General Introduction

Mushroom bodies

When I started my PhD-Thesis in 2000, the mushroom bodies (MB) of the honeybee (*Apis mellifera*) were considered to play a major role in processing and storage of chemosensory information (Menzel et al. 1974, 1994; Erber et al. 1980; Heisenberg et al. 1985; de Belle and Heisenberg 1994; Hammer and Menzel 1995). This concept was based on the fact that, beneath visual and chemosensory information, the main input to the MBs comes from the antennal lobes, the first processing neuropil in the olfactory pathway. The participation of MBs in olfactory learning and memory was subsequently investigated in fruit flies (*Drosophila melanogaster*) and honeybees. Those studies used diverse experimental techniques and simultaneous tests of behavioral responses to trained olfactory stimuli. Using electrophysiological methods in bees, neurons were characterized, taking part in neuronal circuits of the MBs (Hammer 1993; Mauelshagen 1993). Recordings from two identified neurons (VUMmx1 and PE1, see figure 1) during olfactory conditioning confirmed their participation in non-associative and associative olfactory learning. Another approach was temporary blocking of MB function of honeybees by means of local cooling, which led to retrograde amnesia for the period of a few minutes following olfactory learning (Menzel et al. 1974; Erber et al. 1980). Moreover, MB structural mutants obtained by a genetic approach in *Drosophila* reveal impaired olfactory learning and memory (Heisenberg et al. 1985). Furthermore, in *Drosophila,* an ablation procedure has been established to selectively delete MBs (de Belle & Heisenberg 1994). Olfactory conditioning of these animals demonstrated that MBs intercede associative odor learning in fruit flies. All in all, these results indicate a central role of MBs in olfactory learning and memory.

In addition, the MBs are also characterized by their neuronal plasticity during development and even during early adult life. MBs of honeybees are also structurally highly plastic in adult life (Fahrbach et al. 1995). Another study found that the transition of nursing bees to foraging bees 7–10 days after emergence is accompanied by a drastic volume change of the calyx regions (Durst et al. 1994). This structural plasticity is indicative of synaptic reorganization at the input side of the MBs and might reflect the connectivity adaptations related to learning processes in foraging bees.

In this work, bees with mushroom body lesions were produced, in order to investigate the influence of a loss of MB Kenyon cells on olfactory learning within a behavioral learning paradigm. In this approach, the DNA synthesis inhibitor hydroxyurea was applied to first-

instar larvae in this time frame. Defects induced in the bee differ from those described for *Drosophila* in many respects. These differences are likely owed to the number of origin Kenyon cell neuroblasts, to their proliferation pattern, and to the overall structural organization of MBs in bees and flies. Honeybees are not able to reach the adult state without any MBs as *Drosophila* is. Most of the bees that show MB lesions after HU treatment lack one or both median calyces. In the first days of adult life, all bees with double side ablations die. In my experiments with HU treated bees (chapter III), I used bees at an age of 11 days. All ablated bees that are left at this time show single side ablations of one median calyx.

The olfactory pathway of the honeybee

The antennal lobe (AL) is the first-order neuropil in the olfactory pathway, which receives input of 60.000 chemosensory axons. 156 spherical glomeruli form the AL. Here one can find synapses between the input neurons but also local interneurons, which make their connections within but also between the glomeruli. Projection neurons take the information of the AL to the MBs the second-order olfactory neuropil. The MBs receive also visual and mechanosensory input. They consist of 170.000 densely packed and parallel neurons. These intrinsic neurons have widely overlapping dendritic branches in the median and the lateral calyces, but also in the input region of the MBs. The axons of the Kenyon cells form the pedunculus. The calyx is segregated into modality-specific regions; the upper part (lip region) is olfactory, the median part (collar) visual and the lower part is olfactory, visual and mechanosensory (basal ring) (Abel et al. 2001). The output regions of the MB are the α- and β-lobes. These are formed by two collaterals of the intrinsic MB neurons. Coming from the pedunculus and the α-lobe, an intrinsic feedback loop projects back to the calyces. Projection neurons coming from the AL are combined into two neural tracts, one leading medial directly to the calyces and another one leading firstly to the lateral protocerebrum (also called lateral horn) but afterwards also to the calyces. The lateral horn receives also input from the α-lobe. The role of the lateral horn as the third olfactory neuropil is more or less unknown. Descending neurons run from there to the motor centre in the sub-oesophageal ganglion (SOG). The output neuron of the SOG is the ventral unpaired median neuron of the maxillary neuromere 1 (VUMmx1). Stimulation of this neuron replaces the unconditioned stimulus (US, sucrose solution) within a olfactory conditioning. The organization of the honeybee MB constitutes an example of multiple parallel-neuron architecture. This neuropil is composed of multiple aligned neurons that could be flexibly organized into various functional ensembles.

