A Sensory Map of the Odour World in the Moth Brain

Mikael A Carlsson
Department of Crop Science
Alnarp

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Abstract

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The functional organisation of the moth antennal lobe was studied using two species, *Manduca sexta* and *Spodoptera littoralis*, as model organisms. Glomerular activity was investigated at the population level by means of optical imaging techniques.

In the male-specific macroglomerular complex (MGC), responses to pheromone compounds corroborated earlier results obtained from single cell recordings, i.e. different compartments of the MGC responded specifically to one component of the pheromone. Among the sexually isomorphic glomeruli the responses to plant-associated compounds were more distributed but odour-specific and similar across individuals of both sexes. Several glomeruli were activated by a single compound and each glomerulus was activated by several different compounds. Thus, broad tuning and overlapping responses suggest an across-glomerular coding mechanism for non-pheromones. Using series of homologous aliphatic compounds revealed that the highest correlation between activity patterns was always found for compounds with the same functional group and with minimal difference in carbon chain length.

A concentration-dependence of glomerular activity was found. Increased concentration resulted in a recruitment of activated glomeruli. The single most activated glomerulus was often not the same across concentrations. The movement, however, was generally restricted to neighbouring glomeruli. Furthermore, activity patterns elicited by different odorants were more similar at high than at low stimulus doses.

A method to selectively stain a large population of projection neurons with a Ca^{2+} sensitive dye was applied to *S. littoralis*. Also at the output level glomeruli were broadly tuned and activity patterns evoked by different odours overlapped each other. Temporal differences were found both between stimuli and between glomeruli. Glomerular activity patterns evoked by different odours became less similar as a function of time.

Keywords: olfaction, odour representation, spatial coding, temporal coding, receptor neuron, projection neuron, chemical structure

Author's address - Mikael A. Carlsson, Department of Crop Science, SLU, P.O. Box 44, SE-230 53 Alnarp, Sweden. E-mail: mikael.carlsson@vv.slu.se

Contents

Appendix

Papers I-V

This thesis is based on the following papers, which will be referred to by their Roman numerals.

I Carlsson MA, Galizia CG, Hansson BS. 2002. Spatial representation of odours in the antennal lobe of the moth *Spodoptera littoralis* (Lepidoptera: Noctuidae). *Chem. Senses*. 27(3), 231-244.

II Meijerink J, Carlsson MA, Hansson BS. 2003. Spatial representation of odorant structure in the moth antennal lobe: a study of structure response relationships at low doses. *J. Comp. Neurol*. In Press.

III Hansson BS, Carlsson MA, Kalinova B. 2003. Olfactory activation patterns in the antennal lobe of the sphinx moth, *Manduca sexta*. *J. Comp. Physiol. A*. 189(4), 301-308.

IV Carlsson MA, Hansson BS. 2003. Dose-response characteristics of glomerular activity in the moth antennal lobe. *Chem. Senses.* 28(4), 269-278.

V Carlsson MA, Hansson BS. Spatio-temporal dynamics of olfactory output neurons in the moth antennal lobe. Submitted.

Objective

The objective of the thesis was to investigate neural representations of behaviourally relevant olfactory information in the brain of two moth species. It further included an adaptation of optical imaging techniques to these two species.

Introduction

In the central nervous system there is one neuropil that stands out due to its beautiful architecture. It is the primary olfactory centre, the olfactory bulb (vertebrates), the olfactory lobe (molluscs) or the antennal lobe (insects), which is characterised by its glomerular structure. In his famous work on invertebrate neuroanatomy, Bertil Hanström (1928) noticed that glomerular structures were found in phylogenetically diverse species. Actually, within the animal kingdom only few organisms lack these structures (Strausfeld and Hildebrand, 1999). The glomerular structure may have evolved independently several times (Strausfeld and Hildebrand, 1999). Hence, it could be a perfect example of convergent evolution. The functional significance of the glomerular organisation has riddled the scientific community for decades and the question has occupied me for the last four years. In early attempts to reveal the function of the olfactory organs, the Nobel laureate Lord Adrian (1950, 1953) recognised that odours evoked differential activity across the glomerular array. Since then a growing body of evidence from different scientific fields supports the idea that glomeruli are discrete functional units. Still there is a considerable amount of work to do in order to reveal the mechanisms behind olfactory processing. The results from the experiments presented in my thesis are aimed at contributing to the general knowledge and also providing a base for further studies.

Virtually all animals use the sense of smell in vital tasks including foraging, mate-finding and predator avoidance. Although we may not fully appreciate the olfactory sense due to its relatively minor importance in our own species, it is the most essential of the senses in many other organisms. Moths are mainly nocturnal, which means that vision generally plays a minor role. Olfaction, on the other hand, is vital for their survival. It is truly an amazing feat that a male moth can locate a calling female from far distance simply by sensing small amounts of volatile molecules. For example, it was recently demonstrated that five molecules hitting the antenna of the female-emitted pheromone was sufficient to alter the heart rate of a male moth (Angioy et al., 2003). Moreover, under natural conditions the “chemical noise” must be enormous. Odorous molecules are emitted from an array of sources like green leaves, flowers, frass and chemicals involved in communication in other species. These sources contribute to a molecular soup of potential odours.

Why use moths as model organisms? Moths are excellent model organisms due to their relatively easily accessible nervous system, the comparatively simple neural architecture (compared to vertebrates) and the fact that they are easily

cultured. Due to the similarities between the olfactory systems in insects and in vertebrates we may learn more about our own sense of smell by using the moth as a model. Furthermore, many moth species are serious pests on economically important crops. Thus, learning more about how and why they are attracted to specific plant-emitted odours or to conspecific attractants, pheromones, we may proceed towards methods for environmentally acceptable pest management.

In my thesis I have chosen to focus on processing of odour cues in the central olfactory system of two moth species. I used optical imaging techniques, which is an excellent tool for studying neuron population events in the moth brain.

Odour detection and central integration in insects

The detector – the antenna

Morphology

The major olfactory organs in insects are the antennae. In addition, many insects have odour detectors located on e.g. mouthparts (Lee et al., 1985; Bogner et al., 1986). On the third flagellum of the antenna there are numerous cuticular formations, sensilla, containing the sensory cells. These sensilla display different shapes, for instance hair like (*sensilla trichodea*), cone like (*s. basiconica*) or plate like (*s. placodea*) (Keil, 1999). Each sensillum normally houses 2-5 olfactory receptor neurons (ORN) but exceptionally more than 100 (Keil, 1999). As in vertebrates, the ORNs are bipolar and connect directly to the brain without any peripheral synapses. From the cell somata at the sensillar base, the dendritic end extends into an aqueous fluid, the sensillar lymph, which acts as the interface between neuron and environment. As it is generally only the males that have the ability to detect female-emitted pheromones, a marked sexual dimorphism of the antennal ultrastructure is common (Keil, 1999). For example, in the moth *Manduca sexta*, the female antenna is filiform, whereas the male has two large phalanxes carrying sensilla protruding from the margins of the entire antenna (Shields and Hildebrand, 2001a).

Perireceptor events and transduction

Odour molecules enter the sensilla through pores in the cuticular walls (Steinbrecht, 1997). As most odorous volatiles are lipophilic the transfer from the pores to the receptor sites on the ORNs is believed to be facilitated by docking to odour binding proteins (OBP) (Vogt and Riddiford, 1981). These proteins increase the water solubility of the odorants and might be involved in the binding process of odour molecules to receptor sites.

Putative olfactory receptor proteins were first identified in rats (Buck and Axel, 1991) and more recently in insects (Clyne et al., 1999; Vosshall et al., 1999; Hill et al., 2002; Krieger et al., 2002). The receptors are G-protein coupled 7-transmembrane proteins with little homology between phylogenetically divergent groups of organisms (Mombaerts, 1999). There are several different theories

suggesting how odour molecules interact with the receptor protein (Turin and Yoshii, 2002). These theories include receptor binding to structural motifs of the odorous molecule and detection of molecular vibrations.

The number of expressed receptor-coding genes in olfactory organs in *Drosophila melanogaster* is 42 (Vosshall, 2001) and in the mosquito *Anopheles gambie* 79 (Hill et al., 2002). No matter what species, the family of genes coding for olfactory receptor proteins accounts for a substantial part of the entire genome (Mombaerts, 1999). With a few exceptions, each ORN expresses a single receptor type (Clyne et al., 1999; Vosshall et al., 1999; Dobritsa et al., 2003). A different strategy is found in the nematode *Caenorhabditis elegans* in which as much as ~500 receptor proteins are expressed in only 32 neurons (Bargmann and Kaplan, 1998). In the past years we have seen evidence for the functional role of some of the candidate receptors. In *D. melanogaster,* an overexpression of the putative receptor protein Or43a rendered an increased EAG response to a limited set of ligands (Störtkuhl and Kettler, 2001). Another candidate receptor in *D. melanogaster*, OR22a, could be mapped to a physiologically characterised neuron (Dobritsa et al., 2003). Mutants lacking OR22a showed no odour-evoked responses in this neuron. Likewise, Bozza et al. (2002) used calcium imaging in mice to characterise the response profiles of dissociated cells expressing a particular receptor protein. When the coding sequence was replaced by one from the rat the set of effective ligands changed.

Binding of an odour molecule to a receptor protein triggers a second messenger cascade. The major pathway in insects involves generation of inositol 1,4,5,-triphosphate (IP_3) that causes an influx of calcium ions into the dendrite (Stengl et al., 1999). The calcium in turn activates non-specific cation channels. An inflow of cations changes the membrane potential. If the depolarisation exceeds a certain threshold an action potential is evoked at the initiation site near the soma. Action potentials carry information along the axons into the primary olfactory centre of the brain, the antennal lobe (AL) in insects or the olfactory bulb in vertebrates. The frequency of the evoked action potentials in a neuron is proportional to the concentration of the stimulus.

