CHAPTER 8

VISUALIZING A FLY'S NOSE

Genetic and physiological techniques for studying odour coding in Drosophila

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Abstract. Most insect species rely on odours to orient themselves towards resources or escape hazardous environments. Over the past six years studies on odour perception in *Drosophila melanogaster* have rapidly increased our knowledge on the detection of such signals. Due to the availability of relatively straightforward genetic techniques, the cellular elements of the olfactory code in this insect can be manipulated. Olfactory receptor neurons (ORN) in *Drosophila* can be visualized with fluorescent proteins and their physiological properties studied using electrophysiological and optophysiological techniques. The ultrastructure of olfactory sensilla and the odour responses of ORNs in more than half of them have been described. On the molecular level, three large families of genes that provide the basis for these responses have been characterized; olfactory receptors (OR), gustatory receptors (GR) and odour-binding proteins (OBP). OR proteins have been shown to function as odour detectors and they have been mapped to ORN classes to a high degree of completion. Hence, the *Drosophila* olfactory system provides a good basis for studying how odour coding in insects has evolved and how ORNs relay the information present in chemical communication systems.
Keywords: olfaction; *Drosophila*; genetics; sensory physiology; neural coding

INTRODUCTION

Chemical signals are involved in most interactions of insects with their environment. Volatile chemicals (i.e., odours) are signals that have many degrees of freedom and can travel far. Some, such as sex pheromones, can be specific, stable predictors of reproductive success. Because both signal and response are generated by the same genome, highly specialized systems for pheromone synthesis and perception have evolved (Löfstedt 1993). However, most odours are not generated by conspecifics but rather by a large variety of biotic and abiotic factors. In fact, many odours are the result of complex interactions, as for example in weather-dependent microbial decay of plant material. How have these sensory systems evolved to extract reliable chemical information from variable environments? Have olfactory systems evolved as a set of detectors for specific chemical messages or are they designed for efficient detection of a broad range of chemical stimuli? To answer these questions we need

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to determine how a complete olfactory system works, first in one species and then in a comparative way across species.

Encoding of odour information is a two-step process. First, sensory transduction converts chemical information in the environment into a code of action potentials. It takes place in a heterogeneous population of olfactory receptor neurons (ORNs) that determine which volatiles can be detected. Second, from the messages sent out by this array of detectors the brain extracts a percept we call an odour. It is this combined input from many ORN classes that can lead to a behavioural response, depending on the animal's internal state and the integration with other sensory modalities. Most recent research has focused on the first step of this process. The process of capturing and transducing chemical information from the environment was thought to involve G-protein-coupled receptors (Boekhoff et al. 1990), but convincing evidence was lacking. Buck and Axel (Buck and Axel 1991) made a crucial breakthrough when they discovered a large gene family encoding such receptors in vertebrates. Evidence for their crucial role in transducing olfactory information came from studies in *C. elegans* (Sengupta et al. 1996). It was only after genomic sequences of *Drosophila melanogaster* became available that candidate odour receptor proteins were identified in an insect (Clyne et al. 1999; Vosshall et al. 1999). This paper will argue that research on *Drosophila* olfaction has significantly advanced our knowledge on the mechanisms of olfactory perception and should also help in answering more ultimate questions about the ecology and evolution of chemical communication. I will provide an overview of the powerful techniques available in this model organism.

DROSOPHILA OLFACTORY ORGANS

Drosophila melanogaster has rapidly become the favourite model system for studying olfactory coding (Carlson 1996; Vosshall 2000; Stocker 2001; Davis 2004). The reasons for this are many. Its olfactory system is numerically simple, containing only ca. 1300 receptor neurons (Stocker 1994). Furthermore, there are powerful genetic and molecular tools to manipulate the system and determine its genetic underpinnings. Moreover, the *Drosophila* genome has been sequenced and annotated very accurately. Several physiological and genetic techniques are available to peer into the workings of the little fly's sensory organs and associated neuropiles in the central nervous system. Great progress has been made in visualizing neuronal structures and studying neural activity (De Bruyne et al. 1999; 2001; Jefferis et al. 2002; Fiala et al. 2002; Ng et al. 2002; Wang et al. 2003; Wilson et al. 2004). Finally and perhaps most importantly, physiological and genetic analysis can be combined with simple assays for innate or conditioned behaviour.

Drosophila has a relatively simple olfactory system with ORNs distributed over two paired appendages, the antennae, which carry most of the receptor neurons, and maxillary palps (Figure 1A, Stocker 1994; De Bruyne 2003). The *Drosophila* antenna does not have a segmented flagellum like most other insects. Instead all olfactory sensilla are on one segment that does not contain taste or mechanosensory

