General Discussion

Changes in Alpha lobe extrinsic neurons as neuronal correlate for learning

The experimental focus of this thesis was placed on the alpha-lobe-extrinsic neurons [ENs] of the honeybee. The ENs with their extended dendrites integrate information from Kenyon cells [KCs] of the mushroom body [MB] and project to various other brain regions (Mobbs, 1982; Rybak, 1993). The information from approximately 170.000 KCs converges onto a much smaller number of about 400 ENs. One may assume that the EN activity, which forms the output of the MB does not merely represent sensory information about a stimulus, e.g. the identity and intensity of a particular odorant. Rather, they collapse information from the large neuronal KC space. The activity patterns of KCs are highly odor specific and sparse, as has been shown in different insect species (*Drosophila:* Turner et al., 2007, Wang et al., 2004; locusts: Jortner et al., 2007, Perez-Orive et al., 2002, Stopfer et al., 2003; honey bees: Szyszka et al., 2005). Also the ventral unpaired median neuron number1 of the maxillary neuromere [VUM mx1], which mediates reward-related reinforcement in appetitive odor learning (Hammer, 1993) projects into the calyces of the MBs. Thus, the MB integrates olfactory, visual and mechano-sensory information and combines them with the rewarded stimulus.

Since the MBs are thought to be the centers for learning and memory formation in the insect brain (Dujardin, 1850; Strausfeld, 1998, review) the neurons transmitting their output should reflect learned and associated stimuli. Changes in their response patterns over time may be the result of memory formation. One identified and characterized EN of the honeybee MB is the pedunculus-extrinsic neuron one [PE1]. It is known, that electrical stimulation of the KCs leads to a formation of associative long-term potentiation [LTP] in that particular neuron (Menzel and Manz, 2005). Mauelshagen (1993) found, that the PE1 shows an initial decrease in its response to a forward-paired odor (CS+). This decrease developed to be stable as shown in extracellular long term recordings from the same neuron (Okada et al., 2007). Also in other insect species there is increasing evidence, that the ENs reflect learning induced plasticity. E.g. in locusts where spike time dependent plasticity [STDP] occurs between KCs and β-lobe neurons (Cassenaer and Laurent, 2007). In *Drosophila* a delayed memory trace is formed after

30 min only in the vertical (α-lobe) branch of the dorsal paired medial neuron [DPM], a MB extrinsic neuron which is an odor generalist (Yu et al., 2005).

I recorded the extracellular activity from the ventral part of the alpha lobe aiming at the ENs. To meet a prerequisite for the detection of different learning induced changes I characterized in the first chapter of the present thesis the general response characteristics of this neuron type to repeated odor presentations. I found that most of the ENs are odor generalists, similar to the DPM in *Drosophila* (Yu et al., 2005) responding to many different odors (Strube-Bloss et al., 2008a; chapter1). The across-trial reliability of responses was generally low for repeated presentations of the identical stimulus. In a next step, I applied differential conditioning to the animals and recorded simultaneously the activity of the ENs. The presented learning tasks resulted in various kinds of changes in the single neuron responses, 3 hours after classical odor conditioning, as outlined in chapter 2. Some of the ENs (~30%) changed their odor response spectrum into the direction of the rewarded odor (CS+). The reliability was also influenced by learning and memory formation and in some units completely rearranged in the post-conditioning test phase. Although the most increase in reliability was found for the CS+, the individual changes observed in single units were manifold. This indicates that the single neuron contribution to the computation in the entire network is highly individual and indicates how complex the underlying computing mechanisms might be. In the final chapter of my thesis I applied side specific learning tasks to test whether the separate stimulation of only one MB is also reflected at this neuronal level. To test this, I adapted the experiments by Sandoz and Menzel (2001) in which they demonstrated, that an transfer of olfactory information between both MBs occurred three hours after classical conditioning. I could show that after contralateral differential conditioning the CS+ is represented differently in the activity of the same unit depending on the side of its presentation. This result supports the idea that in general, memory traces are build as configurations across both hemispheres (Strube, 2005). There are various ways how single ENs change their response and integrate into the network which processes the stimulus-reward association and which is then able to differentiate a rewarded stimulus from other stimuli, as I could show in chapter 3. One unit may be excited by the side specific CS+ if it is presented on the “correct” antenna (i.e. the side on which the CS+ was presented during training). Presenting the inverse

left/right combination may inhibit the same unit (Strube-Bloss et al., 2008c; chapter3: Fig. 2). This example of neuronal behavior gives an impression of the multidimensionality and the interactions between excitation and inhibition regarding decision making.