Physiology

Since the pioneering work of Schneider (1957) a plethora of electrophysiological studies of insect ORNs have been published. Different degrees of response specificity have been reported. Extremely narrow tuning is often found in pheromone responding neurons. Even a minor deviation from the "required" structure, like e.g. movement of double bonds or replacement of functional groups, may result in a substantially reduced response (e.g. Liljefors et al., 1984, 1985, 1987).

Also, ORNs tuned to plant related odours can be highly selective (Hansson et al., 1999; Larsson et al., 2001; Stensmyr et al., 2001; Röstelien et al., 2000). However, more often ORNs respond to a broader range of stimuli. This fact does not necessarily mean that the neuron is "sloppy". Rather, the neuron is selective to a molecular feature that may be shared by several compounds (Araneda et al., 2000). Due to the limited number of receptor proteins, an insect with exclusively

specific receptors (i.e. tuned to a single compound) will have a low coding capacity. The environment offers a very large number of potentially important odorous molecules with high informative value. Even the same flower, e.g., may change its odour composition over time. Therefore, it would be beneficial to have receptors with broader tuning. Broad and overlapping tuning allows detection and discrimination of an almost infinite number of potential odours. As a comparison, it has been estimated that humans possess about 300 different functional receptor proteins (Mombaerts, 2001). Still, we can detect more than 400 000 different odorous molecules (Mori, 2003). Specific receptors, on the other hand, would be beneficial in order to detect stimuli that are highly predictable, e.g. sexual pheromones, where a failure of correct identification would be fatal.

No matter how selective a sensory neuron is, if the concentration is high enough even structurally unrelated compounds may excite the neuron. As an example, pheromone-selective neurons in the moth *Agrotis segetum* even respond to flower volatiles at very high doses (Hansson et al., 1989; Carlsson and Hansson, 2002). It is, however, unlikely that the animal will encounter such high concentrations under natural conditions.

Evidence from several species indicates a lack of strict topological organisation of ORNs with similar tuning (Mori et al., 1999; de Bruyne et al., 2001). Rather, ORNs seem to be randomly dispersed all over the olfactory organ. Some rough topology exists in that, e.g. pheromone-detecting neurons in moths are often located either in the basal part or along the margins of the male antenna. Also in the mammalian olfactory epithelium there is a zonal subdivision where a given type of receptor is expressed in one of the zones (Mori et al., 1999).

The primary integration centre – the antennal lobe

Morphology

The ALs (Figure 1a) are functional analogs of the vertebrate olfactory bulbs. A substantial part of the insect brain is devoted to olfactory processing. The antennal lobes of moths make up ~25% of the entire brain volume. As a comparison, the human olfactory bulbs represent about 0,01 % of the total brain volume (Stoddart, 1990). As in vertebrates, the insect ALs consist of spheroidal structures called glomeruli. Glomeruli are discrete neuropilar islets, which, to different extents, are separated by glial processes (Hähnlein and Bicker, 1996; Tolbert and Hildebrand, 1981). The glomeruli are normally arranged in a single layer around a central fibre core. The number of glomeruli is species-specific and range from about 30-40 in some dipteran species to 160 in honeybees and more than 1000 in locusts (Bausenwein and Nick, 1998; Laissue et al., 1999; Flanagan and Mercer, 1989; Galizia et al., 1999a; Ignell et al., 2001). Moths normally have ~60 glomeruli (Koontz and Schneider, 1987; Rospars, 1983; Rospars and Hildebrand, 2000). Each ORN generally projects to a single glomerulus (exception Diptera where most ORNs project to one glomerulus in each lobe; Strausfeld, 1976 and locusts, where several small glomeruli contain arborisations from a single ORN, Ignell et al., 2001). Within a species, the majority of the glomeruli are individually identifiable, i.e. shape, size and positions are invariant across individuals. In a

handful of insects, an atlas of identifiable glomeruli has been constructed from anatomical stainings (Rospars and Hildebrand, 2000; Galizia et al., 1999a; Laissue et al., 1999; Sadek et al., 2002; Berg et al., 2002).

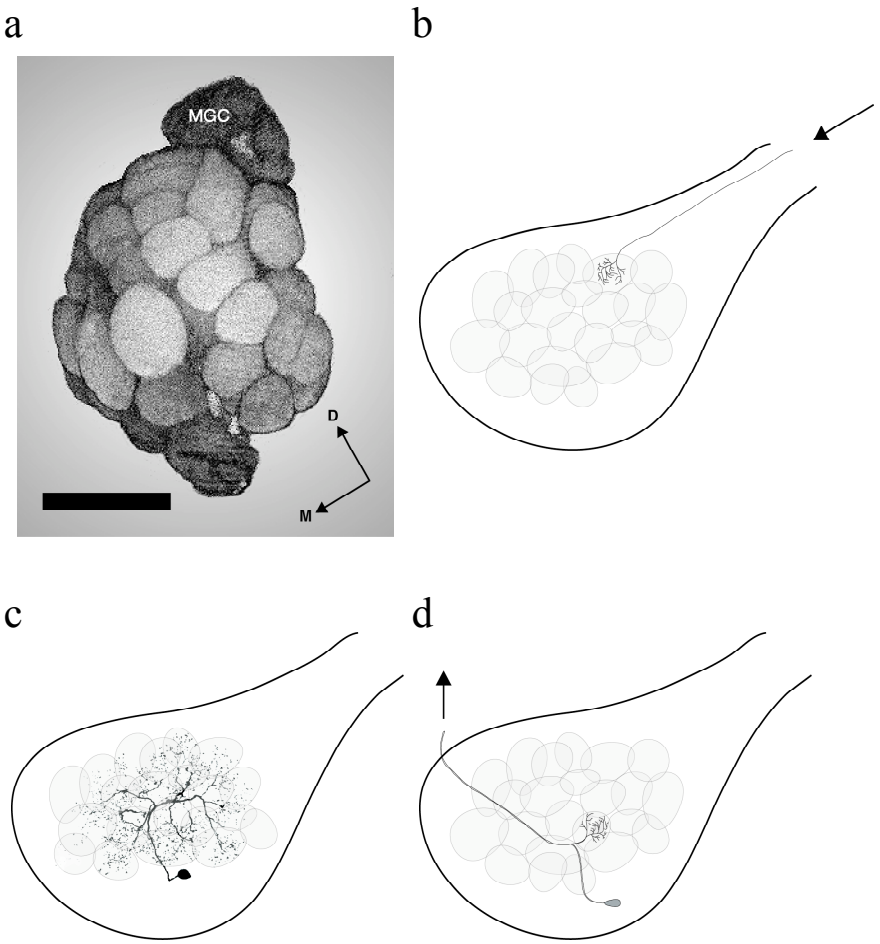

Figure 1. The antennal lobe (AL) and its neural elements. (a) A surface reconstruction of a synapsin immunostained AL of a male *S. littoralis*. The image is based on several optical sections obtained by confocal microscopy. The view is frontal, i.e. the same as in the imaging experiments. In addition to the macroglomerular complex (MGC) about 25 sexually isomorphic glomeruli are visible. (b-d) Schematic drawings of the three major antennal lobe neurons. (b) Receptor neuron with a uniglomerular arborisation. (c) Local interneuron with homogenous arborisation in most glomeruli. The soma is located in a lateral cell cluster. Modified from a Lucifer Yellow stained LN kindly provided by Dr Q. Han (d) Uniglomerular projection neuron with axonal projection to protocerebral brain regions. The soma is located in a lateral cell cluster. D, dorsal; M, medial. Scale bar 100 µm.

As early as 1927, Bretschneider recognised a sexual dimorphism in the moth AL. He showed that males have a cluster of enlarged glomeruli close to the entrance of the antennal nerve. Generally, this macroglomerular complex (MGC) consists of two to four glomeruli but exceptionally as many as seven (Anton and Homberg, 1999). Female moths posses glomeruli that appear to be homologous to the male MGC, called the large female glomeruli (LFG; Rospars and Hildebrand, 2000). These glomeruli are considerably smaller than the MGC and have a different function (see below).

Despite a non-topological organisation in the sensory organs (see above), there is a highly ordered targeting of ORNs in the glomeruli. Receptor neurons expressing a specific putative receptor protein send axons to the same one or two glomeruli in *D. melanogaster* (Gao et al., 2000; Vosshall et al., 2000). These target glomeruli have stereotyped locations across individuals. Also in vertebrates the same type of ORNs converges on, generally, two glomeruli (Ressler et al., 1994; Vassar et al., 1994; Mombaerts et al., 1996).

Neural elements

In most insects, each ORN targets a single glomerulus (Figure 1b) and synapses onto higher order neurons. Chemical synapses allow transmission of information between neurons. Evidence suggests acetylcholine as the principal neurotransmitter of ORNs in moths (Sanes and Hildebrand, 1976). Acetylcholine-synthesising as well as degrading enzymes have been localised to the outer caps of glomeruli, which corresponds to the terminal arborisations of ORNs. The higher order neurons are first of all the output neurons, the projection neurons (PNs). The majority of the moth PNs arborise in a single glomerulus (Figure 1d) whereas a minor part have dendrites in all or several glomeruli (Anton and Homberg, 1999). The PNs leave the AL and send axonal projections to higher brain centres through the antennocerebral tracts.

