Table of contents

Introduction 1

I Three-Dimensional Average-Shape Atlas of the Honeybee Brain and Its Applications 13

II Functional and anatomical features of *Apis mellifera* olfactory interneurons 52

III Age-, task- and experience-dependent structural plasticity in *Apis mellifera* olfactory microglomerular complexes 85

General Conclusions 107

Summary 113

Zusammenfassung 116

Danksagung 119

Introduction

Insect olfaction has long been studied on the behavioral and cellular levels, however, the way the insect brain encodes different odors is still a subject of considerable debate. In this thesis I investigated functional and anatomical features in the olfactory system of the European honeybee, *Apis mellifera*, focussing on olfactory projection neurons and their structural changes associated with the honeybee's elaborate behavior. The honeybee has a discrete system of age-related division of labor proceeding from hive and guard duties to foraging activities. Since foraging honeybees have to discriminate natural stimuli represented as complex blends consisting of a large number of volatile compounds, their evolved olfactory system is an outstanding model for studying odor encoding. A major challenge understanding how olfactory information is processed from the first-order relay station (the antennal lobe) onto the second-order relay station (the mushroom body). This task is achieved by the projection neurons which I'm focussing on in this thesis. Since former studies revealed their sophisticated morphology and their single and compound odor coding strategies, I addressed the issue of how they encode multicompound odor mixtures in relation to their anatomical features. Given that the mushroom bodies play a dominant role in olfactory learning (Heisenberg, 1998; Menzel, 1999), I asked whether the organization of projection neuron output within the mushroom body alters in relation to olfactory experience.

In my thesis I applied a combination of the most recent staining and recording techniques to experimentally approach these problems. The questions I address in the three chapters of my thesis arise against the background of previous re-

search on both vertebrates and invertebrates, aiming to understand how brains process and memorize smells.

The olfactory system as a model for studying anatomical and functional principles of sensory coding

Remarkable chemodetecting systems have been developed over eons of evolutionary time, enabling organisms to interact with an increasingly larger number of odors. Mammals – including humans – can sense more than thousand different odors. Their high dimensionality, based on their different chemical structures, e.g. being aliphatic or aromatic molecules with varied carbon backbones and diverse functional groups, differentiates olfactory stimuli from visual or auditory stimuli which are varied along a single dimension: the light or sound frequencies. Thus, more complex principles seem to be involved in neuronal coding of olfactory information at both the peripheral and central parts of the brain.

Analogous to the vertebrate nose, the insect's antennae have specialized epithelia lined with thousands of ciliated olfactory sensory neurons (OSNs) expressing the olfactory receptors as the molecular units for detecting odorant molecules. In both vertebrates and insects, OSNs send their excitatory neural signals inward to a first relay station in the brain with spherical, glomerular structures, the olfactory bulb or the antennal lobe. Here, cholinergic axons from the antennae (Kreissl and Bicker, 1989) or glutamatergic axons (Aroniadou-Anderjaska et al., 1997) from the vertebrate nasal epithelium project to specific glomeruli. OSNs expressing a common olfactory receptor gene converge onto one or a few common glomeruli (Mombaerts et al., 1996; Vosshall et al., 2000). The number of glomeruli ranges roughly from 50 - 160 in Drosophila and the honeybee (Laissue et al., 1999; Flanagan and Mercer, 1989; Galizia et al., 1999) to about 2000 - 3000 in mice and rats (reviewed by Shipley and

Ennis, 1996). Within each glomerulus, large numbers of OSNs converge upon several types of cells, including the principal neurons that convey information to higher-order brain centers (Mori and Yoshihara, 1995; Shipley and Ennis, 1996; Hansson and Anton, 2000; Abel et al., 2001). In vertebrates, these principal neurons are glutamatergic mitral and tufted cells (Aroniadou-Anderjaska et al., 1999); in insects, they are most frequently cholinergic projection neurons (PNs) (Bicker, 1999). In insects, PNs can be predominantly uniglomerular, as in Drosophila (Jefferis et al., 2001) and the honeybee (Bicker et al., 1993) or multiglomerular, as in locusts (Anton et al., 2002). Both the antennal lobe and olfactory bulb are densely woven with GABAergic inhibitory interneurons that form reciprocal dendrodendritic synapses with the principal neurons. Many insects have both widely-branching and spatially-restricted LNs (Flanagan and Mercer, 1989; Leitch and Laurent, 1996). Taking the honeybee as an example, there are two morphological types of local interneurons which – in contrast to the locust – show spiking activity (Flanagan and Mercer, 1989; Sun et al., 1993; MacLeod and Laurent, 1996). The mammalian olfactory bulb has GABAergic interneurons that branch mainly within the glomerular layer (juxtaglomerular cells) and between mitral cells (granule cells) (Kosaka et al., 1987).

