Introduction

In this thesis I investigated the processing of odor information in the mushroom body (MB) of the honeybee. The MB is a higher order structure of the insect brain, which is important for multisensory integration and learning. I used the Ca^{2+} imaging technique in order to analyze neural activity in the input area of the MB.

Chapter I addresses the issue of neural coding. It reveals transformations that accompany the transmission of odor information from the first odor processing brain area, the antennal lobe (AL) to the MB.

Chapter II deals with the issue of neural plasticity. It investigates the behavior of MB intrinsic Kenyon cells (KC) during and after associative learning. The results inspired a model which suggests a solution to the computational problem of odor learning, exploiting the advantages derived from the coding properties of the KCs.

Chapter III evaluates the application of 2-photon laser scanning microscopy to the study of structure and signaling in the honeybee brain.

The olfactory system as a model for the study of neural coding and neural plasticity

Animals need to create a coherent image of their environment and learn its causal structure in order to generate adequate behavior. These requirements lead to the evolution of nervous systems capable of extracting and representing relevant information about virtually unlimited combinations of physical and chemical stimuli (neural coding). Moreover, neuronal connectivity and excitability can change through experience (neural plasticity). Understanding how neural coding and neural plasticity are concerted is a fundamental goal in neuroscience.

Olfactory systems are particularly well suited for analyzing how neural coding and plasticity act together in order to generate adaptive behavior. On the one hand, the early processing of olfactory information in the insect AL and the vertebrate olfactory bulb (OB) is well understood. On the other hand, memory areas, as the insect MB and the mammal olfactory cortex (OC), are just one synapse away from the AL/OB, simplifying the task of defining the input they receive.

Furthermore, previous studies suggest that the principles of olfaction follow similar rules in diverse phyla, both among invertebrates and vertebrates (reviewed in Hildebrand and Shepherd, 1997; Strausfeld and Hildebrand, 1999). Taking into account the separate evolution of insects and mammals, for example, this finding suggests that the common architectural and functional organization found in both groups provides an optimal solution for common demands presented by the olfactory task.

The role of olfaction and odor mediated behaviors

Most animals rely on the sense of smell to detect and analyze chemical cues in the environment. Olfaction and olfactory memory play an important role in feeding, exploration and navigation. It is especially the strong dependence of odor related behaviors on memory processes that has called researchers' attention in the last decade (reviewed in Wilson and Stevenson, 2003; Davis, 2004).

Vertebrates and insects are able to detect thousands of odors, but with some exceptions (for example pheromones) odors have no inherent meaning *per se*. However, meaningful and non-meaningful odors need to be discriminated and recognized. These tasks are achieved by several forms of non-associative and associative experience dependent plasticity. In humans, for example, the discrimination of similar odors was enhanced by previous experience with the same odors (non-associative perceptual learning) (Jehl et al., 1995).

Associative learning plays a dominant role in feeding behavior and, since food sources are variable, the association between olfactory stimuli and their significance must be highly plastic. Associative odor learning binds odor information with a meaningful appetitive or aversive stimulus (classical conditioning) or with a particular behavior (operant conditioning).

Honeybees, for example, use associative learning during their foraging flights for the identification of flowers that are reliable nectar sources (von Frisch, 1919; von Frisch, 1965). This learning ability can be experimentally approached in classical conditioning paradigms where honeybees learn to associate a neutral odor (conditioned stimulus, CS) with a sucrose reward (unconditioned stimulus, US) (Takeda, 1961; Bitterman et al., 1983; Menzel, 1990). Associative learning in honeybees follows the rules of classical conditioning (Rescorla, 1988) as it only take

place if CS and the US coincide within a critical time interval and if the CS precedes the US (contiguity).

Another form of olfactory memory is formed during a sensitive period defined by a particular developmental or physiological state (imprinting). Olfactory imprinting enables salmons, for example, to return to their natal stream after spending a whole life in the sea (Dittman and Quinn, 1996).

