PHEROMONE SIGNALING IN THE FRUIT FLY *DROSOPHILA MELANOGASTER*

PERCEPTION AND BEHAVIOR

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Summary

The fruit fly *Drosophila melanogaster* has been used as a model species in very diverse branches of science for more than 100 years. The genetic, molecular, and physiological tools that are available for this species offer unique opportunities for experiments that are currently not possible in other organisms.

One important line of study in *D. melanogaster* has been how the chemosensory system of the fly converts information sampled from the outside world to representations in the central nervous system, and how this translates into specific behaviors. Insects rely on chemosensory cues for many aspects of their life, such as locating suitable mates, discovering the presence of a predator, or assessing the quality of a resource or an oviposition site. Communication between individuals of the same species is also often governed by pheromones; molecules that elicit a stereotypic reaction in a conspecific. These molecules can be perceived either by the olfactory system or by the gustatory system.

The aim of this paper is to give an overview over what we know about how the chemosensory system of this fruit fly is organized, how odors are perceived and processed, and the identity and functional role of the pheromones that regulate its behavior.

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1. Introduction

1.1. Pheromones

The term ‘pheromone’ was made by combining the greek words meaning ‘to carry’ and ‘to stimulate or excite’, and this gives an accurate idea of what pheromones are: molecules released by one individual that elicit a specific reaction in another individual of the same species (Wyatt 2003). These molecules are often classified by their function, and well-known examples include sex pheromones involved in reproduction, alarm pheromones released in the presence of an enemy, and pheromones regulating hierarchical interactions in social species.

The use of pheromones is widespread among very diverse taxa, but has been most extensively studied in insects. The first pheromone to be chemically characterized was bombykol, the sex pheromone of the silkworm moth *Bombyx mori* (Butenandt et al. 1959), and the intensive research that followed has led to the identification of hundreds of pheromones from different insect species. The last decades have seen breathtaking advances in our understanding of how these pheromones are perceived and processed by the nervous system, and how they subsequently elicit specific behaviors.

1.2. The fruit fly *Drosophila melanogaster*

Several insect species have become important model organisms for the study of chemosensory signaling. One of the most fundamental is the fruit fly *Drosophila melanogaster* Meigen, a human commensal who, with the exceptions of extremes of altitude or latitudes, has a worldwide distribution. Ever since Thomas Hunt Morgan used *D. melanogaster* for his studies of heredity in the early 1900s, this seemingly inconspicuous Dipteran has been at the forefront of biological research.

There are several reasons for why *D. melanogaster* has achieved this position. On a very practical level there are several aspects of the species’ biology that facilitate its use in experiments, such as ease of rearing, low costs, and a short generation time. *D. melanogaster* has a relatively simple nervous system, but still exhibits complex behaviors. This makes the species ideal for understanding how the nervous system is organized, how specific behaviors are encoded in the nervous system, and how these behaviors can be affected by modulation triggered by external and internal cues.

The genetic tools that have been developed for *D. melanogaster*, such as balancer chromosomes and the GAL4/UAS system for targeted gene expression, allows us to create a vast array of genetically modified fly lines with very different and very specific features. Coupled with sequencing of the complete genome this makes it possible to conduct experiments in flies that are currently impossible in any other species. Despite the obvious visual dissimilarity, there are considerable anatomical and functional parallels with the chemosensory systems of higher animals (Benton and Dahanukar 2011, Thorne et al. 2004, Wang et al. 2004). Therefore, results obtained from studies in *D. melanogaster* are likely to provide broad insight into fundamental principles, also for the human nervous system.

1.3. The chemosensory system of *D. melanogaster*

Insect pheromones receptors are integrated in the chemosensory system. Volatile pheromones are perceived by olfactory receptors on the antennae and maxillary palps, non-volatile contact pheromones by gustatory receptors found on the surface of the fly (fig. 1).

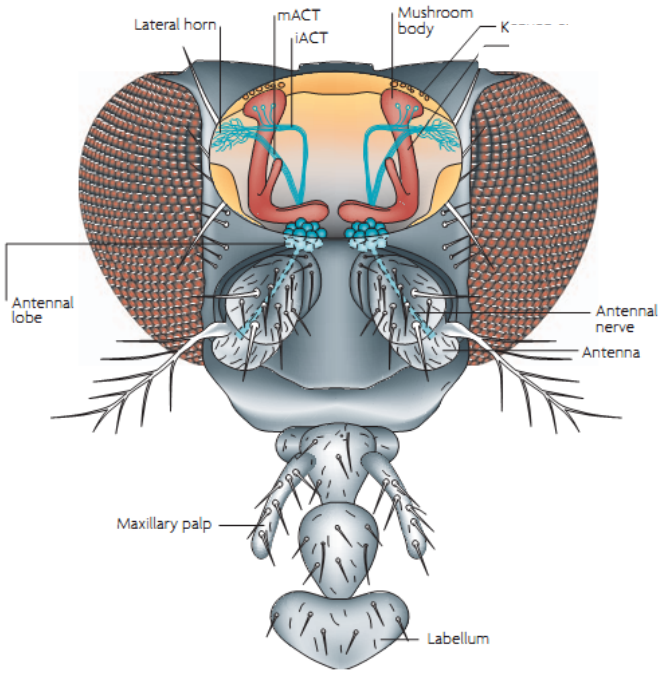

Figure 1: The chemosensory system in *Drosophila melanogaster*. Olfactory stimuli are sensed by neurons on the antennae and maxillary palps that project along the antennal nerve to the antennal lobe. The information passes through the inner and medial antennocerebral tract (iACT and mACT) to the mushroom body and the lateral horn. Gustatory stimuli are sensed by neurons in the labellum on the tip of the proboscis, on the legs, and on the wing margins. These neurons project into the subesophageal ganglion. Modified from Keene and Waddell (2007).

2. Olfaction

Fruit flies detect odors through specialized olfactory sensory neurons (OSNs) on the antennae and maxillary palps. OSNs project to specific glomeruli in the antennal lobe (AL). Glomeruli interact through local interneurons, and the output from the AL is conveyed by projection neurons to down-stream olfactory centers in the protocerebrum, the mushroom bodies and the lateral horn. Combinatorial activation of odorant receptors generates a specific pattern of OSN activity that is relayed to the antennal lobe, where it is transformed into an activation pattern of glomeruli (Ng et al. 2002; Wang et al. 2003). Information contained within these glomerular activation maps is transmitted via projection neurons to the mushroom bodies and lateral horn (Jefferis et al. 2007, Lin et al. 2007, Wong et al. 2002), where it is interpreted, formatted for memory and association with other modalities, modulated by prior experience (Davis 2005), and ultimately drives or modulates the activity of various circuits that control behavior (fig. 2).

