unexpected questions. In particular, can Or be activated without intervention of insect OBPs (expression in *Xenopus* oocytes) or in the presence of different OBPs from the one naturally expressed in the vicinity of the Or (overexpression)? It is

possible that unspecific binding protein(s) that could replace OBP function are present in these systems.

These studies conducted in *Drosophila* provide the first evidence for an *Or* gene function in insects, as well as for ligand determination. Recently, Dobritsa et al. (2003) investigated how the molecular and cellular maps of the *Drosophila* olfactory system are integrated by establishing the correspondence between individual neurons, odorant receptors, and odorants. Using either receptor substitution experiments in a mutant neuron or analysis of strains in which *Or* promoters were used to drive reporter genes, several *Or* genes were shown to confer response to particular odorants or were mapped to particular functional classes of neurons. In particular, *Or22a* maps to a neuron that responds to ethyl butyrate. Through a deletion mutant lacking *Or22a* and transgenic rescue analysis, Dobritsa et al. (2003) demonstrated that *Or22a* is required *in vivo* for response to this compound.

Similarly, Hallem et al. (2004b) have undertaken a systematic functional analysis of a variety of *DOr* genes that combines molecular and electrophysiological approaches. The determination of the odor spectrum of each Or allowed the authors to establish a receptor-to-neuron map of the *Drosophila* antennae by matching receptor spectra to defined ORN spectra. Thirty-one of the 32 *Dor* genes expressed in the antennae have been investigated. Receptors vary in their tuning breadth, and odorants vary in the number of receptors that they activate. In addition, excitation and inhibition, the two modes of olfactory signaling used by ORNs, are determined by the Or they express. Depending on the odorant, a single Or mediates both excitatory and inhibitory responses. Thus, Ors confer not only the odor response spectrum but also the response mode and the response dynamics upon the ORNs that express them (Hallem et al., 2004b). This study greatly advances our understanding of the molecular basis for odor coding in *Drosophila*.

Up to now, only one nondrosophilian Or has been assigned to a ligand. Hallem et al. (2004a) used *Drosophila* as an *in vivo* expression system for the *A. gambiae AgOr1*. *AgOr1* was expressed in *Drosophila* ORNs that normally express *Or22a* and *Or22b*, but these genes were deleted in the experimental construct. Responses to odors by the transformed neurons were assayed using single-cell electrophysiology. Female-specific AgOr1 is thought to participate in host-seeking behavior. Indeed, the functional study revealed that AgOr1 confers a strong response to 4-methylphenol, a component of human sweat (Hallem et al., 2004a).

Functional studies of insect Ors reveal broad odorant selectivity, as observed for vertebrate Ors (Malnic et al., 1999). This leads to a general concept of a combinatorial receptor code for odors with the following precept, verified at least in vertebrates: an Or from an individual neuron recognizes multiple odorants and, reciprocally, a single odorant is recognized by multiple receptors in different neurons. In the latter case, the multiple receptors have variable affinities for the ligand. Combination of specificity and tolerance, correlated with odorant concentration,

establishes a basal level of discrimination as well as the possibility to detect a wide range of various odorants, even with a low population of Ors.