In vertebrates olfactory information is conveyed from the olfactory bulb onto the olfactory cortex, including the piriform cortex, the olfactory tubercle, the anterior olfactory nucleus, and parts of the amygdala and entorhinal cortex (Shipley and Ennis, 1996). While mitral cells innervate the entire olfactory cortex, tufted cells project only to the anterior olfactory nucleus and olfactory tubercle (Shipley and Ennis, 1996; Zou et al., 2001). Insect uniglomerular PNs process olfactory information onto the second-order relay station, the mushroom bodies (MBs), known to be involved in sensory integration and learning (Heisenberg, 1998; Menzel, 1999). The calyces of the MBs receive input from multiple sensory pathways projecting onto spatially distinct subregions. In the honeybee olfaction is known to be confined to the MB lip region (Mobbs, 1982). Fun-

damental features of insect and vertebrate olfactory systems are the converging connections from ORNs to the antennal lobe or the olfactory bulb and the diverging connections onto the MBs or the sensory cortex, respectively. In the honeybee, for example, 60,000 OSNs innervate the AL via four tracts (T1-T4) (Arnold et al., 1985; (Flanagan and Mercer, 1989); Galizia et al., 1999), converging onto about 500 - 1000 PNs which themselves project by means of three tracts: the lateral, the median, and mediolateral antennocerebral tracts (l-, m-, and ml- ACT, respectively) to the MBs and to the lateral horn (Mobbs, 1982; Abel et al., 2001; Mueller et al., 2002; Kirschner et al., 2006). lACT PNs exhibit their somata in the anteroventral, dorsoventral and lateral part of the AL (Schaefer et al., 1988; Abel et al., 2001) and receive input from the T1 glomeruli (Bicker et al., 1993). PNs of the mACT innervate the glomeruli of the T2, T3 or T4 group and their somata are located in the ventro-lateral and dorsal soma clusters of the AL (Bicker et al., 1993). Both types of PNs (l- and mACT) terminate within the calyx lip region, forming a network with around 170,000 postsynaptic Kenyon cell (KC) spines (Witthoeft, 1967; Esslen and Kaissling, 1976; Rybak and Mauelshagen, 1994) as well as reciprocal and feed-forward microcircuits with GABAergic neurons (Ganeshina and Menzel, 2001).

Regarding olfactory encoding, the consequence of glomerular interconnectivity is that olfactory information gets distributed across ensembles of principal neurons. To investigate the spatial component of olfactory encoding, optical imaging methods have been developed that assess afferent or principal neuron responses within the antennal lobe and olfactory bulb. Their results show that odor stimulation evokes spatio-temporal glomerular activity patterns that vary with odor identity (Cinelli and Kauer, 1995; Friedrich and Korsching, 1997; Joerges et al., 1997; Friedrich and Korsching, 1998; Galizia et al., 1999; Sachse et al., 1999; Wachowiak and Cohen, 2001; Meister and Bonhoeffer, 2001; Wachowiak et al., 2002). Electrophysiological recordings confirm the imaging results, and, with higher temporal resolution, characterize an additional response

dimension: temporal patterning in the responses of principal neurons in vertebrates (Wellis et al., 1989; Friedrich and Laurent, 2001) and invertebrates (Laurent et al., 1996; Mueller et al., 2002; Wilson et al., 2004; Mazor and Laurent, 2005). Odor-elicited response patterns can change when a different odor, or a different concentration of an odor, is presented and different principal neurons, simultaneously activated by an odor presentation, can respond with different temporal patterns (Friedrich and Laurent, 2001; Stopfer et al., 2003).