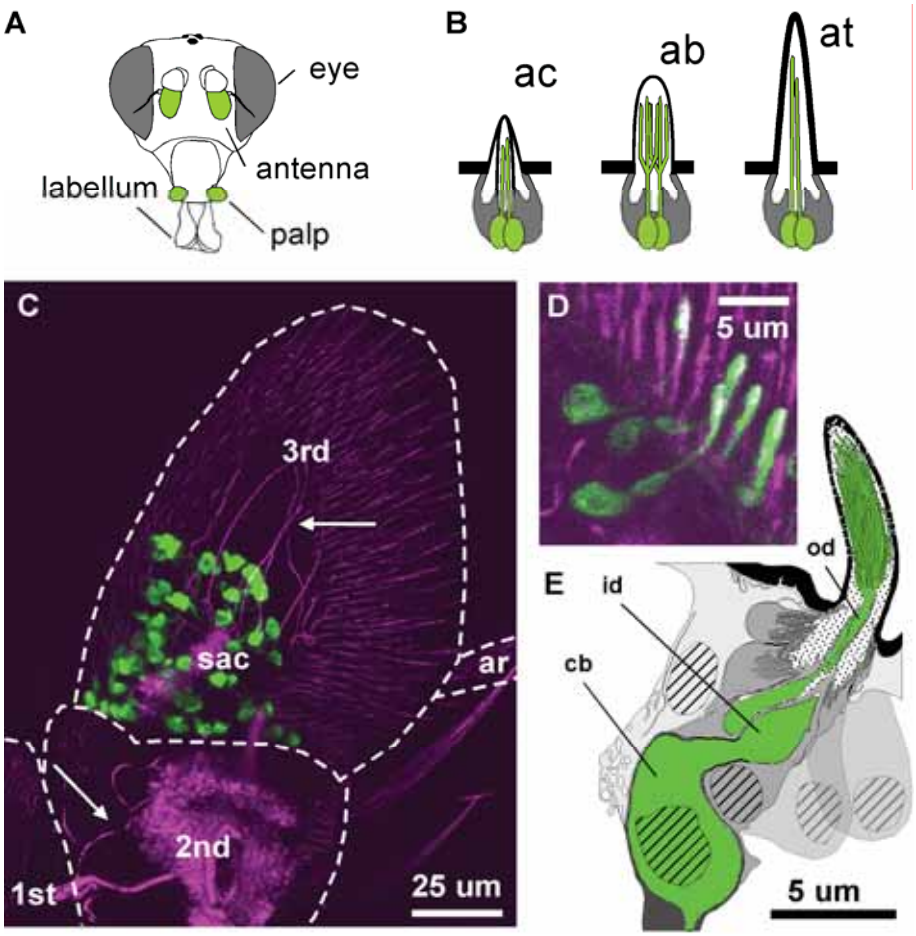

Figure 1. *Visualizing olfactory receptor neurons (ORN) of Drosophila* ***A.*** *Drosophila head with main sensory organs. ORNs (green) can be found on the antennal third segment (funiculus) and the maxillary palp.* ***B.*** *ORNs on the antenna are housed in sensilla made up of a cuticular hair or peg with a pored wall, 1-4 neurons (green) and 3-4 accessory cells (grey, see also under E). There are three structural categories of sensilla: antennal coeloconics (ac), basiconics (ab) and trichoids (at).* ***C.*** *A confocal image of an antenna with ab3A neurons labelled by membrane-bound mCD8::GFP (green) driven by Or22a-Gal4, the regulatory region of a receptor gene. Medial view of three antennal segments (1st, 2nd and 3rd) with cuticular structures visualized by reflected light (magenta). Sac, sacculus; ar, arista. Arrows point to trachaea.* ***D.*** *Detail of GFP-labelled receptor neurons innervating basiconic sensilla.* ***E.*** *Cellular components of a typical basiconic sensillum. Neurons in green, accessory cells in grey, glial cell in dark grey. Epidermal cells are light grey. Note the thin outer dendrite with branches filling the sensillum shaft (od), spindle-shaped inner dendrite (id) and round cell body (cb) very similar to the neurons in D. Drawn to scale after Shanbhag et al. (2001)*

sensilla. In most insects, gustatory receptor neurons (GRN) are mixed with ORNs on the antennae, but *Drosophila* offers the advantage that GRNs are found only on other appendages such as mouthparts and legs (Stocker 1994). As in all insects, the ORNs are housed in sensilla made up of small sets of epithelial cells (Figure 1B). A sensillum is composed of three elements (Figure 1E). First there is a cuticular apparatus, usually a hair or short peg with a pored wall. The accessory cells make up the second element. They supply the hair with a lymph that surrounds the dendrites of the last element; the neurons themselves. *Drosophila* olfactory sensilla contain 1-4 neurons that send their dendrites into the hair and their axons to the antennal lobe in the brain. ORNs of a single class converge on a single member out of a set of ca. 40 glomeruli (Stocker 1994; Laissue et al. 1999; Vosshall 2000, see also Figure 2A); small spherical sub-regions with a high density of synaptic contacts between ORNs, local interneurons and projection neurons. Both the palp and the antennal third segment are small (<100μm) and nearly transparent organs so their sensilla can be visualized under high magnification in a compound microscope. The antennal sensilla fall into two ultrastructural categories, double-walled (dw) and single-walled (sw) (Altner and Prillinger 1980). The dw sensilla of *Drosophila* are known as 'coeloconic' sensilla (Figure 1B, Venkatesh et al. 1984) and have a different developmental origin (Gupta and Rodrigues 1997; De Bruyne 2003) compared to the sw sensilla. They are not – as the term 'coeloconic' implies – situated in pits but merely in slight depressions which can be clearly seen under the microscope as circles. The sw sensilla are more abundant and have traditionally been further categorized as 'basiconic' and 'trichoid' sensilla (Figure 1B, Venkatesh et al. 1984): short peg-shaped with a rounded tip or longer and more hair-like, respectively. Around 550 of all 1200 neurons on the antenna are in basiconic sensilla. The maxillary palp bears only 60 basiconic sensilla housing 120 neurons in pairs. Compared to some other insect species that are important models in olfaction (moths, bees, cockroaches, locusts) *Drosophila* has fewer neurons that are more easily visualized.

CHEMOSENSORY GENES

Three large families of genes providing the molecular basis for detection of chemicals have been characterized: olfactory receptors (OR), gustatory receptors (GR) and odour-binding proteins (OBP). The first members of the *Drosophila* OR gene family were isolated by searching through genomic DNA sequences using algorithms designed to pull out sequences coding for multiple transmembrane domains in the predicted protein (Clyne et al. 1999; Vosshall et al. 1999). The sequencing of the full *Drosophila* genome sequence has revealed 60 OR genes with highly divergent sequences (Vosshall et al. 2000; Robertson et al. 2003). They show no sequence similarity to those of vertebrates or nematodes. However, a common feature of ORs across phyla is that they belong to the superfamily of seven transmembrane domain G-protein-coupled receptors (GPCRs). A second family of