SIGNAL TERMINATION, ADAPTATION, AND MODULATION

Signal reduction–inactivation and cessation are important components of olfactory perception. Different mechanisms may regulate agonist concentration. For example, biotransformation enzymes (see "General Olfactory Process") function to inactivate the signal (Figure 7A). In a recent example, a cytochrome P450 specific to the male antennae of the chafer beetle *Phylloperta diversa*, has been characterized as a potential pheromone-degrading enzyme (Maïbèche-Coisne et al., 2004). Adaptation is another process by which odorant perception depends on the previous experience of the ORNs. Thus, temporal information may be an important part of the chemosensory code. Reduction of receptor signaling is characterized at the molecular level by a process known as desensitization. It can affect the different steps of the transduction cascade or the GPCR itself. GPCR desensitization involves phosphorylation of the receptor by either protein kinases (PK) or G-protein-coupled serine–threonine receptor kinases (GRKs). While PKs are involved in slow desensitization processes, GRKs are responsible for rapid desensitization. GRKs phosphorylate the activated form of the receptor, and this in turn allows binding of an arrestin protein, which further uncouples the receptor from the signaling cascade (Figure 7B). Arrestins also trigger the endocytotic internalization of receptors, which is an integral component of GPCR resensitization in many systems (Figure 7B). For example, arrestins from *Drosophila* have been shown to be involved in visual signaling (Hyde et al., 1990; Smith et al., 1990). In addition to *Drosophila*, arrestins have been cloned in antennal tissues from *H. virescens* and from the migratory locust, *Locusta migratoria* (Raming et al., 1993b), and more recently from *A. gambiae* (Merrill et al., 2002, 2003). The functional significance of the arrestins has been investigated only in *Drosophila* (Merrill et al., 2002). Indeed, *Drosophila* visual arrestins appear to have a bimodal expression, in both photoreceptor and chemosensory neurons. Although their exact role is not yet understood, they are required for proper olfactory function, as arrestin mutants exhibit a decrease in the amplitudes of the electrophysiological responses to olfactory stimuli (Merrill et al., 2002).

Several factors have been shown to modulate the olfactory response of insects from a behavioral and electrophysiological point of view. These include prolonged exposure to plant volatiles (Stelinski et al., 2003), preexposure to sex pheromone (Anderson et al., 2003), circadian rhythms (Krishnan et al., 1999; Page and Koelling, 2003), or physiological states like mating. All of these effects suggest neuronal plasticity. However, the molecular mechanisms underlying these