Despite the overwhelming knowledge about structural and functional properties of identified neurons and neuropils, it is still unresolved how olfactory processing mediates odor-guided behavior and learning of odors. One set of problems arises from the complex nature of natural odors which occur as mixtures of many single compounds and are distributed in the environment in variable spatio-temporal patterns and concentration gradients. Recent studies suggest that neural representations of mixtures are not simple combinations of the representations of their components, and that mixture interactions can result from olfactory computation within the antennal lobe and the olfactory bulb (Joerges et al., 1997; Giraudet et al., 2002; Tabor et al., 2004; Deisig et al., 2006). Thus there are rules of mixture interactions that may explain olfactory behavior and provide a basis for understanding the processing of natural odor stimuli in the antennal lobe and olfactory bulb.

Knowing how neurons are interconnected and how these connections may change as the animal ages and gains experience, it is important to reveal their 3-D morphology within the entire network. Current developments in neuroanatomical methods enable us to characterize the connectivity of neural nets within a common framework.

Development of 3-D neuroanatomical techniques and their significance for olfactory coding research

Santiago Ramón y Cajal (1853-1934) enriched the understanding of neuronal shapes and connections by revealing the orderly arrangement of neurons within the central nervous system. By using a histological staining technique developed by his contemporary Camillo Golgi (1843-1926) he stated an important generalization that became the basis of the neuron doctrine. Since then it has been known that shape and position of a neuron, as well as the origin and destination of its signals in the neural network, supply valuable cues about its function. Thus a realistic spatial representation of anatomical structures requires 3-D imaging and reconstruction techniques mapping the complex organization of the brain's microcircuitry. Confocal or two-photon laser scanning microscopy offer these possibilities by providing 3-D image stacks of fluorescently-stained structures at submicron resolution. The application of fluorophores with distinct spectral excitation and emission wavelengths makes it possible to discriminate multiple selectively-stained structures within the same specimen.

Recent advances in 3-D image analysis algorithms allow partitioning a digital image into multiple regions (segmentation), according to a given criterion. The goal of segmentation is to locate objects of interest within a grey-value image and assign them a label appropriate to the anatomical terminology.
Each volume pixel (voxel) no longer encodes the staining intensity but represents a certain label coding for a particular brain structure.

Several software tools featuring automated digitalization, 3-D reconstruction and geometric analysis of detailed cellular and tissular structures have been developed: Amira (Mercury Computer Systems, Inc, San Diego, CA); ImageJ (NIMH, USA), Neurolucida (Micro-BrightField, Williston, VT, USA), FilamentTracer (Imaris, Bitplane, Zürich; Rodriguez et al., 2003). For 3-D reconstruction of single neurons these tools provide threshold-based tracing methods

which suffer from different limitations dependent on the staining intensity and noise level. 3-D confocal image stacks comprise stained and unstained tissue which differs in strength dependent on the point spread function (PSF). Since cells can be inhomogeneously stained and fine structures exhibit a low contrast to the background, threshold-independent methods counteract these phenomena. Thus a threshold-independent framework assisting the 3-D reconstruction process and comprising a successive gain of metric parameters has been developed (Schmitt et al., 2004; Evers et al., 2005). This technique produces a structural description of individual neurons, including the topology and the exact dendritic lengths and diameters. Since selective staining of brain structures has to be performed on separate preparations leading to multiple 3-D reconstructions with interindividual differences, a whole network can only be composed by a common framework, an anatomical brain atlas. High-resolution anatomical atlases were created for a number of different animal species (for review see Dhenain et al., 2001; Toga et al., 2006; Bai et al., 2006). Since insect brains are smaller and easier accessible the creation of population-based anatomical atlases is feasible and available for the fruit fly *Drosphila melanogaster* (Rein et al., 2002; see also `http://www.neurofly.de`) and the honeybee *Apis mellifera* (Brandt et al., 2005; see also `http://www.neurobiologie.fu-berlin.de/beebrain`). Computing the average of a population of structures requires accurate mapping of corresponding voxels in all images onto each other. This method – referred to as registration – is the process of transforming different sets of data into one coordinate system. Registration via image similarity-based methods consists of a transformation model, which is applied to the reference image to locate corresponding coordinates within the target image. An image similarity metric quantifies the degree of correspondence between features in both images achieved by a given transformation. The most commonly used similarity measure for registration of multimodality images is the Mutual Information and its invariant Normalized Mutual Informa-