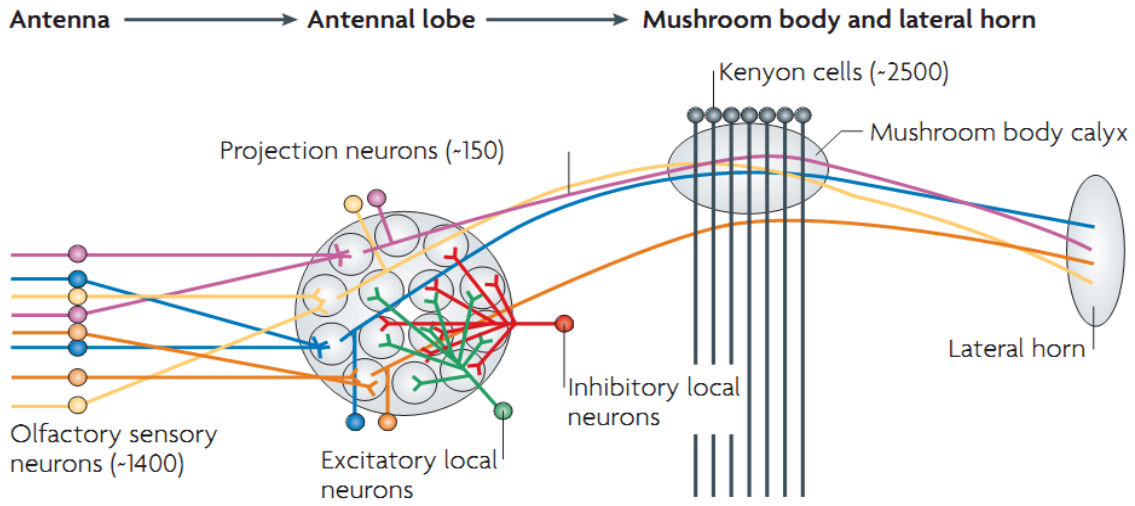

Figure 2: Schematic representation of the olfactory system of *D. melanogaster*. Olfactory sensory neurons in the antennae and maxillary palps send axons to specific glomeruli in the antennal lobe. All olfactory receptor neurons expressing the same odorant receptor (same colour) converge at the same glomerulus. There they form synaptic contacts with projection neurons and excitatory and inihibitory local neurons. Projection neurons send axons either directly to the lateral horn neuropil or terminate in the lateral horn after first projecting to the calyx of the mushroom body, where they form synapses with intrinsic Kenyon cells. From Keene and Waddell (2007).

2.1. Peripheral processes

2.1.1. Olfactory sensilla

Fruit flies detect odors through two specialized paired organs on their head, the third antennal segments and the maxillary palps. These structures are covered by specialized structures called sensilla: hair-like protrusion that contains the dendrites of one or more OSNs (fig. 3). Each antenna contains around 410 sensilla (Shanbag et al. 1999). The maxillary palp is a simpler structure, and contains approximately 60 sensilla (de Bruyne et al. 1999).

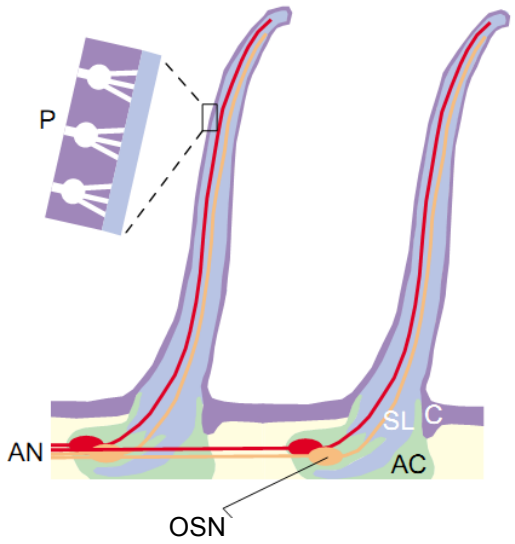

Figure 3: Insect olfactory sensilla. The olfactory sensory neurons (OSNs) are housed within a cuticular hair and surrounded by the sensillum lymph (SL). Each hair is a self-contained unit, owing to a tight seal at the base accomplished by three types of accessory cell (AC). Odor molecules enter the hair through pores (P) in the cuticule (C). ONs project their axons to the brain through the antennal nerve (AN). Modified from Hansson (2002).

The dendrites of the OSNs are bathed in the sensillum lymph. This liquid contains several accessory molecules, among them many different odorant binding proteins (OBPs) and odorant degrading enzymes (ODEs) secreted by accessory cells (Gomez-Diaz et al. 2013, Galindo and Smith 2001, Galizia 2014, Graham and Davies 2002). Swarup et al. (2011) demonstrated that suppression of expression of OBPs in *D. melanogaster* resulted in altered behavioral responses to many odorants, indicating that olfactory binding proteins have a crucial role in odor recognition and for mediating odor-evoked behavior.

Olfactory sensilla can be divided into four distinct morphological classes: club-shaped basiconic sensilla; long and tapered trichoid sensilla; short, peg-shaped coeoloconic sensilla; and intermediate sensilla that combine features of both basiconic and trichoid sensilla. Further morphological features subdivide both basiconic and trichoid sensilla into additional subclasses that are distinguished by the size and density of their odor pores, the number of neurons they house, and how they are distributed across the antennae (Couto et al. 2005, Clyne et al. 1997, Shanbhag et al. 1999, 2000, Stocker 1994). The maxillary palp sensilla are all basiconic sensilla housing two neurons. They can be further subdivided into the three subtypes PB-I, PB-II, and PB-III (Shanbhag et al. 1999). The number of neurons innervating a given sensillum is also well conserved: trichoid sensilla contain one to three neurons, basiconic sensilla mostly have two neurons (though some house four neurons), and coeloconic sensilla typically house two or three neurons (Couto et al. 2005, Shanbhag et al. 1999, Stocker 1994).

The different sensilla types are distributed in highly stereotyped patterns across the surface of the antenna, with large basiconic sensilla found in diagonal bands across the lateral face, trichoid sensilla clustered at the lateral-distal edge, and coeloconic sensilla concentrated at the central face of the antenna (Couto et al. 2005, Shanbhag et al. 1999) (fig.4).