Inhibitory effects at various stages of the network

In the different learning experiments of this thesis we often observed two types of inhibitory effects. On the one hand neuronal responses could be inhibited during the acquisition phase or in the test phase 3 hours after acquisition. On the other hand the spontaneous firing rate could be reduced, i.e. after the first CS/US pairing (cp. Unit1 Fig. 5, chapter 2). The cause of such inhibitory effects could be related to the GABA-immunoreactive (ir) inhibitory feedback neurons (Bicker et al., 1985; Grünewald, 1999) of the protocerebro-calycal-tract (PCTs). They are also related to the ENs and may be effective twice: locally by sending their collaterals down the peduncle and reach the dendritic trees of ENs, and recurrently by leaving the alpha lobe around its lateral midline and projecting to the input region of the mushroom body, the calyces (Okada et al., 2007). During the spontaneous activity after the first CS+ stimulations, down-regulation of the overall activity in the presynaptic MB network might serve to increase the contrast for the associated odor. The cause of such a down regulation may be an increased spontaneous activity of PCT neurons. The local blocking of GABA should than disconnect the inhibitory component from the computing network and the state of the pre-acquisition phase should be observable again. Interestingly, the depression of the spontaneous firing rate after differential odor conditioning in ENs disappeared after reversal learning. In a reversal learning experiment the initially reinforced odor (CS+) was presented in a second conditioning phase to be non reinforced and the non reinforced odor (CS-) vice versa (Fig. 1). In the post test phase [Post test] three hours after the first differential conditioning both odors are presented again without any reinforcement. The response strength is the same, but the spontaneous rate (2 seconds before odor onset) is decreased (Fig. 1; black arrows). During the second differential conditioning the initial reinforceded odor is presented without a reinforcer and the initially non-reinforced odor was now paired with a reinforcer and the spontaneous rate recovered. Note that this recovery could also be influenced by the first post acquisition

test phase between the two differential conditioning phases where the odors were presented alone. This phase in itself could induce extinction. However, both reversal learning and extinction underlying learning processes that reconfigure the initial association between odor and reinforcement. This may have an influence on the integration of the related EN into the computing network.

Another interesting example of inhibitory activity after learning is the side specific inhibition of the “wrong” CS+ shown by the example unit in figure 2 (Strube-Bloss et al., Chapter 3). The odor was presented to be reinforced occurring contralateral to the recording position. The recorded unit is recruited to be excited if the odor is presented to the “correct” antenna and inhibited if the same odor is presented to the ipsilateral antenna related to the recording position. That is the “wrong” side for the initial made association. Also in that example the PCTs may influence the computing network. These neurons could be effective twice: (i) local, by sending there collaterals down the peduncle and reach the dendritic trees of ENs and (ii) recurrent, by leaving the alpha lobe around its lateral midline and projecting to the input region of the MB, the calyces (Okada et al., 2007). (i) The local inhibitory GABA effect onto ENs in the peduncle could drive the observed inhibition. One may speculate that this inhibitory signal influences the bee's decision not to extend the proboscis if the CS+ is presented at the “wrong” side, as illustrated by their behavior (cp. Fig. 2, Chapter 3). (ii) The recurrent path that affects the input region of the MB could be the cause of the down-regulation of spontaneous activity as described before. It would be interesting to test, if the local inhibition of ENs into the peduncle would be also the causation of the phenomenon of blocking between odors in binary mixtures as described by Smith and Cobey (1994) and Thorn and Smith (1997).