There are two main connections between both brain sides inside the honeybee brain: One between the MBs, which is considered to include the main part of information transfer between sides and another one connecting the ALs (Mobbs 1982). The role of this latter association is unknown.

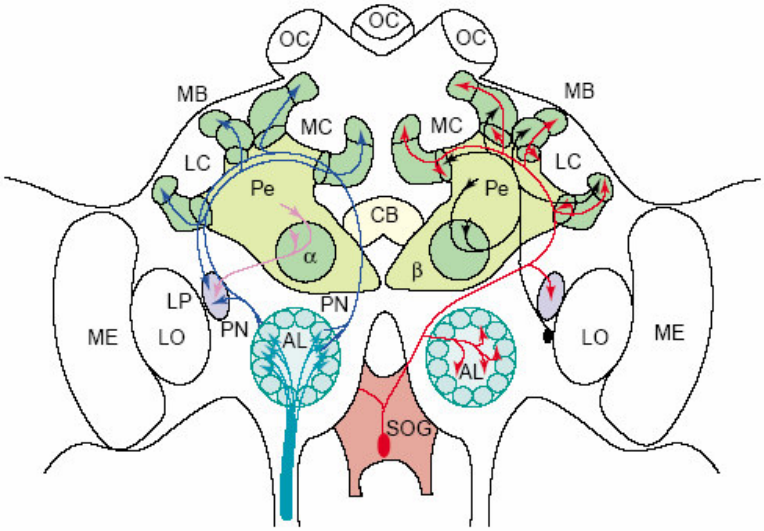

Figure 1: A schematic view of the major neuropils of the central brain area excluding the eyes, showing the olfactory pathway. AL, antennal lobe (light blue); MB, mushroom body (green); MC, median calyx; LC, lateral calyx, Pe, pedunculus; α, α-lobe; β, β-lobe; inhibitory feedback loop (black); PN (blue arrows); LP, lateral protocerebrum (lateral horn); which receives indirect input from the MB via extrinsic neurons (left side, violet arrows); SOG, sub-oesophageal ganglion; VUMmx1, red; CB, central body; LO, lobula; ME, medulla, two visual neuropils; OC, ocelli (three simple eyes). (Taken from Menzel & Giurfa 2001, modified).

Experimental paradigm

The behavioral paradigm that forms the basis of my studies is called the proboscis extension response (PER) (Takeda 1961, Kuwabara 1957, Bitterman et al. 1983). A bee extends its proboscis if one touches their antennae with sucrose solution. In a classical conditioning it is possible to associate the expectation of a reward (food) by presenting sucrose solution (unconditioned stimulus: US) with a former unimportant stimulus (conditioned stimulus: CS). In the case of my experiments only olfactory stimuli have been used. Since foraging honeybees have a highly developed olfactory sense, they are able to distinguish between many odors and relate them to one another. By using olfactory cues foraging bees learn to find flowers and to orientate in an olfactory space. Odors of flowers mostly consist of complex mixtures of odorants. Many single components are part of several flowery odors. Therefore honeybees have to be able to learn about predictive values of odor mixtures not only about their single elements. Honeybees can distinguish very well between different bloom smells. Flowers which ensure their pollination by mistake of pollinators often have no odor (Kunze & Gumbert 2001).

Complex olfactory learning may need MBs

Bees are able to solve non-linear learning paradigms. Only one example has been mentioned in literature at the beginning of my PhD studies: the biconditional discrimination (Hellstern et al. 1995, Chandra & Smith 1998). In this experiment honeybees have to

discriminate binary mixtures of four odor elements. All elements are reinforced (by sucrose solution) in one combination and non-reinforced in another one (AB+ BC- CD+ DA-). The learning problem is therefore only solvable, if the animals learn the information of the combination in addition to the information of every single element. As mentioned, this was the only example of a learning paradigm within the PER conditioning which went ahead mere elemental learning achievement as it is meant by the model of Rescorla and Wagner (1972). This theory assumes the associative strength of a compound as the sum of the associative strengths of the elements that are included in the compound. Thus the first step of my PhD studies was to find a paradigm which makes non-linear learning visible within the PER conditioning. The result of this investigation can be found in chapter I of the work presented here.

What explicitly is the role of the MBs with respect to non-linear learning? A method that was used to interfere with the development of the MB is the hydroxyurea (HU) method that retrains growth of neuroblasts that build up the MB during the larval development. As mentioned above, this method had been used in fruit flies (*Drosophila melanogaster*) (de Belle & Heisenberg 1994) and could be transferred to honeybees (Malun 1998). The most frequent ablation that can be found in the adult animal is the loss of one median calyx and therefore at the first sight a lesion which concerns only one hemisphere of the honeybee brain (See figure 2).