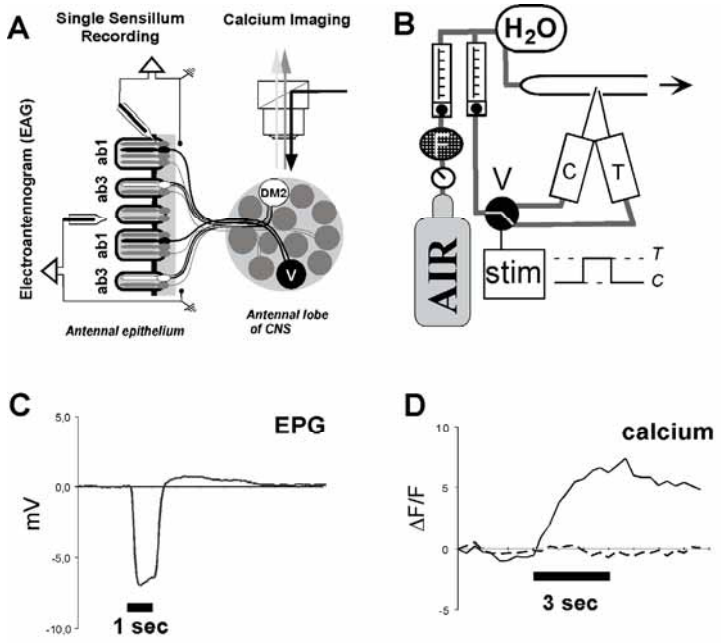

Figure 2. *Recording from olfactory receptor neurons*
A. *Schematic view of three ways to record activity in olfactory receptor neurons (ORNs). Two classes of Drosophila ORNs are indicated here: ab1C neurons (black), which send their axons to the V glomerulus, and ab3A neurons (white), which project to the DM2 glomerulus (see text). In single-sensillum recordings (SSR) electrical activity is measured by bringing an electrode (glass or tungsten) into contact with the lymph of a single sensillum. Electroantennograms (EAG) or electropalpograms (EPG) measure changes in the trans-epithelial potential by depositing a glass electrode on the cuticle. It presumably measures the combined activity of many sensilla but we do not know exactly how this process is accomplished. Finally, neural activity can be assessed by optical measurements on fluorescent-calcium sensors genetically targeted to certain receptor neurons. This can be done through the cuticle of the antenna or (as shown here) on the exposed antennal lobe where all ORNs of a single class converge onto glomeruli (see text) (Fiala et al. 2002).* ***B.*** *Odour stimulation is by delivering controlled pulses of odour-laden air into a constant air stream over the preparation. Air is charcoal-filtered (F), humidified (H_2O) and blown at relatively high speed (180 cm/s) from a glass tube with a small hole in the sidewall. Two syringes have their needles inserted through this hole. One of them (C) is empty and adds a constant flow of clean air. Odours are dissolved in paraffin oil on filter paper placed in another syringe (T). A valve (V), electrically regulated by a stimulator (stim), switches the flow briefly from C to T adding odour to the air without disturbing other properties such as speed, turbulence, humidity or temperature.* ***C.*** *Example of an EPG response to ethyl acetate.* ***D.*** *Example of calcium responses recorded from ab5B neurons expressing cameleon. ΔF/F indicates the increase in the ratio of fluorescence from its two fluorophores relative to the background fluorescence. Pentyl acetate (black line) evokes a change in calcium concentration not observed in a paraffin-oil control (dashed line). Baseline instabilities were corrected for by subtracting responses to clean air.*

such genes was described soon after (Clyne et al. 2000; Dunipace et al. 2001; Scott et al. 2001). These were named gustatory receptors (GR) because most of them are expressed in taste sensilla. The sequences of this family are even more diverse. In *Drosophila* some GR genes are expressed in antennae and may well have an olfactory function as there are no taste sensillae there. The OR genes are thought to have evolved from a subfamily of GR genes (Robertson et al. 2003). Individual members of the OR family are expressed in small subsets of ORNs, with different members expressed in different subsets (Clyne et al. 1999; Vosshall et al. 2000). As in vertebrates, axons of ORNs expressing a particular OR gene converge onto single glomeruli (Vosshall et al. 2000; Gao et al. 2000).

Members of a third gene family that is thought to play a role in mediating odour response variability are generally referred to as odour-binding proteins (OBP). They are not membrane-bound and not neuronal but secreted in large quantities into the extracellular lymph by accessory cells of sensilla or by epithelial cells (Shanbhag et al. 2001). Unlike Ors, the OBPs are not produced exclusively in olfactory sensilla. Nevertheless, they represent a varied set of genes that are differentially expressed in olfactory tissues. This has long been taken as strongly indicative for a role in determining response properties of ORN (Steinbrecht et al. 1995). Evidence for this has recently come from a mutation in one of the *Drosophila* OBPs (obp76a) called *lush* (Xu et al. 2005).

USING THE *DROSOPHILA* TOOLKIT TO STUDY OLFACTORY CODING

Drosophila genetics

The main reason to use *Drosophila* as a model species is of course its amenability to genetic manipulation. There are two ways to study a biological system in *Drosophila* genetically. Classical or 'forward' genetics starts from randomly induced mutations, causing changes in the fly's phenotype that are of interest to the questions at hand. By exposing flies to certain chemicals or radiation, changes in the DNA sequence can be induced (mutagenesis). These mutations are then 'mapped' to a locus in the genome, ideally to a single gene. Nowadays geneticists more often work the other way round. They have identified a candidate gene from the *Drosophila* genome and want to know its effect on a certain phenotype. Attempts to target a particular gene by either reducing or enhancing its function are referred to as reverse genetics.

Several reverse-genetic techniques use transposons, small pieces of DNA that occur naturally and have the property of moving around in the genome by removing and reinserting themselves. The so-called P element is most commonly used. The coding region for the transposase, an enzyme that mediates its mobility, has been removed and a genetic marker gene has been added so that its presence in the genome can be seen in a fly's phenotype. These transposons can be genetically engineered and used to incorporate foreign DNA (transgenes) in the *Drosophila* genome.

The Gal4/UAS system makes it possible to express a transgene in a specific tissue only. Gal4 is a gene originally found in yeast that has no normal function in

Drosophila cells. It encodes a transcription factor; a protein that regulates other yeast genes by binding to DNA at a site called UAS (upstream activating sequence). Brand and Perrimon (1993) cloned the Gal4 gene and the UAS sequence in two separate P-elements, introduced in two separate parental fly lines. One fly line contains the 'driver' element, expressing Gal4 in a particular set of cells and/or at a particular time in development. The other line contains the 'responder' element; the gene of choice under the control of the UAS. When the two are crossed, Gal4 can bind to UAS and activate expression of its transgene, but only in the targeted cells that express Gal4. There are many different Gal4 lines with highly specific expression patterns. Similarly, many useful genes have been introduced in UAS elements. This highly flexible expression system is now widely used as a tool to target specific cell populations such as ORN classes or accessory cells in flies.

In a certain GAL4/UAS approach a P element incorporating Gal4 with a weak promoter is allowed to 'jump' around in the genome by crossing in a chromosome carrying another P element with the transposase. It is then thought to insert randomly, and the transgene is expressed under the influence of regulatory sequences close to the insertion point. Such 'enhancer trap lines' have several disadvantages. For instance, out of thousands of random insertions that were scanned for expression in the brain, very few expression patterns are specific for a particular class of cells (Ito et al. 2003). A more reliable approach is to generate specific Gal4 constructs that include regulatory sequences (promoter and enhancer elements) upstream of a known gene. Such 'promoter constructs' ideally place the Gal4 under the control of the regulatory region of the chosen gene to target the UAS transgene specifically to cells that express it. However, even in this case, care must be taken. The expression pattern can differ from the actual gene's expression because not all enhancing elements were included or suppressing elements were omitted. In addition, Gal4 expression can depend on the insertion point of the P element.