In contrast, communication via chemical signals, another role of olfaction, is based on the fix meaning of some odors for an entire species, and leads to stereotyped behaviors, which are subject to experience dependent plasticity to a lesser degree. Chemical signals, called pheromones, are used to carry social and sexual information (Brennan and Keverne, 2004). The male sphinx moth *Manduca sexta* exhibits a stereotyped mate-seeking behavior after detecting the female sex pheromone (Willis and Arbas, 1991). In this case, as in many others, the effect of the pheromone is achieved through a separate compartment of the olfactory system, containing highly specific sensory neurons and hard-wired circuits (labeled lines) (Hansson et al., 2003).

Characteristics of olfactory stimuli

In order to understand the computational problem of odor processing and how the olfactory system solves it, it is helpful to understand the nature of olfactory stimuli. According to estimations, humans can detect more than 400,000 odorant molecules (Mori and Yoshihara, 1995). Since most odors in the environment are complex blends of molecules, there is an astronomical number of possible smells, leading to a highly multidimensional olfactory stimulus space. This high dimensionality differentiates olfactory stimuli from, for example, visual stimuli. The color of a monochromatic light beam can be uniquely described by a single number, namely the frequency of the light. To sense color, the visual system of humans employs only three types of photoreceptors (cones), which differ in their sensitivity to wavelength. Thus, each color perceived can be defined by three parameters, specifically the activity of the three types of cones. Olfactory stimuli, in contrast, can not be described by a single number. Furthermore, it is not clear which properties of the odor molecules are important in the binding to the corresponding receptor/s. The odor space at the receptor level is, therefore, non-linear and highly complex.

Architecture of olfactory systems

In the following section I will introduce the basic architecture of the insect and vertebrate olfactory systems. A more detailed description of the olfactory system of insects and in particular of the honeybee is given in Chapters I and II.

Odor molecules are sensed by olfactory receptor neurons (ORN) located in the insect antenna and the vertebrate nose. Individual ORNs presumably express only one, or very few, out of a large family of odor receptor genes (~60 in drosophila, ~1300 in mice) (Buck and Axel, 1991; Zhang and Firestein, 2002; Vosshall et al., 2000). Odor receptor proteins are seven-transmembrane G-protein coupled receptors, and have been shown to activate either the cAMP or IP3 second messenger cascades (Restrepo et al., 1996). ORNs expressing the same receptor converge onto one or a few glomeruli within the OB/AL. The arrangement of glomeruli receiving input from the same subpopulation of ORNs is stereotypic across individuals of a given species (Mombaerts et al., 1996; Vosshall et al., 2000).

Glomeruli constitute anatomically discrete subunits (Shipley and Ennis, 1996; Stocker, 1994; Mori and Yoshihara, 1995; Hansson and Anton, 2000). Within each glomerulus, ORNs make synapses with local neurons (insects: LN; vertebrates: periglomerular cells), which are predominantly GABAergic and interconnect glomeruli, and with output neurons (insects: projection neurons, PNs; vertebrates mitral/tufted cells, MTC). Local neurons are reciprocally connected with the PNs/MTCs (Malun, 1991; Mori et al., 1999). A second type of vertebrate local neurons, the granule cells, are reciprocally connected with the MTCs outside the glomeruli (Isaacson and Strowbridge, 1998). PN/MTC can receive input in one or a few glomeruli (Anton and Homberg, 1999; Mori et al., 1999).

PNs/MTCs are divided into distinct subgroups, innervating different higher order brain areas. In insects, most PNs innervate the MB and the lateral horn (LH), while a smaller group projects axons to the lateral horn and protocerebrum bypassing the MB (Mobbs, 1982; Zars et al., 2000; Abel et al., 2001; Marin et al., 2002; Müller et al., 2002; Wong et al., 2002; Tanaka et al., 2004). In the MB neuropil, PNs make synaptic connections with KCs. A particular KC population, the clawed KCs (cKC) has been implicated in short term learning (Zars et al., 2000) and is the focus of this thesis.

In vertebrates output from the OB is transmitted to the OC which is composed of several areas, including the piriform cortex, the olfactory tubercle, the anterior olfactory nucleus, and parts of the amygdala and entorhinal cortex. Mitral cells target the entire OC, while tufted cells innervate only the anterior olfactory nucleus and olfactory tubercle (Shipley and Ennis, 1996; Zou et al., 2001).