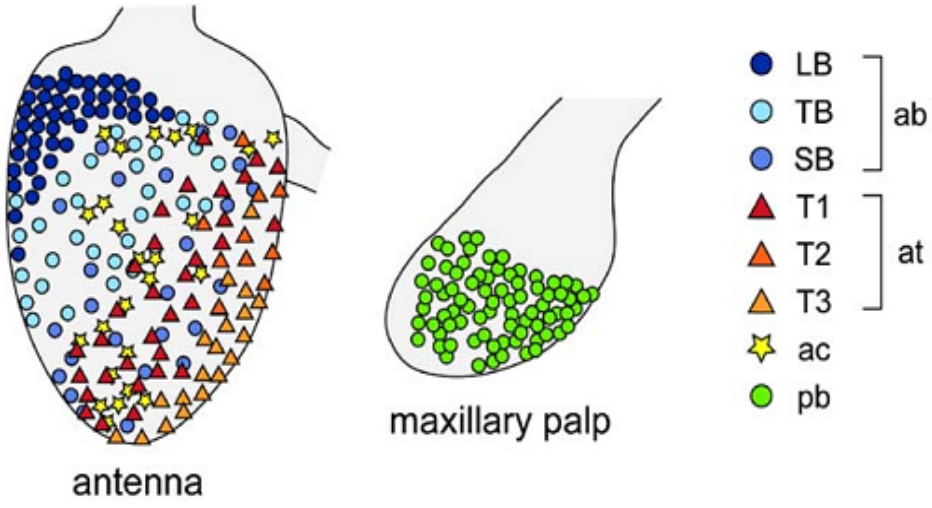

Figure 4: Distribution of different sensilla types on the antenna and maxillary palp of *D. melanogaster*. ab = antennal basiconics. LB, large basiconics; TB, thin basiconics; SB, small basiconics. at = antennal trichoids. T1-3, trichoid sensilla innervated by one, two, or three neurons. ac = antennal coeloconics. pb = palpal basiconics. Modified from Couto et al. (2005).

2.1.2. Olfactory sensory neurons

Each antenna contains approximately 1200 olfactory sensory neurons (de Bruyne et al. 2001, Stocker et al. 1990), each maxillary palp around 120. OSNs are bipolar neurons that extend their dendrites into the shaft of the sensillum, whereas their axons project from the basal end and terminate in the antennal lobe. Through single sensillum electrophysiology (SSR), detailed characterization of most of the OSNs has been achieved (Benton et al. 2009, Dobritsa et al. 2003, Hallem and Carlson 2006, Hallem et al. 2004, Silbering et al. 2011, Yao et al. 2005), demonstrating that the morphological differences between sensillum types also to some extent reflect functional differences: OSNs in basiconic sensilla are tuned to food odors, both in the antennae and in the maxillary palps (Hallem et al. 2004, Hallem and Carlson 2006); OSNs in coeloconic sensilla respond to acids and aldehydes (Benton et al. 2009); and trichoid sensilla house pheromone-sensitive OSNs (Clyne et al.1997, Ha and Smith 2006, Xu et al. 2005). Male flies have more trichoid sensilla than females (Stocker 1994, Shanbhag et al. 1999).

From these studies some general conclusions about the properties of OSNs can also be drawn: most OSNs respond to multiple ligands, and most ligands activate multiple OSNs; OSNs can be broadly tuned, narrowly tuned, or be somewhere in between; and recruitment of OSN types increases with concentration, as OSNs become more broadly tuned at higher concentrations. Olfactory receptor neurons also spike in the absence of ligands. Some ligands are inhibitory, and suppress the spike rate below this spontaneous spiking level. However, since most odors excite some OSNs and at the same time inhibit others, they are not excitatory and inhibitory *per se* (Hallem and Carlson 2006).

2.1.3. Olfactory receptors

Deorphanization efforts have led to the conclusion that all odor responses observed for a given OSN are due to the olfactory receptor (OR) expressed in this neuron (Hallem et al. 2004). Olfactory receptors are a large family of membrane proteins that are selectively expressed in a subset of the OSNs, ranging from two to 50 OSNs for each receptor (Vosshall and Stocker 2007). ORs were first identified in vertebrates in 1991 as a large family of related genes encoding members of the G-protein-coupled receptor (GPCR) superfamily, which couple the binding of a ligand with a cAMP second messenger mechanism that inintiates a signaling cascade (Buck and Axel 1991).

Subsequent efforts to find homologous ORs in insects were unsuccessful until 1999, when three groups separately managed to identify candidate *Drosophila* OR genes (Clyne et al. 1999, Gao and Chess 1999, Vosshall et al. 1999). In *D. melanogaster* there is a total of 62 olfactory receptors that are encoded by a family of 60 genes through alternative splicing (Robertson et al. 2003, Su et al. 2009). The amino-acid homology across this gene family is only about 20 %, indicating that they are of ancient origin (Robertson et al. 2003). Vertebrate and insect ORs differ in topology, with insect ORs adopting an inverted orientation where the N-terminus faces the inside of the cell (Benton et al. 2006, Touhara and Vosshall 2009).

Orco is an unusual member of the OR family. It is the only odorant receptor that is highly conserved among insect species (Jones et al. 2005), is expressed in almost all olfactory neurons, and is crucial for proper functioning of the OSN (Benton et al. 2006, Larsson et al. 2004). This receptor does not confer an independent odorant response (Elmore et al. 2003), but is thought to be an ion channel that either dimerizes or heteromerizes with conventional ORs to form odorant-gated ion channels that are capable of depolarizing the olfactory neuron without relying on a second messenger system (Ha and Smith 2009, Sato et al. 2008, Wicher et al. 2008). Current evidence indicates that at least some insect ORs are ionotropic (ligand-gated ion channels) (Abuin et al. 2011, Benton et al. 2009, Sato et al. 2008, Wicher et al. 2008). This might have important implications for transduction speed: signaling through a second messenger requires activation of the G protein, activation of the effector enzyme, and production and diffusion of a second messenger before the ion channels are opened (Ha and Smith 2009). A direct gating mechanism bypasses these steps, and should theoretically respond faster to olfactory stimuli. In line with this, differences in transduction speed has been observed between vertebrates and fruit flies: in vertebrates, the response to a brief pulse of odor requires 400 ms to peak and 1000 ms to terminate (Bhandawat et al. 2005), whereas *D. melanogaster* OSN responses peak in 30 ms and terminate in 200 ms (Nagel and Wilson 2011). Increased speed of transduction might be more crucial for insects seeing as they often use an odor plume to locate resources crucial for survival and reproduction, meaning that they have to navigate rapidly fluctuating odor filaments dispersed in the surrounding air (Silbering and Benton 2010).

In vertebrates, each OSN expresses only a single olfactory receptor (Malnic et al. 1999), whereas a given insect OSN can co-express up to three conventional ORs in addition to Orco (Abuin et al. 2011, Benton et al. 2009, Couto et al. 2005, Dobritsa et al. 2003, Goldman et al.

2005, Vosshall et al. 2000). Co-expression sometimes is reflected by an additive response of the OSN to the ligands for each OR (Ray et al. 2007), but can also have no apparent functional significance (Dobritsa et al. 2003).