Genetic tools for manipulating olfactory neurons

To target ORNs the most specific sequences that can be used to drive Gal4 expression are those regulating expression of OR genes. A number of OR promotor–Gal4 constructs have been made, which drive expression of Gal4 in a small subset of ORNs (Vosshall et al. 2000; Goldman et al. 2005; Kreher et al. 2005). Several readily available UAS constructs allow the expression of transgenes that visualize cells, allow neuronal-activity monitoring or the inactivation of cells, either permanently or under certain conditions. The gene for green-fluorescent protein (GFP), originally from a jellyfish, has been manipulated to render a cytosolic protein that does not damage the cells and gives strong green (510 nm) fluorescence upon excitation with blue (488 nm) light. It has been coupled to the mouse gene CD8 to give a fusion protein that localizes to the cell membrane (Lee and Luo 1999). Fluorescence can be observed in living flies under a stereomicroscope or in whole mounts under a confocal laser scanning microscope (Figure 1C,D) and the full shape of ORNs can be resolved.

Apart from making cells visible, proteins can be expressed that indicate cellular function. Several UAS transgenes make proteins that change their fluorescent properties with neural activity. Two of these measure intracellular calcium concentrations that usually go up when neurons are depolarized: Cameleon (Fiala et al. 2002) and GcamP (Wang et al. 2003). A third construct, synapto-pHluorin, is localized in synaptic vesicles and increases its fluorescence when neurotransmitters are released during synaptic transmission (Ng et al. 2002).

All these proteins are presumed to have very little influence on the physiology of the cell. Cell function itself can also be manipulated in various ways. A modified version of the bacterial gene for tetanus toxin is used to block synaptic transmission, effectively eliminating neuronal function (Sweeney et al. 1995). Expression of the *rpr* and/or *hid* genes can selectively ablate cells since these genes are part of the cellular mechanism for programmed cell death (Bergmann et al. 1998). One disadvantage of killing or disabling cells is that this can disturb development during embryogenesis or metamorphosis. An alternative is the use of constructs that are only activated under certain conditions. Such as the temperature-sensitive *UAS–Shi* construct (Kitamoto 2001). *Shi* (shibire) is a mutation in the dynamin protein, which is involved in synaptic-vesicle recycling. Expression of this protein is harmless at 25°C, but at 33°C the mutated form effectively blocks the native form and synaptic transmission stops. The advantage is that the flies can develop and behave normally until the temperature is raised during a particular experiment.

Recording neuronal activity

Drosophila's small size can be a challenge for physiologists but also offers distinct advantages. The antennal epithelium is packed with neurons and has a thin, transparent cuticle, so one can see a large set of olfactory sensilla in a single view under a compound microscope. There are three techniques available for recording neuronal responses to olfactory stimuli (Figure 2A). Detailed information on ORN responses can be obtained from single sensillum recordings (SSR), but these are laborious and technically demanding to perform. A quick but less informative approach is the electroantennogram (EAG), where an integrated response from many ORNs is recorded. Finally, specific GAL4 constructs that label ORNs can be used to drive calcium-sensitive proteins in order to measure neuronal activity optically. A reliable and flexible odour delivery system is used, which minimizes contamination between stimuli (Figure 2B). A glass tube continuously supplies humidified air to the preparation while two syringes are inserted through a small hole. A second flow of air (or nitrogen) passes constantly through an empty syringe and can be temporarily switched by a computer-controlled solenoid valve to push odorants from an odour-laden syringe. The delay time in the physiological response after activating the switch is determined partly by the airspeed and the distance from the injection point to the preparation and partly by the physiological response latency. The latter is around 20 ms and can be attributed to physico-chemical events at the air–liquid and liquid–membrane interfaces, as well as the transduction cascade inside the neurons.

Electrophysiological recordings are made from single olfactory sensilla by bringing an electrode (a saline-filled glass capillary with a silver wire or an electrolytically sharpened tungsten wire) in contact with the liquid surrounding the dendrites (Figure 2A). The reference electrode is inserted in the eye or in the thin cuticular folds at the base of the proboscis. This set-up measures extracellular voltage differences across the epithelium between the haemolymph and the sensillum lymph. Because the electrical resistance between individual sensilla is relatively high, only events that take place in the contacted sensillum are recorded. The relative amplitudes of action potentials fired by different ORNs in a single insect sensillum indicate the number of neurons present, and this phenomenon is used to analyse their activity separately (Kaissling 1995). The spikes of the ORNs in *Drosophila* basiconic sensilla can usually be separated reliably this way (Figure 3A,B, De Bruyne et al. 1999; 2001). When recording with glass electrodes one can also record the so-called sensillum potential (SP), an extra-cellular derivative of the membrane potentials of the neurons as well as the ion-pumping activity of the accessory cells that determines the potential difference between the sensillum lymph and the haemolymph (Figure 3C). Changes in this trans-epithelial potential in response to odorant stimulation are thought to reflect receptor potentials of the neurons (Kaissling and Colbow 1987).

Electroantennograms (EAG) can give reliable and more easily obtained information about which odorants are detected by insect ORNs (Figure 2A,C). Changes in the trans-epithelial potential can be recorded from the whole antenna using various electrode arrangements. From large insects such as moths, EAGs are recorded between the base and the cut tip of a severed antenna (Kaissling 1995). In Diptera the reference electrode is generally inserted in the haemolymph of the head and the recording electrode brought into contact with the antennal surface (Guerin and Visser 1980). To record EAG signals from *Drosophila* the fly is left intact, positioned inside a plastic pipette tip and the recording electrode placed on the medio-proximal part of the third antennal segment (Ayer and Carlson 1992). An equivalent signal can also be recorded from the maxillary palp, the electropalpogram (EPG, Ayer and Carlson 1992). The voltage deflections observed in response to odours are a summation of the SPs of many ORNs. They show very similar dynamics but smaller amplitudes. Although EAG recordings supply excellent resolution when comparing wild type to mutant phenotypes, they do not allow conclusions on odour coding per se, because the exact working principles of the EAG are poorly understood. For instance, it is not known how many ORNs are measured or what their relative contribution to the EAG is.