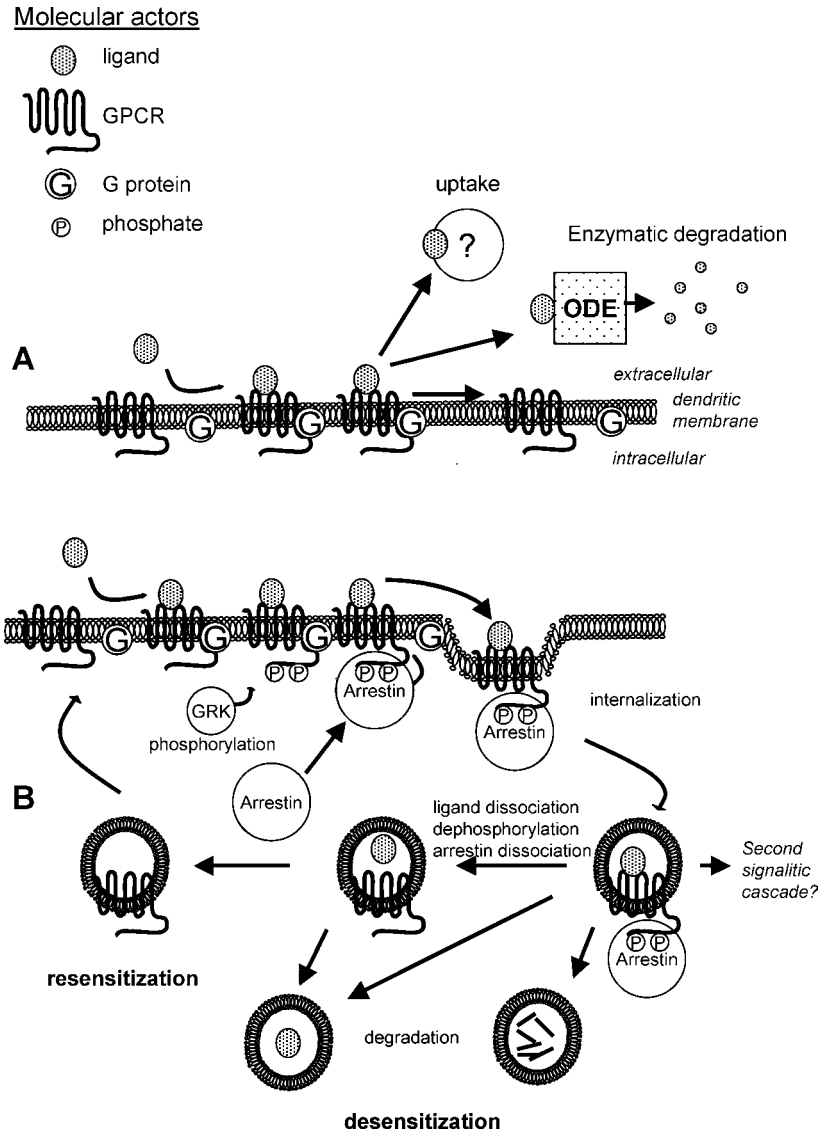

FIG. 7. Hypothetical mechanisms for regulation of agonist concentration in insect olfactory neurons. A: Signal inactivation could result from ligand uptake or enzymatic degradation by ODE (Odorant degrading enzyme). B: GPCR desensitization involves receptor phosphorylation by GRK (G-protein coupled receptor kinase), binding of an arrestin protein, internalization of the receptor, followed by receptor and/or ligand degradation or receptor resensitization.

phenomena are not yet understood. In particular, it is not known if regulation occurs at the peripheral and/or the central level of odorant signal integration. From a chemical ecological point of view, these phenomena are fundamental to understanding olfactory responses at the individual or population levels.

There is a potential role of Ors in olfactory sensitivity. Indeed, sensory experience and sensory activity regulate chemosensory receptor gene expression in *C. elegans* (Peckol et al., 2001). In addition, olfactory sensitivity has been shown to be down regulated after blood feeding in *A. gambiae* (Takken et al., 2001), which could be correlated with down-regulation of mRNA of *AgOr1* in this species (Fox et al., 2001). This *Or* displays female-specific olfactory tissue expression that may indicate a role in establishing host selection. This demonstrates that down-regulation of the expression of a specific gene may, in part, explain the observed decrease in host-seeking behavior, by modification of mosquito odorant response profile.

OTHER POSSIBLE FUNCTIONS FOR Ors

From a developmental standpoint, Ors have been proposed to play a crucial role in building the sensory map in the vertebrate olfactory bulb (Mombaerts et al., 1996; Wang et al., 1998; Strotmann et al., 2004). Specifically, they may determine axonal guidance of ORNs toward their glomerular targets. Indeed, all ORNs expressing the same *Or* converge to the same glomerulus, in vertebrates as well as invertebrates (see "Odor Coding: From Stereochemical Information to an Olfactory Sensory Map in the Brain"). However, *Drosophila Ors* seem to be expressed only after the establishment of synaptic connections (Vosshall et al., 2000). In addition, a recent study conducted in *Drosophila* showed that axonal targeting does not depend on normal *Or* expression (Dobritsa et al., 2003). Indeed, the ab3A neuron finds its normal glomerular target in a mutant that lacks the normally expressed Or in ab3A. Moreover, when other *Ors*, known to be expressed in ORNs that target a distinct glomerulus, were substituted for natural *Ors*, the axons were again observed to project to the normal glomerulus. Dobritsa et al. (2003) concluded that the ab3A neuron finds its glomerular targets through mechanisms independent of Or expression.

Alcedo and Kenyon (2004) have reported an unexpected role for Ors in the nematode *C. elegans* whereby specific subsets of both gustatory and olfactory neurons influence longevity. Some of these neurons inhibit longevity, whereas others promote it. Using RNA-mediated interference (RNAi, see "Future Perspectives, Opportunities, and Challenges of Insect Or Studies"), the authors observed that a decrease in mRNA level for GPCR str-2 in these chemosensory neurons extended life span. This suggests that an environmental cue (although yet unidentified) perceived through str-2 may influence life span. Although this surprising study is limited to *C. elegans*, the sensory system may influence longevity in

other organisms as well. Indeed, the *C. elegans* gustatory neuron influence on life span is likely to be mediated by the insulin/IGF-1 signaling pathway (Alcedo and Kenyon, 2004). This signaling has already been shown to extend life span in *Drosophila* (Tatar et al., 2001) and mice (Holzenberger et al., 2003). In addition, the smell of food has been reported to increase insulin levels in humans (Brand et al., 1982), although the effectors of this odor-stimulated pathway have not been yet deciphered.