2.1.4. Ionotropic glutamate receptors

Not all insect olfactory neurons express ORs: recently Benton et al. (2009) identified a new class of olfactory receptors known as ionotropic glutamate receptors (IRs). Approximately 60 IRs have been identified in Drosophila, 17 of which are expressed in the antenna (Benton et al. 2009). Each IRN expresses two to four IRs, which are thought to form a functional receptor complex (Abuin et al. 2011, Ai et al. 2013, Silbering et al. 2011). ORs and IRs are expressed in non-overlapping populations of olfactory neurons, with IRs expressed in coeloconic sensilla and all ORs (with the exception of Or35a) expressed in basiconic and trichoid sensilla. The neurons expressing these receptors converge in segregated, although interconnected, sets of glomeruli in the antennal lobe. These circuits become extensively interdigitated in higher brain centers (Silbering et al. 2011). ORs are broadly tuned to fruit odors (alcohols, ketones, and esters) and pheromones; in contrast, IRs are primarily tuned to acids and amines amines (Hallem and Carlson 2006, Silbering et al. 2011, Yao et al. 2005).

2.2. The central olfactory circuitry

2.2.1. The antennal lobe

The axons of all the OSNs in the peripheral parts of the olfactory system coalesce into the antennal nerve and project into the brain (Galizia and Rössler 2010). The antennal lobe (AL) is the primary olfactory processing center, and is organized into spherical neuropil structures called glomeruli. All OSNs that express a given odorant receptor converge onto the same glomerulus (Vosshall and Stocker 2007, Galizia 2014). The *D. melanogaster* AL consists of about 50 glomeruli (fig. 5).

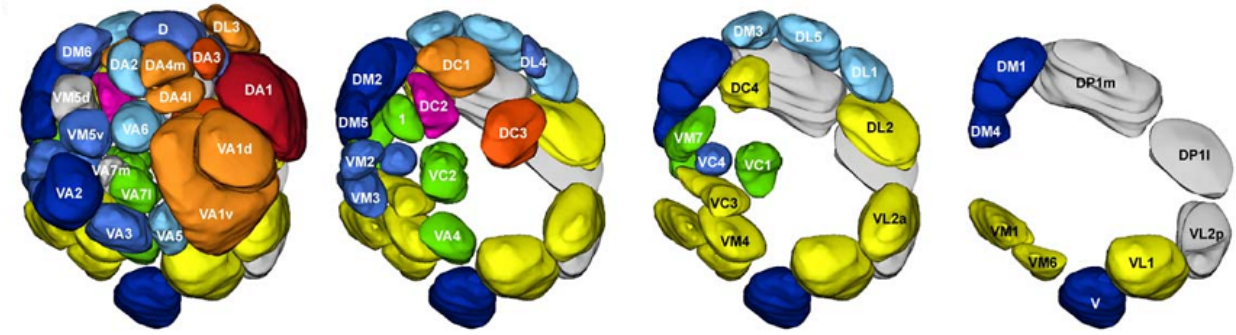

Figure 5: 3D reconstruction of a male *D. melanogaster* antennal lobe, showing the positions of 49 glomeruli. The view is anterior, with the labeled glomeruli removed in each successive panel to reveal the underlying glomeruli. Adapted from Couto et al. (2005).

The AL of *D. melanogaster* exhibits no obvious anatomical divisions, although groups of glomeruli can be distinguished by developmental origins (Jefferis et al. 2001). Projections from the different sensillum types also tend to cluster in the AL, with antennal basiconic neurons innervating the medial edge, antennal trichoids the lateral edge, coeloconic neurons the ventral middle region, and palp basiconics the anterior middle region (Couto et al. 2005, Vosshall and Stocker 2007).

The glomeruli are points of convergence between the OSNs and other classes of neurons: secondary projection neurons (PNs) convey olfactory information from the AL to higher brain centers, and a network of local neurons (LNs) that branch within and between glomeruli and provide lateral interactions among them (Galizia 2014, Gao et al. 2000, Vosshall et al. 2000). The number of LNs in *D. melanogaster* is as of yet unknown, but is at the most within the

range of a couple of hundred (Galizia 2014). Many local neurons are inhibitory (iLNs), but some are excitatory (eLNs). They also differ in terms of their glomerular connectivity: some LNs innervate only a smaller set of glomeruli, but most of them ramify widely throughout the antennal lobe, connecting most or all glomeruli (Chou et al. 2010, Olsen et al. 2007, Seki et al. 2010, Shang et al. 2007, Silbering and Galizia 2007, Wilson et al. 2004, Wilson and Laurent 2005). These interactions indicate that odor representations can be different in the input and the output of the antennal lobe. PNs can be uniglomerular (branch in one glomeruli) or multiglomerular (branch in many glomeruli) (Galizia 2014), and a major role of the LN network is suggested to be synchronization of PN activity, either within a given glomerulus or between multiglomerular PNs (Ng et al. 2002). Several studies have made comparisons between the odor coding in OSNs and their cognate PNs, and have demonstrated a coarse resemblance between their odor responses (Bhandawat et al. 2007, Ng et al. 2002, Silbering et al. 2008, Wilson et al. 2004). However, PNs are in general more broadly tuned than OSNs (Bhandawat et al. 2007, Olsen and Wilson 2008, Wilson et al. 2004).

In addition to the network of LNs, the AL is also a site of modulation through the action of various neuropeptides. Several neuropeptides have been found in the AL of *D. melanogaster*, expressed in subsets of OSNs, LNs, and extrinsic neurons (Carlsson et al. 2010). At present the functional role of most of these neuropeptides is unclear, but it has been demonstrated that certain neuropeptides can modify olfactory behavior related to food odors (Ignell et al. 2009, Nässel and Winther 2010, Root et al. 2011). It is likely that extrinsic neurons releasing either excitatory or inhibitory neuropeptides receive their input from different regions of the brain. This could enable the fly to respond differentially to the same odor in different physiological and behavioral contexts (Carlsson et al. 2010).

2.2.2. The mushroom body and the lateral horn

From the antennal lobe olfactory information is relayed by secondary projection neurons (PNs) to higher-order brain centers in the protocerebrum; the mushroom body (MB) and the lateral horn (LH). Some PNs are polyglomerular, but the majority innervate only a single glomeruli, and therefore only receives input from a single class of OSNs (Strausfeld et al. 2003). Adult *D. melanogaster* have 150 PNs, and they are organized in at least two neural tracts, the inner (i) and medial (m) antennocerebral tracts (ACTs). PNs in the mACT connect only to the LH (Lin et al. 2007), whereas PNs in the iACT terminate in the LH after collateral projections in the mushroom bodies (Galizia and Rössler 2010, Marin et al. 2002). All PNs

that project to the MBs also project to the lateral horn. Most of these are uniglomerular, whereas the PNs that project only to the lateral horn are multiglomerular (Galizia 2014).