Optical imaging has been used extensively in recent years to measure activity from insect brains (Galizia and Menzel 2001, and references therein). In *Drosophila* it is now possible to express various sensors that register either intracellular calcium (Fiala et al. 2002; Wang et al. 2003) or changes in the pH of the synaptic cleft (Ng et al. 2002) because they are available as a UAS construct. To visualize signals in the *Drosophila* brain it is invariably necessary to remove the cuticle and expose the brain (Galizia and Vetter 2005). However, because the antennal cuticle is transparent

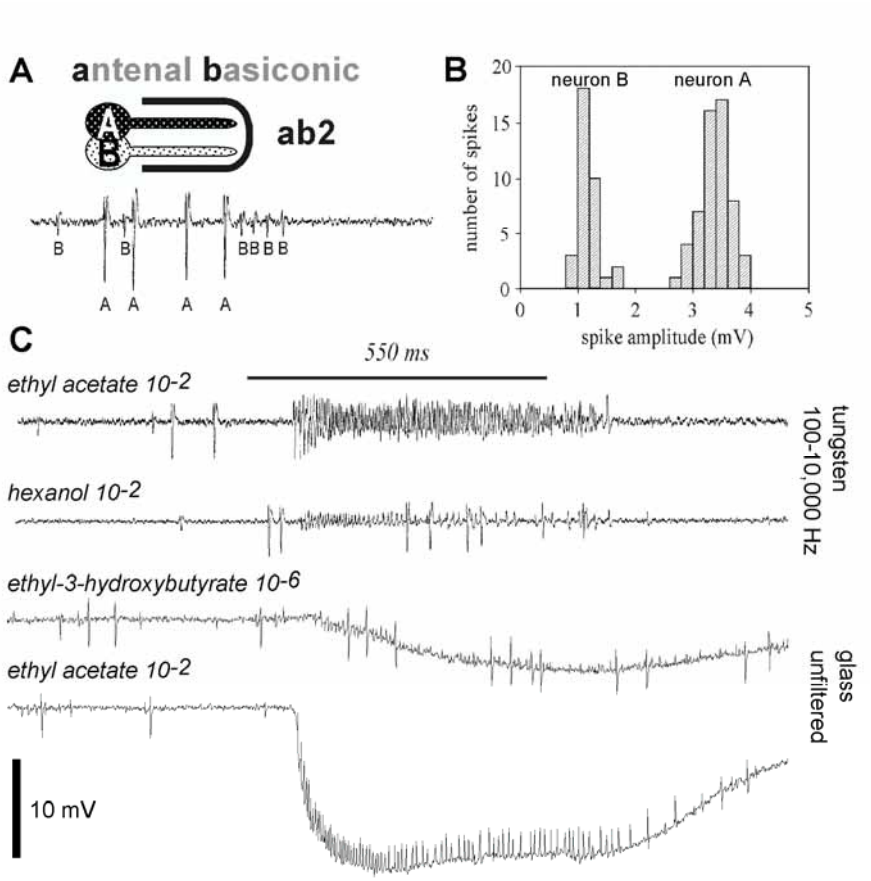

Figure 3. *Single sensillum recording of odour responses in receptor neurons* ***A.*** *Antennal basiconic sensilla of type ab2 contain two neurons that are identified by size and shape of the action potentials they fire as A (the larger) or B (the smaller).* ***B.*** *Frequency histogram of the spike amplitudes shows a bimodal distribution.* ***C.*** *Traces of recordings from ab2 sensilla using either tungsten electrodes with filtering (top two traces) or glass electrodes to reveal slow sensillum potentials. Odorants were dissolved into 20 µl of paraffin oil on filter paper according to the dilution factors indicated. Note that the B neuron is excited by ethyl-3-hydroxybutyrate at a 10,000x lower concentration than hexanol. Partly after de Bruyne et al. 2001, with permission*

it is possible to image ORNs in an intact fly (Figure 2A,D). One of the proteins engineered to indicate calcium concentration is called cameleon (Fiala et al. 2002). It combines calmodulin (a calcium-binding protein) and the calmodulin-binding domain from myosin (M13) with two modified GFP proteins that emit fluorescent light at either 485 nm (cyan) or 535 nm (yellow). An increase in intracellular calcium, such as occurring during an odour response, results in calmodulin binding

to M13 and fluorescent activity moving from the cyan to the yellow GFP. The latter event can be measured by recording light emission at two wavelengths and calculating the ratio. Optophysiological measurements of antennae offer the advantage of being technically less challenging than single sensillum recording. However, the exact relation between calcium signals and action-potential firing rates has not been established and it is of course the latter that drive behavioural output. For instance, calcium signals tend to rise and descend much slower than spike frequencies.

PHYSIOLOGICAL STUDIES REVEAL AN ARRAY OF RESPONSE UNITS

Cell types and distribution

All neural computing that leads to a useful representation of odours in the insect brain must be based on information supplied by receptor neurons. To understand an insect's response to odours, a complete picture of the neural code entering the brain would be desirable. What do typical ORN responses look like and how is neuronal activity in response to a single odorant distributed across all neurons? Figure 3 shows that short stimulations with odours result in increased firing of action potentials. The firing frequency generally increases rapidly (within 100 ms) to a maximum and then falls back due to adaptation (De Bruyne et al. 1999). Most neurons respond to several odorants but with differing sensitivity. Moreover, individual ORNs can be classified into classes with very similar response properties. Recording responses to several odorants from a large number of *Drosophila* basiconic sensilla has revealed 22 different ORN classes (De Bruyne et al. 1999; 2001). Elmore et al. (2003) added 2 more. These 24 neuron classes represent more than 50% of the entire olfactory input to the brain. In trichoid sensilla there are at least a further 6 ORN classes in three different sensillum types (Clyne et al. 1997; Xu et al. 2005). In addition, coeloconic sensilla also house ORNs with response profiles that fall into distinct classes, some responding to small aliphatic acids (Clyne et al. 1997; Park et al. 2002). Thus the *Drosophila* nose is organized in classes of ORNs with distinct response properties, as has also been observed in other insects (e.g., Kaib 1974). The total number of such coding units will probably be around 40-50 because in *Drosophila* ca. 50 glomeruli (including sub-compartments) have been identified (Laissue et al. 1999), 40 OR and a few GR genes are expressed in palps and antennae (Vosshall et al. 2000, and Figure 6) and there is near one-to-one correspondence between ORN classes, OR expression and projection to glomeruli (Vosshall et al. 2000; Goldman et al. 2005; Kreher et al. 2005).