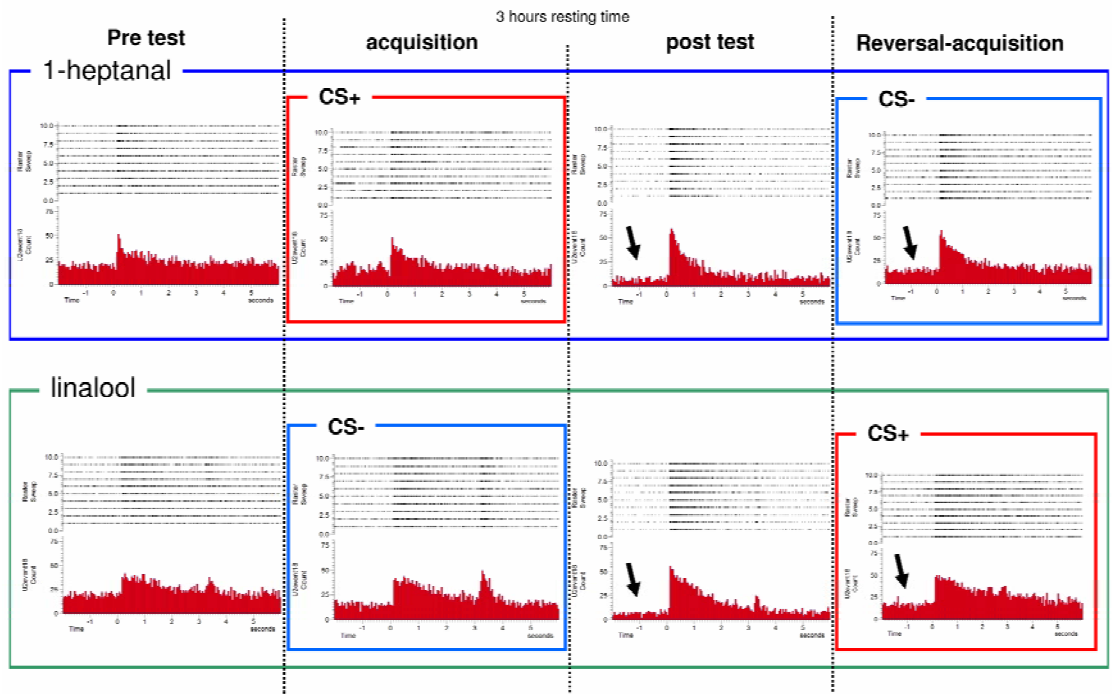

Figure 1. Reversal learning could reverse decrease in spontaneous activity. *Two odors were presented (1-heptanal and linalool) in a pseudo random order in each experimental phase. The dot displays show the time points of spiking for one single unit that is recorded during the complete experimental procedure. The 10 trials per odor and experimental phase are presented on top of each other starting with the first trial (lowest). The peri- stimulus- histograms [PSTH] for each phase and odor are shown below (red, 50ms bins). The odor presentation started at 0ms and lasted for three seconds. In the pre test phase [pre test] before differential conditioning [acquisition] each odor is presented alone. During the acquisition 1-heptanal is paired with the reinforcer [CS+] and linalool is presented alone [CS-]. The reinforcement [US] lasted also 3 seconds and is started 2 seconds after odor onset. Note that the unit showed a clear off response to linalool. During the first acquisition that off response is clearly increased although the odor is presented non reinforced. In the post test phase [Post test] three hours after differential conditioning both odors are presented alone again. The response strength is nearly the same, but the spontaneous rate (2sec. before odor onset) is decreased [black arrow]. During the last experimental phase, differential conditioning were performed reversed, meaning that now linalool is the CS+ and 1-heptanal the CS-. Note, that the spontaneous rate during that phase is already recovered.*

Animal behavior and the network behind

The neuronal network that determines the insect behavior is of enormous complexity that will keep scientists busy for lots of future generations. In honeybees there are several well elaborated behavioral studies that allow fantastic predictions about the neuronal network behind (blocking between odors in binary mixtures, Smith and Cobey, 1994; solving of non-linear olfactory discriminations, Hellstern et al., 1995; Chandra and Smith, 1998; Deisig et al., 2001, 2002, 2003; latent inhibition, Chandra et al., 2000; or the time and training dependent formation of different memory phases, Menzel, 1979; Menzel and Müller, 1996; Menzel et al., 2001; to name only a few). With appropriate physiological access it would be feasible to monitor the computing network over hours while solving complex behavioral learning tasks. One step in this direction regarding the honeybee has been achieved by Okada et al. (2007). He recorded extracellularly the activity of a single neuron (PE1) across hours: before, during and after the bee had built an association. Via his recordings he could make fantastic predictions about the neuronal network behind.