Each mushroom body is composed of a calyx and a stalk (peduncle) connected to vertical and median lobes (Nässel and Winther 2010), and comprise 2500 small intrinsic neurons known as Kenyon cells (Heisenberg 2003). The major input region of the MBs is the calyx, where PNs projecting from the same glomerulus terminate in remarkably similar locations (Jefferis et al. 2001, 2007, Lin et al. 2007, Marin et al. 2002, Wong et al. 2002), and form distinct synapses with the Kenyon cells. The calyx is composed of hundreds of microglomeruli (Yasuyama et al. 2002), and each PN project to 2-11 glomeruli (Wong et al. 2002). The lateral horn, on the other hand, appears to be a diffuse, aglomerular neuropil (Tanaka et al. 2004, Wong et al. 2002). PNs seem to terminate in the LH in a stereotyped pattern that resembles what can be observed in the MBs, and PNs that terminate in the same region of the LH tend to originate in neighboring glomeruli. Together, this demonstrates that a topographic map of olfactory information is retained in the two higher olfactory centers (Jefferis et al. 2007, Marin et al. 2002, Tanaka et al. 2004, Wong et al. 2002). Additionally, pheromones and non-pheromones represent parallel processing systems: pheromone-sensitive PNs project to the anterior-ventral area of the LH, fruit odor-sensitive PNs to the posterior-dorsal part of the LH (Jefferis et al. 2007).

The structural organization of neurons suggests that the MB is capable of integrating a wide range of odorant information across glomeruli, whereas relatively little integration is likely to occur in the LH (Tanaka et al. 2004, Galizia 2014). The MBs have been shown to be involved in diverse functions such as olfactory learning (Davis 2005, Dubnau et al. 2003, Heisenberg 2003), locomotor activity (Martin et al. 1998), male courtship behavior (Sakai and Kitamoto 2006), and sleep regulation (Joiner et al. 2006, Pitman et al. 2006). It has been thought that the MBs are involved in learned behavior and the lateral horn in innate odor-mediated behavior (Keene and Waddell 2007, Tanaka et al. 2004). However, Galizia (2014) recently proposed the idea that the main difference between these two brain centers is that whereas mushroom bodies identify odors, the lateral horn is important for odor evaluation.

3. Gustation

3.1. Gustatory sensilla

Gustatory receptors are widely dispersed over the surface of the fly, including the proboscis, legs, and wing margins (Amrein and Thorne 2005) (fig. 6). Females also have gustatory capacity in the vaginal plate sensilla at the tip of the abdomen, which might be important for oviposition site selection (Falk et al. 1976, Stocker 1994, Yang et al. 2008).

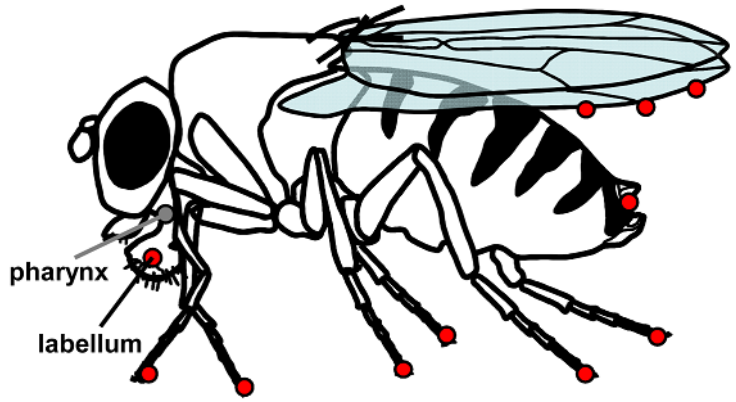

Figure 6: Distribution of gustatory receptors in *Drosophila melanogaster*; on the labellum, pharynx, legs, wing margins, and the ovipositor of the female. Adapted from Montell (2009).

Gustatory sensilla or taste bristles are similar to olfactory sensilla. They contain a single terminal pore where tastants enter the sensillum and dissolve in the hemolymph in its lumen. They have a split lumen connected at the tip, with one side containing two to four chemosensory neurons and the other side only sensillum lymph (Morita 1992). Each sensillum also has one mechanosensory receptor at its root, and is associated with several types of accessory cells (Stocker et al. 1994). The reason why all the gustatory sensilla are associated with one mechanosensory neuron is yet unknown, but mechanoreceptors are likely to detect subtle displacement of the sensilla caused by contact (Miyazaki and Ito 2010). Gustatory sensilla are categorized by size, distribution, and number of innervating neurons into three classes: small (s-type) and long (l-type) sensilla each houses four neurons, whereas intermediate (i-type) sensilla have two neurons each (Hiroi et al. 2002, 2004). In addition to sensilla, *D. melanogaster* also have gustatory receptors housed in smaller structures known as taste pegs. Each peg is associated with one gustatory neuron and one mechanosensory neuron (Falk et al. 1976).

The primary taste organ is the labella, a pair of palps at the distal end of the proboscis. On each of the labelial palps 31 sensilla are situated in four rows, together with about 30 taste pegs (Shanbhag et al. 2001, Vosshall and Stocker 2007). Taste perception by other body parts is mediated by a large number of taste bristles (Stocker 1994). The sensilla found on the legs house two to four gustatory neurons. The first leg has about 50 gustatory sensilla in males and 37 in females, and the sexual dimorphism is due to the presence of specialized male-specific sensilla that detect female pheromones (Bray and Amrein 2003). The second and third leg has 30 and 32 taste sensilla, respectively, with no sexual dimorphism in number or function. The wing margin is covered by 40 taste bristles that each contains four gustatory neurons, and the vaginal plates on the female possess around ten poorly characterized sensilla (Stocker 1994, Vosshall and Stocker 2007).

3.2. Gustatory receptors

Similar to olfactory receptors, each gustatory receptor neuron (GRN) is a bipolar cell with a cell body that lies beneath the surface of the cuticle, and includes a single dendrite that extends to the tip of the sensilla and one axon that projects to down-stream processing centers in the brain. Gustatory sensilla house two to four GRNs. GRNs express members of a family of 68 gustatory receptors (GRs) (Dunipace et al. 2001, Scott et al. 2001). GR gene expression is complex, and GRs are even more divergent than ORs: some share as little as 8% overall amino acid identity (Robertson et al. 2003). GRs have no sequence homology with mammalian taste receptors, but are distantly related to the olfactory receptors in the fly (Clyne et al. 2000, Montell 2009). Together they form a large superfamily of insect chemosensory receptor genes (Robertson et al. 2003).

Several GRs appear to be expressed in multiple cell types that are not associated with contact chemosensation (Montell 2009). At least four members of the GR gene family are expressed in olfactory receptor neurons on the antenna (Dunipace et al. 2001, Jones et al. 2007, Scott et al. 2001), including the two highly related GRs (GR21a and GR63a) that function in the detection of carbon dioxide (Jones et al. 2007, Kwon et al. 2007).

3.3. The gustatory pathway

Gustatory receptor neurons located in different locations on the body all target the subesophageal ganglion (SOG) in the central nervous system, located slightly behind and ventral to the brain (Vosshall and Stocker 2007). However, neurons from different locations terminate in distinct areas of the SOG (Miyazaki and Ito 2010, Thorne et al. 2004). There is also a map of taste categories in the SOG, with separation between sweet and sour tastes (Wang et al. 2004). Some of these spatially distinct neurons express the same receptor, indicating that a given tastant may trigger different behaviors in different contexts (Vosshall and Stocker 2007).

