Introduction

The goal of this thesis is to understand the mechanisms involved in learning and memory formation. These occur in parallel, during which the behavior of a subject changes stepwise to adapt to the currently relevant environmental situation. This adaptation is essential for the animal's survival and is known to start in mammals already in a prenatal state. Particularly, chemosensory information of artificial odorants that enter the womb are perceived by fetal rats, mice, rabbits, and lambs (for reviews, cf. Schaal and Orgeur, 1992; Smotherman and Robinson, 1987) to be later used as cue in postnatal odor directed behavior. In the rat, an in utero negatively reinforced odor continues to be avoided for periods lasting 10 to 16 days in postnatal life (Hepper, 1991; Smotherman, 1982; Stickrod et al., 1982).

The underlying processes of learning and memory formation are going along with the modification of neuronal excitability and synaptic strength between neurons (Milner et al. 1998). These modifications result in changes of the observable neuronal response at different levels of the involved network. In insects, there is much evidence for the mushroom bodies [MB] to be centers for "intelligent" actions (Dujardin, 1850; Strausfeld, 1998). This is supported by experiments using amnesic treatments. In honey bees, the probability for eliciting the conditioned response after a single learning trial is strongly reduced when the MBs have been treated with amnesic agents (Erber et al., 1980; Menzel et al,. 1974). In *Drosophila* the MB-branches (γ-lobe, α/β-lobe) are involved differentially in memory formation (Zars et al., 2000; Pascual and Preat, 2001). Imaging of Kenyon cell [KC] activity showed that a branch specific memory trace is formed within 3 to 9 hours after conditioning only in the alpha-branch of the MB (Yu et al., 2006).

In the this thesis I focused on the output region of the MB, which in the honey bee is represented by about 400 extrinsic neurons [ENs] that read out the activity patterns of the KCs (Rybak and Menzel, 1993). I performed extra cellular long term recordings of these neurons.

In Chapter one I address the question of the general representation of odor stimuli at the level of the ENs by repeated odor stimulation. I analyzed odor specificity by presenting ten different odors. Reliability for each odor was investigated by presenting the same odor 10 times.

In Chapter two the general response properties of the alpha lobe extrinsic neurons, characterized in chapter one, were modified by applying a differential conditioning experiment. I am able to show that previously non-responding ENs are recruited to respond to the odors used during the differential conditioning.

In Chapter three I studied the inter-hemispheric integration of olfactory information at the level of the ENs during the application of side-specific learning tasks. I am able to show that already during the acquisition, the information is processed in a network including both MBs. Contra-lateral differential conditioning leads to recruitment of previously non-responding ENs to the side specific odor stimulus.

The olfactory system as a model for learning related plasticity

The olfactory system in vertebrates, as well as in invertebrates, provides us with many advantages, when studying learning and memory formation by applying olfactory learning paradigms (Davis 2004; Wilson and Mainen, 2006). Both are able to detect thousands of different odors. Most of them have no congenital meaning (as in the case of pheromones) and the animals have to build associations to discriminate meaningful and non-meaningful odor cues. First of all, odors have to be discriminated and recognized. These tasks are achieved by several forms of non-associative and associative experience dependent plasticity. These tasks are influencing each other. In humans, experience with similar odors enhances the ability to discriminate these odors in a learning task (Jehl et al., 1995). Also, experience with word pairs increases the ability to learn these pairs after four weeks, over the ability to learn newly encountered word pairs, although the subjects claimed to have forgotten the initial word pairs (Nelson, 1978). Olfactory associative learning and memory formation has an important influence on feeding behavior. It has to be very plastic, because food sources can vary a

lot, for example due to seasonal differences. The associated odor information can be appetitive or aversive, depending on the stimulus. Whereas in a classical conditioning paradigm most of the time a reflex is linked to the associated odor, in an operant conditioning paradigm a particular self motivated behavior is related to the associated odor stimulus.

The principal organization of the olfactory systems in mammals and insects show many similarities. Both, olfactory epithelium (mammals) and the antenna (insects) are divided into a few large zones, consisting of different olfactory receptor neurons [ORNs]. Each ORN can contain different olfactory receptor [OR] types. In *Drosophila,* some ORNs express two or three ORs, but the same OR is never expressed in more than one ORN type (Vosshall et al., 1999; Hallem et al., 2004a; Couto et al., 2005; Fishilevich and Vosshall, 2005; Goldmann et al., 2005). The different ORN types intermingle widely within each zone (Ressler et al., 1993, de Bryne et al., 2001). This relatively disordered distribution of different ORNs becomes very well structured at the first relay station, the olfactory bulb [OB] (mammals) and the antennal lobe [AL] (insects) which consist of substructures called glomeruli. ORNs expressing the same OR converge onto one, or a few common glomeruli (Mombaerts et al., 1996; Vosshall et al., 2000). The number of glomeruli in different species ranges from 50 - 160 in *Drosophila* and the honey bee (Laissue et al., 1999; Flanagan and Mercer, 1989; Galizia et al., 1999) to about 2000 - 3000 in mice and rats (Shipley and Ennis, 1996). Olfactory information is processed by inter neurons [IR] and principal neurons, glutamatergic mitral and tufted cells in vertebrates (Aroniadou-Anderjaska et al., 1999), and cholinergic projection neurons [PNs] in insects (Bicker, 1999). These principal neurons convey the olfactory information to higher order brain centers (Mori and Yoshihara, 1995; Shipley and Ennis, 1996; Hansson and Anton, 2000; Abel et al., 2001). In mammals, the mitral and tufted cells already project onto higher brain areas like the amygdala or the entorhinal cortex related to emotion and cognition (Wilson and Mainen, 2006). Also in the insects' olfactory pathway, the PNs send collaterals to higher order brain centers via different axonal tracts (Mobbs, 1982; Bicker et al., 1993). The lateral antenno-cerebral tract [l-ACT] and the medial antenno-cerebral tract [m-ACT] are two of them. They are composed of uniglomerular PNs and target the lateral horn [LH] and the MB input region, the Calyx, which consist of Kenyon Cells [KC] (Abel et al., 2001; Müller et al.,