The glomerular map of the AL/OB is not recapitulated in the MB-LH/OC. While inputs from different ORNs are spatially segregated in different glomeruli in the AL/OB they appear to partially overlap in the MB-LH/OC, where information from distinct glomeruli is distributed onto many cells. Both in the MB-LH and the OC the projection patterns of PNs and MTCs appeared stereotyped across individuals of a given species (Zou et al., 2001; Tanaka et al., 2004).

Olfactory processing seems to involve a series of transformations of the sensory inputs that require convergence, divergence and parallel transmission of information. A characteristic feature of insect and vertebrate olfactory systems is a massive convergence from ORNs to the AL/OB and a massive divergence from there to the MB and OC. In the honeybee, for example, ~60,000 ORNs converge onto ~800 PN, which themselves diverge onto roughly 100,000 olfactory KCs (Witthöft, 1967; Esslen and Kaissling, 1976; Rybak, 1994).

Once again, the architectural coincidences may point out to the functional relevance of these common features: the convergence of many ORNs onto fewer PNs/MCs has been implicated in the task of noise reduction. The divergence of PNs onto KC could be the basis of enhanced specificity and sparseness[1] of odor representations (Laurent, 2002), as discussed in Chapter I.

The insect MB and the vertebrate piriform cortex are particularly interesting in the context of odor learning (see below). Both receive neuromodulatory inputs at different sites (Bicker, 1999; Linster and Hasselmo, 2001). In bees, for example, octopamine released by the VUMmx1 neuron has been shown to represent the rewarding function in appetitive associative learning (Hammer, 1993; Hammer and Menzel, 1998), while in vertebrates, cholinergic inputs have been implied in the local modulation of

[1] The term „sparseness“ describes the proportion of active units at any time (population sparseness) and/or the mean tuning width of each neuron (lifetime sparseness). A sparse code is characterized by few neurons active at any time and/or a narrow tuning width (Willmore and Tolhurst, 2001; Olshausen and Field, 2004).

dendrodendritic synaptic microcircuits between MTC and PCs in the piriform cortex, in the context of olfactory memory formation (Patil et al., 1998).

Odor coding

How does the olfactory system generate distinguishable neural representations for such a large variety of odors in order to subserve behavior?

The initial event in olfactory perception is the detection of odorants by ORNs. Odor receptor proteins confer ORNs with different sensitivities to different odorants. Because each ORN responds to multiple odorants, odor information is encoded in odor specific combinatorial patterns of activity across glomeruli (Joerges et al., 1997; Friedrich and Korsching, 1997; Rubin and Katz, 1999; Kauer and White, 2001; Wang et al., 2003).

Within the AL/OB, odor representations are transferred from ORN to PN/MT cells. The role of the AL/OB in the processing and representation of odor information is currently being debated, with different experimental approaches leading to disparate results. It is obvious that the identity of active neurons in the combinatorial pattern is essential for odor coding. However, no agreement has been achieved on the nature of the transformations taking place in the AL. Data from honeybees and rabbits suggest that the AL/OB sharpens the odor-evoked input patterns of the ORN into a narrower response profile of the output neurons (Yokoi et al., 1995; Sachse and Galizia, 2002). In contrast, Wilson and colleagues (Wilson et al., 2004) have described a broadening of the pattern in the output neurons of Drosophila with respect to the ORN input pattern. These results, however, are contradicted by findings from other groups which found no evidence of processing within the AL at all (Ng et al., 2002; Wang et al., 2003).

In addition, many studies point to the relevance of temporal activity patterns in different time scales for the coding of odor information. Slow dynamics of PN/MTC activity, which evolve over some hundreds of milliseconds, lead to more specific representation of the odor identity Galizia et al., 2000; Galán et al., 2004; locust: Stopfer et al., 2003; zebrafish olfactory bulb: Friedrich and Laurent, 2001). Fast oscillatory synchronization of PN/MTCs was found in many insect and vertebrates species reviewed in (Laurent, 2002). In zebrafish, for example, synchronized mitral cells encode information about the odor category (Friedrich et al., 2004).