FUTURE PERSPECTIVES, OPPORTUNITIES, AND CHALLENGES OF INSECT Or STUDIES

Or Sequence Identification and Development of Functional Studies. Since the completion of the *Drosophila* genome, insect genome sequencing is now growing rapidly. The first draft of the *Bombyx mori* genome has been published (Mita et al., 2004), and a draft of the *Apis mellifera* genome was released in January, 2004 (www.hgsc.bcm.tmc.edu/projects/honeybee/). Other insect genomes are on their way for sequencing; species soon to follow include *Drosophila pseudoobscura* (http://www.hgsc.bcm.tmc.edu/projects/drosophila/). This will no doubt lead to the identification of more Or candidates from a variety of species.

Functional studies, as well as ligand determination, will continue to be needed to confirm the role of the newly discovered Ors in odor detection and selection. From heterologous expression systems such as cell cultures, in particular insect cells, to *in vivo* gene expression modifications (overexpression, silencing), insects offer a wide array of genetic tools that can be combined with electrophysiological, pharmacological, and behavioral approaches. In particular, gene invalidation by double-stranded RNA interference (RNAi) allows suppression of gene expression in a sequence-specific manner, by targeting homologous mRNA degradation (for a review see Hammond et al., 2001). Specific gene silencing in targeted tissues allows elucidation of the biological role of the protein product. Although neurons appeared initially to be resistant to RNAi, effective RNAi has been obtained recently in adult *Drosophila* ORNs (Kalidas and Smith, 2002), providing a powerful tool to manipulate *Or* expression. Although *Drosophila* appears as the academic model with an abundance of molecular resources, considerable efforts have been made in developing similar tools in other insect species. Indeed, germline transformation protocols have been expanded to a wide range of species (Handler, 2001), including moths (Tamura et al., 2000) and mosquitoes (Grossman et al., 2001). Together with the development of gene expression modification tools, this will allow functional studies of insect Ors in agricultural pests or disease vectors.

With sequences in hand, the comparison of intra- and interspecific variants of a given Or may provide useful information about structure–activity relationships. Moreover, comparative studies of potential Ors from insects that use different

host–mate selection processes may provide information on their molecular basis. In addition, sex-specific expression in a given species may be relevant. For example, female-specific expression, observed for five *A. gambiae* Ors, could be especially relevant for disease transmission, which only occurs via females during the course of the blood meal. Male-specific Ors in moths may be implicated in response to female sex pheromones.

Identification of Pheromone Receptors. Among Ors, identification of pheromone receptors is a particular challenge, since pheromones mediate complex innate behaviors, most notably courtship and mating behavior. In mammals, pheromone receptors are expressed in a physically distinct sensory neuroepithelium from the main olfactory system: the vomeronasal organ. Only one of the candidate pheromone receptors has been proven to be functional in mice (Del Punta et al., 2002). These receptors show no sequence similarity to vertebrate Ors expressed in the olfactory epithelium, suggesting an independent evolutionary history. In insects, candidate pheromone receptors have been identified in *Drosophila* (Bray and Amrein, 2003) and in the moth *H. virescens* (Krieger et al., 2004). The *Drosophila* sex pheromone is composed of nonvolatile cuticular hydrocarbons, which may be detected by the male through the gustatory system. Bray and Amrein (2003) identified a putative gustatory receptor, Gr68a, which is expressed in chemosensory neurons of gustatory bristles in the forelegs. Using a molecular genetic approach that employs neuron inactivation (tetanus toxin) and gene invalidation (RNAi), in combination with a behavioral assay, they were able to show that Gr68a is an essential component of pheromone-driven courtship behavior in *Drosophila*. Inactivation of Gr68a-expressing neurons, as well as silencing of *Gr68a*, led to significant reduction in male courtship performance. It has been proposed that males use Gr68a to recognize female pheromone in the early steps of courtship, primarily through the tapping of female abdomen by male forelegs.

Potential use in Crop Protection or Animal and Human Health. Herbivorous insects are responsible for 25% of agricultural losses in the world and mosquitoes, as disease vectors, remain one of the leading causes of human mortality. For example, in Africa alone, malaria is responsible for more than 2 million deaths per year. As insects primarily use host volatiles to seek and select potential hosts, deciphering the molecular mechanisms of olfaction is essential for understanding odor discrimination and sensitivity that mediate host preference. This could allow improvement of already employed olfactory-based pest management strategies, as well as development of novel strategies.