Here I adapted and modified the method of extra cellular recording and added behavioral experiments. In Chapter 3 I carried on investigating the phenomenon of side specific integration of the olfactory information of both antennae of the honeybee. Previous studies had shown that bees learn the combinations of side specific olfactory inputs and thus appear to form side overlapping configurations from spatial arrangements of olfactory inputs (Strube, 2005). The prerequisite for building this kind of stable antenna specific memory is the independency of the two antennae regarding there odor reception and first evaluation. That is supported by non-associative learning, e.g. habituation, which was already found to be limited to the stimulated side (Braun and Bicker, 1992). The integration of the information of both antennae is necessary for the building of a compound therefore it is necessary that both brain sides were sensitized. Sandoz et al. (2002) found, that an antenna-US provides unilateral sensitization, restricted to the side of US application, whereas proboscis-US and compound-US (one antennae and proboscis) induced bilateral sensitization. I always presented the US to the contralateral antenna and the proboscis. Both brain sides should be sensitized to integrate the received input of both antennae. During differential conditioning I applied the CS+ and the CS- only to the contralateral antenna related to

the recording position. The other antenna was spatially separated. After three hours I tested if the odor presentation on that antenna, which had no experience with the odor from the learning trials before also leads to the learned behavior (PER). The same behavioral experiment has been carried out by Sandoz and Menzel (2001). They found that bees transfer the olfactory information to the other brain side in a time window between 10 minutes and 3 hours. The advantage of my experimental access was that I was able to observe the activity of single units that are part of the computing network which may generate the observed "transfer" behavior. Anatomical studies support, that first at this stage of the olfactory path neuronal connections allow the crosstalk between the brain sides. Possible candidates can be related to the A7 cluster of ENs (Rybak, 1993). Indeed, I found at this neuronal stage the lateral representation of the received side specific stimuli. Thus, the side specific presentation of the CS+ on the "correct" antennae, where it was also presented during the acquisition evoked different activity in the recorded EN than the CS+ presented on that antenna that had no experience with the odor during the acquisition phase. The additional awareness of the present experiments was that both the behavior and the neuronal activity were observed. That provides us with direct access to at least single units of the computing network. The transfer of the information of only the odor identity from one side to the other did not occur. Possibly such an effect might exist only as a behavioral observation (for detailed discussion cp. Chapter 3). Rather is the different information of both antennae integrated in a side-spanning compound that is certainly different for the "correct" CS+ and the "wrong" CS+ (odor presented to either the correct or to the wrong side).