Although some taste information is sent to higher brain centers (possibly the MBs), simple reflexes such as proboscis extension or food ingestion may rely on local circuitry with fairly limited processing (Vosshall and Stocker 2007). The SOG contains no apparent morphological structural divisions such as the glomeruli, and this has made it harder to elucidate how gustatory information is conveyed by extrinsic neurons. As a result, the information pathway between the SOG and motoric neurons is as of yet unclear, as is the pathway between the SOG and important centers for memory (Miyazaki and Ito 2010).

4. Pheromone signaling in *D. melanogaster*

Pheromone-mediated behaviors in Drosophila are surprisingly complex and involve not only sexual communication, but also a set of social behaviors, the precise roles of which still await detailed description. The *D. melanogaster* pheromones that have been studied so far are mostly involved in sexual communication during courtship. Courtship in fruit flies is a complex innate behavior that involves signals from both the male and the female fly, and is perceived through multiple sensory modalities: vision, audition, and chemosensation (Greenspan and Ferveur 2000, Hall 1994, Villella and Hall 2008). This behavior consists of six steps (fig. 7).

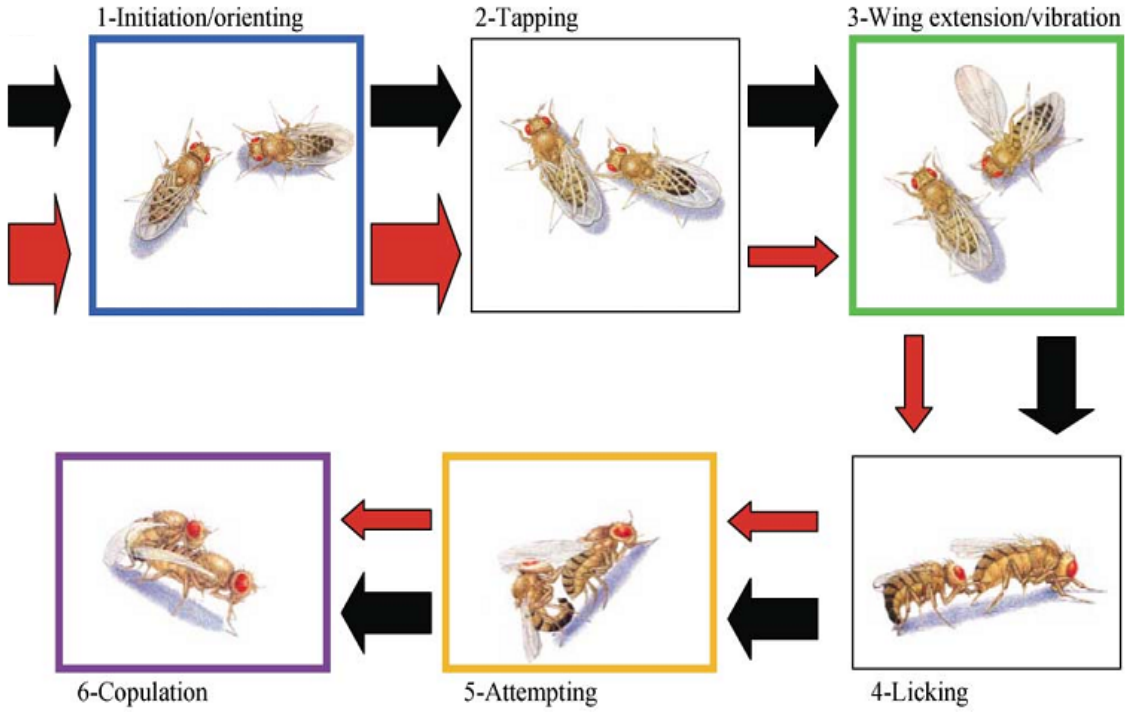

Figure 7: The six steps in *Drosophila melanogaster* courtship. The male (darker pigmented abdomen) is on the right in top three panels (steps 1–3) and on the left in panels at the bottom (step 4–6). The arrows indicate the transition from each step to the next. Modified from Bray and Amrein (2003).

Several of these steps could potentially involve pheromone signaling: courtship is initiated when the male orients towards the female, and this could provide him with the opportunity to assess close-range volatile pheromones. The second step is 'tapping' where the male taps the female with his foreleg, potentially allowing him to sample non-volatile contact pheromones

from the female with specialized taste bristles. The fourth step is completed when the male licks the genitalia of the female with his labial palps and attempts copulation; this might fascilitate sampling of a different set of contact pheromones.

4.1. Olfactory stimuli: 11-*cis*-vaccenyl acetate

One of the most extesively studied pheromone detection systems is the only known volatile *D. melanogaster* pheromone 11-*cis*-vaccenyl acetate (cVA) (Bartelt et al. 1985). This lipid is produced by male flies, though a recent study indicates that it is also produced in minute amounts by females (Yew et al. 2009). It is found on the male cuticle, and is produced in the ejaculatory bulb and transferred with the ejaculate to the female during copulation (Amrein 2004).

Although it is produced by males, cVA is detected by both sexes by specialized olfactory neurons (T1 neurons) on the antennae (van der Goes van Naters and Carlson 2007). The OR Or67d is involved in sensing cVA (Ha and Smith 2006, Jon et al. 2008, Kurtovic et al. 2007, Ronderos and Smith 2010), but it is not the only component required for pheromone detection. It has been demonstrated that detection of cVA requires not only Or67d and Orco, but also the proteins SNMP (Benton et al. 2007) and LUSH (Xu et al. 2005). SNMP is a sensory membrane protein, whereas LUSH is an odorant binding protein secreted from the accessory cells. Studies with mutant fly lines have indicated that SNMP might be an inhibitory subunit in the receptor complex (Benton et al. 2007, Jin et al. 2008). A model for cVA-detection has been proposed where LUSH binds cVA and undergoes a conformational change (Laughlin et al. 2008). Activated LUSH is able to bind to SNMP, where it relieves the Or67d/Orco, complex from SNMP-mediated inhibition, thereby allowing cations to enter the neurons (Ha and Smith 2009). However, Gomez-Diaz et al. (2013) recently presented several lines of evidence that contradicts this model, and demonstrated that high concentrations of cVA can induce neuronal activity even in the absence of LUSH.

Another receptor, Or65a, has also been shown to respond to cVA (Clyne et al. 1997, van der Goes van Naters and Carlson 2007), and has been suggested to be involved in learned suppression of male courtship (Ejima et al. 2007). Or65a also seem to be linked to cVA-induced social regulation through inter-male aggression (Liu et al. 2011).

cVA has diverse behavioral functions, and flies can respond to it in a sex-specific manner. One of the earliest observations was that it acts as an aggregation pheromone for both male and female flies (Bartelt et al. 1985, Wertheim et al. 2002). It has been shown to mediate sexual recognition between individuals (Ejima et al. 2007, Ha and Smith 2006, Kurtovic et al. 2007, Ronderos and Smith 2010), and to enhance receptivity in females and inhibit male courtship of other males or newly mated females (Griffith and Ejima 2009, Jallon et al. 1981, Smith 2012, Wang et al. 2011, Zawistowski and Richmond 1986). Lately, a novel role of cVA has been suggested from studies on aggression in *D. melanogaster*: it seems to play a role in eliciting male-male aggression (Liu et al. 2011, Wang and Andersson 2011).