If the ORNs would simply contact the PNs, the information flow would be isomorphic from input to output, let be a signal strengthening due to the neuronal convergence (see below). However, a third type of neuron confined to the AL, the local interneuron (LN, Figure 1c), is present. These number about 360 in *M. sexta* (Anton and Homberg, 1999). Three morphological types of LNs can be recognised in moths, multiglomerular with homogenous arborisations, multiglomerular with heterogeneous arborisations and oligoglomerular with branches in a small number of glomeruli (Anton and Homberg, 1999). The LNs convey information from the ORNs to the PNs (Boeckh and Tolbert, 1993; Distler and Boeckh, 1996, 1997; Malun, 1991; Sun et al., 1997). They also make reciprocal synapses with PNs and feedback contact with ORNs. Most or all LNs have been found to be GABA-ergic (Hoskins et al., 1986; Homberg et al., 1989). Thus, the LNs have been proposed to have a role in an inhibitory network. One theory is that the LNs constantly inhibit PNs with high spontaneous activity (Christensen et al., 1993). If the LN then is inhibited by another LN the PN will be disinhibited and thus starts spiking.

The number of receptor neurons far outnumbers the interneurons these synapse with (reviewed in Anton and Homberg, 1999). In *M. sexta*, e.g., about 300 000

ORNs contact 66 glomeruli that in turn are innervated by 360 LNs and 900 PNs. Thus, the degree of convergence is in the order of 300:1 from input to output.

Efferent fibres of a fourth type of neuron, the centrifugal neuron, are also found in the AL. As opposed to LNs and PNs, which have most of their somata restricted to peripheral clusters within the AL, these neurons have somata located either in the AL or outside the lobe (Anton and Homberg, 1999). Centrifugal neurons are generally believed to modulate AL activity and have shown immunoreactivity to different biogenic amines (Homberg and Müller, 1999). Modulatory effects for some of these amines have been established. For example, local injection of octopamine in the honeybee AL increased the probability to respond to a conditioned stimulus (Hammer and Menzel, 1995).

The PNs leave the AL through axon bundles called antennocerebral tracts. The tracts project to higher brain centres. At least 5 different tracts have been described in *M. sexta* (Anton and Homberg, 1999). The largest tract, the inner antennocerebral tract, leaves the AL in the medial region, PN axons synapse with Kenyon cells in the calyces of the mushroom bodies and then proceeds further to the lateral protocerebrum. Other tracts run directly to the lateral protocerebrum or to the calyces of the mushroom bodies via the lateral protocerebrum.

Physiology

The functional significance of glomeruli has long been a riddle. Already some fifty years ago it was suggested that odours were represented in the vertebrate olfactory bulbs as spatial patterns of activated glomeruli (Adrian, 1950). More recently, Rospars (1988) suggested an across-glomerular coding strategy in insects after having demonstrated that the AL architecture was invariant across individuals of the same species.

In moths, most electrophysiological studies have focused on the pheromone detecting subsystem, mainly because much is known about the behavioural relevance of identified pheromone components. In 1987, Koontz and Schneider demonstrated that the MGC was innervated by ORNs tuned to sexual pheromones. Meanwhile it was shown that sex pheromone selective PNs in *M. sexta* had their dendritic arborisations restricted to the MGC (Christensen and Hildebrand, 1987). Depending on the specificity of the PN it arborised in either of the two MGC glomeruli in a stereotypic manner (Hansson et al., 1991; Heinbockel et al., 1998). Projection neurons responding to both components arborised in both compartments. In 1992, Hansson et al. demonstrated that each glomerulus in the MGC of the moth *A. segetum* is targeted by ORNs tuned to a specific component of the sexual pheromone. Thus, both ORNs and PNs have specific targets in the MGC. In two closely related moths, *Helicoverpa zea* and *Heliothis virescens,* it was demonstrated that the large MGC glomerulus, the cumulus, was responsible for processing of the same principal pheromone component in both species (Vickers et al., 1998). A smaller glomerulus with similar position in both species was innervated by PNs tuned to a secondary component, which differs between the two moths. Hence, functionality of glomeruli as well as spatial positions seem to be conserved across the species barrier. The input to the MGC in the Heliothine moths seems to match the output. Single cell recordings of ORNs with

subsequent anatomical stainings and optical imaging in *H. virescens* (Hansson et al., 1995; Berg et al., 1998; Galizia et al., 2000) and optical imaging in *H. zea* (Carlsson and Hansson, 2003) indicate response patterns that closely match the "sensory maps" of the output neurons. However, matching of ORN and PN identity in MGC glomeruli is not universal. Anton and Hansson (1999) demonstrated a mismatching of input and output patterns in the moth *Trichoplusia ni*.

Recordings of PNs arborising in the MGC suggest that information is not simply relayed from the ORNs. The strongest evidence comes from the frequently observed blend interactions. A blend interaction occurs when the response to a mixture cannot be predicted based on the responses to its constituents (Laing et al., 1989). Strong synergistic effects to pheromone blends, i.e. when the response to the blend is significantly stronger than the sum of responses to the individual components, have been recorded in e.g. heliothine moths (Christensen et al., 1989, 1991, 1995). Therefore, processing of olfactory information must occur already at the level of the MGC. It has been shown that LNs often connect input and output elements and also glomeruli and thus constitute a potential source for intra/inter glomerular computations (Tolbert and Hildebrand, 1981).

Prior to the studies in my thesis only little information was available concerning representation of plant-associated and other non-pheromonal odours in the moth antennal lobes. Due to practical difficulties, only a single investigation of innervation patterns of individual ORNs responding to non-pheromones is so far published. Todd and Baker (1996) filled physiologically characterised ORNs with cobalt in the moth *T. ni*. The major conclusion that could be drawn from that study was that ORNs responding to aromatic compounds sent axons deeper in the AL than ORNs responding to a terpene compound. This is an interesting finding as it corroborates our data from paper III and extends it to another species.

A handful of investigations have characterised responses of moth PNs responding to plant-associated and other non-pheromonal odours (Anton and Hansson, 1994, 1995; Sadek et al., 2002). Selectivity of these neurons range from very specific to broadly tuned but no consistency in projection patterns, as in the MGC, has been observed. In *M. sexta*, however, one of the two large female glomeruli (LFG), located at a similar position as the MGC in males, is innervated by PNs responding preferentially to the monoterpene linalool (King et al., 2000). The role of linalool as a male pheromone has been established in another species (Landolt and Heath, 1990). Thus, it was suggested that the LFGs are homologous to the male MGCs and process information about male emitted pheromones (King et al., 2000).

Optical imaging as a means to study glomerular activity at a neuron population level

The functional significance of glomeruli has been studied using several different assays. However, a technique allowing visualisation of activity in the entire glomerular array would be preferable. The development of optical imaging techniques has greatly contributed to our knowledge of neuron population events in the olfactory system. Optical recordings are done in real time, which allows visualisation of spatial as well as temporal aspects of odour processing. Furthermore, repeated stimulations can be done in the same animal, which facilitates comparisons of odour-evoked activity patterns.

Intrinsic imaging, which reflects metabolic activity, has mainly been used in the vertebrate olfactory system (Rubin and Katz, 1999; Meister and Bonhoeffer, 2001), whereas the use of fluorescent dyes has been favoured in insects (e.g. Joerges et al., 1997; Galizia et al., 1999b, 1999c, 2000; Papers I-V this thesis). The major advantages with dyes are better signal-to-noise ratio and higher temporal resolution.

Optical responses are seen as localised increases of emitted light that have been shown to originate from individual glomeruli (Galizia et al., 1999b; Belluscio and Katz, 2001; Paper IV). Glomerular activity patterns are bilaterally symmetrical between the lobes or bulbs (Galizia et al., 1998; Belluscio and Katz, 2001) and stereotypic across individuals (Galizia et al., 1999b; Sachse et al., 1999; Belluscio and Katz, 2001), which suggest a hardwired sensory map among glomeruli.

A disadvantage with optical recordings is the limitation to image activity in depth. This problem would be partly solved by the use of confocal imaging (Wang et al., 2003). A second disadvantage has been the inability to distinguish between the neural elements involved in the signals. To overcome this, techniques have recently been developed in insects to selectively measure from specific populations of neurons (Sachse and Galizia, 2002; Fiala et al., 2002; Ng et al., 2002; Wang et al., 2003; Paper V).

Methodology

Staining with a non-selective dye (paper I-IV)

For both methods (see below) a window was cut in the head capsule between the compound eyes (Figure 2a). Glands, muscles and tracheae were removed to uncover the ALs. After dye application, the preparation was incubated in a cooled and dark environment. Recordings were done *in vivo*, which permitted repeatable odour exposures in the same animal.