tion. To achieve anatomical correspondence the registration procedure accounts for global as well as local size and shape differences by applying different transformations, referred to as degrees of freedom (DOF). Rigid Transformation includes global translation and rotation, thereby preserving distances, planarity of surfaces and non-zero angles. Affine Transformation includes global scaling and shearing in addition to rigid transformation, and it preserves planarity of surfaces and parallelism but not the angle. Nonrigid Transformation includes local transforms in addition to the global affine transforms, but it does not preserve distance, planarity, parallelism or the angle (Ashburner and Friston, 1999; Ashburner, 2000; Guimond et al., 2000). For the 'Atlas of the Honeybee Brain' an average shape image was calculated through an iteration of one affine registration, followed by multiple nonrigid registrations (Rohlfing et al., 2001; Rohlfing et al., 2004). Including sophisticated single-cell staining, 3-D reconstruction (Schmitt et al., 2004; Evers et al., 2005) and registration techniques (Rohlfing et al., 2001; Rohlfing et al., 2004) a standardized method for the creation of surface-based averaged brain atlases including their elemental components has been developed (Brandt et al., 2005). This was achieved by using the software Amira (Mercury Computer Systems, Inc, San Diego, CA) and custom modules kindly provided by Jan-Felix Evers (University Cambridge) and the Zuse Institute Berlin. The honeybee olfactory system exemplifies its applications.

The role of odor-guided behavior and brain plasticity

The olfactory sensory system is the most intriguing of the sensory systems mediating olfactory behaviors, which are crucial for reproduction and feeding. Animals analyze the chemical world, extract meaningful information from the environment and form olfactory memories through learning processes which – in turn – can guide olfactory behaviors (Wilson and Stevenson, 2003). This

is achieved by either ontogenetic olfactory learning processes, like imprinting, or olfactory experience as a consequence of behavioral activity and associative learning. Ontogenetic olfactory learning takes place in an important functional context which needs to be kept invariant and exclusively occurs during a "sensitive period" coinciding with a particular developmental stage or physiological state (Hudson, 1993). This so-called olfactory imprinting leads to olfactory-guided behaviors known to produce multiple, dissociable changes in the brain (Brennan and Keverne, 1997). In newborn mammals, a variety of sensitive periods for the acquisition of olfactory information associated with a reinforcer have been described.

During their first postnatal week rats, for example, can associate an odor with tactile stimulations simulating their mother's licking (Woo and Leon, 1987; Leon, 1992). This olfactory conditioning paradigm leads to structural changes occurring within the olfactory bulb. Both the number of juxtaglomerular cells surrounding the glomeruli, and the number of dendritic processes innervating them have shown to be increased (Woo and Leon, 1987; Woo and Leon, 1991). Since these structural changes in the neonatal olfactory bulb are limited to early developmental processes and do not occur after the second or third postnatal week (Woo and Leon, 1987) neonatal olfactory learning may be analogous to the developmentally regulated periods of plasticity (Brunjes and Frazier, 1986). Thus there are two ways that experience could alter the brain: either by modifying existing circuitry or by creating novel circuitry (Kolb and Whishaw, 1998).

One way to examine experience-dependent changes in the brain is to look at the effects of different experiences on neuronal structure by applying two approaches: animals are either placed in a differential environment (enriched or impoverished), or trained in specific types of tasks (Kolb and Whishaw, 1998). For example, dendrites of cortical neurons of rats reared in enriched environments are longer and more branched compared to those of rats reared alone or in typical group laboratory cages (Kolb and Whishaw, 1998).

Experience-related changes in brain structure have also been documented in insects, in particular in the honeybee. Since synaptogenesis is absent in the adult honeybee brain (Fahrbach et al., 1995; Ganeshina et al., 2006) it provides an excellent model system to study experience-dependent modifications of existing circuitry. Most evidence for experience-driven plasticity in the honeybee brain comes from studies on the mushroom bodies, showing that they undergo volume expansion in relation to foraging experience (Durst et al., 1994; Fahrbach et al., 1998; Farris et al., 2001; Fahrbach et al., 2003). It is believed that the growth of the mushroom body neuropil in adult bees occurs throughout adult life and continues after bees begin to forage, indicating effects of age- and experience-dependent plasticity (Farris et al., 2001; Ismail et al., 2005).