2002). Thus, in both, mammals and insects, the olfactory system forms an extremely straight forward processing stream, in which the information about the odor plume has to cross only two synapses before it reaches higher levels. Since there are many similarities between the olfactory systems of vertebrates and insects, it is a great advantage to choose insects as the more simple and manageable model system, to investigate the principles of olfactory coding, learning, and memory formation.

The output of the Mushroom body [MB]

In my thesis, I focused primarily on the mushroom body extrinsic neurons. These ENs form the output of the MB, which consists mainly of Kenyon Cells [KCs] (Heisenberg, 2003). The olfactory information diverges into a high dimensional space of KC activity (about 200,000 in cockroaches, 170,000 in honey bees, 50,000 in locusts and 2,500 in *Drosophila*). KCs are supposed to respond highly odor selective and sparse, in Drosophila (Turner et al., 2007) as well as in locusts (Stopfer et al., 2003; Jortner et al., 2007), and in honey bees (Szyszka et al., 2005). Still, the processing of odors in the mushroom body is poorly understood and largely considered to be a "black box". One experimental access to this "black box" is to focus directly on the KC activity. Using optical imaging Faber and Menzel (2001) have shown that the Ca^{2+} response for the rewarded odor is increased in the MB lip after learning. However, the cells involved have not been identified yet. Therefore it remains unclear, whether and how KCs are involved in the learning process. Imaging studies in *Drosophila* have shown that the different MB-branches (γ-lobe, α/β-lobe) are involved differentially in memory formation (Zars et al., 2000; Pascual and Preat, 2001). Yu et al. (2006) have shown that a branch-specific memory trace is formed within 3 to 9 hours after conditioning, only in the alpha-branch of the MB.

Another possibility to shed light on the MB function is to study the input by focusing on the PNs and compare their activity to the output. There are many studies investigating the input of the MB by focusing on the AL activity, where in general odors are specifically represented in complex spatio-temporal activity patterns of excited and inhibited glomeruli (Sachse and Galizia, 2002). The investigation of learning induced changes at the PN level led to contradicting results. Peele et al. (2006), for example,

found that uniglomerular AL projection neurons in honey bees show no significant difference in odor-evoked activity after classical odor conditioning. By applying differential conditiong Faber et al. (1999) found learning induced changes within the AL represented by an increase in the activity to the rewarded but not to the unrewarded odor. In *Drosophila*, PN synapses can be recruited for a small time window of up to seven minutes (Yu et al., 2004). Also at the output of the MB, which in the honey bee consists of about 400 ENs (Rybak and Menzel, 1993) learning induced changes were investigated. The pedunculus extrinsic neuron [PE1] is one of the most studied, identified extrinsic cells of the MBs alpha lobe (ENs) with large branches collecting information from KCs. During classical conditioning the PE1 changes its response pattern (Mauelhagen, 1993) and moreover electrical stimulation of the KCs leads to the formation of associative long-term potentiation (LTP) in the PE1 (Menzel and Manz, 2005). Extra-cellular long term recordings also document that the PE1 shows a reduction in the response to the rewarded stimulus after the bee has associated an odor with a reward (Okada et al., 2007).

In the present study I focus on different, so far unidentified ENs, by applying extra-cellular recordings to the ventral part of the alpha-lobe of the MB. The neurons recorded at this part of the alpha-lobe, can be related to the A1, A2, A4, A5 and A7 clusters (Rybak and Menzel, 1993). The projection fields of most mentioned EN types are restricted to only one protocerebral hemisphere, where they connect the MB with the neuropiles around the alpha lobe and the lateral protocerebral lobe [LPL]. Only type A7 connect the MBs of both hemispheres (Rybak and Menzel, 1993).