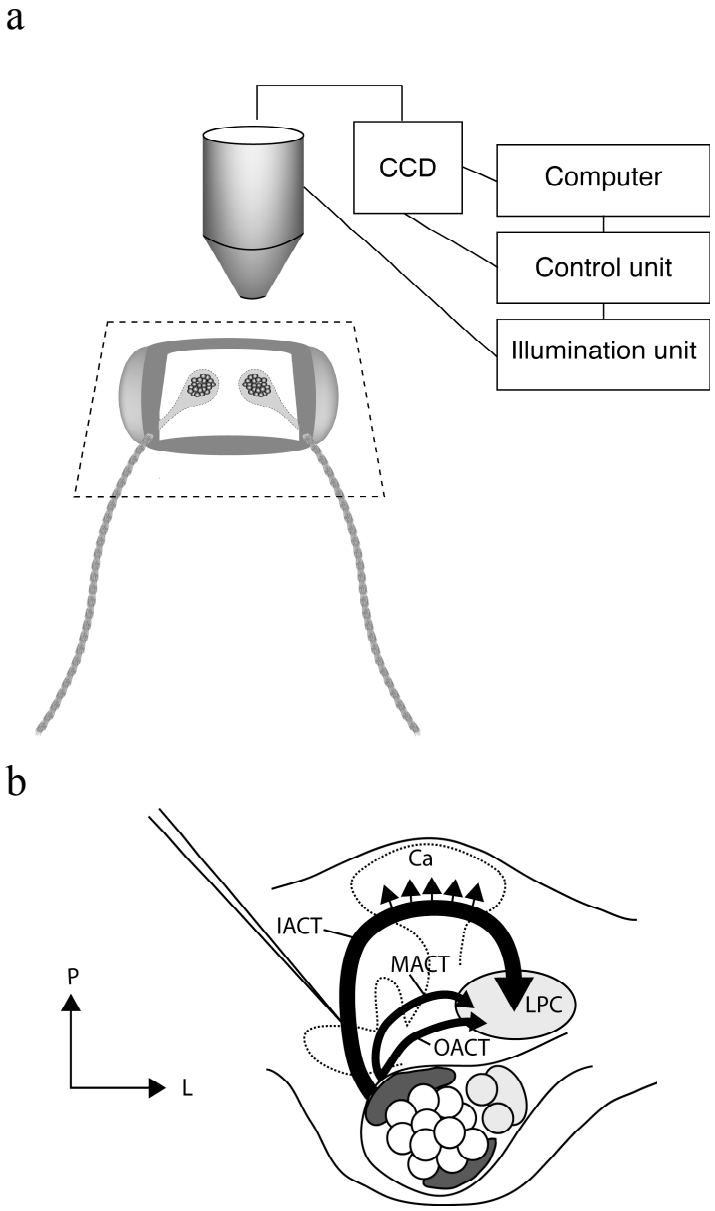

Figure 2. (a) Schematic view of the setup for optical imaging. The moth brain is uncovered and excited with monochromatic light from the illumination unit during the experiment. Fluorescence from the stained ALs is captured by the CCD camera and digitised images stored on the computer. (b) Selective staining of PNs. A frontal view of the moth brain showing the injection site of the dye-coated glass electrode in the IACT. The locations of two additional antennocerebral tracts are indicated, the middle antennocerebral tract (MACT) and the outer antennocerebral tract (OACT). Two major targets of the antennocerebral tracts, the calyces of the mushroom bodies (Ca) and the lateral protocerebrum (LPC) are also indicated. P, posterior; L, lateral.

In papers I-IV we used the calcium-sensitive dye CaGR-2AM (Molecular Probes). The dye is coupled with an acetoxymethyl group, which increases the membrane permeability of the dye complex. Cytosolic esterases split the complex whereby confining the dye to the intracellular space and setting it in an active state. Topical application of the calcium-sensitive dye would hypothetically stain all types of cells in the ALs including afferent neurons, interneurons and supportive cells. However, it is believed that the signals observed originate mainly from ORN activity (Galizia et al., 1998). This assumption is based on the fact that the ORNs outnumber all other types of neurons in the lobe, that the ORNs innervate the rind of the glomeruli whereas the interneurons have the densest innervation in the core and because no inhibitory signals or spontaneous activity have been observed.

The preparation was excited by light of a dye-specific wavelength and fluorescence from the brain tissue was measured by a sensitive CCD camera (Figure 2a). Increased [Ca^{2+}] was visualised as elevated light intensity relative to the background. In a typical experiment, activity was recorded before, during and after odour stimulation. To confirm that the observed localised responses were assigned to individual glomeruli we conducted a second morphological staining after the recordings (paper IV). We used a membrane-bound dye (RH795, Molecular Probes), which visualises the boundaries of glomeruli. The morphological staining was then aligned with images of odour-evoked activity.

PN-selective staining (paper V)

To obtain optimal information from the optical recordings it is necessary to dissect the signals into its neural components. In vertebrates it has been possible to selectively stain ORNs by disrupting the membranes of the cilia, apply the dye and then letting the cilia regenerate (Friedrich and Korsching, 1997, 1998; Wachowiak and Cohen, 2001; Fried et al., 2002; Wachowiak et al., 2002). Recently, the Gal4-UAS system was used to visualise neuronal activity in *D. melanogaster*. Reporter proteins could be expressed in the neuron type of interest by using different Gal4 specific lines (Fiala et al., 2002; Ng et al., 2002; Wang et al., 2003). For similar experiments in moths we have to wait until genetic tools are available.

Instead we adapted a technique formerly used in vertebrate mitral cells (Delaney et al., 2001) and recently in honeybees (Sachse and Galizia, 2002. We coated a glass electrode with a membrane impermeable calcium-sensitive dye (FURA-dextran, Molecular Probes). The glass electrode was inserted in the inner antennocerebral tract (Figure 2b) and the dye was allowed to be retrogradely transported within the axons to the terminals in the glomeruli. The preparation was excited at two independent wavelengths and a ratio was calculated between them. Ratio calculation reduces effects of uneven staining and photo bleaching. A successful staining was manifested in clearly visible somata and glomerular outlines. Furthermore, we often observed a spontaneous PN activity in the absence of stimuli, which altered randomly between glomeruli. Temporal resolution was high enough to permit investigation of slow temporal patterns. The latency period before the transient increase in calcium concentration is comparable to that observed in electrophysiological experiments, i.e. 200-300 ms.

The model animals

Both species of moths used in the experiments in my thesis have been extensively studied from different points of view. Much is known about their pheromone communication system, oviposition behaviour and feeding habits. Furthermore, electrophysiological studies of different levels in the nervous system provide a good background for my experiments and facilitate the interpretation of the results.

Spodoptera littoralis (paper I, II, IV and V)

The Egyptian Cotton Leafworm, *Spodoptera littoralis* (Noctuidae; Boisd.), has a distribution in northern Africa, the Middle East and the Mediterranean countries. It is well known as a serious pest on a wide range of economically important crops, e.g. cotton and alfalfa (Avidov and Harpaz, 1969). It is a true generalist species with a seemingly promiscuous feeding strategy. Feeding has been observed on at least 84 plant species within 40 families (Brown and Dewhurst, 1975).

The sexual pheromone communication system in *S. littoralis* is well studied and the female-emitted pheromone consists of at least two behaviourally active components, (*Z,E*)-9,11-tetradecadienyl acetate (*Z*9, *E*11-14:OAc) and (*Z,E*)-9,12-tetradecadienyl acetate (*Z*9, *E*12-14:OAc) (Kehat et al., 1976). In addition, a compound, (*Z*)-9-tetradecanol (*Z*9-14:OH), is known to inhibit upwind search behaviour in the males (Campion et al., 1980).

Female *S. littoralis* use olfactory cues to locate a suitable site for oviposition or to avoid a less suitable one. A number of oviposition deterrent compounds have been identified, which seem to act synergistically in a blend rather than individually (Anderson et al., 1993).

Electrophysiological studies have been performed both in the periphery (Anderson et al., 1995; Ochieng' et al., 1995, Jönsson and Anderson, 1999) and in the AL (Anton and Hansson, 1994, 1995; Sadek et al., 2002). In addition, a three dimensional anatomical map of a part of the female AL has been constructed (Sadek et al., 2002).

Manduca sexta (paper III)

The sphinx moth, *Manduca sexta* (Sphingidae; L.), has its distribution in North America. As opposed to *S. littoralis, M. sexta* is a more specialised species. Females preferentially oviposit on plants belonging to the family Solanacae (Yamamoto et al., 1969).

The female produces two main pheromone components, *E*10, *Z*12-hexadecadienal (bombykal) and *E*10, *E*12, *Z*12-hexadecatrienal (*EEZ*) (Tumlinson et al., 1989). In addition, a number of minor components emitted from the female have been indicated as behavioural synergists (Starrat et al., 1979; Tumlinson et al., 1989, 1994).

The first intracellular recording of AL interneurons in insects was performed in *M. sexta* (Matsumoto and Hildebrand, 1981). Since then it is probably the most extensively used model insect in neurophysiological experiments. Especially processing in the male MGC of pheromone information has been thoroughly studied (Christensen and White, 2000). Recently, extracellular recordings from ORNs in both males and females using extensive sets of stimuli were made (Kalinova et al., 2001; Shields and Hildebrand, 2001b).

Summary of results

Pheromone-evoked responses are restricted to the MGC (paper I and III)

Due to the established innervation patterns of physiologically characterised neurons in the MGC, optical measurements of pheromone-evoked activity provide an excellent control for the validity of the method. In both *S. littoralis* and *M. sexta* pheromone activity was restricted to the MGC. Plant-associated compounds, on the other hand, evoked no or only weak responses in the MGC. Hence, there is a rough division of labour in the AL, which is in accordance with other studies, and virtually no overlap between the two subsystems. In *S. littoralis*, responses to sexual pheromones were found to corroborate earlier stainings of individual physiologically characterised ORNs (Ochieng' et al., 1995; Hansson, 1997, Figure 3a). The principal pheromone component, (*Z,E*)-9,11-14:OAc, activated a large glomerulus close to the entrance of the antennal nerve, which most likely corresponds to the identified "cumulus", or "a" glomerulus (Figure 3b). Likewise, the secondary pheromone component, (*Z,E*)-9,12-14:OAc, showed highest activity in a glomerulus, which likely corresponds to the "c" glomerulus, whereas a behavioural antagonist, *Z*-9-14:OH, activated the "b" glomerulus (Figure 3b). A third putative pheromone component, *Z*7-12:OAc, activated a fourth glomerulus. This glomerulus was located proximal to the others but does likely not belong to the MGC. Anton and Hansson (1995) found that single PNs responding to *Z*7-12:OAc arborised in an "ordinary" glomerulus.