The sex-specific behavioral effects of cVA are mirrored by sexual dimorphism in the nervous system. cVA-sensitive olfactory neurons expressing Or67d converge on the DA1 glomerulus in the antennal lobe (Couto et al. 2005, Fishilevich and Vosshall 2005). This is one of three glomeruli that are larger in male flies (Kondoh et al. 2003). Projections from the DA1 glomerulus to the protocerebrum are also sexually dimorphic, with a male-specific axonal arbor in the lateral horn (Datta et al. 2008, Ruta et al. 2010). This cVA-activated sexually dimorphic circuit in the protocerebrum suggests a mechanism by which a single pheromone can elicit different behaviors in males and females.

4.2. Gustatory stimuli – cuticular hydrocarbons

Cuticular hydrocarbons (CHs) are synthesized from fatty acid precursors in epidermal cells called oenocytes and deposited on the fly cuticle (Ferveur 1997). Their primary function is to prevent desiccation by reducing water loss (Nelson and Leopold 2003). Species of the *Drosophila* genus qualitatively differ from the majority of other insect species for their much smaller number of CHs (Howard and Blomquist 2005). However, they reveal a great diversity of CHs with regard to chain length, position and number of double bonds, intraspecific sexual dimorphism, and interspecific variation. This diversity is important for sexual isolation between species, and gives the CHs a secondary function as cuticular sex pheromones in *D. melanogaster*.

In *D. melanogaster*, the predominant cuticular hydrocarbons are sexually dimorphic: males produce high levels of monoenes, while females also produce dienes (Ferveur 2005). Only unsaturated hydrocarbons have been shown to have a behavioral role, with the double bond in position 7 in males and in positions 7 and 11 in females. The main pheromones are 7-

tricosene and 7-pentacosadiene in males, and 7,11-heptacosadiene and 7,11-nonacosadiene in females (Everaerts et al. 2005, Wicher-Thomas et al. 2006). Additionally, there are also many minor differences in CH profile between males and females (Yew et al. 2009). The predominant female CHs tend to increase male courtship (Anthony and Jallon 1982, Grillet et al. 2013, Siwicki et al. 2005), while the male hydrocarbon 7-tricosene (7-T) reduces male-male courtship (Ferveur and Sureau 1996, Sureau and Ferveur 1999, Svetec and Ferveur 2005). Female flies also seem to prefer males with higher levels of 7-T (Grillet et al. 2006).

During mating, CHs can be transferred from one partner to the other, and this can lead to changes in the CH profile and subsequently affect behavior. An example is 7-tricosene, which is thought to be transferred to females during mating and leads to reduced courtship from males towards newly mated females (Everaerts et al. 2010). Yew et al. (2009) identified the male-specific sex pheromone CH503 which, also is thought to suppress further male courtship, and was demonstrated to persist on the cuticle for at least 10 days after mating.

In males, the gustatory sensilla on the forelegs, wing margins, and labella are thought to recognize female contact pheromones (Amrein 2004, Villella and Hall 2008). However, few receptors for such pheromones have been identified. The first GR indicated to participate in mating behavior was Gr68a. This receptor is expressed specifically in male-specific sensilla on the forelegs, and inactivation of GR68-expressing GRNs impaired normal courtship (Bray and Amrein 2003). However, the majority of Gr68a-expressing neurons were later shown to be mechanosensory neurons (Ejima and Griffith 2008, Koganezawa et al. 2010), whereas Gr32a, the GR most closely related to Gr68a and similarly expressed in the forelegs, inhibits male-male courtship (Miyamoto and Amrein 2008) and is indicated to play a role in male lateralized wing extension during courtship (Koganezawa et al. 2010). In contrast to wild-type males, which show a diminished propensity to court previously mated females, Gr32a mutant males display similar courtship behavior to virgin and mated females. This might result from a decreased ability to perceive an inhibitory pheromone transferred from males to females during mating. In addition, 7-tricosene has been shown to elicit Gr32a-dependent intermale aggression, and to suppress intermale courtship (Wang et al. 2011). Gr33a, which is essential for bitter taste, is also expressed in GRNs on the forelegs, and is required for suppressing male-male courtship (Moon et al. 2009). Gr32a and Gr33a may function in concert as subunits of a receptor complex required for pheromone reception and for controlling sexual preference in males (Montell 2009). Another GR, Gr39a, is thought to detect female-enriched

CHs (Watanabe et al. 2011). The ion channel Pickpocket 23 (Ppk23) is crucial for CH-activated neurons involved stimulating male courtship of females and inhibiting male-male courtship (Lu et al. 2012, Thistle et al. 2012, Toda et al. 2012). A similar role has been proposed for the ion channel ppk29 (Thistle et al. 2012).

4.3. Seminal fluid proteins and their effect on post-mating behavior

Besides altering the CH profile of a fly, mating can also induce other changes in physiology and behavior. Besides sperm, the male ejaculate contains a combination of seminal proteins that can have multiple effects on both sexes for an extended post-mating period (Nässel and Winther 2010, Ram and Wolfner 2007). An example is the proteins produced by the male accessory glands (Acps), which are small molecules that are produced in the male reproductive organs. After transfer to the vaginal duct they are transported through the hemolymph to target sites in the CNS, where they induce physiological and behavioral changes that can persist for several days (Chen et al. 1988, Amrein 2004). More than a hundred predicted Acps have been identified, including peptides, prohormones, glycoproteins, and enzymes (Chapman and Davies 2004, Ram and Wolfner 2007, Wolfner 2002). Biological functions are not known for all of these, but some have been attributed roles in fertility and reproductive behavior. Their effects include reduced female receptivity, stimulation of egg-laying, and increased feeding (Kubli 2003). One of the most studied Acps is sex peptide (SP), which induces oviposition and oogenesis when expressed in virgin females (Aigaki et al. 1991, Chapman et al. 2003, Chen et al. 1988). Another example is the prohormone ovulin, which also stimulates egg laying (Heifetz et al. 2000, Herndon and Wolfner 1995).

Peptides other than the Acps are also transferred to the female with the seminal fluid; an example is Dup99B, which has been suggested to affect females by stimulating egg laying and reducing sexual receptivity (Rexhepaj et al. 2003).

5. The neural circuitry underlying sex-specific behavior

Studies on *D. melanogaster* has not only given insight into the pheromones this species uses, and how these molecules are perceived by the chemosensory system of the fly: we are also starting to get a better understanding of exactly how features of the nervous system underlie pheromone-induced behavior.