Not only is there consistency in the response properties of ORNs but also in the way they are combined into sensillum types and (at least on the antenna) how sensillum types are distributed over the surface of the antenna. Genetic studies on the development of sensillum morphologies and ORN identities strongly suggest a hierarchy of events determining the layout of the array of odour detectors (for a review see De Bruyne 2003). In spite of this conspicuous pairing there is no

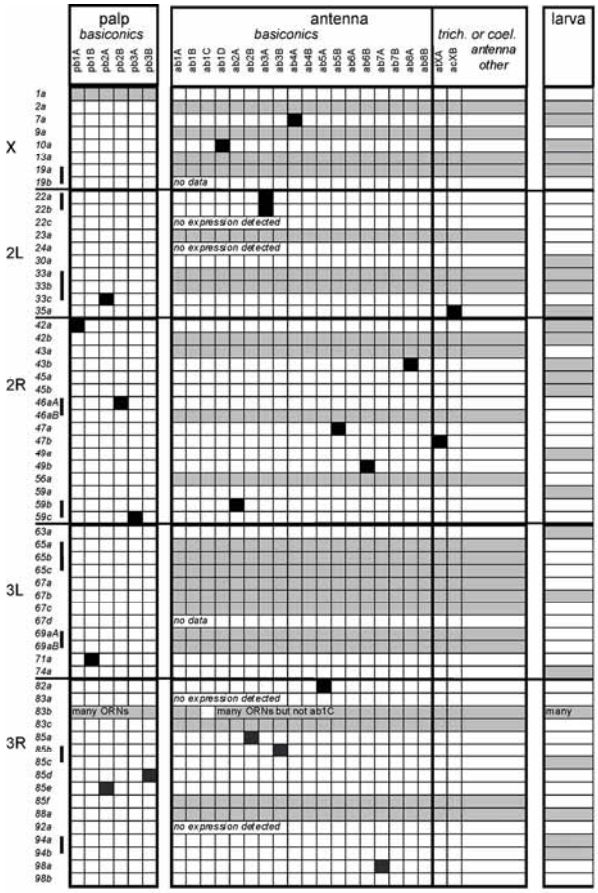

Figure 6. *Mapping OR genes to olfactory receptor neuron classes*
A summary of recent advances in the mapping of OR gene expression to defined response classes of olfactory receptor neurons or to palp, antenna or whole larva. All 60 genes of the Drosophila OR gene family are listed. The Or46a and Or69a genes are alternatively spliced rendering 62 OR proteins (Robertson et al. 2003). The nomenclature (Warr et al. 2000) indicates a gene's location on either the sex chromosome (X) or one of the arms of the autosomes (2L is left arm of 2nd chromosome). Vertical bars label genes that form small clusters. Identified ORN classes on the maxillary palp (De Bruyne et al. 1999) and antenna (De Bruyne et al. 2001; Elmore et al. 2003) are listed on the top and black squares in the matrix indicate positive mapping of a gene to an identified ORN class in the adult (Hallem et al. 2004; Goldman et al. 2005) and/or to a single larval ORN. Note that Or83b is expressed in most ORNs although its expression has not been verified for each class (Larsson et al. 2004). One identified exception is the ab1C neuron, which does not express Or83b. Grey squares indicate that the gene has been mapped to antenna, palp and/or larva but the ORN class has not been identified (Vosshall et al. 1999; Hallem et al. 2004; Kreher et al. 2005). For some genes data are lacking and for others RT-PCR experiments failed to reveal expression in the two adult olfactory organs (Vosshall et al. 1999) or in larvae (Kreher et al. 2005)

evidence for integration of odour information at the level of sensory neurons. When challenging one of the two neurons of a palpal basiconic sensillum (pb1A) with a strong prolonged stimulation it was found that adaptation of this neuron does not affect the response of the other neuron (De Bruyne et al. 1999). It has been suggested that two ORNs sampling the same µl of air via the same sensillum lymph allows more accurate computation of the concentration ratios of components in a mixture (Todd and Baker 1999). For instance, pairing two neurons, sensitive to the components of a pheromone blend, may allow moths to assess their relative concentrations with a very high spatial and temporal resolution. It is conceivable that this is required for split-second decisions about odour quality during upwind flight. Pheromone plumes are mixed with non-relevant odours from the background, but individual odour packets within such a plume are thought to derive from the same source. If this is a general reason for combining certain ORNs in a single sensillum then we should ask ourselves what the functional relations are between the odorants that cohabiting ORNs detect.

Coding properties

Odour stimuli contain three elements of information that are encoded by the ensemble of ORNs. The first is odour identity, i.e., the chemical structure of a single compound or of several components in a blend. The second is the concentration of the odorants. The third is odour variations in time. Typical experimental odour pulses have an onset and an end. By contrast, natural stimuli consist of variations in odour intensity and identity over time. These variations can be long-term (minutes, hours, days) when insects move from one environment to another, or short-term (milliseconds) as, for instance, when a flying insect traverses an odour plume.

The discussion about identity coding in insect ORNs has focused on whether there are so-called 'specialist' and 'generalist' neurons (Schneider 1984; Hildebrand and Shepherd 1997). In *Drosophila* basiconic sensilla we find examples of both (De Bruyne et al. 1999; 2001). Figure 4 shows how an odour stimulus leads to neural activity across an array of ORN classes. The ab2A neuron would be considered a specialist with its specific response to ethyl acetate. However, classification of odour response spectra as narrowly tuned (specialist) or broadly tuned (generalist) to odours depends very much on the set of stimuli used. The same neuron also responds to acetone and 2,3-butanedione. Moreover, its response to ethyl acetate is not very strong and it is likely that as yet unidentified chemicals provide a better stimulus. The data in Figure 4 show that at these relatively high doses some odours stimulate several ORN classes and some ORN classes respond to more than one odour. The general notion is that odour perception would require the CNS to integrate information across ORNs (combinatorial coding). The ab1C neuron could supply what has been described as a 'labelled line'. In *Drosophila* it is the only ORN that responds to CO_2, and CO_2 is the only odorant it responds to. Other ORNs are also narrowly tuned, e.g., ab1D to methyl salicylate, and ab5A to geranyl acetate (not shown). In a combinatorial code (a.k.a. across-fibre pattern) an odour will be