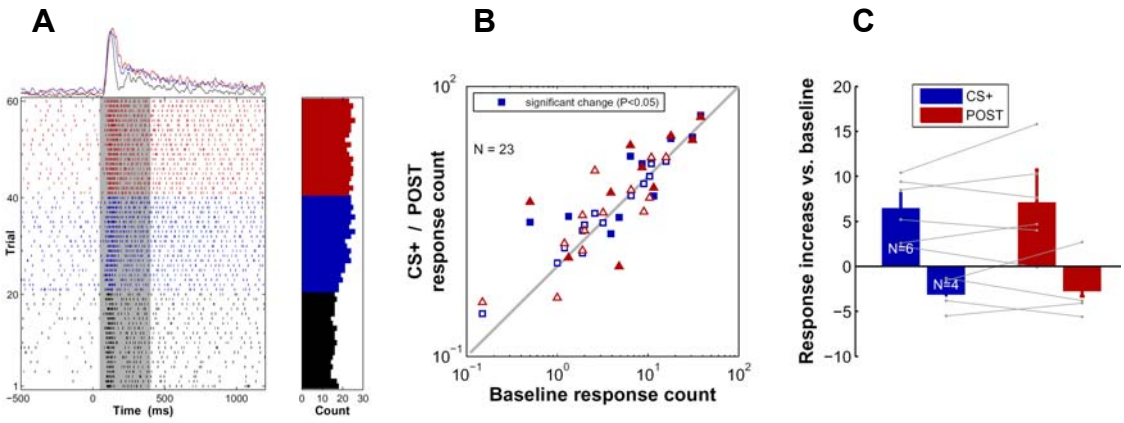

Figure 2. Short term plasticity in ENs after absolute conditioning. ***A:*** *Example of a dot display of a unit that is stimulated 60 times with only one odor (trial). The inter trial interval is 1 minute. The experiment started with the first 20 trials in black, as pre-acquisition test. During the acquisition (trial 21-40, blue) the odor is presented reinforced, meaning that conditioned stimulus (CS; odor) and unconditioned stimulus (US; sugar reward) were presented together (CS+). Each stimulus (CS, US) lasted three seconds. They were presented with one second overlap (CS first). Note, that only the first second of odor presentation is shown starting with 0. In the last phase (trial 41-60, red) the odor is again presented non reinforced. All three phases are separated by 15 minutes. The upper lines illustrate the mean response rate across the 20 trials of each experimental phase. In the "Count"-display the rate response between 50 and 450 ms (grey) after odor onset of each trial is illustrated. Note that after the first CS+ trials the rate increased. That effect remains stable across the post test phase.* ***B:*** *Response count during the three experimental phases in a time window between 50-450 ms after stimulus onset before the conditioning phase (baseline) [x-axis] vs. during the conditioning phase (CS+, blue squares) and the post-conditioning phase (Post, red triangles) [y-axis]. Filled symbols marking significant count changes from baseline (t-test, p<0.05) [left].* ***C:*** *Mean increase (positive) and mean decrease (negative) related to the baseline for the response during the conditioning (blue) and the post-phase (red) for the units that showed significant changes in* ***B****. Grey lines joining the response changes of individual units.*

Different types of memory require different ENs

Since it is known that reward learning in honeybees initiates a sequence of multiple memory phases that leads to a stable long-lasting memory (Menzel and Müller, 1996; Menzel, 1999), scientists try to find the memory trace in the honeybee's brain by either focusing on different neuropiles, or by searching for all memory phases at one higher order stage in the neural network. The investigation of learning induced changes at the PN level led to, at the first glance, contradicting results. Peele et al. (2006), for example, found that uniglomerular AL projection neurons in honeybees show no significant difference in odor-evoked activity after classical odor conditioning. Faber et al. (1999) found learning induced changes and an increase in the activity to the rewarded, but not to the unrewarded odor after differential conditioning in the AL. In *Drosophila*, PN synapses can be recruited for a small time window of up to seven minutes (Yu et al., 2004). The results of Peele (2006) support the idea, that the odor representation in the first order neuropile, the AL is stable, indicating that memory is to be formed only at later processing stages at higher levels in the processing hierarchy. The ENs of the MB show different plastic properties that may originate from the different forms of memory. Mauelshagen (1993) found already that the PE1 showed an initial decrease in its response to a forward-paired odor (CS+). This decrease developed to be stable as shown in extra cellular long term recordings from the same neuron (Okada et al., 2007). This in fact could mean that early long term memory and stable long term memory are represented at the same neuronal level. The PE1 is responding to different odors and different modalities (Mauelshagen, 1993, Menzel and Manz, 2005). Following the classification of this thesis (Strube-Bloss et al. 2008a, Chapter 1) it would be a initially responding EN. I could show that other groups of ENs are also odor unspecific with few exceptions, some of them responding reliable others unreliable to the different odor stimulations (cp. Chapter 1). But there are ENs that are initially non responding and were recruited to respond specifically as a consequence of differential conditioning on both antennae (cp. Chapter 2) or after contralateral odor conditioning (cp. Chapter 3). However, the experimental designs that were chosen for this thesis (Chapter 2 and Chapter 3) did not allow memory tests before the three hour resting time. In a set of additional experiments I therefore tested whether units that initially respond to odors like the PE1, increased or decreased their response rate significantly already in

early memory phases. The preliminary results are presented in figure two. During this experiment only one odor was presented during 60 trials in total: 20 times before, 20 times during and 20 times after absolute odor conditioning. During the conditioning the odor is presented forward paired with a reward (sucrose). Ten out of 23 recorded units changed there rate response during the conditioning significantly (Fig. 2B). For some of them this change remains stable during the 20 trials after conditioning. Other units decreased or increased there response strength again (Fig. 2C). Possibly the short term memory is mirrored by the up and down regulation of the response strength of initially responding units (e.g. the PE1) whereas long term memory is reflected by the recruitment of initially non responding units. This would mean that the different memory phases find reflection in the different individual learning-induced behavior of single alpha-lobe-extrinsic neurons and thus at the same hierarchical network level that performs the read out of the Mushroom bodies exist in parallel.

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