The honeybee allows behavioral manipulations by uncoupling age and experience (Winston, 1987). Focusing on the olfactory input, the mushroom body lip, its microcircuitry can be visualized reflecting presynaptic projection neuron boutons and postsynaptic Kenyon cell spines (Roessler et al., 2002; Yusuyama et al., 2002; Frambach et al., 2004). Thus new methods have to be developed which address the issue about how the mushroom body lip microcircuitry influences mushroom body growth and whether it is associated with age-related sensory stimulation.

The examination of structural plasticity involves measurements such as brain weight, dendritic extent, spine density, synapse formation, glial activity, and altered metabolic activity. The accomplishment of these measurements relies on two important factors. First, the visualization of morphology must provide similar results from animal to animal and study to study. Second, it is essential that only a subset of cells is stained. Traditionally Golgi-stains solved this problem, since only between 1 - 4% of cells can be randomly stained (Pasternak and Woolsey, 1987). With the aid of light microscopy and camera lucida drawings measurements can be performed and both arborization pattern and synaptic

densities can be estimated using various methods (Kolb and Whishaw, 1998).

Recent advances in selective staining and 3-D imaging techniques allow stereological studies to understand the structural inner 3-D arrangement based on the analysis of 2D slices. The common stereological methods used for volume estimation were demonstrated by the mathematician Bonaventura Cavalieri (1598-1647). Cavalieri's method determines the volume in serially sectioned structures by the product of the slice areas and the slice thickness (Gundersen et al., 1983). Thus, the volume of structures is estimated by extrapolation of the slice area accordant to slice thickness. Since this might lead to inaccurate volume measurements advanced 3-D neuroimaging analysis techniques have been developed. More often they comprise semi-automatically drawing the region of interest and calculating the enclosed volume, or more elaborate methods as, for example, Voxel-Based Morphometry (Ashburner and Friston, 1999). Hence the method dealing with structural plasticity on the level of the mushroom body lips'microcircuitry has to employ 3-D quantification and volume measurement techniques to unravel the role of olfaction in mediating behavior and leading to structural plasticity within the brain.

Thesis overview

In my studies I applied 3-D neuroanatomical reconstructions and sophisticated registration methods to investigate the morphology of projection neurons within a spatial reference map, the Atlas of the Honeybee Brain. **Chapter I** addresses the issue of composing components of the honeybee olfactory pathway within a common framework, the Atlas of the Honeybee Brain. It represents a standardized method for the creation of surface-based averaged brain atlases and their applications. Using state-of-art 3-D reconstruction techniques I recreated a model of the neuroanatomical structures with data from several individual brains and compiled them into the Honeybee Brain Atlas.

This spatial reference map provides realistic models of structural relationships within the network of the honeybee brain and is the second standard-atlas in invertebrates along with that of the fly *Drosophila*.

To correlate projection neuron (PN) anatomical features with their physiological properties I performed intracellular recordings from single projection neurons and classified their response profiles on the basis of a large set of odors. **Chapter II** is concerned with the question of how multicompound odor mixture are encoded by the PNs depending on their morphology and topical location within their input site, the antennal lobe, and their output site, the mushroom body. I demonstrate how olfactory PNs located in either the lACT or the mACT exhibit different odor mixture encoding strategies and how it is related to their segregated terminal projections within the mushroom bodies'lips.

To show how the bee olfactory brain restructures with age and behavioral experience, I applied a combination of behavioral manipulations, pre- and post-synaptic staining and 3-D-based quantitative structural and volume measurements to reveal plasticity processes at the output level of olfactory projection neurons, e.g. within the lips of the honeybee mushroom bodies. In **Chapter III** I show that quantitative changes in the mushroom bodies of adult bees take place reflecting re-wiring processes at the synaptic level correlated with both development over early age stages and specific, task-dependent olfactory experience. By double-staining and thus discriminating between presynaptic projection neuron terminals (boutons) and postsynaptic Kenyon cell spines I was able to show clear effects indicating degrading compensatory structural plasticity effects of age-related developmental processes, presumably affecting olfactory perception.