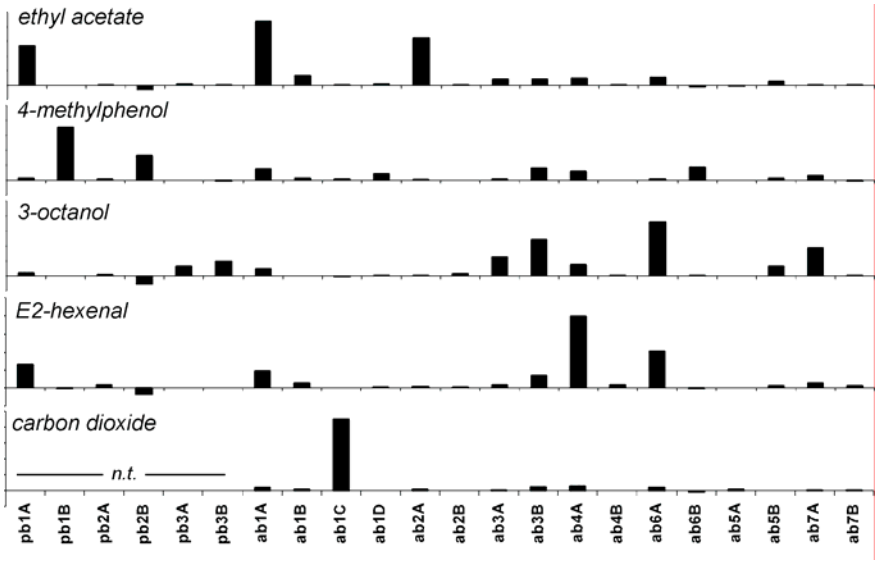

Figure 4. *Odours excite different combinations of receptor neurons*
Excitation patterns across 22 olfactory receptor neuron (ORN) classes for five odorants as extrapolated from single-sensillum recordings using 10^{-2} dilutions. Data are from De Bruyne et al. (1999; 2001). pb…. – palpal ORN; ab….. – antennal ORN. Note that CO_2 was not tested (n.t.) on palpal neurons but electropalpograms show no responses

defined by the combined activity of several ORN classes. In its most extreme form all ORNs would have broad overlapping response spectra and the CNS would be a homogeneous network that converts their activity patterns in odour percepts. The other extreme would be a labelled line system where each odour has a 'dedicated' ORN class defining its perception.

In order to understand principles of odour coding it is important to realize that dose–response relationships are not linear. The typical dose–response curve is sigmoid in shape, rising slowly at lower doses, more or less linear over a range of 2-3 log steps and saturating at spike rates of over 200-300 spikes/s (Figure 5A). As a result, coding of odour identity over a wider range of concentrations would require two or more ORN classes with different sensitivities (De Bruyne 2003). Another consequence of the non-linearity is that odour identity cannot simply be determined from the ratio in firing rates of a set of ORNs because these will be dose-dependent. For our initial characterization of *Drosophila* ORNs, we tested odorants at a relatively high, but not unnatural dose (De Bruyne et al. 2001). When comparing dose–response relations we found that thresholds for the more active stimuli were at least 1000x lower. In order to identify neuron classes one has to find at least one stimulus that elicits a response, but the best ligands for most of these ORNs may not have been in our set of odorants. Stensmyr et al. (2003b) have since identified better stimuli for some of them, such as ethyl-3-hydroxybutyrate, which stimulates the ab2B neuron (Figure 3C).

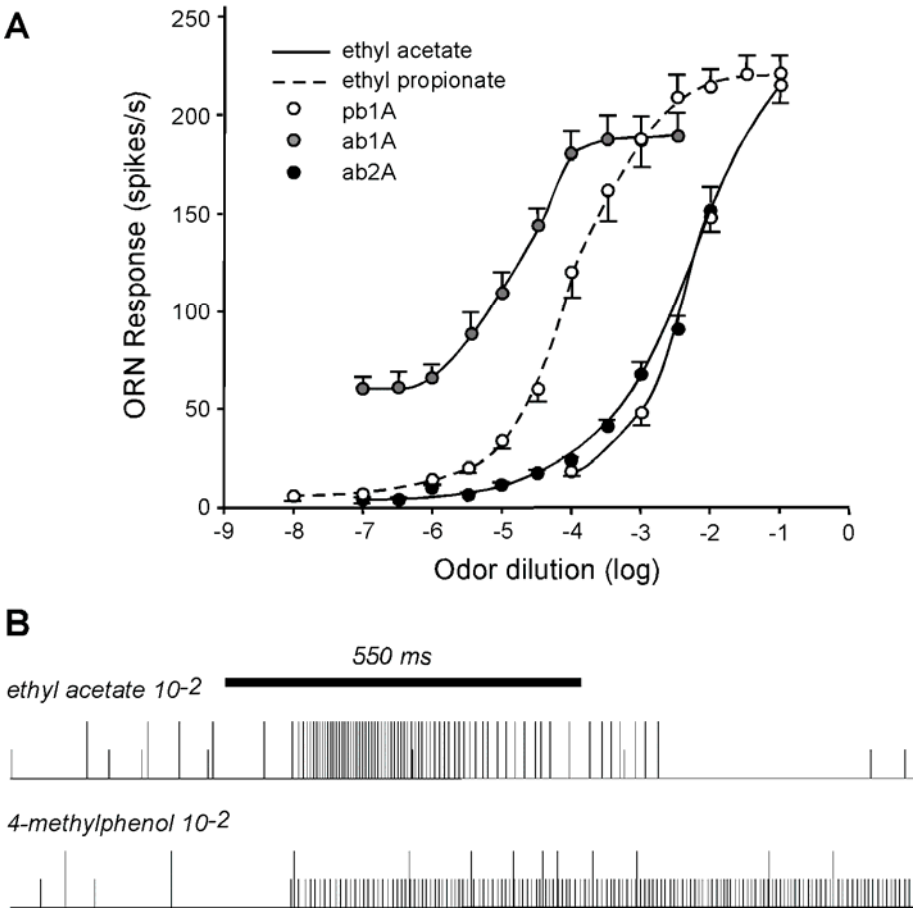

Figure 5. *Coding of odour concentration and odour dynamics*
A. *Dose–response curves of olfactory receptor neuron excitation are sigmoid with sensitivities reaching as low as 10^{-7}. Odour concentration is indicated here as dilutions in 20 µl paraffin oil on filter papers placed in odour cartridges. Concentrations reaching the fly are unknown, as they depend on vapour pressure, but air expelled from these cartridges is diluted another 16-fold. Note that at higher doses three neurons respond to ethyl acetate but only one is sensitive to low doses. Meanwhile the pb1A neuron, though excited by ethyl acetate at high doses, responds better to ethyl propionate at low doses.* ***B.*** *Raster plots of neural activity in pb1 sensilla in response to two odorants. Note the strongly phasic-tonic nature of the pb1A neuron's response to ethyl acetate (large spikes in upper trace) and the more tonic and longer-lasting activation of pb1B by 4-methylphenol (small spikes in lower trace). After De Bruyne et al. (1999; 2001), with permission*

The basis of the olfactory code is that ORNs respond with different sensitivities to different odours. However, response kinetics also vary (Figure 5B). The onset of a typical odour pulse induces a sharp rise in firing frequency, which quickly decreases

(adaptation), but the end of stimulation is either marked by an abrupt decrease in firing or by a much slower decay in firing. These variations in temporal integration of stimulation are specific for stimulation of a particular ORN with a particular odorant (De Bruyne et al. 2001). Theoretically at least, these properties could contribute to odour coding.