Previous studies in *M. sexta* showed that ORNs tuned to the two principal pheromone components arborised in the MGC (Christensen and Hildebrand, 1987). Due to the co-localisation of these two types of ORNs in the same sensillum it has not been possible to investigate their targets in individual subunits of the MGC. The innervation patterns of PNs, however, are known (Hansson et al., 1991, Heinbockel et al., 1998, Figure 3c). We found that *EEZ* preferentially activated the "cumulus" and bombykal the "toroid" (Figure 3d). Further we tested a panel of six putative pheromone components. With one exception, none of these elicited any clear responses in the AL. However, the putative pheromone component *Z*11-hexadecenal evoked clear responses in what was likely the third glomerulus of the MGC, the "horseshoe". No single physiologically characterised PN has been found to innervate this glomerulus. However, ORNs selectively tuned to *Z*11-hexadecenal have recently been

recognised in both male and female *M. sexta* (Kalinova et al., 2001). Thus, there is a correlation between our results and the known innervation patterns of ORNs and PNs. Assuming that a major part of the calcium signals report input activity, our results indicate a matching of input and output in *M. sexta*. Projection patterns of pheromone selective PNs in *S. littoralis* could not be shown to match those of the input patterns (Anton and Hansson, 1995; Ochieng' et al., 1995). Single PNs arborising in the same glomerulus had different tuning and PNs with the same tuning arborised in different glomeruli. Using the selective staining method described in paper V would possibly reveal whether a neuronal matching occurs in *S. littoralis*. Preliminary results indicate that this indeed may be the case (Carlsson and Hansson, unpublished observation).

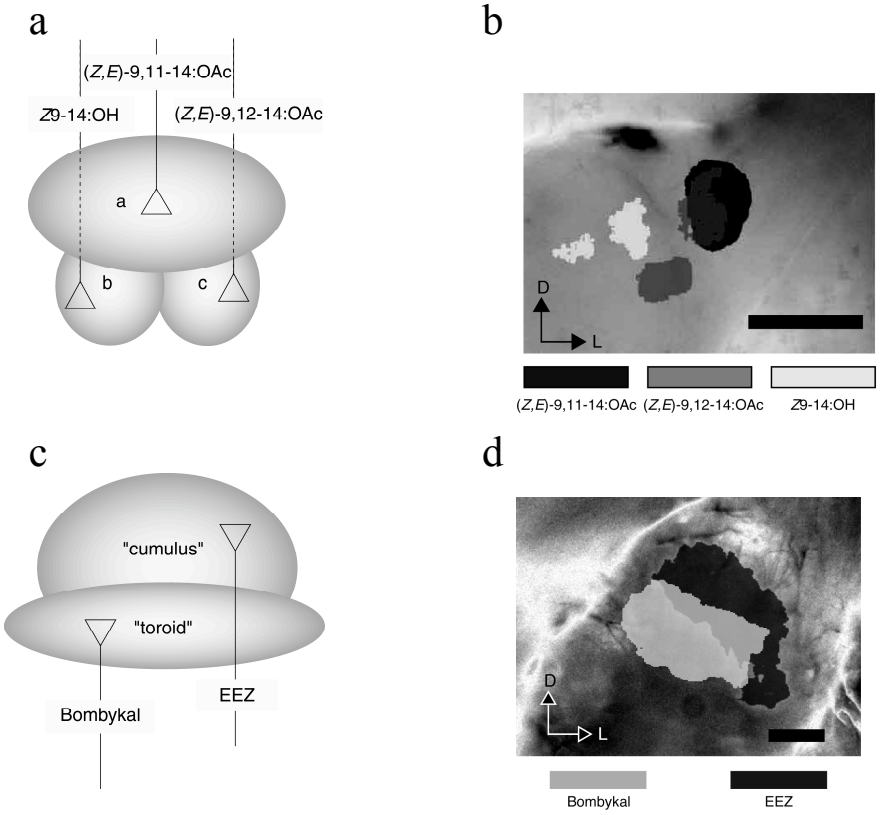

Figure 3. Correlation of imaging results and innervation patterns of single neurons. (a) A schematic illustration of ORN projections in the MGC of *S. littoralis*. Three physiologically identified types of ORNs send axons to specific glomeruli within the MGC. Modified from Hansson (1997). (b) Optical responses in a male *S. littoralis* to the two principal pheromone components and a behavioural antagonist in the same animal. The activity maps have been thresholded at 50% of maximal activity and superimposed on a common image of the AL. (c) A model of PN innervation patterns in the MGC of *M. sexta*. Adapted from Hansson et al., (1991) and Heinbockel et al., (1998). (d) Activity maps of optical responses in a male *M. sexta* to the two principal pheromone components superimposed on an image of the AL from the same animal. Threshold is 50% of maximal activity. Scale bars 100 μm. D, dorsal; L, lateral.

Responses to non-pheromones – combinatorial representations (paper I-IV)

Whereas the functional organisation of the MGC glomeruli is well established, virtually nothing is known about the functionality of the sexually isomorphic glomeruli. Sensory neurons tuned to plant-associated or other non-pheromonal odours are known to innervate the "ordinary" glomeruli (Hansson et al., 1992; Todd and Baker, 1996). However, there is no evidence in moths for a "chemotopic" organisation of these glomeruli, analogous to the MGC organisation.

Several papers have recently reported highly specific ORNs tuned to plant-associated odours (Hansson et al., 1999; Larsson et al., 2001; Stensmyr et al., 2001; Röstelien et al., 2000). Therefore we expected to find glomerulus-specific responses not only to pheromone components but also to plant odours. This was, however, not the case. Responses were distributed across the AL, generally with one or two principal glomeruli being highly activated together with weaker responses in several other glomeruli (Figure 4). Responses to plant-derived compounds were observed among the sexually isomorphic glomeruli in both sexes. There was virtually no overlap with the MGC, i.e. plant odours did no evoke activity in the MGC and pheromones did not evoke activity in the ordinary glomeruli.

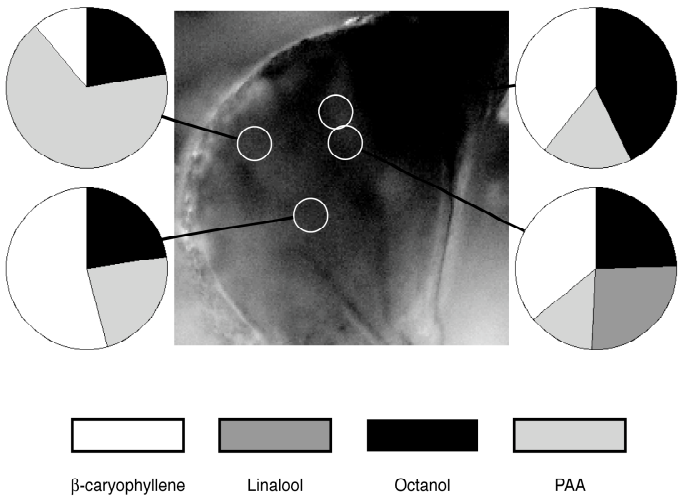

Figure 4. Across-glomerular representations. Measurements of responses to four different compounds in four glomeruli in a single male *S. littoralis*. The graphs show the relative activity each odour evokes in the glomeruli.

About twenty glomeruli are accessible to optical recordings in the moths, which is roughly one third of the total population. Interestingly, all odours tested evoked a response among the accessible glomeruli. As the combinations of activated glomeruli were odour specific it seems that some degree of redundancy may occur in the AL. Alternatively, the inaccessible glomeruli may have different functions or respond to odours not included in the test panel.

The plant odours elicited unique combinations of activated glomeruli. If we extrapolate results obtained in the fruitfly (Gao et al., 2000; Vosshall et al., 2000), where each type of candidate olfactory receptor generally is represented in a single glomerulus, we can draw the conclusion that the odorants we used most likely interact with several different membrane receptors. The degree of interaction is correlated with the concentration (see below). Due to the limited stimulus panel we cannot exclude that highly specific plant-odour tuned receptors and accordingly highly focussed glomerular activation may exist also in the two moth species studied.

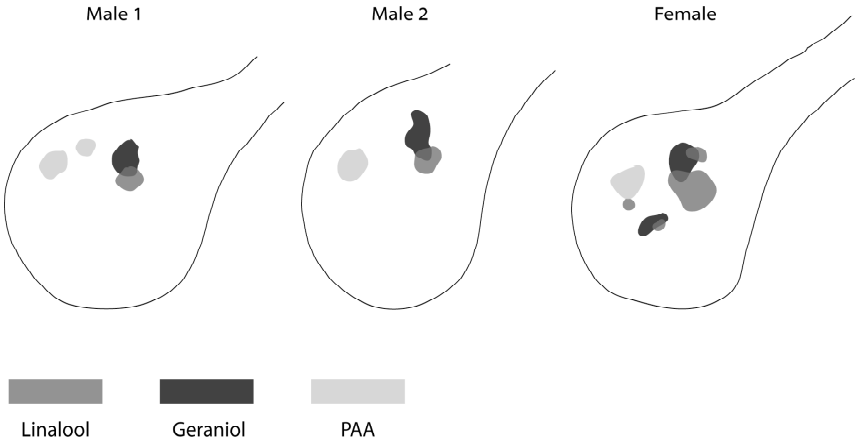

Figure 5. Stereotypy of activity patterns across individuals. Activity maps of responses to three different odours in two male and one female *S. littoralis*. Threshold is 50% of maximal activity. The relative positions of activated glomeruli are maintained across individuals of both sexes. Adapted from Paper I.

In *S. littoralis*, we found similar responses between individuals based on the position of certain key glomeruli that had invariant relative positions across animals (Figure 5). The positions of the principal glomeruli were in addition conserved between males and females. In an attempt to reveal the link between chemical structure and distribution of odour representations we analysed the correlation between activity patterns evoked by different odorants. We found that chemically (structurally) related compounds evoked more similar patterns than odorants with less structural resemblance. For example, the response patterns evoked by benzaldehyde showed the highest correlation with the pattern evoked by phenylacetaldehyde (PAA). Both compounds are aromatic and have an attached aldehyde group. Interestingly, single ORNs activated by PAA do not respond to

benzaldehyde (Anderson et al., 1995). The glomerulus that is most strongly activated by either of these compounds in our recordings appears to be the same. This suggests that (1) more specific glomeruli may be present in regions not accessible for recordings and (2) that additional types of ORNs are present with a broader tuning. Alternatively, it cannot be excluded that ORNs expressing different membrane receptors target the same glomerulus as opposed to the findings in the fruitfly (Gao et al., 2000; Vosshall et al., 2000).