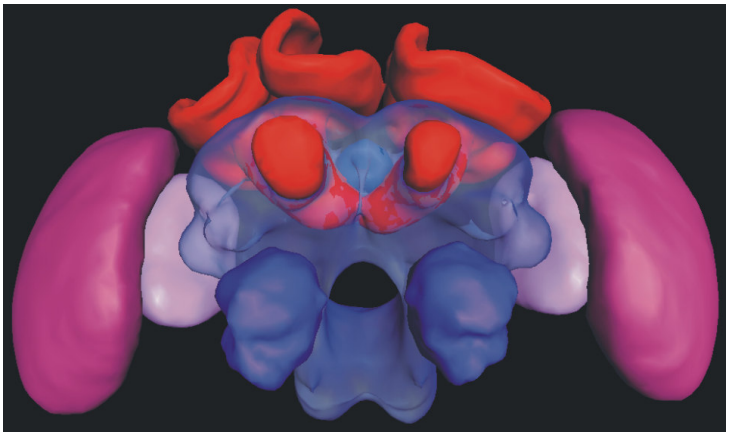

Figure 2: Computer generated 3D reconstruction of a real honeybee brain showing MB lesions caused by HU. In this example, one of the median calyces is lost. This represents the most frequent kind of HU induced ablations (Picture: R. Brandt).

Surprisingly it has been found out, that these kind of ablations seemed to have no influence on learning within the PER conditioning (Malun et al. 2002), meaning that the developed paradigm of chapter 1 was unsuitable for unilateral ablated bees produced by the use of HU. Therefore the next step of my PhD work was to search for a paradigm that shows non-linear learning in single side conditioning.

Single-side olfactory conditioning

For single side PER conditioning we used an experimental design developed by Sandoz and Menzel (2001). In this kind of treatment the antennae of the bees are separated by a small plastic foil, which is fixed with low temperature melting wax between the antennae. Afterwards both antennae can be stimulated separately. Meanwhile another kind of non-linear processing within the olfactory learning of the honeybee had been published (Deisig et al. 2001): negative patterning (NP). Fulfilling this kind of experiment bees have to learn that single elements A and B count more with respect to an expected reward than the combination of both elements (A+B+AB-). In contrast to this stands positive patterning (PP) which includes the same stimuli but can be solved in a mere elemental way, because the sum AB counts more than it parts A and B (A-B-AB+). Both forms of patterning have been used for a side specific PER conditioning in chapter II. This experiment showed that honeybees are not able to fulfill the mentioned non-linear learning problems by the use of only one antenna. Whilst PP could be solved both by using one or two antennae, NP could only be solved by the use of both antennae. Since most of the transfer of olfactory information between hemispheres is considered to take place within the MB (Mobbs 1982), this finding showed that the MB takes part in non-linear learning, through bilateral connections. Again this kind of experiment is not useful for HU treated bees, because the influence of the ablation on non-linear learning should be shown. But non-linear learning does not work in single side conditioning.

Speaking about neuronal connections between sides, it is necessary to mention also neuronal connections between the antennal lobes that may play a role in the transfer of olfactory information between brain sides (Mobbs 1982). These neurons seem to be primarily involved in the retrieval of learned information (Grünewald et al. *in prep.*).

Therefore I tried to use a side specific conditioning as Sandoz and Menzel (2001) have used it on untreated foraging honeybees. Bees are able to separate olfactory learning problems on both antennae from each other. At least 10% of bees solve A+B- on one side and at the same time C+D- on the other side. Three Hours later A and C release the reflex also on the side where they did not have been presented before. Therefore the olfactory memory of both

sides is transferred to the other side or at least accessible from both sides. Getting ambiguous learning problems on both antennae (A+B- on one side B+A- on the other) bees learn **not** to transfer memory information between sides or to add the information of the side to the information of the odor. Thus, honeybees build a side specific memory. This paradigm was used on HU treated bees (chapter III). For comparison an experimental group was used which did not get ambiguous olfactory information between sides (A+B- right / C+D- left). Another experimental group that received four odors bilaterally (A+B-C+D-) was used in order to show, that bilateral olfactory learning is not impaired by single side MB ablations. This is suggested by the work of Malun et al. (2002). The difference to my experiments is different olfactory conditionings given to both antennae within an individual animal.

This last experiment also included an experimental group confronted with the biconditional discrimination problem (AB+ BC- CD+ DA-). Confirming the findings of negative patterning, bees at the age of 11 days that outlasted their adult life within small cages in the hive, are not able to fulfill this complex learning task. This step was unfortunately necessary to separate HU contaminated bees from untreated bees. Therefore this experimental group has not been included in chapter III.

All in all, I tried to find an explanation on the role of the MB within PER conditioning using a non-invasive method. At this time, the HU-method was the only experimental access to cause lesions within the MBs of the honeybee brain in living animals without opening the head capsule before olfactory training.