Much less is known about how odor information is encoded within the MB/OC. Each PN/MTC makes synaptic connections with several KCs/PCs and each KC/PC receives convergent input from several PN/MTC. Electrophysiological recordings in locusts and imaging experiments in *Drosophila* indicate that the principles of odor coding differ remarkably in the AL and the MB (Perez-Orive et al., 2002; Stopfer et al., 2003; Wang et al., 2004). Unlike PNs, KCs respond to odors in a sparse way: KCs appear to be much more odor specific than PNs and generate fewer action potentials in response to a given odor. In contrast to the KCs of insects, PCs in the piriform cortex of vertebrates exhibit overlapping responses to many different odors, and little is known about their integration properties of these neurons (Litaudon et al., 2003).

Experience dependent plasticity of the olfactory system

It is commonly assumed that memories are stored as changes in the pattern and/or strength of synaptic connections (Milner et al., 1998). Thus, identifying sites of plasticity is a useful approach to characterize neural networks that underlie learning. Sites of plasticity can be recognized by comparing neural activity before and after learning. This approach has been applied to the olfactory system in insects and mammals in several studies (reviewed in Davis, 2004). The learning paradigms used include non-associative learning (perceptual learning and habituation) and associative learning (classical and operant conditioning).

In insects, learning induced changes in neural activity have been studied mainly in the AL. A common finding is an increase in responsiveness to the learnt odors after classical conditioning. Ca2+ imaging experiments in the bee AL revealed increased responses to the rewarded odor after learning (Faber et al., 1999). Similarly, extracellular recordings from moths showed a net recruitment of neurons activated by the rewarded odor and a net loss of neurons activated by the unrewarded odor (Daly et al., 2004). In the same direction, optical imaging experiments in Drosophila, using a marker for neurotransmitter release, demonstrated that olfactory learning leads to a short-term increase in the number of PN that are synaptically active after stimulation with the rewarded odor (Yu et al., 2004).

In contrast to the abundant evidence of neural plasticity in the AL, very little is known about learning induced changes in the MB. Faber and Menzel (Faber and Menzel, 2001) have shown with optical imaging that Ca^{2+} responses in the MB lip increased

for the rewarded odor after learning. However the cells involved were not identified. It is therefore not clear whether and how the KCs are involved in the learning process.

At the output of the honeybee MB, two different types of MB extrinsic neurons (PE1, Mauelshagen, 1993; and PCT, Grünewald, 1999) have been shown to undergo associative plasticity. However, it remains to be shown whether these neurons are reflecting changes that take place in the KCs or whether they are the direct substrate of plasticity.

Also in mammals, recordings in the piriform cortex revealed increased population responses to the reinforced stimulus after operant conditioning (Litaudon et al., 1997; Mouly et al., 2001).

The goal of the thesis

Reviewing the current knowledge in olfactory coding and learning, it becomes clear that a lot of information has been collected in the first stage of the olfactory pathway (AL/OB), while very little is known about odor processing in higher brain areas (MB/OC). Defining the transformations that accompany the routing of odor information from the antennal lobes onto the MB and the plasticity underlying olfactory learning is an important next step in expanding the knowledge about olfactory processing. In particular, I was interested in the following questions:

How is olfactory information organized in the MB?

In order to understand the transformations taking place along the olfactory pathway, I compared the population responses and temporal activity patterns in three consecutive neural compartments that represent the input of the MB. First, I recorded odor responses in the dendrites of the antennal lobe PN, next I measured their presynaptic terminals in the MB and finally I characterized their postsynaptic partners, the cKC. The results of these experiments are presented in Chapter I.

Are KCs involved in odor learning?

In order to investigate how MB network activity contributes to odor learning, I trained bees in a differential classical conditioning paradigm and simultaneously recorded selectively stained cKCs in the input region of the MB. The results of these experiments are presented in Chapter II.

The results from Chapters I and II are integrated in a functional model of the MB. This model shows how the sparseness of the cKC code could be exploited by the learning mechanism. It also opens many new questions that can be experimentally addressed in order to further understand odor processing in the insect brain (see Discussion in Chapter I).

Finally, I evaluated the 2PLSM approach in the study of odor evoked activity in the MB in order to overcome the limitations imposed by the reduced spatial and temporal resolution of conventional fluorescence imaging. The results of these experiments are presented in Chapter III.

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