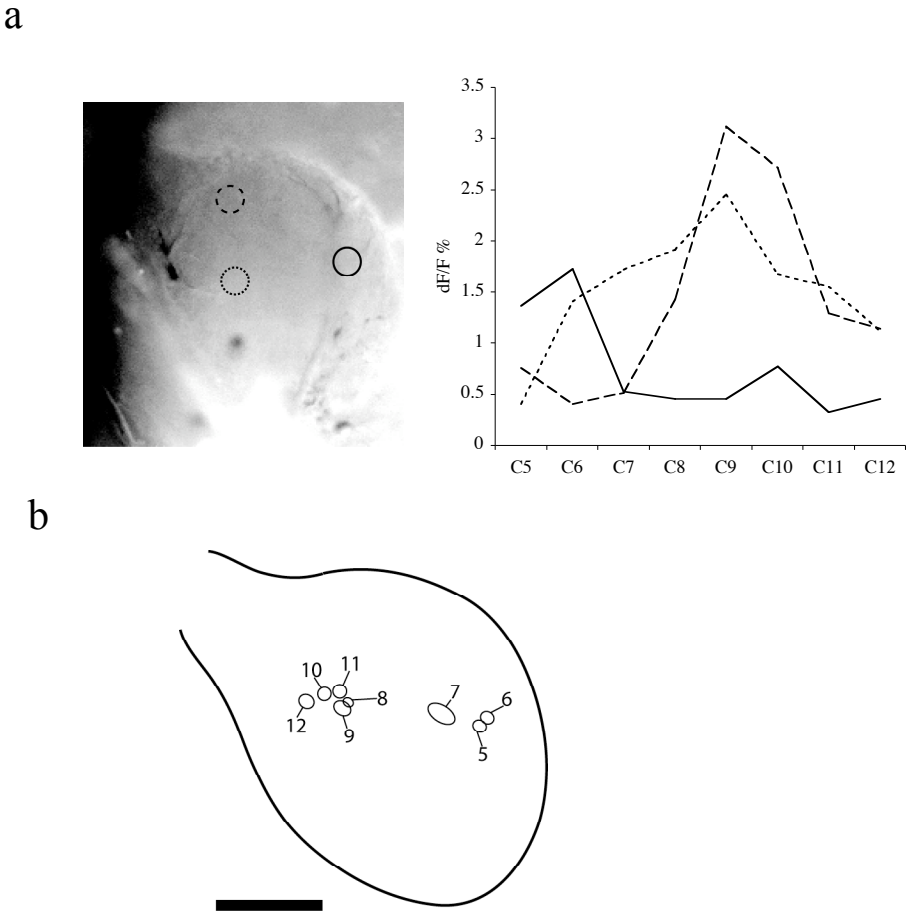

Figure 6. Structure-response relationships. (a) Broad and overlapping tuning curves in three glomeruli in a single female *S. littoralis* to a series of homologous aldehydes (C5-C12). (b) A standardised AL shows the foci of highest activity for a series of homologous aldehydes (C5-C12). The coordinates are averaged across eight animals and corrections were made for differences in AL size. The ovals represent averaged coordinates +/- SEM. Scale bar 100 µm.

In the second paper we extended the analysis of structure-dependency of the glomerular representations to a reduced number of dimensions. We used series of aliphatic alcohols or aldehydes. In a series, each compound differed by a single

carbon atom. In order to make proper comparisons we corrected for differences in volatility. No specific glomerulus was found that exclusively responded to any of the compounds. Rather, the responding glomeruli showed continuous and overlapping properties (Figure 6a). Adding atoms to the carbon chain resulted in a shift in glomerular activity from the medial to the lateral regions of the lobe (Figure 6b). The number of responding glomeruli increased from a chain length of 5 carbon atoms up to 9 atoms where after the number of responding glomeruli decreased again. A possible explanation is that short chain molecules might at low concentrations interact with one or a few olfactory receptor proteins. Longer molecules, on the other hand, may have additional features, which make them more complex and thus increase the probability for interaction with a larger population of receptors. With a further extension of the chain length, molecules might become so inflexible that they can only interact with a smaller population of receptors. A pattern evoked by a given odorant was always most similar to its structural neighbour. We did not find any specific regions or glomeruli for any of the functional groups as has been shown in vertebrates (Uchida et al., 2000; Johnson et al., 2002). The contrast between compounds from the two groups was highest for the lower chain lengths, suggesting that these compounds are easier discriminated by the animal. It is highly likely that the short chain length compounds are more biologically relevant to the moth. Five and six carbon aliphatics are common green leaf volatiles (Visser et al., 1978).

In *S. littoralis* we further conducted an analysis to compare the representations of the two series of aliphatic compounds. All possible permutations were compared and the highest correlation was found when the series were ordered with increasing chain length. Hence, chain length dependency is similarly represented in the AL for aldehydes and alcohols.

In *M. sexta*, plant related odorants activated different combinations of glomeruli, similar to *S. littoralis*. In a previous experiment it was shown that different subgroups of ORNs responded either to aromatic or terpene compounds with virtually no overlap (Shields and Hildebrand, 2001b). Therefore we used a subset of these compounds in our experiment and found two non-overlapping clusters of glomeruli responding to either of the chemical groups. A cluster of glomeruli responding preferentially to aromatics was located in the medial region of the AL. A second cluster with glomeruli responding to terpenes was located more laterally. A similar segregation was found in both males and females. These results suggest a hierarchical organisation with a rough division according to primary structures, e.g. cyclic structure, and a fine-scale division within each cluster. A similar organisation has been proposed in vertebrates (Uchida et al., 2000). A possible alternative explanation is discussed below. To further analyse the conservation of activity patterns across animals we measured the distance in two dimensions to the locations of the highest activity. We found that the positions of activity foci for the aromatics did not significantly differ and had similar positions in both sexes. Similar results were found for four terpenes. A third focus was found for the sesquiterpene β-caryophyllene. The activity focus for this compound was located in a ventral region in both males and females. Control experiments with a structurally similar compound, α-humulene, revealed a similar activity pattern. In conclusion, at least three different principal glomeruli (glomeruli responding with

the highest activity for a certain stimulus) with invariant positions across individuals of both sexes were found responding to aromatics, most of the tested terpenes and β-caryophyllene, respectively.

Responses to plant odours were generally observed in a large fraction of the visible glomeruli. This fact may seem to contradict the assumption that the signals obtained are mainly representing input activity. However, specific presynaptic staining of ORNs in vertebrates also revealed widely distributed glomerular activity to general odours (Friedrich and Korsching, 1997, 1998; Wachowiak and Cohen, 2001; Fried et al., 2002; Wachowiak et al., 2002). Similarly, by using genetically incorporated markers in *D. melanogaster* ORNs a large population of glomeruli responded to a different degree to single odours (Ng et al., 2002; Wang et al., 2003).

Concentration-dependent activity patterns (paper IV)

Odours often occur in plumes consisting of strands of different concentrations. This fact puts an extra pressure on the olfactory system to correctly recognise the identity of the stimulus. A concentration-invariant code would solve the problem. On the other hand, the moth must also be able to discriminate between concentrations. Thus, in the fourth paper we focussed on how stimulus concentrations were represented among glomeruli in *S. littoralis*. First of all, we were able to measure from glomeruli visualised in a second morphological staining. We could confirm that odour-evoked calcium changes were assigned to individual glomeruli. We exposed the animals to a series of concentrations and found that the number of activated glomeruli increased with concentration. This is in accordance with imaging studies in vertebrates (Rubin and Katz, 1999; Johnson and Leon, 2000; Fuss and Korsching, 2001; Wachowiak and Cohen, 2001; Meister and Bonhoeffer, 2001; Fried et al., 2002). A glomerular recruitment might represent a spread of signals through interglomerular connection. It is, however, more likely that it reflects a recruitment of receptors with lower affinity. Selective imaging of ORN activity in vertebrates has revealed similar results, i.e. a higher concentration evokes activity in a larger number of glomeruli (Fuss and Korsching, 2001; Wachowiak and Cohen, 2001; Fried et al., 2002).

We could identify specific glomeruli based on location and sensitivity to each compound at a dose of 100 µg. Hence, we could average responses across animals in these glomeruli. The dynamic range of glomerular responses spanned 3-4 orders of magnitude. This is in accordance with extracellular recordings from single ORNs in *S. littoralis,* which show dose-response dynamics within the same range (Anderson et al., 1995; Ljungberg et al., 1993). Dose-response curves often revealed a sigmoidal function in the most strongly responding glomeruli. This indicates a saturation of responses in certain glomeruli (and likely ORNs) at high concentrations. Similar shapes of dose-response curves are often observed in single antennal neurons (Anderson et al., 1995; Ljungberg et al., 1993). Some glomeruli, however, showed moderate activity at low concentrations but no further increase at higher doses. A plausible explanation is that the observed signals in these glomeruli originate from LNs rather than ORNs. AL interneurons are often less concentration-dependent than ORNs (Anton et al., 1997; Masante-Roca et al.,

2002). An alternative explanation for saturations at low concentration is that responding ORNs innervate these glomeruli but are presynaptically inhibited (Wachowiak and Cohen, 1998, 1999).