All sexually reproducing animals show differences in behavior between males and females, for example related to mating and aggression. These differences in behavior are a consequence of sexually dimorphic nervous systems, of which we have already seen examples in *D. melanogaster*, both in the periphery (male-specific taste bristles, more trichoid sensilla on male antennae) and in the central chemosensory pathway (larger glomeruli in males, sexually dimorphic circuit for cVA).

In *D. melanogaster*, the sex determination pathway ensures sexual differentiation of the nervous system through the expression of sex-specific splice forms of two transcription factors, *fruitless (fru)* and *doublesex* (*dsx*) (Manoli et al. 2013). They both contribute to sexual differentiation of the nervous system, but *fruitless* is the one that has been most intensively studied. Male courtship behavior in Drosophila has been linked to the activity of a set of about 2000 Fru^M-expressing neurons scattered around the nervous system (Cachero et al. 2010), as has male aggression (Vrontou et al. 2006, Wang and Andersson 2010). These neurons encompass sensory cells that detect pheromones (receptor neurons that innervate trichoid sensilla), interneurons in higher brain centers, and motor neurons (Cachero et al., 2010, Demir and Dickson 2005, Kimura et al. 2005, 2008, Manoli et al. 2005, Stockinger et al. 2005, Yamamoto 2008, Yu et al. 2010). Both OSNs and PNs connected with the three sexually dimorphic glomeruli in the antennal lobe express Fru^M (Kondoh et al. 2003, Stockinger et al. 2005). However, specific functions in pheromone-invoked behaviors have at present only been assigned to a small subset of these neurons. Examples include neurons involved in the sexually dimorphic cVA circuitry (Datta et al. 2007, Ha and Smith 2006, Kurtovic et al. 2007, Ruta et al. 2010), neurons driving cVA-induced aggressive behavior in males (Vrontou et al. 2006, Wang and Andersson 2010), initiation of wing vibration during courtship (Kohatsu et al. 2011, von Philipsborn et al. 2011), and neurons involved in conditioning of courtship behavior (Manoli et al. 2005) and in inhibiting of this behavior (Kimura et al. 2008). The

Gr32a-expressing neurons involved in inhibiting male-male courtship also connect with a sexually dimorphic population of *fru*-expressing neurons (Koganezawa et al. 2010).

Recently, Grosjean et al. (2011) described a novel courtship function of the ionotropic receptor Ir84a, a amember of the ionotropic glutamate receptors (Benton et al. 2009). Neurons expressing Ir84a are activated not by fly odors, but by the aromatic food compounds phenylacetaldehyde and phenylacetic acid. Fruit flies use rotting fruit for food, but also aggregate on them to locate mates or oviposition sites. Flies lacking Ir84a show reduced courtship behavior, while male courtship increased in the presence of phenylacetic acid. Ir84a-expressing neurons map to VL2a, one of the sexually dimorphic glomeruli in the antennal lobe. The PN output from this glomerulus express fru^M, and Ir84a thus seems to link feeding and reproductive behavior by coupling food preferences to the activation of Fru^M courtship circuitry.

Several recent studies of the male courtship song have yielded interesting results regarding initiation of courtship (Kohatsu et al. 2011, Pan et al. 2012, von Philipsborn et al. 2011). These identified a cluster of 20 Fru^M-expressing interneurons, named P1, whose activation induced courtship song production, as well as other elements of male courtship behavior. P1 neurons have two important properties: they are located in the lateral protocerebrum, a higher brain center that receives sensory input from olfactory, gustatory, visual, and auditory systems (Miyamoto and Amrein 2008), and they are present only in male flies. These interneurons might therefore be important in integrating multimodal stimuli sampled during courtship, and influence the male's decision of whether to court or not. They also demonstrate that individual groups of Fru^M neurons can control specific components of a complex behavior such as courtship song (Manoli et al. 2013).

6. Concluding remarks and future perspectives

Through a century of using *D. melanogaster* as a model organism advanced genetic, molecular, and physiological techniques have been developed for this species, and their application have resulted in an unprecedented understanding of how chemosensory cues (in particular olfactory cues) are detected and processed by the central nervous system. The extensive work on the fruit fly has aided in elucidating not only specific features of their own olfactory system, but also in establishing general principles for olfactory coding in the insect brain. But work on *D. melanogaster* has also highlighted the complexity of olfactory processing, even in a relatively simple nervous system. This can be easily illustrated by the extensive modulation that occurs in the antennal lobe, the first processing center for olfactory information, both through local interneurons connecting different glomeruli and through the release of different neuropeptides.

Much of what we know about the olfactory system of *D. melanogaster* comes from experiments conducted in a controlled laboratory setting. This has enabled us to identify ligands for olfactory receptors and to create accurate maps of the distribution of sensilla and glomeruli, but has in all likelihood obscured important factors that would affect a fly's behavior in a more natural setting. In real-life situations insects are subjected to a huge amount of different olfactory stimuli simultaneously, and are not only depending on the input they get through their olfactory system; their nervous system has to integrate this input with information relayed through other modalities such as vision and mechanosensation. The ultimate behavioral choice made by the individual insect is also influenced by its internal state, as well as by previous experience. Factors like these are often controlled for in experiments, and this might prevent us from discovering important features of the olfactory system, especially related to modulation and plasticity.

Pheromones have been demonstrated to be important in reproductive behavior in fruit flies, especially during courtship. But fruit flies feed, locate potential partners, mate, and oviposit on fermenting fruit, and compounds produced by the fruit will be present during the most important decisions of a fruit fly's life. As a consequence, reproductive behavior is infleunced by both pheromones and food odors.

One of the most important decisions a female fruit fly has to make is where to lay her eggs. Placing your eggs in a suitable environment ensures the survival of the offspring, and oviposition sites differ in many features that could provide females with ways of assessing their quality. The chemosensory cues that trigger oviposition are largely unknown, although several recent studies have shed some light on this aspect of oviposition behavior in *D. melanogaster*. Joseph et al. (2009) found that acetic acid, a product of fruit fermentation, induced oviposition, but also caused positional avoidance in female flies. This avoidance behavior was stronger in unmated females, indicating the importance of internal state for behavioral decisions. Stensmyr et al. (2012) identified the microbial odorant geosmin as an off-flavor for female flies that indicate the presence of harmful microbes, and inhibits both feeding and oviposition. Finally, Dweck et al. (2013) demonstrated that flies preferred to lay their eggs on *Citrus* fruits, and also that endoparasitoid wasps were repelled by the smell of these fruits, indicating that the preference for this substrate is linked to the need to escape parasitism.

From these findings it is clear that important behavioral decisions in *D. melanogaster* require an accurate assessment of the surroundings. A reductionist approach to fruit fly behavior has generated valuable information about the features of their sensory systems, but to increase our understanding of how detected stimuli actually result in specific behaviors, the complexities of real-life situations must be taken into account – also in a laboratory setting.

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