OR GENES: FUNCTIONAL CHARACTERIZATION AND MAPPING TO ORN CLASSES

The predicted role of the *Drosophila* OR genes as odorant receptors has been confirmed by inducing odour responses after the expression of an OR gene and by their removal in case of mutation. One study uses a Gal4 enhancer trap line to over-express the OR43a gene in *Drosophila* ORNs (Störtkuhl and Kettler 2001) while another uses heterologous expression of the same gene in *Xenopus* oocytes (Wetzel et al. 2001). In both cases it was shown that expression of the OR gene leads to physiological responses to specific odorants. Electrophysiological analysis of mutations in the genes Or22a and Or43b showed that odour responses from ORNs that normally express these genes were no longer observed (Dobritsa et al. 2003; Elmore et al. 2003). Each ORN class is restricted to a particular spatial domain on the antennal surface, although there is considerable overlap between them (De Bruyne et al. 2001). The expression patterns of OR genes in the antenna reflect this organization (Clyne et al. 1999; Vosshall et al. 1999; Gao and Chess 1999). Now, the expression of many OR genes has been mapped to the ORN classes (Figure 6) using two different techniques. In the first technique, OR-Gal4 constructs were used to label sensilla with GFP or delete the targeted ORN with *rpr* (Dobritsa et al. 2003; Goldman et al. 2005). Single sensillum recordings were then used to identify the sensillum type. The second technique makes use of the fact that in Or22a mutants the ab3A neuron no longer responds to odorants but it still functions as a neuron (Dobritsa et al. 2003). Other OR genes can be expressed in this 'empty neuron' (Δab3A), inducing odour responses specific for the expressed OR (Hallem et al. 2004; Kreher et al. 2005). The acquired response is then compared to the established spectra of native ORNs.

Expression of OR genes has also been analysed in *Drosophila* larvae (Kreher et al. 2005). Some ORs are unique to the larval olfactory organ but others are expressed in both adults and larvae. The extensive characterization of ORN response properties, mapping of OR genes and ORN projection patterns indicates that all information about odours is represented across 40-50 units in the adult and ca. 25 in the larva.

The general rule emerging from these studies is that a single functional class of ORN expresses only one receptor gene and a single receptor gene is expressed only in one class of ORN. One notable exception is the Or83b gene, which is expressed in a large number of ORNs (Vosshall et al. 2000). It was recently shown that in most ORNs this special OR is needed to make the other OR functional (Larsson et al. 2004). Mutations in this gene render the fly largely anosmic. Because Or83b probably does not function as an odour receptor, the dogma of one-neuron-one-

receptor is still valid. However, Goldman et al. (2005) have recently demonstrated that pb2B neurons express two 'classical' OR genes (Figure 6). Although both are functional the authors could not show that the response spectrum of the neuron is significantly broadened by this coexpression.

CONCLUDING REMARKS

Chemical ecology of Drosophila

D. melanogaster females lay their eggs on ripe fruit in various stages of fermentation where larvae feed on yeast. Olfaction plays a major role in finding these resources. Simple products of fermentation such as ethanol and acetic acid have long been known to attract *Drosophila* (Barrows 1907; Zhu et al. 2003). Several *Drosophila* ORNs respond to esters, classical components of fruit odours: ethyl acetate, ethyl hexanoate, pentyl acetate, ethyl 3-hydroxybutyrate (De Bruyne et al. 2001; Stensmyr et al. 2003b). However, ovipositing females must not only localize feeding sites with high nutritive value but also with low toxicity. Plant chemical defences could play a role here. Does the presence of a specific receptor for methyl salicylate suggest a role for this compound in the chemical ecology of *Drosophila*? Methyl salicylate is a derivative of salicylic acid and part of volatile distress signals of many plants (Dicke et al. 1990; Shulaev et al. 1997). A predatory mite that has very few ORNs, detects this compound as it is released by the feeding activity of its spider mite prey (De Bruyne et al. 1991; Dicke et al. 1990; De Boer and Dicke 2004). However, ticks also detect it and here the compound is part of an aggregation pheromone emitted when feeding on its mammalian host (Schöni 1987; Diehl et al. 1991), underlining an entirely different role of the same odorant. It is risky to jump to conclusions about the role of specific ORNs in ecological interactions. However, certain odorants may be signals in numerous interactions and their detection a conserved property of many olfactory systems.

Evolution of the olfactory code

Do OR sequences and their associated ORN properties reflect the selective pressure enforced by chemical ecological needs or do they simply vary with phylogenetic distance? Olfactory systems in insects are probably conserved to a certain extent but specific ORNs could be subject to high selective pressure related to shifts in behavioural ecology. The females of several fruit-fly species (Tephritidae) are known to exhibit marked preferences for odours from specific fruits to lay their eggs (Frey and Bush 1990). Although *Drosophila* species are less specific in their selection of oviposition sites, they do show differences in attraction to odours of different fruits (Hoffmann 1985). Within the closely related *melanogaster* species group *D. simulans* shows a lack of preference similar to that of *D. melanogaster*. However, a third member of this group, *D. sechellia*, exhibits remarkable preference for one particular fruit (Higa and Fuyama 1993). A comparison of the response spectra of 8 ORN classes in large basiconic sensilla of the 9 members of this species group indicated that the initial encoding of olfactory information is highly conserved

(Stensmyr et al. 2003a). Only in one ORN class a shift in sensitivity to certain esters was observed in three species, one of them being *D. sechellia*. This species also seems to have replaced one sensillum type with more copies of another. These results show that when changes do occur they can be in the transduction elements themselves (e.g., OR genes) or in genes that regulate the patterning of the antennae. An example of a highly conserved trait is the coexpression of two OR genes, which is also found in *Drosophila pseudoobscura*, a species that diverged ca. 46 million years ago from *D. melanogaster* (Goldman et al. 2005). By contrast, there is only very little sequence homology between OR genes of *D. melanogaster* and a member of the nematoceran Diptera, the mosquito *Anopheles gambiae* (Hill et al. 2002). Detailed knowledge on molecular and cellular elements of the peripheral olfactory system of *Drosophila* is likely to be extremely useful for studying the evolution of odour coding in insects.

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