Synergistic effects of different odorants, olfactory response modulation, ORN adaptation and desensitization, are known phenomena that could interfere with olfactory-based pest management tools such as mass trapping or mating disruption. In particular, the physiological state of the individuals appears important for the efficiency of such strategies. Indeed, after mating (Gadenne et al., 2001) or after feeding (Takken et al., 2001), insects undergo behavioral transitions where an

attractive cue can switch to an inactive or even repulsive one. A better knowledge of the molecular basis of olfactory sensitivity could assist in the improvement of these strategies.

Identification and functional characterization of molecular elements of the olfactory pathway, including Ors but also OBPs, SNMPs, GCs, and arrestins, will open the way toward the development of novel and innovative applications for controlling insects based on olfactory-mediated behaviors. Among them, targeted inhibition of the reception of a specific chemical compound, via Or engineering, would alter the blend ratio and lead to misperception of the chemical message. Experimental verification of predicted GPCRs is an essential step in pursuing these studies, and further exploration of GPCRs will facilitate the discovery of new pharmacological targets. Indeed, it is estimated that drugs that target GPCRs account for more than half of the medicines currently used, whatever the therapeutic domain (Galvez and Pin, 2003). In addition, the important role that Ors are likely to play in mediating host preference suggests that their study will likely contribute to the generation of new insect attractants or repellents for both crop protection and human health.

CONCLUDING REMARKS

From a fundamental and basic point of view, the mechanisms of odor recognition and the ensuing signal transduction seem to be identical or closely related in phylogenetically diverse species. This may reflect a striking evolutionary convergence towards a conserved organization of signaling pathways in olfactory systems. Insects are good models for studies of olfaction, as they offer not only a compartmentalized olfactory system, but also the possibility of using genetic engineering in combination with integrated physiological, pharmacological, and behavioral studies. However, as reflected in this review, we are still far from answering the question of how the olfactory system processes distinct olfactory cues to elicit appropriate behavioral responses. For instance, attractive or repulsive behaviors can be achieved from the same odorant stimulation depending on the species, the physiological state of the individual, or the concentration of the odorant.

Specificity and differential expression of Ors, along with OBPs, ODEs, or SNMPs, establishes unique functional phenotypes for different sensilla. In particular, while mammals express one or two OBPs in their olfactory system, insects express a diversity of OBPs in their antennae (e.g., 25 in *Drosophila*). On the contrary, insects express a relatively low number of Ors (62 DOrs, 79 AgOrs) (Hill et al., 2002; Robertson et al., 2003) when compared with vertebrates (1000 in mammals) or *C. elegans* (800). Although the biological significance of these observations is not known, it could be postulated that combinatorial expression of these different elements allows detection of a large number of odors. Because the

different actors may act in combination to trigger a specific response, studies of Ors should be undertaken to investigate the mechanisms in an integrative manner. Although many of these actors have been discovered and identified, their functional interaction leading to odor recognition is not yet clearly understood. The exact role of OBPs as the first line of discrimination has still not been demonstrated, there is no evidence about the exact nature of the Or agonist (odorant alone or the complex odorant–OBP), most of identified Ors are orphan receptors, and SNMP and GC functions are not yet understood.

The development of promising technologies, like *in vivo* functional imaging or behavioral genetic approaches, along with a growing understanding of the neuroanatomy of the system, will certainly in the near future match molecular structure with biological significance. Two invertebrate models, *D. melanogaster* and *C. elegans*, have proved to be useful in increasing our understanding of olfaction and olfactory-mediated behaviors, either as model animals to study their own receptors or as experimental systems to study *in vivo* heterologous Ors from a wide range of species. With the extension of genomic data and genetic tools, this information is rapidly being transferred to other insects, particularly those of medical or economical importance. This will undoubtedly result in great advances in understanding chemical communication mechanisms as well as in the future development of pest management strategies to reduce the negative effects of insects.

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