Learning related plasticity investigated via extra-cellular long term recordings

The ideal situation to investigate the neuronal correlates of learning is to observe the neuronal network while the subject can communicate its behavior. This allows a direct comparison, between the steady state of single neurons of the neuronal network and the steady state of the behavioral change (learning). In vertebrates, extra-cellular recordings have already been successfully used to monitor neural processes during learning and memory retrieval at the single-neuron level (e.g. in place cells in the rodent hippocampus: Sutherland and McNaughton, 2000; in prefrontal neurons related to

working memory in primates: Goldman-Rakic, 1995; in orbito-frontal neurons related to olfactory learning: Rolls et al. 1996; and in dopamine neurons in the ventral tegmentum of the monkey: Schultz, 1998). In insects, extra-cellular long-term recordings have been successfully used to characterize the activity of single mushroom body [MB] neurons in freely moving cockroaches (Mizunami et al., 1993; Mizunami et al., 1998; Okada et al., 1999). In honey bees extra-cellular long term recording were established to record the activity of the PE1 in a behaving animal, during a classical conditioning experiment (Okada et al., 2007).

Here, I adapted and modified this extra-cellular recording technique (see methods, Chapter 1) to increase the possibility of simultaneously recording more than one single unit and to establish a basis for further studies (Strube-Bloss et al., 2008b, chapter 2; Strube-Bloss et al., 2008c, chapter 3; forthcoming) which will focus on the investigation of the extrinsic neurons' response changes after a classical conditioning experiment. The most popular examples of classical conditioning are the studies on dogs conducted by Ivan Pavlov (1927). In bees olfactory classical conditioning is a robust and well-studied type of learning which is based on the proboscis extension response [PER]: When sucrose solution (unconditioned stimulus; US) is delivered to the antennae or proboscis, hungry bees respond with an extension of their proboscis (Kuwabara, 1957; Menzel et al., 1974; Vareschi, 1971). This reflex is usually paired with olfactory cues (conditioned stimulus; CS). Ideally, the repetition of such pairings leads to a learned behavior related to the CS. After three such conditioning trials, a long-lasting stable memory is formed (Menzel et al., 1991). In a differential conditioning procedure, it has also been shown that bees learn to discriminate between two odors within two to three learning trials (Bitterman et al., 1983). To simultaneously observe the neuronal activity of EN and the steady state of the behavior of the subjects, I recorded the muscle M17 of the bee (Rehder, 1987), which mediates the PER.

The neuronal correlate for side specific representation in the bee brain

The bilateral, symmetric organization of sensory systems is a widely spread phenomenon and allows an improved integration of information from the environment. In general, the received information at the two input sides is slightly different. This difference can be used by the brain to add an accessorial dimension. That means, the two brain sides have to collaborate to build the environmental representation. The honey bee brain is organized in a bilateral symmetric way up to the higher-order integration centers, the MBs (Mobbs et al., 1982). In bees both MBs are involved in memory formation (Menzel et al., 1974; Erber et al., 1980), although unilateral olfactory association is only recallable on the trained antennae (Menzel et al., 1974). Following a retention period this association is also retrievable from the contra-lateral brain side (Sandoz and Menzel, 2001). Thus, in bees both phenomena exist: unilateral and bilateral learning. Since the integration of olfactory information in both hemispheres seems to be time dependent, consolidation may be involved. Not only consolidation time seems to be a prerequisite for the integration of the information of both antennae, also the complexity of the learning task may play an important role. There is for example the solving of non-elemental learning tasks, like negative patterning, which can easily be solved by honey bees (Deisig et al., 2001). In this form of learning, two olfactory components are rewarded when they are presented alone, and not rewarded when presented as a compound. To solve this learning task the input of both brain sides is needed (Komischke et al., 2003), whereas in elemental tasks like positive patterning (Deisig et al., 2001) where the compound of both components is rewarded and the single odors are unrewarded, the processing in one hemisphere seems to be sufficient (Komischke et al., 2003). Furthermore, bees with ablation of one MB are not able to solve side spanning learning tasks, although they are learned in a differential conditioning paradigm (Komischke et al., 2005).

In the second chapter I show that ENs which leave the MB via the ventral alpha lobe were completely recruited after the honey bee has build an association between a conditioned stimulus [CS] and an unconditioned stimulus [US]. The projection fields of most of the ENs that are leaving the MB at the ventral part of the alpha lobe are restricted to only one protocerebral brain side, where they connect the MB with the neuropils around the alpha lobe and with the lateral protocerebral lobe [LPL]. These

cells are related to the A1, A2, A4 and the A5 clusters. Only the ENs related to the A7 cluster are connecting both brain sides (Rybak and Menzel, 1993). Studying these neurons during the application of side-specific learning tasks should give us insight into the integration of the olfactory information between brain sides.

In the third chapter of the present thesis I adapt the behavioral experiments by Sandoz and Menzel (2001) in which they have shown that unilateral differential conditioned information is transferred to the contra-lateral brain side where bees had now experience from the previous trials, after 3 hours. During that task I measured the activity of single ENs. I am able to show that already during the conditioning, ENs of the contra-lateral MB are involved in the computation of the side specific information, which leads to a stable side specific representation of the stimulus 3 hours after resting (consolidation) time in the activity pattern of the ENs.

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