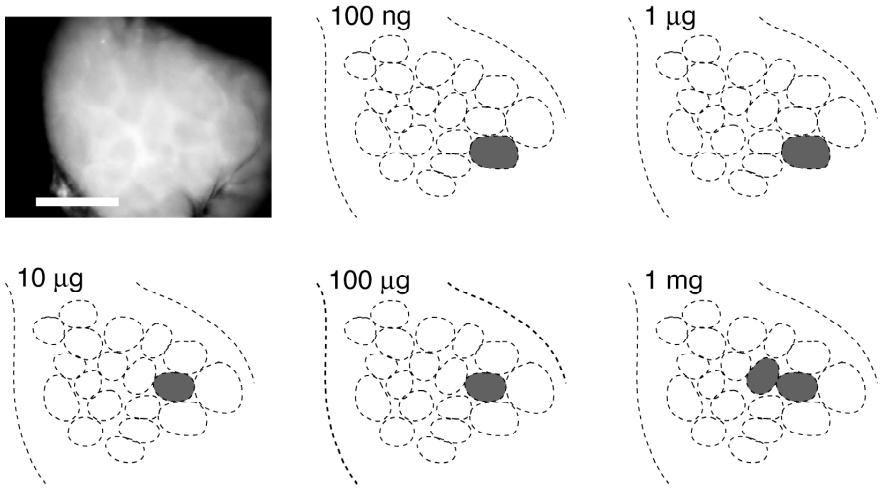

Figure 7. The effect of concentration on glomerular responses. Responses to a concentration series of PAA. Only the principal glomeruli are shaded but these are not identical across concentrations. Movement of the highest activity takes place between neighbouring glomeruli. At the highest concentration two different glomeruli are almost equally activated. The AL image shows the stained glomeruli from which the outlines have been constructed. Scale bar 100 µm.

Even though a large number of glomeruli responded to a stimulus, a principal glomerulus (the most strongly activated) was generally present. However, the principal glomerulus for a certain stimulus varied with concentration (Figure 7). The movement of activity foci was almost exclusively restricted to proximal glomeruli. The explanation for such a movement is the differing dose-response curves. When saturation was reached in one glomerulus the response became stronger in another glomerulus that had a higher saturation level. As the focal movement generally occurred within a limited region further suggests that neighbouring glomeruli have a similar receptive range.

Finally we compared activity patterns evoked by different odours and concentrations. Responses were normalised, thus disregarding absolute intensities. Representations of the same stimulus at different concentrations became less correlated as the difference in concentration increased. When we compared responses evoked by different odours it was revealed that the responses were more similar at high than at low concentrations. This similarity was, however, only significant for three of the odour pairs. These three odours were structurally similar and their glomerular responses were overlapping. The fourth odour, on the other hand, evoked patterns that were virtually non-overlapping with the other compounds. If a glomerular spatial code is read by downstream brain regions our

results implicate that similar odours should be more difficult to discriminate at high concentrations. However, relevant information may still be present in the population of PNs after neuronal processing. Moreover, it cannot be ruled out that the spatial patterns of glomerular activity play a less important role in odour encoding than the temporal characteristics of the signals. If so, odour quality may still be correctly coded in the temporal patterns at different concentrations, no matter if the spatial patterns vary. However, multiunit recordings in *M. sexta* showed that synchrony between responding neurons is concentration-dependent (Christensen et al., 2000). Behavioural data from honeybees indicate that the discriminatory ability actually increases at high concentrations (Bhagavan and Smith, 1997). In future experiments we aim to test the odour pairs used in a differential condition experiment. We further plan to replicate the experiment using the selective method of staining output neurons described in paper V.

Comparison between sexes (paper I and III)

From morphological studies in a number of moth species (Koontz and Schneider, 1987; Rospars, 1983; Rospars and Hildebrand, 2000) we know that the majority of the glomeruli are sexually isomorphic. Our investigations in *M. sexta* and *S. littoralis* further indicate that these glomeruli are also functionally similar across sexes. In *S. littoralis*, the relative positions of the principal glomeruli for three different odours did not vary between males and females (paper I, Figure 5). PAA elicited strongest response in a glomerulus with dorso-medial position. Geraniol and linalool activated two neighbouring glomeruli in the lateral region. Geraniol, however, always evoked strongest response in the more dorsal glomerulus. Interestingly, many of the non-pheromonal compounds used in paper I are known to be involved in female oviposition behaviour (Anderson et al., 1993). The fact that these odours evoke similar activity patterns in both sexes naturally raises the question of their behavioural significance in males.

In *M. sexta* we extended the analysis of sexual similarities by measuring the absolute distances from certain common morphological landmarks to the centre of responding glomeruli (paper III). We showed that the position of glomeruli responding to the same stimuli had invariant positions both across individuals and across sexes.

In *M. sexta*, a female-specific glomerulus, the lateral LFG, is innervated by PNs preferentially responding to linalool and to a lesser degree structurally similar terpenes (King et al., 2000). We found clear responses to linalool among the sexually isomorphic glomeruli. Moreover, similar activity patterns were found in males. It is likely that the female LFGs were inaccessible for recordings, possibly due to a more posterior position than the glomeruli that could be recorded from. King et al. suggested that the female LFGs participated in processing of male-emitted pheromones. In fact, the role of linalool as a male pheromone has been established in another species (Landolt and Heath, 1990). The results from our study and the one by King et al. (2000) show that both males and females possess detectors for linalool and suggest that females have an additional pathway.

Species comparison (paper I-V)

As two distantly related species, *S. littoralis* and *M. sexta* were used in the experiments it is interesting to compare the results. In both species, pheromone responses were restricted to the MGC and plant-associated odours to the sexually isomorphic glomeruli. Furthermore, in both species plant-related odours were represented as across-glomerular activity patterns. We did not find any glomeruli that specifically responded to a limited set of odours. Two major clusters of glomeruli responding to aromatics and terpenes, respectively, were found in *M. sexta*. Similarly, in *S. littoralis* at least two aromatic compounds, PAA and benzaldehyde elicited a strong response in medially located glomeruli (paper I and V). Terpenes, on the other hand preferentially activated more laterally located glomeruli. In a third moth species, *T. ni*, single ORNs tuned to aromatic compounds targeted glomeruli located more medially than glomeruli targeted by neurons tuned to a terpene (Todd and Baker, 1996). Furthermore, preliminary results from optical recordings in two other species, *A. segetum* and *H. zea,* show a similar rough organisation according to chemical structure of the stimulus (Carlsson and Hansson, unpublished observation). Thus, it is not unlikely that the organisation of functionally similar glomeruli is conserved across the species barrier. A more elaborate comparative study of a range of lepidopteran species is currently in progress (Meijerink, Carlsson and Hansson, in preparation).

Comparison of input and output patterns (paper I-V)

In the final paper we employed a different technique in order to selectively stain a large population of output neurons. Responses were observed to a different degree in several glomeruli and responses elicited by different odours often overlapped. The focus of activity was limited to one or a few principal glomeruli. The relative position of the principal glomeruli for a selection of compounds was invariant across individuals. Interestingly, these positions were the same as those obtained with CaGR-2AM. As CaGR-2AM is believed to mainly report ORN activity, a rough correlation of input and output patterns could be discerned. This does not, however, mean that information is relayed unprocessed from ORNs to PNs. Recent imaging experiments in *D. melanogaster* using genetically incorporated indicators in selected neuron types showed no differences in input and output responses (Ng et al., 2002; Wang et al., 2003). It was suggested that information is faithfully transferred from the sensory neurons to the PNs without being processed. However, more carefully performed analyses of these patterns are needed.

Spatiotemporal patterning in output neurons (paper V)

So far the studies in my thesis have focussed on spatial representations averaged over time. The relatively high temporal resolution in recordings using FURA-dextran specifically injected in PNs permitted a more detailed analysis of slow temporal patterns within the stimulus period.

A rapid increase in [Ca^{2+}] about 200-300 ms after stimulus onset reflects latency times similar to those observed with intracellular recording techniques (Anton and

Hansson, 1994, 1995). The following signal decrease differs between stimuli and glomeruli and likely reflects slow temporal patterns in single PNs. Slow temporal patterns in single PNs are odour specific and consist of periods of excitation and inhibition (Laurent et al., 1996).

Odours evoked patterns of activated glomeruli that continuously changed over time, i.e. an initial pattern was often considerably different from a late pattern within the period of stimulation. No new glomeruli were recruited to the pattern but the degree of activity changed heterogeneously among glomeruli. A glomerulus that showed highest activity at the stimulus onset became weaker and other glomeruli took over the role.

In zebrafish it was demonstrated that responses in mitral cells changed within the period of odour exposure (Friedrich and Laurent, 2001). When responses to different odours in a large population of mitral cells were compared it became clear that response patterns initially were similar but decorrelated as a function of time.

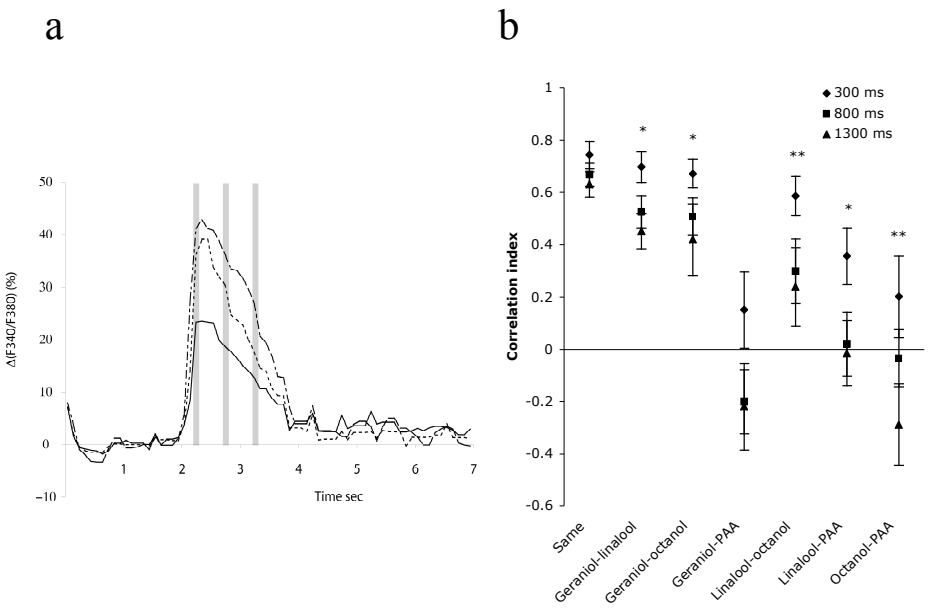

Figure 8. Temporal decorrelation of activity patterns evoked by different compounds. (a) Measurements were done from three time-points (300, 800 and 1300 ms after stimulus onset). (b) Correlations were compared at the three time-points for all odour pairs. Comparisons of correlation indices for patterns evoked by the same compound served as a control. Friedman's test was used for statistical analysis of differences in correlation over time (n=7 animals; * $p<0.05$; ** $p<0.01$).

We hypothesised that glomerular activity patterns evoked by different odours should become less similar over time. Measurements of [Ca^{2+}] were made at three time-points, 300, 800 and 1300 ms after stimulus onset (Figure 8a). As we generally repeated stimulations we could also compare patterns evoked by the same odour, which served as a control. When comparing correlations of repeated

stimulations of the same compound we found no significant difference in correlation between the three time-points. However, when comparing correlations between patterns elicited by different odours we found a significant decrease in correlation from the first measurement (Figure 8b). The only odour pair that did not become less similar was geraniol versus PAA. A possible explanation is that the correlation was already very low at the first measurement.

If brain regions downstream of the AL can decode the spatial origin of signals, our results suggest that there is a sharpening of representations over time that would increase the discrimination ability. Behavioural support for this hypothesis comes from the honeybee. Honeybees need ~500 ms for a response to (non-sexual pheromone) odours but at least one second of stimulation is required for a correct discrimination (J. Klein, unpublished, cited in Galizia et al., 2000). Thus, it appears that time is an important factor in discrimination tasks involving non-pheromonal odours, and the slow temporal patterns could theoretically contribute to an olfactory code. In contrast, these temporal patterns would be too slow to encode information about sexual pheromones. Male moths, e.g., must be able to respond to rapid changes in stimulus intermittency when moving upwind in pheromone plumes in search for a calling female.

Laurent (2002) argues that the slow temporal patterning is crucial for optimising the code but may not be the code *per se*. Rather "the complicated patterning we observe in AL/OB neurons might simply be part of the process through which the format of the message is actively optimised for further processing by downstream areas" (Laurent, 2002).

Chemotopicity (paper I-V)

A principle of receptotopic representation is obvious from these and related studies, which means that ORNs with different tuning innervate different glomeruli. An extension of this principle is chemotopicity (Korsching, 2001). A chemotopic representation implies that ORNs with similar and overlapping tuning project to neighbouring glomeruli. In both *M. sexta* and *S. littoralis* we found that glomeruli responding to a certain odour generally are clustered together. Glomeruli responding to structurally similar odours are found within the same region of the AL. Similar results come from optical recordings in vertebrates and honeybees (Friedrich and Korsching, 1997, 1998; Sachse et al., 1999; Uchida et al., 2000). Moreover, changes in stimulus concentration often resulted in a movement of the principal glomerulus (Figure 7b). The movement was, however, almost always restricted to proximal glomeruli that have overlapping tuning. Further evidence for a chemotopic organisation comes from transgenic mice where it was found that ORNs expressing closely related receptor proteins converged onto neighbouring glomeruli (Tsuboi et al., 1999).

The results from papers I, III and IV favour the idea that related chemical structures are represented in discrete populations of glomeruli. We found that aromatic compounds preferentially activated glomeruli in the medial region whereas terpene compounds activated more laterally located glomeruli. However, there may be an alternative explanation. In paper II we showed that aliphatic

compounds activated different areas of the lobe depending on the carbon chain length (Figure 6b). Short-chain molecules activated mainly a glomerulus in the medial region and there was a shift in activity focus towards the lateral part when increasing the chain-length. The glomeruli activated by these aliphatic compounds overlap with glomeruli activated by aromatic and terpene compounds. It could thus be the molecular length that is detected, as aromatic compounds are generally shorter than the more straight-chained terpenes. This hypothesis should be tested by systematical varying of the length of both aromatic and terpene molecules. In a [^{14}C]2-deoxyglucose experiment in rats, Johnson and Leon (2000) showed that among several parameters the molecular length was the principal factor explaining most of the variance in the activation patterns.

Chemotopicity might be an important feature that facilitates odour discrimination by processes like lateral inhibition. However, lateral inhibition has not been demonstrated in the insect olfactory system. A few experiments have shown indications that lateral inhibition might be a possible mechanism in the vertebrate olfactory bulb (Yokoi et al., 1995; Mori and Yoshihara, 1995) but an alternative framework has been suggested (Laurent, 1999). Moreover, it cannot be ruled out that juxtaposition of glomeruli with similar tuning is simply a consequence of evolutionary constraints on targeting of ORN terminals. That is, a genetic instruction to guide an ORN to "its" glomerulus might have evolved parallel to the receptor the neuron houses. Evidence from mice, however, suggests that it is the receptor protein itself that determines the target of the ORNs (Mombaerts et al., 1996; Wang et al., 1998).

Finally, turn it the other way round, lateral inhibition would likely work even without an organisation of proximal glomeruli with overlapping tuning. As the LNs generally connect a large portion of the glomeruli from all around the AL, proximity of glomeruli may not be necessary for such processes.

General conclusions and future directions

In the five papers presented in this thesis I have described the functional organisation of the moth antennal lobe. Even though a lot of work remains to be done I believe that the initial steps taken in our studies greatly contribute to the general understanding of glomerular mechanisms.

As opposed to other senses, olfaction has to deal with a multidimensional type of stimulus. The visual sense, e.g., can encode the entire spectrum of light simply by a few types of receptors. Olfactory stimuli, however, cannot easily be arranged along a single dimension as wavelength. Parameters as molecular structure, weight, length, volume, electro negativity as well as bonds and functional groups constitute different dimensions. For a sensory system to detect and to discriminate between the vast arrays of potential chemical stimuli a large set of receptors is required. As previously discussed, the gene family coding for olfactory receptor proteins accounts for a substantial part of the entire genome in all animals studied.

The multidimensional properties of olfactory stimuli may have put an evolutionary pressure on developing an architecture suitable for processing odour information. A layered structure, as in other sensory systems may not cope with such requirements. Interglomerular connections should be facilitated by the lobe structure with equidistance from the central core to all glomeruli.

The large number of possible combinations of activated glomeruli suggests a high coding capacity. Furthermore, if in addition temporal features add information about odour identity the animal may be able to discriminate between an almost infinite number of potential odours.

An important question is how glomerular representations relate to perception. Honeybees have an astonishing ability to discriminate even structurally similar compounds (Laska et al., 1999). The discrimination performance is negatively correlated with the structural similarity of odour molecules. Using series of homologous aliphatic compounds, Laska and colleagues (1999) showed that discrimination of odour pairs differing by one carbon atom was significantly more difficult than discrimination of odour pairs that differed by more carbon atoms. The relationship between odour structure and behaviour can be compared with the relation between odour structure and glomerular activity patterns. Sachse et al. (1999) used the same series of aliphatic compounds as Laska et al. (1999) and found that pattern similarities were correlated with similarities in carbon chain length. In rats it was shown that only those pairs of enantiomers that could be spontaneously discriminated by the rat elicited distinguishable patterns of glomerular activity as measured by [^{14}C]2-deoxyglucose uptake (Linster et al., 2001).

Our results suggest an orderly organisation with respect to input selectivity not only in the male-specific MGC but also among sexually isomorphic glomeruli. This means that from randomness on the antennae, the ORN axons regroup and target specific glomeruli in a stereotyped manner. Furthermore, ORNs with similar tuning seem to be represented in neighbouring glomeruli. Assuming a one receptor-to-one glomerulus relationship (Gao et al., 2000; Vosshall et al., 2000) our results propose that a large number of different receptors interact with an odour molecule. Moreover, the degree of interaction is concentration-dependent and it is likely that stimulation at a detection threshold level may result in activation of a single type of ORN and, hence, glomerulus. The role of glomeruli as discrete functional units is conserved at the output level. This fact does not exclude processing of the incoming information as the LNs likely contribute with both intra- and inter-glomerular computations. Further experiments should include careful comparisons of input and output patterns within the same animals.

In order to recognise and discriminate between the vast arrays of potential odours the brain needs to know which combination of receptors is activated. The readout from the AL cannot possibly rely on responses in single PNs. These responses are rather unspecific also when considering the slow temporal patterns. Instead, an across-neuron pattern must be considered. From an observers point of view it is tempting to assign an olfactory code to odour-specific patterns, spatial or temporal. However, I have been reluctant to claim that an odour-evoked representation among glomeruli is a code *per se* that brain regions downstream of

the AL can decode. There has been much controversy regarding the involvement of spatial and temporal features in an olfactory code (Laurent, 1999). I do not think these principles are mutually exclusive but may rather coexist as a spatio-temporal code or as context-dependent strategies.

The studies in my thesis have focussed on several important issues concerning the functional organisation of the moth AL. However, the results raise several new questions to be addressed. Important issues are, e.g. the functional significance of local interneurons, which seem to have a key role in odour information processing. Also, the functional role of a number of neuroactive substances is still elusive. Furthermore, to reveal what neural features contribute to an olfactory code the perception and behavioural outcome must be considered. Solutions to many questions may come with integration of different molecular, pharmacological, electrophysiological, optophysiological and behavioural approaches and with development of new techniques with higher spatial and temporal resolution.

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